Factors contributing to biofilm formation of *Yersinia enterocolitica*

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Abstract

Biofilms of pathogenic bacteria are recognised as a threat to food safety. The aim of the present study was to investigate the potential of *Yersinia enterocolitica* to form biofilms in the pork processing environment and identify the resistance of these biofilms to sanitation. The biofilm formation by *Y. enterocolitica* was monitored at conditions simulating pork processing environment under daily cleaning routine using an impedance method established in this study. Results showed that *Y. enterocolitica* had the potential to form biofilm and become resistant to sanitation in a pork processing environment. An investigation into the factors influencing biofilm formation of *Y. enterocolitica* indicated that the Ca^{2+} ion increased the level of biofilm formation. In addition, the presence of the virulence plasmid pYV is essential for the biofilm Ca^{2+} response. Further analysis of the bacterial cell surface properties and extracellular polymeric substance (EPS) composition suggested that the pYV^{+} cell surfaces are more negatively charged and more hydrophobic than the pYV^{-} cells although no significant difference was observed with the addition of Ca^{2+}. The pYV^{+} cells appear to produce more exopolysaccharide than the pYV^{-} cells regardless of Ca^{2+} concentration. Ca^{2+} was able to increase the yield of extracellular DNA while the presence of pYV appeared to be dispensable in terms of extracellular DNA release. Analysis of cell wall protein revealed one protein expressed in the pYV^{+} cells but absent in the pYV^{-} cells.

List of publications

Wang H, Tay M, Palmer J, Flint S (2016) Biofilm formation of *Yersinia enterocolitica* and its persistence following treatment with different sanitation agents. Food Control 73: 433-437.

Wang H, Palmer J, Flint S (2015) A rapid method for the nonselective enumeration of *Yersinia enterocolitica*, a foodborne pathogen associated with pork. Meat Science 113: 59–61.

List of conference presentations

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List of abbreviations

eDNA: extracellular DNA

EPS: extracellular polymeric substances

ERL: enteric reference laboratory

ESR: environmental science and research

HIC: hydrophobic interactive chromatography

MJ: meat Juice

PCR: polymerase chain reaction

pYV: plasmid of *Yersinia* virulence

pYV^{+}: pYV positive

pYV^{-}: pYV negative

QAC: quaternary ammonium compounds

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

SEM: scanning electron microscopy

TSA: trypticase soy agar

TSB: tryptic soy broth

Yops: *Yersinia* outer membrane proteins

Ysc: Yop secretion

Chapter One. Introduction

Yersinia enterocolitica causes yersiniosis, a predominantly food borne illness. In New Zealand there are approximately 500 notifications of yersiniosis annually, most of which are due to *Y. enterocolitica* (ESR, 2016). Yersiniosis causes diarrhoea, fever and septicaemia, the severity depending on the health of the patient (Bottone, 1997). Patients infected with *Y. enterocolitica* can go on to develop the post-infectious sequelae - reactive arthritis and erythema nodosum (Bottone, 1997). One key factor in *Y. enterocolitica* pathogenesis is the presence of a virulent plasmid (Cornelis et al., 1998).

As *Y. enterocolitica* can be carried by pigs asymptomatically, it poses high risk of contamination of the pork processing environment. The prevalence of *Y. enterocolitica* on fresh pork samples at retail level tested by culture method was within a range of 2% to 5.2 %, tongues showed higher contamination rate than general pork meat (Esnault et al., 2013, Fredriksson-Ahomaa et al., 1999). The isolation rate was higher when polymerase chain reaction (PCR) method was used instead of culture method (Fredriksson-Ahomaa et al., 1999). The presence of *Y. enterocolitica* can provide a source of human infection during the handling and consumption of raw or undercooked meat (Gütler et al., 2005).

Biofilms are often involved in the contamination of food processing environments. Biofilms are bacteria colonising surfaces and they often develop resistance to cleaning and sanitising agents used in maintaining hygiene in food processing. In the food industry, biofilms of pathogenic bacteria are recognised as a threat to food safety (Shi and Zhu, 2009). The potential for *Y. enterocolitica* to form biofilms in a pork processing environment is unknown.

The research question of this study is that what the conditions result in biofilm formation of *Y. enterocolitica*. The hypothesis that biofilms are a possible source of contamination of food with *Y. enterocolitica* was tested. This study was designed to investigate the potential for isolates of *Y. enterocolitica* from the food industry to form

biofilms and identify the resistance of these biofilms to sanitation. The factors leading to biofilm formation were investigated with the aim of identifying the risks in the contamination of a pork processing environment and assist in designing recommendations to reduce the risk of contamination of food and foodborne illness resulting from *Y. enterocolitica.*

Chapter Two. Literature review

2.1 *Yersinia*

Yersinia is a genus of bacteria in the family Enterobacteriaceae. *Yersinia* species are Gram-negative, rod-shaped bacteria and facultative anaerobes. The *Yersinia* genus contains several pathogenic species for human and animal including *Y. enterocolitica*, *Y. pseudotuberculosis*, *Y. pestis*, and *Y. ruckeri*. *Y. enterocolitica* and *Y. pseudotuberculosis* are the causative agents of yersiniosis. *Y. pestis* is the cause of plague, one of the most devastating diseases of human history (Perry and Fetherston, 1997). *Y. ruckeri* causes enteric red mouth disease (ERM) in salmonid fish (Furones et al., 1993).

2.2 Yersiniosis

Yersiniosis is the infectious disease caused by *Y. enterocolitica* and *Y. pseudotuberculosis*. The yersiniosis annual notifications in New Zealand from 1997 to 2015 are shown in Figure 2.1. The notification rate has remained stable (ranging from 9.3 to 14.8 per 100,000) from 2006 to 2015 as the third most common notifiable food borne disease caused by bacteria in New Zealand, after Campylobacteriosis and Salmonellosis (ESR, 2016). The reports of zoonoses monitoring in 32 European countries presents similar results (EFSA and ECDC, 2015). Yersiniosis is a diarrheal disease. The most frequent symptom of yersiniosis, particularly in children, is acute enteritis accompanied by fever and vomiting (Black et al., 1978). Symptoms mimicking appendicitis appear to be a more common in young adults (Chandler and Parisi, 1994). Fatal necrotizing enterocolitis and suppurative mesenteric adenitis may also occur in protracted infection (Barlow and Gandhi, 1981, Braunstein et al., 1971). Patients infected with *Y. enterocolitica* can go on to develop the post-infectious sequelae - reactive arthritis and erythema nodosum (Bottone, 1997). The majority of cases of yersiniosis are caused by *Y. enterocolitica* (ESR, 2016, EFSA and ECDC, 2015).

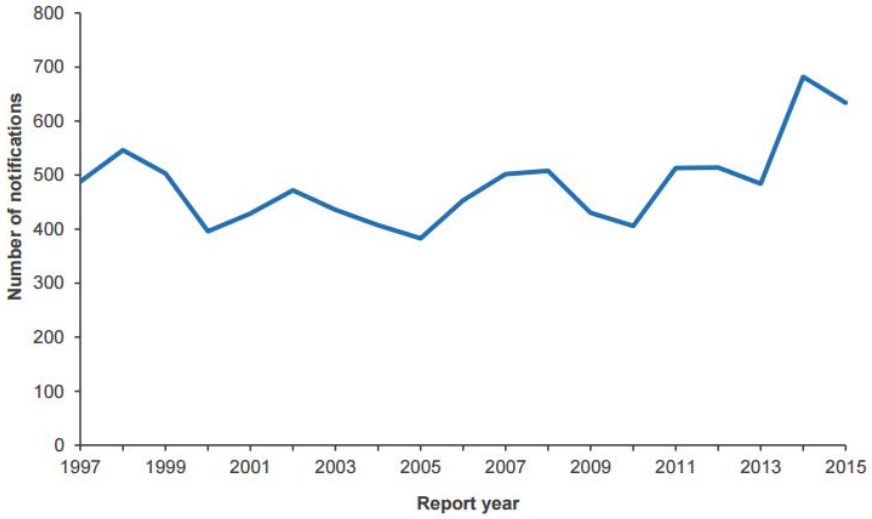

Figure 2.1. Yersiniosis notifications in New Zealand by year, 1997–2015 (ESR, 2016).

2.2.1 Geological distribution

Yersiniosis has been observed worldwide (Bottone, 1999). By the mid-1970s, Mollaret *et al*. (1979) had compiled reports of *Y. enterocolitica* isolates from 35 countries on six continents. Most infections caused by *Y. enterocolitica* are sporadic and outbreaks are uncommon. The largest *Y. enterocolitica* infection outbreak (affecting 1051 persons) was reported in Japan in 1980 following the ingestion of contaminated milk (Maruyama, 1987). Subsequent cases were recorded in the USA and Sweden with a variety of sources including chocolate milk, pasteurised milk, tofu and bean sprouts (Black et al., 1978, Ackers et al., 2000, Tacket et al., 1985, Aber et al., 1982). Overall, it's difficult to compare *Y. enterocolitica* infections among countries worldwide. There is a lack of yersiniosis surveillance in many countries including Africa, Asia, the Middle East, Pacific Islands, Latin America and the Caribbean (Fredriksson-Ahomaa et al., 2006). Although several national agencies in developed countries, particularly those from the member states of the European Union, the United States and New Zealand, do include yersiniosis as a primary surveillance target in their reports of human enteric disease, differences in reporting systems, isolation methods, and availability of strain information greatly complicate comparisons among countries (Fredriksson-Ahomaa et al., 2006).

2.2.2 Food vehicles

Meat and meat products have been implicated as sources in outbreaks of *Y. enterocolitica* infection. In 2006, 11 people fell ill in Norway after ingesting traditional pork products (Grahek-Ogden et al., 2007). Pork products have also been a source of food poisoning in other countries including the USA, Hungary and Japan (Moriki et al., 2010, Lee et al., 1990, Marjai et al., 1986). Besides, chocolate milk, pasteurised milk, tofu, and bean sprouts have also been implicated as sources of *Y. enterocolitica* infection (Black et al., 1978, Ackers et al., 2000, Tacket et al., 1985, Aber et al., 1982). In addition, some *Yersinia* outbreaks have been associated with well water (Thompson and Gravel, 1986, Abraham et al., 1997).

2.3 *Y. enterocolitica*

Y. enterocolitica was initially described in 1939 by Schleifstein and Coleman in the U.S. (Schleifstein and Coleman, 1939) and subsequently designated as *Bacterium enterocoliticum* in 1943(Schleifstein and Coleman, 1943). The name "*Yersinia enterocolitica*" was proposed by Frederiksen in 1964 (Frederiksen, 1964). In 1971, Mollaret for the first time comprehensively described a large number of yersiniosis cases diagnosed over a period of 4 years primarily in Europe(Mollaret, 1971). Since then, awareness of the importance of the microorganism in human infection has heightened.

2.3.1 Taxonomy

Y. enterocolitica is a highly heterogenic species which is designated into 6 biotypes- 1A, 1B, 2, 3, 4 and 5 (Wauters et al., 1987). Of the six biotypes, biotype 1A contains the most serotypes and was believed to be non-pathogenic to humans although it has recently been demonstrated to be able to cause disease in patients with underlying disorders (Tennant et al., 2003). The pathogenic biotypes, namely, 1B, 2, 3, 4 and 5, contain a highly conserved 70-kb virulence plasmid and several chromosomal genes, while biotype 1A is reported to lack the virulence plasmid (Paixão et al., 2013). Biotype 1B is the most pathogenic group whereas biotype 2, 3, 4 and 5 are of equally lower

pathogenicity (Lee et al., 1990, Vantrappen et al., 1977). However, the infections on a worldwide basis are mostly caused by the biotypes with lower virulence and the number of biotype 1B isolations made from symptomatic patients has declined (Bottone, 1999).

2.3.2 Taxonomic distribution of *Y. enterocolitica* infection

Biotype 4 is widespread in Europe, Japan, Canada, Africa and Latin America (Kapperud et al., 1990, Mollaret et al., 1979). Biotype 4 seems to be responsible for the majority of the cases in Denmark, Norway, Sweden, New Zealand and Belgium (Nesbakken, 1997). Biotype 2 is the second most common in Europe. It accounts for a relatively high percentage of the strains isolated in France, Belgium and the Netherlands while only a few strains have been isolated in Scandinavia (Nesbakken, 1997). In the United States, biotype 1B used to be the most frequently reported biotype (Mollaret et al., 1979). However, in recent times biotype 4 has become the major biotype of sporadic *Y. enterocolitica* isolates in outbreaks in some regions of the USA (Nesbakken, 1997).

2.3.3 The prevalence of *Y. enterocolitica* in meat plants

Pigs are usually asymptomatic carriers of *Y. enterocolitica,* and *Y. enterocolitica* are widespread in pigs. The prevalence of *Y. enterocolitica* is high although there is some variation between different countries. In the pig slaughterhouses of Belgium, the positive rate for pathogenic *Y. enterocolitica* is 57.2% for tonsils, 20.0% for rectal contents and 42.2% for pig carcasses (Van Damme and De Zutter, 2011). While in a pig slaughterhouse in France, the pathogenic *Y. enterocolitica* biotype 4 serotype O:3 was detected in 25% of tonsils and 17% of the faecal samples, but not detected from pig carcasses (Mazzette et al., 2015). This difference might be due variations in the prevalence in pig herds or different hygiene practises among slaughterhouses. Swab samples from a pig slaughtering environment have shown 8-20% positive for were also investigated for the presence of pathogenic *Y. enterocolitoca* (Terentjeva and Berzins, 2011). The downstream processing after slaughtering may help reduce the level of pathogenic *Y. enterocolitica* presence to some extent, because the isolation rate seems to be lower at the retail level than in the slaughtering environment (Terentjeva and

Berzins, 2011). Apart from pork, pathogenic *Y. enterocolitica* also exist in other types of meat products such as beef and poultry products. It's worth noting that poultry samples have a very high prevalence of *Y. enterocolitica* biotype 4 serotype O:3 in Iran (Saberianpour et al., 2012).

2.3.4 The virulence plasmid

All biotypes of *Y. enterocolitica* other than biotype 1A contain a virulence plasmid of 70kb called pYV which is also present in *Y. pseudotuberculosis* and *Y. pestis* (Cornelis et al., 1998). Genes on the pYV plasmid can be divided into several categories based on their function. Most importantly, pYV encodes for a set of proteins called Yops which are essential for virulence (Cornelis et al., 1998). Secondly, some of the genes encode for a specific apparatus called Ysc which is required by the secretion of Yops (Galán and Wolf-Watz, 2006). This apparatus is known as the Type III Secretion System (T3SS) which represents an important virulence mechanism in various pathogenic bacteria (Cornelis et al., 1998). Apart from Ysc, Yops secretion also requires chaperons called Syc (for "specific Yop chaperone"), different Yop has its own specific Syc (Wattiau et al., 1994). Through the function of Ysc and Syc, bacteria are able to inject bacterial effector proteins into host cells to inhibit phagocytosis by macrophages and trigger apoptosis of the host cells (Galán and Wolf-Watz, 2006). The pYV plasmid also encodes for an adhesion protein that allows the bacteria to attach to the host cells which is part of the virulence mechanism (Perregaard et al., 1991).

2.3.5 Reservoirs and transmission route

Pigs are the only animal species from which pathogenic *Y. enterocolitica* strains have frequently been isolated (Gütler et al., 2005). Therefore pigs are assumed to be the main reservoir of pathogenic *Y. enterocolitica*. In addition, case reports identified the consumption of raw pork as the risk factors for most cases of yersiniosis (Satterthwaite et al., 1999, Ostroff et al., 1994, Boqvist et al., 2009, EFSA and ECDC, 2015). The transmission of pathogenic *Y. enterocolitica* from pigs on the farm to carcasses post slaughter house was highlighted by Laukkanen *et al.* (2009) in Finland. Therefore, although pathogenic *Y. enterocolitica* have seldom been isolated from foods, which is

possibly due to the low sensitivity of detection methods, *Y. enterocolitica* is thought to be a significant foodborne pathogen and cause infection through ingestion of contaminated pork. (El Qouqa et al., 2011, Rosner et al., 2010, MacDonald et al., 2011). Other foodborne pathogens may have different foods as their transmission vectors. For examples, *Listeria monocytogenes* and *Salmonella spp.* are regularly found in a variety of dairy and meat products, poultry, egg products and fresh produce (Farber and Peterkin, 1991, Leasor and Foegeding, 1989, Heisick et al., 1989, Bouchrif et al., 2009); *Escherichia coli* O157:H7 are commonly found from foods of bovine origin such as beef and milk (Tarr et al., 1997); *Campylobacter jejuni* is generally associated with fresh and undercooked poultry products (EFSA and ECDC, 2015). The diversity in transmission vehicle among different foodborne pathogens may reflect the diversity of reservoirs.

2.3.6 Factors influencing growth and survival in foods

2.3.6.1 Temperature

The optimal growth temperature is 22–29 °C for *Y. enterocolitica*, but it has the capability to grow over a wide range of temperatures (Bresolin et al., 2006, Olsvik and Kapperud, 1982). As a psychrotrophic bacterium, *Y.enterocolitica* has the capability to survive and multiply in cold environments (Annamalai and Venkitanarayanan, 2005, Neuhaus et al., 1999). This property enables *Y. enterocolitica* to grow to clinically significant levels on food surfaces at refrigeration temperatures. It's been shown that in a food with a neutral pH stored at 5°C, *Y. enterocolitica* cell numbers may increase from 10 CFU/ml to 2.8×10^7 CFU/ml after five days (Bhaduri, 2005). In addition, *Y. enterocolitica* can survive in frozen foods for long periods. Differences in survival rates are observed among different serotypes during storage of frozen pork meat at -20 °C, and it is assumed that less virulent *Y. enterocolitica* biotype 2 and 4 can survive longer than the highly virulent biotype 1B (Iliev and Najdenski, 2008).

2.3.6.2 pH

The optimum for growth of *Y. enterocolitica* is between pH 7 and 8 (Stern et al., 1980). *Y. enterocolitica* is not able to grow at pH < 4.2 or >9.0 (Zadernowska et al., 2014).The

flesh of animals prior to slaughter has a pH value of 7.1 and pork reaches the lowest pH of 5.4 to 5.8 at 6-10 hours after slaughter. A growth from 2.5 x 10^4 to 2 x 10^7 CFU of *Y. enterocolitica* can be detected after incubation at pH 4.4 at 4 °C for 21 days (Brocklehurst and Lund, 1990). However, *Yersinia* prefers a less acidic environment at low temperatures (Brocklehurst and Lund, 1990) therefore they are not usually found in fermented food products (Vereecken et al., 2003). However, *Y. enterocolitica* may survive in fermented pork for long periods (Deza et al., 2005).

2.3.6.3 Salt

Y. enterocolitica can growth in the media with up to 5% of salt while it is inhibited and killed at 7% and 9% salt concentrations respectively (Adams and Moss, 2000, Stern et al., 1980). The normal salt concentrations in different pork products vary between 0.06%-1.88% (Limon et al., 2011) therefore *Y. enterocolitica* has the potential to survive and grow in pork. However, salt concentration can affect the temperature profile that *Y. enterocolitica* can grow at. For example, at 3°C the presence of seven percent salt is bactericidal but bacteriostatic at 25 °C (Le Marc et al., 2005). The salt tolerance drops with decreasing temperatures indicating the importance of cold storage in salted products.

2.3.7 Research gap

Y. enterocolitica is relatively susceptible to heating. Its heating susceptibility is strain-dependent with D_{60} values (the time required at a certain temperature to kill 90% of the organisms) in a range from 0.7 to 57.6 s (Adams and Moss, 2000). Heat treatment of food product at 60 °C for 1–3 min effectively inactivates *Y. enterocolitica* (Lee et al., 1980). Therefore, it's unlikely to survive typical cooking, boiling, baking, and frying temperatures. However, the yersiniosis notification rate has remained stable at high level for years, both in New Zealand and internationally (ESR, 2016, EFSA and ECDC, 2015). These heat sensitive bacteria have somehow managed to survive food processing to be responsible for foodborne disease.

It is now generally accepted that bacteria preferentially grow attached to surfaces as biofilms and are commonly surrounded by extracellular polymeric substances, produced by bacteria while growing on the surface (Sutherland, 2001). Biofilms can increase the resistance of bacteria to many physical and chemical factors including heat treatment (Brown and Gilbert, 1993). Therefore, *Y. enterocolitica* may persist on surfaces of pork and the pork processing environment in the form of biofilm, which in turn leads to cross-contamination of pork products and the handling and consumption of these products results in transmission of disease.

2.4 Foodborne pathogens and biofilms in food

2.4.1 The definition of biofilm

Biofilms were first described in 1936 by Zobell and Anderson (1936). The significance and ubiquity of bacterial biofilms was first recognized in 1978 (Costerton et al., 1978). Now biofilms are usually defined as microbial aggregations adhering to biological or non-biological surfaces enclosed in a biofilm matrix (Hall-Stoodley et al., 2004). The development of biofilms generally includes initial attachment, irreversible attachment, maturation and dispersal (Sauer et al., 2002) (Figure 2.2).

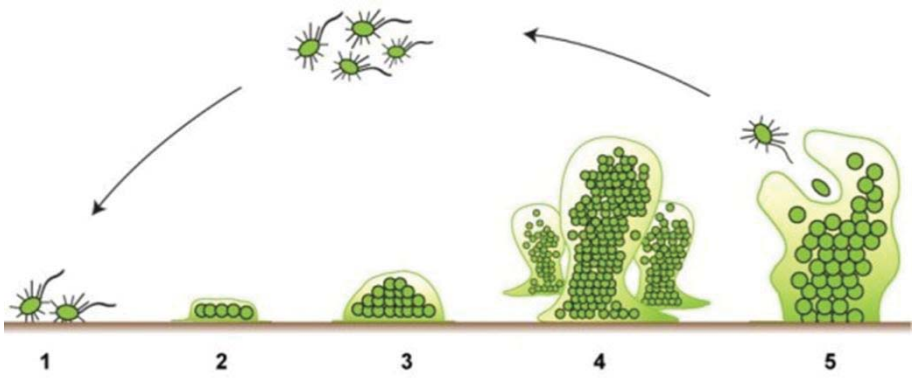

Figure 2.2. A diagram of the five stages of biofilm development: 1 initial attachment, 2 irreversible attachment, 3 early vertical development, 4 maturation, 5 finally dispersal (https://aqua-tech.ca/2016/01/biofilm-in-spas/).

Bacteria, being embedded in matrix, are more resistant than free bacteria to harsh environmental conditions (Mah and O'Toole, 2001). Mature biofilms are highly

coordinated functional communities and have even been described as a city of microbes as a reference to the human world (Watnick and Kolter, 2000). Bacteria within biofilms are much more efficient in terms of nutritional utilization than planktonic bacteria (Costerton et al., 1987).

2.4.1 Biofilm in the food industry

The biofilm mode of growth is widespread and of economic importance in diverse ecological niches. Biofilms formed by foodborne pathogens are a concern to the food industry (Srey et al., 2013). As organic molecules accumulate on surfaces in the food industry, these modified surfaces can facilitate bacterial attachment and biofilm formation due to their higher roughness and more concentrated nutrition (Hall-Stoodley and Stoodley, 2005) (Loeb and Neihof, 1975). For example, biofilms of foodborne pathogens on surfaces with biological soil of porcine serum are more difficult to clean than those without biological soil (Allan et al., 2004).

Biofilms are also shown to play a role in the persistence of microorganisms in food and food processing environments for many foodborne pathogens including *L. monocytogenes*, *Salmonella spp.*, *E. coli* O157:H7 and *C. jejuni* (Djordjevic et al., 2002, Joseph et al., 2001, Ryu and Beuchat, 2005, Joshua et al., 2006). However, more information is yet to be provided for understanding the role biofilms play in the persistence of *Y. enterocolitica*.

2.5 Environmental conditions influencing biofilm formation

Environmental factors are essential in shaping biofilm formation. In this section, the influence of a few environmental factors including ions, temperature, pH and organic coatings on biofilm formation are reviewed. *P aeruginosa* is frequently chosen as an example because it's the most extensively studied Gram-negative bacteria in terms of the intrinsic and extrinsic mechanisms regarding biofilm formation.

2.5.1 Ions

Ion such as Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Na^{+} and K^{+} are common chemical elements in red meat processing environments. Studies on the effect of different ions on biofilm formation are reviewed below.

2.5.1.1 Ca^{2+}

Ca^{2+} supplementation is shown to increase biofilm formation in range of bacteria including *P.aeruginosa* (Sarkisova et al., 2005), *Pseudoalteromonas spp.* (Patrauchan et al., 2005), *Sinorhizobium meliloti* (Rinaudi et al., 2006), *Xylella fastidiosa* (Cruz et al., 2012), *Enterobacter cloacae* (Zhou et al., 2013), *Aeromonas hydrophila* (Das et al., 2014), and *Enterococcus faecalis* (Das et al., 2014). Ca^{2+} acts as a signalling molecule during the biofilm formation of *P. aeruginosa* and regulates the expression of proteins which are involved in the synthesis of alginate, a major extracellular polymeric substance (EPS) component of *P. aeruginosa* biofilm (Sarkisova et al., 2005, Patrauchan et al., 2007). Ca^{2+} also regulates iron uptake and stress responses which have been shown to be important in biofilm development (Banin et al., 2005, Zhang et al., 2007, Patrauchan et al., 2007). In addition, Ca^{2+} increases the mechanical properties of *P aeruginosa* biofilm by crosslinking alginate molecules (Körstgens et al., 2001).

2.5.1.2 Magnesium

Less information exists for the effect of Mg^{2+} on biofilm formation. The influence of Mg^{2+} on biofilm formation varies within different bacteria species. Mg^{2+} increases initial attachment and subsequent biofilm formation of *Pseudomonas fluorescens*, *Sphingomonas paucimobilis* and *Staphylococcus epidermidis* (Song and Leff, 2006, Guvensen et al., 2012, Dunne and Burd, 1992). However, with *P aeruginosa*, it was shown that Mg^{2+} limitation increases exopolysaccharide production and biofilm formation which means the presence of Mg^{2+} shows an inhibitory effect on biofilm formation (Mulcahy and Lewenza, 2011).

2.5.1.3 Iron

Iron plays a critical role in biofilm formation of many bacteria species (Banin et al., 2005, Wu and Outten, 2009, Mey et al., 2005). Experiments using an iron-chelating agent suggest that a critical low level of intracellular iron is needed for the regulation of *P. aeruginosa* biofilm development whereas high concentrations of iron inhibit *P. aeruginosa* biofilm (Banin et al., 2005). From a biochemical point of view, this iron signalling is mediated by a cytoplasmic iron uptake regulator protein called Fur (Banin et al., 2005). From a phenotypical point of view, evidence shows that a certain low level of iron up-regulates extracellular DNA (eDNA), an important constituent of the biofilm matrix (Yang et al., 2007).

2.5.1.4 Zinc

Zinc is usually considered as an inhibitor of biofilm formation of quite a few pathogens including *Actinobacillus pleuropneumoniae*, *E. coli*, *Haemophilus parasuis*, *Salmonella* Typhimurium, *Salmonella* Heidelberg, *S. aureus* and *Streptococcus suis* (Wu et al., 2013). Zinc can inhibit the processing of poly-N-acetylglucosamine (PGA), a component of EPS for *A. pleuropneumoniae* and *S. aureus* biofilms (Wu et al., 2013). However, bacteria species that do not produce PGA are also inhibited in terms of biofilm formation (Wu et al., 2013), indicating that the inhibitory effect of zinc is not solely dependent on the presence of PGA in the biofilm matrix. The mechanism behind the anti-biofilm activity of zinc has yet to be determined.

2.5.1.5 Sodium and potassium

Sodium chloride exerts an inhibitory effect on biofilm formation (Kim and Rhee, 2016, Lee et al., 2013, Martinez, 2011) while little information exists on the influence potassium chloride on biofilm formation. A study with *Pseudomonas spp.* showed that the thinner biofilm formation in the presence of sodium chloride might be due to decreased cell surface hydrophobicity (Kuntiya et al., 2005). However, sodium and potassium chloride are also shown to enhance biofilm production for many staphylococcal isolates (Beckingsale et al., 2011). This discrepancy might be due to different bacteria species, media and cation concentration.

2.5.2 Temperature

The effect of temperature on biofilm formation varies within different bacteria species or strains. The biofilm formation of *Vibrio parahaemolyticus , E. coli* K-12 is significantly higher at room temperatures than 37 °C (Song et al., 2016, White-Ziegler et al., 2008). A number of biofilm related genes in *E. coli* K-12 show increased expression at 23 °C (White-Ziegler et al., 2008). In contrast, *E. coli* O157:H7 was shown to form stronger biofilm at 30 °C and 37 °C than at 25 °C (Uhlich et al., 2014). Studies also show that biofilm formation by *V. parahaemolyticus* increases with rise in temperature from 15 °C to 37 °C (Han et al., 2016). The discrepancy could be due to different strains and test conditions and environments that different bacterial species have adapted to.

2.5.3 pH

Some studies show that higher pH (within a range of 5.5-8.5) leads to biofilm production for *P. aeruginosa*, *Klebsiella pneumonia*, *Vibrio cholerae* (Hoštacká et al., 2010) and *S*. Typhimurium.(Nguyen et al., 2014a). In contrast, higher pH exerts an inhibitory effect on the biofilm formation of *S. aureus* and *S. epidermidis* within pH values of 7.2 to 8.5 (Nostro et al., 2012). This discrepancy may indicate that the response to pH in terms of biofilm formation might be different between Gram-negative and Gram-positive bacteria. However, the biofilm formation of *S. enteritidis* after incubation at 20 °C was found to be independent of the pH values (4.5, 5.5, 6.5, and 7.4) (Giaouris et al., 2005) which may be due to different test conditions (e.g. temperature or media).

2.5.5 Organic coating

Organic materials, such as proteins, in the fluid environment, can be adsorbed onto surfaces forming conditioning films (Loeb and Neihof, 1975). These organic coatings may promote microbial attachment (Murga et al., 2001). For example, conditioning film formed by human blood enhances the biofilm formation by *E. cloacae*, *P. aeruginosa*, and *Pantoea agglomerans* on the inner lumen of needleless central venous catheter connectors (Murga et al., 2001). It is shown that the film formation

increases the hydrophobicity of substrate surfaces which might favour bacteria attachment (Lorite et al., 2011). The surface functional groups resulting from film formation is another mechanism for increased adhesion and biofilm formation (Lorite et al., 2011). In addition, the conditioning film could serve as a nutritional source for bacteria and support their growth on surfaces (Siboni et al., 2007).

2.6 Fundamental factors of the bacterial surface influencing attachment and biofilm formation

2.6.1 Factors involved in initial attachment- surface charge, hydrophobicity and cell surface protein

The attachment of bacterial cells to a surface is the first stage in biofilm formation (Palmer et al., 2007). Bacterial cells first act as inert colloidal particles and come into contact with surfaces driven by their physicochemical properties (Hermansson, 1999). Subsequently, bacteria cells are anchored more firmly to the surface by biological cell surface structureS (Marshall et al., 1971).

For the first phase of attachment, the net interaction between a bacterial cell and substratum involves electrical interactions governed by surface charge, Liftshitz–van der Waal interactions and hydrophobic interaction (Verwey et al., 1999, Derjaguin and Landau, 1941, Marshall and Cruickshank, 1973). The electrical interaction caused by surface potential is usually repulsive because the surfaces of bacterial cells are generally believed to be negatively charged (Hermansson, 1999), as are the majority of surfaces (Hunter and Liss, 1982). Liftshitz–van der Waal interactions, being related to particle size and distance between surfaces, are generally attractive (Hermansson, 1999). Hydrophobic bacteria attach better to hydrophobic surfaces than hydrophilic bacteria. The surface potential induced electrical interaction and hydrophobicity interaction can be influenced by cell surface composition such as polysaccharide and cell surface protein (Kozel et al., 1980, Masuoka and Hazen, 1997). The initial adhesion ability of bacterial cells under certain circumstances is a balance from the above factors and is dominated by the most outstanding interaction factor.

The adhesion strength to a surface increases over time because biologically produced cell surface structures that induce more specific interactions between bacteria and the substrate leading to irreversible attachment (Boks et al., 2008). Cell surface proteins are shown to be essential for bacteria attachment leading to biofilm formation in many species (Cucarella et al., 2001, Toledo-Arana et al., 2001, Corrigan et al., 2007). *S. aure*us has a *bap* gene encoding for a cell wall associated protein. *Staphylococcal* isolates harbouring *bap* are highly adherent and strong biofilm producers (Cucarella et al., 2001). Homologous genes of *bap* are found in Gram-negative (*P. aeruginosa* and *S. enterica*) and Gram-positive (*E. faecalis*) bacteria (Cucarella et al., 2001).

2.6.2 Factors involved in biofilm maturation- exopolysaccharides and extracellular DNA

The biofilm matrix is a crucial interface between bacteria and the surfaces they attach to and is essential for maintaining biofilm structure. Therefore, considerable effort has been put into the understanding of the biofilm matrix and its composition. Exopolysaccharides and eDNA represent two major molecular species in the biofilm matrix (Whitchurch et al., 2002, Vuong et al., 2004). Exopolysaccharides vary greatly in their composition in different bacteria species (Decho, 1990). In addition, many biofilm forming species have the capacity to produce multiple types of polysaccharides with significant strain-to-strain variation. For example, *P. aeruginosa* produces at least three exopolysaccharides, alginate, Pel and Psl (Franklin et al., 2011). Alginate is the primary polysaccharide in the matrix of mucoid strains while non-mucoid strains can use either Pel or Psl as the primary matrix structural polysaccharide (Franklin et al., 2011).

The polysaccharides do not exist alone but may interact with a wide range of other molecular species. eDNA is increasingly identified as a factor playing an important role in the development of biofilm (Whitchurch et al., 2002). DNase I is shown to be effective in reducing biofilms of many foodborne pathogens (Tetz and Tetz, 2010). As one of the major components in EPS, eDNA can adsorb to the cell surface and influence the cell surface properties such as hydrophobicity which will in turn modify the initial attachment and biofilm structure (Okshevsky and Meyer, 2015).

Exopolysaccrides and eDNA are both required for biofilm formation by forming a web structure in *P. aeruginosa* biofilm matrix (Wang et al., 2015).

2.7 Research objective

Y. enterocolitica infection is the third most frequently reported foodborne disease worldwide in spite of its heat sensitivity (EFSA and ECDC, 2015, ESR, 2016). This present study hypothesizes that biofilms play an important role on the survival and persistence of *Y. enterocolitica* in pork processing environments. Therefore, the biofilm forming ability of *Y. enterocolitica* was studied in a media mimicking a pork processing environment. In addition, the influence of ions (Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{2+}, Na^{+} and K^{+}) and temperature on *Y. enterocolitica* biofilm formation was investigated aiming to provide information that will lead to intervention strategies to control *Y. enterocolitica* in the pork industry. Finally, in order to further understand how these environmental factors modify biofilm formation, the fundamental properties that may be important to *Y. enterocolitica* biofilm formation, including surface potential, hydrophobicity, cell surface protein and EPS composition, were characterized.

Chapter Three. Methods and materials

3.1 Source of isolates

A total of 16 *Y. enterocolitica* isolates recovered from food and provided by the Enteric Reference Laboratory in ESR (The Institute of Environmental Science and Research) New Zealand were used in this study (Table 3.1). For three strains from biotype 4, the pYV negative (pYV^-) mutants were prepared after prolonged incubation at 37 °C and the loss of pYV was identified with polymerase chain reaction (PCR) (Section 3.1.2).

Table 3.1. *Y. enterocolitica* Isolates from food used in this study (Provided by ESR[a], Porirua, New Zealand).

Biotypes	Strain number
1A	ERL[b]072345, ERL072346, ERL072347, ERL073947, ERL082059, ERL093846, ERL10460
2	ERL032126
3	ERL104253, ERL112277
4	ERL032122, ERL032123, ERL032124, ERL032125, ERL072344, ERL114165

a: Environmental science and research

b: Enteric reference laboratory

3.1.1 Plasmid curing

The pYV^- derivatives of *Y. enterocolitica* were obtained as described(Bhaduri et al., 1990) with minus modification. *Y. enterocolitica* was incubated at 37 °C on trypticase soy agar (TSA) for 48 hrs. The pYV^- derivatives emerge spontaneously on the edges of pYV positive (pYV^+) colonies.

3.1.2 PCR Confirmation

The three plasmid cured strains of *Y. enterocolitica* were tested for the absence of pYV by the detection of the virF gene using conventional PCR. virF is an important regulatory gene located on pYV, transcription of many pYV genes in dependent on VirF (Cornelis et al., 1989, Skurnik and Toivanen, 1992). The primers (5'-TCATGGCAGAACAGCAGTCAG-3' and 5'-ACTCATCTTACCATTAAGAAG-3') for the detection of the virF gene (located in 430- to 1020-nucleotide region) amplified a 591-base pair (bp) sequence from the virulence plasmid, the PCR was performed in 50-µl volumes of reaction mixture containing 1 µl of each forward and reverse primer, 3 µl of DNA template, 25 µl Maxima Hot Start PCR Master Mix (Invitrogen, 1×), 20 µl of RNAase free water. The thermal cycling conditions were as follows: 1 cycle of denaturation at 94 °C for 10 min; 40 cycles of melting at 94 °C for 1 min, annealing at55 °C for 1 min and elongation at 72 °C for 30 s; and a final extension at 72 °C for 10 min. (Bhaduri and Pickard, 1995).

3.2 Culture preparation

The strains were pre-cultured in Tryptic soy broth (TSB) (Becton Dickinson, New Jersey) for 18 hrs at 24 °C. The cultures were harvested and washed twice by centrifugation (2400 g for 5 min). The pelleted bacteria were re-suspended in 0.85% NaCl to an optical density of 0.5 at 600 nm (equivalent to 10^8CFU/ml) before use.

3.3 Preparation of meat juice

Meat juice (MJ) was prepared referring to the method described by (Birk et al., 2004) and used as the medium for biofilm development to represent the conditions in a meat processing plant. Fresh pork meat was purchased from PAK'nSAVE (Palmerston North, New Zealand) in February 2015 and ground in a meat mincer. The pork mince was repeatedly put through three freeze/thaw cycles (-20 °C/4 °C) and meat drip was collected during each thawing step until little more could be extracted. The MJ was centrifuged at 2400 g for 10 min to remove large particles. The supernatant was

adjusted to pH 6.8 using 0.1M NaOH and then was filter-sterilized using the Minsart® 0.45 micron syringe filter units before being stored at -80 °C.

3.4 Microtiter plate assay

A microtitre plate assay as described by O'Toole (2011) with minor modification was used to screen the biofilm formation of *Y. enterocolitica*. A standardised bacterial solution (20 µl) (OD_{600} 0.5) was inoculated into 200 µl media in each well and incubated for 24 hrs. The absorbance at 600 nm was measured to record bacterial growth using a spectrophotometer plate reader (Spectrostar Nano, BMG Labtech). To measure biofilm formation, the culture was drained and the plate was washed with distilled water three times and dried at 40 °C for 30 min. Then, the plate was stained with 0.5% crystal violet and incubated at room temperature for 15 min before being drained, rinsed and dried again as described above. Finally, the retained crystal violet in each well was solubilized using 95% ethanol and the microtiter plate was incubated at room temperature for 10-15 min before the absorbance at 595nm was measured. The extent of biofilm formation was expressed as the biofilm formation index (BFI) A_{595nm} /A_{600nm}.

The background from the media was subtracted by including the result from un-inoculated media (n=4). The average of A_{595nm} and twice the standard deviation was chosen as the cut-off value. Biofilm formation of each well was expressed as the difference between the tested A595nm value and the cut-off value corresponding to its culture medium. Each strain in each culture medium was tested in quadruplicate.

3.5 Coupon assay

Round coupons (diameter 1cm) were used to study the biofilm formation and initial attachment of *Y. enterocolitica*. Two types of round coupons – stainless steel and polycarbonate, with the diameter of 1 cm, were used. Stainless steel coupons were used to simulate real factory condition (Chapters Four and Five); polycarbonate coupons were chosen to represent the material of microtitre plate assay, because they

were used in further studies based on the results of the microtitre plate assay (Chapter Seven). The coupons were soaked in detergent (Trigene Advanced, 1%) for at least 30 minutes, washed with distilled water and dried before autoclaving for 15 minutes at 121 °C. Coupons were washed with distilled water instead of phosphate buffer to simulate the real practise in the pork industry.

3.5.1 Attachment test

Bacteria were pre-incubated (Section 3.2) under specific test conditions, the cultures were harvested and washed twice in sterile NaCl solution (0.85%) by centrifugation (4400 rpm, 10 min). Each tested bacteria solution was adjusted to OD_{600} = 0.4. Each polycarbonate coupon was placed in a well of the 24-well micrototre plate and incubated in 1 ml of respective bacteria solution for 1hr. Triplicate coupons were used for each experiment. The coupons were washed in distilled water by dipping into three 60 mm petri-dishes full of distilled water consecutively to remove loosely attached cells. The cells left on the coupons were enumerated by impedance (descripted in Section 3.6.1) or plate counting followed by bead-beating (descripted in Section 3.6.2).

3.5.2 Biofilm development

The annular rotating disc reactor (Biosurface Technologies Ltd) was used to develop biofilms in Chapter Four. In the annular reactor, six stainless steel coupons were fitted into a Teflon holder containing a magnetic stir bar in the base. This assembly was autoclaved before being placed on a magnetic stirrer. Planktonic cultures (4 ml) obtained from an overnight culture in TSB grown at 25 °C were inoculated into each biofilm reactor containing 400 ml TSB and incubated at either 10 or 25 °C for 48 hrs with a rotating speed of 150 rpm to generate biofilms.

The 24-well micrototre plate was used to develop biofilm in Chapter Five and Seven. Coupons were put into each well of a 24-well microtitre plate (Falcon®, Corning inc., USA) containing 2 ml MJ or TSB. Overnight cultures of the bacteria were diluted in fresh media to make a bacterial solution of OD_{600}= 0.05. Subsequently, 200 µl of diluted bacterial suspension (OD_{600nm}= 0.05) were inoculated into each well. The

coupons were incubated together with the bacterial solution for 24 hrs. The 24-well plate was incubated in a static environment at specific test conditions to allow biofilm formation before treatment or enumeration.

3.6 Detection method

To detect the cells attached to a coupon (either in a biofilm or initial attachment), two methods were used to quantify the cell number as described below.

3.6.1 Impedance enumeration

An impedance method was developed and used as a fast counting method for *Y. enterocolitica* attached cells or biofilm on coupon surfaces. To enumerate the cells on coupons after attachment or biofilm growth, each coupon was transferred by sterile forceps into an individual BacTrac tube containing 10 ml of TSB. The vials were incubated in the BacTracTM 4000 microorganism growth analyser (SyLab, Austria) at 30 °C. The change of the culture medium impedance (M-value) due to bacterial metabolism was measured every 20 min. The time it takes for the M-value to reach certain threshold (1.5) was recorded as an indicator of cell number. The actual cell number can be calculated by using the detection time and the calibration equation as descripted in Chapter Four.

3.6.2 Bead-beating recovery

The bead beating recovery followed by plate counting was chosen as the reference method to check the reliability of the impedance method.

As described in (Lindsay and Von Holy, 1997) with modification, the washed coupons were put into a 50 ml flask containing 10 ml of NaCl 0.85% and 5 g glass beads (2-5mm in diameter), vortex mixed for 10 min at 180 rpm. The cells removed from each coupon and were counted by plate counting.

3.7 Scanning electron microscopy

Biofilms developed on polycarbonate coupons were analysed by using scanning electron microscopy (SEM) at the Manawatu Microscopy and Imaging Centre. Coupons were firstly incubated in fixative (3% glutaraldehyde and 2% formalde-hyde in 0.1 M phosphate buffer, pH 7.2) for 24 hrs at room temperature. Then, they were washed with 0.1 M phosphate buffer (pH 7.2) for three times before being dehydrated by passing through a graded ethanol series. Finally the coupons were critical point-dried using liquid CO_2, mounted onto aluminium specimen support stubs, sputter-coated with gold, and observed using a scanning electron microscope (FEI Quanta 200; FEI Co., Hillsboro, OR, USA).

3.8 Cell surface properties analysis

To find out why certain incubation conditions increase biofilm formation by *Y. enterocolitica,* the physical surface properties including surface hydrophobicity and surface charge of the cells were investigated.

3.8.1 Cell surface hydrophobicity

The hydrophobic interactive chromatography (HIC) procedure was carried out as described by Dahlback *et al*. (Yang et al., 2003) by passing a bacteria solution through Sepharose CL-4B filled column. Hydrophobic cells are retained on the column and hydrophilic cells are released. The resin and bacteria suspension was prepared in 0.05M phosphate buffer at pH 7 containing 2M NaCl. The resin suspension (1 ml) was filled into a glass Pasteur pipette. A small amount of glass wool was used to block the end of each Pasteur pipette, to stop the resin from leaking. Each resin column was loaded with 300 µl of bacteria culture and was held stationary for 15 min before eluting with 900 µl of the buffer. The absorbance of the loaded bacteria solution and eluted bacteria solution were measured at $OD_{600\,nm}$ using a spectrophotometer. The percentage of bacteria retained in the resin column was calculated from the

absorbance of a ¼ dilution of the loaded bacteria suspension and the absorbance of the eluted bacteria suspension.

3.8.2 Cell surface charge

The bacterial cells were diluted to OD_{600} 0.5 in phosphate-citrate buffers of defined pH ranging from pH 2 to pH 8 (Table 3.2). The surface charge (Zeta potential) of bacteria cells in each buffer was measured by using in a zeta potential analyser (Zetasizer Nano Range, Malvern).

Table 3.2. Citric acid-Sodium phosphate buffer – pH range 2–8.

pH at 25 °C	0.01 M citric acid (ml)	0.02 M Na_2HPO_4 (ml)
2	89	11
3	79.5	20.5
4	61.4	38.6
5	48.5	51.5
6	36.8	63.2
7	17.6	82.4
8	6.4	93.6

3.9 Extracellular polymeric substances analysis

To determine the effect of extracellular polymeric substances (EPS) on the biofilm formation by *Y. enterocolitica*, EPS was extracted from the bacterial culture and the carbohydrate, protein and nucleic acid were analysed.

3.9.1 Alkaline extraction of EPS

Alkaline extraction was chosen in this study. Formaldehyde fixation was performed before alkaline treatment to prevent cell damage. As described by Bales *et al.* (Bales et al., 2013) after incubation of a planktonic culture, 60 µl of formaldehyde (36.5% solution) was added to each 10 ml of culture. The formaldehyde-culture mixture was incubated at 25 °C with gentle shaking (100 rpm) for 1 hr. Next, 4 ml of 1M NaOH was added for each 10 ml culture and incubated at 25 °C for 3 hours with shaking (100 rpm) to extract EPS. Bacteria suspensions were then centrifuged (16800 g) at 4 °C for 1 hr. Absolute ethanol (1.5 volumes) was added to the supernatant containing soluble EPS and the mixture was placed at -20 °C for 20 hrs to precipitate EPS. The solution was then centrifuged (16800 g) at 4 °C for 1 hr. The EPS pellet was tested for carbohydrate, protein and nucleic acid content.

3.9.2 Quantification of exopolysaccharides in EPS

The exopolysaccharides were quantified by the phenol sulfuric acid method as described by Masuko *et al*. (Masuko et al., 2005) with minor modification. Briefly, the EPS pellet was re-suspended in 5 ml of distilled water and mixture was placed in a sonication water bath for 3 min to promote solubilisation of the EPS before testing. A 5 ml volume of 5% phenol was mixed with the exopolysaccharides solution and 25 ml of concentrated sulphuric acid was then rapidly added (the EPS pellet is not completely solubilized in water, but after sulfuric acid is added in it will become even mixture because the concentrated sulfuric acid is strong enough to break down the insoluble bits to soluble substance). The mixture was then given a full vortex mix and allowed to settle at room temperature for 1 hr before measuring the absorption at $OD_{485\ nm}$. This method was calibrated first with glucose standards (10, 100, 200, 300 and 400 µg/ml).

The reaction product of the sample may need to be diluted into the linear interval of the calibration range (10-400 µg/ml) if the concentration is too high to give meaningful reading, the final concentration is then calculated from the measured value of the diluent and the dilution factor.

3.9.3 Quantification of nucleic acid in EPS

Nucleic acid was extracted from the EPS pellet by using the phenol chloroform method described previously (Sambrook and Russell, 2006). Briefly, the EPS pellet was re-suspended in 5 ml of re-suspension buffer, then 5 ml of Phenol: Chloroform (1:1) was added. The Phenol: Chloroform extraction was done in a fume hood at room temperature. The mixture was continually vortex mixed at top speed for 2 min and then centrifuged for 5 min at 3,000 g. The aqueous phase was pipetted into a clean 10 ml centrifuge tube, and an equal volume of phenol/chloroform was added before vortex mixing and centrifugation as described above. The aqueous phase was again pipetted into a clean 10 ml centrifuge tube and equal volume of phenol was added before vortex mixing and centrifugation as described above. Finally, to remove the remaining phenol, an equal volume of chloroform was mixed with the aqueous phase and given a full vortex mixing before centrifugation. Nucleic acid concentration in the liquid phase was then determined by using a Microvolume Spectrometer (Colibri, Titertek Berthold). DNA sample with a concentration of 420 ng/µl was included in the whole extraction process as a positive control which showed that there is little change in DNA concentration in spite of the volume loss due to repeated extraction steps.

3.10 Membrane protein analysis

To find out why certain incubation conditions increase the biofilm formation by *Y. enterocolitica*, the profile of membrane protein was analysed.

3.10.1 Membrane protein extraction

The membrane protein was extracted as described by this article (Portnoy et al., 1984) with minor modification. A 40 ml volume of stationary phase (18 h) culture was harvested and washed twice in 0.85% NaCl. The culture was re-suspended in 1 ml of lysis buffer (Table 3.3) before being lysed by sonication (the culture was constantly kept on ice during sonication and the cell lysis was confirmed by microscope observation). The lysate was centrifuged at 12300 g at 4 °C for 2 min to remove cell debris. The supernatant was then centrifuged at 12300 g at 4 °C for 30 min. The pellet

was incubated in 300 µl of extraction buffer (Table 3.3) at room temperature for 20 min. The membrane proteins which are insoluble in the extraction buffer containing Triton-X100 were collected by centrifugation at 12300 g, 4 °C for 30 min, finally the protein pellet was re-suspended in 250 µl of sample buffer (Table 3.3) and aliquoted to small portions before being stored at -80 °C.

3.10.2 SDS-PAGE

The recipe for preparing the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel is given in Table 3.3.

Table 3.3. Recipes of working buffer for membrane protein extraction

Buffer	Ingredients	Comments
Bacteria lysis buffer	NaCl 0.5 M, NaH_2PO_4 0.065 M, imidazole 0.01 M, PMSF 1 Mm, pH8	100 mM PMSF was prepared in isopropanol, aliquoted and stored at -20 degree. Thaw and vortex before use.
Membrane protein extraction buffer	Tris 0.01 mM, $MgCl_2$ 5 mM, Triton X-100 2%, pH8	None
Sample loading buffer	Tris-HCl 0.05 M, SDS 0.07 M, Bromophenol Blue 15 mM, 2-Mercaptoethanol 1%, pH 6.8	None
Separation gel	Acrylamide 12%, Tris 0.125 M, SDS 0.1%, APS 0.1%, TEMED 0.1%	APS and TEMED was added right before casting the gel
Stacking gel	Acrylamide 5%, Tris 0.375 M, SDS 0.1%, APS 0.1%, TEMED 0.1%	APS and TEMED was added right before casting the gel
Running buffer	SDS 0.1%, Tris 0.05 M, Glycine 0.384 M，pH8.3	None
Coomassie Brilliant	0.1% Coomassie G-250, 1% Phosphoric	The dye will not go completely

Blue stain (stock)	acid, 0.15 M Ammonium sulphate	in solution, mix 4 parts of the stock with 1 part of MeOH before use
Fixative solution	MeOH 40%, Acetic acid 10%	None

When casting gels, the gel glass plates were wiped clean with ethanol and left for 5 min to allow the ethanol to evaporate. Preparation occurred inside a fume hood with positive air flow to keep the working area as dust-free as possible. A volume of 10 µl was loaded. The gel was run in a 4 °C cold room at 80 V for 5 hrs. After the electrophoresis was done, the gel was fixed with fixative solution (Table 3.3) and shaken for 30 min. Then, the fixative was discarded; the gel was covered by deionised water and shaken for 10 min. This step was repeated three times. Subsequently the fixed gel was stained in 0.02% Coomassie Brilliant Blue stain (Table 3.3) and shaken at 40 rpm overnight at room temperature before being distained with deionised water till the background Bromophenol blue faded.

3.11 Transcriptional analysis

The gene expression was analysed by RNA sequencing by the Massey Genome Service.

3.11.1 RNA isolation

Total RNA of *Y. enterocolitica* was isolated by using the NucleoSpin RNA plus kit (Macherey-Nagel), as described by the manufacturer. The culture was grown at 37 °C to early exponential phase (5 hrs) and then collected by centrifugation at 12000 g, 4 °C, for 2 min before RNA extraction.

3.11.2 RNA sequencing

The quality and quantity of the RNA sample prepared above was assessed by using the Agilent 2100 Bioanalyzer before proceeding with genome sequencing at the Massey Genome Service.

A library was prepared by using Illumina TruSeq™ Stranded Total RNA Library Prep Kit at the Massey Genome Service. The MiSeq personal sequencing system was used for sequencing. The sequencing results were mapped to the *Y. enterocolitica subsp. palearctica* Y11 genome. Differential expression was analysed using DESeq2 (Love et al., 2016).

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Haoran Wang

Name/Title of Principal Supervisor: Prof Steve Flint

Name of Published Research Output and full reference:

A rapid method for the nonselective enumeration of Yersinia enterocolitica, a foodborne pathogen associated with pork.

In which Chapter is the Published Work: Four

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate: 70%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Experimental design, all the experimental implementation, data analysis and writing.

Haoran Wang Digitally signed by Haoran Wang Date: 2016.12.15 12:09:55 +13'00'
Candidate's Signature

15th December
Date

Steve Flint Digitally signed by Steve Flint DN: cn=Steve Flint, o=Massey University, ou=SFN, email=s.h.flint@massey.ac.nz, c=NZ Date: 2016.12.15 12:29:24 +13'00'
Principal Supervisor's signature

15/12/16
Date

Chapter Four. Establishment of an impedance method for rapid enumeration of *Y. enterocolitica* biofilm

4.1 Introduction

For enumeration of biofilm cells, the conventional method involves removal and culturing of attached bacteria (Lindsay and Von Holy, 1997). This method is time-consuming and labour- intensive. More limitations arise during the detachment of biofilms- some areas where biofilm can grow may be hard to access. Enumeration method based on impedance microbiology can overcome the above drawbacks as no detachment and plate counting is needed during the detection. Impedance microbiology is a rapid method that enables enumeration of microorganisms by measuring the change in the electrical conductivity of growing media. It has been applied to the detection or enumeration of many species of *Enterobacteriaceae* of concern in food hygiene (Kierek and Watnick, 2003, Yang et al., 2003). However, there are no reports on the use of impedance for the enumeration of *Y. enterocolitica*.

An impedance method was developed to enumerate *Y. enterocolitica* in planktonic culture. This method was also validated to be reliable to enumerate *Y. enterocolitica* in biofilms attached to surfaces. This application provides a faster counting method for *Y. enterocolitica* biofilms and was used in the inactivation study of *Y. enterocolitica* biofilms descripted in Chapter Five.

4.2 Procedures

4.2.1 Development of an impedance calibration

ERL10460 (biotype 1A), ERL104253 (biotype 3) and ERL032123 (biotype 4) from 3 different biotypes were chosen to develop calibration. All the cultures were incubated in TSB (as described in Section 3.2).

Impedance was measured using the BacTrac^{TM} 4000 microorganism growth analyser (SyLab, Purkersdorf-Vienna, Austria). This BacTrac^{TM} 4000 measuring system measures the conductivity change caused by microorganism growth. During microorganism growth, macromolecules are converted to smaller molecules which will in turn change the conductivity/impedance of the media and electrode. The M-value represents the impedance change of the growth medium The E-value represents the impedance change of the electrode surface. There is a linear relationship between the time taken for a change in conductivity/impedance and the numbers of bacteria. This can be used to calibrate the impedance system to estimate the numbers of viable cells of a specific bacterium.

The overnight cultures in TSB at 25 °C of the ERL10460, ERL104253 and ERL032123 were serially diluted in sterile 0.85% NaCl solution (with the dilution factor from 10^{-1} to 10^{-7}). For each dilution, 20 µl of bacteria solution was inoculated into each vial of the BacTrac^{TM} 4000 containing 10 ml of TSB. Triplicate vials were prepared and incubated at 30 °C for 24 hrs. The impedance changes were measured and the time taken for this impedance change to reach 1.5% was recorded. TSA plates were inoculated with 0.1 ml of 10^{-1}, 10^{-2} and 10^{-3} dilution, inverted and incubated at 30 °C for 24 hrs, triplicate plates were done for each dilution. The number of colonies was counted to provide the standard plate count and a calibration equation of plate count against impedance change threshold time was calculated by using the Microsoft Excel program. The number of viable cells of *Y. enterocolitica* could be determined by using the calibration equation.

4.2.2 Validation of impedance method

To validate the impedance method developed, random dilutions of the overnight cultures of each strain were enumerated by using the respective calibration. The actual cell number was determined by plate counting. The calibration was validated by the consistency of the two values (impedance count vs plate count).

4.2.3 Biofilm development

Biofilms were developed by using the annular rotating disc reactor (descripted in 3.4.2). Planktonic cultures (4 ml) obtained from an overnight culture in TSB grown at 25 °C were inoculated into each biofilm reactor containing 400 ml TSB and incubated at either 10 or 25 °C for 48 hrs with a rotating speed of 150 rpm to generate biofilms.

4.2.4 Comparison between the impedance method and the bead-beating recovery technique for the detection of biofilm cells of *Y. enterocolitica*

The stainless steel coupons were removed from the Teflon holder and washed five times with sterile deionized water. Thereafter three of the six coupons were transferred to BacTrac^{TM} vials containing 10 ml of TSB and enumerated by using the impedance method described above. While the other three were tested by the bead-beating technique followed by agar plating which is chosen as the standard recovery method.

The bead-beating recovery technique was performed as previously described with a slight modification (Lindsay and vonHoly, 1997). Each stainless steel coupon was placed in a 50 ml conical flask containing 10 ml of peptone saline solution (0.85% NaCl and 0.1% Peptone (Oxoid)) with 20 g of glass beads (5 mm diameter) and agitated on a shaker (150 strokes min^{-1}) at 25 °C for 10 min, after which the cell number was determined by plate counting.

4.2.5 Statistical analysis

To establish the calibration of each strain, either six or seven calibration points were carried out in triplicate and three experiments were performed, separately in time. For the comparison of the two methods, triplicate stainless steel coupons were separately treated with either the impedance method or the bead-beating recovery technique. The consistency of the data sets was analysed using the t-test.

Data that were statistically significant appeared with a p≤0.05. Excel was used for statistical analysis.

4.3 Results

The impedance measuring system used in this study measured two values-the M-value and the E-value. The results showed that the M-value gave sharp and reproducible growth curves for all *Y. enterocolitica* strains for an accurate estimation of cell numbers when compared to the plate counts (Figure 4.1, Appendix 1). The E-value curves were mostly out-of-shape and poorly reproducible. The M-value was therefore chosen to establish the calibrations. Figures 4.2, 4.3 and 4.4 show the calibration graphs for strains ERL10460, ERL104253 and ERL032123 (Raw data is shown in Appendix 2, Appendix 3 and Appendix 4). The calibration equations (Table 4.1) showed good correlations with the plate counts (r^2> 0.95).

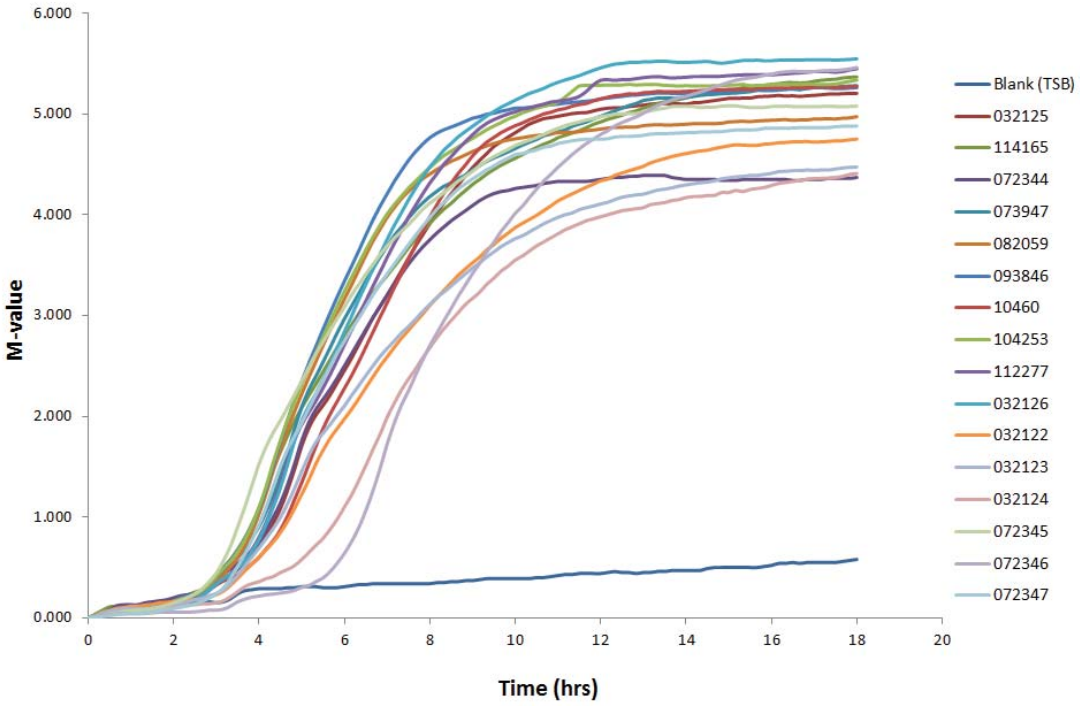

Figure 4.1. Growth curves of 16 strains of *Y. enterocolitica* in TSB at 30 °C using impedance. The straight line at the bottom is un-inoculated TSB; The "S" shape lines

include ERL032122, ERL032123, ERL032124, ERL032125, ERL072344, ERL114165, ERL072345, ERL072346, ERL072347, ERL073947, ERL082059, ERL093846, ERL10460, ERL104253, ERL032126, ERL112277.

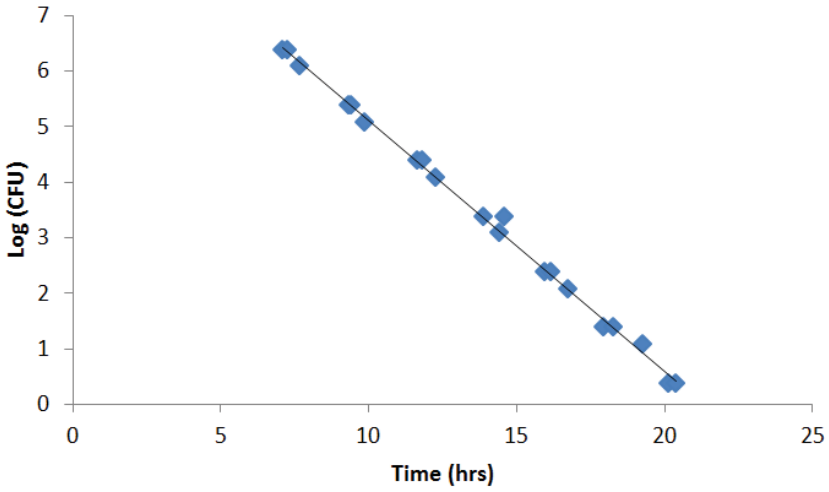

Figure 4.2. Linear Regression of the scatter plot for strain ERL032123: y = -0.4506x + 9.6181 r^2 = 0.997.

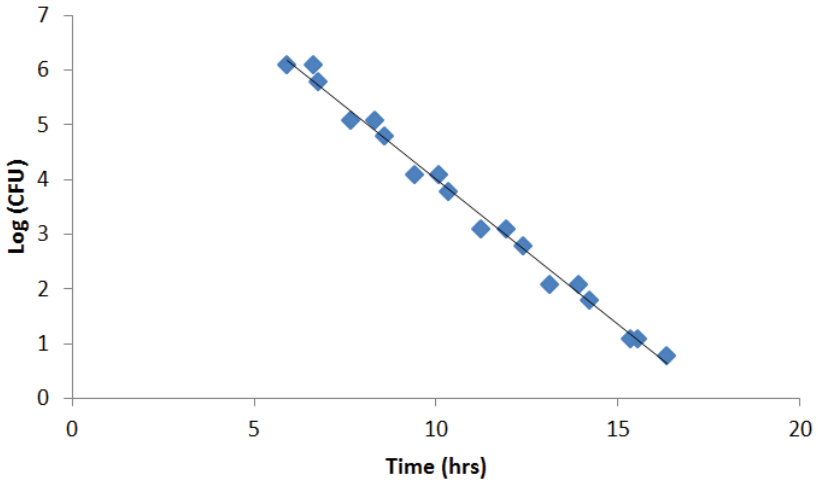

Figure 4.3. Linear Regression of the scatter plot for strain ERL104253: y = -0.5321x + 9.3275 r^2 = 0.9918.

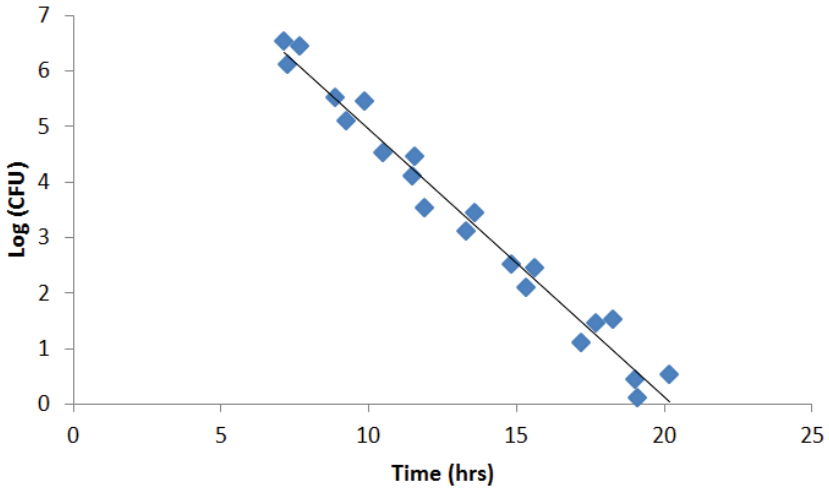

Figure 4.4. Linear Regression of the scatter plot for strain ERL10460: y = -0.4822x + 9.7797 r^2 = 0.9759.

Table 4.1. Comparison of *Y. enterocolitica* strains ERL032123, ERL104253 and ERL10460 measured by impedance and plate counting.

Strain Number	Cell number by plate counting (CFU)	Detection Time (hr)	Regression equation	Cell number by impedance method (CFU)	P-value
ERL10460	$(1.33\sim1.49) \times 10^3$	13.74±0.07	y = -0.4822x + 9.7797	$(1.32\sim1.54) \times 10^3$	0.626
	$(1.33\sim1.49) \times 10^1$	18.09±0.07	r^2 = 0.9759	$(1.10\sim1.20) \times 10^1$	0.073
ERL104253	$(0.78\sim1.34) \times 10^3$	11.60	y = -0.5321x + 9.3275	1.43×10^3	0.412
	$(0.78\sim1.34) \times 10^1$	15.42±0.12	r^2 = 0.9918	$(1.10\sim1.50) \times 10^1$	0.205
ERL032123	$(0.40\sim1.08) \times 10^3$	14.61±0.25	y = -0.4506x + 9.6181	$(0.84\sim1.40) \times 10^3$	0.1
	$(0.40\sim1.08) \times 10^1$	19.29±0.22	r^2 = 0.997	$(0.70\sim1.10) \times 10^1$	0.458

In the validation experiment, the cell number of each strain calculated from their respective equation and the actual cell number determined by plate counting showed good consistency (P>0.05) (Table 4.1). The impedance method based on the M-value enabled reproducible estimates of *Y.enterocolitica* cell in planktonic

culture. Since the calibration equations are strain specific, a separate calibration needs to be developed for every individual strain.

For the *in situ* enumeration of biofilm cells of *Y. enterocolitica* on stainless steel surfaces, the counts calculated by the impedance method was equivalent with that derived from the traditional bead-beating recovery technique ($P>0.05$) (Table 4.2).

Table 4.2. Comparison between impedance and bead-beating recovery for the enumeration of biofilm cells of *Y. enterocolitica* strains ERL104253 and ERL10460.

Sample Number	ERL104253		ERL10460	
	Bead-beating	Impedance	Bead-beating	Impedance
1	$(2.26\sim2.62) \times 10^6$	1.64×10^6	$(0.93\sim2.77) \times 10^6$	1.72×10^6
2	$(2.54\sim4.10) \times 10^6$	2.55×10^6	$(2.18\sim2.32) \times 10^6$	6.10×10^6
3	$(1.86\sim2.14) \times 10^6$	2.92×10^6	$(0.89\sim3.01) \times 10^6$	6.59×10^6
P-value	0.625		0.096	

4.4 Discussion

The BacTrac^{TM} 4000 machine can measure two specific impedance values for each measurement; these values are the M-values or E-values. The M-value is based upon the media impedance and the E-value based upon the electrode impedance. The choice of M-value or E-value used to estimate cell numbers depends on the shape of the curve and reproducibility of the results. In this study it was found that the M-value resulted in reproducible growth curves for *Y. enterocolitica*. The E-value was unsuitable for this study, whereas for other bacteria, it performs well (Flint and Brooks, 2001).

Preliminary trials with TSB showed a rapid and reproducible change in the M-value for all *Y. enterocolitica* strains (Figure 4.1). Thus, TSB was chosen to establish the calibration. *Y. enterocolitica* was pre-cultured at its optimal growth temperature 25 °C. A temperature of 30 °C instead of 25 °C was chosen to develop the calibration to achieve a faster result.

ERL10460 (biotype 1A) and ERL104253 (biotype 3) were chosen to validate the impedance method for biofilm enumeration because they form good biofilms (Figure 5.1). Biotype 1A is mostly non-pathogenic and biotype 3 a less common pathogenic biotype (Tennant et al., 2003), so ERL032123 (biotype 4) was also chosen to cover the most representative pathogenic biotype.

The fact that the impedance change of M-value in TSB was reproducible for all 16 strains and that the calibrations established for 3 strains from different biotypes all performed well, shows the potential use of impedance on the enumeration of all *Y. enterocolitica* strain cells in biofilm or planktonic form.

Bead-beating recovery is a published and widely used method for the recovery of biofilm cells (Lindsay and vonHoly, 1997, Nguyen et al., 2014b), and therefore provided a suitable reference for the impedance method in this study. The impedance method is reliable for the enumeration of *Y. enterocolitica* in planktonic cultures. Also, the results support its use as a tool for quantitative measurement of *Y. enterocolitica* biofilms. The simplicity of the impedance method (<15 hrs for the estimation of 1000 cells and <20 hrs for the estimation of 10 cells) makes it an ideal alternative to enumeration by plate counting. The sensitivity of this method is also increased compared to the bead-beating recovery and plate counting method. Because 10 ml solution will be derived after bead-beating recovery, and to detect the cell number in the 10 ml solution using either pour plating or spread plating, the detection limit will end up to be 10 CFU/cm^2 or 100 CFU/cm^2, whereas in theory, the impedance method is able to detect one cell present on the coupon.

It might be argued that the calibrations performed with planktonic cells may not be relevant for biofilm cells as the impedance method is based on impedance changes resulting from metabolic activity which may differ between planktonic and biofilm cells Comparative studies using planktonic cultures and biofilm for other bacteria have shown some discrepancy between the impedance result and that of the reference method (Flint et al., 1997). However, in this study, there is good agreement between the impedance and plate counting methods for planktonic and

biofilm cells of *Y. enterocolitica.* This might be due to the low recovery rate of the swab recovery method used in the study in 1997 compared to the bead-beating recovery method used in this study. Being able to enumerate biofilm formation facilitates further investigation of *Y. enterocolitica* biofilms- to evaluate the effect of different factors on the growth of biofilm (medium, surface roughness, surface topology, temperature, pH, ion concentration, sanitizers, etc.) by estimating cell number upon completion of various treatments. The biofilm studies could provide more knowledge about how *Y. enterocolitica* biofilms persist during food processing and help with the corresponding control.

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STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Haoran Wang

Name/Title of Principal Supervisor: Prof Steve Flint

Name of Published Research Output and full reference:

Biofilm formation of Yersinia enterocolitica and its persistence following treatment with different sanitation agents.

In which Chapter is the Published Work: Five

Please indicate either:

- The percentage of the Published Work that was contributed by the candidate 60%

  and / or

- Describe the contribution that the candidate has made to the Published Work:

  Experimental design, part of the experimental implementation, data analysis and writing.

Haoran Wang Digitally signed by Haoran Wang Date: 2016.12.15 12:09:55 +13'00'
Candidate's Signature

15th December
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Steve Flint Digitally signed by Steve Flint DN: cn=Steve Flint, o=Massey University, ou=SFN, email=s.h.flint@massey.ac.nz, c=NZ Date: 2016.12.15 12:27:57 +13'00'
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Date

Chapter Five. *Y. enterocolitica* biofilm formation at simulated meat processing conditions and its persistence following treatment with different sanitation agents

5.1 Introduction

Biofilms are a safety concern as they increase the resistance of bacteria to many physical and chemical factors used in controlling hygiene in the food industry (Brown and Gilbert, 1993). The role of biofilms as a source of foodborne human pathogens is well established. However, in case of *Y. enterocolitica* the role of biofilms and the factors leading to biofilm formation remain largely unknown.

In the food industry, chemicals are routinely used to sanitize and disinfect product contact surfaces. These chemicals provide a necessary and required step to ensure that the foods produced and consumed are as free as possible from microorganisms that can cause foodborne illness. According to AOAC Official Methods of Analysis, the sanitization standard for contamination reduction of food contact surfaces is generally accepted as 99.999% (a 5-log reduction) achieved in 30 s (Gaithersburg, 2009). Hypochlorites are the most widely used sanitizers due to their effectiveness and low cost. Hypochlorites kill microorganisms by damaging their outer membrane, losing permeability control and eventual cell lysis (Virto et al., 2005). These compounds also destroy enzymes and DNA. However, hypochlorites cause concern, due to their effects on equipment and human health as they are corrosive to metals and may irritate skin and damage mucous membranes. Hypochlorites may cause environmental contamination because the chlorine compounds in hypochlorites can form toxic compounds after combining with organic materials. Therefore, hypochlorites are recommended for use at low concentrations (<200ppm) (Pfunter, 2011). Quaternary ammonium compounds (QAC) have a relatively mild bactericidal effect compared to the hypochlorites but still function well as a sanitizer. They block the nutritional uptake of bacteria by binding with the acidic phospholipids in the bacterial cell wall (Pfunter, 2011, McBain et al., 2004). They are commonly used because they are usually

noncorrosive and relatively nontoxic to users. Also, they are biodegradable hence pose less burdens on the environment.

Bacteria may become resistant after repeated exposure to chemical treatment due to the inherent resistance of a particular bacterium or populations of bacteria. In addition, the development of a biofilm may provide additional resistance due to the physical protection from EPS that are associated with biofilm. This study aimed to investigate the potential of *Y. enterocolitica* biofilms, formed under conditions that are likely to happen in meat processing plant, to survive sanitizer treatments commonly used in the meat industry. Meat drip (juice) was used as the medium for biofilm development to represent the conditions in a meat processing plant. According to the Processed Meats Code of Practice in New Zealand, operators of meat processing plants are required to develop their own cleaning and sanitation programmes including detergents/sanitizers to be used, their concentrations, application methods and contact times (NZFSA, 2010). In this study, biofilm formation was monitored under an assumed cleaning regime in a pork processing plant over a period of five days. The effectiveness of sodium hypochlorite and QAC, were compared.

In this study, the biofilm formation by *Y. enterocolitica* was studied under conditions simulating a meat processing environment. The persistence of *Y. enterocolitica* biofilms were monitored during repeated treatment simulating the everyday cleaning routine by the impedance method described in Chapter Four.

5.2 Procedures

5.2.1 Microtitreplate biofilm assay

A microtitre plate assay was used to screen the biofilm formation by all 16 isolates in MJ (the preparation of MJ was described in Section 3.3) and TSB at 24 °C as described in Section 3.4.

5.2.2 Biofilm growth on stainless steel under a simulated meat processing regime

Round shape stainless steel coupons (grade 316, 2b, 1 cm dia) were used in this study as it is the most common bench surface material used in the meat processing industry. Coupons were put into each well of a 24-well microtitre plate (Falcon®, Corning inc., USA) containing 2 ml MJ or TSB. Subsequently, 200 µl of designated bacterial suspension (OD_{600}= 0.5) was inoculated into each well. The 24-well plate was incubated in a static culture at 24 °C for 24 hrs to allow biofilm formation. Then, the *Y. enterocolitica* biofilm formed on each stainless steel coupon was subjected to treatment with different sanitizers in a simulated daily cleaning regime for 5 days. Triplicate samples were taken on days 1, 3 and 5. The cell number on each coupon was enumerated by an impedance method established previously (Chapter Three). All cell enumerations for this study were performed using method described in Chapter Three in which the time taken for bacteria to alter the media impedance by 1.5% was recorded (t) and used to calculate the cell number (y) using the following equation: y=-0.4506t + 9.6181. Cell counts ranging from 3 to 2.4×10^7 CFU could be measured using this method.

The daily cleaning regime used in this trial was based on that used in a study on *L. monocytogenes* (Pan et al., 2006) with modifications. As briefly shown in Table 5.1, after pre-growth of biofilm (Day1-Step 1), the coupons were sanitized (Day1-Step 2) then put to starvation (Day1-Step 3). The starved coupons were re-incubated for 7 hrs (Day2-Step 1) before the same sanitation (Day2-Step 2) and starvation (Day2-Step 3) procedures were carried out. The treating procedures at Day 3, Day 4, and Day 5 are repetitions of Day 2. Sampling point following incubation was designated as point 1, following sanitation as Point 2 and following starvation Point 3.

At step 1 in the treatment, coupons were incubated in TSB or MJ for 24 hrs (day1, pre-growth) or 7 hours (day 2, 3, 4 and 5, re-growth following starvation) at 24 °C. Planktonic and loosely attached cells and growth media residues were rinsed off by

Table 5.1. The treatment procedures and sampling points during the cleaning regime.

Treatments	Sampling
Day1-Step 1 : Pre-incubation (24 hrs)	Point 1 (after Day1-Step 1)
Day1-Step 2 : Sanitation	Point 2 (after Day1-Step 2)
Day1-Step 3 : Starvation (16 hrs)	Point 3 (after Day1-Step 3)
Day2-Step 1 : Re-incubation (7 hrs)	
Day2-Step 2 : Sanitation	
Day2-Step 3 : Starvation (16 hrs)	
Day3-Step 1 : Re-incubation (7 hrs)	Point 1 (after Day3-Step 1)
Day3-Step 2 : Sanitation	Point 2 (after Day3-Step 2)
Day3-Step 3 : Starvation (16 hrs)	Point 3 (after Day3-Step 3)
Day4-Step 1 : Re-incubation (7 hrs)	
Day4-Step 2 : Sanitation	
Day4-Step 3 : Starvation (16 hrs)	
Day5-Step 1 : Re-incubation (7 hrs)	Point 1 (after Day5-Step 1)
Day5-Step 2 : Sanitation	Point 2 (after Day5-Step 2)
Day5-Step 3 : Starvation (16 hrs)	Point 3 (after Day5-Step 3)

dipping the coupons into sterile distilled water and gently shaking for a few seconds. After rinsing, each coupon was then transferred to an individual BacTrac tube by sterile forceps before enumeration by the impedance method. Triplicate samples were taken for cell enumeration (Point 1).

At step 2 in the treatment, coupons from point 1 containing biofilm were immediately subjected to sanitation treatment by 50 ppm Sodium hypochlorite (pH 6.5, XY-12, Ecolab) or 200 ppm QAC (22 Multi-Quat sanitizer, Ecolab) for 1 minute and 0.85% NaCl solution was used as control. Coupons were treated with neutralizers for 60 sec at the end of the exposure time to stop the sanitizer from functioning. Sodium thiosulphate-phosphate solution (0.1%, pH 7.0) was used to neutralise the sodium hypochlorite a lecithin solution (0.53% lecithin, 3.75% Tween 80 and 0.05% KH_2PO_4) was used to

neutralise the quaternary ammonium sanitizer. All chemicals were rinsed off before cell enumeration as described in step 1. Triplicate samples were taken for cell enumeration (Point 2).

At step 3 in the treatment, coupons containing biofilm were stored in a sterile petri dish without any nutrition for 16 hrs at room temperature. A small amount of water was kept in the petri dish to prevent the coupons from drying out. The coupons containing biofilm following starvation were rinsed to remove the loosely attached cells before triplicate samples were taken for cell enumeration (Point 3).

5.2.3 Statistical analysis

The microtiter plate assay was carried out in four replicates and the coupon assay in triplicates. Mean values ± 1 standard deviation are reported. T-test in Microsoft Excel software was used to analyse the data sets. Differences that were statistically significant appeared with a $p \leq 0.05$.

5.3 Results

5.3.1 Pathogenic biotype 4 form stronger biofilms in MJ than in TSB after 24 hrs at 24 °C

Biofilm formation was recorded for representatives of biotypes 1A, 2, 3 and 4 in TSB and MJ (Figure 5.1, Appendix 5). Three of the seven biotype 1A strains (ERL072345, ERL072347, ERL093846) and one biotype 4 strain (ERL114165) formed no biofilm both in TSB and MJ. Four of the seven biotype 1A strains (ERL072346, ERL073947, ERL082059, ERL10460) and the two of the biotype 3 strains (ERL104253, ERL112277) showed biofilm formation in TSB, whereas, the only biotype 2 strain (ERL032126) and all the six biotype 4 strains didn't form biofilm in TSB. The TSB-biofilm forming biotype1A strains and one of the biotype 3 strains (ERL104253) showed decreased or similar biofilm formation in MJ compared to TSB. The biotype 2 strain (ERL032126)and the one of the biotype 3 strains (ERL112277) together with five of the six biotype 4

strains (ERL032122, ERL032123, ERL032124, ERL032125, ERL072344) showed increased biofilm formation in MJ compared to TSB.

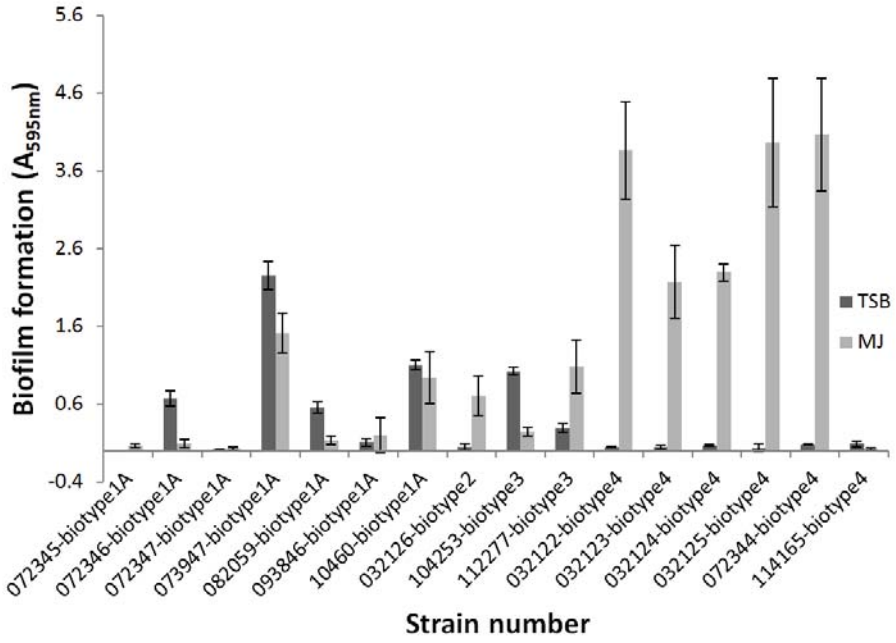

Figure 5.1. Biofilm formation by different biotypes of *Y. enterocolitica* in TSB and MJ at 24 °C after 24 hrs. Error bars represent mean ± standard deviation. The biotype 2 strain (ERL032126)and the one of the biotype 3 strains (ERL112277) together with five of the six biotype 4 strains (ERL032122, ERL032123, ERL032124, ERL032125, ERL072344) showed increased biofilm formation in MJ compared to TSB.

5.3.2 Biofilms Grown under a simulated meat processing regime

One of the biotype 4 strains ERL032123 was chosen to carry out the biofilm study under a Simulated Meat Processing Regime. After 24 hrs incubation the cell number attached to stainless steel coupons was 10^6-10^7CFU/cm^2. The results from the stainless steel coupon assay reflected the results from the microtitre plate assay which showed more biofilm in the presence of MJ than in TSB (Figure 5.2, Appendix 6).

After the control treatment in which 0.85% NaCl was used daily instead of sanitizers, the cells left on both groups of stainless steel coupons incubated in TSB and MJ

reduced to 10^3CFU/cm^2 by day 5. However, MJ coupons maintained higher cell numbers than those from the TSB batch for the first three days (Figure 5.2).

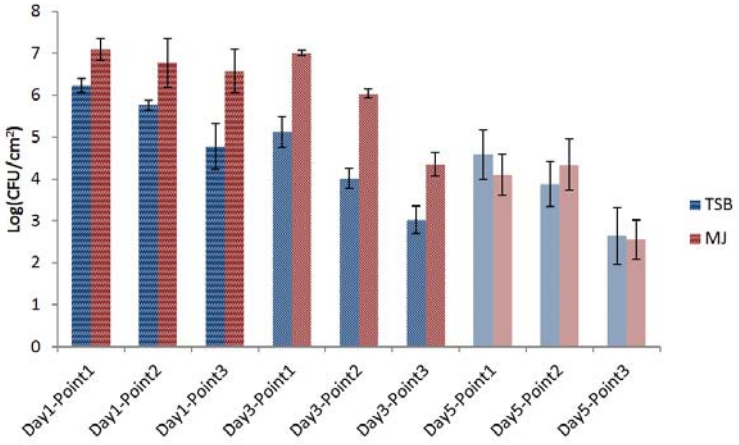

Figure 5.2. Persistence of biofilm of biotype 4, strain ERL032123 formed in TSB and MJ under control treatment using 0.85% NaCl solution at 24 °C. Error bars represent mean ± standard deviation.

When the coupons incubated in TSB were treated with 50 ppm Sodium hypochlorite, the cell number dropped by five log CFU/cm^2 immediately after sanitation and maintained a similar level with slight fluctuations until the end of the trial (Figure 5.3, Appendix 7).

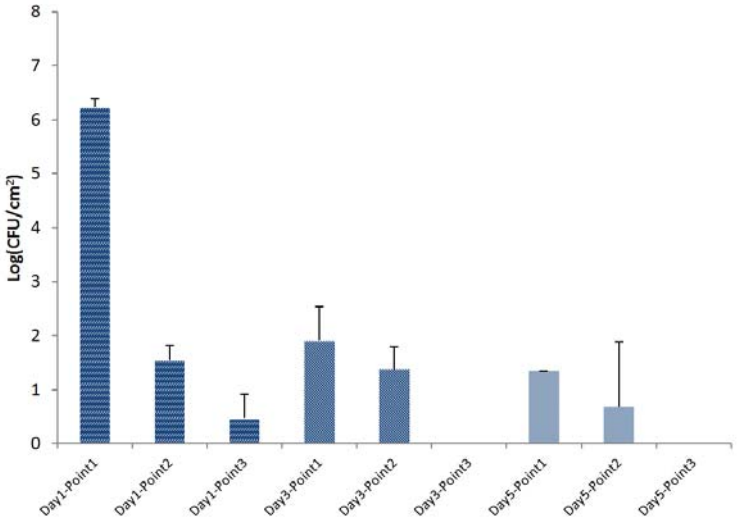

Figure 5.3. Biofilm of *Y. enterocolitica* strain ERL032123 treated with 50 ppm Sodium hypochlorite in TSB at 24 °C. Error bars represent mean ± standard deviation.

When the coupons incubated in MJ were treated with 50 ppm Sodium hypochlorite, there was only a three log drop after sanitation and the cell number remained at similar level at Point 3 After re-incubation at day 3 the cell number on coupons was about 10^4-10^5 CFU/cm^2 and again, after sanitation there was a 3 log drop. Levels of < 3 cells/cm^{2}were detected from day 3 onwards (Figure 5.4, Appendix 7).

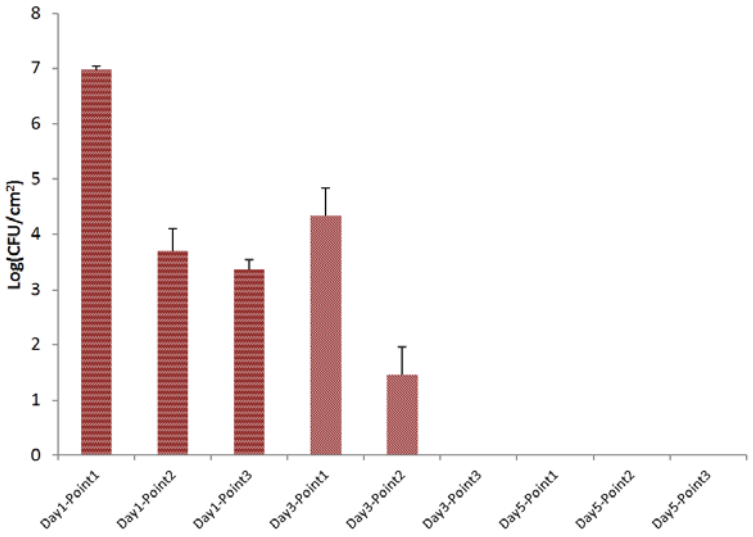

Figure 5.4. Biofilm of *Y. enterocolitica* strain ERL032123 treated with 50 ppm Sodium hypochlorite in MJ at 24 °C. Error bars represent mean ± standard deviation.

When the coupons incubated in TSB were treated by QAC, the cell number dropped by 3-log and kept dropping during starvation. The cell counts were within 10^1-10^3 CFU/cm^2 level until starvation was over on day 3 when no cells were detected. However, the cell number soared almost to the initial level on day 5 and then plummeted down to 10 after sanitation and again was undetectable after starvation (Figure 5.5, Appendix 8).

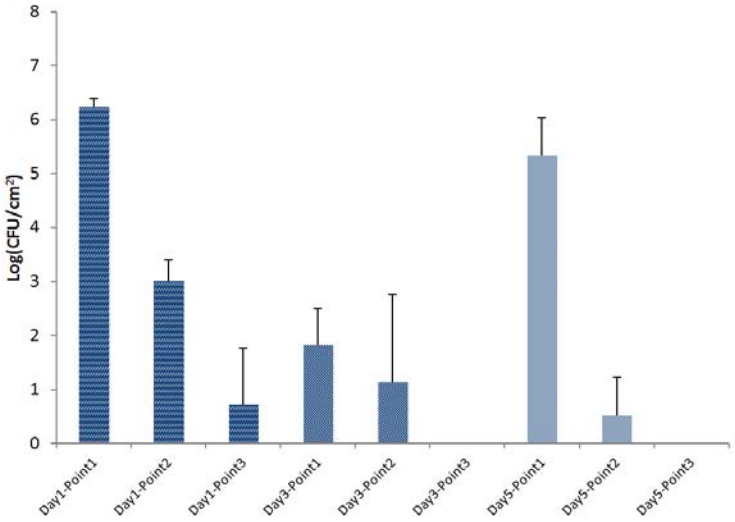

Figure 5.5. Biofilm of *Y. enterocolitica* strain ERL032123 treated with 200 ppm QAC in TSB at 24 °C. Error bars represent mean ± standard deviation.

When coupons incubated in MJ were treated by QAC, the cells dropped by 3-4 log and remained at about 10^3 CFU/cm^2 for the first 3 days. Cell numbers dropped down to 10^2 CFU/cm^2 at day 5. After starvation, no microbial cells were detected (Figure 5.6, Appendix 8).

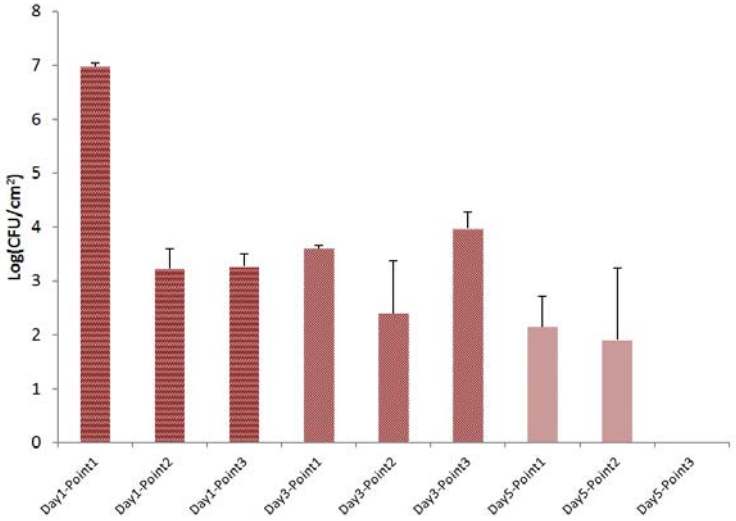

Figure 5.6. Biofilm of *Y. enterocolitica* strain ERL032123 treated with 200 ppm QAC in MJ at 24 °C. Error bars represent mean ± standard deviation.

5.4 Discussion

The biotype 4 of *Y. enterocolitica* which most commonly occurs in porcine sources (Gütler et al., 2005), formed the strongest biofilm in pork meat dripping compared to other biotypes. By natural selection, species will gradually change to adapt to their niche. The results of this study imply that biotype 4 of *Y. enterocolitica* may have become well adapted to its living environment by forming a biofilm.

Under the control treatment (0.85 % NaCl at 24 °C), biofilm cell numbers of strain ERL032123 was reduced from 10^6 CFU/cm^2 in TSB and 10^7 CFU/cm^2 in MJ to 10^3 CFU/cm^2 (Figure 5.2).The cell number on coupons incubated in MJ maintained a higher level of bacteria for longer than those from the coupons incubated in TSB, showing higher persistence against the force of physical washing up until day 3.

When the TSB incubated coupons were treated with sodium hypochlorite, the five log reduction in cells after sanitation would have reflected the killing of the majority of cells. However, some injured or inherently more resistant biofilm cells were still detected after the initial treatment and this is reflected in the results (Figure 5.4). The injured cells appeared to have died during starvation which could explain the even lower cell number after starvation. There was a slight increase in cells after each re-incubation, reflecting some cell recovery, but overall, the cell number remained below 5×10^2 CFU/cm^{2}although did not persist until the last day of the test.

When coupons were incubated in MJ (pH 6.5), more biofilm cells were detected compared with the coupons incubated in TSB (pH 6.5). That might be due to nutrients (e.g. ions) in the MJ that favour the growth of these bacteria. MJ may also contribute to cell attachment by covering and conditioning the abiotic surface with particulates as reported elsewhere (Brown et al., 2014). The higher primary attachment on surfaces may increase the potential for biofilm formation via quorum sensing which has been shown to be involved in the development of biofilms by other bacteria (Davies et al., 1998, Hammer and Bassler, 2003, Tao et al., 2010) Also, the cells may be more robust due to the cell response to the MJ nutrients that may reflect an increase in

extracellular polysaccharide production or a change in the structure of the biofilm that may increase the resistance of these cells to sodium hypochlorite. The production of extracellular polysaccharide and curli increase the resistance of *E. coli* O157:H7 to chlorine (Ryu and Beuchat, 2005). In the present study, the decrease in attached cells (three log) in MJ than in TSB (five log) after sanitation may reflect a more robust biofilm structure and greater resistance to sodium hypochlorite. Starvation appeared to have a less effect on the biofilm cells grown in the presence of MJ compared with TSB and this may reflect the entrapment of some of the nutrients in the biofilm structure when grown in MJ. QAC was less effective in terms of the bactericidal activity compared with sodium hypochlorite. Because QACs block nutritional uptake in bacterium (McBain et al., 2004, Pfunter, 2011), this may result in reduced microbial activity rather than cell death, allowing recovery when conditions improve. During starvation, the metabolism will reduce further but the cells persist on the surface for the first three days. Presumably, at some point between days 3 and 5, the cell surface experienced a change leading to QAC detachment from the cell surface as a study has shown that *E.coli* could become QAC-resistant by the expression of efflux pumps (Lomovskaya and Lewis, 1992). A recovery to normal metabolism may happen after QAC detachment which could help explain the increase in cell numbers at point 1, day 5 in the cells grown as biofilm in TSB. At point 3, day 5, the reduction in cell numbers to an undetectable level may relate to a reduction in hydrophobicity due to the known surfactant activity of the QAC sanitizers, resulting in cell detachment. Again, when the coupons incubated in MJ were treated by QAC, the biofilm structure and concentration of nutrients may have helped the cells to persist through treatment.

The environment simulating conditions in a pork processing plant will support biofilm growth and persistence of *Y. enterocolitica*, especially the biotype that is most commonly found in pigs, pork and human samples. Biofilms grown in a simulated pork processing environment consisting of a cyclical sanitizer treatment and operating condition, are able to persist after being challenged with the two sanitizers, in particular the QAC sanitizer. The persistence is stronger when biofilms are grown in MJ compared with growth in a TSB (laboratory medium) environment. Sodium

hypochlorite is more effective than QAC in cleaning biofilm of *Y. enterocolitica* formed in MJ.

Chapter Six. The influence of ions, temperature and plasmid pYV on *Y. enterocolitica* biofilm formation

6.1 Introduction

The concentrations of ions in the aqueous phase that immerses a biofilm can influence biofilms in many ways. Calcium, magnesium, and iron have all been implicated in biofilm formation. However, the role of ions has been suggested as being nonspecific in some cases (e.g., Ca^{2+} and Mg^{2+}), with these elements playing a role as bridges for the adhesion between negatively charged bacterial cells and surfaces (Leite et al., 2002). Electrostatic interactions between cations and bacterial polymers can influence biofilm formation. In addition, bacteria could physiologically respond to the presence of ions which may change the surface structures. Cell surface structures such as flagella, fimbriae, curli, and polysaccharides can shape the physic-chemical surface properties of bacterial cells, hence influence attachment and biofilm formation (Van Houdt and Michiels, 2010). Ca^{2+} was shown to stimulate a *Pseudoalteromonas spp*. to increase both the amount and the composition of extracellular proteins it expresses, which primes the bacteria for biofilm formation.(Patrauchan et al., 2005). Ca^{2+} levels have also been reported to modulate biofilm structures in *V. cholerae* by interacting directly with the O-antigen polysaccharide (Kierek and Watnick, 2003)

It was proposed that Mg^{2+} may enhance biofilm formation by *P. fluorescens* by influencing the production of flagella and fimbriae or the production and structure of exopolysaccharide (Song and Leff, 2006). For the pathogenic *Yersinia* spp, a temperature upshift from $\leqslant$30 °C to 37 °C induces virulence gene expression which in turn triggers multiple effects in cell morphology and physiology. Changes after a temperature upshift include the repression of flagellum synthesis and induction of a set of plasmid-encoded virulence genes (Kapatral and Minnich, 1995). The virulence plasmid, termed pYV encodes a type III secretion system essential for delivery of additional plasmid-borne anti-host factors collectively referred to as Yops (*Yersinia* outer membrane proteins) (Cornelis et al., 1998). Expression of genes related to Yops

synthesis and delivery requires the transcriptional regulator protein VirF (Cornelis et al., 1989). VirF synthesis is induced by elevated temperature (Rohde et al., 1994).

In this study, the influence of ions, temperature and the plasmid on the biofilm formation by *Y. enterocolitica* was investigated.

6.2 Procedure

6.2.1 Biofilm formation of *Y. enterocolitica* at 24 °C in peptone water supplemented with ions

Biofilm formation was assessed by microtitre plate assay when cells were grown in casein soy digest medium supplemented with ions (casein digest 17g/L, soy digest 3g/L). The casein soy digest medium was prepared in 0.02M Tris-HCl buffer instead of phosphate buffer to avoid phosphate precipitate and was adjusted to pH 6.8 before use. $CaCl_2.2H_2O$ (Univar), $MgSO_4.\ 7H_2O$ (LabServ^{TM}), $ZnSO_4.\ 7H_2O$ (AnalaR), $C_7H_{13}FeNO_7$ (Sigma Aldrich), NaCl (LabServ^{TM}) and KCl (Merck) were added as supplements to the peptone water before the biofilm testing using the microtitre plate assay. These ions were chosen because they are very likely present in higher concentration in MJ than TSB (The ions contents of MJ were analysed by Nutrition Laboratory, Massey Institute of Food Science and Technology as attached in Appendix 9. However, information about ions contents in TSB is not available.). The concentration of these various ions in peptone water was unknown but assumed to be negligible. A row (n = 12) of cells grown in non-supplemented peptone water medium was included as a control. The cut-off value of each supplemented media was generated as described in 3.4. The concentrations of each ion tested were 1.5, 3, 4, 5, 7.5, 10mM.

6.2.2 Preparation of a pYV$^-$ population of *Y. enterocolitica* strain ERL032123

Y. enterocolitica ERL032123 strain (pYV$^-$) was prepared as later results showed that pYV is important in biofilm formation by *Y. enterocolitica*. Distinct colony morphology indicates the loss of the plasmid shown in Figure 6.1. Details of the method were described in 3.1.1. The loss of the plasmid was confirmed by PCR for a plasmid specific gene *vir*F (3.1.2), DNAse free water was used as negative control, the result is shown in

Figure 6.2. The PCR product wasn't further confirmed by DNA sequencing because the level of the PCR product on the E-gel agrees to the molecular weight of *vir*F as shown in a previous publication (Nilsson et al., 1998), moreover, the DNA band only existed on the pYV^+ strain not on the pYV^- mutant.

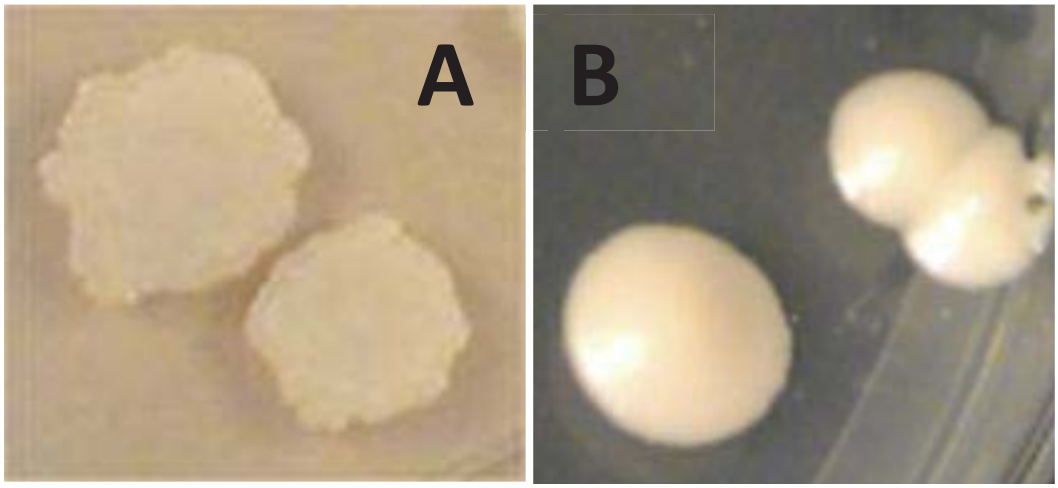

Figure 6.1. The different colony shape before (Picture A) and after (Picture B) the loss of pYV.

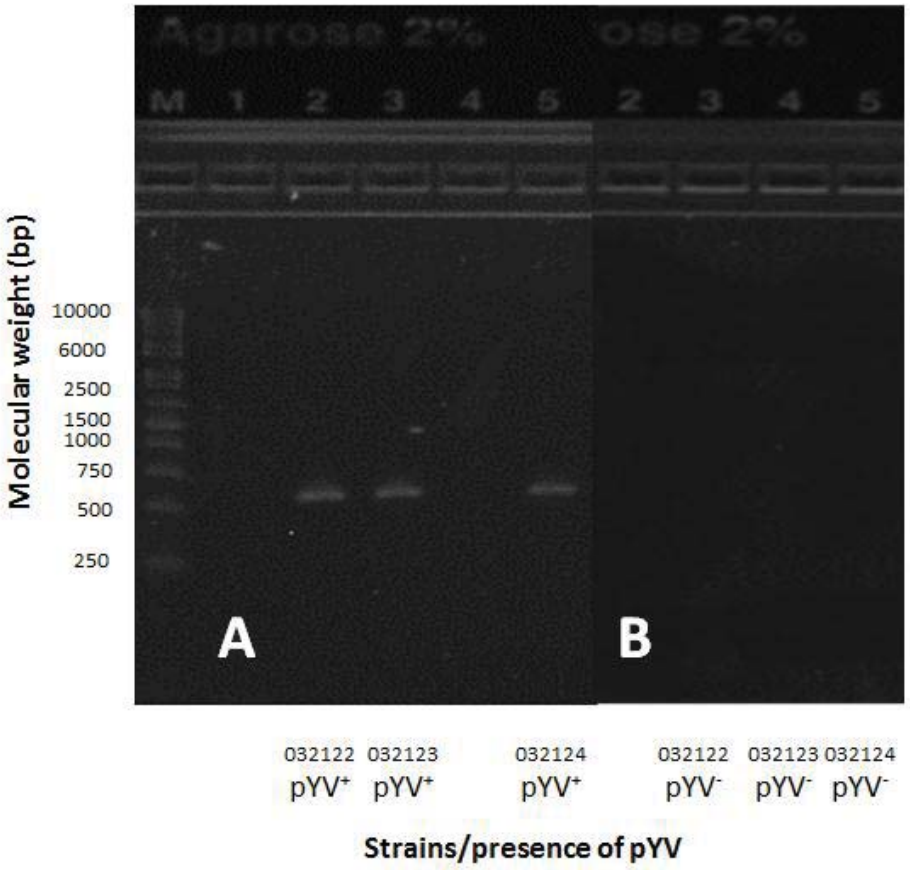

Figure 6.2. Digital photo of the gel. A- Lane2: PCR product of VirF gene from strain 032122, Lane3: PCR product of VirF gene from strain 032123, Lane5: PCR product of VirF gene from strain 032124; B- Lane2: PCR product of VirF gene from the pYV$^-$ mutant of strain 032122, Lane3: PCR product of VirF gene from the pYV$^-$ mutant of strain 032123, Lane5: PCR product of VirF gene from the pYV$^-$ mutant of strain 032124.

6.2.3 Biofilm formation of pYV$^+$ and pYV$^-$ *Y. enterocolitica* strain ERL032123 at room temperature and 37 °C in peptone water supplemented with Ca^{2+}

The biofilm formation of pYV$^+$ *Y. enterocolitica* and its pYV$^-$ counterpart were assessed when cells were grown at 24 °C and 37 °C in the presence of Ca^{2+}. Peptone water was prepared as described in 6.2.1 The Ca^{2+} concentration range tested was 1.5, 3, 4, 5, 7.5, 10, 20, 30, 40 and 50mM.

6.2.4 Statistical analysis

Each treatment in the microtiter plate assay was carried out in four replicates and the coupon microscopic observation in triplicates. Mean values ± 1 standard deviation are reported. T-test in Microsoft Excel software was used to analyse the data sets. Differences that were statistically significant appeared with a $p \leq 0.05$.

6.3 Results

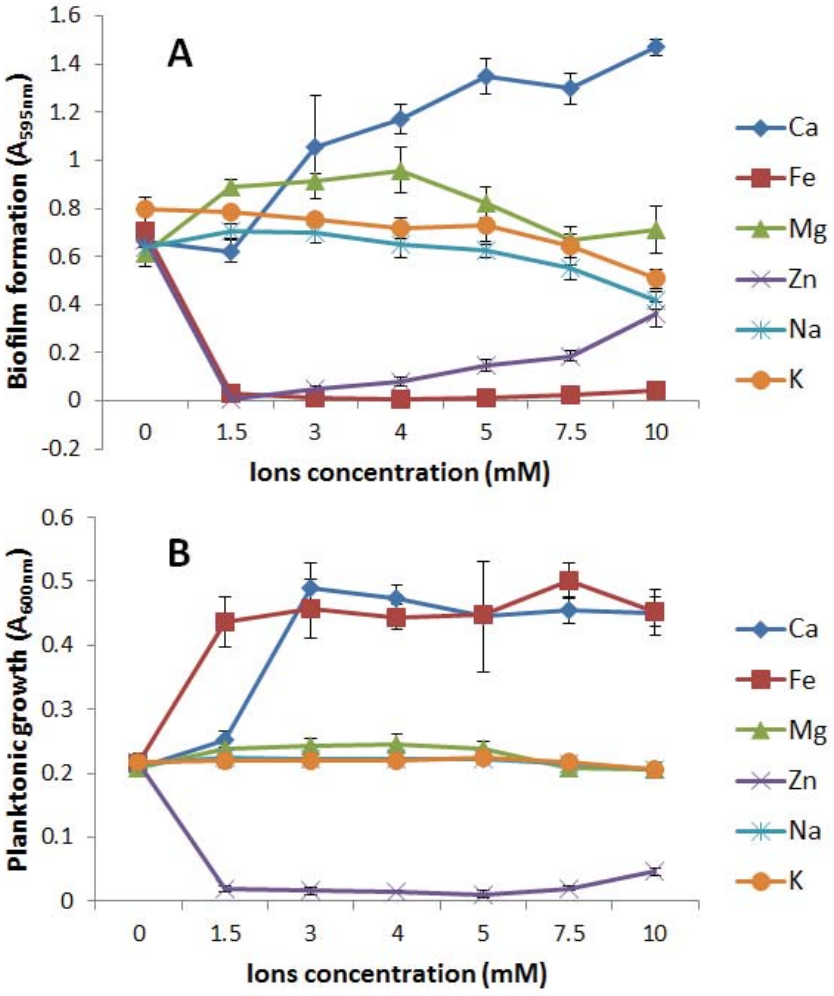

Figure 6.3. Biofilm formation (A) and planktonic growth (B) by *Y. enterocolitica* strain ERL032123 (pYV^+) at 24 °C in the presence of different ions and concentrations. Error bars represent mean ± standard deviation.

As shown in Figure 6.3 (Appendix 10), among the six ions tested, Ca^{2+} produced a large increase in biofilm formation at concentrations between 2-5 mM. The biofilm formation was slightly enhanced when Mg^{2+} was applied at the concentration range of 2-4 mM and gradually dropped to the original level when the Mg^{2+} concentration was increased further. Na^{+} and K^{+} had no influence on biofilm formation at the tested concentration range, while Zn^{2+} and Fe^{2+} inhibited biofilm formation. Planktonic growth increased in the presence of Ca^{2+} and Fe^{2+}, but Na^{+} and K^{+} had no effect on planktonic growth rate. In addition Zn^{2+} inhibited planktonic growth.

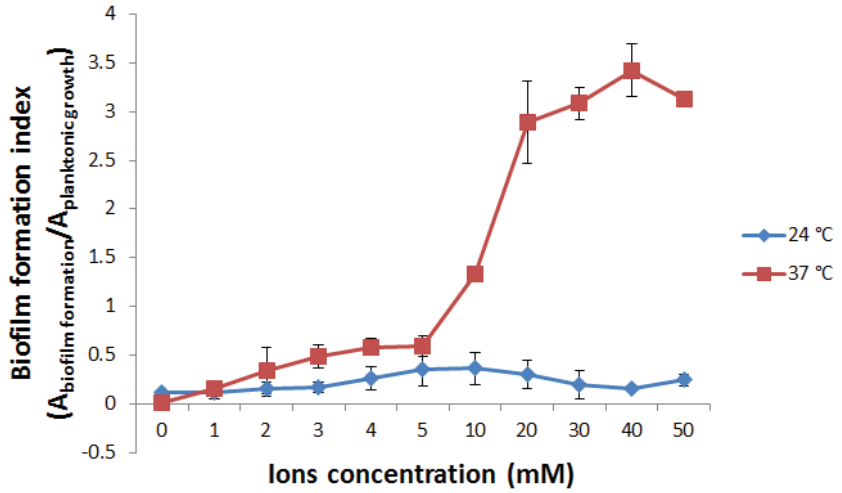

Figure 6.4. Biofilm formation index of *Y. enterocolitica* strain ERL032123 (pYV^{+}) at 24 °C.and 37 °C with the presence of Ca^{2+}. Error bars represent mean ± standard deviation.

Biofilm formation index was used to represent the level of biofilm formation to balance different cell growth at different Ca^{2+} conditions. The biofilm formation index is the ratio of the optical density of the biofilm formation and that of the cells growth in suspended culture. The effect of temperatures of 24° C and 37 °C on biofilm formation in the presence of Ca^{2+} produced very contrasting results (Figure 6.4, Appendix 11). When incubated at 24 °C, the biofilm formation reached the maximum level at about 5-10 mM and reduced at higher Ca^{2+} concentrations. However when the bacteria were incubated at 37 °C, the biofilm formation gradually increased before the

Ca^{2+} concentration reached 5 mM, and sharply increased at Ca^{2+} concentrations higher than 5 mM and maintain a similar level when Ca^{2+} concentrations were higher than 20 mM.

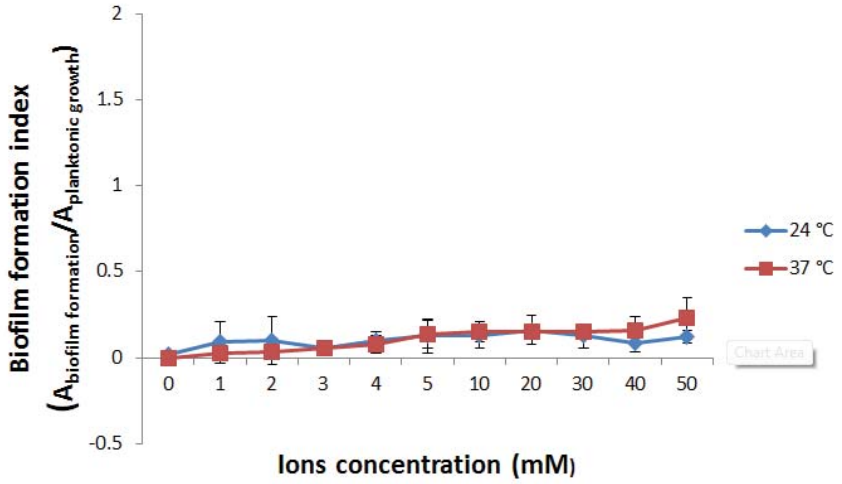

Figure 6.5. Biofilm formation index of *Y. enterocolitica* strain ERL032123 (pYV^-) at 24 °C and 37 °C at different concentrations of Ca^{2+}. Error bars represent mean ± standard deviation.

The loss of the plasmid was confirmed as shown in Figure 6.2. The pYV^- mutant lost the ability to form biofilm at both 24 °C and 37 °C regardless of the Ca^{2+} concentration (Figure 6.5, Appendix 12).

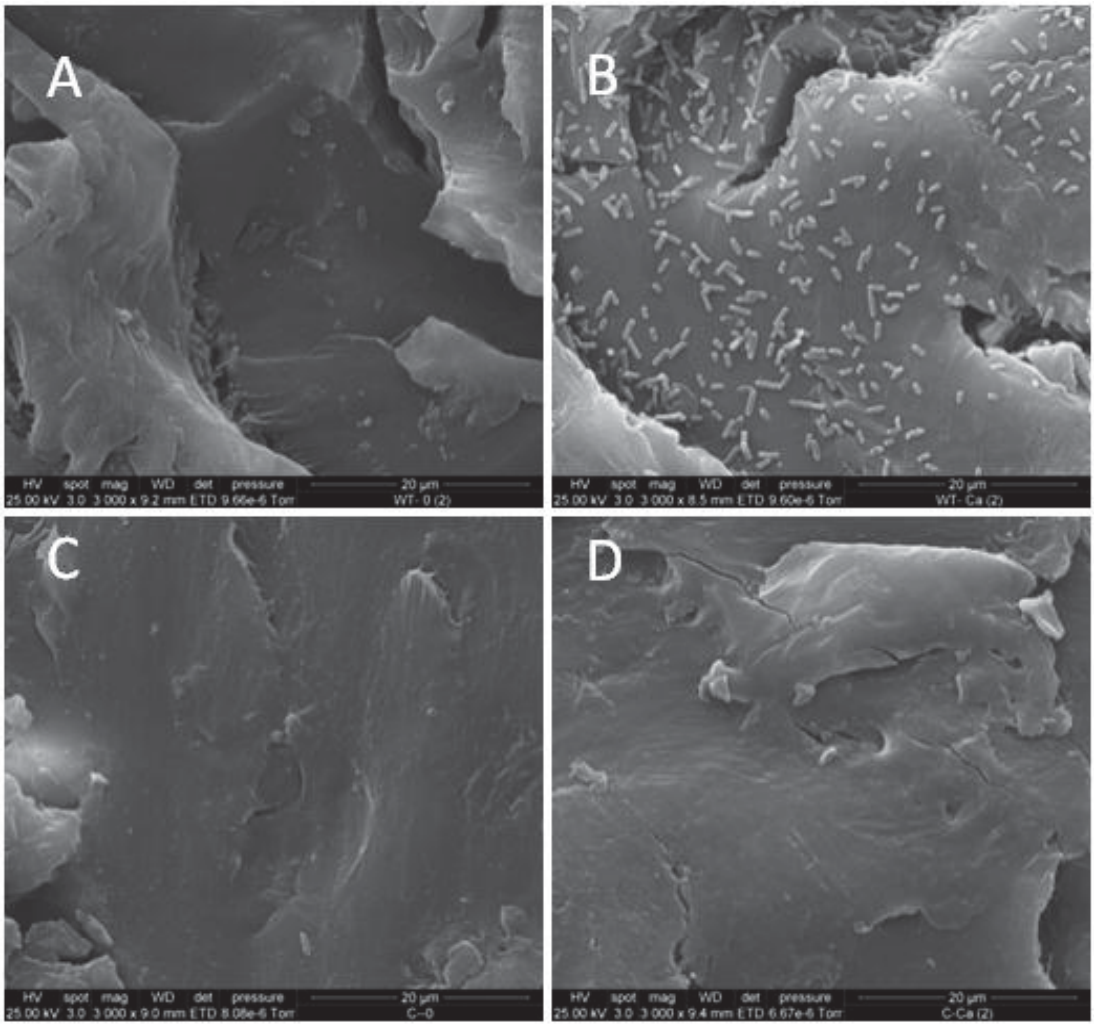

Figure 6.6. SEM microscopy of pYV^+ and pYV^- cells attached to coupons after incubation in the presence and absence of Ca^{2+}at 37 °C, (A)-pYV^+ without Ca^{2+} (B)-pYV^+ with Ca^{2+} (C)-pYV^- without Ca^{2+} (D)- pYV^- with Ca^{2+}. pYV^+ cells attach to surface in the presence of Ca^{2+} while pYV^- cells don't show attachment regardless the presence of Ca^{2+}.

The SEM microscopy agrees with the result from microplate assay with the plasmid positive bacteria in the presence of Ca^{2+} showing the most bacterial attachment (Figure 6.6).

6.4 Discussion

Both Ca^{2+} and Fe^{2+} facilitated planktonic growth of *Y. enterocolitica*. However, only Ca^{2+} enhanced biofilm formation whereas Fe^{2+} showed some inhibition of biofilm. This

implies that, apart from increasing planktonic growth, Ca^{2+} also has some physiological influence on biofilm formation by *Y. enterocolitica.* This Ca^{2+} response is much stronger at 37 °C than at 24 °C. In addition, the presence of the pYV plasmid is essential to this Ca^{2+} induced biofilm formation.

Most of the genes on the plasmid encode for the Type III Secretion System which represents an important virulence mechanism in various pathogenic bacteria (Cornelis et al., 1998). The hallmark of Type III Secretion Systems is a needle-like apparatus located on the bacteria cell wall (Galán and Wolf-Watz, 2006). By this structure, bacteria are able to inject bacterial effector proteins into the host cells to inhibit phagocytosis by macrophages and trigger apoptosis of the host cells (Galán and Wolf-Watz, 2006). The plasmid in *Y. enterocolitica* pYV also encodes for an adhesion potential that allows the bacteria to attach to the host cells which is an important virulence mechanism for *Y. enterocolitica* (Perregaard et al., 1991). This is the first report of this pYV plasmid being involved in adhesion and biofilm development on non-biological surfaces. This adhesion ability conferred by pYV is important for *Y. enterocolitica* to attach to surfaces, including those involved in food preparation such as meat slaughter plants, butchers and packaging companies, leading to biofilm formation. This enhanced attachment may also be important in the pathogenicity of a *Y. enterocolitica* infection, especially as the influence on attachment is most pronounced at 37 °C. Interestingly, this plasmid is differentially expressed at 37 °C (Cornelis et al., 1998), which may also correlate to the fact shown in my study that the increased biofilm formation with the presence of Ca^{2+} was only observed at 37 °C.

The observation about the physiological influence of Ca^{2+} on *Yersinia spp.* can be tracked back to the mid-1950s when *Yersinia pestis* was found to stop growing in Ca^{2+} - deprived media at 37 °C (Burrows and Bacon, 1956, Higuchi and Smith, 1961). This property of pathogenic *Yersinia spp.* can be lost after the loss of pYV - when pYV is lost the bacteria can now grow in low Ca^{2+} concentrations (Higuchi and Smith, 1961) It is believed that under the growth limiting conditions (37 °C and low Ca^{2+} concentration), the pYV is expressed and synthesize the Type III Secretion System proteins (Cornelis et

al., 1998). Later studies showed that this Ca^{2+} dependency phenotype and its correlation with the virulence plasmid also existed in *Yersinia. pseudotuberculosis* and *Y. enterocolitica* (Zink et al., 1980, Gemski et al., 1980). However, it is generally accepted that, *in vitro*, *Yersinia spp.* secrete Yops only in the absence of Ca^{2+}, Yops secretion is down-regulated in the presence of Ca^{2+}(Cornelis, 2002). With this trend, decreased adhesion ability would be expected with an increase in Ca^{2+} concentration in the extracellular medium. However, this hypothesis contradicts the observation in this study with increased Ca^{2+} increasing biofilm formation. To unravel the puzzle, the influence of pYV and the presence of Ca^{2+} have on cell surface characteristics (including surface charge, hydrophobicity and cell surface proteins), EPS content and gene expression was examined.

Chapter Seven. Investigation of Ca^{2+} induced, plasmid mediated biofilm formation by *Y. enterocolitica* at 37 °C

7.1 Introduction

Attachment is the initial and critical step for any biofilm formation (Palmer et al., 2007).It is generally believed that the adhesion process can be divided into two phases. The first phase is the initial reversible sorption step governed by physio-chemical interactions between the bacterium and the surface (Hermansson, 1999). In this phase, bacteria are seen as more or less inert colloidal particles without biological activities. The second phase is the irreversible absorption step in which biological processes like growth and phenotype adaptation occurs and the bacteria were transferred from an unbound state into a firmly attached state (Boks et al., 2008).

The physicochemical surface properties (e.g. surface charge, hydrophobicity and cell surface proteins) have been associated with the increased adhesion (the synonym of "adhesion", which is used to describe the adherence to other cells, while "attachment" usually means attachment to abiotic surfaces.) ability of many bacteria including other *Enterobacteriaceae* (Terada et al., 2012, Zhang et al., 2007, Sousa et al., 2009). These properties may also play an important role in modulating adherence of *Y. enterocolitica* as several cell surface adhesion proteins such as Inv, YadA and Ail, are present in virulent *Y. enterocolitica* (Bottone, 1997). EPS, which are biopolymers produced and secreted by microorganisms into their environment, are considered the "house" of biofilm cells, establishing functionally and structurally mature biofilms (Flemming and Wingender, 2010).

In this study, various factors normally associated with bacterial cells and biofilm formation were investigated including physical cell surface properties such as charge and hydrophobicity, cell surface proteins and the production and composition of EPS. Gene expression analysis aimed to provide further information about the role of pYV and Ca^{2+} in biofilm formation.

7.2 Procedures

For all experiments described below, bacteria were grown at 37 °C in casein soy digest medium and Ca^{2+} supplemented casein soy digest medium as described in 6.2.1.

7.2.1 Surface charge

According to the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory, the net interaction between a cell and a flat surface can be described as a balance between van der Waals interactions and the repulsive interactions from the overlap between the electrical double layer of the bacterial cell and the surface (Hermansson, 1999). van der Waals interactions are usually a constant when the radius of the cell and the distance between the cell and the surface remain constant, while the repulsive interaction can be related to the surface charge. Therefore, surface charge could be used to predict microbial adhesion. Although the correlation between surface charge and adhesion may not be straightforward due to the biological heterogeneity of different bacterial strains (Hermansson, 1999), the availability of isogenic pairs of *Y. enterocoltica* in this study could overcome this problem by giving a more consistent result. The method used for surface charge measurement is described in Section 3.8.2. The bacterial cells were diluted to OD600 0.5 in phosphate-citrate buffers of defined pH ranging from pH 2 to pH 8 (Table 3.2). The surface charge (Zeta potential) of bacterial cells in each buffer was measured by using in a zeta potential analyser (Zetasizer Nano Range, Malvern).

7.2.2 Hydrophobicity

Cell surface hydrophobicity represents another adhesion concept (Marshall and Cruickshank, 1973). Generally, hydrophobic bacteria prefer hydrophobic surfaces and hydrophilic bacteria prefer hydrophilic surfaces. The polystyrene surface used in my study is hydrophobic. Therefore in theory, bacteria with a higher surface hydrophobicity tend to attach better than the ones that are less hydrophobic. The hydrophobicity of each bacterial culture was measured at its respective isoelectric point by using HIC which is described in Section 3.8.1. The bacteria solution was passed through a Sepharose CL-4B filled column. Hydrophobic cells are retained on the

column and hydrophilic cells are released. Each column was loaded with 300 µl of bacteria culture and was held stationary for 15 min before eluting with 900 µl of the buffer. The absorbance of the bacteria loaded and eluted was measured at OD600 nm using a spectrophotometer. The percentage of bacteria retained in the resin column was calculated from the absorbance of a ¼ dilution of the loaded bacteria suspension and the absorbance of the eluted bacteria suspension.

7.2.3 Cell wall protein profile analysis

Cell surface structures are believed to be involved in bacterial adhesion (Ryder et al., 2007). From a physico-chemical point of view, some surface structures could extend out from the cell surface and bridge the energy barrier that separates the cell and the surface (Jucker et al., 1997). The tips of these structures have much smaller radius and therefore less repulsion than the cell itself. This character could help anchor the cell firmly to the surface. In this section, the profile of cell surface proteins of plasmid-bearing and plasmid-less cell culture incubated in the presence and absence of Ca^{2+} was analysed by electrophoresis. The protein extraction method and SDS-PAGE are described in Section 3.10.1 and 3.10.2. A stationary phase culture was harvested and lysed by sonication. Cell debris was removed from the lysate by quick centrifugation (12300×g at 4 °C for 2 min). The supernatant was then centrifuged at 12300×g at 4 °C for 30 min. The pellet was incubated in Triton-X100 containing extraction buffer and the membrane proteins which are insoluble in the extraction buffer were collected by centrifugation at 12300×g, 4 °C for 30 min, finally the protein pellet was re-suspended in sample buffer (Table 3.3) and aliquoted to small portions before being stored at -80 °C.

7.2.4 EPS analysis

After irreversible adhesion, bacteria will secrete bio-polymers which help glue the cell and its daughter cells onto the surface facilitating biofilm maturation (Flemming and Wingender, 2010). EPS are mostly composed of polysaccharides and proteins, whereas in some cases, they also comprise of a surprising amount of eDNA (Flemming et al., 2007). In this section, the content of carbohydrate and nucleic acid in EPS was

compared within the plasmid-bearing and plasmid-less cell cultures incubated in the presence and absence of Ca^{2+}. The EPS extraction and the quantitative analysis methods are described in Section 3.9.1 and Section 3.9.2. Planktonic cultures were fixed by formaldehyde before extracted by NaOH solution. After 3 hrs extraction, bacteria suspensions were then centrifuged to remove bacteria cells. Absolute ethanol was added to the supernatant to precipitate EPS. EPS was harvested by centrifugation and quantified by the phenol sulfuric acid method as described in section 3.9.2.

7.2.5 Gene expression analysis by RNA sequencing

The gene expression patterns in the following three conditions were analysed by RNA sequencing- the plasmid-bearing cells in the absence of Ca^{2+} (Sample 1), the plasmid-bearing cells in the presence of Ca^{2+} (Sample 2), and the plasmid-less cells in the presence of Ca^{2+} (Sample 3). The sequence data was mapped to ASM25317v1 assembly for *Y. enterocolitica subsp. palearctica Y11.* The differential expression between Samples 1 and Sample 2 and between Samples 2 and Sample 3 were analysed aiming to find possible clues related to the Ca^{2+} effect at a transcriptional level.

7.2.6 Statistical analysis

The measurement of Zeta potential, hydrophobicity, polysaccharide and nucleic acid concentration in EPS was carried out in four replicates and the coupon microscopic observation in triplicates. Mean values ± 1 standard deviation are reported. T-test in Microsoft Excel software was used to analyse the data sets. Differences that were statistically significant appeared with a $p \leq 0.05$. SDS-PAGE was performed twice, separately in time.

7.3 Results

7.3.1 Surface charge

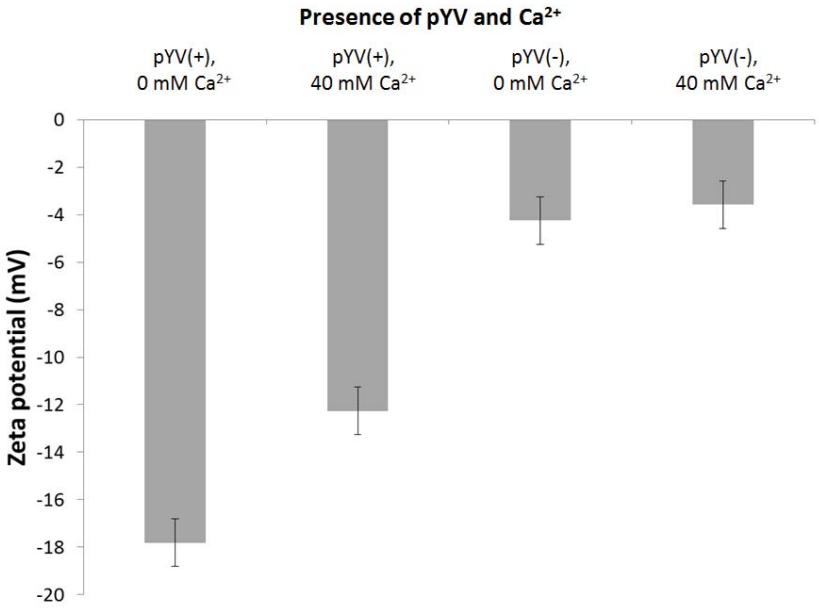

Figure 7.1. Zeta potential of pYV^+ and pYV^- cells at pH 7 after incubation in the presence and absence of Ca^{2+}at 37 °C. Error bars represent mean ± standard deviation.

Figure 7.1 (Appendix 13) shows the surface charge results at pH 6. The pYV^+ cells have a greater negative Zeta potential than the pYV^- cells (p=0.046). The addition of Ca^{2+} decreased the Zeta potential of pYV^+ cells (p=0.011) but demonstrated no effect on that of pYV^- cells (p=0.07).

7.3.2 Hydrophobicity

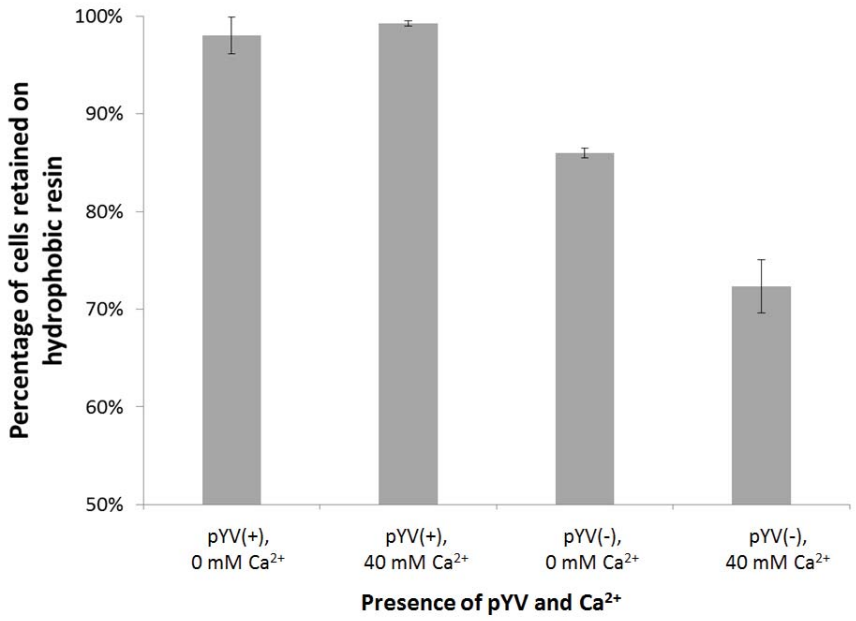

Figure 7.2. Hydrophobicity of pYV^+ and pYV^- cells after incubation in the presence and absence of Ca^{2+} at 37 °C. Error bars represent mean ± standard deviation.

Figure 7.2 (Appendix 14) shows the hydrophobicity of the four cultures at their respective isoelectric point. The pYV^+ cells showed higher hydrophobicity than the pYV^- cells regardless of the growth conditions (p=0.002). There was a moderate reduction in hydrophobicity of the pYV^- cells in the presence of added Ca^{2+} (p=0.008). A previous study (Lachica and Zink, 1984) show a moderate reduction in cell surface hydrophobicity of the pYV^+ cells when grown in the presence of added Ca^{2+}, on the other hand, pYV^- cells show less hydrophobicity regardless of growth conditions. My study presented a similar result.

7.3.3 Profile of cell wall proteins

As shown in Figure 7.3, one band at 45kD-56kD was observed in the pYV^+ cell wall protein mixture but not in that of the pYV^- cells. The addition of Ca^{2+} had little effect on the profile of cell wall proteins for both strains.

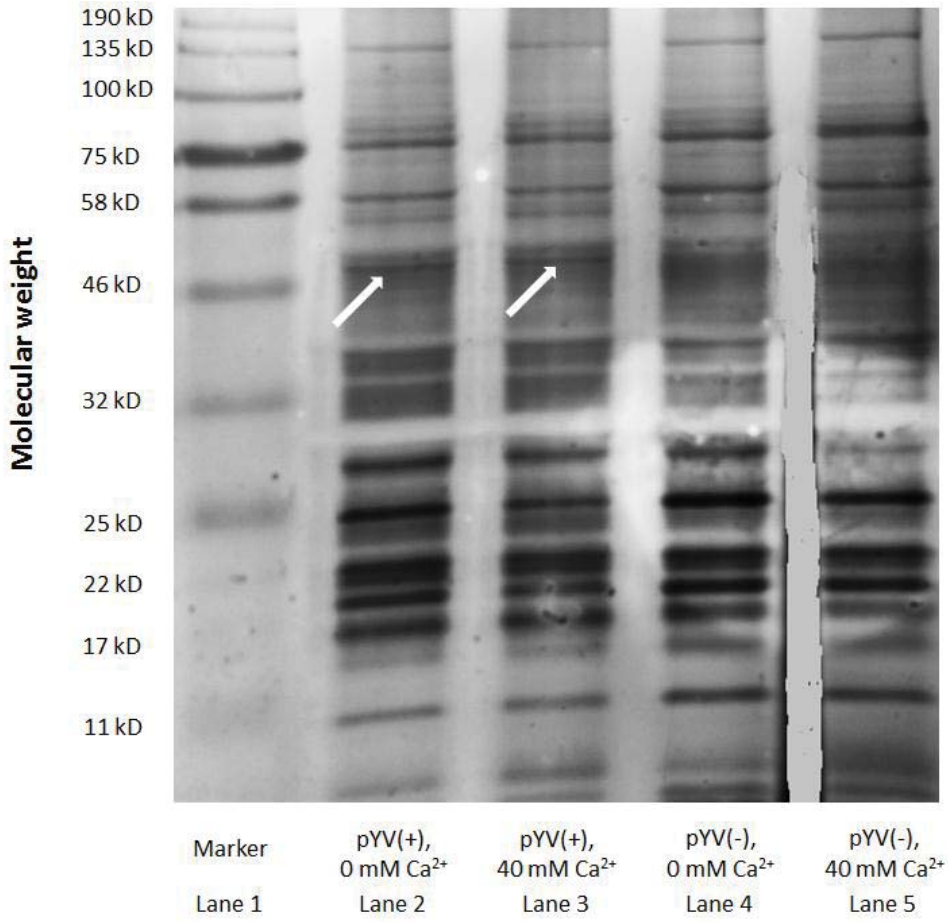

Figure 7.3. Cell wall protein profiles of pYV^+ and pYV^- cells after incubation in the presence and absence of Ca^{2+} at 37 °C by SDS-PAGE, the white arrows show the bands differentially expressed in pYV^+ cells.

7.3.4 Exopolysaccharide content in EPS

The amount of polysaccharide in EPS recovered from plasmid bearing cells was 0.99 mg $\pm$0.08 mg/10^{10} CFU when incubated without Ca^{2+} and 1.04 mg $\pm$0.06 mg/10^{10} CFU when incubated in the presence of Ca^{2+} (p=0.905). The carbohydrate production decreased with the loss of plasmid to 0.47 mg $\pm$0.05 mg/10^{10} CFU and 0.54 mg $\pm$0.01 mg/10^{10} CFU with the absence (p=0.112) and presence (p=0.005) of Ca^{2+} respectively (Figure 7.4, Appendix 15).

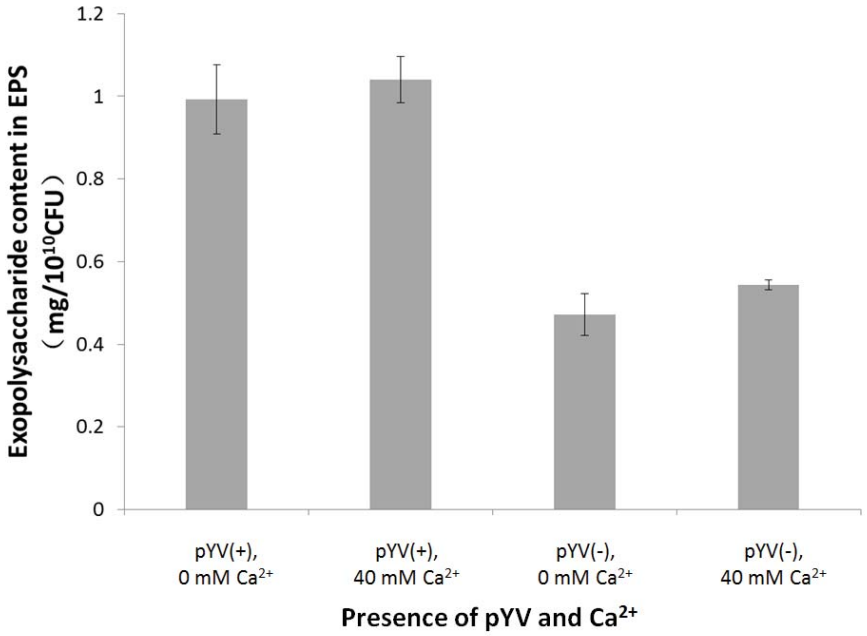

Figure 7.4. Exopolysaccharide content in the EPS of pYV^+ and pYV^- cell cultures after incubation in the presence and absence of Ca^{2+} at 37 °C. Error bars represent mean ± standard deviation.

7.3.5 Nucleic acid content in EPS

The amount of nucleic acid in EPS recovered from pYV^+ cells was 0.71 mg/10^{10} CFU ± 0.002 mg/10^{10} CFU when incubated without Ca^{2+} and increased to 1.25 mg/10^{10}CFU ± 0.008 mg/10^{10} CFU when incubated in the presence of Ca^{2+} (p=0.02). The values for pYV^- cells were 0.58 mg/10^{10}CFU ±0.002 mg/10^{10} CFU and 0.89 mg /10^{10} CFU ±0.017 mg/10^{10} CFU with the absence and presence of Ca^{2+} respectively (p=0.076) (Figure 7.5, Appendix 16).

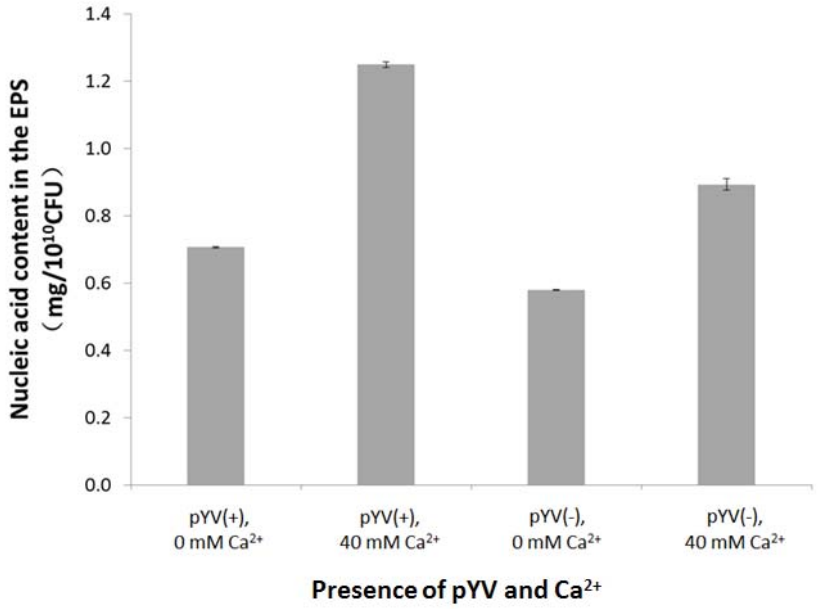

Figure 7.5. Nucleic acid content in the EPS of pYV^+ and pYV^- cell cultures after incubation in the presence and absence of Ca^{2+} at 37 °C. Error bars represent mean ± standard deviation.

7.3.6 Gene expression

The differential gene expressions analysis (where the genes are tested individually for expression differences between conditions) between Samples one and two (the plasmid bearing cells incubated in the absence or presence of 40mM Ca^{2+}) showed that on the genome, about 1500 genes were up-regulated and 1700 were down regulated. It was notable that there was a general trend of up-regulation of the genes on pYV with the addition of Ca^{2+} with a few exceptions (Table 7.1). The comparison between Samples two and three (plasmid bearing and plasmid negative cell incubated in the presence of Ca^{2+}) showed that 740 genes were up-regulated and 1100 genes were down-regulated with the loss of pYV. It is worth noting that a putative capsular polysaccharide transport protein YegH was down-regulated with the loss of pYV indicating that less effective transportation might be responsible for the decreased exopolysaccharide production with the loss of pYV.

Table 7.1. Differential expressions of the genes on pYV after addition of Ca^{2+}; green shows the up-regulated genes, red shows the down-regulated genes.

Gene location	Annotation	Without Ca2+	With Ca2+	Fold Change	p-value
gene:Y11_p0411	description:chromosome (plasmid) partitioning protein ParA	144.141	848.547	5.887	0.026
gene:Y11_p0891	description:type III secretion host injection protein (YopB)	126.345	609.155	4.821	0.042
gene:Y11_p0881	description:type III secretion chaperone protein for YopD (SycD)	61.393	259.622	4.229	0.066
gene:Y11_p0871	description:type III secretion cytoplasmic LcrG inhibitor (LcrV,secretion and targeting control protein, V antigen)	151.259	588.925	3.893	0.066
gene:Y11_p0601	description:type III secretion effector protein (YopR,encoded by YscH)	33.811	152.851	4.521	0.070
gene:Y11_p0571	description:type III secretion cytoplasmic protein (YscK)	5.339	43.832	8.211	0.071
gene:Y11_p0431	description:hypothetical protein	2.669	29.221	10.947	0.086
gene:Y11_p0421	description:chromosome (plasmid) partitioning protein ParB	70.291	254.002	3.614	0.091
gene:Y11_p0931	description:type III secretion possible injected virulence protein (YopM) / internalin, putative	13.346	67.434	5.053	0.092
gene:Y11_p0581	description:type III secretion bridge between inner and outermembrane lipoprotein (YscJ,HrcJ,EscJ, PscJ)	20.464	89.912	4.394	0.096
gene:Y11_p0761	description:type III secretion protein (YscP)	225.108	730.537	3.245	0.097
gene:Y11_p0051	description:type III secretion injected virulence protein (YopP,YopJ, induces apoptosis, prevents cytokine induction,inhibits NFkb activation)	3.559	30.345	8.526	0.101
gene:Y11_p0751	description:type III secretion	67.621	233.772	3.457	0.102

	inner membrane protein (YscQ,homologous to flagellar export components)				
gene:Y11_p0091	description:type III secretion injected virulence protein (EC 3.4.22.-,YopT,cysteine protease,depolymerizes actin filaments of cytoskeleton,causes cytotoxicity)	189.518	590.049	3.113	0.108
gene:Y11_p0611	description:type III secretion spans bacterial envelope protein (YscG)	40.929	144.983	3.542	0.113
gene:Y11_p0561	description:type III secretion cytoplasmic protein (YscL)	15.126	66.310	4.384	0.115
gene:Y11_p0831	description:required for Yop secretion	24.913	91.036	3.654	0.129
gene:Y11_p0031	description:type III secretion injected virulence protein (YopO,YpkA,serine-threonine kinase)	259.809	732.785	2.820	0.132
gene:Y11_p0821	description:putative type III secretion protein YscX	56.944	177.577	3.118	0.135
gene:Y11_p0741	description:type III secretion inner membrane protein (YscR,SpaR,HrcR,EscR,homologous to flagellar export components)	56.055	166.338	2.967	0.151
gene:Y11_p0811	description:putative type III secretion protein SycN	85.417	241.639	2.829	0.152
gene:Y11_p0591	description:type III secretion cytoplasmic protein (YscI)	17.795	65.186	3.663	0.153
gene:Y11_p0861	description:type III secretion cytoplasmic plug protein (LcrG)	95.204	265.241	2.786	0.154
gene:Y11_p0141	description:transposase	0.890	14.611	16.421	0.158
gene:Y11_p0841	description:type III secretion inner membrane channel protein (LcrD,HrcV,EscV,SsaV)	224.219	577.686	2.576	0.164
gene:Y11_p0641	description:type III secretion inner membrane protein (YscD,homologous to flagellar export components)	42.708	124.753	2.921	0.170
gene:Y11_p0771	description:type III secretion spans bacterial envelope protein (YscO)	105.881	277.604	2.622	0.173
gene:Y11_p0851	description:type III secretion low calcium response protein (LcrR)	21.354	68.558	3.211	0.183

gene:Y11_p0681	description:hypothetical protein	8.008	33.717	4.211	0.190
gene:Y11_p0671	description:type III secretion chaperone protein for YopN (SycN,YscB)	28.472	84.293	2.961	0.191
gene:Y11_p0101	description:type III secretion chaperone protein for YopT (SycT)	11.567	42.708	3.692	0.192
gene:Y11_p0901	description:type III secretion host injection and negative regulator protein (YopD)	304.297	714.802	2.349	0.198
gene:Y11_p1091	description:replication protein	43.598	113.514	2.604	0.216
gene:Y11_p0781	description:type III secretion cytoplasmic ATP synthase (EC 3.6.3.14, YscN,SpaL,MxiB,HrcN,EscN)	613.932	1356.551	2.210	0.220
gene:Y11_p0801	description:type III secretion outermembrane negative regulator of secretion (TyeA)	96.094	224.781	2.339	0.227
gene:Y11_p0491	description:transposase and inactivated derivatives	72.960	173.081	2.372	0.231
gene:Y11_p0711	description:type III secretion inner membrane protein (YscU,SpaS,EscU,HrcU,SsaU, homologous to flagellar export components)	54.275	131.497	2.423	0.236
gene:Y11_p0211	description:hypothetical protein	2.669	15.735	5.895	0.243
gene:Y11_p0981	description:hypothetical protein	11.567	1.124	0.097	0.244
gene:Y11_p0261	description:transposon resolvase	64.952	150.603	2.319	0.249
gene:Y11_p0621	description:type III secretion cytoplasmic protein (YscF)	24.913	64.062	2.571	0.266
gene:Y11_p0551	description:type III secretion negative regulator of effector production protein (LcrQ,YscM, YscM1 and YscM2)	72.960	159.594	2.187	0.275
gene:Y11_p0021	description:hypothetical protein	53.385	120.258	2.253	0.276
gene:Y11_p0661	description:type III secretion outermembrane pore forming protein (YscC,MxiD,HrcC, InvG)	125.456	260.745	2.078	0.282
gene:Y11_p0791	description:type III secretion outermembrane contact sensing protein(yopN,Yop4b,LcrE)	389.713	776.617	1.993	0.282

gene:Y11_p0441	description:putative transposase remnant	23.134	55.071	2.381	0.316
gene:Y11_p0221	description: adhesin (YadA)	30.252	66.310	2.192	0.338
gene:Y11_p0631	description:type III secretion protein (YscE)	9.787	25.850	2.641	0.370
gene:Y11_p0541	description:type III secretion injected virulence protein (YopH,tyrosine phosphatase of FAK and p130cas, prevents phagocytosis)	242.014	428.207	1.769	0.373
gene:Y11_p0131	description:transposase	0.000	4.496	4.496	0.386
gene:Y11_p0081	description:type III secretion negative modulator of injection (YopK,YopQ,controls size of translocator pore)	97.873	176.453	1.803	0.389
gene:Y11_p0071	description:putative transposase	49.826	91.036	1.827	0.420
gene:Y11_p0471	description:type III secretion injected virulence protein (YopE)	1226.08	748.519	0.610	0.421
gene:Y11_p0451	description:Orf73 (o91); similar to gi|1806202	133.463	76.425	0.573	0.428
gene:Y11_p0731	description:type III secretion inner membrane protein (YscS,homologous to flagellar export components)	22.244	43.832	1.971	0.442
gene:Y11_p0921	description:hypothetical protein	0.890	5.620	6.316	0.447
gene:Y11_p0201	description:transposase	0.000	3.372	3.372	0.455
gene:Y11_p0721	description:type III secretion inner membrane protein (YscT,HrcT,SpaR,EscT,EpaR1,homologous to flagellar export components)	47.157	82.045	1.740	0.463
gene:Y11_p0301	description:arsenic resistance protein ArsH	8.898	20.230	2.274	0.472
gene:Y11_p0331	description:transposase	2.669	8.991	3.368	0.474
gene:Y11_p0281	description:arsenic efflux pump protein	10.677	22.478	2.105	0.493
gene:Y11_p0391	description:putative integrase protein	0.890	4.496	5.053	0.525
gene:Y11_p0911	description:ISSod5, transposase	0.890	4.496	5.053	0.525
gene:Y11_p1021	description:COG1112: Superfamily I DNA and RNA	0.890	4.496	5.053	0.525

	helicases and helicase subunits				
gene:Y11_p0111	description:ORF61 (o129)	0.000	2.248	2.248	0.544
gene:Y11_p0941	description:hypothetical protein	0.000	2.248	2.248	0.544
gene:Y11_p0171	description:type III secretion negative regulator of effector production protein (LcrQ,YscM, YscM1 and YscM2)	92.535	139.364	1.506	0.548
gene:Y11_p0061	description:IncF plasmid conjugative transfer surface exclusion protein TraT	56.944	87.664	1.539	0.555
gene:Y11_p0191	description:transposase for transposon Tn3	85.417	123.629	1.447	0.591
gene:Y11_p1031	description:IncF plasmid conjugative transfer pilin acetylase TraX	1.780	0.000	0.000	0.601
gene:Y11_p0461	description:ParE toxin protein	213.542	158.470	0.742	0.644
gene:Y11_p0991	description:transposase	7.118	3.372	0.474	0.662
gene:Y11_p0121	description:ORF61 (o129)	0.890	0.000	0.000	0.680
gene:Y11_p0961	description:hypothetical protein	6.228	3.372	0.541	0.727
gene:Y11_p1081	description:replication protein	29.362	21.354	0.727	0.731
gene:Y11_p0011	description:transposase and inactivated derivatives	4.449	2.248	0.505	0.742
gene:Y11_p0971	description:Orf60 (f139)	2.669	1.124	0.421	0.752
gene:Y11_p0181	description:transposase for transposon Tn3	90.755	80.921	0.892	0.870
gene:Y11_p0161	description:transposase	1.780	1.124	0.632	0.878
gene:Y11_p0241	description:hypothetical protein	1.780	1.124	0.632	0.878
gene:Y11_p0251	description:transposase	1.780	1.124	0.632	0.878
gene:Y11_p0001	description:ISPsy4, transposition helper protein	2.669	2.248	0.842	0.941

7.4 Discussion

In this study, the intrinsic mechanism was investigated by analysing various factors normally associated with biofilm formation.

Bacteria are usually negatively charged at neutral pH(Hermansson, 1999). In my study, the pYV^+ strain was shown to have lower Zeta potential than the pYV^- strain at pH6. The addition of Ca^{2+} decreased the negative surface charge of pYV^+ cells but had no influence on the charge of the pYV^- cells. This observation partially agrees with a previous study (Lachica and Zink, 1984) in that pYV^+ cells have a higher negative charge than pYV^- cells. But in this study, Ca^{2+} increased instead of decreased the surface charge of pYV^+ cells. This difference might be due to the growth medium or strain used in the study resulting in different cell surface properties. More negatively charged pYV^+ cells would be expected to be more repulsive to surfaces. However, the immediate attachment of pYV^+ strains is higher than that of pYV^- strains regardless of Ca^{2+} addition (data is shown in Appendix 17). This could be explained in several ways. Firstly, the surface charge was measured by measuring electrophoretic motility of bacteria which only gives an overall surface potential. However, there might be localized positively charged areas on the cell surface which accounts for the higher adhesion (Weiss and Harlos, 1972). Secondly, among the factors that are believed to have an impact on cell adhesion, surface charge may not be the key one. Another study showed that the effect of surface charge was less important for the adhesion of hydrophobic cells than hydrophilic cells (Van Loosdrecht et al., 1987). Considering that the cell surfaces of the four cultures used in the present trial are all hydrophobic, the cell surface hydrophobicity compared to surface charge might be more substantial for irreversible adhesion. Another consideration is the presence of organic matter in the growing media that may form a conditioning layer on the substrate and bacterial cell surface which in turn modifies the surface properties and may influence the bacterial attachment.

pYV^+ cells produced more polysaccharide in their EPS than pYV^- cells indicating that pYV is important for the production and/or delivery of exopolysaccharide. This observation agreed with the RNA sequencing result that a putative capsular polysaccharide transport protein YegH was down-regulated with the loss of pYV. Plasmid-linked exopolysaccharide phenotypes are also reported in other bacteria (Kranenburg et al., 1997, Peiris and Dlamini, 1999). However, difference is the

polysaccharide is encoded by plasmid in these bacteria while no polysaccharide related genes on pYV was identified. A possible explanation might be that a certain protein on pYV with unknown function is involved in the polysaccharide synthesis or a transportation pathway instead of directly synthesizing polysaccharide. The Ca^{2+} addition didn't significantly influence the level of exopolysaccharide produced. A similar result was observed in a biofilm study of *X. fastidiosa* (Cruz et al., 2012). However, Ca^{2+} was shown to increase the production of alginate (a kind of extracellular polysaccharide) in *P. aeruginosa* which contributed to stronger biofilm architecture (Körstgens et al., 2001). Therefore, the mechanism of Ca^{2+} induced biofilm formation may vary between different species.

The amount of nucleic acid in the EPS was increased by the addition of Ca^{2+} with both pYV^{+} and pYV^{-} cells. It has been shown that eDNA is one of the important components in the biofilm matrix of many bacteria (Flemming et al., 2007). A study was carried out to investigate the influence of Ca^{2+} on eDNA release and biofilm formation of a variety of bacteria including *P. aeruginosa*, *Aeromonas hydrophilla, E. coli, S. aureus*, *S. epidermidis*, *E. faecalis* (Das et al., 2014). The results showed that the presence of eDNA itself favours bacterial aggregation and Ca^{2+} further increases the aggregation via cationic bridging, however, Ca^{2+} had no influence on eDNA release. The present study is the first to show that Ca^{2+} increases eDNA production.

Genes on the pYV plasmid can be divided into several categories based on their function. Most importantly, pYV encodes for a set of proteins called Yops which were initially described as outer membrane proteins but later found to be secreted proteins essential for virulence (Cornelis et al., 1998). In addition, a specific apparatus called Ysc (for Yop secretion) is required by the secretion of Yops (Michiels et al., 1990). Apart from Ysc, Yops secretion also requires chaperons called Syc (for "specific Yop chaperone"), different Yop has its own specific Syc (Wattiau et al., 1994). It is worth noting that, being separately located from other groups of genes, a gene called *yadA* encodes for an adhesin. The adhesive ability of YadA is essential for virulence (Cornelis et al., 1998). Besides the virulence related function, the pYV of certain stains of *Y.*

enterocolitica also contains an *ars* transposon which confers arsenite and arsenate resistance (Neyt et al., 1997). This *ars* transposon will be discussed more in Chapter Eight. The rest of the genes are responsible for replication and partitioning of the plasmid during bacteria multiplication (Vanooteghem and Cornelis, 1990).

YadA which has already been shown to be an adhesin to host cells may also have potential for attachment to abiotic surfaces. The cell wall protein analysis in this study showed a protein band differentially expressed in pYV^+ cells with a molecular weight of 45kD-56kD which is suspected to be YadA. The presence of this protein on the pYV^+ cell surface might be related to the higher hydrophobicity compare to pYV^+ cells. Apart from YadA, certain Ysc proteins (YscC, YscD, YscR, YscU, YscV) which are located on the outer or inner membrane of the cell wall could also potentially facilitate bacteria attachment (Cornelis et al., 1998). The Yops have been shown to be only secreted under Ca^{2+} deprived conditions at 37 °C and the presence of Ca^{2+} inhibited Yops secretion (Cornelis, 2002). However, YopN is an exception because it can be detected in both the absence and presence of Ca^{2+} (Cornelis et al., 1998, Forsberg et al., 1991). It was suggested that YopN is a putative plug closing the secretion channel (Forsberg et al., 1991). Under low Ca^{2+} conditions, it's released to the culture supernatant, while in the presence of $Ca^{2+}$the protein is not released but exposed at the cell surface (Forsberg et al., 1991) which may block the secretion channel but favour bacteria attachment. Apart from Yops, the expression of other proteins on the pYV might change in without the influence of Ca^{2+}. For example, it's already known that the production of YadA is independent of Ca^{2+} concentration (Kapperud et al., 1985). The membrane located Ysc proteins might be independent of Ca^{2+} concentration too as there is evidence showing that Yops and Ysc might be regulated by different regulatory network (Michiels et al., 1991, Rouvroit et al., 1992). In addition, there is no clear differential expression pattern between the absence and presence of Ca^{2+} shown by the cell wall protein analysis in this study.

RNA-seq was conducted to identify changes in gene (especially genes on pYV) expression in *Y. enterocolitica* grown in medium supplemented with Ca^{2+}. Results show

that most of the genes on the pYV were up-regulated after the addition of Ca^{2+} with a few exceptions although the fold change (a measure describing how much a quantity changes going from an initial to a final value, it is calculated as the ratio of the final value and the initial value. When a gene is up-regulated, fold change is greater than one. When a gene is down-regulated, fold change is less than one) is not very high (Table 7.1). Ca^{2+} stimulation of T3SS gene expression was reported in *V. parahaemolyticus* (Gode-Potratz et al., 2010). In addition, some of the RNA-sequencing results in the present study are in line with the phenotype observation made in the present or other studies. For example, the transcription of a putative capsular polysaccharide transport protein YegH was down-regulated with the loss of pYV which could possibly explain the decrease in exopolysaccharide in Section 7.3.4. The transcription of YopE, one of the important effectors, is down-regulated which agrees with the reported trend (effector proteins are down-regulated at protein level with the presence of Ca^{2+}). However, the up-regulation or down-regulation trend cannot be seen in SDS-PAGE analysis which might be due to the low sensitivity of SDS-PAGE to detect minor differential expression. In addition, as no replication was done for the RNA-sequencing due to limited sample number that can be processed by the Illumina MiSeq Sequencing System and funding, further confirmation is needed to confirm the genes of interest.

Based on the experimental results so far, several hypotheses could be proposed to explain the fact that both pYV and Ca^{2+} are needed for biofilm formation of *Y. enterocolitica.* Firstly, the plasmid encoded cell surface structures (certain proteins such as YadA and/or exopolysaccharide) are required but not enough for biofilm formation and Ca^{2+} may function as a bridging agent to cross link these structures to surfaces as shown in other studies. Many bacterial exopolysaccharides contain negatively charged uronic acid residues that bind Ca^{2+} ions by ionic interactions allowing cross-linking and gelling of exopolysaccharide (Körstgens et al., 2001). Secondly, the plasmid encoded cell surface structures may be sufficient to provide the ability for firm attachment. The role of Ca^{2+} is to promote the planktonic growth of *Y. enterocolitica* that may result in a stronger biofilm colonizing on surfaces. Another

possibility is that exopolysaccride and eDNA are both needed for biofilm formation of *Y. enterocolitica.* A study showed that exopolysaccride and eDNA can form a web which facilitates biofilm formation by *P. aeruginosa* (Wang et al., 2015). Therefore, the pYV linked exopolysaccride production and Ca^{2+} induced increase in eDNA release may end up contributing to biofilm formation of *Y. enterocolitica*. A reverse experiment with enzyme treatment (such as protease and DNAse) is needed to confirm the involvement of certain cell surface structures on biofilm formation of *Y. enterocolitica.*

Chapter Eight. Final discussion and conclusions

Although the biofilm formation by *Y. enterocolitica* has been evaluated previously using lab media (Kim et al., 2008, Raczkowska et al., 2011), the present study is the first report of *Y. enterocolitica* biofilm formation in a simulated pork processing environment. *Y. enterocolitica* formed much stronger biofilm in pork meat juice than in lab media (TSB). A similar phenomenon was observed with *S. enterica* which produced more biofilm in meat juice than in TSB (Wang et al., 2013). Furthermore, this study showed that biofilm formed in pork meat juice demonstrated higher resistance to physical washing and starvation as well as sanitization treatment than that in TSB which may be due to the biofilm structure. This speculation is supported by a confocal laser scanning microscopy investigation of *S. enterica* biofilm showing that *Salmonella spp.* biofilms grown in a meat thawing-loss broth showed a cloud shaped morphology, whereas it appeared reticulum shaped when grown in TSB (Wang et al., 2013). The results from sanitizer treatment showed that QAC was less effective than sodium hydroxide in removing *Y. enterocolitica* biofilm therefore the right cleaning agent needs to be chosen for effective cleaning of *Y. enterocolitica* biofilm. In addition, this study is the first to suggest that biotype 4 of *Y. enterocolitica,* which is most commonly found from porcine sources (Gütler et al., 2005), formed the strongest biofilm in pork meat juice compared with other biotypes. This suggests that *Y. enterocolitica* biotype 4 may have become adapted to its living environment by forming biofilms, one such environment is the pork processing plant, which could lead to increased risk of *Y. enterocolitica* contaminating processed pork products. A study of *Pseudomonas spp.* isolates supports this hypothesis showing that biofilm formation by meat associated isolates are more common than environmental isolates (Robertson et al., 2013)

Two studies on the molecular mechanism of biofilm formation by *Y. enterocolitica* biotype 1B and biotype 2 showed that motility played a critical role in biofilm formation (Raczkowska et al., 2011, Kim et al., 2008). Therefore, the motility of all 16 strains used in this study was tested in a preliminary experiment (data shown in Appendix 18). Five out of the six strains (3 biotype 1A, 2 biotype 3) forming biofilm in

TSB and showed motility in semi-solid TSA with one biotype 1A exception. However, four motile strains (2 x biotype 1A, 1 x biotype 2 and 1 x biotype 4) didn't form biofilm in TSB. These results showed that the correlation between motility and biofilm formation seems to be strain specific, rather than biotype specific. In addition, the above two studies also implied different molecular regulatory mechanisms for biofilm formation between *Y. enterocolitica* biotype 1B and biotype 2, because OmpR, a transcriptional response regulator controlling the expression of a wide range of genes in *Enterobacteriaceae*, was shown to be important to the biofilm formation of *Y. enterocolitica* biotype 2 while dispensable for that of biotype 1B (Raczkowska et al., 2011). Therefore, as a highly heterogeneous species, the mechanism of biofilm formation of different biotypes might differ.

In this study, pork meat juice was shown to enhance biofilm formation of *Y. enterocolitica.* The ions including Ca^{2+},Fe^{2+}, Mg^{2+}, Zn^{2+}, Na^{+}, K^{+} were investigated for their influence on the biofilm formation of *Y. enterocolitica* biotype 4. Ca^{2+} supplementation produced a stronger biofilm than the other ions tested. Ca^{2+} is one of the metal elements producing essential functions in all living organisms and is required by bacteria in various cellular processes(Guragain et al., 2013, Gode-Potratz et al., 2010, Wang et al., 2008, Werthén and Lundgren, 2001, Kim et al., 1999). However, its spectrum of functions in bacterial cells is less clear than the transition metals such as Fe, Zn, and Mn (Andrews et al., 2003, Diaz-Ochoa et al., 2015). Although it has been established that Ca^{2+} acts as a secondary messenger in eukaryotic cells (Johnson et al., 2011), it only became clear recently that Ca^{2+} performs homologous signalling functions in prokaryotes (Shemarova and Nesterov, 2005, Case et al., 2007). Ca^{2+} has been reported to regulate a variety of bacterial activities including biofilm formation. Ca^{2+} supplementation has been shown to increase biofilm formation in quite a few bacteria including *P.aeruginosa* (Sarkisova et al., 2005), *Pseudoalteromonas spp.* (Patrauchan et al., 2005), *S. meliloti* (Rinaudi et al., 2006), *X. fastidiosa* (Cruz et al., 2012), *E. cloacae* (Zhou et al., 2013), *A. hydrophila* (Das et al., 2014), and *E. faecalis* (Das et al., 2014).

In this study, the effect of Ca^{2+} on biofilm formation was much stronger at 37 °C than at 24 °C and the presence of pYV was essential to this Ca^{2+} induced biofilm formation at 37 °C. Ca^{2+} influences some other phenotypes of *Y. enterocolitica* such as to stop growth at low Ca^{2+} concentration under 37 °C, and adhesion/invasion ability to host cells (Zink et al., 1980). These influences are related to the pYV plasmid (Zink et al., 1980). However, the present study is the first report demonstrating the Ca^{2+} effect on *Y. enterocolitica* biofilm formation on abiotic surfaces.

These results have important implications in the control of *Y. enterocolitca* in the food industry. Colonisation of surfaces is likely to be enhanced at warmer temperatures so keeping temperatures cool during the handling of pork will reduce the likely colonisation of surfaces and reduce the risk of spreading contamination. Chelating agents may assist in improving cleaning of meat processing areas by limiting the availability of Ca^{2+} in the environment, helping to remove *Y. enterocolitica* biofilm and reducing the risk of re-colonising surfaces.

The present study also made a further effort to explore the intrinsic mechanism for the Ca^{2+} induced biofilm formation conferred by the pYV plasmid. The Ca^{2+} response in biofilm formation varied between the wild type and plasmid cured strain, suggesting that the role Ca^{2+} plays in the biofilm formation of *Y. enterocolitica* is more than just physical-chemical interactions. This suggestion is in line with the growing evidence in other bacteria showing that Ca^{2+} also plays a regulatory role in modulating bacterial biofilm. Apart from analysing the physical and chemical properties of the cell surface, the extracellular polymeric substances as well as gene expression were also analysed. Motility has been ruled out as a factor correlated to *Y. enterocolitica* biotype 4 biofilm formation by a preliminary experiment (Appendix 18) where the biotype 4 strain showed no motility at 37 °C regardless of Ca^{2+} supplementation. The results demonstrated that the presence of the pYV plasmid did alter the profile of cell surface proteins and the amount of exopolysaccharide which may in turn influence the cell surface charge and hydrophobicity. These are all factors that can influence biofilm formation. Electrophoresis showed the presence of a protein, which is believed to be

YadA,on the cell wall of the pYV^+ cells but not on that of the pYV^- cells. Further confirmation of this protein band needs to be performed by Mass spectrometry. Ca^{2+} supplementation did not produce any detectable change the cell surface physical-chemical properties, the profile of cell wall protein or the amount of exopolysaccharide, however, Ca^{2+} did show a tendency to increase the amount of eDNA. eDNA is key component of the EPS in many biofilms (Whitchurch et al., 2002). Study shows that eDNA can be a result of the release of genomic DNA by lysed bacterial cells (Mulcahy et al., 2008). Das et al. (2014) claim that Ca^{2+} has no role in the release of eDNA but is important in interacting with Ca^{2+} to promote cell aggregation and biofilm formation. The results from the present trial suggest that Ca^{2+} may influence DNA release, at least for *Y. enterocolitica.* It would be interesting to explore this further. This could be linked with changes in gene expression associated with the presence of Ca^{2+}. RNA-seq identified changes in gene (especially genes on pYV) expression in *Y. enterocolitica* grown in supplemental Ca^{2+}. The results showed that most of the genes on pYV were up-regulated after the addition of Ca^{2+} with a few exceptions although the fold change is not very high (Table 7.1). The Ca^{2+} stimulation of T3SS gene expression noted in this study was also reported in *V. parahaemolyticus* (Gode-Potratz et al., 2010). In addition, some of the RNA-sequencing results are in accordance with the phenotype observation made in the present or other studies. For example, a putative capsular polysaccharide transport protein YegH was down-regulated with the loss of pYV which could possibly explain the decrease in exopolysaccharide. YopE, one of the important effectors, was down-regulated; however, neither up-regulation nor down-regulation could be seen in SDS-PAGE analysis which might be due to the low sensitivity of SDS-PAGE to detect minor differential expression. In addition, as no replication has been done for RNA-sequencing, these results should be regarded as indicative rather than definitive until more samples can be processed by the Illumina MiSeq Sequencing System. Further confirmation is needed for the genes of interest.

This study is the first to address the importance of pYV to the biofilm formation by *Y. enterocolitica*. It was suggested that the *ars* transposon with arsenic resistance located

on pYV might have contributed to the establishment of *Y. enterocolitica* in pigs as arsenic compounds were widely used to protect pigs from diarrhoea caused by *Serpulina hyodysenteriae* before World War Ⅱ (Neyt et al., 1997, Cornelis et al., 1998). This *ars* transposon is not present in *Y. enterocolitica* biotype 1B which doesn't occur in pigs (Neyt et al., 1997). Therefore, it might be rational to assume that pYV is the key to help *Y. enterocolitica* to establish niche in pigs, initially by arsenic resistance then by biofilm formation.

The availability of the isogenic pair for this study allowed the investigation of the intrinsic mechanisms of biofilm formation by *Y. enterocolitica* biotype 4 to provide further information building on existing biofilm studies for *Y. enterocolitica* biotype 1B and biotype 2 (Kim et al., 2008, Raczkowska et al., 2011). Both studies were carried out at 24 °C showing that motility is important for biofilm formation although the molecular mechanism behind is different. Motility seems to be dispensable for the biofilm formation of *Y. enterocolitica* biotype 4 under the test conditions in this study. In addition, flagella are encoded by chromosome genes (Fauconnier et al., 1997) which further demonstrate that the mechanisms of biofilm formation of different biotypes under different test condition are distinct.

Much work could be done to fully understand how *Y. enterocolitica* manage to survive as biofilms in the pork processing environment. For example, as environmental biofilms are usually formed by multiple bacterial species, the interaction between *Y. enterocolitica* and other bacteria commonly found in pork processing environment in a biofilm needs to be investigated. Other factors that may have an impact on biofilm formation such as quorum sensing could also be investigated to provide further understanding on the intrinsic mechanism of biofilm formation by *Y. enterocolitica.*

In conclusion, the pork processing environment has the potential to support *Y.enterocolitica* biofilm formation. Ca^{2+} promotes biofilm formation through the function of the pYV plasmid.

For future work, the protein that was differentially expressed in pYV^{+} cells needs to be identified. RNA-sequencing needs to be repeated to verify the change in gene expression with the presence of pYV and Ca^{2+}. COG analysis of the up/down-regulated

genes needs to be carried out to identify potential functional groups involved in *Y. enterocolitica* biofilm formation (Tatusov et al., 2000). What's more, to develop controlling strategy for *Y. enterocolitica* biofilm, the ability of a Ca^{2+} chelating agent, proteinase and nuclease in removing *Y. enterocolitica* biofilm needs to be investigated.

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Appendix 1. M-values of 16 *Y. enterocolitica* strains in TSB at 30 °C over 18 hrs

Time (hr)	Blank (TSB)	032122	032123	032124	032125	114165	072344	032126	104253	114165	072345	072346	072347	073947	082059	093846	10460
0.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.2	0.03	0.02	0.02	0.01	0.02	0.04	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.02	0.01	0.01	0.01
0.3	0.06	0.06	0.05	0.03	0.05	0.08	0.05	0.03	0.03	0.03	0.03	0.03	0.01	0.04	0.03	0.04	0.03
0.5	0.08	0.07	0.07	0.04	0.07	0.11	0.08	0.04	0.05	0.05	0.04	0.03	0.02	0.06	0.05	0.06	0.05
0.7	0.08	0.10	0.08	0.05	0.10	0.12	0.12	0.07	0.08	0.07	0.05	0.04	0.03	0.08	0.06	0.08	0.07
0.8	0.09	0.10	0.09	0.07	0.11	0.12	0.13	0.08	0.09	0.08	0.07	0.04	0.04	0.09	0.07	0.09	0.08
1.0	0.10	0.12	0.10	0.08	0.11	0.12	0.13	0.09	0.09	0.08	0.07	0.04	0.04	0.10	0.07	0.10	0.09
1.2	0.11	0.12	0.10	0.07	0.11	0.12	0.13	0.09	0.09	0.09	0.08	0.04	0.04	0.11	0.08	0.10	0.09
1.3	0.12	0.13	0.09	0.06	0.12	0.14	0.15	0.11	0.12	0.11	0.08	0.04	0.05	0.12	0.10	0.13	0.11
1.5	0.13	0.13	0.10	0.07	0.13	0.14	0.16	0.12	0.13	0.12	0.09	0.05	0.06	0.13	0.11	0.13	0.11
1.7	0.13	0.15	0.11	0.09	0.13	0.15	0.17	0.12	0.13	0.11	0.11	0.06	0.07	0.14	0.12	0.14	0.12
1.8	0.14	0.15	0.12	0.10	0.14	0.16	0.18	0.13	0.14	0.12	0.13	0.06	0.08	0.14	0.13	0.14	0.13
2.0	0.14	0.16	0.13	0.11	0.15	0.18	0.20	0.14	0.16	0.13	0.15	0.06	0.09	0.17	0.14	0.17	0.14
2.2	0.14	0.17	0.14	0.11	0.17	0.21	0.22	0.16	0.19	0.16	0.17	0.06	0.11	0.20	0.16	0.19	0.17
2.3	0.14	0.18	0.16	0.13	0.18	0.23	0.23	0.18	0.20	0.17	0.20	0.06	0.12	0.22	0.18	0.22	0.18
2.5	0.15	0.18	0.18	0.13	0.20	0.26	0.24	0.19	0.23	0.18	0.24	0.06	0.14	0.24	0.20	0.23	0.20
2.7	0.15	0.19	0.19	0.14	0.22	0.28	0.26	0.22	0.27	0.22	0.29	0.07	0.17	0.26	0.23	0.27	0.22
2.8	0.16	0.21	0.22	0.14	0.28	0.34	0.32	0.28	0.33	0.27	0.36	0.08	0.20	0.32	0.29	0.33	0.28
3.0	0.15	0.23	0.24	0.15	0.34	0.42	0.38	0.34	0.41	0.34	0.45	0.08	0.24	0.39	0.36	0.41	0.33
3.2	0.16	0.27	0.28	0.17	0.39	0.50	0.44	0.39	0.49	0.38	0.57	0.09	0.30	0.46	0.43	0.49	0.37
3.3	0.19	0.32	0.34	0.21	0.43	0.58	0.48	0.43	0.56	0.43	0.71	0.12	0.39	0.52	0.51	0.57	0.39
3.5	0.24	0.39	0.42	0.26	0.48	0.68	0.54	0.49	0.66	0.50	0.90	0.16	0.51	0.60	0.60	0.67	0.43
3.7	0.27	0.47	0.52	0.31	0.55	0.79	0.60	0.57	0.78	0.58	1.10	0.19	0.63	0.69	0.71	0.79	0.48
3.8	0.28	0.54	0.60	0.34	0.62	0.93	0.67	0.66	0.92	0.68	1.31	0.21	0.77	0.79	0.85	0.91	0.54
4.0	0.29	0.61	0.69	0.36	0.71	1.09	0.75	0.78	1.10	0.80	1.53	0.22	0.92	0.90	1.03	1.08	0.60
4.2	0.29	0.68	0.79	0.39	0.81	1.28	0.87	0.93	1.30	0.95	1.70	0.23	1.10	1.05	1.23	1.28	0.68
4.3	0.29	0.75	0.89	0.41	0.94	1.48	1.00	1.10	1.54	1.13	1.84	0.24	1.30	1.21	1.46	1.51	0.77
4.5	0.29	0.85	1.00	0.45	1.08	1.65	1.14	1.29	1.76	1.34	1.96	0.25	1.50	1.41	1.67	1.74	0.87
4.7	0.30	0.96	1.13	0.48	1.27	1.81	1.32	1.52	1.96	1.56	2.08	0.26	1.67	1.63	1.87	1.96	1.01
4.8	0.30	1.09	1.29	0.52	1.48	1.95	1.53	1.76	2.14	1.77	2.22	0.27	1.81	1.88	2.05	2.17	1.18
5.0	0.31	1.23	1.46	0.58	1.71	2.09	1.76	1.96	2.32	1.92	2.35	0.30	1.94	2.09	2.22	2.35	1.34
5.2	0.31	1.37	1.61	0.65	1.89	2.21	1.94	2.10	2.48	2.04	2.49	0.32	2.08	2.26	2.39	2.53	1.53
5.3	0.31	1.53	1.74	0.72	2.01	2.32	2.07	2.23	2.63	2.15	2.62	0.36	2.22	2.40	2.54	2.69	1.70
5.5	0.30	1.66	1.83	0.79	2.11	2.44	2.17	2.37	2.79	2.28	2.74	0.40	2.36	2.54	2.70	2.86	1.88
5.7	0.30	1.78	1.92	0.88	2.21	2.56	2.27	2.52	2.94	2.42	2.86	0.46	2.50	2.68	2.86	3.03	2.02
5.8	0.30	1.88	2.01	0.98	2.33	2.69	2.38	2.68	3.09	2.56	2.97	0.54	2.63	2.83	3.02	3.19	2.15
6.0	0.31	1.97	2.10	1.10	2.45	2.80	2.50	2.84	3.24	2.71	3.08	0.64	2.74	2.97	3.16	3.34	2.28
6.2	0.32	2.07	2.21	1.22	2.58	2.91	2.62	2.99	3.38	2.86	3.20	0.76	2.86	3.09	3.30	3.49	2.40
6.3	0.33	2.17	2.30	1.36	2.71	3.02	2.74	3.16	3.51	3.00	3.31	0.89	2.97	3.22	3.45	3.64	2.54
6.5	0.33	2.28	2.40	1.51	2.84	3.12	2.86	3.31	3.65	3.14	3.41	1.06	3.09	3.34	3.60	3.80	2.69
6.7	0.34	2.39	2.50	1.67	2.98	3.22	2.98	3.46	3.78	3.29	3.51	1.26	3.20	3.46	3.74	3.95	2.83
6.8	0.34	2.50	2.59	1.82	3.09	3.31	3.09	3.61	3.90	3.43	3.60	1.48	3.31	3.58	3.86	4.08	2.98
7.0	0.34	2.59	2.67	1.99	3.20	3.40	3.20	3.76	4.01	3.58	3.69	1.72	3.41	3.69	3.97	4.21	3.13
7.2	0.34	2.68	2.75	2.13	3.32	3.49	3.31	3.89	4.10	3.71	3.77	1.93	3.51	3.79	4.07	4.33	3.28
7.3	0.34	2.77	2.83	2.26	3.44	3.58	3.41	4.02	4.18	3.84	3.83	2.11	3.61	3.88	4.16	4.44	3.41
7.5	0.34	2.85	2.89	2.37	3.57	3.67	3.51	4.15	4.26	3.98	3.90	2.25	3.70	3.97	4.23	4.54	3.56
7.7	0.34	2.94	2.96	2.47	3.69	3.76	3.60	4.26	4.33	4.09	3.98	2.41	3.80	4.05	4.30	4.63	3.70
7.8	0.34	3.01	3.04	2.58	3.80	3.84	3.68	4.37	4.40	4.21	4.05	2.56	3.89	4.12	4.36	4.70	3.84
8.0	0.34	3.10	3.11	2.68	3.91	3.91	3.75	4.47	4.47	4.31	4.11	2.70	3.97	4.18	4.40	4.76	3.98
8.2	0.35	3.18	3.18	2.78	4.02	3.99	3.82	4.56	4.53	4.41	4.17	2.83	4.05	4.23	4.45	4.81	4.10
8.3	0.35	3.25	3.23	2.87	4.12	4.06	3.88	4.64	4.59	4.50	4.22	2.95	4.12	4.28	4.49	4.84	4.22
8.5	0.36	3.33	3.29	2.95	4.21	4.11	3.94	4.71	4.63	4.58	4.28	3.07	4.19	4.32	4.53	4.87	4.31
8.7	0.36	3.40	3.35	3.03	4.30	4.18	3.99	4.77	4.67	4.65	4.33	3.19	4.26	4.36	4.56	4.90	4.41
8.8	0.37	3.46	3.41	3.11	4.39	4.24	4.04	4.83	4.72	4.72	4.38	3.30	4.31	4.39	4.59	4.93	4.49
9.0	0.37	3.52	3.47	3.17	4.47	4.30	4.09	4.88	4.76	4.79	4.43	3.42	4.36	4.44	4.63	4.96	4.58

The table is continued on the next page.

Time (hr)	Blank (TSB)	032122	032123	032124	032125	114165	072344	032126	104253	112277	072345	072346	072347	073947	082059	093846	10460
9.2	0.38	3.58	3.52	3.23	4.54	4.35	4.14	4.93	4.80	4.86	4.49	3.52	4.40	4.49	4.66	4.97	4.66
9.3	0.39	3.65	3.58	3.31	4.61	4.40	4.18	4.98	4.84	4.91	4.54	3.63	4.45	4.53	4.68	5.00	4.72
9.5	0.39	3.70	3.63	3.37	4.68	4.45	4.21	5.02	4.88	4.94	4.58	3.73	4.48	4.56	4.71	5.01	4.77
9.7	0.39	3.77	3.68	3.44	4.72	4.49	4.23	5.07	4.91	4.97	4.61	3.83	4.53	4.59	4.73	5.03	4.81
9.8	0.39	3.82	3.72	3.49	4.77	4.53	4.24	5.10	4.95	5.00	4.65	3.92	4.56	4.62	4.74	5.04	4.85
10.0	0.39	3.87	3.76	3.55	4.81	4.56	4.26	5.14	4.98	5.02	4.69	4.01	4.60	4.66	4.75	5.06	4.88
10.2	0.39	3.91	3.79	3.59	4.86	4.60	4.27	5.17	5.01	5.05	4.71	4.08	4.61	4.69	4.76	5.06	4.91
10.3	0.39	3.95	3.83	3.64	4.90	4.63	4.28	5.20	5.03	5.06	4.74	4.17	4.63	4.72	4.78	5.07	4.94
10.5	0.40	3.99	3.87	3.69	4.93	4.67	4.29	5.23	5.06	5.08	4.77	4.25	4.64	4.75	4.79	5.07	4.97
10.7	0.40	4.04	3.91	3.73	4.95	4.70	4.31	5.25	5.08	5.10	4.80	4.33	4.67	4.78	4.79	5.08	5.00
10.8	0.41	4.09	3.94	3.77	4.96	4.73	4.32	5.29	5.09	5.12	4.83	4.40	4.68	4.80	4.81	5.09	5.02
11.0	0.42	4.14	3.97	3.81	4.98	4.76	4.33	5.31	5.12	5.13	4.86	4.47	4.71	4.82	4.81	5.10	5.04
11.2	0.43	4.17	4.00	3.85	4.99	4.79	4.33	5.34	5.17	5.14	4.88	4.53	4.72	4.84	4.82	5.11	5.05
11.3	0.43	4.21	4.02	3.88	5.00	4.81	4.33	5.36	5.22	5.15	4.90	4.60	4.73	4.88	4.82	5.11	5.08
11.5	0.44	4.24	4.05	3.91	5.01	4.83	4.33	5.38	5.28	5.17	4.92	4.65	4.74	4.90	4.82	5.12	5.09
11.7	0.44	4.27	4.07	3.94	5.01	4.86	4.33	5.41	5.28	5.22	4.94	4.71	4.75	4.92	4.83	5.13	5.10
11.8	0.44	4.30	4.09	3.96	5.03	4.90	4.34	5.43	5.28	5.29	4.95	4.75	4.75	4.95	4.84	5.13	5.13
12.0	0.44	4.34	4.11	3.98	5.05	4.92	4.35	5.46	5.28	5.33	4.97	4.80	4.75	4.98	4.85	5.15	5.15
12.2	0.45	4.35	4.12	4.00	5.06	4.95	4.36	5.48	5.29	5.34	4.98	4.83	4.75	5.00	4.86	5.16	5.17
12.3	0.46	4.39	4.15	4.02	5.06	4.97	4.37	5.49	5.29	5.33	5.00	4.88	4.77	5.03	4.87	5.17	5.18
12.5	0.45	4.41	4.17	4.04	5.06	4.99	4.37	5.50	5.29	5.34	5.00	4.91	4.78	5.06	4.87	5.17	5.19
12.7	0.45	4.44	4.19	4.05	5.07	5.01	4.38	5.51	5.29	5.34	5.00	4.94	4.78	5.08	4.87	5.18	5.20
12.8	0.44	4.46	4.20	4.06	5.08	5.03	4.38	5.51	5.29	5.35	5.00	4.97	4.78	5.10	4.87	5.19	5.20
13.0	0.45	4.48	4.20	4.07	5.09	5.06	4.39	5.52	5.29	5.36	5.02	5.01	4.79	5.13	4.88	5.19	5.21
13.2	0.45	4.51	4.22	4.10	5.10	5.08	4.39	5.52	5.29	5.37	5.03	5.05	4.80	5.14	4.89	5.20	5.21
13.3	0.46	4.54	4.24	4.11	5.10	5.10	4.39	5.52	5.29	5.37	5.05	5.08	4.80	5.16	4.89	5.21	5.22
13.5	0.46	4.56	4.25	4.12	5.10	5.12	4.39	5.52	5.28	5.36	5.06	5.11	4.80	5.16	4.89	5.21	5.22
13.7	0.47	4.58	4.27	4.14	5.10	5.13	4.37	5.52	5.28	5.36	5.07	5.14	4.81	5.16	4.89	5.20	5.22
13.8	0.47	4.59	4.28	4.15	5.10	5.14	4.36	5.51	5.28	5.36	5.07	5.16	4.81	5.15	4.89	5.20	5.22
14.0	0.47	4.61	4.30	4.17	5.10	5.16	4.35	5.51	5.28	5.36	5.07	5.17	4.81	5.16	4.90	5.20	5.22
14.2	0.47	4.62	4.31	4.18	5.11	5.18	4.35	5.51	5.28	5.37	5.07	5.20	4.81	5.17	4.90	5.21	5.23
14.3	0.47	4.63	4.32	4.18	5.11	5.20	4.35	5.52	5.28	5.37	5.06	5.22	4.82	5.18	4.90	5.22	5.23
14.5	0.49	4.64	4.32	4.19	5.12	5.20	4.35	5.52	5.28	5.37	5.07	5.24	4.82	5.19	4.90	5.22	5.24
14.7	0.50	4.66	4.34	4.19	5.13	5.22	4.35	5.51	5.28	5.37	5.07	5.27	4.82	5.19	4.90	5.22	5.24
14.8	0.50	4.67	4.36	4.22	5.14	5.23	4.34	5.50	5.28	5.37	5.08	5.29	4.82	5.20	4.91	5.22	5.24
15.0	0.50	4.68	4.37	4.22	5.15	5.24	4.34	5.51	5.29	5.38	5.08	5.31	4.83	5.20	4.92	5.22	5.24
15.2	0.50	4.69	4.38	4.24	5.15	5.25	4.34	5.52	5.29	5.38	5.08	5.33	4.84	5.21	4.92	5.23	5.24
15.3	0.50	4.69	4.38	4.23	5.16	5.26	4.35	5.53	5.29	5.39	5.07	5.35	4.84	5.21	4.93	5.23	5.25
15.5	0.50	4.69	4.39	4.25	5.16	5.27	4.35	5.53	5.28	5.39	5.06	5.36	4.84	5.22	4.92	5.23	5.24
15.7	0.50	4.69	4.39	4.26	5.17	5.27	4.35	5.53	5.28	5.39	5.06	5.37	4.84	5.23	4.93	5.24	5.25
15.8	0.51	4.70	4.40	4.27	5.17	5.28	4.35	5.53	5.28	5.39	5.06	5.38	4.86	5.23	4.93	5.24	5.26
16.0	0.52	4.70	4.41	4.29	5.18	5.29	4.35	5.53	5.29	5.39	5.07	5.39	4.86	5.23	4.94	5.24	5.26
16.2	0.54	4.71	4.43	4.31	5.18	5.30	4.35	5.53	5.29	5.39	5.07	5.41	4.86	5.24	4.94	5.24	5.26
16.3	0.54	4.72	4.43	4.32	5.18	5.31	4.35	5.53	5.29	5.40	5.08	5.42	4.86	5.24	4.94	5.25	5.26
16.5	0.55	4.72	4.44	4.33	5.18	5.31	4.34	5.53	5.29	5.40	5.07	5.42	4.86	5.25	4.94	5.25	5.26
16.7	0.54	4.72	4.44	4.34	5.17	5.31	4.34	5.53	5.29	5.41	5.07	5.42	4.86	5.24	4.94	5.25	5.26
16.8	0.55	4.73	4.44	4.35	5.18	5.31	4.35	5.54	5.29	5.41	5.07	5.42	4.86	5.25	4.94	5.25	5.26
17.0	0.55	4.72	4.44	4.36	5.18	5.32	4.35	5.54	5.29	5.42	5.07	5.42	4.86	5.26	4.95	5.26	5.26
17.2	0.55	4.72	4.44	4.36	5.19	5.33	4.35	5.54	5.29	5.42	5.07	5.43	4.86	5.26	4.95	5.26	5.27
17.3	0.55	4.73	4.44	4.36	5.20	5.34	4.36	5.54	5.29	5.42	5.07	5.43	4.86	5.26	4.95	5.26	5.27
17.5	0.55	4.73	4.45	4.37	5.20	5.34	4.36	5.53	5.30	5.41	5.07	5.43	4.87	5.27	4.95	5.25	5.26
17.7	0.56	4.73	4.46	4.38	5.20	5.36	4.36	5.54	5.31	5.42	5.07	5.44	4.88	5.27	4.96	5.25	5.26
17.8	0.57	4.74	4.47	4.40	5.20	5.36	4.36	5.54	5.32	5.44	5.07	5.45	4.88	5.28	4.96	5.26	5.27
18.0	0.58	4.75	4.47	4.41	5.20	5.37	4.37	5.55	5.34	5.45	5.08	5.46	4.88	5.28	4.97	5.26	5.27

Appendix 2. Raw data for linear regression of the scatter plot for strain ERL032123

Detection time by impedance method (hr)	Cell counts by standard plate counting (CFU)
20.1	2.40
20.4	2.40
18.0	2.40×10^{1}
18.3	2.40×10^{1}
19.3	1.20×10^{1}
16.0	2.40×10^{2}
16.2	2.40×10^{2}
16.8	1.20×10^{2}
13.9	2.40×10^{3}
14.4	1.20×10^{3}
14.6	2.40×10^{3}
11.6	2.40×10^{4}
11.8	2.40×10^{4}
12.3	1.20×10^{4}
9.3	2.40×10^{5}
9.4	2.40×10^{5}
9.9	1.20×10^{5}
7.1	2.40×10^{6}
7.3	2.40×10^{6}
7.7	1.20×10^{6}

Appendix 3. Raw data for linear regression of the scatter plot for strain ERL104253

Detection time by impedance method (hr)	Cell counts by standard plate counting (CFU)
16.3	6.00
15.6	1.20
15.4	1.20×10^{1}
14.2	6.00×10^{1}
13.9	1.20×10^{2}
13.1	1.20×10^{2}
12.4	6.00×10^{2}
11.9	1.20×10^{3}
11.2	1.20×10^{3}
10.3	6.00×10^{3}
10.1	1.20×10^{4}
9.4	1.20×10^{4}
8.6	6.00×10^{4}
8.3	1.20×10^{5}
7.7	1.20×10^{5}

Appendix 4. Raw data for linear regression of the scatter plot for strain ERL10460

Detection time by impedance method (hr)	Cell counts by standard plate counting (CFU)
20.2	3.34
19.1	1.26
19.0	2.80
18.3	3.34×10^{1}
17.7	2.80×10^{1}
17.2	1.26×10^{1}
15.6	2.80×10^{2}
15.3	1.26×10^{2}
14.8	3.34×10^{2}
13.6	2.80×10^{3}
13.3	1.26×10^{3}
11.9	3.34×10^{3}
11.6	2.80×10^{4}
11.5	1.26×10^{4}
10.5	3.34×10^{4}
9.9	2.80×10^{5}
9.2	1.26×10^{5}
8.9	3.34×10^{5}
7.7	2.80×10^{6}
7.3	1.26×10^{6}
7.1	3.34×10^{6}

Appendix 5. Raw data for biofilm formation by different biotypes of *Y. enterocolitica* in TSB and MJ at 24 °C.

	TSB		MJ	
	Mean (A_{595nm})	Standard deviation (A_{595nm})	Mean (A_{595nm})	Standard deviation (A_{595nm})
072345-biotype1A	0.000	0.000	0.066	0.032
072346-biotype1A	0.669	0.098	0.092	0.049
072347-biotype1A	0.007	0.013	0.026	0.021
073947-biotype1A	2.255	0.183	1.512	0.262
082059-biotype1A	0.556	0.078	0.134	0.053
093846-biotype1A	0.113	0.049	0.204	0.225
10460-biotype1A	1.099	0.066	0.940	0.330
032126-biotype2	0.053	0.041	0.709	0.257
104253-biotype3	1.018	0.047	0.241	0.056
112277-biotype3	0.289	0.058	1.085	0.344
032122-biotype4	0.054	0.008	3.861	0.631
032123-biotype4	0.046	0.021	2.171	0.468
032124-biotype4	0.073	0.014	2.292	0.117
032125-biotype4	0.037	0.056	3.961	0.830
072344-biotype4	0.083	0.007	4.067	0.725
114165-biotype4	0.088	0.034	0.026	0.016

Appendix 6. Raw data for biofilm of biotype 4, strain ERL032123 formed in TSB and MJ under control treatment using 0.85% NaCl solution at 24 °C.

	TSB		MJ	
	Mean (CFU)	Standard deviation (CFU)	Mean (CFU)	Standard deviation (CFU)
Day1-Point1	1.70×10^{6}	1.45	1.26×10^{7}	1.82
Day1-Point2	5.75×10^{5}	1.29	5.89×10^{6}	3.80
Day1-Point3	6.03×10^{4}	3.55	3.80×10^{6}	3.24
Day3-Point1	1.32×10^{5}	2.34	1.00×10^{7}	1.17
Day3-Point2	1.02×10^{4}	1.70	1.10×10^{6}	1.29
Day3-Point3	1.07×10^{3}	2.14	2.24×10^{4}	1.91
Day5-Point1	3.80×10^{4}	3.89	1.26×10^{4}	3.09
Day5-Point2	7.59×10^{3}	3.47	2.19×10^{4}	4.07
Day5-Point3	4.47×10^{2}	4.79	3.63×10^{2}	2.95

Appendix 7. Raw data for biofilm of strain ERL032123 treated with 50 ppm Sodium hypochlorite in TSB at 24 °C.

	TSB		MJ	
	Mean (CFU)	Standard deviation (CFU)	Mean (CFU)	Standard deviation (CFU)
Day1-Point1	1.70×10^{6}	1.45	9.55×10^{6}	1.15
Day1-Point2	3.55×10^{1}	1.86	5.01×10^{3}	2.51
Day1-Point3	2.95	2.82	2.29×10^{3}	1.51
Day3-Point1	8.13×10^{1}	4.27	2.19×10^{4}	3.16
Day3-Point2	2.40×10^{1}	2.63	2.82×10^{1}	3.24
Day3-Point3	0	0	0	0
Day5-Point1	2.24×10^{1}	0	0	0
Day5-Point2	4.90	1.58	0	0
Day5-Point3	0	0	0	0

Appendix 8. Raw data for biofilm of strain ERL032123 treated with 200 ppm QAC in TSB at 24 °C.

	TSB		MJ	
	Mean (CFU)	Standard deviation (CFU)	Mean (CFU)	Standard deviation (CFU)
Day1-Point1	1.70×10^{6}	1.45	9.55×10^{6}	1.15
Day1-Point2	1.02×10^{3}	2.45	1.70×10^{3}	2.34
Day1-Point3	5.37	1.10×10^{1}	1.91×10^{3}	1.66
Day3-Point1	6.61×10^{1}	4.79	3.98×10^{3}	1.15
Day3-Point2	1.38×10^{1}	4.17×10^{1}	2.51×10^{2}	9.55
Day3-Point3	0	0	9.33×10^{3}	2.04
Day5-Point1	2.14×10^{5}	5.01	1.41×10^{2}	3.72
Day5-Point2	3.24	5.25	8.13×10^{1}	2.14
Day5-Point3	0	0	0	0

Appendix 9. Ion content in MJ

	MJ
Mg^{2+}	95 µmol/l
Ca^{2+}	75 µmol/l
Zn^{2+}	22 µmol/l
Fe^{2+}	14 µmol/l
Na^{+}	1.36 mmol/l
K^{+}	118 µmol/l

Appendix 10. Raw data for biofilm formation and planktonic growth of *Y. enterocolitica* strain ERL032123 (pYV+) at 24 °C in the presence of different ions.

Biofilm formation

Ion concentration (mM)	Ca^{2+}		Fe^{2+}		Mg^{2+}	
	Mean (A_{595nm})	Standard Deviation (A_{595nm})	Mean (A_{595nm})	Standard deviation (A_{595nm})	Mean (A_{595nm})	Standard deviation (A_{595nm})
0	0.663	0.013	0.704	0.021	0.611	0.050
1.5	0.623	0.045	0.029	0.000	0.889	0.031
3	1.056	0.215	0.014	0.006	0.913	0.032
4	1.171	0.059	0.008	0.011	0.959	0.093
5	1.348	0.072	0.014	0.009	0.824	0.064
7.5	1.297	0.064	0.023	0.013	0.670	0.026
10	1.469	0.033	0.045	0.017	0.712	0.097

Ion concentration (mM)	Zn^{2+}		Na^{+}		K^{+}	
	Mean (A_{595nm})	Standard Deviation (A_{595nm})	Mean (A_{595nm})	Standard deviation (A_{595nm})	Mean (A_{595nm})	Standard deviation (A_{595nm})
0	0.667	0.031	0.639	0.049	0.795	0.051
1.5	0.007	0.002	0.705	0.028	0.786	0.026
3	0.048	0.016	0.698	0.041	0.757	0.021
4	0.079	0.018	0.648	0.052	0.718	0.041
5	0.146	0.023	0.624	0.030	0.731	0.066
7.5	0.187	0.020	0.550	0.045	0.642	0.079
10	0.359	0.050	0.419	0.035	0.508	0.040

Planktonic growth

Ion concentration (mM)	Ca^{2+}		Fe^{2+}		Mg^{2+}	
	Mean (A_{600nm})	Standard Deviation (A_{600nm})	Mean (A_{600nm})	Standard deviation (A_{600nm})	Mean (A_{600nm})	Standard deviation (A_{600nm})
0	0.208	0.011	0.216	0.015	0.207	0.013
1.5	0.253	0.013	0.436	0.039	0.239	0.013
3	0.489	0.040	0.457	0.045	0.243	0.010
4	0.474	0.020	0.444	0.021	0.245	0.016
5	0.445	0.086	0.448	0.008	0.237	0.012
7.5	0.455	0.021	0.501	0.027	0.208	0.007
10	0.451	0.036	0.452	0.022	0.207	0.007

Ion concentration (mM)	Zn^{2+}		Na^{+}		K^{+}	
	Mean (A_{600nm})	Standard Deviation (A_{600nm})	Mean (A_{600nm})	Standard deviation (A_{600nm})	Mean (A_{600nm})	Standard deviation (A_{600nm})
0	0.217	0.005	0.214	0.010	0.217	0.005
1.5	0.020	0.004	0.225	0.019	0.219	0.010
3	0.016	0.005	0.223	0.014	0.220	0.008
4	0.015	0.002	0.222	0.014	0.220	0.005
5	0.011	0.005	0.223	0.015	0.224	0.008
7.5	0.020	0.004	0.216	0.009	0.216	0.008
10	0.046	0.005	0.207	0.005	0.206	0.010

Appendix 11. Raw data for biofilm formation of *Y. enterocolitica* strain ERL032123 (pYV$^+$) at 24 °C.and 37 °C with the presence of Ca^{2+}.

Ca^{2+} concentration (mM)	24 °C		37 °C	
	Mean (BFI)	Standard Deviation (BFI)	Mean (BFI)	Standard deviation (BFI)
0	0.110	0.023	0.005	0.009
1	0.116	0.062	0.154	0.063
2	0.153	0.074	0.342	0.234
3	0.167	0.051	0.488	0.115
4	0.261	0.122	0.580	0.086
5	0.356	0.179	0.597	0.107
10	0.363	0.160	1.331	0.066
20	0.303	0.143	2.891	0.417
30	0.196	0.139	3.085	0.167
40	0.156	0.026	3.425	0.269
50	0.244	0.062	3.134	0.058

Appendix 12. Raw data for biofilm formation of *Y. enterocolitica* strain ERL032123 (pYV$^-$) at 24 °C and 37 °C at different concentrations of Ca^{2+}.

Ca^{2+} concentration (mM)	24 °C		37 °C	
	Mean (BFI)	Standard Deviation (BFI)	Mean (BFI)	Standard deviation (BFI)
0	0.017	0.025	0.000	0.000
1	0.089	0.121	0.023	0.033
2	0.098	0.139	0.031	0.009
3	0.054	0.045	0.056	0.035
4	0.098	0.052	0.077	0.054
5	0.125	0.096	0.135	0.082
10	0.132	0.075	0.152	0.059
20	0.162	0.084	0.153	0.022
30	0.126	0.067	0.151	0.038
40	0.083	0.053	0.160	0.077
50	0.122	0.039	0.229	0.121

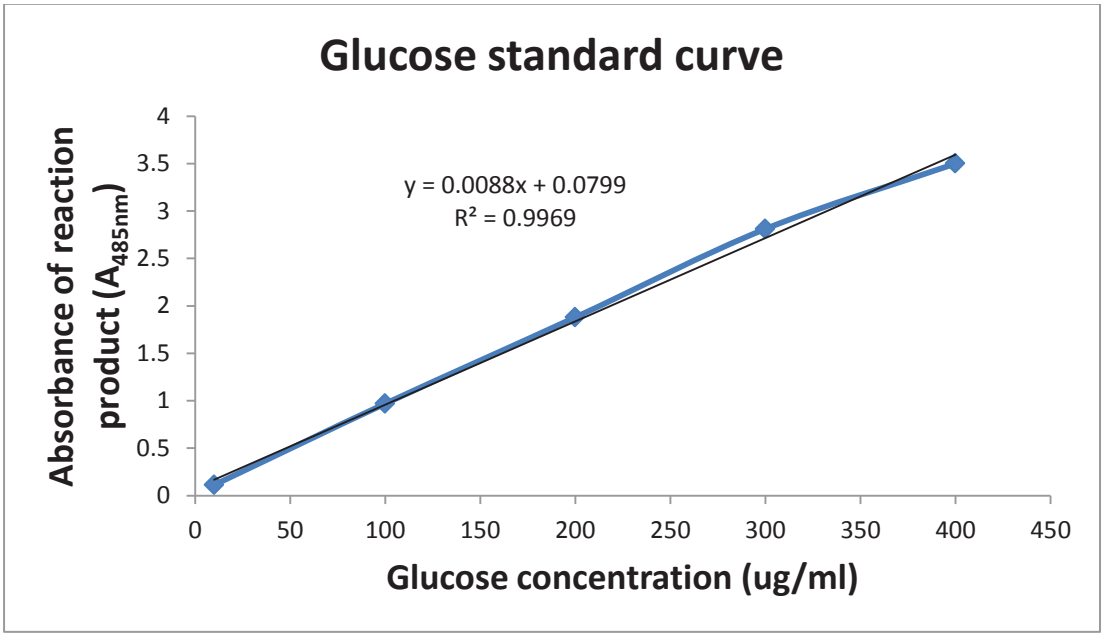
Appendix 13. Raw data for surface Zeta potential of pYV^+ and pYV^- cells at pH 7 after incubation in the presence and absence of Ca^{2+}at 37 °C.

pH	pYV^+, no Ca^{2+}		pYV^+, with Ca^{2+}		pYV^-, no Ca^{2+}		pYV^-, with Ca^{2+}	
	Mean (mV)	Standard Deviation (mV)	Mean (mV)	Standard Deviation (mV)	Mean (mV)	Standard Deviation (mV)	Mean (mV)	Standard deviation (mV)
8	-8.860	0.818	-6.217	0.301	-4.940	0.282	-4.323	0.180
7	-8.823	0.257	-5.627	0.316	-4.827	0.695	-4.130	0.416
6	-19.133	2.501	-12.867	1.601	-4.453	0.777	-3.610	0.403
5	-7.537	0.517	-7.053	0.549	-2.660	0.240	-2.203	0.050
4	-1.813	0.251	-2.357	0.249	-0.009	0.179	-0.682	0.264
3	4.810	0.243	1.180	0.115	2.327	0.178	2.050	0.231

Appendix 14. Raw data for hydrophobicity of pYV^+ and pYV^- cells after incubation in the presence and absence of Ca^{2+} at 37 °C.

	pYV^+, no Ca^{2+}	pYV^+, with Ca^{2+}	pYV^-, no Ca^{2+}	pYV^-, with Ca^{2+}
A_{600nm} of original bacteria solution	1.385	1.321	1.619	1.437
	1.361	1.324	1.607	1.468
	1.375	1.321	1.597	1.455
A_{600nm} of eluted bacteria solution	0.017	0.012	0.221	0.357
	0.008	0.012	0.235	0.432
	0.057	0.006	0.221	0.417
Percentage of retained cells	98.8%	99.1%	86.3%	75.2%
	99.4%	99.1%	85.4%	70.6%
	95.9%	99.5%	86.2%	71.3%
Average	98.0%	99.3%	86.0%	72.4%
Standard Deviation	1.89%	0.25%	0.50%	2.46%

Appendix 15. Raw data for exopolysaccharide measurement in the EPS of pYV^+ and pYV^- cell cultures after incubation in the presence and absence of Ca^{2+} at 37 °C.



	pYV^+, no Ca^{2+}	pYV^+, with Ca^{2+}	pYV^-, no Ca^{2+}	pYV^-, with Ca^{2+}
A_{485nm} of EPS reaction product after phenol sulphuric acid assay	2.973	2.835	1.562	2.023
	2.545	2.632	1.924	1.965
	2.915		1.737	
Total carbohydrate content calculated using glucose standard curve(µg)	3287.955	3130.795	1683.636	2207.841
	2801.250	2900.000	2095.568	2142.500
	3221.477		1883.409	
Total cell counts (CFU)	3.25×10^{10}	3.19×10^{10}	2.75×10^{10}	3.52×10^{10}
	3×10^{10}	2.97×10^{10}	2.75×10^{10}	3.41×10^{10}
	3×10^{10}	2.53×10^{10}	5.25×10^{10}	4.62×10^{10}
Carbohydrate content per 10^{10}CFU (mg/10^{10}CFU)	1.012	0.981	0.612	0.627
	0.934	0.976	0.762	0.628
	1.074		0.359	
Mean (mg/10^{10}CFU)	1.007	0.979	0.578	0.628
Standard Deviation (mg/10^{10}CFU)	0.070	0.004	0.204	0.001

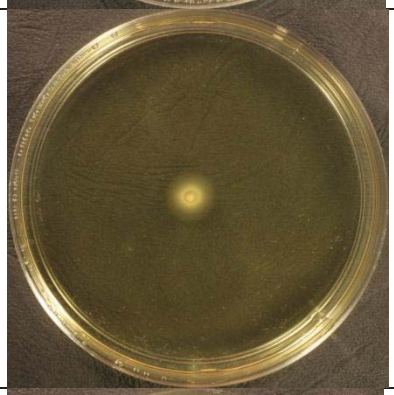
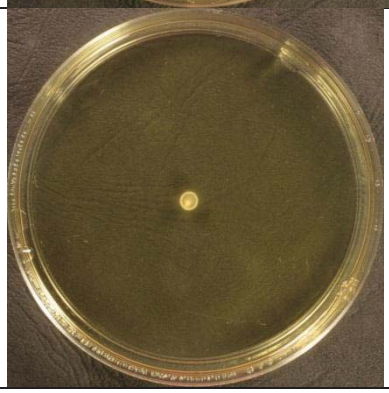
Appendix 16. Raw data for nucleic acid measurement in the EPS of pYV^+ and pYV^- cell cultures after incubation in the presence and absence of Ca^{2+} at 37 °C.

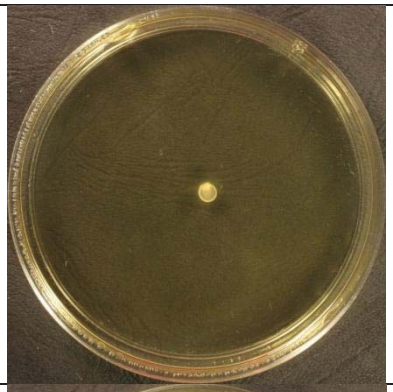
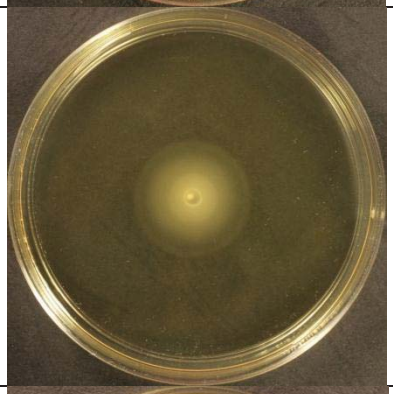
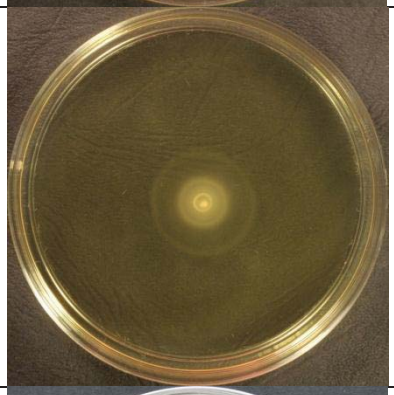
	pYV^+, no Ca^{2+}	pYV^+, with Ca^{2+}	pYV^-, no Ca^{2+}	pYV^-, with Ca^{2+}
Total nucleic acid content (ng)	971210.7	2430794	801764.8	2186901
	977011.7	2448562	796986.3	2210386
	976694.3	2461901	800513.8	2269735
Total cell counts (CFU)	1.45×10^{10}	2.1×10^{10}	1.38×10^{10}	2.3×10^{10}
	1.56×10^{10}	1.8×10^{10}	1.6×10^{10}	2.9×10^{10}
	1.32×10^{10}	2.05×10^{10}	1.25×10^{10}	3×10^{10}
Nucleic acid content per 10^{10}CFU (mg/10^{10}CFU)	0.670	1.158	0.581	0.951
	0.626	1.360	0.498	0.762
	0.740	1.201	0.640	0.757
Mean (mg/10^{10}CFU)	0.679	1.240	0.573	0.823
Standard Deviation (mg/10^{10}CFU)	0.057332	0.10678	0.071469	0.110562

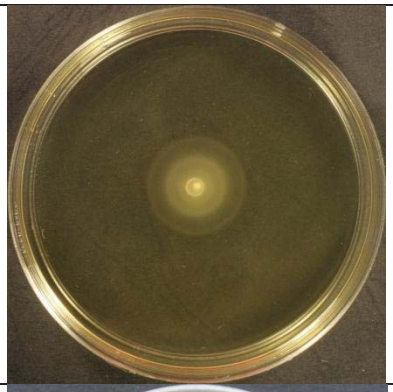
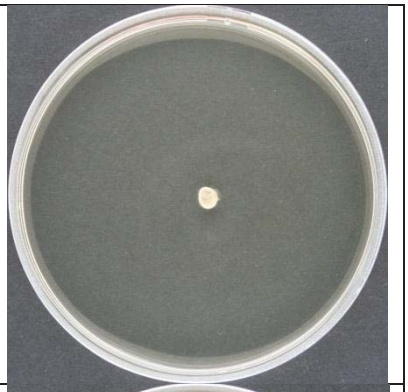
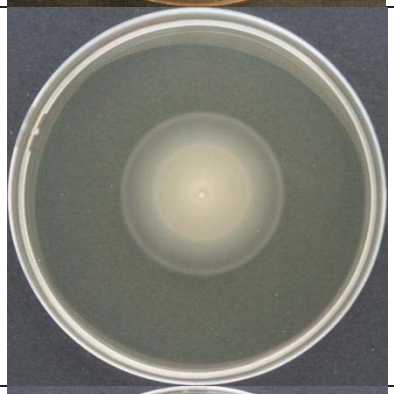
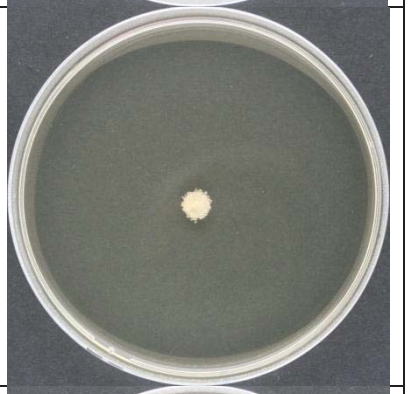
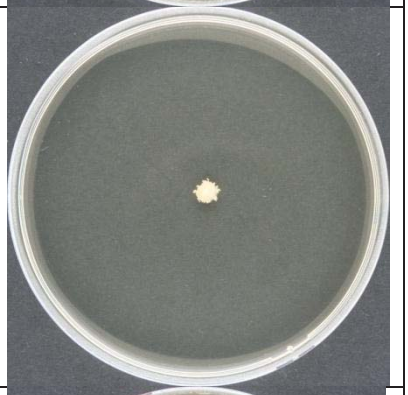
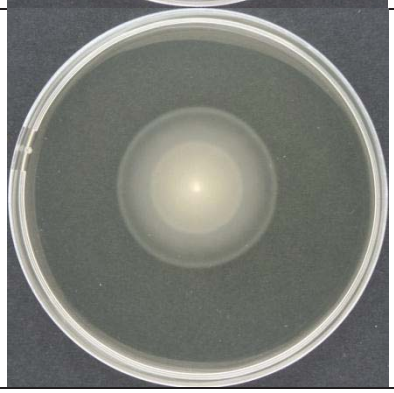
Appendix 17. The immediate attachment to coupon surfaces (1 hr) of pYV^+ and pYV^- cells after incubation in the presence and absence of Ca^{2+} at 37 °C.

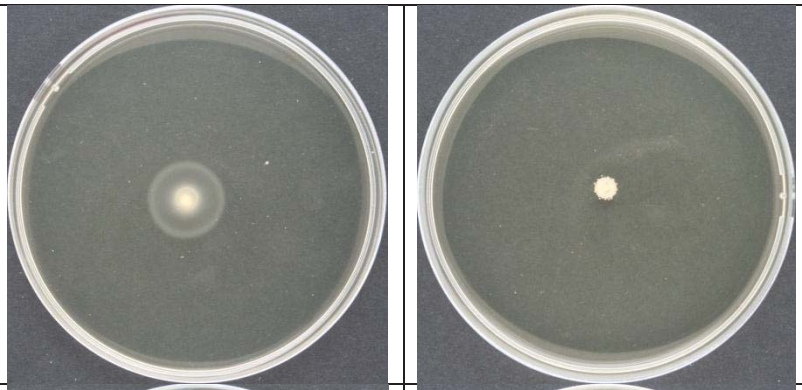
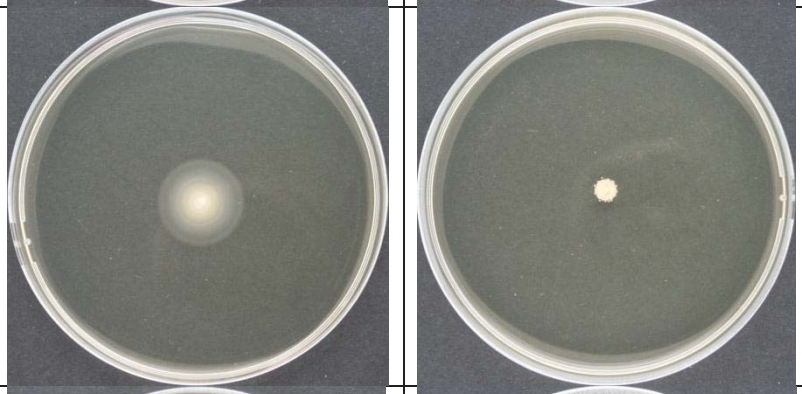
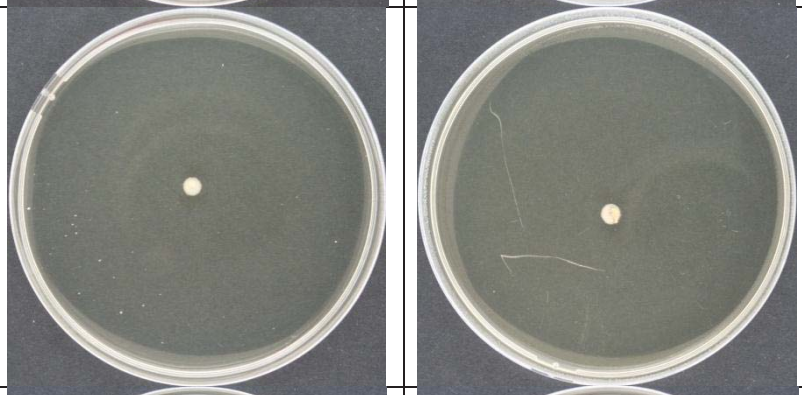
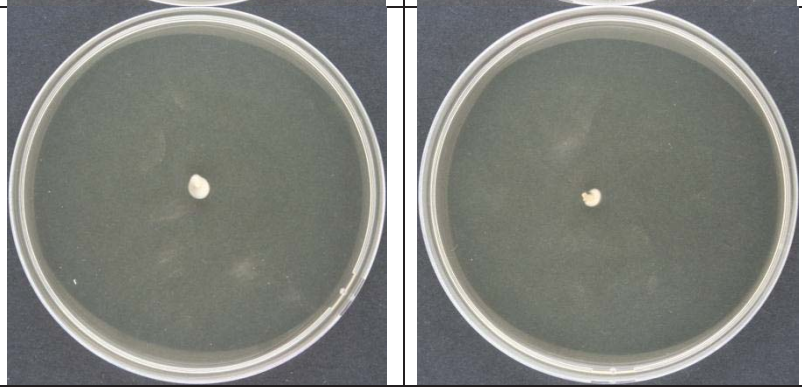
	pYV^+, no Ca^{2+}	pYV^+, with Ca^{2+}	pYV^-, no Ca^{2+}	pYV^-, with Ca^{2+}
Attached cell number (CFU)	2.36×10^6	1.86×10^6	7.8×10^5	2.8×10^5
	2.03×10^6	2.4×10^6	3.6×10^5	2.73×10^5
	1.9×10^6	1.16×10^6	3.03×10^5	1.83×10^5
Mean (CFU)	2.10×10^6	1.81×10^6	4.81×10^5	2.45×10^5
Standard Deviation (CFU)	0.24×10^6	0.62×10^6	2.61×10^5	0.54×10^5

Appendix 18. The motility of all 16 strains.

	24 °C	37 °C
ERL 032122 (biotype 4)		
ERL 032123 (biotype 4)		
ERL 032124 (biotype 4)		
ERL 032125 (biotype 4)		

ERL 072344 (biotype 4)		
ERL 114165 (biotype 4)		
ERL 032126 (biotype 2)		
ERL 104253 (biotype 3)		

ERL 112277 (biotype 3)		
ERL 072345 (biotype 1A)		
ERL 072346 (biotype 1A)		
ERL 072347 (biotype 1A)		

ERL 073947 (biotype 1A)	
ERL 082059 (biotype 1A)	
ERL 093846 (biotype 1A)	
ERL 10460 (biotype 1A)	

Appendix 19. A rapid method for the nonselective enumeration of *Yersinia enterocolitica*, a foodborne pathogen associated with pork.

(Pages 132-134)

Appendix 20. Biofilm formation of *Yersinia enterocolitica* and its persistence following treatment with different sanitation agents.

(Pages 136-140)