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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