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ARTICLE

Antimicrobial potential of myristic acid against *Listeria monocytogenes* in milk

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Abstract

Listeria monocytogenes (*L. monocytogenes*), an important food-borne pathogenic microorganism, has resistance immune function to many commonly used drugs. Myristic acid is a traditional Chinese herbal medicine, but it has been rarely used as a food additive, limiting the development of natural food preservatives. In this study, the antibacterial activity and mechanism of myristic acid against *L. monocytogenes* were studied. The minimum inhibitory concentration (MIC) of myristic acid against 13 *L. monocytogenes* strains ranged from 64 to 256 μg ml^{-1}. The time-kill assay demonstrated that when myristic acid was added to dairy products, flow cytometry confirmed that myristic acid influenced cell death and inhibited the growth of *L. monocytogenes*. Transmission electron microscopy (TEM), scanning electron microscopy (SEM), and NPN uptake studies illustrated that myristic acid changed the bacterial morphology and membrane structure of *L. monocytogenes*, which led to rapid cell death. Myristic acid could bind to DNA and lead to changes in DNA conformation and structure, as identified by fluorescence spectroscopy. Our studies provide additional evidence to support myristic acid being used as a natural antibacterial agent and also further fundamental understanding of the modes of antibacterial action.

Introduction

Listeria monocytogenes (*L. monocytogenes*) is a food-borne pathogenic microorganism [1]. According to previous studies, ~2500 cases of human illness and more than 500 deaths result from this pathogen every year in the United States [2]. In addition, food poisoning caused by *L. monocytogenes* has a 30% rate of mortality among patients, which can be higher for people with weak immune systems or during pregnancy [3]. Therefore, the development of food preservatives has garnered attention by us, and we should pay more attention to the biological food preservatives.

Myristic acid is obtained from *Myristica fragrans*, which is a tropical herbal plant. *M. fragrans* has good antibacterial activity, and it is used to treat deficiency enterorrhea, cold dysentery, abdominal distention and pain, dyspepsia, and other symptoms.

Myristic acid usually accounts for small amounts of total fatty acids in animal tissues, but it is more abundant in milk fat or in copra and palmist oils. Myristic acid utilization has been mostly studied in vivo when added to the diets of animals [4, 5] and humans [6, 7]. In addition, we queried a large number of data and found that myristic acid has been widely confirmed to have strong antitumour effects, which induce apoptosis of many kinds of tumor cell, such as breast cancer cells, prostate cancer cells, stomach cancer cells, liver cells, and others. Therefore, myristic acid has broad prospects as a new type of efficient and safe antitumour drug, and it is also a safe food additive.

However, as far as we know, little is known about the antibacterial activity and possible antibacterial mechanism of myristic acid against *L. monocytogenes* strains. The present study is the first study of the antibacterial effects of myristic acid, and we explored its antibacterial activities and

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mode of action against *L. monocytogenes* strains in a food system to provide data establishing myristic acid as an alternative natural food preservative and additive.

Materials and methods

Chemicals

Myristic acid and other reagents were purchased from Sigma-Aldrich (St. Louis, USA). Coomassie Brilliant Blue R-250 was purchased from Beyotime Biotechnology (Shanghai, China), and agarose was obtained from Biowest (France). All reagents were the highest grade commercially available.

Bacterial strains and growth conditions

L. monocytogenes strains were obtained from the Jilin Entry-Exit Inspection and Quarantine Bureau. Bacterial cells were cultured at 37 °C in TSB broth or agar (Oxoid, Basingstoke, UK), with aeration to test the antibacterial activity of the antibacterial agents.

Determination of minimum inhibitory concentrations

MICs of myristic acid against the *L. monocytogenes* strains were determined using a broth microdilution assay (CLSI, 2010). The specific step of this test can be referred to one that nisin and p-Anisaldehyde are against *L. monocytogenes* [8]. All tests were performed in triplicate.

Time-kill curves assay

The bactericidal activity of myristic acid against *L. monocytogenes* ATCC 19115 was evaluated by measuring the reduction in the numbers of Colony Forming Unit (CFU) according to previous studies [9].

Measurement of cell injury

The cell damage was detected by annexin V-FITC (luoresceinisothiocyanate)/PI (propidium iodide) double-staining. Logarithmic-phase bacteria were exposed to myristic acid with 1/4 × MIC (16 μg ml^{-1}), 1/2 × MIC (32 μg ml^{-1}), and 1 × MIC (64 μg ml^{-1}) for 3 h. And then 100 μl of untreated and three treated groups were added into the mixture of 5 μl Annexin V labeled-FITC and 5 μl PI with a concentration of 20 μg ml^{-1}. The obtained mixture was kept still at 37 °C for 30 min. The fluorescence intensity was detected within an hour in a flow cytometer Flow cytometer (BD FACSAria, Biosciences, USA).

Scanning electron microscopy

Logarithmic-phase bacteria were allowed to adhere to polylysine-coated coverslips for 12 h and were exposed to myristic acid with 1/4 × MIC (16 μg ml^{-1}), 1/2 × MIC (32 μg ml^{-1}), and 1 × MIC (64 μg ml^{-1}) for 3 h. Before the bacteria cells were fixed for 30 min at 4 °C with 500 μl 2.5% glutaraldehyde, they needed to be washed three times with PBS. At last, cells were observed using scanning electron microscopy (SEM; Hitachi S-3400N). The bacterial cells not exposed to antibacterials were similarly processed and used as controls. All tests were performed in triplicate.

Transmission electron microscopy

The preparation and treatment of target indicators in transmission electron microscopy (TEM) were the same as those in SEM analysis, and TEM analysis was performed following the guidelines in the literature with slight modifications [10]. The resulting pellets were subjected to a series of treatments according to the guidelines in the literature to perform TEM analysis.

NPN uptake

The *N*-phenyl-1-naphthylamine (NPN) uptake assay was conducted following the method reported by [11] with some modifications. The fluorescence value was measured immediately by a fluorescence spectrophotometer at the excitation wavelengths of 360 nm and emission wavelengths 460 nm.

The effect of myristic acid on bacterial genomic DNA

Bacteria genomic DNA was extracted using TIAN amp Bacteria DNA Kit (Tiangen Biotech, co., LTD) according to the operation instruction. The purity of the extracted genomic DNA was evaluated by the optical density ratio of 260 and 280 nm, and then the effect of myristic acid with DNA was carried out by competitive binding assays using RF-5301 fluorescence spectrophotometer (Hitachi High-Technologies, Tokyo, Japan) according to the method described by Magdalena T., Agnieszka P., Jan M. & Zygmunt W [12].

Statistical analyses

All data were presented as mean ± standard deviation of at least three independent experiments. Statistical analyses were conducted using SPSS 11.5 statistical Software. $^{*}P < 0.05$ was considered statistically significant.

Table 1 In vitro myristic acid against food-borne isolates of *L. monocytogenes*

Strains	Median MIC of myristic acid (μg ml^{-1})
L. monocytogenes. JL-10002	256
L. monocytogenes. JL-10003	128
L. monocytogenes. JL-10004	128
L. monocytogenes. JL-10005	256
L. monocytogenes. JL-10006	128
L. monocytogenes. JL-10007	128
L. monocytogenes. JL-10008	128
L. monocytogenes. JL-10009	256
L. monocytogenes. JL-100010	256
L. monocytogenes. JL-100011	128
L. monocytogenes. JL-100012	128
L. monocytogenes. JL-100013	128
L. monocytogenes ATCC 19115	64

Results

MIC determination

The MIC values of myristic acid against 13 *L. monocytogenes* strains were investigated, and the results are shown in Table 1. In the present study, myristic acid demonstrated different antibacterial activities against the tested strains based on the calculated MICs. The MIC values for myristic acid against 13 strains ranged from 64 to 256 μg ml^{-1}. The MIC values of myristic acid against the ATCC 19115 strain were 64 μg ml^{-1}.

Time-kill assay

The bactericidal kinetics of myristic acid were studied in pasteurized milk containing 1/4 × MIC (16 μg ml^{-1}), 1/2 × MIC (32 μg ml^{-1}), and 1 × MIC (64 μg ml^{-1}) of myristic acid, with an initial bacteria inoculum of 1×10^5 CFU ml^{-1}. The results of the time-kill curves are demonstrated in Fig. 1, which indicated that myristic acid significantly inhibited the growth of *L. monocytogenes* in pasteurized milk. The bacteria grew quickly from 3.5-log$_{10}$ to 5.6-log$_{10}$ in milk during storage under refrigeration without myristic acid, and when the bacteria were exposed to myristic acid with 1/4 × MIC (16 μg ml^{-1}), the amplitude of the growth of *L. monocytogenes* decreased. In addition, when the bacteria were treated with myristic acid with 32 μg ml^{-1}, the number of bacterial species remained at ~3.4-log$_{10}$ for 21 days, which demonstrated that myristic acid effectively inhibited the growth of *L. monocytogenes*. Finally, when the concentration of myristic acid was increased to 64 μg ml^{-1}, the number of bacteria dropped to 1.4-log$_{10}$ after 15 days,

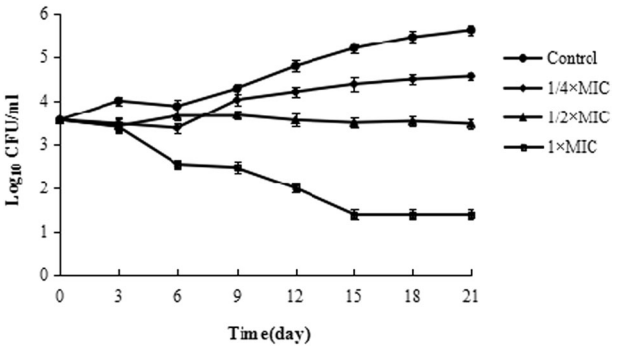

Fig. 1 Time-kill curves for different concentrations of myristic acid against *L. monocytogenes* ATCC 19115. Values are expressed as the mean ± S.D. of three different experiments performed in triplicate. *Error bars* indicate S.D

which indicated that myristic acid effectively killed *L. monocytogenes*. These results demonstrated that myristic acid inhibited bacterial growth in a dose-dependent manner.

Effect of myristic acid on cell injury of L. monocytogenes

Cell injuries were analysed by flow cytometry (FCM) by dual staining of *L. monocytogenes* with FITC and propidium iodide (PI). The scatter plot demonstrated four different types of cells (Fig. 2) and was divided into four regions, including the Q1 area, which contained necrotic cells and debris; the Q2 area, which had dead cells; the Q3 area, which contained living cells; and the Q4 area, which had injured and apoptotic cells. The proportion of different cell areas is shown in Table 2. As shown in Fig. 3 and Table 2, live cells in the blank control group (Q3) accounted for 80.24% of all stained cells, but after treatment with 1/4 × MIC myristic acid for 3 h, the percentage of live cells of *L. monocytogenes* decreased to 26.42%, while the percentage of dead cells (Q2) increased to 29.64%. Moreover, damage became more important as the myristic acid concentration increased, and treatment of *L. monocytogenes* with 1/2 × MIC and 1 × MIC resulted in a significant decrease in live cells to 21.88% and 12.28%, with the proportion of dead cells reaching 32.11% and 61.48%, respectively. These results indicated that treatment with a higher concentration of myristic acid (1 × MIC) clearly increased cell death and decreased the growth of *L. monocytogenes*.

Effect of myristic acid on bacterial morphology

SEM

To understand the mode of action of myristic acid, morphological changes of *L. monocytogenes* cells were

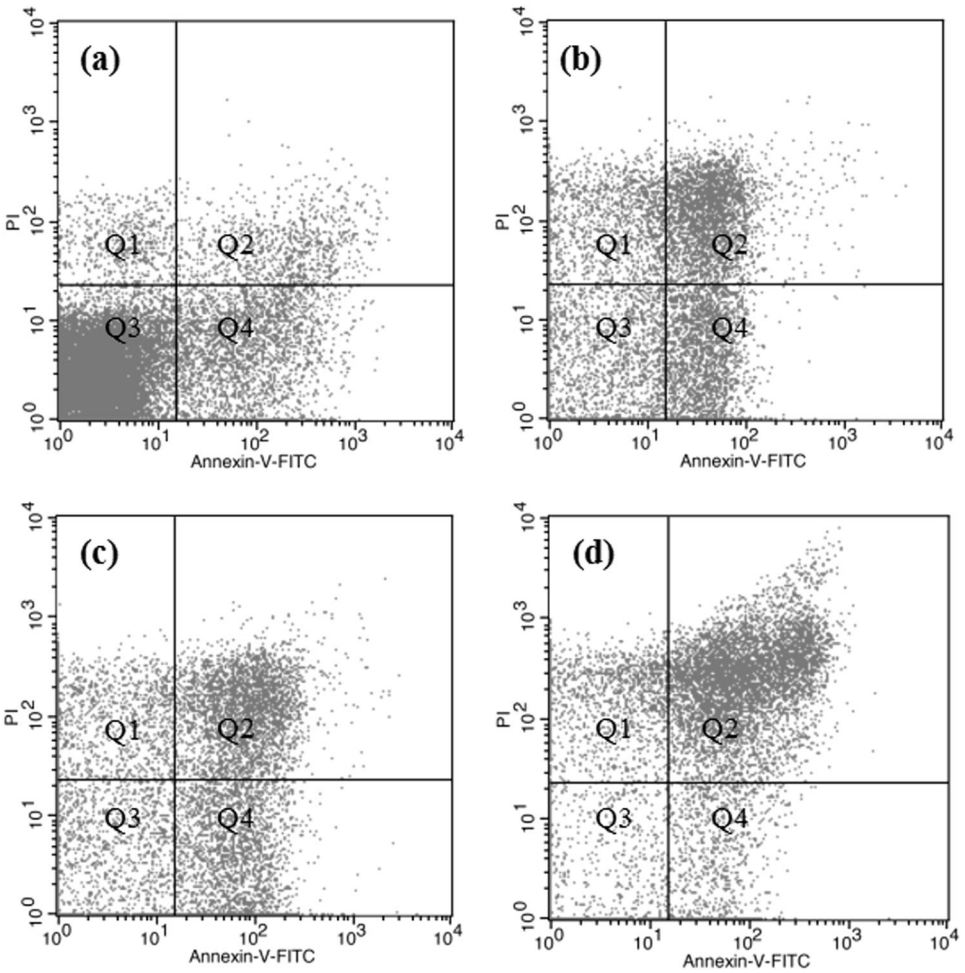

Fig. 2 FCM of *L. monocytogenes* ATCC 19115: **a** blank group and groups treated with **b** 16 μg ml^{-1} myristic acid, **c** 32 μg ml^{-1} myristic acid, and **d** 64 μg ml^{-1} myristic acid

Table 2 Proportion of various cell areas on Annexin V-FITC/PI double labeling of *L. monocytogenes*

The proportion of each area %	Q1	Q2	Q3	Q4
0 × MIC (a)	2.70	4.40	80.24	12.65
1/4 × MIC (b)	17.26	29.64	26.42	26.68
1/2 × MIC (c)	18.10	32.11	21.88	27.91
1 × MIC (d)	15.61	61.48	12.28	10.74

observed using SEM. The bacterial cells treated with different concentrations of myristic acid which are 1/4 × MIC (Fig. 3b), 1 × MIC 1/2 × MIC (Fig. 3c), 1 × MIC (Fig. 3d). According to four pictures, the higher the concentration of myristic acid, the more damaging the *L. monocytogenes* cells. When the concentration of myristic acid is 64 μg ml^{-1}, most of the outermost layer of the bacterial cells had disappeared and *L. monocytogenes* cells have lost a lot of protect which can cause cell death. The results demonstrated that myristic acid may have severe effects on the cell wall and cytoplasmic membrane. However, more detailed observations were still needed.

TEM

A previous study reported that antibacterial agents can directly interact with bacterial cell membranes and then increase the membrane permeability, causing rapid cell death [13]. Therefore, to further characterize the bactericidal effects of myristic acid, TEM analysis was conducted to visualize the morphological changes of *L. monocytogenes* cells exposed to 1/4 × MIC, 1/2 × MIC, or 1 × MIC myristic acid. As shown in Fig. 4a, which is without myristic acid treated, *L. monocytogenes* cells were normal and surrounded by cell membranes with a compact surface, which is showing a well-defined cell membrane and a uniform cytoplasm region, without the release of intracellular components. However, the integrity and permeability of the cell membrane changed with increasing myristic acid concentrations, until the bacterial cells were empty (Fig. 4d).

NPN uptake by cell membranes

The effect of myristic acid on the uptake of NPN by bacteria is shown in Fig. 5. Compared with the control, the addition

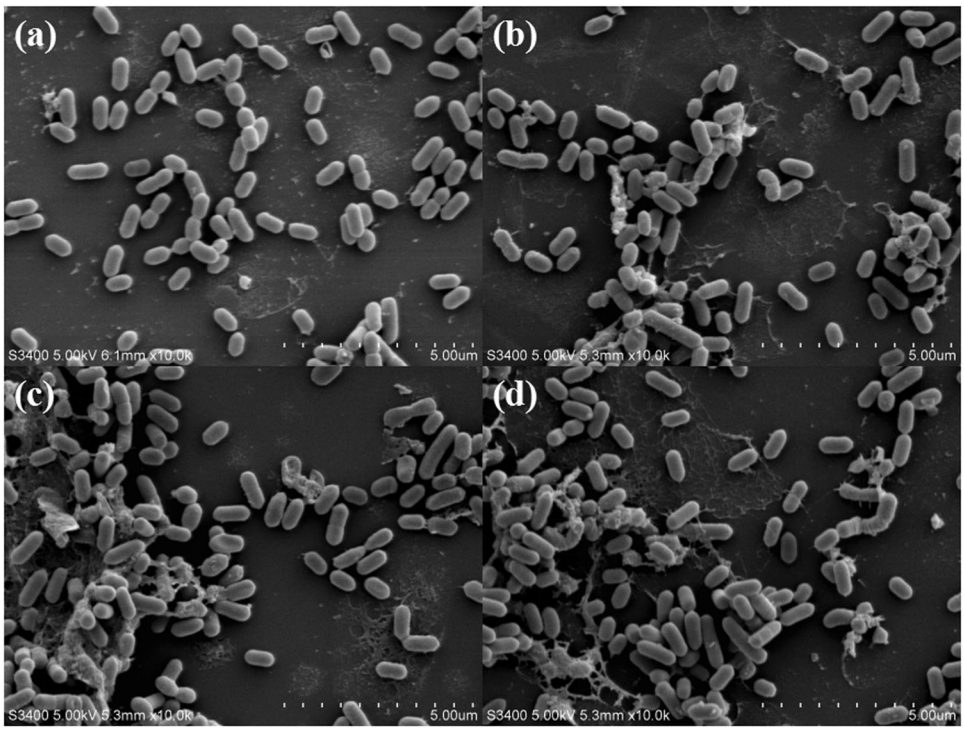

Fig. 3 SEM of *L. monocytogenes* ATCC 19115: **a** blank group and groups treated with **a** 16 μg ml^{-1} myristic acid, **b** The bacterial cells treated the concentration of myristic acid which are 1/4 × MIC, **c** 32 μg ml^{-1} myristic acid, and **d** 64 μg ml^{-1} myristic acid

of myristic acid to *L. monocytogenes* suspensions caused a sharp increase in fluorescence intensities (Fig. 5). The fluorescence intensity of *L. monocytogenes* treated with myristic acid significantly increased by 11.2%, 20.7%, and 50.9%, respectively, at 1/4 × MIC, 1/2 × MIC, and 1 × MIC treatment levels, compared with the control. This indicated that different concentrations of myristic acid damaged the bacteria cell membrane by different degrees, which agreed with the results of cell membrane integrity assay. When *L. monocytogenes* treated with myristic acid, bacterial cells were changed. Therefore, NPN was easily collected from the supernatant into hydrophobic structures, such as the phospholipid bilayer, resulting in clear fluorescence enhancement.

Effect of myristic acid on DNA of *L. monocytogenes*

DNA is most important to the cell, and if the structure of the body's DNA is altered, it will be leading to the blocking of normal enzyme and receptor synthesis, and causing the death of bacteria at last. Thus, the purity of extracted genomic DNA of *L. monocytogenes* was 1.83 (OD_{260nm}/OD_{280nm} = 1.83), and in order to obverse the the interaction of myristic acid with DNA, we used the most sensitive techniques for DNA-fluorescence spectroscopy. As shown in Fig. 6, the addition of myristic acid exhibited significant fluorescence quenching in *L. monocytogenes*, indicating that myristic acid can probably bind to DNA and lead to changes in the DNA conformation and structure. The present study observed that, in addition to the cell wall and membrane, the bacterial genome might be another antibacterial target of myristic acid, elucidating a possible antibacterial mechanism of myristic acid.

Discussion

The food-borne pathogenic microorganism *L. monocytogenes* can cause serious food poisoning and stillbirth; therefore, drugs to kill *L. monocytogenes* are in high demand. However, repeated use of drugs to treat *L. monocytogenes* can cause immune function, so new drugs to suppress it are required. Consequently, we studied the antibacterial activity of myristic acid against *L. monocytogenes*. Myristic acid is very rarely used as a natural preservative in foods, including dairy products. George A. Burdock and Ioana G. Carabin [14] assessed the safety of myristic acid as a food ingredient and reported that a safe daily dose of myristic acid is up to 35.07 mg day^{-1}. Therefore, the current use of myristic acid to flavor food does not pose a health risk to humans. Our research demonstrated that the MIC value of myristic acid against *L. monocytogenes* ATCC 19115 was 64 μg ml^{-1}, and this dose is safe for patients. These data indicated that myristic acid is a potentially effective antibacterial agent against *L. monocytogenes* strains. For comparison, the MIC_{50} values for myristic acid against *Staphylococcus epidermidis* is 0.86 μg ml^{-1} [15], and no research on the MIC values of

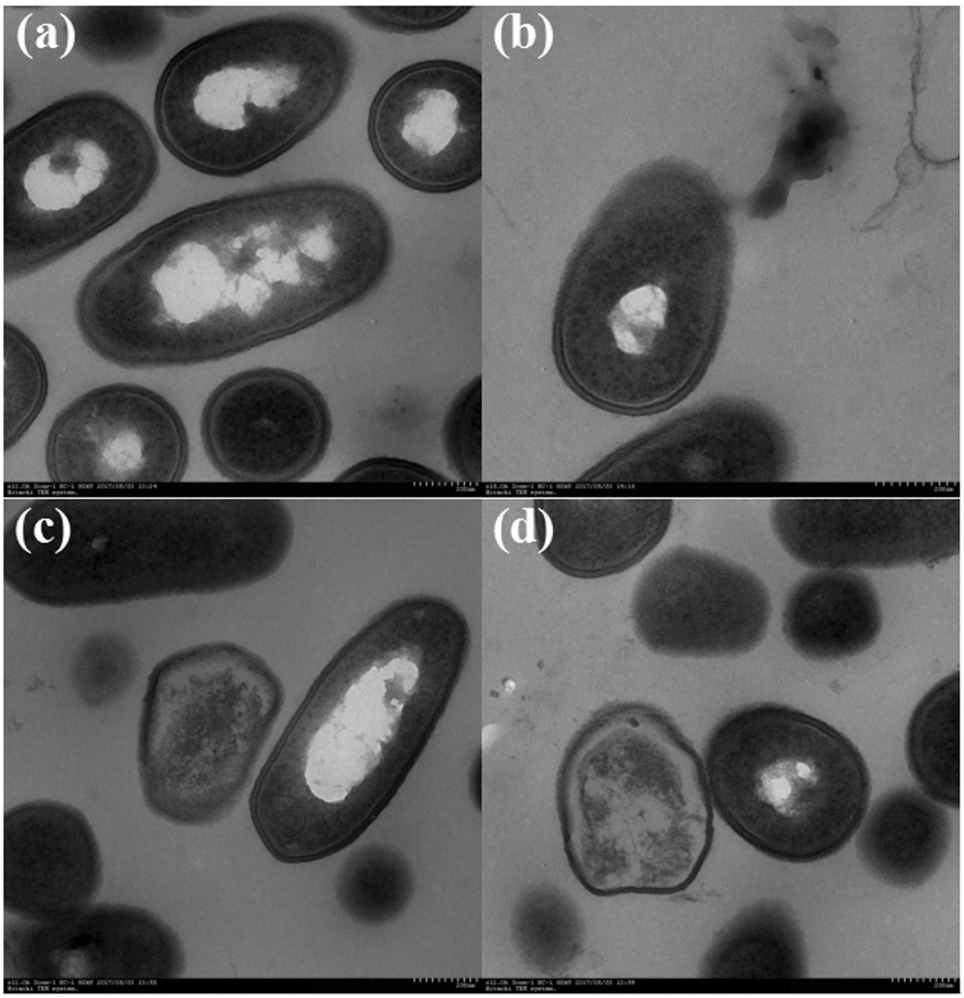

Fig. 4 TEM of *L. monocytogenes* ATCC 19115: **a** blank group and groups treated with **b** 16 μg ml^{-1} myristic acid, **c** 32 μg ml^{-1} myristic acid, and **d** 64 μg ml^{-1} myristic acid

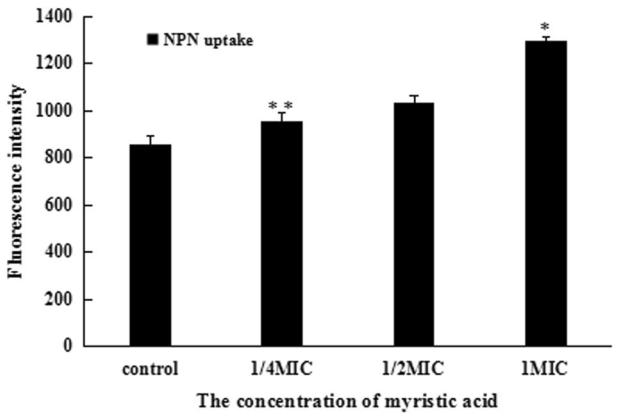

Fig. 5 NPN uptake of *L. monocytogenes* treated with myristic acid

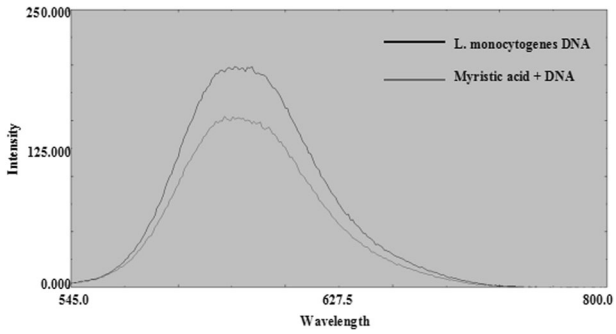

Fig. 6 Fluorescence spectra of 64 μg ml^{-1} myristic acid with DNA from *L. monocytogenes*

myristic acid against some food-borne pathogenic bacteria and clinical isolates of bacteria has been reported. Therefore, myristic acid has great potential as a new food preservative.

We studied the bactericidal kinetics of myristic acid against *L. monocytogenes* in pasteurized milk. Our study indicated that adding myristic acid can effectively control *L. monocytogenes* growth, as supported by trends in the modern food industry. Food preservatives have been considered useful alternatives to control foodborne pathogens, including in dairy products. No research involving myristic acid to inhibit the growth of *L. monocytogenes* in pasteurized milk at 4 °C for 21 days had been performed, despite the practical application value. In addition, we studied the

antibacterial mechanism of myristic acid against *L. monocytogenes*. Through the result of the experiment, when myristic acid acts on *L. monocytogenes*, the bacteria cell wall, membrane permeability, and genomic DNA have been changed, which might have resulted in the deaths of *L. monocytogenes*.

The literature [16] suggested that the active components of some food preservatives might bind to the cell surface and then penetrate to the target sites possibly the plasma membrane and membrane-bound enzymes, leading to the disruption of cell wall structures. In addition, other reports have revealed that the changes or disruption in the membrane usually occur due to membrane lipid composition alterations and are thought to be a compensatory mechanism to counter the lipid disordering effects of the treatment agent [17]. The study [18] confirms that the antibacterial agent of *L. monocytogenes* has two mechanisms: one is to act on the cell membrane system so that the pores or membrane are cleaved in the cell membrane, thereby causing the cell contents to leak and causing cell death; the other way is by acting on the genetic material, which inhibits the synthesis of DNA, so that cells are in the R phase, which inhibits cell division and inhibits bacterial activity.

However, no previous research has involved testing myristic acid for the influence on the structure of *L. monocytogenes* membranes. In our study, SEM observation demonstrated that myristic acid induced an obvious change in the shape of *L. monocytogenes*, and TEM observation and cell membrane NPN uptake demonstrated that the bacteria had become an empty shell and that all bacterial content had been lost, which illustrated that the membrane structure of *L. monocytogenes* had changed. Therefore, we need to continue to explore how the chemical reactions between the polyacids and membrane proteins in the cell change the membrane permeability. In addition, myristic acid also affected the structure of the bacteria genome DNA, but we failed to demonstrate how myristic acid changed the genomic DNA of *L. monocytogenes*. Regarding the change in bacterial DNA, we explored whether myristic acid could replace a component of DNA. However, there is no theory to support this speculation. Therefore, the antibacterial mechanism of myristic acid to *L. monocytogenes* also needs to be further studied.

Conclusions

In summary, myristic acid demonstrated an effective bactericidal effect against *L. monocytogenes* strains in vitro, and the MIC value of myristic acid against *L. monocytogenes* ATCC 19115 was 64 μg ml^{-1}. In addition, flow cytometry indicated that myristic acid clearly killed and inhibited the growth of *L. monocytogenes*. Based on the results of this study, myristic acid likely changed the permeability and integrity of bacterial cell membranes, leading to the leakage of intracellular materials, as identified in the SEM, TEM, and NPN uptake assays. Furthermore, myristic acid changed the structure of genomic DNA, causing the death of *L. monocytogenes*, which provides a new mechanism for antibacterial effects. In addition, myristic acid inhibited the growth of bacteria in pasteurized milk under 4° of preservation; therefore, myristic acid can be used as a natural antimicrobial food preservative to control foodborne pathogens in food industries.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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