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Antibacterial activity and mechanism of phillyrin against selected four foodborne pathogens

Junshun ZHANG[1], Mingkun GAO[2], Jiayuan LUO[1], Yang GUO[1], Yihong BAO[1,3]*, Tianzhi YANG[4]

Abstract

The antibacterial activity of phillyrin on *Escherichia coli* ATCC8739 (*E. coli* ATCC8739), *Bacillus subtilis* BS08, *Staphylococcus aureus* ATCC6538 (*S. aureus* ATCC6538) and *Salmonella* ATCC14028 was determined by filter paper method. The minimum inhibitory concentration (MIC) of the four tested bacteria was investigated by plate coating method to evaluate antibacterial ability. The antibacterial mechanism was further investigated by measuring growth curve, electric conductivity, nucleic acid content, Na^+/K^+-ATPase activity and polyacrylamide gel electrophoresis (SDS-PAGE). The results indicated that phillyrin had antibacterial effects and the MICs against *Escherichia coli* ATCC8739, *Bacillus subtilis* BS08, *Staphylococcus aureus* ATCC6538 and *Salmonella* ATCC14028 were 2.15, 3.16, 3.02 and 3.40 μg/mL respectively, and the antibacterial effect on *Escherichia coli* ATCC 8739 was more significant ($p < 0.05$) than that on other bacteria. Scanning electron microscopy (SEM) indicated that phillyrin destroyed the morphology of the cells and the cells ruptured. The leakage of intracellular substances led to an increase in nucleic acid content and an increase in electric conductivity in the bacterial suspension; SDS-PAGE analysis indicated that phillyrin could inhibit protein synthesis; in addition, phillyrin could reduce Na^+/K^+-ATPase activity. Therefore, phillyrin had obvious antibacterial ability and inhibited the expression of bacterial proteins by destroying the cell membrane structure, resulting in the death of the cells.

Keywords: phillyrin; antibacterial mechanism; antibacterial activity.

Practical Application: Determination of antibacterial activity and mechanism of phillyrin against selected four foodborne pathogens and phillyrin has the potential to be the natural bacteriostatic agent.

1 Introduction

Nowadays, food contamination caused by foodborne pathogens is a serious threat to people's health (Rembischevski & Caldas, 2020), and food spoilage is widespread around the world (Jing et al., 2022). Increase in food-borne outbreaks has become public health concern worldwide. Approximately, 48 million people after consuming contaminated food develop various symptoms of illness, with 128,000 hospitalization and 3,000 deaths annually (Xedzro et al., 2022). In China, food safety incidents caused by contamination of food-borne bacteria account for more than half of all food safety incidents. Chemical synthesized preservatives have been widely used in food in order to prevent the growth of foodborne pathogens (Zhao & Talha, 2022). However, the use of chemical preservatives has been a controversial topic because they are deemed to have carcinogenic and teratogenic attributes as well as residual toxicity (Hugo & Hugo, 2015). Therefore, it is necessary to develop a kind of safe and natural food preservatives (Vinci et al., 2015), which can effectively inhibit food-borne pathogens and maintain the shelf quality of food products.

The use of natural antibacterial compounds in food has received extensive attention from consumers and the food industry (Gyawali & Ibrahim, 2014). As a result, the demands for natural products that can be used as alternatives to chemically synthesized food preservatives is increasing (Tajkarimi et al., 2010). Thus, it is particularly important to inhibit the growth of food-borne spoilage bacteria to prolong the shelf life of food. *Forsythia suspense* [(Thunb.) Vahl (Oleaceae)] is an ascending plant that is widely dispersed throughout China, Korea, Japan, and many European nations, and is a well-known ingredient of traditional Chinese medicines. Phillyrin is an active natural lignan compound and the main pharmacological component of *Forsythia suspensa*, mainly extracted from the fruit of *Forsythia suspensa*. Phillyrin is an active constituent found in many medicinal plants and certain functional foods. As a matter of fact, phillyrin is commonly used as an important ingredient in the food, beverage, and cosmetic industries. Modern pharmacological studies have shown that phillyrin can perform a number of biological functions such as antioxidant (Du et al., 2019), anti-inflammatory (Zhong et al., 2013), anti-hyperlipidemia (Kong et al., 2008), anti-virus (Qu et al., 2016) and antipyretic activities (Zhang et al., 2012). Furthermore, some researchers have found that phillyrin has anti-obesity

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[1] *School of Forestry, Northeast Forestry University, Harbin, PR, China*
[2] *State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing, PR, China*
[3] *Key Laboratory of Forest Food Resources Utilization of Heilongjiang Province, Harbin, PR, China*
[4] *Department of Agricultural, Food and Nutritional Science, University of Alberta, Edmonton, Alberta, Canada*
*Corresponding author: baoyihong@163.com

activity in vivo (Do et al., 2013) and an in vivo study showed that phillyrin exerted anti-obesity effects in nutritive obesity mice (Zhao et al., 2005). The latest research shows phillyrin can not only inhibit the replication of the two viruses, but also has a good anti-inflammatory effect, which is expected to become a candidate compound against COVID-19 and influenza (Lai et al., 2021). Besides, phillyrin treatment alleviates intracerebral hemorrhage injury-induced apoptosis and oxidative stress via activation of the Nrf2 signaling pathway, highlighting a potential role for phillyrin as an intracerebral hemorrhage therapeutic (Guo et al., 2021). Moreover, a study concluded that phillyrin had antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, which had potential as an antimicrobial agent for the control of infectious pathogens (Qu et al., 2008). However, the antibacterial mechanism of phillyrin is still unclear. In this study, the antibacterial properties of phillyrin extracted form the leaves of *Forsythia suspensa* and its antibacterial mechanism against four tested bacteria commonly found in foods including *Bacillus subtilis*, *Escherichia coli*, *Salmonella and Staphylococcus aureus* (Dong et al., 2015). This study is a supplement to the antibacterial mechanism of phillyrin and provides a theoretical basis for its useful application in the future.

2 Materials and methods

2.1 Materials

Phillyrin (≥ 98% HPLC purity) was purchased from Shanghai yuanye Bio-Technology Co., Ltd., (Shanghai, China). *Bacillus subtilis* BS08, *Escherichia coli* ATCC8739, *Salmonella* ATCC14028 and *Staphylococcus aureus* ATCC6538 were obtained from the Microbiology Laboratory of Northeast Forestry University (Harbin, China). All the kits were purchased from Nanjing Jiancheng Bioengineering Institute. All the reagents and chemicals were analytical grade and were purchased from Tianjin Guangfu Fine Chemical Research Institution.

2.2 Diameter of inhibition zone (DIZ)

The antimicrobial activities of phillyrin were investigated by the DIZ (Iwansyah et al., 2021), in which a filter paper of 6 mm diameter was prepared and sterilized. Under sterile conditions, the medium was poured into a sterile plate, cooled and solidified. 100 μL of the bacterial suspension was spread evenly on the medium, and 4 sterilized filter paper (6 mm in diameter) were added 10 μL of phillyrin with a concentration of 3.50 μg/mL. The bacteria were incubated in 37 °C for 12 h and then DIZ was measured.

2.3 Determination of MIC

MIC was measured according to the previous described method (Felhi et al., 2017). The tubes were sterilized and labeled, and the extract was diluted by two-fold dilution method to prepare eight concentration gradients. The concentrations of phillyrin were 0.039, 0.078, 0.156, 0.313, 0.625, 1.250, 2.500, and 5.000 μg/mL, numbered from 1 to 8, respectively. A 96-well plate was sterilized, followed by the addition of 20 μL (1×10^7 CFU/mL) of the bacterial solution, 90 μL of the liquid medium, and 90 μL of the extract with different concentration gradients into each well. Sterile water was added as control. 100 μL of solution was taken from per well and spread evenly on the medium, incubated for 12 h to observe the growth of the four kinds of bacteria. The concentration of phillyrin in the first single colony plate was the lowest inhibitory concentration for each bacterium.

2.4 Growth curve assay

The growth curve was measured according to the previous described method (Lee & Je, 2013). After diluting the concentrations to 1×10^7 CFU/mL, 90 μL of 1 MIC phillyrin was added to the 96-well plate, followed by 20 μL of selected bacterial solution. The control group did not contain phillyrin. The tested suspension was placed in a constant temperature shaking incubator at 37 °C and 200 rpm, sampled every 2 h during the culture, and the OD values were measured at a wavelength of 660 nm.

2.5 Study on the mechanism of inhibition

Determination of bacterial morphology

According to the previous described method (Cai et al., 2019). Four selected bacteria were inoculated into a liquid medium, cultured at 37 °C for 12 h to logarithmic phase, and the concentration of diluted bacterial solution was 1×10^7 CFU/mL. 3 mL of the bacterial solution was added to 1 MIC phillyrin, and the control group was performed without phillyrin. After incubating at 37 °C and shaking at 200 rpm for 4 h, the cells were collected by centrifugation at 5000 rpm for 15 min, washed three times with sterilized 0.1 M phosphate buffer saline (PBS, pH 7.0), and placed at 4 °C for 4 h. Dehydration was carried out sequentially using 30%, 50%, 70%, 90% ethanol and absolute ethanol. After vacuum freezing at -40 °C for 12 h, the cells were sprayed with gold and observed by SEM.

Permeability of the cell membrane

The permeability of the cell membrane was investigated using the previously described method of measuring electric conductivity (Zhang et al., 2017). Four selected bacteria were cultured at 37 °C for 12 h to logarithmic phase, centrifuged at 5000 rpm for 10 min, and the cells were washed with sterile distilled water until the electric conductivity was close to that of sterile distilled water. 1 MIC phillyrin was added to each of bacterial suspension as experimental group. The control group was without phillyrin treatment. The samples were incubated at 37 °C and 120 rpm for 6 h. The conductivity was measured every 1 h.

Determination of nucleotide leakage

The relative electric conductivity was used to assessed permeability of bacteria membrane according to the described method (Kong et al., 2008). The four selected bacteria were inoculated into 100 mL of liquid medium and cultured to logarithmic growth phase. 10 mL of bacterial solution was centrifuged at 5000 rpm for 15 min, and the cells were collected, washed three times with sterilized 0.1M PBS (pH 7.0), and the

volume was adjusted again. 10 mL, 1 MIC of phillyrin was added before bacteria were cultured in a 37 °C constant temperature shaker for 3 h. After 3 h, 3 mL of the sample solution and the centrifuge tube were centrifuged at 5000 rpm for 5 min, and the supernatant was taken at a wavelength of 260 nm to measure the absorbance.

Confocal laser scanning microscopy

To evaluate the damage of treatment with phillyrin to the membranes of four selected bacteria, CLSM (LEICA TCS SP5 II, Leica Microsystems, Germany) analyses with fluorescent staining propidium iodide (PI) were conducted as privious reported (Mei et al., 2013). Bacterial cells of experimental group were treated with phillyrin at 1 MIC concentrations. Bacterial were examined using the CLSM at 515/488 nm in an argon laser.

Effect of phillyrin on bacterial protein synthesis

The effect of phillyrin on bacterial protein synthesis was studied according to a previously reported method (Kang et al., 2008) with some modifications. 1 MIC of phillyrin was added to the bacterial suspension of four tested bacteria before cultured at 37 °C for 12 h to logarithmic phase and centrifuged at 5000 rpm for 15 min. The supernatant was discard, and the pellet was washed three times with sterilized 0.01 M PBS (pH 7.0) 20 μL of the loading buffer solution was added and boiled for 5 min, followed by centrifuged at 5000 rpm for 15 min to remove the supernatant and perform polyacrylamide gel electrophoresis according to the electrophoresis sample preparation procedure.

Determination of cell membrane Na^+/K^+-ATPase activity

The activity of cell membrane Na^+/K^+-ATPase was evaluated based on the previous report (Lin et al., 2017) . Four selected bacteria were inoculated into a sterilized liquid medium, cultured at 37 °C for 12h to logarithmic growth phase, centrifuged at 5000 rpm for 10 min, washed and resuspended in sterilized 0.01 M phosphate buffered saline (PBS) buffer (pH 7.0) to 1×10^7 CFU/mL, 1 MIC of phillyrin was added into the bacterial suspension. Then the suspension was cultured at 37 °C for 6 h, centrifuged at 5000 rpm for 10 min, The activity of Na^+/K^+-ATPase of the four tested bacteria was determined by the ATPase kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions on a microplate reader, and each sample was tested in three parallel experiments.

2.6 Statistical analysis

All experiments were performed in triplicate and the results were expressed as the mean ± standard deviation. One-way analysis of variance (ANOVA) test and SPSS software (SPSS Inc., USA) were used to conduct statistical analyses.

3 Results and discussion

3.1 Antibacterial activity of phillyrin

Antibacterial activity of phillyrin against the four tested bacteria was determined. The sterile water was used as a control. As shown in Table 1, no inhibition zone was observed in the control. However, the inhibition zone appeared in the experimental group added with phillyrin, indicating that phillyrin inhibited the four selected bacteria. The minimum inhibitory concentration of *E. coli* ATCC8739 was 2.15 μg/mL, and the diameter of the inhibitory zone of phillyrin to *E. coli* ATCC8739 was 12.1 ± 0.12 mm, followed by *Bacillus subtilis* BS08 >*S. aureus* ATCC6538 > *Salmonella* ATCC14028. In general, phillyrin had a more significant ($p < 0.05$) antibacterial effect against *Escherichia coli* ATCC8739.

3.2 Growth curve study

It can be seen from Figure 1 that the growth curves of the four tested bacteria in the control group were all typical S-type, but the growth of bacteria was stable, with evident logarithmic phase and stable growth phase. After adding 1 MIC concentration of phillyrin, compared to the control group, the growth rate of the four test bacteria in the experimental group was significantly slowed down and the logarithmic period was delayed. However, the lag phase was significantly increased. Moreover, the growth of *Salmonella* 14028 had no significant logarithmic phase, and the logarithmic phase of *Staphylococcus aureus* ATCC6538 was delayed for about 9 hours; the maximum biomass was also significantly lower than that of the control group. After 16 hours, the growth curve entered a stable period and tended to be flat or even decreased. The phillyrin had inhibitory effects on growth and reproduction of the bacteria, delaying the growth period, reducing the maximum biomass, and inhibiting growth during the stationary phase and even leading to the decline of the decay period.

3.3 Scanning electron microscope observation

According to the results of scanning electron microscopy in Figure 2, the cells of the four tested bacteria without phillyrin in the control group were intact, and the surface of the cell membrane was slightly shrunk, which was more rounded and fuller than the cells added with phillyrin in the experimental group. When 1 MIC of phillyrin was added into the bacteria of experimental group, the cells of the four tested bacteria produced a large degree of shrinkage, and were distorted. The cell membrane was not intact, and the cell membrane of *E. coli* ATCC8739 and *S. aureus* ATCC6538 cells obviously ruptured, leading to the leakage of intracellular material and further bacterial cell lysis death (Bajpai et al., 2009). Those results indicated that phillyrin acted on and damage the cell membrane, which led to an incomplete structure of the bacterial cells, thus exerting antibacterial action. The results of the SEM assay were similarly consistent with those of previous antimicrobial tests performed on *E. coli* ATCC8739 and *S. aureus* ATCC6538 (Chen et al., 2017).

Table 1. MIC and DIZ of phillyrin against 4 tested bacteria.

Bacteria	DIZ (mm)	MIC (μg/mL)
Escherichia coli	9.85 ± 0.11[a]	2.15
Bacillus subtilis	8.81 ± 0.18[b]	3.16
Staphylococcus aureus	8.33 ± 0.13[c]	3.02
salmonella	8.85 ± 0.14[b]	3.40

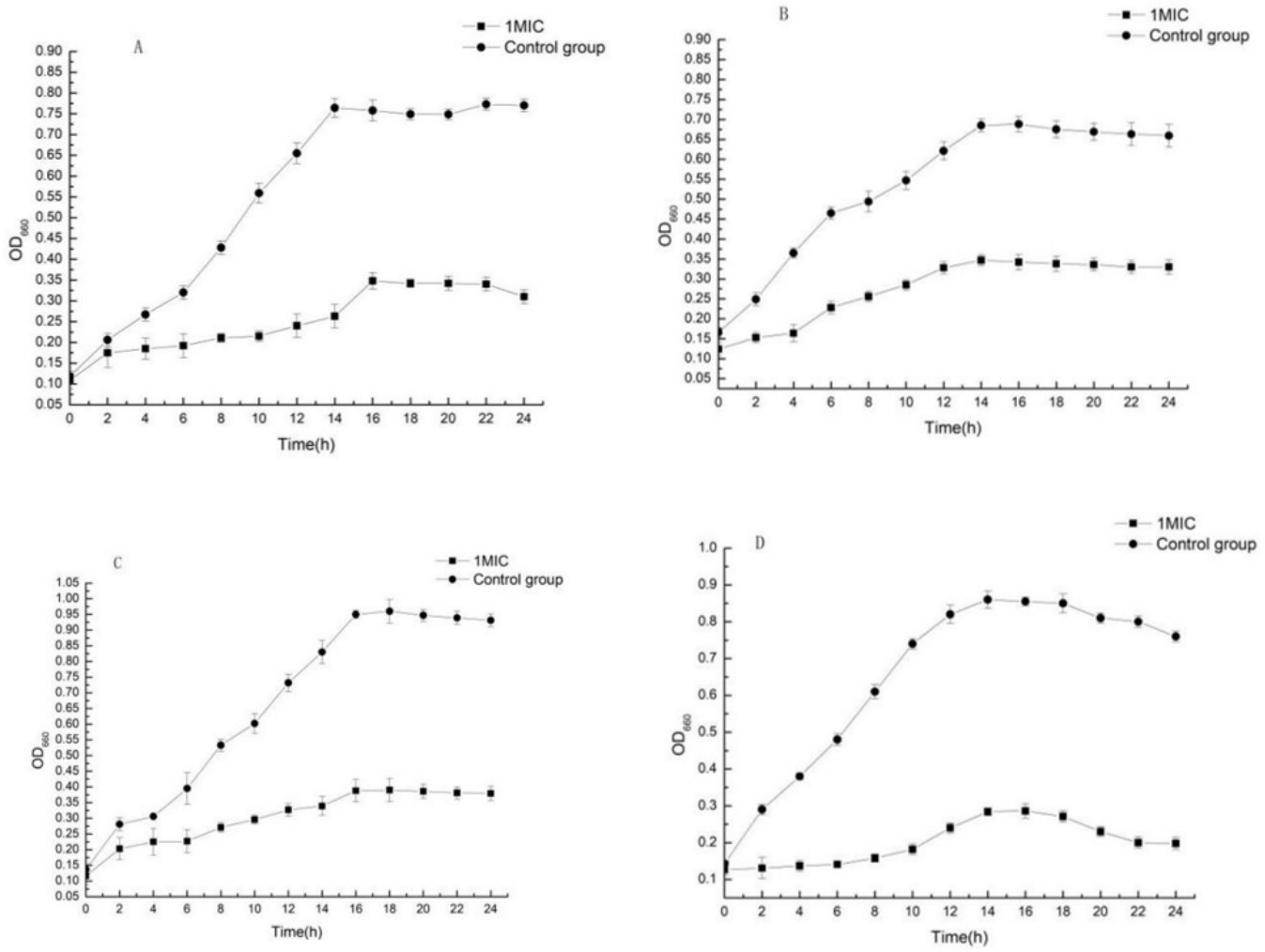

Figure 1. Inhibitory effect of phillyrin on the growth curve of four tested bacteria. (A) St*aphylococcus aureus* ATCC6538; (B) *Escherichia coli* ATCC8739; (C) *Salmonella* ATCC14028; (D) *Bacillus subtilis* BS08.

3.4 Effect of phillyrin on bacterial nucleotide leakage

After adding antibacterial chemicals, the cell membrane looked to be irreversibly destroyed, and cellular components were discharged. The integrity of cell membrane appeared to be damaged permanently after adding antibacterial agents, and the cellular substances were released, such as nucleotide (Zhang et al., 2016) which is a signal for determining membrane integrity (Shen et al., 2015).

The effect of phillyrin on the nucleotide leakage of the four tested bacteria is demonstrated in Figure 3. The absorbance values at 260 nm of the experimental group added with 1 MIC of phillyrin increased significantly compared with those of the control group ($p < 0.01$). It was indicated that the amount of nucleotide released in the bacteria was increased, that is to say, phillyrin could damage the integrity of the cell membrane, and the leakage of macromolecules such as nucleotide led to impaired cell metabolism, loss of protection of the cells, and ultimately the death of the bacteria, which was also shown by the results of scanning electron microscopy. The results of phillyrin on bacterial nucleotide leakage were also consistent with the results from experiments testing other antimicrobials on common foodborne pathogens (Zhang et al., 2019).

3.5 Effect of phillyrin on membrane permeability

The cell membrane is a crucial structural component of bacteria and a key target for antibacterial medicines (Eom et al., 2014). The cell membrane is barrier to the passage of small ions such as K^+ and Na^+, which is fundamental to maintain cell functions such as energy metabolism and metabolic regulation, and tiny changes in the integrity of bacterial cell membranes will lead to cell death (Cox et al., 2001). As shown in Figure.4, conductivity increased steadily and showed a slight increase trend in control group, which was caused by the natural decline and dissolution of the cells (Diao et al., 2014). However, the conductivity of the four selected bacteria in the experimental group increased from 0 h and was significantly higher than that of the control group at 3 h ($p < 0.01$). Conductivity reflected the effect of phillyrin on cell membrane permeability. K^+, Na^+,

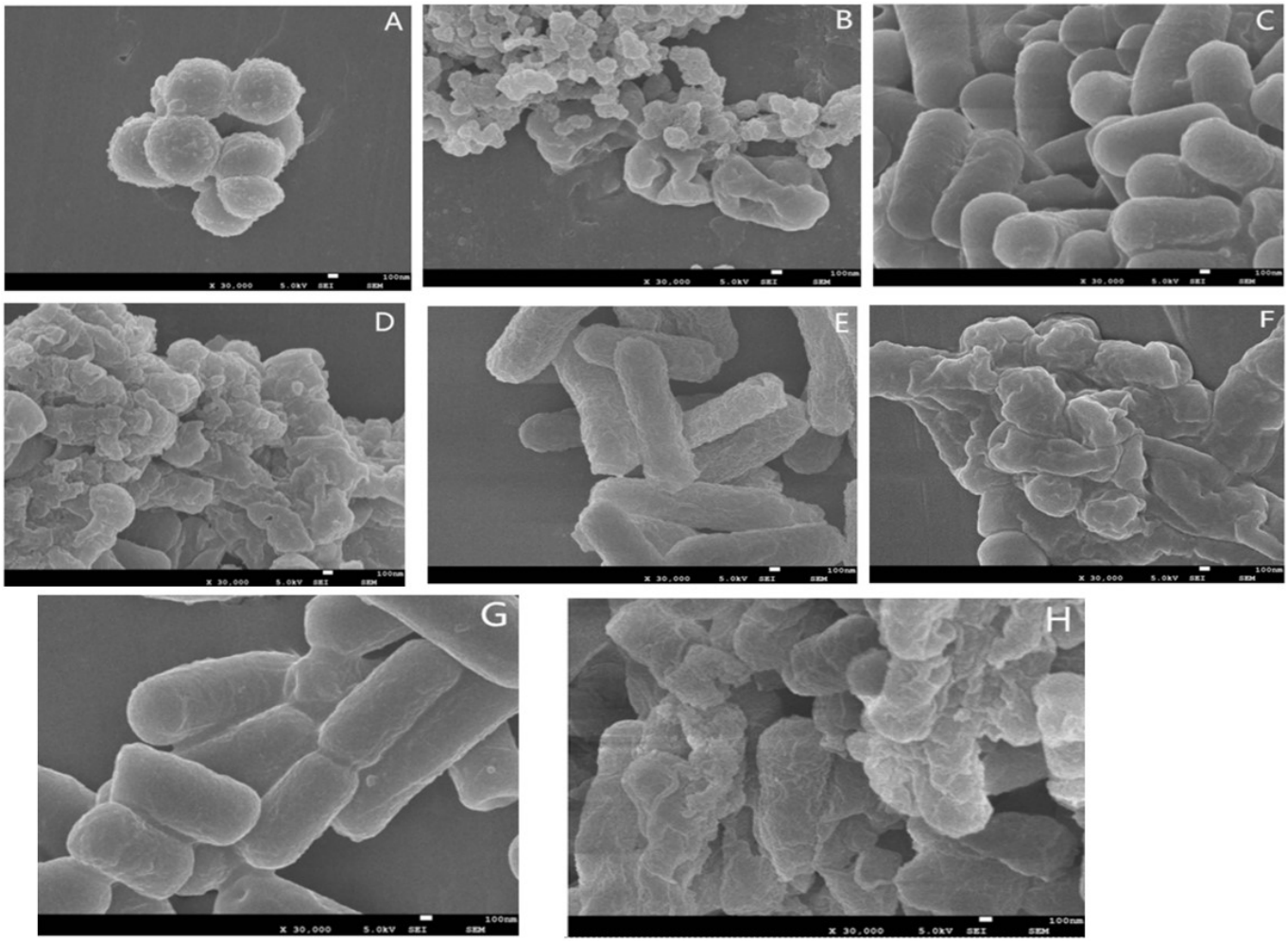

Figure 2. Scanning electron micrographs of four tested bacteria. A, C, E and G demonstrate the *Staphylococcus aureus* ATCC6538, *Escherichia coli* ATCC8739, *Salmonella* ATCC14028, *Bacillus subtilis* BS08 respectively in the experimental group; B, D, F and H show the *Staphylococcus aureus* ATCC6538, *Escherichia coli* ATCC8739, *Salmonella* ATCC14028, *Bacillus subtilis* BS08 respectively in the control group.

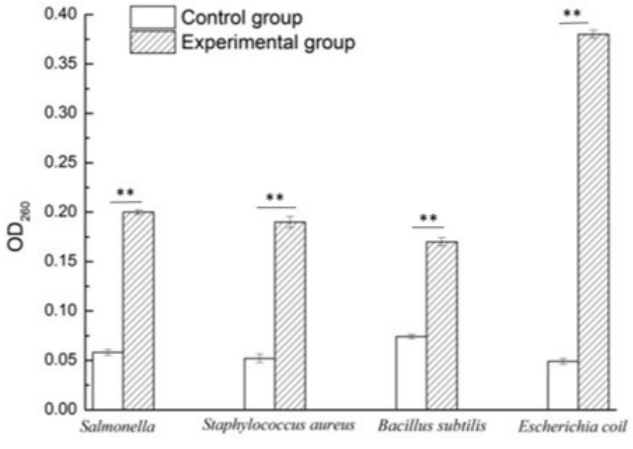

Figure 3. Effect of phillyrin on bacterial nucleotide leakage. ** indicates significant differences ($P < 0.05$) between different samples.

H^+, etc. are fundamental to maintain cell membrane potential as well as the normal metabolism and function of the cells, which entered the cell through cell membrane, these ions . However, phillyrin could rupture the cells, destroy the ionic homeostasis in the cells, and leak the electrolytes inside the cells. As a consequence, the fundamental metabolism of the cells could not normally carry out, which eventually brought about the death of the cells.

3.6 Confocal laser scanning microscopy

CLSM was used to further observe the overall survival of the four bacteria. According to the principle of staining, PI is a macromolecular fluorescent dye that can hardly pass through the intact cell membrane and thus cannot stained intact cells. Destruction, death, or permeability changes of cell membrane can contribute to the PI passing through the cell membrane to bind to the nucleic acid material and fluoresce

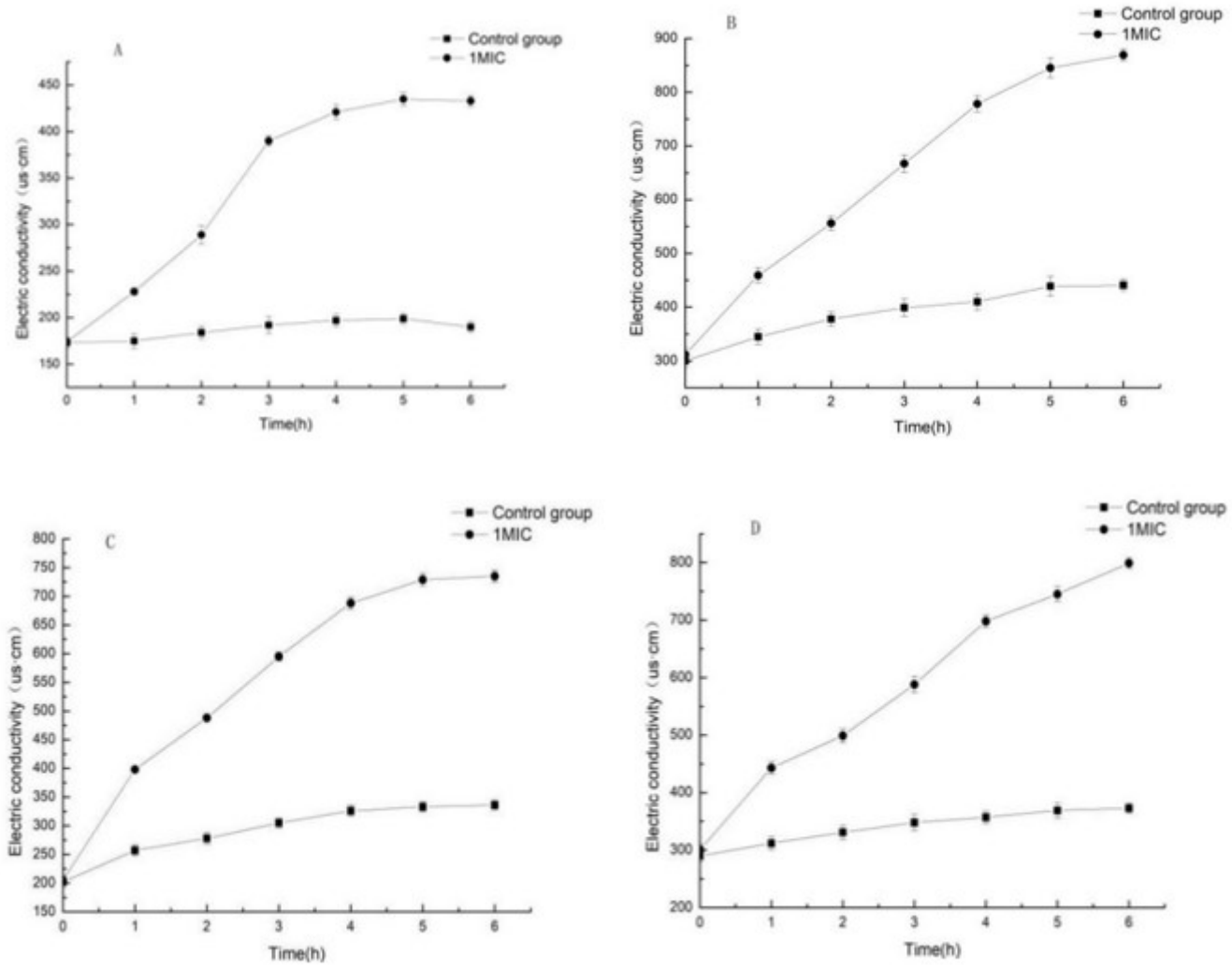

Figure 4. Effect of phillyrin on electric conductivity. (A: *Staphylococcus aureus* ATCC6538, B: *Escherichia coli* ATCC8739, C: *Salmonella* ATCC14028, D: *Bacillus subtilis* BS08).

(Milillo et al., 2011). Dead cells were stained by PI and emited red fluorescence, while living cells cannot be stained and do not emit fluorescence, so PI is usually used to confirm cell viability (Hameed et al., 2016). As shown in Figure 5, the four tested bacteria added with 1 MIC phillyrin emitted a large area of red fluorescence, indicating that the cells were dead, while the control group did not fluoresce, indicating that the cells were still alive. The staining rates of *Staphylococcus aureus* ATCC6538, *E. coli* ATCC8739, *Salmonella* ATCC14028 and *Bacillus subtilis* BS08 in the control group were 10.83%, 0%, 29.17% and 3.18%, respectively, which might be due to the normal decline of cells. After four tested bacteria were treated with 1MIC phillyrin, the cell membranes were damaged and ruptured, resulting in the increase of permeability. The staining rates increased to 84.44%, 59.46%, 95.63%, and 78.63%, respectively, and were significantly higher than those of control group. It demonstrated that phillyrin could destroy the cell membranes of four tested bacteria, leading to bacterial decline, which was consistent with the analysis of SEM results.

3.7 SDS-PAGE analysis

The bacteria in experimental group added with phillyrin were different from the control group without phillyrin (Figure 6). The number of bands in the control group was larger than that in the experimental group, and the bands were clear and obvious. The bands of *E. coli* ATCC 8739, *Bacillus subtilis* BS08, *Staphylococcus aureus* ATCC6538 and *Salmonella* ATCC 14028 in the experimental group were lighter than those of the control group. The bands of *Bacillus subtilis* BS08 in the experimental group at 116 KDa and 97.2 KDa were not observed. The band of *Salmonella* ATCC 14028 in the experimental group at 66.4 KDa was lighter than the band of the control group, indicating that phillyrin inhibited the expression of intracellular proteins in the tested bacteria.

3.7 Effect of phillyrin on enzyme activity of Na^+/K^+ -ATPase

The effect of phillyrin on Na^+/K^+-ATPase activity is shown in the Figure 7. The Na^+/K^+-ATPase activity of each type of

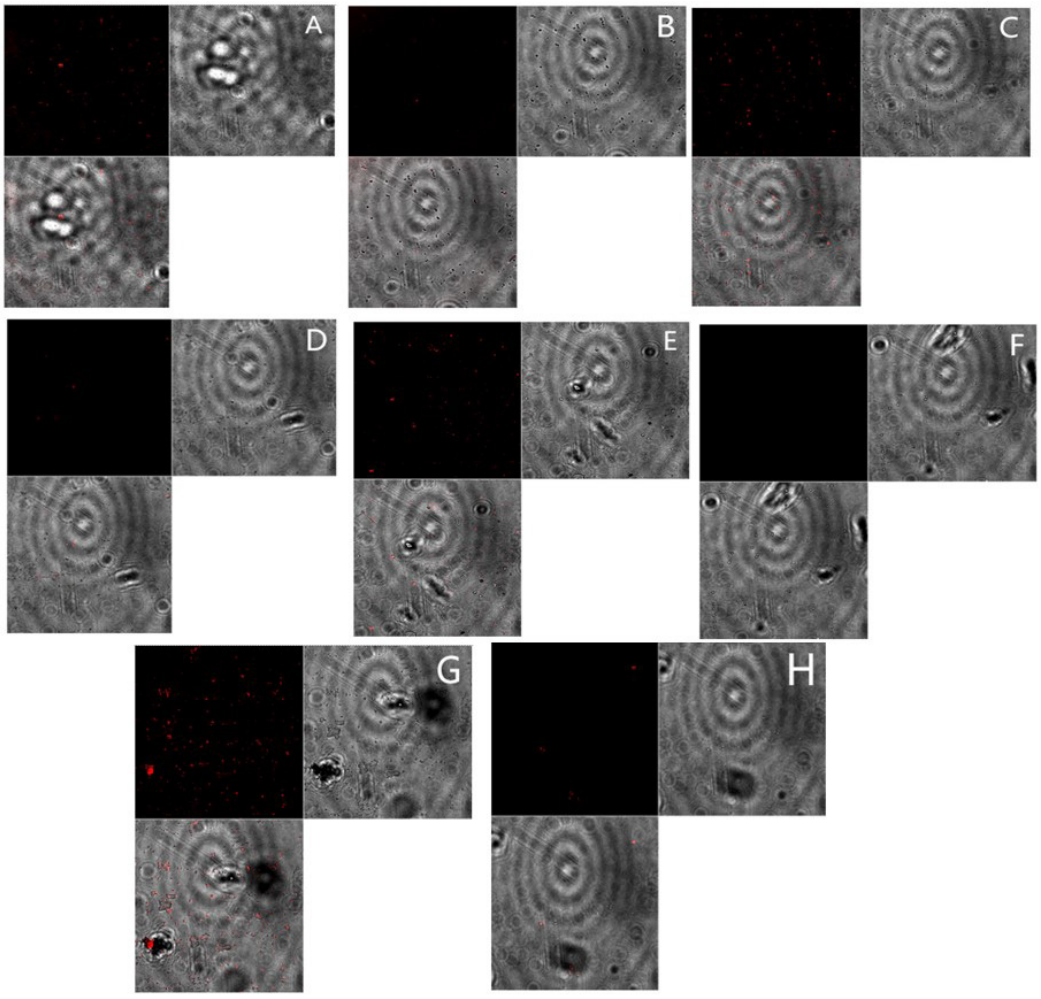

Figure 5. Confocal laser scanning microscopy of four tested bacteria. A, C, E and G show the *Bacillus subtilis* BS08, *Staphylococcus aureus* ATCC6538, *Escherichia coli* ATCC8739, *Salmonella* ATCC14028 respectively in the experimental group; B, D, F and H show the *Bacillus subtilis* BS08, *Staphylococcus aureus* ATCC6538, *Escherichia coli* ATCC8739, *Salmonella* ATCC14028 respectively in the control group.

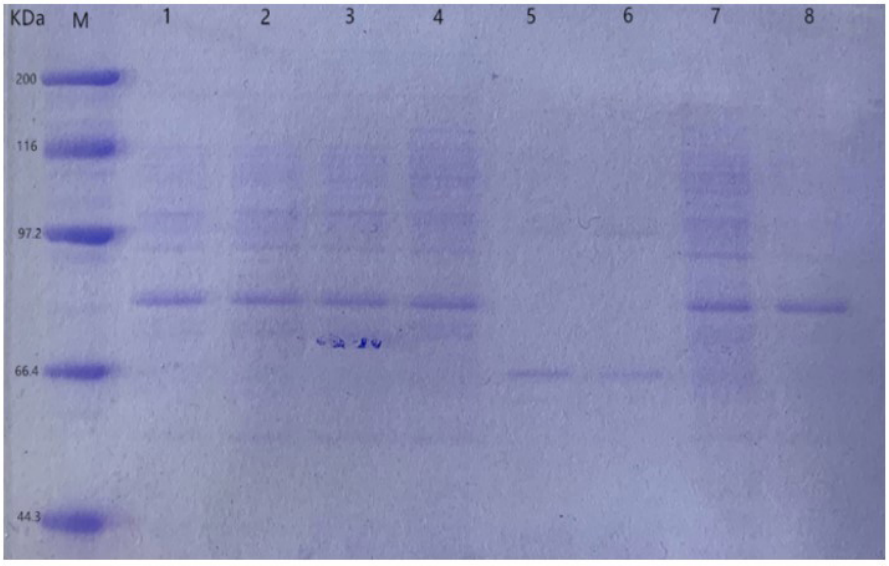

Figure 6. SDS-PAGE analysis M represents the marker; 1, 3, 5, 7 represent the control group of *Staphylococcus aureus* ATCC6538, *Escherichia coli* ATCC8739, *Salmonella* ATCC14028, *and Bacillus subtilis* BS08 respectively. 2, 4, 6, 8 represent the experimental groups of *Staphylococcus aureus* ATCC6538, *Escherichia coli* ATCC8739, *Salmonella* ATCC14028, and *Bacillus subtilis* BS08 respectively.

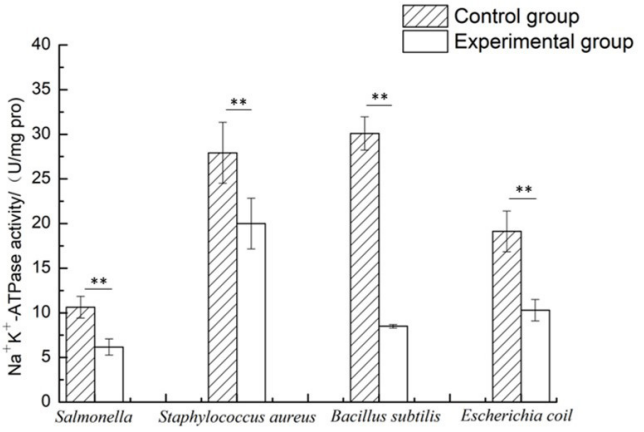

Figure 7. Effect of phillyrin on enzyme activity of Na^+/K^+ -ATPase. ** indicates significant differences ($P < 0.05$) between different samples.

bacteria treated with 1 MIC phillyrin in the experimental group was significantly ($p<0.05$) lower compared to that of the control group without phillyrin. The Na^+/K^+ -ATPase activity of *Salmonella* ATCC14028 decreased from 10.63 ± 1.21 U/mg pro to 6.16 ± 0.92U/mg pro with the incorporation of phillyrin; the Na^+/K^+-ATPase activity of *Staphylococcus aureus* ATCC6538 decreased from 27.93 ± 3.40 U/mg pro to 20 ± 0.42 U/mg pro; the Na^+/K^+-ATPase activity of *E. coli* ATCC8739 decreased from 19.1 ± 2.29 U/mg pro to 10.3 ± 1.22 U/mg pro; the Na^+/K^+-ATPase activity of *Bacillus subtilis* BS08 decreased the most from 30.08 ± 1.86U/mg pro to 8.5 ± 0.20 U/mg pro, indicating the inhibitory effect on *Bacillus subtilis* BS08 was the best. This might reveal that phillyrin had an inhibitory effect on the protein, thereby inhibiting the enzyme activity, so phillyrin has an inhibitory effect on Na^+/K^+-ATPase activity.

4 Conclusion

The antibacterial mechanism of phillyrin as a natural bacteriostatic agent was investigated. Phillyrin effectively inhibited the four foodborne pathogens, which had potential to be applied as a kind of natural food preservative and a promising antibacterial compound in the food industry. This study provided a basic theoretical basis for the development and utilization of phillyrin in edible preservatives.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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