Aquaculture and Fisheries xxx (2018) 1–7

Contents lists available at ScienceDirect

Aquaculture and Fisheries

journal homepage: www.keaipublishing.com/en/journals/aquaculture-and-fisheries/

Antibacterial mechanism of *Ginkgo biloba* leaf extract when applied to *Shewanella putrefaciens* and *Saprophytic staphylococcus*

Nannan Zhang [a, b, 1], Weiqing Lan [a, b, *, 1], Qian Wang [a, b], Xiaohong Sun [a, b], Jing Xie [a, b, **]

[a] College of Food Science & Technology, Shanghai Ocean University, Shanghai 201306, China
[b] Shanghai Engineering Research Center of Aquatic Product Processing and Preservation, Shanghai 201306, China

ARTICLE INFO

Article history:
Received 2 January 2018
Received in revised form
11 May 2018
Accepted 30 May 2018
Available online xxx

Keywords:
Ginkgo biloba leaf extracts (GBLE)
Shewanella putrefaciens
Saprophytic staphylococcus
Antibacterial mechanism

ABSTRACT

The antimicrobial mechanism of *Ginkgo biloba* leaf extracts (GBLE) when applied to predominant spoilage bacteria (*Shewanella putrefaciens* and *Saprophytic staphylococcus*) on refrigerated pomfret and minimal inhibitory concentrations (MICs) were measured by the plate counting method. GBLE at MIC and 2MIC were prepared in tryptic soy broth (TSB) medium and equivalent amounts of sterile distilled water were used in place of GBLE as a control group. The impact of GBLE on the growth of bacteria, the permeability of cell membrane, and cell wall were also investigated by growth curve of bacteria, alkaline phosphates activity (AKP), and electrical conductivity. A scanning electron microscope (SEM) was used to study the effects of GBLE on the cellular structure of *S. putrefaciens* and *S. staphylococcus*. The results showed that the MICs of GBLE when applied to *S. putrefaciens* and *S. staphylococcus* were 100 mg/mL, the inhibitory rates of MIC and 2MIC concentrations of GBLE when applied to *S. putrefaciens* were 36.11% and 100%, while 27.78% and 62.22% for *S. staphylococcus*. Meanwhile, GBLE inhibited the growth of *S. putrefaciens* and *S. staphylococcus* until the number of cells at 2MIC values decreased to 0 and 4.29 log CFU/mL, respectively, after 24 h. The electrical conductivity of bacteria increased with GBLE treatment, which was followed by an increased leakage of AKP. The SEM revealed that the structure of bacterial cells was destroyed and the bacteria began to be adhere to each other. The inhibition effect of GBLE when applied to *S. putrefaciens* and *S. staphylococcus* was related to the damage of cell membrane and cell wall. It was also revealed that GBLE damages the morphology of bacteria and had stronger effects on the cell membrane of *S. putrefaciens* than that of *S. staphylococcus*.

1. Introduction

Food, no matter the source, is always subject to spoilage, mainly due to microorganisms, particularly bacteria (Patra, Das, & Baek, 2015). *Pseudomonas*, *Aeromnas*, *Shewanella*, *Staphylococcus* are common organisms cultured from freshwater fish (He, Guo, Song, Li, & Zhang, 2017; Kačániová et al., 2017; Yano et al., 2015; Zhao, Zhu, Ye, Ge, & Li, 2016). *S. saprophyticus* has been identified as a major cause of spoilage in pomfret. Specific spoilage organisms (SSO) participate in the spoilage process at its onset. It is known that *Shewanella baltica* is identified to be an SSO for temperate fish species (Gu, Fu, Wang, & Lin, 2013; T.; Li, Li, & Hu, 2013; Zhao et al., 2016; Zhu, Zhao, Feng, & Gao, 2016). The growth of SSO is a matter of great concern. He, Guo, Song, Li, & Zhang. (2017) reported that 1% Chitosan combined with 0.6% Nisin had significant inhibitory effects on the growth and biofilm formation of *S. putrefaciens* and *Shewanella* algae in *P. crocea*. Studies have shown that plants and their byproducts are rich sources of phenolic substances possessing antimicrobial and antioxidant capabilities. Siwe, Krause, van Vuuren, Tantoh, & Olivier. (2014) examined the antibacterial activity of *Alchornea floribunda* extract when applied to *S. saprophyticus* through a micro-dilution assay and found that the minimum inhibitory concentration (MIC) value was 63 μg/mL. Al-Zoreky, & Al–Taher. (2015) studied the activity of extracts from *spathe* (date palm) applied to *S saprophyticus* through an agar diffusion assay and the inhibition zones and the minimum

Abbreviations: GBLE, Ginkgo biloba leaf extracts; MIC, minimal inhibitory concentration; TSB, tryptic soy broth; AKP, alkaline phosphates activity; SEM, scanning electron microscope.

* Corresponding author. 999 Huchenghuan Road, Shanghai Ocean University, Shanghai 201306, China.
** Corresponding author. 999 Huchenghuan Road, Shanghai Ocean University, Shanghai 201306, China.
E-mail addresses: wqlan@shou.edu.cn (W. Lan), jxie@shou.edu.cn (J. Xie).
[1] Equal contribution to the first author.

https://doi.org/10.1016/j.aaf.2018.05.005

inhibitory concentration (MIC) were 31 mm and 3 mg/mL, respectively. Plant extracts have shown considerable promise for a range of applications in the food industry.

Ginkgo biloba is a unique tree species in China and a living fossil that has existed for 200 million years (Boonkaew & Camper, 2005; López-Gutiérrez, Romero-González, Vidal, & Frenich, 2016; Zhang, Wang, Tao, & Fang, 2016). *Ginkgo biloba* leaf extract contains 24% flavonoids, 6% terpene trilactones, and less than 5 ppm of ginkgolic acids (van Beek & Montoro, 2009), which belong to a group of natural antioxidants (Boonkaew & Camper, 2005; Goh & Barlow, 2002). Previous studies indicated that extract from yellow and green *Ginkgo* (*Ginkgo biloba*) leaves have in vitro model systems with strong antioxidant activity. Joanna, Ewa, Magdalena, &, Dominik. (2014). Demonstrated that extracts of *Ginkgo biloba* leaves had stabilizing effects on lipids and cholesterol in pork meatballs over 21 days of refrigerated storage. Other studies, such as Xie, Hettiarachchy, Jane, &, Johnson. (2003), reported that GBLE and sodium ethylenediaminetetraacetic acid (EDTA) effectively inhibited the growth of *Listeria monocytogenes*, demonstrating the antibacterial capacity of GBLE. Q. U. Li, Yong-Mei, Song, Yan, & Han-Wei. (2014) studied the antibacterial activity of GBLE on *Staphylococcus aureus* and *Escherichia coli*, measured the antimicrobial circle diameter and indicated that GBLE showed potential for development and utilization as a natural antibacterial agent and antiseptic (Sati, Pandey, & Palni, 2012). mentioned that GBLE displayed antibacterial activities, with minimum inhibitory concentrations (MIC) reported to range between 300-400 and 400−600 μg/mL for Gram-positive and Gram-negative bacteria, and between 500-600 and 700−900 μg/mL for actinomycetes and fungi, respectively. It has been demonstrated that GBLE antibacterial activity is dependant on the strain examined and on their growth phase, GBLE concentration, pH, temperature, and the composition of the medium (Sati et al., 2012). Although the chemical compositions and antimicrobial properties of GBLE have been studied, there is little work reported on the mechanism that prevents spoiling bacteria from growing in aquatic products.

In this study, *S. putrefaciens* and *S. staphylococcus* were isolated from previously spoiled pomfret that were stored at 4 °C. The aim was to investigate the spoiling potential of *S. putrefaciens* and *S. staphylococcus* based MIC, inhibitory rate, the growth curve of bacteria, and permeability of cell membrane measurements, AKP assay, and SEM observation. This research suggests that GBLE should be used as a natural bacteriostatic agent for aquatic product storage.

2. Materials and methods

2.1. Materials

The fresh, pest-free, and fan-shaped *Ginkgo biloba* leaves were collected in main loop around Shanghai Ocean University, Shanghai, in August 2016. These leaves were cleaned, dried, and crushed into powder for use. 10 g *Ginkgo biloba* leaf powder was added with 500 mL 60% ethanol by an ultrasonic-assisted method and the conditions were extracted after 30 min at 60 °C. The ethanol extract was centrifuged with 6000 r/min at 4 °C for 20 min. Then, the filtrates were rotary-evaporated to remove ethanol and water and concentrated to a final volume of filtrates to 20 mL. Therefore, the concentration of GBLE was measured the absorbance at 510 nm and compared with rutin concentration. *S. putrefaciens* and *S. saprophyticus* were separated and purified from spoiled pomfret, identified, and preserved by the Shanghai Engineering Research Center of Aquatic Product Processing and Preservation.

2.2. Culture preparation

Strains of *S. putrefaciens* and *S. saprophyticus* were activated and inoculated into TSB (Sinopharm Chemical Reagent Co., Ltd.) medium, cultivated with shaking with 150 rpm at 37 °C for 7 h, to produce a final cell concentration of about 10^6–10^7 CFU/mL.

2.3. Determination of minimal inhibitory concentrations (MICs)

The antimicrobial effects of GBLE on *S. putrefaciens* and *S. saprophyticus* were determined by analyzing MICs via the method of plate counting (Liu et al., 2016). Serial dilutions of GBLE were prepared to obtain the final concentrations of 150, 125, 100, 75, 50, and 25 mg/mL in TSB mediums. The same amount of sterile distilled water was used as the control. Suspended *S. putrefaciens* and *S. saprophyticus* were inoculated to different concentrations of GBLE and mixed well to obtain a mixture with a final microbial content of 10^6 CFU/mL. 100 μL of the mixture was diluted 10-fold with sterile physiological saline in 1.5 mL centrifuge tube, then 100 μL of the appropriate dilution was uniformly coated on the TSA plates and incubated at 37 °C for 24 h, after which the colonies were counted (0 h GBLE treatment). At the same time, the mixtures were placed in shaker and incubated at 37 °C for 24 h. After that, the bacterial suspensions were diluted, coated, incubated and counted one more time (24 h GBLE treatment). The MICs were determined to be the lowest concentrations of GBLE at which the growth of tested bacteria was inhibited completely.

2.4. Determination of inhibitory rate

MIC and 2MIC concentrations of GBLE were added to sterile 96-well plates. Each well was inoculated with 100 μL of prepared bacteria suspended to the final concentration of 10^6 CFU/mL, then mixed well and cultured in a microplate oscillator at 37 °C for 12 h. Then the 96-well plates were measured by a Microplate Reader at 600 nm value. The inhibitory rate was defined as formula (1) (du Toit & Rautenbach, 2000):

$$Inhibitory\ Rate(\%) = \frac{OD_R - OD}{OD_R - OD_B} \quad (1)$$

where OD_R is absorbance value of a control well, OD is absorbance value of a sample well, and OD_B is absorbance value of a blank well.

2.5. Growth curves of bacteria

The *S. putrefaciens* and *S. saprophyticus* suspensions were added to GBLE at the concentration of MIC and 2MIC, and an equivalent amount of bacterial suspension without GBLE was used as control group. The bacteria were cultured at 37 °C in a 150 r/min mixer for 24 h. The bacteria growth was indexed by measuring the number of colonies on the plate surfaces every 2 h.

2.6. Cell membrane permeability assay

The effects of GBLE on cell membrane permeability of *S. putrefaciens* and *S. saprophyticus* were characterized by changes in electric conductivity. The prepared test bacteria suspensions were mixed with GBLE to final concentrations of MIC and 2MIC, and the control group was maintained without GBLE. The samples were mixed well and incubated in a 150 r/min mixer for 12 h at 37 °C, after which the conductivity was measured every 2 h with an electrical conductivity meter (Tao,Qian, & Xie, 2011).

Please cite this article in press as: Zhang, N., et al., Antibacterial mechanism of *Ginkgo biloba* leaf extract when applied to *Shewanella putrefaciens* and *Saprophytic staphylococcus*, Aquaculture and Fisheries (2018), https://doi.org/10.1016/j.aaf.2018.05.005