THE ROLE OF NON-IONIC SURFACTANT-EXTRACELLULAR POLYMERIC SUBSTANCES IN REMOVAL LISTERIA MONOCYTOGENES BIOFILM FORMED ON DIFFERENT CONTACT SURFACES

By

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ABSTRACT OF THE DISSERTATION

ROLE OF SURFACTANT-EXTRACELLULAR POLYMERIC SUBSTANCES IN THE REMOVAL OF *LISTERIA MONOCYTOGENES* BIOFILM FORMED ON DIFFERENT CONTACT SURFACES

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The occurrence of biofilm in food processing environments can lead to spoilage and transmission of diseases, this aided in the development of this study. *Listeria monocytogenes* can cause serious and possibly fatal illnesses in humans and animals after ingestion the contaminated food. Spinach can be contaminated by *L. monocytogenes* during harvest. The effectiveness of six nonionic surfactants (Pluronic F68, Pluronic F127, Tween 20, Tween 40, Tween 80, and Brij 58) were evaluated in disrupting *L. monocytogenes* biofilms on the surface of spinach leaves. Wells were washed with surfactants after incubation and then mixed on a platform shaker for 1, 5, 15, and 30 min. Then, the wells were rinsed with distilled water to remove dead cells, and fixation was conducted at 30 min at 60 °C. Our findings showed that Brij 58 most effectively removed the *L. monocytogenes* biofilm on spinach, followed by Pluronic F127, Tween 80, Tween 40, Tween 20, and Pluronic F68.In the second experiment, the effectiveness of different types of nonionic surfactants such as Pluronic F68, Pluronic F127, Tween 20, Tween 40, Tween 80 and Brij 58 against the *Listeria monocytogenes* biofilm cells formed on food contact surfaces made of Low-Density Polyethylene (LDPE), Polypropylene (PP), Low-

Density Polyethylene and Polypropylene (LDPE-PP), Low-Density Polyethylene and Ethylene Vinyl Acetate (LDPE-EVA), Stainless-Steel and Aluminum was determined.Acridine Orange staining (AOS) to quantify the amount of the *L. monocytogenes* biofilm cells that were destroyed by the nonionic surfactants after different time intervals of 1, 5, 15 and 30 minut.

Keywords: Listeria monocytogenes biofilm, fresh-cut produce, food contact surfaces, extracellular polymeric substances, nonionic surfactants

Dedications

For my precious country Iraq who supports me especially the ministry of higher education of Iraq to conduct my Ph.D. research in the United States

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Chapter I General Background, Significance of a Study, Objectives, and Hypothesis

1. Introduction

The consumption of food products contaminated with pathogenic microorganisms such as bacteria and viruses have led to the development of many cases of foodborne diseases in the united stated (Cartwright et al., 2013). Among the most common factors helping to increase the number of foodborne diseases are inappropriate food preparation, handling, and storage techniques. Cartwright et al. stated that increasing cases of the foodborne diseases are considered important threats to the public health; hence, there is an urgent need to ensure they are controlled.

One of the most common types of foodborne disease is listeriosis, which caused by *L. monocytogenes* bacteria (Zhu, Gooneratne, & Hussain, 2017). Though there have been many significant approaches to eradicate foodborne pathogens, their application has not been effective because these microorganisms have developed strategies to survive in different environments (Cahoon & Freitag, 2014). *L. monocytogenes* is a rod-shaped, gram-positive bacterium that can survive in both the presence and absence of oxygen gas (Shi & Zhu, 2009). The *L. monocytogenes* has been declared as one of the important public U.S. health threats.

According to Colagiorgi et al. (2017), the formed *L. monocytogenes* biofilms can persists on food contact surfaces for several years. *L. monocytogenes* biofilms are composed of community of microbial cells are permanently attached to a substrate, interface, or to each other, embedded in a matrix of extracellular polymeric substances (Donlan &Costerton, 2002; Leong et al.,2014),that form a protective layer against

different types of cleaning agents (Silva, Teixeira, Oliveira, & Azeredo, 2008), leading to increased resistance of *L. monocytogenes* to different types of disinfectants. Furthermore, these biofilms enable the *L. monocytogenes* cells to attach themselves firmly on different types of food contact surfaces on which they undertake their pathogenic activities leading to food contamination (Zhu et al., 2017).

Stewart and Franklin (2008) found *L. monocytogenes* are able to attach and survive on different surfaces, such as those of freshly cut produce and food contact surfaces made of materials such as stainless steel, aluminum, and polypropylene. Because *L. monocytogenes* are psychotropic bacteria, they have the ability to grow and survive on different freshly cut vegetables. Therefore, the *L. monocytogenes* will easily attach on waxy parts of the freshly cut produce. Botticella et al. (2013) determined that the presence of hydrophobic pockets and folds in the surfaces of freshly cut vegetables will enhance the survival of the *L. monocytogenes* cells on such surfaces because they will act as protective layers that prevent the disinfectants from coming into contact with these bacterial cells (Omac, 2014).

According to Yuan, Hays, Hardwidge, and Kim (2017), the attachment of the *L. monocytogenes* on different food contact surfaces is dependent on the physical properties of the surfaces such as roughness and charges. In other words, the adhesion of the bacterial cells depends on surface characteristics (Cortés, Bonilla, & Sinisterra, 2011; Van Houdt & Michiels, 2010). According to Ronner and Wong (1993), the surfaces with high energy and high moisture content influence attachment of *L. monocytogenes* cells. More cells of the *L. monocytogenes* will be attached on hydrophilic surfaces, such as stainless steel, than hydrophobic surfaces such as Teflon (Myszka & Czaczyk, 2011).

2. Significance of the Study

Biofilms are specifically problematic in food industry areas such as dairy processing, brewing, poultry processing, fresh produce, and red meat processing (Simões, Simoes, & Vieira, 2010).The growing rate of biofilms is helping to increase resistance to antimicrobial products, making the removal process from the food processing facilities a complex issue. In addition to a rise in foodborne diseases, the formation of the listeria biofilm on dairy industry equipment can also result in economic losses, driving the need to come up with measures on how to control the growth and formation of listeria biofilms. Additionally, prevention of contamination through management of listeria biofilm can reduce the high number of cases related to listeriosis (Simões, Simoes, & Vieira, 2010).

3. Study Hypothesis

Biofilms have presented challenges in various sectors of the food industry including brewing, dairy processing, fresh produce, poultry processing, and red meat processing (Simões et al., 2010). Simões et al. noted an emergence of biofilms resistant to conventional antimicrobial treatments, which indicates a need for investigation of novel designs for washing procedures to achieve better food safety. Various formulations of nonionic wetting agents have been widely used because they control foaming and are good emulsifiers. This has led to the formulation of this study's main hypothesis that non-ionic surfactants do not form complexes with extracellular polymeric substances (EPS) constituents and can effectively remove *L. monocytogenes* biofilms from leaf surfaces and food contact surfaces.

4. Research Objectives

1. EPS promote biofilm formation by:

a) Measuring the amount of protein and polysaccharide in EPS;

b) Measuring the leaf surface hydrophobicity by contact angle measurement; and

c) Evaluating the effect of pre-conditioning the surface of spinach leaves with extracellular polymeric substances (EPS).

2. Non-ionic surfactants can effectively remove listeria biofilm from the leaf surface of spinach, regarding which this research seeks:

a) To investigate the effect of anti-adhesive/ant-biofilm and antimicrobial properties of non–ionic surfactants in disrupting pre-formed biofilms of *L. monocytogenes* on spinach leaf surfaces; and

b) To identify the effect interaction of non-ionic surfactants with and without listeria EPS.

3. Non-ionic surfactants can effectively remove listeria biofilm on food contact surfaces (LDPE, PP, LDPE + PP, LDPE + EVA, stainless steel, aluminum), for which we seek:

a) To assess the ability of *L. monocytogenes* to form biofilms on different food-contact surfaces with regard to different surfactants;

b) To investigate effectiveness of non-ionic surfactants in disrupting preformed biofilms of *L. monocytogenes* on food contact surfaces; and

c) To measure surface energy of food contact surfaces.

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Chapter II. Literature Review

2.1. Foodborne Disease Outbreak

The outbreak of different types of foodborne diseases arises from consumption of pathogenically contaminated foods, whether bacterial, viral, or parasitic. The occurrence of foodborne diseases is often facilitated by different factors such as improper handling of food, poor preparation techniques, and improper food storage (Zhu, Gooneratne, & Hussain, 2017). To reduce the chances of contacting foodborne illnesses, handlers should apply proper hygiene practices before, during, and after the preparation of food. The increased number of foodborne diseases in the public domain has made consumers raise an alarm about the credibility of the food processing, handing, and storage facilities (Anand, Holmen, Neely, Pannaraj, & Bard, 2016). Pathogenic microorganisms facilitating outbreaks of foodborne diseases can be grouped into categories such as the infectious pathogens, including *L. monocytogenes*, which cause listeriosis and toxigenic pathogens such as *Bacillus cereus* and toxic-infectious pathogens such as *Clostridium perfringens* (Sidney & Kings, 2015)..

Though there has been significant development of novel strategies and technologies to improve food quality by eliminating potential pathogenic contaminants, incidences of foodborne diseases in recent years have not been decreasing (Zhu et al., 2017). In many cases, there have been incidences of listeriosis connected to the intake of contaminated Mexican-style cheese and turkey frankfurters (Cartwright et al., 2013).According to the information from the Centers for Disease Control and Prevention (CDC), approximately 1,662 new incidences of listeriosis take place every year in the United States, leading to

about 1,520 hospitalizations and 266 related deaths (Cartwright et al., 2013).Listeriosis outbreak was observed in Canada in 1981 after the infected people consumed contaminated coleslaw. In the United States, a listeriosis outbreak was observed in 1983 and was related to the intake of contaminated pasteurized milk (Cartwright et al., 2013).

2.2. L. monocytogenes

L. monocytogenes is currently one of the most dangerous foodborne pathogens and a leading cause of death form the foodborne illnesses in the United States. *L. monocytogenes* is a gram-positiv rod-shaped bacterium that can persist in the absence or presence of oxygen and the causative agent of listeriosis, an infection that results from eating *L. monocytogenes*-contaminated foods (Colagiorgi et al., 2017). Listeriosis has been identified as an essential public health burden in the United States. In most cases, listeriosis greatly affects individuals with compromised immune systems, pregnant women, and newborns (Zhu et al., 2017). It is a non-spore forming bacterium broadly spread in the environment. Because *L. monocytogenes* does not have a specific host, it considered a non-host specific pathogen.

The two most important clinical manifestations of listeriosis include meningitis and sepsis. A microscopic analysis of the *L. monocytogenes* culture has shown its small rod shape presents primarily as a series of short chains; a direct smear of the *L. monocytogenes* culture makes them appear in a coccoid shape that can sometimes be identified as streptococci (Di Ciccio et al., 2012). *L. monocytogenes* has been identified in dairy forms such as raw milk and pasteurized milk. Other foods have been linked to listeriosis outbreaks (Leong, Alvarez-Ordóñez, & Jordan, 2014), including coleslaw, ice cream, soft cheese, and sliced cold cuts.

Because *L. monocytogenes* is a facultative anaerobe, it can grow in a harsh anaerobic condition with an altered metabolism of carbon (Lobel et al., 2015). In the presence of oxygen, *L. monocytogenes* partially oxidizes glucose to produce acetate and lactate. *L. monocytogenes* produces lactate as its major fermentation product along with other minor products such as carbon dioxide, ethanol, and formate (Wallace, Newton, Abrams, Zani, & Sun, 2017). The level of oxygen in *L. monocytogenes* plays a significant role in its carbon metabolism regulation.

2.3. Virulence and Pathogenicity

Strains of *L. monocytogenes* are ubiquitous yet the bacteria can seriously affect few people because there is an adequate immune response to it by most healthy people (Cahoon & Freitag, 2014). Analysis of the *L. monocytogenes* serotypes, isolated from 1,363 patients, has indicted that the 4b serotype is the most common (being present in 64% of the cases), and serotype 1/2a, 1/2b, and 1/2c have been identified in 15%, 10% and 4% of the reported cases, respectively (Rychli et al., 2014). Additionally, the serotype 4b was determined to be commonly found in pregnant women.

The ability of the *L. monocytogenes* to cause severe illness is linked to the fact that it can induce self-phagocytosis by the host cells, replicate within the already infested host cells, and direct transfer to neighboring cells (Rychli et al., 2014). *L. monocytogenes* moves to the other parts of the host body while protected from different forms of host immune response components such as antibodies and the complement system. The ingested listeria following the consumption of *L. monocytogenes*-contaminated foods is taken up by the enterocytes, or M cells, located in the small intestines; it then multiplies within the underlying phagocytic cells (Cahoon & Freitag, 2014). The transfer of the *L.*

monocytogenes cells from the intestine within the macrophage cells or lymph node to the liver and spleen leads to the destruction of a large number of these infectious bacterial cells by neutrophils in collaboration with the Kupffer cells.

In a situation with an inadequate T cell-mediated immune response, the listeria will be able to multiply within the hepatocytes and macrophages and thus transport through the blood to the different parts of the body including the brain and the uterus, a condition that lets them penetrate through the blood-brain barriers and placental barriers with pregnant women (Lobel et al., 2015). To ensure that each step of the invasive process takes places effectively, *L. monocytogenes* produces a series of virulent factors, with the gene coding for these factors' being assembled on the chromosome and regulated by the prfA gene (Lobel et al., 2015). These virulence factors are internalins, surface protein p104, Listeriolysin O, ActA protein, phospholipases, metalloprotease, Clp proteases and ATPase, and Protein p60.

2.4. *L. Monocytogenes* in food industry

The adhesion of *L. Monocytogenes* biofilms on food contact surfaces poses many concerns in the food industries because they caused contamination of the food products (Di Ciccio et al., 2012). Different studies on the formation of the *L. Monocytogenes* biofilms in the food environment have shown that these biofilms have the capacity of persisting in the food environment for several years (Shi & Zhu, 2009)

According to Colagiorgi et al. (2017), *L. monocytogenes* are made up of dominant and nondividing cells that enable them to increase their survival in the food industry. The persistence of *L. monocytogenes* in the food industry can also be attributed to improper cleaning of food contact surfaces. Furthermore, Leong et al. (2014) argued that *L.*

monocytogenes persistence can be related to the biofilm formation because cells formed within the biofilms are more resistant to different cleaning agents and stressful conditions associated with the food industry (Shi & Zhu, 2009)

Silva, Teixeira, Oliveira, and Azeredo (2008) determined the attachment capability of various segregates of *L. monocytogenes* on different surfaces including marble, glass, stainless steel, and polypropylene and found *L. monocytogenes* adhere to all evaluated surfaces. However, various studies have been noted differences in attaching pathogens to surfaces (Cortés, Bonilla, & Sinisterra, 2011; Garrett, Bhakoo, & Zhang, 2008; Teughels, Van Assche, Sliepen, & Quirynen, 2006). The adhesion and colonization of bacteria on surfaces often lead to important modifications that may influence the viability of the microorganisms. Studies have shown that various bacteria of biological importance including *L. Monocytogenes*, *Escherichia coli*, and Salmonella can survive for hours and even days on biological surfaces (Lindsay & Von Holy, 2006; Silva et al., 2008). These surfaces may be important sources of foodborne pathogens, especially in food-handling environments (Feng et al., 2015; Silva et al., 2008).

Shi and Zhu (2009) and Yuan, Hays, Hardwidge, and Kim (2017), determined that the physical properties of the contact surfaces in the food-manufacturing industries play an essential role in the promotion of the biofilm adhesion. Additionally, the bacterial adhesion depends on critical surface characteristics. Surfaces with high free energy and moister, for example, will promote bacterial adhesion. Therefore, more bacterial cells will be attached to the hydrophilic surfaces such as stainless steel than hydrophobic surfaces such as Buna-N rubber and glass (Shi & Zhu, 2009). Additionally, the surface chemistry and surface energy of different categories of food exchange surfaces, such as

stainless steel and aluminum, can influence the *L. monocytogenes* attachment and survival in the food industry (Di Ciccio et al., 2012).

2.5. Survival and Growth of *L. monocytogenes* on Leafy Green Vegetables

The contamination of the fresh leafy green vegetables by *L. monocytogenes* can occur after application of sewage water as fertilizers to plant crops. Thus, different forms of *L. monocytogenes* serotypes may circulate among soil, vegetables, and human beings contaminated with feces (Sant'Ana, Franco, & Schaffner, 2014). The survival mechanism of the *L. monocytogene*s is enhanced by its ability to have a saprophytic life while in soil and vegetables and pathogenic life once it enters human or animal cells, a process influenced by the PrfA proteins and the availability of a carbon source (Zhu et al.,2017). *L. monocytogenes* can survive in normal green vegetables with survival's being boosted in the presence high-moisture-containing green vegetables such as fresh leafy spinach. Additionally, the physical vegetable leaf influence the attachment of the *L. monocytogenes*, thus preventing them from being washed away (Zhu et al.,2017).

Therefore, it is should be noted that contaminated soils and nutrient levels on vegetable leaves influence the survival of the *L. monocytogenes*. Fresh vegetables are exposed to different surfaces during processing and transportation, facilitating formation of *L. monocytogenes* biofilm on the leaves of these vegetables (Sant'Ana et al., 2014). According to Zhu et al. (2017), the survival of *L. monocytogenes* on fresh vegetables is influenced by the level of biofilm that they produce because this helps them attach to the vegetable surfaces. Therefore, their survival can be inhibited by disruption of the *L. monocytogenes* biofilms. Sant'Ana et al. (2014) showed that the persistence of the *L. monocytogenes* on the ready-to-eat vegetables resulted from existing harborage sites

formed following the formation of the biofilms. The complex and delicate nature of the vegetable surfaces obstructs the elimination of the *L. monocytogenes* after contamination. *L. monocytogenes* is a psychotropic bacterium and therefore can grow in fresh and freshly cut vegetables for an extended period (Sant'Ana et al., 2014). Colonization of spinach leaves by *L. monocytogenes* takes place in areas where the waxy cuticles are destroyed during harvesting and storing of the produce (Babic et al., 1996). Additionally, hydrophobic pockets and folds in the surfaces of the spinach leaves help in the provision of a protective mechanism for *L. monocytogenes* during the disinfection process (Zhu et al., 2017,Zhang&Farber, 1996). The ability of the *L. monocytogenes* to produce biofilms enables it to be firmly attached to leafy green vegetable surfaces (Zhu et al., 2017). Furthermore, the biofilm is important in creating chemical and physical barriers for the *L. monocytogenes* cells.

According to Botticella et al. (2013), the freshly cut produce is implicated in a higher rate of contamination from *L. monocytogenes* than whole produce. Biofilms formed by *L. monocytogenes* are a possible menace to the safety of freshly cut produce with chances of recurrent contamination and the infection of consumers; hence the need to inhibit the growth and formation of the pathogens. Omac (2014) argued for a number of reasons that the growth of *L. monocytogenes* in spinach and other green leafy vegetables is persistent because biofilms on fresh produce develop as clusters of bacterial cells joining in exopolysaccharide materials. The materials act as a prevention mechanism for harm that might result from environmental stressors, in addition to containing desiccation and bactericidal agents. The phyllosphere areas of the plant also protect the pathogen against washing or surface sanitization techniques such as chlorine and water washing. The

internalization of pathogens into the plant vascular system is an essential issue because decontaminants such as chlorine are not effective in diminishing pathogens during washing procedures.

2.6. Biofilm Composition

Biofilms consist of a cluster of microbes embedded in an organic polymeric matrix, referred to EPS (Donlan&Costerton,2002).This group of microbes is made up of different components that facilitate the formation of the microcolonies containing water channels. The extracellular polymeric substances of the biofilm are composed of proteins, nucleic acids, polysaccharides, phospholipids, and other polymeric substances (Ronner & Wong, 1993).

According to Di Ciccio et al. (2012), the biofilms formation can take place on both biotic and abiotic environments such as living tissues, food processing contact surfaces, and natural aquatic systems. The ability of biofilms to attach themselves to different surfaces depends on the nature of a series of adhesions. Some of the most common types of surface adhesions include polysaccharide adhesions, fimbrial, and nonfimbrial adhesions. The biofilms are made up of heterogeneous microcolonies that enable them to survive in a stressful environment and spread to the neighboring environments as well as ensuring their reproductive success. The ability to respond to stress is one of the most significant characteristics of the biofilms (Shi & Zhu, 2009). The survival of the *L. monocytogenes* in the stressed environment has been facilitated by the ability of its biofilm to induce and oxidative stress response (Di Ciccio et al., 2012).

The arrangement of the biofilm cells helps determine the physiology and physical properties of the biofilms (Shi & Zhu, 2009). Many factors influence the development of

L. monocytogenes biofilms: (a) medium, including composition, presence of antimicrobial agents, and temperature; (b) the inoculums comprising the identity of the organism and the number of cells; (c) hydrodynamics entailing the presence of shear, flow rate, retention time, and batch versus open system; and (d) the type of substrate characteristics like chemistry, roughness, and conditioning films. The factors influence the different steps in biofilm formation from initial attachment to maturation and detachment (Campanac, Pineau, Payard, Baziard-Mouysset, & Roques, 2002).

2.7. Mechanism of Biofilm Formation

The mechanism of biofilm establishment is a multiple-step process facilitated by both biological and physiological factors. According to Myszka and Czaczyk (2011) , the biofilm production takes place in a five-step process that includes: initial reverse attachment of the planktonic microorganism to a solid surface, production of EPS, biofilm architectural development, transformation of the micro-colonies into mature biofilm, and spreading of the biofilm cells to the surrounding environments. The attachment of the single bacterial cells on the abiotic surface initiates the formation of biofilms, a process that largely depends on time and that can be further classified into two stages: reversible and irreversible (Van Houdt & Michiels, 2010).

Biofilm formation is governed by adhesive and cohesive forces determined by various biological, chemical, and physical properties (Stewart & Franklin, 2008). When bacterial cells arrive on the surface for attachment between the distances of 2–50 nm, the reversible phase of bacterial attachment will be developed (Ronner & Wong, 1993). These bacterial cells will be attached to the surface using weak van der Waals and electronic forces (Van Houdt & Michiels, 2010). Depending on the electronic charges

present on the bacterial cells and the contact surfaces, the developed electronic forces between the contact surface and the bacterial cells can lead to the establishment of either repulsive or attractive forces (Mafu, Roy, Goulet, & Savoie, 1991). The development of the irreversible bacterial attachment phase is facilitated by the occurrence of different forces such as the hydrophobic, ion-ion, covalent bonds, and hydrogen exchanges (Myszka & Czaczyk, 2011).

Additionally, the ability of the bacterial cells to contact the abiotic surface depends on their ability to produce surface structures like the extracellular polymers of flagella, fimbriae, and pili. The irreversible attachment of the bacterial cells on the surface are followed by maturation of the biofilm cells (Ronner & Wong, 1993). In the bacterial cells, the detachment process is controlled by a cluster of gene expression regulated by a cell-to-cell regulatory molecule like the acylated homoserine lactones (AHLs) and specific peptides (Davies et al., 1998).

The attached bacterial cells will then mature and divide through use of the nutrients available in the surrounding environment and adapting films, leading to the formation of microcolonies that increase and unite to develop a sheet of cells that will eventually cover the surface (Van Houdt & Michiels, 2010). Furthermore, the attached bacterial cells will synthesize additional extracellular polymers, which will be used by the cells to strongly attach themselves to the contact surface and protect them from the variations of the environment. The maturation of the biofilm cells is a slow process influenced by the nutritional content of the surrounding environment (Myszka & Czaczyk, 2011). As the biofilm cells age, they can detach and scatter away from the biofilm to help them to persist in the harsh conditions and to colonize new niches.

2.8. EPS Composition and Biosynthesis of EPS

The extracellular polymeric substances are natural polymers secreted by microorganisms such as bacteria into their environment and of high molecular weight. Extracellular polymeric substances influence the establishment of the structural and functional integrity of the bacterial biofilms (González-García et al., 2015). They are thus significant components of the bacterial cells that help in the determination of the physiochemical properties of the biofilms. The EPS compounds are components of diverse types of macromolecules such as proteins, polysaccharides, nucleic acids, glycoproteins, and phospholipids. Moreover, EPS elements may be found in varying categories in a given bacteria species (Flemming & Wingender, 2003). Proteins, polysaccharides, and DNA form the extracellular matrix of most biofilms. The molecules are concerned with sticking to the exterior, organization within the biofilm, and collecting bacterial cells (Hefford et al., 2005).The EPS of numerous pure cultures has carbohydrate as the main constituent, while protein is predominant in sludges of various wastewater treatment reactors. Microbial exopolysaccharides contain either homopolysaccharides or heteropolysaccharides. Homopolysaccharides comprise a single monosaccharide type D-glucose or L-fructose (Flemming & Wingender, 2003). Homopolysaccharides are categorized into three groups: D-glucans produced by *Leuconostoc mesenteroides*, D-glucans synthesized by pediococcus, and fructans produced by *Streptococcus salivarius* (Zhang, Bishop, & Kupferle, 1998).

The EPS may be hydrophilic or hydrophobic, with a large number being hydrophilic and containing more than 95% water by weight (McSwain, Irvine, Hausner, & Wilderer, 2005). The tertiary structure of the EPS depends on the chemical

composition and functional group present. The tertiary structure establishes whether the EPS is in a cohesive gel or in a colloidal form (D'Abzac, 2009). Stal (2012) concluded that the structure and composition of EPS are diverse within a wide range of microorganisms. A single strain of the microorganisms can develop over one form of EPS concurrently or at varying growth stages. A large number of the polysaccharides found in EPS are heteropolysaccharides set in recurring units. The EPS has uronic acids such as D-glucuronic acid, D-galacturonic acid, and D-mannuronic acid (Dogsa, Kriechbaum, Stopar, & Laggner, 2005). The uronic acids are essential functional groups containing carboxyl groups in charge of interlinking with other EPS molecules or joining of metals.

The biosynthesis of the EPS is facilitated by a high carbon-to-nitrogen ratio and nutritional limitations of phosphorous, iron, potassium and nitrogen. The production of an extracellular polymer will be higher in an environment characterized by high glucose and low nitrogen contents. According to Czaczyk and Myszka (2007), some bacteria can simultaneously synthesize extracellular polymeric substances and polyhydroxyalkanoates (PHAs) because the conditions for the synthesis of PHA are quite similar to those for EPS.

The enzymes required for the synthesis of the extracellular polymer precursors are under separate control from those for the mechanisms of gene expression linked to the biosynthesis of the EPS molecules (González-García et al., 2015). Czaczyk and Myszka (2007) observed that microorganisms of different taxonomies in the presence of extreme environmental conditions could produce the same or almost identical forms of extracellular compounds.

The biosynthesis and production of the extracellular polymer molecules are controlled by a group of genes such as algAgene, which codes for the GDP-mannose pyrophosphorylase, algD that codes for GDP-mannose dehydrogenase, and algE that programs the membrane proteins, which are involved in the exportation of the alginate (Czaczyk & Myszka, 2007). In different species of bacteria, the biosynthesis of the extracellular polymeric substances is controlled via mega-plasmids instead of in the chromosome (Czaczyk & Myszka, 2007)..

2.9. Importance of EPS Matrix

The generation of the extracellular polymeric substances plays an important role during the formation of the microbial biofilms through the promotion of the biofilm attachment in the adhesion or irreversible adhesion phase of development (Donlan & Costerton, 2002).Therefore, extensive production of EPS takes place during the adhesion stage. The morphological arrangement of the extracellular polymeric substance molecules helps ensure a strengthened interaction between the bacterial biofilm cells and the attachment sites, leading to the establishment of cell clusters on the contact surface (Czaczyk & Myszka, 2007).

Czaczyk and Myszka (2007) established that most of the extracellular proteins, exopolysaccharides, and extracellular DNA are key determinants of the structural morphology of the biofilm matrix. Furthermore, Donlan (2002) determined the proteins produced within the extracellular matrix to have an influential role in the attachment process of microbial biofilms on different solid surfaces because these extracellular proteins can be easily adsorbed onto the contact surface, leading to the formation of a

protein layer on the solid surface favorable for the bacterial cells attachment (Czaczyk & Myszka, 2007).

Additionally, this protein layer formed on the solid surface can transform the solid-medium boundary into a gel-like region, hence enhancing the contact between the microbial cells and the solid surface (Czaczyk & Myszka, 2007).The secreted extracellular proteins within the extracellular polymeric substance matrix also play a key role in the escalation of the bacterial binding process by anchoring e the bacterial cells on the contact surfaces (Czaczyk & Myszka, 2007). The adsorption abilities of the extracellular proteins and the contact surface are determined by the interfacial reorganization of the charged groups and the hydration alterations that take place within the proteins, contact surfaces, and cell surfaces (Donlan, 2002).

The attachment of bacterial biofilm cells on the compact surface is therefore influenced by the tertiary extracellular structure of the protein and the intensity of the molecular exchange between the conjugated pili and the solid surface. To effectively demonstrate its ability to colonize the contact surfaces, the bacterial biofilm cells will rely on the activities of the extracellular matrix proteins (Donlan, 2002).

L. monocytogenes can survive in harsh conditions given their ability to tolerate acidic and osmotic stresses and to grow in cold temperatures (Chae & Schraft, 2000; Köseoğlu et al., 2015). Some of these features have been attributed to specific transcriptional factors, alternative sigma factors, stress tolerance systems, transport proteins, and two-component systems (Chae & Schraft, 2000). Köseoğlu et al. showed that *L. monocytogenes* produce EPS, which makes the cells inside it highly tolerant of disinfectants and long-term desiccation. Listerial EPS is, therefore, an important factor in

listerial persistence in the environment and food safety. There is an assumption that the EPS matrix restricts biocides from getting to the intended microorganism in the biofilm using diffusion limitations or chemical contact with EPS molecules (Flemming & Wingender, 2003). In effect, the impact of EPS on the reaction of bacteria from biocides differs based on the aspects of the biocide applied.

2.10. Factors Influencing the Attachment of Bacterial Cells on Solid Surfaces

2.10.1 Physiochemical properties of the bacterial cells.

Different physiochemical characteristics of the bacterial cells have influential roles in the capacity of these bacterial cells to adhere to solid surfaces (Van Houdt & Michiels., 2010).According to Mafu et al. (1991), the level of interaction between the bacterial complementary surfaces such as the polymers' interactions or the physiochemical characteristics of the bacterial cells such as the charge and the free energies of the surface are some of the physiochemical factors that influence bacterial cell adhesion. Additionally, the application of different methods used in analyzing the physiochemical characterization of *L. monocytogenes* has indicated that this bacterium is hydrophilic (Mafu et al., 1991). Therefore, factors such as cell surface hydrophobicity, surface charges, and the existence of exopolymers play important roles to ensure that these bacterial cells are attached on different surfaces such as stainless steel, glass, polypropylene, and rubber (Mafu et al., 1991).

2.10.2 Solid surface properties

The physiochemical properties of solid surfaces also determine the level of the bacterial cell adhesion. According to Myszka and Czaczyk (2011), the adhesion of the

bacterial cells on solid surfaces is due to the existence of a rough surface, which has a greater surface area and the depressions in the roughened materials, which enhances conducive sites for bacterial colonization. Additionally, Ronner and Wong (1993) established that roughness of the solid surface promotes the level of bacterial adhesion. The rough solid surfaces will lead to an increased surface area onto which the bacterial cells will attach themselves (Van Houdt & Michiels, 2010).

In addition, the initial bacterial cells' attachment is affected by the hydrophobicity and hydrophilicity of the solid surface. According to Myszka and Czaczyk (2011), the metallic surfaces have high surface energies with negative charges, making them highly hydrophilic and making the attachment of microorganism easier than the hydrophobic surface. Additionally, Myszka and Czaczyk established that hydrophobic materials are more resistant to the adhesion of the bacterial cells than hydrophilic surfaces. The bacterial cells have net negative charges contributes to an electrostatic repulsive force, and due to the existence of cellular components such as capsules, lipopolysaccharides, and proteins, the repulsive force between two surfaces is reduced, thus initiating the surface adhesion (Shi & Zhu, 2009).The deposition of the bacterial cells onto the solid surface is initially influenced by the electrostatic repulsive force.

2.11. *L. monocytogenes* Biofilms and Antibacterial Agents

The contamination of food by *L. monocytogenes* can occur within the food processing environments because of their ability to persist in stressful environment following their attachment to food contact surfaces. The adhesion and colonization of the surrounding environment by the *L. monocytogenes* are facilitated by its ability to produce biofilms (Sadekuzzaman, Yang, Mizan, & Ha, 2015). Even though the state of the *L.*

monocytogenes biofilm cells shows decreased vulnerability to the antimicrobial agents, there are still some different types of antibacterial agents being used to destroy or control the growth of the *L. monocytogenes* cells, some of which involve the use of bio-solutions such as enzymes, phages, interspecies interactions, and antimicrobial molecules (Sadekuzzaman et al., 2015).

Additionally, the application of plant extracts, such as essential oils, and surfactants have been applied in recent years to destroy *L. monocytogenes* biofilms, though they have not been fully effective. These surfactants can be classified into anionic, nonionic, cationic, and amphoteric. The applicability and efficiency of each type of surfactant in the removal of the bacterial biofilms depend on the surface charges of both the bacterial cells and contact surfaces (Santos, Rufino, Luna, Santos, & Sarubbo, 2016).

Schramm, Stasiuk, and Marangoni (2003) stated that nonionic surfactants are the second-most produced agents in the industry. Nonionic surfactants do not ionize in aqueous solutions because their hydrophilic group is of a nondissociable form, such as phenols, alcohols, and esters. Nonionic surfactants are hydrophilic because of the polyethylene glycol chain, gained through polycondensation of ethylene oxide. The polycondensation process of propylene oxide creates a polyether that is relatively hydrophobic. The polyether chain is applied as the lipophilic group within PolyEO–PolyPO and blocks copolymers that are in a different class, such as polymeric surfactants (Schramm et al., 2003).

The abilities of surfactants to lower the surface tension between two liquids or between a liquid and solid make the compounds suitable not only for laundry, but also in the removal of biofilms from surfaces. Both surfactants, as well as the biofilms, contain

charges, and therefore form an electrostatic bond (Simões, Pereira, & Vieira, 2005). The electrostatic bond causes the cells in the biofilm to stretch, and then undergo lysis, which causes cell death. The activities of the bacteria are destroyed, and they are removable once the cells are dead. Surfactants also function by destroying the permeability of the biofilm's cell wall, which reduces intake of important nutrients and can lyse the cells. Surfactants can also cause protein denaturation resulting in cell death (Simões et al., 2005). Simões et al. (2005) studied the control of biofilms using surfactants, cetyltrimethylammonium bromide (CTAB), and sodium dodecyl sulfate (SDS), a cationic and an anionic surfactant. The authors concluded that CTAB application by itself did not promote the detachment of biofilms from the surface, whereas SDS used in high concentrations limited the growth and formation of biofilm. Van Houdt and Michiels (2010) concluded that there is increased resistance by biofilm cells to biocides because of the interference of the exopolymeric matrix. However, the use of disinfectants to kill biofilms increased in efficiency with the addition of quaternary ammonium compounds. For instance, peroxy acid disinfectants were more effective than chorine in the deactivating of the multispecies biofilms of pseudomonas sp. and *L. monocytogenes* on stainless steel (Fatemi & Frank, 1999). Dupard (2005) concluded that (CPC) can successfully be used as a washing solution to inhibit *L. monocytogenes* growth on the surface of shrimp, both cooked and raw. The highest reduction of *L. monocytogenes* counts on CPC-treated shrimp was 1.69 Log CFU/g found on the surface of headless, shell-on cooked shrimp. A similar pattern was observed when *L. monocytogenes*-inoculated CPC-treated shrimp were exposed to a water rinse then stored at - 20°C for 90

days. However, CPC has not yet been approved by the FDA as an antimicrobial agent for seafood (Dupard, 2005).

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Chapter III. Role of Surfactant-Extracellular Polymeric Substances in the Removal of *Listeria monocytogenes* Biofilm formed on fresh-cut produce

ABSTRACT

Bacterial attachment to surfaces and consequent biofilm formation have serious implications in the food, environmental, and medical fields. When ingested, *Listeria monocytogenes* can cause serious and possibly fatal illnesses in humans and animals. Spinach can get contaminated by *L. monocytogenes* during harvest. This study aimed to evaluate the effectiveness of six nonionic surfactants (Pluronic F68, Pluronic F127, Tween 20, Tween 40, Tween 80, and Brij 58) in disrupting *L. monocytogenes* biofilms on the surface of spinach leaves. Wells were washed with surfactants after incubation and then mixed on a platform shaker for 1, 5, 15, and 30 min. Then, the wells were rinsed with distilled water to remove dead cells, and fixation was conducted at 30 min at 60 °C. Our findings showed that Brij 58 most effectively removed the *L. monocytogenes* biofilm on spinach, followed by Pluronic F127, Tween 80, Tween 40, Tween 20, and Pluronic F68. The amount of polysaccharides and proteins secreted by *Listeria* increased with time. Moreover, addition of extracellular polymeric substances changed the hydrophobic properties of the leaves, which was necessary for adhesion and biofilm formation on the spinach leaf surface.

Keywords: Listeria monocytogenes biofilm, fresh-cut produce, extracellular polymeric substances, nonionic surfactants

1. INTRODUCTION

Regulations to prevent food spoilage and foodborne illnesses (FBIs) encompass various guidelines for the handling, preparation, and storage of foods (Rodrigues et al. 2017). Most FBI outbreaks are caused by bacterial contamination of fruits and vegetables, especially lettuce, spinach, sprouts, and cantaloupes. Food safety and the increased significance of FBIs is a critical concern internationally. Partially or fully prepared fresh spinach and other leafy greens contaminated with *Sal**monella* spp., *Escherichia coli*, and *Listeria monocytogenes* are the primary causes of FBI outbreaks in the US.

L. monocytogenes is a nonsporing, gram positive, facultative anaerobic bacillus causing often-fatal listeriosis in humans. *L. monocytogenes* perpetuates contamination by establishing biofilms, especially on fresh-cut lettuce (Costa, 2016). Fresh-cut produce, such as spinach, is more susceptible to contamination by *L. monocytogenes* than unprocessed whole spinach products. According to Botticella et al. (2013), *L. monocytogenes* can actively proliferate on fresh-cut spinach and cause FBIs.

A biofilm is a group(s) of heterogeneous or homogenous bacteria and other microorganisms able to adhere to other biotic or abiotic surfaces and remain in the matrix of extracellular polymeric substances (EPSs) secreted by constituent populations. Although ubiquitous it is sometimes difficult to eradicate biofilms from produce (Gupta et al. 2016). Bacterial cells are embedded in the EPS matrix, an important component of biofilms, and are classified as soluble and insoluble. Soluble EPSs help in bacterial adhesion and accumulation; insoluble EPSs contribute to rigidness and structural changes

(De Sousa et al. 2015). The matrix contains proteins, polysaccharides, and extracellular DNA, enabling biofilm functions (Colagiorgi et al. 2016).

Surfactants can effectively prevent biofilm formation. Brandl and Huynh (2014) reported that Tween 80 inhibited *Pseudomonas aeruginosa* growth. Nonionic surfactants are widely applied in many food-processing industries to remove and prevent *L. monocytogenes* and other bacterial biofilms because these substances are biodegradable, less toxic, and cost effective (Nielsen et al. 2016). Nonionic surfactants can absorb at the surface and destroy biofilms formed on the spinach leaf surface. This is improved by increasing the critical micelle concentration on the surfactant structure (Olkowska et al. 2014).

Attachment of microorganisms to surfaces and subsequent biofilm formation are very complex processes. Strategies using nanotechnology, detergents, quorum quenching, natural substances, and enzymes to inhibit biofilm formation have been investigated (Sadekuzzaman et al. 2015). Understanding the biofilm formation process of *L. monocytogenes* on plant surface and the role of nonionic surfactants in disrupting early attachment of *L. monocytogenes* is critical to ensure food safety. Nonionic surfactants are the second commonest agents used for cleaning produce. Although studies have documented that various surfactants can destroy bacterial biofilms, we focused on the removal of *L. monocytogenes* biofilm from the spinach leaf surface by washing with Pluronic F68 and 127, Brij 58, Tween 20, 40, and 80.

Because EPS composes the microbial biofilm and can promote biofilm formation, this study aimed to determine the contribution of EPS to biofilm formation and to determine how nonionic surfactants disrupt *Listeria* from preformed biofilms by (1)

measuring the amounts of proteins and polysaccharides in the EPS matrix, (2) determining the effect of EPSs on leaf surface hydrophobicity, (3) evaluating effects of preconditioning the surface of spinach leaves with EPS, (4) investigating effect of anti-adhesive/anti-biofilm antimicrobial properties of nonionic surfactants on the disruption of preformed biofilms of *L. monocytogenes* on the spinach leaf surface, and (5) identifying effects of the interactions of nonionic surfactants with *L. monocytogenes* EPSs.

2. Material and Methods

2.1. Bacterial culture preparation

L. monocytogenes strain Scott A was purchased from D. Portnoy-The University of California, Berkeley, CA, USA. Freshly acquired *L. monocytogenes* was maintained in brain heart infusion (BHI) broth (Difco laboratories, Franklin Lakes, NJ, USA) in a sterile flask incubated at desired temperatures. All strains were stored in BHI broth supplemented with 30% glycerol at (−20°C) until use (Gorski et al. 2003). The inoculum used for various experiments were subcultured from the original culture.

2.2. Preparation of spinach leaf surfaces

Spinach was commercially purchased and was freshly cut during each experiment. Each leaf was washed with distilled water to eliminate debris, disinfected with sodium hypochlorite (6.25 mL/L deionized water), and then rinsed again with distilled water to remove disinfectant residues. The leaves were air dried on filter paper. When cutting and adjusting the leaves, a cork was fitted to the bottom of each well of a 24-wellmicroplate.

2.3. Preparation of surfactant solutions

Brij 58 and Tween 20, 40, and 80 were obtained from Merck KGaA (Darmstadt, Germany). Pluronic F127 and F68 were purchased from Spectrum Chemical Mfg. corp. (New Brunswick, NJ, USA). Six different surfactants (Pluronic F127, Pluronic F68, Brij 58, and Tween 20, 40, and 80) were prepared by dissolving the surfactant in water at 25°C to the appropriate critical micelle concentration 0.8,1, 0.08, 0.0499, 0.0333, and 0.015 mM, respectively **Figure 1**.

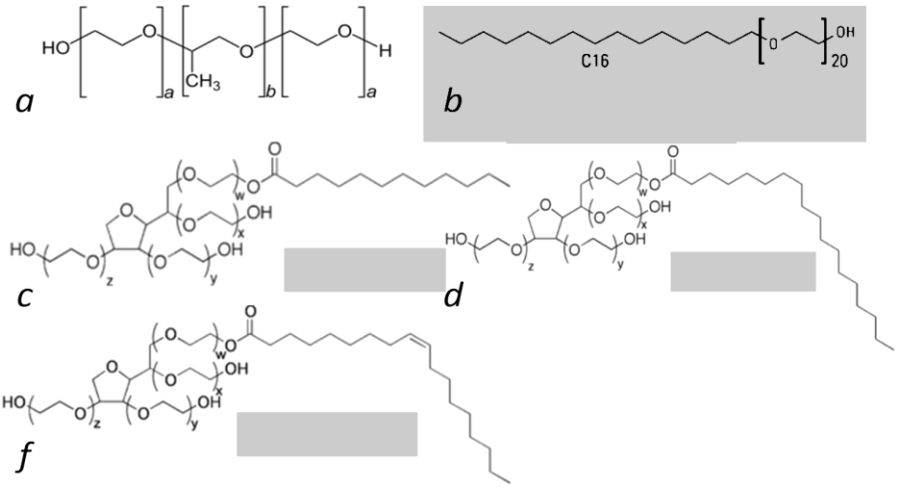

Figure 1. Structural formulae of surfactants used: (a) Pluronic (F68 a = b = 127 a = b =), (b) Brij 58, (c) Tween 20, (d) Tween 40, and (f) Tween 80; A chemical structure of six non-ionic surfactants that used in the experiments.

2.4. Extraction of soluble EPS fraction from *L. monocytogenes* culture

Soluble EPSs are readily dissolvable and digestible in water, whereas insoluble EPSs are not. Compared with insoluble EPSs, soluble EPSs enable easy extraction from a biofilm as they are readily digestible (De Sousa et al. 2015).

Here, EPS was extracted from *L. monocytogenes* planktonic cells using two methods. As the first method, EPS was extracted using a modification of the protocol reported by Liu

and Fang (2002). *L. monocytogenes* was cultured overnight in BHI broth, after which a 100 µL aliquot was transferred into a 50 ml tube containing 10 mL BHI broth to achieve a concentration of 10^7 CFU/mL. The extracts were purified by centrifugation at 13000 rpm for 20 min at 4°C without adding any chemical extractant (Maree and Viljoen, 2012), and then diluted and stored in vials. After centrifugation, the extract was filtered via a 0.2-µm membrane to remove microbial cells and low molecular weight metabolites (do Valle Gomes and Nitschke, 2012).

As the second method, EPS was extracted at different bacterial growth stages. *L. monocytogenes* cells were cultured overnight in BHI broth (Colagiorgi et al. 2016). A 100 µL aliquot of it was then transferred to a 50 ml test tube containing 10 ml BHI broth to reach a concentration of 10^7 CFU/mL (Combrouse et al. 2013). For sample preparation, 5 ml diluted culture was collected at 0, 1, 3, 6, 7, 8, and 24 h. Bacterial samples collected at different times were transferred to a new10 ml tube and centrifuged at 13000 rpm for 20 min at 4°C without chemical extractants (Liu and Fang, 2002). Afterward, the membrane was separated to remove microbial cells. Improved Bradford assay was used to measure EPS protein content with bovine serum albumin (BSA) as standard (Ernst and Zor, 2010).

2.5. Determination of protein content

To measure humic substance and protein contents in EPS, modified Bradford assay (Ernst and Zor, 2010) that uses BSA as the standard was performed (Kruger, 2010). A detailed description of the Bradford assay is provided by Ernst and Zor (2010); BSA was used as the standard solution. In all experiments, 0.1 mg/ml stock solution was diluted to 0.05, 0.025, and 0.0125 mg/ml (Brouwer et al. 1998). A 96-well microplate

reader was used to measure absorbance. The Bradford reagent was diluted by 2.5 fold with deionized water, followed by addition of 50 µL of 0.1, 0.05, 0.025, and 0.0125 mg/ml BSA to each well in triplicates. Deionized water was used to complement each concentration of BSA to achieve 100 µL/well (Ernst and Zor, 2010). Next, 100 µL of the unknown proteins were added in triplicates in different wells. To each well containing BSA and unknown samples, 100-µL diluted Bradford reagent was added to obtain total volume of 200 µL per well. As a negative control, 200 µL deionized water was added to three wells. As reported by Ernst and Zor (2010), absorbance at 450 and 595 nm after 10 and 30 min were measured and a calibration graph was prepared by dividing values of the two wavelengths. Unknown sample concentrations were calculated according to the liner equation of the calibration curve.

2.6. Determination of total polysaccharide content in the supernatant

The phenol-sulfuric acid colorimetric method with glucose as the standard was used to measure EPS carbohydrate content. All samples were assayed in duplicates. Briefly, 0, 2, 4, 6, 8, and 10 µL of 10 mg/mL standard solution were dispensed into the wells of a 96-well plate to generate standards of 0, 4, 8, 12, 16, and 20 µg per well. Water was added to each well to achieve final volume of 30 µL.

Next, 10 µL of the unknown sample and 20 µL of deionized water were added to individual wells. Finally, 150 µL concentrated sulfuric acid was added to each well (Sigma-Aldrich, 2014), and the plate was placed on horizontal shaker to mix the contents. The plate was covered to block light and was incubated for 15 min at 90°C. Finally, 30 µL of 80% phenol was added to each well, and the plate was placed on a horizontal

shaker, and the contents were mixed for 5 min at room temperature. After the contents were mixed for an additional minute by pipetting, absorbance was read at 490 nm.

2.7. Measurement of the surface tension of EPS-surfactant interaction

Surface tensions of surfactant solutions were measured using pendant drop method in an optical goniometer (Rame-Hart Instrument Co., Succasunna, NJ, USA). Six surfactant solutions were added to the BHI broth and EPS supernatant mixture in a 1:1(v/v) ratio and vortexed. The resultant solutions with six surfactants and the control (without surfactant) were drawn at room temperature into a 1 cm^3 glass syringe through a stainless steel needle with an outer diameter of 2.4 mm. Drops that formed on the syringe tip were observed under light microscope equipped with a video camera (CAM101; KSA Instrument Ltd. Helsinki, Finland) connected to a frame grabber card. For each sample, the average surface tension of 20 droplets was calculated by fitting the calculated profile to the Young–Laplace equation (Berry et al. 2015).

2.8. Contact angle measurement

Spinach leaf hydrophobicity was calculated by measuring contact angle. Two leaves were placed on a glass slide, and one was treated with EPS for 6 h, whereas the other was used as untreated control, 10 µL drops of water deposited on the surface of each leaf were observed under a microscope equipped with a video camera (CAM101; KSA Instrument Ltd., Helsinki, Finland) connected to a frame grabber card. Contact angles were measured on acquired images.

2.9. Cell adhesion and biofilm formation assay

After overnight culture in BHI broth, 100 µL of the suspension was transferred into a 15-mL test tube and combined with 10-mL fresh broth to achieve a concentration of 10^7 CFU/mL. Then, 30 µL of 1% wax was added to each well of a 24-well tissue culture plate to create a spinach leaf paste. After adding 400 µL EPSs to each well and 400-µL BHI broth to each control well, the plate was incubated for 24 h at room temperature. After incubation, the EPSs and BHI broth were removed without rinsing and 400 µL of diluted bacteria was added and incubated for 6 and 24 h to develop a biofilm. The cell suspension was then removed and washed twice. Totally, 400 µL of buffered water was added and the cells were fixed for 30 min at 60°C.

2.10. Biofilm removal assay

Biofilm formation on spinach leaves was conducted according to Stiefel et al. (2016) and do Valle Gomes and Nitschke (2012) with some modifications. Briefly, 30 µL of 1% wax (Docosanal) was added to each well of a 24-well plate to ensure that the spinach leaves were attached to the well bottom. *L. monocytogenes* was cultured overnight in BHI broth. BHI broth supplemented with 1% glucose was used to prepare *Listeria* cell suspension dilution. A 100 µL aliquot of the cell suspension was transferred to a 15 mL test tube containing 10 mL of BHIG 1% broth to achieve a concentration of 10^7 CFU/mL (Stiefel et al. 2016). Then, 400 µL of diluted bacteria was transferred by pipette into the wells of a sterilized 24-well polystyrene flat-bottom tissue culture plate, which was incubated for 24 h at 37°C. To avoid contamination and prevent light penetration, the microplate was covered (do Valle Gomes and Nitschke, 2012; Ledala et al. 2010).

After incubation, each well was washed twice with 400-µL buffered water to remove dead cells. To disrupt the biofilm, 400 µL of each surfactant was added in triplicates to the wells of a 24-well plate. As a control, 400-µL buffered water was added to each well in a row. The plate was vortexed for 1, 5, 15, and 30 min to acquire dynamic culture. Afterward, the spent media and superfluous planktonic cells were discarded, and the adherent cells were gently rinsed twice with buffered water. Then, 400-µL buffered water was added to each well (do Valle Gomes and Nitschke, 2012). The cells were then fixed for30 min at 60°C.

2.11. Biofilm quantification

Acridine orange staining was performed as described by Stiefel et al. (2016), but with some modifications. The acridine orange solution comprised 2% acridine orange dissolved in water at 1:100 (v/v) in Walpole's buffer. Then, 400 µL of it was added to each well of a 24-well tissue culture plate. After 15 min incubation in the dark, the solution was discarded, and the plate was gently rinsed twice with 400 µL 0.9 % NaCl solution. After discarding the washing solution, fluorescence intensity was measured using an emission filter at 527/20 nm and an excitation filter at 485/20 nm (Stiefel et al. 2016) using the Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT, USA).

2.12. Epifluorescense microscopy

Following spectrophotometry of *L. monocytogenes* biofilms on spinach leaves stained with 2% acridine orange for 15 min, leaves that were treated for 30 min with appropriate surfactant solution were placed on a glass slide for examination using epifluorescense microscopy.

3. RESULTS

3.1. Kinetics of EPS soluble fraction formation in *L. monocytogenes*

EPS was extracted from planktonic *L. monocytogenes* cells at 0, 1, 3, 6, 7, 8, 24, and 30 h. Polysaccharide and protein contents of EPS were determined using phenol-sulfuric acid colorimetry and Bradford assay, respectively. Figure 2 and Figure 3 indicates that extracted polysaccharides and protein amounts had increased with time. The smallest increase in these amounts was observed between 0 and 1 h, whereas the largest increases were observed between 24 and 30 h. At 0 h, 0.095 mg/mL polysaccharides were extracted, which gradually increased to 0.118 mg/mL at 8 h and stabilized at 0.15 mg/mL between 8 and 24 h. After 30 h, only a slight increase in extracted polysaccharides (0.157 mg/mL) was observed. As shown in Figure 2, the rate of protein accumulation was highest between approximately 8 and 20 h, which then stabilized at approximately 20 h, indicating that the maximum amount of proteins had been synthesized. The amount of proteins in the EPS of *L. monocytogenes* was 0.0049 mg/mL at 0 h and gradually increased to 0.0085 mg/mL at 6 h. Between 6 and 24 h, the amount of synthesized proteins had increased by 0.0085 to 0.0148 mg/mL.

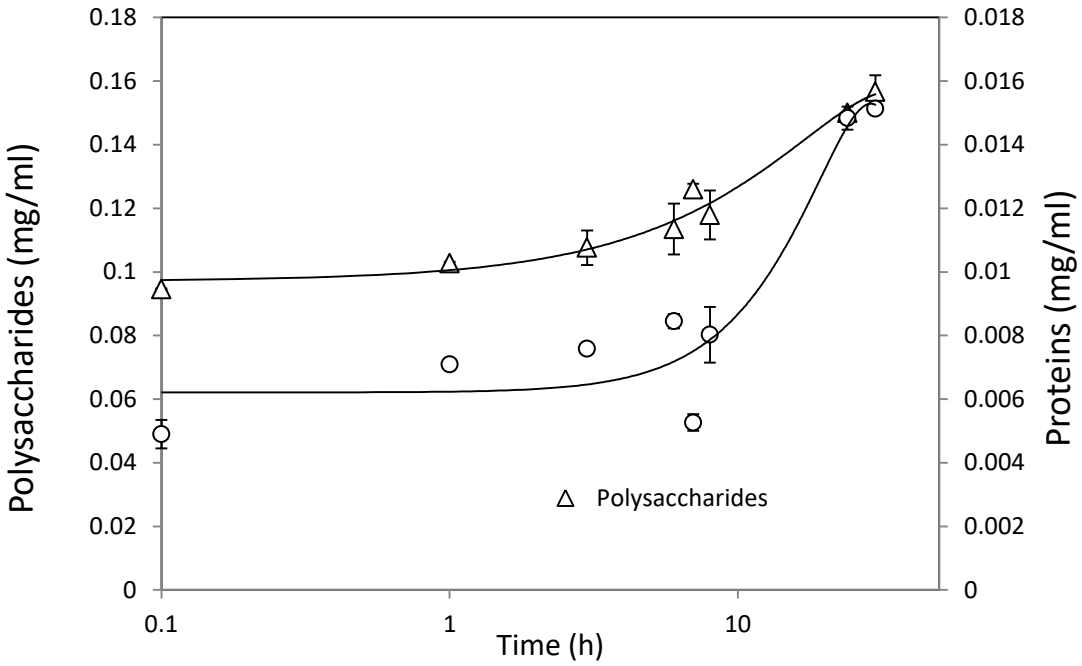

Figure 2. Changes in polysaccharide and protein contents in *L. monocytogenes* EPS

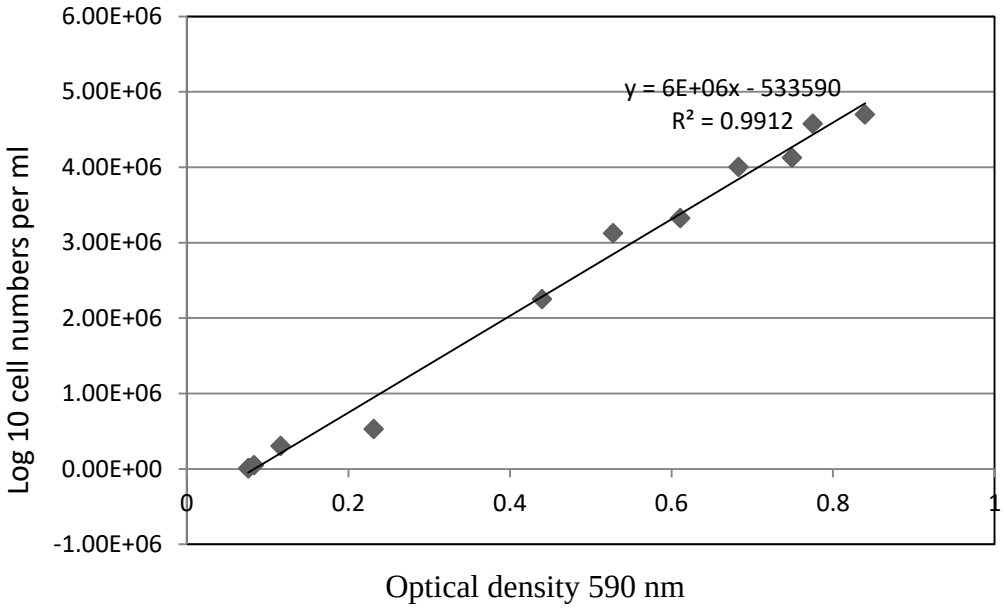

Figure 3. Bacterial calibration curve.

A growth curve was measured using direct growth measurements (serial dilutions and standard plate counts at 30 min intervals) and indirect measurement (spectrophotometric measurements at 30 min intervals).

The rate of carbohydrate content increase (0.0019 mg/mL/h) surpasses that of the protein content (0.0003 mg/mL/h), which explains the variation in protein and polysaccharide contents of *L. monocytogenes* EPS, although both concentrations tended to increase with time.

3.2. Contact angle measurement

The images obtained in this study demonstrated that spinach leaves treated with EPSs exhibited hydrophilic features, which offers further evidence that the EPSs of preconditioned biofilms can change the hydrophobicity of spinach leaves **Figure 4**.

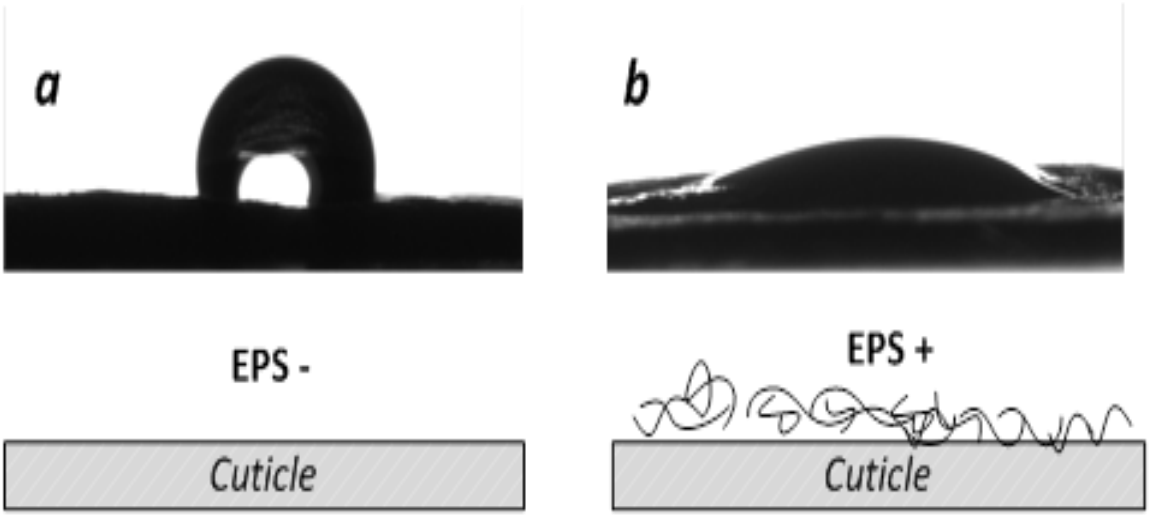

Figure 4. Leaf surface hydrophobicity

Leaf hydrophobicity was measured by contact angle measurement using the pendant drop method performed in an optical Rame-Hart goniometer. Two spinach leaves were placed on a glass slide, and one was treated with EPSs for 6 h, and the other was used as an untreated control (treated with BHI broth only). Images of 10 µL drops deposited on the surface of each leaf were observed under a light microscope equipped with a video camera connected to a frame grabber card. Contact angles were measured on the acquired images. The experiment was repeated twice.

3.3. EPS preconditioned of biofilm promoted cell adhesion and biofilm formation in spinach leaves

The results showed that EPS addition effectively increased the rate of biofilm formation by *L. monocytogenes* on spinach leaf surfaces. EPSs extracted from *L. monocytogenes* planktonic cells demonstrated the ability to enhance the degree of biofilm formation on spinach leaves. The biofilm intensity without adding EPS was 117.1907 mg/mL at 6 h and 162.283 mg/mL after 24 h.

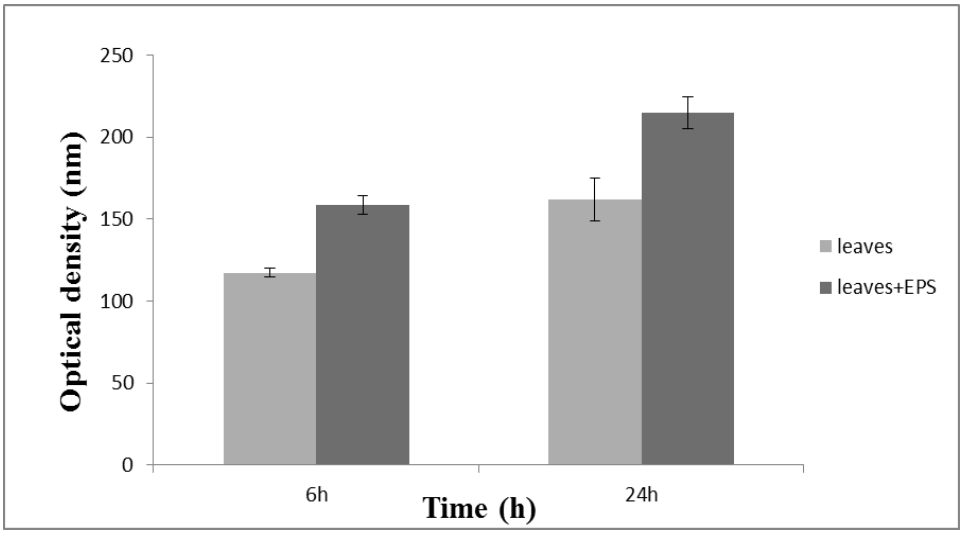

Figure 5. Effectiveness of EPS synthesized by *L. monocytogenes* on the degree of *L. monocytogenes* biofilm formation in spinach leaves

As shown in **Figure 5**, the increase in EPSs was accompanied with an increase in biofilm thickness on the spinach leaf surface, suggesting that EPSs promote bacterial attachment and abundant biofilm formation on solid surfaces. Moreover, presence of EPSs influenced the secretion of more compact and thicker *L. monocytogenes* biofilms. EPSs had an apparent impact on the biofilm formation capability of *L. monocytogenes.* After incubation for 6 h on the spinach leaf surface, the amount of formed biofilm was greater with EPSs than without (158.6257 vs. 117.1907 mg/mL, respectively). Similar results were obtained after 24 h of incubation (214.891 vs. 162.283 mg/mL, respectively).

3.4. Surfactants promoted biofilm removal from leaf surfaces

Surfactants can be used to remove bacterial biofilms from leaf surfaces. This study focused on the effectiveness of six surfactants to disrupt biofilm formation to reduce the growth and proliferation of *L. monocytogenes* on spinach leaf surfaces. As

shown in **Figure 6**, the effectiveness of five of the six surfactants increased with time from 5 to 30 min, as there was little change in the effectiveness of Pluronic F127.

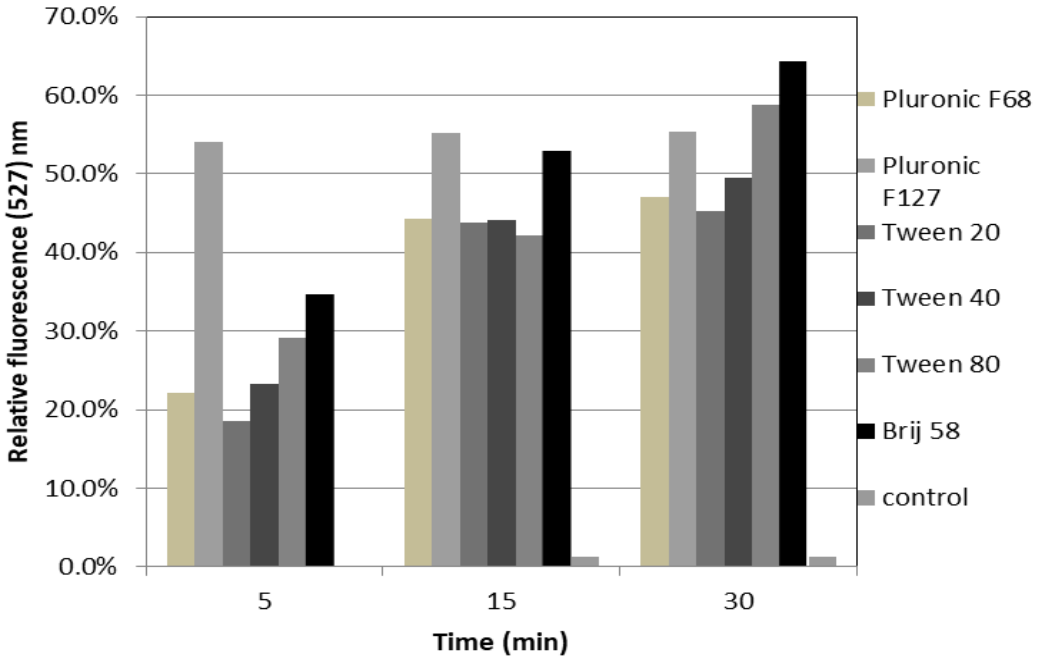

Figure 6. Total cell quantification using acridine orange staining (AOS).

No surfactant caused any significant changes in the number of *L. monocytogenes* cells at 1 min, whereas there were noticeable differences at 30 min, suggesting that the most effective time to remove *L. monocytogenes* biofilms was after 30 min**.** Among the surfactants, Pluronic F127 most effectively reduced the total bacterial cell number after the first 5 min, which decreased by approximately 54.1%, whereas Brij 58 decreased the number of cells by 34.7%. The number of bacterial cells reduced by Pluronic F127 appeared to stabilize from 15 to 30 min (55.1% and 55.2%, respectively). These results suggest that Pluronic F127 was the most effective surfactant in the first few minutes of contact with *L. monocytogenes* and its ability to destroy the biofilm increased with time. Brij 58 was the most effective in disrupting biofilm formation and growth (34.7%,

52.9%, and 64.4% at 5, 15, and 30 min, respectively). Brij 58 resulted in the greatest reduction in bacterial cells after 30 min, compared with other surfactants.

Tween 80 was the third most effective surfactant. At 5, 15, and 30 min, it reduced the percentage of cells in the biofilm by 29.2%, 42.1%, and 58.8%, respectively. Efficacy of Tween 80 against *L. monocytogenes* also increased with time. Tween 40 was the fourth most effective surfactant against bacterial cell growth as indicated by reduction in cell number over time (23.3%, 44.1%, and 49.4% at 5, 15, and 30 min, respectively).

Pluronic F68 also effectively destroyed *L. monocytogenes* cells in the biofilm; however, it was fifth in effectiveness (22.2%, 44.3%, and 47.1% at 5, 15, and 30 min, respectively). Therefore, unlike Pluronic F127, which tended to stabilize after 15 min, Pluronic F68 was less effective; its activities increased with time. Tween 20 was the least effective (18.5% and 45.3% at 5 and 30 min, respectively).

The results of these experiments indicate that, on average, the largest reduction in the bacterial cell number occurred between 5, 15, and 30 min. In fact, the effectiveness of all surfactants increased after 15 min. However, the activity of Pluronic F127, which was the most effective surfactant, had mostly stabilized after 15 min. These results also indicate that two or more surfactants could be combined to enhance efficacy against biofilms. Pluronic F127 was most effective within the first 5 min, whereas Brij 58 activity increased with time. Therefore, a combination of Pluronic F127 and Brij 58 may enhance bacterial cell removal from the spinach leaf surfaces ,Figure 7

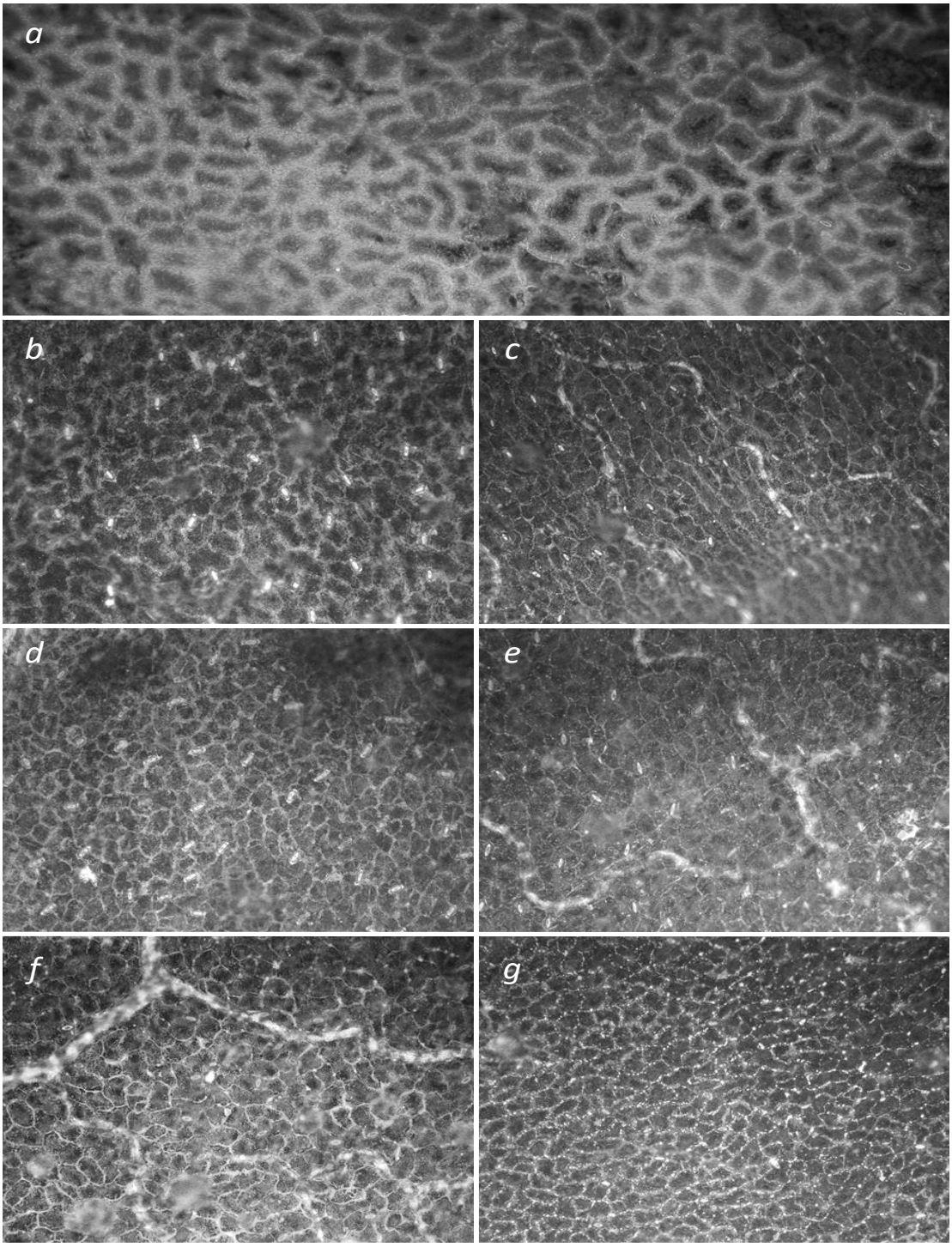

Figure 7. Fluorescent images of the *L. monocytogenes* biofilm on a spinach leaf after 24 h of growth (a) as a control and after consequent washing with nonionic surfactants: Tween 20 (b), Tween 40 (c), Tween 80 (d), Brij 58 (e), Pluronic F68 (f), and Pluronic 127 (g).

Following spectrophotometric measurement of L. monocytogenes biofilms on spinach leaves stained with 2% acridine orange solution for 15 min and after 30 min of treatment

with the surfactant, the leaves were mounted on a glass slide and examined under an epifluorescence microscope.

3.5. Interaction of the *L. monocytogenes* EPSs and nonionic surfactants at the water–air interface

All nonionic surfactants that did not develop complexes with EPSs efficiently removed the biofilm. As shown in **Figure 8**, the difference in surface tension was greatest between the control BHI broth and EPSs. Specifically, the control experiment indicated that the surface tension of EPSs (−) was 36.907 dyne, whereas that of EPSs (+) was 31.581 dyne. Tween 20 was the least effective surfactant to dislodge the biofilm. Its surface tension was 29.917 dyne for EPS (−) and 27.723 dyne for EPS (+). For EPS (−) and (+), the surface tension of Tween 80 was 36.818 and 35.573 dyne, respectively, and that of Tween 40 was 34.41937 and 33.85488 dyne, respectively. The difference in the activity between the BHI and EPS (+) surfactants on surface tension values indicated that Pluronic F 127 was more efficient than either of the Tween compounds. Moreover, for EPS (−) and (+), the surface tension of Pluronic F 127 was 24.904 and 24.202 dyne, respectively, whereas that of Pluronic F68 was 30.947 and 30.651 dyne, respectively. These data also showed that Brij 58 had the least significant difference between surface tension values of EPS (+) and (−) surfactants (26.061 vs. 26.074 dyne, respectively).

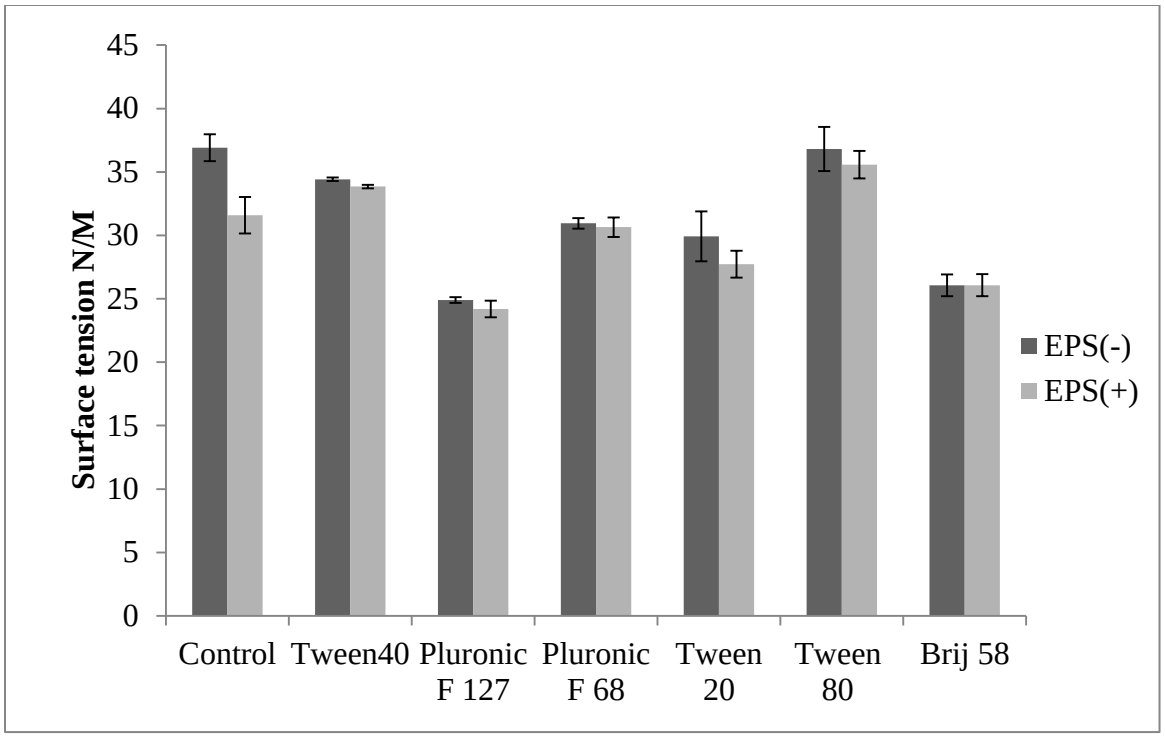

Figure 8. Surface tensions of various surfactant interaction with *L. monocytogenes* EPSs

The surface tension of surfactant solutions were measured using the pendant drop method in an optical Rame–Hart goniometer. Six surfactant solutions mixed 1:1 (v/v) with BHI broth and with EPS supernatant separately and the control (without surfactant) were vortexed and drawn at room temperature into a 1 cm^3 glass syringe through a stainless steel needle with an outer diameter of 2.4 mm. Drops that formed on the syringe tip were observed under a light microscope equipped with a video camera connected to a frame grabber card. For each sample, the average surface tension of 20 droplets was calculated by fitting the calculated profile to the Young–Laplace equation. The error bars represent the standard deviations of the results of three independent experiments.

As shown in **Figure 9**, after 1 min; there was no change in the number of *L. monocytogenes* cells in the biofilms formed on the spinach leaves. As time increased from 5 to 15 min and then to 30 min, there was a significant interaction between surfactants and a *substrate*. The correlation between surfactant exposure and bacterial contamination of spinach leaves over time is illustrated in **Figure 9**. Notably, the increase in the time of exposure of the biofilms to the nonionic surfactants was directly proportional to the number of *L. monocytogenes* cells in the biofilm that were destroyed,

with the greatest correlation between surfactant exposure and cell death occurring at 30 min.

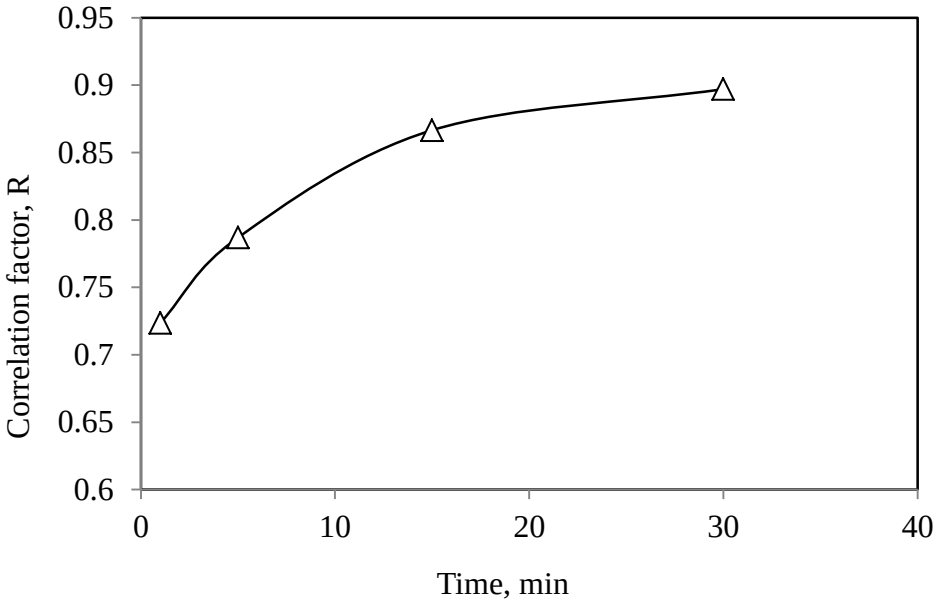

Figure 9. Correlation between surface tension data and biofilm removal assay data.

Surface tension data and biofilm removal assay data were used to see the efficacy of non-ionic surfactant activity through the time based on the surface tension data.

4. DISCUSSION

4.1. Kinetics of EPS soluble fraction formation in *L. monocytogenes*

The assay results demonstrated that polysaccharides and proteins comprise the EPS matrix; however, the rates of synthesis of these molecules differ. Other constituents of the EPS matrix included nucleic acids and lipids. The proteins and polysaccharides in the EPS matrix contribute to the mechanical stability of biofilms. The EPS matrix is an important constituent of microbial biofilms (Flemming and Wingender, 2010).

Flemming and Wingender (2010) showed that the rate of polysaccharide synthesis in the EPS matrix was far greater than that of proteins; the rate of polysaccharide

synthesis was 0.0019 mg/mL/h, whereas that of proteins was 0.0003 mg/mL/h, which provides an explanation as to why polysaccharides constitute a larger portion of structural components in the EPS matrix, as compared with proteins. The present study indicates that polysaccharides, rather than proteins, are major constituents of the EPS matrix. Proteins and polysaccharides serve as attachment molecules of bacterial cells and protect the biofilm from external damaging factors, such as dehydration and chemical/physical damage.

As shown in **Figure 2** and **3**, differences in the extracted polysaccharide and protein volumes were caused by various factors. In the initial stages of bacterial growth, few polysaccharides were produced because much of this volume was consumed during the growth process as a carbon source. In the next stage, the rates of polysaccharide and protein production were similar (Flemming and Wingender, 2010), which would therefore make the values, for both polysaccharides and proteins, move closer to the stationary phase of bacteria. There was a high amount of polysaccharides being produced following the occurrence of bacterial cell metabolic activity. Both polysaccharides and proteins are very important in the formation of biofilms, because both formed the leaf surface with attached bacterial cells. The findings of a majority of studies are consistent with the findings our study, which found that the significant protein and polysaccharide concentrations were the major components of the *L. monocytogenes*-secreted EPS matrix.

This study determined that both polysaccharides and proteins constitute the largest portions of nutrients in the EPS matrix. Polysaccharides and proteins are major factors in microbial biofilms, facilitating cell-to-cell aggregation, *L. monocytogenes* cell attachment, and subsequent generation of a protective biofilm. Another finding is that polysaccharides

were synthesized at greater rates than proteins. Future research should focus on rates of polysaccharide and protein synthesis in *L. monocytogenes* to further understand biofilm formation.

4.2. EPS preconditioning of biofilms promotes cell adhesion and biofilm formation on spinach leaves and leaf surface hydrophobicity

As shown in **Figure 4**, the existence of the EPS matrix renders the surface of *L. monocytogenes* cells hydrophilic. Spinach leaf cuticle is naturally waxy and, thus, hydrophobic (Brandl and Huynh, 2014). This characteristic is important in promoting defense against *L. monocytogenes* cell adhesion (Brandl and Huynh, 2014). When the leaf is exposed to EPSs, the hydrophobic leaf becomes hydrophilic. This indicates that the preconditioning nature of the film changed the leaf's surface characteristics.

Refer to Figure 5, this study revealed that a biofilm formed at a deposition of 158.6257 mg/mL (SD=2.7143) after 6 h and 214.891 mg/mL (SD=9.793) after 24 h. Biofilm formation increased by approximately 135% (214.891/158.6257×100) over an 18 h period (6–24 h) relative to the initial amount of 158.6257 mg/mL. The EPS matrix promoted biofilm formation by *L. monocytogenes*. As shown in Figure 5, presence of EPSs promoted the formation of *L. monocytogenes* biofilm with time.

The findings of the present study agree with those of other studies that reported that synthesis of biofilms is dependent on surface nature and the concentration or availability of essential nutrients (Pechook et al. 2015; Salas et al. 2016; Van Houdt and Michiels, 2010). *L. monocytogenes* synthesizes biofilms containing EPSs. EPSs are categorized as either soluble or insoluble. Soluble EPSs are lighter and smaller than insoluble EPSs (Da Silva and De Martinis, 2013). The soluble EPS matrix covers

molecules, and is responsible for filming over a surface to enhance attachment of insoluble EPSs. They move to the substrate via diffusion, whereas insoluble EPSs move to the film surface via chemotaxis (Figure 10; Da Silva and De Martinis, 2013; Morgan et al. 2013). In agreement with previous studies, the findings of the present study demonstrated that soluble EPSs extracted from *L. monocytogenes* promoted biofilm formation with time, as compared with controls.

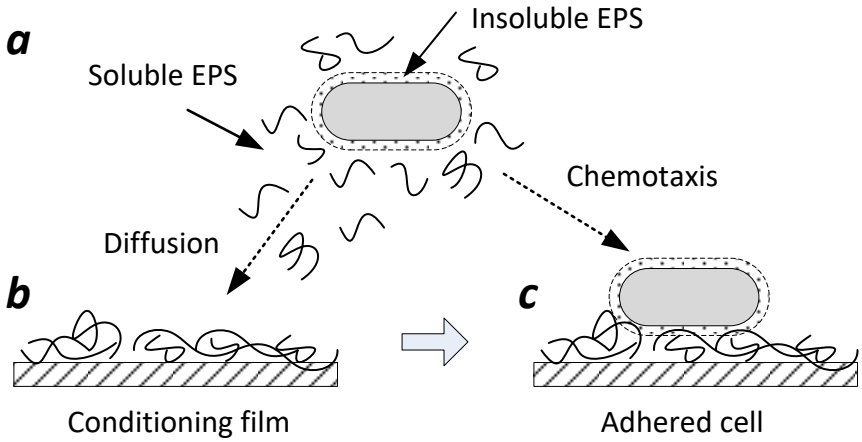

Figure 10. EPS-mediated adhesion of cells to the spinach leaf surface as the first step in biofilm formation

EPS contributes to biofilm formation by conditioning surface and change surface hydrophobicity.

The role of exopolysaccharides in bacterial cell aggregation and reduction of cell migration is consistent with the findings of this study (Figure 5) showing that the EPS matrix was highly involved in biofilm formation. The findings of this study offer a unique insight because previous studies did not satisfactorily describe the role of EPSs in biofilm formation. A major conclusion of this study was that the EPS matrix is an important element needed for biofilm formation.

4.3. Surfactant addition promoted biofilm removal from the leaf surfaces

Surfactants are agents applied for the removal of *L. monocytogenes* biofilms that form on food surfaces and containers to eliminate FBIs resulting from bacterial contamination. Results of Pluronic F127 application can be linked to the hydrophilic nature of the surfactant. Pluronic F127 was found to be anti-adhesive; however, its effectiveness depends on the duration of pathogenic growth, pathogen type, and specific surface. Using Pluronic F127 on medical implants was effective in reducing bacterial adherence and associated infection (Veyries et al. 2000). Efficacy of Pluronic F127 is directly related to the duration of pathogenic growth and ability to reduce cell number, thereby inhibiting growth with time. Pluronic F127 is effective for washing fresh-cut spinach, as it produces surfaces resilient to microbial adhesion. Veyries et al. (2000) reported that using Pluronic F127 effectively reduced the adherence of *Staphylococcus epidermidis* and *Staphylococcus aureus* to polymethylmethacrylate.

Effectiveness of using Tweens in biofilm removal could be attributed to the surfactant's ability to dissolve soluble biofilm material. Tweens 20, 60, and 80 are effective in dissolving spironolactone, because of their ability to disperse solids (Akbari et al. 2015). These can be used to remove the *L. monocytogenes* biofilm as these substances have no negative effects on human health. In support, Parkar et al. (2004) established that Tween 80 is effective because of its surfactant properties that increase biofilm wettability. Brandl and Huynh (2014) established that Tween 80 causes detachment and dispersal of *Salmonella enterica* serovar Thompson by decreasing the total percentage of pathogens.

The antimicrobial activities of Tween 80 for removal of *P. aeruginosa* from lettuce were superior to the activities of other Tween solutions Toutain-Kidd et al. (2009). Contrarily, Nielsen et al. (2016) established the effects of Tween 80 on growth and the antimicrobial susceptibility of *Pseudomonas fluorescens*, *L. monocytogenes*, and *S. aureus* and found that Tween 80 did not significantly affect the colony-formation ability of *L. monocytogenes*. Tween 80 treatment resulted in the formation of fewer biofilms by *L. monocytogenes*. Meanwhile, Li et al. (2002) established that Tween 20 influenced the lipid composition and fluidity of the *L. monocytogenes* membrane. The use of Tween 20 had a greater capability to alter *L. monocytogenes* Nisin sensitivity, as compared with Tweens 60 and 80, suggesting that Tween 20 was most effective (Li et al. 2002).

Brij 58 is a nonionic surfactant that is combined with various solutions for cleaning biofilms. Most nonionic surfactants are considered hydrophilic because of the existence of a polyethylene glycol chain, gained through polycondensation of ethylene oxide. Our results were consistent with those of Zaidi et al. (2011) who established that Brij58 inhibited biofilm formation much more strongly than Tween 20. Brij58 seemed effective in the removal of the *L. monocytogenes* biofilm by washing spinach when compared with the other surfactants used in the study.

The present study indicates that Brij 58 was the most effective in removing *L. monocytogenes* biofilms at 30 min. Pluronic F127 was the most effective at 5 and 15 min. These results are consistent with those of previous studies, which indicate that a short duration of contact with biosurfacants disrupted the biofilms more effectively (do Valle Gomes and Nitschke, 2012). Omac (2014) stated that apart from disinfection

treatments, there are limited control measures to prevent or remove pathogenic microorganisms prior to the produce being consumed. Therefore, development of new and better techniques is required to eliminate pathogenic microorganisms from spinach and other fresh produce.

The results of surface tension interactions of nonionic surfactants with the EPS matrix demonstrated that nonionic surfactants that did not develop complexes with the EPS matrix constituents were vital in the removal of biofilms from leaf surfaces compared with the complex-forming nonionic surfactants (Figure 8). There was a small difference in the anti-biofilm formation activities of the EPS (+) and EPS (−) surfactants. Surfactants that did not form complex compounds with the EPSs were the most efficient. The non-ionic surfactants replaced the polysaccharides in the EPSs formed on the spinach leaf surfaces, thus completely removing the biofilm (Figure 11). According to Valle Gomes and Nitschke (2012), for the optimal removal of biofilm, the surfactants must enter the interface between the solid substrate and the biofilm to ease adsorption at the interface and to minimize the interfacial tension. Consequently, the attractive interactions between the bacterial surfaces and solid areas might be reduced, easing the process of biofilm removal (do Valle Gomes and Nitschke, 2012; Darvas et al. 2011).

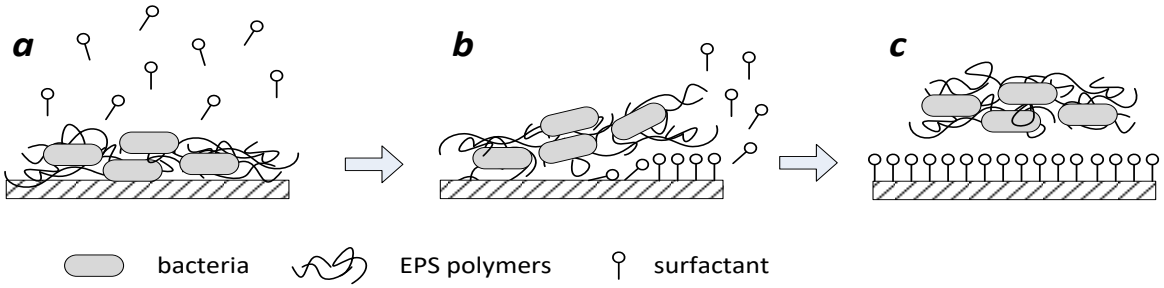

Figure 11. Proposed mechanism of biofilm removal by nonionic surfactants
Non-ionic surfactants were adsorbed on the leaf surface, removing the biofilm.

Refer to Figure 9, the increase in time of contact between the surfactant and biofilms led to the increase in the death of cells in the biofilm. Provision of enough time of contact between the biofilms and the surfactant is very important to optimize the effectiveness of the surfactant against the biofilm by ensuring that a sufficient volume of the surfactant can penetrate the biofilm (De Rienzo et al. 2016). As shown in Figure 9, the increase in time led to an increase in the number of destroyed cells in the biofilm, because of an increased interaction level between the surfactant and biofilm. This was attributed to the fact that an increased surfactant volume, which allowed penetration of the biofilm, led to increase in the ability of the surfactant to interfere with the functions of normal *L. monocytogenes* cells.

5. CONCLUSION

The amount of polysaccharides and proteins increased with time. The amount of polysaccharides was compared with that of proteins. Addition of EPSs changed the hydrophobic nature of the spinach leaves, which is necessary for adhesion and biofilm formation. The abilities of six surfactants to remove *L. monocytogenes* biofilms from the surfaces of spinach leaves were tested. The average numbers of bacterial cells that remained after washing for 1, 5, 15, and 30 min were recorded. The results indicated that Pluronic F127 and Brij 58 were the most effective surfactants, whereas Tween 20 and Pluronic F68 were the least effective. Nonionic surfactants do not form complexes with EPS constituents, and therefore, can effectively remove biofilms from the leaf surfaces. The results showed that the greatest correlation between surfactants and leaf surface occurred at 30 min, indicating that the surfactants start to occupy the leaf surface and can completely remove the biofilm with time.

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Chapter IV. Controlling *Listeria monocytogenes* Biofilm on Food Contact Surfaces by Non-Ionic Surfactants

Abstract

The current cleaning methods which are used in the food processing industries are not always sufficient to prevent or disperse the *Listeria monocytogenes* biofilm cells. The formation of the biofilms has enabled the *L. monocytogenes* to survive in different types of unfavorable conditions hence making the process of eliminating them from such surfaces to be difficult. The poor management of these biofilm cells has therefore led to the development of different challenges regarding the fulfillment of the expected safety and high quality of the food products. This has therefore posed serious health-related threats to the customers. To prevent or limit these risks, it is necessary to ensure food safety in the food processing industries by creating conditions which do not favor the thriving of the *L. monocytogenes* on the surfaces of the equipment used in the food processing. In this experiment, the effectiveness of different types of nonionic surfactants such as Pluronic F68, Pluronic F127, Tween 20, Tween 40, Tween 80 and Brij 58 against the *Listeria monocytogenes* biofilm cells formed on food contact surfaces made of Low-Density Polyethylene (LDPE), Polypropylene (PP), Low-Density Polyethylene and Polypropylene (LDPE-PP), Low-Density Polyethylene and Ethylene Vinyl Acetate (LDPE-EVA), Stainless-Steel and Aluminum was determined. The quantification of the amount of the *L. monocytogenes* biofilm cells that were destroyed by the nonionic

surfactants was done using the Acridine Orange staining (AOS) method after different time intervals of 1, 5, 15 and 30 minutes.

Keywords: biofilm, food contact surfaces, *Listeria monocytogenes*, nonionic surfactants.

1. Introduction

The food processing industries have been encountering many challenges regarding maintaining the expected safety and high quality of food products in the recent years. This is as a result of the occurrence of undesirable microbial growth which leads to contamination of the food supply. Microorganisms have the ability to thrive in all of the products of agricultural origin (Bower, McGuire, & Daeschel, 1996). Food safety is considered a major priority in the food processing industries based on the increased consumption of processed foods. Therefore, there is need to eliminate any factor which can promote food spoilage which in turn leads to spread of foodborne diseases (Van Houdt & Michiels, 2010).

The pathogenic microorganisms are the key factors that lead to food-borne infections, and they largely include viruses, parasites, molds, and bacteria (Everson, 1988). There are different types of pathogenic microorganisms, which found on food surfaces. The attachment of these undesirable microorganisms on the food processing surfaces have created a lot of concerns since they can result in product contamination leading to serious economic and health problems (Myszka & Czaczyk, 2011).

Listeria monocytogenes is one of the most common pathogenic microorganisms, which found in the food processing industries (Chen, Pyla, Kim, Silva, & Jung, 2010). It is a Gram-positive bacterium which is rod-shaped, and that can survive in the presence or absence of oxygen. It is broadly disseminated in the environment, and it does not form

spores(Chen, Pyla, Kim, Silva, & Jung, 2010).This bacterium has no defined particular host hence it is identified as a non-host specific pathogen (Chen, Pyla, Kim, Silva, & Jung, 2010). *L.monocytogenes* causes listeriosis, which can occur epidemically or sporadically (Chen, Pyla, Kim, Silva, & Jung, 2010). Even though the incidents of listeriosis are minimal, it is still considered as a public health concern due to its high mortality rate (Shoughy & Tabbara, 2014).

It has been established that *L. monocytogenes* have the potential to form biofilms on food contact surfaces such as glass, rubber, plastic, stainless steel and polypropylene (Bendinger, Rijnaarts, Altendorf, & Zehnder, 1993). Biofilms are composed of a series of the bacterial community, which attached to a surface by produce sticky, sugary substances, which incorporate bacteria in a matrix (Olszewska, Kocot, Stanowicka, & Łaniewska-Trokenheim, 2016).

The food processing environments provide favorable conditions such as the presence of nutrients, moisture, and inoculation of microorganisms from raw materials, which promote the formation of biofilms (Ksontini, Kachouri, & Hamdi, 2013). Biofilms and microbial adhesion have played a great role in the food industry and found on different food contact surfaces.

Biofilms tend to display a wide range of phenotypes depending on the precise systems examined and the type of the microorganism (Morinaga, 2017). *L. monocytogenes* biofilms grown in constant flow conditions comprises of spherically shaped micro-colonies, which are enclosed, with a network of knitted chains made up of elongated cells. On the other hand, *L. monocytogenes* biofilms, which grown in stable conditions, made up of a uniform layer of cells or microcolonies, hence showing

morphology, which is similar to that of planktonic cells (Morinaga, 2017). The biofilms form in both food processing environment and finished food products hence leading to cross-contamination and post-process contamination respectively. Some of the potential sources of biofilms in the food processing industry include floors, stainless steel surfaces, waste water pipes, conveyor belts and rubber, Buna-N and Teflon seals. The consequences of biofilm growth are very many, and they pose impending dangers to everyone and every surface in the food processing industries (Poulsen, 1999). *L. monocytogenes* have the ability to produce biofilms on hydrophilic surfaces such as stainless steel in the presence of complex growth nutrients. These bacteria therefore have the ability to accumulate on these surfaces to a level, which might enhance the spread of these pathogenic microorganisms in the food-processing plant (Blackman & Frank, 1996).

Active bacterial adhesion on the industrial equipment surfaces is influenced by physicochemical properties of the cell surface (Zhang, Wang, & Levänen, 2013). Most of the bacterial surfaces are negatively charged, and the negativity is dependent on the growth environment (Zhang, Wang, & Levänen, 2013). These negative charges produce electrostatic repulsive forces, which are keys for bacterial adhesion. The hydrophobic nature of bacterial cells plays a vital role determining the physiological status of the bacterial cells (Lyklema, Norde, Van Loosdrecht, & Zehnder, 1989). The existence of lipopolysaccharides (LPS), fimbriae and flagella on the bacterial cell surfaces ensure the formation of the hydrophobic surface, which reduces the force repulsion, which promotes the interface between the two exteriors and bacterial adhesion (Shi & Zhu, 2009). The physical characteristics of solid surfaces play a significant role in the biofilm formation

since they impact initial attachment of bacterial cells (Lyklema et al., 1989). The existence of wet surfaces and high free energy play a key role in bacterial adhesion (Zhang et al., 2013). Most of the cells/ are therefore expected to bond themselves to hydrophilic faces like steel and glassware than hydrophobic exteriors made of plastic polymers such as Buna-N (Bendinger et al., 1993; Sinde & Carballo, 2000).

The standard materials which are used to make the food processing surfaces such as stainless steel and polypropylene have been known to provide suitable surfaces for bacterial attachments (Słotwińska, 2013). Factors such as bacterial surface characteristics; either Gram-positive or Gram negative bacteria and the nature of the material used for making the content play significant roles in determining the degree of bacterial adhesion to inert surfaces (Medilanski, Kaufmann, Wick, Wanner, & Harms, 2002). Surface characteristics such as acidity and basicity of different polymers such as LDPE, LDPE-EVA, polypropylene (PP) and LDPE-PP influence bacterial surface adhesion (Słotwińska, 2013). This is because they contain specific factors, which play a great role in bacterial attachment and growth. Characteristics such as being hydrophilic negatively charged and susceptive to contamination make the stainless steel to be rarely clean (Zhang et al., 2013).

L. monocytogenes which is attached to the food processing surfaces show increased resistance to commonly used disinfectants such as quaternary ammonium compounds and anionic acid sanitizers (Macgowan, Reeves, & Mclauchlin, 1990). Other groups of biofilms have also been observed to offer resistance to sanitizers made of formaldehyde, peracetic acid, and mercuric chloride. In a situation whereby dangerous bio-fouling process takes place; there is the formation of thick biofilms, which contains many

metabolically dormant or dead cells, which alters the bacterial growth rate and physiological characteristics, leading to increased resistance to antibacterial constituents (Macgowan et al., 1990).

In the recent years, different food processing industries have adopted the use of more biodegradable and less toxic compounds like surfactants to control or prevent the formation of *L.monocytogenes* biofilms. These substances have the ability to absorb at surfaces and interfaces (Anand, Singh, Avadhanula, & Marka, 2014). The surfactants tend to change the surface properties of the materials on which the bacterial cells adhere to, hence are added to increase the washing effects of the sanitation practices. Additionally, they have the ability to lower the surface and interfacial tensions of the aqueous fluids that interfere with the propensity to solubilize fatty materials and wet the surfaces (Eriksone, 2015).

Surfactants are characterized by properties such as critical micelle concentration (CMC), chemical structure and charge, and hydrophile-lipophile balance (HLB) which enable them to control the growth of biofilms (Garnier, Laschewsky, & Storsberg, 2006).They can, therefore, be categorized into various types such as amphoteric, non-ionic, anionic and cationic surfactants, based on the charge of the hydrophilic structural element. They have the ability to wield toxic effects by promoting membrane disruption, which leads to cellular lysis, altering the physical membrane structure, therefore, compromising the normal membrane functions and increasing permeability of the membrane, which leads to metabolite leakage (Panswad, Sabatini, & Khaodhiar, 2012).

Application of nonionic surfactants in controlling the growth of *L. monocytogenes* biofilms can provide potent hydrophobic antibacterial, which is insoluble in water. This

helps to ensure that problems associated with inactivation of active compounds found in the top layers of biofilms are taken care of effectively (Bajpai & Tyagi, 2010). The nonionic surfactants have the ability to act on the microbial growth through the destruction of the integrity of the bacterial cells hence eliminating problems associated with evaporation and corrosion of the traditional sanitizers (Garnier et al., 2006).

Different studies have been done to investigate the action of different surfactants, which are cationic and anionic surfactants, in controlling bacterial biofilms (M Simões, Pereira, & Vieira, 2005). Furthermore, there are new studies, such as bioelectric approach, bactericidal coating, nanotechnology, quorum sensing and enzymatic disruption, which had been conducted with an aim of finding effective alternatives, which can be used for the prevention, and control of biofilms (Sadekuzzaman, Yang, Mizan, & Ha, 2015). In this study, there will be focus on removing *L. monocytogenes* biofilms found on different food contact surfaces made of Low-Density Polyethylene (LDPE), Polypropylene (PP), Low-Density Polyethylene and Polypropylene (LDPE-PP), Low-Density Polyethylene and Ethylene Vinyl Acetate (LDPE-EVA), Stainless-Steel and Aluminum while using non-ionic surfactants such as Pluronic F68 and 127, Brij 58, Tween 20, 40, and 80 by washing method.

To effectively complete the tasks of removing or dispensing listeria biofilm on food contact surfaces by nonionic surfactants, it is necessary to accomplish the following overall research objectives, which make the study approach of the project. (i) To investigate effect of anti-adhesive/ant-biofilm and antimicrobial properties of non –ionic surfactants in disrupting pre-formed biofilms of *L.amonocytogenes* on food contact surfaces. (ii) To measure surface energy of food contact surfaces.The results obtained

from this study will help in developing recommendations, which would help the food processors in choosing the optimal operating conditions, which should be considered in a cleaning operation using nonionic surfactants.

2. Materials and Methods

2.1. Preparation of bacterial culture

The *L. monocytogenes* strain Scott A was obtained from D. Portnoy – The University of Carlifonia, Berkeley, CA, USA. The maintenance of the freshly *L. monocytogenes* broth was achieved using the Brain Heart Infusion (BHI) broth (Difco laboratories, Franklin Lakes, NJ - USA) contained in a sterile flask that was then incubated at preferred temperatures. At -20^0 C, There was complete complementation between the bacterial strains stored in the BHI and 30% glycerol (Gorski, Palumbo, & Mandrell, 2003).The sub-culturing of the original culture allowed for the formation of inoculum, which were used in the experiment.

2.2. Preparation of Surfactants solution

Both Pluronic F127 and F68 were obtained from Spectrum Chemical MFG. corp. while Brij 58, Tween 20, Tween 40 and Tween 80 were acquired from Merck KGaA (Darmstadt, Germany). The preparation of Pluronic F127, Pluronic F68, Tween 20, Tween 40, Tween 80 and Brij 58 were achieved by dissolving the surfactants in water at 25^0C to the critical micelle concentration of 0.8,1, 0.0499, 0.0333, 0.015, and 0.08mM, respectively.

2.3. Preparation of food contact surface coupons

Food contact surfaces were used in the experiment was LDPE, PP, LDPE+PP, LDPE+EVA, stainless,-steel, aluminum. LDPE: Low-density polyethylene, PP: Polypropylene, EVA: Ethylene vinyl acetate. Food material washed with sterile distilled water, left to dry and cleaned with % 70 ethanol (v/v), then rinsed again with sterile distilled water .Fitting Cork was used when cutting and adjusting the coupons to bottom of a 24 –well microplate.

2.4. Contact angle measurement

To investigate the surface energy of common food processing and packaging materials, contact angle measurements were conducted. Six different surface samples including Low density polyethylene (LDPE), Polypropylene (PP), LDPE+PP, LDPE+ Ethylene vinyl acetate (EVA), stainless-steel, aluminum were tested. Samples were cut using a fitting cork into circle with 10mm diameter and then attached evenly on a double side tape and a glass slide. Four samples were attached to one glass microscope slide with 5 mm gap between each other. Double-sided tape was used to ensure a flat viewing surface.

The surface energy is obtained by using static contact angle method on an optical Rame-Hart goniometer (KSV CAM101). Contact angles were defined by the tangent angle formed at the surface. Two liquids with the known dispersive and polar components, Ethylene Glycol (polarity surface tension of dispersion: 32.8 and surface tension of polar: 16.0) (Angle, 1993) and water (surface tension of dispersion: 22.6 and surface tension of polar: 50.2) (wu S, 1982) were chosen to be liquids to perform contact angle measurement. A 5 µL droplet of each liquid was dropped from the micro pipette

(Finnpipette® 1 µL-10 µL) and then the contact angle was measured. Four droplets were measured for each liquid on each sample, the values were averaged.

2.5. Biofilm removal assay

The biofilm formation on food contact surfaces was conducted according to Stiefel et al. (2016) and some modifications made by do Valle Gomes and Nitschke (2012).The culturing of the *L. monocytogenes* was done overnight. This was followed by transferring of 100µL aliquot of the cell suspension into 15mL test tube composed of 10mLof BHI broth to form a concentration of 10^7CFU (Stiefel et al., 2016). 400µL of diluted bacteria was pipetted into sterilized 24-well polystyrene flat- bottoms tissue culture plate, then incubated at 37^0C for 24 hours. Covering of the microplate was important in preventing contamination and light penetration.

Non-adhered cells were removed by washing each well twice using 400µL buffered water. The disruption of the biofilm was achieved by adding 400µL of each surfactant to triplicate wells of a 24-well plate while 400µL of buffered water was as a control for the experiment. To form a dynamic culture, the pale was shaken for 1, 5, 15 and 30 minutes. The surplus planktonic cells and spent media were discarded while the adherent cells were gently rinsed twice using buffered water. Thereafter, 400µL of buffered water was added to each well of the microplate (do Valle Gomes & Nitschke, 2012). The cells were finally fixed after 30 minutes at 60^0C.

2.6. Biofilm quantification

Even though the acridine orange staining method was performed as described by Stiefel et al. (2016), there need of modifying the process to meet objectives of this study.

The acridine orange solution was prepared by dissolving 2% acridine orange in water at a ration 1:100(v/v) in Walpole's buffer. 400 µL of acridine orange was added to each well of a 24-well plate. The plate was gently rinsed twice-using 400 µL of 0.9% NaCl solution after 15 minutes of incubation in the dark. Determination of the fluorescence intensity was achieved by using an emission filter at 527/20nm and an excitation filter at 485/20nm (Stiefel et al., 2016).

2.7. Epifluorescense microscopy

After measuring the spectrophotometry of 2% acridine stained *L. monocytogenes* biofilms on spinach leaves for 15 minutes as illustrated by Stiefel et al. (2016),the leaves which were treated using the appropriate solution for 30 minutes were taken up and placed on a glass slide for examination under an epifluorescense microscope.

3. Results

3.1. Surfactant addition promotes biofilm removal from the food contact surfaces

This study focuses on an investigation to determine the effectiveness of six different non-ionic surfactants, Pluronic F68 and F127, Brij58, Tween 20, 40, and 80, in removing *L. monocytogenes* biofilms found on six different food contact surfaces made of Low-Density Polyethylene (LDPE), Polypropylene (PP), Low-Density Polyethylene and Polypropylene (LDPE+PP), Low-Density Polyethylene and Ethylene Vinyl Acetate (LDPE+EVA), Stainless-Steel and Aluminum. The effects of the surfactants and time of contact with the surfactant were evaluated. The results on the effects specific surfactants on different food contact surfaces were recorded over a time interval of 1, 5, 15 and 30 minutes. The effectiveness of different surfactants used in the experiment could be

established through observing the total number of *L. monocytogenes* cells regarding percentage they destroyed after different time intervals. The efficacy of non-ionic surfactant in removing listeria biofilm on different food surfaces shown in Table 1 and Figure 2.

Table 1 Efficacy of non-ionic surfactant in removing listeria biofilm on different food surfaces

Surface	Surfactant Efficacy
Stainless-Steel	Brij 58 > Pluronic F127 > Tween 20 > Pluronic F68 > Tween 80 > Tween 40
Aluminum	Brij 58 > Tween 80 > Pluronic F68 > Tween 40 > Pluronic F127 > Tween 20
LDPE	Brij 58 > Pluronic F127 > Tween 80 > Tween 40 > Tween 20 > Pluronic F68
PP	Pluronic F127 > Brij 58 > Tween 80 > Pluronic F68 > Tween 40 > Tween 20
LDPE+PP	Brij 58 > Tween 80 > Pluronic F127 > Tween 40 > Pluronic F68 > Tween 20
LDPE+EVA	Tween 80 > Brij 58 > Pluronic F127 > Tween 40 > Pluronic F68 > Tween 20

In addition, this study assessed the biofilm formation by *L.monocytogenes* on different food surfaces. Our results demonstrated that listeria biofilm can be attached more easily on stainless-steel compared to other food surface as shown in Figure 1

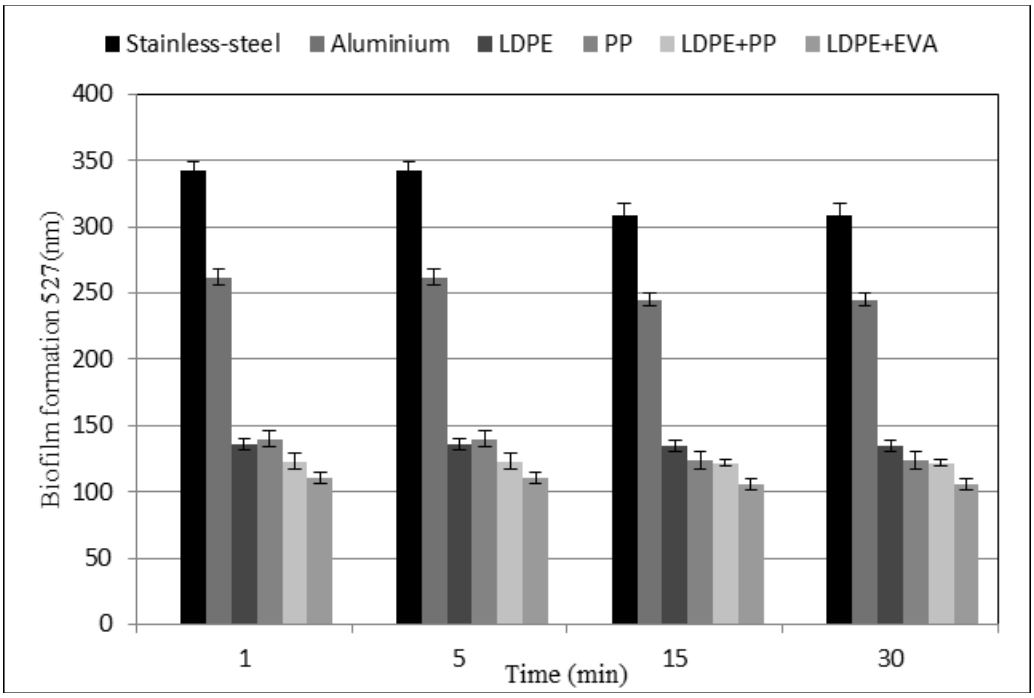


Figure 1 Biofilm formation on different food contact surface

3.1.1. Stainless-Steel Surface

Refer to Figure 2 (a), there was no observable change caused by any of the surfactants on the listeria biofilm cell count, which was formed on the stainless-steel food contact surface after one minute. From the results, it can, therefore, be noted that the most effective time for removing *L. monocytogenes* biofilms was after 30 minutes of contact with the surfactants. After this time, all of the surfactants were able to eliminate more than 50% of the listeria biofilms formed on the food contact surfaces made of stainless steel.

Brij58 was placed in the first place in terms of the overall ability to destroy the Listeria biofilms. After 5 minutes, Brij58 showed the most effectiveness against Listeria biofilms by destroying 34.13, 29.14, 23.79, 21.90, 24.24, and 23.26 % respectively of the

L. monocytogenes cells. This trend continued even after 15, where Brij58, Pluronic F127, Tween 20, Tween 40, Tween 80, Pluronic F68 destroyed 59.88, 45.59, 41.45, 35.65, 31.03, 24.86% respectively while after 30 minutes, they reduced 78.50, 59.87, 57.31, 50.14, 51.48, 56.17% respectively of the *L. monocytogenes* biofilm cells. The ability of these surfactant to remove the Listeria biofilms increased with the increase in time just as shown by the increasing percentage of Listeria biofilm cells destroyed over time.

A more effective surfactant against the Listeria biofilms can be achieved through a combination of two or more surfactants, which have distinct properties. For example, a combination of Brij58 that showed the highest overall effectiveness against Listeria biofilm cells and Tween 20, which was easily stabilized after 15 minutes, can lead to the development of a surfactant, which can destroy a large percentage of *L. monocytogenes* over a short period.

3.1.2. Aluminum Surface

Refer to Figure 2 (b), the results from this experiment involving the aluminum surface indicate that there was no significant change in cell count after one minute. Therefore, the most effective time for eliminating the Listeria biofilms was after 30 minutes since, at this point, all of the surfactants were able to eliminate more than 50% of the *L. monocytogenes* biofilms. Brij58 showed the most effectiveness against the Listeria biofilms followed by Pluronic F127, Tween 80, Tween 40, Tween 20, Pluronic F68. After 5 minutes, the Brij58, Pluronic F127, Tween 80, Tween 40, Tween 20, Pluronic F68, they were able to remove 28.42, 23.86, 27.81, 24.62, 17.83, and 4.60 % respectively of the biofilms. After 15 minutes, Brij58, Pluronic F127, Tween 80, Tween 40, Tween 20, Pluronic F68 destroyed 44.06, 51.61, 39.80, 36.78, 32.73, and 33.93 % respectively

of the Listeria biofilms. This trend changed after 30 minutes since they were able to eliminate 64.79, 56.48, 62.31, 60.47, 56.24, and 61.48 respectively % of the Listeria biofilms hence making the Brij58 was the most effective surfactant against *L. monocytogenes* biofilms compared to the other surfactants used in the experiment.

The results show that Tween 20 has the least ability to remove Listeria biofilms compared to other surfactants belonging to the Tween family used in the experiment at all of the time intervals. According to the results, Pluronic F68 has the least effectiveness against Listeria biofilms as compared to the other surfactants hence was placed in the sixth position. There was further improvement in the effectiveness of Pluronic F68 after 30 minutes. From the results, it can be shown that a combination of two or more surfactants can improve the rate of Listeria biofilm elimination. For example, a combination of Pluronic F68 which gains its effectiveness over time and Brij58 which is more effective after 5 minutes can lead to the improvement of Listeria biofilm cells elimination.

3.1.3. Low-Density Polyethylene Surface (LDPE)

Refer to Figure 2 (c), there was no observable change caused by any of the surfactants on the listeria biofilm cell count, which was formed on the Low-Density Polyethylene Surface (LDPE) food contact surface after one minute (data not shown). From the results, it can, therefore, be noted that the most effective time for removing Listeria biofilms was after 30 minutes. Tween 40 showed the most effectiveness against Listeria biofilm cells in the first minutes. After 5 minutes, Tween 40, Tween 20, Brij58, Pluronic F127, Pluronic F68, Tween 80 eliminate 35.23, 37.65, 8.11, 10.83, 11.25, and 8.50 % respectively of *L. monocytogenes* biofilms. The results of the experiment indicate that the

effectiveness of surfactant against Listeria biofilms increases with time. After 15 minutes, Tween 40, Tween 20, Brij58, Pluronic F127, Pluronic F68, Tween 80 eliminated 47.05, 40.49, 47.96, 41.50, 44.61, and 38.85 % respectively of the Listeria cells. The effectiveness of the surfactant, against Listeria biofilms, increased over time. This can be explained based on the fact that after 30 minutes, Tween 40, Tween 20, Brij58, Pluronic F127, Pluronic F68, Tween 80 eliminated 52.20, 47.18, 61.58, 54.82, 46.78, and 53.88 % respectively of the Listeria biofilm cells. This trend indicates that Tween 40 was able to gain its stability much faster after 5 minutes compared to the other surfactants. This is explained by the decreasing effectiveness ranking over time.

The results further show that Pluronic F68 was not able to eliminate more 50% of the biofilms at all of the time intervals. Taking into consideration of Pluronic surfactant family used in the experiment, it can be established that Pluronic F68 was the least effective against Listeria biofilms.

3.1.4. Polypropylene Surface (PP)

Refer to Figure 2 (d), the results observed that there was no significant change in the number of Listeria biofilm cells formed on the Polypropylene Surface (PP) after the first minute and hence an excellent time for removing the *L. monocytogenes* biofilms was after 30 minutes. Brij58 was the most effective surfactant against Listeria biofilm cells. After 5 minutes, Brij58, Pluronic F127, Pluronic F68, Tween 80, Tween 20, Tween 40 eliminated 21.88, 22.23, 27.75, 14.61, 20.14, and 0.98% of the Listeria biofilms. The effectiveness of Brij58, Pluronic F127, Pluronic F68, Tween 80, Tween 20, and Tween 40 against Listeria biofilms increased with the increase in time since they removed 51.32, 38.16, 39.94, 31.82, 21.85, and 25.72% respectively of the Listeria biofilm cells after 15

minutes while after 30 minutes they were able to eliminate 56.42, 58.41, 44.79, 45.42, 32.04, 35.58 % respectively of the Listeria biofilms.

According to the results, Pluronic F127was the most effective surfactant against Listeria biofilms amongst the Pluronic surfactant family used in the experiment regarding the ability to destroy the Listeria biofilms cells from Polypropylene surface. The results further indicate that Pluronic F68 showed the least ability to remove Listeria biofilms as compared to Pluronic F127. According to the results obtained on Polypropylene surface, the Tween family showed the least effectiveness against Listeria biofilm cells.Tween 80 was the most effective surfactant against Listeria biofilm cells as compared to the other Tween family surfactants used in the experiment.

The most effective surfactant against Listeria biofilms can be achieved through a combination of two or more surfactants which show different positive characteristics towards the ability to destroy the *L. monocytogenes* biofilms. For example, a combination of Pluronic F68 which is the most effective in the first 5 minutes and Pluronic F127 which is the most effective against Listeria biofilms after 30 minutes can lead to the establishment of surfactant which would eliminate a large percentage of Listeria biofilms after between 5 and 30 minutes.

3.1.5. Low-Density Polyethylene and Polypropylene Surface (LDPE+PP)

Refer to Figure 2 (e), the results from the Low-Density Polyethylene and Polypropylene Surface indicate that there was no significant change observed in the Listeria biofilm cell counts after 1 minute; hence an excellent time for removing the *L. monocytogenes* biofilms was after 30 minutes. Brij58 was the most effective surfactant against Listeria biofilm cells. After 5 minutes, Brij58, Tween 80, Pluronic F127, Tween

40, Pluronic F68, Tween 20 reduced the Listeria Biofilms by 14.61, 12.90, 15.51, 13.59, 16.38, and 14.44 % respectively of the biofilm cells. The effectiveness of Brij58, Tween 80, Pluronic F127, Tween 40, Pluronic F68, and Tween 20 increased with the increase in time since after 15 minutes they reduced the number of Listeria biofilm cells by 46.70, 39.65, 30.90, 34.30, 20.25, and 21.88 % respectively while after 30 minutes they removed 53.53, 48.83, 45.72, 36.25, 33.87, and 29.69 % respectively of the biofilm cells. Brij58 was the only surfactant which managed to eliminate more than 50% of the Listeria biofilm cells hence considered as the most effective surfactant amongst those used for the experiment.

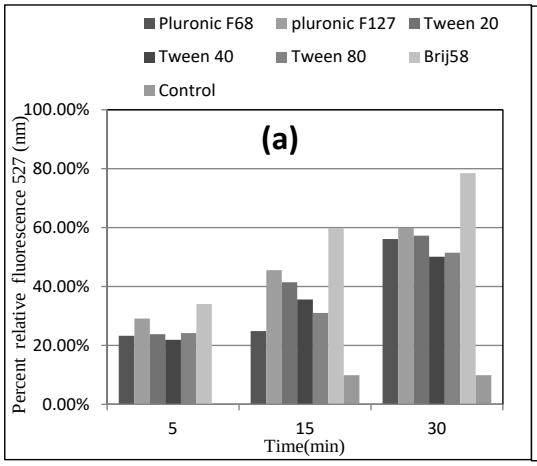
At all of the time intervals, except after 5 minutes, Tween 20 showed the least effectiveness against Listeria biofilms as compared to the other surfactants belonging to the Tween family used in the experiment. A more effective surfactant against Listeria biofilm cells can be obtained through the combination of two or more surfactants. For example, a combination of Brij58 which showed the highest effectiveness against Listeria biofilms and Pluronic F68 which showed the highest effectiveness against Listeria biofilms during the first 5 minutes of contact with the Listeria biofilm cells.

3.1.6. Low-Density Polyethylene and Ethylene Vinyl Acetate Surface (LDPE+EVA)

Refer to Figure 2 (f), from the results, it is observed that there was no significant change in the number of Listeria biofilm cells formed on the Low-Density Polyethylene and Ethylene Vinyl Acetate Surface (LDPE+EVA) after the first minute and hence an excellent time for removing the *L. monocytogenes* biofilms was after 30 minutes. Among all of the surfactants used in the experiment, Brij58 showed the most effectiveness surfactant against *L. monocytogenes* biofilm cells. After the first 5 minutes, Brij58,

Tween 40, Tween 80, Pluronic F127, Pluronic F68, Tween 20 destroyed 14.48, 17.93, 7.58, 11.26, 3.55, and 8.49% respectively of the Listeria biofilms. The ability of the surfactant to eliminate Listeria biofilms increased with the increase in time as indicated by the results. After 15 minutes, they destroyed 41.39, 34.31, 37.57, 30.34, 36.97, and 30.93 % respectively while after 30 minutes; they destroyed 65.44, 59.01, 65.46, 63.24, 55.80, and 55.50% respectively of the Listeria biofilms.

The results show that Tween 20 surfactant did not only show the least effectiveness among the Tween group, but also amongst all of the other surfactants used for the experiment. The results show that most of the *L. monocytogenes* cells were destroyed between 5 minutes, 15, and 30 minutes. In all the surfactants, the amount of the Listeria biofilm cells eliminated by the surfactants reduced after 15 minutes and the activity of Tween 20, which is the most effective surfactant almost, stabilized after 15 minutes. The image of listeria biofilm on different food contact surfaces shown in Figure 3


Pluronic F68
pluronic F127
Tween 20
Tween 40
Tween 80
Brij58
Control
(a)
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80.00%
60.00%
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20.00%
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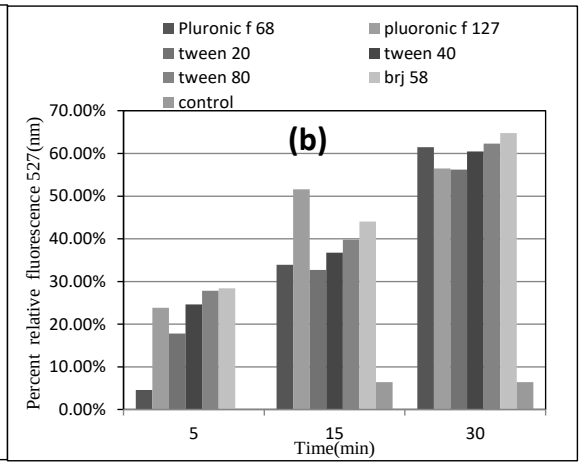
5
15
30
Time(min)


Pluronic f 68
pluoronic f 127
tween 20
tween 40
tween 80
brj 58
control
(b)
Percent relative fluorescence 527(nm)
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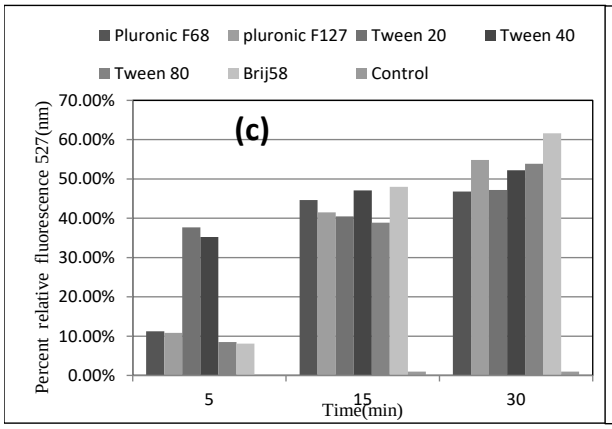
60.00%
50.00%
40.00%
30.00%
20.00%
10.00%
0.00%
5
15
30
Time(min)


Pluronic F68
pluronic F127
Tween 20
Tween 40
Tween 80
Brij58
Control
(c)
Percent relative fluorescence 527(nm)
70.00%
60.00%
50.00%
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30.00%
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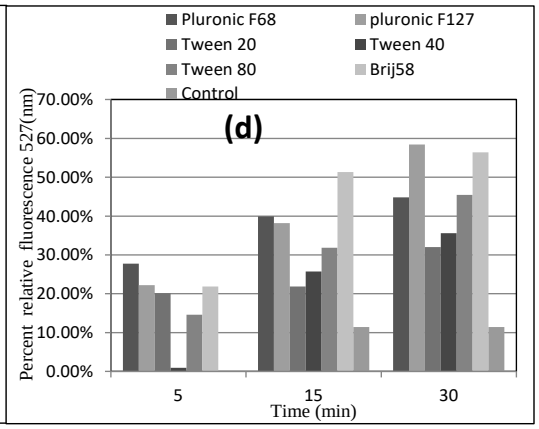
Pluronic F68
pluronic F127
Tween 20
Tween 40
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Brij58
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Percent relative fluorescence 527(nm)
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Time (min)

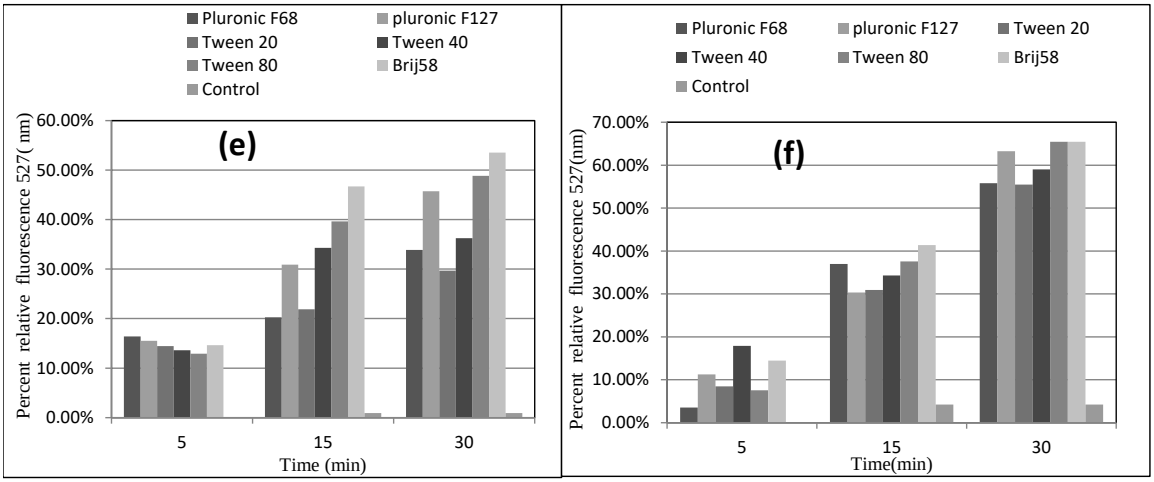
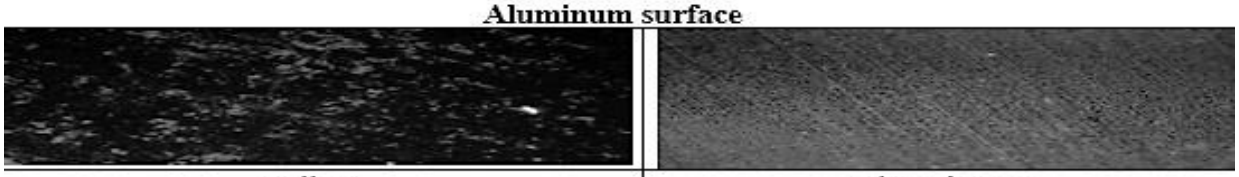


Figure 2: Total cell quantification by Acridine Orange staining. *L. Monocytogenes* biofilm on (a) Stainless-Steel,(b) Aluminum, (c) Low-Density Polyethylene surface (LDPE), (d) Polypropylene Surface (PP), (e) Low-Density Polyethylene and Polypropylene (LDPE+PP), (f) Low-Density Polyethylene and Ethylene Vinyl Acetate (LDPE+EVA.

Food Surfaces were treated with different Surfactants at Different Times (1, 5, 15, and 30) min. The BHI medium was used to grow the *L. monocytogenes* culture overnight, a 100µL aliquot of the cell suspension was transferred into 15mL test tube composed of 10mLof BHI broth. 400µL of diluted bacteria was pipetted into sterilized 24-well polystyrene flat- bottoms tissue culture plate then incubated at 37^0C for 24 hours. After incubation, each well was washed with buffered water. Each well was filled with 400 µL of each surfactant in triplicates. After 1,5,15, and 30 min, each well was rinsed gently twice using buffered water. Finally, fixation was carried out for 30 min at 60^oC then the wells were stained by Acridine Orange, fluorescence intensity was measured using an emission filter at 527/20 nm and excitation filter at 485/20 nm. The experiment repeated three times, the average was calculated and converted to a percentage.

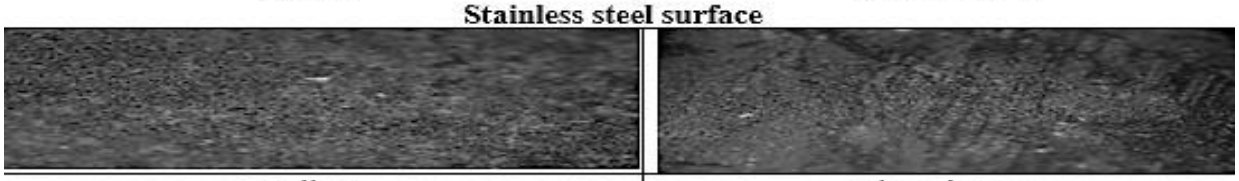
Control

Aluminum surface



Brij 58	Pluronic F68

Stainless steel surface

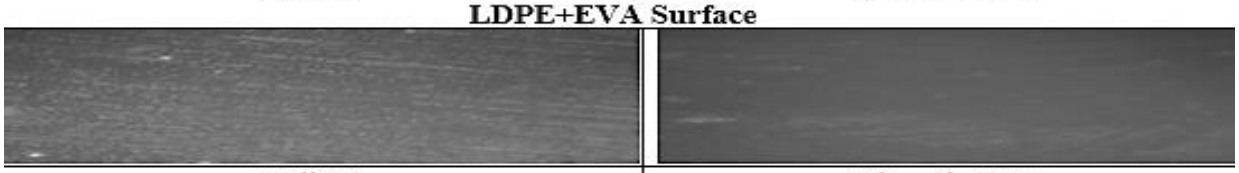


Brij 58	Pluronic F68

LDPE Surface

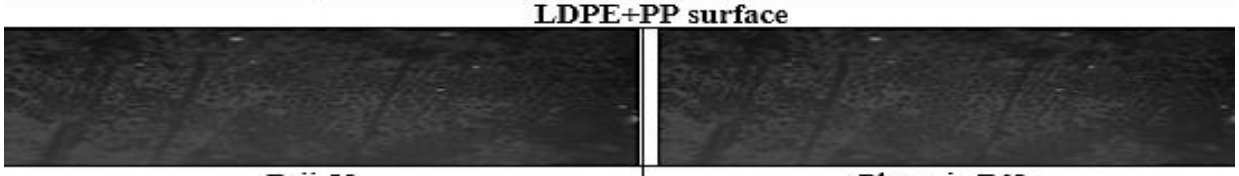
Brij 58	Pluronic F68

LDPE+EVA Surface



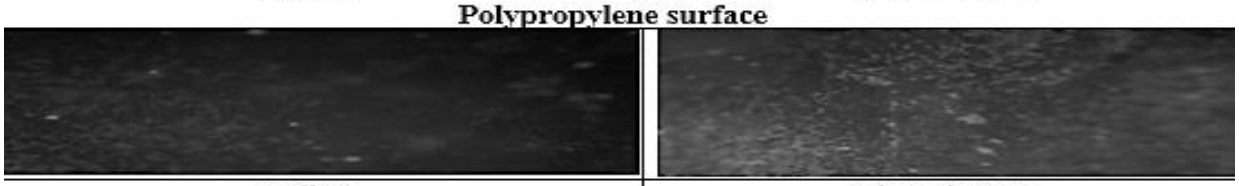
Brij 58	Pluronic F68

LDPE+PP surface



Brij 58	Pluronic F68

Polypropylene surface



Brij 58	Pluronic F68

Figure 3 Fluorescent images of the *L. monocytogenes* biofilm on different food surfaces after 24 h of growth (a) as a control and after consequent washing with nonionic surfactants: Tween 20 (b), Tween 40 (c), Tween 80 (d), Brij 58 (e), Pluronic F68 (f), and Pluronic 127 (g).

3.2. Surface energy of food contact surface

Refer to Table 2 and Figure 4 presents the total surface energy and its corresponding dispersive and polar components of selected packaging material surfaces by using Wu method. The result indicates that steel has the highest surface energy compared to the rest of the materials (25.94 mJ/m^2). PP has the second highest surface energy due to the high polar component (19.41 mJ/m^2), with the low dispersive component of surface energy. The rest of the materials have similar surface energy ranging from 18 to 20 mJ/m^2. Additionally, stainless steel had the highest dispersive components (14.04 mJ/m^2) while PP had the lowest dispersive component (2.688 mJ/m^2). The variation in the amount of dispersive component, polar component and total surface energy among the six contact surfaces influenced bacterial adhesion and contact angle on those surfaces.

Table 2 Surface energy of packaging materials and its polar and dispersive component

Solids	γ(mJ/m2)	γD(mJ/m2)	γP(mJ/m2)
LDPE+EVA	20.29492	6.155548	14.13937
PP	22.10045	2.688131	19.41232
Aluminum	20.50752	6.072506	14.43501
Steel	25.90435	14.03863	11.86572
PP+LDPE	18.06195	4.552486	13.50947
LDPE	18.14277	6.809631	11.33313

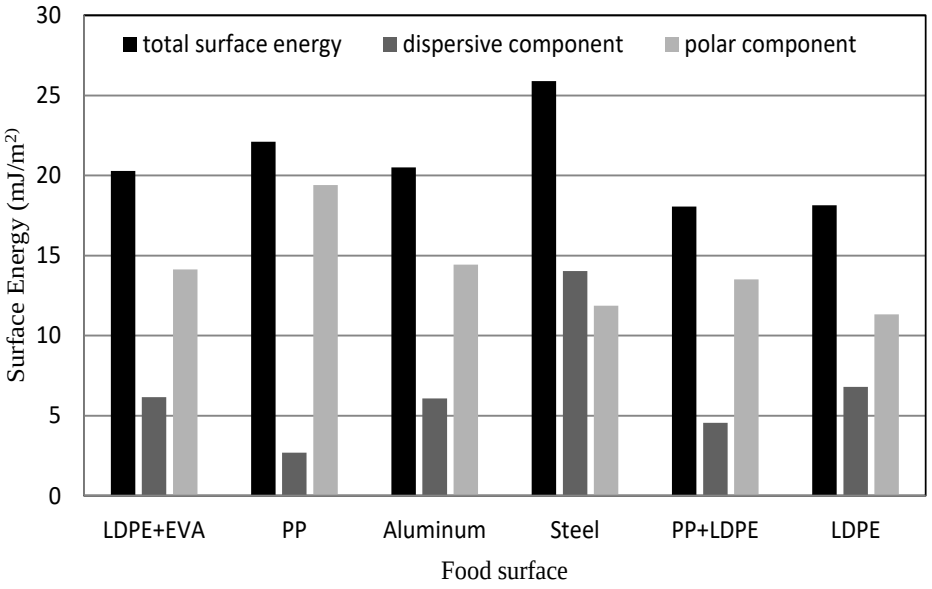


Figure 4 surface energy measurement of food contact surface.

4. Discussion

Food contact surfaces provide conducive environments, which promote the formation of biofilms (do Valle Gomes & Nitschke, 2012). Sanitizing and cleaning of these food contact surfaces is, therefore, one of the most important steps that should be taken to prevent the risks associated with foodborne diseases (Niemira, Boyd, & Sites, 2014). The ability of the surfactant to remove the biofilms from the food contact surfaces faster depends on the type of material used to make the food contact surfaces and the kind of surfactant used, the time allowed for the contact between the surfactant and the *bacterial* cells (Aggarwal, Stewart, & Hozalski, 2015).

The ability of the surfactant to penetrate into the bacterial cells played an important role in reducing the time taken for the elimination of the *L. monocytogenes* from different

food contact surfaces (Microbiology Series & Méndez-Vilas). According to the results that shown in Figure 2, there was no observable change caused by any of the surfactants on the listeria biofilm cells which was formed on all of the six food contact surfaces at 1 min. This was a clear indication that after the first one minute, the surfactants were still penetrating into the *L. monocytogenes* biofilms from where they would later interfere with the normal Listeria cell functions (de Candia, Morea, & Baruzzi, 2015). These results are in agreement with the study conducted by Khelissa, Abdallah, Jama, Faille, and Khelissa, Abdallah, Jama, Faille, and Chihib (2017), which involved the use of Alkylbenzenesulphonate surfactant to destroy *L. monocytogenes* biofilm cells formed on the food contact surface made of stainless steel. In the experiment, the authors indicated that the major reasons which propelled the occurrence of such observations were; failure of the surfactant to diffuse properly into the biofilm, the phenotypic adaptations of the biofilm cells to the sub-lethal concentrations of the surfactant and the presence of the surfactant-adapted and persisted cells (Cortés, Bonilla, & Sinisterra, 2011).

Refer to Figure 2, the results further showed that in general, the least amount of *L. monocytogenes* cells was destroyed after 5 minutes irrespective of the type of the food contact surface on which the biofilms were formed or the type of the surfactant used in the elimination process. This was a clear indication that as much as limited time was allowed for the contact between the surfactant and the *L. monocytogenes* biofilms, the effectiveness of the surfactants against Listeria biofilms had already begun to be felt (Sarjit, Tan, & Dykes, 2015). This observation also helped to indicate that the activity of the surfactants on the *L.monocytogenes* biofilm cells increased with the increase in time.

Refer to Figure 2, as time was increasing from 5 to 15 and then to 30 minutes, the amount of the *L. monocytogenes* biofilm cells which was destroyed also increased irrespective of the type of surfactant which was used in the elimination process or the type of food contact surface onto which the *L. monocytogenes* biofilm cells were formed. These results, therefore, justified that the effectiveness of the surfactants was directly proportional to the time which was allowed for them to act on the *L. monocytogenes* biofilm cells (Harrison, Schratzberger, Sapp, & Osborn, 2014). According to M Simões, Simoes, and Vieira (2010),there is need to provide for enough time which would allow for the complete penetration of the surfactants into the biofilm matrix, thus leading to the achievement of an effective cleaning technique of the bacterial biofilm cells.

According to the results that were obtained, different surfactants showed different levels of effectiveness against the *L. monocytogenes* biofilm cells. This led to the formation of the following sequence of surfactants in a decreasing order regarding the effectiveness of the *L. monocytogenes* biofilm cells; Brij 58 >Pluronic F127 > Tween 80 > Tween 40 > Tween 20 >Pluronic F68 as shown in Table 1. This trend is a clear indication that Brij 58 surfactant was able to destroy more *L. monocytogenes* biofilm cells as compared to the other surfactants used in the experiment. This type of variation about the effectiveness against the Listeria biofilm was because of the readiness of the Brij 58 surfactant to easily penetrate into the cellular components of the *L. monocytogenes* biofilm cells and hence interfering with the normal cellular activities. These results are in agreement with the study conducted by Epstein, Hochbaum, Kim, and Aizenberg (2011) in which the anionic surfactant such as Sodium Dodecylsulphate (SDS) and the cationic surfactant such as N-Dodecyltrimethylammonium Bromide (CTAB) were used to test

their effectiveness against the bacterial biofilm. In that experiment, it was established that the high adsorption rate of the N-Dodecyltrimethylammonium Bromide (CTAB) played an important role in ensuring that it eliminated large volumes of bacterial cells.

Refer to Figure 4, however, Stainless steel showed the highest surface energy, aluminum, and Polypropylene surfaces had higher values of free surface energies compared to Low-Density Polyethylene and Ethylene Vinyl Acetate Surface, Low-Density Polyethylene and, Low-Density Polyethylene and Polypropylene. Refer to Figure 1, our results demonstrated that *L. monocytogenes*can be attached more easily on hydrophilic than thehydrophobic surface. Our results were in disagreement with findings of Absolom et al. (1983),which based on the thermodynamic concept regarding the applicability of the surface free energies in relation to the polar and dispersion components, the adhesion of the *L. monocytogenes*on the food contact surfaces is energetically more favored on hydrophobic surface that has lower surface energy than hydrophilic surface that has high surface energy.

The type of the material used in the construction of the food contact surfaces played a key role in determining the total number of *L. monocytogenes* biofilm cells, which were destroyed by the surfactants (BAKTERIJ, 2014). In a decreasing order regarding the ability of all of the surfactants used in the experiment to destroy the *L. monocytogenes* biofilm cells formed on different food contact surfaces, this was the established sequence; Stainless Steel > Aluminum > LDPE > LDPE+EVA > Polypropylene > LDPE+PP as shown in Figure 2. It was, therefore, justifiable to state that the surfactants used in the experiment showed more effectiveness against the *L. monocytogenes* biofilm cells formed on stainless steel surfaces than on any other surface since the inert nature of the

Stainless Steel played a major role in facilitating easy elimination of the *L. monocytogenes* biofilm cells which were formed on them.This type of variation regarding the ability of the surfactants to destroy the *L. monocytogenes* biofilm cells formed on different surfaces was as a result of various factors such as surface physiochemical properties which include the hydrophobicity and roughness of the materials used in the manufacturing of these food contact surfaces (Yu et al., 2016).Refer to Figure 2 and Figure 4, *Listeria monocytogenes* biofilms formed on the food contact surfaces with high surface energies, such as stainless steel, were easily destroyed by the non-ionic surfactants used in the experiment than the other food contact surfaces with low surface energies such as Low-Density Polyethylene and polypropylene, and Low-Density Polyethylene surfaces; these results were in agreement with the study conducted by Mafu, Roy, Goulet, Savoie, and Roy (1990)and Mafu, Roy, Goulet, and Savoie (1991),who showed that biofilm formed on hydrophilic surface can be easily removed than hydrophobic surface.

According to Teixeira, Silva, Araújo, Azeredo, and Oliveira (2007), which showed that the ability of the disinfectants to clean up the *L. monocytogenes* biofilms formed on different on surfaces of different substances which are used in the kitchen that are made of various types of materials depended on the physiochemical properties such as surface topography of the materials. Refer to Figure 2, our results demonstrated also that the efficacy of surfactant on LDPE,LDPE+EVA, Polypropylene, LDPE+PP were less effective compared to Stainless Steel and Aluminum. Properties such as roughness and hardness of the Low-Density Polyethylene and Ethylene Vinyl Acetate (LDPE-EVA) provided conducive conditions which facilitated firm attachment of the *L. monocytogenes*

biofilms and hence led to the reduced number of *L. monocytogenes* biofilm cells destroyed from the surface, the same factors which were behind the poor destruction of biofilm cells on PP and LDPE+PP surfaces (Chavant, Martinie, Meylheuc, Bellon-Fontaine, & Hebraud, 2002; Manuel Simões, Simões, Machado, Pereira, & Vieira, 2006).These results are in agreement with the study conducted by Jerônimo et al. (2012), which indicated that the bacterial biofilm cells which were formed on the polypropylene surfaces were not easily destroyed by sodium hypochlorite and peracetic acid which acted as sanitizers as compared to those which were formed on the stainless steel surfaces. Mafu et al. (1990), have shown that *L. monocytogenes* biofilm cells have formed on stainless steel and glass were easier to clean by sanitizing agents as compared to polypropylene and rubber.The results also are in agreement with that study conducted by Teixeira et al. (2007), it was established that it is hard to destroy *L. monocytogenes* biofilms which were formed on bowls made of Polypropylene than on those which were made of Stainless Steel (Teixeira et al., 2007; Vasiljević, Simončič, & Kert, 2015). This was because the formation of the biofilms on the surfaces of the bowl made of Polypropylene has holes that acted as a harborage that protects biofilm cell from the access of surfactant when itcontacts with surface compared to those made of Stainless Steel (Mafu et al., 1990).

According to dos Reis-Teixeira, Alves, and de Martinis (2017), the relationship between the bacterial adhesions on the surface is directly proportional to the removability of those biofilms from that particular surface. Therefore, it can be justified that the presence of favorable factors on the food contact surface, which facilitated the bacterial adhesions, would also affect the process of bacterial biofilm elimination from such

surfaces since they were firmly held on those surfaces. About the results that were obtained from the experiment, it could be established that there were specific factors which influenced the removal of the *L. monocytogenes* biofilm cells more easily from one food contact surface to the other (BAKTERIJ, 2014; Cortés et al., 2011).

To ensure efficient destruction of the *L. monocytogenes* biofilms by non-ionic surfactant, it was important to all for much time of contact between the surfactant and the food surface and that was the reason as to why much of the *L. monocytogenes* biofilms cells were destroyed after 30 minutes. The efficiency of the surfactant against the *L. monocytogenes* biofilm cells could, therefore, be improved by increasing the time of contact and by combining two or more surfactants.

5. Conclusion

As the research has demonstrated, it can be justified that the removal of the *L. monocytogenes* largely depended on the type of food contact surface onto which the biofilms were formed, the type of surfactant used in the destruction of the biofilm cells and the time allowed for the contact between the surfactant and the *L. monocytogenes* biofilm cells. The effectiveness of all of the surfactants against the *L. monocytogenes* biofilm cells increased with the increase in time and therefore this was the reason as to why the largest number of the *L. monocytogenes* biofilm cells destroyed were obtained after 30 minutes in all of the surfactants.

From the research, it was established that different surfactants showed different effectiveness against the *L. monocytogenes* biofilms. This, therefore, led to the form of the following sequence in a decreasing order regarding the ability to destroy the biofilm cells; Brij 58 > Pluronic F127 > Tween 80 > Tween 40 > Tween 20 > Pluronic F68.

Regarding the easiness to destroy the biofilm cells formed on different surfaces was; Stainless Steel > Aluminum > LDPE > LDPE+EVA > Polypropylene > LDPE+PP.

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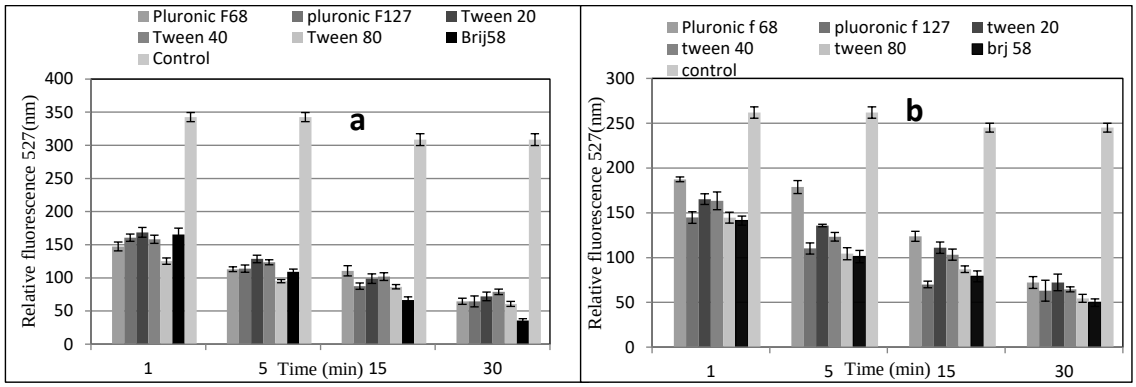
Appendix I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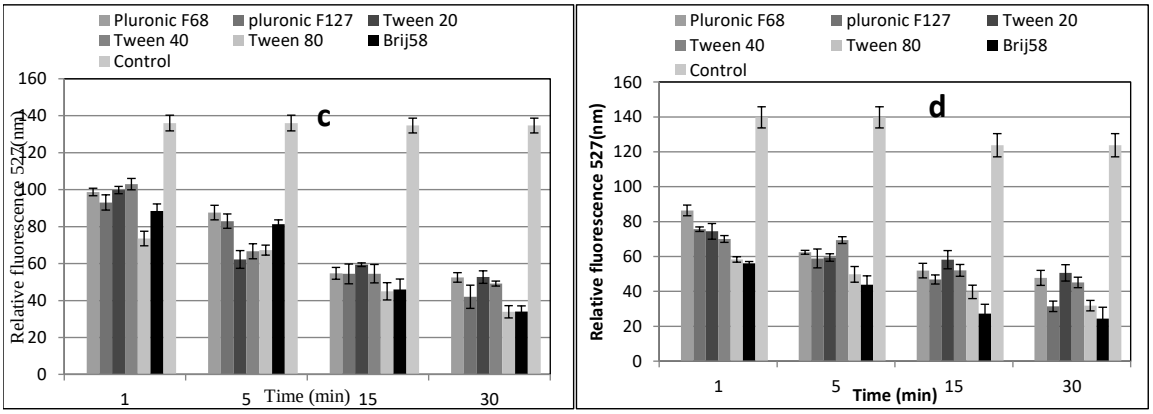
pluronic F127
Tween 20
Tween 40
Tween 80
Brij58
Control
Relative fluorescence 527(nm)
a
Time (min)
Pluronic f 68
pluoronic f 127
tween 20
tween 40
tween 80
brj 58
control
b


Pluronic F68
pluronic F127
Tween 20
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Tween 80
Brij58
Control
Relative fluorescence 522(nm)
c
Time (min)
Relative fluorescence 527(nm)
d
Time (min)

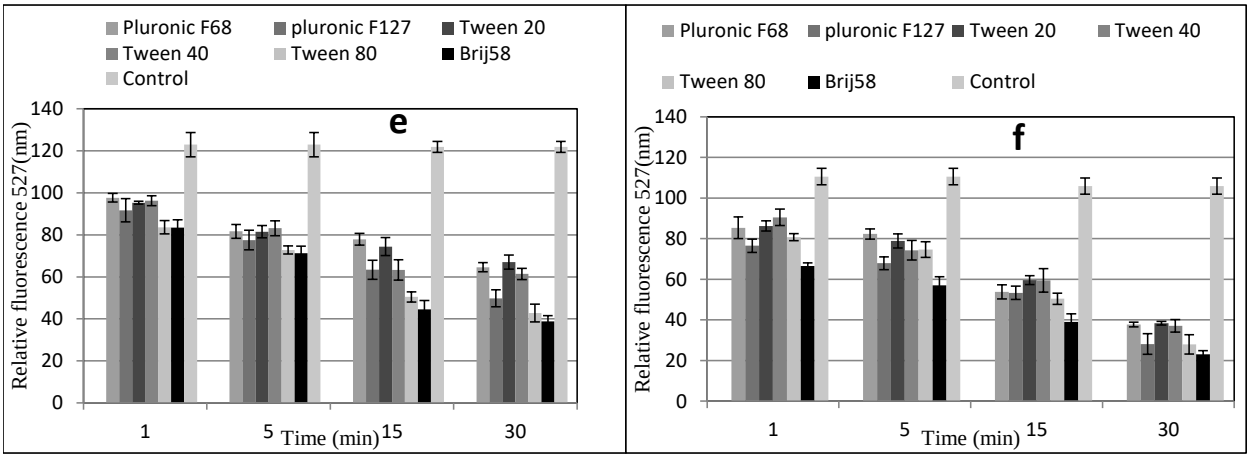

Figure 16 Total cell quantification by Acridine Orange staining. L. Monocytogenes biofilm on (a) Stainless-Steel,(b) Aluminum, (c) Low-Density Polyethylene surface (LDPE), (d) Polypropylene Surface (PP), (e) Low-Density Polyethylene and Polypropylene (LDPE+PP), (f) Low-Density Polyethylene and Ethylene Vinyl Acetate (LDPE+EVA.

Food Surfaces were treated with different Surfactants at Different Times (1, 5, 15, and 30) min. The BHI medium was used to grow the *L. monocytogenes* culture overnight, a 100µL aliquot of the cell suspension was transferred into 15mL test tube composed of 10mLof BHI broth. 400µL of diluted bacteria was pipetted into sterilized 24-well polystyrene flat- bottoms tissue culture plate then incubated at 37^0C for 24 hours.After incubation, each well was washed with buffered water.Each well was filled with 400 µL of each surfactant in triplicates.After 1,5,15, and 30 min, each well was rinsed gently twice using buffered water. Finally, fixation was carried out for 30 min at 60^oC then the wells were stained by acridine orange, fluorescence intensity was measured using an emission filter at 527/20 nm and excitation filter at 485/20 nm. The experiment repeated three times, the average was calculated.

Chapter V Recommendation and Future studies

1. Even though this study was very successful in the determination of the ability of the various types of non-ionic surfactants in the removal of the *L. monocytogenes* biofilms from the leaves of Spinach and food contact surfaces, there are still some adjustments, which needed to be done in order to improve the effectiveness of the whole experiment. Because different non-ionic surfactants used in the experiment showed different effectiveness against the *L. monocytogenes* biofilms, the study could have included the determination of the effectiveness of a combination of two or more surfactants against the Listeria biofilms. Based on this limitation, future studies should, therefore, focus on the ability of a combination of two or more non-ionic surfactants in the removal of the Listeria biofilms from the spinach leaves and food contact surface.

2. In the experiment, 30 minutes was the highest amount of time allowed for the contact between the surfactant and the *L. monocytogenes* biofilm cells, yet still, there was no point in which all of the biofilm cells were destroyed. Future research on this experiment should, therefore, allow much time to determine if there will be the possibility of the nonionic surfactants destroying all of the *L. monocytogenes* biofilm cells formed on the food contact surfaces.

3. From the experiment, it was observed that different surfactants showed different effectiveness against the *L. monocytogenes* biofilm cells at different time intervals and with some gaining their stability much faster than the others gain. Since this research used a single surfactant at a time, future research should there be conducted using the mixture of these surfactants that have different effectiveness and stabilities against the *L. monocytogenes* biofilm cells with some natural antimicrobial agents such nisin in order to

determine whether much of the cells would be destroyed after the provided time intervals as compared to those destroyed using a single surfactant.

4. This study approved the removal of listeria biofilm on spinach leaves by the non-ionic surfactants; further studies require evaluating the effectiveness of non-ionic surfactants on removal listeria biofilm on other fresh-cut produces.

5. Future studies require evaluating other types of surfactants such cationic and anionic surfactants against listeria biofilm and other food-borne pathogens.

6. The application of nanoemulsion as an antimicrobial agent is a new and promising innovation.Nanoemulsions are two phased colloidal systems constituting of water, oil, and surfactant in nano-sized droplets of less than 100 microns.The EO nanoemulsion has a broad spectrum activity against bacteria, future study require to use the non-ionic surfactants to make emulsion of Eos and evaluation against listeria biofilm.