Mechanism of Action of Essential Oils Extracted from Bamboo (*Phyllostachys heterocycla cv. pubescens*) Leaves: Chemical Composition and Antimicrobial Activity against Four Food-related Microorganisms

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The antimicrobial activities and potential mechanisms of natural essential oils (EOs) derived from the leaves of *Phyllostachys heterocycla cv. pubescens* (bamboo) against a broad range of food-borne pathogens, which included Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria and yeast (*Saccharomyces cerevisiae*), were investigated. The chemical characterization of bamboo leaf essential oils (BLEOs) was determined by gas chromatography-mass spectrometry (GC-MS). Hexadecanoic acid (19.35%) and phytol (10.54%), the antimicrobial ingredients that have been reported in previous studies, were found to be the major components in the BLEOs. According to the antimicrobial tests, *Escherichia coli* was most sensitive to BLEOs, showing the largest DIZ (12.77 ± 0.25 mm) and the lowest MIC (0.56 mg/mL). In addition, the pathogen growth in the presence of BLEOs at two-times the minimum inhibitory concentration (MIC) revealed that the EOs were bacteriostatic after 12 h to all tested strains. According to the results from the cellular constituents, the fatty acids profiles, and the atomic force microscope (AFM) observations, the membranes of the treated cells were severely damaged. Therefore, it was concluded that the mechanism of action of BLEOs could be described as a disruption to the pathogen's membrane integrity. The results indicated that the EOs from the leaves of bamboo could be a potential source of antimicrobial agents in the future.

Keywords: Phyllostachys heterocycla cv. pubescens; Essential oils; Antimicrobial activities; Mechanism of action

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INTRODUCTION

Despite modern developments in food hygiene and production techniques, foodborne diseases caused by pathogens are still a considerable threat to the human health (Gould *et al.* 2013). Notable pathogens causing foodborne diseases include *Staphylococcus aureus*, *Listeria monocytogenes*, *Escherichia coli*, *Salmonella typhimurium*, *Vibrio parahaemolyticus,* and *Clostridium perfringens* (Lee *et al.* 2014), which damage food quality and can also lead to serious health issues to the people that consume the contaminated food. With increasing demand for food that is free from added synthetic

preservatives, natural, effective, and nontoxic food additives with a broad antimicrobial activity spectrum have garnered much attention (Burt 2004). It has been reported that chitosan-nanocellulose films have the potential to extend shelf life of ground meat (Dehnad *et al.* 2014). It is important to find more antibacterial agents against food pathogens to improve food safety.

It is known that aromatic plants' essential oils (EOs) and their components have a wide range of applications in ethnomedicine, food preservation, food flavorings, and perfumes and fragrances (Burt 2004; Bakkali *et al.* 2008). Several investigators have reported about the activities of natural essential oils as antimicrobial agents in food systems, and the essential oils were considered as an additional intrinsic determinant to increase the safety and shelf life of foods (Sagdic *et al.* 2003; Salgueiro *et al.* 2010). *Phyllostachys heterocycla cv. pubescens*, which belongs to the family Gramineae (approximately 300 species), is widely distributed in China, Japan, and Southeastern Asian countries (Peng and She 2014); it is one of the most important economic bamboo resources in the world. It has been reported that the EOs from bamboo leaves exhibit various biological activities, such as antioxidant (Kweon *et al.* 2001; Jin *et al*. 2011) and antimicrobial (Jin *et al.* 2011; Tao *et al.* 2017). However, there are few published investigations that report about the mechanism of the antimicrobial activity of EOs from bamboo leaves. In addition, broad-spectrum antimicrobial activities of bamboo leaf essential oils (BLEOs) still need to be studied in detail. Thus, the objectives of this work were to investigate the antimicrobial effect of BLEOs on major food-related microorganisms, and to determine the mechanism of antimicrobial activity of BLEOs.

EXPERIMENTAL

Materials

Plant materials and chemicals

Bamboo leaves from *Phyllostachys heterocyla cv. pubescens* were collected in Nanping City, Fujian Province, China. The leaf samples were sent to the College of Biological Sciences and Biotechnology, Beijing Forestry University (Beijing, China) for species confirmation. Nutrient agar (NA) and yeast peptone dextrose agar (YPDA) were purchased from Beijing Aoboxing Bio-tech Co., Ltd. (Beijing, China). All reagents used in the experiments were of analytical grade.

Microbial strains and culture

The antimicrobial activities of the EOs were tested on four food-related microorganisms, which included two Gram-positive bacteria (*Staphylococcus aureus* CICC 10384 and *Bacillus subtilis* CICC 20034), one Gram-negative bacteria (*Escherichia coli* CICC 23845), and one yeast (*Saccharomyces cerevisiae* CICC 1346). These strains were provided by the College of Biological Sciences and Biotechnology, Beijing Forestry University (Beijing, China), and were cultured at 37 °C in NA for bacteria and at 28 °C in YPDA for the yeast.

Methods

Extraction of BLEOs

The bamboo leaves were ground and extracted in a modified EOs extraction device described by Lv *et al.* (2010). A schematic diagram of the device is displayed in Fig 1.

Briefly, the dried bamboo leaves were ground and hydrodistilled (liquid-to-solids ratio of 25:1) for 4.5 h. During the heating, the steam containing the EOs was condensed. Then, the condensed water droplets were passed through *n*-hexane, and essential oils distilled in the condensed water were extracted into *n*-hexane. The water after *n*-hexane extraction was recirculated back to the distillation flask. Finally, the *n*-hexane was collected and dried over anhydrous sodium sulfate. The dried *n*-hexane was evaporated using a rotary evaporator (Manufacturer, City, Country), where the temperature (*T*) was < 40 °C under a vacuum, to yield the extracted EOs. The BLEOs obtained were stored in dark vials at 4 °C that were tightly closed until needed.

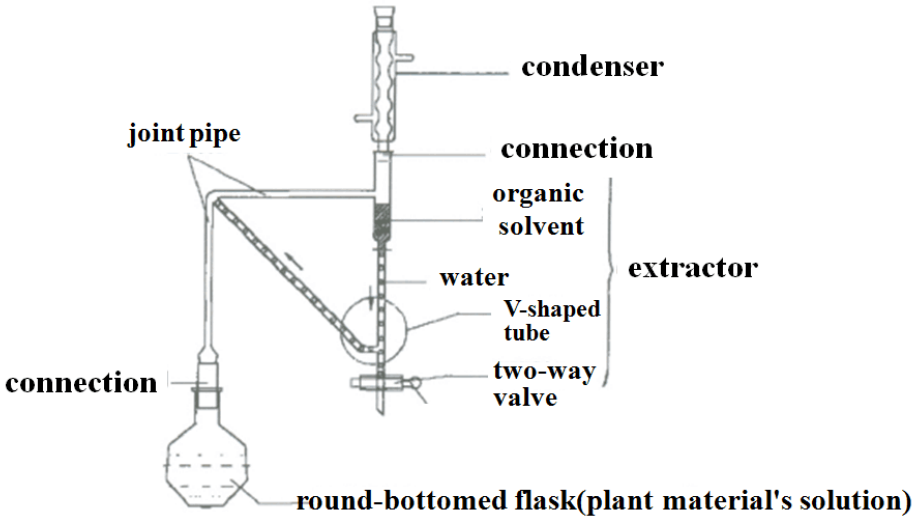

Fig. 1. The schematic diagram of the device used for the extraction of BLEOS

Chemical characterization analysis of the BLEOs

Chemical characterization of the BLEOs was carried out according Tao's method (Tao *et al*. 2017) using gas chromatography-mass spectrometry (GC-MS; GCMS-QP2010 Ultra; Shimadzu, Kyoto, Japan). The device was equipped with an Rtx-5MS quartz capillary column (30 m × 0.25 mm × 0.25 μm). The oven temperature was programmed to the following schedule: 60 °C for 2 min, raised to 220 °C at a rate of 3 °C/min, ramped to a rate of 15 °C/min to 280 °C, and held at 280 °C for 10 min. The injector and detector temperatures were 280 °C. The extracted essential oils (0.4 μL) were manually injected at a split mode of 15:1. High-purity helium was used as the carrier gas at a flow rate of 1 mL/min. The mass selective detector was operated in electron-impact ionization (EI) mode with a mass scan range from *m*/*z* 33 to 500 at 70 eV. The BLEOs constituents were qualitative compared to the published mass spectra data, as well as to the National Institute of Standards and Technology (NIST) mass spectra library data. The components of the BLEOs were determined as a relative percentage of the total oil by peak area.

Agar punching diffusion assay

The essential oils were completely dissolved in *n*-hexane at a concentration of 36.00 mg EOs/mL *n*-hexane. Antimicrobial tests were then performed by the agar punching diffusion method. A suspension (100 μL) that contained 10^5 to 10^6 CFU/mL of microbes was swabbed onto the surface of sterile NA or YPDA medium using a sterile cotton swab. A plastic straw with a diameter of 5 mm was then inserted into the inoculated agar, and 30

μL of essential oils were added by micropipette. The diameter of inhibition zone (DIZ) was measured after 24 h of incubation at 37 °C for bacteria, and 48 h of incubation at 28 °C for yeast; *n*-hexane was used as a negative control. The tests were performed in triplicate.

Minimum inhibitory concentration (MIC)

The MIC was determined according to the method described by Lee *et al.* (2014) with minor modifications. Two-fold serial dilutions of BLEOs were prepared in melted NA or YDPA medium in sterile test tubes; the final concentrations of the samples ranged from 0.56 to 18 mg EOs/mL medium. The test tubes were inoculated with 100 μL of the test pathogen suspension to yield a microbe concentration of 10^5 to 10^6 CFU/mL. The mixtures were poured into plates and then inoculated as described earlier. The MIC values were reported as the lowest concentration of the BLEOs that inhibited microbial growth. A control test was also performed that contained the inoculated medium with only *n*-hexane.

Growth curve determination

The growth curve assay was determined in accordance with the method described by Muroi and Kubo (1993) with some modifications. Briefly, the cultivation with the EOs at two times the MIC value was executed in the same way as the above assay. The cultures were incubated at 37 °C for bacteria and 28 °C for yeast. The samples were shaken on a rotary shaker at 120 rpm. At selected time intervals, the absorbance was measured at a wavelength of 600 nm (OD_{600nm}) for bacteria and 560 nm (OD_{560nm}) for yeast.

Release of cellular materials

The release of cellular materials was measured and assessed in accordance with previous literature reports with some modifications (Oussalah *et al.* 2006; Xu *et al*. 2010; Moshayedi *et al.* 2013). In brief, the tested strains were cultured to a logarithmic growth phase and then centrifuged (at 8000 rpm for 20 min) to collect the microbial cells. These were then washed three times with sterile water and then resuspended in 0.1 M phosphate buffer saline (PBS (pH 7.2)). The essential oils were added into the cell suspensions to procure the required concentration of two times the MIC value. After incubation for 6 h at 37 °C for bacteria (or 28 °C for yeast), 20 mL of each sample was collected and centrifuged at 12,000 rpm for 5 min. The supernatants obtained were analyzed to determine the concentrations of proteins and sugar in accordance with the method described by Xu *et al.* (2010). In addition, 3 mL of the supernatant was gathered; the ultraviolet (UV) absorption at 260 nm of the supernatant was recorded to measure the concentration of nucleic acids released by the cellular material.

Analysis of membrane fatty acid composition

The cultured broths of each microorganism were centrifuged for 15 min at 5000 rpm, the solids were washed three times, and the washed solids were resuspended in 0.1 M phosphate buffer saline (PBS (pH 7.0)). After the addition of BLEOs and incubation for 3 h, the cell pellets were harvested and subjected to membrane fatty acid extraction. Extractions of fatty acids from the cellular materials were performed as described by Lv *et al.* (2011) with some modifications. Lipid samples were transmethylated for analysis of their acyl groups as fatty acid methyl esters (FAME) *via* a GC-MS (GCMS-QP2010 Ultra, Shimadzu, Kyoto, Japan) analysis. The oven temperature was programmed to the following schedule: initial temp at 120 °C for 2 min, then raised up to 200 °C at a rate of 3 °C/min, then ramped at an increment of 5 °C/min to 250 °C for 2 min, and raised up to 280 °C at a

rate of 8 °C/min, finally holding for 10 min at 280 °C. The injector and detector temperatures were 280 °C. The EOs (1 μL) were manually injected at a split mode of 20:1. High-purity helium was used as the carrier gas at a flow rate of 1 mL/min. The mass selective detector was operated in electron-impact ionization (EI) mode with a mass scan range from *m*/*z* 33 to 500 at 70 eV.

Atomic force microscope (AFM)

The effects of BLEOs on the microorganism's cell surface were examined by AFM (Bruker Multimode 8 AFM with Nanoscope V Controller, Bruker Corp., Karlsruhe, Germany). Cells were first collected by centrifuging the microbial suspension (10^5 to 10^6 CFU/mL) at 4000 rpm for 10 min; the solids were washed with 0.1 M PBS (pH 7.2) three times and resuspended in 0.1 M PBS (pH 7.2). The treated microbial suspension samples were incubated at a concentration of two times the MIC of essential oils for 3 h. After the incubation, the cells were collected, washed twice, and immobilized overnight in the PBS that contained 2.5% glutaraldehyde. The control samples (without essential oils) and treatment samples were both applied on a freshly cleaved mica surface, which was followed by air-drying. All samples were prepared at the same time and were exposed to the same environmental conditions. The AFM images were obtained in tapping mode using a silica probe (NP-S20; Veeco Instruments, Plainview, NY, USA) with the settings of 512 pixels/line and 1.5 Hz scan rate (Ziaee *et al.* 2010; Campos *et al.* 2012).

Statistical analysis

All values were expressed as means ± standard deviation (SD) of three experiments. The Student's *t*-test was used for the statistical analysis, and a value of $\rho < 0.05$ was considered statistically significant. The photographs of AFM and figures were only the representative. Microsoft Excel 2016 software (Redmond, WA, USA), Origin 8.5 software (Origin Lab, Northampton, MA, USA), and SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA) were employed for the data analysis.

RESULTS AND DISCUSSION

Chemical Composition of the EOs

Qualitative and quantitative analyses of the volatile compounds in the essential oils were determined *via* GC-MS; the results are presented in Table 1. In total, 39 components were identified, which accounted for 95.83% of the total detected constituents in the essential oils from the leaves of *P. heterocyla cv. pubescens*. Hexadecanoic acid (19.35%), phytol (10.54%), and pentacosane (9.89%) were the major components, followed by lauric acid (4.65%), 4-hydroxy-2-methylacetophenone (4.27%), tetratriacontane (4.16%), isophytol (3.43%), *α*-Ionone (3.23%), dibutyl phthalate (2.65%), 6,10,14-trimethyl-2-pentadecane ketone (2.64%), eicosane (2.47%), and damascenone (2.09%). Other identified compounds that were present were at concentrations lower than 2%. Organic acids, alcohols, hydrocarbons, and ketones were the main constituents. The profile obtained in this study was very similar to previous results reported by Tao *et al.* (2017) with only slight differences in concentrations. Conversely, Jin *et al*. (2011) reported that the main components comprising the essential oils of bamboo leaves were *cis*-3-hexenol (27.11%), 3-methyl-2-butanol (6.8%), (*E*)-2-hexenal (5.32%), and hexadecanoic acid (3.19%); these

reported literature values are very different than the chemical compositions obtained from this study. These differences might arise from geographical origins, cultivation varieties, seasonal variations, and extraction methods, as well as genetic differences of bamboo species (Perry *et al.* 1999).

Table 1. Chemical Composition of BLEOs

No.	Compound	Molecular Formula	Retention Index	Concentration (Peak Area %)
1	Hexanol	$C_6H_{14}O$	860	1.01
2	Leaf alcohol	$C_6H_{12}O$	868	1.75
3	Cyclohexanol	$C_6H_{12}O$	908	0.34
4	5,6-Diethyl-1,3-cyclohexadiene	$C_{10}H_{16}$	1005	0.67
5	2,3-Dihydrobenzofuran	C_8H_8O	1036	0.78
6	1-Nonen-4-ol	$C_9H_{18}O$	1068	1.29
7	Benzeneacetaldehyde	C_8H_8O	1081	1.67
8	Nonanal	$C_9H_{18}O$	1104	0.8
9	Menthone	$C_{10}H_{18}O$	1148	0.53
10	Safranal	$C_{10}H_{14}O$	1186	0.3
11	4-Ethylbenzaldehyde	$C_9H_{10}O$	1195	0.45
12	2,6-Dimethyl-1-cyclohexen-1-yl acetate	$C_{10}H_{16}O_2$	1208	1.76
13	Nonanoic acid	$C_9H_{18}O_2$	1272	0.37
14	2-Undecenal	$C_{11}H_{20}O$	1311	0.34
15	4-Hydroxy-2-methylacetophenone	$C_9H_{10}O_2$	1363	4.27
16	Geranylacetone	$C_{13}H_{22}O$	1420	1.24
17	*α*-Ionone	$C_{13}H_{20}O$	1429	3.23
18	Damascenone	$C_{13}H_{18}O$	1440	2.09
19	*β*-Ionone	$C_{13}H_{20}O$	1457	1.02
20	Ionone epoxide	$C_{13}H_{20}O_2$	1479	0.85
21	Lauric acid	$C_{12}H_{24}O_2$	1570	4.65
22	Dicyclohexyl ketone	$C_{13}H_{22}O$	1576	0.53
23	6,10,14-trimethyl-2-pentadecane ketone	$C_{18}H_{36}O$	1754	2.74
24	Myristic acid	$C_{14}H_{28}O_2$	1769	0.86
25	Isophytol	$C_{20}H_{40}O$	1899	3.43
26	Farnesyl acetone	$C_{18}H_{30}O$	1902	1.58
27	Hexadecanoic acid	$C_{16}H_{32}O_2$	1968	19.35
28	Stearaldehyde	$C_{18}H_{36}O$	1999	1.16
29	Eicosane	$C_{20}H_{42}$	2009	2.47
30	Dibutyl phthalate	$C_{16}H_{22}O_4$	2037	2.65
31	Phytol	$C_{20}H_{40}O$	2045	10.54
32	Methyl oleate	$C_{19}H_{36}O_2$	2085	2.03
33	Geranyl linalool	$C_{20}H_{34}O$	2192	0.77
34	Pentacosane	$C_{25}H_{52}$	2506	9.89
35	Hexacosane	$C_{26}H_{54}$	2606	0.89
36	1-Hexacosanol	$C_{26}H_{54}O$	2848	0.57
37	Tetratriacontane	$C_{34}H_{70}$	3401	4.16
38	Tetracosane	$C_{24}H_{50}$	3501	0.95
39	Hexatriacontane	$C_{36}H_{74}$	3600	1.85
Total identified components				95.83

Hexadecanoic acid (19.35%), phytol (10.54%), and pentacosane (9.89%) were the three major components in the BLEOs, and have been reported to have antimicrobial activity in previous studies (Karthikeyan *et al.* 2014; Lee *et al.* 2016). These literature

findings corroborate the reported observations of this study regarding the antimicrobial efficacy of the EOs from the leaves of *P. heterocyla cv. pubescens*.

DIZ, MIC, and Growth Curves of the BLEOs

The antimicrobial activities of the BLEOs were tested on four different microorganisms that were selected based on their relevance as food contaminants. The DIZ and the MIC values of the essential oils are presented in Table 2. The results showed that the essential oils had certain antimicrobial effects on all of the tested microorganisms. The DIZ values of the oil on the tested microorganisms ranged from 8.77 ± 0.25 mm to 12.77 ± 0.25 mm, and MIC values ranged from 0.56 to 2.25 mg oil/mL medium. Among these tested microorganisms, the essential oils exhibited the greatest antimicrobial effect against the Gram-negative bacteria *E. coli*, with both the highest DIZ value (12.77 mm) and the lowest MIC value (0.56 mg oil/mL medium). The inhibition zones of the essential oils on the test microorganisms showed a marked correlation with MIC values ($p < 0.05$). Comparing the DIZ and MIC values, *E. coli* was considerably the most sensitive microorganism; this was followed by *B. subtilis*, *S. cerevisiae*, and *S. aureus*, which showed similar susceptibility as was indicated by the same MIC value (2.25 mg oil/mL medium).

The effects of the BLEOs on the growth rate of the tested microorganisms, as measured by the OD_{600nm} for the bacterial strains and by the OD_{560nm} for the yeast strain, are shown in Fig. 1. For all the studied food pathogens, the treatment resulted in an appreciable decrease in the maximum growth rate in the log phase when compared to the control. Moreover, all of the samples treated with EOs had visibly lower stationary phase levels than the control, with *E. coil* showing the greatest reduction.

The results obtained indicated that the EOs from bamboo leaves had strong inhibitory effects to all of the tested food-borne pathogens, which included Gram-positive stains, Gram-negative strains, and yeast. The antimicrobial effects of the EOs derived from *P. heterocyla cv. pubescens* leaves to food-borne strains, such as *Staphylococcus aureus, Escherichia coli, Bacillus subtilis, Pseudomonas fluorescens*, and *Flavobacterium columnare*, have been reported by some recent investigations (Jin *et al.* 2011; Tao *et al.* 2017).

These literature reports are congruent with the findings of this study. The growth curves of the microorganisms determined by measuring the viable cell counts indicated that the BLEOs had a negative effect on the cell viability of all the tested strains. *E. coil* was the most sensitive microorganism, which was in accordance with the DIZ and MIC results. Similar results were also obtained in Guo's work that the Gram-negative bacteria *E. coil* showed the lowest MIC and MBC values when acted upon by *Amomum tsaoko* essential oil (Guo *et al*. 2017).

The Gram-negative bacteria are generally considered less sensitive than the Gram-positive ones to the essential oil (Gill and Holley 2006), while several studies have also founded that some Gram-positive bacteria are less or equally sensitive to Gram-positive bacteria. Therefore, the antibacterial activity of essential oil probably depended on the type of essential oil more than the bacteria (Dorman *et al.* 2000). The exposure of microorganisms to EOs mainly affected the logarithmic growth phase. It was inferred that the normal division and reproduction of cells was inhibited by the BLEOs.

Table 2. DIZ and MIC Values with the Essential Oils from the Leaves of *P. heterocyla cv. pubescens* Against the Tested Microorganisms

Test Microorganisms	DIZ (mm)	MIC (mg Oil/mL Medium)
Bacillus subtilis	11.97±0.29[a]	1.12
Staphylococcus aureus	8.77±0.25[b]	2.25
Escherichia coli	12.77±0.25[a]	0.56
Saccharomyces cerevisiae	9.77±0.46[b]	2.25

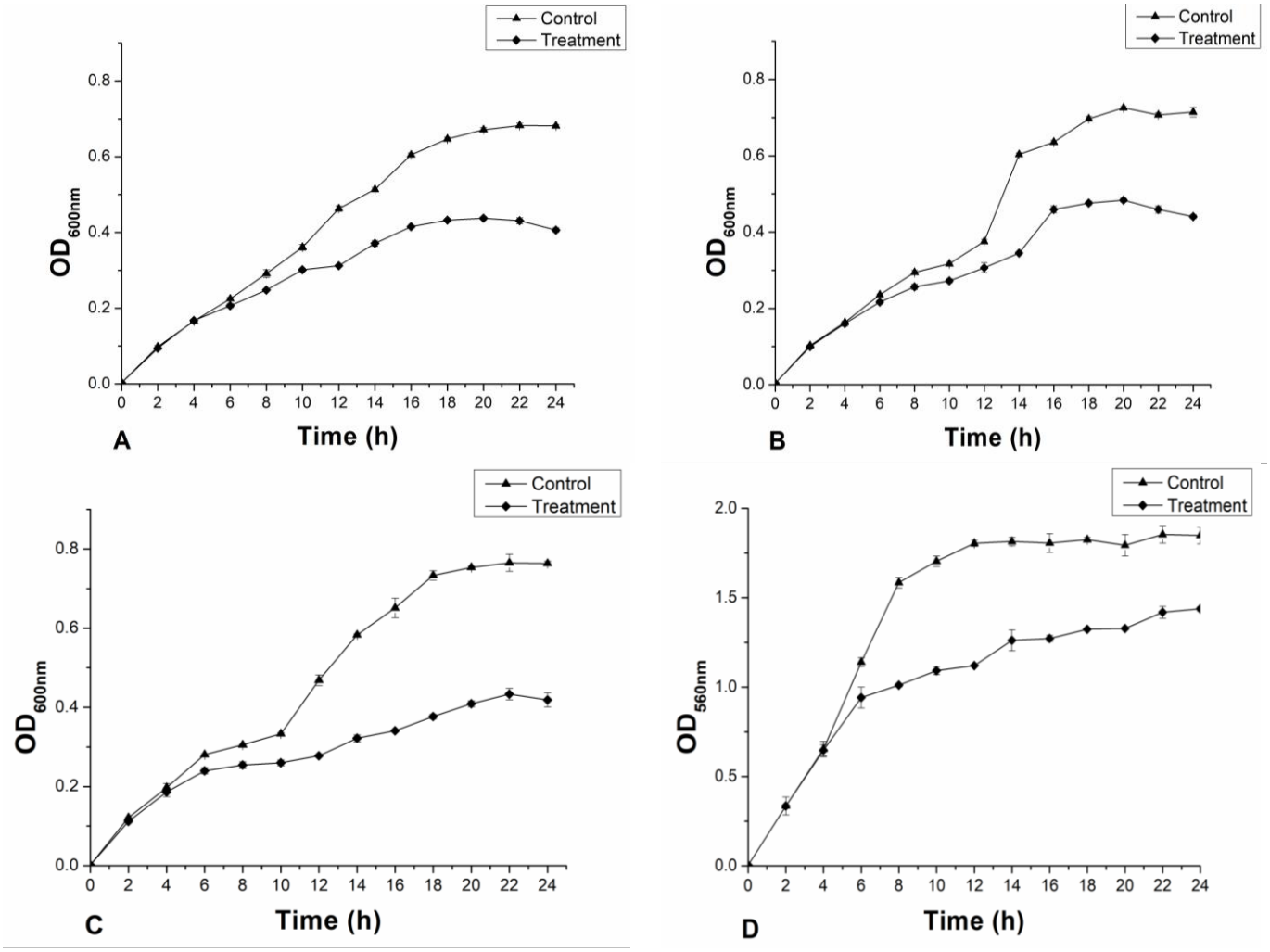

Fig. 2. The effect of BLEOs on the growth curves of the tested microorganisms at two times the MIC value for: (A) *B. subtilis*; (B) *S. aureus*; (C) *E. coli*; and (D) *S. cerevisiae*

Antimicrobial Mechanism of BLEOs

To further study the antimicrobial mode of the BLEOs, the major cell constituents and the profiles of the membranes' fatty acids were examined to note changes to the permeability and integrity of cell membranes. An AFM analysis was used to determine the surface characteristics of the microorganisms. Furthermore, AFM imaging is a highly suitable tool for the study of bacteria, making this technique extremely advantageous for the antibacterial effect. (Gaboriaud *et al*. 2005).

Table 3. Effect of the Essential Oils on Release of Cell Constituents by Tested Microorganisms

	Protein (μg/mL)				Sugar (μg/mL)				Cell Constituents (OD_{260})			
Treatment	*B. subtilis*	*S. aureus*	*E. coli*	*S. cerevisiae*	*B. subtilis*	*S. aureus*	*E. coli*	*S. cerevisiae*	*B. subtilis*	*S. aureus*	*E. coli*	*S. cerevisiae*
Control	46.6 ± 2.1a	41.0 ± 1.1[a]	55.4 ± 3.1[a]	50.7 ± 3.3[a]	29.6 ± 1.8[a]	28.2 ± 2.3[a]	33.1 ± 1.3[a]	992.4 ± 13.5[a]	0.180 ± 0.007[a]	0.162 ± 0.005[a]	0.160 ± 0.013[a]	0.113 ± 0.004[a]
2×MIC	54.4 ± 3.6[b]	59.5 ± 1.8[b]	68.6 ± 4.2[b]	71.5 ± 4.3[b]	35.8 ± 2.2[b]	33.2 ± 3.0[b]	38.2 ± 2.8[b]	1131.1 ± 18.3[b]	0.277 ± 0.005[b]	0.188 ± 0.004[b]	0.349 ± 0.006[b]	0.222 ± 0.006[b]

Values represent mean values of three independent replicates (with ± standard deviations). Different superscripted letters within a column indicate statistically significant differences between the two means ($p < 0.05$) *via* the Tukey's paired comparison statistical test; 2×MIC stands for twice the MIC concentration of BLEOs.

Table 4. The Effect of BLEOs on the Fatty Acids Profiles of the Tested Microorganisms

	E. coli		*S. aureus*		*B. subtilis*		*S. cerevisiae*	
FAME*	Control	Treated	Control	Treated	Control	Treated	Control	Treated
C10:0	ND[b]	ND	ND	ND	ND	ND	5.22 ± 0.113	4.275 ± 0.077
C12:0	3.51 ± 0.02[a]	6.63 ± 0.2917	ND	3.635 ± 0.007	ND	7.48 ± 0.064	4.47 ± 0.467	7.145 ± 0.120
C14:1	ND	ND	ND	ND	ND	ND	1.61 ± 0.035	1.645 ± 0.035
C14:0	6.47 ± 0.057	6.92 ± 0.304	ND	4.92 ± 0.014	ND	5.24 ± 0.106	2.95 ± 0.707	3.87 ± 0.757
C15:0	2.12 ± 0.014	2.30 ± 0.106	ND	1.675 ± 0.007	9.22 ± 0.057	4.06 ± 0.085	ND	0.645 ± 0.007
C16:1	10.95 ± 0.085	5.27 ± 0.240	ND	4.195 ± 0.007	ND	ND	34.80 ± 2.451	29.44 ± 2.345
C16:0	45.58 ± 1.280	60.37 ± 1.435	56.41 ± 4.27	59.54 ± 2.234	57.3 0± 2.34	55.82 ± 0.900	32.67 ± 3.405	33.49 ± 3.340
C17:1 *cis*	5.48 ± 0.042	2.30 ± 0.106	ND	1.925 ± 0.007	ND	ND	ND	ND
C18:2	ND	ND	ND	2.805 ± 0.104	ND	2.99 ± 0.064	ND	1.68 ± 0.102
C18:1	6.17 ± 0.049	6.06 ± 0.269	ND	6.575 ± 0.242	ND	7.15 ± 0.141	11.15 ± 1.233	11.37 ± 1.220
C18:0	19.73 ± 1.004	10.17 ± 2.758	43.59 ± 4.27	14.73 ± 2.305	33.48 ± 1.24	17.27 ± 1.358	7.15 ± 0.922	6.45 ± 0.655

* FAME is fatty acid methyl ester; [a] Percentage values, means of duplicate measurements (with ± standard deviations); [b] Not detected

Release of cellular materials

The release of cellular material from the four tested strains into the medium was measured and assessed to determine whether the cells' membranes were damaged after being treated by the BLEOs. The major cellular constituents included proteins and sugar; the UV absorbance at 260 nm was also measured, which is regarded as an index of cell lysis (Kasi *et al.* 2010). The results of these measurements are presented in Table 3. The results showed that after the addition of twice the MIC concentration of BLEOs for 6 h, the release of cell constituents significantly increased ($p < 0.05$) when compared to the control under all conditions. The increase in the measured values indicated that the microorganism exposure to the BLEOs resulted in the damage of cells' membranes and appreciable cellular leakage.

Changes of membrane fatty acids profiles

It has been shown that the microbial cells would regulate the membrane's fluidity by changing its fatty acid chain length and the fatty acid composition, when being in the attack from the antimicrobial compounds (Pasqua *et al*. 2006). Therefore, the profile alterations to membrane fatty acids profiles were examined from the cellular extracts after microorganism's exposure to BLEOs for 3 h. The extracted fatty acids were analyzed by GC-MS once they were derivatized to their corresponding FAMEs. The major fatty acids were identified and their concentrations were determined in the extracts of the control and the treatment for each microorganism; the results are presented in Table 4. The results showed that the presence of BLEOs affected the lipid profiles of all microorganisms examined, both in the quantity and the fatty acid types. As shown in Table 4, the fatty acid compositions of *S. aureus* and *B. subtilis* were strongly affected by the presence of BLEOs, especially in the fatty acid types. The *E. coli* cells, after treatment by BLEOs, showed a significant increase in its lipid profile ($p < 0.05$) of saturated C16 fatty acids, and a decrease of the saturated C18 fatty acids. However, the fatty acids profile of the yeast (*S. cerevisiae*) after treatment showed little alteration when compared to the control.

AFM observations

Application of AFM for spatial resolution surface imagining has been recommended as a useful tool for gaining insight across the range from subcellular structures to multi-cellular assemblies (Kaminskyj and Dahms 2008). The AFM method is theoretically capable of higher resolution imaging than other microscopes and delivering quantitative results. Morphological characterizations of the microorganisms of both the untreated (control) and essential oils treated are presented in Fig. 2. The left column of graphs (control) displayed typical rod-shaped cells for *B. subtilis* and *E. coli*, and cocci-shaped cells for *S. aureus* and *S. cerevisiae.* The images of the samples treated with essential oils at a concentration of twice the MIC concentration for 3 h are shown in the right column of Fig. 2.

When compared to the corresponding control, all the images showed notable differences in cell structure; treated microorganisms exhibited cellular collapse and rougher surfaces. These observations were confirmed by the measurement of the membrane roughness (Table 5).

Table 5. Measured AFM Parameter Roughness from the Tested Microorganisms

Tested Microorganisms	R_q (nm)	SD
B. subtilis	21.54	6.88
B. subtilis-treated	33.43	2.10
S. aureus	28.22	3.31
S. aureus-treated	43.88	8.95
E. coli	21.18	8.01
E. coli-treated	43.66	7.88
S. cerevisiae	10.77	2.09
S. cerevisiae-treated	56.32	3.90

R_q represents the root mean square roughness and SD represents the standard deviation

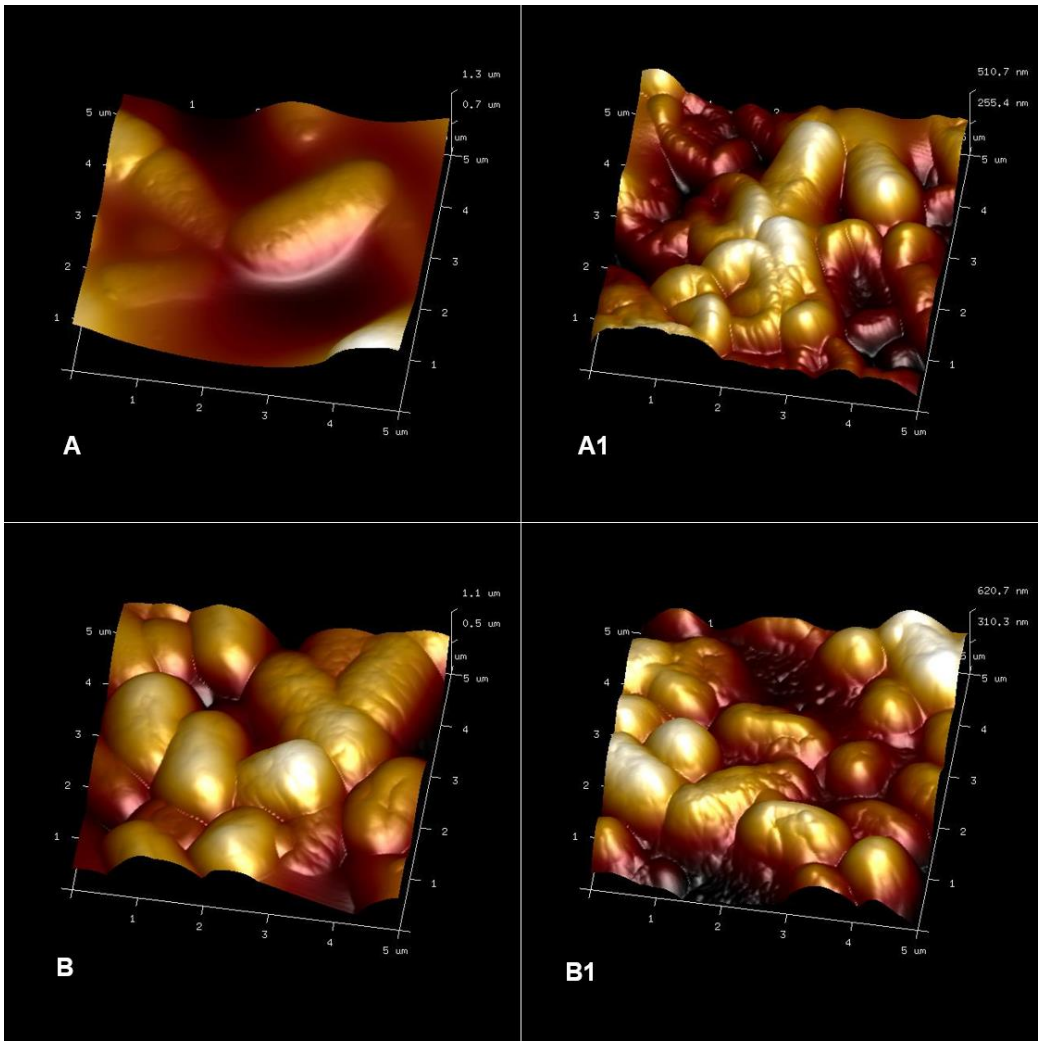

Fig. 3 (part 1). AFM images (in tapping mode) of the effect of BLEOs on cell morphology for: untreated (A) B. *subtilis*, (B) *S. aureus*, (C) *E. coli*, and (D) *S. cerevisiae*; and treated with essential oils (A1) *B. subtilis*, (B1) *S. aureus*, (C1) *E. coli*, and (D1) *S. cerevisiae*; treatment was performed with essential oils at two times the MIC concentration for 3 h

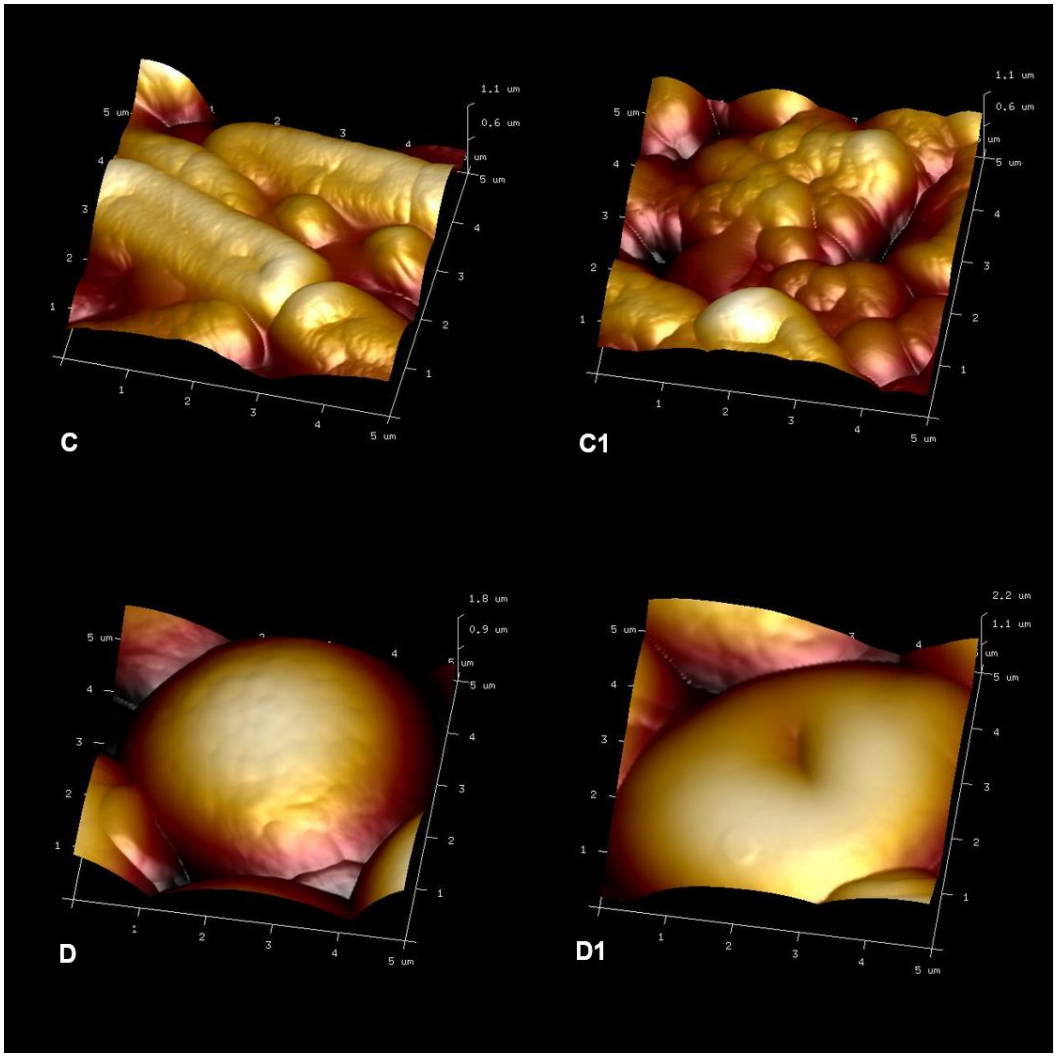

Fig. 3. (part 2). AFM images (in tapping mode) of the effect of BLEOs on cell morphology for: untreated (A) B. *subtilis*, (B) *S. aureus*, (C) *E. coli*, and (D) *S. cerevisiae*; and treated with essential oils (A1) *B. subtilis*, (B1) *S. aureus*, (C1) *E. coli*, and (D1) *S. cerevisiae*; treatment was performed with essential oils at two times the MIC concentration for 3 h

Significant variations in the roughness values were found between the BLEO treated and BLEO untreated cells of all the tested strains. Nevertheless, there were also some undamaged cells in the tested strains. In all the cases tested, the treated *E. coli* was observed to be more deformed, which indicated cellular rupture and lysis of the membranes (Fig. 2C1).

The results presented here showed that the release of constituents from the microorganisms increased and the profile of the fatty acids changed when the cells were treated with BLEOs. This observation indicated an increase in cell membrane permeability. Some authors have suggested that the distortion of the cell walls and cytoplasmic membranes causes the expansion and destabilization of the membranes, which increases membrane permeability that results in the leakage of various vital intracellular constituents, leading to cell death (Lv *et al.* 2011). These findings indicated that irreversible damage to the cytoplasmic membranes occurred after exposure to BLEOs, which was supported by the AFM observations. In addition, some studies reported that the active ingredients of the

EOs might bind to the cell wall surfaces and then penetrate the phospholipid bilayer of the cytoplasmic membrane (Rhayour *et al.* 2003). However, to determine the accurate action site that the BLEOs impart damage to cells, deeper research is needed. Additionally, different changes to the fatty acids profiles occurred between the bacteria and the yeast, which could be inferred that BLEOs exhibited antimicrobial activities toward the bacteria and the yeast due to different mechanisms of action. The AFM images showed notable morphological alterations appeared in the cell walls and membranes of all the tested microorganisms. Similar cellular damage has been reported for various kinds of microorganisms when exposed to different essential oils (Diao *et al.* 2014; Hu *et al.* 2017). In line with the findings of cellular leakage and with the fatty acid profiles, the EOs were likely to interact with the cell walls that results in the disruption of the cell walls and the consequent release of intracellular contents. However, it is possible that other sites of action besides the cytoplasmic membrane exist. Therefore, further work is necessary to fully understand the mechanisms involved.

CONCLULSIONS

1. The chemical composition of bamboo leaves EOs were identified, and hexadecanoic acid (19.35%) and phytol (10.54%) were the major constituents.
2. The bamboo leaves EOs possessed promising antimicrobial activities to four selected foodborne pathogens.
3. According to MIC and DIZ tests, *Escherichia coli* was most sensitive to BLEO, showing the largest DIZ (12.77 ± 0.25 mm) and the lowest MIC (0.56 mg/mL).
4. The antimicrobial action of BLEOs is thought to be related to how the EOs interact with the cell surfaces to damage membrane integrity, which leads to the leakage of the intracellular contents until the cell lysis.

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