In vitro Antibacterial Effect of Polyglycerol Monolaurates against Gram-Bacteria and Understanding the Underlying Mechanism

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Abstract: Polyglycerol monolaurates are generally recognized as safe food additives and are commonly used as food emulsifiers. In this study, the antimicrobial effect of four polyglycerol monolaurates on two Gram-positive bacteria (Staphylococcus aureus and Bacillus subtilis) and two Gram-negative bacteria (Escherichia coli and Pseudomonas aeruginosa) were investigated. The minimum inhibitory concentration (MIC) of diglycerol monolaurate (PG2ML), triglycerol monolaurate (PG3ML), hexaglycerol monolaurate (PG6ML), and decaglycerol monolaurate (PG10ML) against S. aureus was 0.16, 0.32, 0.63, and 1.25 mg/mL, respectively. The MIC of PG2ML, PG3ML, PG6ML, and PG10ML against B. subtilis was 0.32, 0.63, 1.25, and 3.75 mg/mL, respectively. No apparent antimicrobial effect of these four polyglycerol monolaurates on E. coli and P. aeruginosa was observed even up to 10.00 mg/mL. The underlying mechanism was investigated by assessing cell membrane permeability, the integrity of cell membrane, and morphology. We concluded that polyglycerol monolaurates might eliminate Gram-positive bacteria by disrupting the cell membrane, thereby increasing cell membrane permeability, releasing the cellular contents, and altering the cell morphology.

Key words: antimicrobial effect, food-related bacteria, polyglycerol monolaurate, underlying mechanism

1 Introduction

Food is susceptible to microbial infections during processing, production, packaging, and transportation, leading to food spoilage. Microbial contamination is considered a significant hazard in terms of food safety. Microorganisms, including bacteria, fungi, and yeast, can be easily ingested by consuming contaminated food, thereafter they can not only attack human tissues and cells, but also release toxins, leading to serious infectious diseases. To ensure food safety and extend their shelf life, synthetic preservatives, such as benzoates and sorbates, are commonly used in food products. However, the use of these preservatives is significantly limited, as they can be harmful to the human body if present in higher than the permitted limits. Therefore, the development of novel and safe alternatives is urgently required.

Fatty acids and their corresponding esters, such as monoglycerides and sugar esters, are a group of chemicals derived from natural oils, which are considered to be non-toxic and antimicrobial in nature. Among all fatty acid esters, medium-chain monoglycerides, including monolaurin and monocaprin, have been commonly used as food emulsifiers and have been reported to exhibit broad spectrum of inhibitory effects against food-borne bacteria, fungi, and yeast. However, the industrial applications of monoglycerides in liquid-type food are still limited because of their poor aqueous solubility.

Polyglycerol fatty acid esters, composed of several glycerol units, are considered an alternative to monoglycerides because of their high aqueous solubility. In contrast to the extensive studies on the antimicrobial properties of monoglycerides, information regarding the use of polyglycerol fatty acid esters as food preservatives is limited. Previous studies have shown that polyglycerol fatty acid esters can inhibit the growth of several Gram-positive bacteria, but did not affect Gram-negative bacteria. However, a recent study showed that triglycerol monolaurate can effectively inhibit both Gram-positive and Gram-negative bacteria.
bacteria\textsuperscript{197}. Besides, diglycerol monolaurates exerted inhibitory effect on yeast such as \textit{Saccharomyces cerevisiae}, \textit{Candida utilis}, and \textit{Candida albicans}\textsuperscript{20, 21}.

The antimicrobial effect and action mechanism of polyglycerol fatty acid esters vary with different tested strains, carbon-chain lengths, glycerol polymerization degrees, and culture conditions, requiring further in-depth study. With respect to different carbon-chain lengths, lauroyl ester was found to be more effective against microorganisms\textsuperscript{20, 21}.

Therefore, in the present study, the antibacterial activities of four polyglycerol monolaurates against \textit{S. aureus}, \textit{B. subtilis}, \textit{E. coli}, and \textit{P. aeruginosa} were assessed. In addition, the possible action mechanism of these polyglycerol monolaurates against these bacteria was investigated.

2 Experimental Procedures

2.1 Materials

Polyglycerol monolaurates (food grade), including diglycerol monolaurate (PG2ML), triglycerol monolaurate (PG3ML), hexaglycerol monolaurate (PG6ML), and deca-glycerol monolaurate (PG10ML) were obtained from Shandong Binzhou GIN&ING New Material Technology Co., Ltd. (Shandong, China). Nutrient broth and fluorescein diacetate (FDA) were purchased from Sigma-Aldrich China (Shanghai, China). Coomassie Brilliant Blue G250 was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

2.2 Bacterial strains and culture conditions

\textit{S. aureus} (AS1.89), \textit{B. subtilis} (AS1.1849), \textit{E. coli} (AS1.90), and \textit{P. aeruginosa} (AS1.10452) were obtained from the China General Microbiological Culture Collection Center (Beijing, China). Nutrient broth (1.8 g) was added to 100 mL physiological saline, and then sterilized at 121°C and 0.1 MPa for 20 min, giving nutrient broth culture medium. Cultures were routinely grown by subculturing 100 μL stock culture in 100 mL sterilized culture medium and incubating at 37°C for 15 h.

2.3 Determination of minimum inhibitory concentration (MIC)

The MIC of four polyglycerol monolaurates was determined using a broth dilution method\textsuperscript{20} with slight modifications. The four strains in the logarithmic growth phase were suspended in nutrient broth medium at 10^{-6} CFU/mL. Different concentrations of polyglycerol monolaurates were prepared via two-fold serial dilution. Bacterial suspension (30 μL) was mixed with 180 μL nutrient broth medium and 30 μL of different polyglycerol monolaurate dilutions, such that the final concentrations of polyglycerol monolaurates were 10.00, 5.00, 2.50, 1.25, 0.63, 0.32, 0.16, and 0.08 mg/mL. The same volume of physiological saline was added instead of the polyglycerol monolaurate solution in the negative control. The medium was incubated at 37°C for 36 h in the Bioscreen C system (Oy Growth Curves, Helsinki, Finland), and the optical density was measured at 492 nm (OD\textsubscript{492}) and recorded at 1-h intervals. OD\textsubscript{492} was plotted against time to obtain the growth curve of the tested bacteria. The MIC was defined as the lowest concentration of polyglycerol monolaurates that did not increase the OD\textsubscript{492} values for any replicate compared to that of the negative control after 36 h. All tests were performed in triplicate.

2.4 Determination of cell membrane permeability

Cell membrane permeability of the tested bacteria was determined using the FDA fluorescent dye. FDA is a non-polar ester which can easily pass through cell membranes. Upon hydrolysis by esterases present in viable cells, it can produce fluorescein, accumulating inside the cell and fluorescing under UV light. When the bacteria’s cell membrane is disrupted, the fluorescein molecules leak out from the cell and the fluorescence intensity decreases drastically. Thus, according to a published procedure, the permeability of the cell membrane of bacteria was determined by measuring the change in fluorescence intensity\textsuperscript{20}. Briefly, the four cultured strains in the logarithmic growth phase were suspended in a nutrient broth medium at 10^{-6} CFU/mL, followed by the addition of MIC and 2×MIC polyglycerol monolaurates. The negative control was prepared by adding the same amount of physiological saline. After incubation at 37°C and 120 rpm for 12 h, 4 mL bacterial suspension was taken and centrifuged at 8000 rpm and room temperature for 6 mins. After discarding the supernatant, bacterial cells were collected and washed twice with 10 mL PBS, followed by the addition of 400 μL FDA-acetone solution (2 mg/mL). The mixture stood for 20 min at room temperature and then washed twice with PBS. Another 4 mL PBS was added, and the fluorescence intensity was measured by a fluorescence spectrophotometer (F-4600, Hitachi, Tokyo, Japan), using an excitation wavelength of 490 nm and an emission wavelength of 526 nm.

2.5 Release of intracellular biomacromolecules

The cell membrane is a natural protective barrier for bacteria. Once exposed to antibacterial agents, the integrity of the bacterial cell membrane would be damaged, resulting in leakage of intracellular biomacromolecules, including nucleic acids and proteins. The release of cellular contents was determined according to our previously reported method\textsuperscript{20} with minor modifications. Briefly, 10 mL bacterial suspension was centrifuged at 8000 rpm for 6 min. The absorbance of the supernatant at 290 nm was measured using a UV-visible spectrophotometer (UV-3600, Shimadzu, Japan), revealing the amount of nucleic acids released from bacterial cells.
After discarding the supernatant, bacterial cells were collected and washed twice with PBS, and then resuspended in 10 mL distilled water. The intracellular proteins were obtained after sonic disruption at 25 kHz and 600 W for 15 min. 2 mL intracellular proteins were mixed with 3 mL Coomassie Brilliant Blue G-250 and incubated for 20 min at room temperature. The absorbance at 595 nm was determined using a UV-visible spectrophotometer.

2.6 Observing cell morphology using a scanning electron microscope (SEM)

Polyglycerol monolaurates were added to 100 µL bacterial suspension at the final concentration corresponding to the MIC and 2×MIC. After incubated at 37°C for 12 h, the mixture was centrifuged at 8,000 rpm for 6 min. Then, the bacterial cells were washed three times with PBS and fixed with 2.5% (v/v) glutaraldehyde solution at 4°C for 3 h. After dehydration with 50%, 70%, 90%, and 100% ethanol, the cells were resuspended in ethanol for 10 min. Finally, the bacterial cells were lyophilized and sputter-coated with gold before observation using a SEM-SU8010 scanning electron microscope (Hitachi, Japan).

3 Results and Discussion

3.1 Antimicrobial effect

The antibacterial growth curves of *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* treated with four polyglycerol monolaurates was investigated using Bioscreen C. The growth curves of *S. aureus* are shown in Fig. 1. The OD₄₉₂ of the control (not treated with polyglycerol monolaurates) increased rapidly at the beginning, and then plateaued after 17 h. After treatment with polyglycerol monolaurates, the OD₄₉₂ of the *S. aureus* suspension remained unchanged when the concentration was higher than a specific value. For example, *S. aureus* was almost unable to grow after treatment with 0.16 mg/mL or higher concentration of PG2ML, suggesting that PG2ML completely inhibited this bacterium’s growth. Therefore, the MIC of PG2ML against *S. aureus* was determined to be 0.16 mg/mL.

Similarly, the MIC values of PG3ML, PG6ML, and PG10ML against *S. aureus* were determined to be 0.32 mg/mL, 0.63 mg/mL, and 1.25 mg/mL, respectively. These results showed that the antimicrobial effect of polyglycerol monolaurates against *S. aureus* correlated with the extent of glycerol polymerization. The antimicrobial effect decreased with increase in the extent of glycerol polymerization of polyglycerol monolaurates. Figure 1 also shows that the growth of *S. aureus* was not inhibited or was partially inhibited when the concentration of polyglycerol monolaurates was lower than the MIC values.

The results obtained using *B. subtilis* as the test strain are displayed in Fig. 2. Polyglycerol monolaurates inhibited *B. subtilis* in a concentration-dependent manner, in addition, the inhibitory effect correlated negatively with the...
extent of glycerol polymerization of polyglycerol monolaurates. The MICs of PG2ML, PG3ML, PG6ML, and PG10ML against *B. subtilis* were determined to be 0.32 mg/mL, 0.64 mg/mL, 1.25 mg/mL, and 3.75 mg/mL, respectively.

No antimicrobial effect was observed for the Gram-negative bacteria *E. coli* and *P. aeruginosa* after treatment.

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**Fig. 2** Growth curves of *B. subtilis* treated with different concentrations of polyglycerol monolaurates: (a) PG2ML; (b) PG3ML; (c) PG6ML; (d) PG10ML.

**Fig. 3** Growth curves of *E. coli* treated with different concentrations of polyglycerol monolaurates: (a) PG2ML; (b) PG3ML; (c) PG6ML; (d) PG10ML.
with polyglycerol monolaurates up to 10.00 mg/mL. At all the tested concentrations of polyglycerol monolaurates, the growth curves of *E. coli* and *P. aeruginosa* were similar to those of the control sample. These results indicated that the MICs of the four polyglycerol monolaurates against *E. coli* and *P. aeruginosa* were higher than 10.00 mg/mL.

All the MICs of polyglycerol monolaurates against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* obtained from the above growth curves are summarized in Table 1. Among the four tested polyglycerol monolaurates, PG2ML displayed the best antibacterial effect against *S. aureus* and *B. subtilis*, with MICs of 0.16 mg/mL and 0.32 mg/mL, respectively. Similar results were obtained with the other three polyglycerol monolaurates, with MICs increasing with the degree of glycerol polymerization. For PG10ML, MICs against *S. aureus* and *B. subtilis* were 1.25 mg/mL and 3.75 mg/mL, respectively. Reports showed that the MIC of triglycerol monolaurate (namely PG3ML) against *S. aureus* was 0.08 mg/mL, while in the present study, it was 0.32 mg/mL. Besides, the MICs of diglycerol monolaurate (namely PG2ML) on *S. cerevisiae* and *C. albicans* at pH 7.0 were 0.02% and >4.00% (mass fraction), respectively, after incubation for 2 d, and the inhibitory effect varied with different tested strains, carbon-chain lengths of diglycerol fatty acid esters, and culture conditions. Similarly, among the diglycerol esters of fatty acids with different carbon chain lengths, diglycerol monolaurate was the most active against *L. monocytogenes*. Based on the results of the present study, the inhibitory effect of polyglycerol monolaurates also varied with different extents of glycerol polymerization.

We concluded that polyglycerol monolaurates inhibited the growth of Gram-positive bacteria, such as *S. aureus* and *B. subtilis*, but displayed low inhibitory effects on Gram-negative bacteria, such as *E. coli* and *P. aeruginosa*.
Similar results have been reported in literature, in which a series of polyglycerol esters inhibited Gram-positive organisms, although Gram-negative organisms were not affected\(^1\). This phenomenon was similar to the antibacterial effect of monoglycerides observed in our previous work\(^2\), which was probably due to the differences in cell wall structure between Gram-positive and Gram-negative bacteria.

### 3.2 Permeability of cell membrane

FDA is a cell-permeant esterase substrate, yielding fluorescein upon hydrolysis by intracellular esterases. It can act as a viability probe for measuring cell membrane permeability, which may affect the intracellular retention of fluorescent molecules. As shown in Fig. 5, compared to the negative control, the fluorescence intensity of *S. aureus* suspension decreased significantly \((p < 0.05, p\) value was calculated with the maximum fluorescence intensity) after treatment with MIC of polyglycerol monolaurates for 12 h. These results indicated a notable increase in membrane permeability in the presence of polyglycerol monolaurates at MIC. The fluorescence intensity was considerably lower when treated with 2\(\times\)MIC of polyglycerol monolaurates, indicating increase in membrane permeability. When treated with MIC of PG3ML, the maximum fluorescence intensity of *S. aureus* and *B. subtilis* decreased by 23% and 53%, respectively. When treated with 2\(\times\)MIC of PG3ML, the maximum fluorescence intensity of *S. aureus* and *B. subtilis* decreased by 68% and 71%, respectively. However, no noticeable tendency was observed in the different types of polyglycerol monolaurates. Similar results were observed for the other Gram-positive bacterium *B. subtilis* (Fig. 6).

The fluorescence intensity of the two Gram-negative bacteria, *E. coli* and *P. aeruginosa*, decreased by less than 10% compared to that of the control group (Fig. 7). This finding indicated that change in cell membrane permeability was negligible after treatment with 10.00 mg/mL PG3ML, which is consistent with the antibacterial effect of PG3ML against Gram-negative bacteria.

### 3.3 The integrity of the cell membrane

The integrity of the cell membrane was investigated by determining the release of cellular contents, including proteins and nucleic acids. In the present study, the proteins retained inside the cells and the nucleic acids that leaked out from the cells were quantified. As shown in Table 2, for the Gram-positive bacteria, *S. aureus* and *B. subtilis*, the absorbance of proteins decreased significantly \((p < 0.05)\) compared to that of negative control after treatment with MIC of polyglycerol monolaurates for 12 h. Simultaneously, the absorbance of nucleic acids increased significantly \((p < 0.05)\) compared to that of the negative control. In addition, this phenomenon became more evident when the concen-

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**Fig. 5** Effect of polyglycerol monolaurates on cell membrane permeability of *S. aureus*: (a) PG2ML; (b) PG3ML; (c) PG6ML; (d) PG10ML. The test strain suspensions containing FDA were treated with polyglycerol monolaurates at MIC (short dash) and 2\(\times\)MIC (dash-dot) or sterilized physiological saline (solid).

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*J. Oleo Sci.* **70**, (4) 571-580 (2021)
The concentration of polyglycerol monolaurates was doubled ($2 \times \text{MIC}$). These results indicated that macromolecular cellular contents leaked out from the cells, implying that the integrity of the cell membrane was disrupted by the polyglycerol monolaurates, leading to inhibition of cell growth. However, when *E. coli* and *P. aeruginosa* were treated with 10.00 mg/mL PG3ML, the absorbance of proteins and nucleic acids changed slightly (Table 3, $p > 0.5$), indicating negligible effect on the integrity of the cell membrane of Gram-negative bacteria. The tendency observed was in accordance with the results of antibacterial activity.

### 3.4 Morphological changes of bacteria

In addition to the determination of cell membrane damage in terms of permeability and integrity, the changes in cell surface morphology after treatment with polyglycerol monolaurates were observed using SEM. As shown in Fig. 8, the cell surfaces of *S. aureus* were smooth and complete in the negative control group. However, after treatment with the MIC of four polyglycerol monolaurates, most cells remained intact, although the cell surfaces appeared apparently sunken and slightly rough. Further increasing the concentration of polyglycerol monolaurates to $2 \times \text{MIC}$ led to noticeable rupture of *S. aureus* cells, and most of the cells were severely damaged and disintegrated.
completely. We speculated that the polyglycerol monolaurates disrupted the cell membrane of *S. aureus*, thereby resulting in leakage of bacterial cell content and eventual bacteriolysis.  

Figure 9 shows that the cells of *B. subtilis* not treated with the polyglycerol monolaurates were rod-shaped, and that the surfaces were smooth. However, after adding MIC of polyglycerol monolaurates, most cells became shrunken, and the cell surfaces appeared visibly rough. After treatment with 2×MIC of polyglycerol monolaurates, the *B. subtilis* cells wizened and almost dried up, indicating that the integrity of the cell membrane was disrupted, releasing the cellular contents.

The rod-shaped cells of Gram-negative bacteria *E. coli* and *P. aeruginosa* were regular and intact. No apparent change in cell morphology was observed when treated with 10.00 mg/mL PG3ML for 12 h (Fig. 10), indicating that PG3ML exerted negligible antibacterial effect on *E. coli* and *P. aeruginosa* when the concentration was not higher than 10.00 mg/mL.

**Table 2**  
Release of proteins and nucleic acids of Gram-positive bacteria, *S. aureus* and *B. subtilis*, treated with polyglycerol monolaurates at MIC and 2×MIC. The absorbance of proteins retained in the cells and nucleic acids that leaked out from cells were measured.

| Polyglycerol monolaurates | Concentration | Proteins (595 nm) | Nucleic acids (290 nm) |
|---------------------------|---------------|-------------------|------------------------|
|                           |               | *S. aureus*       | *B. subtilis*          | *S. aureus* | *B. subtilis* |
| Control                   | -             | 0.193 ± 0.002     | 0.338 ± 0.006          | 0.618 ± 0.007 | 0.615 ± 0.001 |
| PG2ML                     | MIC           | 0.143 ± 0.003     | 0.102 ± 0.002          | 0.685 ± 0.006 | 0.743 ± 0.028 |
|                           | 2×MIC         | 0.061 ± 0.002     | 0.080 ± 0.001          | 0.708 ± 0.005 | 0.804 ± 0.017 |
| PG3ML                     | MIC           | 0.131 ± 0.007     | 0.156 ± 0.003          | 0.693 ± 0.014 | 0.674 ± 0.011 |
|                           | 2×MIC         | 0.079 ± 0.006     | 0.022 ± 0.001          | 0.870 ± 0.017 | 0.715 ± 0.010 |
| PG6ML                     | MIC           | 0.095 ± 0.004     | 0.034 ± 0.001          | 0.781 ± 0.015 | 0.735 ± 0.010 |
|                           | 2×MIC         | 0.029 ± 0.001     | 0.026 ± 0.002          | 0.977 ± 0.035 | 0.772 ± 0.008 |
| PG10ML                    | MIC           | 0.152 ± 0.003     | 0.080 ± 0.001          | 0.897 ± 0.048 | 0.758 ± 0.010 |
|                           | 2×MIC         | 0.084 ± 0.007     | 0.011 ± 0.001          | 0.979 ± 0.034 | 0.789 ± 0.020 |

**Table 3**  
Release of proteins and nucleic acids from Gram-negative bacteria, *E. coli* and *P. aeruginosa*, treated with 10.00 mg/mL PG3ML.

| Polyglycerol monolaurates | Concentration | Proteins (595 nm) | Nucleic acids (290 nm) |
|---------------------------|---------------|-------------------|------------------------|
|                           |               | *E. coli*         | *P. aeruginosa*        | *E. coli* | *P. aeruginosa* |
| Control                   | -             | 0.707 ± 0.001     | 0.703 ± 0.001          | 0.679 ± 0.007 | 0.743 ± 0.003 |
| PG3ML                     | 10.00 mg/mL   | 0.700 ± 0.001     | 0.698 ± 0.002          | 0.686 ± 0.008 | 0.749 ± 0.005 |
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4 Conclusions

The antibacterial effect of four polyglycerol monolaurates with different extents of glycerol polymerization on four food-related bacteria was evaluated. Polyglycerol monolaurates efficiently inhibited the growth of two Gram-positive bacteria, *S. aureus* and *B. subtilis*, and the antibacterial effect decreased with increase in the extent of glycerol polymerization. However, no inhibitory effect was observed on two Gram-negative bacteria, *E. coli* and *P. aeruginosa*, at all the tested concentrations of the polyglycerol monolaurates. Polyglycerol monolaurates disrupted *S. aureus* and *B. subtilis*, increasing cell membrane permeability, releasing cellular contents, and changing cell morphology. However, the effect of polyglycerol monolaurates on *E. coli* and *P. aeruginosa* was not noticeable. In conclusion, polyglycerol monolaurates can effectively act against Gram-positive bacteria, *S. aureus* and *B. subtilis*, and may be utilized as a safe preservative in food systems or other suitable systems.

Acknowledgment

This work was financially supported by the National Natural Science Foundation of China (21676003, 21902004), the National Key R&D Program of China (No. 2017YFB0308701), the Beijing Natural Science Foundation of China (2204076), and the Science and Technology Program Key Project of the Beijing Municipal Commission of Education (KZ201510011010).

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