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Characterization of antimicrobial efficacy of soy isoflavones against pathogenic biofilms

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ABSTRACT

Pathogenic biofilms that form on food processing equipment/surfaces are of great concern, because these can readily lead to food spoilage, bio-fouling, food-borne illness, and their recalcitrance can result in the acquisition of multi-drug resistance. Currently available coatings do not completely inhibit microbial growth and an increased demand for such coatings means that new products will need to be developed. The unique properties of antimicrobial soy isoflavones, including their biodegradability, biocompatibility, and lack of toxicity as edible products, make their application more appealing than artificial polymer or chemical-based coatings. In this study, we evaluated the antimicrobial efficacy of soy isoflavones against pathogenic biofilms of *Listeria monocytogenes*, *Escherichia coli*, *Pseudomonas aeruginosa*, and Methicillin-Resistant *Staphylococcus aureus* (MRSA) using microtiter plate assays (MPAs), scanning electron microscopy (SEM), and atomic force microscopy (AFM). Ultrasonication technique yielded 491 μg of isoflavones per gram of soy flour sample. MPA assays and the imaging experiments revealed that the establishment of *L. monocytogenes* and *E. coli* biofilms was inhibited by 10 μg/mL and 100 μg/mL soy isoflavones, while MRSA and *P. aeruginosa* were largely unaffected.

1. Introduction

The incidence of foodborne illnesses, food contamination, spoilage, fouling, and wastage have increased considerably in recent years, incurring significant losses to food processing and manufacturing industries (Public Health Agency of Canada, 2014). The Public Health Agency of Canada (PHAC) estimated that one in eight Canadians acquire foodborne illnesses every year. Biofilm formation on food products and equipment surfaces for food processing is the leading cause of food spoilage and contamination (Shi & Zhu, 2009). Food spoilage is defined as changes in food that render it unfit and even harmful for human consumption, which is most commonly caused by microbial contamination (Gram et al., 2002).

Microbes frequently reside within dynamic, complex, multicellular communities referred to as biofilms (Wolcott, Costerton, Raoult, & Cutler, 2013). Some pathogens commonly found in the food industry are *Escherichia coli*, found in fresh produce and ready-to-eat food and meat products, *Listeria monocytogenes*, found in fresh produce, dairy, fish, and read-to-eat foods, and *Salmonella* spp., found commonly in poultry and poultry products (Srey, Jahid, & Ha, 2013). Pathogens can enter processed foods through the contamination of raw materials or ingredients, ventilation or water systems, food contact surfaces, personnel working in the processing plants, and pests, such as insects and rodents (Beuchat et al., 2011).

Biofilm control strategies are used to prevent food contamination and spoilage (Srey et al., 2013). Some of these include clean-in-place (CIP), disinfection, and chemical-based decontamination strategies (Srey et al., 2013). Antimicrobial agents inhibit the growth of and/or kill microorganisms and could ideally be used as a coating or film for food-processing surfaces or even as an additive in food to prevent contamination. Common chemical antimicrobials include sodium benzoate, sorbates, and benzoic acid, while natural antimicrobials include agents such as lysozyme, polypeptides (e.g. nisin), and essential oils (Corbo et al., 2009). Although there are a number of antimicrobial coatings, films, and agents currently available, those used in the food industry are largely ineffective due to the high level of resistance present in common food contaminants, especially when these form a biofilm (Fransisca,

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Zhou, Park, & Feng, 2011). For example, chlorine and 1% hydrogen peroxide are completely ineffective in preventing *E. coli* contamination in the fresh produce industry (Fransisca et al., 2011), while alternatives benzalkonium chloride and cetrimide do not inhibit *L. monocytogenes* (Mereghetti, Quentin, Marquet-Van Der Mee, & Audurier, 2000). Overall, commercial disinfectants have been found to be largely ineffective against several species found on surfaces that contact food during processing, including *L. monocytogenes, Pseudomonas aeruginosa*, and *Staphylococcus aureus* (Pan, Breidt, & Kathariou, 2006). At the same time, synthetic food preservatives (e.g. sodium benzoate) may be harmful to health and have been attributed to neurological and genetic disorders in children (Kaplan, 2010). The demand for food preservatives is estimated to reach 2.7 billion US dollars by 2018 (King, 2014). With the increasing demand for antimicrobials, increasing pathogenic resistance, and the limited effectiveness of current preparations, which may also be fraught with health risks, it is imperative that we develop new and effective antimicrobials that are ultimately safe for consumption.

Isoflavones are a group of phytoestrogenic compounds that are found in high quantities in soybeans and other legumes (Albulesco & Popovici., 2007). Isoflavones are comprised of nine glucosides, namely genistin, daidzin, glycitin, malonylgenistin, malonyldaidzin, malonylglycitin, acetylgenistin, acetyldaidzin, and acetylglycitin and three aglycones, namely genistein, daidzein and glycitein (Albulesco & Popovici., 2007). Isoflavones have been extensively studied for their potential use in preventing cancer, cardiovascular disease, and menopausal hot flashes (Messina, 1999). Certain forms of isoflavones (genistein and daidzein) have also been found to exhibit antimicrobial properties against *S. aureus*, MRSA, and *Vibrio harveyi* (Hong, Landuer, Foriska, & Ledney, 2006; Ulanowska, Tkaczyk, Konopa, & Wegrzyn, 2006). Soy isoflavones possess unique therapeutic and biological properties, as well as offer several advantages in terms of availability, biocompatibility, biodegradability, and edibility (Ullah et al., 2011).

Isoflavones have been extracted from numerous soybean products, including seeds, tofu, milk, and soy flour; the yield and the composition varies among these (Pyo, Yoo, & Surh, 2009; Rostagno, Araujo, & Sandi, 2002). Optimizing the extraction process is critical, because of differences in the stability of bio-active compounds present in plant matrices (Negi, 2012). Solvent extraction is the most conventional method to isolate isoflavones (Pyo et al., 2009; Rostagno et al., 2002). The objective of this study was to isolate and test the efficacy of bio-active isoflavones from low fat soy flour using an ultrasonication technique and ethanol solvent.

2. Materials and methods

2.1. Soy flour sample

Low fat soy flour (fat: 1.5 g per 100 g sample) was purchased from a wholesale store (Bulkbarn, Canada). Upon receipt, the sample material was stored in the freezer (<−20 °C) until used for extraction experiments.

2.2. Chemicals

The following chemicals and materials were used in this study: deionized water from a Barnstead Nanopure Diamond lab water system (APS Water Services Corporation, USA), 100% Anhydrous ethyl alcohol (Sigma−Aldrich, USA), 10 cm × 10 cm weighing paper (Fisher Scientific, Canada), Polyvinylidene Fluoride (PVDF) 0.45 μm H_2O filters (Fisher Scientific, Canada), 98% formic acid for mass spectrometry (Sigma−Aldrich, Canada), HPLC grade methanol (Fisher Scientific, USA), HPLC grade dimethyl sulphoxide (DMSO) (Sigma−Aldrich, USA) and 96-well polystyrene microtiter plates (Fisher Scientific, USA).

2.3. Solvent extraction

A 3 g sample of low fat soy flour was weighed and mixed thoroughly with 25 mL of 50% (v/v) ethanol in a centrifuge tube. The Symphony ultrasonic bath (35 KHz, VWR, USA) was set at 60 °C. Centrifuge tubes were placed inside the ultrasonic bath for 20 min with shaking once at 10 min. After 20 min, the tubes were centrifuged (Mandel Scientific Company Inc., Canada) at 5950 rpm (Scilogex Model D3024, Connecticut, USA) for 10 min. The supernatant was removed and extractions were performed in triplicate. A nitrogen blow down evaporator (N-EVAP, Organomation Associates Inc., USA) was used to remove any solvent from the crude extract. The extract was then freeze-dried (<−4 °C) using a bulk tray dryer (Labconco, USA). The dried sample was dissolved in 70% methanol and then analyzed using high performance liquid chromatography (HPLC).

2.4. High performance liquid chromatography (HPLC)

An Agilent 1100 series HPLC system with a quaternary pump, vacuum degasser, auto-sampler, column compartment and a diode array detector (DAD) (Agilent Technologies, USA) was used to identify and analyze extracted isoflavones. Isoflavones were separated using a reverse phase C_{18} Luna column (Phenomenex, USA, 250 × 4.6 mm; 5 μm). The mobile and stationary phases were solvent A-methanol:formic acid (95:5 v/v) and solvent B- formic acid:water (5:95 v/v). A constant flow of mobile phase at 0.8 mL/min was set up in the column at 254 nm. Initially, the condition was set to 85% B. A gradient was set to increase A from 15% to 35% for 50 min and after a 10 min hold time, it was brought back to 15%. The injection volume was 20 μL. Identification of isoflavones was performed by comparing the obtained results with the standard results. Retention times were also observed and compared with the standard curve (Kim et al., 2014). Quantification was carried out by integrating the peak areas. The HPLC curves of isoflavone extract curves are shown in Fig. 1.

2.5. Microtiter plate assay (MPA)

L. monocytogenes (LMC379), *P. aeruginosa* (PA76), *E. coli* (ATCC 25922), and Methicillin Resistant *S. aureus* (MRSA M0535) were obtained from the Ontario Veterinary College (University of Guelph, Canada) and streaked separately onto 5% sheep's blood agar plates from −80 °C frozen stocks. Plates were incubated at 37 °C for 24 h. Plate cultures were used to inoculate liquid cultures for MPA inoculation. *L. monocytogenes* was grown in brain heart infusion broth (BHI), while *E. coli*, MRSA, and *P. aeruginosa* were grown in tryptic soy broth (TSB). A single, isolated colony of *E. coli, P. aeruginosa,* MRSA, and *L. monocytogenes* was individually inoculated into a tube containing liquid media and grown overnight at 37 °C. MPA assays were then performed as described by O'Toole, 2011. Overnight cultures were diluted to a 0.5 McFarland Standard and used to inoculate 96-well polystyrene plates (250 μL per well with at least three wells per replicate for all treatments and controls were incubated). Wells were treated with controls or isoflavone treatments, which contained 10 μg/mL or 100 μg/mL soy isoflavones from stock solution (v/v). All reagents including chemicals namely PBS and Sorensen's buffer were purchased from Sigma−Aldrich (Sigma−Aldrich, Oakville, Canada). Freeze dried isoflavone samples were dissolved in DMSO, creating a 10 mg/mL stock solution for 1% treatments and 100 mg/mL for 10% treatments. After incubation at 37 °C for 24 h, the wells were washed with phosphate

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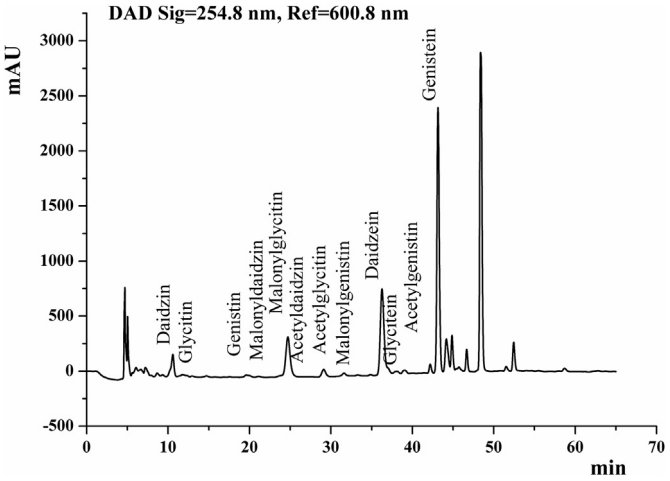

Fig. 1. High Performance Liquid Chromatography curve plotted in time versus normalized area curves showing peaks of: 1. Daidzin; 2. Glycitin; 3. Genistin; 4. Malonyldaidzin; 5. Malonylglycitin; 6. Acetyldaidzin; 7. Acetylglycitin; 8. Malonylgenistin; 9. Daidzein; 10. Glycitein; 11. Acetylgenistin; 12. Genistein.

buffer solution (PBS composition: 0.01 M Phosphate buffer, 0.0027 M potassium chloride, and 0.137 M sodium chloride with a pH of 7.4) and stained with 0.1% crystal violet. Optical density readings at 600 nm were measured using an ELISA plate reader (Serial Number 80-2115-80 Amersham Biosciences Corp., USA). During the MPA experiment, a well containing ampicillin (50 μg/mL) was used as the positive control along with two wells containing culture medium with 1% DMSO and culture medium only as the negative controls.

2.6. Scanning electron microscopy (SEM)

A Hitachi S-570 Scanning Electron Microscope (Hitachi High Technologies, Japan) was used to visualize the antimicrobial effect of isoflavones on *L. monocytogenes*. After cultures were grown and sub-cultured to assess cells in log-phase, three approaches were followed to prepare stainless steel coupons with a 10 μg/mL isoflavones, either pre-coated, post-coated, or added simultaneously (relative to inoculation). These approaches were chosen to see if the addition of 100 μg/mL isoflavones before, with, and after addition of bacteria to stainless steel had any effect on the ability of bacteria to adhere and grow. Stainless steel (alloy 430) discs 10 mm in diameter (Ted Pella, Redding, CA) were used as the substrate. The 430 type stainless steel is widely used in food industries for splash backs, housing and equipment enclosure applications. For pre-coated stainless steel samples, coupons were first coated with 800 μl of isoflavone extract for 30 min. Then, 7.2 mL of culture was added to a petri dish with pre-coated coupons ($n = 3$) and incubated at 37 °C. In the second approach (simultaneous addition), 7.2 mL of culture was mixed thoroughly with 800 μL isoflavones in a test tube, which was then added to a petri dish with stainless steel coupons and incubated at 37 °C. In the final approach (post-coating), the steel coupons were incubated overnight with 7.2 mL bacterial culture. After incubation, 800 μl isoflavone extract was added and incubated for an additional 4–24 h. At three different time points (4, 10, and after 24 h), coupons were fixed, washed with Sorenson's buffer (50:50 (v/v) dibasic sodium phosphate 0.07 M and monobasic potassium phosphate 0.07 M), dehydrated with ethanol at a gradient ranging from 50% to 100% for 10 min each, critical point dried with carbon dioxide, and gold sputtered for about 15 nm. Then the coupons were imaged using scanning electron microscope at varying magnifications.

2.7. Atomic force microscopy (AFM)

An AFM/SPM 5500 with Olympus ILM, MAC Mode atomic force microscope (Agilent Technologies, USA) was used to observe the structure of isoflavones on a nanoscale and evaluate the antimicrobial effect of isoflavones on *L. monocytogenes*. Gelatin-coated mica sheets were placed in cell culture dishes. Bacteria were grown in cell culture dishes (2 mL) with and without 10 μg/mL and 100 μg/mL soy isoflavones (v/v) from the stock. After 24 h, the mica substrates were washed with 1 mL deionized water and dried completely. Then the mica sheets were imaged using atomic force microscopy. At the same time, 50 μL of isoflavone stock solution alone (100 mg/mL in DMSO) was pipetted onto a mica substrate without gelatin. After 20 min, the substrate was washed gently with 1 mL of deionized water, then dried and imaged.

2.8. Statistics

One-tailed t-tests were performed using the R open source statistical programming software. The significance level was set at $P < 0.05$. Results presented are averages of 3 replicates ± s.e.m and are representative of three independent experiments.

3. Results and discussion

3.1. Extraction of isoflavones

In order to investigate the antimicrobial efficacy of soy isoflavones against pathogens, isoflavones were isolated from low fat soy flour using ultrasonication technique. Ultrasonication was chosen, because it affords better extraction efficiency when compared to other methods (Pyo et al., 2009; Rostagno et al., 2002). Ultrasonic waves disrupt the cell wall of the sample, facilitating transport of solvents into the cells for better extraction of any bioactive compounds (Yasui, Tuziuti, & Iida, 2005). Mechanical vibrations also provide better penetration of the solvent into sample by increasing the contact surface area (Yasui et al., 2005). The total amount of isoflavones obtained in this study was 491 μg per gram of soy flour sample (Table 1). The HPLC curve of extracted isoflavones is shown in Fig. 1. Our yield appears to be within a reasonable range, as various groups using different source materials and techniques have yielded varying amounts of isoflavones (~300–2400 μg/g starting material).

Rostagno et al. (2002) showed a recovery of 311.55 μg/g soy flour using ultrasonication technique with 70% methanol as the solvent, while Achouri, Boye, and Belanger (2005) demonstrated that sonication for 15 min yielded 3770 μg/g soy meal sample and 1755 μg/g soy protein isolate sample using 80% ethanol in 0.1 N HCl, after a total of five extractions. Lee and Row (2006) showed that ultrasonication at 20 kHz with 60% aqueous ethanol solvent yielded 2354.92 μg/g Korean soybean seeds sample.

The quantity of isoflavones in any sample matrix depends on storage, harvest, processing, and regional and climatic conditions (Griffith & Collison, 2001; Kao, Su, & Lee, 2004; Klejdus et al., 2005; Lee et al., 2004; Murphy, Barua, & Hauck, 2002; Wu, Wang, Sciarappa, & Simon, 2004). The variations in yield emphasizes that optimization is a critical step when developing protocols for isolating plant extracts. In general, isoflavones are present in both glycosidic and aglycone forms (Rostagno et al., 2002). The glycoside form of isoflavone is not preferred as it is less bioactive than the aglycone form due to its hydrophilicity (Klejdus et al., 2005). Among the aglycones, a lower yield of daidzein compared to genistein could be attributed to its low solubility in the solvent used (Albulesco & Popovici., 2007; Rostagno et al., 2002). From our

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Table 1
Isoflavone compounds extracted using ultrasonication technique. Standard error calculated with n = 3 on the mean values.

Compounds	Amount (μg/g)	Standard error of mean (μg/g)
Daidzin	23.70	0.38
Glycitin	2.12	0.44
Genistin	2.45	0.64
Malonyldaidzin	2.24	0.12
Malonylglycitin	144.03	1.62
Acetyldaidzin	0.90	0.28
Acetylglycitin	11.67	0.24
Malonylgenistin	7.12	0.27
Daidzein	116.81	1.72
Glycitein	3.47	0.22
Acetylgenistin	27.95	1.99
Genistein	148.56	1.34
Total	**491.02**	

extracted samples, we obtained 54.75% of aglycones while glycosides accounted for 45.25%.

3.2. Antimicrobial efficacy of soy isoflavones using microtitre plate assay (MPA)

MPA was performed to investigate the antimicrobial efficacy of soy isoflavones against MRSA, *L. monocytogenes, E. coli*, and *P. aeruginosa* with 10 μg/mL and 100 μg/mL soy isoflavones. Antimicrobial activity of soy isoflavones against these bacterial species are reported in terms of the resulting OD_{600} value (Fig. 2). Isoflavone treatment reduced the biomass of *L. monocytogenes* reflected in the reduced optical density. However, these isoflavones had little to no effect on MRSA, *E. coli*, and *P. aeruginosa* biofilms. In general soy isoflavones likely prevents nucleic acid synthesis through reduction of RNA and DNA of bacterial cells (Ulanowska et al., 2006; Verdrengh, Collins, Bergin, & Tarkowski, 2004; Wang, Wang, & Xie, 2010). Isoflavones could have inhibited the DNA and RNA synthesis of *L. monocytogenes* as reflected in the reduction of optical density. It was also interesting to note that even a 10-fold increase in treatment concentration did not affect inhibition of *L. monocytogenes* very much. This could be attributed to the limitations of MPA assay in terms of accuracy and cross contamination. It could also be possible that a sub population within the culture could pose resistance to an increasing concentration of isoflavones. The isoflavones at a higher concentration could have formed aggregates which might have decreased their antimicrobial efficacy.

Genistein and daidzein have been studied extensively for their antimicrobial properties. Ulanowska et al. (2006) investigated the effect of genistein and daidzein against *E. coli, V. harveyi, and Bacillus subtilis* at a treatment concentration of 0.1 mM (about 27 μg/mL genistein and 25 μg/mL daidzein). The results showed that genistein had strong inhibitory effects on *V. harveyi*, a gram-negative bacterium, while it had intermediate inhibitory effects on *B. subtilis*, a gram-positive bacterium; there were no significant effects for *E. coli*. Hong et al. (2006) evaluated the direct effect of genistein on the growth of *Lactobacillus reuteri*, *E coli*, *Shigella sonnei*, *S. aureus, Klebsiella pneumonia*, and *Bacillus anthracis*. At 100 μM (27 μg/mL) concentration of genistein, *S. aureus* and *B. anthracis* were inhibited, but the other bacterial strains were not inhibited. All these studies showed that *E. coli* was largely not inhibited by genistein and daidzein, while *S. aureus* (including some MRSA strains) were inhibited by genistein. The MPA results from this work showed that *E. coli* was not inhibited by soy isoflavones which is comparable to these previous results. It is reported that the general target of drugs in *E. coli* is *gyrase*, which is a type II topoisomerase (Verdrengh et al., 2004). Therefore it could be

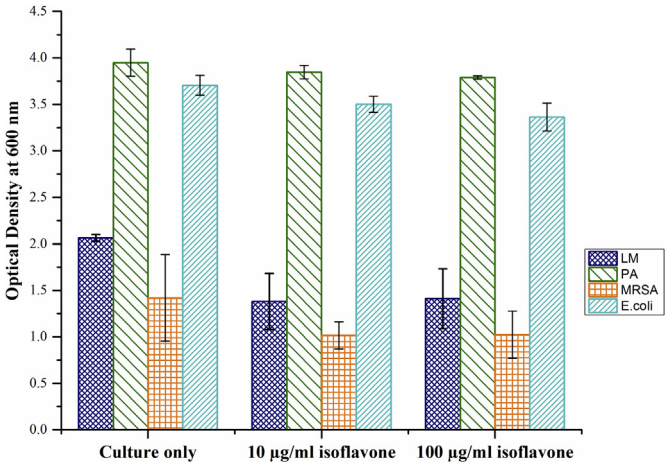

Fig. 2. Antimicrobial Efficacy of 10 and 100 μg/mL Soy isoflavones against *L. monocytogenes, P. aeruginosa*, MRSA, and *E. coli*. *Error bars* are expressed as mean ± SEM (standard error of mean) with n ≥ 3 ($p \leq 0.05$).

speculated that the isoflavones used in this study do not target *gyrase*.

MRSA and *P. aeruginosa* were not inhibited by soy isoflavones in our study, although other groups have reported that certain forms of isoflavones like genistein could inhibit *Staphylococcus* species. The reasons for differences in bacterial sensitivity to isoflavones could be attributed to the differences in the strains, culture conditions, type of assays used to test antimicrobial efficacy, and/or the specific isoflavone composition. The isoflavone extract used in this study was a composition of many forms of isoflavones (including glycosides), whereas previous studies used a single isoflavone (genistein and/or daidzein). The amount of genistein isolated in this study was comparatively less than the pure solutions (in several μg/mL) used in other studies, which might have impacted its effectiveness against these bacteria.

Structural prerequisites could be a factor that determines the antimicrobial efficacy of isoflavones. The presence of a prenyl group at C-6 position and hydroxyl groups at C-5 or C-7 position is associated with high antimicrobial activity (Mukhne, Vishwanathan, & Phadatare, 2011). Cyclization of prenyl and hydroxyl groups decreases the antimicrobial activity of isoflavones (Hatano et al., 2000). Perhaps this could explain the lack of efficacy against *E. coli, P. aeruginosa*, and MRSA in our current study. Hydroxyl groups promote enzyme inhibition and the inhibition of biosynthetic pathways in bacteria, as they have a high affinity for protein (Alcaraz, Blanco, Puig, Tomas, & Ferretti, 2000; Tsuchiya et al., 1996). Prenyl groups can increase lipophilicity, which in turn increases the compound's interaction with the bacterial cell by disturbing and damaging its functions (Mukne et al., 2011).

Crude extracts contain conjugated or glycosidic forms of isoflavones in addition to free or aglycone forms. It has been reported that the presence of sugar decreases the activity of isoflavones against bacteria (Kapoor, Narain, & Misra, 2007). In this study, we investigated the antimicrobial activity of isoflavones as a whole (including conjugated forms) which could have reduced its activity against bacterial species.

3.3. Scanning electron microscopy (SEM) analysis

In order to visualize the effect of soy isoflavones on biofilms formed by *L. monocytogenes*, SEM and AFM imaging were performed. Fig. 3 shows the effects of isoflavones on *L. monocytogenes* bacterial biofilms and cells (the three approaches did not

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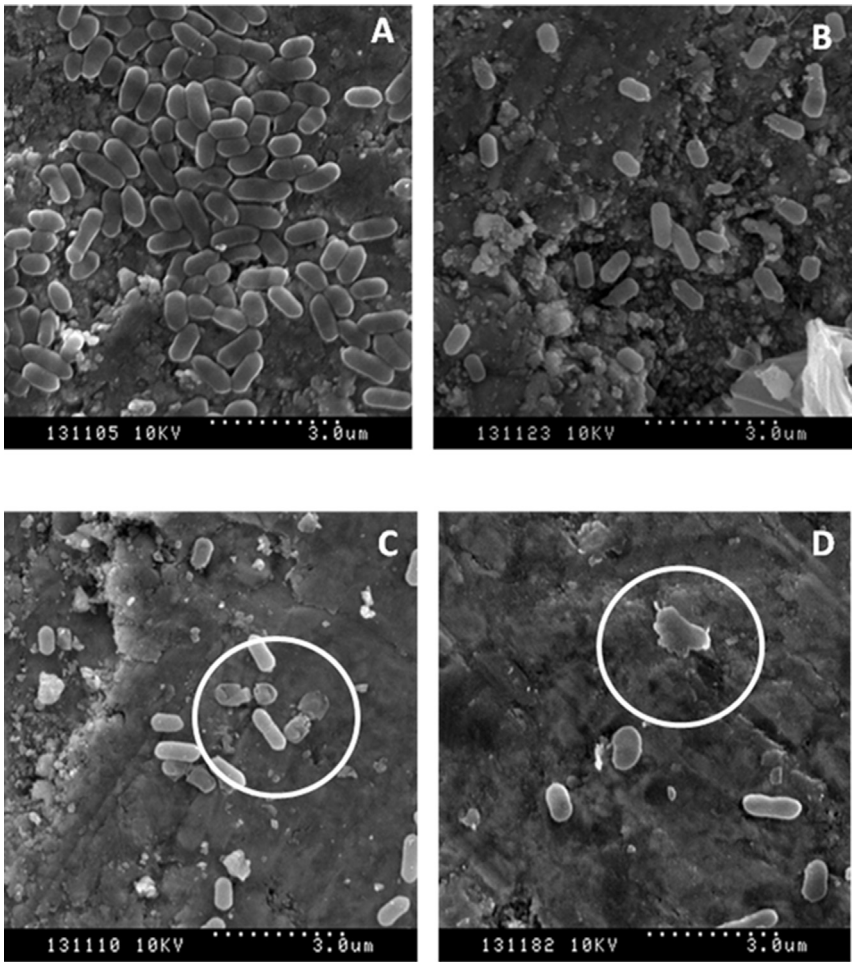

Fig. 3. Scanning Electron Microscopy images of *L. monocytogenes* treated with and without 100 μg/mL isoflavone. Treatments on stainless steel at different time points are shown; damaged cells (putative mode of action) are highlighted within white circles. Panel A is a control *L. monocytogenes* biofilm. Panel B is an image taken at 4 h showing dispersion of cells. Panel C is a picture at 10 h showing cell breakage, while panel D is an image after 24 h showing cell leakage.

significantly differ from one another). As described, SEM imaging was performed to understand if the antibacterial effect of isoflavones differs in relation to the time of addition. It could be viewed that the number of cells in each panel gradually reduced from biofilm (with numerous cells) in panel A (control) to about 21 cells in panel B (treated with isoflavones at 4 h) to 14 cells in panel C (image taken at 10 h) to about 6 cells in panel D (after 24 h). From this study, could be speculated that isoflavone treatment induces cell leakage and breakage, along with generalized cell dispersion of *L. monocytogenes* on stainless steel (Fig. 3).

3.4. Atomic force microscopy (AFM)

AFM image analysis revealed that isoflavone particles ranged from 100 to 600 nm in diameter and about 10–15 nm in thickness (Fig. 4). Further analysis of 100 μg/mL isoflavone-treated *L. monocytogenes* grown in liquid culture showed that isoflavones were scattered across the mica substrate, along with the extra polymeric substances that surrounded the bacterial cells. This indicates that isoflavones were not completely solubilized in the bacterial media. Solubility of isoflavones (or the uptake) in the bacterial growth media may play a role in the inhibition of the food-borne pathogenic bacterial biofilms. Flagella appeared to be disconnected from the bacterial cell wall upon treatment with 100 μg/mL soy isoflavones, while flagella were intact at 10 μg/mL isoflavone treated sample (Fig. 4). In *L. monocytogenes*, motility is considered important for the initial steps of finding and attaching to a surface in their biofilm growth (Pilchova et al., 2014). *Listeria* usually has multiple polar flagella. The results from AFM images (Fig. 4 Panels D and E) shows that the isoflavone treated *Listeria* had detached flagella implying that isoflavones may impede the adherence by altering or arresting flagellar motility.

The effect of isoflavones on gene expression of various metabolic pathways will affect susceptibility of different bacterial genera and species, especially gram negative vs. gram positive bacteria. Enzymes involved in DNA unwinding may allow replication and transcription of the bacterial species to occur (Robinson & van Oijen, 2013). It is possible that the isoflavones may affect the gene expression, which could explain the differences observed on the effect of isoflavone treated bacterial species. Differences in the cell wall and the cell membrane and/or the differences in the transcriptional targets are possible reasons behind the differences in the inhibition of soy isoflavones against the *L. monocytogenes*, MRSA, *E. coli*, and *P. aeruginosa*.

In vitro and *in vivo* evaluation of soy flavones by Huang, Hung, Lin, and Fang (2008) showed that genistein exhibited greater skin absorption than daidzein and concluded that the topical delivery of soy isoflavones might help to efficiently treat photo-damage and photo-aging of the skin. Therefore, the working mechanism of isoflavones against the pathogenic bacterial biofilms likely depends

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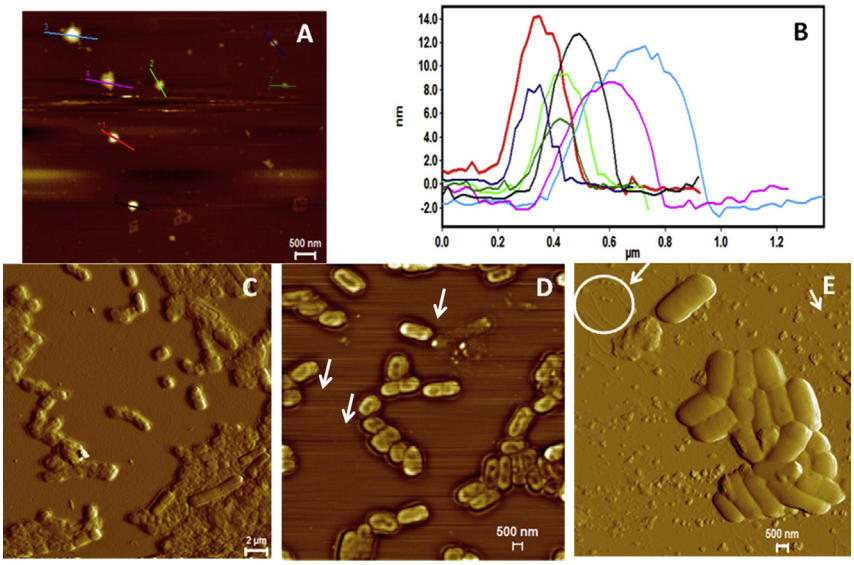

Fig. 4. Atomic force Microscopy (AFM) Images. The colored lines on Panel A shows the morphology of isoflavones observed on a mica substrate. AFM line profile (Panel B) shows the corresponding size distribution of isoflavones (Panel A) on the mica sheet substrate. Panel C is a control image of *L. monocytogenes* biofilm on a gelatin-coated mica substrate. Panel D shows 10 μg/mL of soy isoflavone treated with *L. monocytogenes* where flagella remain attached to the cells (highlighted by arrows). Panel E shows 100 μg/mL soy isoflavone treated with *L. monocytogenes* where the flagella have become disconnected and a damaged cell covered by media, extra polymeric substances, and soy isoflavone is visible (highlighted by circle and arrows). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

on the mode of delivery and application. When ingested orally, the solubility of isoflavones in the blood stream could be enhanced due to enzymatic processing and the low pH of the stomach. Isoflavones may inhibit bacterial growth indiscriminately, regardless of whether they are pathogenic or not. Supplementing in solution (and ingested by human) may cause unexpected harm to normal gut microflora; as coating on food contact surfaces, however, would minimize release and consumption, thus is safer to use at higher, effective concentrations.

3.5. Antimicrobial mode of action

The antibacterial activity of plant extracts in general is associated with the adsorption of polyphenols to bacterial cell membranes, leading to disruption of the membrane and subsequent leakage of the cell contents. However, the only studies that have proposed a putative mechanism for isoflavones have looked at their effect on enzymes involved in DNA replication. One study concluded that the primary target of genistein is bacterial topo IV by stabilizing topoisomerase-II DNA cleavage complex but gyrase was not affected by genistein (Verdrengh et al., 2004). Soy isoflavones likely prevents nucleic acid synthesis by influencing topoisomerase I and II (Wang et al., 2010) or through topoisomerase IV (Verdrengh et al., 2004) through reduction of the quantities of RNA and DNA of bacterial cells. Cell morphology analysis by Ulanowska et al. (2006) shows that isoflavones, namely daidzein and genistein drastically inhibits the synthesis of both the DNA and RNA along with protein for *V. harveyi* and *Bacillus subtilis*, however the cell morphology and synthesis of nucleic acids were unaffected in *E. coli*. The soy isoflavone genistein inhibits the exotoxin of the bacterial cell and thereby influence bacterial pathogenesis and eventually kills the bacteria (Ulanowska, Majchrzyk, Moskot, Jakóbkiewicz-Banecka, and Węgrzyn (2007)). Ulanowska et al. (2007) showed a very thorough study of the generation (doubling) time as a measurement of the effects of various isoflavones on growth of 12 different bacteria. Cushnie and Lamb (2011) reviewed on the potential mechanisms of flavonoids, and suggested that the cell leakage may be the results of programmed cell death due to inhibition of DNA/RNA synthesis, not from effects on cell membrane integrity. Although the mode of antibacterial action of soy isoflavones is relatively unknown, significant efforts are in place for creating synthetic derivatives of genistein considering its potential antimicrobial and anti-parasitic activities (Rusin et al., 2010).

4. Conclusions

Microbial biofilms are a serious threat to the food and medical industry, due to their spoilage potential and contribution to antimicrobial resistance. Most antimicrobial agents used in the food industry today are synthetic and are not necessarily safe for human consumption/exposure. This research provides insights into the isolation of isoflavones from soy flour and their potential antimicrobial efficacy against foodborne and antibiotic resistant pathogens. Ultrasonication technique provided 491 μg of isoflavones per gram of soy flour sample. Our assays demonstrate that isoflavones have antimicrobial properties against *L. monocytogenes* and *E. coli*, at 10 and 100 μg/mL concentration levels, but had no significant effect against MRSA, and *P. aeruginosa* biofilms. Hence, soy isoflavones show promise for developing biocompatible, biodegradable, and eco-friendly antimicrobial agents for the food industry.

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