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Investigating the Attachment of *Salmonella enterica* Strains to Bacterial Cellulose-based Plant Cell Wall Models

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Notice 1

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This work in this thesis has been submitted to peer reviewed journals as five research papers, one has been published, another one has been accepted while three publications have been submitted and are under review. The core theme of the thesis is the investigation on factors involved in the attachment of *Salmonella enterica* strains to cut plant cell walls and the potential use of sonication as a control for its attachment to fresh produce. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the School of Science under the supervision of Professor Gary A. Dykes and Professor Sadequr Rahman.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of Chapters 2 to 6, my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status	Nature and extent (%) of students contribution
Chapter 2	Relationship between Cell Concentration and *Salmonella* Attachment to Plant Cell Walls	Published	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
Chapter 3	Pectin and Xyloglucan Influence the Attachment of *Salmonella enterica* and *Listeria monocytogenes* to Bacterial Cellulose-Derived Plant Cell Wall Models	Published	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
Chapter 4	Attachment of *Salmonella* Strains to a Plant Cell Wall Model is Modulated by Surface Characteristics and Not by Specific Carbohydrate Interactions	Submitted	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
Chapter 5	Role of Fimbriae, Flagella and Cellulose on the Attachment of *Salmonella* Typhimurium ATCC 14028 to Plant Cell Wall Models	Published	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.
Chapter 6	Sonication Reduces the Attachment of *Salmonella* Typhimurium ATCC 14028 Cells to Bacterial Cellulose-Based Plant Cell Wall Models and Cut Plant Material	Submitted	I was responsible for 100% of analysis and interpretation of data and 90% of drafting and writing.

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: **Date:**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

Main Supervisor signature: [redacted] **Date:**

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Abstract

In this study, the role that different plant cell wall (PCW) components (cellulose, pectin and xyloglucan) play in *Salmonella* (*S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4) attachment to cut PCWs was investigated using bacterial cellulose (BC)-derived PCW models. Pectin significantly increased *Salmonella* attachment (~0.5 to 0.9 log CFU/cm^2) whereas xyloglucan had no effect on *Salmonella* attachment but significantly increased *Salmonella* attachment when present with pectin (~0.5 to 1.1 log CFU/cm^2). Attachment of *Listeria monocytogenes* ATCC 7644, which was used as a control strain in the study, to the PCW models was influenced by pectin and xyloglucan in a different way to *Salmonella*. This suggests that interactions between the PCW components and bacterial species are specific.

Attachment studies with monosaccharides making up pectin and xyloglucan polymers indicated that *Salmonella* attachment to plants was not mediated by receptor-ligand interactions involving carbohydrates and bacterial surface adhesins. The mechanism was instead suggested to be due to the effect of pectin and xyloglucan on the surface morphology of the PCW models. *Salmonella* attachment was not directly determined by the physicochemical properties of the attachment surface (hydrophobicity) nor the bacterial surface (hydrophobicity and aggregation abilities).

This study established that flagella, fimbriae and cellulose all play a role in *Salmonella* attachment to plant surfaces. The effect of incubation temperature on the role of cell surface components in attachment was investigated. All three cell surface components were produced at 28^{o}C but only flagella were produced at 37^{o}C. Of these structures, flagella appeared to be most important (a reduction of ~1.5 and 0.9 log CFU/cm^2 when grown at 28^{o}C and 37^{o}C respectively). Using this knowledge, the effectiveness of using ultrasonic waves which can disrupt bacterial surface structures (including flagella) to reduce levels of bacterial

attachment to PCWs was investigated. Sonication proved to have potential use as the process resulted in significantly lower numbers of *Salmonella* cells attach to PCW models and cut plant materials (between 0.5 to 1.0 log CFU/cm^2).

A linear mathematical model was constructed to predict the numbers of *Salmonella* cells that would attach to natural PCWs based on cell concentration. This was achievable as the number of *Salmonella* cells attaching to the PCW models increased linearly with cell concentration. Validation of the model for a range of variables (different natural plant tissues, different subspecies of *S. enterica* strains and other common human foodborne pathogens) supported its robustness. Development of the mathematical model has the potential to feed into risk-assessment tools for the control of *Salmonella* associated with the processing of fresh produce.

List of Abbreviations

AFM	Atomic force microscopy
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BATH	Bacterial adhesion to hydrocarbon
BC	Bacterial cellulose
BCP	BC-Pectin
BCPX	BC-Pectin-Xyloglucan
BCX	BC-Xyloglucan
BTB	Bromothymol Blue
CBB	Coomassie Brilliant Blue
CDC	Centers for Disease Control and Prevention
c-di-GMP	cyclic-di(3'→5')-guanylic acid
CFU	Colony forming units
CLSM	Confocal laser scanning microscopy
CR	Congo Red
CW	Calcofluor White
FAO	Food and Agriculture Organization
GC	Gas chromatography
HS	Hestrin and Schramm
LSA	Listeria selective agar
MAMP	Microbe-associated molecular patterns
OD	Optical density
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCW	Plant cell wall
rdar	Red, dry and rough
RMS	Root mean square
saw	Smooth and white
SEM	Scanning electron microscopy
SPIP	Scanning Probe Image Processor
SPSS	Statistical Package for the Social Sciences
TSA	Tryptic soy agar

TSB	Tryptic soy broth
UV	Ultraviolet
WHO	World Health Organization
XLDA	Xylose lysine deoxycholate agar

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Chapter 1

Literature Review

1 Literature Review

1.1 Overview

In recent years minimally processed fresh produce has been implicated as a major source of microbial foodborne diseases around the world (Brandl, 2006). The cut surfaces of minimally processed fresh produce expose plant cell walls (PCWs) that are vulnerable to human foodborne pathogens, particularly *Salmonella enterica* (Klerks et al., 2007; Kroupitski et al., 2009b). According to the World Health Organization (WHO), none of the currently available decontamination strategies can completely eliminate pathogens on produce without damaging its organoleptic qualities (López-Gálvez et al., 2010). Pathogenic bacteria associated with fresh produce may establish themselves on plant surfaces and cause disease and therefore the initial process of bacterial attachment is a crucial step in their transmission (Bordas et al., 1996; Lu and Walker, 2001). A study by Saggers et al. (2008) suggested that PCW components at the PCW junction, particularly pectin, may provide receptor sites for bacterial attachment. In addition to the structural components of the PCW, bacterial attachment can also be influenced by many other factors such as bacterial attachment structures (Barak et al., 2007, 2005; Berger et al., 2009; Kroupitski et al., 2009b), surface topography (Han et al., 2000; Macarisin et al., 2013) and hydrophobicity of the bacterial cell and the attachment surface (An and Friedman, 1998; Benito et al., 1997; Lima et al., 2013). Little is known about how PCWs exposed at cut surfaces interact with foodborne bacteria that become attached to them. A better understanding of how human pathogens attach to PCWs is required to aid in the development of new and more effective ways of preventing and removing attached bacteria. Therefore, the objective of this literature review and thesis is to investigate the factors involved in the attachment of *Salmonella enterica* to cut PCWs on fresh produce.

1.2 Increased consumption of fresh fruit and vegetables

Over the last few decades, there is a fast growing trend towards the consumption of fresh produce (such as fruits and vegetables) mainly due to heightened consumer awareness of the benefits of a healthy diet (Heaton and Jones, 2008; Jacxsens et al., 2010). According to the WHO, about 1.7 million deaths (2.8%) worldwide annually are linked to low fresh produce consumption (Rekhy and McConchie, 2014). Governments around the world have been encouraging the consumption of fruits and vegetables to prevent diseases such as diabetes, heart diseases, strokes, eye diseases and cancers (Berger et al., 2010). This growth is also driven by consumer demand for convenience and variety, thus fresh-cut, ready-to-eat or minimally processed foods are fast gaining popularity (Berger et al., 2010; Heaton and Jones, 2008; Jacxsens et al., 2010).

The Food and Agriculture Organization (FAO) of the United Nations reported that the global yearly production of fruits and vegetables increased by 94% from 1980 to 2004 and still continues to grow (FAO/WHO, 2008). Minimally processed vegetables accounted for an increasing percentage of the total fresh produce sales in Western Europe with an estimated growth of 10 to 25% per year since 1990, and for example more than 50% of the fresh produce market turnover in Belgium (Ragaert et al., 2004; Viazis et al., 2011). The fresh-cut industry in Southeast Asian countries such as Korea and Japan is also experiencing fast growth. The fresh-cut produce market in Korea was about $1.1 billion in 2006 which was about double the volume of sales made in 2003 (Kim, 2007). In Japan fresh-cut produce sales have increased from about $1 billion in 1999 to $2.6 billion in 2005 and accounted for 10% of the total fresh produce sales (Izumi, 2007).

1.3 Fresh produce-associated foodborne outbreaks

The increased consumption of minimally processed fresh produce has been accompanied by a simultaneous increase in the prevalence of foodborne illness (Tauxe, 1997; Viazis et al., 2011). Fresh produce has been implicated as the major cause of microbial foodborne disease around the world (Brandl, 2006).

In the United States, fresh produce has become the leading cause of foodborne outbreaks (46%) and hospitalizations (38%) (Painter et al., 2013) which is a huge increase from a mere 0.7% of outbreaks in the 1970s (Sivapalasingam et al., 2004). Foodborne outbreaks associated with fresh produce in the European Union doubled within a year, with the figure increasing from 4.4% in 2009 to 10% in 2010 (EFSA, 2010). A study of general outbreaks of infectious intestinal diseases in England and Wales between 1992 and 2006 found that out of these, 23% of the reported outbreaks were of foodborne origin and 4% of the foodborne outbreaks were associated with cut and prepared salad (Berger et al., 2009; Little and Gillespie, 2008).

In addition to causing serious health consequences in many countries, foodborne outbreaks caused major economic losses to the produce industry due to recall costs, unsaleable produce, medical costs and lost productivity (Heaton and Jones, 2008; Li et al., 2010). It has been estimated that in the United States alone produce causes 20 million cases of foodborne illnesses with about 3000 deaths which cost about $38.6 billion every year (Olaimat and Holley, 2012).

1.3.1 Factors causing increase in human pathogen spread

Many factors contribute to the spread of human pathogens on fresh produce. Fresh produce can be contaminated both pre-harvest and post-harvest. Pre-harvest contamination sources include soil, dust, faeces, reconstituted fungicides and insecticides, contaminated irrigation water, raw or insufficiently composted manure, wild or domestic animals and human handling (Doyle and Erickson, 2008; Newell et al., 2010; Olaimat and Holley, 2012; Tauxe, 1997). Primary reservoir of human pathogens present in soil and manure can also contaminate water systems through sewage systems that are not functioning properly, sewage overflows, polluted storm water run-off and the run-off from animal pastures (Tang et al., 2012).

Fresh produce can also be easily contaminated post-harvest during storage, rinsing, cleaning, cutting and transporting (Berger et al., 2010). Since these post-harvest processes require human handling, infected food handlers can also contaminate fresh produce (Berger et al., 2010). Cross-contamination during post-harvest processing also poses a serious problem for the fresh produce industry (Elizaquível et al., 2012; Pérez-Rodríguez et al., 2008). Fresh produce is usually submerged in a large rinse tank and the processing wash water is usually reused to save cost. This may result in the accumulation of microbial load in the wash water, making the wash water an excellent medium for transferring pathogens and allowing cross-contamination to occur. Specifically pathogens present in the wash water or contaminated produce can be transferred to other previously non-contaminated produce (Gil et al., 2009; Pérez-Rodríguez et al., 2008).

1.3.2 Ineffectiveness of current decontamination methods

Since microbial contamination can occur during any step in the farm-to-fork continuum, sanitation methods which can guarantee the safety of the fresh produce are very important. Currently in produce-processing facilities, washing is the only step used to remove soil and debris, with sanitizers usually added to the wash water to reduce microbial populations (Herdt and Feng, 2009). Chlorine, which is the most commonly used sanitizer in fresh produce processing, has limited effectiveness and generally only achieve bacterial reductions of 1 to 2 logs (Delaquis et al., 2002; Takeuchi and Frank, 2000). Chlorinated water is only effective in keeping the wash water free of contaminants and preventing cross-contamination (Aruscavage et al., 2006; Delaquis et al., 2002; Takeuchi and Frank, 2000). The use of surfactants enhance the effectiveness of chlorine by improving surface contact of chlorine with the pathogen but the addition of surfactants affects organoleptic qualities of produce (Escudero et al., 1999).

The limited effectiveness of chlorine and other chemical reagents may be due to dependence of the action of these chemical antimicrobial agents on many factors, such as pH, temperature, and the amount of free available chlorine and organic matter in the solution, which are hard to control and optimize (Doyle and Erickson, 2008). In addition, these chemical sanitizers are generally not environmental-friendly and may cause adverse health effects due to the formation of carcinogenic by-products, such as trihalomethanes, chloramines, haloketones, chloropicrins, and haloacetic acids caused by the reaction of residual chlorine with organic matter (Ölmez and Kretzschmar, 2009; Parish et al., 2003). The use of chlorine on minimally processed fresh produce is banned in some European countries such as Sweden, Germany and Belgium (Pérez-Gregorio et al., 2011).

Physical methods, such as thermal treatments, irradiation and high pressure processing, can also be used to inactivate microbes in food products. Irradiation is able to inactivate pathogens on intact and minimally-processed produce but the dosage needed to achieve this causes undesirable softening of plant tissues (Fonseca, 2006). Thermal pasteurization and sterilization are also commonly used to process food, however heating of food products is energy-intensive and reduces food nutritional content (Piyasena et al., 2003).

An emerging technology in food processing is ultrasonication which has been used to clean medical equipment and also for non-destructive quality evaluation of agricultural produce and beef products (Ngadi et al., 2012; Piyasena et al., 2003; Rodgers and Ryser, 2004). Furthermore, ultrasound has been used in the food and beverage industry to assist in the extraction of metabolites from plant materials, for example, extraction of lipids and proteins from plant seeds and oil from palm fruit, corn germ and citrus fruit (Sampedro and Zhang, 2012). Surface treatment and cleaning of food products using ultrasound has also been investigated (Rediske et al., 1998). Some studies (Sagong et al., 2011; Scouten and Beuchat, 2002; Seymour et al., 2002; Zhou et al., 2009) have shown that power ultrasound was successful in sanitizing fresh produce, especially when used in combination with other processing steps (such as using heat and pressure) during the washing step. However, this technology has not yet seen widespread use in the fresh produce industry (Rodgers and Ryser, 2004). The use of ultrasonication offers many advantages over current practices such as lower usage of wash water, lower cost, faster processing time and no production of hazardous chemical by-products (São José et al., 2014).

1.4 Salmonella

Salmonella is the most commonly identified human pathogen associated with fresh produce (Klerks et al., 2007; Kroupitski et al., 2009b). Other human pathogens associated with fresh produce include *Listeria monocytogenes* and *Escherichia coli* O157:H7 (Berger et al., 2010; Takeuchi et al., 2000). These human pathogens have traditionally been associated with foods of animal origin, however, recently they have been found to occur on various parts of plants, including roots, leaf surfaces and even within plant tissues (Klerks et al., 2007; Kutter et al., 2006; Solomon et al., 2002).

1.4.1 Physiology and taxonomy

Salmonella belongs to the Enterobacteriaceae family and are a genus of Gram-negative, facultatively anaerobic and non-spore forming rods. Most strains are motile by peritrichous flagella except for *Salmonella* Gallinarum and *Salmonella* Pullorum (Bailey and Maurer, 2001). *Salmonella* are resilient mesophiles that can grow over a wide range of temperatures (from 7 to 48^{o}C), has high pH tolerance (pH 4 to 8) and water activities above 0.93 (Baird-Parker, 1991). *Salmonella* is commonly found in the intestinal tracts of animals and humans and can survive for a long time in the environment. For example, *Salmonella* was able to survive up to six months in cold cattle manure (Forshell and Ekesbo, 1993) and for up to nine months in pond and stagnant water (Pelzer, 1989). *Salmonella* that persisted on contaminated soil for more than six months led to the contamination of lettuce and parsley crops (Islam et al., 2004).

Salmonella comprises of two species, namely *Salmonella bongori* and *Salmonella enterica.* The species *S. enterica* can further be categorized into 6 subspecies, which include *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houtenae* (IV), and *indica* (VI). *S. bongori*, previously categorized as a subspecies of *S. enterica*, is denoted with the Roman

numeral V (D'Aoust and Maurer, 2007). There are currently over 2600 serovars described within the genus *Salmonella* and each serovar is differentiated using the Kauffmann-White scheme based on the antigenic variability of their LPS O-antigens (O) and flagellar (H) antigens (Levantesi et al., 2012).

1.4.2 Epidemiology

S. enterica subsp. *enterica* encompasses over 2300 of the known *Salmonella* serovars and is the only subspecies which infects mammals (Porwollik et al., 2004). About 90% of human infections are associated to only about 50 serovars, all within the subspecies *enterica* and consist of both typhoidal and non-typhoidal serovars (Gal-Mor et al., 2014). Typhoidal serovars (e.g. Typhi, Paratyphi) can only infect humans and water contaminated with faeces of infected persons is one of the main source of enteric fever infections. Enteric fever infections are therefore more common in less developed countries that lack access to clean water (Levantesi et al., 2012). Globally, over 27 million cases of enteric fever with more than 200000 deaths are detected every year (Buckle et al., 2012; Crump et al., 2004).

Most *Salmonella* serovars are non-typhoidal and exhibit less host specificity and can colonize both humans and animals. Non-typhoidal serovars are more frequently linked to foodborne transmission and tend to cause acute but usually self-limiting gastroenteritis (Rabsch et al., 2001). The infectious dose for salmonellosis can be as few as 10 cells and extend up to 10 logs of cells depending on the variation among organisms, the age and health of the infected individuals (Blaser and Newman, 1982; Gorden and Small, 1992) . Symptoms of gastroenteritis normally appear within 12 to 72 h after ingestion and usually cause nausea, vomiting, diarrhoea, abdominal pain and fever. In immunocompromised individuals these non-typhoidal serovars can become life-threatening due to septicaemia (*Salmonella* infecting

body organs through the blood stream) and serious dehydration from excessive diarrhoea (Brands and Alcamo, 2006; Scheld et al., 2008).

It has been estimated that there are about 93.8 million cases of gastroenteritis yearly and results in about 155000 deaths around the world (Majowicz et al., 2010). *Salmonella* was isolated in 48% of foodborne outbreak cases in the United States between 1973 and 1997 (Sivapalasingam et al., 2004) and from 41% of foodborne illness cases in the United Kingdom between 1992 and 2000 (Heaton and Jones, 2008).

1.4.3 Prevalence of *Salmonella* on fresh produce

While non-typhoidal serovars such as *S.* Enteritidis and *S.* Typhimurium have been historically linked to the majority of the outbreaks from food of animal origins, a number of *Salmonella* serovars (e.g. Typhimurium, Enteritidis, Newport, Dublin, Senftenberg and Saintpaul) have now been strongly linked to outbreaks from fresh produce (Klerks et al., 2007; Lynch et al., 2009; Shi et al., 2007). Some *S. enterica* genes which are required for virulence in animals are also needed for *Salmonella* attachment to plants (Barak et al., 2005). Fresh produce has been linked to 27% and 37% of *Salmonella* outbreaks and cases in the United States (Olaimat and Holley, 2012). Sampling of several farms in Mexico found that about 43% of the produce was contaminated with *S. enterica* and 91% of the strains isolated were *S.* Typhimurium (Gallegos-Robles et al., 2008).

Salmonellosis outbreaks have been linked to a wide range of fresh produce including alfalfa sprouts, peppers, lettuce, tomatoes, melons and parsley (Lapidot et al., 2006). *S. enterica* was found to be responsible for 76%, 60% and 30% of outbreaks in the United States linked to fruits, seed sprouts and leafy vegetables respectively (Brandl, 2006). The Centers for Disease Control and Prevention (CDC) (2007) reported that 510 people in 26 states were infected by *S.* Newport contamination on tomatoes in 2002 and the same strain

caused another outbreak in 2005 which infected 72 people in 16 states. In 2008, a large salmonellosis outbreak linked to the consumption of hot peppers contaminated by *S.* Saintpaul affected 1442 victims in 43 states in the United States and Canada (CDC, 2008). There was another salmonellosis outbreak linked to the consumption of alfalfa or spicy sprouts between late 2010 and early 2011 where 140 cases were reported across 26 states in the United States (CDC, 2011). *Salmonella* contamination of fresh produce has been suggested to be serovar-dependent and this could explain why only a narrow range of serovars are involved in the outbreaks. Shi et al. (2007) found that certain *Salmonella* serovars (Newport, Montevideo and Hadar) grew and persisted better on ripened tomatoes than others (Enteritidis, Typhimurium and Dublin). Another study by Klerks et al. (2007) demonstrated a significant serovar-cultivar interaction of *S. enterica* serovars with different lettuce cultivars.

1.5 Bacterial attachment

Bacterial attachment is a crucial step in the colonization and transmission of human pathogens. Human pathogens associated with fresh produce have to establish themselves on plant surfaces or in plant tissue before they can cause infections and diseases in humans (Bordas et al., 1996; Lu and Walker, 2001). Bacteria with high adhesion rates are able to establish themselves on a surface better and tend to have higher infectivity (Ofek and Doyle, 1994). Human pathogens attach rapidly to produce surfaces (Iturriaga et al., 2003) and can be hard to remove using the current washing methods (Soon et al., 2012).

Cut surfaces of fresh-cut produce are especially vulnerable to attachment by human pathogens as these surfaces lack the waxy cuticle which repels water that could carry pathogens (Aruscavage et al., 2006; Kroupitski et al., 2009). For example, the attachment of

S. Chester has been reported to occur mainly on the injured surface of green pepper but rarely on the intact skin (Liao and Cooke, 2001). These cut surfaces also exude nutrients and water which are favourable for the growth and survival of the pathogens. Some human pathogens are able to penetrate the internal tissues after attaching to cut plant surfaces which could protect them from chemical sanitizers (Takeuchi et al., 2000). Another study by Liao and Sapers (2000) also found that almost 20% more *Salmonella* cells remain adhered to apple disks when the apple skin was removed as compared to those that still had intact skin.

1.5.1 Factors involved in bacterial attachment

The attachment of *Salmonella* to plant surfaces can be influenced by many factors. Some factors associated with the bacteria include cell surface components (Barak et al., 2007, 2005; Berger et al., 2009; Kroupitski et al., 2009b), bacterial concentration (Liao and Cooke, 2001; Takeuchi and Frank, 2000) and bacterial hydrophobicity (Benito et al., 1997; Mitik-Dineva et al., 2006) and aggregation abilities (Burdman et al., 2000; Del Re et al., 2000). Factors associated with the plant surface which can affect bacterial attachment include surface topography (Wang et al., 2009; Zhang et al., 2013), surface hydrophobicity (Lima et al., 2013; São José et al., 2014) and chemical composition of the plant surface (Saggers et al., 2008). The influence of these factors on the attachment of *Salmonella* to surfaces of fresh produce is largely unknown as the occurrence of human pathogens on fresh produce was only recently noted as of concern (Brandl et al., 2013). Investigation of these factors on fresh produce is not straightforward as fresh produce has complex surface properties which are dependent on its morphology, hydrophobicity and constitutional characteristics (Park et al., 2012).

1.5.1.1 Bacterial surface components

Most human pathogens rely on surface structures and appendages, which associate with proteins on the PCW, for successful attachment to plant surfaces (Sapers et al., 2005). Curli fimbriae, flagella, O-antigen capsule and cellulose have been shown to play important roles in *Salmonella* adhesion to plants (Barak et al., 2007, 2005; Berger et al., 2009; Kroupitski et al., 2009b). Different *Salmonella* strains may have strain-specific attachment mechanisms (Berger et al., 2009).

Flagella are long, thin surface appendages that extends up to 20µm and which are important for motility and chemotaxis (Wiedemann et al., 2015). Pathogens use flagella to move along the plant surface before finding a favourable attachment site (Cooley et al., 2003). Studies by Berger et al. (2009) and Kroupitski et al. (2009) showed that flagella play a significant role in the attachment of *S.* Senftenberg and *S.* Typhimurium to basil and lettuce leaves, respectively. Rossez et al. (2014) showed that the *E. coli* flagella mediate binding to the plant plasma membrane. Flagella also mediate chemotaxis which can guide planktonic cells to swim towards sites with nutrients or towards cells attached on a surface (Pratt and Kolter, 1998). Klerks et al. (2007) showed that *S. enterica* serovars actively moved toward root exudates of a lettuce cultivar by chemotaxis.

Curli, which is shorter in length, straighter and more numerous than flagella in numbers does not play a role in *Salmonella* motility. The function of adhesins on the curlin tip in determining its specific attachment properties has been demonstrated on animal cells (Weening et al., 2005). Lectins, found on curli and flagella structures, are able to recognize oligosaccharide units on the animal cells for specific attachment (Rodríguez-Navarro et al., 2007), these could be involved in the bacterial attachment to plant surfaces. Cellulose has been hypothesized to aid attachment through its involvement in cell-to-cell interactions and

the formation of bacterial aggregates which favours bacterial attachment to a surface (Barak et al., 2007; Rodríguez-Navarro et al., 2007). In addition to its role in attachment, cellulose also promotes bacterial persistence in the environment by conferring cells with resistance to adverse conditions such as exposure to chlorine and bleach (Solano et al., 2002).

Production of curli, cellulose and O-antigen capsule are mainly regulated by the *csgD* gene in *Salmonella*. The CsgD regulator also controls the production of the extracellular matrix which contributes to biofilm formation and is important for the environmental persistence of many *Salmonella* serovars including enhancing its ability to avoid desiccation stress (Gibson et al., 2006). *Salmonella* that use fimbriae and cellulose to form biofilms on cut lettuce leaves have a greater persistence and survival rate as compared to poor biofilm formers (Jonas et al., 2007; Kroupitski et al., 2009). Fimbriae, cellulose and O-antigen capsules were also shown to be important for *S. enterica* attachment to alfalfa sprouts (Barak et al., 2007).

Bacterial attachment to plants is usually explained by a two-step model. According to Lapidot and Yaron (2009), the initial reversible adhesion to plants is mediated by weak forces, such as van der Waals forces, electrostatic forces and hydrophobic interactions (Van Loosdrecht et al., 1987). During this phase, cells are attached to the surface loosely via a single pole and may readily detach. Bacterial surface appendages such as flagella and curli fimbriae may help bacterial cells in making the first physical contact with the surface by overcoming the hydrodynamic boundary layer and also by overcoming the repulsive force between bacteria and the surface. This is followed by stronger irreversible attachment which is mediated by strong forces such as covalent bonds, hydrogen bonds and strong hydrophobic interactions (Van Oss, 1989). Extracellular polysaccharides such as cellulose and O-antigen capsule allow bacteria to attach strongly to the plant surface (Barak et al., 2007). Specific

interactions between bacterial surface structures and plant ligands have also been shown. Rossez et al. (2014a) demonstrated that the *E. coli* common pilus targeted arabinosyl residues in PCWs to mediate its persistence on fresh produce plants. Another study by Rossez et al. (2014b) showed that the *E. coli* flagella interacted with phospholipids and sulpholipids in plasma membranes of spinach plants and *A. thaliana.*

1.5.1.2 Bacterial cell concentration

It is well established that increased bacterial density allows more bacterial cells to attach to a surface. Examples of this in the literature (Barak et al., 2002; Dickson and Daniels, 1991; Fratamico et al., 1996) all show that the numbers of bacteria attaching to a surface increased proportionally to inoculum size. This is not, however, always the case as once a threshold is reached, fewer bacterial cells are able to attach due to the occupancy of the available attachment sites. Liao and Cooke (2001), for example, found that the attachment of *S.* Chester to green pepper discs initially showed proportional increase to the initial inoculum concentration but slowed down and reached saturation at high initial inoculum concentration (more than 9 log CFU/mL). The relationship between bacterial density and attachment also depends on the presence of available attachment receptors and the way in which bacteria attach (Fletcher, 1977). For example, a study by Takeuchi and Frank (2000) showed less pronounced differences in the preferential attachment of *E. coli* O157:H7 cells to cut tissues over intact surface tissues at higher inoculum levels. This feature may be because *E. coli* O157:H7 cells attach to less favourable attachment sites once favourable attachment sites are occupied.

1.5.1.3 Hydrophobicity

Another important factor which can influence levels and strength of bacterial attachment is hydrophobicity. Hydrophobicity is a relative measurement which can be

determined through methods such as bacterial adherence to hydrocarbons (BATH) assays and water contact angle. A surface is considered hydrophilic when it has contact angle of less than 90^{o} whereas the value of contact angle for a hydrophilic surface is above 90^{o} (Bhushan and Jung, 2006). Bacterial surface hydrophobicity is significantly affected by the chemical composition and structures on the cell surface (Vacheethasanee et al., 1998). Proteins (such as adhesins and antigens) at the bacterial surface confer higher hydrophobicity whereas bacterial surface polysaccharides confer higher hydrophilicity (Collado et al., 2007).

As a general rule of thumb, hydrophobic bacteria attach preferentially to hydrophobic surfaces whereas hydrophilic ones prefer hydrophilic surfaces (An and Friedman, 1998). Most plant surfaces are covered by waxy cuticle which prevents plant dehydration (Aruscavage et al., 2006; Kroupitski et al., 2009). The hydrophobic nature of the cuticular wax also determines surface wettability and the plant's interaction with microbes (Fernandes et al., 2014). This hydrophobic waxy cuticle on the plant surface repels water that could carry pathogens, making it difficult for bacteria to attach (Aruscavage et al., 2006; Kroupitski et al., 2009). A number of studies (Han et al., 2000; Liao and Sapers, 2000) have shown that human pathogens attached better to hydrophilic cut plant surfaces as compared to the intact hydrophobic surfaces. In contrast to this, São José et al. (2014) found that the adhesion of *S.* Enteritidis and *E. coli* were greater to the hydrophobic green pepper surface compared to the hydrophilic surface of melons. Lima et al. (2013) also noted that *S.* Enteritidis showed four times greater attachment to hydrophobic lettuce leaves grown using a hydroponic system than the slightly hydrophilic lettuce leaves grown conventionally.

According to Van Oss and Giese (1995), attachment occurring in an aqueous environment favours adhesion to hydrophobic surfaces due to the exclusion of water molecules. As bacterial cells approach the attachment surface, intervening water molecules

act as a barrier to attachment (Van Loosdrecht et al., 1990). Hydrophobicity facilitates the process of removing water from the surface and it is easier to remove water molecules between two hydrophobic surfaces (Araújo et al., 2009). This explains why hydrophobic cells generally adhere to surfaces to a much greater extent than hydrophilic cells and why higher hydrophobicity confers higher strength of attachment (Benito et al., 1997). Hydrophobic groups on fimbriae, lipopolysaccharides or outer membrane proteins could also assist bacterial cells to get closer to the attachment surface (Mitik-Dineva et al., 2006). In addition, hydrophobic plant surfaces also make it difficult for chemical sanitizers to contact the plant surface structures (Bhushan and Jung, 2006). The addition of surfactants which lowers the surface tension of sanitizing solutions also increases the contact of the solutions on hydrophobic produce surfaces, therefore also increase effectiveness of the sanitizer (Choi et al., 2002).

1.5.1.4 Aggregation

Cell aggregation is defined as the clustering of cells under certain physiological conditions to form relatively stable, contiguous and multicellular associations (Burdman et al., 2000). Aggregation between bacterial cells of the same strain (auto-aggregation) and between different strains (co-aggregation) is reportedly linked to bacterial adhesion (Collado et al., 2007). Cell surface hydrophobicity has been suggested to influence auto-aggregation with increased hydrophobicity leading to higher cell-to-cell adhesion which in turn results in greater auto-aggregation (Collado et al., 2007; Liu et al., 2004b). Co-aggregation ability has been shown to be dependent on auto-aggregation (Collado et al., 2007). Bujnakova and Kmet (2002), for example, showed that only auto-aggregating strains co-aggregate with other strains.

Bacterial cell surface components, such as lipoteichoic acid, proteins, carbohydrates and soluble proteins appear to influence the ability of bacterial cells to aggregate (Collado et al., 2007). These polymeric molecules form bridges between bacterial cells, the main forces involved in the cell-to-cell adhesion are hydrogen bonds, ionic bonds, Van der Waals forces and hydrophobic forces (Daniels, 1980). Cellulose and curli fimbriae also play a role in bacterial cell-cell interactions and contribute to multicellular behaviour in microorganisms (Gerstel and Römling, 2003). *S. enterica* produces an extracellular matrix with curli as the major proteinaceous component. Curli are amyloid fibers which are also involved in environmental persistence and biofilm formation (Jonas et al., 2007). Cellulose is also secreted by *S. enterica* and *E. coli* as a second matrix component (Jonas et al., 2007). Auto-aggregation has been shown to influence bacterial adhesion (Burdman et al., 2000; Del Re et al., 2000) but some other studies found no such relationship (Goulter-Thorsen et al., 2011; Ryu et al., 2004). Cookson et al. (2002) and Goulter et al. (2010) found that the influence of bacterial surface properties on attachment may be strain-specific.

1.5.1.5 Surface topography

Surface topography has been suggested to influence bacterial attachment to surfaces (Goulter et al., 2011; Medilanski et al., 2002; Wang et al., 2009). Surface topography is the local deviations of a surface from a perfectly flat plane and the mapping of features on a surface (Wang et al., 2012). According to Bhattacharjee et al. (1998), surface roughness affects the surface interaction energy between the particle and the substratum surface, especially at short separation distances. Rough surfaces provide a larger surface area available for attachment and more surface irregularities that can protect bacteria from shearing forces (An and Friedman, 1998; Katsikogianni and Missirlis, 2004).

Macarisin et al. (2013) found that lettuce with the greatest leaf roughness favoured the attachment *E. coli* O157:H7. Han et al. (2000) also observed that *E. coli* O157:H7 attached better to coarse, porous or injured surfaces of green peppers compared to intact surfaces. Similarly, Wang et al. (2009) observed a positive linear correlation between average surface roughness of fruit skin surface and the attachment rate of *E. coli* O157:H7, whereas a negative correlation between surface roughness and the efficacy of microbial inactivation by chemical sanitizers was apparent. Grooves and cavities on fruits provide a rough and irregular surface which protect the cells from washing treatments (Wang et al., 2009). Liao and Sapers (2000) observed higher attachment of *S.* Chester to wounded tissues compared to intact skin on apples. They suggested that differences in surface topography and physicochemical properties could have caused the difference in bacterial attachment. *S.* Typhimurium showed favourable attachment to potato with a rough and hydrophilic surface in comparison to the tomato which has a smooth and hydrophobic surface (Zhang et al., 2013).

There are some reported discrepancies on the influence of surface roughness on bacterial attachment. Fernandes et al. (2014) found that the attachment of *S.* Typhimurium to mango, which had significantly rougher surface as compared to tomato, were similar to both fruit surfaces. They also found that hydrophobicity of the fruits surfaces did not affect the efficacy of different sanitizers in removing attached bacterial cells. Medilanski et al. (2002) and Verran et al. (2010) both reported an optimal surface roughness value for bacterial attachment with the number of attached bacteria drops proportionally below or above this value. Roughness also affects surface tension and surface hydrophobicity (Quirynen et al., 1990) and therefore the effect of these other factors could complicate the contribution of surface roughness in attachment.

It is important to note that bacterial attachment is unlikely to be controlled by any one aspect in isolation but it is much more likely to be controlled by a combination of several factors. Therefore, the contribution and importance of each factor need to be investigated.

1.6 The plant cell wall (PCW)

Plant cell walls (PCWs) play many important roles in plants including their involvement in plant growth, intercellular communication, control of water balance and defence against potential pathogens in addition to providing mechanical support to various plant cells (Keegstra, 2010). There are two forms of PCWs, namely the primary cell wall which surround growing cells and the secondary cell wall which forms within the primary cell wall to surround specialized cells.

Most plants, including fresh produce, have Type I primary cell walls whereas only cereals and grasses have Type II primary cell walls. Fresh produce has Type I primary cell walls which consist primarily of polysaccharides, namely cellulose (~25%), hemicellulose (~25%) and pectin (~35%) with small amounts of structural proteins (~1 to 8%), minerals (~1 to 5%) and phenolic esters (~2%) (% of dry mass) (shown in Figure 1.1), the composition of these walls, however, varies among different plant species (Cybulska et al., 2010a; Taiz and Zeiger, 2002). Cellulose is synthesized at the plasma membrane by cellulose synthase enzyme complexes that extrude microfibrils to the outer side of the membrane and which then become embedded in a matrix of pectin and hemicelluloses (Lack and Evans, 2005). These matrix polysaccharides (pectin and hemicelluloses) synthesized in the Golgi apparatus are packed into vesicles and then transported to the plasma membrane (Cosgrove, 2005). Unlike cellulose microfibrils, which only sit on the surface, matrix polysaccharides can

diffuse into the cell wall with the assistance of turgor pressure that increases cell wall porosity thus easing its diffusion (Cosgrove, 2005).

As previously mentioned a study by Saggers et al. (2008) suggested that PCW components at the PCW junction, particularly pectin, may provide receptor sites for bacterial attachment. From scanning electron micrographs, these authors observed that *S.* Typhimurium cells appeared to attach preferentially to the pectin layer at the PCW junction. Pectin was discovered to provide special interaction sites that benefit attachment of *Pseudomonas* and *Agrobacterium* (Rao et al., 1982). Although there is widespread occurrence of foodborne outbreaks on fresh produce, very few studies (Rao et al., 1982; Saggers et al., 2008) have investigated the role of PCW components on bacterial attachment to fresh produce.

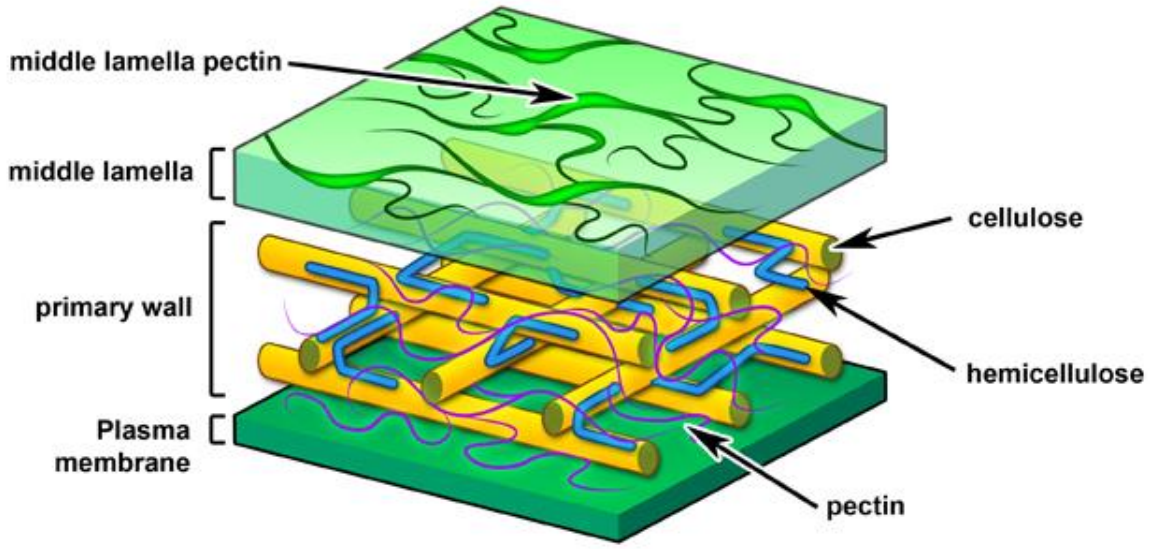


Figure 1.1 PCW structure and its major components (cellulose, hemicelluloses and pectin) (adapted from Sticklen, 2008).

1.6.1 Cellulose

One of the most abundant polymers in nature, cellulose $(C_6H_{10}O_5)_n$ is mostly located in PCWs. This polymer consists of β(1-4)-linked glucose units (shown in Figure 1.2) which form glucan chains (also known as microfibrils) that are linked by a network of hydrogen bonds and Van der Waals interactions to produce a crystalline structure with a high degree of polymerization (Waldron et al., 2003). Cellulose has the greatest influence on the structural properties of the primary cell wall amongst the PCW polysaccharides (Waldron et al., 2003; Cosgrove, 2005). The tight and inaccessible arrangement in the highly organized crystalline cellulose structures protects its glycosidic bonds from chemical and enzymatic hydrolysis (Marga et al., 2005; Deguchi et al., 2006).

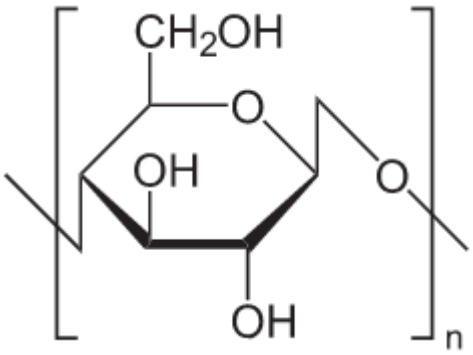


Figure 1.2 Chemical structure of cellulose, $(C_6H_{10}O_5)_n$ (adapted from Nishiyama et al., 2002).

1.6.2 Hemicellulose

Xyloglucan is the major type of hemicellulose found in primary cell walls which consists of D-glucose (~45%), D-xylose (~34%), D-galactose (~18%) and L-arabinose (~3%). Xyloglucan has a similar backbone to cellulose but with slight modifications. Compared to cellulose, this polysaccharide has additional xylose molecules on 3 out of its 4 glucose monomers and is sequentially supplemented with galactose and fucose residues

(shown in Figure 1.3) (Cosgrove, 2005). This polymer also forms cross-links with cellulose microfibrils to form a strong and extensible framework which is believed to be the major load-bearing structure in the primary cell wall. The strength of the cross-links is thought to be controlled by expansins which can break the hydrogen bonds between both polysaccharides to allow cell extension (Bowes and Mauseth, 2008). Xyloglucan which coats cellulose microfibrils not only prevents the agglutination of microfibrils, it also determines the spacing between microfibrils (Lloyd, 1991).

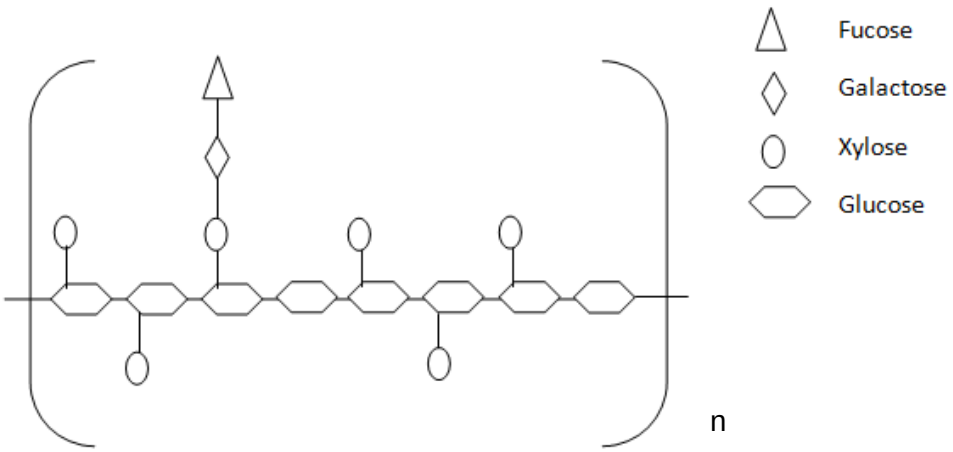


Figure 1.3 A schematic representation of the structure of xyloglucan (adapted from Yamatoya and Shirakawa, 2003).

1.6.3 Pectin

Pectin consists primarily (~90%) of α(1-4)-linked D-galacturonic acid residues ($C_6H_{10}O_7$) although it also contains other monosaccharides including D-galactose (~38%), D-glucose (~3%), D-xylose (~1.9%), D-mannose (~0.7%), L-rhamnose (~0.3%) and L-arabinose (~0.3%) (shown in Figure 1.4). There are three major pectic polysaccharide domains; namely homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II which are covalently linked to each other (Brett et al. 2005). These three polysaccharide

domains have been suggested to covalently link each other to form a pectic network throughout the primary cell wall matrix and middle lamella. Cell wall-based enzymes are able to modulate the structure of the pectic network (Willats et al., 2001).

Homogalacturonan, a linear homopolymer of α(1-4)-linked D-galacturonic acid, is the most abundant pectic polysaccharide. The homogalacturonan structure is partially methyl esterified at the C-6 carboxyl, may be O-acetylated at O-2 or O-3. It may also contain other potential crosslinking esters of uncertain structure. Homogalacturonan is thought to contain about 100 to 200 galacturonic acid residues (Mohnen, 2008). Rhamnogalacturonan I is an acidic pectic domain which consist of about 100 repeats of the disaccharide (1-2)-α-L-rhamnose-(1-4)-α-D-galacturonic acid. It is heterogeneous, abundant and generally thought to be glycosidically attached to homogalacturonan domains. Another domain, rhamnogalacturonan II, is a highly conserved and widespread domain but it is not structurally related to rhamnogalacturonan I. It is a branched pectin domain containing a homogalacturonan backbone. The rhamnogalacturonan II appears to be the only major pectic domain which does not have significant structural differences from its fine structure (Willats et al., 2001).

Although the cellulose-xyloglucan networks are generally recognized as the major support within the primary wall, pectin also forms another network around the cellulose-xyloglucan network to provide additional mechanical reinforcement. The pectin network also regulates cell wall porosity and wall thickness (Reiter, 2002). Additionally, pectin has other important roles in the adhesion of adjoining cells, ionic regulation of the cell wall, stomatal function and plant defence (Caffall and Mohnen, 2009; Carpita et al., 1979; Jarvis et al., 2003).

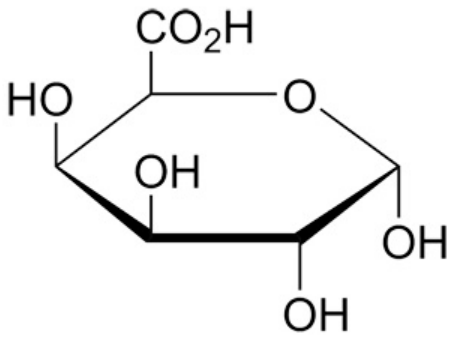


Figure 1.4 Chemical structure of D-galacturonic acid, $C_6H_{10}O_7$ (adapted from Willats et al., 2001).

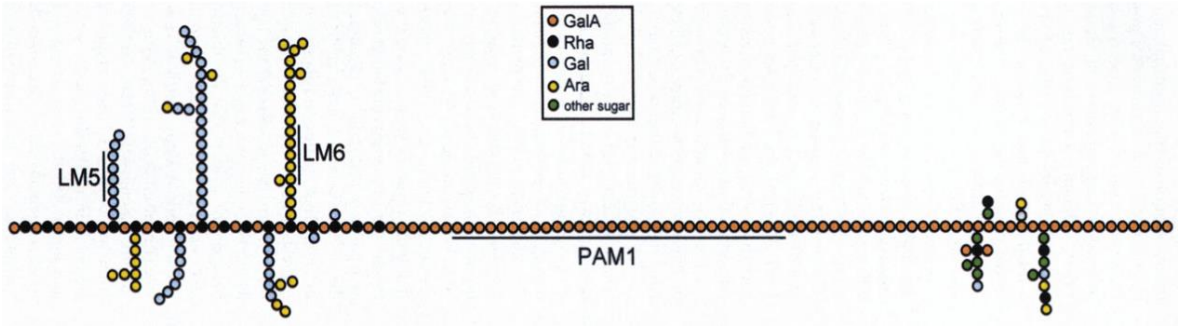


Figure 1.5 Simplified schematic diagram which indicates some of the major features of the three major polysaccharide domains of pectin (obtained from Willats et al., 2001).

1.7 PCW models

Bacterial cellulose (BC) is a carbohydrate polymer produced by, among others, strains of *Gluconacetobacter xylinus* which have been used with the addition of PCW components (xyloglucan and pectin) to construct a PCW model. *G. xylinus* is a rod-shaped, aerobic, Gram-negative bacterium which was first discovered to produce cellulose in 1886 (Brown, 1886). From then on, the bacterium has been applied as the model microorganism for experimental studies on cellulose due to its ability to use a wide range of carbon and nitrogen sources to produce relatively high levels of the polymer.

Cellulose synthesis by the *G. xylinus* cell is a precisely and specifically regulated multi-step process which involves many enzymes and complex of catalytic and regulatory proteins (Chawla et al., 2009). The process begins with the formation of uridine diphosphoglucose, followed by glucose polymerization into the β-(1-4)-glucan chain and a nascent chain which forms ribbon-like structure of cellulose chains, their extrusion outside the bacterial cell and finally, self-assembly into cellulose fibrils. The cellulose synthesis process is tightly associated with catabolic processes of oxidation and consumes as much as 10% of energy derived from catabolic reactions (Chawla et al., 2009).

Possessing similar organizational characteristics as Type I primary cell wall, the PCW model produced by *G. xylinus* has been used as a novel *in vitro* approach to understanding the molecular assembly, mechanical properties and behaviour of higher plant cell walls (Whitney et al. 1995; Whitney 1998; Chanliaud & Gidley 1999; Whitney et al. 1999; Chanliaud et al. 2002; Kacuráková et al. 2002; Tokoh et al. 2002; Astley 2003; Cybulska et al. 2010; Cybulska et al. 2011).

The first PCW model, consisting of only BC and xyloglucan was used to study the interactions between cellulose and xyloglucan (hemicellulose) as well as their involvement in the assembly of the native Type I PCW (Whitney et al. 1995). Findings showed that the proposed model closely resembles the native PCW in terms of the presence and lengths of cross-links, molecular organization of cellulose microfibrils and also the amount of xyloglucan incorporated into it (Whitney et al. 1995). Interestingly, the form of BC produced became more alike higher plant cellulose forms in the presence of hemicellulose (Uhlin et al. 1995). The model was later improved through the addition of pectin which formed interpenetrating networks with cellulose (typical of Type I primary walls) that resulted in decreased porosity of the model (Chanliaud & Gidley 1999; Cybulska et al. 2011). A further

refined model, consisting of BC with both pectin and xyloglucan incorporated into it, has a chemical composition and microstructure most similar to native PCWs (Cybulska et al. 2011). The incorporation of both cell wall polysaccharides was found to increase the elasticity while reducing the mechanical strength of the PCW model (Chanliaud et al. 2002; Astley 2003).

In order to investigate the effect of PCW components on the attachment of human pathogens, a bacterial cellulose (BC)-based PCW model was used in this study due to many difficulties in working with native PCWs. Part of the difficulties in identifying the mechanisms of bacterial attachment to PCWs is the reliance on working with cut plant material under highly variable and uncontrolled conditions (Burton et al., 2010; Jarvis, 1992). The heterogeneous composition of native PCWs complicates the study of how individual PCW components affect bacterial attachment as PCW composition differs considerably between species, among plant parts and even for cell types within each tissue (Burton et al., 2010; Fuller, 2004). In addition, it is also difficult to work with native PCWs as the whole PCW structure collapses when specific PCW components are extracted through physical or chemical methods, which means that their composition cannot be manipulated (Jarvis, 1992).

The bacterial cellulose (BC)-based PCW model is produced by culturing *Gluconacetobacter xylinus*, a BC-producing species, in growth medium with the addition of PCW components (xyloglucan and pectin). Formation of the PCW model mimics the natural phenomenon of PCW deposition in native plants (Chanliaud and Gidley, 1999). This model provides a realistic picture of what occurs within the actual PCW as it is based on a constructive approach in comparison to the destructive chemical and physical treatments used to obtain PCW fractions (Mikkelsen et al., 2011). The PCW model can also be produced in relatively large quantities and at a desired thickness which makes it convenient to work with

(Chanliaud et al., 2002; Padayachee et al., 2012). The model has similar molecular and architectural properties to native primary PCWs, and has been used as a novel *in vitro* approach to understanding the molecular assembly, mechanical properties and behaviour of higher PCWs (Astley et al., 2003; Chanliaud and Gidley, 1999; Chanliaud et al., 2002; Cybulska et al., 2011, 2010b; Kacuráková et al., 2002; Tokoh et al., 2002; Whitney et al., 1999, 1995).

We have previously developed and extended the use of this model to study bacterial attachment on PCW surfaces (Tan et al., 2013). This presents a novel approach to improve our understanding of the bacterial attachment process on PCWs. More importantly, the chemical composition of the BC-based PCW model can be easily manipulated through the specific addition and removal of PCW components. This enables the direct investigation of the effect of varying levels of specific PCW components on pathogenic bacterial attachment in this study.

1.7.1 Limitations of the PCW model

Although BC-derived PCW models resemble native PCWs to a certain degree there are still some limitations to its use. For example, PCW models have defined molecular features but they lack the heterogeneity and complexity of native PCWs (Mikkelsen et al., 2009). Fresh-cut fruits and vegetables have different chemical compositions and surface morphologies to each other and this presents diverse ecological niches for bacteria (Kroupitski et al., 2009b). The diversity in phenotypic characteristics of the host plant due to differences in plant characteristics such as leaf water content, phosphorus content, phenolics and mesophyll thickness may create “hot-spots” of microbial growth which are not reproducible on the PCW models. Also, most cells of all higher plants have the same six major polysaccharides which are xyloglucan, xylan, homogalacturonan, rhamnogalacturonan

I and rhamnogalacturonan II. However, there are several other polysaccharide which can be found in certain species, these include mannan, xylogalacturonan and mixed-linkage glucans (Mohnen et al., 2008). In addition, the presence of other elements in the native PCW, such as structural proteins, minerals, and phenolic esters which could affect bacterial attachment also presents another limitation. Another difference is that the presence of structures and tunnels within the BC structure has conferred it with very high water retention capacity which is almost 17 times greater than typical plant cellulose (Yoon et al., 2006). In addition, BC fibrils are approximately 100 times thinner than those of plant cellulose, thus giving BC a higher degree of polymerization, higher crystallinity, greater tensile strength and better shape retention compared to plant cellulose (Chawla et al., 2009; Yano et al., 2005; Yoshinaga et al., 1997). In addition, *G. xylinus* cells used in the construction of the BC-based PCW models are not present in the native PCWs and this could also affect interactions with human pathogens.

One further major difference is this. Native plants have an innate immune system with pattern recognition receptors which can recognize pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) and elicit an immune response (Garcia et al., 2014). However, the defence mechanism of native plants to colonization of human pathogens also cannot be tested using the model. Although the aforementioned limitations exist in the BC-based PCW model, it is still important and very useful in helping us to understand the contribution of each factor without interference from other components. The PCW model may provide us with insights into questions which would be difficult to address *in situ* on plants.

1.8 Objectives of the research

Although the problem of foodborne outbreaks linked to the consumption of fresh produce has become increasingly serious, the issue has not garnered the attention it should be

getting. Since the occurrence of human pathogens on fresh produce has only been recently highlighted, relatively little is known about the mechanisms which allow *Salmonella* to attach to plant surfaces.

Based on a review of the literature, the following questions were identified as important:

1. What are the factors associated with the PCW and with bacteria which can affect the attachment of *Salmonella* to the PCWs?
2. How do these factors affect attachment?
3. What can be done to reduce the attachment of *Salmonella* to the PCWs?

Based on these questions, the objectives of the research presented in this thesis were as follow:

1) To investigate the effect of inoculum size on *Salmonella* attachment to BC-based PCW models and native PCWs and to develop a mathematical model to predict *Salmonella* attachment based on its inoculum size (Chapter 2)
2) To investigate the effects of chemical components of the PCW (pectin and xyloglucan) on *Salmonella* attachment to the BC-based PCW models (Chapter 3)
3) To investigate the roles of carbohydrates and surface properties of the PCW models on *Salmonella* attachment to the BC-based PCW models (Chapter 4)
4) To investigate the roles of bacterial surface components (flagella, fimbriae, cellulose) on *Salmonella* attachment to the BC-based PCW models (Chapter 5)
5) To investigate the potential use of ultrasonication to control attachment of *Salmonella* to PCWs of fresh produce (Chapter 6)

Chapter 2

Effect of Cell Concentration on the Attachment of *Salmonella enterica* to Plant Tissues

The work presented in this chapter represents the following publication accepted for peer review:

Tan, M.S.F., Rahman, S. and Dykes, G.A. (2016). Relationship between cell concentration and *Salmonella* attachment to plant cell walls. Food Control. Available at: http://dx.doi.org/10.1016/j.foodcont.2016.02.039

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of chapter 2, the nature and extent of my contributions to the work was as following:

Nature of contribution	Extent of contribution (%)
I was responsible for 100% of data analysis and 90% of drafting and writing	90%

The following co-authors contributed to the work and the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%)
Sadequr Rahman	SR was responsible for 2% of writing and review of the manuscript.	2%
Gary Dykes	GD was responsible for 8% of writing and review of the manuscript.	8%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature	[redacted]	**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author(s) to agree on the respective contributions of the authors.

2 Effect of Cell Concentration on the Attachment of *Salmonella enterica* to Plant Tissues

2.1 Introduction

Fresh produce is an important vehicle for the transmission of human pathogens worldwide, especially for *Salmonella enterica* which has been recognized as the human pathogen most commonly found on fresh produce (Klerks et al., 2007; Kroupitski et al., 2009b). The initial attachment of human pathogens on fresh produce surfaces is a crucial step in their transmission through the processing chain (Bordas et al., 1996; Lu and Walker, 2001). Preparation of minimally processed fresh produce in the fresh-cut industry involves cutting, shredding, dicing or peeling. These post-harvest processes create wounds in the plant tissues which are especially vulnerable to bacterial attachment (Doyle and Erickson, 2008; Kroupitski et al., 2009b).

It is well established that increased bacterial density allows more bacterial cells to attach to a surface, however, the dynamics of this are also dependent on the way in which bacteria attach and the presence or absence of receptors for the bacteria (Fletcher, 1977). Some studies (Barak et al., 2002; Dickson and Daniels, 1991; Fratamico et al., 1996) have found a proportional relationship between inoculum size and bacterial attachment numbers whereas others (Liao and Cooke, 2001; Takeuchi and Frank, 2000) did not.

A number of mathematical models have been developed based on inoculum size and used, for example, to predict bacterial transfer rates to potato tissues (Garrood et al., 2004), bacterial transfer rates between lettuce and knives (Zilelidou et al., 2015), pseudomonad attachment rates to polystyrene (Fletcher, 1977) and bacterial survival after sanitization (Keeratipibul et al., 2011). However, the effect of varying cell concentration on the attachment of *Salmonella* specifically to cut PCWs has not been systematically investigated. This is particularly

important in the context of cross-contamination during washing in which a small proportion of contaminated products can cause a large batch to become contaminated (Doyle and Erickson, 2008; López-Gálvez et al., 2010a). Fresh produce processing water is an important source of cross-contamination. In order to save costs, processing wash water is often reused and this may result in the build-up of microbial loads which could lead to subsequent batches of fresh produce becoming contaminated (Gil et al., 2009).

Our attachment studies were carried out using bacterial cellulose (BC)-based PCW models which have been demonstrated in a previous study (Tan et al., 2013) to be useful for understanding the attachment of pathogens to cut surfaces of fresh produce. These PCW models were used to simulate attachment to natural cut PCWs as these easily usable PCW models have been shown to possess similar chemical composition and microstructure to natural PCWs (Chanliaud et al., 2002; Tan et al., 2013; Whitney et al., 1999).

In a previous study (Tan et al., 2013), we have investigated the effect of incubation time on *Salmonella* attachment. It is in our interest to investigate how bacterial inoculum size affect bacterial attachment to PCWs. Hence, this study aimed to provide quantitative data on the effect of inoculum size on the attachment of three *Salmonella* strains to BC-based PCW models. The data obtained were then used to develop a simple mathematical model to predict how many *Salmonella* cells will attach to the PCW models at a given inoculum concentration. Finally, the model was validated using natural PCWs from cut plant materials, different subspecies of *Salmonella* and other pathogens (*Escherichia coli* and *Listeria monocytogenes*).

2.2 Materials and methods

2.2.1 Bacterial strains

Salmonella enterica subspecies *enterica* serovar Enteritidis ATCC 13076, *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, *Listeria monocytogenes* ATCC 7644, *L. monocytogenes* ATCC 19112, *Escherichia coli* ATCC 25922, *E. coli* O157:H7 ATCC 700728 and *Gluconacetobacter xylinus* ATCC 53524 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). *Salmonella enterica* subsp. *indica* M4 was isolated from lettuce in Malaysia and used in this study as a fresh produce isolate. Two strains each from four groups of *Salmonella* subspecies, namely *S. enterica* subsp. *enterica* (I) (R11, U5), *S. enterica* subsp. *arizonae* (IIIa) (R32, U3), *S. enterica* subsp. *diarizonae* (IIIb) (U68, R1) and *S. enterica* subsp. *indica* (VI) (U56, U61) were all isolated from lizards in Malaysia (Cheng et al., 2014).

All bacteria (except *G. xylinus*) were grown aerobically at 37^{o}C on tryptic soy agar (TSA; Merck, Darmstadt, Germany) or in tryptic soy broth (TSB; Merck, Darmstadt, Germany) under shaking incubation (150 rpm) (Lab Companion SK-600 benchtop shaker, Medline, UK). *G. xylinus* ATCC 53524 was grown as described below.

2.2.2 Production of BC-based plant cell wall (PCW) models

A primary inoculum of *G. xylinus* ATCC 53524 was prepared and then used for the production of all BC composites as described (Tan et al., 2016). The primary inoculum prepared by culturing *G. xylinus* ATCC 53524 at 30^{o}C for 72 h in Hestrin and Schramm (HS) broth medium containing 2% (w/v) glucose, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.27% (w/v) Na_2HPO_4 and 0.115% (w/v) citric acid and adjusted to pH 5.0 (Hestrin and Schramm, 1954). The BC composites were produced in enclosed plastic containers (1.5 cm x 1.5 cm x 1.5

cm) incubated statically at 30ºC for 72 h depending on the HS medium composition. Harvested BC composites (1.5 cm x 1.5 cm, ~ 2 mm thickness) were purified by rinsing in 6 mM $CaCl_2$ at 100 rpm for 1 h to remove media components. The four different types of PCW models produced were:

- BC
- BC-Pectin (BCP) produced by adding 0.1% w/v apple pectin (Classic AU710) with a degree of methyl esterification of about 30% (Herbstreith & Fox, Neuenbürg, Germany) to the HS medium and 3 mM $CaCl_2$ was added to allow incorporation of pectin in the composite (R&M Chemicals, Malaysia).
- BC-Xyloglucan (BCX) produced by adding 0.1% w/v xyloglucan (Megazyme, County Wicklow, Ireland) to the HS medium.
- BC-Pectin-Xyloglucan (BCPX) produced by adding 0.1% w/v pectin, 3 mM $CaCl_2$ and different concentrations of xyloglucan (0.1%, 0.3% and 0.5% w/v) to the HS medium.

2.2.3 Effect of inoculum size on the attachment of *Salmonella* strains

Early stationary phase cultures of *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4 (18 h incubation) were centrifuged at 5500 x *g* (Hettich D-78532, Tuttlingen, Germany) for 10 min at 4ºC. Early stationary phase was selected for harvesting bacteria as bacterial numbers stay the most constant during this phase of bacterial growth. It is more difficult to gauge bacterial numbers during the log phase where exponential growth occurs and also during the death phase when bacterial population declines. Therefore, results obtained using early stationary phase cultures in the attachment studies are less likely to be affected by physiological states of the bacterial cells. Bacteria were cultured at 37ºC which resembles the temperature within the animal body where it is normally found. Centrifuging the bacterial

suspension at 4°C was done to speed up the process of pelleting bacterial cells and separating out TSB media components which may affect its growth. Different temperatures may have different effect on the bacterial surface. It was found that lower temperatures reduced bacterial attachment ability probably because the medium or bacterial surface polymer becomes more viscous at lower temperatures while higher temperatures favor physical adsorption of bacteria to surfaces (Fletcher, 1977). However, this is not a concern as all bacterial cells have been treated in the same way and this would not significantly affect our results.

The pellet was washed twice with phosphate buffered saline (PBS) (pH 7.4) (1st BASE, Singapore) and suspended in PBS to an optical density (OD) at 600nm (UV/Vis spectrophotometer, Shimadzu UV mini-1240, USA) which corresponds to 8 log CFU/mL for each isolate (Appendix I). Serial 10 fold dilutions of the bacterial suspensions were carried out to obtain the desired range of bacterial concentrations of 3, 4, 5, 6, 7 and 8 log CFU/mL with a 0.2 log CFU/mL margin of error. The range of concentration used in this study is similar to that of other studies.

Each rinsed BC composite was incubated in 10 mL of pathogenic bacterial suspension of different concentrations for 20 min with gentle shaking (100 rpm) at 25°C. Incubation time of 20 minutes was selected to allow attachment levels of these pathogens reach a stable saturation point as shown in an earlier study (Tan et al., 2013). This condition simulates the occurrence of irreversible bacterial attachment to plant surfaces in real life as plants are usually exposed to pathogens for a longer period of time. This was followed by gentle rinsing (100 rpm) in 6 mM $CaCl_2$ for 1 min to remove loosely attached cells. Each composite was then placed in a stomacher bag with 50 mL PBS and pummelled for 1 min at 8 strokes/s in a stomacher (BagMixer 400; Interscience, France). The number of *Salmonella* cells attached to the BC composite was

enumerated by serial dilution of the stomached fluid followed by the plating of appropriate dilutions on xylose lysine deoxycholate agar (XLDA; Oxoid, UK). Numbers of attached bacterial cells were expressed as CFU/cm^2 composite. The experiment was performed in triplicate (three independently grown bacterial cultures).

2.2.4 Linear model development

Data points obtained for the attachment of the three *Salmonella* strains (*S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *Salmonella enterica* M4) were plotted on the same graph to assess the overall trend of *Salmonella* attachment over a range of inoculum concentrations. Since the overall trend was linear within the data range studied, these data were modelled using a linear regression approach. The 95% confidence interval, 95% prediction interval and the equation of the best fit line were obtained. The aim of constructing this simple mathematical model was to enable prediction of the numbers of *Salmonella* cells that will attach on the PCW models if their initial inoculum concentration was within the range of 3 to 8 log CFU/mL.

2.2.5 Verification of the mathematical model

Tests across a range of parameters were carried out to verify the validity of the linear regression model in predicting the numbers of attached *Salmonella* cells to the PCW models and more importantly, to natural PCWs. For this purpose, natural plant tissues were used to assess the applicability of the model in predicting *Salmonella* attachment to different types of plants. Comparisons between different *Salmonella enterica* subspecies and two other common foodborne pathogens (*E. coli* and *L. monocytogenes*) were also made to assess whether the model can predict the attachment of these pathogens to the PCW models. Data from each parameter was

plotted on a graph overlaid with 95% confidence and prediction intervals obtained from the linear regression model.

Potato (*Solanum tuberosum*) tuber, apple (*Malus domestica*) fruit and lettuce (*Lactuca sativa*) leaves were obtained from a retail outlet in Selangor, Malaysia. These three plant tissues were used since they represent a range of different plant species that *Salmonella* have been isolated from and which have also been associated with cases of salmonellosis (Beuchat, 2002; Quiroz-Santiago et al., 2009). The outer surfaces of the potato, apple and lettuce leaves were lightly wiped down with 70% ethanol solution before cutting out approximately 1.5 cm x 1.5 cm pieces of the plant materials with a sterile scalpel. The surfaces of the lettuce leaves were lightly grazed to expose the upper and lower epidermis.

Suspensions of *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4 were prepared by spinning down the TSB broth culture after 18 h incubation and then suspended at their original concentration before carrying out serial 10 fold dilutions to prepare six different concentrations of the bacterial suspensions (about 4 to 9 log CFU/mL). Subsequently, attachment assays were carried out as described above (Section 2.2.3) but using cut plant materials instead of BC composites and using sixconcentrations of the *Salmonella* suspensions. Concentrations of the initial inoculum and also the final attached *Salmonella* cells on the BC composites were determined by spread plating on XLDA.

The attachment of four *S. enterica* subspecies [subsp. *enterica* (I), *arizonae* (IIIa), *diarizonae* (IIIb) and *indica* (VI)] to the BC composites was tested with two *Salmonella* strains for each subspecies: *S. enterica* subsp. I (R11, U5); *S. arizonae* subsp. IIIa (R32, U3); *S. diarizonae* subsp. IIIb (U68, R1) and *S. indica* subsp. VI (U56, U61). Four different

concentrations of each bacterial suspension were prepared using serial 10 fold dilutions of the bacterial suspensions as described above. Again, attachment assays were performed as outlined in Section 2.2.3 but different BC composites were haphazardly assigned to each bacterial suspension. The concentrations of the initial inoculum and also the final attached cells on the BC composites were determined by spread plating on XLDA.

Two strains each of *E. coli* (*E. coli* O157:H7 ATCC 700728, *E. coli* ATCC 25922) and *L. monocytogenes* (*L. monocytogenes* ATCC 19112, *L. monocytogenes* ATCC 7644) were used in this part of the study. Four different concentrations of each bacterial suspension were prepared and used for the attachment assays. Each suspension was incubated with haphazardly assigned BC composites. The concentrations of the initial inoculum and also the final attached cells on the BC composites were determined by spread plating on TSA.

2.2.6 Statistical analysis

Statistical analysis of results was performed using Statistical Package for the Social Sciences (SPSS) (SPSS Inc., USA). Two-way analysis of variance (ANOVA) was used to determine the significance of the effects of bacterial strains (*S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *Salmonella enterica* M4) and different types of BC composites on the final numbers of attached cells for data collected from Section 2.2.3. Using the equation obtained from the linear regression model, a table to compare the predicted and actual attachment of *Salmonella* strains from different subspecies was constructed. One-way ANOVA was performed to determine the significance of differences between strains from different *Salmonella* subspecies. In order to test the validity of the model, data obtained from various tests with different *S. enterica* subspecies, other pathogens and natural plant tissue were plotted against the

95% prediction and confidence intervals constructed from the linear regression model. Differences among the means were determined using Tukey's method at 95% confidence level.

2.3 Results and discussion

2.3.1 Effect of initial inoculum concentration on the attachment of *Salmonella* strains and linear model development

Attachment of the three *Salmonella* strains, namely *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4, to the BC-based PCW models were carried out using a series of initial inoculum concentrations. From Figure 2.1, it was apparent that higher inoculum size resulted in higher numbers of attached cells on the BC composites. None of the *Salmonella* strains used in this study reached a saturation point with respect to attachment even up to an initial inoculum concentration of 8 log CFU/mL. We hypothesized that there are an abundance of attachment sites on the BC composites which were not fully occupied even at high densities of *Salmonella* cells up to 8 log CFU/mL. Barak et al. (2002) showed that *S. enterica* serovar Newport attached to alfalfa sprouts in a linear fashion within the range of 3 to 6 log CFU/mL. In our study, the linear trend may indicate the ability of *Salmonella* cells to attach to the BC composites and also to the bacterial cells bound on the composites as suggested by Barak et al. (2002). Liao and Cooke (2001) found that higher concentrations of *Salmonella* in the suspension proportionally increased the number of bacteria attaching to green pepper discs within the range of 5 to 7 log CFU/mL. In their study, the extent of attachment, however, decreased and was near saturation at concentrations higher than 9 log CFU/mL. Iturriaga et al. (2003) also showed that the degree of attachment of *Salmonella* to tomato surfaces decreased at higher inoculum concentrations. This feature may be caused by the difficulty faced by pathogens

in attaching to less favourable sites after the preferred attachment sites were occupied. Another possible explanation for the decreased attachment numbers at high bacterial densities is that both these studies used fresh produce samples with intact surface which are less favourable for bacterial attachment as compared to cut surfaces.

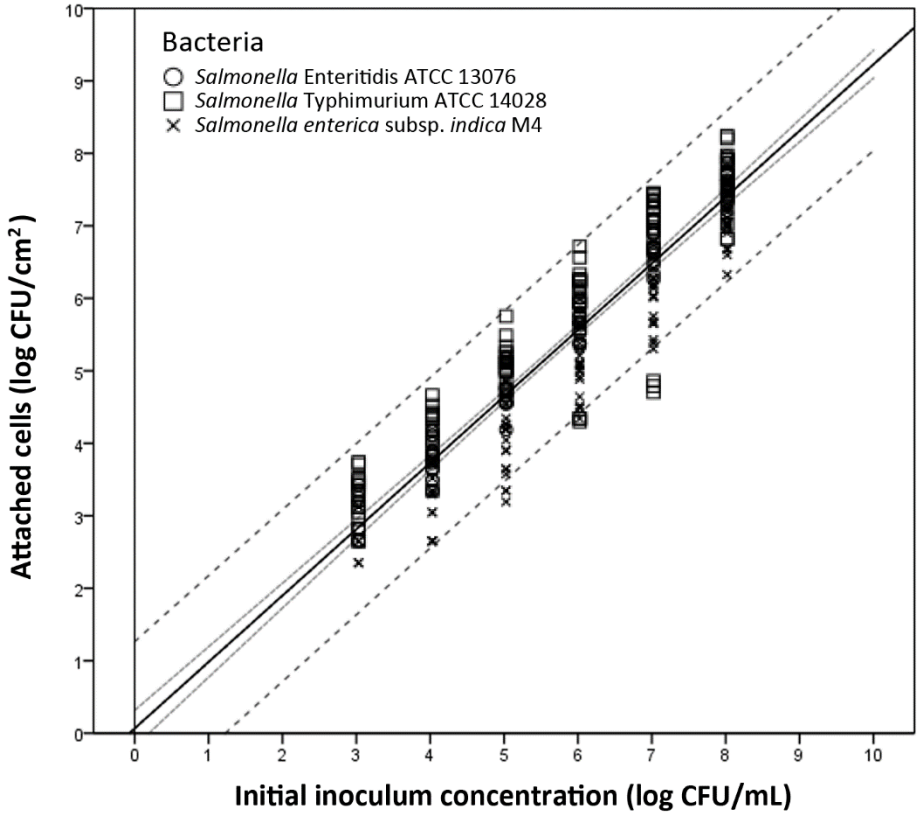


Figure 2.1 Linear regression model of the number of attached *Salmonella* cells to BC composites over a range of initial inoculum concentration (3 to 8 log CFU/mL) plotted with confidence (-----) and prediction (- - -) intervals at 95% confidence limit. The final equation for the model: y = 0.916x.

Since the attachment of these *Salmonella* strains increased linearly with increasing bacterial density, a linear regression model was fitted to the data. Initially, an intercept was included in the linear model to account for any bias in the model to fulfil the assumption that the

mean of the residuals should be 0. The initial equation obtained for the model was y = 0.916x + 0.067. Since the intercept (0.067) was found to not be statistically significant (linear regression model, p= 0.599), it was excluded from the final equation. This is because logically no bacteria are available to attach (y= 0) when the initial inoculum concentration is 0 (x=0).

The final equation for the linear model was: y = 0.916x, with x within the range of 3 to 8 log CFU/mL (R^2 = 86.4%, $F_{1, 289}$= 1837.86, p<0.001). The concentration of 3 log CFU/mL was used as it was the lowest limit of detectable range. The R^2 value of the equation is high (86.4%) and the initial inoculum concentration reliably predicts the final attached *Salmonella* cells ($F_{1, 289}$= 1837.86, p<0.001). Both bacterial strains and type of composites, however, significantly affected attachment (two-way ANOVA, p<0.05) and may account for the other 13.6% of the R^2 value which were not contributed by the predictor used (inoculum size). The prediction interval gives an estimation of where most (95%) of future observations will fall. The confidence interval, on the other hand, measures the reliability of an estimate and was also included in the graph with a confidence level of 95%. The narrow confidence interval of the model confirmed that the linear model represents the true relationship and is good for prediction within the range of 3 to 8 log CFU/mL.

2.3.2 Verification of the linear model

Attachment of *Salmonella* cells (*S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4) to different natural plant tissues fall mostly within the 95% prediction interval (Figure 2.2). This supports the validity of using the linear regression model to predict *Salmonella* attachment to cut natural PCWs. Counts of *Salmonella* attached to apple tissue fell very closely to the best-fit line of the linear model. This may be because the pectin used for the production of the BC composites was also extracted from apples. The attachment of *Salmonella*

to lettuce leaves, on the other hand, was found to be at the lower limit of the prediction interval. This could be due to the difference in the thickness of lettuce leaves (<1 mm) used for the attachment study as compared to the potato and apple tissues which were much thicker (~2 mm). The thin lettuce leaves provide less surface for bacterial attachment which could have resulted in low attachment numbers (Katsikogianni and Missirlis, 2004). Variations in surface roughness and compactness of plant cells on the surfaces of the three plant materials may also account for differences in bacterial attachment to these surfaces. Saggers et al. (2008) showed that 6.6 log CFU/g *S.* Typhimurium LT-2 attached to potato cell wall material after incubation in an inoculum with a starting concentration of 9 log CFU/mL for 10 min. In another study, the attachment of *E. coli* O157:H7 to cut edges of lettuce leaves (~6.5 log CFU/cm^2) was slightly higher than to apple skin (~6.2 log CFU/cm^2) (Hassan and Frank, 2004). However, the results of their study were not directly comparable to ours as the apple tissues we used were much thicker than the thin apple peel they used.

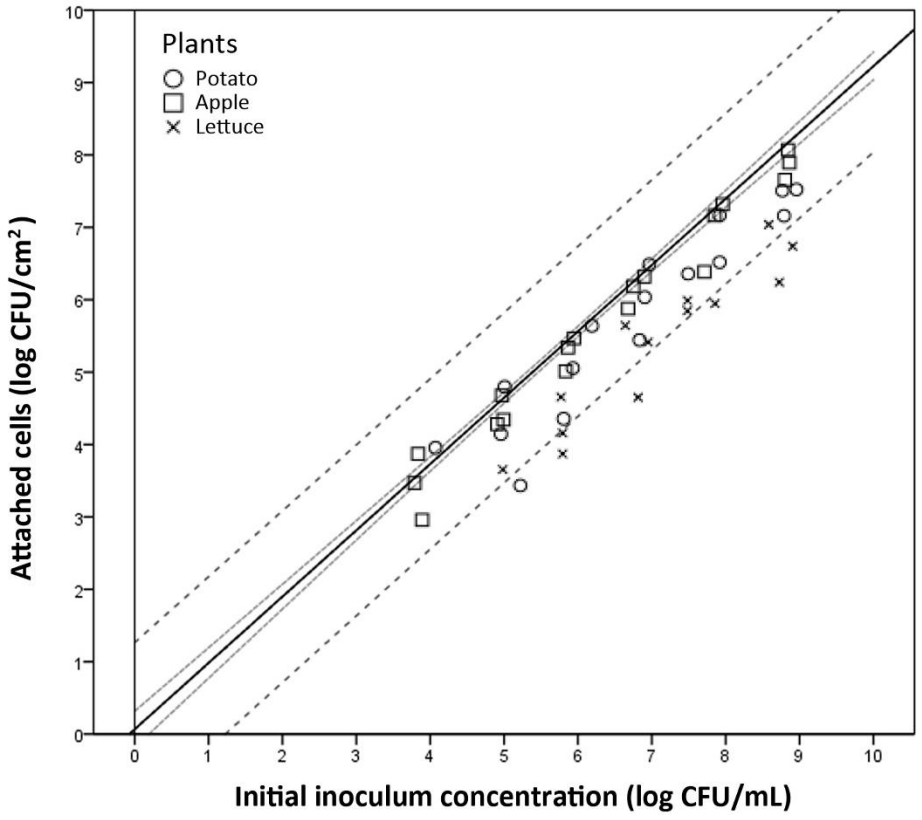


Figure 2.2 Attachment of three *Salmonella* strains (*S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* subsp. *indica* M4) to natural PCWs (potato tuber, apple fruit and lettuce leaves) plotted against confidence (----) and prediction (- - -) intervals at 95% confidence limit obtained from the linear regression model (y=0.916x).

It should be noted that both strains of *S.* Enteritidis and *S.* Typhimurium used in earlier experiments belong to *Salmonella* subsp. I. Of the six subsp., only *Salmonella* subsp. I is associated with disease in warm-blooded animals and accounts for more than 99% of salmonellosis in humans (Kingsley et al., 2002). The other *Salmonella* subsp. are commonly found in cold-blooded vertebrates and the *S. enterica* M4 strain belongs to subsp. VI (Kingsley et al., 2002). The graph of attachment associated with the different *Salmonella* subspecies (Figure 2.3) showed that all data points were found within the 95% prediction interval. This indicates that the linear regression model can be used to predict the attachment of *Salmonella*, regardless of the different subspecies (I, IIIa, IIIb, VI), within the range of 3 to 8 CFU/mL.

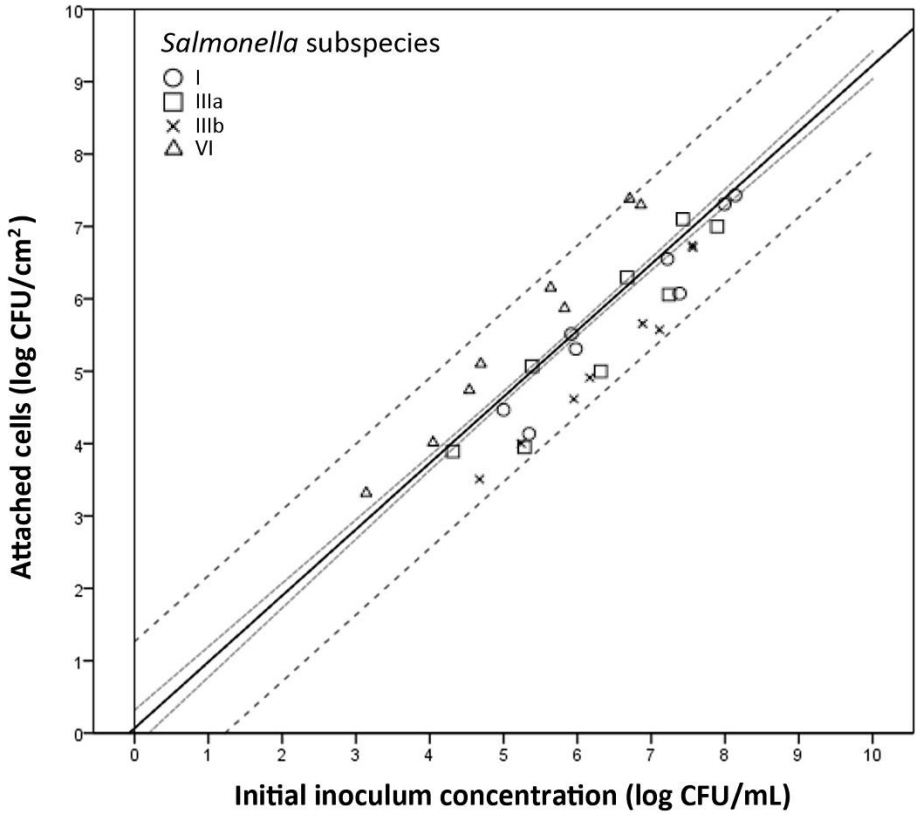


Figure 2.3 Attachment of different *Salmonella* subspecies (I, IIIa, IIIb, VI) to BC composites plotted against confidence (——) and prediction (- - -) intervals at 95% confidence limit obtained from the linear regression model (y = 0.916x).

The equation from the linear regression model y = 0.916x was also tested by calculating the expected attachment of the different *Salmonella* subspecies for a given initial inoculum concentration and then comparing it to the actual attachment numbers in Table 2.1. We found that the differences in subspecies had no significant effect on *Salmonella* attachment (one way ANOVA, $p>0.05$). It was noted that most cases of *Salmonella* subsp. VI (U56 and U61) attached at least 0.5 log higher than the expected value, whereas the model generally overestimated the attachment of *Salmonella* subsp. IIIb, although these were still within the 95% prediction interval. Both *Salmonella* subsp. I and IIIa showed attachment numbers which were close to the predicted values.

The attachment data for the *E. coli* and *L. monocytogenes* strains also fits within the 95% prediction interval (Figure 2.4). Unlike the linear attachment trend for *Salmonella,* however, the attachment trends for *E. coli* O157:H7 ATCC 700728, *E. coli* ATCC 25922 and *L. monocytogenes* ATCC 7644 appeared to plateau out at higher inoculum concentrations. Simiarly, both Takeuchi and Frank (2000) and Garrood et al. (2004) found that the numbers of an *E. coli* O157:H7 and a *L. monocytogenes* strain attached to plant material did not increase proportionally with inoculum size and were lower than expected at high inoculum concentrations.

In a study by Jablasone et al. (2005), a *L. monocytogenes* strain showed significantly higher attachment to lettuce (~6.2 log CFU/g) when compared to the two *E. coli* O157:H7 strains used (~5 log CFU/g). Of the *E. coli* and *L. monocytogenes* strains used in our study, only the attachment of *L. monocytogenes* ATCC 19112 was found at the lower border of the 95% prediction interval. This could be due to strain-specific differences in *L. monocytogenes* attachment as observed by Gorski et al. (2004). Barak et al. (2002) found that *E. coli* O157:H7 strains attached at levels of about 3 log CFU per alfalfa sprout after 4 h inoculation in the starting concentration of 3 log CFU/mL. Ells and Hansen (2006) found that *Listeria* spp. strains attached at an average of 5.7 log CFU/cm^2 to cut cabbage tissues after being inoculated using 6 log CFU/mL suspensions for 3 h.

Although the trend for the attachment of *E. coli* and *L. monocytogenes* strains appeared to be different from *S. enterica*, the linear regression model is deemed applicable to predict the attachment of these pathogens given their bacterial density is within the range of 3 to 8 log CFU/mL.

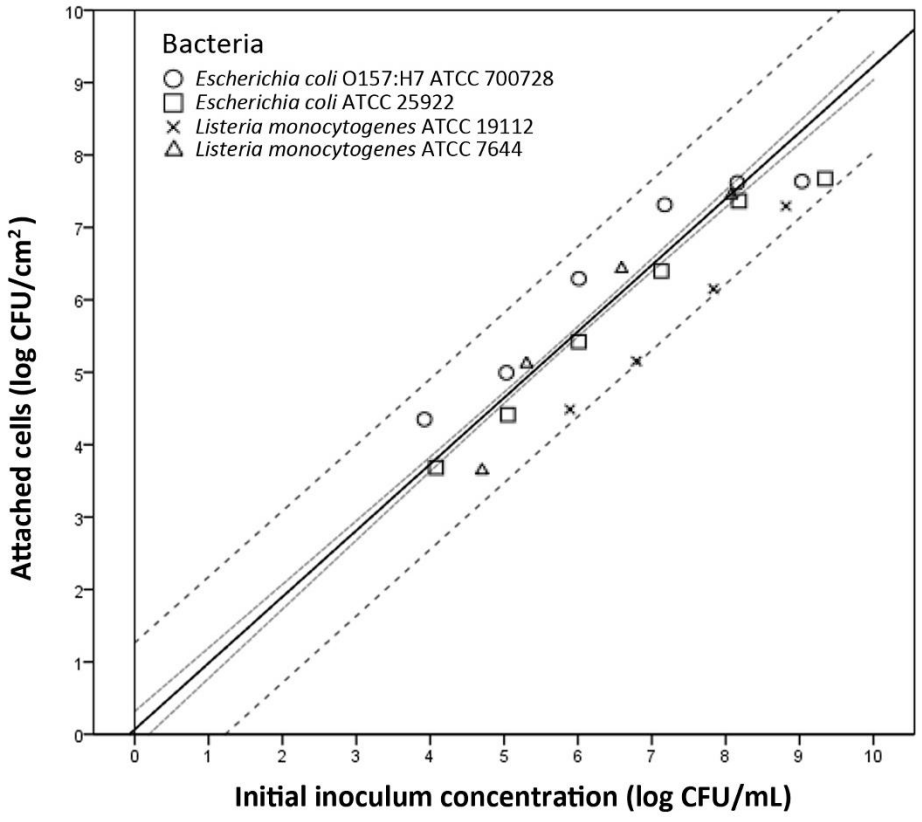


Figure 2.4 Attachment of different strains of *E. coli* and *L. monocytogenes* to BC composites plotted against confidence (----) and prediction (- - -) intervals at 95% confidence limit obtained from the linear regression model (y=0.916x).

Table 2.1 Comparison of the actual and expected (using equation from linear regression model, y=0.916x) attachment of different *Salmonella* subspecies.

Subsp.	Bacteria	Composites	Initial inoculum (log CFU/mL)	Attached cells (log CFU/cm^2)	Expected attachment (log CFU/cm^2)	95% confidence interval
I	R11	BC	8.000	7.311	7.328	6.217 - 8.574
		BC	7.230	6.551	6.623	5.512 - 7.865
		BCX	5.920	5.508	5.423	4.320 - 6.669
		BCX	5.010	4.457	4.589	3.480 - 5.830
	U5	BCP	8.150	7.433	7.465	6.351 - 8.708
		BCP	7.390	6.071	6.769	5.663 - 8.016
		BCPX(0.5%)	5.990	5.301	5.487	4.377 - 6.726
		BCPX(0.5%)	5.350	4.125	4.901	3.797 - 6.146
IIIa	R32	BCPX(0.1%)	7.900	7.000	7.236	6.129 - 8.485
		BCPX(0.1%)	7.250	6.054	6.641	5.533 - 7.885
		BCPX(0.3%)	6.320	4.990	5.789	4.686 - 7.035
		BCPX(0.3%)	5.290	3.938	4.846	3.741 - 6.090
	U3	BCPX(0.5%)	7.440	7.103	6.815	5.703 - 8.056
		BCPX(0.5%)	6.680	6.296	6.119	5.012 - 7.363
		BCX	5.390	5.063	4.937	3.831 - 6.180
		BCX	4.320	3.878	3.957	2.851 - 5.202
IIIb	U68	BC	7.570	6.709	6.934	5.824 - 8.178
		BC	7.120	5.567	6.522	5.414 - 7.766
		BCX	6.180	4.903	5.661	4.550 - 6.899
		BCX	5.250	3.990	4.809	3.700 - 6.049
	R1	BCP	7.560	6.739	6.925	5.820 - 8.174
		BCP	6.890	5.652	6.311	5.205 - 7.556
		BCPX(0.5%)	5.960	4.607	5.459	4.352 - 6.701
		BCPX(0.5%)	4.680	3.493	4.287	3.180 - 5.531
VI	U56	BCPX(0.1%)	6.720	7.384	6.156	5.044 - 7.395
		BCPX(0.1%)	5.830	5.865	5.340	4.236 - 6.585
		BCPX(0.5%)	4.700	5.095	4.305	3.196 - 5.547
		BCPX(0.5%)	4.050	4.000	3.710	2.603 - 4.957
	U61	BCPX(0.5%)	6.870	7.301	6.293	5.184 - 7.535
		BCPX(0.5%)	5.640	6.153	5.166	4.062 - 6.411
		BCX	4.540	4.729	4.159	3.054 - 5.405
		BCX	3.146	3.301	2.882	1.874 - 4.151

2.4 Conclusions

This study investigated the effect of inoculum size on the attachment of *Salmonella* cells. It provided quantitative data regarding the attachment of *Salmonella* cells to the BC-based PCW models with an increase in inoculum concentration. From this, a linear regression model was constructed to predict the attachment of *Salmonella* cells to natural PCWs within the range of 3 to 8 log CFU/mL and the applicability of the model was validated with different tests. The model satisfactorily predicted *Salmonella* attachment to different natural plant tissues and was able to predict the attachment of different subspecies of *S. enterica* strains. The linear model also appeared to be applicable to other common pathogens, *E. coli* and *L. monocytogenes*. Therefore, the model has potential to contribute to assessing the risks associated with attachment of pathogens in liquid to the cut surfaces of fresh produce.

Chapter 3

Effect of Plant Cell Wall Components on the Attachment of *Salmonella enterica* to Plant Cell Wall Models

The work presented in this chapter represents the following peer reviewed publication:

Tan, M.S.F., Rahman, S. and Dykes, G.A. (2016). Pectin and xyloglucan influence the attachment of *Salmonella enterica* and *Listeria monocytogenes* to bacterial cellulose derived plant cell wall models. Appl. Environ. Microbiol. 82, 680–688.

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of chapter 3, the nature and extent of my contributions to the work as following: **Nature of contribution**	**Extent of contribution (%)**
I was responsible for 100% of data analysis and 90% of drafting and writing	90%

The following co-authors contributed to the work and the extent of their contribution in percentage terms:

Name	**Nature of contribution**	**Extent of contribution (%)**
Sadequr Rahman	SR was responsible for 2% of writing and review of the manuscript.	2%
Gary Dykes	GD was responsible for 8% of writing and review of the manuscript.	8%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author(s) to agree on the respective contributions of the authors.

3 Effect of Plant Cell Wall Components on the Attachment of *Salmonella enterica* to Plant Cell Wall Models

3.1 Introduction

Minimally processed fresh produce which are usually consumed raw have been identified as a major cause of microbial foodborne outbreaks worldwide (Brandl, 2006). Between 1992 and 2006, a study of general outbreaks of infectious intestinal diseases in England and Wales recorded that 23% of the outbreaks were foodborne outbreaks and 4% of the foodborne outbreaks were traced to prepared salad (Little and Gillespie, 2008).

Cut surfaces of minimally processed fresh produce are especially vulnerable to the attachment of human foodborne pathogens, especially *Salmonella enterica* (Klerks et al., 2007; Kroupitski et al., 2009a). A study by Saggers et al. (2008) suggested that structural components, and particularly pectin, in plant cell walls (PCWs) exposed on broken or cut surfaces may provide receptors for bacterial attachment. To the best of our knowledge, there were no other in-depth studies conducted to investigate the role of PCW components on *Salmonella* attachment to fresh produce.

In addition to the structural components of the PCW, the physicochemical properties (such as hydrophobicity and charge) of both the attachment surface and the attaching bacteria also influence bacterial adhesion. For example, it has been suggested that more hydrophobic bacteria attach preferably to hydrophobic surfaces whereas more hydrophilic ones prefer hydrophilic surfaces (An and Friedman, 1998). Other studies have suggested that higher bacterial hydrophobicity favours bacterial adhesion to most surfaces (Dykes et al., 2003; Voravuthikunchai and Suwalak, 2009). Increased hydrophobicity also favours cell-to-cell

adhesion which in turn leads to greater auto-aggregation (Collado et al., 2007; Liu et al., 2004b). Similarly, co-aggregation has been shown to be dependent on auto-aggregation (Collado et al., 2007). Bujnakova and Kmet (2002), for example, showed that only auto-aggregating strains co-aggregate with other strains.

In this study, we investigated the role of the major structural components of the PCW (cellulose, pectin and xyloglucan) and the physicochemical properties (hydrophobicity and aggregation abilities) of surfaces on the attachment of *S. enterica* strains. A Gram-positive *Listeria monocytogenes* strain used in a previous study (Tan et al., 2013) was also included in this study for comparison as it showed different attachment ability compared to the Gram-negative *Salmonella* strains.

3.2 Materials and methods

3.2.1 Bacterial strains

S. Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028, *S. enterica* M4, *L. monocytogenes* ATCC 7644 and *G. xylinus* ATCC 53524 were obtained from the same sources and grown in the same way as mentioned earlier in Sections 2.2.1 and 2.2.2.

3.2.2 Production of bacterial cellulose (BC)-based PCW models

The method for preparation of the BC-based PCW models were as described earlier in Section 2.2.2, however in this study a wider range of the PCW models were produced using varying concentrations of pectin and/or xyloglucan:

- BC

- BC-Pectin (BCP) composite produced by adding 0.1%, 0.3% and 0.5% w/v apple pectin with a degree of methyl esterification of about 30% (Herbstreith & Fox, Neuenbürg, Germany) to the HS medium and calcium chloride (R&M Chemicals, Malaysia) (3 mM $CaCl_2$ for 0.1% w/v pectin, 6 mM $CaCl_2$ for 0.3% w/v pectin and 12.5 mM $CaCl_2$ for 0.5% w/v pectin).
- BC-Xyloglucan (BCX) composites produced by adding 0.1%, 0.3% and 0.5% w/v xyloglucan (Megazyme, County Wicklow, Ireland) to the HS medium.
- BC-Pectin-Xyloglucan (BCPX) composites produced by adding different combinations of pectin and xyloglucan (0.1%, 0.3% and 0.5% w/v) and 3 mM, 6 mM and 12.5 mM $CaCl_2$ corresponding to the amount of pectin added respectively.

3.2.3 Chemical composition analysis

Chemical composition of the BC composites were analysed as described by Mikkelsen et al. (2011). BC composites were first cryo-grounded using a 6850 SPEX freezer/mill (SPEX, Metuchen, USA). Saeman hydrolysis was then carried out to hydrolyze the cryo-ground composites into monosaccharides (Saeman, 1945). Derived monosaccharides were reduced to their corresponding alditol acetates before determination by gas chromatography (GC; Shimadzu GC-17A Instrument, Kyoto, Japan). This allows the determination of the percentage of each PCW component incorporated into the composites.

3.2.4 Attachment assays for individual strains of bacteria

Early stationary phase cultures *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028, *S. enterica* M4 and *L. monocytogenes* ATCC 7644 (18 h for the *Salmonella* strains and 42 h for the *L. monocytogenes* strain) were prepared as described in Section 2.2.3 to a final concentration which corresponds to 8 log CFU/mL for each isolate. Attachment studies with the BC composites were also as aforementioned, with an additional step of enumerating attached *L. monocytogenes* cells by spread plating appropriate dilutions of stomached liquid on *Listeria* selective agar (LSA; Oxoid, UK) added with supplement SR0140.

3.2.5 Attachment assays for pairwise combination of two bacteria

In order to determine whether the bacterial attachment to BC composites were stochastic or based on bacterial characteristics, attachment assays were carried out using pairwise combination of two bacteria with different attachment characteristics as described by Chia et al. (2011). Our previous study (Tan et al., 2013) showed that *S.* Typhimurium ATCC 14028 (~7.3 log CFU/cm^2) and *L. monocytogenes* ATCC 7644 (~5.7 log CFU/cm^2) attached in significantly different numbers from each other. Three different pairwise ratios (0.428 [30:70], 1 [50:50], 2.333 [70:30]) of *S.* Typhimurium ATCC 14028 with *L. monocytogenes* ATCC 7644 (ST:LM) were prepared to a total density of 8 log CFU/mL for the attachment assay. Attachment study for different combinations of the ST:LM bacterial pair to different BC composites [BC, BCP, BCX, BCPX(0.1%), BCPX(0.3%), BCPX(0.5%)] were carried out as described above (Section 2.2.3) in the attachment studies for individual strains of bacteria. BC composites were incubated in a total of 10mL mixed ST:LM pathogenic bacterial suspension prepared at a given pairwise ratio (8 log CFU/mL) for 20 min with gentle shaking at 100 rpm and 25^{o}C. This was followed by gentle rinsing in $CaCl_2$ solution for 1 min before the composite was pummelled in 50 mL

phosphate buffered saline (PBS) solution. Enumeration of attached bacterial cells (CFU/cm^2 composite) were carried out by spread plating appropriate dilutions of the stomached liquid on xylose lysine deoxycholate agar (XLDA; Oxoid, UK) and LSA.

Similarly, attachment of the three different pairwise ratios of ST:LM to abiotic surfaces of different hydrophobicity, namely glass slides (Premier slides, Azer Scientific, Morgantown, USA) stainless steel coupons (SSC; type 302, no. 4 finishing, 1 mm thickness) and Teflon coupons (Tekdon, Florida, USA), was also carried out. Each slide (75 mm x 25 mm) was sterilized before incubation for 20 min in 20 mL of the mixed ST:LM bacterial suspension (8 log CFU/mL) in ESCO Airstream Horizontal Laminar Flow Clean Bench (ONBoard Solutions, Australia) and then rinsed in $CaCl_2$ before swabbing the bottom part of the slide. The cotton swab was then placed in 50 mL PBS and pummeled for 1 min in the stomacher before spread plating on XLDA and LSA. Attachment ratios of CFU numbers of the ST:LM pair was calculated by dividing the number of attached *S.* Typhimurium cells (log CFU/cm^2) by the number of attached *L. monocytogenes* cells (log CFU/cm^2).

3.2.6 Bacterial surface hydrophobicity

Bacterial surface hydrophobicity was determined by the bacterial adhesion to hydrocarbon (BATH) assay as described by Rosenberg et al. (1980) with modifications. Briefly, bacteria were pelleted and washed twice with PBS and then suspended in PBS to achieve optical density of 1 ± 0.1 at 550 nm. One mL of xylene (Fischer, Leicestershire, UK) was added to the 3 mL cell suspension, the mixture was vortexed (VTX-3000L, LMS, Japan) for 2 min and the phases were allowed to separate for 1 h. The absorbance of the aqueous phase was measured at 550 nm before (A_0) and after (A_1) addition of xylene. The hydrophobicity index was expressed as: % attachment to xylene = $(1 – A_1/A_0) \times 100\%$. As described by Ahumada et al. (2001),

bacterial hydrophobicity can be classified into three groups depending on their attachment to xylene: strongly hydrophobic (71-100%), moderately hydrophobic (36-70%) and weakly hydrophobic (0-35%).

3.2.7 Aggregation assays

The auto-aggregation assay was performed as described by Collado et al. (2007) with slight modifications. Briefly, bacterium were pelleted, washed and resuspended in PBS to an optical density of 0.25 ± 0.05 at 600 nm. An aliquot of 1.5 mL bacterial suspension was then incubated at 25^{o}C and absorbance of the suspended cells was measured after 6 h. The co-aggregation assay was performed as described by Grześkowiak et al. (2012) with slight modifications. Three different pairwise ratios (0.428 [30:70], 1 [50:50], 2.333 [70:30]) of *S.* Typhimurium ATCC 14028: *L. monocytogenes* ATCC 7644 were prepared at a total density of 8 log CFU/mL each. Suspensions of the three pairwise ratios were diluted with PBS to achieve an optical density of 0.25 ± 0.05 at 600 nm (A_i) and absorbance was measured after 6 h (A_f). Auto-aggregation and co-aggregation were expressed as: % Aggregation = 100 - $[(A_f/A_i) \times 100]$. As described by Binetti et al. (2013), strains were classified into three groups based on their auto-aggregation and co-aggregation abilities: high (>60%), moderate (30-60%) and low (<30%).

3.2.8 Data analysis

All experiments were performed in triplicate (three independently grown bacterial cultures). Statistical analysis of results was performed using Statistical Package for the Social Sciences (SPSS) (PASW Statistics 18, SPSS Inc., USA). Attachment of bacterial cells to the BC composites were expressed as CFU/cm^2 and the data obtained were parametric. The significant differences between the overall attachments of all four strains to the BC composites were determined using one-way analysis of variance (ANOVA). A one-way ANOVA was also used to

establish the significant differences between the types of BC composites, as well as the different levels of PCW structural components used within each type of composite, on the attachment of each bacterial strain. A paired t-test was used to determine the significance of differences between the initial inoculum ratio and the final attached ratio for all surfaces. A two-way ANOVA was used to establish the significance of differences in the attachment between *S.* Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 7644 when attached individually and at different initial inoculum ratios on the BC composites and abiotic surfaces. Pearson's correlation was used to determine the correlation between bacterial physicochemical properties and their attachment. Differences between the means were determined using Tukey's method at 95% confidence level.

3.3 Results and discussion

3.3.1 Chemical composition analysis

We examined the effect of varying the concentration of PCW components added into the growth media on their incorporation within the PCW models. The chemical composition analysis (Table 3.1) showed that the incorporation of pectin and xyloglucan increased when higher concentrations of these components were added into the HS medium. Pectin was found to be incorporated at a relatively higher percentage in the composites as compared to xyloglucan.

According to Zykwinska et al. (2008), the amount of pectin absorbed onto cellulose decreases as the level of xyloglucan increases, with no pectin bound to cellulose at xyloglucan concentrations above 500 µg/mL; however, we observed that the incorporation of both pectin and xyloglucan increased when more of the PCW components were added into the media, even

up to a concentration of 5000 µg/mL. Zykwinska et al. (2008) measured the binding of pectin and xyloglucan to cellulose by mixing the polysaccharide solutions with cellulose, however, our study uses a constructive approach that resembles the formation of native PCWs which may be more representative of the occurrence in nature.

Our BC composites were shown to have similar compositions to other BC-based PCW models reported in the literature (Mikkelsen et al., 2009; Cybulska et al., 2010). Our BCPX composites also showed similar chemical composition to the average native PCW (approximately 35% cellulose, 40% pectin and 15% xyloglucan) (Cybulska et al., 2010). This is important because our BC composites are designed to be used as models to understand the interactions of pathogenic bacteria with native PCWs.

Table 3.1 Chemical composition analysis of BC composites.

Composites	Components added (% w/v)			Percentage incorporation (%)	
	Pectin (P)	Xyloglucan (X)	Bacterial cellulose (BC)	Pectin (P)	Xyloglucan (X)
BCP	0.1	0	44.7	55.3	0
	0.3	0	43.9	56.1	0
	0.5	0	42.8	57.2	0
BCX	0	0.1	76.5	0	23.5
	0	0.3	72.3	0	27.7
	0	0.5	67.7	0	32.3
BCPX with 0.1% X	0.1	0.1	34.0	41.5	24.5
	0.3	0.1	33.9	44.7	21.4
	0.5	0.1	32.5	48.5	18.3
BCPX with 0.3% X	0.1	0.3	33.9	40.7	25.4
	0.3	0.3	33.2	43.1	23.7
	0.5	0.3	32.9	47.8	19.3
BCPX with 0.5% X	0.1	0.5	33.5	39.4	26.7
	0.3	0.5	33.1	42.6	24.3
	0.5	0.5	33.2	47.4	20.1

3.3.2 Effects of pectin and xyloglucan on the attachment of bacteria to PCW models

An overall comparison of the attachment of *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028, *S. enterica* M4 and *L. monocytogenes* ATCC 7644 showed that the bacterial strains attached in significantly different numbers from each other to the BC composites ($p<0.05$). Specifically, *L. monocytogenes* attached in the lowest numbers (mean of attachment to all composites, ~5.7 log CFU/cm^2 composite), followed by *S. enterica* M4 (~ 6.5 log CFU /cm^2 composite) and lastly *S.* Enteritidis (~7.0 log CFU /cm^2 composite) and *S.* Typhimurium (~7.3

log CFU /cm^2 composite) which both attached in highest numbers and which were not statistically different from each other ($p>0.05$).

We found that all *Salmonella* strains attached to BC composites in significantly greater numbers than did *L. monocytogenes* ATCC 7644 ($p<0.05$), which is in agreement with our previous findings (Tan et al., 2013). Jablasone et al. (2005) showed that *S.* Typhimurium was internalized into plant tissues while *L. monocytogenes* attached only to the plant surface and was not internalized in seedlings. One possible reason that may account for the greater attachment of *Salmonella* strains is the availability of a larger number of adhesive appendages, such as flagella, fimbriae, thin aggregative fimbriae (tafi), lipopolysaccharide and outer membrane proteins produced by *Salmonella* for attachment (Barak et al., 2007, 2005; Wagner and Hensel, 2011). In contrast, *Listeria* attachment generally relies only on flagella (Dussurget, 2008). It should be noted, however, that Takeuchi et al. (2000) and Jablasone et al. (2005) observed greater *L. monocytogenes* attachment than *Salmonella* species attachment on cut lettuce leaves and on various vegetable seedlings.

Generally, the presence of pectin and xyloglucan in the BCP and BCX composites affected the attachment of *L. monocytogenes* differently from the *Salmonella* strains (Figure 3.1). The presence of pectin in the BCP was associated with an increase in the attachment of the *Salmonella* strains whereas *L. monocytogenes* attachment was reduced. Higher levels of pectin in the BCP composite increased the attachment of all *Salmonella* strains in our study. This result is consistent with the findings of Saggers et al. (2008), who observed that another strain of *S.* Typhimurium attached preferentially to the pectin layer at the potato cell wall junction, while less attachment was observed where less pectin was present. Xyloglucan in the BCX composites significantly lowered the attachment of *L. monocytogenes* ($p<0.05$) but had no significant effect

on the attachment of the *Salmonella* strains ($p>0.05$). Attachment of *L. monocytogenes* ATCC 7644 to the BCP and BCX composites was significantly lower than that to the BC, but we have yet to determine a reason for this occurrence.

Higher levels of pectin increased the attachment of only the *Salmonella* strains and not the *Listeria* strain, which suggests specific interactions between *Salmonella* cells and the pectin molecules. Most of the receptor-ligand interactions required for bacterial adhesion are mediated by carbohydrates and bacterial surface adhesins (Odenbreit, 2005; Schmidt et al., 2003). Extensive research has been undertaken regarding the role of sugar residues that serve as receptors for the binding of animal pathogens to animal cells (Brandley and Schnaar, 1986; McHan et al., 1989). However, only a few studies have focused on the role of sugar residues in the attachment of human pathogens to plants. Klerks et al. (2007) found that root exudates that contain many monosaccharides, such as fructose and glucose, cause chemotaxis of *Salmonella* strains, which use these signals to move toward the plants. Root exudates also conditioned *S. enterica* cells for attachment and colonization of the plant roots. Another study proposed that exudates of germinating seeds and developing roots trigger *Escherichia coli* O157 to colonize seedlings (Quilliam et al., 2012).

There was no significant interaction between pectin and xyloglucan in the BCPX on the attachment of all four bacterial strains ($p>0.05$). This indicates that within the BCPX composite, the effect of the level of pectin on bacterial attachment is independent of the level of xyloglucan present and vice versa. When comparing the effects of pectin and xyloglucan levels within the BCPX composites on bacterial attachment, xyloglucan had a greater influence on bacterial attachment as compared to pectin. Xyloglucan significantly affected the attachment of all strains ($p<0.05$) whereas pectin only significantly affected the attachment of the *S.* Typhimurium strain

($p<0.05$). Interestingly, all *Salmonella* strains showed the highest attachment to the BCPX (0.3%) composites which has 0.3% w/v xyloglucan added into the media, regardless of the amount of pectin present in the growth media.

Based on these data, we hypothesize that pectin and xyloglucan could have interacted physically and given the BCPX composites surface structures that are distinct from those of the BCP and BCX composites. This may also explain why all *Salmonella* strains showed the highest levels of attachment to the BCPX composite with 0.3% (w/v) xyloglucan, regardless of the amount of pectin added to the growth media. The BCPX composite with 0.3% (w/v) xyloglucan may have distinct structural features (such as porosity and surface roughness) that are ideal for the attachment of *Salmonella* cells. The effects of various concentrations of pectin and xyloglucan on the structural properties of BC composites have not been determined, hence mechanical studies and surface profiling of the BC composites to study this were performed and discussed in the following chapter (Chapter 4).

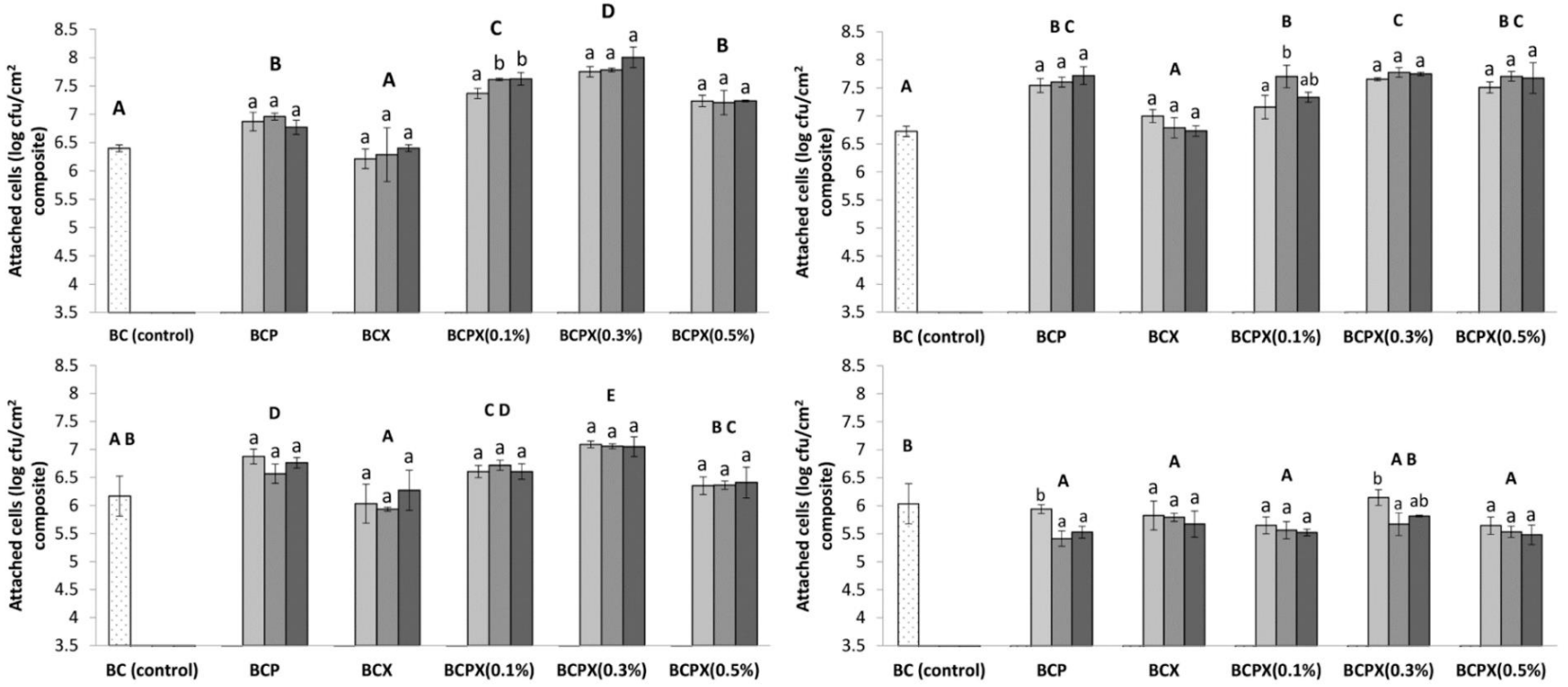


Figure 3.1 Attachment of (a) *S.* Enteritidis ATCC 13076, (b) *S.* Typhimurium ATCC 14028, (c) *S. enterica* M4 isolated from lettuce, and (d) *L. monocytogenes* ATCC 7644 to different BC composites. Different uppercase letters indicate significant differences between types of composites, whereas different lowercase letters indicate significant differences within each type of composite (one-way ANOVA and Tukey's pairwise comparison, $p<0.05$). ☐, 0% PCW components (for BC); ☐, 0.1% PCW components; ■, 0.3% PCW components; ■, 0.5% PCW components added into the growth medium.

3.3.3 Attachment of pairwise combinations of *Salmonella* Typhimurium and *Listeria monocytogenes* to BC composites and abiotic surfaces

In the second part of this study, a protocol developed by Chia et al. (2011) was used to determine the stochasticity (or randomness) of the attachment process. Two bacteria with different attachment characteristics (*S.* Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 7644) (ST:LM) were allowed to attach to the BC composites and abiotic surfaces in different pairwise ratios (0.428, 1, 2.333). If the attachment process is stochastic, the numbers of bacteria attached to the substratum is dependent on the concentration of the initial bacterial suspension. Attachment is therefore considered stochastic when the initial ratio of the bacterial pair in a bacterial suspension does not differ significantly from the final ratio of the bacterial pair attached on the attachment surfaces ($p>0.05$). Similarly, attachment is deemed non-stochastic when the process is influenced by other factors which cause both initial and final ratios of the bacteria pair before and after attachment to be significantly different from each other ($p<0.05$).

Several studies have suggested that bacterial attachment can be influenced by the physicochemical properties of the attachment surface (Lima et al., 2013; São José et al., 2014). To investigate this, assays of the attachment of the ST:LM bacterial pair to both BC composites and abiotic surfaces with different hydrophobicities [glass slides, Teflon coupons and stainless steel coupons (SSC)] were carried out. Our findings showed that bacterial attachment to all BC composites and abiotic surfaces was non-stochastic (Tables 3.2 and 3.3), as the numbers of the attached *S.* Typhimurium and *L. monocytogenes* strains did not depend on the levels of bacterial strains present in the initial inoculum (Figure 3.2). An increased concentration of *S.* Typhimurium ATCC 14028 in the bacterial suspension (higher initial inoculum ratio of ST:LM) led to significant increases in the final ST:LM attached ratio in most cases. Differences between the attachment of *S.* Typhimurium and *L. monocytogenes*

strains were generally greater at higher initial inoculum ratio of ST:LM. Hence, the attachment of the bacteria pair to the BC composites was shown to be strongly influenced by individual bacterial attachment characteristics, especially for *S.* Typhimurium ATCC 14028. In addition, the individual attachment of both *S.* Typhimurium and *L. monocytogenes* strains were not significantly different from the attachment of the ST:LM bacteria pair at an initial inoculum ratio of 1 (50% ST, 50% LM) ($p>0.05$). The finding that the two strains did not interact with each another and attached similarly when they were present together or separately supported the importance of the role of individual bacterial attachment characteristics in adhesion.

It was also shown that attachment surface hydrophobicity did not play an important role in bacterial attachment to the different surfaces ($p>0.05$) as attachment of the bacterial pair to glass, SSC and Teflon which have different hydrophobicities were not significantly different from each other ($p>0.05$). However, the attachment surface had a significant effect on the attached cell ratio ($p<0.05$)**.** For most BC composites, a higher initial inoculum ratio of ST:LM resulted in a significantly higher ($p<0.05$) attached ST:LM ratio whereas for abiotic surfaces the trend was less distinct (Tables 3.2 and 3.3).

The differences in the numbers of both *S.* Typhimurium and *L. monocytogenes* attached to the various surfaces were also examined. As compared to their attachment to the BC composites, *S.* Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 7644 generally showed significantly lower differences in numbers attached to abiotic surfaces ($p<0.05$) with the exception of BCPX(0.5%) (Figure 3.2). This was because *S.* Typhimurium ATCC 14028 attached at about 2 log CFU/cm^2 less to abiotic surfaces as compared to the BC composites. *L. monocytogenes* ATCC 7644, on the other hand, attached similarly to both BC composites

and abiotic surfaces. We also observed that the pairwise attachment of ST:LM was not significantly different on the different abiotic surfaces ($p>0.05$).

Another alternative to test stochasticity is to attach a small number of bacteria to two types of spherical beads. as described by Behkam and Sitti (2008). "Patterned" beads have half of their surface treated with air plasma, whereby bacterial attachment characteristics have been modified and only attachment to the treated hemisphere is favoured. "Unpatterned" beads have unmodified bacterial cells attached over their entire surface, the difference in attachment ability between "patterned" and "unpatterned" beads will then be evaluated to determine stochasticity.

Table 3.2 Attachment ratios of CFU numbers for pairwise combinations of *S.* Typhimurium ATCC 14028: *L. monocytogenes* ATCC 7644 (ST:LM) at different initial inoculum ratios (0.428, 1, 2.333) to BC composites. Different lowercase letters indicate significant differences between final attached ratios for pairwise combinations of ST:LM within the same BC composite (One-way ANOVA, Tukey's pairwise comparison at $p<0.05$).

Composites	**Components added (% w/v)**		**Final attached ratio of ST:LM**		
	Pectin (P)	**Xyloglucan (X)**	**0.428**	**1**	**2.333**
BC	-	-	1.230 ± 0.013 [a]	1.225 ± 0.002 [a]	1.342 ± 0.021 [b]
BCP	0.1	-	1.308 ± 0.008 [a]	1.379 ± 0.014 [b]	1.475 ± 0.033 [c]
	0.3	-	1.516 ± 0.041 [a]	1.512 ± 0.057 [a]	1.560 ± 0.040 [a]
	0.5	-	1.516 ± 0.019 [a]	1.504 ± 0.045 [a]	1.524 ± 0.039 [a]
BCX	-	0.1	1.321 ± 0.015 [a]	1.284 ± 0.027 [a]	1.384 ± 0.009 [b]
	-	0.3	1.323 ± 0.045 [a]	1.372 ± 0.020 [a]	1.360 ± 0.025 [a]

	-	0.5	1.216 ± 0.068 [a]	1.397 ± 0.050 [b]	1.389 ± 0.014 [b]
	0.1	0.1	1.194 ± 0.100 [a]	1.188 ± 0.031 [a]	1.378 ± 0.055 [b]
BCPX with 0.1% X	0.3	0.1	1.173 ± 0.026 [a]	1.229 ± 0.045 [a]	1.334 ± 0.017 [b]
	0.5	0.1	1.244 ± 0.032 [a]	1.225 ± 0.015 [a]	1.310 ± 0.029 [b]
	0.1	0.3	1.180 ± 0.016 [a]	1.230 ± 0.041 [a]	1.337 ± 0.011 [b]
BCPX with 0.3% X	0.3	0.3	1.296 ± 0.052 [a]	1.279 ± 0.016 [a]	1.364 ± 0.066 [a]
	0.5	0.3	1.304 ± 0.030 [b]	1.230 ± 0.025 [a]	1.373 ± 0.023 [c]
	0.1	0.5	1.128 ± 0.026 [a]	1.191 ± 0.028 [ab]	1.227 ± 0.032 [c]
BCPX with 0.5% X	0.3	0.5	1.116 ± 0.048 [a]	1.231 ± 0.026 [b]	1.286 ± 0.020 [b]
	0.5	0.5	1.109 ± 0.023 [a]	1.218 ± 0.009 [b]	1.306 ± 0.019 [c]

Table 3.3 Attachment ratios of CFU numbers for pairwise combinations of *S.* Typhimurium ATCC 14028: *L. monocytogenes* ATCC 7644 (ST:LM) at different initial inoculum ratios (0.428, 1, 2.333) to abiotic surfaces of different hydrophobicities. Different lowercase letters indicate significant differences between final attached ratio of ST:LM on different abiotic surfaces for the same initial inoculum ratio of ST:LM used (One-way ANOVA & Tukey's pairwise comparison at $p<0.05$).

Initial inoculum ratio for ST:LM	**Final attached ratio of ST:LM**		
	Glass	**SSC**	**Teflon**
0.428	1.073 ± 0.082^{a}	1.247 ± 0.113^{a}	1.371 ± 0.082^{a}
1	1.140 ± 0.085^{a}	1.211 ± 0.134^{a}	1.547 ± 0.070^{b}
2.333	1.145 ± 0.029^{a}	1.172 ± 0.036^{ab}	1.293 ± 0.091^{b}

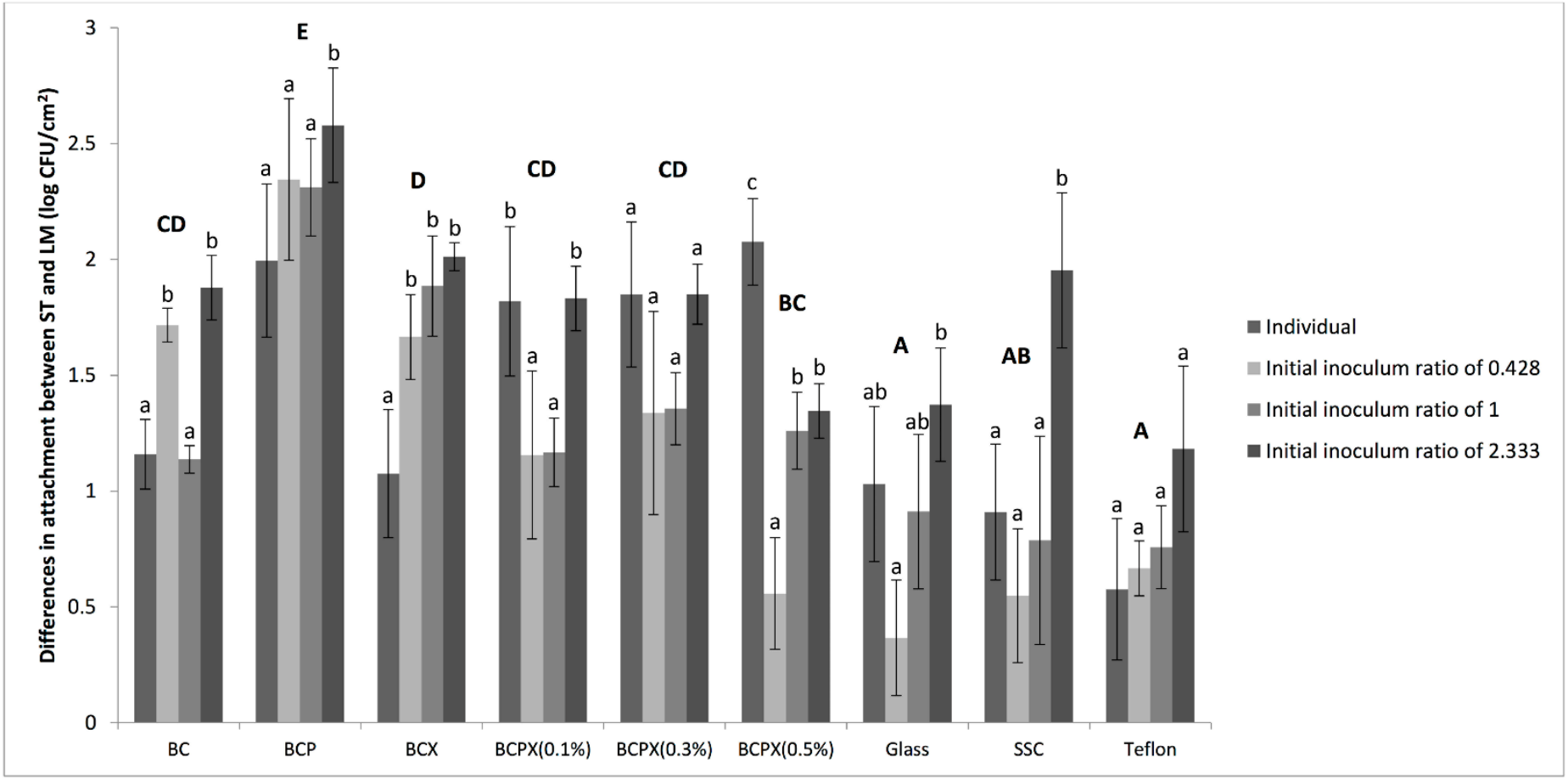


Figure 3.2 Differences in attachment numbers between *S.* Typhimurium ATCC 14028 (ST) and *L. monocytogenes* ATCC 7644 (LM) when present individually and at different initial ST:LM inoculum ratios (0.428, 1, or 2.333) on BC composites and abiotic surfaces. SSC, stainless steel coupons. Different uppercase letters indicate significant differences between attachment surfaces, whereas different lowercase letters indicate significant differences between individual bacterial attachment and bacterial attachment at different initial inoculum ratios for the same attachment surface (two-way ANOVA and Tukey's pairwise comparison, $p<0.05$).

3.3.4 Role of bacterial physicochemical properties in the attachment of four pathogens to BC composites

In addition to the physicochemical properties of the attachment, there have been some findings (Liu et al., 2004; Collado et al., 2007) which also suggest that the physicochemical properties of the bacteria influence bacterial attachment to various surfaces. As mentioned earlier, there is strong evidence that auto-aggregation is correlated with the ability to attach to surfaces (Collado et al., 2007; Del Re et al., 2000; Kos et al., 2003). However, *S.* Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 7644 demonstrated hydrophobicities and auto-aggregation levels that were not significantly different from each other, although their abilities to attach to surfaces were significantly different.

The hydrophobicity of all bacterial strains used in this study was relatively low and most strains were classified as weakly hydrophobic (*S.* Enteritidis ATCC 13076, 34.70%; *S.* Typhimurium ATCC 14028, 15.00%; *L. monocytogenes* ATCC 7644, 9.13%). *S. enterica* M4 (42.53%) was considered moderately hydrophobic. No significant correlation between hydrophobicity and bacterial attachment was found (Pearson's correlation, $p>0.05$).

Most strains used in our study also showed low auto-aggregation (*S.* Enteritidis ATCC 13076, 25.60%; *S. enterica* M4, 27.60%; *L. monocytogenes* ATCC 7644, 28.40%), with only *S.* Typhimurium ATCC 14028 (34.80%) displaying moderate auto-aggregation ability. We found a very weak positive correlation between hydrophobicity and auto-aggregation (Pearson's correlation: $r = 0.27$, $R^2 = 7.29\%$, $p>0.05$). Overall, there was no significant correlation between hydrophobicity, auto-aggregation and adhesion ability (Pearson's correlation, $p>0.05$).

The co-aggregation assays showed that the pairwise combinations of the ST:LM had intermediate co-aggregation abilities which were not significantly different at all initial

inoculum ratios ($p>0.05$) (ST:LM at ratio of 0.428, 39.87%; ratio of 1, 39.47%, ratio of 36.40%). The resulting co-aggregation values for the two strains were very similar to the auto-aggregation values obtained for *S.* Typhimurium (~35%) but not to those for *L. monocytogenes* (~28%). The ability to co-aggregate did not significantly affect the final ST:LM attachment ratios at all initial inoculum ratios ($p>0.05$). This again emphasizes that some specific characteristics of the *S.* Typhimurium strain, including its auto-aggregation and attachment ability, strongly influence the attachment of the bacterial pair.

There were no significant correlations of hydrophobicity, auto-aggregation, co-aggregation and adhesion. In addition to our study, other studies have come to the conclusion that these physicochemical characteristics vary in importance for the attachment of different species of bacteria (Flint et al., 1997; Sinde and Carballo, 2000). In contrast to Hood and Zottola (1995) who found that cell surface charge, polysaccharide production and hydrophobicity affect bacterial attachment to surfaces, Flint et al. (1997) observed that the role of these factors appears to be species-specific. Their study was unable to show an association between bacterial attachment and any of the three factors. Similarly, a study by Oliveira et al. (2007) showed that cell surface hydrophobicity did not play a major role in the attachment of *Salmonella* strains to stainless steel surfaces. This indicates that other factors may be more important for *Salmonella* attachment than the physicochemical properties of the bacterial cells and attachment surface.

3.4 Conclusions

In summary, our findings demonstrate that PCW components significantly affect bacterial attachment. Pectin in the BCP composite and xyloglucan in association with pectin in the BCPX composites were shown to increase *Salmonella* attachment significantly. We confirmed that the attachment of the bacterial strains, particularly *S.* Typhimurium ATCC 14028, to the BC composites was not stochastic and was most likely controlled by specific interactions between the bacteria and the attachment surface. We also found that bacterial attachment was not significantly influenced by the hydrophobicity of the attachment surface or the physicochemical properties of bacteria. It is still, however, unclear whether the attachment of the *Salmonella* strains to the BC composites was due to the presence of potential attachment receptors for the PCW polysaccharides on the bacterial surface or the influence of the PCW polysaccharides on the physical and structural characteristics of the BC composites. The effect of these factors on *Salmonella* attachment to the PCWs has been investigated and will be discussed in the following chapter.

Chapter 4

Effect of Surface Characteristics and Carbohydrates on the Attachment of *Salmonella enterica* to Plant Cell Wall Models

The work presented in this chapter represents the following manuscript submitted for peer review:

Tan, M.S.F., Moore, S.C., Tabor, R.F., Fegan, N., Rahman, S. and Dykes, G.A. (Submitted). Attachment of *Salmonella* strains to a plant cell wall model is modulated by surface characteristics and not by specific carbohydrate interactions. BMC Microbiol.

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of chapter 4, the nature and extent of my contributions to the work was as following:

Nature of contribution	Extent of contribution (%)
I was responsible for 100% of data analysis and 90% of drafting and writing	90%

The following co-authors contributed to the work and the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%)
Sean Moore	SM was responsible for 1% of writing and review of the manuscript.	1%
Rico Tabor	RT was responsible for 1% of writing and review of the manuscript.	1%
Narelle Fegan	NF was responsible for 1% of writing and review of the manuscript.	1%
Sadequr Rahman	SR was responsible for 2% of writing and review of the manuscript.	2%
Gary Dykes	GD was responsible for 5% of writing and review of the manuscript.	5%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**

Main Supervisor's Signature		**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author(s) to agree on the respective contributions of the authors.

4 Effect of Surface Characteristics and Carbohydrates on the Attachment of *Salmonella enterica* to Plant Cell Wall Models

4.1 Introduction

Plant cell walls (PCWs) play many important roles in plant growth, intercellular communication, control of water balance and in providing mechanical support to plant cells (Keegstra, 2010). The three major components of the PCW include cellulose, pectin and hemicellulose (mostly xyloglucan). Due to their surface location, PCWs also play an important role in plant-microbe interactions and intact PCWs are important to provide a physical barrier against potential pathogens.

Bacterial attachment can be influenced by chemical composition and physical properties of the attachment surfaces (Boyd et al., 2002). It has been shown that exposed cut surfaces of PCWs are prone to bacterial attachment (Han et al., 2000; Liao and Sapers, 2000). These cut surfaces of the PCWs leach different nutrients and substances which may affect bacterial colonization on the plant, including sugars, sugar alcohols, pectic substances, minerals, amino acids, plant hormones and vitamins (Aruscavage et al., 2006).

Our previous study (Tan et al., 2016) suggested that the presence of carbohydrates on PCW components may provide sites for the attachment of *Salmonella enterica* strains which agrees with the findings of Saggers et al. (2008) which suggested that PCW polysaccharides, and particularly pectin, may provide receptor sites for *Salmonella* cells to attach. Nonetheless, the mechanisms which caused increased *Salmonella* attachment to the PCW components have not been elucidated. The role of sugar residues as receptors for the binding of animal pathogenic bacteria to animal cells has long been accepted, for example, it was discovered almost 40 years ago that D-mannose inhibited *Escherichia coli* from binding to

human epithelial cells by blocking mannose-specific bacterial hemagglutinins or lectins Ofek et al., 1977). McHan et al. (1989) found that a number of sugar molecules including D-galactose, L-fucose, L-arabinose and D-mannose reduced the attachment of *S.* Typhimurium to ceca in one week old chicks. Very few studies (Rodríguez-Navarro et al., 2017; Rossez et al., 2014a; Rossez et al., 2014b) have focused on the role of sugar residues in plants on the attachment of human pathogens. It has also been suggested in our previous study (Tan et al., 2016) that varying concentrations of the PCW polysaccharides may have an effect on the structural features of the attachment surface. Physical properties, such as roughness, porosity and hydrophobicity, have also been shown to affect bacterial attachment (An and Friedman, 1998).

This study aimed to investigate whether the sugar residues of PCW components (cellulose, pectin and xyloglucan) provide sites for *S. enterica* attachment. The study also aimed to establish the effect of these PCW polysaccharides on the structural properties of the bacterial cellulose (BC)-based PCW model and the associated attachment of *S. enterica* to these surfaces.

4.2 Materials and methods:

4.2.1 Bacterial strains

S. Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028, *S. enterica* M4, *L. monocytogenes* ATCC 7644 and *G. xylinus* ATCC 53524 were obtained from the same sources and grown in the same way as mentioned earlier in Section 2.2.1.

4.2.2 Production of BC-based PCW models

The BC-based PCW models were produced and prepared as described earlier in Section 2.2.2, a variety of the PCW models were produced with different combinations of pectin and/or xyloglucan:

- BC
- BC-Pectin (BCP) with 0.5% w/v pectin and 12.5 mM $CaCl_2$
- BC-Xyloglucan (BCX) with 0.5% w/v xyloglucan
- BC-Pectin-Xyloglucan (BCPX) composites, including BCP(0.1%)X(0.1%), BCP(0.1%)X(0.3%), BCP(0.1%)X(0.5%) and BCP(0.25%)X(0.25%) were produced by adding different combinations of pectin and xyloglucan (% w/v). The concentration of $CaCl_2$ added is determined by the amount of pectin added. BCPX composites with 0.1% and 0.25% w/v pectin were supplemented with 3 mM and 6 mM $CaCl_2$ respectively, regardless of the xyloglucan concentrations.

4.2.3 Effect of sugar molecules on the attachment of *Salmonella* strains

Early stationary phase cultures of *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4 were centrifuged at 5500 x *g* (Hettich D-78532, Tuttlingen, Germany) for 10 min at 4^{o}C. Pelleted cells were then washed twice with phosphate buffered saline (PBS) (pH 7.4) (1st BASE, Singapore) and resuspended in PBS containing 1% w/v of each monosaccharide sugar composing pectin and xyloglucan (Table 4.1) to a final OD_{600nm} which corresponds to 8 log CFU/mL for each isolate. Pelleted cells resuspended in PBS only (8 log CFU/mL) served as controls for this experiment. The sugars used (Sigma-Aldrich, USA) were D-galacturonic acid, D-mannose, L-rhamnose, L-arabinose, D-galactose, D-glucose and D-xylose.

Table 4.1 List of monosaccharides found in the major PCW components.

Cellulose	Xyloglucan	Pectin
D-glucose	D-glucose	D-glucose
	D-xylose	D-xylose
	D-galactose	D-galactose
	L-arabinose	L-arabinose
		L-rhamnose
		D-mannose
		D-galacturonic acid

Bacterial suspensions prepared with sugar solutions were left at 25ºC for 1 h to allow sugar molecules to attach to potential sugar receptors on the *Salmonella* cell surface. Subsequently four BC composites [BC, BCP(0.5%), BCX(0.5%), BCP(0.25%)X(0.25%)] were exposed to the suspensions for 20 min. Rinsing of the incubated BC composites with 6mM $CaCl_2$ was carried out for 1 min to remove loosely attached cells. Treated BC composites were then placed in a stomacher bag containing 50mL PBS and pummeled for 1 min in a stomacher (BagMixer 400; Interscience, France) at a rate of 8 strokes/sec. An aliquot of the PBS was serially diluted before spread plating on xylose lysine deoxycholate agar (XLDA; Oxoid, UK) to enumerate the numbers of *Salmonella* cells that were attached to the composites. Attached numbers of bacterial cells were expressed as log CFU/cm^2 composite.

Preliminary results showed that D-galacturonic acid killed all bacteria at 1%w/v as the pH of the D-galacturonic acid solution was more acidic (~pH 2.6) as compared to the other sugar solutions which were only slightly acidic (~pH 4.5). For this reason, the pH of the D-galacturonic acid solution was then adjusted to pH 4.5 to eliminate any pH effect on bacterial survival during the attachment assays. At 1%w/v, D-galacturonic acid appeared to have decreased the attachment in the *Salmonella* strains to some of the composites containing

pectin and therefore, a higher concentration of D-galacturonic acid (2% w/v) was used in subsequent attachment assays. Viable counts of *Salmonella* cells were performed by spread plating on XLDA after treatment with 1% and 2% w/v galacturonic acid. Another treatment on the *Salmonella* cells was carried out with 1% w/v sodium metaperiodate ($NaIO_4$; Sigma-Aldrich, USA), a chemical which cleaves the C-C bond between neighbouring hydroxyl groups in sugars, before incubation with BC composites to further confirm the role of sugar interactions in *Salmonella* attachment to BC composites.

4.2.4 Microscopy

4.2.4.1 Confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) was used to visualize the cross-sectional view of the BC composites in its native hydrated state without any modification. The 3D microstructure of the BC composites [BC, BCP(0.5%), BCX(0.5%), BCP(0.1%)X(0.1%), BCP(0.1%)X(0.3%), BCP(0.1%)X(0.5%)] were examined. Different concentrations of the Calcofluor White stain (CW; Sigma-Aldrich, USA) were used to stain the BC composites for 1 min as the presence of pectin and xyloglucan can impede uptake of the stain by cellulose fibrils. BC was stained with 0.01% w/v CW, BCX and BCPX were stained with 0.02% w/v CW whereas BCP was stained with 0.1% w/v CW. After staining, BC composites were rinsed in distilled water for 1 min before adding 10% w/v potassium hydroxide for another min then viewed at 25^{o}C using a Leica TCS SP5 CLSM (Leica Microsystems, Germany) under a HCX PL APO 100x objective. The CW dye was excited by a Diode 405 nm laser and the emitted light was collected from 427 to 477 nm.

In order to ascertain whether *Salmonella* cells can penetrate and attach inside the matrix of the BC composites, *S.* Typhimurium ATCC 14028 was grown for 18 h in Bromothymol Blue (BTB; Sigma-Aldrich, USA) broth which contains 1% (w/v) casein peptone, 0.5% (w/v) sodium chloride and 0.0025% (w/v) BTB dye. *Salmonella* cells grown

in and subsequently dyed with BTB broth were pelleted at 5500 x g for 10 min at 4^{o}C and resuspended in PBS. The BCP(0.1%)X(0.1%) composite was dyed with CW stain as described above before incubation with BTB dyed *Salmonella* cells for 20 min. The BTB dye was excited by an Argon 488 nm laser and the emitted light was collected from 707 to 741 nm.

4.2.4.2 Atomic force microscopy (AFM)

BC composites [BC, BCP(0.5%), BCX(0.5%), BCP(0.1%)X(0.1%), BCP(0.1%)X(0.3%), BCP(0.1%)X(0.5%)] were air dried for three days after harvesting and rinsing. Tapping mode was used on the atomic force microscopy (AFM;JPK Nanowizard 3 AFM, Germany) with a Bruker NCHV model cantilevers (California, USA). AFM height images were analysed using Scanning Probe Image Processor (SPIP) software (NanoScience, USA). The diameter of the microfibrils were determined using the cross section profile tool which extracts height profiles. The width of the peaks obtained represent the width of the microfibrils as illustrated by Cybulska et al. (2010). The mean diameter of microfibrils for each composite was determined from 10 distinctive microfibrils lying on relatively flat surfaces of the BC composites (without large scale features, such as *G. xylinus* cells) chosen from each of three images for each type of sample. The average roughness and root mean square (RMS) roughness were determined by choosing 10 relatively flat areas from each of three images for every type of BC composite.

4.2.4.3 Scanning electron microscopy (SEM)

BC composites [BC, BCP(0.5%), BCX(0.5%), BCP(0.1%)X(0.1%), BCP(0.1%)X(0.3%), BCP(0.1%)X(0.5%)] with and without *S.* Typhimurium ATCC 14028 cells attached to them were rinsed in 6 mM $CaCl_2$, air dried and fixed with 4% (v/v) glutaraldehyde (Sigma-Aldrich, USA) in PBS for 40 min. Samples were gradually

dehydrated in a series of ethanol concentrations (20%, 40%, 60%, 80% and 100% v/v ethanol in water; 10 min for each concentration) before drying the samples in a desiccator for three days. Samples were gold-sputtered using a sputter coater (Q150RS: Quorum, UK) and viewed under a scanning electron microscope (SEM; S-3400N, Hitachi, Japan).

4.2.5 Statistical analyses

All experiments were conducted in triplicate with independent cultures. Statistical analysis of results was performed using Statistical Package for the Social Sciences (SPSS) (SPSS Inc., USA) at a 95% confidence level. A one-way ANOVA was performed to determine significance of the effects of different sugar solutions on the attachment of each *Salmonella* strain among the types of BC composites, as well as among the different levels of PCW components used within each type of BC composite. Another one-way ANOVA was carried out to compare significant differences in microfibril diameters and surface roughness between different BC composites.

4.3 Results and discussion:

4.3.1 Effect of sugar molecules on the attachment of *Salmonella* strains

The BC composites have previously been chemically analysed as shown in Table 3.1 and published in our study (Tan et al., 2016). When more pectin and/or xyloglucan was present in the HS media, more of these PCW components were incorporated within the BC composites. Of the different types of BC composites, the chemical composition of the BCPX composites (on average with ~33% cellulose; ~44% pectin; ~23% xyloglucan) were most similar to the average native PCWs (~25% cellulose; ~35% pectin; ~25% hemicellulose) (Cybulska et al., 2010a).

Previous experiments (Tan et al., 2016) suggested that *Salmonella* may harbour specific carbohydrate-binding receptors on the cell surface that bind preferentially to complementary carbohydrate molecules. This postulation was made after we observed that the presence of pectin significantly increased the attachment of the *S. enterica* strains to the BCP composite ($p<0.05$) whereas xyloglucan had no significant effect on *Salmonella* attachment to the BCX composite ($p>0.05$). Interestingly, among the different BC composites, *S. enterica* strains attached at the highest numbers to the BCP(0.1%)X (0.3%) composite which contained 0.3% w/v xyloglucan in the media regardless of the amount of pectin present. This suggests that xyloglucan at the optimal concentration of 0.3% (w/v) favours *Salmonella* attachment when in association with pectin in the BCPX composite.

To further investigate the role of sugar molecules on the attachment of *Salmonella* cells to PCW components, the bacterial cells were incubated in sugar solutions prepared from the monosaccharides making up the PCW components. This allows the monosaccharides of interest to potentially block lectins on *Salmonella* cells (which may act as attachment receptors) from associating with complementary sugar molecules (which could act as receptor sites) on the PCW models. If the monosaccharide of interest successfully blocks the attachment receptors on *Salmonella* cells, the attachment of *Salmonella* cells to the BC composites which contain the same monosaccharide will also be inhibited.

An overall comparison of the attachment of *S.* Typhimurium ATCC 14028 (6.90 ± 0.53 log CFU/cm^2), *S.* Enteritidis ATCC 13076 (6.66 ± 0.45 log CFU/cm^2) and *S. enterica* M4 (6.57 ± 0.35 log CFU/cm^2) to the various BC composites were not significantly different from each other ($p>0.05$). Preliminary experiments showed that the addition of D-galacturonic acid (makes up 90% of pectin) at 1% w/v reduced the attachment of all three *Salmonella* strains to certain BC composites containing pectin. D-galacturonic acid at 1% w/v

reduced the attachment of *S.* Enteritidis ATCC 13076 to the BCPX composite, *S.* Typhimurium ATCC 14028 to both BCP and BCPX composites and *S. enterica* M4 to the BCP composite respectively, when compared to their attachment without addition of any sugars. This initial finding agreed with the postulation that the *Salmonella* strains may harbour attachment receptors which are specific to D-galacturonic acid residues in pectin. This may also explain the phenomenon of increased *Salmonella* attachment in the presence of pectin as observed in our previous study (Tan et al., 2016).

Higher concentration of D-galacturonic acid (2% w/v) was used to further investigate its role in *Salmonella* attachment to the BC composites. The viability of all *Salmonella* strains were not affected (Appendix II). Compared to 1% w/v D-galacturonic acid, 2% w/v of the sugar significantly reduced the overall attachment of *S.* Enteritidis ATCC 13076 to all four types of BC composites ($p<0.05$), whereas the overall attachment of *S. enterica* M4 to the four types of BC composites was significantly increased ($p<0.05$) (Figure 4.1). The overall attachment of *S.* Typhimurium ATCC 14028 to the BC composites was not significantly different at either sugar concentrations ($p>0.05$) (Figure 4.1). If D-galacturonic acid complements the attachment receptors on *Salmonella* cells, the sugar should specifically reduce the attachment of *Salmonella* cells only to BC composites which contain pectin. This was not the case as the attachment of *S.* Enteritidis ATCC 13076 and *S.* Typhimurium ATCC 14028 to the BC and BCX composites which did not contain pectin was also reduced significantly ($p<0.05$) in some cases. This suggests that D-galacturonic acid does not competitively block bacterial cell receptors and could have affected the bacterial cell in other ways which have yet to be investigated. As the addition of sodium metaperiodate which cleaves the C-C bond between vicinal hydroxyl groups in sugars did not cause a significant decrease in the attachment of all strains ($p>0.05$), this further confirmed suggestions that the attachment of *Salmonella* cells to BC composites is not likely to be mediated by specific

binding of bacterial cell receptor to complementary sugar residues under the conditions tested here.

Some sugars significantly increased the attachment of the *S. enterica* strains, for example, L-rhamnose and D-xylose significantly increased the attachment of *S.* Enteritidis ATCC 13076 and *S. enterica* M4 ($p<0.05$). We propose that these sugar molecules could have increased the ability of the strains to auto-aggregate which can be tested in future studies. Planktonic *Salmonella* cells in the bacterial suspension could have attached and aggregated onto the bacterial cells which were already attached to the composite surface. Sugars produced during gluconeogenesis have been shown to cause *S.* Typhimurium cells to aggregate (White et al., 2010). In another study, the Gram-negative bacterium *Azospirrilum brasilense* increased its capacity to aggregate in the presence of exopolysaccharide which consists of different sugars such as xylose, rhamnose and arabinose (Burdman et al., 1998). While these results are intriguing, we have yet to elucidate the mechanism of action of these sugars on *Salmonella* attachment in our study. More in-depth using plant probes and antibodies can be done in the future to investigate the presentation and accessibility of exposed glycans which could act as specific receptors for bacterial surface factors.

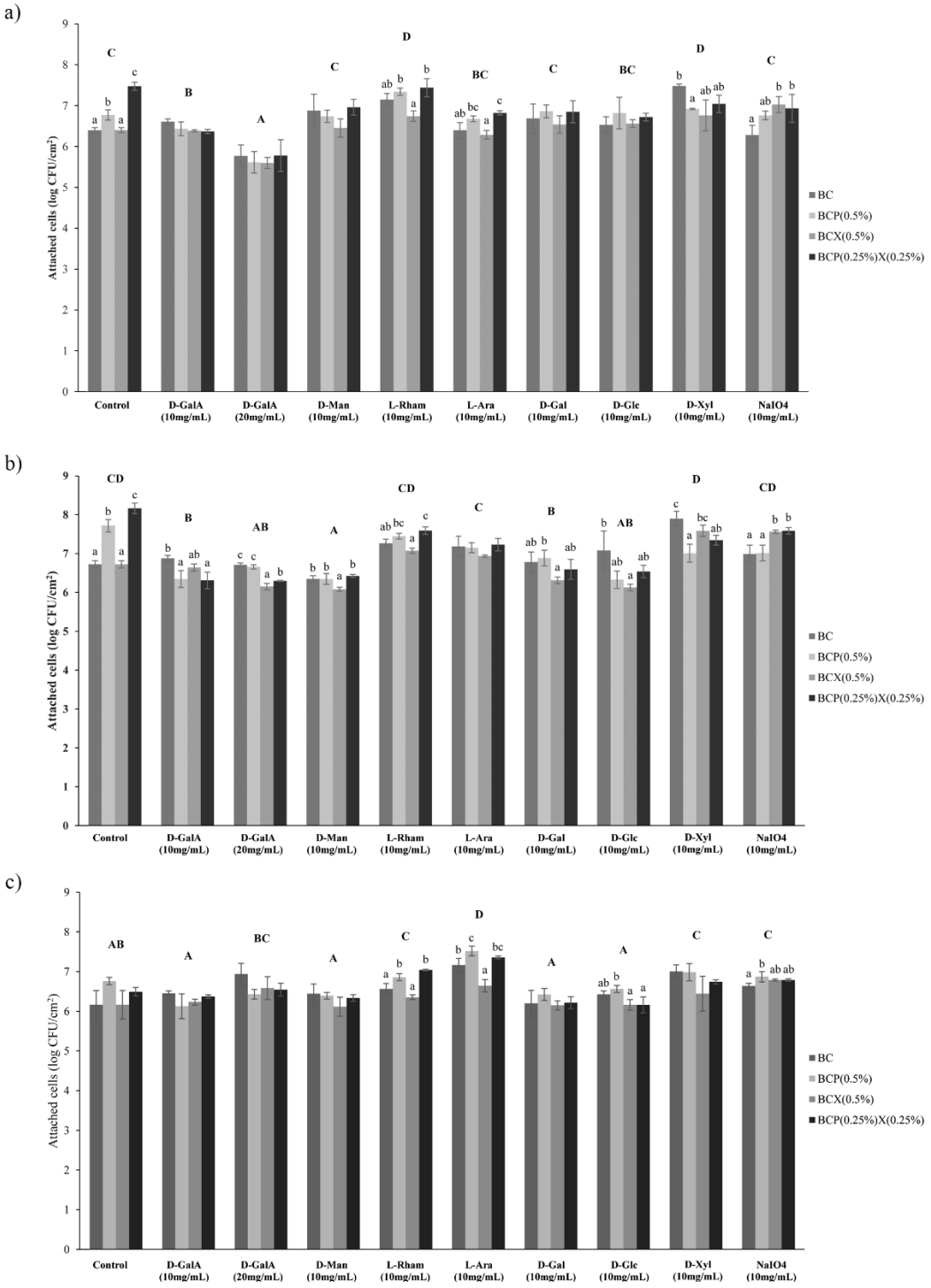


Figure 4.1 Effect of monosaccharides (D-Galacturonic acid, D-GalA; D-Mannose, D-Man; L-Rhamnose, L-Rham; L-Arabinose, L-Ara; D-Galactose, D-Gal; D-Glucose, D-Glc; D-Xylose, D-Xyl) and sodium metaperiodate ($NaIO_4$) on the attachment of (a) *S.* Enteritidis ATCC 13076, (b) *S.* Typhimurium ATCC 14028 and (c) *S. enterica* M4 cells to BC composites [BC, BCP(0.5%), BCX(0.5%), BCP(0.25%)X(0.25%)]. Different uppercase letters indicate significant differences in bacterial attachment between types of composites. Different lowercase letters indicate significant differences in bacterial attachment within each type of composite whereas the absence of lowercase letters indicate no significant differences in bacterial attachment within each type of composite (One-way ANOVA & Tukey's pairwise comparison at $p<0.05$).

4.3.2 Microscopy

We have shown that the attachment of *Salmonella* cells to the BC composites do not involve receptor-ligand interactions mediated by carbohydrates and bacterial surface adhesins under the conditions tested here. Their attachment to the BC composites were therefore most probably non-specific and stochastic in nature and instead could be governed by the physical properties of the attachment surface. Very few studies have investigated on the effect of varying levels of pectin and xyloglucan on the structural properties of BC-based PCW models. We have used three microscopic techniques to obtain structural information on the BC composites.

4.3.2.1 Confocal laser scanning microscopy (CLSM)

CLSM images (Figure 4.2) showed that the cellulose fibrils in all BC composites were tightly packed and appeared to be in a wavelike arrangement, creating gaps within the structure. Cellulose fibrils stained by CW appeared as red strands while *G. xylinus* cells which can also be stained by CW appear as bright red dots on the images. The BCP composite had a different appearance from the other BC composites with visible dark patches and very bright dots in its image. We suggest that the high amounts of pectin in the BCP composite coated the cellulose fibrils and fibrils were unable to take up the CW dye readily. Hence, bright red dots of *G. xylinus* cells which absorbed the CW dye and stained better than the fibrils were more obvious against the dark background.

In order to establish whether the *S.* Typhimurium ATCC 14028 cells could penetrate and attach inside the thick and tightly arranged BC composites, the *S.* Typhimurium cells were dyed with BTB and later allowed to attach to the BCPX composite. More *S.* Typhimurium ATCC 14028 cells attached inside the composite than at the surface, even to a depth of 8 µm below the composite surface (Figure 4.3). This may be because *Salmonella*

cells at the BC composites' surface are exposed to shear force which can detach adhered bacteria, whereas bacteria attached within the composites were shielded from shear force (An and Friedman, 1998). Internalization of human pathogens has also been shown to occur within native plants (Jablasone et al., 2005; Kroupitski et al., 2009a; Solomon et al., 2002). For example, Solomon et al. (2002) observed *E. coli* O157:H7 cells trapped 20 to 100 µm below the lettuce plant surface in stomata and cut edges.

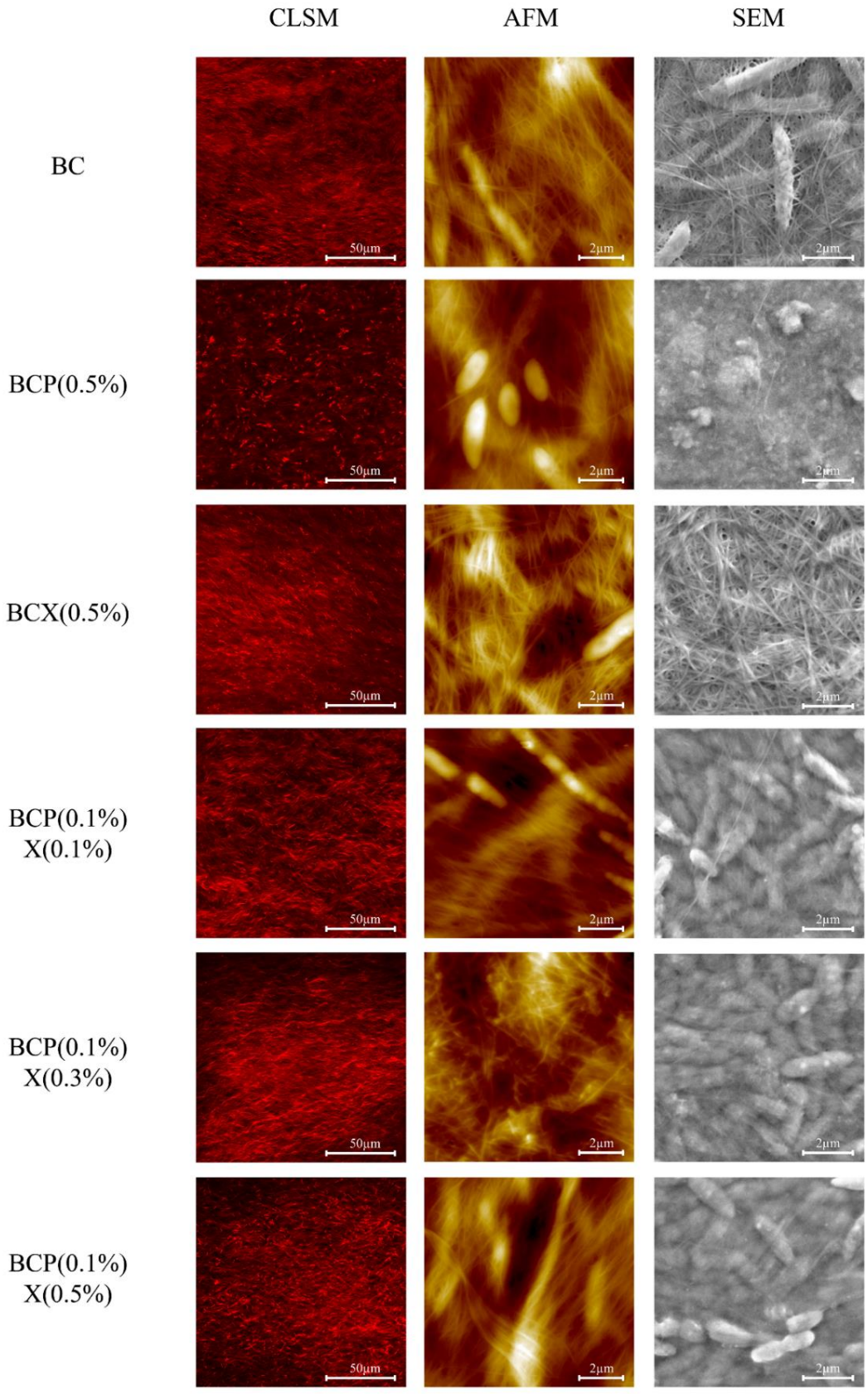


Figure 4.2 CLSM images, AFM height images and SEM images of the BC, BCP(0.5%), BCX(0.5%), BCP(0.1%)X(0.1%), BCP(0.1%)X(0.3%) and BCP(0.1%)X(0.5%) composites.

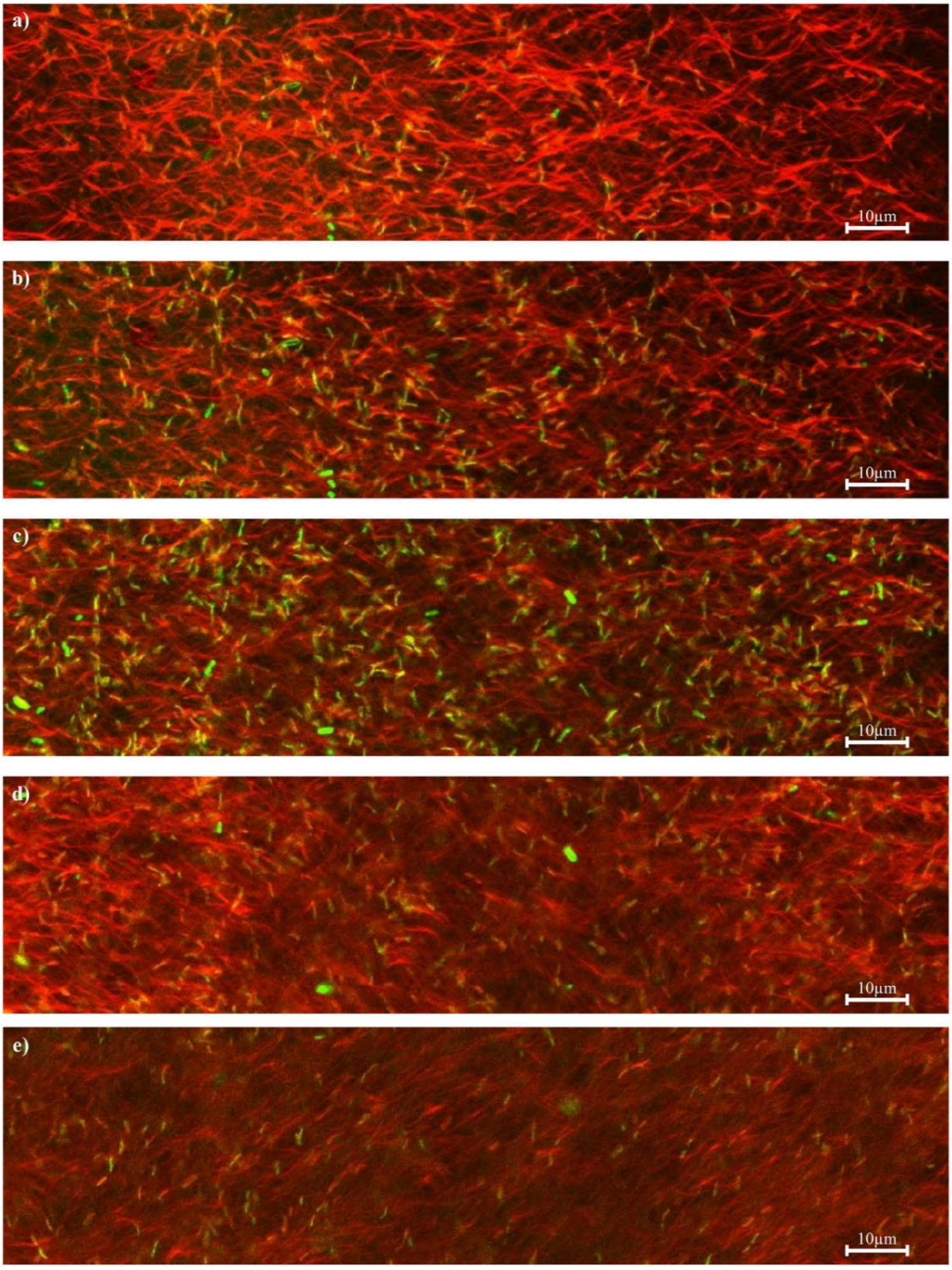


Figure 4.3 CLSM micrographs showing *S.* Typhimurium ATCC 14028 cells (green dots) (a) on the BCP(0.1%)X(0.1%) composite surface (cellulose fibrils shown in red) and at (b) 2 µm, (c) 4 µm, (d) 6 µm and (e) 8µm below the surface.

4.3.2.2 Atomic force microscopy (AFM)

Analysis of AFM height images (Figure 4.2) showed that out of the six composites, BCP(0.1%)X(0.3%) had the thickest fibril diameter (180.0 ± 24.7 nm) and this was significantly higher than the other composites ($p<0.05$) whereas BC had the thinnest fibril (103.3 ± 10.4 nm) ($p<0.05$) (Table 4.2). The imaged fibrils are macrofibrils consisting of bundles of microfibrils (<5nm) strongly linked to one another. Our past results showed that *Salmonella* strains exhibited the highest attachment to the BCPX composite with 0.3% (w/v) xyloglucan regardless of the amount of pectin added into the growth media (Tan et al., 2016). The significantly thicker fibrils in the BCPX(0.3%) composite may have provided a greater surface area for bacterial attachment.

Although the addition of pectin and xyloglucan both increased fibril thickness, these polymers differed in the way they interact with cellulose fibrils. Formation of the BC-based PCW model has been shown to mimic the natural phenomenon of PCW deposition in native plants (Chanliaud and Gidley, 1999). After synthesis in the Golgi apparatus, PCW polysaccharides such as pectin and xyloglucan are secreted separately into the extracellular matrix in plant cells (Chanliaud and Gidley, 1999). In the native PCW, xyloglucan coats the cellulose microfibrils (Lloyd, 1991) while pectin forms a network around the cellulose-xyloglucan network (Reiter, 2002). In reference to this, we postulate that xyloglucan increases fibril diameter by forming strong cross-links with cellulose and coating the cellulose fibrils. Pectin increases fibril diameters as it also coats the fibrils but its primary function is to fill up the gaps between cellulose fibrils.

Showing similar results to our study, Fanta et al. (2011) also measured cellulose fibril diameters of ~100 nm. They found that the fibril diameters of BC (110 ± 33 nm) and BCPX (123 ± 29nm) were not significantly different from each other; but in contrast to our results,

the fibril diameter for their BCP composite was much lower (45 ± 9 nm). These authors suggested that pectin may have resisted microfibril association but this was not apparent in our study. Another study by Cybulska et al. (2010) supported our finding that cellulose fibrils for BCPX (~75 nm) was significantly thicker than both BC (~37 nm) and BCP (~46 nm). The difference in the size of cellulose fibrils between this study and ours may be related to the strains of *G. xylinus* used.

Another factor that could affect bacterial attachment is the surface roughness of the BC composites and this was measured from the AFM height images (Figure 4.2). Rough surfaces have greater surface area and more surface irregularities which provide favourable sites for bacterial adhesion (An and Friedman, 1998). We found that the average and root mean square (RMS) roughness were not significantly different between the BC composites, this suggests that surface roughness has no significant effect on the attachment of *Salmonella* cells to the composites. Similarly, Cybulska et al. (2010) did not observe significant differences in RMS roughness between BC, BCP and BCPX composites.

AFM phase images give an indication of the sample hardness, chemical composition and elasticity (Raghavan et al., 2000). Interestingly, the phase image of BCPX(0.3%) exhibited more heterogeneity compared to the other BCPX(0.1%) and BCPX(0.5%) composites (Figure 4.4) although chemical compositions of the three different BCPX composites were previously shown to be similar (Tan et al., 2016). This suggests that pectin and xyloglucan interact differently at particular concentrations and cause distinct physical and structural changes to the composites which also influence bacterial attachment. It is still unclear how different concentrations of PCW components influence these specific structural changes.

Table 4.2 Measurements obtained from AFM height images of the BC composites. Data are presented as mean ± SD where n=30. Different lowercase letters indicate significant differences between types of BC composites whereas absence of lowercase letters indicate no significant differences between types of BC composites within the same row (One-way ANOVA & Tukey's pairwise comparison at $p<0.05$).

	BC	**BCP (0.5%)**	**BCX (0.5%)**	**BCP(0.1%)X(0.1%)**	**BCP(0.1%)X(0.3%)**	**BCP(0.1%)X(0.5%)**
Microfibril diameters (nm)	103.3 ± 10.4 [a]	130.9 ± 17.2 [b]	141.5 ± 16.6 [b]	143.1 ± 23.6 [b]	180.0 ± 24.7 [c]	145.7 ± 25.6 [b]
Average roughness (nm)	63.8 ± 23.2	79.1 ± 20.3	65.9 ± 12.3	77.2 ± 22.1	91.4 ± 24.3	73.9 ± 6.7
Root mean square roughness (nm)	75.1 ± 22.0	97.3 ± 24.8	76.5 ± 13.5	88.5 ± 23.9	110.6 ± 28.9	87.7 ± 9.7

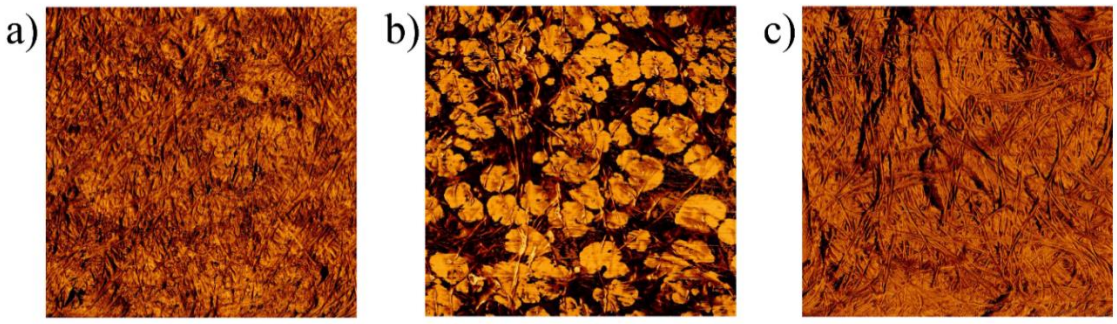


Figure 4.4 AFM phase images of (a) BCP(0.1%)X(0.1%), (b) BCP(0.1%)X(0.3%) and (c) BCP(0.1%)X(0.5%) composites.

4.3.2.3 Scanning electron microscopy (SEM)

SEM was used to complement the results from AFM imaging. Unlike the AFM, SEM is able to analyse a large surface area and has a large depth of field which allows it to be used on relatively rough surfaces. Of the composites, the BC composite was clearly the most porous with the most number of black regions and the BCX composite was also more porous compared to the rest (Figure 4.2). It was difficult to determine the porosity and pore size from the images as the BC composites were too thick. Cybulska et al. (2010) and Fanta et al. (2011) both found that the BC has the highest porosity, BCP has lower porosity after the addition of pectin whereas BCPX has the lowest porosity and greatest compactness amongst the composites.

According to Shah et al. (2013), a porous BC matrix can easily trap liquid substances and small particles. Surface porosity has been found to favour bacterial attachment, this was probably due to the increased available area for attachment (Katsikogianni and Missirlis, 2004). However, this was not the case in our previous study (Tan et al., 2016) as the more compact BCP and BCPX composites had a greater number of bacteria attached to it (more than 0.5 log CFU/cm^2 higher) compared to the more porous BC and BCX composites. We hypothesize that pectin fills up the gaps between cellulose fibrils in the BCP and BCPX

composites and creates a complex matrix within the composites which can trap *Salmonella* cells which enter through the pores. Within the matrix, weak forces such as van der Waals forces, electrostatic forces and hydrophobic interactions (Van Loosdrecht et al., 1987) may mediate the initial reversible attachment. These weak forces draw bacterial cells closer to the attachment surfaces which then allow other stronger interactions to occur, such as covalent bonding, hydrogen bonding and cation bridging (Van Oss, 1989). Although carbohydrates are generally considered to be highly polar, they still contain hydrophobic regions which allow non-specific hydrophobic bonding. Calcium bridges formed between free carboxyl groups of pectin chains (Willats et al., 2001) may favour the attachment of negatively charged *Salmonella* cells. On the other hand, fewer *Salmonella* cells (~2 µm) attach inside the BC and BCX composites which do not contain the pectin matrix as they were able to swim more freely through their pores (~100 µm after 3 days incubation) (Quero et al., 2010).

Pectin masks cellulose fibrils and forms clumps on the surface of the BCP composite. Cellulose fibrils in the BC and BCX composites were more randomly arranged while those in the BCPX composites appeared to be more unidirectional, however, individual fibrils cannot be distinguished clearly as they were coated with pectin and xyloglucan. Similarly, Cybulska et al. (2010) also observed that microfibrils in the BCPX composite did not cluster in distinct bundles compared to those seen on the BC and BCP composites.

4.4 Conclusions

This study suggested that the attachment of *Salmonella* cells to native PCWs were not mediated by receptor-ligand interactions involving carbohydrates and bacterial surface adhesins. This was demonstrated when carbohydrate molecules did not selectively inhibit the attachment of *Salmonella* cells to the BC-based PCW models. Instead, it was suggested that

Salmonella attachment to the BC-based PCW models could be mediated by structural properties of the attachment surface. This study found that pectin and xyloglucan interacted differently at varying concentrations which conferred BC composites with distinct physical and structural characteristics that could have influenced the extent of bacterial attachment to these surfaces. Similar to the phenomenon of PCW deposition in native PCW, pectin also filled in the gaps between cellulose fibrils within the BC-based PCW models. The presence of pectin reduced porosity of the composites, whereby pore sizes were sufficiently large to allow internalization of *Salmonella* cells while creating a matrix that was able to retain *Salmonella* cells. On the other hand, addition of xyloglucan increased cellulose fibril diameter and also the attachment surface area, thus allowing more *Salmonella* cells to attach when it is in association with pectin. Our results are consistent with the hypothesis that a combination of the effects of surface roughness and porosity determines the attachment of *Salmonella* to PCWs.

Chapter 5

The Role of Cell Surface Structures in the Attachment of *Salmonella enterica* to Plant Cell Wall Models

The work presented in this chapter represents the following manuscript submitted for peer review:

Tan, M.S.F., White, A.P., Rahman, S. and Dykes, G.A. (2016). Role of curli, flagella and cellulose on the attachment of *Salmonella* Typhimurium ATCC 14028 to plant cell wall models. PLoS ONE. 11, e0158311.

Declaration for Thesis Chapter 5

Declaration by candidate

In the case of chapter 5, the nature and extent of my contributions to the work was as following:

Nature of contribution	Extent of contribution (%)
I was responsible for 100% of data analysis and 90% of drafting and writing	90%

The following co-authors contributed to the work and the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%)
Aaron White	AW was responsible for 1% of writing and review of the manuscript.	1%
Sadequr Rahman	SR was responsible for 2% of writing and review of the manuscript.	2%
Gary Dykes	GD was responsible for 7% of writing and review of the manuscript.	7%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature	[redacted]	**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author(s) to agree on the respective contributions of the authors.

5 The Role of Cell Surface Structures in the Attachment of *Salmonella enterica* to Plant Cell Wall Models

5.1 Introduction

Fresh produce is now recognized as the main cause of foodborne outbreaks (46%) and cases of hospitalizations (38%) associated with foodborne outbreaks in the United States (Painter et al., 2013). It was initially thought that enteric pathogens, which are usually found in the intestinal tracts of animals, would survive poorly on plant surfaces where microorganisms encounter harsh environmental conditions such as drastic temperature fluctuations, desiccation, sunlight and nutrient limitation but recent research (Barak and Schroeder, 2012; Holden et al., 2008; Krtinić et al., 2010; Tyler and Triplett, 2008) has shown otherwise. *Salmonella* in particular was previously largely only reported to be associated with foods of animal origin but is now the most commonly identified human bacterial pathogen associated with fresh produce (Klerks et al., 2007; Kroupitski et al., 2009b). However, the adaptations required for the attachment of *Salmonella* cells which are naturally occurring in animal intestines to plant surfaces which present different environment conditions have not yet been investigated in detail.

Studies have found that bacterial cell surface components such as cellulose, flagella and curli are important for the attachment of pathogens to fresh produce (Barak et al., 2005; Berger et al., 2009; Kroupitski et al., 2009a; Lapidot and Yaron, 2009). These cell surface components may harbour surface epitopes which enable pathogens to preferentially attach to cut surfaces and natural openings such as stomata which expose nutrients produced during photosynthesis (Klerks et al., 2007; Kroupitski et al., 2009). Flagella are long, thin surface appendages that extends up to 20 µm and which are important for motility and chemotaxis (Wiedemann et al., 2015). Bacteria use flagella to move along the plant surface before

finding a favourable attachment site (Cooley et al., 2003). Curli are fine, hair-like protein appendages which contain adhesins on their tips with affinity to different sugar molecules and can be up to several micrometers long (Yaron and Römling, 2014). Cellulose, which consists of β(1-4)-linked glucose units secreted by bacterial cells, can hinder flagellar rotation and limit bacterial motility (Zorraquino et al., 2013). Production of both curli and cellulose are regulated by the *csgD* gene in *Salmonella.* Expression of these structures are associated with biofilm formation and are important for the environmental persistence of *Salmonella* including enhancing its ability to avoid desiccation stress (Gibson et al., 2006; White et al., 2006a). Temperature regulation of the expression of cellulose and curli takes place through the control of its transcription level by products of the *csgD* gene (Romling et al., 1998). The *csgD* gene was the first enteric bacterial regulator identified to play a more influential role in enteric bacterial interaction with plants than within the animal host (Barak and Schroeder, 2012).

The specific mechanisms involved in the association between bacterial cell structures and plant cell wall (PCW) components exposed on cut PCWs have yet to be elucidated and up till now, very few genetic elements have been identified to be important for the attachment of human foodborne pathogens to plants. However, the ability to form biofilm has been correlated to better survival and stronger attachment to fresh produce (Yaron and Römling, 2014), for example, *Salmonella* isolates sampled during tomato outbreaks produced biofilms and attached better to tomato leaflets compared to non-biofilm producing strains (Cevallos-Cevallos et al., 2012).

In this study we aimed to investigate how cell surface structures (flagella, curli and cellulose) influence *Salmonella* attachment to major structural components of the PCW (cellulose, pectin and xyloglucan) after growth at 28^{o}C (average environmental temperature)

and 37°C (animal and human body temperature) using bacterial cellulose (BC)-based PCW models.

5.2 Materials and methods

5.2.1 Bacterial strains

Gluconacetobacter xylinus ATCC 53524 and *Salmonella enterica* subspecies *enterica* serovar Typhimurium ATCC 14028 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Gene knockout mutants of *S.* Typhimurium ATCC 14028 used in attachment experiments are listed in Table 5.1.

Table 5.1 Genotype and characteristics of *S.* Typhimurium ATCC 14028 mutant strains used in this study.

Genotype	Characteristics	Source or reference
Δ*fliC fljB*	Lacks phase 1 and 2 flagellin	Miao et al. (2006)
ΔbcsA	Lacks cellulose	White et al. (2006)
ΔcsgA	Lacks curli	White et al. (2006)
ΔcsgA bcsA	Lacks cellulose and curli	This study
ΔcsgD	Missing the major biofilm transcriptional regulator coding sequence, lacks cellulose and curli	MacKenzie et al. (2015)

G. xylinus ATCC 53524, a BC-producing strain, was cultured as aforementioned in Section 2.2.2. For the attachment experiments, wild type and mutant strains of *S.* Typhimurium ATCC 14028 were grown at either 28°C or 37°C in tryptic soy broth (TSB; Merck, Darmstadt, Germany) under shaking incubation (150 rpm) (Lab Companion SK-600 benchtop shaker; Medline, UK).

Production of cellulose and curli by these strains at 28ºC or 37ºC were confirmed by monitoring their colony morphology on Luria-Bertani (LB; Merck, Darmstadt, Germany) medium without salt supplemented with 40 µg/mL of Congo Red (CR; Sigma-Aldrich, Missouri, USA) and 20 µg/mL Coomassie Brilliant Blue (CBB; Sigma-Aldrich, Missouri, USA) when grown at these two temperatures. Cellulose production was further confirmed using LB medium without salt supplemented with 50µg/mL of calcofluor white (CW; Sigma-Aldrich, Missouri, USA), colonies which produce cellulose will fluoresce under ultraviolet (UV) light. Flagella production at both temperatures were determined using Ryu's flagella stain as described by Kodaka et al. (1982). *Salmonella* strains were maintained on tryptic soy agar (TSA; Merck, Darmstadt, Germany) at 4ºC.

5.2.2 Generation of the *S.* Typhimurium ATCC 14028 Δ*bcsA csgA* double mutant strain

The *S.* Typhimurium ATCC 14028 Δ*bcsA* strain was generated as previously described (White et al., 2006a). *S.* Typhimurium Δ*bcsA* cells were transformed with the pHSG415/Δ*csgA* (formerly Δ*agfA*) construct prepared from S. Enteritidis 27655-3b genomic DNA (White et al., 2001) and selected on LB agar supplemented with 100 µg/mL ampicillin. The Δ*csgA* mutation was successfully performed through allelic exchange following established procedures (White et al., 2007). Final ampicillin-sensitive *S.* Typhimurium Δ*bcsA csgA* colonies were differentiated from Δ*bcsA* colonies by growth at 28ºC on agar media (1% tryptone, 1.5% agar) supplemented with 100 µg/mL Congo red; Δ*bcsA csgA* colonies appeared light pink, whereas Δ*bcsA* colonies appeared orange or red. Polymerase chain reaction (PCR) was used to confirm the Δ*csgA* mutation, using primers TAFPF (TACGCCAGGAAGGATCAAAACTAT) and TAFPR (GCCGTCGCGCACAGAGA); PCR products were purified and confirmed by DNA sequencing (Eurofins MWG Operon, Kentucky, USA).

5.2.3 Production of BC-based PCW models

The different types of BC-based PCW models [BC, BC-Pectin (BCP), BC-Xyloglucan (BCX), BC-Pectin-Xyloglucan (BCPX)] were produced as described in Section 3.2.2.

5.2.4 Attachment study

Early stationary phase cultures (18 h incubation) of *S.* Typhimurium ATCC 14028 and its mutants were prepared to a final concentration of 8 log CFU/mL were used for the subsequent attachment studies as aforementioned in Section 2.2.3.

5.2.5 Data analysis

Experiments were performed in triplicate with three independently grown bacterial cultures. Statistical analysis of results was performed using Statistical Package for the Social Sciences (SPSS) (PASW Statistics 18, SPSS Inc., USA). One-way analysis of variance (ANOVA) was used to compare significant differences between wild type and mutant strains of *S.* Typhimurium ATCC 14028 grown at the same temperature of their overall attachment to the BC composites. Another one-way ANOVA was carried out individually for each strain grown at a specific temperature to compare significant differences in its attachment numbers to different BC composites. Independent samples t-tests were also conducted to determine significant differences for the same strain in its attachment to the BC composites when grown at two different temperatures. Differences among the means were determined using Tukey's method at 95% confidence level.

5.3 Results and discussion

5.3.1 Production of cell surface components by *S.* Typhimurium ATCC 14028 strains

S. Typhimurium ATCC 14028 produced flagella when grown at 28°C and 37°C (Figure 5.1). However, the *S.* Typhimurium strain displayed temperature-dependent expression of cellulose and curli as can be seen on CR and CW plates (Figure 5.2). As described by Römling et al. (1998), the wild type strain of *S.* Typhimurium was able to produce cellulose and curli at 28°C and showed rough, dry and red (rdar) phenotypes on the CR plate. Wild type colonies grown on the CW plate fluoresced under UV light. When grown at 37°C however, the strain lost the ability to produce these structures and appeared as smooth and white colonies (saw) on the CR plate and colonies formed on the CW plate did not fluoresce. White et al. (2006a) also noted that *Salmonella* only produce these extracellular structures at incubation temperatures of below 30°C and under nutrient-limited conditions at low osmolarity. According to Kader et al. (2006), temperature regulation of the rdar morphotype is mediated by the temperature gradient in cyclic-di(3'→5')-guanylic acid (c-di-GMP) concentrations. The c-di-GMP secondary messenger regulates cellulose and curli production by affecting both CsgA and CsgD expression on the transcriptional and post-transcriptional levels respectively. Therefore, wild type *Salmonella* strains are expected to have lower levels of c-di-GMP at 37°C than at 28°C, this could be caused by either increased phosphodiesterase activity or reduced diguanylate cylase activity (Kader et al., 2006).

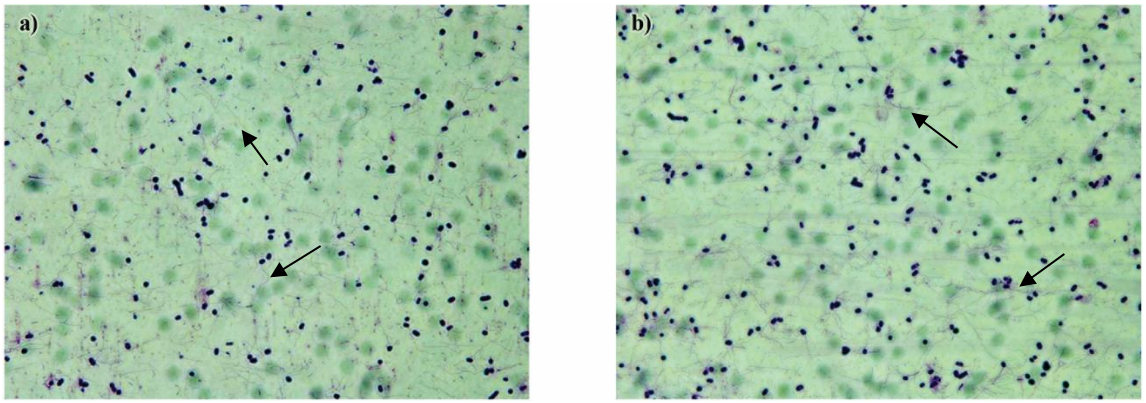


Figure 5.1 Flagella (indicated by arrows) of *S.* Typhimurium ATCC 14028 cells grown at (a) 27°C and (b) 37°C stained with Ryu's stain.

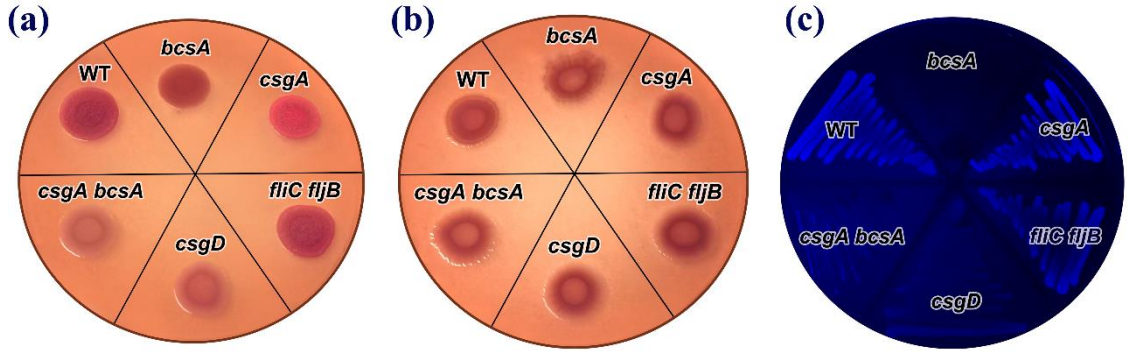


Figure 5.2 Colony morphology of *S.* Typhimurium ATCC 14028 wild type and mutant strains on (a) Congo Red (CR) agar plate grown at 28°C, (b) CR agar plate grown at 37°C and (c) Calcofluor White (CW) agar plate grown at 28°C.

5.3.2 Effect of PCW components and temperature on *Salmonella* attachment to BC composites

We found in this study that the varying levels of pectin and xyloglucan did not have significant effect on *S.* Typhimurium attachment to the BC composites, hence attachment numbers for each type of composite were collated and presented as an average value in Figure 5.3 for easy comparison (full representation in Appendix III). We initially expected the *S.* Typhimurium strains grown at 28^{o}C (especially the wild type strain and the Δ*fliC fljB* mutant which can produce cellulose and curli at 28^{o}C) to have higher attachment levels to the BC composites than the same strains grown at 37^{o}C. However, of the six strains only the wild type *S.* Typhimurium strain showed significant difference in the number of cells attached to the BC composites when grown at the two different temperatures ($p<0.05$) whereas others showed similar attachment for both temperatures ($p>0.05$). In most cases, strains grown at 28^{o}C did not show significant differences in attachment to the various BC composites ($p>0.05$) except for the Δ*fliC fljB* mutant which showed the highest attachment to BC alone. This suggests that flagella may interact with pectin and xyloglucan, with the loss of flagella also decreasing *Salmonella* attachment to the BC composites containing these PCW components. As Warriner and Namvar (2010) have pointed out, human pathogen attachment to plants may involve specific recognition interactions between the bacterial cell surface and physical structures on the leaf. Saggers et al. (2008) also observed that fewer *S.* Typhimurium cells were attached when less pectin was present in the potato tissue. It was also expected that the attachment for all *S.* Typhimurium strains grown at 37^{o}C (except for the Δ*fliC fljB* mutant) would be similar to each other since cellulose and curli are not produced at this temperature. This was not, however, the case as significant variations in the attachment of different strains were observed ($p<0.05$). Variations in the attachment of *S.* Typhimurium strains grown at different temperatures were most probably not due to the effect of cellulose and curli structures but could be caused by other factors we have yet to identify.

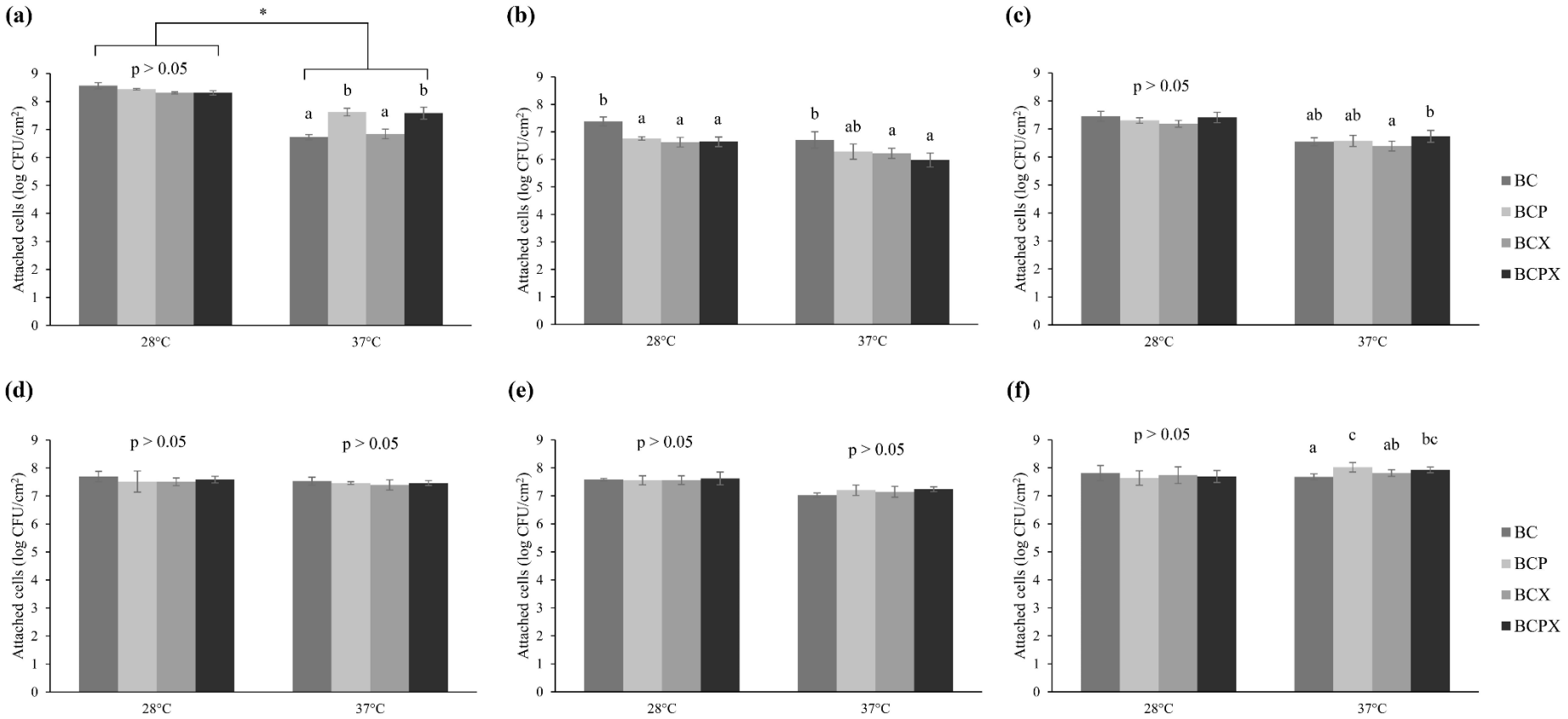


Figure 5.3 Attachment of *S.* Typhimurium ATCC 14028 (a) wild type, (b) Δ*fliC fljB* mutant, (c) Δ*bcsA* mutant, (d) Δ*csgA* mutant, (e) Δ*csgA bcsA* mutant and (f) Δ*csgD* mutant grown at 28°C and 37°C to various BC composites (BC, BCP, BCX, BCPX). Different lowercase letters indicate significant differences in attachment numbers between different BC composites within each strain grown at a specific temperature (One-way ANOVA & Tukey's pairwise comparison at $p<0.05$). Asterisk signs indicate significant differences in attachment numbers for the same strain grown at two different temperatures (28°C and 37°C) (Independent samples t-test at $p<0.05$).

5.3.3 Role of cell surface structures in *Salmonella* attachment to BC composites

An overall comparison on the average number of cells of the *S.* Typhimurium ATCC 14028 wild type and mutant strains attached to the BC composites are presented in Figure 5.4. These results indicated that overall the strains attached in significantly different numbers to the composites from each other ($p<0.05$). Specifically, after growth at 28°C the wild type strain attached in significantly higher numbers as compared to all mutants ($p<0.05$). This indicates that cellulose, curli and flagella were all involved in the attachment of *S.* Typhimurium to the PCW models. Lapidot and Yaron (2009) suggested that bacterial surface appendages, such as flagella and curli, may influence the initial reversible adhesion to plants, which is mediated by van der Waal interactions and hydrogen bonds. This initial adhesion is followed by stronger irreversible attachment which is mediated by electrostatic forces and dependent on extracellular components, such as bacterial cellulose.

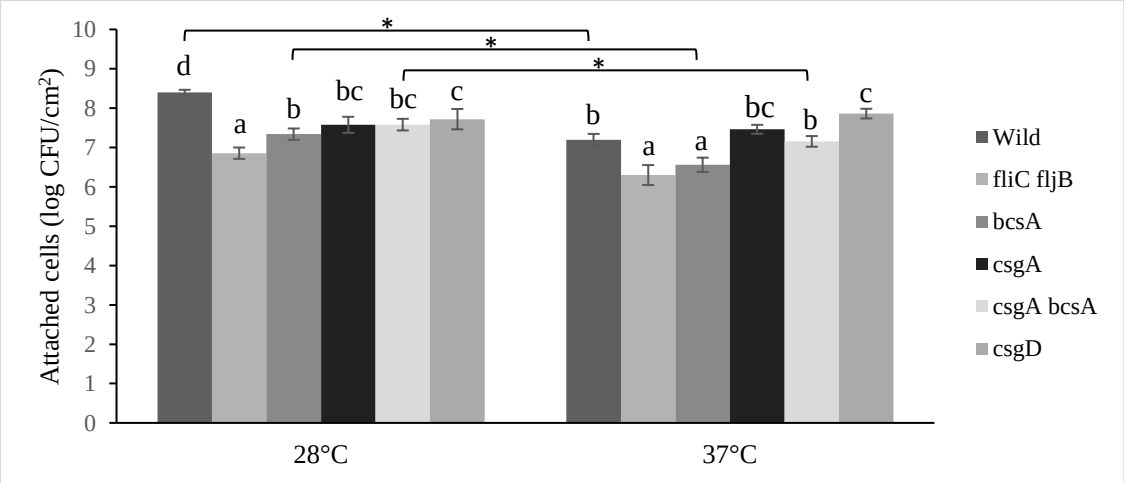


Figure 5.4 Average values obtained for attachment of *S.* Typhimurium ATCC 14028 wild type and mutant strains to various BC composites (BC, BCP, BCX, BCPX). Different lowercase letters indicate significant differences in bacterial attachment between different strains grown at the same temperature (One-way ANOVA & Tukey's pairwise comparison at $p<0.05$). Asterisk signs indicate significant differences in attachment numbers for the same strain grown at two different temperatures (28°C and 37°C) (Independent samples t-test at $p<0.05$).

5.3.3.1 Role of flagella

Of the bacterial surface structures studied, flagella appeared to have the most important role in attachment as the Δ*fliC fljB* mutant attached at the lowest number of all mutants to PCW models when grown at both temperatures. As compared to the wild type, attachment of the Δ*fliC fljB* mutant was reduced by ~1.5 and 0.9 log CFU/cm^2 when grown at 28^{o}C and 37^{o}C, respectively. Flagella are known to be important for the motility of *S.* Typhimurium cells (Moens and Vanderleyden, 1996) and allow the bacteria to reach the attachment surface faster (Reina et al., 2002). Flagella also mediate chemotaxis which can guide planktonic cells to swim towards sites with nutrients or towards cells attached to a surface (Pratt and Kolter, 1998). Similarly, motility enables pathogens to enter and colonize stomata, wounds and openings in plants (Kroupitski et al., 2009b). Flagella and motility mutants of *S.* Typhimurium failed to invade lateral root junctions of the *Arabidopsis thaliana* plants, which may be explained by the inability of the mutants to find entry points into the plant (Cooley et al., 2003). It was also found that mutations which impaired bacterial motility also reduced the ability of *Salmonella* to be internalized by plants (Kroupitski et al., 2009b). We suggest that flagella enable the cells to move within the matrix of the BC composites where attachment can occur. There is also a possibility that the long flagella filament (which extends up to 20 μm) may cause entanglement of *Salmonella* cells (~2 μm) within the BC matrix. A study by Berger et al. (2009) has shown that *S.* Senftenberg requires flagella to attach to salad leaves. In contrast to the results of our study, these authors found that the deletion of the *fliC* gene did not affect *S.* Typhimurium attachment to basil leaf epidermis. The different outcome of this previous study and ours may be due to the fact that the *fljB* gene was not deleted in the previous study. The normal expression of *fljB* (which encodes the phase-2 flagellin) could have substituted for the loss of phase-1 flagellin (encoded by *fliC*) and thus the ability of the *S.* Typhimurium to attach was not affected in their study. In

addition to the use of flagella for motility and chemotaxis, *S.* Typhimurium also uses the flagella to sense external environment in order to regulate its own biogenesis and virulence (Wang et al., 2005). It has been demonstrated that the flg22 peptide conserved in the *Salmonella* flagellin activates the plant immune system which then inhibits *Salmonella* colonization. Iniguez et al. (2005) showed that the *S.* Typhimurium Δ*fliC fljB* mutant were more successful in colonizing roots in alfalfa, wheat and *Arabidopsis* plants and this could be due to the inability of the plant defence system in detecting colonization by the pathogens. Although the role of flagella in *Salmonella* attachment to PCWs could be studied using the PCW models and cut plant material, these models could not be used to investigate plant response to *Salmonella* cells and this is a limitation of our study.

5.3.3.2 Roles of cellulose and curli

In addition to the importance of flagella, our results also showed that cellulose and curli were involved in the attachment of *S.* Typhimurium to PCWs. Single and double mutants of cellulose and curli (Δ*bcsA*, Δ*csgA*, Δ*csgA bcsA*) which were grown at 28^{o}C all displayed significantly lower attachment compared to the wild type strain ($p<0.05$). When these mutants were grown at 37^{o}C, the Δ*csgA* and Δ*csgA bcsA* attached in similar numbers as compared to the wild type ($p>0.05$). Only the Δ*bcsA* mutant attached at a significantly lower level ($p<0.05$). The expression of cellulose and curli which are regulated by the *csgD* gene are known to contribute to aggregative multicellular behaviour, biofilm formation and protection against harsh environmental conditions (Gu et al., 2011; Shaw et al., 2011). The *csgD* gene is also involved in the synthesis of O antigen capsule which has been shown to modulate bacterial attachment to plants (Barak et al., 2007). Some studies have shown that cellulose and curli were involved in *Salmonella* attachment to parsley and alfalfa sprout seedlings (Barak et al., 2007; Lapidot and Yaron, 2009). Solomon et al. (2005) found that high proportions of *Salmonella* strains isolated from fresh produce possess cellulose and

curli, which further supports this finding. Lapidot and Yaron (2009) showed that curli was more important than cellulose in *S.* Typhimurium transfer from contaminated irrigation water to parsley. Another study by Barak et al. (2007) showed that the roles of cellulose and curli in attachment are additive, whereby initial attachment of the Δ*csgB bcsA* double mutant was further reduced compared with the Δ*bcsA* single mutant. Barak et al. (2005) also observed that mutation in *csgB*, not *csgA*, reduced the ability of *S.* Newport to attach to alfalfa sprouts. The *csgA* gene (with the ordered locus name STM1144) codes for a major curlin subunit. The *csgBA* operon encodes for the CsgA protein which is secreted as an unpolymerized protein into the extracellular environment and the CsgB protein which has been proposed to induce polymerization of CsgA at the cell surface (Barnhart and Chapman, 2006). They suggested that the curli fimbrial nucleator (encoded by *csgB*) may facilitate the initial attachment of *Salmonella* to plants even without the production of curli. The published data of the role of curli in promoting binding to alfalfa has yet to be fully clarified as there are some significant differences between reported studies. Curli is known to form cross-links between bacterial cells but it might not necessarily bind directly to a target receptor in the same manner as a classic adhesin. The amyloid fibre could promote binding of another fimbrial adhesin, however this needs to be further investigated.

Besides being involved in the regulation of cellulose and curli expression, the *csgD* gene is also involved in the synthesis of the O antigen capsule and colanic acid which have been shown to modulate bacterial attachment to plants (Barak et al., 2007). Colanic acid has been found to be associated with curli in *E. coli* and is involved in the formation of *Salmonella* biofilms on animal cells. The O antigen capsule has been shown to protect bacterial cells from desiccation (Gibson et al., 2006). It was interesting to note that in our study the Δ*csgD* mutant grown at both 28^{o}C and 37^{o}C had significantly higher ability to attach to the composites as compared to the Δ*bcsA* mutant ($p<0.05$). Studies (Marshall and

Gunn, 2015; Ogasawara et al., 2011) have shown that Δ*csgD* mutants lacking cellulose and curli have increased *fliE* (encoding the flagellum basal body) promoter activity and production of FliC protein as compared to wild type cells. This can be explained as the synthesis of cellulose and curli or flagella production are mutually exclusive from each other due to opposite regulation by the signalling molecule cyclic di-GMP (Mika and Hengge, 2013). Increased production of flagella-related genes could therefore have helped the Δ*csgD* mutant to attach better to the BC composites. Further studies are required to confirm this.

5.4 Conclusions

Taken together the results of our study demonstrate that *S.* Typhimurium cells grown in the animal host (at 37ºC) do not produce cellulose and curli and that these biofilm-forming structures will only form if the pathogens are released into the external environment which has a lower temperature (e.g.: 28ºC). Flagella, curli and cellulose all contribute to the interaction of *Salmonella* with intact plants in the environment, but we have shown that these components are not the only factors most important for the attachment of *Salmonella* to the BC composites which may be influenced by many other factors.

Chapter 6

The Potential Use of Sonication to Reduce *Salmonella* Attachment to Plant Cell Walls

The work presented in this chapter represents the following manuscript submitted for peer review:

Tan, M.S.F., Rahman, S. and Dykes, G.A. (Submitted). Sonication reduces the attachment of *Salmonella* Typhimurium ATCC 14028 cells to bacterial cellulose-based plant cell wall models and cut plant material. Food Microbiol.

Declaration for Thesis Chapter 6

Declaration by candidate

In the case of chapter 6, the nature and extent of my contributions to the work was as following:

Nature of contribution	Extent of contribution (%)
I was responsible for 100% of data analysis and 90% of drafting and writing	90%

The following co-authors contributed to the work and the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%)
Sadequr Rahman	SR was responsible for 2% of writing and review of the manuscript.	2%
Gary Dykes	GD was responsible for 8% of writing and review of the manuscript.	8%

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work*.

Candidate's Signature		**Date**
Main Supervisor's Signature	[redacted]	**Date**

*Note: Where the responsible author is not the candidate's main supervisor, the main supervisor should consult with the responsible author(s) to agree on the respective contributions of the authors.

6 The Potential Use of Sonication to Reduce *Salmonella* Attachment to Plant Cell Walls

6.1 Introduction

One of the main problems in the fresh produce industry is cross-contamination that occurs during fresh-cut processing operations which involve cutting, shredding, washing and packaging of the produce (Artés-Hernández et al., 2013). According to the World Health Organization (WHO), none of the decontamination methods commonly used today can achieve complete elimination of microorganisms on fresh produce without affecting its organoleptic quality (López-Gálvez et al., 2010).

In the fresh-cut industry, generally a pre-washing step is performed by dipping fresh produce in water to remove dust and tissue exudates (López-Gálvez et al., 2010). This is then followed by a washing step involving chemical sanitizers for decontamination of the produce. Since water is used extensively in most aspects of fresh produce processing, the fresh-cut industry tend to recycle wash water in order to minimize water consumption and to save costs. Reusing processing water may result in the build-up of microbial densities and cause cross-contamination problems, whereby a small lot of contaminated produce can cause the contamination within a large batch and among sequentially washed batches (Artés-Hernández et al., 2013; Gil et al., 2009). Cross-contamination can also occur through direct contact with the contaminated product or through indirect contact with contaminated equipment surface (Doyle and Erickson, 2008; Kaneko et al., 1999; Moore et al., 2003).

Currently, chlorine is most commonly used in the fresh-cut industry for the sanitation of fresh produce (Aruscavage et al., 2006; Parish et al., 2003; Seymour and Appleton, 2001). However, chlorine has limited decontamination effectiveness and was only effective in keeping the wash water free of contaminants and for preventing cross-contamination

(Aruscavage et al., 2006; Delaquis et al., 2002; Takeuchi et al., 2000). Besides, chlorine-based sanitizers are corrosive, cause skin and respiratory tract irritations and may form carcinogenic by-products (Ölmez and Kretzschmar, 2009). The use of chlorine also caused environmental concerns and has been banned in a few European countries (São José et al., 2014). Therefore, there is a need to find better methods to decontaminate fresh produce. One of the emerging technologies is ultrasonication which has been used to clean equipment surfaces and reduce microbial contamination in the medical and poultry industries but this technology has not yet seen widespread use in the fresh produce industry (Rodgers and Ryser, 2004).

In this study, we investigated the importance of cell surface components in *Salmonella* attachment to cut surfaces of plant cell walls (PCW). The attachment of *S.* Typhimurium ATCC 14028 cells, which had their flagella and other surface structures removed through sonication, on cut plant materials and bacterial cellulose (BC)-based PCW models were then evaluated. Besides, the potential of sonication, as a proof of concept, to be applied in the processing steps of the fresh produce industry was also evaluated.

6.2 Materials and methods:

6.2.1 Bacterial strains

S. Typhimurium ATCC 14028 and *G. xylinus* ATCC 53524 were obtained from the same sources and grown in the same way as mentioned earlier in Section 2.2.1.

6.2.2 Production of BC-based PCW models

The BC-based PCW models were produced and prepared as described earlier in Section 2.2.2, a variety of the PCW models were produced with different combinations of pectin and/or xyloglucan added into the HS medium:

- BC
- BC-Pectin (BCP) with 0.5% w/v pectin and 12.5 mM $CaCl_2$
- BC-Xyloglucan (BCX) with 0.5% w/v xyloglucan
- BC-Pectin-Xyloglucan (BCPX) composites produced by adding 0.25% w/v pectin, 0.25% w/v xyloglucan and 12.5 mM $CaCl_2$

6.2.3 Preparation of cut plant material

Potato (*Solanum tuberosum*) tuber, apple (*Malus domestica*) fruit and lettuce (*Lactuca sativa*) leaves were obtained from a retail outlet in Selangor, Malaysia. These three plant tissues were used since they represent a range of different plant species that *Salmonella* have been isolated from and have also been associated with cases of salmonellosis (Beuchat, 2002; Quiroz-Santiago et al., 2009). The outer surfaces of the potato, apple and lettuce leaves were lightly wiped down with 70% ethanol solution before cutting out approximately 1.5 cm x 1.5 cm pieces of the plant materials with a sterile scalpel. The surfaces of the lettuce leaves were lightly grazed to expose the upper and lower epidermis to resemble cut surfaces of fresh produce after fresh-cut processing.

6.2.4 Preparation of bacterial suspensions and sonication treatment

Early stationary phase culture of the *S.* Typhimurium ATCC 14028 strain was prepared to a concentration of 8 log CFU/mL using protocols described in Section 2.2.3. Universal bottles each carrying 10 mL *S.* Typhimurium ATCC 14028 suspensions (8 log CFU/mL) were subjected to sonication (20 kHz at 20% amplitude) for a series of time periods (5 s, 15 s, 30 s, 45 s, 60 s and 90 s) at 25^{o}C with an ultrasonic processor (Cole Palmer 750-Watt, 115 VAC, 50/60 Hz, USA). Flagella counts and hanging drop motility assays were performed on the sonicated bacterial suspensions. Flagella counts of the cell suspensions were determined using Ryu's flagella stain in combination with direct microscopic count.

Ryu's flagella stain was prepared according to protocols described by Kodaka et al. (1982). Briefly, 50 fields were selected randomly and examined using a compound microscope (Motic, BA 200, Wetzlar, Germany) at 1000x magnification. Each field was taken to quantitatively represent 2% of the population; flagella was regarded to represent in 2% of the population when they were present within a field (2%) whereas flagella was regarded as being absent in 2% of the population when they were not observed within a field (0%). The percentage of flagella in a sample was estimated by summing up the percentages for all 50 fields. Hanging drop motility method was used as an additional assay to ascertain that bacterial motility was reduced after sonication treatment. Cell viability was tested after sonication by serial dilution and spread plating on xylose lysine deoxycholate agar (XLDA; Oxoid, UK).

From this preliminary study, a suitable parameter used to sonicate bacterial cells was selected based on the conditions that bacterial surface appendages (including flagella) can be sufficiently removed from the cells, bacteria had reduced motility and that cell viability was not affected by the sonication process. This is to ensure that the effect of sonication on bacterial attachment was solely due to the disruption of bacterial surface structures.

6.2.5 Attachment study

Based on the results from preliminary tests (Appendix IV), sonication for 60 s at 20 kHz was chosen as the suitable condition for subsequent attachment studies. Each rinsed BC composite or cut plant material was incubated in 10 mL of sonicated *S.* Typhimurium suspension (8 log CFU/mL) for 20 min with gentle shaking (100 rpm) at 25^{o}C. This was followed by gentle rinsing (100 rpm) in 6 mM $CaCl_2$ for 1 min to remove loosely attached cells. Each composite was then placed in a stomacher bag with 50 mL PBS and pummelled for 1 min at 8 strokes/s in a stomacher (BagMixer 400; Interscience, France). The number of

Salmonella cells attached to the BC composite was enumerated by serial dilution of the stomached fluid followed by the plating of appropriate dilutions on XLDA. Numbers of attached bacterial cells were expressed as CFU/cm^2 composite. The experiment was performed in triplicate (three independently grown bacterial cultures).

6.3 Results and discussion

6.3.1 Flagella count and cell viability after sonication

S. Typhimurium ATCC 14028 cells showed a reduction in flagella counts after being subjected to sonication. Flagella were visibly sheared into small fragments (Figure 6.1). After 5 s of sonication, fewer flagella were observed in the population but flagella counts did not change significantly ($p>0.05$) at any sonication time up to and including 45 s (Figure 6.2). When cells were sonicated for 60 s there was a significant ($p<0.05$) decrease in flagella counts to ~55%, a level which then did not change significantly ($p>0.05$) until 90 s at which time point the experiment was terminated (Figure 6.2). The results of the hanging drop method indicated that the bacterial cells were less motile after 60 s compared to the control. Viability of the *S.* Typhimurium cells was not affected after 60 s of sonication treatment but was significantly ($p<0.05$) reduced after 90 s of sonication (Figure 6.2). For this reason, sonication at 20 kHz for 60 s was selected as the parameter used to treat *S.* Typhimurium cells for subsequent attachment assays as flagella could be sheared without compromising cell viability.

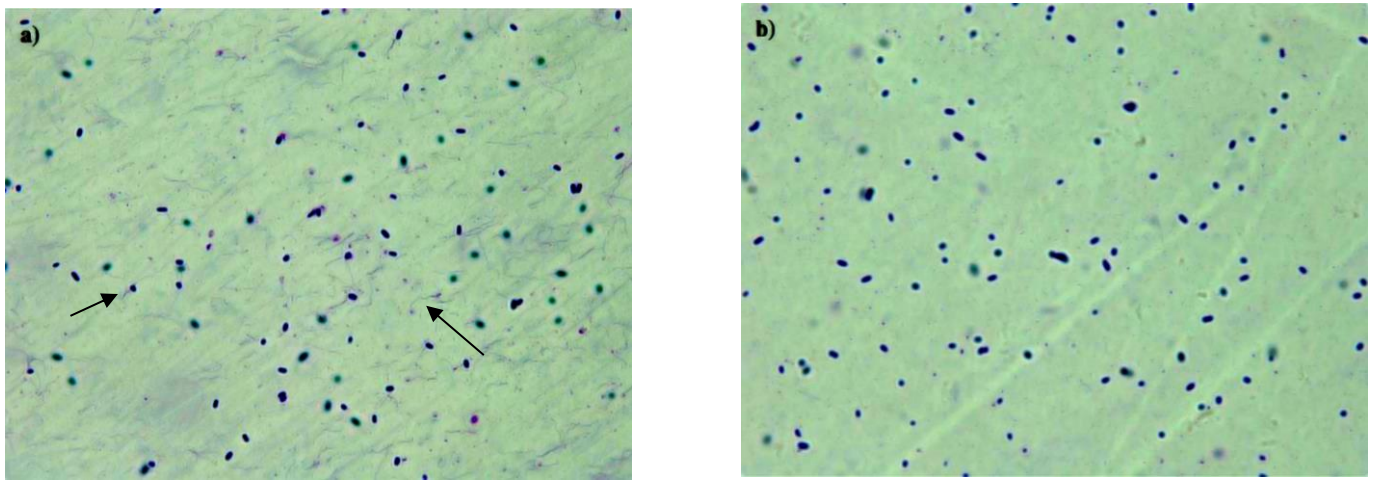


Figure 6.1 (a) Flagella (indicated by arrows) of *S.* Typhimurium ATCC 14028 cells stained with Ryu's stain before sonication and (b) no flagella observed after 60 s of sonication at 20 kHz.

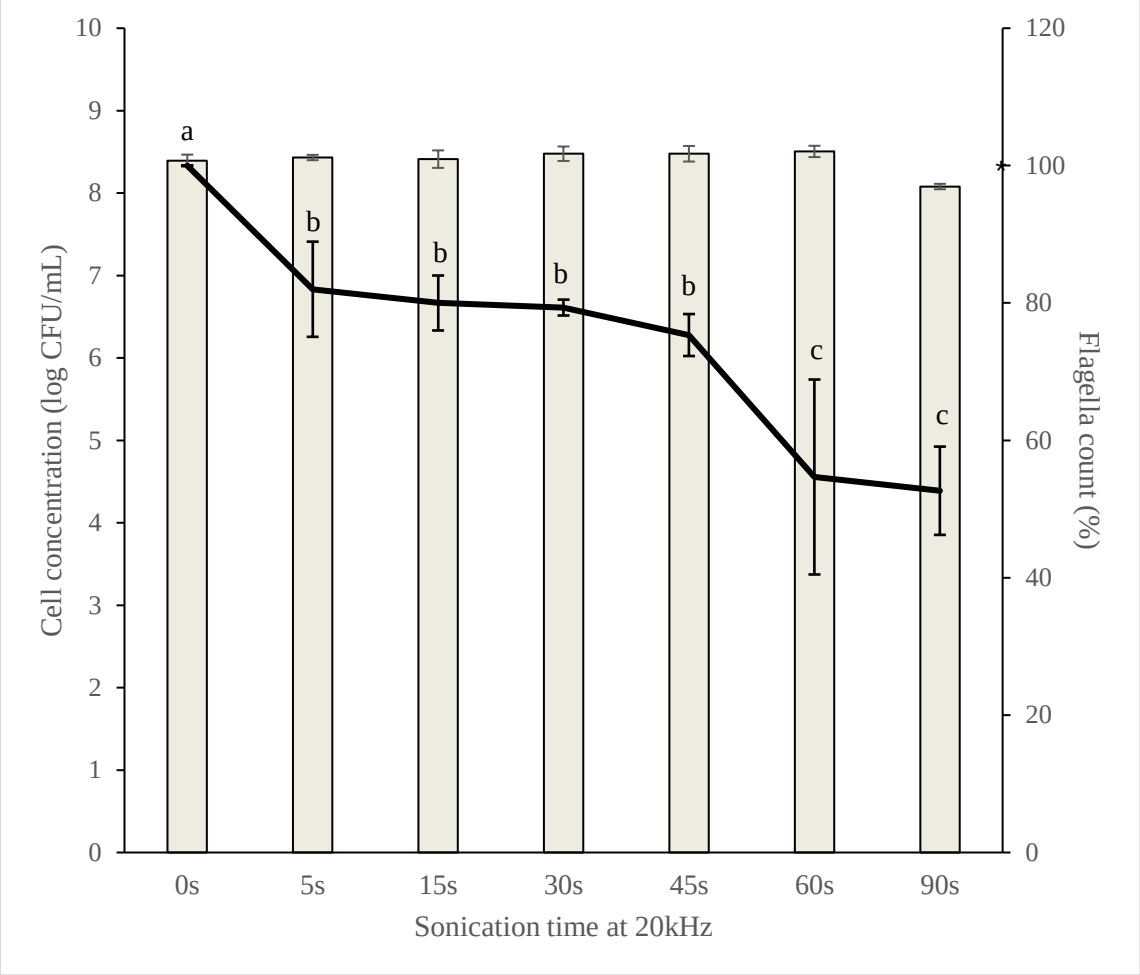


Figure 6.2 *S.* Typhimurium ATCC 14028 cell viability (log CFU/mL) and flagella count (%) after sonication at 20 kHz over a series of duration. Different lowercase letters indicate significant differences in flagella counts between cells sonicated for different time periods. Asterisk sign indicates significant reduction in cell viability compared to the control *S.* Typhimurium cells which were not subjected to sonication.

6.3.2 Attachment assays with *S.* Typhimurium ATCC 14028 cells before and after sonication

The overall attachment of the *S.* Typhimurium strain was similar to all BC composites ($p>0.05$). Treatment with sonication significantly reduced the attachment abilities of *S.* Typhimurium ATCC 14028 cells to attach to all BC composites and cut plant materials except to the BC ($p<0.05$) (Figure 6.3). Since sonication was carried out at a level that does not affect cell viability, reduced attachment ability of the sonicated *S.* Typhimurium cells is most probably due to the disruption of bacterial surface structures which include flagella and curli (Jimenez-sanchez et al., 2012; Sojar et al., 1997).

We have previously highlighted the importance of flagella (in Chapter 5) for bacterial motility and bringing bacterial cells close enough to the substrata surface for attachment to occur. This chapter confirms the role of flagella in attachment as our earlier observation (Chapter 5) showed that the attachment of *fliCfljB* mutants (lacking flagella) grown at 37ºC were also significantly lower ($p<0.05$) to most BC composites compared to the wild type. The loss of cell surface structures, including flagella, did not affect the attachment of *S.* Typhimurium cells to BC only, whereas its attachment to other BC composites and plant material containing either of these PCW components was reduced. Similarly, our previous results (in Chapter 5) showed that the *fliCfljB* mutant attachment in similar numbers to BC when compared to the wild type strain, however, its attachment to other BC composites were significantly lowered. This again suggests that bacterial surface structures, particularly flagella, could have specific interactions with pectin and xyloglucan molecules.

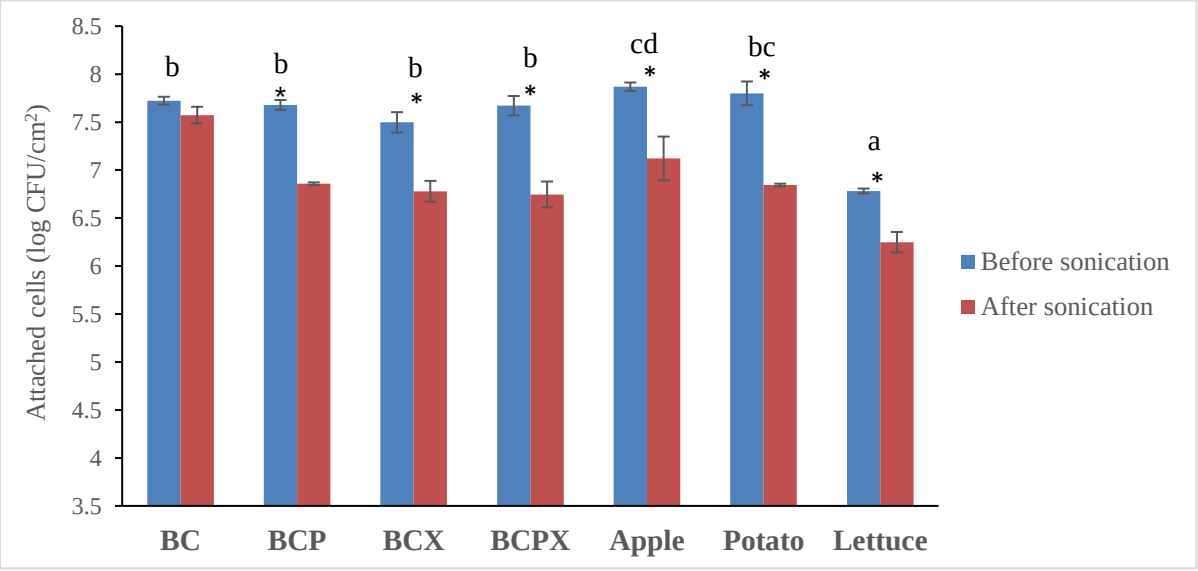


Figure 6.3 Attachment of *S.* Typhimurium ATCC 14028 cells (log CFU/cm^2) to BC composites (BC, BCP, BCX, BCPX) and native plant tissues (apple, potato, lettuce) before and after sonication (20 kHz for 60 s). Different lowercase letters indicate significant differences in attachment numbers between different attachment surfaces. Asterisk signs indicate significant differences in attachment numbers before and after sonication for the same attachment surface.

Among the various attachment surfaces, lettuce had the lowest number of bacteria attached to its surface. The lettuce samples used in the attachment study was much thinner compared to the other BC composites and cut plant materials. Hence, the thin lettuce leaves have lower surface area for bacteria to attach (Katsikogianni and Missirlis, 2004). In another study by Hassan and Frank (2004), they found that there were more *E. coli* O157:H7 cells attached to lettuce leaves (~6.5 log CFU/cm^2) compared to apple skins (~6.2 log CFU/cm^2). The difference in the results from both their study and ours can be explained as the apple tissues we used were much thicker compared to the apple peel used in their study.

Sonication is useful for microbial inactivation and acts by disrupting bacterial cell membranes, breaking cell walls and causing damage to DNA by producing free radicals (Butz and Tauscher, 2002; Mason et al., 2003). In our study we were able to achieve ~0.5 to 1 log CFU/cm^2 reduction in bacterial attachment just by detaching bacterial surface structures, especially shown with the flagella. We expect greater microbial reduction when higher frequency and duration of ultrasound is used and the application of ultrasound for this purpose should be further investigated. Other studies have found that sonication works best in combination with other processing steps for microbial decontamination of fresh produce. Ajlouni et al. (2006) showed that sonication did not significantly reduce microbial load on Cos lettuce when the vegetable tissues were sonicated in wash water. A study by Seymour et al. (2002) found that sonication in a chlorine solution increased the antimicrobial activity of the chlorine with respect to the removal of *S.* Typhimurium cells on iceberg lettuce, but was not effective in reducing microbial load during large-scale trials. Wong et al. (2012) reported that sonication alone was insufficient to reduce *Salmonella* levels in orange juice but a significant reduction in microbial levels was achieved when sonication was used in combination with storage at high osmotic pressure. Sagong et al. (2011) found that combining ultrasound with organic acids resulted in an additional 0.8 to 1.0 log reduction of

foodborne pathogens compared to the individual treatments. Johnson et al. (1998) and Rediske et al. (1999) combined sonication (70 kHz) with the antibiotics gentamicin sulfate and erythromycin, respectively and observed enhanced reduction of bacterial cell numbers as sonication increased the diffusion of the antibiotic through the cell membrane. The effectiveness of using sonication in combination with other processing steps, such as heat and pressure, should also be tested.

6.4 Conclusions

Findings from our study serve as a proof of concept which supports the use of sonication in processing fresh produce. Ultrasonic processing is becoming important for food processing and this technology can be scaled up for commercial use to generate enough income to cover investments (São José et al., 2014). Unlike chlorine which is most commonly used in the fresh produce industry, this technology does not compromise food organoleptic properties, corrode equipment surfaces nor exude carcinogenic by-products (São José et al., 2014). The sonication process can be applied at bactericidal levels to treat wash water used for fresh produce sanitation and the process water can be recycled back into the system again after another round of sonication (Elizaquível et al., 2012). This helps to save cost, reduce wastewater usage and is more environmentally-friendly compared to the conventional method using chlorine (Ajlouni et al., 2006; Elizaquível et al., 2012). In order for this technology to be applied by the fresh-cut industry, it is important to optimize the sonication conditions to achieve satisfactory decontamination levels without causing physical damage to the produce. Therefore, sonication treatment at a non-bactericidal level which was sufficient to remove bacterial surface components (including flagella) is important as sonication at this level is less aggressive and can be applied together on fresh produce in

wash water. Large-scale trials using commercial sonicators and further investigations testing the effectiveness of sonication in reducing microbial load when fresh produce is sonicated together with the wash water are required.

Chapter 7

General Discussion and Conclusions

7 General Discussion and Conclusions

7.1 Major findings and contribution of this study

The primary aims of this project were to investigate: (i) the role of chemical components of the plant cell wall (PCW) in the attachment of *Salmonella enterica* to PCWs; (ii) the role that physicochemical properties and other surface properties associated with the bacterial cell and plant surface play in these interactions and (iii) the use of practical methods to reduce *Salmonella* attachment to PCWs.

From the results obtained from this project, we conclude that the attachment of *S. enterica* strains to PCWs is not controlled by a single factor but it is a complicated process involving a combination of different factors. Chemical components in the PCW affect the ability of *Salmonella* strains to attach to the bacterial cellulose (BC)-based PCW models, with pectin alone or pectin in association with xyloglucan significantly increasing *Salmonella* attachment (Chapter 3). Enhanced attachment of *Salmonella* strains in the presence of these PCW polysaccharides was most probably due to the effect of these polysaccharides on the surface morphology of the PCW model. It is unlikely this effect was caused by receptor-ligand interactions involving carbohydrates and bacterial surface adhesins (Chapter 4). *Salmonella* cells were small enough to pass through pores on the PCW model surfaces and attach inside the composites (Chapter 4) and this may enhance the internalization of bacteria into plant tissue. The physicochemical properties of the PCW model and bacterial cell (hydrophobicity and aggregation) did not play a significant role in its attachment (Chapter 3).

The numbers of *Salmonella* cells attaching to PCW models increased linearly with cell concentration (Chapter 2). From the data generated a linear mathematical model that is able to predict the attachment of *Salmonella* cells to natural PCWs (within the range of 3 to 8 log CFU/mL) was constructed. The model was validated for a range of variables (different

plant tissues, *Salmonella enterica* subspecies and other foodborne bacteria) and appeared to be robust (Chapter 2). The use of the model could contribute to assessing the risks associated with the attachment of foodborne pathogens in liquids to cut surfaces of PCWs.

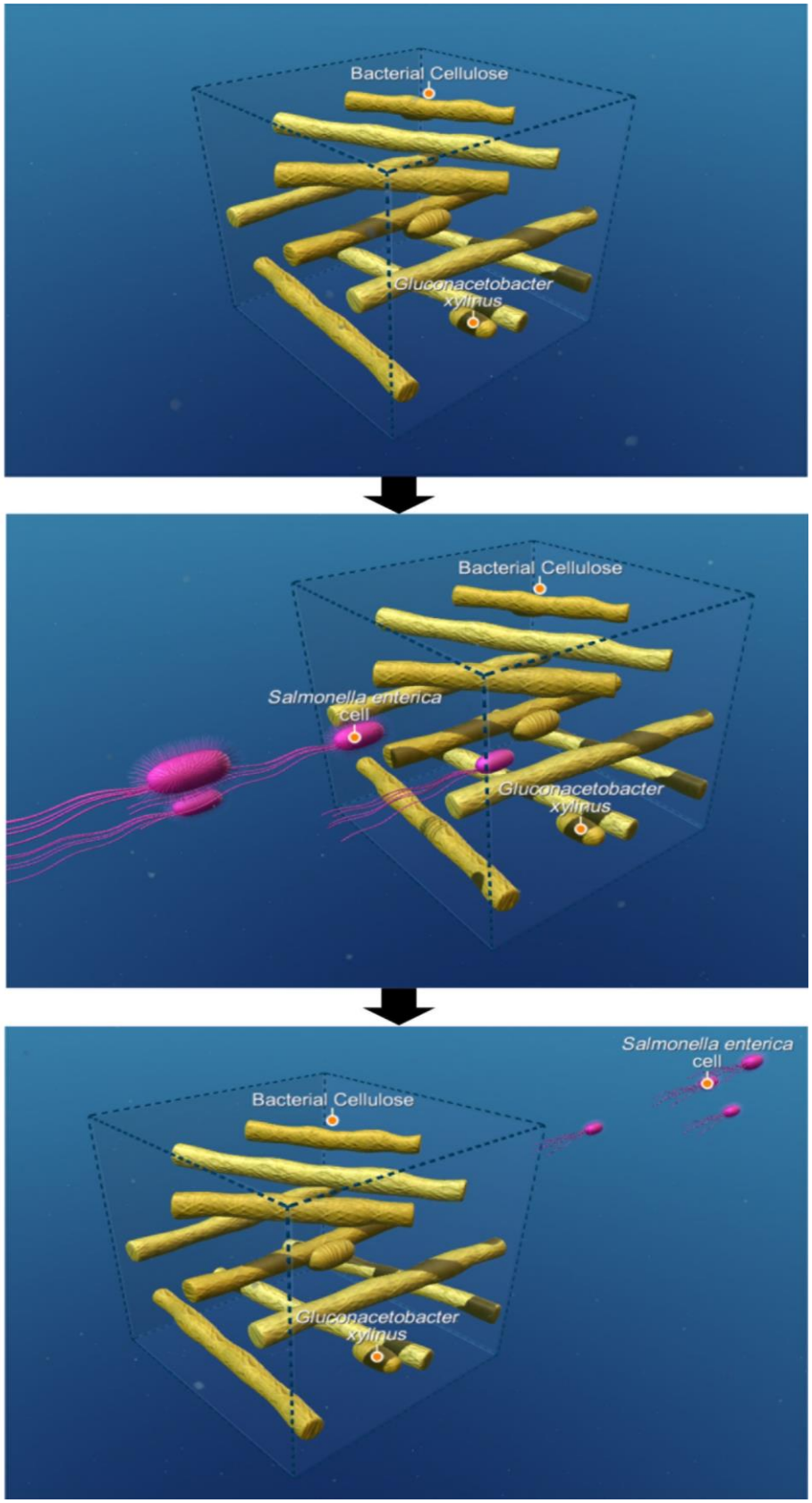
The role of bacterial cell surface components (flagella, curli and cellulose) in the attachment of the *S.* Typhimurium ATCC 14028 strain to PCWs was established (Chapter 5). At 37^{o}C, only flagella were produced whereas the expression of curli and cellulose structures only occurred at a lower temperature (28^{o}C). Of these structures, flagella appeared to play a more important role in *Salmonella* attachment as compared to cellulose and curli (Chapter 5). It is suggested that *Salmonella* use flagella for attachment within the animal host (37^{o}C) and it employs other structures such as curli and cellulose for its attachment only after it is released into the external environment with a lower temperature (28^{o}C).

Using the knowledge gained from this project, removal of key surface structures by sonication was evaluated as a practical method to reduce bacterial contamination on fresh produce (Chapter 6). The intensity of the ultrasonic waves was used at a level which removes bacterial surface structures (particularly flagella) without affecting cell viability. This was sufficient to significantly reduce the ability of sonicated *S.* Typhimurium cells to attach to PCW models and native plant materials (Chapter 6). In the fresh-cut industry, wash water which is commonly reused for the washing of fresh produce can be treated with sonication to reduce the risk of cross-contamination and bacterial attachment. This helps to save cost and reduce wastewater usage (Ajlouni et al., 2006; Elizaquível et al., 2012).

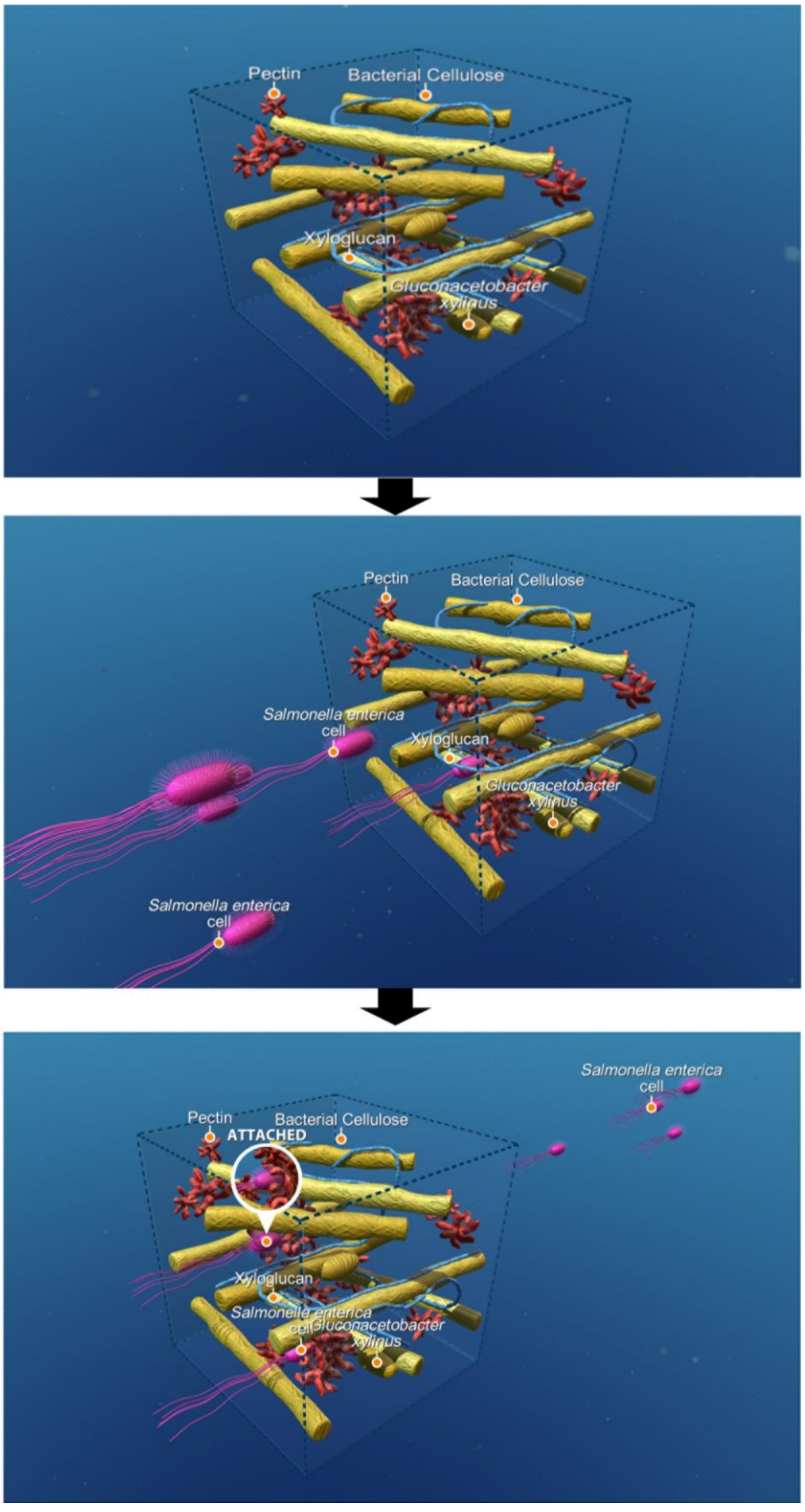
Based on the findings of this study, a visual model of the interaction of *Salmonella* with PCWs has been developed using an interactive software (Unity) to allow better visualization of the interaction. The proposed scenarios on the attachment of *Salmonella* cells

to cross sections of the BC and BCPX composites were summarized and presented in Figure 7.1.

a)


Bacterial Cellulose
Gluconacetobacter xylinus
Bacterial Cellulose
Salmonella enterica cell
Gluconacetobacter xylinus
Salmonella enterica cell
Bacterial Cellulose
Gluconacetobacter xylinus

b)


Pectin
Bacterial Cellulose
Xyloglucan
Gluconacetobacter xylinus
Pectin
Bacterial Cellulose
Salmonella enterica cell
Xyloglucan
Gluconacetobacter xylinus
Salmonella enterica cell
Salmonella enterica cell
Pectin
Bacterial Cellulose
ATTACHED
Xyloglucan
Salmonella enterica cell
Gluconacetobacter xylinus

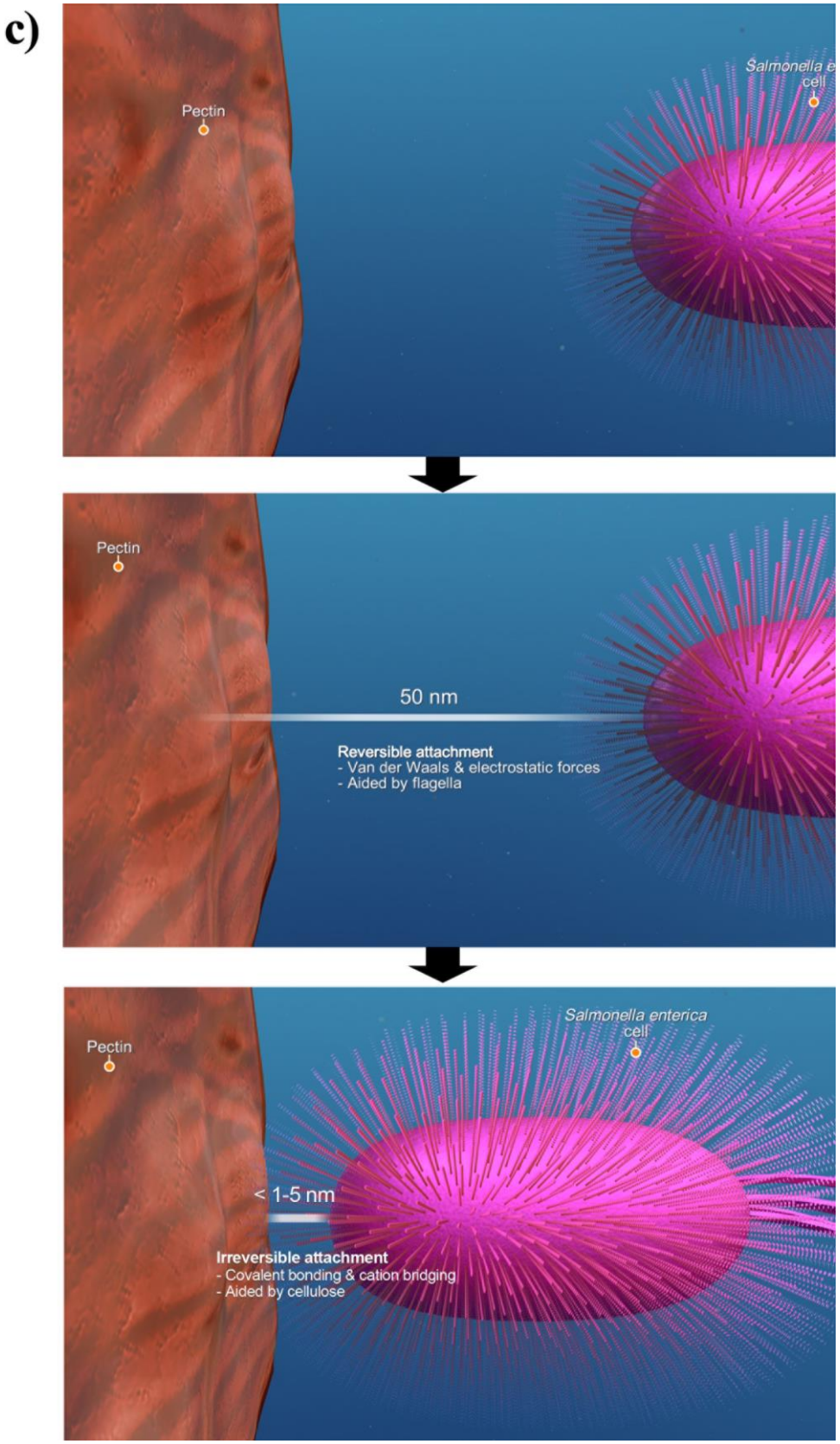


Figure 7.1 Visual model showing a proposed model for attachment of *S. enterica* cells to: (a) a BC composite, (b) a BCPX composite and (c) a close-up view of its reversible and irreversible attachment to the BCPX composite with the forces involved in the process.

7.2 Future directions

This project provides a better understanding on the different factors involved in the attachment of *Salmonella* to PCWs. Listed below are the potential areas of future research that could be conducted to gain more insight into these interactions in order to help develop effective strategies to minimize bacterial attachment to fresh produce.

7.2.1 Investigate the role of pectin and xyloglucan in the BC network

The additions of pectin and xyloglucan increased the thickness of cellulose fibrils and reduced the porosity of the BC composites. These structural changes could be responsible for the increased attachment of *Salmonella* strains to the PCW model surfaces (Chapter 4). The formation of the BC-based PCW models has been shown to mimic the formation of PCWs in native plants (Chanliaud and Gidley, 1999). With reference to the process of PCW deposition in native plants, it was suggested that xyloglucan increases fibril diameter by coating the cellulose fibrils while pectin has an additional role of filling the gaps between cellulose fibrils. The mechanisms behind this effect have not yet been properly investigated. In future research the specific interactions between pectin and xyloglucan, and the cellulose network should be established in order to understand the broader picture of how these components affect the attachment of foodborne pathogenic bacteria.

7.2.2 Study the effects of sugar molecules on *Salmonella* attachment to plants

In our study we used monosaccharides which make up the PCW polymers to block potential receptor sites on *Salmonella* cells (Chapter 4). We observed that higher concentrations of D-galacturonic acid (only found in pectin of the polymers we studied) reduced *Salmonella* attachment but the effect was not specific to BC composites containing pectin only. The specificity of sugar molecules associated with PCWs as receptor sites for *Salmonella* attachment can be further confirmed using antibodies which have higher specificity to the sugar molecules as compared to the exogenous monosaccharides used in

this study. If found to be specific, these sugars can be used for microbial competitive exclusion of *Salmonella* in plants as has been suggested for application to control this pathogen in poultry (McHan et al., 1990).

7.2.3 Understand the interactions between cell surface components and chemical components of the PCW

As suggested in Chapters 5 and 6, flagella appeared to have specific interactions with pectin and xyloglucan. The loss of flagella (in the *ΔfliC fljB* mutant and through sonication respectively) did not affect *Salmonella* attachment to the BC-only composite whereas its attachment to other BC composites containing pectin and xyloglucan was significantly reduced. These interactions could be further investigated using atomic force microscopy (AFM). The AFM cantilever tip could be functionalized with *S.* Typhimurium cells and scanned over the surface of a variety of PCW models (with/without pectin and/or xyloglucan). Measurements of the force of interaction between the tip and the PCW model surface can be compared between the wild type cells and mutants of different cell surface components. Results from such a study could allow us to better gauge the strength of interactions between surface structures and the PCW components.

The mechanism causing the overall attachment of the Δ*csgD* mutant (which lacks both cellulose and curli) at 37^{o}C to be significantly higher than the wild type to all BC composites is not yet understood (Chapter 5). This feature may be due to increased *fliE* (encoding the flagellum basal body) promoter activity and production of FliC protein when compared to wild type cells (Marshall and Gunn, 2015; Ogasawara et al., 2011). Western blot analysis could be employed to compare the expression of the FliC protein between the Δ*csgD* mutant and wild type cells.

7.2.4 Understand the relationship between *Salmonella* and background microbiota on fresh produce

The mathematical model generated in this study (Chapter 2) to predict *Salmonella* attachment to PCWs has limitations as other factors, such as the presence of plant pathogens and background microbiota, on plant surfaces were not taken into account. Natural microbiota have shown potential to be a means of biocontrol as these microbes inhibit pathogenic attachment to fresh produce (Babic et al., 1997; Carlin et al., 1996; Liao, 2007). It would be useful to include these factors in future attachment studies to better predict the attachment of *Salmonella* strains in practical situations under variable conditions.

7.2.5 Applying sonication in the fresh produce industry

The potential effectiveness of sonication in reducing *Salmonella* attachment to both PCW models and cut plant materials was established (Chapter 6). In order for this technology to be applied in the fresh-cut industry, further work needs to be carried out. This includes large-scale trials using commercial sonicators, sonicating wash water together with fresh produce and also using sonication in combination with other processing steps (such as heat and pressure). Different durations and intensities of the sonication process need to be tested and optimized to achieve satisfactory decontamination without causing physical damage to the produce (São José et al., 2014).

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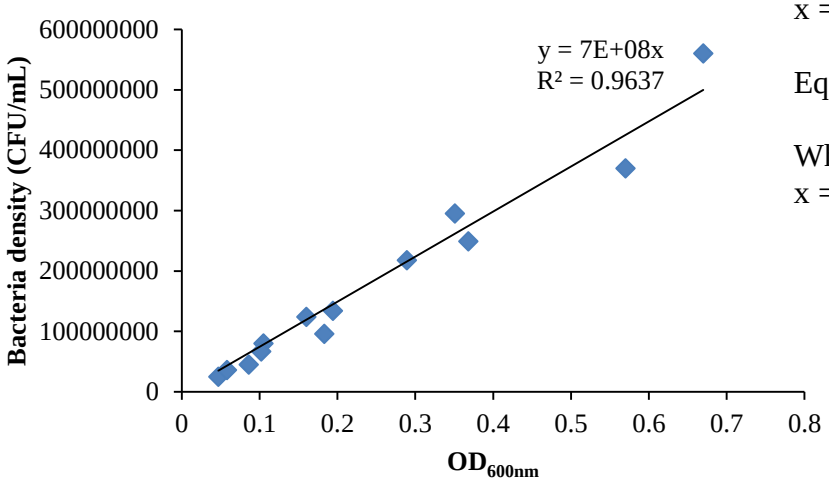
Appendix I

Standard Curves of Optical Density (OD_{600nm}) against Bacterial Density

The following appendix contains results of viable count and optical density of bacterial suspensions (Chapters 2 to 6) to deduce the OD_{600nm} required to obtain a concentration of 8 log CFU/mL.

a) ***Salmonella* Enteritidis ATCC 13076**

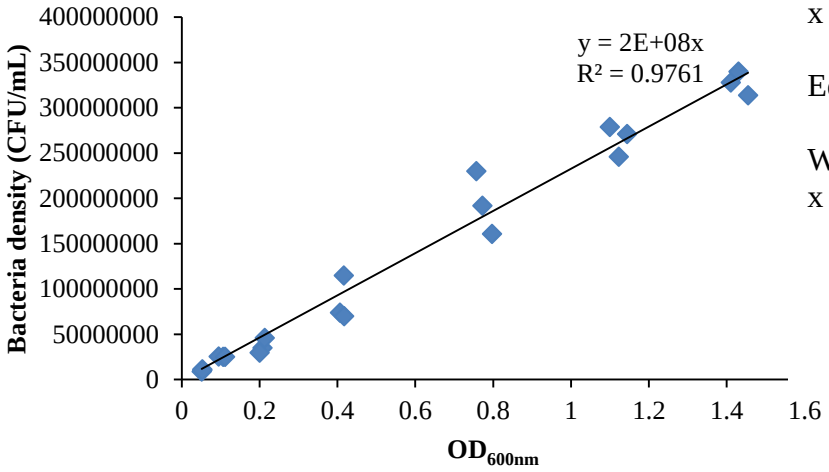


y = bacteria density,
x = OD_{600nm}

Equation: $y = 7(10^8)x$

When y = 8 log CFU/mL,
x = 0.143

b) ***Salmonella* Typhimurium ATCC 10428**

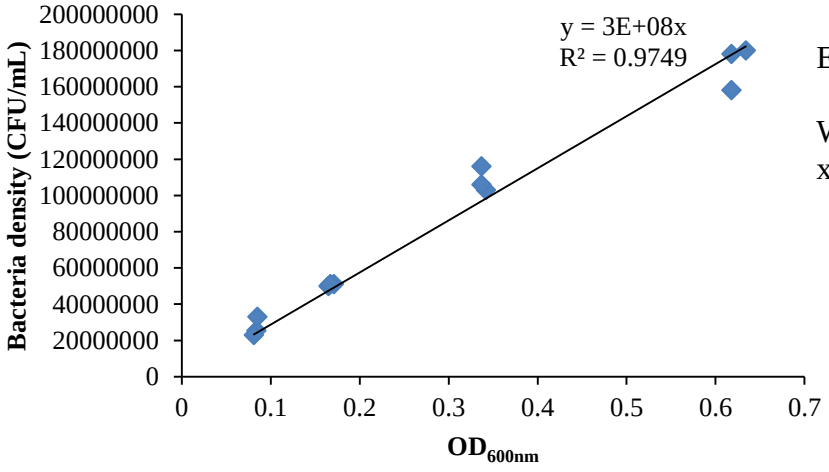


y = bacteria density,
x = OD_{600nm}

Equation: $y = 2(10^8)x$

When y = 8 log CFU/mL,
x = 0.500

c) ***Salmonella indica* M4**

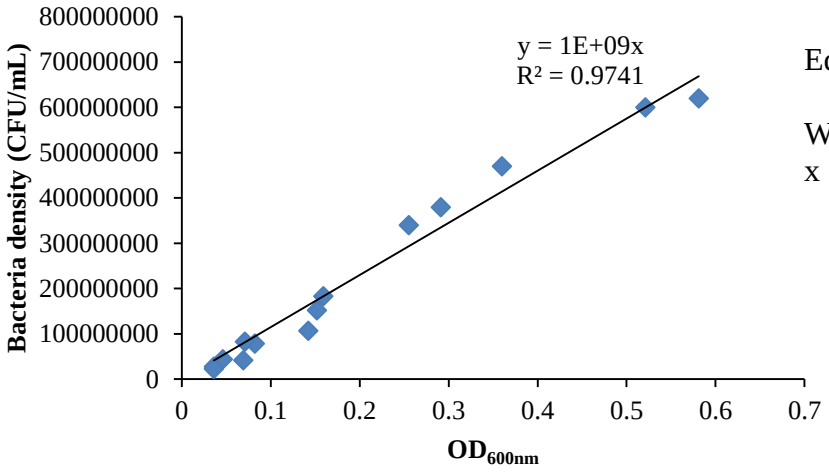


y = bacteria density,
x = OD_{600nm}

Equation: $y = 3(10^8)x$

When y = 8 log CFU/mL,
x = 0.333

d) ***Listeria monocytogenes* ATCC 7644**

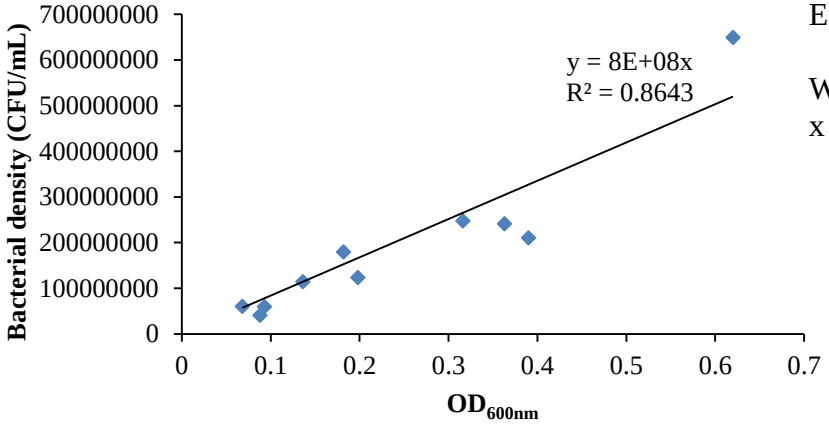


y = bacteria density,
x = OD_{600nm}

Equation: $y = 1(10^9)x$

When y = 8 log CFU/mL,
x = 0.100

e) ***S.* Typhimurium ATCC 14028 Δ*fliC fljB* mutant**

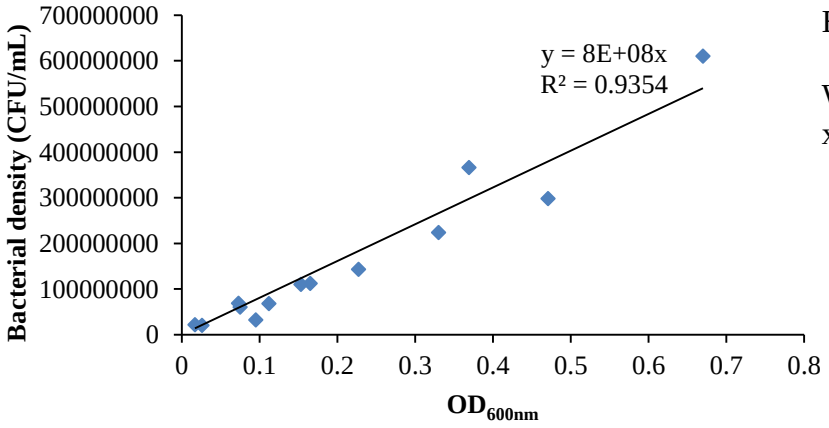


y = bacteria density,
x = OD_{600nm}

Equation: $y = 8(10^8)x$

When y = 8 log CFU/mL,
x = 0.125

f) **S. Typhimurium ATCC 14028 Δ*bcsA* mutant**

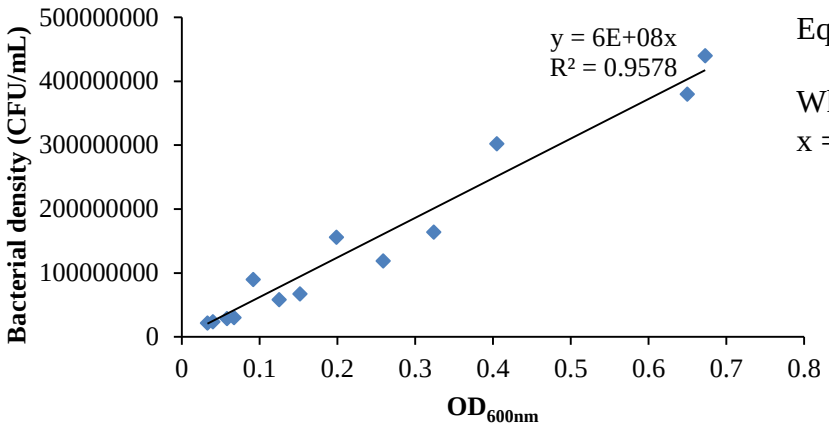


y = bacteria density,
x = OD_{600nm}

Equation: $y = 8(10^8)x$

When y = 8 log CFU/mL,
x = 0.125

g)

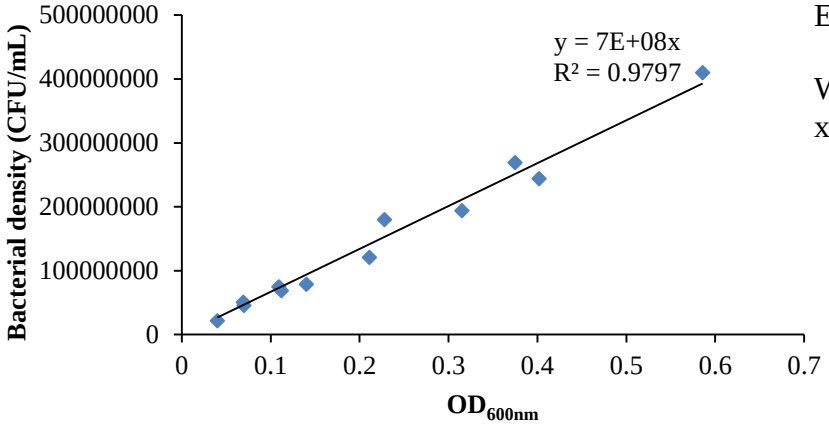


y = bacteria density,
x = OD_{600nm}

Equation: $y = 6(10^8)x$

When y = 8 log CFU/mL,
x = 0.167

h)

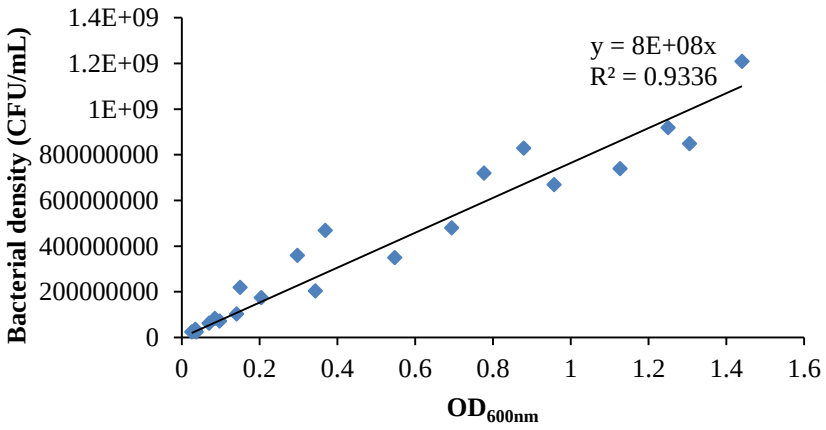


y = bacteria density,
x = OD_{600nm}

Equation: $y = 7(10^8)x$

When y = 8 log CFU/mL,
x = 0.143

i)



y = bacteria density,
x = OD_{600nm}

Equation: $y = 8(10^8)x$

When y = 8 log CFU/mL,
x = 0.125

Figure A.1 Standard curves of OD_{600nm} against bacterial density for different bacterial strains (a—i) and equations derived from the graphs.

Appendix II

Viable Counts of *Salmonella* Cells after Treatment with D-galacturonic Acid

The following appendix contains results of viable counts of *Salmonella* cells (initial concentration of 8 log CFU/mL) after incubation in suspensions prepared with 1% and 2% w/v D-galacturonic acid (Chapter 3).

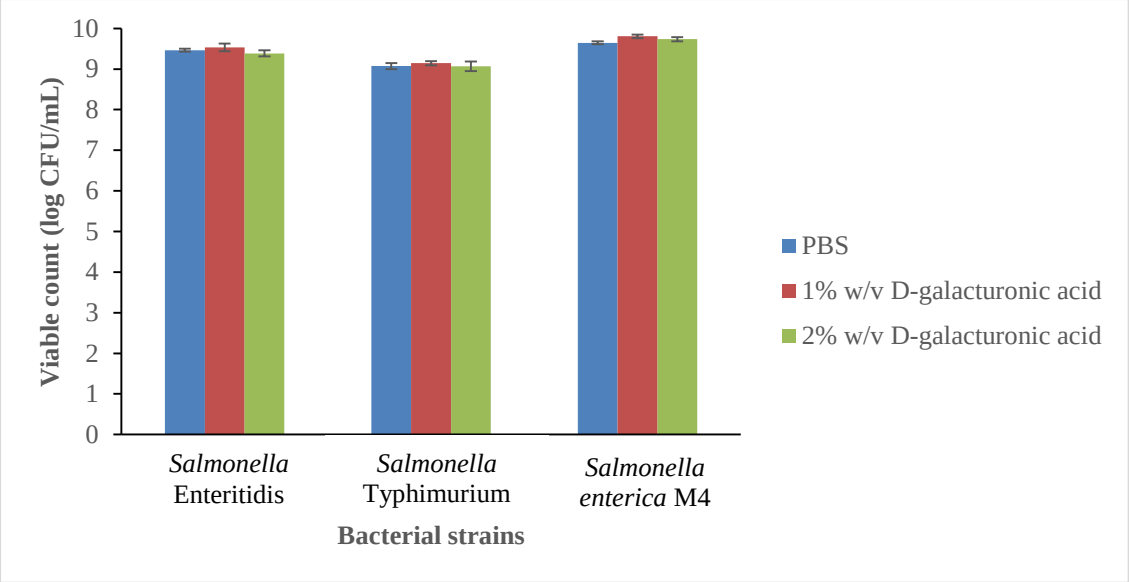
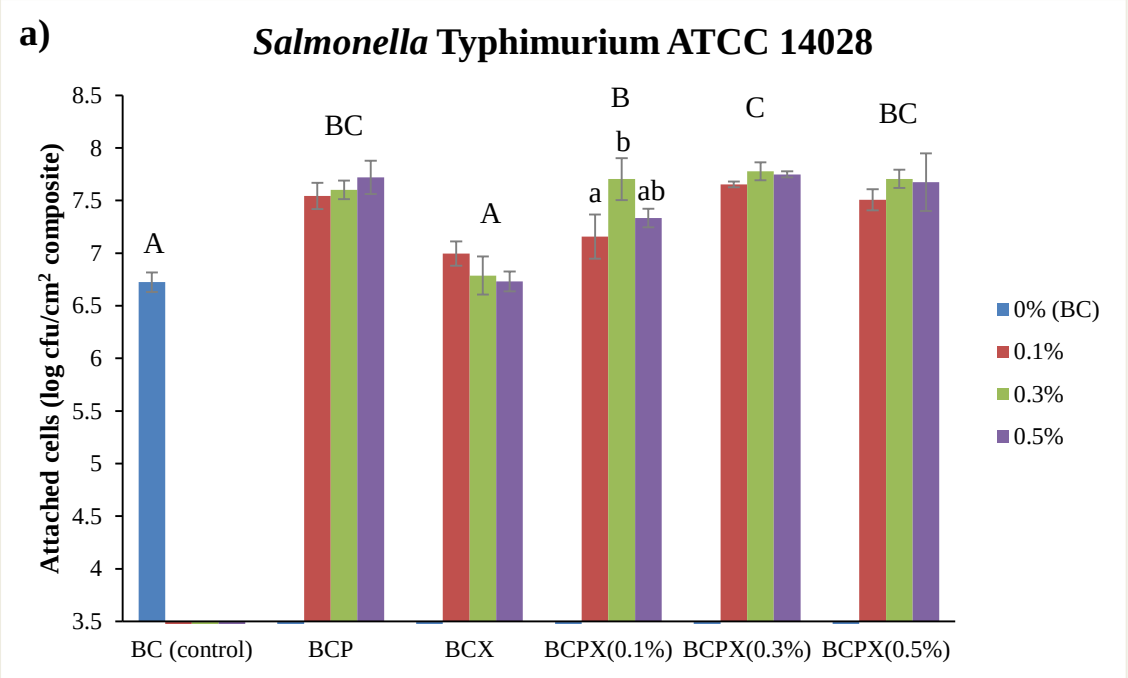


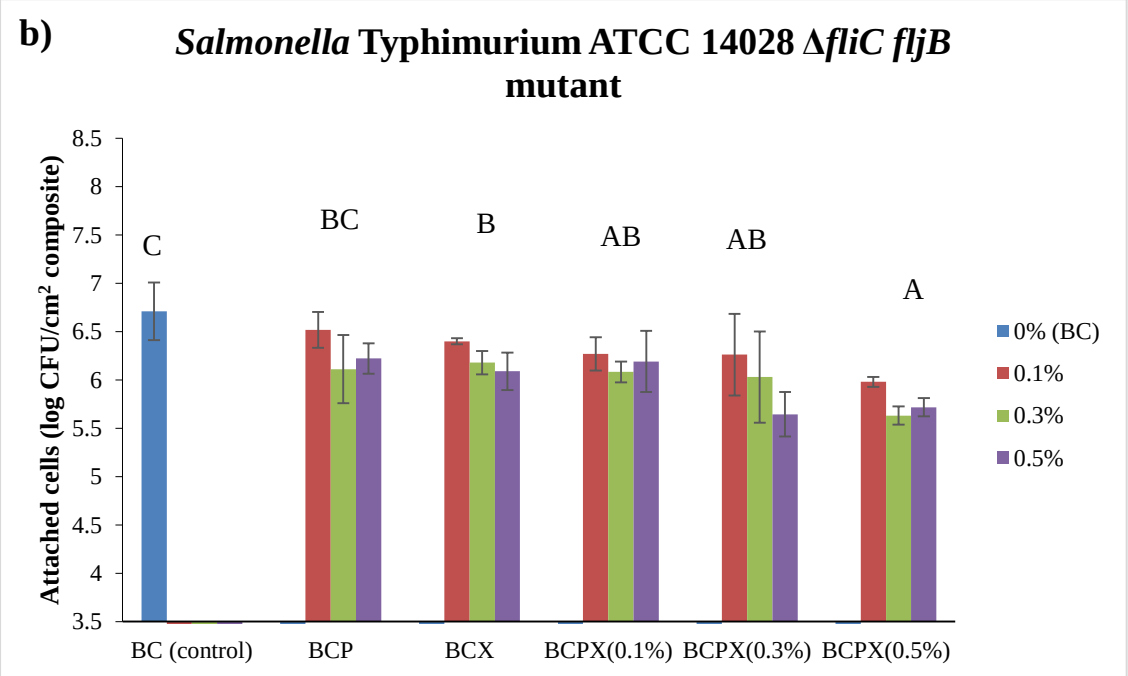
Figure A.2 Viable counts (log CFU/mL) of *Salmonella* strains after treatment with PBS (control), 1% and 2% w/v D-galacturonic acid.

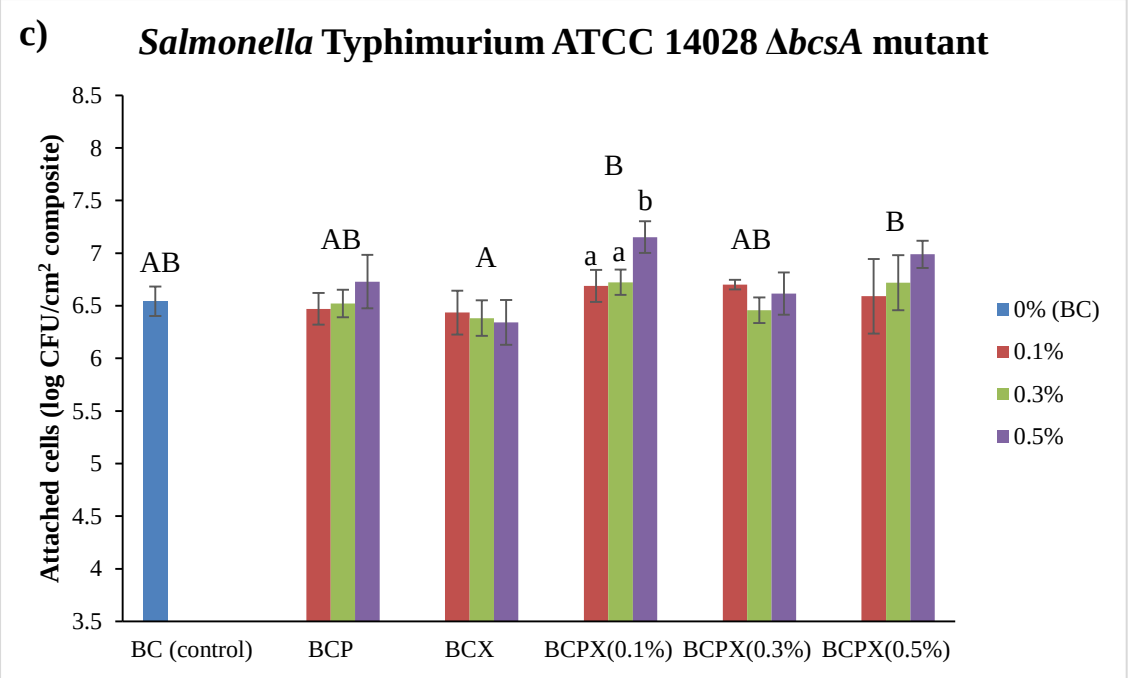
Appendix III

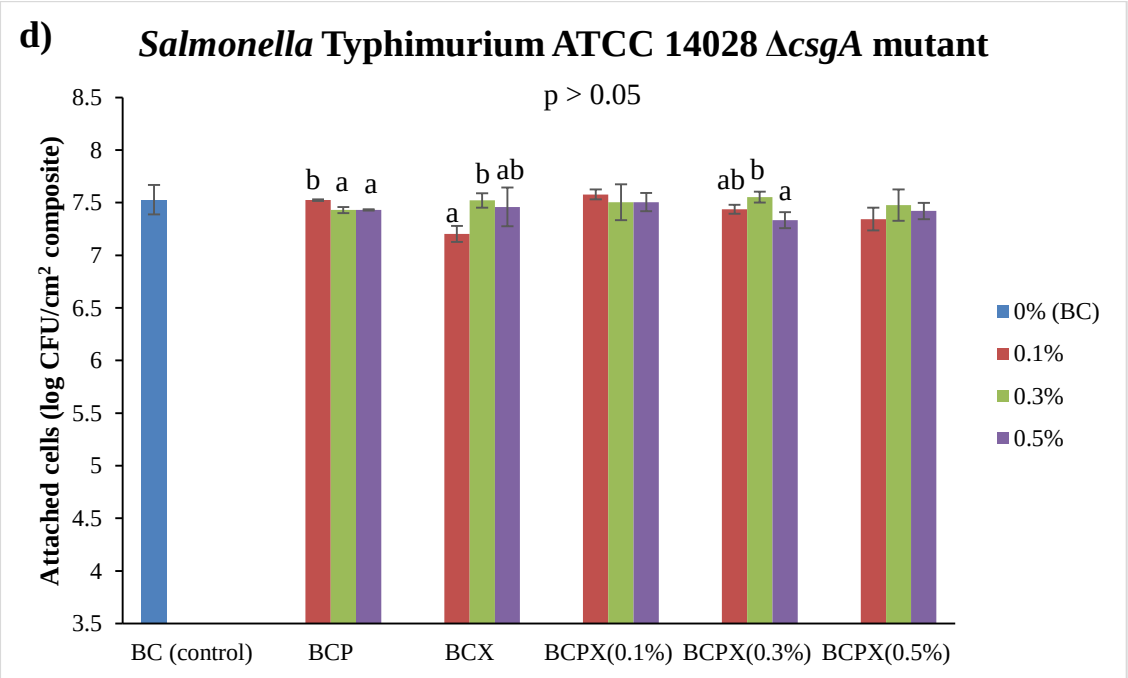
Attachment of Wild Type and Mutant Strains of *Salmonella* Typhimurium ATCC 14028 to a Range of Bacterial Cellulose (BC) Composites

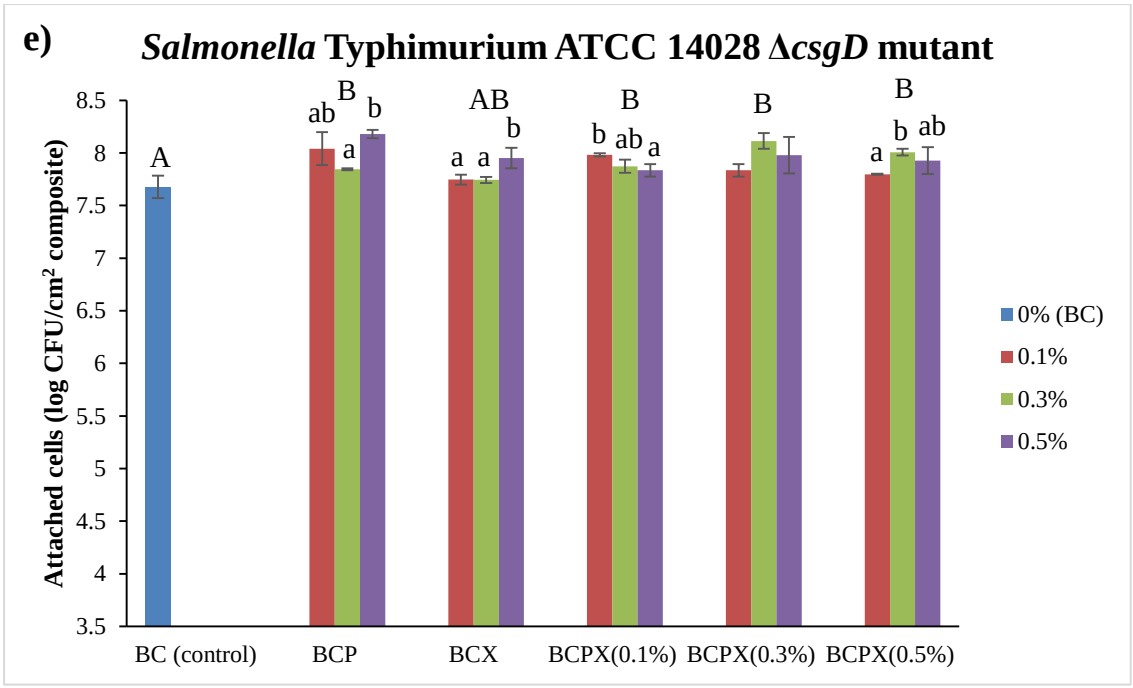
The following appendix contains results of the numbers of cells of wild type and mutant strains of *Salmonella* Typhimurium ATCC 14028 attached to BC composites containing different concentrations of pectin and xyloglucan (Chapter 5).


a)
Salmonella Typhimurium ATCC 14028
Attached cells (log cfu/cm² composite)
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
A
BC
A
B
b
a
ab
C
BC
BC (control)
BCP
BCX
BCPX(0.1%)
BCPX(0.3%)
BCPX(0.5%)
0% (BC)
0.1%
0.3%
0.5%


b)
Salmonella Typhimurium ATCC 14028 Δ*fliC fljB* mutant
Attached cells (log CFU/cm² composite)
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
C
BC
B
AB
AB
A
BC (control)
BCP
BCX
BCPX(0.1%)
BCPX(0.3%)
BCPX(0.5%)
0% (BC)
0.1%
0.3%
0.5%


c)
Salmonella Typhimurium ATCC 14028 ΔbcsA mutant
Attached cells (log CFU/cm² composite)
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
AB
AB
A
B
a a
b
AB
B
BC (control)
BCP
BCX
BCPX(0.1%)
BCPX(0.3%)
BCPX(0.5%)
0% (BC)
0.1%
0.3%
0.5%


d)
Salmonella Typhimurium ATCC 14028 ΔcsgA mutant
p > 0.05
Attached cells (log CFU/cm² composite)
8.5
8
7.5
7
6.5
6
5.5
5
4.5
4
3.5
b a a
a
b ab
ab b
a
BC (control)
BCP
BCX
BCPX(0.1%)
BCPX(0.3%)
BCPX(0.5%)
0% (BC)
0.1%
0.3%
0.5%



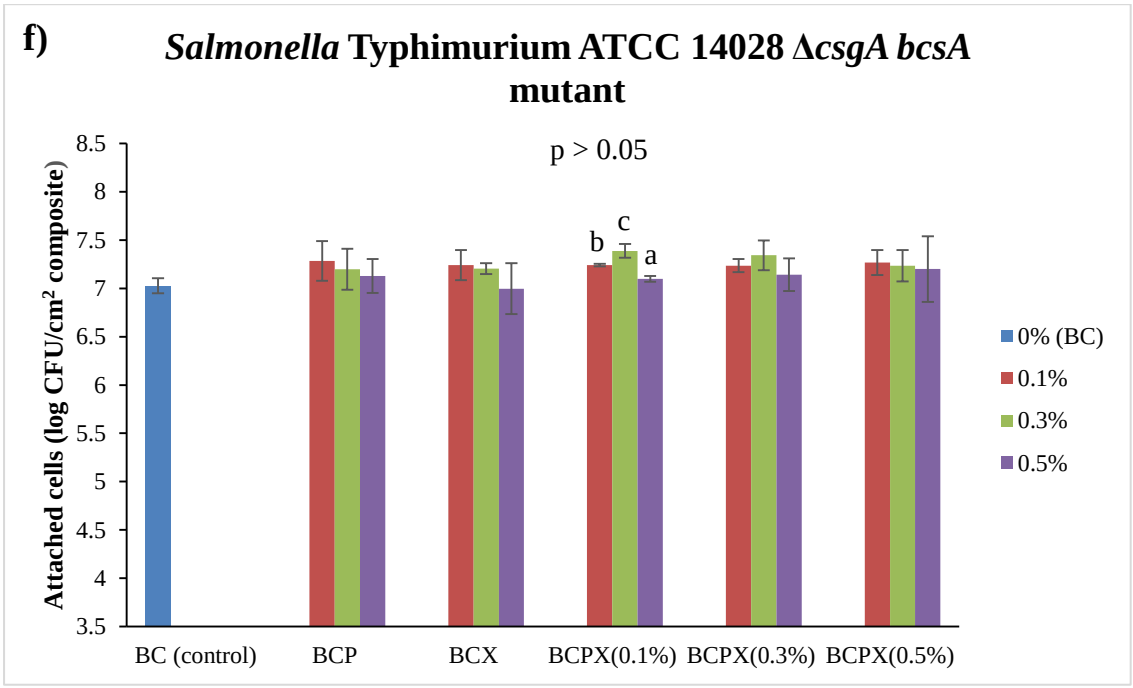


Figure A.3 Attachment numbers for wild type and mutant strains of *S.* Typhimurium ATCC 14028 cells (a – f) to BC composites containing different concentrations of pectin and xyloglucan. Different uppercase letters indicate significant differences in bacterial attachment between types of composites (One-way ANOVA & Tukey's pairwise comparison at $p<0.05$). Different lowercase letters indicate significant differences in bacterial attachment within each type of composite whereas the absence of lowercase letters indicate no significant differences in bacterial attachment within each type of composite (One-way ANOVA & Tukey's pairwise comparison at $p<0.05$).

Appendix IV

Sonication Conditions to Remove Bacterial Surface Structures without Affecting Cell Viability

The following appendix contains preliminary results on the viable counts of *Salmonella* Typhimurium ATCC 14028 cells after being subjected to different ultrasonic conditions (Chapter 6).

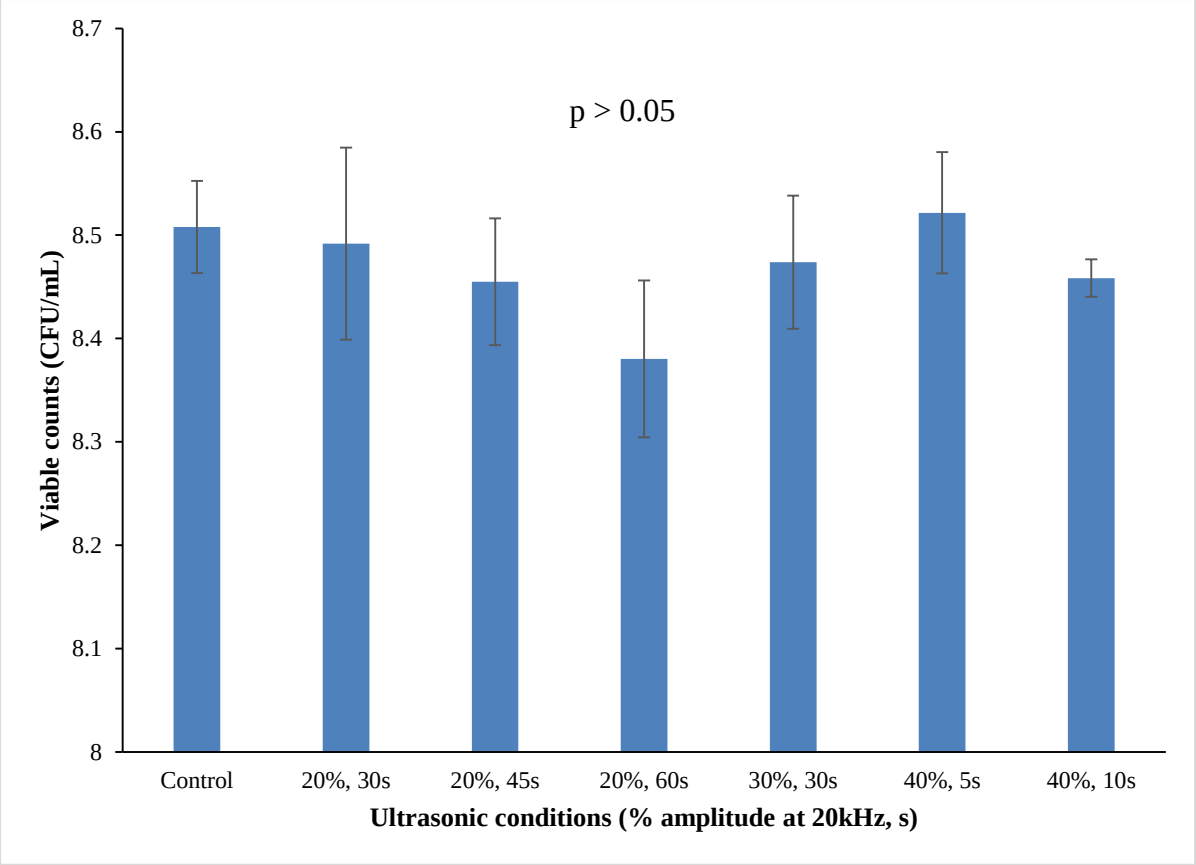


Figure A.4 Viable counts of *S.* Typhimurium ATCC 14028 cells after being subjected to different ultrasonic conditions.

Appendix V

Publication Related to This Thesis

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Relationship between cell concentration and *Salmonella* attachment to plant cell walls

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ABSTRACT

Cut surfaces of fresh produce, which directly expose plant cell walls to the environment, are particularly susceptible to contamination by pathogens, including *Salmonella enterica*. The effect of *Salmonella* cell concentration in liquids (such as rinses or washes) on their attachment to plant cell walls was investigated using bacterial cellulose-based plant cell wall models. Numbers of *Salmonella* cells attaching to the plant cell wall models increased linearly with cell concentration. A simple linear model ($y = 0.916x$) was constructed to predict the number of *Salmonella* cells that will attach to per unit surface area of plant cell wall (CFU/cm^2), assuming their initial inoculum concentration lies within the range of 3 log to 8 log CFU/mL. The linear regression model generated from the model surfaces was validated for a range of variables (different plant tissues, *S. enterica* subspecies and other foodborne bacteria). The validation supported the use of the linear regression model in predicting *Salmonella* attachment to plant cell walls regardless of the *Salmonella* subspecies. The use of the model can also be extended to other bacteria within the cell concentration range. This work may contribute to generating risk-assessment tools for control of *Salmonella* associated with the processing of fresh produce.

1. Introduction

Fresh produce is an important vehicle for the transmission of human pathogens worldwide. Globally it is estimated that 93.8 million cases of salmonellosis occur in humans with 155,000 deaths every year (Majowicz et al., 2010). *Salmonella enterica* is now the most commonly identified human pathogen from fresh produce with *Salmonella* Enteritidis and *Salmonella* Typhimurium being the most prevalent serovars (Klerks, Franz, van Gent-Pelzer, Zijlstra, & van Bruggen, 2007; Warriner, Huber, Namvar, Fan, & Dunfield, 2009). *S. enterica* usually contaminates fresh produce through direct or indirect contact with faeces, sewage, untreated irrigation and surface water (Takeuchi, Matute, Hassan, & Frank, 2000).

Contamination of fresh produce by human pathogens can occur both pre-harvest and post-harvest. Pre-harvest application of raw or insufficiently composted animal faeces or sewage as fertilizer may be a possible vehicle for the spread of human pathogens (Doyle & Erickson, 2008; Newell et al., 2010; Tauxe, 1997). Post-harvest processes including storage, rinsing, cleaning and cutting of fresh produce can also introduce pathogens, often through cross-contamination (Berger et al., 2010).

Cut surfaces of plant cell walls (PCWs) are especially vulnerable to human foodborne pathogen attachment (Kroupitski, Pinto, Brandl, Belausov, & Sela, 2009). Pathogens need to establish themselves on the fresh produce surface before they can cause disease and therefore bacterial attachment is a critical step in the colonization and transmission of pathogens through the food processing chain (Bordas, Balebona, Zorrilla, Borrgo, & Morinigo, 1996; Lu & Walker, 2001).

It is well established that increased bacterial density allows more bacterial cells to attach to a surface but the dynamics of this are dependent on the way in which bacteria attach and the presence or absence of receptors for the bacteria (Fletcher, 1977). A number of mathematical models have been developed based on cell concentrations and used, for example, to predict bacterial transfer rates to potato tissues (Garrood, Wilson, & Brocklehurst,

Abbreviations: PCW, plant cell wall; BC, bacterial cellulose; BCP, bacterial cellulose-pectin; BCX, bacterial cellulose-xyloglucan; BCPX, bacterial cellulose-pectin-xyloglucan.

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0956-7135/

2004), bacterial transfer rates between lettuce and knives (Zilelidou, Tsourou, Poimenidou, Loukou, & Skandamis, 2015), pseudomonad attachment rates to polystyrene (Fletcher, 1977) and bacterial survival after sanitization (Keeratipibul, Phewpan, & Lursinsap, 2011). The effect of varying cell concentration on the attachment of *Salmonella* specifically to cut PCWs has not been systematically investigated. This is particularly important in the context of cross-contamination during washing in which a small proportion of contaminated products can cause a large batch to become contaminated (Doyle & Erickson, 2008; López-Gálvez, Gil, Truchado, Selma, & Allende, 2010).

Our attachment studies were carried out using bacterial cellulose (BC)-based PCW models which have been demonstrated in a previous study (Tan, Wang, & Dykes, 2013) to be useful for understanding the attachment of pathogens to cut surfaces of fresh produce. These PCW models were used to simulate attachment to natural cut PCWs as these easily usable PCW models have been shown to possess similar chemical composition and microstructure to natural PCWs (Chanliaud, Burrows, Jeronimidis, & Gidley, 2002; Tan et al., 2013; Whitney, Gothard, Mitchell, & Gidley, 1999).

This study aimed to: i) provide quantitative data on the effect of cell concentration on the attachment of three *Salmonella* strains to the BC-based PCW models; ii) develop a simple mathematical model to predict how many *Salmonella* cells will attach to the PCW models at a given inoculum concentration and iii) test if the model is applicable to natural PCWs, different subspecies of *Salmonella* and other bacteria.

2. Materials and methods

2.1. Bacterial strains

S. enterica subspecies *enterica* serovar Enteritidis ATCC 13076, *S. enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, *Listeria monocytogenes* ATCC 7644, *L. monocytogenes* ATCC 19112, *Escherichia coli* ATCC 25922, *E. coli* O157:H7 ATCC 700728 and *Gluconacetobacter xylinus* ATCC 53524 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). *S. enterica* subsp. *indica* M4 was isolated from lettuce in Malaysia and used in this study as a fresh produce isolate. Two strains each from four groups of *Salmonella* subspecies, namely *S. enterica* subsp. *enterica* (I) (R11, U5), *S. enterica* subsp. *arizonae* (IIIa) (R32, U3), *S. enterica* subsp. *diarizonae* (IIIb) (U68, R1) and *S. enterica* subsp. *indica* (VI) (U56, U61) were all isolated from lizards in Malaysia (Cheng, Wong, & Dykes, 2014).

All bacteria (except *G. xylinus*) were grown aerobically at 37 °C on tryptic soy agar (TSA; Merck, Darmstadt, Germany) or in tryptic soy broth (TSB; Merck, Darmstadt, Germany) under shaking incubation (150 rpm) (Lab Companion SK-600 benchtop shaker, Medline, UK). *G. xylinus* ATCC 53524 was grown as described below.

2.2. Production of bacterial cellulose-based plant cell wall models

A primary inoculum of *G. xylinus* ATCC 53524 was cultured in Hestrin and Schramm (HS) broth medium containing 2% (w/v) glucose, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.27% (w/v) Na_2HPO_4 and 0.115% (w/v) citric acid (Hestrin & Schramm, 1954). The pH of the medium was adjusted to 5.0 with 5 M NaOH and incubated statically at 30 °C. The primary inoculum was used for the production of different PCW composites as described by Mikkelsen, Gidley, and Williams (2011):

- Bacterial cellulose (BC)
- BC-Pectin (BCP) produced by adding 0.1% w/v apple pectin with a degree of methyl esterification of about 30% (kindly donated by Herbstreith & Fox, Neuenbürg, Germany) to the HS medium and 3 mM $CaCl_2$ was added to allow incorporation of pectin in the composite (R&M Chemicals, Malaysia).
- BC-Xyloglucan (BCX) produced by adding 0.1% w/v xyloglucan (Megazyme, County Wicklow, Ireland) to the HS medium.
- BC-Pectin-Xyloglucan (BCPX) produced by adding 0.1% w/v pectin, 3 mM $CaCl_2$ and different concentrations of xyloglucan (0.1%, 0.3% and 0.5% w/v) to the HS medium.

The composites were produced in enclosed plastic containers (1.5 cm × 1.5 cm × 1.5 cm) that were incubated statically for 72 h. All harvested BC composites (BC, BCP, BCX, BCPX) (1.5 cm × 1.5 cm, ~ 2 mm thickness) were rinsed in 6 mM $CaCl_2$ at 100 rpm for 1 h to remove media components.

2.3. Effect of cell concentration on the attachment of Salmonella strains

Stationary phase cultures of *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4 (18 h incubation) were centrifuged at 5500 × *g* (Hettich D-78532, Tuttlingen, Germany) for 10 min at 4 °C. The pellet was washed twice with phosphate buffer saline (PBS) (pH 7.4) (1st BASE, Singapore) and suspended in PBS to an optical density at 600 nm (UV/Vis spectrophotometer, Shimadzu UV mini-1240, USA) which corresponds to 8 log CFU/mL for each isolate. Inoculum concentration was enumerated by spread plating appropriate dilutions on xylose lysine deoxycholate agar (XLDA; Oxoid, UK). Serial 10 fold dilutions of the bacterial suspensions were carried out in order to obtain the desired range of bacterial concentrations of 3, 4, 5, 6, 7 and 8 log CFU/mL with a 0.2 log CFU/mL margin of error. The range of concentrations used in this study is similar to that of other studies. Although bacterial concentrations of 3–8 log CFU/mL are not common in real life situations, these high levels of bacterial cells were used to allow better measurement of how the trend of *Salmonella* attachment varied according to different inoculum concentrations. Once this is established, further work at lower levels of inoculum would be appropriate.

Each rinsed BC composite was incubated in 10 mL of pathogenic bacterial suspension of different concentrations for 20 min with gentle shaking (100 rpm) at room temperature. An incubation time of 20 min was selected to allow the levels of attachment of these pathogens to reach a stable saturation point as shown in an earlier study (Tan et al., 2013). This condition simulates the occurrence of irreversible bacterial attachment to plant surfaces in real life as plants are usually exposed to pathogens for a longer period of time. This was followed by gentle rinsing (100 rpm) in 6 mM $CaCl_2$ for 1 min to remove loosely attached cells. Each composite was then placed in a stomacher bag with 50 mL PBS and pummelled for 1 min at 8 strokes/sec in a stomacher (BagMixer 400; Interscience, France). The number of *Salmonella* cells attached to the BC composite was enumerated by serial dilution of the stomached fluid followed by the plating of appropriate dilutions on XLDA. Numbers of attached bacterial cells were expressed as CFU/cm^2 composite. The experiment was performed in triplicate (3 independently grown bacterial cultures).

2.4. Linear model development

Data points obtained for the attachment of the three *Salmonella* strains (*S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4) were plotted on the same graph to assess the overall trend of *Salmonella* attachment over a range of inoculum concentrations. Since the overall trend was linear within the data range studied, these data were modelled using a linear regression

approach. The 95% confidence interval, 95% prediction interval and the equation of the best fit line were obtained. The aim of constructing this simple mathematical model was to enable prediction of the numbers of *Salmonella* cells that will attach on the PCW models if their initial inoculum concentration was within the range of 3–8 log CFU/mL.

2.5. Verification of the mathematical model

Tests across a range of parameters were carried out to verify the validity of the linear regression model in predicting the numbers of attached *Salmonella* cells to the PCW models and more importantly, to natural PCWs. For this purpose, natural plant tissues were used to assess the applicability of the model in predicting *Salmonella* attachment to different types of plants. Comparisons between different *S. enterica* subspecies and two other common foodborne pathogens (*E. coli* and *L. monocytogenes*) were also made to assess whether the model can predict the attachment of these pathogens to the PCW models. Data from each parameter was plotted on a graph overlaid with 95% confidence and prediction intervals obtained from the linear regression model.

Potato (*Solanum tuberosum*) tuber, apple (*Malus domestica*) fruit and lettuce (*Lactuca sativa*) leaves were obtained from a retail outlet in Selangor, Malaysia. These three plant tissues were used since they represent a range of different plant species that *Salmonella* have been isolated from and which have also been associated with cases of salmonellosis (Beuchat, 2002; Quiroz-Santiago et al., 2009). The outer surfaces of the potato, apple and lettuce leaves were lightly wiped down with 70% ethanol solution before cutting out approximately 1.5 cm × 1.5 cm pieces of the plant materials with a sterile scalpel. The surfaces of the lettuce leaves were lightly grazed to expose the upper and lower epidermis.

Suspensions of *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4 were prepared by spinning down the TSB broth culture after 18 h incubation and then suspending at their original concentration before carrying out serial 10 fold dilutions to prepare six different concentrations of the bacterial suspensions (about 4–9 log CFU/mL). Subsequently, attachment assays were carried out as described in Section 2.3 but using cut plant materials instead of BC composites and using six concentrations of the *Salmonella* suspensions. Concentrations of the initial inoculum and also the final attached *Salmonella* cells on the BC composites were determined by spread plating on XLDA.

The attachment of four *S. enterica* subspecies [subsp. *enterica* (I), *arizonae* (IIIa), *diarizonae* (IIIb) and *indica* (VI)] to the BC composites was tested with two *Salmonella* strains for each subspecies: *S. enterica* subsp. I (R11, U5); *S. arizonae* subsp. IIIa (R32, U3); *S. diarizonae* subsp. IIIb (U68, R1) and *S. indica* subsp. VI (U56, U61). Four different concentrations of each bacterial suspension were prepared using serial 10 fold dilutions of the bacterial suspensions as described above. Attachment assays were performed as outlined in Section 2.3 but different BC composites were randomly assigned to each bacterial suspension. The concentrations of the initial inoculum and also the final attached cells on the BC composites were determined by spread plating on XLDA.

Two strains each of *E. coli* (*E. coli* O157:H7 ATCC 700728, *E. coli* ATCC 25922) and *L. monocytogenes* (*L. monocytogenes* ATCC 19112, *L. monocytogenes* ATCC 7644) were used in this part of the study. Four different concentrations of each bacterial suspension were prepared and used for the attachment assays. Each suspension was incubated with randomly assigned BC composites. The concentrations of the initial inoculum and also the final attached cells on the BC composites were determined by spread plating on TSA.

2.6. Statistical analysis

Statistical analysis of results was performed using Statistical Package for the Social Sciences (SPSS) (SPSS Inc., USA). Two-way ANOVA was used to determine the significance of the effects of bacterial strains (*S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4) and different types of BC composites on the final numbers of attached cells for data collected from Section 2.3. Using the equation obtained from the linear regression model, a table to compare the predicted and actual attachment of *Salmonella* strains from different subspecies was constructed. One-way ANOVA was performed to determine the significance of differences between strains from different *Salmonella* subspecies. In order to test the validity of the model, data obtained from various tests with different *S. enterica* subspecies, other pathogens and natural plant tissue were plotted against the 95% prediction and confidence intervals constructed from the linear regression model. Differences among the means were determined using Tukey's method at 95% confidence level.

3. Results and discussion

3.1. Effect of initial inoculum concentration on the attachment of Salmonella strains and linear model development

Attachment of the 3 *Salmonella* strains, namely *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4, to the BC-based PCW models were carried out using a series of initial inoculum concentrations. From Fig. 1, it is apparent that higher initial inoculum concentrations resulted in higher numbers of attached cells on the BC composites. None of the *Salmonella* strains used in this study reached a saturation point with respect to attachment even up to an initial inoculum concentration of 8 log CFU/mL. We hypothesize that there are an abundance of attachment sites on the BC composites which were not fully occupied even at high densities of *Salmonella* cells up to 8 log CFU/mL. Since the BC composites are porous to different degrees it is difficult to predict at what stage saturation may be reached. This is a feature that requires further investigation at higher densities of inoculum. Barak, Whitehand, and Charkowski (2002) showed that *S. enterica* serovar Newport attached to alfalfa sprouts in a linear fashion within the range of 3–6 log CFU/mL. In our study, the linear trend may indicate the ability of *Salmonella* cells to attach to the BC composites and also to the bacterial cells bound on the composites as suggested by Barak et al. (2002). Liao and Cooke (2001) found that higher concentrations of *Salmonella* in the suspension proportionally increased the number of bacteria attaching to green pepper discs within the range of 5–7 log CFU/mL. In their study, the extent of attachment, however, decreased and was near saturation at concentrations higher than 9 log CFU/mL. Iturriaga, Escartín, Beuchat, and Martínez-Peniche (2003) also showed that the degree of attachment of *Salmonella* to tomato surfaces decreased at higher inoculum concentrations. This feature may be caused by the difficulty faced by pathogens in attaching to less favourable sites after the preferred attachment sites were occupied. Another possible explanation for the decreased attachment numbers at high bacterial densities is that both these studies used fresh produce samples with intact surface which are less favourable for bacterial attachment as compared to cut surfaces.

Since the attachment of these *Salmonella* strains increased linearly with increasing bacterial density, a linear regression model was fitted to the data. Initially, an intercept was included in the linear model to account for any bias in the model to fulfil the assumption that the mean of the residuals should be zero. The initial equation obtained for the model was $y = 0.916x + 0.067$.

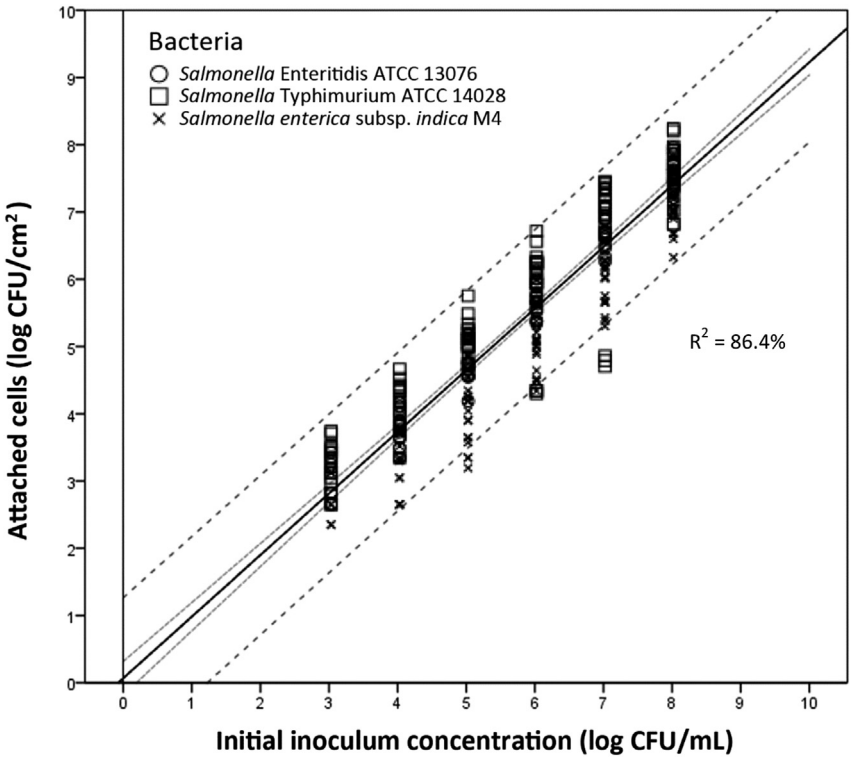

Fig. 1. Linear regression model of the number of attached Salmonella cells to BC composites over a range of initial inoculum concentration (10^3–10^8 CFU/mL) plotted with confidence (-----) and prediction (- - -) intervals at 95% confidence limit. The final equation for the model: y = 0.916x.

Since the intercept (0.067) was found to not be statistically significant (Linear regression model, p = 0.599), it was excluded from the final equation. This is because logically no bacteria are available to attach (y = 0) when the initial inoculum concentration is zero (x = 0).

The final equation for the linear model was: y = 0.916x, with x within the range of 3–8 log CFU/mL, R^2 = 86.4%, $F_{1, 289}$ = 1837.86, p < 0.001. The R^2 value of the equation is high (86.4%) and the initial inoculum concentration reliably predicts the final attached *Salmonella* cells ($F_{1, 289}$ = 1837.86, p < 0.001). Both bacterial strains and type of composites, however, significantly affected attachment (p < 0.05) and may account for the other 13.6% of the R^2 value which were not contributed by the predictor used (initial inoculum concentration). The prediction interval gives an estimation of where most (95%) of future observations will fall. The confidence interval, on the other hand, measures the reliability of an estimate and was also included in the graph with a confidence level of 95%. The narrow confidence interval of the model confirmed that the linear model represents the true relationship and is good for prediction within the range of 3–8 log CFU/mL.

3.2. Verification of the linear model

Attachment of *Salmonella* cells (*S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028 and *S. enterica* M4) to different natural plant tissues fall mostly within the 95% prediction interval (Fig. 2). This supports the validity of using the linear regression model to predict *Salmonella* attachment to cut natural PCWs. Counts of *Salmonella* attached to apple tissue fell very closely to the best-fit line of the linear model. This may be because the pectin used for the production of the BC composites was also extracted from apples. The attachment of *Salmonella* to lettuce leaves, on the other hand, was found to be at the lower limit of the prediction interval. This could be due to the difference in the thickness of lettuce leaves (less than 1 mm) used for the attachment study as compared to the potato and apple tissues which were much thicker (~2 mm). The thin lettuce leaves provide less surface for bacterial attachment which could have resulted in low attachment numbers (Katsikogianni & Missirlis, 2004). Variations in surface roughness and compactness of plant cells on the surfaces of the three plant materials may also account for differences in bacterial attachment to these surfaces. Saggers, Waspe, Parker, Waldron, and Brocklehurst (2008) showed that 6.6 log CFU/g *S.* Typhimurium LT-2 attached to potato cell wall material after incubation in an inoculum with a starting concentration of 9 log CFU/mL for 10 min. In another study, the attachment of *E. coli* O157:H7 to cut edges of lettuce leaves (~6.5 log CFU/cm^2) was slightly higher than to apple skin (~6.2 log CFU/cm^2) (Hassan & Frank, 2004). However, the results of their study were not directly comparable to ours as the apple tissues we used were much thicker than the thin apple peel they used.

The graph of attachment associated with the different *Salmonella* subspecies (Fig. 3) showed that all data points were found within the 95% prediction interval. This indicates that the linear regression model can be used to predict the attachment of *Salmonella*, regardless of the different subspecies (I, IIIa, IIIb, VI) within the range of 3–8 CFU/mL.

The equation from the linear regression model y = 0.916x was also tested by calculating the expected attachment of the different *Salmonella* subspecies for a given initial inoculum concentration and then comparing it to the actual attachment numbers in Table 1. We found that the differences in subspecies had no significant effect on *Salmonella* attachment (p > 0.05). It was noted that most cases of *Salmonella* subsp. VI (U56 and U61) attached at least 0.5 logs higher than the expected value, whereas the model generally overestimated the attachment of *Salmonella* subsp. IIIb, although

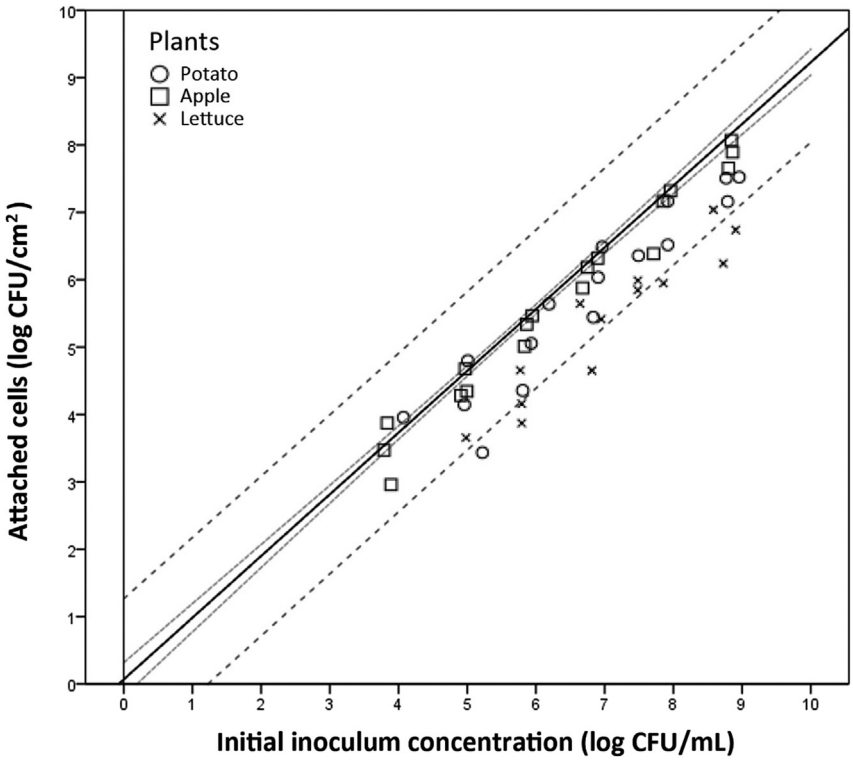

Fig. 2. Attachment of three Salmonella strains (S. Enteritidis ATCC 13076, S. Typhimurium ATCC 14028 and *Salmonella enterica* subsp. indica M4) to natural PCWs (potato tuber, apple fruit and lettuce leaves) plotted against confidence (-----) and prediction (- - -) intervals at 95% confidence limit obtained from the linear regression model (y = 0.916x).

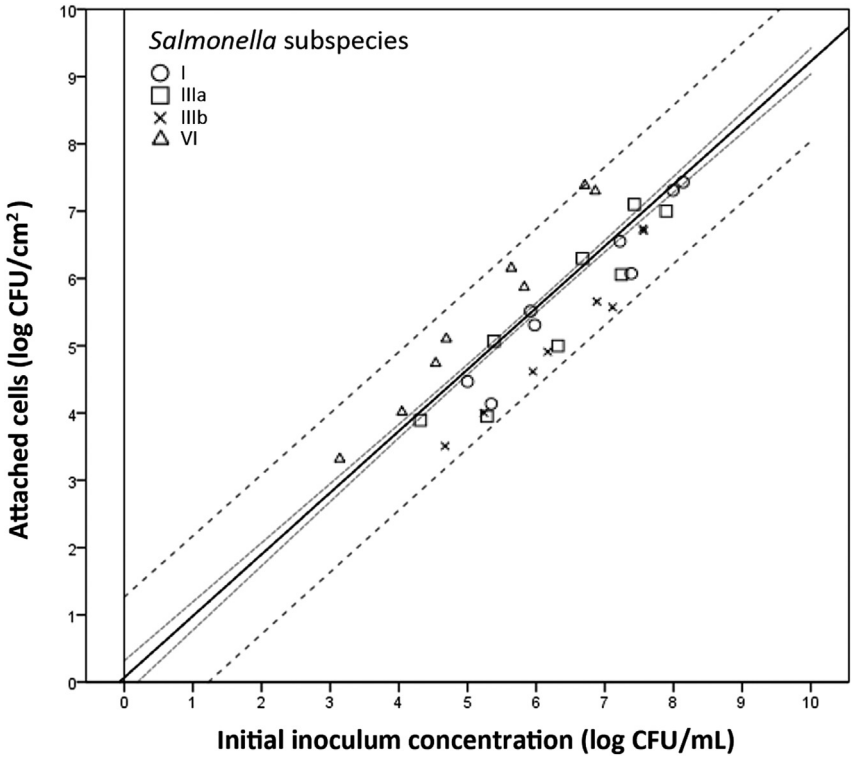

Fig. 3. Attachment of different Salmonella subspecies (I, IIIa, IIIb, VI) to BC composites plotted against confidence (-----) and prediction (- - -) intervals at 95% confidence limit obtained from the linear regression model (y = 0.916x).

these were still within the 95% prediction interval. Both *Salmonella* subsp. I and IIIa showed attachment numbers which were close to the predicted values.

The attachment data for the *E. coli* and *L. monocytogenes* strains

Table 1
Comparison of the actual and expected (using equation from linear regression model, y = 0.916x) attachment of different *Salmonella* subspecies.

Subsp.	Bacteria	Composites	Initial inoculum (log CFU/mL)	Attached cells (log CFU/cm^2)	Expected attachment (log CFU/cm^2)	95% Confidence interval
I	R11	BC	8.000	7.311	7.328	6.217–8.574
		BC	7.230	6.551	6.623	5.512–7.865
		BCX	5.920	5.508	5.423	4.320–6.669
		BCX	5.010	4.457	4.589	3.480–5.830
	U5	BCP	8.150	7.433	7.465	6.351–8.708
		BCP	7.390	6.071	6.769	5.663–8.016
		BCPX(0.5%)	5.990	5.301	5.487	4.377–6.726
		BCPX(0.5%)	5.350	4.125	4.901	3.797–6.146
IIIa	R32	BCPX(0.1%)	7.900	7.000	7.236	6.129–8.485
		BCPX(0.1%)	7.250	6.054	6.641	5.533–7.885
		BCPX(0.3%)	6.320	4.990	5.789	4.686–7.035
		BCPX(0.3%)	5.290	3.938	4.846	3.741–6.090
	U3	BCPX(0.5%)	7.440	7.103	6.815	5.703–8.056
		BCPX(0.5%)	6.680	6.296	6.119	5.012–7.363
		BCX	5.390	5.063	4.937	3.831–6.180
		BCX	4.320	3.878	3.957	2.851–5.202
IIIb	U68	BC	7.570	6.709	6.934	5.824–8.178
		BC	7.120	5.567	6.522	5.414–7.766
		BCX	6.180	4.903	5.661	4.550–6.899
		BCX	5.250	3.990	4.809	3.700–6.049
	R1	BCP	7.560	6.739	6.925	5.820–8.174
		BCP	6.890	5.652	6.311	5.205–7.556
		BCPX(0.5%)	5.960	4.607	5.459	4.352–6.701
		BCPX(0.5%)	4.680	3.493	4.287	3.180–5.531
VI	U56	BCPX(0.1%)	6.720	7.384	6.156	5.044–7.395
		BCPX(0.1%)	5.830	5.865	5.340	4.236–6.585
		BCPX(0.5%)	4.700	5.095	4.305	3.196–5.547
		BCPX(0.5%)	4.050	4.000	3.710	2.603–4.957
	U61	BCPX(0.5%)	6.870	7.301	6.293	5.184–7.535
		BCPX(0.5%)	5.640	6.153	5.166	4.062–6.411
		BCX	4.540	4.729	4.159	3.054–5.405
		BCX	3.146	3.301	2.882	1.874–4.151

also fits within the 95% prediction interval (Fig. 4). Unlike the linear attachment trend for *Salmonella*, however, the attachment trends for *E. coli* O157:H7 ATCC 700728, *E. coli* ATCC 25922 and *L. monocytogenes* ATCC 7644 appeared to plateau out at higher inoculum concentrations. Both Takeuchi and Frank (2000) and Garrood et al. (2004) found that the numbers of an *E. coli* O157:H7 and a *L. monocytogenes* strain attached to plant material did not increase proportionally with inoculum size and were lower than expected at high inoculum concentrations.

In a study by Jablasone, Warriner, and Griffiths (2005) a *L. monocytogenes* strain showed significantly higher attachment to lettuce (~6.2 log CFU/g) when compared to the two *E. coli* O157:H7 strains used (~5 log CFU/g). Of the *E. coli* and *L. monocytogenes* strains used in our study, only attachment of *L. monocytogenes* ATCC 19112 was found at the lower border of the 95% prediction interval. This could be due to strain-specific differences in *L. monocytogenes* attachment as observed by Gorski, Palumbo, and Nguyen (2004). Barak et al. (2002) found that *E. coli* O157:H7 strains attached at levels of about 3 log CFU per alfalfa sprout after 4 h of inoculation in the starting concentration of 3 log CFU/mL. Ells and Hansen (2006) found that *Listeria* spp. strains attached at an average of 5.7 log CFU/cm^2 to cut cabbage tissues after being inoculated using 6 log CFU/mL suspensions for 3 h.

Although the trend for the attachment of *E. coli* and *L. monocytogenes* strains appeared to be different from *S. enterica*, the linear regression model is deemed applicable to predict the attachment of these pathogens given their bacterial density is within the range of 3–8 log CFU/mL. In this study only the attachment of potentially pathogenic bacterial strains was investigated. Further studies investigating the ability of non-pathogenic strains and species to attach could be useful to establish if the effects observed here are ubiquitous.

Bacterial attachment to plant cell walls is a complicated process which involves many factors and that is influenced by both specific and non-specific binding to these surfaces. We have shown here that bacterial cell concentration (largely influencing non-specific attachment) plays an important role in determining the bacterial attachment numbers to the plant cell walls, with attachment levels falling within the 95% confidence level which encompasses a wide range of about 2 log CFU/cm.^{2} In another study using a *Salmonella* strain and associated isogenic mutants for different structural appendages, such as flagella, we found that these structures also influence the extent of bacterial attachment of up to 1.5 log CFU/cm^2 (unpublished results). This attachment study was only undertaken at one inoculum level (~8 log CFU/mL) and the effect of removing bacterial structural appendages at different inoculum levels has not been tested. Based on these findings, it appears that both specific and non-specific attachment play important roles in bacterial attachment to plant cells.

The mathematical model generated in this study to predict *Salmonella* attachment to PCWs has limitations and may not fully represent real life situation. The effect of background microbiota on plant surfaces which may influence the attachment of Salmonella to plant surfaces was excluded in this study to better evaluate the effect of cell concentrations on *Salmonella* attachment without the interference of another factor. It may, however, be useful to investigate this in future studies.

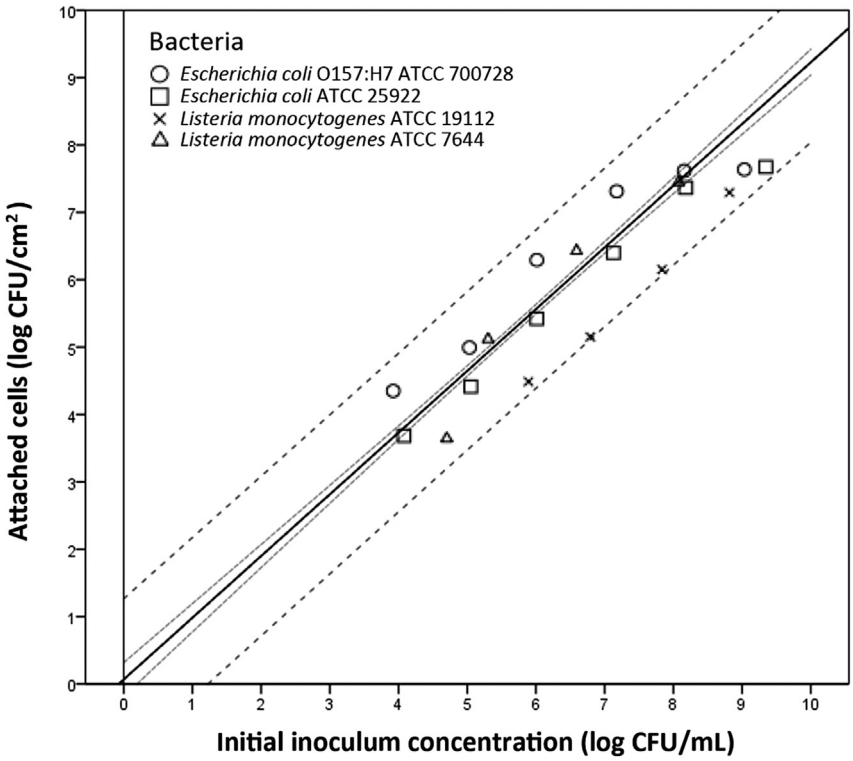

Fig. 4. Attachment of different strains of *E. coli* and *L. monocytogenes* to BC composites plotted against confidence (——) and prediction (- - -) intervals at 95% confidence limit obtained from the linear regression model (y = 0.916x).

4. Conclusion

The present study investigated the effect of initial inoculum concentration on the attachment of *Salmonella* cells. It provided quantitative data regarding the attachment of *Salmonella* cells to the BC-based PCW models with an increase in inoculum concentration. From this a linear regression model was constructed to predict the attachment of *Salmonella* cells to natural PCWs within the range of 3–8 log CFU/mL and the applicability of the model was validated with different tests. The model satisfactorily predicted *Salmonella* attachment to different natural plant tissues and was able to predict the attachment of different subspecies of *S. enterica* strains. The linear model appeared to be applicable to other common pathogens, *E. coli* and *L. monocytogenes*. The model has potential to contribute to assessing the risks associated with attachment of pathogens in liquid to the cut surfaces of fresh produce.

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Pectin and Xyloglucan Influence the Attachment of *Salmonella enterica* and *Listeria monocytogenes* to Bacterial Cellulose-Derived Plant Cell Wall Models

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Minimally processed fresh produce has been implicated as a major source of foodborne microbial pathogens globally. These pathogens must attach to the produce in order to be transmitted. Cut surfaces of produce that expose cell walls are particularly vulnerable. Little is known about the roles that different structural components (cellulose, pectin, and xyloglucan) of plant cell walls play in the attachment of foodborne bacterial pathogens. Using bacterial cellulose-derived plant cell wall models, we showed that the presence of pectin alone or xyloglucan alone affected the attachment of three *Salmonella enterica* strains (*Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, and *Salmonella enterica* subsp. *indica* M4) and *Listeria monocytogenes* ATCC 7644. In addition, we showed that this effect was modulated in the presence of both polysaccharides. Assays using pairwise combinations of *S.* Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 7644 showed that bacterial attachment to all plant cell wall models was dependent on the characteristics of the individual bacterial strains and was not directly proportional to the initial concentration of the bacterial inoculum. This work showed that bacterial attachment was not determined directly by the plant cell wall model or bacterial physicochemical properties. We suggest that attachment of the *Salmonella* strains may be influenced by the effects of these polysaccharides on physical and structural properties of the plant cell wall model. Our findings improve the understanding of how *Salmonella enterica* and *Listeria monocytogenes* attach to plant cell walls, which may facilitate the development of better ways to prevent the attachment of these pathogens to such surfaces.

According to the World Health Organization (WHO), almost 1.5 million cases of human salmonellosis are reported globally every year (1–3). Fresh produce is an important vehicle for the transmission of human pathogens and a major source of foodborne microbial outbreaks worldwide. While *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium were historically linked to the majority of the outbreaks from food of animal origin, a number of *Salmonella* serovars have now been strongly linked to fresh produce (4–6).

Pathogenic bacteria associated with fresh produce may establish themselves on plant surfaces and cause disease; therefore, the initial process of bacterial attachment is a crucial step in their transmission (7, 8). A study by Saggers et al. (9) suggested that plant cell wall (PCW) components at the PCW junction, particularly pectin, may provide receptor sites for bacterial attachment. In addition to the structural components of the PCW, the physicochemical properties (such as hydrophobicity and charge) of both the attachment surface and the attaching bacteria influence bacterial adhesion. For example, it has been suggested that bacteria that exhibit greater surface hydrophobicity than other strains attach preferably to hydrophobic surfaces, whereas bacteria that exhibit greater surface hydrophilicity than other strains prefer hydrophilic surfaces (10). Other studies have suggested that greater bacterial hydrophobicity favors bacterial adhesion to most surfaces (11, 12). Increased hydrophobicity also favors cell-to-cell adhesion, which leads to greater autoaggregation (13, 14). Similarly, coaggregation has been shown to be dependent on autoaggregation (14). Bujnakova and Kmet (15), for example, showed that only autoaggregating strains coaggregate with other strains.

We have used a bacterial cellulose (BC)-based PCW model to investigate the effects of PCW components on the attachment of pathogenic bacteria (16). The BC-based PCW model is produced by culturing *Gluconacetobacter xylinus*, a BC-producing bacterium, in Hestrin-Schramm (HS) growth medium with the addition of pectin and/or xyloglucan. Formation of the PCW model mimics the natural phenomenon of PCW deposition in native plants (17). The PCW model has been shown to possess molecular and architectural properties similar to those of native primary PCWs (17, 18). This model gives a more realistic picture of what occurs within actual PCWs because it is based on a constructive approach, in comparison with destructive chemical and physical treatments used to obtain PCW fractions (19). The PCW model can also be produced in relatively large quantities and at desired thicknesses, which makes it convenient to use (20, 21). More importantly, the chemical composition of the BC-based PCW model can be easily manipulated through the specific addition or removal of PCW components; this enables direct investigation of the effects of different levels of specific PCW components on pathogenic bacterial attachment. The PCW model used in this study has limitations and cannot fully replace the study of bacte-

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rial attachment to plant tissues *in situ*. It is subject to less variability, however, and can provide a substantial amount of information that can be used subsequently to design better experiments for plant tissue studies.

In this study, we investigated the roles of the major structural components of the PCW (cellulose, pectin, and xyloglucan) in the attachment of three *Salmonella enterica* strains to PCW models. The effect of attachment surface hydrophobicity on *Salmonella* attachment was also investigated, using abiotic surfaces of different hydrophobicities. A Gram-positive *Listeria monocytogenes* strain that was used in a previous study (16) was included in this study for comparison, as it showed different attachment characteristics than did the Gram-negative *Salmonella* strains. The overall aim of this study was to understand how bacteria attach to PCWs, to aid in the development of decontamination strategies that can effectively eliminate pathogens on fresh produce without damaging the organoleptic qualities of the produce (22).

MATERIALS AND METHODS

Bacterial strains. *Salmonella enterica* subsp. *enterica* serovar Enteritidis ATCC 13076, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028, *Listeria monocytogenes* ATCC 7644, and *Gluconacetobacter xylinus* ATCC 53524 were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). *Salmonella enterica* subsp. *indica* M4 was isolated from lettuce in Malaysia and was used in this study as a control and fresh produce isolate. The *Salmonella* and *Listeria* strains were grown aerobically at 37°C on tryptic soy agar (Merck, Darmstadt, Germany) for maintenance or in tryptic soy broth (Merck), with shaking (150 rpm), for experiments (Lab Companion SK-600 benchtop shaker; Medline, United Kingdom). The *Gluconacetobacter* strain was grown as described below.

Production of BC-based PCW models. A primary inoculum was prepared and then used for the production of all BC composites, as described by Mikkelsen and Gidley (23). The primary inoculum was prepared by culturing *G. xylinus* ATCC 53524 at 30°C for 72 h in Hestrin-Schramm (HS) broth containing 2% (wt/vol) glucose, 0.5% (wt/vol) peptone, 0.5% (wt/vol) yeast extract, 0.27% (wt/vol) Na_2HPO_4, and 0.115% (wt/vol) citric acid and adjusted to pH 5.0 (24). The different BC composites were prepared individually and grown at the same time. Triplicates of each type of BC composite were grown for each attachment assay. These composites were produced in enclosed plastic containers (1.5 cm by 1.5 cm by 1.5 cm) and incubated statically at 30°C for 72 h. Harvested BC composites (1.5 cm by 1.5 cm; thickness, ~2 mm) were purified by rinsing in 6 mM $CaCl_2$ at 100 rpm for 1 h, to remove medium components. The different types of PCW models produced were as follows: (i) bacterial cellulose (BC); (ii) BC-pectin (BCP) composites produced by adding 0.1%, 0.3%, or 0.5% (wt/vol) apple pectin, with a degree of methyl esterification of about 30% (Herbstreith & Fox, Neuenbürg, Germany), to the HS medium with calcium chloride (R&M Chemicals, Malaysia) (3 mM $CaCl_2$ for 0.1% [wt/vol] pectin, 6 mM $CaCl_2$ for 0.3% [wt/vol] pectin, or 12.5 mM $CaCl_2$ for 0.5% [wt/vol] pectin); (iii) BC-xyloglucan (BCX) composites produced by adding 0.1%, 0.3%, or 0.5% (wt/vol) xyloglucan (Megazyme, Bray, Ireland) to the HS medium; and (iv) BC-pectin-xyloglucan (BCPX) composites produced by adding different combinations of pectin and xyloglucan (0.1%, 0.3%, or 0.5% [wt/vol]) with $CaCl_2$ (3 mM, 6 mM, or 12.5 mM, respectively, corresponding to the amount of pectin added).

Chemical composition analysis. The chemical compositions of the BC composites were analyzed as described by Mikkelsen et al. (19). BC composites were first cryoground using a 6850 SPEX freezer/mill (SPEX, Metuchen, NJ, USA). Saeman hydrolysis was then performed to hydrolyze the cryoground composites into monosaccharides (25). The derived monosaccharides were reduced to their corresponding alditol acetates before analysis by gas chromatography (GC-17A system; Shimadzu, Kyoto, Japan); this allowed determination of the percentage of each PCW component incorporated into the composites.

Attachment assays with individual strains of bacteria. Early-stationary-phase cultures of *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028, *Salmonella enterica* M4, and *L. monocytogenes* ATCC 7644 (18 h for the *Salmonella* strains and 42 h for the *L. monocytogenes* strain) were centrifuged at 5,500 × *g* (1620A rotor; Hettich, Tuttlingen, Germany) for 10 min at 4°C. The pellet was washed twice with phosphate-buffered saline (PBS) (pH 7.4) (1st BASE, Singapore) and suspended in PBS to optical densities at 600 nm (OD_{600}) (Shimadzu UVmini-1240 UV-visible spectrophotometer) of 0.143 for *S.* Enteritidis ATCC 13076, 0.500 for *S. Typhimurium* ATCC 14028, 0.333 for *Salmonella enterica* M4, and 0.100 for *L. monocytogenes* ATCC 7644, which corresponds to 10^8 CFU/ml for each isolate.

BC composites (BC, BCP, BCX, and BCPX) were incubated in 10 ml of pathogenic bacterial suspension for 20 min at 25°C, with gentle shaking (100 rpm), followed by gentle rinsing (100 rpm) with 6 mM $CaCl_2$ solution for 1 min to remove loosely attached cells. Each composite was then placed in a stomacher bag with 50 ml of PBS and pummeled for 1 min at 8 strokes/s in a BagMixer 400 (Interscience, France). The numbers of pathogenic bacteria attached to the BC composites were enumerated by serial dilution of the stomacher bag fluid followed by plating of appropriate dilutions on xylose lysine deoxycholate agar (XLDA) (Oxoid, United Kingdom) (for *Salmonella* strains) or *Listeria* selective agar (LSA) with supplement SR0140 (Oxoid, United Kingdom) (for the *L. monocytogenes* strain). The numbers of attached bacterial cells were expressed as CFU per square centimeter of composite.

Attachment assays with pairwise combinations of bacteria. In order to determine whether the bacterial attachments to BC composites were stochastic or based on bacterial characteristics, attachment assays were carried out using pairwise combinations of two bacteria with different attachment characteristics, as described by Chia et al. (26). Our previous study showed that *S.* Typhimurium ATCC 14028 (~7.3 log CFU) and *L. monocytogenes* ATCC 7644 (~5.7 log CFU) attached in significantly different numbers (16). Three different pairwise ratios (i.e., 0.428 [30:70], 1 [50:50], and 2.333 [70:30]) of *S.* Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 7644 were prepared at a total density of 10^8 CFU/ml for the attachment assay. Studies of the attachment of different combinations of the *S.* Typhimurium-*L. monocytogenes* bacterial pair to different BC composites (BC, BCP, BCX, 0.1% BCPX, 0.3% BCPX, and 0.5% BCPX) were carried out as described above for the attachment studies with individual strains of bacteria. BC composites were incubated for 20 min at 25°C with a total of 10 ml of mixed *S.* Typhimurium-*L. monocytogenes* pathogenic bacterial suspension, prepared at a given pairwise ratio (10^8 CFU/ml), with gentle shaking at 100 rpm. Incubation was followed by gentle rinsing with $CaCl_2$ solution for 1 min before the composite was pummeled in 50 ml of PBS. Enumeration of attached bacterial cells (CFU per square centimeter of composite) was carried out by spread plating appropriate dilutions of the stomacher bag liquid on XLDA and LSA.

Similarly, attachment of *S.* Typhimurium-*L. monocytogenes* at the three different ratios to abiotic surfaces of different hydrophobicities, namely, glass slides (Premier slides; Azer Scientific, Morgantown, PA, USA), stainless steel coupons (type 302, no. 4 finishing, 1-mm thickness), and Teflon coupons (Tekdon, Myakka City, FL, USA), was carried out. Each slide (75 mm by 25 mm) was sterilized before incubation for 20 min in 20 ml of the mixed *S.* Typhimurium-*L. monocytogenes* bacterial suspension (10^8 CFU/ml), in an ESCO Airstream horizontal laminar flow clean bench (ONBoard Solutions, Australia), and then rinsed in $CaCl_2$ before swabbing of the bottom part of the slide. The cotton swabs were placed in 50 ml of PBS and pummeled for 1 min in the stomacher before spread plating on XLDA and LSA. Attachment ratios for the *S.* Typhimurium-*L. monocytogenes* pair were calculated by dividing the number of attached *S.* Typhimurium cells (log CFU per square centimeter) by the number of attached *L. monocytogenes* cells (log CFU per square centimeter).

TABLE 1 Chemical compositions of BC composites

Composite	Component(s) added (% [wt/vol])		Incorporation (%)		
	Pectin	Xyloglucan	Bacterial cellulose	Pectin	Xyloglucan
BCP	0.1	0	44.7	55.3	0
	0.3	0	43.9	56.1	0
	0.5	0	42.8	57.2	0
BCX	0	0.1	76.5	0	23.5
	0	0.3	72.3	0	27.7
	0	0.5	67.7	0	32.3
BCPX with 0.1% xyloglucan	0.1	0.1	34.0	41.5	24.5
	0.3	0.1	33.9	44.7	21.4
	0.5	0.1	32.5	48.5	18.3
BCPX with 0.3% xyloglucan	0.1	0.3	33.9	40.7	25.4
	0.3	0.3	33.2	43.1	23.7
	0.5	0.3	32.9	47.8	19.3
BCPX with 0.5% xyloglucan	0.1	0.5	33.5	39.4	26.7
	0.3	0.5	33.1	42.6	24.3
	0.5	0.5	33.2	47.4	20.1

Bacterial surface hydrophobicity. Bacterial surface hydrophobicity was determined with the assay of bacterial adhesion to hydrocarbons described by Rosenberg et al. (27), with modifications. Briefly, bacteria were pelleted, washed twice with PBS, and then suspended in PBS to achieve an OD_{550} of 1 ± 0.1. One milliliter of xylene (Fischer, Leicestershire, United Kingdom) was added to the 3-ml cell suspension, the mixture was vortex-mixed (VTX-3000L; LMS, Japan) for 2 min, and the phases were allowed to separate for 1 h. The absorbance of the aqueous phase was measured at 550 nm before (A_0) and after (A_1) the addition of xylene. The hydrophobicity index was expressed as follows: % attachment to xylene = $(1 - A_1/A_0) \times 100\%$. As described by Ahumada et al. (28), bacterial hydrophobicity can be classified into three groups according to the attachment to xylene, as follows: strongly hydrophobic, 71 to 100%; moderately hydrophobic, 36 to 70%; weakly hydrophobic, 0 to 35%.

Aggregation assays. The autoaggregation assay was performed as described by Collado et al. (14), with slight modifications. Briefly, bacteria were pelleted, washed, and resuspended to an OD_{600} of 0.25 ± 0.05. A 1.5-ml aliquot of bacterial suspension was then incubated at room temperature, and optical density was measured after 6 h. The coaggregation assay was performed as described by Grześkowiak et al. (29), with slight modifications. *S.* Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 7644 at three different pairwise ratios (i.e., 0.428 [30:70], 1 [50:50], and 2.333 [70:30]) were prepared at a total density of 10^8 CFU/ml of each bacterium. Suspensions of the strain combination at the three pairwise ratios were diluted with PBS to achieve an OD_{600} of 0.25 ± 0.05 (A_i), and absorbance was measured after 6 h (A_f). Autoaggregation and coaggregation were expressed as follows: % aggregation = $100 - [(A_f/A_i) \times 100]$. As described by Binetti et al. (30), strains were classified into three groups according to their autoaggregation and coaggregation abilities, as follows: high, >60%; moderate, 30 to 60%; low, <30%.

Data analysis. All experiments were performed in triplicate (3 independently grown bacterial cultures). Statistical analysis of results was performed using SPSS software (PASW Statistics 18; SPSS Inc., USA). The attachment of bacterial cells to the BC composites was expressed as CFU per square centimeter, and the data obtained were parametric. Significant differences in the overall attachments of the four strains to the BC composites were determined using one-way analysis of variance (ANOVA). One-way ANOVA was also used to establish significant differences among the types of BC composites, as well as among the different levels of PCW structural components used within each type of composite, in the attachment of each bacterial strain. Paired *t* tests were used to determine the significance of differences between the initial inoculum ratio and the final ratio of attached bacteria for all surfaces. Two-way ANOVA was used to establish the significance of differences in the attachment of *S.* Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 7644 when attached individually and at different initial inoculum ratios on the BC composites and abiotic surfaces. Pearson's correlation was used to determine the correlations between bacterial physicochemical properties and attachment. Differences between the means were determined using Tukey's method at a 95% confidence level.

RESULTS

Chemical composition analysis. The chemical composition analysis showed that the incorporation of pectin and xyloglucan increased when higher concentrations of these components were added to the HS medium (Table 1). Pectin was found to be incorporated in the composites at a relatively higher percentage than that of xyloglucan.

Effects of pectin and xyloglucan on attachment of bacteria to PCW models. An overall comparison of the attachment of *S.* Enteritidis ATCC 13076, *S.* Typhimurium ATCC 14028, *Salmonella enterica* M4, and *L. monocytogenes* ATCC 7644 showed that the bacterial strains attached to the BC composites in significantly different numbers. Specifically, *L. monocytogenes* attached in the lowest numbers (mean attachment to all composites, ~5.7 log CFU/cm^2 composite), followed by *Salmonella enterica* M4 (~6.5 log CFU/cm^2 composite) and lastly *S.* Enteritidis (~7.0 log CFU/cm^2 composite) and *S.* Typhimurium (~7.3 log CFU/cm^2 composite), which attached in the highest numbers and were not statistically different from each other.

Generally, the presence of pectin and xyloglucan in the BCP and BCX composites affected the attachment of *L. monocytogenes* differently from that of the *Salmonella* strains (Fig. 1). The presence of pectin in the BCP composites was associated with increased attachment of the *Salmonella* strains, whereas *L. monocytogenes* attachment was reduced. Xyloglucan in the BCX composites significantly reduced the attachment of *L. monocytogenes* but had no significant effect on the attachment of the *Salmonella* strains.

There was no significant interaction between pectin and xyloglucan in the BCPX composites with respect to the attachment of the four bacterial strains. This indicates that the effect of the level of pectin on bacterial attachment is independent of the effect of

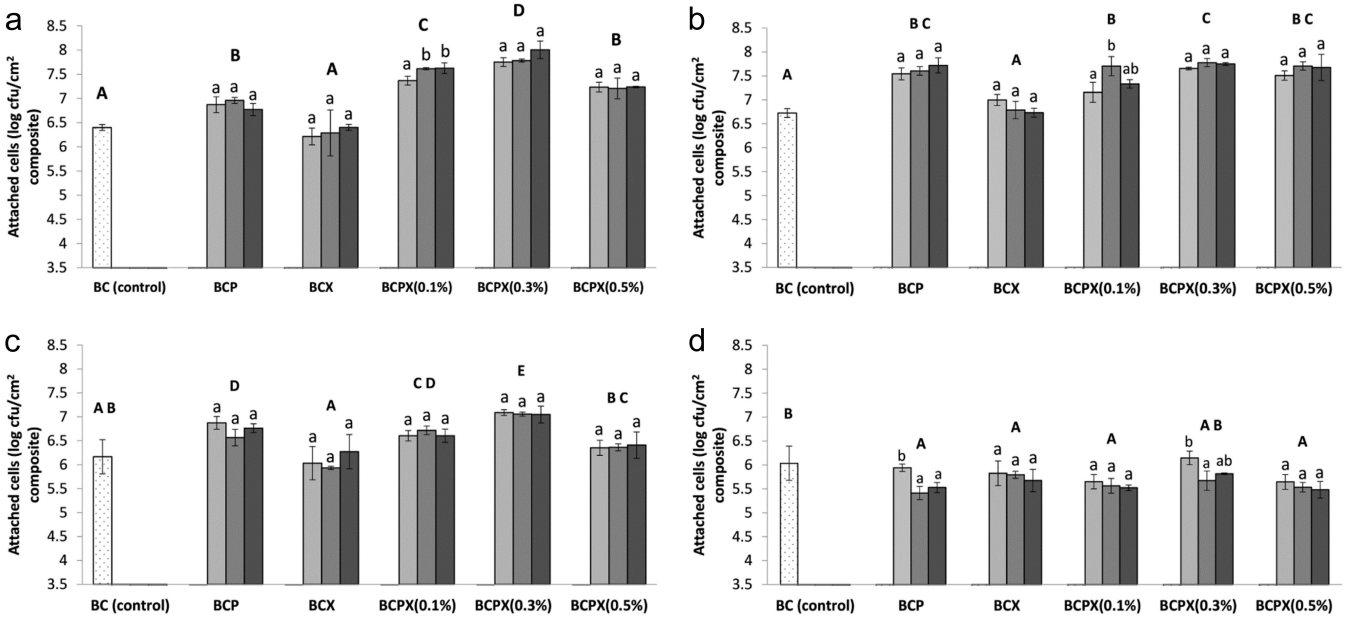

FIG 1 Attachment of *Salmonella* Enteritidis ATCC 13076 (a), *Salmonella* Typhimurium ATCC 14028 (b), *Salmonella enterica* M4 isolated from lettuce (c), and *Listeria monocytogenes* ATCC 7644 (d) to BC, BCP, BCX, 0.1% BCPX, 0.3% BCPX, and 0.5% BCPX. Different uppercase letters indicate significant differences between types of composites, whereas different lowercase letters indicate significant differences within each type of composite (one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$). Stippled bars, 0% PCW components (for BC); light gray bars, 0.1% PCW components; medium gray bars, 0.3% PCW components; dark gray bars, 0.5% PCW components added into the growth medium.

the level of xyloglucan and vice versa. When the effects of pectin and xyloglucan levels in the BCPX composites on bacterial attachment were compared, xyloglucan had a greater influence on bacterial attachment than did pectin. Xyloglucan significantly affected the attachment of all strains, whereas pectin significantly affected only the attachment of the *S.* Typhimurium strain. Interestingly, all *Salmonella* strains showed the greatest attachment to the 0.3% BCPX composite, which had 0.3% (wt/vol) xyloglucan added to the medium, regardless of the amount of pectin present in the growth medium.

Attachment of pairwise combinations of *Salmonella* Typhimurium and *Listeria monocytogenes* to BC composites and abiotic surfaces. A protocol developed by Chia et al. (26) was used to determine the stochasticity (or randomness) of the attachment process. Two bacteria with different attachment characteristics (*S.* Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 7644) were allowed to attach to the BC composites and abiotic surfaces in different pairwise ratios (0.428, 1, and 2.333). If the attachment process is stochastic, then the numbers of bacteria attached to the substratum are dependent on the concentration of the initial bacterial suspension. Therefore, attachment is considered stochastic when the initial ratio of the bacterial pair in a bacterial suspension does not differ significantly from the final ratio of the bacterial pair attached to the attachment surfaces. Similarly, attachment is deemed nonstochastic when the process is influenced by other factors which cause the initial and final ratios of the bacterial pair before and after attachment to be significantly different from each other.

Bacterial attachments to all BC composites and abiotic surfaces in this study were nonstochastic, and the attachment surface had a significant effect on the attached cell ratio (Tables 2 and 3). A higher initial *S.* Typhimurium/*L. monocytogenes* inoculum ratio resulted in a significantly higher *S.* Typhimurium/*L. monocytogenes* attachment ratio for most BC composites, whereas the trend was less distinct for abiotic surfaces. We also observed that the pairwise attachment of *S.* Typhimurium-*L. monocytogenes* was not significantly different on the different abiotic surfaces.

The numbers of *S.* Typhimurium and *L. monocytogenes* cells attached to the various surfaces were also examined. Compared to the attachment of *S.* Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 7644 to the BC composites, both bacteria generally showed significantly smaller differences in the numbers attached to abiotic surfaces, with the exception of 0.5% BCPX; this was because *S.* Typhimurium ATCC 14028 attached at $\sim 10^2$ CFU/cm^2 less to abiotic surfaces than to the BC composites. In contrast, *L. monocytogenes* ATCC 7644 cells attached at similar levels to BC composites and abiotic surfaces.

Differences in the attachment of *S.* Typhimurium and *L. monocytogenes* strains were generally greater at higher initial *S.* Typhimurium/*L. monocytogenes* inoculum ratios. In addition, the individual attachments of *S.* Typhimurium and *L. monocytogenes* strains were not significantly different from the attachment of the *S.* Typhimurium-*L. monocytogenes* pair at an initial inoculum ratio of 1 (50% *S.* Typhimurium and 50% *L. monocytogenes*).

Influence of bacterial physicochemical properties on attachment of four pathogens to BC composites. The hydrophobicity of all bacterial strains used in this study was relatively low, and most of the strains were classified as weakly hydrophobic (*S.* Enteritidis ATCC 13076, 34.70%; *S.* Typhimurium ATCC 14028, 15.00%; *L. monocytogenes* ATCC 7644, 9.13%). *Salmonella enterica* M4 (42.53%) was considered moderately hydrophobic. No significant correlation between hydrophobicity and bacterial attachment was found.

Most strains used in our study also showed low levels of auto-

TABLE 2 Attachment ratios for pairwise combinations of *S.* Typhimurium ATCC 14028-*L. monocytogenes* ATCC 7644 at different initial inoculum ratios on BC composites[a]

Composite	Component(s) added (% [wt/vol])		Final attachment ratio of *S.* Typhimurium to *L. monocytogenes* with an inoculum ratio of[b]:		
	Pectin	Xyloglucan	0.428	1	2.333
BC	0	0	1.230 ± 0.013 A	1.225 ± 0.002 A	1.342 ± 0.021 B
BCP	0.1	0	1.308 ± 0.008 A	1.379 ± 0.014 B	1.475 ± 0.033 C
	0.3	0	1.516 ± 0.041 A	1.512 ± 0.057 A	1.560 ± 0.040 A
	0.5	0	1.516 ± 0.019 A	1.504 ± 0.045 A	1.524 ± 0.039 A
BCX	0	0.1	1.321 ± 0.015 A	1.284 ± 0.027 A	1.384 ± 0.009 B
	0	0.3	1.323 ± 0.045 A	1.372 ± 0.020 A	1.360 ± 0.025 A
	0	0.5	1.216 ± 0.068 A	1.397 ± 0.050 B	1.389 ± 0.014 B
BCPX with 0.1% xyloglucan	0.1	0.1	1.194 ± 0.100 A	1.188 ± 0.031 A	1.378 ± 0.055 B
	0.3	0.1	1.173 ± 0.026 A	1.229 ± 0.045 A	1.334 ± 0.017 B
	0.5	0.1	1.244 ± 0.032 A	1.225 ± 0.015 A	1.310 ± 0.029 B
BCPX with 0.3% xyloglucan	0.1	0.3	1.180 ± 0.016 A	1.230 ± 0.041 A	1.337 ± 0.011 B
	0.3	0.3	1.296 ± 0.052 A	1.279 ± 0.016 A	1.364 ± 0.066 A
	0.5	0.3	1.304 ± 0.030 B	1.230 ± 0.025 A	1.373 ± 0.023 C
BCPX with 0.5% xyloglucan	0.1	0.5	1.128 ± 0.026 A	1.191 ± 0.028 AB	1.227 ± 0.032 C
	0.3	0.5	1.116 ± 0.048 A	1.231 ± 0.026 B	1.286 ± 0.020 B
	0.5	0.5	1.109 ± 0.023 A	1.218 ± 0.009 B	1.306 ± 0.019 C

[a] Initial inoculum ratios were 0.428, 1, and 2.333.
[b] Different letters indicate significant differences between final attachment ratios for pairwise combinations of *S.* Typhimurium and *L. monocytogenes* with the same BC composite (one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$).

aggregation (*S.* Enteritidis ATCC 13076, 25.60%; *Salmonella enterica* M4, 27.60%; *L. monocytogenes* ATCC 7644, 28.40%), with only *S.* Typhimurium ATCC 14028 displaying moderate autoaggregation (34.80%). We found a very weak positive correlation between hydrophobicity and autoaggregation (Pearson's correlation: $r = 0.27$, $R^2 = 7.29\%$, $P > 0.05$). Overall, there were no significant correlations of hydrophobicity, autoaggregation, and adhesion ability.

The coaggregation assays showed that the pairwise combinations of *S.* Typhimurium and *L. monocytogenes* had intermediate coaggregation abilities which were not significantly different at different initial inoculum ratios (*S.* Typhimurium/*L. monocytogenes* ratio of 0.428, 39.87% coaggregation; ratio of 1, 39.47% coaggregation; ratio of 2.333, 36.40% coaggregation). The resulting coaggregation values for the two strains were very similar to the autoaggregation values obtained for *S.* Typhimurium (~35%) but not to those for *L. monocytogenes* (~28%). The ability to coaggregate did not significantly affect the final *S.* Typhimurium/*L. monocytogenes* attachment ratios for all initial inoculum ratios.

DISCUSSION

Chemical composition analysis. A BC-based PCW model was used in our study, as there are many difficulties associated with using native PCWs to study bacterial adhesion. For example, the use of physical or chemical extraction of PCWs distorts the PCW structure, while the heterogeneous nature of PCW compositions makes it difficult to study how individual PCW components affect bacterial attachment (31, 32).

We examined the effects of varying the concentrations of PCW components added to the growth medium on their incorporation within the PCW models. According to Zykwinska et al. (33), the amount of pectin absorbed onto cellulose decreases as the level of xyloglucan increases, with no pectin being bound to cellulose at xyloglucan concentrations above 500 μg/ml; however, we observed that the incorporation of both pectin and xyloglucan increased when greater amounts of the PCW components were added to the medium, even up to a concentration of 5,000 μg/ml. Zykwinska et al. (33) measured the binding of pectin and xyloglucan to cellulose by mixing the polysaccharide solutions with cellulose; however, our study used a constructive approach that resembled the formation of native PCWs, which may be more representative of the occurrence in nature.

Our BC composites were shown to have compositions similar to those of other BC-based PCW models reported in the literature (34, 35). Our BCPX composites also showed chemical composi-

TABLE 3 Attachment ratios for pairwise combinations of *S.* Typhimurium ATCC 14028-*L. monocytogenes* ATCC 7644 at different initial inoculum ratios on abiotic surfaces of different hydrophobicities[a]

Initial inoculum ratio of *S.* Typhimurium to *L. monocytogenes*	Final attachment ratio of *S.* Typhimurium to *L. monocytogenes* on[b]:		
	Glass	Stainless steel	Teflon
0.428	1.073 ± 0.082 A	1.247 ± 0.113 A	1.371 ± 0.082 A
1	1.140 ± 0.085 A	1.211 ± 0.134 A	1.547 ± 0.070 B
2.333	1.145 ± 0.029 A	1.172 ± 0.036 AB	1.293 ± 0.091 B

[a] Initial inoculum ratios were 0.428, 1, and 2.333.
[b] Different letters indicate significant differences between final attachment ratios of *S.* Typhimurium to *L. monocytogenes* on different abiotic surfaces for the same initial inoculum ratio of *S.* Typhimurium to *L. monocytogenes* (one-way ANOVA and Tukey's pairwise comparison, $P < 0.05$).

tions similar to those of average native PCWs (approximately 35% cellulose, 40% pectin, and 15% xyloglucan) (36). This is important because our BC composites are designed to be used as models for understanding the interactions of pathogenic bacteria with native PCWs.

Effects of pectin and xyloglucan on attachment of pathogenic bacteria to PCW models. We found that all *Salmonella* strains attached to BC composites in significantly greater numbers than did *L. monocytogenes* ATCC 7644, which is in agreement with our previous findings (16). In another study, Kutter et al. (37) found that *Salmonella enterica* was able to colonize barley roots while *Listeria* strains were not. Jablasone et al. (38) showed that *S.* Typhimurium was internalized into plant tissues while *L. monocytogenes* attached only to the plant surface and was not internalized in seedlings. One possible reason that may account for the greater attachment of *Salmonella* strains is the availability of a larger number of adhesive appendages, such as flagella, fimbriae, thin aggregative fimbriae (tafi), lipopolysaccharides, and outer membrane proteins (39–41), produced by *Salmonella* for attachment. In contrast, *Listeria* attachment generally relies only on flagella (42). It should be noted, however, that Jablasone et al. (38) and Takeuchi et al. (43) observed greater *L. monocytogenes* attachment than *Salmonella* species attachment on cut lettuce leaves and on various vegetable seedlings.

Higher levels of pectin in the BCP composite increased the attachment of all *Salmonella* strains in our study. This result is consistent with the findings of Saggers et al. (9), who observed that another strain of *S.* Typhimurium attached preferentially to the pectin layer at the potato cell wall junction, while less attachment was observed where less pectin was present. Xyloglucan did not significantly affect the attachment of *Salmonella* strains in the BCX composites, but it significantly affected the attachment of all *Salmonella* strains when present with pectin in the BCPX composites.

Higher levels of pectin increased the attachment of only the *Salmonella* strains and not the *Listeria* strain, which suggests specific interactions between *Salmonella* cells and the pectin molecules. Most of the receptor-ligand interactions required for bacterial adhesion are mediated by carbohydrates and bacterial surface adhesins (44, 45). Extensive research has been undertaken regarding the role of sugar residues that serve as receptors for the binding of animal pathogens to animal cells (46, 47). However, only a few studies have focused on the role of sugar residues in the attachment of human pathogens to plants. Klerks et al. (4) found that root exudates that contain many monosaccharides, such as fructose and glucose, cause chemotaxis of *Salmonella* strains, which use these signals to move toward the plants. Root exudates also condition *S. enterica* cells for attachment and colonization of the plant roots. Another study proposed that exudates of germinating seeds and developing roots trigger *Escherichia coli* O157 to colonize seedlings (48).

Based on these data, we hypothesized that pectin and xyloglucan could have interacted physically and given the BCPX composites surface structures that are distinct from those of the BCP and BCX composites. This may also explain why all *Salmonella* strains showed the highest levels of attachment to the BCPX composite with 0.3% (wt/vol) xyloglucan, regardless of the amount of pectin added to the growth medium. The BCPX composite with 0.3% (wt/vol) xyloglucan may have distinct structural features (such as porosity and surface roughness) that are ideal for the attachment of the *Salmonella* cells. Cybulska et al. (35) found that the cellulose fibrils of BCPX (~75 nm) were significantly thicker than those of BC (~37 nm) and BCP (~46 nm); the thicker fibrils may provide additional surface area for the attachment of bacteria. Very few studies have investigated the effects of pectin and xyloglucan on the structural properties of BC composites. Fanta et al. (49) found that the fibril diameters for BC (110 ± 33 nm) and BCPX (123 ± 29 nm) were similar, while the fibril diameter for BCP was much smaller (45 ± 9 nm). Both Cybulska et al. (35) and Fanta et al. (49) found that BC has the highest porosity, BCP has lower porosity after the addition of pectin, and BCPX has the lowest porosity and greatest compactness among the composites. The effects of various concentrations of pectin and xyloglucan on the structural properties of BC composites have not been determined, however, and mechanical studies and surface profiling of the BC composites need to be performed in future studies.

Attachment of *L. monocytogenes* ATCC 7644 to the BCP and BCX composites was significantly lower than that to BC, but we have yet to determine a reason for this occurrence. A possible explanation is that the BC matrix, which is more porous than the BCP and BCX composites, has increased area for bacterial attachment and its porous structure enables it to trap liquids and small particles (50, 51).

Attachment of pairwise combinations of *Salmonella* Typhimurium and *Listeria monocytogenes* to BC composites and abiotic surfaces. Our findings showed that bacterial attachment to all BC composites was nonstochastic, as the numbers of attached cells of the *S.* Typhimurium and *L. monocytogenes* strains did not depend on the levels of the bacterial strains present in the initial inoculum (Fig. 2). Instead, the attachment of the bacterial pair to the BC composites was shown to be strongly influenced by individual bacterial attachment characteristics, especially for *S.* Typhimurium ATCC 14028. An increased concentration of *S.* Typhimurium ATCC 14028 in the bacterial suspension (higher initial *S.* Typhimurium/*L. monocytogenes* inoculum ratio) led to significant increases in the final *S.* Typhimurium/*L. monocytogenes* attachment ratio in most cases. The finding that the two strains did not interact with each other and attached similarly when they were present together or separately supported the importance of the role of individual bacterial attachment characteristics in adhesion.

Several studies have suggested that bacterial attachment can be influenced by the physicochemical properties of the attachment surface and the attaching bacteria (13, 14). To investigate whether the strong attachment of the *Salmonella* strain to BC composites was influenced by these factors, assays of the attachment of the bacterial pair to abiotic surfaces with different hydrophobicities and other physicochemical properties were carried out. We established that the numbers of *S.* Typhimurium ATCC 14028 cells that attached to different abiotic surfaces were similar to those of *L. monocytogenes* ATCC 7644, whereas the numbers that attached to the BC composites were far greater. This finding suggests that the hydrophobicity of the attachment surface does not greatly affect the attachment of *S.* Typhimurium ATCC 14028 and thus that other features of bacterial and surface interactions are responsible for the attachment of *Salmonella* strains to the BC composites.

Role of bacterial physicochemical properties in attachment of four pathogens to BC composites. There have been some findings suggesting that the physicochemical properties of bacteria influence bacterial attachment to various surfaces. As mentioned earlier, there is strong evidence that autoaggregation is correlated

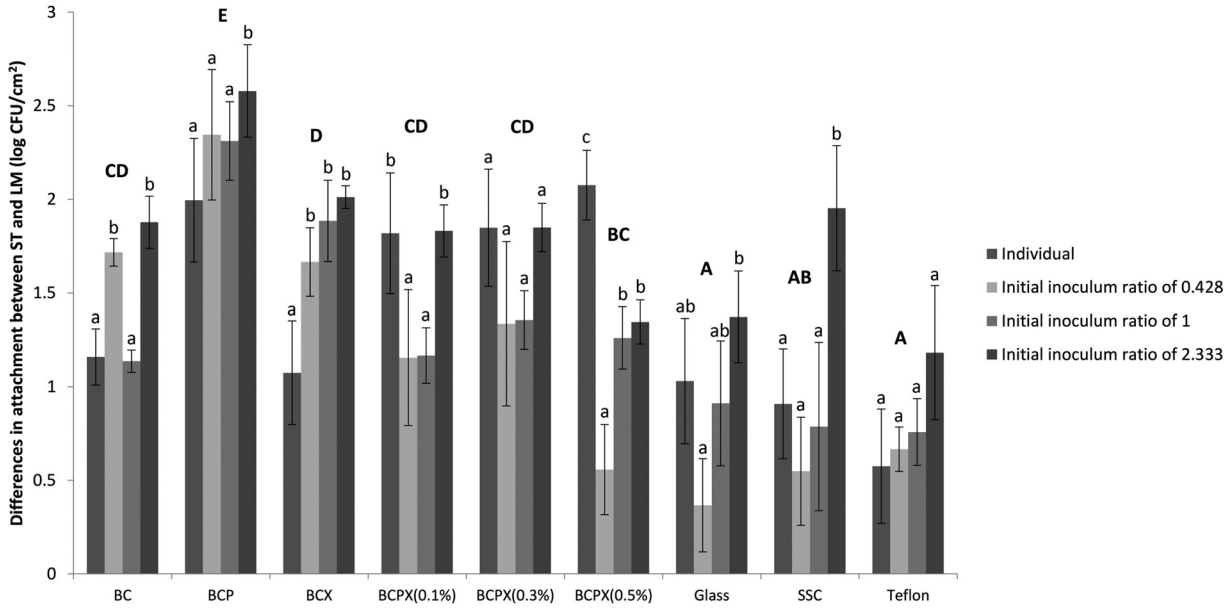

FIG 2 Differences in attachment numbers between *S.* Typhimurium ATCC 14028 (ST) and *L. monocytogenes* ATCC 7644 (LM) when present individually and at different initial *S.* Typhimurium/*L. monocytogenes* inoculum ratios (0.428, 1, or 2.333) on BC composites and abiotic surfaces. SSC, stainless steel coupons. Different uppercase letters indicate significant differences between attachment surfaces, whereas different lowercase letters indicate significant differences between individual attachment and attachment at different initial inoculum ratios for the same attachment surface (two-way ANOVA and Tukey's pairwise comparison, $P < 0.05$).

with the ability to attach to surfaces (14, 52, 53). However, *S.* Typhimurium ATCC 14028 and *L. monocytogenes* ATCC 7644 demonstrated hydrophobicities and autoaggregation levels that were not significantly different from each other, although their abilities to attach to surfaces were significantly different. As for the pairwise combinations of *S.* Typhimurium and *L. monocytogenes*, coaggregation values obtained for the bacterial pair were similar to the autoaggregation value obtained for *S.* Typhimurium ATCC 14028. This again emphasizes that some specific characteristics of the *S.* Typhimurium strain, including its autoaggregation and attachment abilities, strongly influence the attachment of the bacterial pair.

There were no significant correlations of hydrophobicity, autoaggregation, coaggregation, and adhesion. In addition to our study, other studies have come to the conclusion that these physicochemical characteristics vary in importance for the attachment of different species of bacteria (54, 55). In contrast to Hood and Zottola (56), who found that cell surface charge, polysaccharide production, and hydrophobicity affect bacterial attachment to surfaces, Flint et al. (55) observed that the role of these factors appears to be species specific. Their study was unable to show an association between bacterial attachment and any of the three factors. Similarly, a study by Oliveira et al. (57) showed that cell surface hydrophobicity did not play a major role in the attachment of *Salmonella* strains to stainless steel surfaces. This indicates that other factors may be more important for *Salmonella* attachment than the physicochemical properties of the bacterial cells and the attachment surface.

Only a limited number of bacterial strains were investigated in this study. Ideally, a larger number of individual strains or a cocktail of strains could be used. The data provided in this study represent a resource for further studies of this type, as well as providing insights into differences in strain interactions with the composites and ultimately PCWs.

Overall, our findings demonstrated that PCW components significantly affect bacterial attachment. Pectin in the BCP composites and xyloglucan in association with pectin in the BCPX composites were shown to increase *Salmonella* attachment significantly. We confirmed that the attachment of the bacterial strains, particularly *S.* Typhimurium ATCC 14028, to the BC composites was not stochastic and was most likely controlled by specific interactions between the bacteria and the attachment surface. We also found that bacterial attachment was not significantly influenced by the hydrophobicity of the attachment surface or the physicochemical properties of the bacteria. It is still unclear, however, whether the attachment of the *Salmonella* strains to the BC composites was due to the influence of the PCW polysaccharides on the physical and structural characteristics of the BC composites.

Surface roughness and porosity are known to favor bacterial attachment by providing a greater surface area for attachment (50). The effects of these factors on *Salmonella* attachment to PCWs remain to be investigated. The findings of this study improve our understanding of how bacteria attach to PCWs. This may in turn aid in the development of more effective methods for fresh produce decontamination, as the current sanitation method using chlorine has limited effectiveness (58).

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PLOS ONE

RESEARCH ARTICLE

Role of Fimbriae, Flagella and Cellulose on the Attachment of *Salmonella* Typhimurium ATCC 14028 to Plant Cell Wall Models

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Abstract

Cases of foodborne disease caused by *Salmonella* are frequently associated with the consumption of minimally processed produce. Bacterial cell surface components are known to be important for the attachment of bacterial pathogens to fresh produce. The role of these extracellular structures in *Salmonella* attachment to plant cell walls has not been investigated in detail. We investigated the role of flagella, fimbriae and cellulose on the attachment of *Salmonella* Typhimurium ATCC 14028 and a range of isogenic deletion mutants (Δ*fliC fljB*, Δ*bcsA*, Δ*csgA*, Δ*csgA bcsA* and Δ*csgD*) to bacterial cellulose (BC)-based plant cell wall models [BC-Pectin (BCP), BC-Xyloglucan (BCX) and BC-Pectin-Xyloglucan (BCPX)] after growth at different temperatures (28°C and 37°C). We found that all three cell surface components were produced at 28°C but only the flagella was produced at 37°C. Flagella appeared to be most important for attachment (reduction of up to 1.5 log CFU/cm^2) although both cellulose and fimbriae also aided in attachment. The *csgD* deletion mutant, which lacks both cellulose and fimbriae, showed significantly higher attachment as compared to wild type cells at 37°C. This may be due to the increased expression of flagella-related genes which are also indirectly regulated by the *csgD* gene. Our study suggests that bacterial attachment to plant cell walls is a complex process involving many factors. Although flagella, cellulose and fimbriae all aid in attachment, these structures are not the only mechanism as no strain was completely defective in its attachment.

Introduction

Over the past few decades there has been a fast growing and world-wide trend of greater consumption of fresh produce, such as fruits and vegetables, mainly due to a heightened consumer awareness of the benefits of a healthy diet [1,2]. Governments around the world have also encouraged the consumption of fresh produce in an attempt to proactively prevent various diseases such as heart disease, strokes, eye diseases and stomach cancers [3]. The prevalence of foodborne illness associated with consumption of minimally processed produce has, however,

also been increasing rapidly [4,5]. Between 1996 and 2005, the consumption of leafy greens in the United States increased by 9% but the incidence of foodborne outbreaks associated with it increased by 39% [6]. Fresh produce is now recognized as the main cause of foodborne outbreaks around the world [7].

It was initially thought that enteric pathogens, which are usually found in the intestinal tracts of animals, would survive poorly on plant surfaces where microorganisms encounter harsh environmental conditions such as drastic temperature fluctuations, desiccation, sunlight and nutrient limitation but recent research [8–11] has shown otherwise. *Salmonella* in particular was previously largely only reported to be associated with foods of animal origin but is now the most commonly identified human bacterial pathogen associated with fresh produce [12,13].

Human foodborne pathogens need to establish themselves on surfaces, including plants, as a precursor to causing foodborne disease and therefore bacterial attachment is a crucial step in their transmission [14,15]. Cut surfaces of plant cell walls (PCWs) are especially vulnerable to the attachment of human foodborne bacterial pathogens as these surfaces lack the waxy cuticle which repels water that could carry pathogens [16,17]. These cut surfaces also exude nutrients and water which are favourable for the growth and survival of the pathogens. Some human pathogens are able to penetrate the internal tissues after attaching to cut plant surfaces which could protect them from chemical sanitizers [18]. Saggers et al. [19] suggested that PCW components at the PCW junction, particularly pectin, may provide receptor sites for *Salmonella* attachment. Our previous findings [20] also showed that pectin alone and pectin in combination with xyloglucan both increased the attachment of *Salmonella* strains.

Studies have found that bacterial cell surface components such as cellulose, flagella and fimbriae are important for the attachment of pathogens to fresh produce [17,21–23]. Flagella are long, thin surface appendages that extend up to 20μm and which are important for motility and chemotaxis [24]. Bacteria use flagella to move along the plant surface before finding a favourable attachment site [25]. Fimbriae are fine, hair-like protein appendages which contain adhesins on their tips with affinity to different sugar molecules and can be up to several micrometers long [26]. Cellulose, which consists of β(1–4)-linked glucose units secreted by bacterial cells, can hinder flagellar rotation and limit bacterial motility [27]. Production of both fimbriae and cellulose are regulated by the *csgD* gene in *Salmonella*. Expression of these structures are associated with biofilm formation and are important for the environmental persistence of *Salmonella* including enhancing its ability to avoid desiccation stress [28,29]. Temperature regulation of the expression of cellulose and fimbriae also takes place at the transcription level of *csgD* [30]. The *csgD* gene was the first enteric bacterial regulator identified to play a more influential role on enteric bacterial interaction with plants than in their animal hosts [10].

The specific mechanisms involved in the association between bacterial cell structures and PCW components exposed on cut PCWs have yet to be elucidated and up to now, very few genetic elements have been identified to be important for the attachment of human foodborne pathogens to plants. However, the ability to form biofilms has been correlated to better survival and stronger attachment to fresh produce [26], for example, *Salmonella* isolates sampled during tomato outbreaks produced biofilms and attached better to tomato leaflets compared to non-biofilm producing strains [31].

No studies reporting the interactions between bacterial surface components and specific PCW components have been reported. Most studies have focused only on the interactions between bacterial components and whole plant tissues. In this study we aimed to investigate how *Salmonella* cell surface structures (flagella, fimbriae and cellulose) influence attachment to major structural components of the PCW (cellulose, pectin and xyloglucan) after growth at 28°C (average environmental temperature) and 37°C (animal and human body temperature). Cases of salmonellosis usually peak during summer in most countries [32]. Most fresh produce

have optimal growth temperatures within the range of 21°C to 32°C. For this reason, a growth temperature of 28°C was chosen as it coincides with the average summer temperatures in many countries, including China which is the top global producer of fresh vegetables [33].

In order to investigate this, a bacterial cellulose (BC)-based PCW model was used. The PCW model was produced by culturing *Gluconacetobacter xylinus*, a BC-producing bacterium, in Hestrin and Schramm (HS) growth medium with the addition of pectin and/or xyloglucan. Formation of the PCW model mimics the natural process of PCW deposition in native PCWs [34]. The PCW model was also found to possess similar structural and chemical properties to native PCWs [34,35] and has been previously optimized for studying bacterial attachment to PCWs [36]. Another study [37] has shown that the trend and numbers of *Salmonella* cells attaching to the natural PCWs (potato tuber, apple fruit and lettuce leaves) and to BC composites were similar to each other. In comparison to the heterogeneous composition of native PCWs, the PCW model is more versatile as its chemical composition can easily be manipulated. This allows direct investigation on how bacterial cell surface components interact with individual components in the PCW without the interference of other factors which may complicate the study.

Materials and Methods

Bacterial strains and culture conditions

Gluconacetobacter xylinus ATCC 53524 and *Salmonella enterica* subspecies *enterica* serovar Typhimurium ATCC 14028 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Gene knockout mutants of *S*. Typhimurium ATCC 14028 used in attachment experiments and their sources are listed in Table 1. Gene expression in these mutants has been previously well confirmed and characterised [38–40].

The *G. xylinus* strain which produces BC was cultured statically at 30°C for 72h in Hestrin and Schramm (HS) broth containing 2% (w/v) glucose, 0.5% (w/v) peptone, 0.5% (w/v) yeast extract, 0.27% (w/v) Na_2HPO_4 and 0.115% (w/v) citric acid [41]. *G. xylinus* was maintained on HS agar at 4°C which was prepared by adding 1.5% agar to the HS medium.

For the attachment experiments, wild type and mutant strains of *S*. Typhimurium ATCC 14028 were grown at either 28°C or 37°C in tryptic soy broth (TSB; Merck, Darmstadt, Germany) under shaking incubation (150rpm) (Lab Companion SK-600 benchtop shaker; Medline, UK). Production of cellulose and fimbriae by these strains at 28°C or 37°C were confirmed by monitoring their colony morphology on Luria-Bertani (LB; Merck, Darmstadt, Germany) medium without salt supplemented with 40μg/mL of Congo Red (CR; Sigma-Aldrich, Missouri, USA) and 20μg/mL Coomassie brilliant blue (CBB; Sigma-Aldrich, Missouri, USA) when grown at these two temperatures. Cellulose production was further

Table 1. Genotype and characteristics of *S*. Typhimurium ATCC 14028 mutant strains used in this study.

Genotype	Characteristics	Source or reference
Δ*fliC fljB*	Lacks phase 1 and 2 flagellin	Miao et al. [38]
Δ*bcsA*	Lacks cellulose	White et al. [29]
Δ*csgA*	Lacks fimbriae	White et al. [29]
Δ*csgA bcsA*	Lacks cellulose and fimbriae	As described in methods
Δ*csgD*	Missing the major biofilm transcriptional regulator coding sequence, lacks cellulose and fimbriae	MacKenzie et al. [39]

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confirmed using LB medium without salt supplemented with 50μg/mL of Calcofluor White (CW; Sigma-Aldrich, Missouri, USA), colonies which produce cellulose will fluoresce under UV light. Flagella production at both temperatures was determined using Ryu's flagella stain as described by Kodaka *et al.* [42]. *Salmonella* strains were maintained on tryptic soy agar (TSA; Merck, Darmstadt, Germany) at 4°C.

Generation of *S*. Typhimurium 14028 Δ*csgA bcsA* double mutant strain

The *S*. Typhimurium ATCC 14028 Δ*bcsA* strain was generated as previously described [29]. *S*. Typhimurium Δ*bcsA* cells were transformed with the pHSG415/Δ*csgA* (formerly Δ*agfA*) construct prepared from S. Enteritidis 27655-3b genomic DNA [43] and selected on LB agar supplemented with 100μg/mL ampicillin. The Δ*csgA* mutation was successfully performed through allelic exchange following established procedures [44]. Final ampicillin-sensitive *S*. Typhimurium Δ*csgA bcsA* colonies were differentiated from Δ*bcsA* colonies by growth at 28°C on agar media (1% tryptone, 1.5% agar) supplemented with 100μg/mL Congo red; Δ*csgA bcsA* colonies appeared light pink, whereas Δ*bcsA* colonies appeared orange or red. PCR was used to confirm the Δ*csgA* mutation, using primers TAFPF (`TACGCCAGGAAGGATCAAAACTAT`) and TAFPR (`GCCGTCGCGCACAGAGA`); PCR products were purified and confirmed by DNA sequencing (Eurofins MWG Operon, Kentucky, USA).

Production of bacterial cellulose-based plant cell wall models

BC-based PCW models were produced as described in our previous paper [20]. Briefly, a primary inoculum of *G. xylinus* ATCC 53524 was prepared by transferring a colony grown on HS agar into HS broth which was incubated statically at 30°C for 72h. The primary inoculum was used for the scale-up production of all BC composites and was added to fresh HS medium with or without combinations of pectin and/or xyloglucan as shown below:

- BC was produced with only HS medium without additional additives.
- BC-Pectin (BCP) was produced by adding 0.1%, 0.3% and 0.5% w/v apple pectin (kindly donated by Herbstreith & Fox, Neuenbürg, Germany) to the HS medium and an optimal concentration of $CaCl_2$ was added to form a low degree of esterification (DE) pectin gel, i.e. 3mM $CaCl_2$ for 0.1% w/v pectin, 6mM $CaCl_2$ for 0.3% w/v pectin and 12.5mM $CaCl_2$ for 0.5% w/v pectin (R&M Chemicals, Malaysia).
- BC-Xyloglucan (BCX) was produced by adding 0.1%, 0.3% and 0.5% w/v xyloglucan (Megazyme, County Wicklow, Ireland) to the HS medium.
- BC-Pectin-Xyloglucan (BCPX) was produced by adding different combinations of pectin and xyloglucan (0.1%, 0.3% and 0.5% w/v), varying concentrations of calcium chloride was added according to the amount of pectin present as shown earlier.

Composites were produced in enclosed plastic containers (1.5cm x 1.5cm x 1.5cm) incubated statically for 72h depending on the HS medium composition. During harvest, BC composites occur as a gelatinous layer floating above the growth medium. Harvested composites were rinsed in 6mM $CaCl_2$ at 100rpm for 1h to remove media components. The range of pectin and xyloglucan concentrations were selected based on work carried out previously [20,36,37,45,46] which produced composites with characteristics that fall within average native PCW component concentrations. Chemical composition analysis of the BC composites were consistent when compared to each other [20].

Attachment to BC composites

Attachment experiments were carried out as described previously [20]. Early stationary phase cultures of *S*. Typhimurium ATCC 14028 and its mutants grown for 18h at either 28°C or 37°C were centrifuged at 5500 x *g* (Hettich D-78532, Tuttlingen, Germany) for 10 min at 4°C. The pellet was washed twice with phosphate buffer saline (PBS) (pH 7.4) (1st BASE, Singapore) and resuspended in PBS to an optical density at 600nm (UV/Vis spectrophotometer, Shimadzu UV mini-1240, USA) which corresponds to 10^8 CFU/mL for each strain.

After rinsing, BC composites (BC, BCP, BCX, BCPX) (1.5cm x 1.5cm, ~ 2mm thickness) were incubated in 10mL of each pathogenic bacteria suspension (10^8 CFU/mL) for 20 mins with gentle shaking (100rpm) at 25°C. After incubation, gentle rinsing (100rpm) was carried out in 6mM $CaCl_2$ solution for a minute to remove loosely attached cells. Each composite was then placed in a stomacher bag filled with 50mL PBS and pummelled for a minute at 8 strokes/sec in BagMixer 400 (Interscience, France). The resulting stomached fluid was then serial diluted and appropriate dilutions were plated on xylose lysine deoxycholate agar (XLDA; Oxoid, UK) to enumerate the number of pathogenic bacteria attached to the BC composite (CFU/cm^2 composite). There may be some variability in the surface chemistry of the surface exposed to the air compared to the other surfaces exposed to the HS medium. We have reasonably assumed that this will not significantly affect the attachment results as all surfaces of the composites were exposed to bacteria when the composites were fully immersed in the bacterial suspension.

Data analysis

All experiments were performed in triplicates with three independently grown bacterial cultures. Statistical analysis of results was performed using Statistical Package for the Social Sciences (SPSS) (PASW Statistics 18, SPSS Inc., USA). One-way analysis of variance (ANOVA) was used to compare significant differences between wild type and mutant strains of *S*. Typhimurium ATCC 14028 grown at the same temperature (either 28°C or 37°C) for their overall attachment to the BC composites. Another one-way ANOVA was carried out individually for each strain grown at a specific temperature (either 28°C or 37°C) to compare significant differences in numbers attaching to different BC composites. Independent sample t-tests were also conducted to determine significant differences for the same strain for its attachment to the BC composites when grown at two different temperatures (comparing 28°C and 37°C). Differences among the means were determined using Tukey's method at 95% confidence level.

Results and Discussion

Production of cell surface components by *S*. Typhimurium ATCC 14028 wild type strains

S. Typhimurium ATCC 14028 produced flagella when grown at 28°C and 37°C (shown in S1 Fig). However, the *S*. Typhimurium strain displayed temperature-dependent expression of cellulose and fimbriae as can be seen on CR and CW plates (Fig 1). As described by Romling et al. [47], the wild type strain of *S*. Typhimurium was able to produce cellulose and fimbriae at 28°C and showed rough, dry and red (rdar) phenotypes on the CR plate. Wild type colonies grown on the CW plate fluoresced under UV light. When grown at 37°C however, the strain lost the ability to produce these structures and appeared as smooth and white colonies (saw) on the CR plate and colonies formed on the CW plate did not fluoresce. White et al. [29] also noted that *Salmonella* only produce these extracellular structures at incubation temperatures of below 30°C and under nutrient-limited conditions at low osmolarity. According to Kader et al. [48],

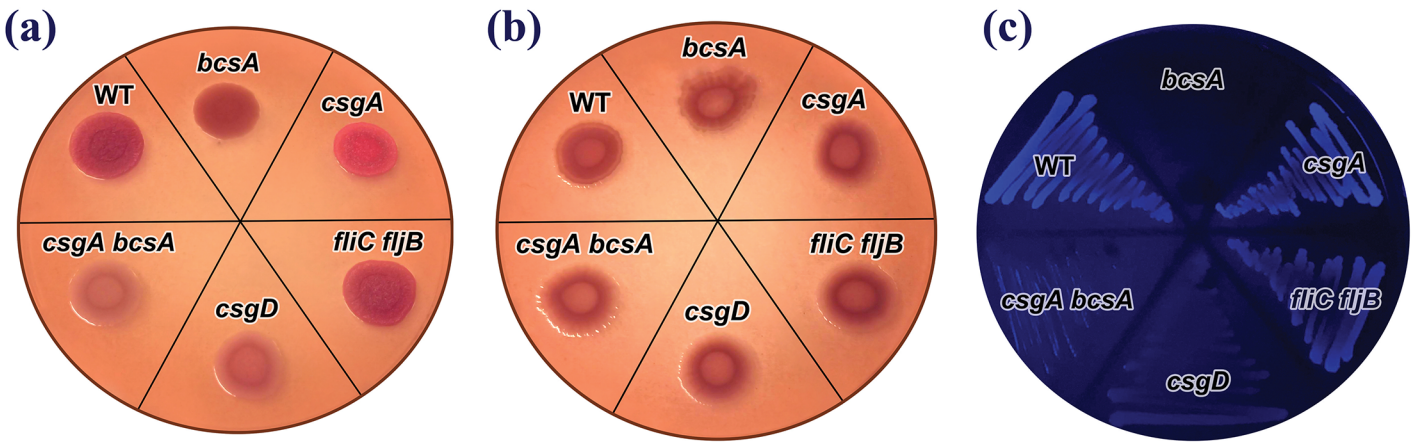

Fig 1. Colony morphology of *Salmonella* Typhimurium ATCC 14028 wild type and mutant strains. Colonies formed on (a) Congo Red (CR) agar plate grown at 28°C, (b) CR agar plate grown at 37°C and (c) Calcofluor White (CW) agar plate grown at 28°C.

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temperature regulation of the rdar morphotype is mediated by the temperature gradient in cyclic-di(3'→5')-guanylic acid (c-di-GMP) concentrations. The c-di-GMP secondary messenger regulates cellulose and fimbriae production by affecting both CsgA and CsgD expression on the transcriptional and post-transcriptional levels respectively. Therefore, wild type *Salmonella* strains are expected to have lower levels of c-di-GMP at 37°C than at 28°C, this could be caused by either increased phosphodiesterase activity or reduced diguanylate cylase activity [48].

Effect of PCW components and temperature on *Salmonella* attachment to BC composites

We found in this study that the varying levels of pectin and xyloglucan (0.1%, 0.3% and 0.5%) did not have significant effect on *S*. Typhimurium attachment to the BC composites (shown in S2 Fig), hence attachment numbers for each type of composite were collated and presented as an average value in Fig 2 for easy comparison. We initially expected the *S*. Typhimurium strains grown at 28°C (especially the wild type strain and the Δ*fliC fljB* mutant which can produce cellulose and fimbriae at 28°C) to have higher attachment levels to the BC composites than the same strains grown at 37°C. However, of the 6 strains only the wild type *S*. Typhimurium strain showed significant difference in the number of cells attached to the BC composites when grown at the two different temperatures ($p<0.05$) whereas others showed similar attachment for both temperatures ($p>0.05$). All strains grown at 28°C did not show significant differences in their attachment to the various BC composites ($p>0.05$) except for the Δ*fliC fljB* mutant which showed the differential attachment to the BC composites. The Δ*fliC fljB* mutant attached at lower levels compared to the other strains and its attachment to the BC composites containing pectin and/or xyloglucan was significantly higher than to the BC-only composite ($p<0.05$). This suggests that flagella may interact with pectin and xyloglucan, with the loss of flagella decreasing *Salmonella* attachment to the BC composites containing these PCW components. As Warriner and Namvar [49] have pointed out, human pathogen attachment to plants may involve specific recognition interactions between the bacterial cell surface and physical structures on the leaf. Cell surface components (such as cellulose, flagella and fimbriae) may harbour surface epitopes which enable pathogens to preferentially attach to cut surfaces and natural openings, such as stomata, which expose nutrients produced during photosynthesis [12,17]. A study by Saggers et al. [19] also indicated interactions between bacterial cells and

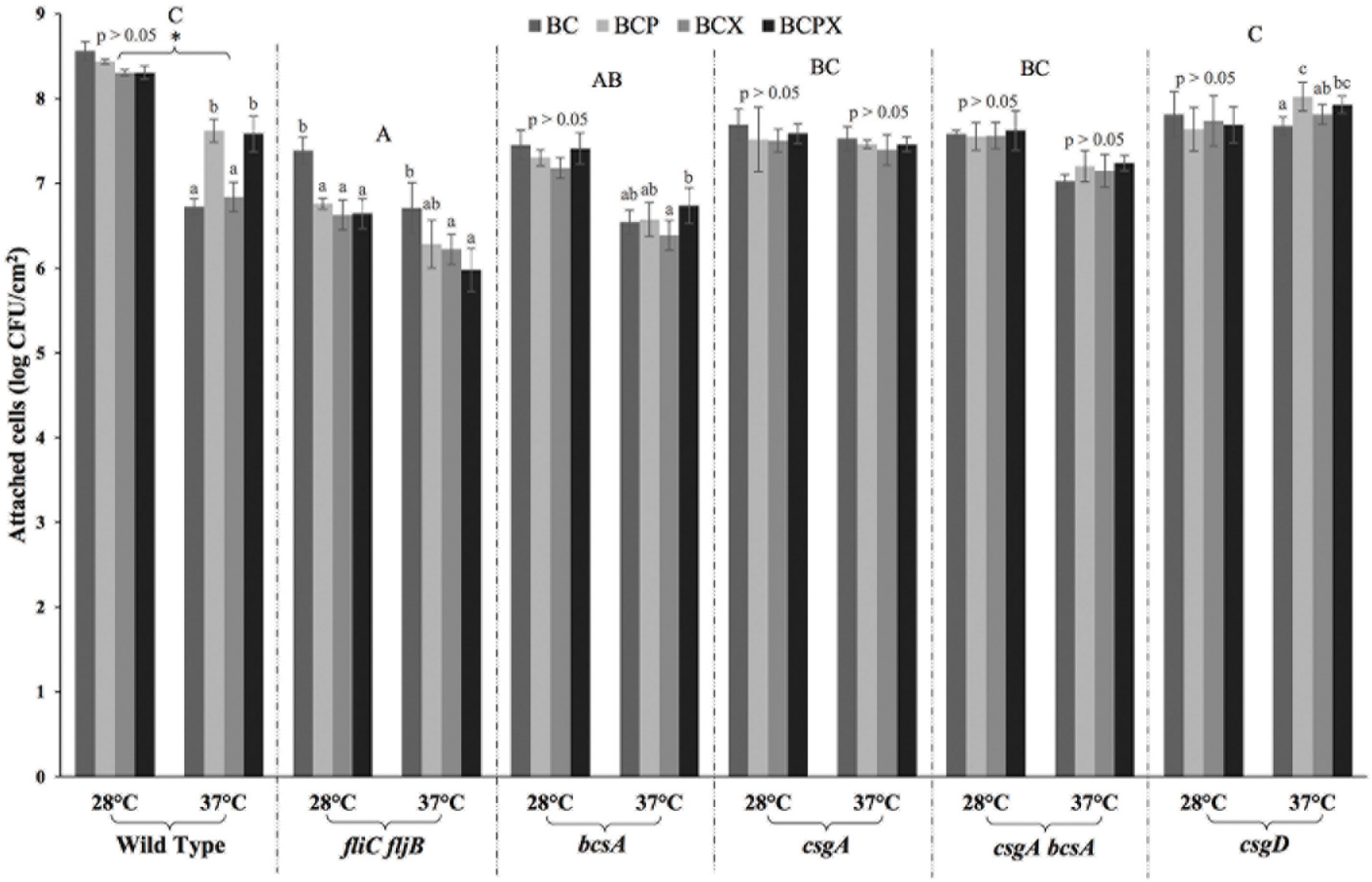

Fig 2. Attachment of *S*. Typhimurium ATCC 14028 (a) wild type, (b) Δ*fliC fljB* mutant, (c) Δ*bcsA* mutant, (d) Δ*csgA* mutant, (e) Δ*csgA bcsA* mutant and (f) Δ*csgD* mutant grown at 28°C and 37°C to various BC composites (BC, BCP, BCX, BCPX). Different lowercase letters indicate significant differences in attachment numbers between different BC composites within each strain grown at a specific temperature (One-way ANOVA & Tukey's pairwise comparison at $p<0.05$). Different uppercase letters indicate significant differences in attachment numbers to BC composites between different strains (One-way ANOVA & Tukey's pairwise comparison at $p<0.05$). Asterisk sign indicates significant differences in attachment numbers for the same strain grown at two different temperatures (28°C and 37°C) (independent samples t-test at $p<0.05$).

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PCW components as fewer *S*. Typhimurium cells were attached when less pectin was present in the potato tissue. It was also expected that the attachment for all *S*. Typhimurium strains grown at 37°C (except for the Δ*fliC fljB* mutant) would be similar to each other since cellulose and fimbriae are not produced at this temperature. This was not, however, the case as significant variations in the attachment of different strains were observed ($p<0.05$). Variations in the attachment of *S*. Typhimurium strains grown at different temperatures were most probably not due to the effect of cellulose and fimbriae structures but could be caused by other factors that have not yet been identified.

Role of cell surface structures in *Salmonella* attachment to BC composites

An overall comparison on the number of cells of the *S*. Typhimurium ATCC 14028 wild type and mutant strains attached to the BC composites presented in Fig 2 indicated that the different strains attached in significantly different numbers to the composites ($p<0.05$). It was shown that overall, the Δ*fliC fljB* and Δ*bcsA* mutants displayed significantly lower attachment

levels compared to the wild type strain ($p<0.05$). Another comparison carried out on strains grown at 28°C showed that the wild type strain attached in significantly higher numbers as compared to all mutants ($p<0.05$). This indicates that cellulose, fimbriae and flagella were all involved in the attachment of *S*. Typhimurium to the PCW models. Lapidot and Yaron [23] suggested that bacterial surface appendages, such as flagella and curli fimbriae, may influence the initial reversible adhesion to plants, which is mediated by van der Waal interactions and hydrogen bonds. This initial adhesion is followed by stronger irreversible attachment which is mediated by electrostatic forces and dependent on extracellular components, such as bacterial cellulose.

Role of flagella. Of the bacterial surface structures studied, flagella appeared to have the most important role in attachment as the Δ*fliC fljB* mutant attached in lowest numbers of all mutants to PCW models when grown at both temperatures. As compared to the wild type, attachment of the Δ*fliC fljB* mutant was reduced by ~1.5 and 0.9 log CFU/cm^2 when grown at 28°C and 37°C, respectively. Flagella are known to be important for the motility of *S*. Typhimurium cells [50] and allow the bacteria to reach the attachment surface faster [51]. Flagella also mediate chemotaxis which can guide planktonic cells to swim towards sites with nutrients or towards cells attached to a surface [52]. Similarly, motility enables pathogens to enter and colonize stomata, wounds and openings in plants [13]. Flagella and motility mutants of *S*. Typhimurium failed to invade lateral root junctions of the *Arabidopsis thaliana* plants, which may be explained by the inability of the mutants to find entry points into the plant [25]. It was also found that mutations which impaired bacterial motility also reduced the ability of *Salmonella* to be internalized by plants [13]. We suggest that flagella enable the cells to move within the matrix of the BC composites where attachment can occur. There is also a possibility that the long flagella filament (which extends up to 20μm) may cause entanglement of *Salmonella* cells (~2μm) within the BC matrix. A study by Berger et al. [22] has shown that *S*. Senftenberg requires flagella to attach to salad leaves. In contrast to the results of our study, these authors found that the deletion of the *fliC* gene did not affect *S*. Typhimurium attachment to basil leaf epidermis. The different outcome of this previous study and ours may be due to the fact that the *fljB* gene was not deleted in the previous study. The normal expression of *fljB* (which encodes the phase-2 flagellin) could have substituted for the loss of phase-1 flagellin (encoded by *fliC*) and thus the ability of the *S*. Typhimurium to attach was not affected in their study. In addition to the use of flagella for motility and chemotaxis, *S*. Typhimurium also uses the flagella to sense external environments in order to regulate its own biogenesis and virulence [53]. It has been demonstrated that the flg22 peptide conserved in the *Salmonella* flagellin activates the plant immune system which then inhibits *Salmonella* colonization. Iniguez et al. [54] showed that the *S*. Typhimurium Δ*fliC fljB* mutant was more successful in colonizing roots in alfalfa, wheat and *Arabidopsis* plants. This could be due to the inability of the plant defence system in detecting colonization by the pathogens. Although the role of flagella in *Salmonella* attachment to PCWs can be studied using the PCW models and cut plant material, these models cannot be used to investigate the plant immune response to human pathogens. Interactions between plants and human pathogens may cause changes in the plant such as the production of free radicals, lignification, pH change and also phenolic and cellulose appositions in the PCW. This represents a limitation of our study and it is necessary to investigate this using native PCWs in future studies to obtain a complete picture of these interactions.

Roles of cellulose and fimbriae. In addition to the importance of flagella, our results also showed that cellulose and fimbriae were involved in the attachment of *S*. Typhimurium to PCWs. Single and double mutants of cellulose and fimbriae (Δ*bcsA*, Δ*csgA*, Δ*csgA bcsA*, Δ*csgD*) which were grown at 28°C all displayed significantly lower attachment compared to the wild type strain ($p<0.05$). When these mutants were grown at 37°C, the Δ*csgA* and Δ*csgA bcsA*

attached in similar numbers as compared to the wild type ($p>0.05$). The Δ*fliC fljB* and Δ*bcsA* mutants attached at significantly lower levels ($p<0.05$) whereas only the Δ*csgD* mutant showed significantly higher attachment numbers compared to the wild type ($p<0.05$). The roles of cellulose and fimbriae in human pathogenic attachment to plants are not well understood yet but both attachment structures are known to contribute to aggregative multicellular behaviour, biofilm formation and protection against harsh environmental conditions [55,56].

Fimbriae, which are shorter in length, straighter and more numerous than flagella in numbers do not play a role in *Salmonella* motility. The function of adhesins on the fimbrial tip in determining its specific attachment properties to animal cells has been demonstrated [57]. Lectins, found on fimbriae and flagella structures, are able to recognize oligosaccharide units on the animal cells allowing specific attachment [58]. A similar process could be involved in bacterial attachment to plant surfaces. Cellulose play a role in cell-to-cell interactions and the formation of bacterial aggregates which may in turn favour bacterial attachment to a surface [58,59]. In addition to its role in attachment, cellulose also promotes bacterial persistence in the environment by conferring cells with resistance to adverse conditions, such as exposure to chlorine and bleach [60].

Some studies have shown that cellulose and fimbriae were involved in *Salmonella* attachment to parsley and alfalfa sprout seedlings [23,59]. A number of studies have found that *S. enterica* uses cellulose to adhere to plant roots [59,61]. Solomon et al. [62] found that high proportions of *Salmonella* strains isolated from fresh produce possess cellulose and fimbriae, which further supports this finding. Lapidot and Yaron [23] showed that both cellulose and fimbriae affect bacterial colonization although fimbriae played a more important role than cellulose in *S*. Typhimurium transfer from contaminated irrigation water to parsley. Another study by Barak et al. [59] also showed that the roles of cellulose and fimbriae in attachment are additive, with the initial attachment of a Δ*csgB bcsA* double mutant further reduced compared with a Δ*bcsA* single mutant. Barak et al. [21] also observed that mutation in *csgB*, not *csgA*, reduced the ability of *S*. Newport to attach to alfalfa sprouts. They suggested that the curli fimbrial nucleator (encoded by *csgB*) may facilitate the initial attachment of *Salmonella* to plants even without the production of fimbriae.

In our study it appeared that the four cellulose and fimbriae mutants behaved similarly. This feature could be explained by the smaller roles these structures have in bacterial attachment such that the loss of either one or both of these structures did not greatly affect attachment of the strains. To the best of our knowledge, no study has a pleiotropic effect of these gene mutations on the general structure of the bacterial cell wall.

In addition to being involved in the regulation of cellulose and fimbriae expression, the *csgD* gene is also involved in the synthesis of the O antigen capsule and colanic acid which have been shown to modulate bacterial attachment to plants [59]. Colanic acid has been found to be associated with fimbriae in *E. coli* and is involved in the formation of *Salmonella* biofilms on animal cells. The O antigen capsule has been shown to protect bacterial cells from desiccation [28]. It was interesting to note that in our study the Δ*csgD* mutant grown at both 28°C and 37°C had significantly higher ability to attach to the composites as compared to the Δ*bcsA* mutant ($p<0.05$). Studies [63,64] have shown that Δ*csgD* mutants lacking cellulose and fimbriae have increased *fliE* (encoding the flagellum basal body) promoter activity and production of the FliC protein as compared to wild type cells. This can be explained as the synthesis of cellulose and fimbriae or flagella production are mutually exclusive from each other due to opposite regulation by the signalling molecule cyclic di-GMP [65]. Increased production of flagella-related genes could therefore have helped the Δ*csgD* mutant to attach better to the BC composites. Further studies are required to confirm this.

Conclusions

Taken together the results of our study demonstrate that *S*. Typhimurium cells grown in the animal host (at 37°C) do not produce cellulose and fimbriae and that these biofilm-forming structures will only form if the pathogens are released into the external environment which has a lower temperature (e.g.: 28°C). Flagella, fimbriae and cellulose all contribute to the interaction of *Salmonella* with intact plants in the environment, but we have shown that these structures are not the most important mechanisms for attachment of *Salmonella* to the BC composites which may be influenced by many other factors. Although the results from our study do not fully represent the real life phenomenon occurring on whole plants, a better understanding of the role of bacterial structures on the attachment of *Salmonella* to plants will aid in finding ways to remove pathogens from fresh produce more effectively.

Supporting Information

S1 Fig. Flagella (indicated by arrows) of *S*. Typhimurium ATCC 14028 cells grown at (A) 28°C and (B) 37°C stained with Ryu's stain.
(TIF)

S2 Fig. Attachment of wild type and mutant strains of *S*. Typhimurium ATCC 14028 grown at (A) 28°C and (B) 37°C to BC composites with varying levels of pectin and xyloglucan (0.1%, 0.3% and 0.5%).
(PDF)

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Author Contributions

Conceived and designed the experiments: MSFT GAD SR. Performed the experiments: MSFT. Analyzed the data: MSFT. Contributed reagents/materials/analysis tools: APW. Wrote the paper: MSFT GAD SR APW.

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