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To cite this article: Zhiling Tang *et al* 2020 *IOP Conf. Ser.: Earth Environ. Sci.* **512** 012080

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Study on Antibacterial Activity of *Amomum tsao-ko* Extracts against *Bacillus subtilis* and *Listeria monocytogenes* and Preliminary Investigation of Its Antibacterial Mechanism

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Abstract: The main objective of this study was to preliminarily investigate the antibacterial activity and mechanism of action of *Amomum tsao-ko* (*A. tsaoko*) extracts against *Bacillus subtilis* and *Listeria monocytogenes*. The results showed that the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *A. tsaoko* extracts against *B. subtilis* and *L. monocytogenes* were 1.25 mg/mL. Bacterial growth curves revealed that the *A. tsaoko* extracts had an obvious antibacterial activity against *Bacillus subtilis* and *L. monocytogenes*. Furthermore, the mechanism of action of *A. tsaoko* was evaluated by analyzing its effects on the integrity of cell membrane and cell morphology. The leakage of alkaline phosphatase (AKP) confirmed that the *A. tsaoko* extracts caused serious damage to the wall structure of *B. subtilis* and *L. monocytogenes*. Results of scanning electron microscopy (SEM) manifested that the destructive effect of *A. tsaoko* extracts on cell morphology and cell integrity of bacterial cells. The results implied that *A. tsaoko* extracts showed relatively good antibacterial effect on *B. subtilis* and *L. monocytog.*

1. Introduction

Food-borne pathogens have become one of the most serious problems that invade the body and cause a wide range of public health problem. The prevalence of spores is a major problem in the food industry, because the resurrection of certain microbial spores can cause food spoilage or foodborne diseases [1]. In the early 1980s, *Bacillus subtilis* (*B. subtilis*) was considered to be the culprit responsible for the corruption of baked goods [2]. *Listeria monocytogenes* (*L. monocytogenes*) is a gram-positive, non-spore-forming bacterium, which is considered as one of the most dangerous food pathogens [3]. Aiming at pathogenic and spoilage bacteria, pathogenic and spoilage bacteria, various antibacterial agents were adopted in recent years. Among them, natural antibacterial agents have become one of the hottest spots of research [4].

Amomum tsao-ko (*A. tsaoko*) is a zingiberaceous plant widely distributed in the southwest of China (Guangxi, Guizhou, Yunnan). *A. tsaoko* are currently used to remove the fishy smell because of its special pungent odor. *A. tsaoko* is used as not only an essential seasoning in daily life, but also a traditional Chinese medicine in the treatment of diarrhea, malaria, throat infections, stomach disorders, dyspepsia, nausea [5]. *A. tsaoko* is widely used to food preservation and antibacterial due to its



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broad-spectrum bactericidal, safe and non-toxic side effects. Moreover, some researchers have demonstrated that the *A. tsaoko* extracts can significantly inhibit the growth of bacteria such as two Gram-negative bacteria and five Gram-positive bacteria [6]. In addition, the essential oil of *A. tsaoko* showed good inhibitory effect on *Bacillus subtilis*, *Staphylococcus albicans*, *Escherichia coli*, *Aspergillus oryzae*, *Rhizopus*, *Penicillium* [7]. However, few studies have investigated the antibacterial mechanism of *A. tsaoko* extracts.

The purpose of this study was attempted to explore the antibacterial activity and its antibacterial mechanism of *A. tsaoko* extracts against *Bacillus subtilis* and *L. monocytogenes*. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were used to evaluate the antibacterial activity of *A. tsaoko* extracts. The growth curves of *Bacillus subtilis* and *L. monocytogenes* were determined for evaluating the antibacterial effect on the growth and reproduction of *Bacillus subtilis* and *L. monocytogenes*. In addition, the alkaline phosphatase (AKP), and scanning electron microscopy (SEM) were determined to study the integrity of cell membrane and cell morphology of *Bacillus subtilis* and *L. monocytogenes* and make a preliminary exploration about the antibacterial mechanism of *A. tsaoko* extracts. It will provide the theoretical basis for the *A. tsaoko* extracts as the natural preservative.

2 Materials and methods

2.1 Materials and chemicals

The samples of *A. tsaoko* were purchased in dongmen market (Haikou, Hainan, China). Using ultrasonic extraction methods, the ethyl acetate extracts of *A. tsaoko* was extracted from *A. tsaoko* and stored at 4°C for use [8,9]. Beef extract-peptone medium and Phosphate buffered saline (PBS, 0.1 M, PH 7.2) were purchased by Beijing Solarbio Science and Technology Company (Beijing, China). All chemicals were of analytical grade.

2.2 Bacterial strain

B. subtilis and *L. monocytogenes* were provided by microbe laboratory of Food Science and Technology in Hainan University (Haikou, Hainan, China). After subculture and activation of *B. subtilis* and *L. monocytogenes* in the nutrient gravy agar medium, the bacteria were cultured in 37 °C incubator for 18~24 hours, and the concentration of the bacterial solution was adjusted to $10^6 \sim 10^7$ CFU/mL by McFarland turbidimetry [10].

2.3 Determination of minimum inhibitory concentration (MIC)

The agar dilution method was used to examine the antimicrobial activity of *A. tsaoko* extracts against *B. subtilis* and *L. monocytogenes*. *A. tsaoko* extracts were dissolved in acetone and diluted to 200, 100, 50, 25, 12.5, 6.25 and 3.125 mg/mL. The antibacterial solution (2 mL) and nutrient broth culture medium (18 mL) were mixed in plates so that the final concentrations were 20, 10, 5, 2.5, 1.25, 0.625 and 0.3125 mg/mL respectively. Then, the bacterial suspension (0.2 mL) with concentration of $10^7 \sim 10^8$ CFU/mL was spread evenly on every plate. Sterile water and pure acetone were marked as blank control and negative control respectively. After inoculation, those plates were inverted in constant temperature incubator at 37 °C for 24 hours.

2.4 Determination of minimum bactericidal concentration (MBC)

The specific method was the same as determination of MIC. Instead, those plates were inverted in constant temperature incubator at 37 °C for 48 hours.

2.5 Determination of growth curves

The growth curves of *B. subtilis* and *L. monocytogenes* was determined by the ultraviolet spectrophotometry method [11]. First, the bacterial solution (5%) was added to nutrient broth culture medium at a concentration of $10^7 \sim 10^8$ CFU/mL and grown to logarithmic growth phase and then

cultured with *A. tsaoko* extracts at MIC (shaken in an oscillator, 180 rpm). Then the culture medium cultivated at 30°C with shaking 180 rpm. Finally, at selected time intervals, the OD 600 values of the samples were determined. Transverse axis represents time (0 h to 24 h). The vertical axis represents value of OD 600.

2.6 Determination of alkaline phosphatase

A. tsaoko extracts (1×MIC) were added to the logarithmic phase bacteria suspension of *B. subtilis* and *L. monocytogenes* and cultured at 30°C and 37°C, respectively. After incubation for 0, 2, 4, 6, 8 h, the bacteria suspension was collected by centrifugation, then the OD 520 of different samples were measured by a microplate reader (Flash Spectrum Biological Technology Co., Ltd., Shanghai, China). According to the requirements of AKP kit, the values of AKP activity were calculated.

2.7 Scanning electron microscope (SEM)

The morphological changes of bacteria were observed by SEM (S-4800, Hitachi, Tokyo, Japan) [12]. *B. subtilis* and *L. monocytogenes* that were cultured to their respective logarithmic growth phase were incubated with *A. tsaoko* extracts (1×MIC) for 4 h and 8 h at 30°C and 37°C, respectively. Then bacteria cells were collected by centrifugation at 6000 rpm for 10 min. Subsequently, the cells were washed with PBS (0.1 M, pH 7.2, 3 times) and were dehydrated by 20%, 40%, 60%, 80% and 100% ethanol. Then, the dehydrated samples were freeze-dried overnight by a freeze dryer (Han Mei Ecology Instrument Co., Ltd., Beijing, China). Finally, the samples were sputter-coated with gold under vacuum and observed by SEM.

3. Results and discussion

3.1 The minimum inhibitory concentration

The minimum dilution concentration for invisible bacterial growth was defined as the MIC [13]. As shown in Table 1, *B. subtilis* and *L. monocytogenes* could not grow when the concentration of *A. tsaoko* extracts was 1.25 mg/mL. Therefore, the MIC against both strains was 1.25 mg/mL, and acetone had no effect on the growth of the two strains. In addition, at the same concentration, the inhibitory effect of *A. tsaoko* extracts on *L. monocytogenes* was stronger than that on *B. subtilis*.

Table 1. The growths of *B. subtilis* and *L. monocytogenes*

Bacterial	control group		concentration of <i>A. tsaoko</i> extracts (mg/ml)						
	Sterile water	acetone	0.3125	0.625	1.25	2.5	5	10	20
<i>B. subtilis</i>	+++	+++	+++	++	-	-	-	-	-
<i>L. monocytogenes</i>	+++	+++	++	+	-	-	-	-	-

“—” represents no bacteria; “++” represents a medium number of bacteria; “+++” represents a large number of colonies.

3.2 The minimum bactericidal concentration

The minimum bactericidal concentration (MBC) refers to the minimum drug concentration required to kill 99.9% of the tested microorganisms [14]. The MBC of *A. tsaoko* extracts to *B. subtilis* and *L. monocytogenes* was 1.25 mg/mL, which was the same as the MIC.

Table 2. The growths of *B. subtilis* and *L. monocytogenes*

Bacterial	control group		concentration of <i>A. tsaoko</i> extracts (mg/ml)						
	Sterile water	acetone	0.3125	0.625	1.25	2.5	5	10	20
<i>B. subtilis</i>	+++	+++	+++	++	-	-	-	-	-
<i>L. monocytogenes</i>	+++	+++	+++	++	-	-	-	-	-

“—” represents no bacteria; “++” represents a medium number of bacteria; “+++” represents a large number of colonies.

3.3 Growth curves of *B. subtilis* and *L. monocytogenes*

In a certain wavelength range, the concentration of bacterial suspension is in direct proportion to the OD value [15]. Therefore, the inhibitory effect of *A. tsaoko* extracts on *L. monocytogenes* and *B. subtilis* could be judged by observing the changes of OD value. Sterile water (blank control group) was marked as control 1, acetone (negative control group) was marked as control 2. As shown in Fig. 1, bacterial growth in the control groups followed the model s-shaped growth curve in general. Blank control groups of *L. monocytogenes* and *B. subtilis* reached the logarithmic period after 8 h and 10 h, respectively. Besides, negative control groups of *L. monocytogenes* and *B. subtilis* reached the logarithmic period after 10 and 12 hours, respectively. Even though the growth of *L. monocytogenes* and *B. subtilis* in negative control groups was slower than that in blank control groups, there was no significant effect on the final growth status of bacteria. However, the OD 600 values of *L. monocytogenes* and *B. subtilis* treated by *A. tsaoko* extracts (1×MIC) were significantly lower than that of the control groups and increased slowly, which indicated that the *A. tsaoko* extracts had inhibitory effect on the *L. monocytogenes* and *B. subtilis*.

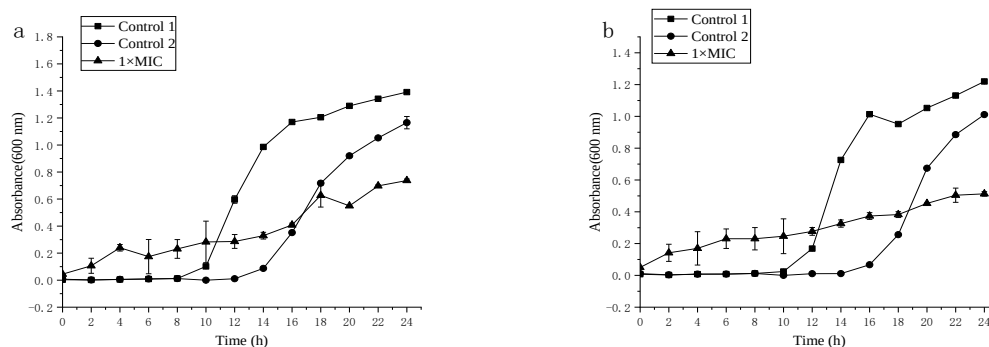


Fig.1. Effect of the MIC of *A. tsaoko* extracts on bacterial dynamic growth curves. (a) *L. monocytogenes*, (b) *B. subtilis*

3.4 Cell wall permeability of *L. monocytogenes* and *B. subtilis*

Alkaline phosphatase (AKP) is a kind of intracellular enzyme that exists between the cell wall and membrane of many prokaryotes [16]. Under normal circumstances, AKP activity is not detected extracellularly unless the cell wall is destroyed and AKP leaks into the extracellular environment [17]. As shown in Fig. 2a, there was no significant difference in the AKP activity of *L. monocytogenes* treated with *A. tsaoko* extracts compared to the control groups during 4 h. The AKP activity of *L. monocytogenes* treated with the *A. tsaoko* extracts (1×MIC) increased rapidly and was much higher than that of the control groups after 4 hours. As shown in Fig. 2b, the AKP activity of *B. subtilis* treated with the *A. tsaoko* extracts was always higher than that of the control groups with a rapid growth. At 8 h, the AKP activity of *B. subtilis* treated with *A. tsaoko* extracts was 4 times higher than that of the blank control group at 8.143U. The results showed that *A. tsaoko* extracts could damage the

integrity of cell wall, increase the permeability of cell wall, and lead to leakage of a large amount of AKP.

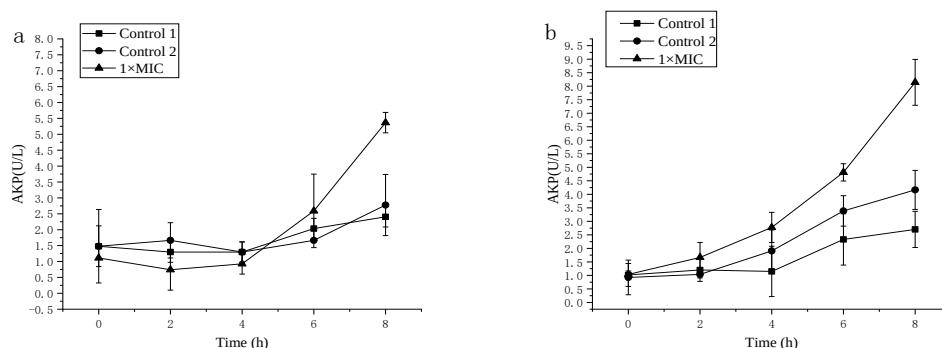
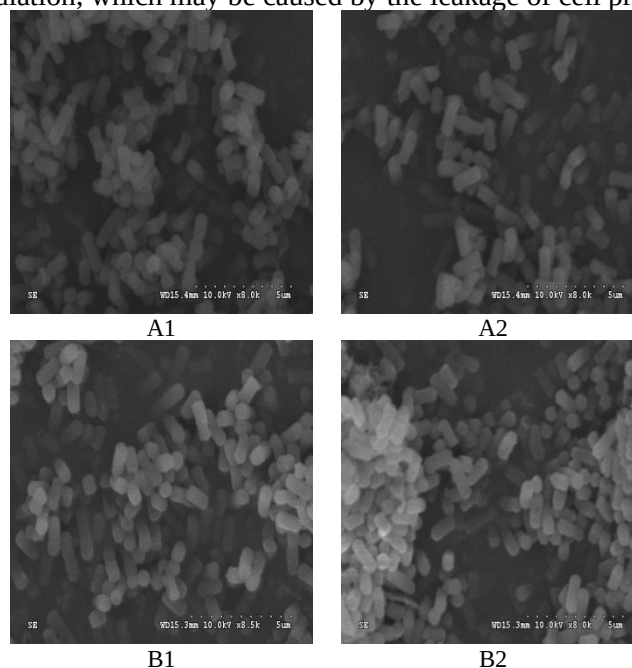


Fig. 2. Changes of Alkaline phosphatase (AKP) activity of *L. monocytogenes* and *B. subtilis* treated with *A. tsaoko* extracts. (a) *L. monocytogenes* (b) *B. subtilis*.

3.5 The effect of *A. tsaoko* extracts on the cell ultrastructure of *L. monocytogenes* and *B. subtilis*

Scanning electron microscopy clearly showed that most of the cells in the control groups (blank control group and negative control group) were complete and smooth with uniform appearance of cytoplasm, clear cell boundary (Fig. 3 A-B, D-E). The *B. subtilis* cells that were exposed to *A. tsaoko* extracts at 1× MIC for 4 h were shriveled and rough (Fig. 3 F1). Some of the cells were distorted, which may be due to the destruction of cell wall and cell membrane. However, the morphological changes of *L. monocytogenes* were not significant (Fig. 3 C1). According to Figure 2A, the effect of *A. tsaoko* extracts on *L. monocytogenes* was slow during the first 4 h. After 8 h, *L. monocytogenes* and *B. subtilis* treated with *A. tsaoko* extracts showed deformed cell morphology (Fig. 3 C2, F2), shrunken cells and rough and uneven cell surface. In addition, *B. subtilis* showed the phenomenon of cell aggregation and accumulation, which may be caused by the leakage of cell protoplasm.



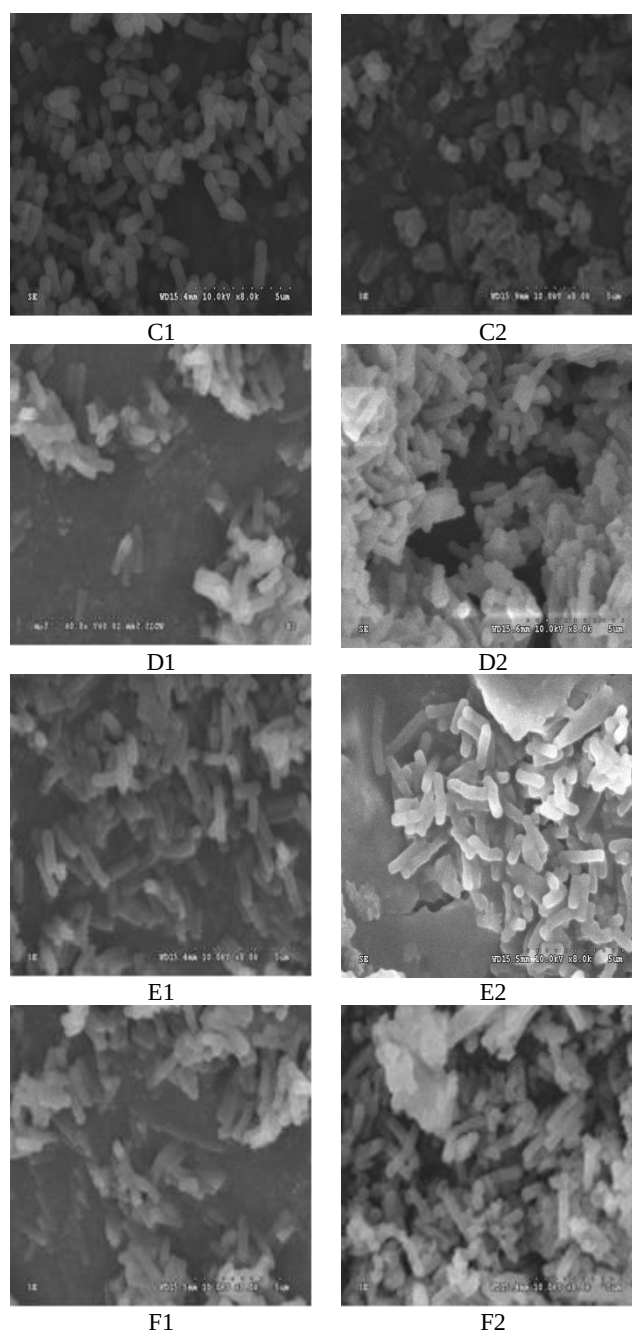


Fig. 3. Scanning electron microphotographs of *L. monocytogenes* and *B. subtilis*. *L. monocytogenes* untreated for 4 h (A1), untreated for 8 h (A2), treated with acetone for 4 h (B1), treated with acetone for 8 h (B2), treated with *A. tsaoko* extracts at 1× MIC for 4 h (C1), treated with *A. tsaoko* extracts at 1× MIC for 8 h (C2). *B. subtilis* untreated for 4 h (D1), untreated for 8 h (D2), treated with acetone for 4 h (E1), treated with acetone for 8 h (E2), treated with *A. tsaoko* extracts at 1× MIC for 4 h (F1), treated with *A. tsaoko* extracts at 1× MIC for 8 h (F2)

4. Conclusions

In summary, the current work confirmed that the *A. tsaoko* extracts have strong inhibition effects on *L. monocytogenes* and *B. subtilis*. The results revealed that the MIC and MBC of the *A. tsaoko* extracts against *L. monocytogenes* and *B. subtilis* were 1.25 mg/mL, and the growth of the two kinds of bacteria treated with *A. tsaoko* extracts at 1×MIC was obviously inhibited. In addition, the *A. tsaoko*

extracts inhibited *L. monocytogenes* and *B. subtilis* by destroying the cell wall, cell morphology and the integrity of the bacterial cells. In conclusion, this study confirmed that *A. tsaoko* extracts can act as a natural inhibitor against *L. monocytogenes* and *B. subtilis*, but it is necessary to further explore the antibacterial mechanism of *A. tsaoko* extracts in the future.

Acknowledgements

This research is supported by the Natural Science Foundation of Hainan Province of China (318MS016) and the National Natural Science Foundation of China (31760480).

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