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ANTIBACTERIAL ACTIVITY AND MECHANISM OF CINNAMON ESSENTIAL OIL AND ITS APPLICATION IN MILK

H. Y. Cui[1], H. Zhou[1], L. Lin[1*], C. T. Zhao[1], X. J. Zhang[1], Z. H. Xiao[2] and C. Z. Li[2*]

[1]School of Food & Biological Engineering, Jiangsu University, Zhenjiang 212013 P.R. China
[2]Hunan Academy of forestry, Changsha 410007, P. R. China
[*]Corresponding authors e-mail: linl@ujs.edu.cn; lichangzhu2013@aliyun.com

ABSTRACT

Essential oils present a fine antibacterial performance against a variety of bacteria. The main component of the cinnamon oil was eugenol (75.52%). In this study, the goal was to evaluate the antimicrobial activity and reveal bactericidal mechanism of cinnamon oil. Firstly, the minimum inhibitory concentration (MIC) and the minimum bactericide concentration (MBC) of cinnamon oil were tested. The results showed the lowest MIC and MBC values of 0.025% and 0.05% respectively. The cinnamon oil exhibited high antibacterial activity against several bacteria *in vitro*. Meanwhile, the application of cinnamon oil in milk also proved its good antibacterial properties. Subsequently, the bactericidal mechanism of cinnamon oil was investigated. The SEM images, TEM images and the loss of 260 nm-absorbing material indicated that the cinnamon oil could damage microbial cell membrane. In addition, the loss of ATP and DNA were detected, which also implied that the direct damage of cinnamon oil to microbial cell membrane was major antibacterial mechanism.

Key words: Cinnamon Essential oil, Antibacterial Activity, Milk, antibacterial Mechanism.

INTRODUCTION

The research area of food technology still face great challenges in finding effective ways of adding value to produce foodstuffs and, moreover, in maintaining their quality and safety (Ayala-Zavala *et al*., 2013). *Staphylococcus aureus* (*S. aureus*) is a ubiquitous organism that causes a variety of foodborne diseases in humans and animals (Holmes and Zadoks, 2011). It is considered the third most common pathogen that causes food poisoning in the world (Can and Çelik, 2012). In animals, *S. aureus* is the most frequent causative agent of mastitis, especially in cattle, sheep and goats and this makes it a common contaminant of raw milk (Mhone *et al*., 2011). Milk contaminated by high levels of spoilage bacteria usually becomes unsuitable for further processing since it does not meet the consumer's expectations in terms of health (nutritional value), safety (hygienic quality) and satisfaction (sensory attributes) (Takahashi *et al*., 2005). Hence, many antibacterial agents have been developed to inhibit *S. aureus* in milk.

However, the growing demand of consumers for safe and natural products, without chemical preservatives, has resulted in thorough investigations from food authorities and researchers to assess the feasibility of mild preservation techniques and to improve the microbial quality and safety of products, while maintaining their good nutritional and organoleptic properties (Kuang *et al*., 2011). Thus, the quest for safer alternatives has led to studies on plant extracts, such as essential oils, that may have potential as natural food additives (Goni *et al*., 2009; Cantore *et al*., 2009).

Recently, spices have attracted a lot of attentions in their useful physiological functions and antimicrobial activity. Among the many spices, cinnamon is one of the most popular and the oldest spices used for foods. It belongs to *Lauraceae* family and usually grows in South and South-East Asia (Sathishkumar *et al*., 2009; Elaissi *et al*., 2011).The essential oil from cinnamon is commonly used in the food industry because of its special aroma in addition to its medicinal properties. In recent years, some studies have reported that cinnamon oil had a broad range of antimicrobial activities against gram-positive and gram-negative bacteria (Tyagi and Malik, 2011). These study results provided a possibility for the application of cinnamon oil in the food preservation.

But so far, there are rare research reports on the antibacterial properties of cinnamon oil in milk and its possible mechanism. They are very important for the future application of cinnamon oil in dairy products. For this reason, the objective of this study is to evaluate the antibacterial activity of cinnamon oil on foodborne pathogens and its potential antibacterial application in milk. On this basis, the possible antibacterial mechanism of cinnamon oil will be discussed as well.

MATERIALS AND METHODS

Bacteria strains: The following foodborne bacteria strains were selected for this study: *Escherichia coli* ATCC 25922, *Staphyloccocus aureus* ATCC 25923,

*Salmonella typhi*B11, *Klebsiella pneumonia* ATCC 13883, *Bacillus subtilis* IFO 3457, *Pseudomonas aeruginosa* ATCC 27853 , *Bacilluspumilus* ATCC 27142. These strains were cultured in Nutrient Broth (NB) at 37 °C for 24 h and 48 h respectively, and stored at -80 °C.

Essential oil: The cinnamon essential oil was bought from J.E International (French). The chemical compositions of cinnamon oil were determined by GC-MS (Agilent 6890GC/5973NMSD, NYSE: A, USA). A fused silica capillary Agilent Technology HP-5ms (5% phenyl methyl siloxane) column (30 m× 0.25 mm i.d., film thickness 0.1 μm) was used for the separation. The injector temperature and the detector temperature were 150 °C and 250 °C, respectively. The initial temperature was kept at 100 °C for 4 min, and the temperature was gradually increased to 130 °C at a rate of 5 °C /min and was then held for 20 min at 130 °C. The linear velocity of the helium carrier gas was 1.2mL/min at a split ratio of 30:1; EI was used as the ion source, and the ion source temperature was 230 °C. The sector mass analyzer was set to scan from 30 to 550 amu, scan time, 1 s. 1.0 μL samples were injected for analysis (Wang *et al*., 2009; Li *et al*., 2013).

Determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC): Cinnamon oil was added into tubes containing NB to obtain the concentrations of 0.1%, 0.05%, 0.025%, 0.0125% and 0.00625% (v/v) respectively. Subsequently, the tubes were inoculated with the freshly prepared bacterial suspension in order to maintain the initial bacterial concentration of 10^3-10^4 CFU/mL, and then incubated in a rotary shaker at 150 rpm and 37 °C for 24-48 h. The lowest concentration of essential oil showing growth inhibition (as seen visually) was considered as the minimum inhibitory concentration. The MBC was recorded as the lowest concentration of essential oil that showed no growth on Nutrient Agar (NA) plates after spot inoculation and incubation at 37 °C for 24-48 h (Ruparelia *et al*., 2008; Otero *et al*., 2014).

Kill time analysis of cinnamon oil: The plate colony-counting method was used to analyze the kill time of cinnamon oil. Cinnamon oil was diluted into tubes with PBS to obtain a concentration of 0.04%. Subsequently, the tubes were inoculated with the freshly prepared bacterial suspension in order to maintain the initial bacterial concentration of 10^5 CFU/mL and cultured at 150 rpm and 37 °C. Numbers of viable bacteria were enumerated at 0 h, 0.5 h, 1 h, 2 h, 4 h and 8 h by counting the number of bacterial colonies grown on the plate. As a control, the bacterial suspension in sterile phosphate buffer solution (PBS) without cinnamon oil was also tested (Nakayama *et al*., 2012).

Antimicrobial activity assay in milk: Four kinds of common milk (MengNiu Milk, Bright Dairy Milk, YiLi Milk, MengNiu Low-fat Milk) were bought from local supermarket. They were added into high-borosilicate bottles respectively and then autoclaved. Subsequently, 0.1% cinnamon oil was added in the bottles filled with milk. The bottles without cinnamon oil were tested as control groups. Finally, the bottles were inoculated with the freshly prepared *S. aureus* suspension (approximately 10^2-10^3 CFU/mL) and cultured at 150 rpm and 30 °C. Numbers of viable bacteria were enumerated at 0 h, 24 h and 48 h by counting the number of bacterial colonies grown on the plate. All the determinations were repeated three times (Liu and Yang, 2012).

The integrity of the cell membrane: The size and morphology of the bacteria were examined by Scanning electron microscopy (SEM, JSM-7001F, JEOL, Japan) and Transmission electron microscopy (TEM, JEM-2010HR, Hitachi, Tokyo, Japan) (Sondi and Sondi, 2004).

Loss of 260nm-absorbing material: *E. coli* and *S. aureus* were cultured in NB and incubated at 37 °C for 24 h and 48 h respectively. After incubation, the suspensions were centrifuged at 4000 rpm for 15 min at 4 °C and the pellets were collected. The pellets were washed three times with PBS and resuspended to make up bacterial suspensions of 10^8 CFU/mL. Different concentrations of essential oil [0.0125%, 0.025%, 0.05%, 0.1% and 0.2% (v/v)] were added to the cell suspension. Samples without essential oil treatment were used as control groups. The bacterial suspensions were cultured under agitation at 150 rpm, 37 °C for 20 h. After incubation, the suspensions were centrifuged at 4000 rpm for 15 min at 4 °C and the supernatant were filtered by microporous membrane filter. The filtrate was determined by ultraviolet spectrophotometer (UV-1801, Beijing, China) at 260 nm (Sharma *et al*., 2013; Lee *et al*., 2014).

Observed DNA and RNA with Fluorescent staining method: The nucleic acids were observed by fluorescent staining method. 0.05% cinnamon oil was added into the bacterial suspension and cultured at 150 rpm and 37 °C. After 28 h, an equal volume of diluted 4'6-diamidino-2-phenylindole (DAPI) (10 μg/mL, Roche Diagnostics GmbH, Germany) and cell sample were mixed, and then placed on a micro slide and kept in the dark for 10 min. The fluorescence of DAPI in cells was detected by inverted fluorescence microscope. As a control group, the bacterial suspension without cinnamon oil was also observed (Leica TCS SP5) (Wang *et al*., 2010).

Quantification of DNA: The suspensions were centrifuged at 4000 rpm for 15 min at 4 °C and the pellets were harvested. The pellets were washed three times with PBS and resuspended in the buffer containing 0.05% cinnamon oil to prepare bacterial suspensions (10^8

CFU/mL). The samples were cultured at 150 rpm and 37 °C. The 800μL of the suspensions were removed after culture for 0 h、4 h、8 h、12 h、16 h、20 h、24 h、28 h、32 h、36 h、40 h and immediately poured into 2400μL of DAPI solution (10 μg/mL, Roche Diagnostics GmbH, Germany) and kept in the dark for 10 min. The fluorescence intensity of DNA was estimated by fluorescence spectrophotometer (Cary Eclpise, USA) with the excitation wavelength of 364 nm. The bacterial suspension in sterile PBS without cinnamon oil was tested as a control group (Liu *et al*., 2004; Diao *et al*., 2014).

Measurement of cellular ATP concentrations: The cellular ATP concentrations were measured according to the method described by Turgis *et al*., (2009). The broth of *E. coli* and *S. aureus* (10^8 CFU/mL) were centrifuged for 10 min at 8000 rpm. The cell pellets were washed three times and resuspended in the buffer to make up 10^8 CFU/mL suspensions of bacteria. After 0.05% cinnamon oil was added, the samples were cultured at 150 rpm and 37 °C for 30 min. Then, the samples were centrifuged for 10 min at 8000 rpm, and the cell pellets were collected. Finally, the cellular ATP concentrations of samples were determined by the Clean Sense TM Surface Hygiene Test Kit (LEYU Biotechnology, Shanghai, China). As a control group, the samples without cinnamon oil were tested (Turgis *et al*., 2009; Finger *et al*., 2012).

RESULTS AND DISCUSSION

Chemical compositions: The 10 chemical components of cinnamon oil were presented in Table 1. The main components are eugenol (75.520%) and eugenyl acetate (4.403%). Previous studies reported that eugenol and eugenyl acetate are the major bioactive constituents of cinnamon oil. These volatile phenolic compounds could disrupt the membranes of bacterial cells, leading to the cell death (Turgis *et al*., 2009; Fu, Chen *et al*., 2009; He *et al*., 2010; Adisakwattana *et al*., 2011).

Determination of the MIC and the MBC: To evaluate the antimicrobial activity of cinnamon oil, the MIC and MBC of seven pathogens were tested. The MIC and MBC values were given in Table 2. For the all bacterial strains, the MICs values ranged from 0.025% to 0.05%, and the MBCs values varied from 0.05% to 0.1%. Based on above results, ranged from 0.025% to 0.05%, and the MBCs values varied from 0.05% to 0.1%. Based on above results, cinnamon oil showed very well antibacterial effect on the foodborne pathogen.

Table 1.Chemical compositions of cinnamon oil

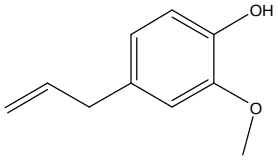
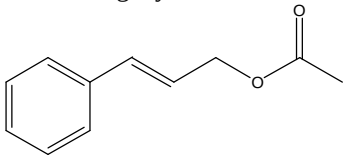
Composition	Proportion (%)	Composition	Proportion (%)
 eugenol	75.520	eugenyl acetate 	4.403
alpha-pinene	1.300	para-cymene	1.458
linalol	1.167	(E)-cinnamaldehyde	1.654
beta-caryophyllene	2.570	cis-cinnamyl acetate	3.020
benzyl benzoate	2.169	alpha-phellandrene	0.985

Table 2. MICs and MBCs of cinnamon oil against pathogens

Test strain	Cinnamon oil	
	MIC (%)	MBC (%)
Escherichia coli	0.05	0.05
Staphylococcus aureus	0.05	0.05
Bacillus subtilis	0.025	0.05
Salmonella typhi	0.05	0.05
Klebsiella pneumonia	0.05	0.1
Pseudomonas aeruginosa	0.05	0.1
Bacilluspumilus	0.025	0.05

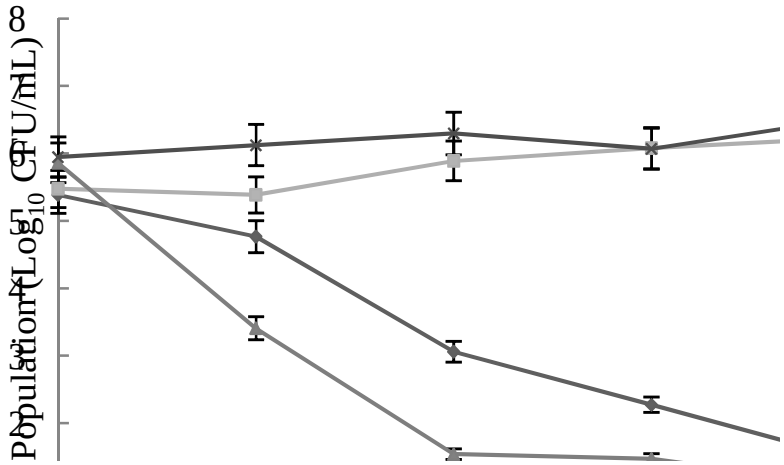


Fig. 1 The antimicrobial activities of cinnamon oil both for *E. coli* and *S. aureus*

The antimicrobial activity of cinnamon oil: In order to investigate the relation between antibacterial time and effect, the kill time analysis of cinnamon oil was carried out.As we can see from the Fig.1, cinnamon oil exhibited satisfactory antimicrobial activity both for *E. coli* and *S. aureus*. About 99.999% and 99.9% reduction in population were observed in *E. coli* and *S. aureus* respectively after 2 h treatment of cinnamon oil. And after 8 h treatment, almost 100% reductions in population were achieved both in *E. coli* and *S. aureus*. It indicated that cinnamon oil can get satisfactory results against both Gram-positive and Gram-negative strains of bacteria in a short time.

Antibacterial activity of cinnamon oil in milk: As a nutrient-rich food, milk is easy to be infected by *S. aureus* in its processing. In the current work, four well-known brand milks were used to examine the antibacterial activity of cinnamon oil and the results were shown in Fig. 2. The results displayed that cinnamon oil had a marked effect on the inhibition of *S. aureus*. For instance, compared to the control groups, about 95.3% and 97.7% reduction in population were observed after 48 h in MengNiu and Bright Dairy milk groups (Fig.2A, Fig.2B). Moreover, after 48 h, *S. aureus* could be completely inactivated in Yi Li and Meng Niu Low-fat groups (Fig.2C, Fig.2D). The better results in Yi Li and Meng Niu Low-fat groups may be due to the influence of the initial bacterial concentration. Logically, higher bacterial population required a greater amount of essential oil than the lower one to obtain the same inhibitory effect (Liu and Yang, 2012). Comparatively, the bactericidal concentration of cinnamon oil in milk is higher than in the broth. This is because milk can provide abundant nutrients to support the bacterial growth (Liu *et al*., 2010). Except this, fats could protect bacteria from antimicrobial agents, thus decreasing the bactericidal action (Liu and Yang, 2012). However, the effect of fat did not clearly show between pure milk and low-fat milk in this experience.

The integrity of the cell membrane: The morphology change of bacteria is the most direct evidence to reveal the antibacterial mechanism of cinnamon oil. SEM was used to investigate the surface morphology of *E. coli* and *S. aureus* before (Fig.3A, Fig.3C) and after cinnamon oil treatment (Fig.3B, Fig.3D) in NB medium. In the control samples, the surfaces were smooth and damage-free. However, treated bacterial cells were significantly damaged. TEM images of *E. coli* and *S. aureus* gave the similar results. The healthy bacterial (Fig.3E and Fig.3G) were morphologically intact with clear membrane. After treated, the cell membrane injury and intracellular material leakage were observed (Fig.3F and Fig.3H). Above results indicated that cinnamon oil could destroy the bacterial cell membrane, resulting in cell lysis and death.

Loss of 260nm-absorbing material: The measurement of release of 260-nm absorbing materials from the bacteria treated with cinnamon oil is an index of cell lysis (Devi *et al*., 2010). As shown in Fig.4, the absorbance of *E. coli* and *S. aureus* was higher with the increase of cinnamon oil concentration. After treatment with 0.0125% and 0.2% cinnamon oil, the optical density (OD) increased approximately more than eightfold. This implied that cinnamon oil disrupted the membrane and caused subsequent loss of intracellular constituents required to sustain life.

Determination of the amount of DNA in bacterial cells: DAPI is a fluorescent dye that binds both DNA and RNA. The dye increases in its fluorescence with increasing in the quantity of nucleic acids (Wang *et al*., 2010). The Fig.5 showed that the fluorescence intensities of *E. coli* and *S. aureus* in the control groups (Fig.5A, Fig.5C) were significantly higher than those in the cinnamon oil treatment groups (Fig.5B, Fig.5D). The

fluorescence spectrophotometer measurements indicated that the DNA content in *S. aureus* was significantly reduced to 57.71% compared to the control group (Fig.5E), and the DNA content in *E. coli* was reduced to 46.42% (Fig.5F). This was because cinnamon oil destroyed the cell membrane leading to the loss of nucleic acid in the cell. In addition, cinnamon oil inhibited nucleic acid synthesis, which made nucleic acid content in cinnamon oil group significantly lower than the control group.

Measurement of cellular ATP concentrations: To observe the level of cell membrane damage caused by cinnamon oil, the content of the residual ATP after treatment for 30 min was measured (Fig. 6). Compared to the control groups, the ATP concentration of *E. coli* and *S. aureus* both sharply declined after cinnamon oil treatment. The ATP concentration in *E. coli* group was dropped from 4485 RLU to 26 RLU. In *S. aureus* group, the ATP concentration was 158 RLU, down from 5188 RLU. There are two possible reasons: (1) Due to the cell membrane damage, the massive extracellular release of ATP leads to decline of ATP bioluminescence value. (2) After cinnamon oil get inside the bacteria cell, it might inhibit the ATPase activity and impede the synthesis of ATP. However, more experiments are needed to prove this hypothesis.

Through the above experimental results, the main antibacterial mechanism of cinnamon oil can be summarized in following pathway. As Fig.7 shown, the volatile phenolic compounds in cinnamon are able to destroy the membranes of bacterial cells. Cell is unable to maintain the stability of the membrane potential and their high osmotic pressure after the cell membrane damaged, what is more, the active transport is collapsed (Ates and Erdogrul, 2003; Soković *et al*., 2010; Marina Soković *et al*., 2010). The intracellular substances, such as ATP and DNA, lose through impaired cell membrane. At the same time, cinnamon oil gets inside bacteria cell through impaired cell membrane. It inhibits the synthesis of nucleic acid and ATP. Both they participate in a wide range of metabolic functions and they are key factors in keeping bacterial healthy (Nazzaro *et al*., 2013, Gutierrez *et al*., 2009). Considering the complex compositions of essential oils, it seems unlikely that there is only one bactericidal mechanism. (Adisakwattana *et al*., 2011; Fisgin *et al*., 2009; Arruda *et al*., 2006). Thus, further studies are needed to understand the mechanisms involved in order to evaluate the efficacy of cinnamon oil in suitable food systems

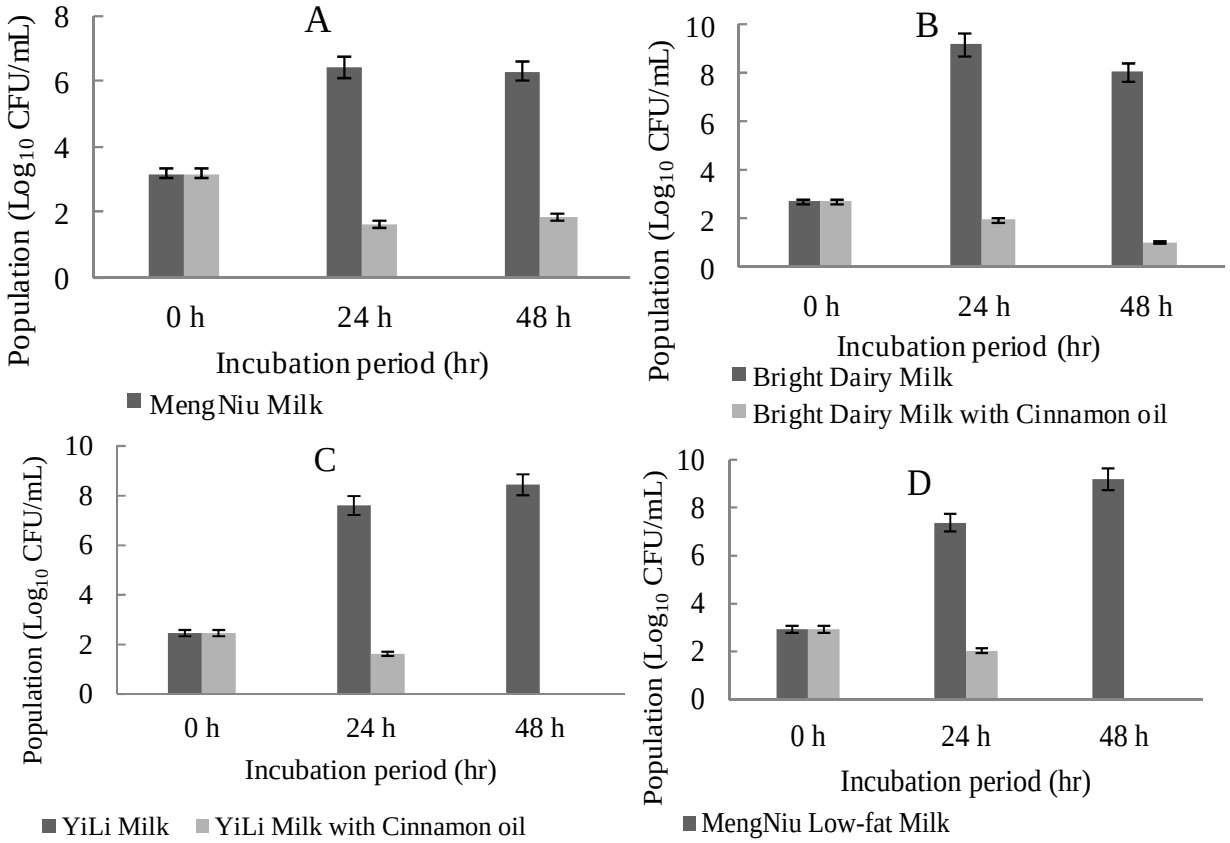


Fig. 2 The antibacterial activity of cinnamon oil in milk (A) MengNiu milk, (B) Bright Dairy milk, (C) YiLi milk, (D) MengNiu Low-fat milk

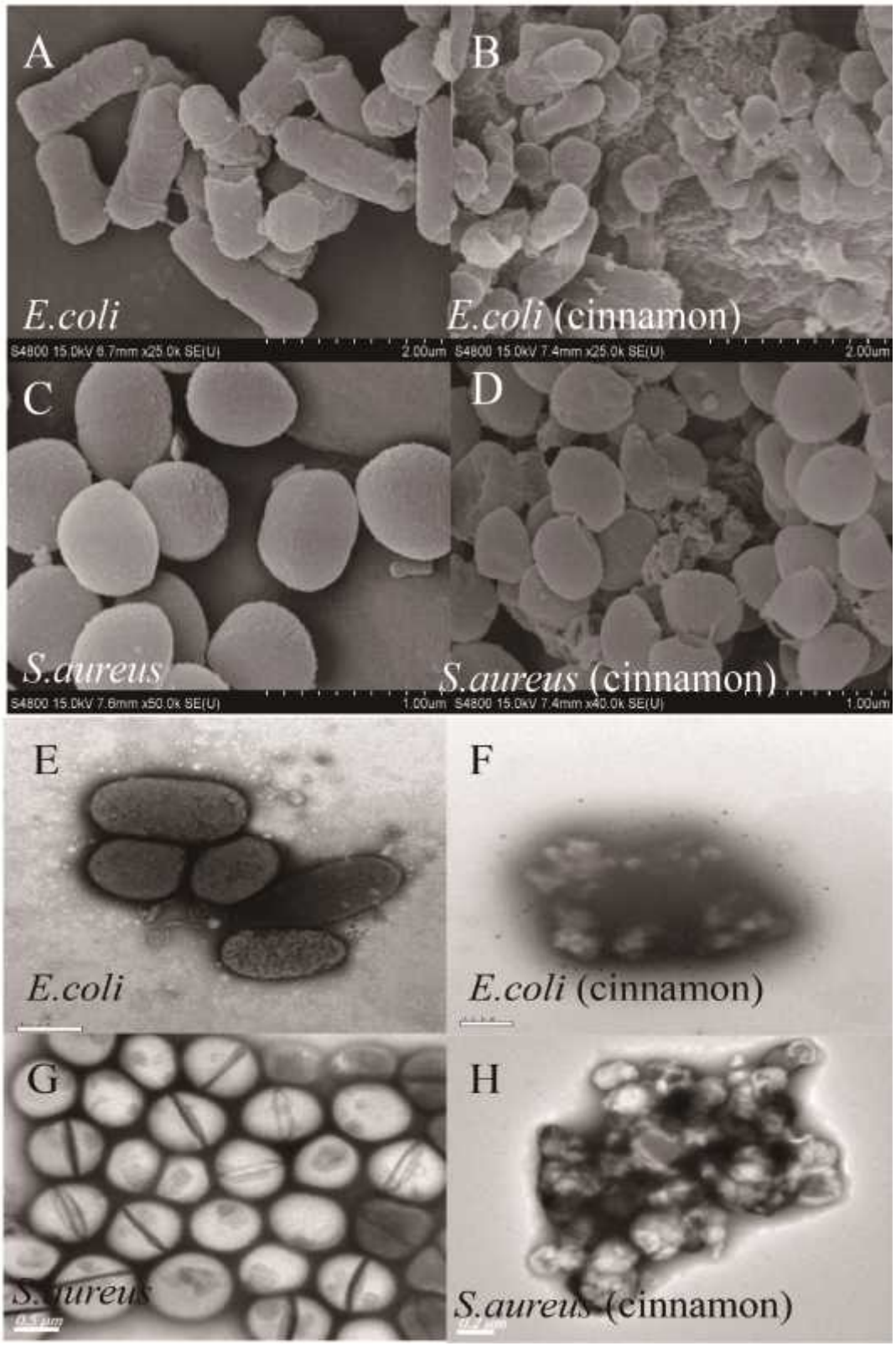


Fig. 3SEM image of *E. coli* before (A) and after (B)cinnamon oil treatment; SEM image of *S. aureus* before (C) and after (D) cinnamon oil treatment; TEM image of *E. coli* before (E) and after (F) cinnamon oil treatment; TTEM image of *S. aureus* before (G) and after (H) cinnamon oil treatment

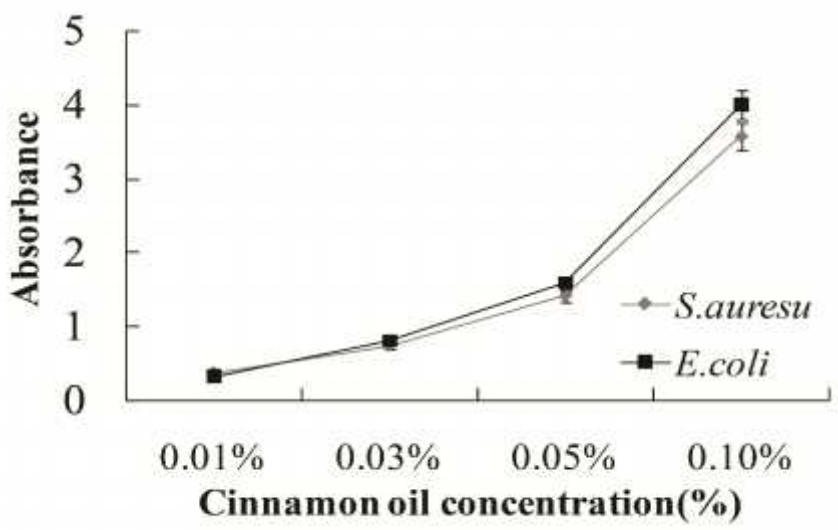


Fig. 4 The change of the absorbance of *E. coli* and *S. aureus* cell suspension treated with cinnamon oil.

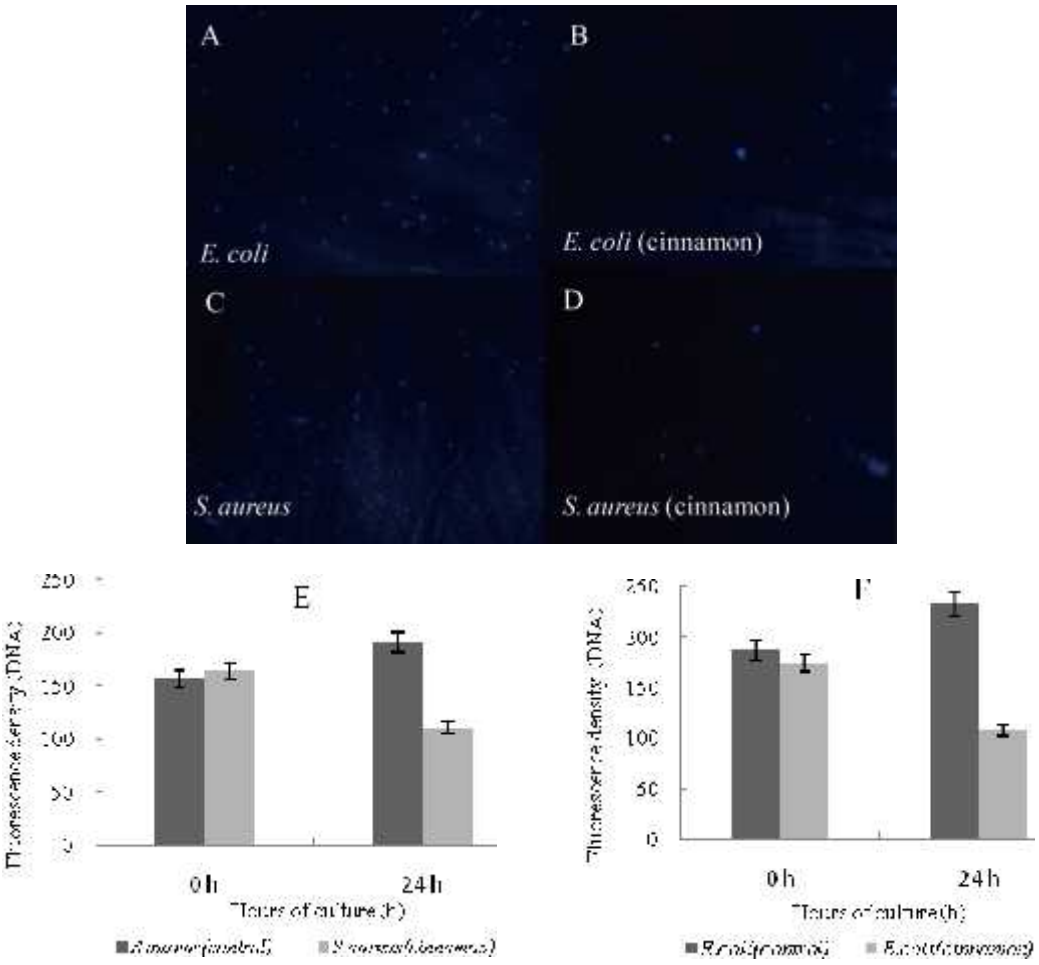


Fig. 5 LSCM image of *E. coli* before (A) and after (B) cinnamon oil treatment; LSCM image of *S. aureus* before(C) and after (D) cinnamon oil treatment; The fluorescence density of DNA of *S. aureus* (E); The fluorescence density of DNA of *E. coli* (F)

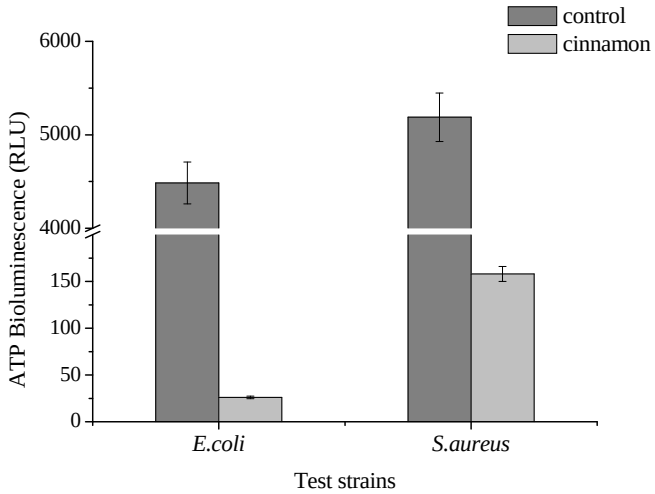


Fig. 6 The cellular ATP concentrations of *E. coli* and *S. aureus* before and after cinnamon oil treatment.

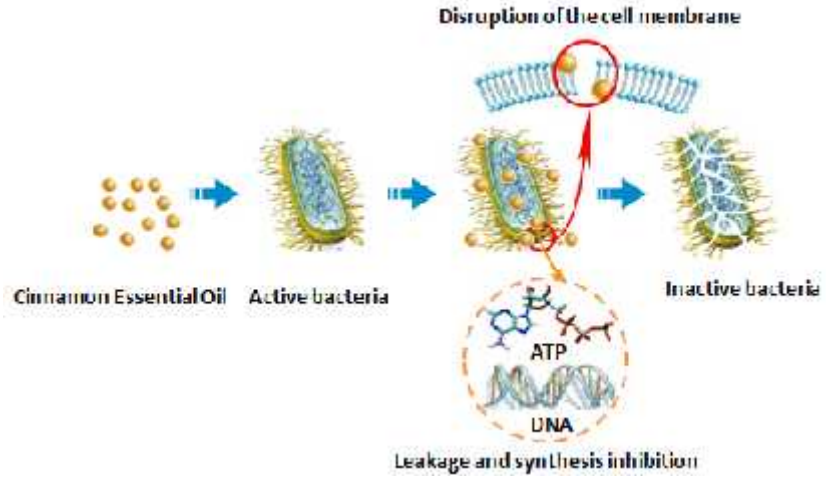


Fig.7 The antibacterial mechanism of cinnamon oil

Conclusion: This study focused on evaluating the bactericidal performance and revealing the bactericidal mechanism of cinnamon oil. Through this study, cinnamon oil displayed very well antibacterial activity to several pathogens. Its antibacterial performance was also been tested in milk. As a kind of natural, safe spice, cinnamon oil will have good prospects in the preparation of antibacterial agent especially in food industry.

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