Desiccation Survival of *Listeria monocytogenes* in Mixed Biofilms with *Pseudomonas fluorescens*, *Serratia liquefaciens* and *Shewanella putrefaciens*

By

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ABSTRACT

Listeria monocytogenes has been found to withstand harsh environmental conditions including desiccation. The pathogen is also known to form biofilm when in co-culture with other bacteria found in food products. This study investigated the desiccation survival of *L. monocytogenes* in mixed biofilms with *Pseudomonas fluorescens*, *Serratia liquefaciens* and *Shewanella putrefaciens*. To this end, mono- or binary species biofilms were formed and desiccated (43% relative humidity, 21 days at 15°C) on stainless steel coupons and the double Weibull model was fitted to the resulting survivor curves.

The presence of the competitor Gram-negative food spoilage bacteria with the exception of *Sh. putrefaciens* suppressed ($p<0.05$) *L. monocytogenes* during biofilm formation (100% relative humidity, 15°C and 48 h) and subsequently decreased ($P<0.05$) the desiccation survival in *L. monocytogenes* without affecting the resistance of individual cells. Microscopic approaches revealed different biofilm forming capabilities in the mono- and binary bacterial combinations.

LIST OF ABBREVIATIONS AND SYMBOLS USED

$\times g$	Gravity force on Earth
$\Delta Log_{10}(N/N_0)$	Absolute loss of the microbial populations
ABS_{450nm}	Absorbance at 450 nanometer
ABS_{600nm}	Absorbance at 600 nanometer
ASPS	Acid shock proteins
ATR	Acid tolerance response
a_w	Water activity
BHI	Brain heart infusion
BHIA	Brain heart infusion agar
BHIA+erm	Brain heart infusion agar supplemented with (8 or 150 µg/ml) erythromycin
CFU/cm^2	Colony forming unit per square centimeter
CFU/g	Colony forming unit per gram
CFU/ml	Colony forming unit per millilitre
CLSM	Confocal laser scanning microscope
DAPI	4', 6-diamidino-2-phenylindole
dH_2O	Distilled water
EPS	Extracellular polymeric substances

FDA	Food and Drug Administration of the United States
gfp-L. monocytogenes	*Listeria monocytogenes* harbouring the green fluorescent protein (on plasmid)
HSPS	Heat shock proteins
IU/ml	International unit per millilitre
KCl	Potassium chloride
KH_2PO_4	Mono potassium phosphate
Lm 568	*Listeria monocytogenes* 568
M	Molar concentration
mM	Millimolar
MPD	maximum population density
Mu	Mega unit (for antibiotics)
Na_2PO_4	Sodium potassium dibasic phosphate
NaCl	Sodium chloride (table salt)
P	Shape factor
PBS	Phosphate buffered saline
Pen G.	Penicillin G
ppm	Part per million (μg/ml)
PPS	Physiological peptone saline

RH	Relative humidity
RTE	Ready-to-eat
SD	Standard deviation
SEM	Surface scanning electron microscope
SS	Stainless steel
SSR	Starvation survival response
Tm	Melting temperature
TSB	Tryptic soy broth
TSB-glu	Tryptic soy broth supplemented with 1% (w/v) glucose
v/v	Volume per volume
V_{acce}	Acceleration voltage
VRBGA	Violet red bile glucose agar
W	Watt
w/v	Weight per volume
w/w	Weight per weight
α	Logarithmic proportion of two subpopulations 1 and 2
σ^B	Alternative sigma factor
$\boldsymbol{\delta}_1$	First decimal reduction time for subpopulation 1
$\boldsymbol{\delta}_2$	First decimal reduction time for subpopulation 2

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CHAPTER 1 INTRODUCTION

1.1. Introduction

Listeria monocytogenes is a rod-shaped Gram-positive bacterium from the genus *Listeria*. The bacterium usually measures 0.4-0.5 by 1-2 µm and was first described as the causative agent of monocytosis in laboratory rodents in United Kingdom in 1926 (Liu, 2008). Later, the bacterium was also isolated from infected desert rats in South Africa and the name *Listerella* was suggested for this genus in honour of Dr. Lord Lister; however, for taxonomic reasons the name ultimately was changed to *Listeria monocytogenes* (Liu, 2008).

The genus *Listeria* is comprised of eight species including *L. monocytogenes sensu stricto*, *L. ivanovii*, *L. innocua*, *L. marthii, L. rocourtiae, L. seeligeri*, *L. welshimeri* and *L. grayi*. However, normally only the first two species are considered pathogenic (Liu, 2008, den Bakker et al. 2010). Employing different genetic subtyping techniques, *L. monocytogenes* is further classified into three lineages. The lineages are also separated to various serovars or serotypes (both refer to same subject) based on their serological reactions. Most strains from serovars 1/2b, 3b, 4b, 4d, and 4e belong to Lineage I while serovars 1/2a, 1/2c, 3a and 3c are placed in lineage II. The lineage III is composed of serovars 4a and 4c (Liu, 2008).

As for the first human cases, in 1929 *L. monocytogenes* was identified in patients with mononucleosis-like infection in Denmark. Later in 1936, *L. monocytogenes* was determined as the causative agent of sepsis or meningitis in newborn infants and adults in the United States (Liu, 2008). The disease remained uncommon or just not recognized until 1980s where several outbreaks of human and animal listeriosis were discovered in Europe and North America. Finally, it was after a serious outbreak of listeriosis in Halifax, Nova Scotia (1981) that the connection between consumption of contaminated foods and the disease was established (Liu, 2008). Since then, *L. monocytogenes* has been recognized as a food-borne pathogen, which can seriously affect food safety especially for ready-to-eat (RTE) foods in situations where sanitation and/or food preservation is

inadequate. *L. monocytogenes* presents a serious concern in the production of RTE foods; possibly due to the fact that the cold-tolerant bacterium survives many of hurdles commonly used in food preservation (e.g., low temperature, high salinity, low pH, low a_w and so on) and will resume growth once the conditions become suitable again. The contaminated food then acts as a vehicle for the pathogen to enter the human body where it can cause serious infections. Individuals with an impaired immune system, infants, elderly people and pregnant women belong to the groups most at risk of contracting listeriosis; although the pathogen may also affect healthy people if the level of contamination exceeds the infectious dose (Ray, 2004; Liu, 2008). The infective dose of *L. monocytogenes* varies based on the strain and susceptibility of the affected individual, however, for the at risk groups it is believed to be fewer than 1000 bacteria (FDA, 2012). Encephalitis, abortion and septicemia are severe illnesses that have been linked to the listeriosis infection (Liu, 2008). Milder symptoms of gastro-enteritis have also been linked to exposure to *L. monocytogenes* in healthy adult people.

The explosion in the size of the population in various countries and the ensuing increase in demand for food, particularly minimally processed RTE foods, has increased the number of listeriosis outbreaks during recent years (WHO, 2007). The high costs associated with huge recalls of contaminated food products and the human health risk have caused food microbiologists to devote considerable research efforts into finding ways to prevent the contamination of food products with this food-borne pathogen (WHO, 2007). The continued threat of food-borne listeriosis outbreaks was exemplified by the 2008 Maple Leaf outbreak in Canada where contaminated meat slicers harbouring *L. monocytogenes* biofilms cross-contaminated several types of RTE luncheon meat products. Subsequently consumption of these products led to 56 confirmed cases of human illness and 22 deaths (Weatherill, 2009). This outbreak served as a severe wake-up call to the industry, regulators, food inspection system and consumers and clearly demonstrated that current food processing and sanitation practices are inadequate for the control of *Listeria* contamination, biofilm formation, survival and growth.

L. monocytogenes can colonise food processing plants resulting in the same genotype being re-isolated for years despite cleaning efforts and periods with inactivity

(Wulff et al. 2006). Survival of bacterial pathogens on food contact surfaces increases subsequent cross-contamination of foods (Midelet et al., 2006; Rodriguez et al., 2007; Keskinen et al., 2008). It was recently shown that *L. monocytogenes* can survive desiccation for three months in a simulated food processing environment (Vogel et al., 2010). As persistence may be described by the relationship between survival/growth and removal (Carpentier & Cerf, 2011), it is tempting to hypothesize that the bacterium's persistence in food plants may be related to desiccation tolerance.

In the food processing environment, the food-borne pathogen can be found either as planktonic cells (single cells) or as attached cells in biofilms (Gandhi & Chikindas, 2007). The *L. monocytogenes* biofilms can be described as a community consisting of a biologically active matrix of bacterial cells that are adhering to an abiotic or biotic surface and enclosed in the extracellular polymeric substances (EPS) (O'Toole et al., 2000). The EPS will affect the conduct of heat, mass and momentum at the substratum. Changes in transport rates can affect the performance of industrial equipment (fouling), reduce the effectiveness of biocide treatments (sanitation and disinfection) and create exclusive niches within biofilm for proliferation of diverse microbial species (Characklis et al., 1990). It has also been reported that the EPS can protect the bacteria from dehydration due to its water holding capacity (Kumar et al., 1998). Other physical, chemical and biological parameters such as salt concentration (Vogel et al., 2010), temperature (Berry et al., 2010), food-soil (Vogel et al., 2010; Takahashi et al., 2011), biofilm (Truelstrup Hansen & Vogel, 2011) and genetic factors (Schnider-Keel et al., 2001; Stockwell et al., 2009) have also been found to be associated with listerial resistance to desiccation. However, it is not known how *L. monocytogenes* behaves when desiccated in mixed biofilms consisting of the bacterium in co-culture with important Gram-negative spoilage bacteria, e.g., those common in seafood and meat products including *Pseudomonas fluorescens*, *Serratia liquefaciens* and *Shewanella putrefaciens* (Carpentier & Chassaing, 2004; Girard, 2004).

Pseudomonas fluorescens belongs to the genus *Pseudomonas*, which are Gram-negative psychrotroph motile rods (0.5×5 μm) (Ray, 2004). The bacterium is ubiquitous in nature and due to its capability to metabolize a wide variety of carbohydrates, proteins

and lipids in foods; it is recognized as an important aerobic food spoilage bacteria (Ray, 2004). No food-borne infection of *P. fluorescens* has been reported so far. Nevertheless, due to its opportunistic nature several outbreaks of *P. fluorescens* have been reported in the United States, including a multistate outbreak in 2005 where a heparinized saline flush was found to have been contaminated with *P. fluorescens* and to be the causative agent of blood infection (bacteremia) in exposed individuals (Gershman et al., 2008).

Serratia liquefaciens is a small motile Gram-negative coccoid to rod-shaped (0.5×1.5 μm) bacterium that belongs to the *Serratia* genus within the family of Enterobacteriaceae (Ray, 2004). The microorganism is widespread in nature and essentially known as a food spoilage bacterium (Ray, 2004). Infections of humans with *S. liquefaciens* have been reported, including cases of transfusion-related septicemia (Roth et al., 2000), ophthalmological infections due to the contaminated contact lenses (Pinna et al., 2011), post-operational infections in surgery departments (Dubouix et al., 2005) and hemodialysis centers (Sullivan, 2001). In food industry, however, the main concern is its spoilage potential which leads to the loss of good sensory properties upon growth in a wide range of foods.

The motile and rod shaped Gram-negative *Shewanella putrefaciens* is similarly known as a food spoilage bacterium commonly found in aerobically stored seafoods and meat products (Adams & Moss, 2008). The bacterium comes from the family of Shewanellaceae that are particularly common in the aquatic environment. All members of this family reduce trimethylamine oxide (TMAO) to trimethylamine (TMA) as well as nitrate to nitrite and cause the development of off odours and flavours in marine products (Nollet & Toldra, 2011). They are also known to synthesise H_2S from thiosulfate (Nollet & Toldra, 2011). Similar to the above mentioned microorganisms, the bacterium is an unusual human pathogen, although some cases of soft tissue infections and bacteremia have been reported (Pagani et al., 2003). Nevertheless, no *Shewanella* spp. associated food-borne infection has been reported so far.

1.2. Thesis Objectives

The overall aim of this study was to obtain an understanding of the desiccation survival of *L. monocytogenes* when present in binary (dual-species) biofilms with selected Gram-negative food spoilage bacteria (*P. fluorescens*, *S. liquefaciens* and *Sh. putrefaciens*). Microbial behaviour (biofilm growth and desiccation inactivation) in single and dual-species biofilms was observed and mathematically modeled to determine the growth and survival kinetics for each bacterium in mono- and dual species biofilms. Also, the biofilm structures were studied qualitatively using scanning electron microscopy (SEM) and epi-fluorescence microscopy.

The long-term objective of this research is to contribute to the development of improved strategies to eliminate *L. monocytogenes* from the food chain.

The main research question to be addressed in this thesis was: How will the presence of selected Gram-negative food spoilage bacteria influence the kinetics of biofilm growth and subsequently desiccation survival of *L. monocytogenes* on food grade stainless steel surfaces?

Therefore the specific objectives of this work were to:

- Compare biofilm formation kinetics of *L. monocytogenes* in single or binary biofilms with the selected Gram-negative food spoilage bacteria using a non-linear logistic with lag phase growth model.
- Qualitatively study the micro-structure of mono- or dual-species biofilms consisting of *L. monocytogenes* and/or Gram-negative food spoilage bacteria using SEM and epi-fluorescence microscopy techniques.
- Assess the desiccation survival kinetics of *L. monocytogenes* in single and binary biofilms with *P. fluorescens*, *S. liquefaciens* and *Sh. putrefaciens* using the non-linear double Weibull inactivation model, which assumes the presence of two subpopulations with different sensitivity to a given treatment.

CHAPTER 2 LITERATURE REVIEW

2.1. Importance of *Listeria monocytogenes* in the Food Industry

2.1.1. Food Safety and Outbreaks of Listeriosis

New improved methods of isolation and detection of microbes have allowed for the identification of many previously unrecognized microorganisms as food-borne pathogens, i.e., pathogens that are transmitted by foods and harmful to human health (Meng & Doyle, 1997). Examples of such relatively recently recognized food-borne human pathogens are *Cyclospora*, *Arcobacter butzleri*, Shiga toxin-producing *Escherichia coli*, *Helicobacter pylori*, *Cryptosporidium parvum* and *Listeria monocytogenes* (Meng & Doyle, 1997). Although current food safety protocols based on preventative concepts such as the hazard analysis of critical control points (HACCP) are useful tools in protecting foods from contamination; new products, processes, handling and more importantly ever-changing pathogens are likely factors that cause the food-borne diseases to remain a serious public health concern (Meng & Doyle, 1997; Schlech III, 2000).

L. monocytogenes became widely recognized as a food-borne bacterial pathogen after a food-borne outbreak of listeriosis took place in Halifax, Nova Scotia in 1981. Since then many research efforts have focussed on obtaining a better understanding of the ecology, prevalence and epidemiology of this bacterium. The spectrum of disease is so broad that it affects both healthy and the most at risk individuals, i.e., children, immune-compromised individuals, the elderly and pregnant women.The disease symptoms varies from a mild stomach-like flu in healthy adults to meningitis and still-born babies in the vulnerable individuals (Schlech III, 2000). The pathogen is found widespread in nature and has been isolated from fresh-water sediments, sewage, soil, effluents, plant tissues and intestinal tracts of animals and humans (Farber & Peterkin, 1991; Meng & Doyle, 1997). This ubiquitous nature allows the bacterium to easily access food products during different stages of manufacturing and find its way into the human body through contact with contaminated foods (Farber & Peterkin, 1991; Meng & Doyle, 1997).

Several foods have been documented as the transmission vehicle of *L. monocytogenes*. Fresh vegetables, dairy, meat and seafood products had all been linked to listeriosis outbreaks (Greenwood et al., 1991; Schuchat et al., 1992; Wilson, 1995; Schlech III, 2000). The first confirmed food-borne listeriosis outbreak took as mentioned above place in 1981 at the Grace Maternity Hospital in Halifax, Nova Scotia, Canada, where the coleslaw prepared from contaminated cabbage was found to be the vector of the pathogen (Weatherill, 2009). Dr. Walter Schlech, who played a major role in the 1981 outbreak investigation and later became a professor in Infectious Diseases in the Faculty of Medicine at Dalhousie University, defined listeriosis as "a disease state with an invasive infection with *Listeria monocytogenes* not just limited to colonization of the gastro-intestinal tract by the organism" (Weatherill, 2009). Since then, numerous listeriosis outbreaks have been reported worldwide, with most of these involving foods as the main route of transmission.

In France two major outbreaks were reported in 1992 and 1993 due to consumption of the RTE pork products (Meng & Doyle, 1997). Another food-borne outbreak of listeriosis was reported in Italy where rice salad was determined to be the source of infection (Meng & Doyle, 1997). In the United States of America (USA) several listeriosis outbreaks have been linked to contaminated meat and dairy products (Meng & Doyle, 1997; CDC report, 2000). More recently in 2011, consumption of contaminated cantaloupes caused a large outbreak of listeriosis leading to 146 cases of serious illnesses and 30 cases of deaths spread over 28 U.S. states (CDC report, 2011). The Center for Disease Control and Prevention (CDC) has estimated that approximately 2500 cases of serious listeriosis illnesses lead to 500 deaths occur in the USA each year. In Canada, the number of food-borne listeriosis illnesses has increased from 85 cases in 2003 to 239 cases in 2008 (Figure 1) (Weatherill, 2009). In 2002 and 2008 two foodborne outbreaks took place in Quebec where contaminated dairy products were traced as the causative agent of listeriosis (Weatherill, 2009). This was in addition to the 2008 Maple Leaf outbreak mentioned above, that involved luncheon meats.

Table 1. Foods associated with listeriosis outbreaks (CFIA, 2012; Liu, 2008)

Meat and fish	Vegetables	Dairy products
Deli meat (cold cuts or RTE)	Coleslaw salad	Soft and semi soft cheese (Brie,
Hot dogs	Fresh-cut vegetables	camembert, blue-veined cheese)
Refrigerated pate and meat	(lettuce, mushroom,	Milk (unpasturized)
spreads	onion, shelled peas,	Ice cream/soft cream
Cooked chicken	etc.)	Butter
Turkey frankfurters	Salted mushroom	
Sausages	Alfalfa sprouts	
Refrigerated smoked seafood	Raw vegetables	
products	Pickled olives	
Shellfish	Rice salad	
Shrimp	Cut fruits (e.g.,	
	cantaloupe)	

The common denominator among most cases of listeriosis is the involvement of refrigerated and lightly processed RTE foods that are consumed without prior cooking or reheating, and allows for growth of the bacterium (Meng & Doyle, 1997).

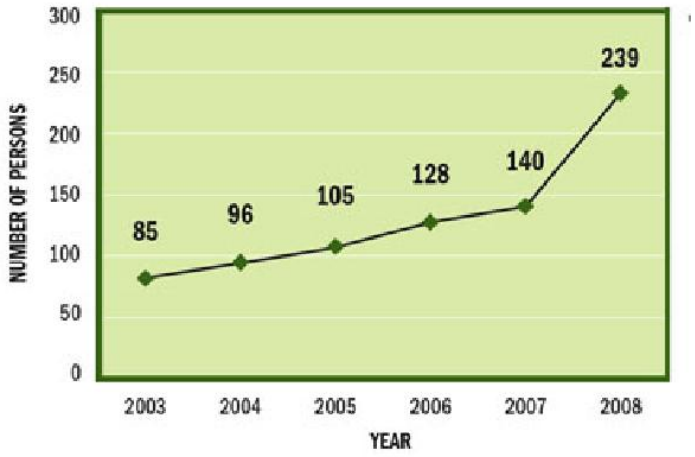

Figure 1. Reported cases of listeriosis in Canada. Cases in 2008 are preparatory and may change. The graph encompasses the 57 cases associated with the 2008 outbreak (adapted from Weatherill, 2009, with formal permission attached in Appendix B).

2.2. Processing Hygiene and *L. monocytogenes* Cross-Contamination of Food Products during Processing

The occurrence of *L. monocytogenes* greatly increases as foods pass from the farm and through the food processing plants generally because of cross-contamination (Thevenot et al., 2006). In fact, the food processing premises appear to be the basic provenance of food contamination by *L. monocytogenes* (Kathariou, 2002). Due to the ubiquitous nature of the pathogen, food processing environments can easily become contaminated from the unprocessed food stuffs that harbour the pathogen. Since the bacterium can become attached to different food contact surfaces and grow at low temperatures, it may then persist in these industries and cause cross-contamination of processed products (Salvat et al., 1995; Carpentier & Cerf, 2011).

L. monocytogenes may survive due to insufficient process inactivation or contaminate the processed foods through direct contact with contaminated unprocessed ingredients, contaminated surfaces and/or infected people where the latter two methods of transfer are recognized as the main routes of cross-contamination (Samelis et al., 1998; Chasseignaux et al., 2001; Reij and De Aantrekker, 2004). Also, poor personal hygiene

and improper hand washing have been identified as main routes of the pathogen's transmission (Thevenot et al., 2006).

Several studies have highlighted the importance of proper implementation of cleaning and sanitizing processes in food processing plants. Sufficient application of disinfectants and sanitizers in combination with mechanical cleaning steps effectively removes both persistent and non-persistent strains of *L. monocytognes* from the food processing environments; nevertheless, improper use of cleaners and/or sanitizers may negatively affect the elimination of the pathogen. Quaternary Ammonium Compounds (QAC), for instance, are disinfectants that due to their non-toxic and non-corrosive nature find common use in food industry; however, the constant exposure has boosted the resistance of some *L. monocytogenes* strains to QACs over the years (Mereghetti et al., 2000). Also, the recommended concentration of sodium hypochlorite that is more than sufficient for elimination of planktonic cells was found to be ineffective on multispecies biofilms consisting of *L. monocytogenes* and other bacteria on stainless steel coupons (Norwood & Gilmour, 2000). In most food processing plants, however, proper cleaning practices followed by sanitizing at correct concentrations on a daily base should effectively eliminate all adhering bacteria from the food processing premises (Norwood & Gilmour, 2000) and therefore, reduce the risk of cross contamination. In this connection, proper design of food processing lines and equipment plays a key role in better application of sanitizing programs (Thevenot et al., 2006).

2.3. *L. monocytogenes* Colonization of and Survival in the Processing Environment

Microbial ecology is a necessary component in studies that concern safety in food processing environments. It is now established that *L. monocytogenes* can colonize most premises and in particular the wet areas in food processing plants (World Health Organization, 2004; Carpentier & Cerf, 2011). In the presence of sufficient organic compounds and enough time for adherence, growth and colonization of the bacteria is very likely. A study in the United States showed that there is a direct link between the

rate of listeriosis outbreaks and colonization of virulent *L. monocytogenes* strains in the production line (Tompkin, 2002).

2.3.1. Competition with the Commensal Microflora and Spoilage Organisms

The growth and colonization of *L. monocytogenes* on solid surfaces in food processing premises may serve as a reservoir for the transfer of the bacteria on to food products which then become contaminated with the pathogen. The ability of *L. monocytogenes* to grow and survive both in the processing environment and on the cross-contaminated food products will depend on the food environmental extrinsic and intrinsic factors including the interactions with the indigenous microflora.

Among several environmental factors that affect the adherence and growth of *L. monocytogenes* on either biotic or abiotic surfaces is the presence of a competitive microflora. For example, the indigenous microflora of minimally processed fresh produce and RTE fresh products such as lettuce and endive are believed to greatly affect the growth and virulence of *L. monocytogenes* (Ryser, 2007). In a study that investigated the effect of a combination of environmental factors on growth of *L. monocytogenes* on endive leaves, Carlin and co-workers (1995) showed that different storage temperatures significantly influenced the growth rate and interaction between the indigenous microflora and *L. monocytogenes*. At 10 and 20°C, listerial growth was similar to that of the natural aerobic microflora while the pathogen's growth was slower at 6 and 30°C (Carlin et al., 1995). The research team subsequently used a chemical sanitizing step to reduce or eliminate the indigenous microflora on the endive leaves before inoculating with *Listeria*. This elimination of background microflora resulted in higher populations of *L. monocytogenes* (increased by 1.5 Log CFU/g) developing on the endive leaves, indicating the role of microbial competition on listerial growth and colonization (Carlin et al., 1996). Finally, it was demonstrated that the pathogen grew to a lower final population size (3 Log CFU/g after 7 days at 10°C) when found in presence of high numbers (10^6-10^7 CFU/g) of indigenous bacteria (Carlin et al., 1996).

An investigation by Li and co-workers (2002) demonstrated that the abuse heating process (at 50 °C) enhanced growth of *L. monocytogenes* on the iceberg lettuce leaves during the subsequent storage at 5 or 15°C. They presumed that the reduction in populations of competing microflora might have led to the enhanced growth of the pathogen, although they did not provide any evidence. Another study indicated that there was a significant correlation between the temperature, native microflora and out-competition of *L. monocytogenes* in RTE foods. Hwang and Sheen (2011) demonstrated that the native microflora on RTE ham reduced the *Listeria* growth at refrigeration temperatures (4-8°C); however, this inhibition declined during temperature abuse storage conditions (10-12°C).

2.3.2. Biofilm Formation and Adverse Effects on Food Safety

Biofilm formation negatively affects many industries including water treatment plants and processing industries such as the food and paper industry (Poulsen, 1999). Upon formation, biofilm can cause energy waste and changes in heat transmission efficiency, blockade of pipes, water reservoirs and cooling towers and corrosion of process equipment (LeChevallier et al., 1987; Poulsen, 1999). Furthermore, in food processing environments biofilm formation is also a potential source of microbial contamination leading to food spoilage, cross-contamination by detachment and transmission of diseases (Wong, 1998).

In general, sessile biofilm microbes are more resistant to disinfectants and sanitizers. Several parameters are involved in this increased resistance, with the delay in the penetration of sanitizing compounds through the biofilm, alterations in growth kinetics and physicochemical properties of biofilm cells in biofilms being the key factors (Blaschek et al., 2007). This particularly creates a significant problem when pathogenic and/or spoilage bacteria survive the cleaning and sanitizing steps to form a biofilm from where cells subsequently detach to cross-contaminate finished products.

Several food-borne pathogens are known to develop biofilms on food contact surfaces such as: *Shigella* spp., *Salmonella enterica*, *Escherichia coli* and *L.*

monocytogenes (Knechtges, 2012). Similar to the other bacteria *L. monocytogenes* can attach to stainless steel (SS), glass, polypropylene and rubber, which are all widely used surfaces in food processing facilities, followed by the development of sanitizer-resistant biofilms (Herald et al., 1988a; Mafu et al., 1990; Helke et al., 1993; Norwood & Gilmour, 1999). These biofilms are resistant to chlorine, iodine, anionic and QACs (Frank et al., 1990). The enhanced resistance poses a major risk in the food industry as even with the common clean in-place (CIP) protocols, the bacteria remain on equipment surfaces (Dunsmore, et al., 1981). With respect to the hazards related to biofilm formation by *L. monocytogenes*, further studies of its biofilm formation as well as the control and removal of biofilms are necessary.

2.3.3. Resistance to Environmental Stresses Including Low Relative Humidity Conditions

L. monocytogenes encounters several harsh environmental conditions during its life cycle in food processing plants. The pathogen's ability to adapt itself to these stresses and cope with the harsh conditions is therefore of great importance to the industry. The microorganism may experience any of following stresses including but not limited to heat, acid, osmotic or low RH conditions during its establishment in a food processing premises. The response of the pathogen to these stresses and its subsequent survival has been the subject of several studies elucidating the role of stress response proteins and the regulatory stress response genes (Liu, 2008).

In the case of lethal or sublethal heat stresses, the expression or accumulation of heat shock proteins will increase inside the cells to protect the microorganism (Liu, 2008). GroES, GroEL, Dnak, HtrA and Clp are some of the heat shock proteins (HSPs) that maintain the integrity of cellular proteins during exposure to the stressful conditions (Liu, 2008). The acid tolerance response (ATR) is another adaptation mechanism that helps the bacterium to cope with acidic conditions. The ATR is initiated after exposure to a sublethal dose of acid and leads to the expression of acid shock proteins (ASPs) and upon the acquisition of acid tolerance, the pathogen now can survive the higher acid concentrations or even pH-levels that are normally lethal to the bacterium (Liu, 2008).

The glutamate decarboxylase system is known to be involved in this stress tolerance (Liu, 2008).

Low moisture or relative humidity (RH) conditions are other stresses that *L. monocytogenes* typically encounters in a food processing plant. Several parameters including presence of organic soils, biofilm, salt, air circulation and RH have been demonstrated to influence the survival kinetics of *L. monocytogenes* when desiccated on SS surfaces (Vogel et al., 2010; Truelstrup Hansen & Vogel, 2011). The bacterial response to exposure to "matric stress", which can be defined as the difference between the water potential of the gaseous phase surrounding the cell and its intracellular water potential, may be closely related to cellular events taking place during "osmotic stress", where the difference in water potential exists between the extra- and intracellular aqueous phases. Factors which affect the resistance of *L. monocytogenes* as well as of other microbial cells during exposure to matric and osmotic stresses, are discussed in more details later in this chapter.

2.4. Biofilm Formation as a Survival Strategy for *L. monocytogenes*

2.4.1. General Characteristics of Biofilms

Biofilms are defined as communities consisting of cells that are adhering to an abiotic or biotic surface and sheltered in the extracellular polymeric substances (EPS) generated by the microorganisms (Costerton, 1987; O'Toole et al., 2000; Davey & O'Toole , 2000). Biofilms can be referred to microbial fouling or biofouling. Biofouling is the undesired establishment of a layer (biofilm) consisting of living microorganisms and their catalytic products which partially or totally covers structures in contact with liquid media. The biofilm confers several benefits to the embedded bacteria in contrast to the planktonic cells. For example in a biofilm, the survival of bacteria is not dependent upon rapid proliferation since the cells are protected in a selected microenvironment (O'Toole et al., 2000; Davey & O'Toole, 2000). This is of great advantages for microbial cells that are living in environments with turbulent currents such as in aquatic systems (Jefferson, 2004). Also, the biofilm not only increases the resistance of microbial cells to disinfectants, grazing and desiccation (Mah & O'Toole, 2001; Sutherland, 2001;

Jefferson, 2004; Fux et al., 2005; Matz & Kjelleberg, 2005), but also enhances the cell-cell interactions such as horizontal gene transfer and co-metabolism (Jefferson, 2004; Molin & Tolker-Nielsen, 2003; Sørensen et al., 2005.)

2.4.2. Biofilm Formation and Mechanism of Microbial Adherence

In establishing the biofilm and its complex micro-environment on a surface, 4 well-defined steps (i.e., attachment, formation of microcolonies, maturation and finally detachment or dispersal) are involved with the first step being the bacterial adherence to the surface. The conditioning of the surface is of great importance. In presence of bacteria, formation of a biofilm most often occurs along with adsorption of other organic and inorganic compounds such as milk and meat proteins to the target surface (Kumar et al., 1998). The agglomeration of molecules at the solid-liquid junction on food contact surfaces creates the conditioning film (Kumar et al., 1998). The conditioning phenomenon influences the food contact surface properties which in turn affects the ensuing microbial events. Alteration of the physicochemical attributes of the target surface, electrostatic charges and surface free energy are side effects of this phenomenon (Kumar et al., 1998).

Although surface conditioning is a major determinant for the attachment of biomass to the target surface, it is not the only factor. Along with surface conditioning, micro-topography of the surface also plays a key role. Scanning electron micrographs have demonstrated that biofilm formation is enhanced on surfaces with deep channels and crevices that can shelter the microorganisms. Such structures can be found on most surfaces in the food processing environment and therefore add to the risk of accumulation and persistence of spoilage and pathogenic microorganisms. As mentioned above, biofilm formation and development has been reported on all surfaces commonly used in the food industry including Teflon seals and nylon materials, Buna N rubber, glass, aluminum and SS (Blackman et al., 1996; Mafu et al., 1990; Herald et al., 1988 a,b).

While some environmental conditioning factors favour bacterial adherence, some biological agents act to inhibit this process. In a study by Al-Makhlafi and colleagues

(1995) it was shown that adsorption of certain soluble macromolecules can negatively affect the attachment of microorganisms. The authors demonstrated that albumin-containing protein films decrease the level of *L. monocytogenes* adhesion due to their passivating characteristics and changes in the surface hydrophobicity. In a similar study by Helke and co-workers (1993), the effect of milk and individual milk components including casein, α-lactalbumin and β-lactoglobulin on attachment of *L. monocytogenes* and *Salmonella* Typhymurium to SS and Buna N was investigated. In spite of their high affinity for attachment to these two surfaces in the presence of phosphate buffered saline (PBS), *L. monocytogenes* and *S.* Typhimurium showed significant reduction in CFU/cm^2 when the target surfaces (SS and Buna N) were treated by whole milk and individual milk components prior to the attachment experiment (Helke et al., 1993). The results of both these experiments illustrated that adherence process involves the interactions between the bacterial cell, the surface and the surrounding microenvironment (surface conditioning). Bacterial motility along with diffusion forces also plays a role in the adherence process. These factors determine whether the adhesion process is active or passive (Kumar et al., 1998). Hydrophobic interactions along with electrostatic and van der Walls forces are the natural forces that govern the initial weak adhesion process (Kumar et al., 1998; Marshall, et al., 1971). This initially reversible phase is characterized by an equilibrium between the electrical double layer repulsion energies, distinct electrolyte concentrations and the van der Waals adsorption forces (Kumar et al., 1998; Marshall et al., 1971). During this stage, microorganisms still show Brownian movement and can easily be removed by the fluid shear forces, e.g., merely by rinsing (Marshall, et al., 1971).

After the first reversible adherence has taken place with or without conditioning of the surface, the next step in biofilm formation is the time-dependent irreversible adhesion (or attachment) of microbial cells to the target surface (Kumar et al., 1998). In this stage although repulsive energies still challenge the adhesion of microorganisms to the target surfaces, other forces come into play and aid in the irreversible adhesion of the cells. In a study carried out by Jones and colleagues (1983), it was found that fimbriae which constituted the proteinaceous products play the most important role in non-

reversible adhesion of the studied bacteria to the target surface. Other scientists contemplated the influence of other elements on the adhesion process and found that flagella, pili, EPS and fibrils (biological factors) along with hydrogen, ionic and covalent bonds, dipole-dipole and hydrophobic interactions and some environmental factors such as pH, and temperature (chemo-physical factors) all influence the irreversible bacterial surface adherence (Marshall et al., 1971; Stanley 1983; Herald et al., 1988a; Ronner et al., 1990; Hood et al., 1995; Gorski et al., 2003; Mai et al., 2007).

In presence of enough nutrients on the conditioned surface, the irreversibly attached microorganisms will commence to grow towards different directions and thereby expand their surface coverage (Characklis et al., 1990). In this process bacterial EPS, fimbriae and flagella aid in the stabilization of the newly settled microcolonies and protect them from fluctuation of the environment (Characklis, et al., 1990; Kumar, et al., 1998). Early on, it was also observed that different nutrient conditions would affect the EPS production by the bacterial cells and therefore the development of biofilm (Ombaka et al., 1983; Uhlinger et al., 1983).

The next step in biofilm formation involves the production of complex three dimensional architectural structures, pores and redistribution of microbial cells far from the substratum. At this maturation phase, production of the chemically diverse biosynthetic EPS is essential for the formation of a mature biofilm and may also serve to entrap other planktonic bacterial cells (Kumar et al., 1998; Stoodley et al., 2002). The structure and architecture as well as the composition of microbial biofilms may be influenced by a multitude of environmental factors that will be discussed in more details later in section 2.4.4.

2.4.3. Detachment and Dispersal of Biofilms

The final step ensuing maturation of a biofilm is the detachment of cells and other particles from the biofilm (Characklis et al., 1989). The cause of detachment can be divided into four distinctly different processes: Grazing or predator harvesting, erosion which is the continuous detachment of small particles from the surface of the biofilm,

abrasion which is caused by the collision and/or rubbing together of particles and sloughing that is the periodic loss of large patches of biofilm (Characklis et al., 1989). Several studies were conducted to evaluate the factors that affect the detachment of bacterial cells from the biofilm. Among these factors are the nature of the substratum (Smoot et al., 1998), the shape of microbial cells (Gómez-Suárez et al., 2001), the existence of a conditioning film (Busscher et al., 1995) and microbial growth rate (Poimenidou et al., 2009), flow regime (turbulent vs. laminar direction vs. no flow) (Boyle & Lappin-Scott, 2007), boundary layers (planktonic cells yield) (Bester et al., 2009), and shear stress (Picioreanu et al., 2001; Choi & Morgenroth, 2003). It is only recently that the influence of biofilm cell detachment on the cross-contamination of foods was investigated focussing on *L. monocytogenes* (Midelet et al., 2004). Poimenidou and colleagues (2009) recently evaluated the detachment of *L. monocytogenes* cells from SS coupons and found that the transfer of the attached cells of *L. monocytogenes* to equipment surfaces may pose great risk due to possible subsequent multiplication in foods. In their report, they proved that the detached cells of *L. monocytogenes*, although old or injured, can still pose an important public health threat to the food industry. They also concluded that detached cells may express higher tolerance to the stress than cell in suspension (Poimenidou et al., 2009).

2.4.4. Effects of Environmental Factors on the Development of Biofilm

As previously discussed, most bacterial species go through well-defined developmental phases in biofilm formation which include i) initial adherence to a surface followed by ii) the development of microcolonies, iii) maturation of microcolonies into an EPS-wrapped biofilm and finally iv) detachment and dispersal of biofilm. The formation of biofilms begins when bacteria sense specific environmental changes (Davey & O'Toole, 2000). The impact of environmental parameters that govern this transition differ remarkably among organisms; however, there is general agreement that these factors initiate signalling pathways leading to the passage between planktonic and biofilm growth (Davey & O'Toole, 2000).

Many studies have focussed on the effect of temperature on adherence of microorganisms to the food contact surfaces. Most scientists believe that the microorganisms' growth at optimum temperature increases biofilm formation (Helke et al., 1993; Mai et al., 2007; Margesin, 2009).

In a recent effort by Pan and others (2010), the correlation of temperature, sodium chloride and glucose (as nutritive environmental factors) and their simultaneous impact on biofilm formation by *L. monocytogenes* was investigated. The authors demonstrated that biofilm establishment by *L. monocytogenes* is generally enhanced with rising temperatures at defined levels of salt and sugar, suggesting that the increased cell surface hydrophobicity at high temperature (e.g., 37 °C) along with more generation and secretion of EPS in response to temperature and other environmental parameters may enhance the initial cell adherence leading to the higher biofilm density (Pan et al., 2010).

In other studies the effect of temperature alone or in combination with other environmental factors such as pH, sodium chloride and sodium nitrate (as commonly used food preservatives) was investigated and it was concluded that irrespectively of the other factors temperature always played a major role on bacterial growth, biofilm formation and microbial activity (Buchon et al., 2000; Mai et al., 2007).

The effect of available water on bacterial growth and activity has drawn the interest of researchers for years. In media with low osmotic pressure (i.e., high water activity) water is freely available to the microorganisms, but in media with high content of water binding solids (i.e., lower water activity) or in solid-air interfaces access to water becomes a key parameter in determination of the potential for microbial growth and biofilm formation. On food commodities surrounded by air with a RH of 60 % or less, microbial growth and hence biofilm formation does not occur. The RH must be nearly 70% or higher to permit for the growth of molds and they must be even higher to allow growth of yeast (80%), Gram-positive and eventually Gram-negative bacteria (85-95%) (Rahman et al., 1999; de Goffau et al., 2009). The concept of RH is directly related to water activity (a_w) since the a_w of an object in equilibrium with its surroundings is equal to the RH divided by 100. In the food industry a_w is known as one of the main

environmental parameters that govern microbial growth along with temperature and pH (Baranyi et al., 2004).

Giaouris and co-workers (2005) examined the effect of environmental factors, including a_w on biofilm formation by *Salmonella enterica* on SS. They showed that an a_w of 0.94 (10.5 % NaCl) clearly inhibited the adhesion of cells to the SS coupons while at the optimum growth conditions (20°C, pH 7.4 and 0.5% NaCl, i.e., higher a_w) adhesion and biofilm formation was so potent that caused ordinary cleaning methods to become inadequate (Giaouris et al., 2005).

As stated before, adhering microorganisms sheltered in biofilms or microorganisms hidden in cracks or crevices can escape cleaning and disinfection procedures and become the source of cross-contamination of foods during processing (Hilbert et al., 2003). Since the substrate composition has major impact on microbial growth and therefore biofilm development, an understanding of how substrate properties affect the adhesion of bacterial cells may help in designing or modifying substrates that are inhibitory to bacterial biofilm formation (Flint et al., 2000).

The perceived correlation between surface topography and bacterial adherence has received the attention of regulatory authorities leading to regulations in regards to acceptable use of different types of surfaces in food processing plants (Flint et al., 1997). However, the standpoints vary considerably among researchers as to the influence of surface roughness, bacterial adherence and growth. While some researchers have reported a positive correlation between increased surface roughness and adherence (Boulange-Petermann et al., 1997; Radford et al., 1998; Taylor et al., 1998), retention of microorganisms and cleanability of substrates (Holah et al., 1990; Bollen et al., 1997; Verran et al., 2001), others reported no direct relation between the surface roughness and microbial adherence (Tide et al., 1999; Flint et al., 2000; Rodriguez et al., 2008). Flint and colleagues (1997) suggested that other factors such as bacterial species, method used to detach bacteria from the surface, surface physicochemical characteristics and the bulk fluid phase would influence the impact of different surface integrity profiles on bacterial adherence.

In a natural environment, fluctuation in availability of nutrients is likely. To survive prolonged periods of nutrient limitation, bacteria such as *L. monocytogenes* deploy a physiological adaptation to insufficient nutrient conditions called starvation survival response (SSR) that enables persistence and survival in the environment until the conditions become permissive for growth again (Watson et al., 1998; Herbert et al., 2001; Lungu et al., 2010). Several studies have shown that the alternative sigma factor, SigB, contributes to prompt adaptation, survival and resistance in Gram-positive bacteria such as *Bacillus subtilis*, *Staphylococcus aureus* and *L. monocytogenes* (Hilbert et al., 2003; Lungu et al., 2010). In particular, the ability to adapt to nutrient limitations may contribute to *Listeria*'s survival and persistence in nature and food production premises and to subsequent cross-contamination (Lungu et al., 2008). External stresses such as nutrient limitation are shown to increase the bacterium's resistance to other stresses such as low pH, heat and oxidative stress possibly through the activation of the SigB regulon (Herbert et al., 2001; Ferreira et al., 2003; Lungu et al., 2008; Lungu et al., 2010). Synthesis of proteins needed for cell maintenance and viability appears to be an important part of the effective response to nutrition deprivation (Helloin et al., 2003). Considering that the process of protein synthesis is energy dependent, it was postulated by Lungu and others (2010) that *L. monocytogenes* has to remain metabolically active to express an effective SSR during the stress. As a result even in stress conditions (in this case nutrition deprivation) *L. monocytogenes* may be metabolically active and remain infectious.

2.4.5. Extracellular Polymeric Substances in Biofilm

Biofilms are composed of two major components: microbial cells and the EPS. Thus the biofilm can be thought of as an organic polymer gel with embedded living organisms. The gel (EPS) has properties that influence the transport of momentum, heat and mass in the substratum. Due to the water binding capacity of many types of EPS, biofilm bacteria may be protected from dehydration due to extra water found in the EPS (Kumar et al., 1998).

Roberson and colleagues (1992) investigated the relationship between desiccation and production of EPS using *Pseudomonas* spp. isolated from soil. Although they could not confirm the role of EPS in protecting microorganisms from desiccation, they provided evidence in support of this. Measuring water potential changes using a pressure plate apparatus (Soil Moisture Equipment Corporation, Santa Barbara, CA, USA), they showed that the EPS provided a microenvironment that held water and caused the water loss to be slower than the loss from the EPS-free surroundings. They hypothesized that the bacteria respond to desiccation stress by channeling energy and nutrients into production of water binding extracellular polysaccharides. Jain and colleagues (2005) also reported the enhanced desiccation survival in some strains of *Thraustochytrids*, which are group of marine stramenopilan protists known for their EPS production. They suggested that the EPS protected the cells from rapid dehydration and therefore enhanced the desiccation survival in the examined species (Jain et al., 2005)

The role of EPS in growth physiology of the hyperthermophilic archaeon *Thermococcus litoralis* was studied by Rinker and colleagues (1996), who demonstrated that EPS plays a key role in adaptability and biofilm formation by this bacterium when subjected to hostile sulfur-free defined medium (Rinker et al., 1996). In a recent investigation by Chae and co-workers (2006) the production of EPS during *L. monocytogenes* biofilm development was confirmed for the first time. By assessing total sugars produced by *L. monocytogenes* strains during biofilm formation on glass, they concluded that the high level of sugar production by *L. monocytogenes* strains is associated with biofilm formation (Chae et al., 2006). As previously stated, the EPS protects but may also provide and/or help in trapping the nutrients that are necessary for the biofilm growth.

2.4.6. Role of Quorum Sensing

Many bacteria communicate with each other to form structured microscopic groups. This communication results in the agglomeration of signaling molecules in the surrounding environment and enables a single cell to realize the cell density has reached a level that allows the entire population to make a harmonic response (Davies et al., 1998;

Diggle et al., 2007). It is now believed that many of social behaviours of bacteria (phenotypes) such as conjugated biofilm maturation, antibiotic resistance, plasmid transfer, swarming, motility and virulence are controlled by quorum sensing systems found in both Gram-negative and Gram-positives (Figure 2) (Diggle et al., 2007).

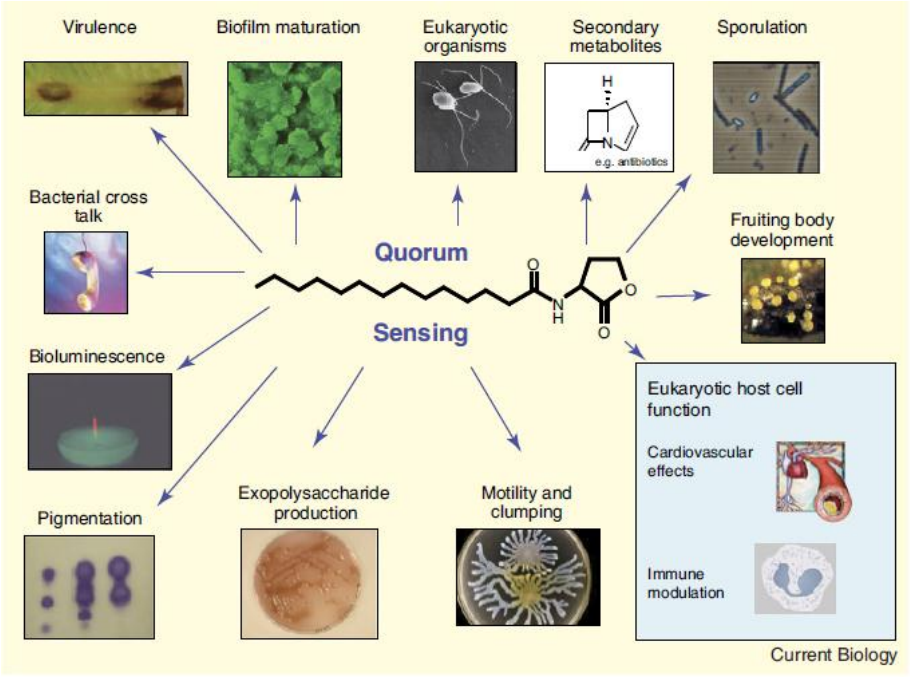

Figure 2. Phenotypic responses initiated by quorum sensing in Gram-negative and Gram-positive bacteria (adapted from Diggle et al., 2007, with formal permission attached in Appendix B).

In past studies, the role of autoinducer-2 (AI-2) as a signaling molecule involved in quorum sensing and its regulatory gene (luxS) in biofilm development has been studied in different bacteria including *L. monocytogenes* (Merritt et al., 2003; Cole et al., 2004; Wen et al., 2004; Parsek et al., 2005; Challan Belval et al., 2006). The Agr system is another quorum sensing mechanism which co-orients population dependent gene expression (Lyon et al., 2004). Its function in *Staphylococci* has been well established (Novick et al., 2003); however, its role in *L. monocytogenes* is being investigated. Recently Riedel and co-workers (2009) investigated the role of the Agr system on global gene expression, biofilm formation, invasion and virulence in *L. monocytogenes* by creating a deletion mutant in *agrD*, which is the structural gene for the putative quorum

sensing peptide. Their results showed that the deletion of the quorum sensing peptide led to defects in global gene expression profiles, less biofilm formation and impaired invasion and virulence response in the *L. monocytogenes* Δ*agrD* strain during both the saprophytic and parasitic lifecycles (Riedel et al., 2009).

2.4.7. Multispecies Biofilms

In natural environments one would expect different microbial cells to aggregate on surfaces leading to the development of a heterogeneous biofilm. In this multispecies community, cells may affect each another positively and in some cases negatively. Interactions that are beneficial in this mutual relationship are those that help in co-aggregation of cells, conjugation and protection from sanitizers (Rickard et al., 2003; Matz & Kjelleberg, 2005; Sharma et al., 2005).

These benefits may induce cooperative biofilm formation among strains that are not able to develop a biofilm alone. In a study by Burmølle and others (2006), it was demonstrated that multispecies biofilms induced synergistic effects that enhanced biofilm biomass and resistance to antimicrobial agents. In that study, four bacteria (*Microbacterium phyllosphaerae*, *Shewanella japonica*, *Dokdonia donghaensis*, and *Acinetobacter lwoffii*) were isolated from the surface of the marine alga *Ulva australis* and investigated for synergistic effects in the multispecies biofilm. Interestingly, a more than 167% increase in the biofilm biomass was observed in the multispecies biofilm which led to the resistance of the biofilm to hydrogen peroxide and tetracycline to increase accordingly (Burmølle et al., 2006). When the four species biofilm was exposed to *Pseudoalteromonas tunicata***,** an antibacterial protein producer, survival was significantly improved compared to survival in single species biofilms (Burmølle et al., 2006). Furthermore, the results of this study indicated that the characteristics of a multispecies biofilm such as biomass and environmental fitness are not necessarily the aggregates of the characteristics of each single biofilm (Burmølle et al., 2006). Similarly, Filoche and co-workers (2004) showed that *Lactobacillus rhamnosus* and *Lactobacillus plantarum*, which are both poor biofilm formers, develop a more mature biofilm when grown in co-culture with *Actinomyces naeslundii* and *Actinomyces gerencseriae*. The

success of individual bacterial species in multispecies biofilm ecosystems in nature, however, depends on the interaction with other competing bacteria and not all the interactions are beneficial to each member of the bacterial community as mentioned before. Competition between *Streptococcus mutans* and *Streptococcus sanguinis* (Kreth et al., 2005), *Candida albicans* and *Candida dubliniensis* (Kirkpatrick et al., 2000) and *L. monocytogenes* and *Lactococcus lactis* (Habimana et al., 2011) are some examples of natural inhibition of one strain by the co-culture partner.

2.4.8. Biofilms Formed by *L. monocytogenes*

It is known that *L. monocytogenes* can survive various harsh conditions either on solid surfaces or in foods. The bacterium may be harboured in niches and remote areas in food processing facilities and once established lead to contamination of the food products and subsequently harm consumers. Persistent strains of the pathogen have been isolated over months or in some cases over years from a same facility (Kathariou, 2002; Blaschek et al., 2007; Carpentier & Cerf, 2011). It is believed that the biofilm formation plays a key role in the bacterium's persistence and colonization of food processing premises.

2.4.9. General Characteristics of *Listeria* Biofilms

Most of our knowledge about the *L. monocytogenes* biofilm characteristics and attributes has been gained from the studies of single species biofilms. Like other bacteria, biofilm formation by *L. monocytogenes* involves initial adherence followed by development of microcolonies, maturation and detachment as the final step; nevertheless, new aspects from the *Listeria* biofilm formation are discovered each year. SEM micrographs taken regularly over 6 h and up to a 7-day period of growth/biofilm formation (37 or 20 °C) have shown the formation of three-dimensional honeycomb structures in well-developed biofilms (Chavant et al., 2002; Marsh et al., 2003); however, at lower temperatures (8°C) *Listeria* only formed a monolayer of cells (Chavant et al., 2002)

As discussed above, biofilm formation begins with adherence of the pathogen's cells to the substratum. The adherence is fairly fast (usually 3-5 seconds depending on the

type of surface) and leads to formation of microcolonies. Although this adherence is reversible at the very early stage, the number of irreversibly adhering cells increases over time (Beresford et al., 2001; Takhistov & George, 2004; Blaschek et al., 2007). It is believed that the flagella are an essential tool for attachment of *L. monocytogenes* to the surface; although the findings vary significantly. While one study showed a 10-fold difference between the attached populations of non-flagellated mutants and the corresponding wild type at 22 °C in the very early stage of adhesion (Vatanyoopaisarn et al., 2000), other reports demonstrated no obvious differences between flagellated and non-flagellated cells (Meylheuc, 2001). Chae and Schraft (2000) found that the rate of initial adherence does not affect the amount of biofilm formed on glass. It appears that other factors are involved in initial adherence of *Listeria* cells similar to reports for other bacteria.

Some studies suggest that the substratum physicochemistry and cell surface charges affect the rate of initial adherence to abiotic (Briandet et al., 1999; Takahashi et al., 2010) and biotic surfaces (Ukuku & Fett, 2002) while others reported no correlation between cell hydrophobicity and adherence (Smoot & Pierson, 1998; Meylheuc et al. 2001; Dykes et al., 2001). Nevertheless, the role of cell wall proteins has been demonstrated in initial adherence of the cells to the tested surfaces (Smoot & Pierson, 1998). It turns out that the conflicting results may be due to types of mediums that were used for the growth of the pathogen such as brain heart infusion (BHI) broth in Meylheuc and co-workers' study (2001) as compared to the tryptone soy broth (TSB) supplemented with 0.6% of yeast extract in the work by Takahashi and others (2010). The interaction between the attractive electrostatic forces of growth medium and the hydrophobicity may be an important determinant of the rate of initial adherence (Dykes et al., 2001).

Following the initial adherence to the surface, *L. monocytogenes* cells begin to generate the EPS. The EPS production varies between different strains of *L. monocytogenes* and this affects the biofilm formation capability of each strain. Borucki and co-workers (2003) found that there is direct correlation between the biofilm-forming capability of each strain and its production of EPS. They examined 80 different strains of *L. monocytogenes* and showed that the strains that form more biofilm generate

remarkably more EPS as well (Borucki et. al., 2003). Beside the innate properties of the cells, some environmental factors also affect the EPS production. The temperature, for instance, is a determining factor in EPS production. Herald and Zottola (1988a) demonstrated that *L. monocytogenes* produces EPS on SS at 21°C but not at 10 or 35 °C. Also, growth conditions are found to play a key role in this phenomenon. Static incubation of the biofilms induces the EPS production while the growth under continuous flow systems negatively affects the process (Sasahara & Zottola, 1993).

As the *Listeria* biofilm gets older, EPS becomes weak up to the point that the whole biofilm will detach from the surface (Blaschek et al., 2007). It is believed that with time, cells begin to consume the EPS as a source of energy and once the EPS was used up, the cells easily detach from the surface (Takhistov & George, 2004).

2.4.10. Effect of Environmental Factors on *Listeria* Biofilm Formation

Temperature, presence of nutrients and food preservatives, acidity and substratum are the primary environmental factors that affect the re-growth and biofilm formation by *L. monocytogenes.* Several commonly used surfaces in food industry have been evaluated for their capability to support adherence of *L. monocytogenes*. Glass, PVC, Buna N and SS have all been shown to be good substrates for *L. monocytogenes* (Beresford et al., 2001). SS in particular has been demonstrated to be the best substratum for the bacterium adherence (close to 5.50 Log CFU/cm^2) (Smoot & Pierson 1998; Meylheuc et al., 2001; Schwab et al., 2005) while Buna N was shown to be inhibitory to *Listeria* adherence (Helke et al., 1994; Smoot & Pierson 1998).

Unlike many other bacteria, *L. monocytogenes* tends to form biofilm when the nutrients are readily available. A comparison between *Salmonella* spp. and *L. monocytogenes* revealed that while *Salmonella* tends to form more biofilm in diluted TSB, the *Listeria* generates more biofilm in the undiluted medium (Stepanovic et al., 2004). Also, it was shown that the bacterium grows toward areas on the surface where more nutrients and lower population densities are presents (Takhistov & George, 2004). It is also worth noting that the role of nutrients is different during the various stages of

biofilm formation. While some compounds such as phosphate or different carbon sources have no effect on the initial adherence, presence of soytone, ascending concentrations of ammonia and descending iron will reduce the primary adhesion (Kim & Frank, 1994). Moreover, different strains of *L. monocytogenes* have varying nutritional requirements perhaps explaining why biofilm formation varies significantly among different strains subjected to different experimental conditions (Moltz & Martin, 2005; Blaschek et al., 2007).

As a psychrotroph, *L. monocytogenes* has been shown to develop biofilm at temperatures ranging from 4 up to 37 °C. The formation of biofilm by the pathogen at refrigeration temperature (4°C) poses a severe risk for food safety since this ability enhances the survival of microbial cells under low temperature conditions. Although the lower temperatures prolong the process of adherence to the surface, biofilm formation is not inhibited. Mafu and colleagues (1990) pointed out that while at room temperature the pathogen adheres to surfaces within 20 min this same process takes 60 min at refrigeration temperature. It is worth noting that lower temperatures does not always have a negative impact as in some cases survival of biofilm cells is sometimes comparatively improved (Bremer et al., 2001). This appears to be due to less evaporation from the biofilms at refrigeration temperature (Helke et al., 1994). Other researchers found that in general 32-37°C is the best temperature range for biofilm formation by *L. monocytogenes* (Duffy & Sheridan, 1997; Djordjevic et al., 2002); although, there are some disagreements over the effect of this temperature range (32-37°C) on the initial bacterial adherence. One reason being the repression of flagella production at high temperatures (37 °C) which in some reports appeared to elicit a role in the initial adherence of *Listeria* cells onto the surface (Vatanyoopaisarn et al., 2000).

Acidity can also affect *Listeria* biofilm formation; however, the influence is mainly observed during the initial stage of adherence. Herald and Zottola (1988a) explained that slightly alkaline conditions (pH=8) induce the bacterial adherence to SS as well as the maximum amount of EPS production. Conversely Smoot and Pierson (1998) reported lower rate of *Listeria* adhesion to Buna-N rubber under alkaline conditions (pH=9) although the detachment of adhered cells was not affected by the medium pH.

In summary, the environmental factors that affect the growth of the bacteria also greatly influence its biofilm formation. Furthermore, individual strains of *L. monocytogenes* and the microenvironment surrounding the cells determine the pathogen's biofilm and EPS production.

2.4.11. Biofilm Formation by *L. monocytogenes* in Competition with Other Bacteria Found in the Food Processing Environment

A variety of environmental factors such as temperature, a_w, pH and RH affect the growth of *L. monocytogenes* in monocultures, however, the effect of other factors such as competing microorganisms may also influence the pathogen's biofilm formation ability in food processing premises. Indigenous microorganisms are often derived from the natural environment of the raw food material and may act as either the primary surface colonizers or later biofilm partners in multi-special biofilms (Kolenbrander, 2000; Blaschek et al., 2007).

One would therefore expect that in natural environments as well as in food processing facilities, *L. monocytogenes* would commonly be found in a mixed biofilm consisting of naturally occurring commensal bacteria (Blaschek et al., 2007). Studies of biofilms consisting of common food spoilage bacteria including *Pseudomonas* spp. and *Flavobacterium* have demonstrated these to significantly increase the adherence of *L. monocytogenes* to the test surfaces (Sasahara & Zottola, 1993; Bremer et al., 2001; Hassan et al., 2004). While some food spoilage microorganisms such as *Pseudomonas putida*, *P. fragi*, *P. fluorescens* and *Flavobacterium* enhance the growth, colonization and biofilm formation by *L. monocytogenes*, other competing strains will negatively impact the pathogen's biofilm formation capability (Blaschek et al., 2007). In presence of antagonistic microorganisms, the maximum population density of *L. monocytogenes* will decrease, although, the magnitude of suppression is influenced by other factors particularly the temperature (Buchanan & Bagi, 1999).

The concept of natural competition is widely being investigated as a potential hurdle for control of food-borne pathogens including *L. monocytogenes*. Lactic acid

bacteria (LAB), for instance, have been shown to effectively inhibit the growth of several food-borne pathogens including *Aeromonas hydrophila*, *L. monocytogenes*, *Salmonella enterica* serovar Typhimurium, and *Staphylococcus aureus* in fresh produce without any negative impact on sensory properties of the foods (Vescovo et al., 1996). In food processing plants, the growth of *L. monocytogenes* on abiotic surfaces may similarly be influenced by bacteriocin producing LAB. In a study by Winkelstroter and co-workers (2011), the bacteriocin-producing bacterium *Lactobacillus sakei* 1 and its neutralized cell-free supernatant containing sakacin 1 was found to inhibit the initial adherence of *L. monocytogenes* to the SS surfaces. This finding agrees with a study looking at the antilisterial effects of bacteriocin-producers such as *Lac. plantarum* and *Enterococcus casseliflavus* which reported that these microorganisms inhibited *L. monocytogenes* adherence to biotic or solid surfaces (Guerrieri et al., 2009).

In another study it was demonstrated that the adhesion and subsequent biofilm formation of *L. monocytogenes* on SS is greatly affected by presence of *Staphylococcus sciuri*. The biofilm of the competitor bacterium prevented the population of adherent *L. monocytogenes* to increase within the joint biofilm; leading to the conclusion that an antagonistic microflora in combination with other hurdles can enhance the control of *Listeria* in the processing environment (Leriche & Carpentier, 2000). The EPS generated by *Staph. sciuri* and competition for nutrients was indicated as the main factors responsible for the observed antagonistic activity (Leriche & Carpentier, 2000).

Overall these findings suggest that the indigenous microflora found in a food processing plant can have major impact on the likelihood of *L. monocytogenes* adherence, colonization and persistence. The exact environmental conditions will determine whether *L. monocytogenes* has the potential to outcompete the background microflora or vice versa.

2.4.12. Control and Removal of *L. monocytogenes* and Other Bacterial Biofilms

Adoption of effective cleaning and sanitation programs and protocols would inhibit the accumulation of bacterial cells on equipment surfaces and their subsequent

biofilm formation (Kumar et al., 1998; Dunsmore et al., 1981). Dunsmore and others (1981) in their review article defined the cleaning system as those practices which maintain the product-contact surfaces of processing equipment in a condition that ensure they do not impair the quality of the food product. Based on this definition, a cleaning system includes a specific sequence of detergents and sanitizers applied by defined physical techniques and complemented by particular conditions between use of the equipment (Dunsmore et al., 1981).

A number of factors are involved in the effectiveness of a cleaning system. Ronner and Wong (1993) showed that the nutrient level of the bacterial growth medium, presence of EPS, type of substratum and species or strain differences all can affect the removal of biofilms by cleaning. Other studies show that good design practices, quality and smoothness of the equipment, proper choice of equipment materials and accessories, process layout and process automation are also critical in the control of biofilm formation and/or inhibition of biotransfer/cross-contamination in food processing equipment such as tanks, pipelines, joints and the accessories (Lelieveld, 1985; Kumar et al., 1998).

In general, three approaches are widely used in the control and removal of biofilms including the physical methods, chemical methods and biological methods (Kumar et al., 1998). But before proceeding to these methods, it seems appropriate to quickly review the role of biofilm on the resistance of bacteria to antimicrobial compounds. It is well-established that bacterial biofilms exhibit an increased resistance to antimicrobial agents compared to planktonic cells growing freely in a suspension (Mustapha et al., 1989; Krysinski et al., 1992). The mechanisms by which the microorganisms in a biofilm evade the inactivation by biocides, disinfectants and antibiotics are of obvious applied interest and are still being discovered. Clearly numerous resistance mechanisms exist by which the embedded biofilm cells can escape from the sanitizing programs. One of these mechanisms is the spatial heterogeneity structure of biofilm which if disrupted; the resistance would be lost (Hoyle et al., 1992). The other parameters involved in this resistance were early on thought to be reduction of effective diffusion coefficient of solutes in biofilms in comparison to pure water (Stewart et al., 1998), reduction of inherent mobility of antimicrobial agents within the biofilm

because of the 3-dimensional structure of biofilms (Dodds et al., 2000), less susceptibility of slow-growing or starved cells within biofilm (Holah et al., 1990; McFeters et al., 1995), age of bacterial cells within biofilm (Lee et al., 1991; Frank et al., 1990) and production of microbial degradation enzymes, e.g., beta-lactamases (Giwercman et al., 1991) that are responsible for enhanced resistance of bacterial cells to beta-lactam antibiotics such as penicillin (Livermore, 1995).

2.4.12.1. Physical Removal Methods

So far, a variety of physical methods have been tried to remove or at least control the biofilm formation. Most of the physical techniques used for this purpose are based on electrical and sound currents methods such as ultrasound (Oulahal et al., 2004), pulsed magnetic fields (Benson et al., 1994), low-voltage pulsed electrical fields (Perez-Roa et al., 2006), high voltage Arc discharge and high-intensity pulsed electrical fields (Rastogi, 2003).

In a study by Perez-Roa and colleagues (2006), the application of low-voltage pulsed electric fields as an anti-biofouling technology was investigated for the first time. In that experiment, the researchers used a test platform consisting of micro scale electrodes which facilitated the creation of high strength electric fields while maintaining the applied voltage below 5 V (Perez-Roa et al., 2006). The test organism was an environmental strain of *P. aeruginosa* isolated from nitro aromatic contaminated waste (Perez-Roa et al., 2006). The authors concluded that the low voltage pulsed electric fields method, in some cases, reduces biofouling formation depending on the applied duty ratio, i.e., percentage of pulsing time over one cycle (Perez-Roa et al., 2006). They showed that low duty ratios would inhibit biofilm formation; however, the mechanism is not completely understood and is the subject of continuous investigation at the authors' laboratory.

2.4.12.2. Biological Removal Methods

Biological techniques generally are applied to the biocontrol of biofilm formation by employment of different means (Kumar et al., 1998). Among the old methods in this context are bacteriocins. Many strains of starter culture bacteria have been reported to produce metabolites that are inhibitory to other bacteria, including *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* (Ray, 1992). Researchers in the past century recognized that some strains of *Streptococci* from group N (now called *Lactococcus*) produce proteinaceous inhibitors that are effective against the growth of anaerobic spore-forming bacterial species (Ray, 1992). To date, numerous reports have been published on the production, purification, properties, assay, antimicrobial spectrum and mode of action of nisin, which is an acidic anti-microbial peptide with post-translational modified sulfur-containing amino acid effective against Gram-positive bacteria including *L. monocytogenes* and *Clostridium botulinum* (Ray, 1992). In a recent report, the surface application of 10^3 IU/ml nisin solution was shown to reduce the biofilm formation on SS coupons by 5.6 CFU/cm^2 (Minei et al., 2008). In that study, nisin was found to totally inhibit biofilm formation during the first 9 h of incubation at 37°C; however, the effect was lost after 24 h with growth resuming and persisting for the duration (48 h) of experiment (Minei et al., 2008). Although, the short term positive effect of nisin on control of biofilm formation by *L. monocytogenes* was shown, the anti-listerial activity was lost after longer periods (Minei et al., 2008). The advantage of applying nisin as a bio-reagent in the control or removal of *Listeria* biofilm from food processing premises may be limited due to its high price and short impact time; nevertheless, the concept of applying natural antimicrobials to surfaces to inhibit *L. monocytogenes* biofilm formation may hold promise for future inventions.

Enzymes have also proven to be effective in biofilm control and inactivation. Essentially enzymes can aid in the removal of extracellular polymers which form the biofilm matrix and thus help in removal of microbial biofilms (Potthoff et al., 1997; Lequette et al., 2010). Using a micro titer plate assay to screen seven proteases and polysaccharidases for removal of biofilms made by 16 species belonging to *P.*

fluorescens and *Bacillus cereus* group, Lequette and colleagues (2010) showed that application of a combination of enzymes that target several components of EPS (such as proteases and polysaccharide degrading enzymes) together with dispersing and chelating agents would be an efficient alternative to ordinary methods of cleaning. For *L. monocytogenes* where the extracellular DNA (eDNA) constitutes a major part of the biofilm matrix, Harmsen and colleagues (2010) showed that treatment of *Listeria* biofilms grown in dilute BHI (2.5% v/v at 37°C) with DNase I decreased the biomass by 84.3%.

2.4.12.3. Chemical Removal Methods

For better elimination of microorganisms and their corresponding biofilms by disinfectants, it is critical to detach as many microorganisms as possible from the surface. Therefore, the first role of chemical cleaners should be to dissolve the EPS and release the cells from the surface to the bulk liquid phase so the free cells are now more susceptible to the subsequent sanitizers (Blaschek et al., 2007). In cases where the EPS matrix remains intact, the chemical sanitizers should ideally be able to penetrate the underlying cells and biologically inactivate them in a way to ensure the successful removal of *Listeria* from the food processing premises.

An integral part of cleaners is chelating agents (sequestrants) that decrease the water hardness via binding minerals (Blaschek et al., 2007). Presence of chelating agents like Ethylenediaminetetraacetic acid (EDTA) and ethylene glycol-bis (β-aminoethyl ether) n,n,n',n'-tetra-acetic acid (EGTA) helps in the removal of biofilms by binding magnesium and calcium ions and destabilizing the outer membrane of the cells (Turakhia et al., 1983; Hood et al., 1995). Recently another function for EDTA has been introduced where low concentrations (0.1 mM) were found to be inhibitory to the initial adherence of *Listeria* cells onto a poly vinyl carbonate (PVC) surface (Chang et al., 2012). The observed phenomenon was not attributed to the chelating function of EDTA but rather to its putative effect on cell-cell and cell-surface interactions (Chang et al., 2012).

Other detergent ingredients are bactericidal, while other may even depolymerize the EPS, which taken together would promote the detachment of biofilms from the surface (Kumar et al., 1998). Examples of effective detergents are oxidants such as peracetic acid (Holah et al., 1990; Fatemi & Frank, 1999; Aarnisalo et al., 2007; van der Veen & Abee, 2010), chlorine (Characklis et al., 1990; Bremer et al., 2002; Belessi et al., 2011), iodine (Cargil et al., 1992) and ozone (Baumann et al., 2009)

Several studies have been conducted to evaluate the impact of different sanitizers on inactivation and removal of listerial biofilm. A study by Ronner and Wong (1992) showed that chlorine and anionic acid sanitizers generally remove extracellular material from *L. monocytogenes* biofilm better than iodine and quaternary ammonium detergent sanitizers. However, they found that the resistance to sanitizers is strongly influenced by the type of surface. Oh and Marshall (1992, 1993) in a joint effort found that monolaurin (glycerol monolaurate) is inhibitory against *L. monocytogenes* when applied at low concentrations. In a later study, they also demonstrated that monolaurine (50 μg/ml) combined with heat treatment at 65 °C for 5 min completely destroyed the biofilm formed by *L. monocytogenes* (Oh & Marshall, 1996).

Recent sanitizing strategies focus on the combination of physical and chemical removal techniques where first the spatial organization of EPS structure in *L. monocytogenes* biofilm is destroyed followed by chemical inactivation of released cells by use of effective sanitizers. Berrang and colleagues (2008) investigated the application of chemical sanitizers with or without ultrasonication on *L. monocytogenes* biofilms in PVC pipes. They reported that a 30 s application of ultrasonication (20 kHz & 750 W) before the sanitizing step improves the efficacy of listerial biofilm removal and inactivation by 26, 15 or 36% following sanitation in quaternary ammonium compound (quat), peroxide or chlorine solutions, respectively (Berrang et al., 2008).

2.5. Desiccation Tolerance and Survival in the Processing Environment of Pathogenic and Spoilage Bacteria

Water is essential for all living organisms since most of the chemical and molecular interactions only take place in presence of liquid water. Nevertheless, organisms often face conditions that remove the water available to cells through evaporation (air drying) or dispersion (osmolysis) and depending on the severity of conditions, the cells must survive or die (Storey & Storey, 2000). Organisms including bacteria, fungi, yeasts and certain plants and animals have specific mechanisms that enables them to survival such harsh environmental conditions. The understanding of mechanisms that permit desiccation tolerance particularly in bacteria may suggest new methods for control and removal of food-borne pathogens from the food chain.

2.5.1. Desiccation Tolerance in *L. monocytogenes*

Traditionally it was believed that the dry conditions in a food processing plant serve to ensure food safety and proper hygienic conditions. Therefore, industrial efforts have focused on keeping the food contact surfaces dry. However, several bacteria strains including *L. monocytogenes* have been isolated repeatedly from food contact surfaces that were sanitized and maintained in dry conditions (Møretrø & Langsrud, 2004; Gudmundsdóttir et al., 2006; Keto-Timonen et al., 2007). This realization has subsequently triggered studies into the mechanisms and kinetics of desiccation survival in *L. monocytogenes* when subjected to conditions resembling those found in the processing environment.

Several studies have been conducted to find the mechanisms by which *L. monocytogenes* can continue to exist under either dry air (desiccation stress) or hyperosmotic stresses as defined in section 2.3.3. It is likely that several parameters are involved in the bacterial tolerance to these water stresses, some of which are related to environmental conditions while others relate to the biology and genetics of the cells.

The role of some of the environmental factors such as bacteriological substrate, food soils, salt, air circulation and moisture content on desiccation survival of *L.*

monocytogenes has previously been investigated (Vogel et al., 2010; Truelstrup Hansen & Vogel, 2011; Takahashi et al., 2011). Vogel and colleagues (2010) investigated the effects of substrate, organic soils, salt, air circulation and RH on survival of persistent and presumably non-persistent strains of *L. monocytogenes* when desiccated on SS. They demonstrated a positive correlation between the presence of increasing amounts of food soils, complex growth substratum and salt on desiccation survival of *L. monocytogenes* (Vogel et al., 2010). However, no significant difference was observed between the desiccation tolerance of persistent or presumably non-persistent listerial strains (Vogel et al., 2010). In the study of Takahashi and co-workers (2011), the pathogenic bacteria (*L. monocytogenes, Staph. aureus* and *S.* Typhimurium) were inoculated (10^7 CFU/coupon) onto SS coupons previously coated with organic soils (minced tuna, ground pork, and cabbage) and stored up to 30 days inside centrifuge test tubes at 25°C. The results showed that presence of food soils increased the tolerance of all bacteria to dehydration. Interestingly, *L. monocytogenes* showed greater resistance to dry conditions as compared to the other strains. Moreover, the maximum desiccation tolerance was observed during the first 14 days after which the survival decreased as the end of the desiccation period approached (Takahashi et al., 2011).

Osmo-adaptation is also known to enhance desiccation survival of *L. monocytogenes*. After 23 days of desiccation at 43% RH, the osmoadapted bacteria cells (the *L. monocytogenes* cells that had been grown in TSB-glu with 5% NaCl) showed only 1.4 and 1 log reduction during desiccation in low or high initial salt level broths, respectively, while the non-osmoadapted cells decreased by 2 and 1.3 log during desiccation under the same conditions (Truelstrup Hansen & Vogel, 2011). Another finding of this study was that the desiccation survival of biofilm cells was significantly higher than that of non-biofilm cells, strongly suggesting a protective effect of biofilm during desiccation of *L. monocytogenes*. The authors suggested that shifts in cell metabolism, cell envelope composition and/or protective effect of the biofilm EPS matrix might be factors that enhance the bacterial survival under low RH stress conditions (Truelstrup Hansen & Vogel, 2011).

Other factors that affect the desiccation survival of *L. monocytogenes* are accumulation of compatible solutes (osmolytes) such as glycine betaine (GB), carnitine, proline and trehalose (Sleator & Hill, 1999; Dreux et al., 2008; Ells & Truelstrup Hansen, 2011, Huang, 2011). In general, compatible solutes are low molecular weight organic compounds that accumulate at high concentrations in the cytoplasm without disrupting the vital cellular processes and help to maintain the turgor pressure inside bacterial cells under hyper-osmotic or desiccation stress conditions (Holland et al., 2003).

In *L. monocytogenes*, two mechanisms are known to be involved in the accumulation of compatible solutes. The osmolytes can be either synthesized by the cells or taken up from the external environment via transporters (Dreux et al., 2008). The GB, for instance, is transported into the cytoplasm through secondary transporters BetL or GbuABC, where the latter is an ATP-binding cassette (ABC) (Dreux et al., 2008). Similarly, carnitine uptake is regulated by the OpuC ABC transporter (Angelidis & Smith, 2003). Deletion of genes *(ΔBetL, GbuABC* or *OpuC*) that encode the GB and carnitine transporters described above, renders *L. monocytogenes* incapable of importing GB or carnitine (Wemekamp-Kamphuis et al., 2002; Angelidis & Smith, 2003), although this did not have any significant impact on the desiccation survival of the mutant in presence of exogenous GB (Dreux et al., 2008).

Dreux and co-workers (2008) demonstrated that the exogenous GB increased the desiccation survival of *L. monocytogenes*; nevertheless, the mutants with impaired GB uptake systems (BetL, Gbu and OpuC) behaved similarly as the parent strains in absence or presence of exogenous GB on parsley leave. These results showed that the role of GB in desiccation tolerance of *L. monocytogenes* is independent of the osmolyte intracellular accumulation (Dreux et al., 2008). Huang (2011) also observed that the exogenous osmolytes (GB and carnitine) protect the listerial cells under osmotic or desiccation stress conditions irrespective of the uptake system. In the presence of exogenous osmolytes, a *L. monocytogenes* Δ*sigB* mutant was observed to behave similarly to the wild type strains when subjected to desiccation stress conditions, although pre-culturing with the compatible solutes prior to the desiccation had the most protective effect (Huang, 2011).

The alternative sigma-factor, sigma B, was shown to be involved in transcription of *opuCA* when grown in media with 5% (w/v) NaCl.

Ells and Truelstrup Hansen (2011) showed that the accumulation of trehalose in a mutant strain of *L. monocytogenes* (*L. monocytogenes* 568:ΔTreA) led to higher resistance of the pathogen to the harsh conditions including desiccation. They showed that although the pathogen naturally does not accumulate intracellular trehalose, its accumulation in the mutant cells enhances survival under hyper-osmolarity, matric and defrost cycling stress conditions (Ells & Truelstrup Hansen, 2011). Although not a naturally occurring process, the study helped to better understand the role of osmolytes including trehalose in enhanced survival of *L. monocytogenes* subjected to dry conditions.

Unlike the other compatible solutes, proline is believed to be synthesized intracellularly in *L. monocytogenes* (Sleator et al., 2003). Three enzymatic reactions catalyzed by γ-glutamyl kinase, γ-glutamyl phosphate reductase and Δ-pyrroline-5-carboxylate reductase are involved in the synthesis of proline from glutamate (Sleator et al., 2003). Proline enhances both osmo- and cryo-tolerance in *L. monocyto*genes (Bayles & Wilkinson, 2000). However, in the study of Huang (2011) proline was found to have a minimal protective effect on the desiccation survival of the bacterium.

2.5.2. Desiccation Tolerance of other Food-borne Pathogens

Tolerance to desiccation varies among pathogenic bacteria of relevance to the food industry. While some pathogens such as *L. monocytogenes* exhibits enhanced tolerance to the matric or hyper-osmotic stress conditions, others do not display this attribute. *E. coli* O157:H7 for instance, did not exhibit enhanced heat tolerance after being subjected to sublethal desiccation stress conditions consisting of an initial drying period at 42°C for 1.5 h and then incubation under dry conditions for 4 days at 21°C (Shen et al., 2011). The study may suggest *E. coli* O157:H7 is rather sensitive to desiccation although further investigation is required.

A Gram-positive food-borne pathogen that is renowned for its resistance to desiccation and hyper-osmotic conditions is *Staph. aureus*. Food poisonings cases of *Staph. aureus* are commonly occurring because the pathogen can survive the elevated salt concentrations or reduced water activities found in many foods (Adams & Moss, 2008). While 5-7% salt is lethal to some microorganisms, *Staph. aureus* can readily grow in media containing such salt concentrations. In fact, some strains of the pathogen grow in reduced RH of 83% and at an elevated osmolarity up to 20% NaCl (Adams & Moss, 2008). Several factors are known to be involved in desiccation survival of *Staph. aureus*, i.e., temperature, growth phase and cell density, desiccation time and presence of osmoprotectants (Chaibenjawong & Foster, 2011). During the screening of a transposon library looking for genetic characteristics involved in the desiccation and hyper-osmolarity tolerance of *Staph. aureus*, *clpX*, *yjbH* and *sigB* were found to contribute to desiccation tolerance in *Staph. aureus* (Chaibenjawong & Foster, 2011). The alternative sigma factor, σ^B, controls 251 genes in the bacterium (Bischoff et al., 2004) and has an essential role in its stress response (Kullik & Giachino, 1997). Also, σ^B governs the biosynthesis of golden pigments in *Staph. aureus*, also known as staphyloxanthin, that are required for the oxidative resistance of the pathogen (Pelz et al., 2005). It is believed that during exposure to and recovery from the matric stress, extensive changes take place in metabolism of the cells which may lead to the oxidative stress (Franca et al., 2007). This may be the reason why *sigB* plays an important role in stress adaptation, tolerance and subsequent recovery mechanisms.

YjbH and *clpX* were previously found to be required for resistance to both ionic and matric stresses in *Staph. aureus* (Frees et al., 2004). The *clpX* gene encodes a protein which is a part of the ClpXP protease which controls the protein turnover in response to ionic and oxidative stresses (Frees et al., 2003; Frees et al., 2004; Frees et al., 2005, Chaibenjawong & Foster, 2011). The function of *yjbH* is believed to be linked to ClpXP (Chaibenjawong & Foster, 2011). At this point it is not fully understood what makes *Staph. aureus* so comparatively resistant to water potential stresses.

Another food-borne pathogen that exhibits an unusual capability to survive in desiccated or hyper-osmotic environments is *Enterobacter sakazakii*. The pathogen is

recognized as the causative agent of severe infections such as meningitis, cerebritis and enterocolitis in infants (Adams & Moss, 2008). The bacterium is believed to survive the elevated heat (pasteurization) and spray drying processes used during preparation of infant milk formula to later cause clinical infections in infants (Adams & Moss, 2008). Survival in dried infant formula with a_w equal to 0.2 is basically dependent on the desiccation resistance of the pathogen but is majorly influenced by the presence of compatible solutes where trehalose holds a central role (Breeuwer et al., 2003). As previously explained, trehalose is an osmolyte that helps in better adaptation of bacterial cells to the stress conditions including matric stresses. Contrary to *L. monocytogenes*, trehalose is synthesized by *Ent. sakazakii* and stationary phase cells have been found to contain more trehalose, perhaps providing an explanation for the increased resistance of *Ent. sakazakii* stationary phase cells (Breeuwer et al., 2003). Shaker and co-workers (2008) examined the role of starvation, heat, cold and desiccation stresses on the subsequent heat (58°C) inactivation kinetics of *Ent. sakazakii*. Interestingly, they demonstrated that prior heat and desiccation stresses did not cross-protect *Ent. sakazakii* as the resistance of the cells to the subsequent heating process decreased. Cold and starvation stresses had no significant impact on the lethality process of the pathogen (the F_{value}) (Shaker et al., 2008).

Several outbreaks of gastroenteritis have been reported worldwide due to the consumption of dry foods contaminated with *Salmonella* spp. (Hiramatsu et al., 2005). Consumption of dried squid chips contaminated with *Salmonella enterica* serovars Oranienburg and Chester caused an outbreak of gastroenteritis in 1999 in Japan (Tsushima et al., 2000). The pathogen was found to resist the low water activity in the dried chips (a_w of 0.5 to 0.6) and remain infectious to consumers (Tsushima et al., 2000). Further investigations by Hiramatsu and co-workers (2005) revealed that the pathogen survived for up to 2 years at refrigeration temperature following desiccation at 35°C for 24 h on paper disks. They postulated that the osmolytes such as trehalose or sucrose are involved in preservation of the structure of essential cell wall proteins during the exposure to low RH conditions enabling the pathogen to survive the dry and subsequent cold storage conditions (Hiramatsu et al., 2005). The authors reported that the presence of

sucrose increases the tolerance of the pathogen to desiccation by 10 to 79 times depending on the strain (Hiramatsu et al., 2005).

Several other salmonellosis outbreaks have been reported in which besides meat, poultry, egg fish and dairy products that are known causative agents of human salmonellosis, low moisture foods have also been involved in the disease transmission (Carrasco et al., 2012). The capacity of *Salmonella* to survive low a_w (Hiramatsu et al., 2005) appears to have caused several outbreaks where low moisture foods such as powdered milk, infant food, bakery products, nut and seed products, spray dried foods such as coffee, tea, eggs and cereals and spices have been directly involved (Carrasco et al., 2012). Low a_w but high fat content products such as chocolate (Food Standards Agency, 2006) and peanut butter (Nummer et al., 2012) have also been reported as *Salmonella* vectors. The severity of problem is so grave that the Grocery Manufacturers Association (2008) has published a seven steps guideline on how to control *Salmonella* in low-moisture foods (Carrasco et al., 2012).

2.5.3. Survival of Food Spoilage Bacteria during Exposure to Dry Conditions

The majority of primitive food preservation techniques depend upon reductions in the a_w of products (Adams & Moss, 2008). Solar drying, salting, smoking and presence of high sugar concentrations were empirically developed as a_w-controlling food preservation methods to preserve the foods such as meat, fish, vegetables, rice, wheat, sorghum and maze from decay (Adams & Moss, 2008). In fact, mankind has for millennia been familiar with the concept of drying and dehydration, although empirically, to ensure access to an adequate supply of food as needed.

A variety of microorganisms are involved in process of biological degradation of foods, including yeasts, fungi and bacteria. The food spoilage bacteria, however, have different requirements for the minimum a_w at which they can actively grow (Adams & Moss, 2008). The minimum a_ws for the majority of Gram-negative or Gram-positive bacteria are 0.97 and 0.90 respectively (Adams & Moss, 2008). As the minimum a_w, that

allows growth, varies among microorganisms, variations in desiccation survival may also be expected among food spoilage bacteria.

2.5.3.1. Desiccation Tolerance of Gram-negative Spoilage Bacteria

In general, dehydration negatively impacts the biological activities of Gram-negative food-spoilage bacteria due to the impairment of DNA, denaturation of proteins and transition of the liquid crystalline phased membrane to its gel phase due to the increase in the melting temperature (Tm) (Ramos et al., 2001). In order to cope with the desiccated conditions, Gram-negative bacteria have developed various strategies. The synthesis or accumulation of compatible solutes such as trehalose is a universal stress response to the dry conditions (Ramos et al., 2001). In most of the Gram-negative bacteria including *E. coli*, the production and/or uptake of osmolytes is governed by the alternative sigma (σ) factors (Ramos et al., 2001). The mechanism by which trehalose protects the cells is not well known; however, the formation of an intracellular inert glass and hydrogen bonds with lipids in cell membrane are believed to play the main roles as proteins are protected from denaturation (Welsh & Herbert, 1999; Ramos et al., 2001; Moran, 2009).

Other strategies such as changes in membrane permeability due to alteration in membrane fatty acid composition are also employed by the Gram-negative bacteria to survive low RH stress conditions (Ramos et al., 2001). Some Gram-negative strains such as *Pseudomonas putida* are using this strategy to overcome the desiccation. The reversible increase in cis-trans isomerization of monosaturated fatty acids that maintains the liquid crystalline phase of membrane during matric or ionic stress is reported to be crucial for the cellular resistance to the mentioned stresses (Junker & Ramos, 1999)

2.5.3.2. Desiccation Survival Response of *Pseudomonas fluorescens*

The role of gene expression and sigma factors in environmental fitness and desiccation stress tolerance of *Pseudomonas fluorescens* has been studied during recent years. It is now generally recognized that all stationary phase bacteria cells express stress

response genes, in which alternative sigma factors play a key role as central regulators of gene expression. The alternative sigma factor RpoS, a central regulator of stationary phase gene expression in *P. fluorescens*, is known to have an essential role in stress response and environmental fitness of the food spoilage bacterium (Stockwell et al., 2009). The role of this regulator in osmotic and oxidative stress resistance of microorganism has been established previously; however, recent studies have found a new function for this sigma factor in response to dry conditions (Stockwell et al., 2009). In an earlier study by Stockwell and Loper (2005), a mutant strain of *P. fluorescens* that lacked the sigma factor RpoS (*ΔrpoS*), was examined for its response to desiccation and it was found that mutant survivor counts were significantly decreased compared to the parent strain, thus confirming the role of sigma factor RopS in *Pseudomonas* desiccation tolerance. In addition to RpoS, a study by Schnider-Keel and colleagues (2001) for the first time demonstrated that another sigma factor AlgU in co-operation with RpoS regulates the adaptation of *P. fluorescens* to high osmolarity (high concentration of NaCl or sorbitol) and desiccation conditions. When activated, the sigma factor may act as an on-off switch that controls the expression of other stress response genes.

2.5.3.3. Desiccation Survival Response of *Shewanella putrefaciens*

To our knowledge, the desiccation survival of *Shewanella putrefaciens* has not previously been investigated, however, the salt tolerance response and hyper-osmotic adaptation of this food spoilage bacterium have been investigated (Leblanc et al., 2003). In this study, two strains of *Sh. putrefaciens* showed increased resistance to hyper-osmotic stresses after prior exposure to sub-lethal concentrations of NaCl or cold stress. Leblanc and co-workers (2003) also demonstrated that this adaptation involved the synthesis of polypeptides and proteins that help the bacteria to tolerate the subsequent osmotic stresses.

Although air-drying and osmotic pressure are two different stresses, the main effect on the bacteria cells is still a decrease in the a_w of the surrounding environment. Elements of the stress response to matric/desiccation and osmotic pressure stresses may therefore be overlapping.

2.5.3.4. Desiccation Survival Response of *Serratia liquefaciens*

Exposure to desiccation and high salinity greatly influence growth and survival of *S. liquefaciens* similarly to other microorganisms. Berry and colleagues (2010) demonstrated that bacterial populations would decrease by about 4 log CFU/ml after desiccation (in simulated Mars soils with <2% RH) for 7 days at 24°C. The high salt concentrations (>10%) used in that study was shown to be inhibitory to the survival and replication of the bacterium; however, in contrast lower salt concentrations (<10%) proved to have either a neutral or positive effect on bacterial survival and/or growth patterns (Berry et al., 2010). The mechanism of this "low-salt" protection is thought to be both complex and species dependent and may be related to the general stress response of the organism and/or its natural marine habitat. The low salt concentrations decreased the volatility and freezing point of water thereby making liquid water available to surround the microbial cells in a wider range of environmental conditions and as a result protecting them from rapid desiccation (Berry et al., 2010). These findings again underscored the key role of low salt stress on the increased bacterial survival towards the desiccation. Although the aim of study by Berry and colleagues (2010) was to evaluate the survival and growth of *S. liquefaciens* under simulated Mars conditions, the results of that study could help in achieving a better understanding of its survival response to the dried conditions in the food industry.

Losantos and others (2000) isolated thirty strains of *S. liquefaciens* or *Proteus vulgaris* (both belong to the family of Enterobacteriaceae) from spoiled cured-hams and demonstrated that *S. liquefaciens* can grow during salting step and continue to grow in the ham until inhibitory a_w-values were reached during post salting or drying. Their results indicated that *S. liquefaciens* grew down to an a_w level of 0.949 (Losantos et al., 2000), which is in contrast to the minimum a_w of 0.97 for active growth of most Gram-negative bacteria (Adams & Moss, 2008).

Although these data do not directly demonstrate the desiccation survival of *S. liquefaciens* on an abiotic surface, they do indicate that the food-spoilage bacterium has a

larger than average tolerance towards desiccation/matric stress when compared to other Gram-negative bacteria.

2.5.4. Desiccation Tolerance of Gram-positives including *Brochothrix thermosphacta*, *Bacillus* spp. and Spoilage Lactic Acid Bacteria

In general, Gram-positive bacteria will due to their cell wall structure survive desiccation better than the Gram-negative strains (Bale et al., 1993; Janning et al., 1994). Bale and co-workers (1993) compared the desiccation tolerance of seven Gram-negative and three Gram-positive species on hydrophobic (polypropylene) and hydrophilic (glass) surfaces and showed that over a 2-week period the Gram-positive species (*Enterococcus* spp.) survived the dry conditions much better than the Gram-negative species. They concluded that the types of surface along with the intrinsic characteristics of the cells (Gram-positive or -negative) are key determinants in resistance to desiccation (Bale et al., 1993).

Brochothrix thermosphacta is an aerobic to facultative anaerobic Gram-positive nonproteolytic food-spoilage bacteria that cause sliminess and production of off-odours and off-flavours in meat products due to creation of short chain fatty acids from glucose during spoilage (Jay & James, 1992; Adams & Moss, 2008; Kilcher et al., 2010). The bacterium is relatively CO_2 tolerant and grows well on pork, lamb and vacuum packed meat products; however, due to its sensitivity to salt it usually cannot grow in cured meat products (Varnam & Sutherland, 1995). Although little is known about the desiccation or hyper-osmotic resistance of *Bro. thermosphacta,* it is still an important concern where it has caused several economic losses in meat industry.

The spore-forming Gram-positive rod bacteria, *Bacillus cereus* is known to cause food-borne poisoning outbreaks (Adams & Moss, 2008). The ability to produce spores makes the bacteria resistant to most environmental factors such as drying and heat during the food production (Adams & Moss, 2008). The spores of *B. cereus* can survive the heating process used during spray drying in infant foods and dried milk products (Becker et al., 1994) or pasteurization to cause ‘sweet curdling’ or ‘bitty cream’ spoilage

in fresh milk products stored in abuse temperatures (Adams & Moss, 2008). The spores are also resistant to dry conditions found in cereals and flours which has led to serious outbreaks in various countries (Blakey & Priest, 1980; Adams & Moss, 2008). Under the conditions that do not allow for efficient sporulation such as high osmolarity or low cell density, the vegetative bacterial cells utilize other strategizes to cope with stresses and continue their vegetative life (Grossman & Losick, 1988; Ruzal et al., 1998). The alternative sigma factor (σ^B) has been found to play a key role in enhanced survival of *B. subtilis* (a non-pathogenic *Bacillus* spp.) vegetative cells subjected to a range of stresses and energy limitation. Volker and co-workers (1999) demonstrated that the *sigB* mutants of *B. subtilis* showed 10 to 100-fold increase in susceptibility towards the experimental stresses such as heat (54°C), ethanol (9%), osmotic (10%), acid (pH 4.3), freezing (-20°C) and lyophilization (desiccation).

As stated before, bacteriocin producing LAB may hold promise as natural hurdles for inhibition of pathogenic and spoilage bacteria. However, not all LAB produce bacteriocins or inhibit competing bacteria and in some cases they contribute to the spoilage of food products. For example, LAB have found to contribute to the spoilage of refrigerated RTE seafood products such as cold-smoked salmon with 3.6-4.6% salt in water phase (Truelstrup Hansen et al., 1995b; Truelstrup Hansen & Huss, 1998; Truelstrup Hansen et al., 1998). LAB have also been involved in spoilage of some food products with higher level of salinity such as RTE dry cured beef (Rubio et al., 2007) with 10.6% salt content or 0.89 a_w (Hui, 2012) or cured hams (2% NaCl, a_w~0.97) during long term refrigerated storage (50 days) (Blesa et al., 2008).

To our knowledge, little is known about desiccation survival of LAB on solid surfaces and no study has addressed this issue so far, however, extensive studies have been done on how to best lyophilize LAB to preserve these for use as starter cultures.

2.6. Methods for Studying Bacterial Biofilms

When studying biofilms, physical conditions such as grooves, crevices, dead ends, corrosion patches makes the sampling of the surfaces difficult, while environmental

stresses such as starvation, chemicals, heat, cold and desiccation can injure the cells and make them non-culturable. It is therefore necessary to use a combination of methods to quantitatively and qualitatively study the microorganisms in their biofilms.

2.6.1. Bacterial Detection and Quantification Techniques

Enumeration of viable bacteria entrapped in the biofilms is a technique that helps in determination of the source, extent of contamination and the type(s) of microorganisms involved in the microbial community (Kumar et al., 1998).

2.6.2. Culture based Enumeration

A simple approach to study and quantify the biofilms is to enumerate the individual microbial cells. This requires the physical separation of surface attached cells after disruption of the biofilm structure into individual cells (Fuqua & Matthysse, 2001). Depending on the thickness and concentration of the formed biofilms, various techniques may be utilized to remove the bacteria from surfaces. Mechanical forces such as abrasive removal with scrapers, glass beads, vortexing or direct swabbing are regularly used to remove and homogenize the microbial biofilms from the surfaces (Kumar et al., 1998; Frank et al., 1990; Mustapha et al., 1989). However, in most cases different scraping techniques are combined with a short ultrasonic treatment to effectively remove the surface attached cells while avoid cell lysis (Flemming et al., 2000). In our lab, we have chosen to use a combination of sonication and vortexing to release the biofilm bacteria as this method has previously been shown to yield the highest counts (Leriche and Carpentier, 2000). Another approach is to use agar contact enumeration methods, which is a technique that widely used for *in situ* sampling and enumeration of biofilms on processing equipment in the industry.

The free biofilm cells are then cultivated on appropriate culture media that can be selective for a specific bacterium or general to allow growth of "all" microorganisms. Plate counts and the most probable number counts (MPN) are two techniques frequently used in the culture based enumerations, although increasingly specific populations may

also be enumerated by quantitative polymerase chain reaction (Q-PCR), however, this technique will not be further discussed in this review.

2.6.3. Chemical Characterization of Biofilms

The food industry has great need for easy to use rapid techniques that allows for the detection of biofilms left behind on processing equipment due to inadequate sanitation protocols. Although culture methods are simple and low in cost, they are labour intensive and results are not immediately available. To acquire more quick answers, rapids methods based on chemical properties of the microbial biofilm cells have been developed.

Nowadays, most of analytical procedures for characterization of biofilms are focused on EPS composition and the processes occurring within biofilms (Denkhaus et al., 2007). Water, microbial cells, EPS and other organic particles are creating the main components of a biofilm; therefore most effort in chemical analysis of biofilms has focused on techniques to identify and quantify each component rapidly and efficiently (Denkhaus et al., 2007). In this relation some of biofilm EPS components such as polysaccharides, proteins, nucleic acid, phospholipids, humic substances and individual organic or inorganic compounds have been isolated using analytical techniques such as extraction, derivatization, field flow fraction, chromatography and electrophoresis (Denkhaus et al., 2007).

Microsensors are other techniques that successfully have been utilized in study of biofilms. This method requires utilization of miniaturized electrochemical and fiber-optic sensors with tip diameters smaller than 20 μm (Denkhaus et al., 2007). Different stages in formation of biofilms, influx or efflux of nutrients and other organic particles, metabolic activity (von Ohle et al., 2010), diffusion (Kuhl, 1996) and mass-transport rate (McLamore et al., 2011) are some subjects of studies where microsensors have played a key role. These methods, however, only rely on chemical composition of biofilms and do not give any details about the structures, morphology and interaction of among the biofilm associated microbial cells.

2.6.4. Structural Characterization Techniques

While the cultural and chemical based methods enumerate or quantify the presence of bacteria cells and their metabolic by-products, microscopy techniques enable the visualization of microorganisms and biofilm structures. A multitude of different microscopy approaches have been utilized in biofilm studies. Scanning electron microscopy was one of the first instruments used for this purpose, and is still widely used. Direct fluorescence microscopy (DFM) (Holah et al., 1988; Wirtanen et al., 1993), interference reflection microscopy (IRM), scanning probe microscopy (SPM), atomic force microscopy (AFM), confocal laser scanning microscopy (CLSM) are alternative methods that are used in parallel with or have be substituted for the SEM (Beech, 1996; Beer et al., 1997; Kumar et al., 1998).

In an advancement of the SEM technique, the environmental scanning electron microscopy (ESEM) is another approach which has faciltated observations of unaltered native biofilms without the need to use traditional microscopy sample preparation procedures like dehydration, fixation and staining. By taking the advantage of this method, researchers are able to observe many of the highly hydrated structures associated with biofilm samples including hydrated cells, the spatial EPS architecture of biofilms and its adherence to the substratum (Little et al., 1991; Kumar et al., 1998).

2.6.4.1. Surface Scanning Electron Microscopy (SEM)

Scanning electron microscopy employs high energy beam of electrons to produce magnified images (Echlin, 2009). Most microbial specimens are light sensitive and poor conductors of electron beams, and in order to get a clear image, the scattered signals from the interaction of high energy beam and the sample must be collected and analyzed by the instrument. As a result, all specimens that are photographed by the SEM need some form of sample preparation to become sufficiently electrically conductive to ensure that the high energy electron beam penetrates down to the surface (Echlin, 2009).

Depending on nature and type of specimens several protocols have been developed to ensure that the samples that go inside the SEM column are sufficiently dry and electrically conductive. All protocols contain at least four steps: Staining, Dehydrating, Drying and Coating. These four steps are highly similar among different fixation protocols. It is the type of fixative(s), concentrations and the order of use that make a protocol appropriate or unsuitable for a specific sample.

Using the SEM approach, structure and spatial arrangement of numerous microbial biofilms including but not limited to *Streptococcus crista* (Sutton et al., 1994), *Candida albicans* (Sangetha et al., 2009), *Staph. aureus* (Soe et al.,2010), *Acinetobacter baumannii* (Pour et al., 2011), *Pseudomonas aeruginosa* (Abdi-Ali et al., 2006), *E. coli* (Chen et al., 2010), *Staph. aureus* and *Staph. epidermidis* (Gad et al., 2009; Atshan et al., 2011), *Enterobacter sakazakii* (Zain & Binti, 2009), *Salmonella* spp. (Prouty et al., 2002; Annous et al., 2005), *L. monocytogenes* (Zameer et al., 2010; Latorre et al., 2010; Minei et al., 2008; Borucki et al., 2003; Hefford et al., 2005), *P. fluorescens* (Simoes et al., 2007), *Serratia marcescens* (Geron et al., 1988) and *Sh. putrefaciens* (Larsen et al., 1998; Ona-Nguema et al., 2004) have been investigated so far.

As stated before, the SEM instrument is commonly used in the study of biofilms due to its fast operation, ease of use, large depth of field, high magnification power and versatile outputs. Nevertheless, since the sample preparation involves desiccation, fixation and staining of samples that is both labour intensive and destructive to the spatial arrangement of EPS and other biofilm related compounds, researchers have tried to find either improved sample preparation methods or other microscopy methods to supplement SEM and overcome these issues.

2.6.4.2. Epifluorescence Microscopy of Biofilms

The use of epifluorescence microscopy in visualization of microbial communities dates backs to 1974 when Zimmerman and Meyer-Reil used acridine orange with epifluorescence illumination to observe a microbial habitat (Poindexter & Leadbetter, 1989). All fluorescence microscopy techniques work is based on the use of fluorescent

dyes or labels that absorb the excitation light and in return emit light at a different (usually longer) wavelength (Wilkinson & Schut, 1998). The illumination system, therefore, has to a high extent been similar in different generations of the fluorescence microscopes, however, the dyes and staining techniques have improved dramatically during recent years. As stated earlier, one of the first dyes employed in fluorescence microscopy was acridine orange. The dye is a nucleic acid binding stain that makes DNA and RNA emit green and orange/red light, respectively, under fluorescent light (optimal excitation wavelengths of 460-500 nm) (Poindexter & Leadbetter, 1989). However, the dye is non-selective and binds to other particles such as clays, colloids and extracellular polymers and produces an orange background that makes the bacterial detection difficult (Poindexter & Leadbetter, 1989). Acridine orange was later replaced by other fluorescent dyes such as 4',6-diamidino-2-phenylindole (DAPI), crystal violet (CV), and SYTO 9 that were more selective (Poindexter & Leadbetter, 1989); even though, they have their own problems as well. Among the major problems associated with use of DAPI or SYTO 9 are "nonspecific cytoplasmic fluorescence, scattering of strong emission light, and fading of the fluorescence under UV excitation" (Hamada & Fujita, 1983) and underestimation in populations of bacteria cells in biofilms due to failure of the dye in penetrating to live bacteria sheltered in the EPS matrix (Flemming et al., 2000).

Another commonly used approach in fluorescence microscopy and bacterial localization is to insert a gene sequence into the target cell that encodes for a fluorescent protein. Subsequent expression of this protein enables the visualization of fluorescent bacteria. Green fluorescent protein (GFP) is one of the most popular fluorescent tags and a number of different expression systems have been developed. However, the need to transform the bacterial strains to enable expression of the protein is time consuming and labour intensive and the influence of certain environmental factors including oxygen and pH on the quality of the fluorescent signal are some of restrictions of this methods (Almeida et al., 2011; Hansen et al., 2001). Finally, if the fluorescent label is delivered on a plasmid (*trans*-expression), then the burden of the maintaining plasmid may change the growth and metabolic kinetics of the organism rendering comparative studies meaningless.

Fluorescence *in situ* hybridization (FISH) has emerged as a molecular alternative approach that could overcome the deficiencies associated with the old fluorescent labeling techniques. FISH uses fluorochrome-labeled oligonucleotide probes that bind specifically to 16 or 23S rRNA genes in target bacteria (Daims & Wagner, 2007). The method is to highly specific and can be applied to environmental samples (no need to cultivate cells in advance); however, permeability, hybridization affinity (cross reactivity with other probes) and target site accessibility are factors that restrict the general application of this labeling technique (Amann & Fuchs, 2008).

These limitations induced the researchers to find a nucleic acid probe that not only specifically hybridize to the complementary DNA or RNA sequences but also easily penetrates the cells without disruption of the bacterial morphology. These attempts led to creation of Peptide Nucleic Acid (PNA) molecules, which are pseudopeptides with DNA-binding capabilities (Stender et al., 1999). The PNA strand consists of the organic nucleic bases that are covalently attached to *N*-(2-aminoethyl) glycine units, and the order and length of the nucleic base sequence can be designed to match any target genome (Stender et al., 1999). Interestingly, the hydrophobic nature of PNA allows the molecule to diffuse through biofilm matrix (Egholm et al., 1993; Stender et al., 1999). Taking advantage of PNA molecules in combination with improved microscopy techniques, the study of spatial organization of microbial populations in biofilms without disturbing the biofilm structure has become possible. One of these improved microscopy techniques is confocal laser scanning microscopy that will be discussed in more details in the next section.

2.6.4.3. Confocal Laser Scanning Electron Microscopy (CLSM) in Biofilm Characterization

The idea of confocal imaging first came about when a postdoctoral fellow replaced the conventional microscope condenser with a lens identical to the objective lens (Pawley, 2006). A pinhole was situated on the microscope axis to limit the field of illumination and the new condenser projected the reduced image of this pinhole onto the specimen (Pawley, 2006). The second (or exit) pinhole, which is placed confocally to the

first pinhole and the projected spot in the specimen, also limits the field of view (Pawley, 2006).

Based on the experimental objective and type of microscopy different illumination systems are used in confocal microscopy. The best known optical source in microscopy is visible light that is usually generated by a mercury lamp; however, other types of beams such as epi-illumination that enables fluorescence visualization and laser beams have successfully been utilized in confocal microscopy. The laser beam has unique characteristics that make it particularly suitable for microscopic studies. The attendant long coherence length which is the distance at which the laser beam can be shifted without any transmittancy and high degree of monochromaticity, intensity and polarization, make the laser one of the most favoured optical systems in the confocal microscopy (Pawley, 2006).

Using an oscillating objective lens and taut steel wired specimen holder, researchers employ the laser beam for Z-axis and X-Y plane stage-scanning purposes. Its high degree of clarity and resolution specifically in 3D fluorescence applications has induced microbiologists to take the advantage of this technology in their studies. The CLSM is used in different fields of microbial studies including studies of biofilms. In combination with other microscopy approaches, the CLSM has been employed to study the EPS structures in heterotrophic biofilms (Wagner et al., 2009), identify various bacteria in a heterogeneous biofilm (Kives et al., 2005; Dige et al., 2007; Wouters et al., 2010; Almeida et al., 2011), investigate the biofilm structures (Manz et al., 1999; Chae & Schraft, 2000; Takenaka et al., 2001; Pereira et al., 2002; Xavier et al., 2003; Mohle et al., 2007; Rieu et al., 2008; Garny et al., 2010; Villena et al., 2010) and assess the influence of different natural and chemical compounds on biological features of different bacterial biofilms (Korber et al., 1994; Seo & Frank, 1999; Akiyama et al., 2004; Song & Leff, 2006; Dynes et al., 2009; Leonard et al., 2010; Pérez-Conesa et al., 2011; Nyila et al., 2012).

In spite of all applications and advantages that the CLSM has provided to microbiologists, the method should not be treated as a cure-all (Pawley, 2006). As every

other technique, CLSM has some restrictions that limit the application of this microscopy method in biofilm studies. Most of these limitations in natural biofilms studies are related to the size, thickness and density of biofilm samples where especially in fluorescence integrated studies the penetration of the excitation beam is to a large degree dependent on the thickness and density of the biofilm sample (Pawley, 2006). In thick biofilms, the excitation beam cannot efficiently penetrate through the EPS structure and therefore, the naturally or artificially fluorescently labeled embedded bacteria in the EPS would not be clearly observed. Moreover, the instrument is rather expensive and requires regular maintenance and intensive training.

2.6.4.4. Spectroscopic Characterization (Fourier Transform Infra Red (FT-IR)) Microscopy of Biofilms

The combination of microscopy and infrared spectroscopy has been developed to enable researchers to gain better insight into the nature and concentration of molecules found in microscopic structures (Smith, 2011). Infrared spectroscopy is "the study of the interaction of infrared light with the matter" and FTIR is a specific instrument based on this technology (Smith, 2011). In identification of unknown samples, the peaks obtained from infrared spectroscopy are compared against the peak library of known materials that have been measured and recorded over the years and the type of molecules then can be identified with this comparison (Smith, 2011). When the nature of sample was identified, then the concentration of desired molecules can easily be calculated using Beer's law and by constructing a calibration curve interrelating absorbance to the concentration (Smith, 2011).

The infrared spectroscopy has great advantages such as universality, good sensitivity and ease of use; though, as with every other technology it has its own disadvantages as well (Smith, 2011). The technique cannot detect some materials such as noble gases, mono-atomic ions and homonuclear di-atomic molecules that do not exhibit detectable vibrations when exposed to infrared spectra. Also, in presence of complex compounds, the spectrum becomes complicated making it hard to detect which peaks

belongs to which molecule and last but not least, water molecules in samples make the spectra so broad that peaks of other molecules are masked, therefore leading to the misinterpretation of results for dissolved solutes (Smith, 2011) specifically in biofilm studies with hydrated nature.

The application of FTIR in food microbiology has lead to non-destructive real-time monitoring, classification, identification and characterization of yeast, fungi, algae and more importantly food-borne pathogens including *Yersinia*, *Bacillus* spp., *Staphylococcus*, *Brucella* , *Salmonella* spp., *Listeria* spp., *Klebsiella*, *Escherichia*, *Enterobacter*, *Citrobacter* and *Cronobacter sakazakii* (Ray & Bhunia, 2008; Alvarez-Ordonez et al., 2011).

As mentioned earlier, using IR beam the instrument gives information about the molecular composition of cell wall and the cytoplasm of the microorganisms (Ray & Bhunia, 2008) and due to its non-destructive, real-time monitoring nature, the FT-IR was recently utilized in studies of cellular and physiological changes occurring in both single-species and multispecies biofilms of various bacteria such as *E. coli* (Holman et al., 2009), *P. aeruginosa* (Comeau et al., 2009), *P. fluorescens* (Delille et al., 2007; Manuzon & Yabes, 2009), *Sa. enterica* serovar Typhimurium (Xu et al., 2010), and *B. subtilis* (Marvasi et al., 2010); however, to date no study has investigated the biofilm formation characteristics of *L. monocytogenes* using this novel technique.

CHAPTER 3 MATERIALS AND METHODS

3.1. Bacterial Strains, Preparation of Stocks and Inoculums

Four different bacterial strains were used in this study: *Shewanella putrefaciens* A2 (Ravn Jørgensen, 1986), *Serratia liquefaciens* 2R4 (Truelstrup Hansen, 1995a), *Pseudomonas fluorescens* ATCC 13525 (ATCC®, Manassas, VA, USA) and *Listeria monocytogenes* 568 (serotype 1/2a, food plant isolate, Hefford et al., 2005). For long-term storage of bacteria, each bacterial strain were grown on Brain Heart Infusion agar (BHIA), consisting of BHI (Difco, BD Canada, Oakville, ON) supplemented with technical agar (1.5%, Difco) as solidifying agent. A single colony from BHIA was picked up and mixed with 0.75 ml of Tryptic Soy Broth (Bacto, BD Canada, Oakville, ON) and 0.25 ml glycerol, transferred into cryonic vials and stored at -75°C.

For routine experiments, a working stock of each bacterial strain was created on BHIA each month. For this purpose, 5 µl from frozen bacterial stock solution was inoculated into 5 ml of TSB (1:1000), incubated at room temperature (~20°C) for 48 h and streaked out onto BHIA. *L. monocytogenes* and *S. liquefaciens* plates were incubated at 37°C for 24 h while *P. fluorescens* and *Sh. putrefaciens* plates were stored at room temperature for 48 h. The plates were subsequently kept in refrigerator at 4°C.

In order to be able to harvest cells in the early stationary phase, it was necessary to determine the growth kinetics of each bacterial strain at 15°C. It is worth noting that the Combase ("a combined database for predictive microbiology" available at: http://www.combase.cc/index.php/en/) has useful information about growth patterns of various food-borne bacteria; however, no information about growth kinetics at 15°C could be found for the specific bacterial strains used in this study. To assess the growth kinetics of each strain at 15°C, firstly a stock solution of each strain was prepared by inoculating a single colony into 5 ml of TSB supplemented with 1% (w/v) glucose (D-glucose anhydrous, Fisher Scientific, ON, Canada). The glucose solution was filter

sterilized (syringe filter, 0.2 µm pore size, Corning Incorporated, Germany) and added to sterile TSB using aseptic technique. Following growth at 15°C for 48 h, the cells were pelleted by centrifugation (3396 × *g*, 15 min, 4°C, Hettich Universal 32R, Andreas Hettich Co., Tuttlingen, Germany) and resuspended in fresh TSB+1% glucose. The populations of each bacterial strain were adjusted to 10^3 CFU/ml using absorbance measurements at 450 nm (UV/visible spectrophotometer, Ultraspec 1100 Pro, Amersham Biosciences Corp., Piscataway, NJ, USA) and incubated at 15°C in aliquots of 1 ml. At specific time intervals (0, 4, 8, 12, 20, 24, 28, 44, 48, 52, 72 and 84 h) the duplicates/triplicates of bacterial suspensions were serially diluted in sterile peptone physiological saline (PPS, 0.1 g bacteriological peptone [Oxoid Canada, Nepean, ON] and 0.85 g sodium chloride [Fisher Scientific] per 100 ml) followed by spread plating and enumeration on BHIA. The growth curves were constructed and early stationary phase was determined to occur after 24 h for *P. fluorescens* and *S. liquefaciens* and 48 h for *Sh. putrefaciens* and *L. monocytogenes*, respectively (data not shown here).

3.2. Preparation of Stainless Steel Coupons

Stainless steel (SS 316, type 4 finish) was chosen as substratum for bacterial regrowth and biofilm formation. The SS sheet was cut into 0.5×0.5 cm coupons, degreased and disinfected first by boiling in 1% (w/v) SDS (Sodium dodecyl sulfate, Bio-Rad Laboratories Canada, Mississauga, ON) for 10 min. The coupons then were rinsed with dH_2O (3 times) and submerged and sonicated (Elmo Ultrasonic bath, 50/60 Hz, Fisher Scientific) in 15% (v/v) Decon solution (CiDecon® concentrated phenolic disinfectant, Decon Labs, Fisher Scientific) for 1 h. Following the second rinse in dH_2O, the coupons were soaked in isopropanol (10 min), rinsed again with dH_2O and autoclaved for 15 min at 121°C. The sterile coupons then were dried and finally stored aseptically in 95% ethanol for future use.

3.3. Inoculation of Stainless Steel Coupons and Formation of Mono- and Binary-Culture Biofilms

A single colony from each bacterial strain was inoculated into 5 ml TSB+1% glucose and incubated at 15°C for 24 (*P. fluorescens* and *S. liquefaciens*) or 48 h (*L. monocytogenes* and *Sh. putrefaciens*) to obtain early stationary phase cells. Following growth at 15°C, the bacterial cells were pelleted (3396 × *g*, 15 min, 4°C) and resuspended in fresh TSB+1% glucose. The concentration was adjusted to ≈10^9 CFU/ml ($ABS_{450\ nm}$=1) and serially diluted in fresh medium to yield a population of 10^6 CFU/ml for each strain. Ten µl of each prepared inoculum was directly deposited onto the cooled flame sterilized, SS coupons to yield an initial concentration of 10^3 CFU/cm^2. The inoculated coupons then were transferred to the desiccation chamber (Fisher Scientific) with the relative humidity (RH) adjusted to 100% by placing 3 petri dishes filled with dH_2O in the bottom of the chamber, and incubated at 15°C for 48-72 h to yield single species biofilms.

For binary biofilms, 5 µl from one of the suspensions of the Gram-negative strains (*P. fluorescens*, *S. liquefaciens* and *Sh. putrefaciens*) was mixed with 5 µl of *L. monocytogenes* suspension (50:50) to yield an initial concentration of 10^3 CFU/cm^2 when directly deposited onto the cooled flame sterilized SS coupons. Biofilm formation followed during incubation under the same conditions (15°C, 100% RH, 72 h).

3.4. Evaluation of Growth Kinetics in Mono- or Binary-Culture Biofilms

The growth kinetics of the bacteria in the mono- or binary-culture biofilms was determined by enumeration of the populations developing on the SS coupons sampled at specific time intervals (0, 12, 24, 36, 48, 60 and 72 h), on suitable general (BHIA) or selective agars (Oxford, Pseudomonas C-F-C, VRBG, Iron agar+Pen. G, see below).

For mono-culture biofilms, 3 coupons from each strain were randomly selected at each time interval, gently rinsed 3 times in PPS to remove the loosely attached cells from the surface and placed in test tubes containing 0.99 ml PPS. The tubes then were vortexed for 30 s, sonicated for 4 min, vortexed again for 30 s, serially diluted in PPS and spread

plated onto both BHIA and the suitable selective media. This detachment technique follows the procedure developed by Leriche and Carpentier (1995) with some modifications. *Listeria* selective agar base (Oxford formulation, CM0856) with *Listeria* selective supplement (SR0140) was used for enumeration of *L. monocytogenes*. Specific counts of *P. fluorescens* were obtained on *Pseudomonas* agar base (CM0559) supplemented with *Pseudomonas* C-F-C (SR0103), while *S. liquefaciens* was enumerated on Violet Red Bile Glucose (VRBG) agar (CM1082). All culture media and supplements were purchased from Oxoid Canada (Nepean, ON). *Sh. putrefaciens* was counted as black (H_2S-producing) colonies on Iron agar (Gram et al., 1987) supplemented with 0.6 ppm penicillin (Pen. G potassium salt 10 MU, Sigma-Aldrich, Oakville, ON, Canada). Briefly, the Iron agar medium consisted (per litre) of peptone (20.0 g), Lab-lemco powder (3.0 g) (LP0029, Oxoid Canada), yeast extract (3.0 g) (Oxoid Canada), ferric-citrate (0.3 g) (Sigma-Aldrich), sodium thiosulphate (0.3 g) (Nichols Chemical company, Amherstburg, ON, Canada), sodium chloride (5.0 g) and technical agar (12.0 g). The pH was adjusted to 7.4±0.2 and the medium was autoclaved (15 min, 121°C), cooled and supplemented with 1 ml of a filter sterilized cysteine hydrochloride monohydrate solution (44.25 mg/ml, Fisher Scientific) per 100 ml medium. As *L. monocytogenes* also grows on Iron agar and thereby could mask the black *Shewanella* colonies, it was necessary to add 0.6 ppm Penicilium G (penicillin G potassium salt 10 MU, Sigma-Aldrich) to the Iron agar. This concentration was in preliminary experiments found to be inhibitory to *Listeria* while supportive of *Sh. putrefaciens*. All plates were incubated 24 h at 37°C for 48 h at room temperature.

The entire experiment was performed twice (biologically independent replicates) for each bacterial biofilm using triplicate samples (n=6). Colonies were enumerated (10-300 colonies per plate), converted into Log CFU/cm^2 (eq. 1) before a non-linear growth model was fitted to the results to obtain estimates for growth kinetic parameters.

$$N = \frac{X}{V \times d \times a} \qquad \text{(eq. 1)}$$

N= CFU/cm^2

X= colony count per plate

V= volume of sample plated (0.1 ml)

d= dilution factor

a= surface area of one side of the SS coupons

For binary-culture biofilms (n=6), the same protocol was utilized except samples were not spread plated onto BHIA but only on the suitable selective media, i.e., Oxford agar and one of the selective media for the Gram-negative bacteria. The results indicated that cells in mono- or binary-culture biofilms reached the early stationary phase after 48 h. For this reason, 48 h biofilms were employed for further analysis of cultures in desiccation experiments.

3.5. Desiccation of Mono- and Binary-Culture Biofilms

Desiccation experiments were carried out on both mono- and binary-culture biofilms. For each biofilm, bacterial stocks were prepared and deposited onto the SS coupons as described above in section 3.3. After incubation at 15°C and 100% RH for 48 h to allow for the biofilm formation, the coupons were transferred into the desiccation chamber (Mini desiccators, W×D×H: 224 mm×200 mm×168 mm, Bohlender**,** Grünsfeld**,** Germany) equipped with 4 petri dishes filled with saturated potassium carbonate (Fisher Scientific) to serve as desiccant (43% RH). The filled desiccation chamber was placed in an incubator set at 15°C. The temperature and RH was monitored using data logger (TV-4500, Tinytag Canada, Markham, ON) and remained constant throughout the experimental desiccation period of 21 d.

At specific time intervals (-2 [initiation of biofilm formation], 0 [end of biofilm formation, beginning of desiccation period], 1, 3, 5, 7, 14, 21 d) three coupons from each biofilm were randomly selected, and the survivors enumerated on BHIA in the case of mono-culture biofilms as described in section 3.4. In order to evaluate the desiccation survival of the two bacterial species in the binary-culture biofilms, selective media were

used as described above (i.e., Oxford agar for enumeration of *L. monocytogenes*, VRBG agar for *S. liquefaciens*, Pseudomonas C-F-C agar for *P. fluorescens* and Iron agar+Pen. G for *Sh. putrefaciens*) to obtain specific counts of the co-cultured strains.To negate the influence of position inside the desiccation chamber (right, left, front or back) on desiccation rate, the coupons were regularly relocated to different positions. The experiments were repeated in two independent runs (n=6).

For mono-culture biofilms of *L. monocytogenes* and *S. liquefaciens*, another desiccation experiment was designed with narrower time intervals (-2, 0, 1, 2, 3, 4, 5, 6, 7, 9, 11, 13, 15, 17, 19, 21) to gain a better understanding of these two microbes' desiccation behaviour. The same experimental protocols as previously described were employed.

3.6. Modeling of the Growth and Formation of Mono- and Binary-Culture Biofilms on SS surfaces

Populations of single or dual-species biofilms of *L. monocytogenes* and the selected Gram-negative bacterial strains on SS coupons were converted into $Log_{10}CFU/cm^2$ and presented as mean±standard deviation for each strain (n=6). The growth curves were plotted using SigmaPlot® software for Windows ver. 11.0 (2008, Systat software Inc., San Jose, CA, USA). The non-linear growth models, then were fitted to the growth data using Systat software for Windows ver. 12.02 (2007, Systat software Inc.) or Solver add-in for Microsoft® Office Excel® (2007, Microsoft Corporation, Redmond, WA, USA).

Using the data from each biofilm, four commonly used growth models (Logistic with lag phase, Baranyi with fixed lag parameter, modified Gompertz) were initially fitted to the growth curves. The models were first assessed for their i) scientific plausibility (i.e., the best-fit values are sensible or not based on the visual investigation of growth curves), ii) precision of the best fit values (i.e., level of certainty of the best fit as indicated by parameter values or simply the standard error of the mean for estimated model parameters), iii) violation from any of the non-linear regression assumptions (i.e.,

X and Y-axis units, normal distribution of "Y" values at any particular "X" value, homoscedasticity or uniform variance all way along the curve and finally independent observations) as described by Motulsky and Christopoulos (2004). If the models could satisfy these prerequisites, then they were assessed for the goodness of fit as explained by den Besten and co-workers (2006) and Ells and others (2009). The fitting performance of the models was evaluated based on statistical analysis on three indices (r^2, MSE_{model} and the F_{test}). These three indices that were considered equally important in selection of the best fitted model, were calculated by fitting the models to all data from the replicates at once (n=6).The adjusted r^2 was acquired from the modeling software while the MSE_{model} and f_{value} were calculated using the following equations:

$$MSE_{model} = \frac{RSS}{DF} = \frac{\sum_{i=1}^{n}(Log_{10}N^{i}_{observed} - Log_{10}N^{i}_{fitted})^2}{n-s} \quad \text{(eq. 2)}$$

MSE_{model}= mean square error of the model

RSS= residual sum of squares

DF= degrees of freedom

n= number of data points

s= number of parameters of the model

$N^{i}_{observed}$= the observed populations level (CFU/cm^2)

N^{i}_{fitted}= the fitted populations level (CFU/cm^2)

$$f = \frac{MSE_{model}}{MSE_{data}} \quad \text{(eq. 3)}$$

MSE_{model}= mean square error of the model

MSE_{data}= mean square error of the data for replicate values

The MSE_{data}, which indicates the measuring error, was determined as follow:

$$MSE_{data} = \frac{RSS}{DF} = \frac{\sum_{i=1}^{m}\sum_{j=1}^{k}(\text{average } Log_{10}N^{i} - Log_{10}N^{ij})^2}{n-m} \quad \text{(eq. 4)}$$

MSE_{data}= the mean square error of the data for replicate values

RSS= residual sum of squares

DF= degrees of freedom

n= number of data points

m= number of time points (sampling times)

k= the number of replicates at each time point

average N^{i}= the mean value of the populations at time point *i* (CFU/cm^2)

N^{ij}= the populations at time point *i* for specific replicate *j* (CFU/cm^2).

The obtained f_{value} was compared against an *F* table value at 95% degree of confidence. If the measured f_{value} was less than the *F* table value ($F_{DF_{data}}^{DF_{model}}$), then the *F*-test was accepted, as it indicated that the model goodness of fit was suitable. Additionally, the model with the best goodness of fit should have the highest adjusted r^2 and the smallest MSE_{model}. Furthermore, Tukey's Studentized Range (HSD) Test at 5% significance level was performed over the MSE_{model} and adjusted r^2 of all models using SAS software V. 9.2 (SAS Canada, Toronto, ON). The analysis of the suitability of the different models to accurately describe the dataset showed that the Logistic with lag phase, Baranyi with fixed lag parameter and modified Gompertz models were best suited. However, the simple Logistic model with lag phase was found to be the better choice for description of the growth kinetics (further explanation is presented in the discussion) and as a result it was used for further analysis of growth kinetics.

The Logistic model with lag phase model is parameterized as follows (Dalgaard, 2009):

$$\begin{cases} Log_{10}(N_t)=Log_{10}(N_0) & t\leq \lambda \\ Log_{10}(N_t)=Log_{10}\left(\frac{N_{max}}{1+\left[\frac{N_{max}}{N_0}-1\right]\times \exp\left(-\mu_{max}\times(t-\lambda)\right)}\right) & t>\lambda \end{cases} \quad \text{(eq. 5)}$$

N_{max} = maximum populations (CFU/cm^2)

μ_{max} = maximum specific growth rate that can also be interpreted as maximum slope of the curve (1/h)

λ = lag time (h)

N_o = initial populations at t=0 (CFU/cm^2)

t = time (h)

N_t= number of cells at any time (CFU/cm^2)

The Systat software or Microsoft® Office Excel® were used to generate the estimates for model parameters (i.e., N_{max}, μ_{max}, N_o, λ) for each of the six replicates. The model parameter estimates were then analyzed by multiple comparisons of means using Tukey's Studentized Range (HSD) test at the 5% significance level using SAS software V. 9.2.

The Baranyi model is parameterized as follows (Toldrá, 2009):

$$Log_{10}(Nt)=Log_{10}(N_{max})+Log_{10}(N_0)+Log_{10}\left\{\frac{-1+\exp(\mu_{max}.\lambda)+\exp(\mu_{max}.t)}{\exp(\mu_{max}.t)-1+\exp[\mu_{max}.\lambda+Ln(N_{max})-Ln(N_0)]}\right\}$$

(eq. 6)

N_{max}= maximum populations (CFU/cm^2)

μ_{max} = maximum specific growth rate that can also be interpreted as maximum slope of the curve (1/h)

λ= lag time that was fixed for each model and calculated based on the focus of growth curve and the time axes (h)

N_o = initial populations at t=0 (CFU/cm^2)

t= time (h)

N_t= number of cells at any time (CFU/cm^2)

The modified Gompertz model is formulated as follows (Garthright, 1991):

$$Log_{10}(N_t)= A + D \times \exp \{-\exp [-B\times (t - M)]\} \quad \text{(eq. 7)}$$

A = value of the lower asymptote (initial population at t = 0, Log_{10}(CFU/cm^2)

D = difference in value between the upper and lower asymptote (maximum minus initial populations in Log_{10}(CFU/cm^2)

B = slope factor of the curve (1/h)

M = time at which the exponential growth rate is maximal (h)

t = time (h)

N_t = number of cells at any time (CFU/cm^2)

3.7. Modeling of Bacterial Desiccation Survival in Mono- and Binary-Culture Biofilms on SS Surfaces

Survivor counts obtained for *L. monocytogenes* and the Gram-negative bacterial strains during desiccation of single and dual-species biofilms on SS coupons were normalized by Log_{10}-tranformation, converted into $Log_{10}(N_t/N_0)$ and presented as

mean±standard deviation for each strain (n=6). The survivor curves were plotted using the SigmaPlot software and analysed using the free non-linear curve-fitting tool GInaFiT (version 1.5), for Microsoft® Office Excel® available at the KULeuven/BioTec-homepage (http://cit.kuleuven.be/biotec/downloads.php) and developed by Geeraerd and colleagues (2005). Using each replicate, inactivation models commonly used in predictive food microbiology were fitted to survivor curves (see details below) except models with a shoulder/lag phase were not considered since no lag phase was observed in the graphed survivor curves. The models were assessed for the goodness of fit as described before (section 3.6). The fitting performance of models was evaluated first based on GInaFiT curve-fitting tool (version 1.5) output from each generated model (r^2, MSE_{model}) while the f_{value} was computed manually as described above (section 3.6). These three indices were obtained by fitting the models to all replicates at once (n=6). The calculated f_{value} was compared against an *F*-table value at 95% degree of confidence as also outlined above in section 3.6.

The double Weibull model, which assumes the presence of two different subpopulations (1 and 2) with differing resistance to the inactivation treatment, is parameterized as follows (Coroller et al., 2006):

$$\mathrm{Log}_{10}\left(\frac{N_t}{N_0}\right) = \mathrm{Log}_{10}\left[10^{-\left[\left(\frac{t}{\delta_1}\right)^p + \alpha\right]} + 10^{-\left(\frac{t}{\delta_2}\right)^p}\right] - \mathrm{Log}_{10}(1 + 10^{\alpha}) \qquad \text{(eq. 8)}$$

$\frac{N_t}{N_0}$= number of relative total survivors

α = the Logit of "f " which may also be shown as $\mathrm{Log}_{10}(N_{01}/N_{02})$

$$\alpha = \mathrm{Log}_{10}\left(\frac{f}{1-f}\right) \qquad \text{(eq. 9)}$$

$\boldsymbol{\delta}_1$= first decimal reduction time for subpopulations 1 (d)

$\boldsymbol{\delta}_2$ = first decimal reduction time for subpopulations 2 (d)

t= time (d)

p= shape factor

The GInaFiT curve-fitting application for Microsoft® Office Excel® 2007(version 1.5) was used to generate the estimates for model parameters (i.e., α, $\boldsymbol{\delta}_1$, $\boldsymbol{\delta}_2$, and p) for each of the six replicates.

The equation for the Biphasic model is expressed as follows (Cerf, 1977):

$$\mathrm{Log}_{10}\left(\frac{N_t}{N_0}\right)=\mathrm{Log}_{10}\ \{f \times \exp^{(-k_1 \times t)}+[1-f] \times \exp^{(-k_2 \times t)}\} \qquad \text{(eq. 10)}$$

$\frac{N_t}{N_0}$ = number of relative total survivors

f= initial proportion of the less resistant fraction ($f=N_{01}/N_0$)

1-f= initial proportion of the more resistant fraction ($1-f=N_{02}/N_0$)

$$\left.\begin{array}{l} k_1 = \text{death rate constant for } N_1(t) \\ k_2 = \text{death rate constant for } N_2(t) \end{array}\right\} \ k_1 > k_2 \geq 0$$

$N_1(t)$= populations of the less resistant fraction at any time (CFU/cm^2)

$N_2(t)$= populations of the more resistant fraction at any time (CFU/cm^2)

t= time (d)

The biphasic model assumes that the bacterial populations consist of two subpopulations or fractions that are inactivated independently and irreversibly by first order, constant rate kinetics (Cerf, 1977; Xiong et al., 1999).

The Weibull model with tail is parameterized as follows (Albert & Mafart, 2005):

$$\mathrm{Log}_{10}(N_t)=\mathrm{Log}_{10}\{(N_0-N_{res}) \times 10^{[1-(\frac{1}{\delta})^p]}+N_{res}\} \qquad \text{(eq. 11)}$$

N_t= number of relative total survivors (N/N_0)

N_0= initial relative total population at t=0 (N_0/N_0=1)

N_{res}= relative residual population at the end of experimental period ($Log_{10}(Nt_{final}/N_0)$)

$\boldsymbol{\delta}$= first decimal reduction time for the population other than N_{res} (d)

p= shape factor

And the equation for the singleWeibull model described by Mafart and colleagues (2002) is as follows:

$$Log_{10}(\frac{N_t}{N_0}) = -(t/\delta)^P \quad \text{(eq. 12)}$$

$\frac{N_t}{N_0}$ =number of relative total survivors

t= time (d)

$\boldsymbol{\delta}$= first decimal reduction time (d)

P=shape factor

The selected inactivation models were compared in terms of how many *F*-test with acceptable values were obtained for the seven biofilm treatments (4 mono- and 3 binary-culture biofilms, for a total of 10 inactivation curves), and the model with the highest number of suitably modeled biofilm treatments, as determined by the *F*-test, was picked to be used in the further analysis of the results.

3.8. Microscopic Evaluation of Adhesion and Biofilm Formation

3.8.1. Surface Scanning Electron Microscopy: Fixation and Observation

Three fixation protocols were used to prepare the SS coupons harbouring single or binary biofilms for observation under the scanning electron microscopy (SEM) instrument.

3.8.1.1. Basic Fixation in Cacodylate Buffer

In order to observe the mechanisms of attachment and biofilm formation, mono- and binary-culture biofilms were formed as before (48 h, 15°C, 100% RH) on SS coupons (see section 3.3). To ensure the bacterial populations were similar to those subjected to the desiccation experiments, 3 coupons from each biofilm treatment were randomly selected and the number of cells enumerated on BHIA or suitable selective agars as described above.

For microscopic analysis, the biofilm coupons were prepared based on the protocol previously described by Austin and Bergeron (1995) with some minor changes in solution concentrations, pH and treatment times. Briefly, two coupons from each mono- or binary-species biofilm were randomly selected. The coupons were then transferred into 0.1 M sodium cacodylate trihydrate (Fisher Scientific) solution with 2% (w/v) glutaraldehyde (50% w/w, Fisher Scientific) (pH=7.2). Following 2 h of immersion in the buffer solution, the coupons were rinsed (3×10 min) in 0.1 M cacodylate buffer supplemented with 3% (w/v) glucose (pH=7.2) and submerged in 1% (v/v) osmium tetroxide solution (4% aqueous solution, Electron Microscopy Science (EMS), Cedarlane, Burlington, ON, Canada) in 0.1 M cacodylate buffer (pH=7.2) for 4 h. The coupons then were rinsed in pure 0.1 M cacodylate buffer (3×10 min) and dehydrated in an ascending ethanol (Fisher Scientific) gradient series (35, 50, 70, 90 and 100%, 15 min each except the last one which was repeated 3 times). The fixed and dehydrated coupons then were dried in a HMDS/ethanol (hexamethyldisilazane, EMS) mixture series (25:75, 50:50, 75:25 and 100:0, 15 min for each step except the last one which was repeated twice). The fixed, dehydrated and chemically dried coupons were air dried for 2 h, mounted onto the aluminum mounts (slotted head, tapered pin, EMS) using carbon adhesive tabs (9 mm diameter, EMS) and sputter coated (Polaron-SC7620 mini sputter coater, Quorum Technologies LTD Canada, Montréal, Québec) with Au/Pd nanoparticles (SC502-314B gold/palladium sputter target, 0.1 mm thick, Quorum Technologies LTD Canada, Montréal, Québec). The prepared coated coupons were protected in universal reversible

mount holders (EMS) and stored under almost dried conditions (3-4% RH) for future observations by the SEM instrument.

3.8.1.2. Modified Fixation in Cacodylate Buffer, Aqueous Tanic Acid and Uranyl Acetate

Another cacodylate buffer fixation method based on the method by Dekker and co-workers (1991) was also employed. Following procedures described above (section 3.3) to grow the biofilms, the SS coupons were transferred into 0.2 M cacodylate buffer in 2.5% (w/v) glutaraldehyde and 0.05 M calcium chloride (Fisher Scientific) (pH=7.2) for 2 h. The coupons then were rinsed (3×10 min) in 0.1 M cacodylate buffer supplemented with 5% sucrose (Fisher Scientific) (pH=7.2) and immersed for 4 h in 1% (v/v) osmium tetroxide solution in cacodylate/sucrose buffer. After the second rinse (3×10 min) in cacodylate/sucrose buffer that was followed by 2×5 min rinses in filter sterilized dH_2O, the coupons were transferred into 1% aqueous tannic acid (Mallinckrodt Canada, Pointe-Claire, QC) for 30 min, rinsed (3×10 min) in dH_2O, and submerged in 2% aqueous uranyl acetate (Taab laboratories, Canton de Gore, QC) for 30 min. Following 3×10 min rinsing in dH_2O, the fixed biofilms were dehydrated, dried, mounted, coated and stored using same protocols presented in section 3.8.1.1.

3.8.1.3. Fixation in FC-72 Solvent

Allan-Wojtas and colleagues (1997) reported on an anhydrous solvent-based fixation protocol for SEM observation of intestinal mucus layers. Based on this protocol, the single or binary biofilms were first formed (48 h, 15°C, 100% RH) on SS coupons as before and then transferred into 1% (w/v) osmium tetroxide (crystalline, EMS) dissolved in FC-72 solvent (3M™ Flourinert™ Electronic Liquid, 3M, London, ON, Canada) and left in the solution for 4 h. After immersion in pure FC-72 solvent for 30 min, the coupons were incubated in cacodylate buffer solution (0.2 M) in glutaraldehyde (2.5% w/v) and calcium chloride (0.05 M) for 2 h. Subsequently, the coupons were rinsed in dH_2O (2×5 min), submerged in 1% aqueous tannic acid (30 min), rinsed again in dH_2O (3×10 min), immersed in 2% aqueous uranyl acetate (30 min) and rinsed one last time in

dH_2O (3×10 min). The fixed coupons then were dehydrated, dried, mounted, coated and stored using same protocols as stated in section 3.8.1.1.

3.8.2. Observation of Fixed and Coated Biofilms under the SEM

The stubs containing the fixed and coated coupons were mounted on the microscope specimen holder and placed inside the microscope chamber according to the operating instructions. The biofilm structures were visualized under the field-emission surface scanning electron microscope (Hitachi S-4700 FE-SEM) with operational conditions of 10 kV acceleration voltage (V_{acc}), 20 µA emissions current, 7.5-8.5 mm working distance and the UHR-A lens mode. Micrographs were taken at different magnification powers (1, 2, 5 and 10 kV, also if applicable: 20, 40 and 60 kV) from diverse spots of each coupon (2 coupons per biofilm treatment and 3 random pictures from random spots of each coupon at various magnifications).

3.8.3. Epifluorescence Microscopy

In order to discriminate the bacterial strains within dual-culture biofilms, epifluorescence microscopy method was employed. To this end the cultures must first become fluorescently visible. In this study two methods were utilized; one where *L. monocytogenes* 568 carrying a green fluorescent protein containing plasmid (pNF8) was combined with DAPI counter staining of all bacteria and the other where the fluorescent Gram-stain was used.

3.8.3.1. Green Fluorescent Protein (GFP) Labeling of *L. monocytogenes* and DAPI Staining of the Gram-negative Co-Cultures.

Plasmid Preparation. The *E. coli* HB101P harbouring the *gfp*-containing plasmid PNF8 was kindly provided by Dr. Rafael A. Garduno (Departments of Microbiology & Immunology and Medicine, Dalhousie University). Following Ma and others (2011) the *E. coli* cells harbouring the pNF8 was first streaked onto BHIA+ 8 µg/ml erythromycin (Sigma-Aldrich) and incubated at 37°C for 24 h. A single colony

was then inoculated in 1 ml of BHI/glycerol (80:20) and stored at -75°C for future use. For routine experiments, 5 µl from the frozen stock was inoculated into LB Broth Miller (EMD Inc., Mississauga, ON, Canada) containing 150 µg/ml erythromycin and incubated with shaking at 37°C for 24 h. The bacteria were streaked onto BHIA+8 µg/ml erythromycin and incubated at 37°C for 24 h. The reference plate was subsequently stored in the refrigerator and re-cultured on a regular base.

Preparation of *L. monocytogenes* Competent Cells. Using the method of Alexander and others (1990) and Ma and colleagues (2011), briefly, a single colony of the parent strain (Lm 568) was inoculated into BHI and incubated overnight at 37°C with shaking. Three ml of this suspension ($ABS_{600nm} > 1.600$) was inoculated into 100 ml preheated (37°C) BHI/sucrose (0.5 M) and incubated for 2-3 h at 37°C until the ABS_{600nm} reached 0.2- 0.3. One hundred µl of Pen. G stock (10 mg/ml) was added followed by incubation with shaking until the culture reached absorbance values (600 nm) of 0.5 - 0.6. The bacteria suspension then was centrifuged (8217 × *g*, 10 min, 0°C), washed twice in an ice cold sterile glycerol/sucrose (10%: 0.25 M) suspension and re-suspended gently in 100 ml of the glycerol/sucrose solution. The last step was repeated in 50 ml glycerol/sucrose solution and finally the collected cells were resuspended in 200-300 µl glycerol/sucrose solution, divided into 100 µl aliquots and frozen at -75°C for future use.

Plasmid Isolation and Insertion into *L. monocytogenes* Competent Cells. The pNF8 plasmid was extracted from the *E. coli* host cells by the alkaline lysis method (Sambrook et al., 1989; Ma et al., 2011). A single colony of the *E. coli* harbouring the pNF8 was inoculated into LB broth Miller+150 µg/ml erythromycin and incubated overnight at 37°C. The plasmid was subsequently extracted using Ultra Clean® 6 minute mini plasmid prep kit (MO BIO laboratories Inc., Carlsbad, CA, USA). The kit employs the alkaline lysis method to extract the DNA plasmids from the host cells. The final volume of the isolated DNA was 50 µl; however, since this plasmid suspension turned out to be too dilute for efficient insertion into Lm 568 competent cells, the plasmid DNA was further concentrated based on the manufacturer's protocol.

Fifty µl aliquots of the thawed competent Lm 568 cells was added directly into the microcentrifuge tube containing the concentrated plasmid DNA solution, mixed thoroughly but gently, transferred into the sterile ice cold gene pulser cuvette (0.1 cm electrode gap, Bio-Rad Laboratories Canada, Mississauga, ON) and electroporated (1.4 kV, 4 ms) using the micropulser electroporation apparatus (Bio-Rad Laboratories). One ml ice cold BHI/sucrose was quickly added to the cuvette, mixed and transferred into a microcentrifuge test tube and incubated at 30°C for 2 h. The suspension was serially diluted in PPS, spread plated onto BHIA+8 µg/ml erythromycin and incubated at 37°C for 48 h. Only transformed Lm 568 cells harbouring the pNF8 would grow on this medium.

Plasmid Burden Assay. To determine the metabolic burden of pNF8 plasmid in the GFP-labeled Lm 568, following the method by Ma and co-workers (2011) the growth curves of the GFP-labeled strain in single and binary biofilms with the Gram-negative strains were obtained and the Logistic model with lag phase was fitted to results as described above. Lm 568 pNF8 was enumerated on BHIA+8 µg/ml erythromycin. The logistic model estimates were then compared to those obtained for the parent Lm 568 strain, using the t-test (SAS software V. 9.2.).

Plasmid Stability Assay. To ensure that the pNF8-plasmid was stable in the GFP-labeled strain, 5 µl of an overnight culture of GFP-labeled Lm was inoculated into 5 ml fresh TSB+1% glucose without antibiotics (1:1000). One ml from this suspension was immediately withdrawn, diluted in PPS and spread plated onto both Oxford agar and BHIA+8 µg/ml erythromycin. The remained suspension (4 ml) was incubated at 15°C for 24 h, followed by enumeration and dilution. This process repeated for 5 consecutive days in fresh TSB+1% glucose without antibiotics. Equation 13 (Todar, 2004) was used to calculate the number of generations that the bacterium would go through in the specified period:

$$n = 3.3\ \mathrm{Log}(b/B) \qquad \text{(eq. 13)}$$

n= number of generations (number of times the cell population doubles during the time interval)

b= number of bacteria at the end of the time interval (CFU/ml)

B= number of bacteria at the beginning of a time interval (CFU/ml)

Bacteria counted on the Oxford agar will represent all *L. monocytogenes* cells whereas populations counted on the BHIA+erm will only represent Lm 568 pNF8 cells allowing for comparisons of counts using the paired t-test (SAS software V. 9.2.). Based on the results from this experiment, it was calculated that this time interval (5 d with daily transfers) corresponded to approximately 50 generations of the GFP-labeled strain with ~10 generations per transfer as the initial population of 10^6 CFU/ml rose to nearly 10^9 CFU/ml after 24 h (=1 transfer period).

DAPI staining and Epifluorescence Observation. To visualize and localize the individual bacterial species in binary-culture biofilms, biofilms of GFP-labeled Lm 568 and the Gram-negative bacteria were formed on SS coupons according to the protocol provided in section 3.3 (15°C, 100% RH, 48 h). The coupons were subsequently rinsed in PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1000 ml dH_2O), counter stained with DAPI (slow fade, gold antifade reagent with DAPI, Life Technologies Inc., Burlington, ON, Canada), quickly rinsed again in PBS and mounted on microscope slides (double frosted, Fisher Scientific) using carbon adhesive tabs. The microscope cover glass (18×18 mm, #1, Fisher Scientific) was placed above the coupons; however, to protect the biofilms from the direct contact and the weight of the cover slips, a rubber perfusion chamber (1 mm deep, 9 mm diameter, EMS, Cedarlane, Burlington, ON, Canada) was employed as a holder to make a tiny distance between the cover slip and the coupons. This gap was filled with non-fluorescing immersion oil (for fluorescence and general microscopy, type DF, Cargille Laboratories Inc., Cedar Grove, NJ, USA). The slides were placed under an epifluorescence microscope (Nikon Eclipse 80i, Nikon Canada) and the imaging performed in the epi-fluorescence illumination mode (multi band filter block, 350-750 nm) and using 100× magnification oil objective lens (Nikon-

plan 100X 1.25 oil ph3 DL ∞/0.17 WD 0.2). Under the fluorescent light, the GFP-labeled strain fluoresces green while the unlabeled Gram-negative strains fluoresce blue due to being stained with DAPI.

3.8.3.2. Fluorescent Gram-staining and Microscopic Observation

Another method of differentiating fluorescence staining was also employed to visualize the bacterial biofilms on SS coupons.

The Live BacLight™ Bacterial Gram stain kit (L-7005, Life Technologies Inc.) was used to tag the bacteria found in the biofilms on SS coupons based on their Gram-reaction. The staining kit contains of two components: Component A which is the green fluorescing SYTO 9 (3.34 mM, excitation/emission: 480 nm/500 nm) in anhydrous DMSO and component B that consists of the red fluorescing hexidium iodide (4.46 mM, excitation/emission: 480 nm/625 nm) in anhydrous DMSO. The green fluorescent SYTO9 will stain all live Gram-negative and positive cells whereas the red fluorescent dye hexidium iodide effectively enters and replaces (by quenching) SYTO9 in Gram-positive cells. Single and binary biofilms (15°C, 100% RH, 48 h) were formed as previous outlined using the parental strain of *L. monocytogenes* and the selected Gram-negative strains as indicated. Using the Live BacLight™ Bacterial Gram dyes, the live Gram-positive strain *(L. monocytogenes)* fluoresced red while the other live Gram-negative strains (*P. fluorescens*, *S. liquefaciens* or *Sh. putrefaciens*) fluoresced green. Dead cells may stain variably.

CHAPTER 4 RESULTS

4.1. Modeling of the Growth of Different Bacterial Strains during Formation of Mono- and Binary-Culture Biofilms on SS Coupons.

The growth curves obtained for the individual bacteria during the formation of single and binary biofilms on SS coupons were fitted by the Logistic with lag phase, Baranyi with fixed lag phase and modified Gompertz models, which are three commonly used growth models in food microbiology. The non-linear growth models were first assessed based on the three primary criteria for an acceptable model as described in section 3.6 and then analyzed for their goodness of fit using three statistical indices (MSE_{model}, adjusted r^2 and F-test). The analysis of models' goodness of fit performance showed that none of the model fits passed the F-test (Table 2). Since the three statistical indices were equally considered in selection of the best model, the F-test was dropped and only the MSE_{model} and r^2 were employed for assessment of the goodness of fit. Comparison of the MSE_{model} and r^2 values revealed that it was the Logistic with lag phase that resulted in the model fit (the highest values for r^2 and the smallest for MSE_{model}, Table 2). Consequently, the Logistic with lag phase model was chosen to describe and compare the biofilm growth kinetics.

Table 3 shows the parameter estimates obtained from fitting the Logistic model with lag phase to the data. This model, which describes the growth kinetics based on a piecewise non-linear regression analysis, enabled the most accurate estimation of lag time (λ), specific growth rate (μ_{max}) as well as the other parameters (N_{max} and N_0) in comparison to the Baranyi and modified Gompertz models.

Table 2. Statistical indices describing the goodness of fit for three commonly used non-linear models (Logistic with lag phase, Baranyi with fixed lag phase and the modified Gompertz) as applied to characterize the growth during formation of mono- and binary-culture biofilms on stainless steel coupons (15°C, 100% RH). The growth models were fitted to all data ($Log_{10}(CFU/cm^2)$)at once (n=6).

Bacterial biofilm	**Statistical indices***	**Logistic with lag phase**	**Baranyi with fixed lag phase**	**Modified Gompertz**
Single *L. monocytogenes*	MSE_{model}	0.063	0.063	0.072
	r^2	0.984	0.981	0.979
	F	6.607	7.169	7.976
Single *P. fluorescens*	MSE_{model}	0.049	0.044	0.019
	r^2	0.984	0.986	0.994
	F	3.151	3.520	3.507
Single *S. liquefaciens*	MSE_{model}	0.015	0.013	0.014
	r^2	0.994	0.995	0.995
	F	2.748	3.130	3.294
Single *Sh. utrefaciens*	MSE_{model}	0.034	0.030	0.026
	r^2	0.989	0.990	0.991
	F	2.493	2.620	2.148
Binary biofilm *L. monocytogenes* (with *P. fluorescens*)	MSE_{model}	0.037	0.044	0.036
	r^2	0.980	0.972	0.978
	F	2.832	3.968	3.124
Binary biofilm *L. monocytogenes* (with *S. liquefaciens*)	MSE_{model}	0.052	0.099	0.086
	r^2	0.979	0.956	0.962
	F	4.042	8.210	7.005

Continued on the next page

Table 2. Statistical indices describing the goodness of fit for three commonly used non-linear models (Cont'd).

Bacterial biofilm	Statistical indices	Logistic with lag phase	Baranyi with fixed lag phase	Modified Gompertz
Binary biofilm *L .monocytogenes* (with *Sh. putrefaciens*)	MSE_{model}	0.057	0.074	0.046
	r^2	0.991	0.986	0.991
	F	4.585	7.858	4.779
Binary biofilm *P. fluorescens* (with *L. monocytogenes*)	MSE_{model}	0.059	0.072	0.092
	r^2	0.988	0.983	0.982
	F	5.047	7.833	7.475
Binary biofilm *S. liquefaciens* (with *L. monocytogenes*)	MSE_{model}	0.104	0.178	0.187
	r^2	0.973	0.956	0.962
	F	6.783	19.719	15.529
Binary biofilm *Sh. putrefaciens* (with *L. monocytogenes*)	MSE_{model}	0.072	0.104	0.064
	r^2	0.988	0.976	0.987
	F	5.090	10.997	5.082

* None of the F-values were significant (P>0.05) as F>F-table value. $F_{(26, 25)}$ table value for single *P. fluorescens*, single *S. liquefaciens* and single *Sh. putrefaciens* is 1.92, whereas for all other biofilms the $F_{(38, 35)}$ table value is equal to 1.74.

Table 3. Logistic model with lag phase parameter estimates to describe the growth of *L. monocytogenes* and three Gram-negative bacteria during formation of mono- or binary-culture biofilms. Cells were grown (100% RH, 15°C) in TSB+1% glucose on SS and enumerated at specific time intervals on BHIA and/or suitable selective agars. The Logistic with lag phase model was fitted to the growth curves (Log_{10}(CFU/cm^2 [n=6] vs. time) .

Bacteria and Biofilm Treatment	N_{max}[I] Log_{10}(CFU/cm^2)	μ_{max}[II] (1/h)	N_0[III] Log_{10}(CFU/cm^2)	λ[IV] (h)
Single *L. monocytogenes*	6.83 [dV]±0.08[VI]	0.36 [b, c]±0.03	2.85 [c]±0.05	19.12 [b]±0.85
Single *P. fluorescens*	7.25 [c]±0.06	0.38 [b]±0.02	3.04 [b]±0.06	0.00 [e]±0.00
Single *S. liquefaciens*	7.46 [b]±0.06	0.31 [d]±0.01	3.57 [a]±0.06	0.00 [e]±0.00
Single *Sh. putrefaciens*	7.84 [a]±0.13	0.30 [e, d]±0.01	3.61[a]±0.10	0.00 [e]±0.00
Binary *L. monocytogenes* (with *P. fluorescens*)	5.57 [f]±0.02	0.32 [d]±0.03	2.85 [c]±0.08	12.00 [c]±0.00
Binary *L. monocytogenes* (with *S. liquefaciens*)	5.85 [e]±0.07	0.22 [f]±0.02	2.58 [e, d]±0.10	12.00 [c]±0.00
Binary *L. monocytogenes* (with *Sh. putrefaciens*)	7.29 [c]±0.10	0.59 [a]±0.03	2.52 [e, d]±0.10	21.84 [a]±0.36
Binary *P. fluorescens* (with *L. monocytogenes*)	7.75 [a]±0.09	0.32 [d]±0.01	2.62 [d]±0.14	4.78 [d]±0.62
Binary *S. liquefaciens* (with *L. monocytogenes*)	7.55 [b]±0.07	0.27 [e]±0.00	2.87 [c]±0.09	0.12[e]±0.02
Binary *Sh. putrefaciens* (with *L. monocytogenes*)	7.56 [b]±0.08	0.33 [c, d]±0.01	2.42 [e]±0.04	12.37 [c]±0.45

[I] N_{max} denotes the maximum population density.
[II] μ_{max} represents the maximum specific growth rate.
[III] N_0 is the initial population level.
[IV] λ denotes the lag time.
[V] Values in the same column followed by same letters are not significantly different (P > 0.05) from each other (data should be interpreted pairwise based on the Tukey test at α=0.05).
[VI] Model estimate ± standard error of mean.

Table 4. Overview of the effect that the presence of a Gram-negative bacteria had on the growth and biofilm formation of *L. monocytogenes* and vice versa using pairwise comparison of Logistic model with lag phase parameter estimates from Table 3.

Biofilm Comparisons	N_{max}	μ_{max}	N_0	λ
L. monocytogenes (with *P. fluorescens*) VS. *L. monocytogenes*	Decreased[a]	Decreased	Similar[b]	Decreased
L. monocytogenes (with *S. liquefaciens*) VS. *L. monocytogenes*	Decreased	Decreased	Decreased	Decreased
L. monocytogenes (with *Sh. putrefaciens*) VS. *L. monocytogenes*	Increased[c]	Increased	Decreased	Increased
P. fluorescens (with *L. monocytogenes*) VS. *P. fluorescens*	Increased	Decreased	Decreased	Increased
S. liquefaciens (with *L. monocytogenes*) VS. *S. liquefaciens*	Similar	Decreased	Decreased	Similar
Sh. putrefaciens (with *L. monocytogenes*) VS. *Sh. putrefaciens*	Decreased	Similar	Decreased	Increased

a - Decreased means that the model parameter value was significantly ($P<0.05$) lower for the bacteria in the binary-culture biofilm compared to the mono-culture biofilm.

b - No significant ($P>0.05$) difference was found between the parameter values for the bacteria in the mono- and binary-culture biofilms.

c - Increased means that the model parameter value was significantly ($P<0.05$) higher for the bacteria in the binary-culture biofilm compared to the mono-culture biofilm.

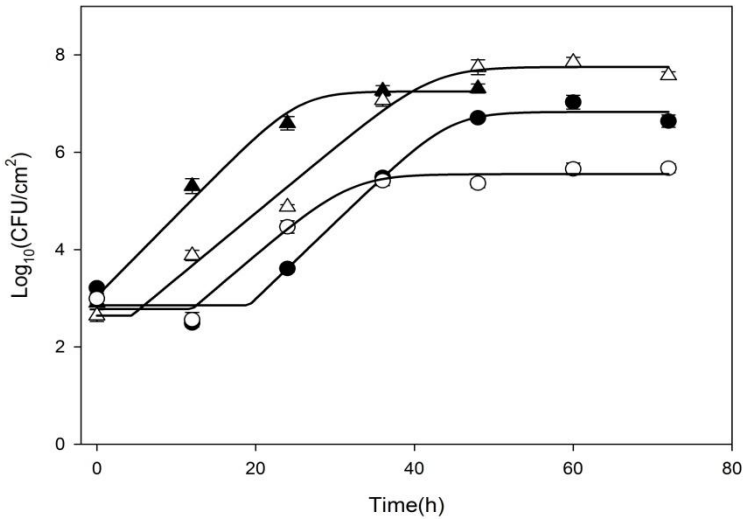

Figure 3. Growth of *L. monocytogenes* and *P. fluorescens* in mono- or binary-culture biofilms. The biofilms were formed on SS coupons (100% RH, 15°C, 48 or 72 h) and the microbial populations were enumerated on general or selective agars (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* mono-culture biofilm, (▲) represents *P. fluorescens* mono-culture biofilm, (○) is *L. monocytogenes* in binary-culture biofilm with *P. fluorescens* and (Δ) represents *P. fluorescens* in binary-culture biofilm with *L. monocytogenes*. Lines in the graph represent the numbers of microbial cells predicted by the Logistic model with lag phase fits.

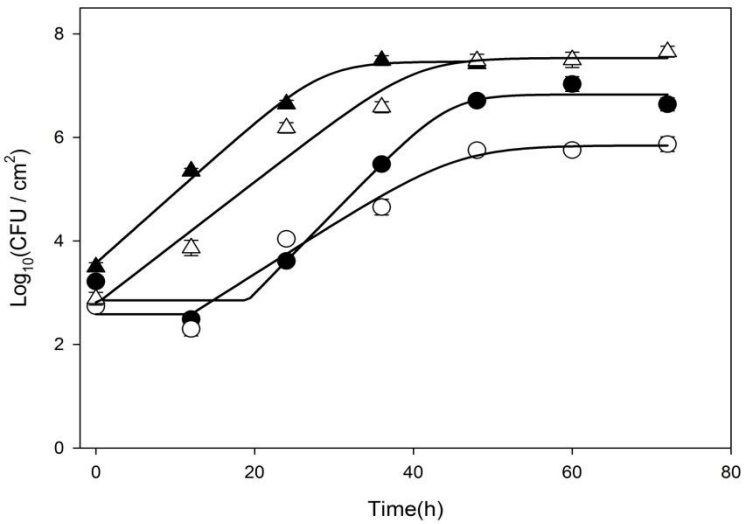

Figure 4. Growth of *L. monocytogenes* and *S. liquefaciens* in mono- or binary-culture biofilms. The biofilms were formed on SS coupons (100% RH, 15°C, 48 or 72 h) and the microbial populations were enumerated on general or selective agars (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* mono-culture biofilm, (▲) represents *S. liquefaciens* mono-culture biofilm, (○) is *L. monocytogenes* in binary-culture biofilm with *S. liquefaciens* and (Δ) represents *S. liquefaciens* in binary-culture biofilm with *L. monocytogenes*. Lines in the graph represent the numbers of microbial cells predicted by the Logistic model with lag phase fits.

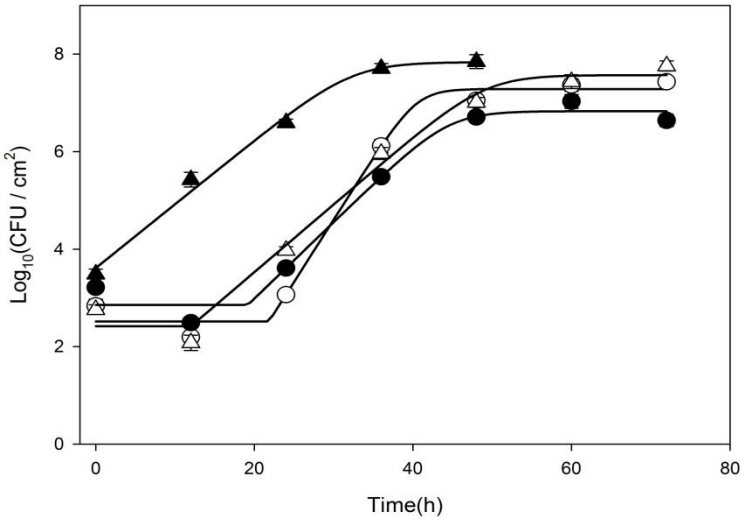

Figure 5. Growth of *L. monocytogenes* and *Sh. putrefaciens* in mono- and binary-culture biofilms. The biofilms were formed on SS coupons (100% RH, 15°C, 48 or 72 h) and the microbial populations were enumerated on general or selective agars (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* mono-culture biofilm, (▲) represents *Sh. putrefaciens* mono-culture biofilm, (○) is *L. monocytogenes* in binary-culture biofilm with *Sh. putrefaciens* and (Δ) represents *Sh. putrefaciens* in binary-culture biofilm with *L. monocytogenes*. Lines in the graph represent the numbers of microbial cells predicted by the Logistic model with lag phase fits.

The population of *P. fluorescens* rose at the highest maximum specific growth rate (μ_{max} = 0.38) observed among all mono-culture biofilms, although statistically this μ_{max} was not significantly different (P>0.05) compared to the μ_{max} of *L. monocytogenes*. The μ_{max} of *S. liquefaciens* and *Sh. putrefaciens* in single species biofilms were similar and significantly (P<0.05) lower than those of the other two bacteria (Table 3). The maximum population density (MPD) was significantly different (P<0.05) for the four strains in the monoculture biofilms, with *Sh. putrefaciens* reaching the highest levels of $10^{7.8}$ CFU/cm^2 followed by *S. liquefaciens*, *P. fluorescens* and then *L. monocytogenes* with levels of $10^{6.8}$ CFU/cm^2 (Table 3, Figures 3-5). Also, *L. monocytogenes* was the only bacterium that showed a lag phase in the mono-culture biofilm (Table 3). N_0 values predicted by the model came close to those actually spotted on to the SS coupons of ~10^3 CFU/cm^2 (Table 3 and Figures 3-5).

When grown in binary-culture biofilms on SS surfaces, the competitor Gram-negative strains had diverse impacts on the growth kinetics of *L. monocytogenes* (Table 3 and 4). The different interactions among *L. monocytogenes* and the Gram-negative strains resulted in different patterns of changes to μ_{max}. While *Sh. putrefaciens* significantly ($P<0.05$) increased the μ_{max} of *L. monocytogenes* in binary biofilms, *P. fluorescens* and *S. liquefaciens* significantly ($P<0.05$) reduced μ_{max} of *L. monocytogenes* in co-cultures with these Gram-negative food-spoilage bacteria. Similarly, the *Listeria* strain significantly ($P<0.05$) reduced the μ_{max} of *P. fluorescens* and *S. liquefaciens* while the influence was not significant ($P>0.05$) on that of *Sh. putrefaciens*.

In terms of MPD, while *P. fluorescens* and *S. liquefaciens* significantly ($P<0.05$) decreased the N_{max} achieved by *L. monocytogenes* in joint biofilms as compared to the control, *Sh. putrefaciens* significantly ($P<0.05$) increased the maximum populations reached by the pathogen. In contrast, presence of *L. monocytogenes* significantly ($P<0.05$) reduced or increased the maximum populations of *Sh. putrefaciens* and *P. fluorescens*, respectively, while the pathogen did not have any significant ($P>0.05$) influence on N_{max} of *S. liquefaciens* (Table 3 and 4).

In terms of lag time, the model yielded estimates that seemed close to the graphical presentation of the results (Figures 3, 4 and 5). The presence of *P. fluorescens* and *S. liquefaciens* in binary-culture biofilms significantly ($P<0.05$) decreased the lag time to 12 h for *L. monocytogenes*, while *Sh. putrefaciens* increased ($P<0.05$) the lag time to 22 h of the Gram-positive bacterium in comparison to the lag time of 19 h in mono-culture biofilms (Table 3). On the other hand, the co-culture with *L. monocytogenes* introduced a significant ($P<0.05$) lag time of 4.8 and 12.4 h for *P. fluorescens* and *Sh. putrefaciens*, respectively, while none of the strains exhibited a lag phase in single species biofilms. In the case of *P. fluorescens*, the time between invidual sampling points (12 h) made it difficult to verify the model estimate of $\lambda=4.8$ h from the graph (Figure 3). The lag time remained close to 0 h for *S. liquefaciens* in co-culture with *Listeria* (Table 3, Figure 4).

The other parameter that was estimated by the Logistic model with lag phase was initial level of populations (N_0) (Table 3). Experimentally the initial bacterial populations were adjusted to around 10^3 CFU/cm^2; however, to account for the decrease in populations observed in some samples after 12 h the model fit resulted in estimated N_0-values lower than those initially spotted (Figures 3-5).

4.2. Modeling of the Survival of Different Bacterial Strains during Desiccation in Mono- and Binary-Culture Biofilms on SS Coupons.

The desiccation survivor curves were constructed (see appendix A) and the non-linear inactivation models fitted to the data as described above (section 3.7). Only inactivation models lacking a lag phase parameter were considered because no lag time was observed in the survivors' curves. An assessment of the models' goodness of fit based on MSE_{model}, r^2, F-test showed that the double-Weibull model provided the best fit to the data (Table 5). Therefore this inactivation model was employed in the analysis of the desiccation inactivation kinetics observed in the different biofilms. The data from the data loggers showed the desired RH and temperature continuously existed in the desiccation chambers throughout the 21-day desiccation period with the exception of brief (< 2 h) increases in temperature and RH caused by the sampling events (Figure 39, Appendix A).

The normalized ($Log_{10}N/N_0$) survivor curves with the double-Weibull model fits are shown in Figures 6-8. The model parameter estimates generated by the GInaFiT curve-fitting tool are tabulated in Table 6. The pair wise comparisons of model parameter means are summarized in Table 7

Table 5. Statistical indices value for different inactivation models fitted to the desiccation survivor curves obtained for the mono- and binary-culture biofilms on SS coupons.

Bacterial biofilm	Statistical indices	Inactivation models			
		Weibull	**Weibull+tail**	**Double Weibull**	**Biphasic**
Single *L. monocytogenes*	r^2	0.949	0.961	0.960	0.945
	MSE_{model}	0.021	0.016	0.017	0.023
	F [a]	1.784	1.394	1.418	1.957
	F-test status[b]	Rejected	Accepted	Accepted	Rejected
Single *P. fluorescens*	r^2	0.913	0.945	0.945	0.898
	MSE_{model}	0.309	0.194	0.196	0.362
	F	37.312	23.473	23.724	43.748
	F-test status	Rejected	Rejected	Rejected	Rejected
Single *S. liquefaciens*	r^2	0.950	0.965	0.974	0.974
	MSE_{model}	0.022	0.015	0.011	0.011
	F	2.431	1.688	1.251	1.251
	F-test status	Rejected	Rejected	Accepted	Accepted
Single *Sh. putrefaciens*	r^2	0.952	0.973	0.973	0.459
	MSE_{model}	0.504	0.283	0.286	5.623
	F	2.157	1.213	1.223	24.075
	F-test status	Rejected	Rejected[c]	Accepted	Rejected
Binary biofilm *L. monocytogenes* (with *P. fluorescens*)	r^2	0.980	0.989	0.989	0.987
	MSE_{model}	0.023	0.013	0.013	0.015
	F	1.842	1.056	1.069	1.204
	F-test status	Rejected	Accepted	Accepted	Accepted
Binary biofilm *L. monocytogenes* (with *S. liquefaciens*)	r^2	0.982	0.988	0.988	0.983
	MSE_{model}	0.018	0.012	0.012	0.018
	F	1.790	1.138	1.149	1.713
	F-test status	Rejected	Accepted	Accepted	Accepted
Binary biofilm *L .monocytogenes* (with *Sh. putrefaciens*)	r^2	0.974	0.987	0.987	0.985
	MSE_{model}	0.022	0.011	0.012	0.013
	F	2.273	1.160	1.170	1.346
	F-test status	Rejected	Accepted	Accepted	Accepted

Continued on the next page

Table 5. Statistical indices value for different inactivation models fitted to the desiccation survivor curves obtained for the mono- and binary-culture biofilms on SS coupons. (Cont'd).

Bacterial biofilm	Statistical indices	Weibull	Weibull+tail	Double Weibull	Biphasic
Binary biofilm *P. fluorescens* (with *L. monocytogenes*)	r^2	0.961	0.990	0.990	0.938
	MSE_{model}	0.193	0.048	0.049	0.303
	F	16.948	4.175	4.293	26.518
	F-test status	Rejected	Rejected	Rejected	Rejected
Binary biofilm *S. liquefaciens* (with *L. monocytogenes*)	r^2	0.944	0.943	0.979	0.963
	MSE_{model}	0.031	0.032	0.012	0.021
	F	3.697	3.794	1.401	2.424
	F-test status	Rejected	Rejected[c]	Accepted	Rejected
Binary biofilm *Sh. putrefaciens* (with *L. monocytogenes*)	r^2	0.962	0.996	0.996	0.969
	MSE_{model}	0.475	0.052	0.053	0.388
	F	70.933	7.779	7.955	57.941
	F-test status	Rejected	Rejected	Rejected	Rejected
	Score	0/10	4/10	7/10	4/10

a- $F_{(86, 75)}$ table value for single *L. monocytogenes* and single *S. liquefaciens* is 1.52 and for all others $F_{(38, 35)}$ table is equal to 1.74

b- Model fit was accepted if F-values<F-table values and rejected if F-values>F-table values

c- $Log_{10}(N_{res})$ is less than the minimal measured value. Model with tailing is unlikely for these data.

Table 6. Bacterial inactivation kinetics observed in mono- or binary-culture biofilms consisting of *L. monocytogenes* and three competitor bacterial strains during desiccation (43% RH, 15°C) in TSB+1% glucose on SS coupons. Survivors were enumerated on general or selective agars, converted into $Log_{10}(N/N_0)$ (n=6) and the double Weibull model fitted to the resulting survivor curves.

Bacterial Biofilm	**α^{I}**	**δ_1^{II} (days)**	**p^{III}**	**δ_2^{IV} (days)**	**$\Delta Log_{10}(N/N_0)^{V}$**
Single *L. monocytogenes*	1.94 [cVI]±0.89[VII]	3.06 [c, b] ±0.19	0.58 [c, d] ±0.17	266.10 [a]±10.21	2.12 [a] ±0.14
Single *P. fluorescens*	4.93 [b]±0.48	0.35 [e, d] ±0.08	0.50 [c, d]±0.22	232.77[a,b]±24.87	5.24 [d]±0.10
Single *S. liquefaciens*	1.35 [c]±0.07	2.39 [c]±0.07	1.04 [b]±0.09	25.14 [e]±0.29	2.23 [a]±0.12
Single *Sh. putrefaciens*	8.63 [a]±0.34	0.04 [e]±0.00	0.38 [d]±0.07	44.04 [e]±10.43	9.27 [f]±0.00
Binary *L. monocytogenes* (with *P. fluorescens*)	2.84 [c]±1.39	4.28 [a]±0.11	0.87 [c, b]±0.17	134.16 [d]±24.11	3.04 [c]±0.08
Binary *L. monocytogenes* (with *S. liquefaciens*)	2.32 [c]±1.21	3.37 [b]±0.14	0.73 [c,b,d]±0.15	105.01 [d]±11.90	2.87 [c]±0.14
Binary *L. monocytogenes* (with *Sh. putrefaciens*)	2.24 [c]±0.42	4.31 [a]±0.09	0.84 [c,b]±0.14	187.16 [c]±27.21	2.60 [b]±0.15
Binary *P. fluorescens* (with *L. monocytogenes*)	5.82 [b]±0.22	0.24 [e, d]±0.04	0.49 [c,d]±0.09	109.75 [d]±8.12	6.32 [e]±0.12
Binary *S. liquefaciens* (with *L. monocytogenes*)	1.15 [c]±0.05	2.63 [c]±0.12	2.47 [a]±0.20	20.89 [e]±0.03	2.26 [a]±0.03
Binary *Sh. putrefaciens* (with *L. monocytogenes*)	9.33 [a]±0.14	0.78 [d]±0.20	0.79 [c,b,d]±0.08	206.65 [b,c]±11.26	9.46 [f]±0.00

[I] α denotes the proportion of subpopulation 1 to subpopulation 2.

[II] δ_1 represents the time to first decimal reduction in subpopulation 1.

[III] P is the shape factor.

[IV] δ_2 denotes the first decimal reduction time for subpopulation 2.

[V] $\Delta Log_{10}(N/N_0)$ is not a model parameter estimate; however, it shows the total loss of viable cells during the course of desiccation experiments.

[VI] Values in the same column followed by same letters are not significantly different (P > 0.05) from each other (Data should be interpreted pairwise based on the Tukey test at α=0.05).

[VII] Model estimate ± standard error of mean.

Table 7. Pairwise comparison of double Weibull model parameters' estimates of desiccation survival in mono- and binary-culture bacterial biofilms on SS coupons derived from Table 6.

Bacterial biofilms comparison	**α**	**δ_1**	**P**	**δ_2**	**$\Delta Log_{10}(N/N_0)$**
L. monocytogenes (with *P. fluorescens*) VS. *L. monocytogenes*	Similar [a]	Increased [b]	Similar	Decreased [c]	Increased
L. monocytogenes (with *S. liquefaciens*) VS. *L. monocytogenes*	Similar	Similar	Similar	Decreased	Increased
L. monocytogenes (with *Sh. putrefaciens*) VS. *L. monocytogenes*	Similar	Increased	Similar	Decreased	Increased
P. fluorescens (with *L. monocytogenes*) VS. *P. fluorescens*	Similar	Similar	Similar	Decreased	Increased
S. liquefaciens (with *L. monocytogenes*) VS. *S. liquefaciens*	Similar	Similar	Increased	Similar	Similar
Sh. putrefaciens (with *L. monocytogenes*) VS. *Sh. putrefaciens*	Similar	Increased	Similar	Increased	Similar

a– No significant ($P>0.05$) difference was found between the parameter values for the mono- and binary-culture biofilms

b– Increased means that the model parameter value was significantly ($P<0.05$) higher for the bacteria in the binary-culture biofilm compared to the mono-culture biofilm

c– Decreased means that the model parameter value was significantly ($P<0.05$) lower for the bacteria in the binary-culture biofilm compared to the mono-culture biofilm.

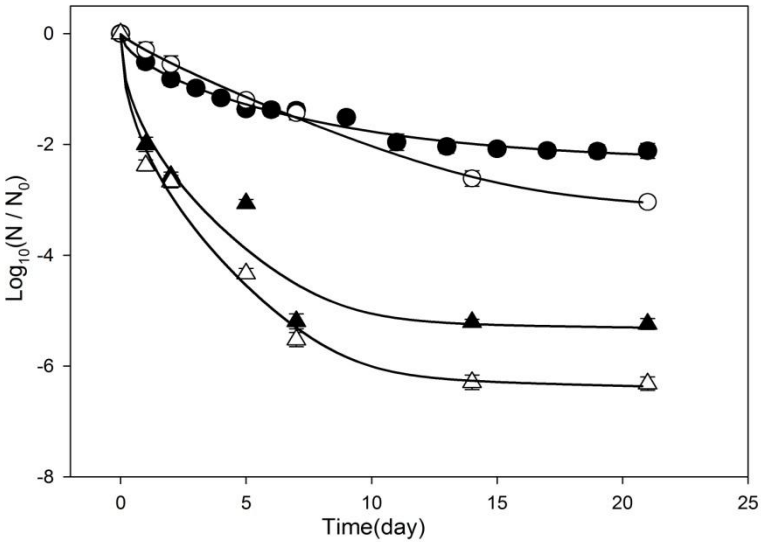

Figure 6. Desiccation survival of *L. monocytogenes* and *P. fluorescens* in mono- and binary-culture biofilms. The SS coupons carrying previously formed biofilms (100% RH, 15°C, 48 h, started on day -2) were desiccated (43% RH, 15°C, 21 d) and survivors were enumerated on general or selective agars (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* mono-culture biofilm, (▲) represents *P. fluorescens* mono-culture biofilm, (○) is *L. monocytogenes* in binary-culture biofilm with *P. fluorescens* and (Δ) represents *P. fluorescens* in binary-culture biofilm with *L. monocytogenes*. Lines in the graph represent the numbers of survivors predicted by double-Weibull model fits (modeling began on day 0).

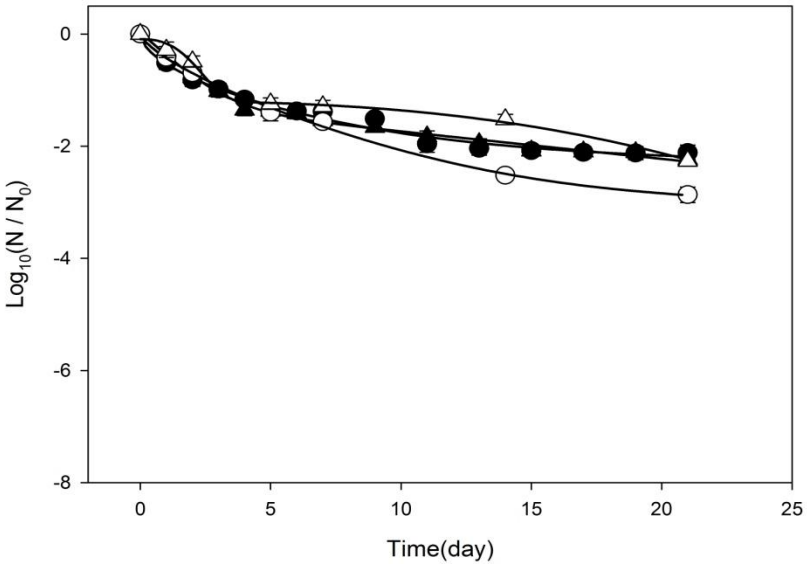

Figure 7. Desiccation survival of *L. monocytogenes* and *S. liquefaciens* in mono- and binary-culture biofilms. The SS coupons carrying previously formed biofilms (100% RH, 15°C, 48 h, started on day -2) were desiccated (43% RH, 15°C, 21 d) and survivors were enumerated on general or selective agars (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* mono-culture biofilm, (▲) represents *S. liquefaciens* mono-culture biofilm, (○) is *L. monocytogenes* in binary-culture biofilm with *S. liquefaciens* and (Δ) represents *S. liquefaciens* in binary-culture biofilm with *L. monocytogenes*. Lines in the graph represent the numbers of survivors predicted by double-Weibull model fits (modeling began on day 0).

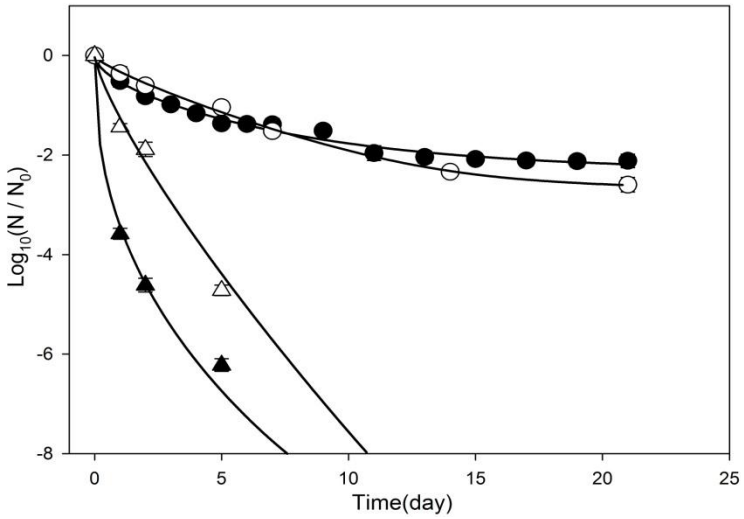

Figure 8. Desiccation survival of *L. monocytogenes* and *Sh. putrefaciens* in mono- and binary-culture biofilms. The SS coupons carrying previously formed biofilms (100% RH, 15°C, 48 h, started on day -2) were desiccated (43% RH, 15°C, 21 d) and survivors were enumerated on general or selective agars (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* mono-culture biofilm, (▲) represents *Sh. putrefaciens* mono-culture biofilm, (○) is *L. monocytogenes* in binary-culture biofilm with *Sh. putrefaciens* and (Δ) represents *Sh. putrefaciens* in binary-culture biofilm with *L. monocytogenes*. Lines in the graph represent the numbers of survivors predicted by double-Weibull model fits (modeling began on day 0). The detection limit is -8 $Log_{10}(N/N_0)$ in this experiment.

As can be seen in Table 5, the double-Weibull model was the model with the highest number (7 out of 10) of biofilm treatments that passed the strict F-test for the curve fit, and thus this model was selected to describe the inactivation kinetics in mono- or binary-culture biofilms during desiccation at 43% RH and 15°C. The chosen model was fitted to individual inactivation data points (i.e., the survivors) to generate estimates for the model parameters (model parameter ± standard error of mean). The double-Weibull model assumes that the bacterial populations consist of two subpopulations 1 and 2 where the subpopulations 1 are more sensitive to the specific stress while subpopulation 2 is more resistant (Coroller et al., 2006).

The double-Weibull α parameter, which estimates the proportion of subpopulations 1 and 2 [$Log_{10}(N_{01}/N_{02})$], was significantly ($P<0.05$) higher in the mono-culture biofilm made by *Sh. putrefaciens* followed by *P. fluorescens*, *L. monocytogenes* and *S. liquefaciens* where the latter two were not statistically different ($P>0.05$) from each another (Table 6). The $\boldsymbol{\delta}_1$ (i.e., the time to the first decimal reduction in subpopulation 1) was similar ($P>0.05$) for the mono-species biofilms made by *L. monocytogenes* (3 days) and *S. liquefaciens* (2.4 days). Similar resistance patterns ($P>0.05$) were observed for *P. fluorescens* and *Sh. putrefaciens* ($\boldsymbol{\delta}_1$=0.4 days); which meant that *L. monocytogenes* and *S. liquefaciens* in mono-culture biofilms were significantly ($P<0.05$) more resistant to desiccation than *P. fluorescens* and *Sh. putrefaciens* (Table 6).

The $\boldsymbol{\delta}_2$ that represents the first decimal reduction time in subpopulation 2 differed significant ($P<0.05$) among the mono-culture biofilms (Table 6). In this regard, *L. monocytogenes* and *P. fluorescens* exhibited similar ($P>0.05$) $\boldsymbol{\delta}_2$ values of 233-260 days which were significantly higher ($P<0.05$) than the 25-44 days obtained for *S. liquefaciens* and *Sh. putrefaciens*. The absolute reduction in number of bacterial populations [$\Delta Log_{10}(N/N_0)$] was higher ($P<0.05$) in mono-culture biofilms consisting of *Sh. putrefaciens* (>8 orders of magnitude) followed by *P. fluorescens* (5.3 orders of magnitude), *S. liquefaciens* and *L. monocytogenes*, where the reduction for the latter two (2 orders of magnitude) were not significantly different ($P>0.05$) from each other (Table 6, Figures 6-8). The shape factor (P) was similar ($P>0.05$) among all mono-culture biofilms with the exception *S. liquefaciens* biofilms which had a higher value ($P<0.05$) (Table 6).

Comparison of α parameters [$Log_{10}(N_{01}/N_{02})$] obtained for the mono- and binary-culture biofilms did not reveal any significant ($P>0.05$) differences (Table 6 and 7). This pattern of no difference ($P>0.05$) was also observed among all matching pairs of mono- and binary-culture biofilms.

Contrary to the α parameter, calculated values for $\boldsymbol{\delta}_1$ and $\boldsymbol{\delta}_2$ showed diverse trends. The $\boldsymbol{\delta}_1$ increased significantly ($P<0.05$) for *L. monocytogenes* in binary-culture

biofilms with *Sh. putrefaciens* or *P. fluorescens* ($\boldsymbol{\delta}1$=4.3 days in both) and *Sh. putrefaciens* in binary-culture biofilms with *L. monocytogenes* ($\boldsymbol{\delta}1$= 0.8 day) as compared to the controls (Table 6). None of the other binary-culture biofilm $\boldsymbol{\delta}_1$-values, however, differed significantly (P>0.05) from those obtained in the respective single species biofilms (Table 6 and 7). The $\boldsymbol{\delta}_2$ only increased significantly (P<0.05) for *Sh. putrefaciens* in the dual-species biofilm with *L. monocytogenes* as compared to the mono-culture *Sh. putrefaciens* biofilm. For all other matched biofilm treatments $\boldsymbol{\delta}_2$ elicited a significant (P<0.05) decrease. The resistance of subpopulation 2 in the binary-culture biofilm consisting of *S. liquefaciens* and *L. monocytogenes* remained similar to the control single species biofilms as no significant (P>0.05) differences were found in the $\boldsymbol{\delta}_2$ values (Table 6 and 7).

The shape parameters (p) were generally not significantly (P>0.05) different when comparing single and dual-species biofilms. The one exception was the binary-culture biofilm made by *S. liquefaciens* and *L. monocytogenes* where the p value significantly (P<0.05) increased for *S. liquefaciens* in comparison to the value in its single species biofilm (Table 6 and 7).

Although the $\Delta Log_{10}(N/N_0)$ is not a double-Weibull model parameter; it helped to gain a better understanding of the overall size of the reduction in survivors during the desiccation period (43% RH and 15°C). With the exception of the unchanged (P>0.05) survival of the Gram-negative bacteria in the binary-culture biofilms consisting of *S. liquefaciens* or *Sh. putrefaciens* with *L. monocytogenes*, the survival of *L. monocytogenes* and *P. fluorescens* was significantly (P<0.05) reduced in the binary-culture biofilms as compared to the controls (Table 6, Figures 6-8). *L. monocytogenes* and *S. liquefaciens* showed similar $\Delta Log_{10}(N/N_0)$ numbers ranging from 2.1 to 3 orders of magnitude while populations of *Sh. putrefaciens* and *P. fluorescens* were reduced by between 6.3 and >8 Log_{10} CFU/cm^2 (Table 6).

4.3. Microscopic Evaluation of Adhesion and Biofilm Formation

4.3.1. Surface Scanning Electron Microscopy (SEM)

As described in section 3.8.1 three different fixation protocols were employed in an attempt to protect the biofilm structures on the substratum (SS coupons) and allow for subsequent observation under the microscope and comparison of these method's ability to protect and display the biofilm structures.

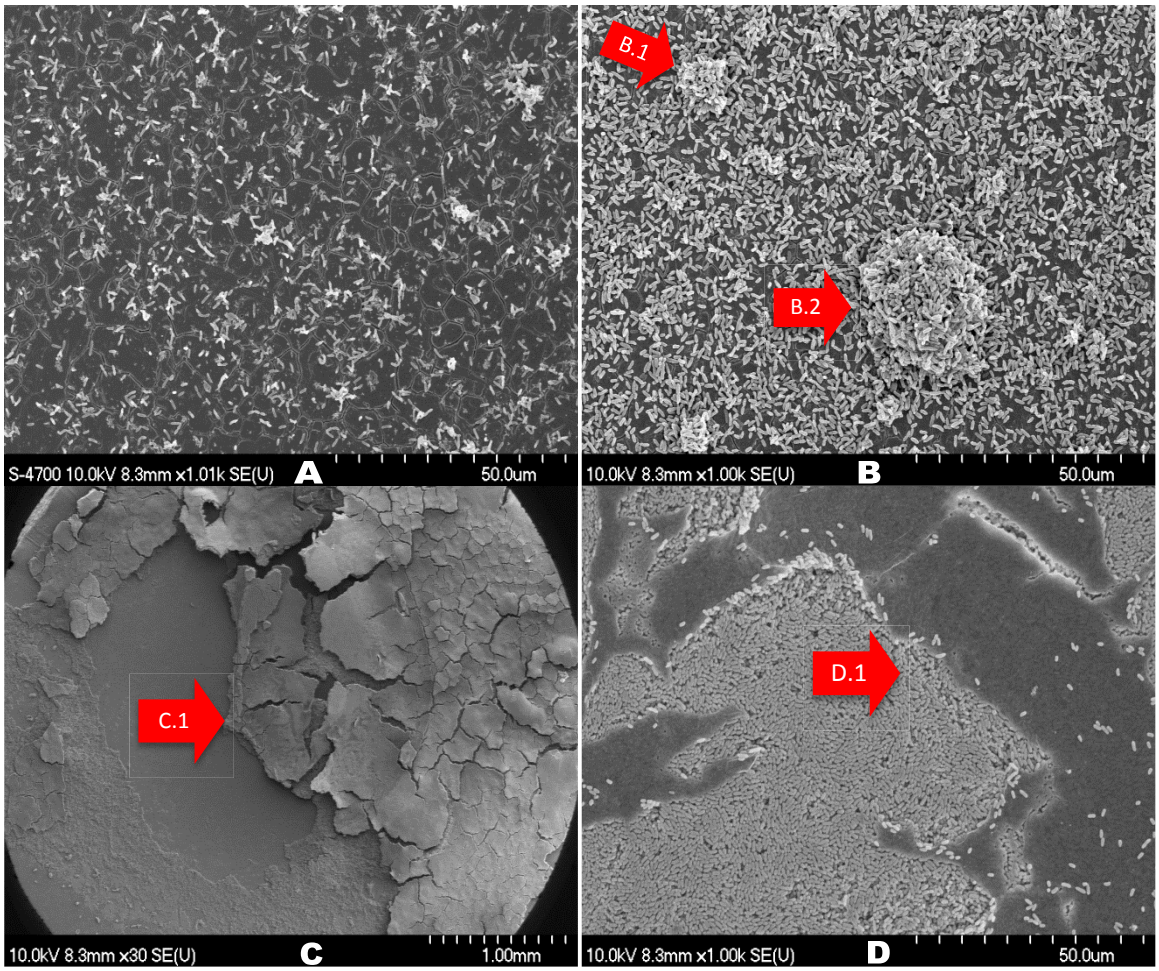

Figure 9. Scanning Electron Micrographs taken of *P. fluorescens* mono-culture biofilms (magnification: 30 or 1k, V_{acce}: 10 kV). The biofilm was developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B), or fixation in FC-72 solvent (C and D). The arrow B.1 shows a microcolony. The arrows C.1 and D.1 indicate the crust and amorphous EPS-like material encasing cells observed when using the organic solvent fixation method at two different magnifications.

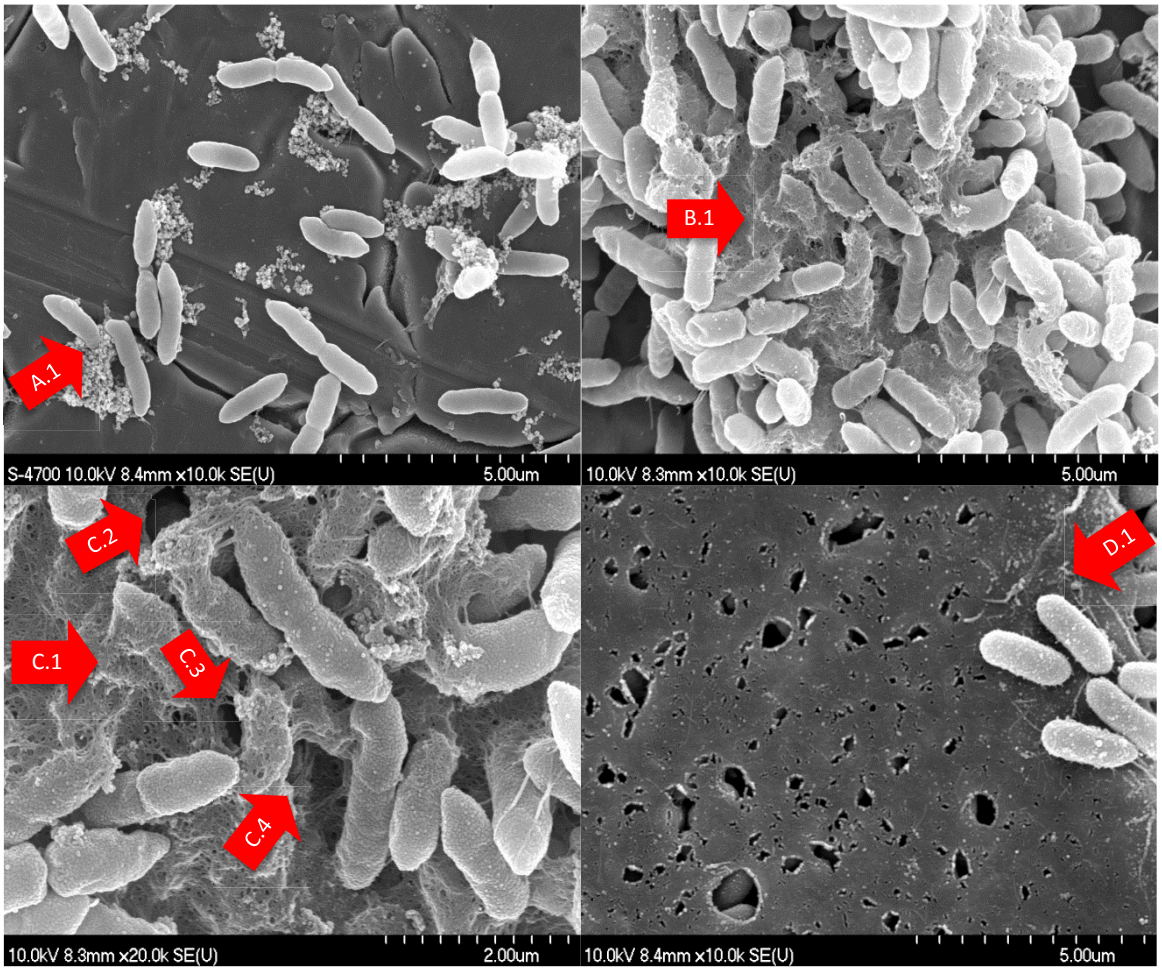

Figure 10. SEM pictures taken of *P. fluorescens* mono-culture biofilm (magnification: 10k or 20k, V_{acce}: 10 kV). The biofilm was developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B and C), or fixation in FC-72 solvent (D). The arrow A.1 shows cells interspersed with remnants of presumptive damaged EPS on the surface. Other arrows (B.1 and C.1) show at two different magnifications layers of presumptive EPS surrounding the cells. The arrows C.2 – C.4 point to pores in the biofilm structure and the arrow D.1 displays cells completely encased in a presumed EPS mass with a few free surface cells.

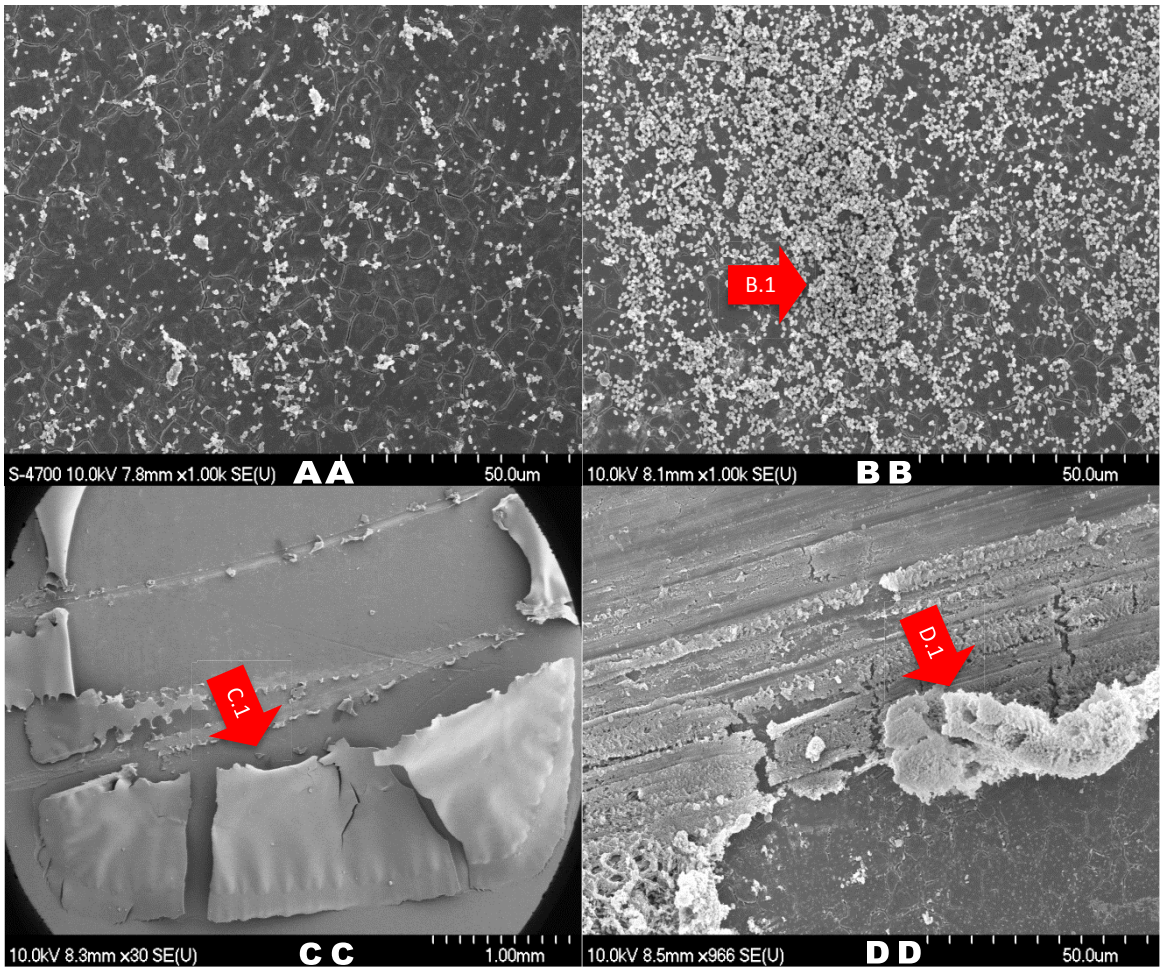

Figure 11. SEM pictures taken of *S. liquefaciens* mono-culture biofilms (magnification: 30 or 1k, V_{acce}: 10 kV). The biofilm was developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B), or fixation in FC-72 solvent (C and D). The arrow B.1 shows the formation of microcolonies. The arrows C.1 and D.1 indicate the layers of biofilm fixed using the organic solvent fixation at two different magnifications.

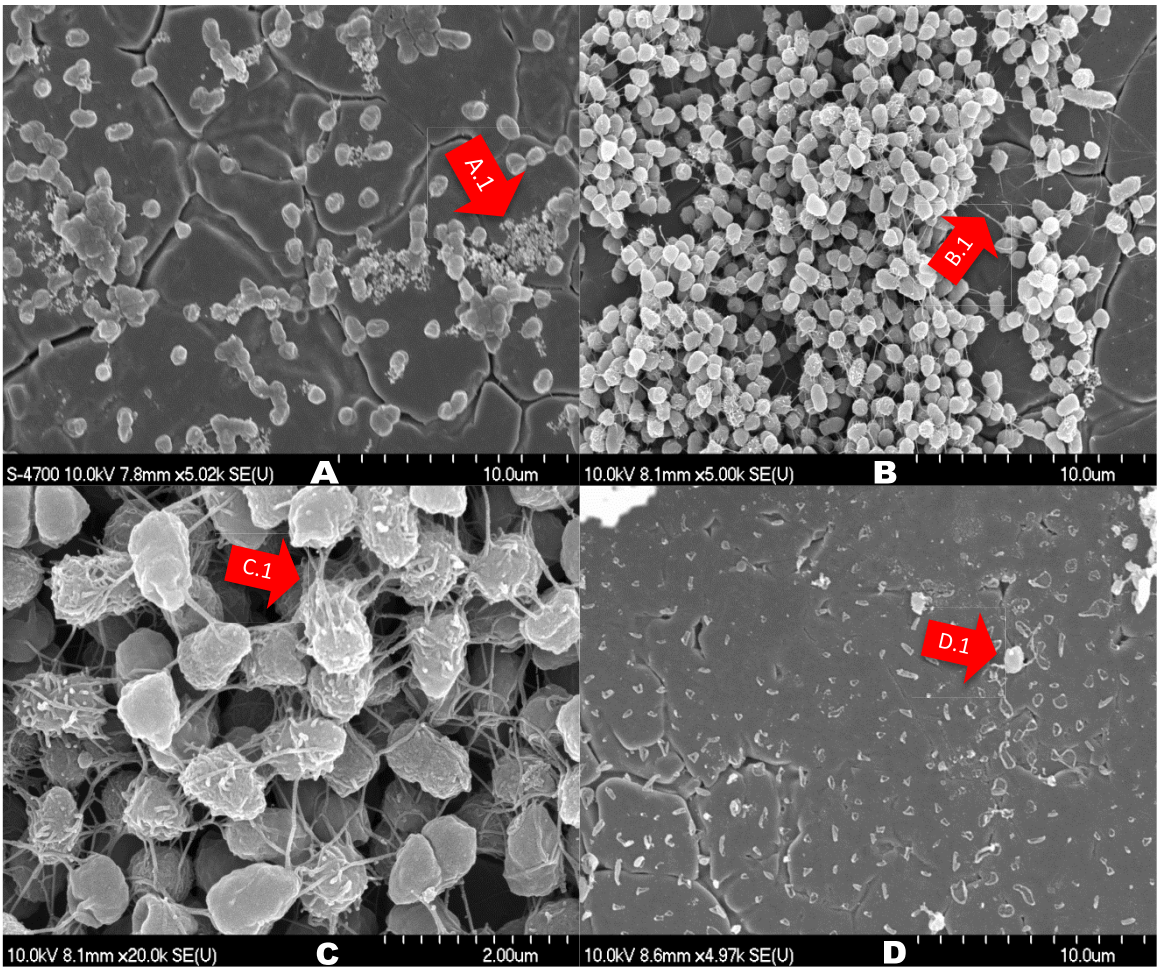

Figure 12. SEM pictures taken of *S. liquefaciens* mono-culture biofilms (magnification: 5k or 20k, V_{acce}: 10 kV). The biofilm was developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B and C), or fixation in FC-72 solvent (D). The arrow A.1 shows the remnants of biofilm structure on the surface. The arrows B.1 and C.1 demonstrate fibril-like junctures that appear to connect cells onto the surface and to each other at two different magnifications. The arrow D.1 displays the remnants of cells preserved on the SS surface after fixation with the FC-72 solvent.

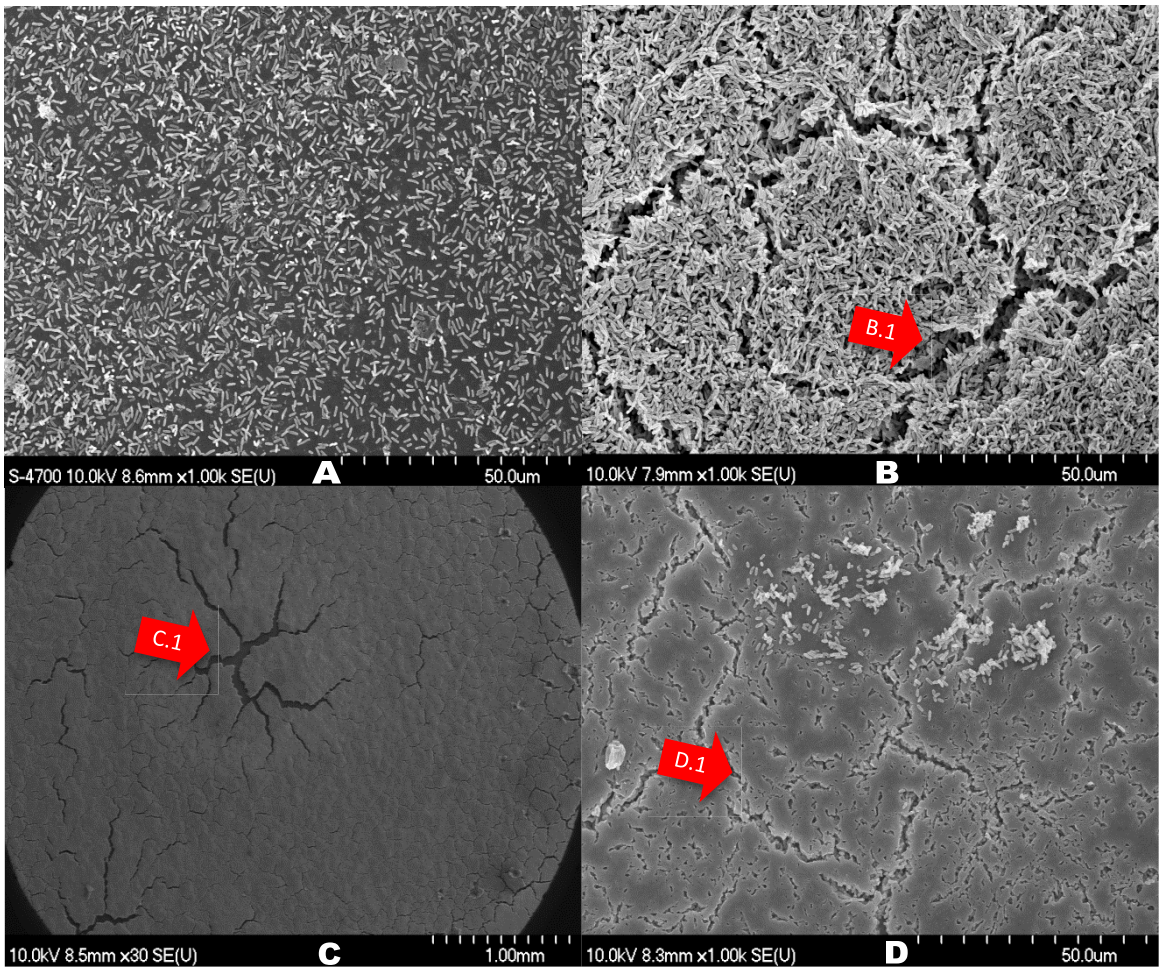

Figure 13. SEM pictures taken of *Sh. putrefaciens* mono-culture biofilms (magnification: 30 or 1k, V_{acce}: 10 kV). The biofilm was developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B), or fixation in FC-72 solvent (C and D). The arrow B.1 shows the border between microcolonies. Fixation in the organic solvent revealed (the arrows C.1 and D.1 at two different magnifications) the cracks in the otherwise complete coverage of the SS-surface by the biofilm.

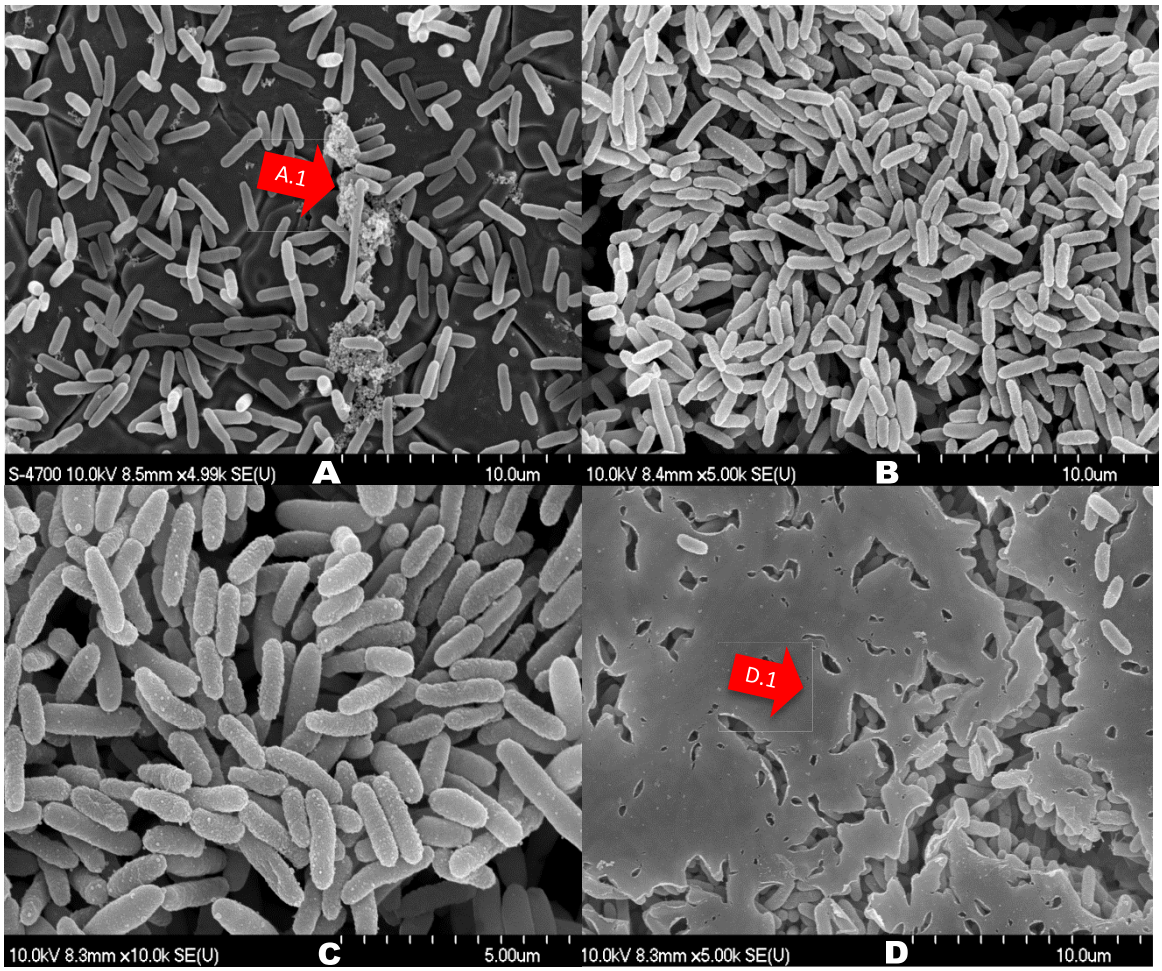

Figure 14. SEM pictures taken of *Sh. putrefaciens* mono-culture biofilms (magnification: 5k or 10k, V_{acce}: 10 kV). The biofilm was developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B and C), or fixation in FC-72 solvent (D). The arrow A.1 shows remnants of EPS, microbial appendices and/or any other cellular compounds on the surface. The arrow D.1 displays cells encased in a layer of amorphous material, presumably EPS.

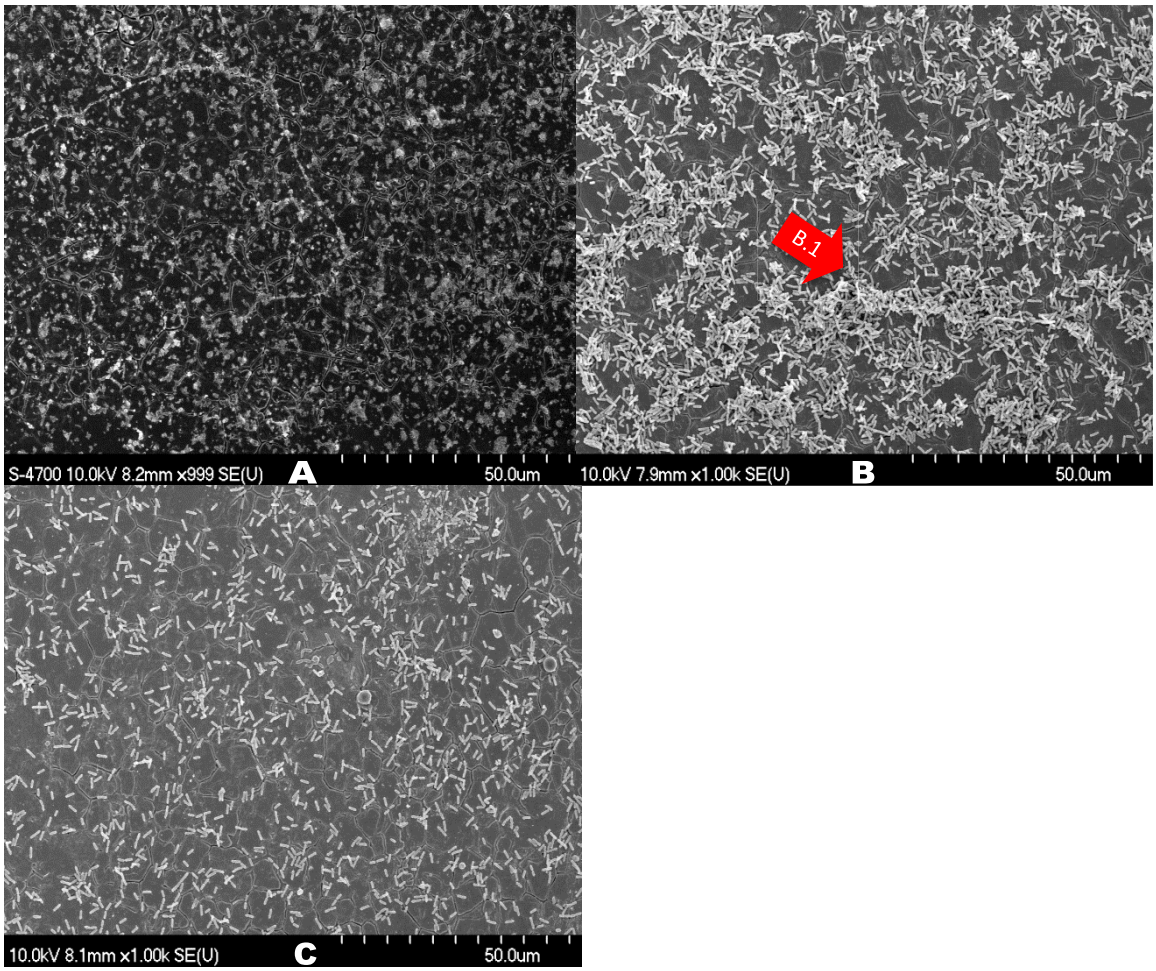

Figure 15. SEM pictures taken of *L. monocytogenes* mono-culture biofilms (magnification: 1k, V_{acce}: 10 kV). The biofilm was developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B), or fixation in FC-72 solvent (C). The arrow B.1 shows the formation of microcolonies.

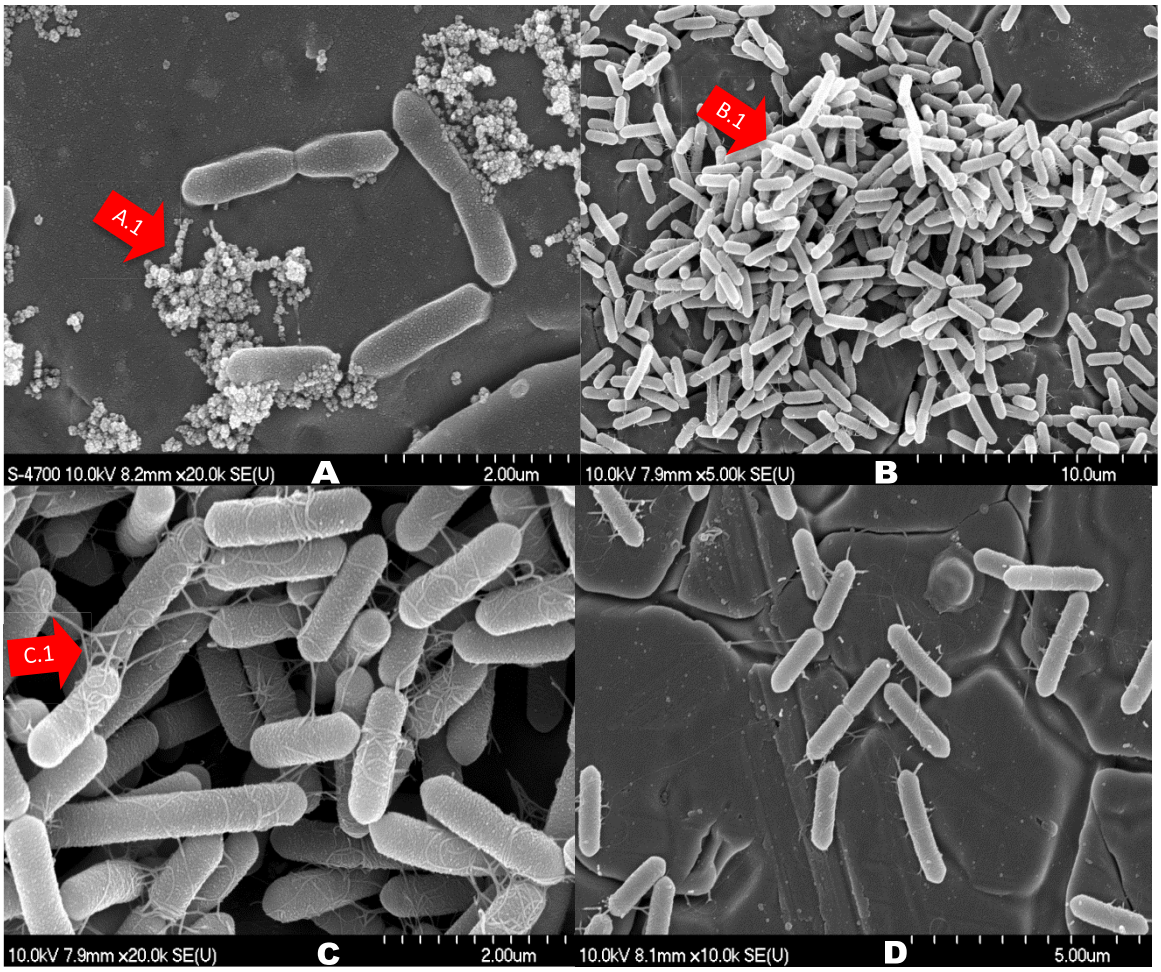

Figure 16. SEM pictures taken of *L. monocytogenes* mono-culture biofilms (magnification: 5k, 10k or 20k, V_{acce}: 10 kV). The biofilm was developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B and C), or fixation in FC-72 solvent (D). The arrow A.1 shows the remnant bacteria or EPS on the surface. The arrow B.1 demonstrates the formation of microcolony around niches and crevices on the surface. The arrow C.1 displays fibrils connecting cells indicating the primary steps in biofilm formation.

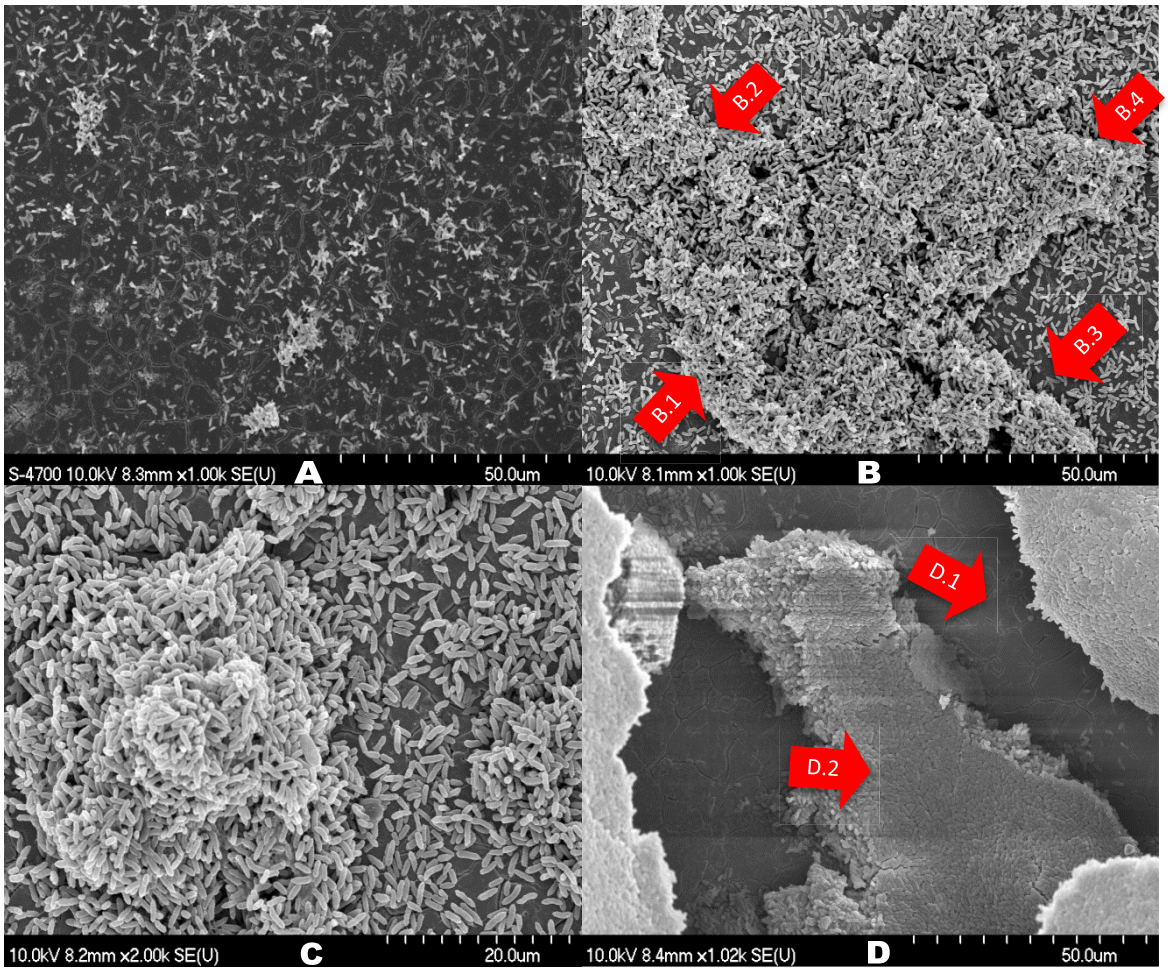

Figure 17. SEM pictures taken of *L. monocytogenes* and *P. fluorescens* binary-culture biofilms (magnification:1k or 2k, V_{acce}: 10 kV). The biofilms were developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B and C), or fixation in FC-72 solvent (D). The arrows B.1-B.4 show the formation and expansion of microcolonies in different directions. The arrows D.1 and D.2 indicate cracks in the biofilm layer and cells being embedded in the presumed EPS, respectively.

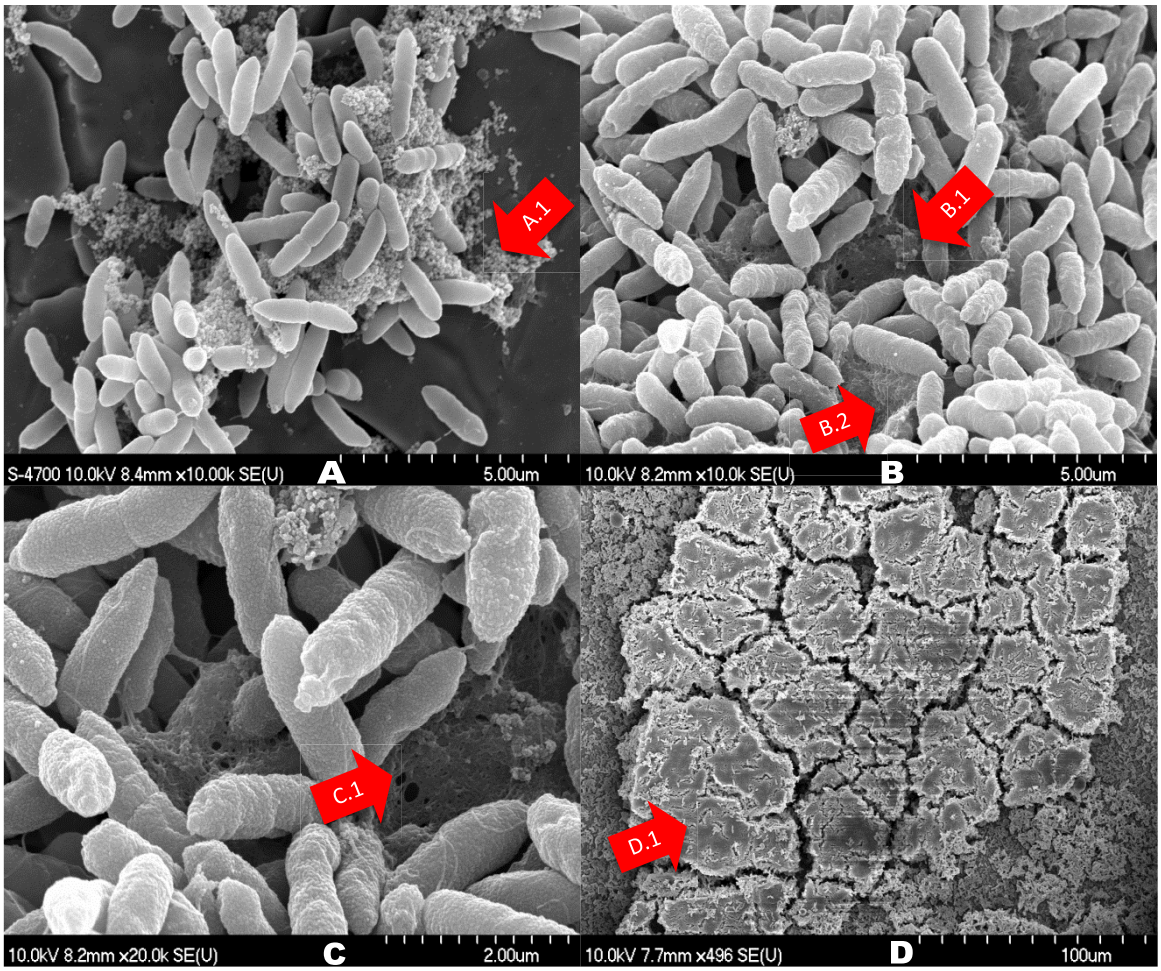

Figure 18. SEM pictures taken of *L. monocytogenes* and *P. fluorescens* binary-culture biofilms (magnification: 500, 10k or 20k, V_{acce}: 10 kV). The biofilms were developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B and C), or fixation in FC-72 solvent (D). The arrow A.1 shows the remnant of presumably EPS on the surface due to inappropriate post fixation. The arrows B.1 and B.2 show the remnants EPS that presumably has covered the microbial cells. The arrow C.1 indicates the pores in the biofilm structure. The arrow D.1 demonstrates the presumed EPS encasement of cells in the biofilm as preserved by the organic fixation method.

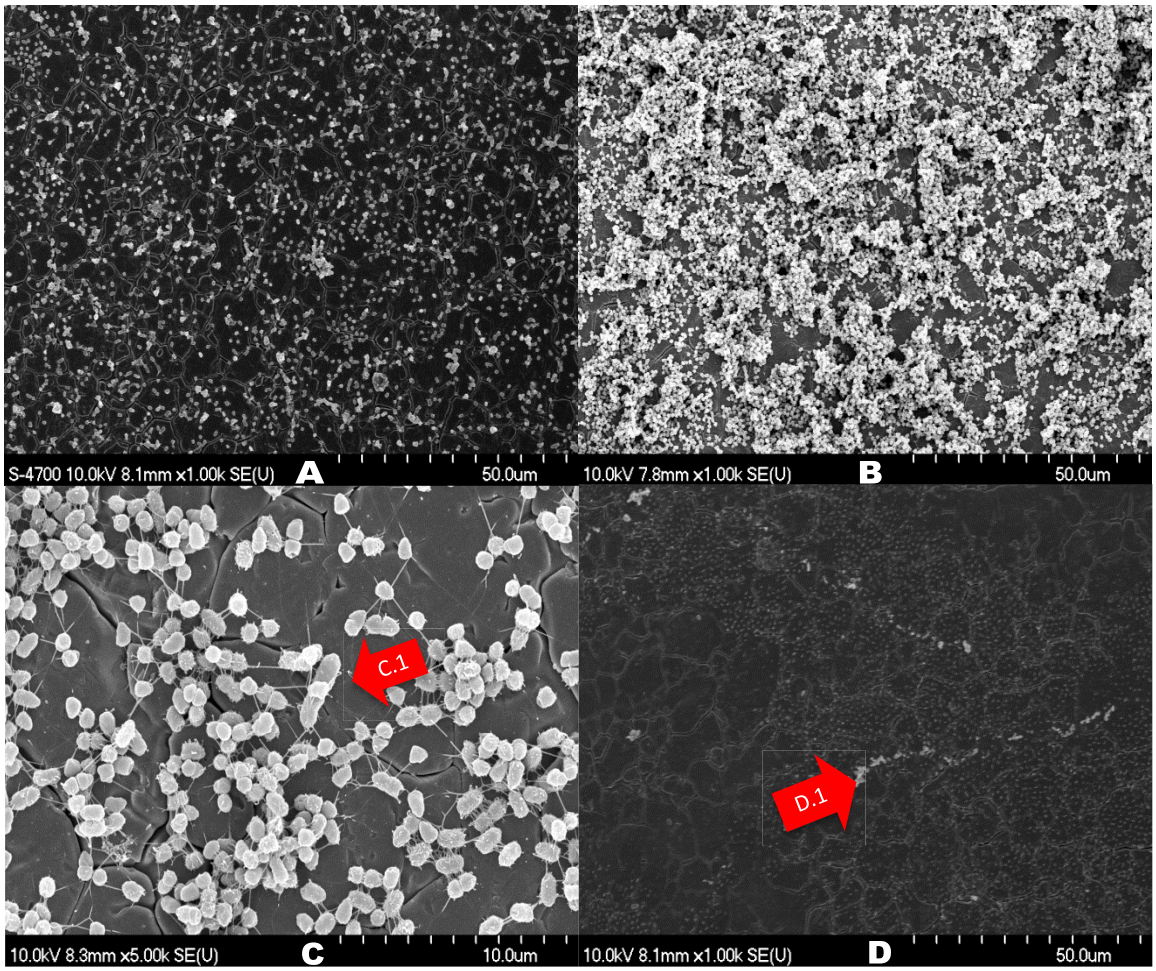

Figure 19. SEM pictures taken of *L. monocytogenes* and *S. liquefaciens* binary-culture biofilms (magnification:1k or 5k, V_{acce}: 10 kV). The biofilms were developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B and C), or fixation in FC-72 solvent (D). The arrow C.1 indicates, based on the difference in cell morphology, a *L. monocytogenes* cell adjacent to the *S. liquefaciens* microcolony. As shown by arrow D.1, fixation in the organic solvent appeared to dissolve and remove the *L. monocytogenes* and *S. liquefaciens* binary-culture biofilm leaving only a few adhering cells behind.

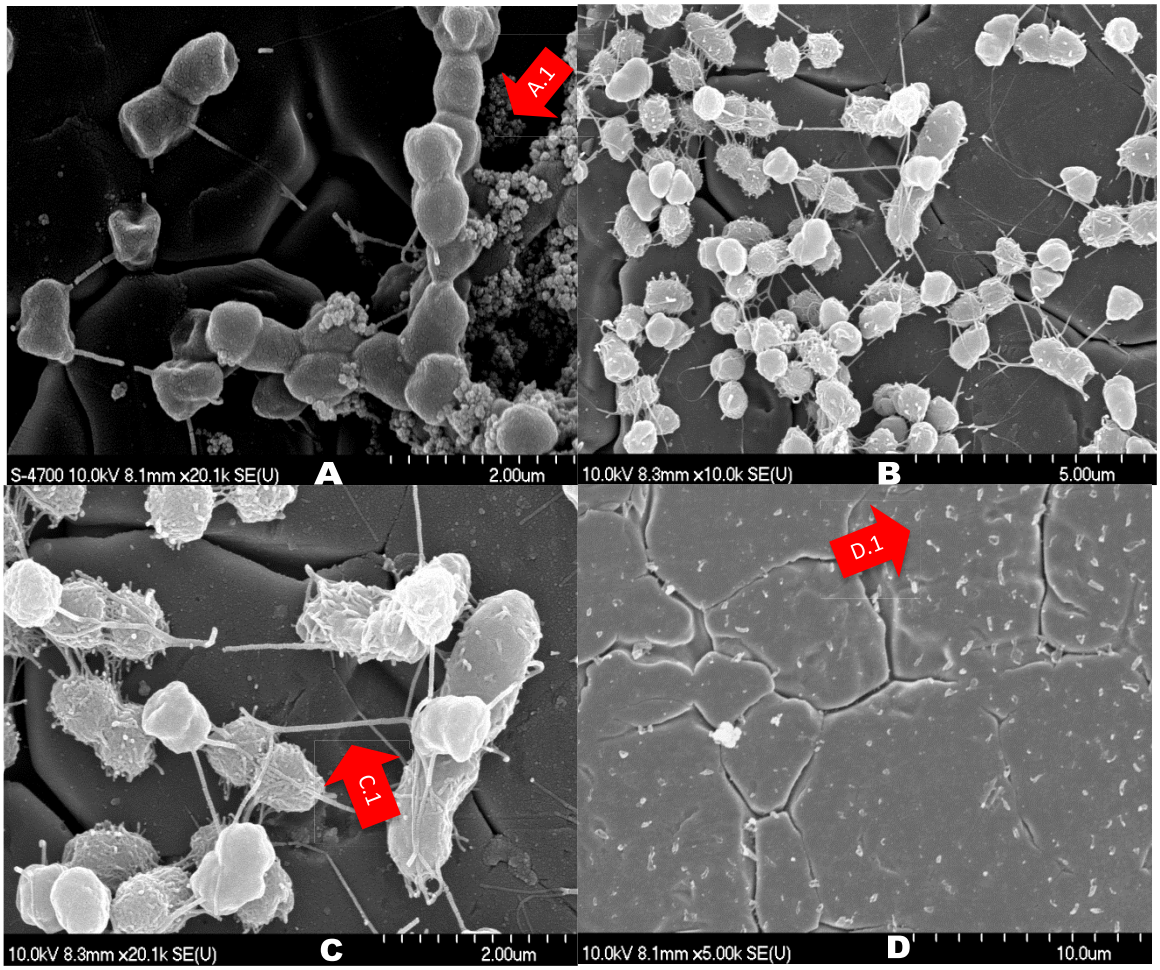

Figure 20. SEM pictures taken of *L. monocytogenes* and *S. liquefaciens* binary-culture biofilms (magnification: 5k, 10k or 20K, V_{acce}: 10 kV). The biofilms were developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B and C), or fixation in FC-72 solvent (D). The arrow A.1 shows cells with remnants of fibrils and/or EPS on the surface. The arrow C.1 demonstrates the fibril junctures that connect the microbial cells onto the surface and to each another. The arrow D.1 indicates the removal of the microbial cells due to the solubilization of the biofilm by the organic fixation.

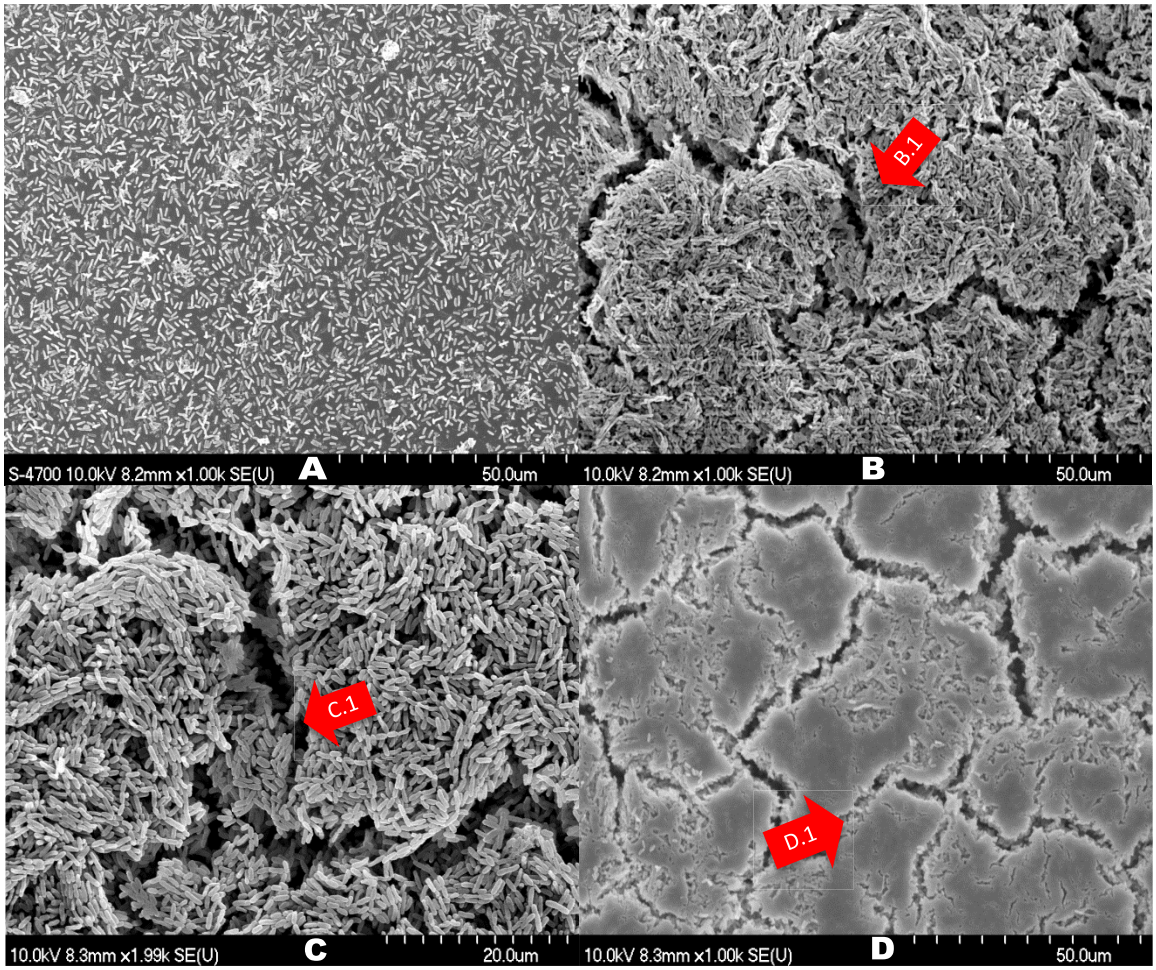

Figure 21. SEM pictures taken of *L. monocytogenes* and *Sh. putrefaciens* binary-culture biofilms (magnification: 1k or 2k, V_{acce}: 10 kV). The biofilms were developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B and C), or fixation in FC-72 solvent (D). The arrows B.1 and C.1 show the microcolonies and cracks in the biofilm layer. The arrow D.1 indicates the same biofilm structure with cracks; however, cells are visibly encased in an amorphous EPS-like mass.

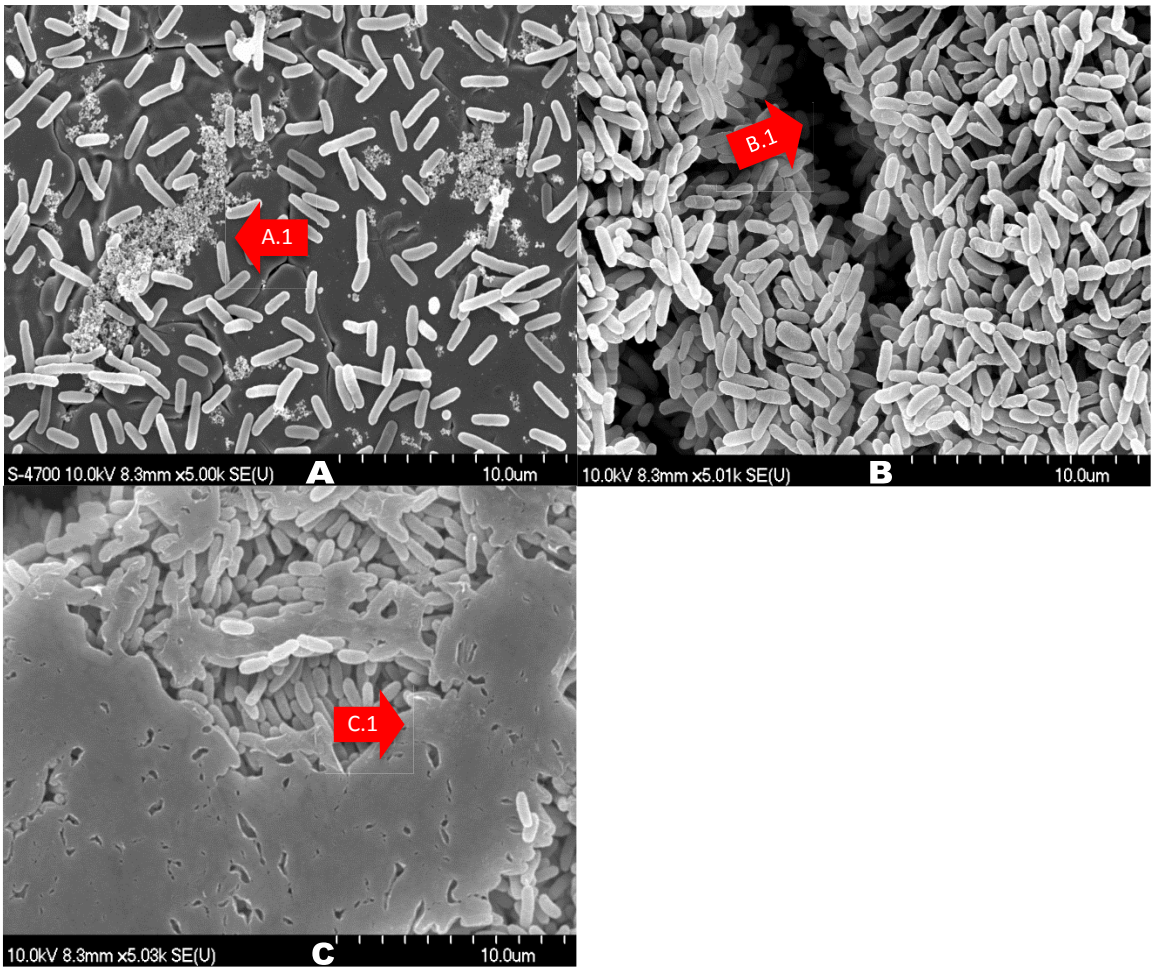

Figure 22. SEM pictures taken of *L. monocytogenes* and *Sh. putrefaciens* binary-culture biofilms (magnification: 5k, V_{acce}: 10 kV). The biofilms were developed on SS coupons (48 h, 100% RH and 15°C) and post fixed using basic fixation in cacodylate buffer (A), modified fixation in cacodylate buffer (B), or fixation in FC-72 solvent (c). The arrow A.1 shows the cells dispersed on the surface surrounded by remnants of fibrils and/or EPS. The arrow B.1 indicates the microcolonies and the gaps between them. The arrow C.1 points to cells encased in the amorphous gel-like EPS preserved by the organic solvent fixation.

The fixation and observation methods for the SEM approach were explained in detail previously in section 3.8.1. Although the aim of all fixation protocols was to firstly preserve the biofilms structure on the SS surfaces and secondly induce the electrical conductivity in samples in order to pass the electrons beam deep into the biofilm structure and collect the reflections for observation by the SEM, not all fixation protocols satisfied both objectives. The basic fixation method could not preserve the 3D structure of various biofilm combinations on the SS coupons. The remnants of presumed EPS or other cells appendices were evident on the surface once fixed with this protocol. Therefore, the SEM pictures taken from the samples that were prepared by the basic fixation in cacodylate buffer protocol may not reflect the real structure and/or cellular distribution during biofilm formation on the SS surfaces.

On the other hand, the modified fixation in cacodylate buffer and FC-72 protocol gave in better results.These fixation protocols appeared to preserve the 3D structure of biofilms, EPS matrix and fibril-like junctures connecting cells onto the surface and to each other and indicating the primary steps in biofilm formation for *L. monocytogenes* and the presence of more mature biofilm structures in all other mono- or binary-culture biofilms. Distribution of cells, formation of microcolonies and expansion in various directions on the surface and last but not least the cell morphology (coccobacilli shaped *S. liquefaciens* versus the rod shaped *L. monocytogenes*, *P. fluorescens* and *Sh. putrefaciens*) were other features that could also be observed using these two fixation protocols. However, due to the nature of solvents (aqueous buffer versus FC-72 organic solvent) used to in the chemical fixative phase, the micrographs looked different. For instance, while the modified fixation protocol preserved the cellular distribution and boundary between microcolonies in mono- or binary-culture biofilms of *Sh. putrefaciens* with *L. monocytogenes*, the FC-72 protocol preserved the EPS gel that seemed to encase the entire biofilm structure (Figures 13, 14, 21 and 22). This finding highlights the importance of employing several different fixation protocols to gain as much details as possible when using SEM to study the biofilm structure.

4.3.2. Epi-fluorescence Microscopy

To complement the SEM images and to locate the individual members in the binary-culture biofilms, two different epifluorescence microscopy techniques were attempted, one which used GFP-labelled *Listeria* cells while the other used the fluorescent Gram-stain**.**

4.3.2.1. Plasmid Burden Assay

To assess the influence of the *gfp*-plasmid on the metabolism of mutant *gfp- L. monocytogenes* 568, growth of the mutant in mono- or binary-culture biofilms was determined (see section 3.8.3.1) and the Logistic model with lag phase fitted to the growth curves. The generated model parameter estimates for mutant *gfp- L. monocytogenes* 568 (Table 8) were subsequently compared to that of the parental strain using the t-test (Table 9).

Table 8. Kinetics of the formation of mono- and binary-culture biofilms with *gfp-L. monocytogenes* 568 and three competitor bacterial strains. Cells were grown (100% RH, 15 °C, 72 h) in TSB+1% glucose on SS coupons and enumerated at specific time intervals on BHIA+8μg/ml erythromycin or other suitable selective media. The Logistic model with lag phase was fitted to the transformed data ($Log_{10}(CFU/cm^2$, n=6).

Bacteria Biofilm	**N_{max}[I] Log(CFU/cm^2)**	**μ_{max}[II] (1/h)**	**N_0[III] Log(CFU/cm^2)**	**λ[IV] (h)**
Single *gfp-L. monocytogenes*	5.63±0.02[V]	0.21±0.01	2.64±0.11	20.19±0.72
Binary *gfp-L. monocytogenes* (with *P. fluorescens*)	6.46±0.04	0.29±0.01	2.53±0.09	17.14±1.77
Binary *gfp-L. monocytogenes* (with *S. liquefaciens*)	5.23±0.10	0.22±0.02	2.87±0.09	12.03±0.07
Binary *gfp-L. monocytogenes* (with *Sh. putrefaciens*)	7.52±0.06	0.28±0.02	2.76±0.07	17.27±0.54
Binary *P. fluorescens* (with *gfp-L. monocytogenes*)	8.23±0.07	0.31±0.01	3.00±0.10	8.26±1.07
Binary *S. liquefaciens* (with *gfp-L. monocytogenes*)	7.21±0.05	0.41±0.01	2.84±0.08	7.94±0.34
Binary *Sh. Putrefaciens* (with *gfp-L. monocytogenes*)	7.46±0.10	0.33±0.01	2.37±0.06	15.53±0.90

[I] N_{max} denotes maximum population density.

[II] μ_{max} represents maximum specific growth rate (slope of the curve).

[III] N_0 is the initial bacterial population.

[IV] λ denotes the lag time.

[V] Model estimate ± standard error of mean.

Table 9. Pairwise t-test results for Logistic model with lag phase model parameter estimates obtained for growth in mono- and binary-culture biofilms made by *gfp-L. monocytogenes* 568 alone or together with one of three Gram-negative bacterial strains as compared to model values obtained for the parental *L. monocytogenes* 568 strain (Table 3). The t-test (two tailed) was performed at α=0.05.

Bacteria Biofilm	N_{max}	μ_{max}	N_0	λ
Single *gfp-L. monocytogenes* VS. single *L. monocytogenes*	<0.001*	<0.001	0.002	**0.110***
Binary *gfp-L. monocytogenes* (with *P. fluorescens*) VS. binary *L. monocytogenes* (with *P. fluorescens*)	<0.001	0.038	<0.001	0.001
Binary *gfp-L. monocytogenes* (with *S. liquefaciens*) VS. binary *L. monocytogenes* (with *S. liquefaciens*)	<0.001	**0.673**	<0.001	**0.360**
Binary *gfp-L. monocytogenes* (with *Sh. putrefaciens*) VS. binary *L. monocytogenes* (with *Sh. putrefaciens*)	0.004	<0.001	<0.001	<0.001
Binary *P. fluorescens* (with *gfp-L. monocytogenes*) VS. binary *P. fluorescens* (with *L. monocytogenes*)	<0.001	**0.409**	0.007	0.001
Binary *S. liquefaciens* (with *gfp-L. monocytogenes*) VS. binary *S. liquefaciens* (with *L. monocytogenes*)	<0.001	<0.001	**0.498**	<0.001
Binary *Sh. putrefaciens* (with *gfp-L. monocytogenes*) VS. binary *Sh. putrefaciens* (with *gfp-L. monocytogenes*)	**0.130**	**0.409**	**0.123**	<0.001

* P-values obtained from the two tailed t-test (α=0.05) between model parameter estimates for mono- and binary-culture biofilms of parental Lm and *gfp-L. monocytogenes*.

** P-values above 0.05 mean there was no statistically significant change between the paired strains. These P-values were indicated in bold.

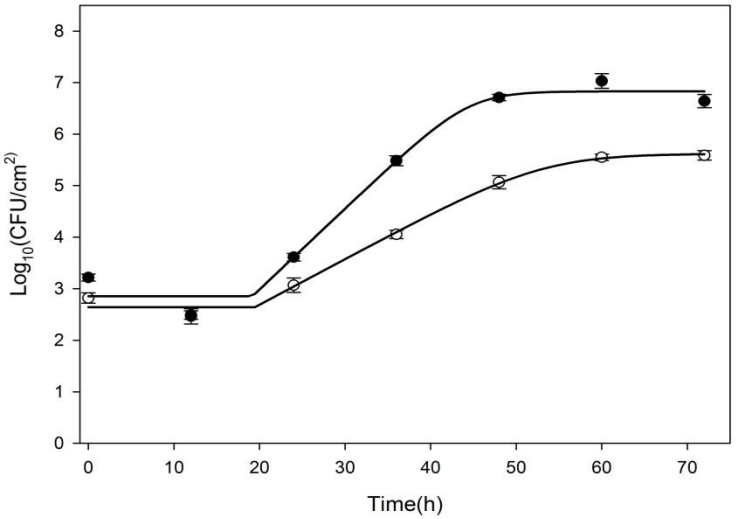

Figure 23. Growth of *gfp-L. monocytogenes* 568 and its parent strain on SS coupons (100% RH, 15 °C). Cells were enumerated at specific time intervals on BHIA+8 µg/ml erythromycin or Oxford agar (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* 568 parental strain and (○) represents *gfp-L. monocytogenes* 568. Lines in the graph represent the populations of microbial cells predicted by Logistic model with lag phase fits.

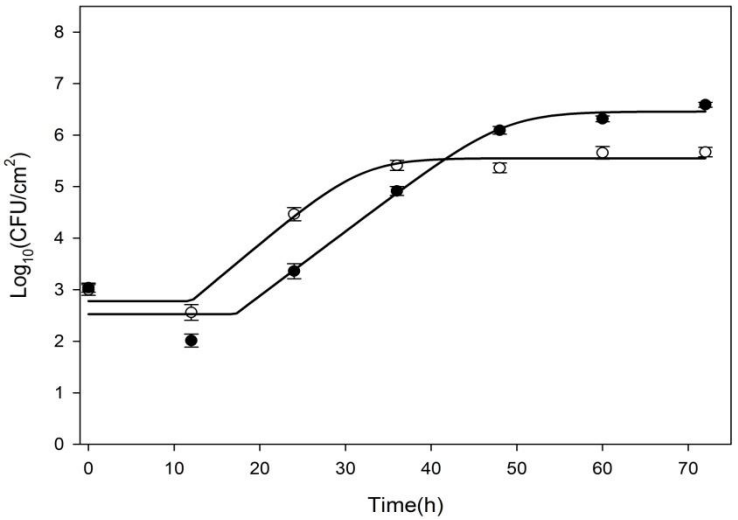

Figure 24. Growth of *gfp*- and wild type *L. monocytogenes* 568 in binary-culture biofilms with *P. fluorescens* on SS coupons (100% RH, 15 °C). Cells were enumerated at specific time intervals on BHIA+8 µg/ml erythromycin or Oxford agar (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* 568 in binary biofilms with *P. fluorescens* and (○) represents *gfp-L. monocytogenes* 568 in dual-species biofilm with *P. fluorescens*. Lines in the graph represent the populations of microbial cells predicted by Logistic model with lag phase fits.

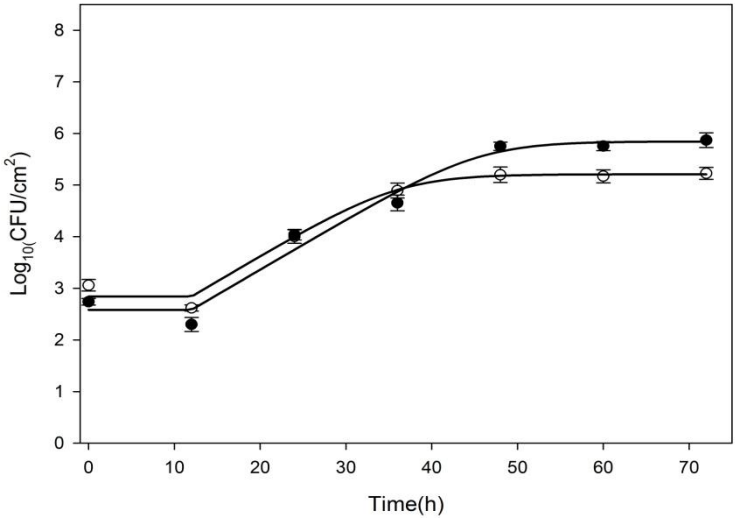

Figure 25. Growth of *gfp*- and wild type *L. monocytogenes* 568 in binary-culture biofilms with *S. liquefaciens* on SS coupons (100% RH, 15°C). Cells were enumerated at specific time intervals on BHIA+8 µg/ml erythromycin or Oxford agar (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* parental strain in binary biofilms with *S. liquefaciens* and (○) represents *gfp-L. monocytogenes* in dual-species biofilm with *S. liquefaciens*. Lines in the graph represent the populations of microbial cells predicted by Logistic model with lag phase fits.

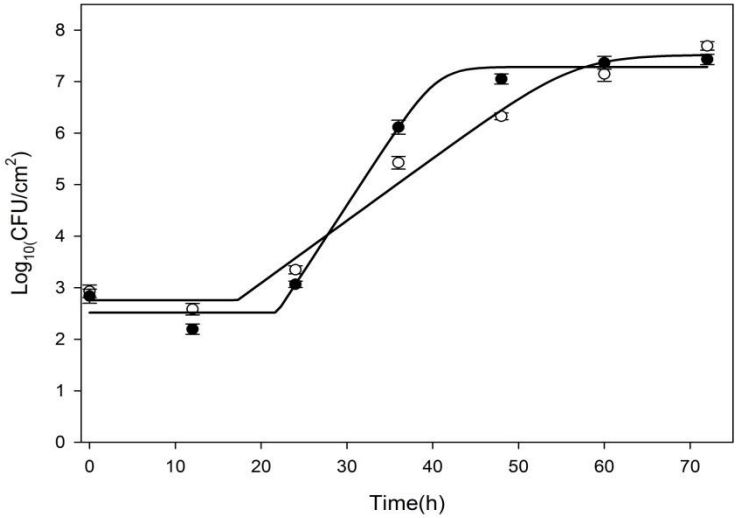

Figure 26. Growth of *gfp*- and wild type *L. monocytogenes* 568 in binary-culture biofilms with *Sh. putrefaciens* on SS coupons (100% RH, 15°C). Cells were enumerated at specific time intervals on BHIA+8 µg/ml erythromycin or Oxford agar (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* parental strain in binary biofilms with *Sh. putrefaciens* and (○) represents *gfp-L. monocytogenes* in dual-species biofilm with *Sh. putrefaciens*. Lines in the graph represent the populations of microbial cells predicted by Logistic model with lag phase fits.

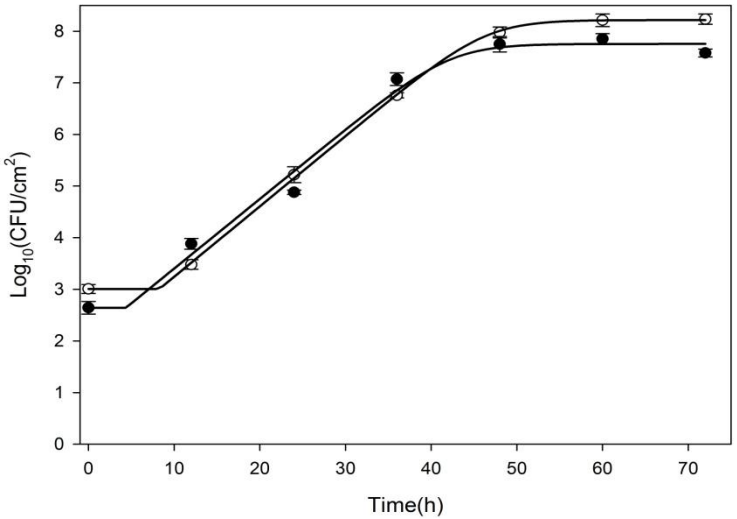

Figure 27. Growth kinetics of *P. fluorescens* in binary-culture biofilms with *gfp*- or wild type *L. monocytogenes* 568 on SS coupons (100% RH, 15°C). Cells were enumerated at specific time intervals on Pseudomonas C-F-C agar (n=6, ±SD, representing two independent experiments). (●) denotes *P. fluorescens* in binary biofilm with parental strain *L. monocytogenes* and (○) represents *P. fluorescens* in dual-species biofilm with *gfp-L. monocytogenes*. Lines in the graph represent the populations of microbial cells predicted by Logistic model with lag phase fits.

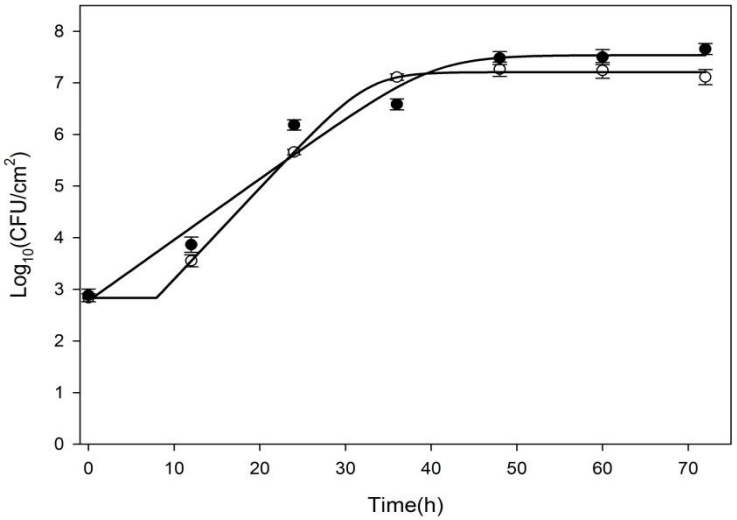

Figure 28. Growth of *S. liquefaciens* in binary-culture biofilms with *gfp*- or wild type *L. monocytogenes* 568 on SS coupons (100% RH, 15°C). Cells were enumerated at specific time intervals on VRBG selective agars (n=6, ±SD, representing two independent experiments). (●) denotes *S. liquefaciens* in binary biofilm with parental strain *L. monocytogenes* and (○) represents *S. liquefaciens* in dual-species biofilm with *gfp-L. monocytogenes*. Lines in the graph represent the populations of microbial cells predicted by Logistic model with lag phase fits.

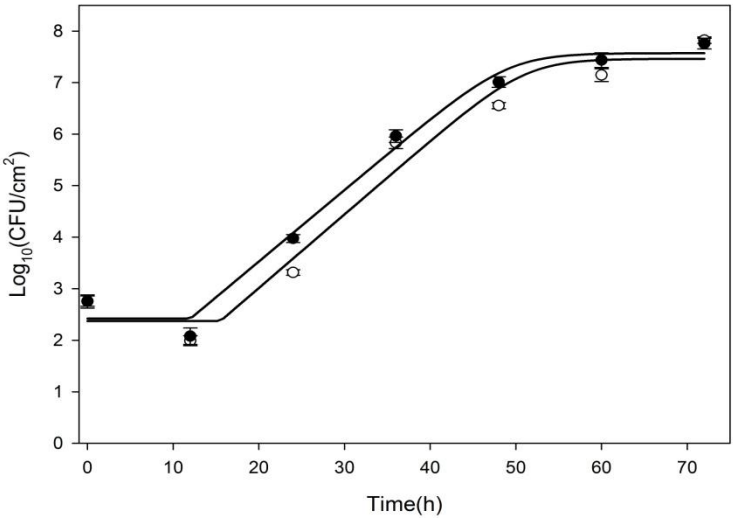

Figure 29. Growth of *Sh. putrefaciens* in binary-culture biofilms with *gfp*- or wild type *L. monocytogenes* 568 on SS coupons (100% RH, 15°C). Cells were enumerated at specific time intervals on Iron + PenG agar (n=6, ±SD, representing two independent experiments). (●) denotes *Sh. putrefaciens* in binary biofilm with parental strain *L. monocytogenes* and (○) represents *Sh. putrefaciens* in dual-species biofilm with *gfp-L. monocytogenes*. Lines in the graph represent the populations of microbial cells predicted by Logistic model with lag phase fits.

The t-test results in Table 9 demonstrated that with exception of μ_{max} and λ in *gfp-L. monocytogenes* in binary-culture biofilm with *S. liquefaciens* ($P>0.05$), there were significant ($P<0.05$) changes between the parameter estimates for growth of the parent strain and the *gfp-L. monocytogenes* in mono- and binary-culture biofilms with other Gram-negatives. Plotting of the biofilm growth curves obtained for the parent and GFP mutant strains of *L. monocytogenes* also revealed the differences in growth kinetics (Figures 23-26). In other words, the presence of the pNF8 plasmid significantly ($P<0.05$) reduced the N_{max} and μ_{max} of *gfp-L. monocytogenes* in the single or binary-species biofilms (Table 8) as compared to the values obtained for the wild type (Table 3). Although significant ($P<0.05$) changes were observed for some growth model parameter estimates for the Gram-negative strains co-cultured with *gfp-L. monocytogenes* in comparison to values obtained when co-cultured with the parent strain (Tables 3, 8 and 9), visual comparison of the growth curves (Figures 27-29) revealed these differences to

be more subtle. In fact, the P-values in t-tests of the Gram-negative strains was close to the 0.05 significance level (Table 9).

4.3.2.2. Plasmid Stability Assay

The plasmid stability assay was performed based on the method mentioned in section 3.8.3.1 and the results were presented in Table 10.

Table 10. Plasmid stability assay. The *gfp- L. monocytogenes* cells were cultured in TSB+1% glucose (15°C) and transferred to the fresh broth (inoculation level of 1:1000) on a daily base for 5 consecutive days (~50 generations). The cells were enumerated on Oxford agar and BHIA+8 µg/ml erythromycin and counts were converted to Log_{10} CFU/ml (n=2).

Day	Population on Oxford agar	Populations on BHIA+erm
0	5.80±0.02*	5.84±0.02
1	7.14±0.05	7.03±0.06
2	6.29±0.35	6.43±0.02
3	6.09±0.01	5.94±0.08
4	6.30±0.00	6.11±0.10
5	6.25±0.04	5.52±0.03

*Log_{10} CFU/ml±SD

The paired t-test was done between the counts on Oxford agar that represents the *Listeria* cells with or without the GFP-plasmid whereas the populations on the BHIA+erm that indicates the cells solely with the GFP-plasmid. The result showed that there is not any significant ($P>0.05$) difference among the groups and this means that the GFP-plasmid is highly stable in the microbial cells over 50 generations of the test.

Although the GFP-plasmid appeared stable in the harbouring cells, most of the growth model parameters were found to be significantly ($P<0.05$) different for *gfp-L. monocytogenes* in mono- or binary-culture biofilms when compared with the parent strain. Since the growth kinetics of the plasmid bearing strain had become altered and no longer mimicked that of the wild strain, results from the microscopy did not reflect the

dynamics observed in biofilms with the wild-type strain. A few images to demonstrate the potential usefulness of the concept of the *gfp* cellular labeling technique are presented in Appendix A (Figure 33 and 34).

4.3.2.3. Gram-staining and Fluorescence Microscopy

Using the fluorescent Gram-stain, biofilms with the parental strain of *L. monocytogenes* was cultivated in co-culture with the Gram-negative strains as before and subsequently stained with fluorescent nucleic acid dyes to enable discrimination between the Gram-positive and -negative bacteria with epifluorescence microscopy.

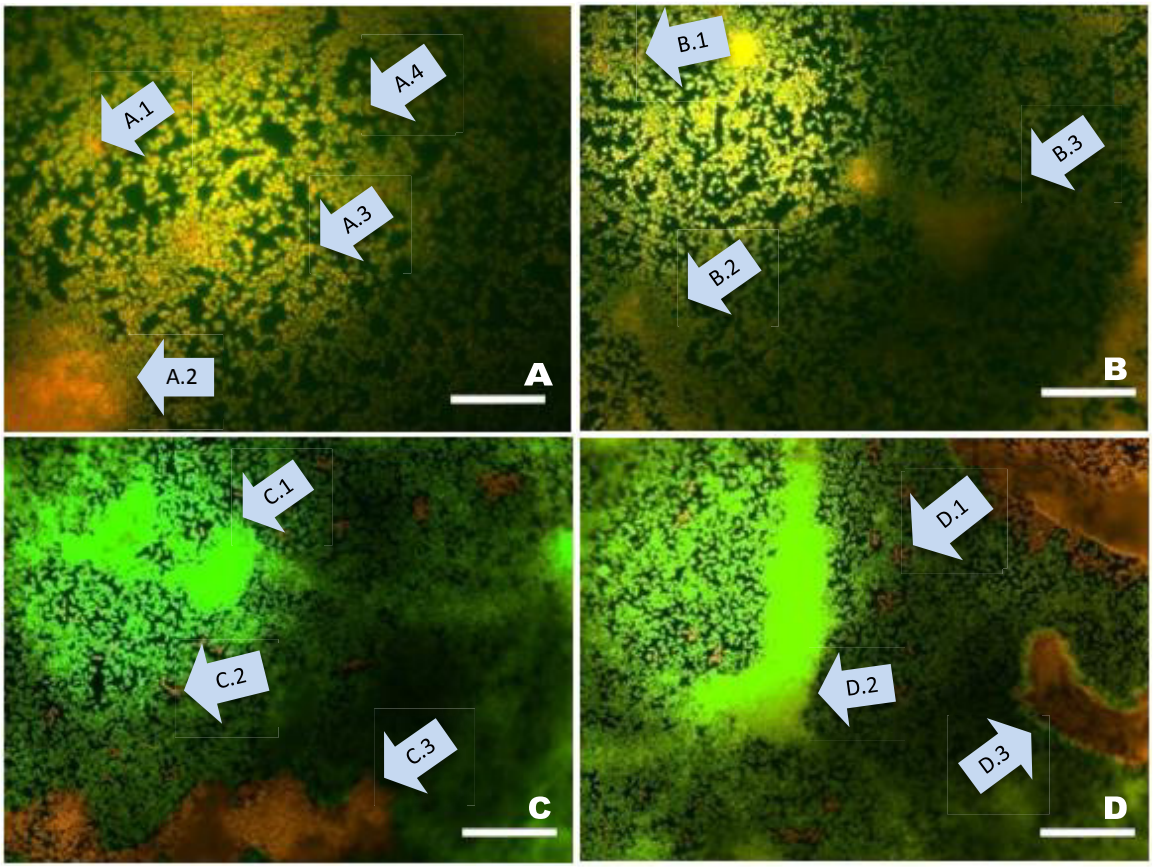

Figure 30. Epifluorescence micrographs taken of *L. monocytogenes* and *P. fluorescens* binary-culture biofilms (magnification ×1000). The biofilms were developed on SS coupons (48 h, 100% RH and 15°C) and stained using fluorescent nucleic acid dyes SYTO9/hexidium iodide (20:80). The green areas represent the Gram-negative *P. fluorescens* individual cells (arrow A.4) or microcolonies (arrows A.3, B.2, C.1, D.2) and the red zones indicate the Gram-positive *L. monocytogenes* individual cells (arrows A.1, B.1, C.2, D.1) or microcolonies (the arrows A.2, B.3, C.3, D.3) (scale bar, 10 µm).

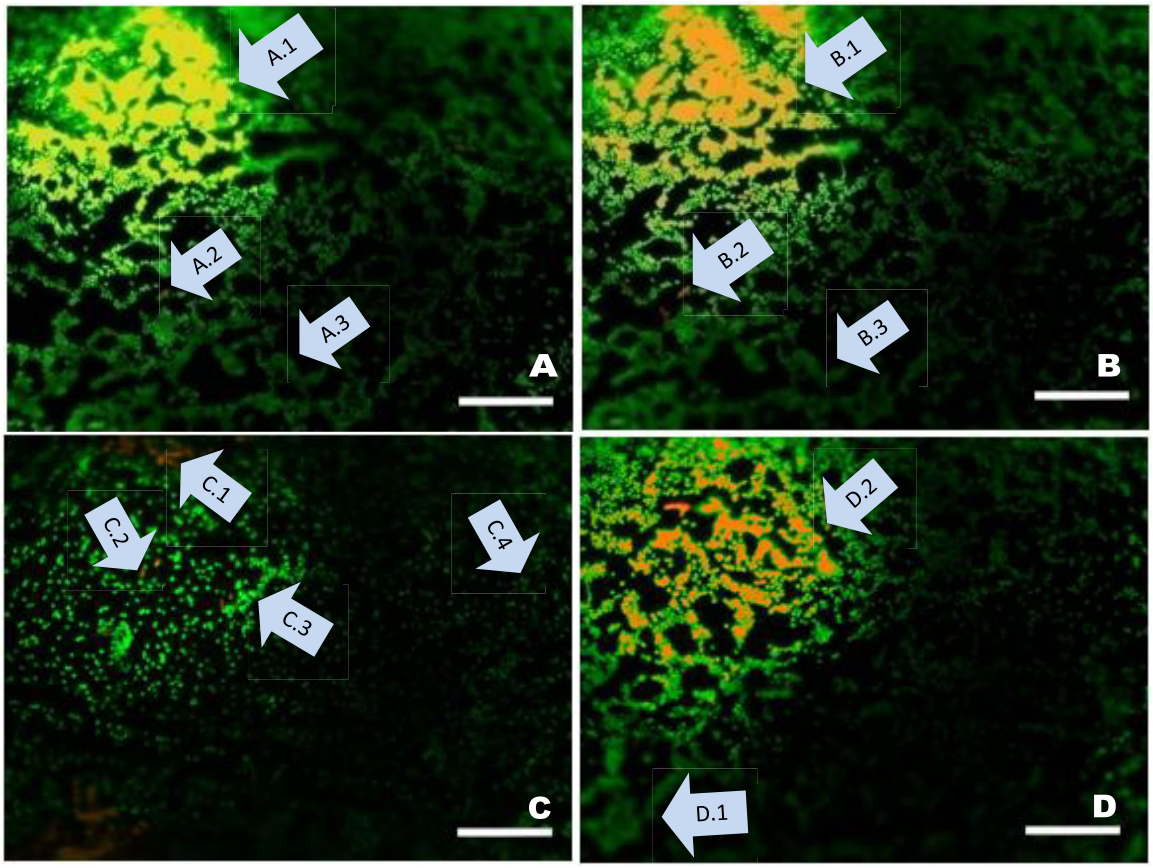

Figure 31. Epifluorescence micrographs taken of *L. monocytogenes* and *S. liquefaciens* binary-culture biofilms (magnification ×1000). The biofilms were developed on SS coupons (48 h, 100% RH and 15°C) and stained using the fluorescent nucleic acid dyes SYTO9/hexidium iodide (20:80). The green areas represent the Gram-negative *S. liquefaciens* individual cells (arrow C.4) or microcolonies (arrows A.3, B.3, C.3 and D.1) and the red zones indicate the Gram-positive *L. monocytogenes* individual cells (arrows A.2, B.2 and C.2) or microcolonies (arrows A.1, B.1, C.1 and D.2) (scale bar, 10 µm).

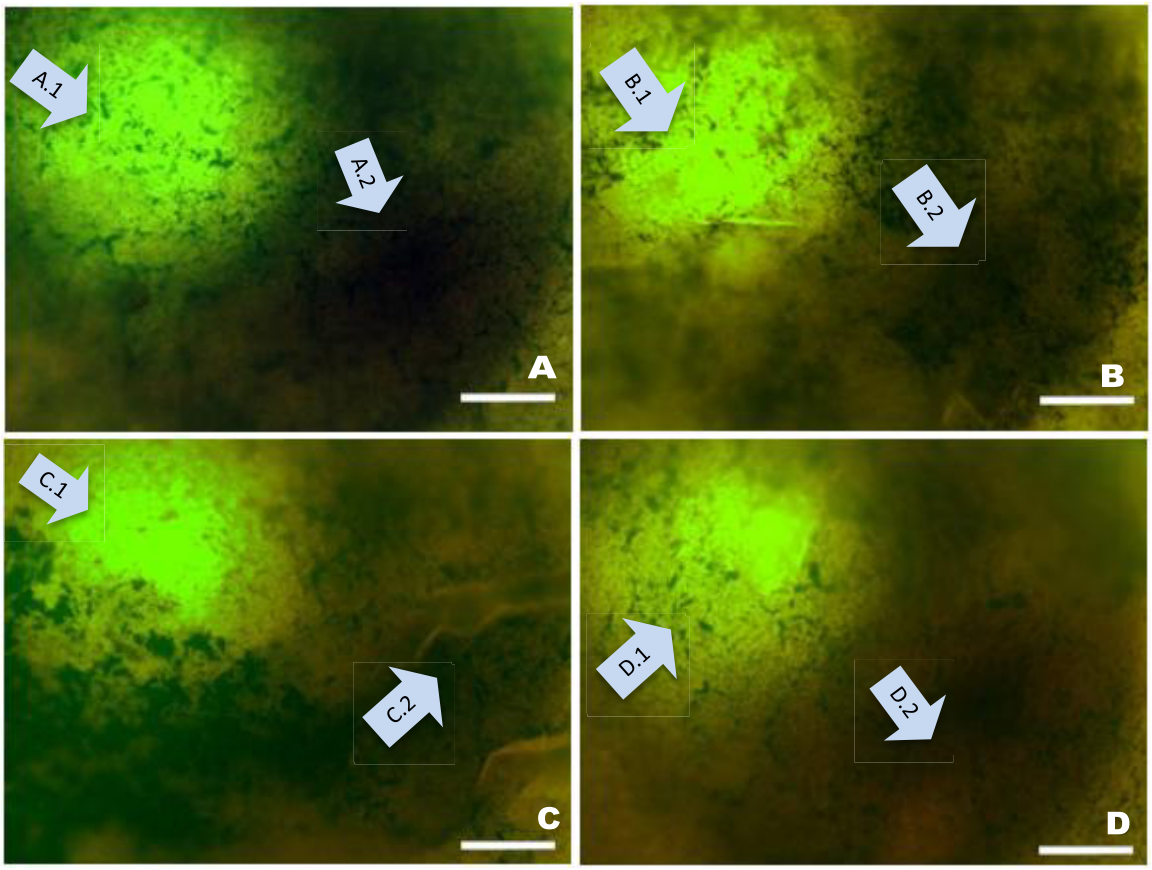

Figure 32. Epifluorescence micrographs taken of *L. monocytogenes* and *Sh. putrefaciens* binary-culture biofilms (magnification ×1000). The biofilms were developed on SS coupons (48 h, 100% RH and 15°C) and stained using the fluorescent nucleic acid dyes SYTO9/hexidium iodide (20:80). The green areas represent the Gram-negative *Sh. putrefaciens* microcolonies (arrows A.1, B.1, C.1and D.1) and the red zones indicate the Gram-positive *L. monocytogenes* microcolonies (the arrows A.2, B.2, C.2 and D.2). The individual cells of both strains were not clearly visible due to the high population density (scale bar, 10 μm).

CHAPTER 5 DISCUSSION

The statistical analysis of the growth model parameter estimates for biofilm growth in this study showed a significant ($P<0.05$) shift in the growth kinetics of *L. monocytogenes* and the Gram-negatives when conditions were changed from single species to binary species biofilms. The interactions varied among the strains. While *P. fluorescens* and *S. liquefaciens* significantly ($P<0.05$) reduced the N_{max} of *L. monocytogenes*, co-culture with *Sh. putrefaciens* significantly ($P<0.05$) increased the maximum populations in *L. monocytogenes*. The effect of co-culture strains on μ_{max} was quite similar to the N_{max} pattern. *Pseudomonas* and *Serratia* decreased ($P<0.05$) the μ_{max} of *Listeria* while *Shewanella* significantly ($P<0.05$) raised the maximum growth rate in *L. monocytogenes* (Table 4).

L. monocytogenes on the other hand, caused N_{max} to be elevated or lowered significantly ($P<0.05$) for *P. fluorescens* or *Sh. putrefaciens*, respectively, while the bacterium had no impact on that of *S. liquefaciens* ($P>0.05$). The pathogen also significantly ($P<0.05$) decreased the μ_{max} for *P. fluorescens* and *S. liquefaciens* while μ_{max} remained unchanged for *Sh. putrefaciens*. These results proved that the interactions among microbial species may impact the growth kinetics of each another in dramatically different ways. In binary-culture biofilms, while one strain may be beneficial to the growth of its partner, another strain may have antagonistic activity and effectively limit or inhibit growth of its co-culture partner.

In this study, *P. fluorescens* and *S. liquefaciens* behaved antagonistically against *L. monocytogenes* growth and in both cases limited the biofilm formation by *L. monocytogenes*. However, the degree of inhibition differed among the antagonistic strains. While *P. fluorescens* reduced the *L. monocytogenes* μ_{max} by 11%, *S. liquefaciens* decreased this parameter by 39%. The estimated maximum populations of *L. monocytogenes* were decreased by 18 and 14% in presence of *P. fluorescens* or *S. liquefaciens*, respectively. This meant that the *Pseudomonas* and *Serratia* strains not only limited the maximum population levels of *L. monocytogenes* in mixed binary biofilm but also negatively affected the maximum growth rate and therefore the time that the

pathogen required to reach the stationary phase. Other researchers have observed similar antagonistic activities among multi-species biofilms.

Buchanan & Bagi (1999) reported that the maximum population density of *L. monocytogenes* was greatly reduced in presence of antagonistic microorganisms; however, other environmental parameters particularly the temperature affected the magnitude of suppression. Bacteriocin producing bacteria such as *Lactobacillus sakei* (Winkelstroter et al., 2011), *Lactobacillus plantarum* and *Enterococcus casseliflavus* (Guerrieri et al., 2009) have also been reported to inhibit *L. monocytogenes* growth and biofilm formation on live or solid surfaces. Leriche and Carpentier (2000) also demonstrated that once co-cultured, *Staphylococcus sciuri* would lower the *L. monocytogenes* maximum population size. The extracellular polysaccharides generated by *Staph. sciuri* and competition for nutrients were found to be the main factors involved in this antagonistic process (Leriche & Carpentier, 2000).

The natural competition between *L. monocytogenes* and *P. fluorescens* has specifically been investigated in diverse environmental conditions. In one study, different species of Pseudomonads including *P. fluorescens*, *P. chlororaphis* and *P. putida* did not demonstrate considerable influence on the planktonic growth kinetics of *L. monocytogenes* cultivated in minimum media at 10°C (Campo et al., 2001). With the same growth conditions four species of Enterobacteriaceae family significantly suppressed the MPD of *L. monocytogenes*. The same trend was observed in diluted yeast extract medium with or without supplementation of amino acids and glucose. In the absence of amino acids and glucose in diluted yeast extract, the Enterobacteriaceae species did not modify *L. monocytogenes* growth kinetics. Competition for amino acids and glucose were presumed to be the key determinants for this phenomenon (Campo et al., 2001). In another study, the inhibitory effect of several *Pseudomonas* spp. isolated from fresh or spoiled fish was investigated on six target strains including *E. coli*, *Sh. putrefaciens*, *Aeromonas sobria*, *P. fluorescens*, *L. monocytogenes* and *Staph. aureus* (Gram, 1993). The siderophores producing Pseudomonads exhibited the most pronounced inhibitory action against most of the target strains in an agar well assay. However, dense growth of *L. monocytogenes* and *Staph. aureus* was reported around the

wells containing antagonistic *Pseudomonas* strains presumably due to the greater supply of iron and other low-molecular-weight nutrients in the outskirts of the wells (Gram, 1993).

Buchanan and Bagi (1999) also evaluated the influence of initial pH, salt content and temperature on interactions between *L. monocytogenes* and *P. fluorescens*. The authors observed a significant decrease in MPD of *L. monocytogenes* (>4.0 Log cycles) co-cultured with the Gram-negative bacterium at low temperature (4°C) and two different salt concentrations (5 and 25 g/L). This pattern was quite different at higher temperatures (12 and 19°C) and at salt concentrations of 25 and 45 g/L, where reversely a slight increase in MPD of *L. monocytogenes* was observed (Buchanan & Bagi, 1999). Considering the impact of other factors such as strain differences and initial level of inoculation on the interaction of *L. monocytogenes* and other competitors, these authors suggested that the behaviours of competitor bacteria may dramatically vary at different environmental conditions. In the present study, the temperature, RH and nutrient conditions were kept constant (15°C, 100% RH and TSB+1% glucose), meaning that the observed differences for co-culture interactions may change if biofilm were to be formed under alternative environmental conditions.

In this respect, the 'Jameson Effect' that refers to the inhibition of growth of competitor strains by the dominant bacterium once it has reached its maximum cell concentration in a batch culture, can also explain the observed competition pattern in binary-culture biofilms of *P. fluorescens* and *L. monocytogenes* in the current study. The initial level of inoculation was shown to be important for the Jameson Effect due to its effect on the time it takes for a bacterium to reach its N_{max} in the culture (Mellefont et al., 2008). In their study, the effect of initial inoculation levels in dual-cultures, *L. monocytogenes* was in particular suppressed by the other strain (*E. coli*, *P. fluorescens* or *Lactobacillus plantarum*), when its initial concentration was lower than that of the co-culture, proposing a non-specific inhibition (Mellefont et al., 2008). Competition for the nutrients was suggested as the general explanation for the observed 'Jameson Effect'; however, other more complex interactions such as species-specific pH limits and diverse utilization of nutrients should also not be forgotten (Mellefont et al., 2008).

In the current study, in spite of the similarity of μ_{max} of *L. monocytogenes* and *P. fluorescens* in the binary biofilm together with the almost equal N_0 of both strains, the difference in the lag phase of the two strains (12 h in *L. monocytogenes* versus 4.8 h lag in *P. fluorescens*) would lead to a ~7-h difference in the time that it took *P. fluorescens* to reach the stationary phase/MPD allowing it to out-compete the pathogen (Figure 3). The SEM pictures also confirmed this observation, as the micrographs showed that binary biofilms of the two species (Figures 17B&C) resembled the mono-culture *P. fluorescens* biofilm with its developed EPS and biofilm structure more than the *L. monocytogenes* mono-culture species biofilm (Figures 10B&C and 18B&C). A similar pattern has previously been reported where extracellular polysaccharides generated by *Staph. sciuri* prevented *L. monocytogenes* from reaching its MPD modifying the balance existing between planktonic and biofilm populations (Leriche & Carpentier, 2000).

Little is known about the competition pattern between *L. monocytogenes* and *S. liquefaciens*. However, as the Tables 3 and 4 showed, *S. liquefaciens* dominated the Gram-positive pathogen once co-cultured in a binary-culture biofilm. The μ_{max} of of *L. monocytogenes* in mono-species biofilm was significantly ($P<0.05$) higher than that of *S. liquefaciens* but, when co-cultured in the binary biofilm μ_{max} of both species significantly ($P<0.05$) decreased. The *Serratia* μ_{max} parameter was, however, statistically higher ($P<0.05$) than that of *Listeria* in the joint biofilm (Table 3). The 'Jameson Effect' could similarly explain the suppression of *Listeria* growth. By having a greater ($P<0.05$) μ_{max} and short lag time (0.12 h), *S. liquefaciens* reached the stationary phase sooner than *L. monocytogenes* (λ=12 h), making *S. liquefaciens* the winner of the 'Jameson Effect' race (Figure 4). The domination of *S. liquefaciens* is clearly observable in both SEM and epifluorescence micrographs of the binary-culture biofilms (Figures 19B&C, 20B&C and 31). *S. liquefaciens* has previously been reported to suppress growth of *Botrytis cinerea* (Whiteman & Stewart, 1998). Although the aim of that study was to investigate the antifungal behaviour of *S. liquefaciens* against the sporulation of *B. cinerea* on living tissues, their findings do indicate the association of antagonistic activity with *S. liquefaciens*.

Not all bacterial relationships are antagonistic, however. Unlike *P. fluorescens* and S. *liquefaciens*, *Sh. putrefaciens* had no negative impact on *L. monocytogenes* growth kinetics, and instead significantly ($P<0.05$) increased the MPD and μ_{max} of the pathogen as compared to the control (Tables 3 and 4). Nonetheless, this relationship was not beneficial to the Gram-negative bacteria as the *Shewanella* N_{max} decreased ($P<0.05$) while μ_{max} remained unchanged ($P>0.05$) during growth in the co-culture biofilm compared to the control (Table 3 and 4). Furthermore the lag time for *Sh. putrefaciens* shifted from zero h in the single species biofilm to 12.4 h in the binary-culture biofilm with *L. monocytogenes* which is equivalent of a 14% increase in the lag phase of the pathogen from 19.12 h in single to 21.84 h in binary biofilm(s) with *Sh. putrefaciens* (Table 3). Nevertheless, irrespectively of the pathogen's greater maximum specific growth rate, the shorter lag-time of *Shewanella*, 13 h versus 22 h for *L. monocytogenes*, caused the maximum population to become significantly higher for the Gram-negative bacteria with N_{max} equal to 7.6 Log CFU/cm^2 compared to 7.3 for *L. monocytogenes*. It appeared that unlike the previous two scenarios, the 'Jameson Effect' race played no role in this bacterial interaction as the presence of *Sh. putrefaciens* helped *L. monocytogenes* to grow to higher populations than its single species biofilm.

The SEM and epifluorescence micrographs also confirmed this feature. *L. monocytogenes* cells were observed to produce fibrils and extracellular substances that helped in adhesion to the surface and linkages to other cells in the thin single species biofilm (Figure 16). *Sh. putrefaciens* on the other hand formed a thick biofilm layer with large amounts of EPS as visualized with 2 of the SEM fixation methods (Figures 14B, C and D). Since the Gram-negative bacteria reached a slightly but significantly higher MPD, SEM pictures taken of binary-culture biofilm with *L. monocytogenes* and *Sh. putrefaciens* resembled the mono-culture biofilms of the latter (Figures 21B and C). In addition, unlike the previous epifluorescence pictures (Figures 30 and 31), the populations of co-cultured bacteria appeared to be almost evenly distributed (Figure 32), which was in agreement with the nearly same sized MPD observed in quantitative assays (Table 3 and Figure 5). The microbial cells were growing in separate microcolonies

(Figure 32), a feature that was observed in other binary-culture biofilms as well (Figures 30 and 31).

The finding of antagonistic/synergistic interactions in *L. monocytogenes* co-culture biofilms has previously been reported and includes reports showing that common food spoilage bacteria such as some strains of *Pseudomonas* spp. and *Flavobacterium* spp. significantly increased the adhesion and biofilm formation by *L. monocytogenes* (Sasahara & Zottola, 1993; Bremer et al., 2001; Hassan et al., 2004). In particular, the food spoilage microorganisms such as *Pseudomonas putida* and *P. fragi* were demonstrated to enhance the growth, colonization and biofilm formation by *L. monocytogenes*; however, no studies have reported the commensalistic interaction between *L. monocytogenes* and *Sh. putrefaciens*.

The antagonistic activity of some *Pseudomonas* sp. in general (Gram, 1993) and *P. fluorescens* in particular against *Sh. putrefaciens* and *B. thermosphacta* has previously been elucidated (Tsigarida et al., 2003). It was shown that while *P. fluorescens* is inhibitory to *Sh. putrefaciens*, it promotes the growth of *B. thermosphacta* at 5°C in broth culture (Tsigarida et al., 2003). It was also pointed out that the siderophores produced by *P. fluorescens* and competition for glucose were the main factors responsible for this phenomenon (Tsigarida et al., 2003). A remarkable aspect of that study was the increase in growth rate of *B. thermosphacta* in co-culture with *Sh. putrefaciens* (Tsigarida et al., 2003). Although the authors did not present any explanation for this phenomenon, it seems that the end metabolites of *Sh. putrefaciens,* i.e., formic acid and two unidentified organic acids (Tsigarida et al., 2003), played a role in this interaction.

The findings by Tsigarida and co-workers (2003) are important for two reasons; firstly the negative impact of siderophores, i.e., by-products of the *P. fluorescens* metabolism, on growth patterns of a variety of microbial species such as *B. thermosphacta* and *Sh. putrefaciens* may be extended to its effect on *L. monocytogenes* and secondly the positive effect of *Sh. putrefaciens* on the growth rate of *B. thermosphacta* may similarly support the observed behaviour on the pathogen in the current study (Table 3, Figure 5).

As discussed earlier, it was demonstrated that the biofilm formation, differences in the structures of biofilm and distribution of various bacteria species in binary-culture biofilm are important factors in mutual relationships of bacteria in a bigger microbial community. In the following section various aspects of the SEM and epifluorescence micrographs will be discussed in more detail.

Three protocols were employed to fix and visualize the *P. fluorescens* biofilm on SS coupons. The best pictures were acquired when using the modified fixation in cacodylate buffer and the FC-72 protocols. Both protocol preserved the microbial cells as well as the biofilm structure and cellular distribution on the surface (Figures 9B, 9D, 10B, 10C and 10D). The other protocol, i.e., basic fixation in cacodylate buffer, was not able to efficiently preserve neither the cells nor the EPS matrix and led to destruction of spatial organization of the cells (Figures 9A & 10A).

The formation of microcolonies and maturation in EPS-matrix were shown at lower and higher magnifications in Figures 9B, 10B, 10C and 10D, respectively. In other words, the present SEM pictures supported the role of EPS in biofilm structure. The spatial organization and chemical profile of *P. fluorescens* biofilm has been investigated in particular. Baum and colleagues (2009) characterized the mature biofilm structure of the Gram-negative bacterium. They observed heterogeneous self-organized fibrillary clusters of EPS constructions that had created honeycomb-like chambers enveloped in thin sheets. These authors also reported up to 50% of proteins and 3% (by dry weight) accumulated calcium in the mature biofilm and suggested that this divalent metal is crucial in biofilm formation (Baum et al., 2009). Their structural findings are in agreement with the observations in the current study as visualized in the SEM pictures (Figures 9B, 9D, 10B, 10C and 10D).

The SEM images taken of the dual species biofilm of *P. fluorescens* and *L. monocytogenes* (Figures 17 and 18) displayed similar structure to the *P. fluorescens* single biofilm images (Figures 9 and 10). Again the best images were acquired when using the modified fixation in cacodylate buffer and FC-72 protocols (Figures 17B, 17C, 17D, 18B, 18C and 18D). The main limitation of the SEM micrographs was the inabillity

to discriminate different microbial species within the dual species biofilm community. Therefore, other visualization technique was employed to enable differentiation of the bacterial cultures based on the Gram-reaction.

To this end two labelling techniques were employed to stain and visualize the mixed binary biofilms on SS coupons. As mentioned earlier (section 4.3.2), insertion of the pNF8 plasmid with the GFP label caused *L. monocytogenes* to grow significantly ($P<0.05$) slower in both the mono- and binary-culture biofilms in comparison to the parent strain. Therefore the images taken by this staining procedure were excluded from further analysis and presented in appendix A only for information. The nucleic acid Gram-staining method was, however, successfully applied with the best pictures obtained using the stains in a 20:80 proportion of SYTO9:hexidium iodide. The fluorescence labeling of *P. fluorescens* and *L. monocytogenes* effectively differentiated the microbial species based on their Gram reaction. The different microbial communities were evident in the micrographs which were dominated by *P. fluorescens* (green areas) with a few red *L. monocytogenes* areas interdispersed (Figure 30), indicating that two species created their own niches in the bigger microbial community.

Other researchers have also investigated the spatial arrangement of multi-species biofilms. In a study by Almeida and co-workers (2011), using CLSM it was demonstrated that in a binary-culture biofilm, *E. coli* and *L. monocytogenes* created two well defined separate layers. This pattern was also observed in the binary biofilm of *E. coli* and *S. enterica*; however, conversely to the other biofilms, *Salmonella* and *Listeria* did not generate two separate layers (Almeida et al., 2011). Although the spatial distribution of the dual-species biofilms varied in that study (Almeida et al., 2011), the formation of distinct microcolonies was evident in the epifluorescence and CLSM images, a similar finding to what was observed in Figures 30, 31 and 32.

The biofilm structure of *S. liquefaciens* in single or dual-species biofilms with *L. monocytogenes* was presented in SEM pictures in Figures 11, 12 or 19 and 20, respectively. The honey comb structure was evident in all the micrographs. However, the comparison of these pictures with mono- or binary-culture biofilms containing *P.*

fluorescens revealed great differences in both cell morphology and biofilm matrix. In *Pseudomonas* biofilm, the microbial cells were embedded in a spatial matrix of extracellular substances while in *S. liquefaciens* biofilms the cells were anchored onto the substratum by fibril like appendages. These junctures also linked the cells together and formed a spatial honey comb-like matrix. Xu and others (2010) demonstrated that both *S. liquefaciens* and *L. monocytogenes* are strong biofilm producers and the rate of biofilm production expressed as specific biofilm formation (SBF) index increases considerably with extension of the incubation from 24 h to 36 h. The SEM images were also taken of the individual biofilms of these species; however, the spatial organization and structure of biofilms were not clear and, *L. monocytogenes* was reported to only form a net-like structure and a mono-layer biofilm (Xu et. al., 2010). To the best of our knowledge, no previous work has assessed *L. monocytogenes* and *S. liquefaciens* in binary-culture biofilms. The SEM micrographs (Figures 11, 12, 19 and 20) obtained in the current study clearly displayed the biofilm EPS matrix (Figures 12C, 20B and 20C), cellular arrangement and formation of microcolonies in the single and dual-species biofilms. The epifluorescence pictures also revealed a pattern similar to what was observed for binary-culture biofilms made by *P. fluorescens* and *L. monocytogenes* (Figures 30 and 31) where the two species created separate microcolonies.The findings in current study for binary *L. monocytogenes* and *S. liquefaciens* biofilms resemble those described by Almeida et al. (2011).

Bagge and co-workers (2001) investigated the biofilm formation features of *Sh. putrefaciens* and pointed out that the food-spoilage bacterium can readily adhere and form biofilm on food contact surfaces. High density of microbial cells in biofilm matrix was reported when adequate nutrients were provided, although the availability of carbohydrates (lactate or glucose) and iron starvation were found to influence the thickness and rate of biofilm formation by the bacterium. Presence of a competitor bacteria (*P. fluorescens*) was shown to affect the rate of biofilm formation and decrease the population of *Sh. putrefaciens* in the mixed biofilm (Bagge et al., 2001). The thick layers of *Sh. putrefaciens* biofilm on SS coupons were observed in SEM pictures both in single and co-culture with *L. monocytogenes* (Figures 13, 14, 21and 22). The cellular

arrangement were similar to that observed by Bagge and co-workers (2001) and also similar to their finding for *P. fluorescens*, the *Listeria* reduced the MPD of *Sh. putrefaciens* in binary biofilm in comparison to the MPD obtained in the single species biofilm (Tables 3 and 4).

While the quantification techniques indicated 3.57% (0.28 Log_{10} CFU/cm^2) decrease ($P<0.05$) in MPD of *Sh. putrefaciens* in the binary biofilm, the visualization methods did not reveal any obvious difference mainly due to the high population density of *Sh. putrefaciens* in both biofilms (Figures 13 and 21). The epifluorescence micrographs exhibited an almost even distribution of the Gram-negative and Gram-positive cells on the substratum where separate formation of microcolonies was also evident (Figure 32) similar to images obtained for the other binary-culture biofilms (Figures 30 and 31). To our knowledge, this study is the first work that has quantitatively and qualitatively assessed the binary-culture biofilm formed by *L. monocytogenes* and *Sh. putrefaciens*.

In other studies, the biofilm structure of *L. monocytogenes* in mono-culture has been subject of intensive interest. Hefford and co-workers (2005) reported multicellular layers of *L. monocytogenes* 568 (same strain as was used in the current study) adhering to various test surfaces and embedded in a carbohydrate containing EPS matrix. The presence of extracellular materials was confirmed using microscopy with fluorescent-conjugated concanavalin A. Rieu and others (2008) observed a biofilm consisting of a knitted network under nutrient flow conditions, although in absence of adequate nutrients and flow an unstructured biofilm with a few layers of cells was reported. The authors concluded that the growth conditions and availability of enough nutrients highly affect the spatial organization and gene expressions that govern the biofilm formation (Rieu et al., 2008). Under continuous flow conditions, the elongation of cells and formation of knitted network in the biofilm structure was later found to be governed by SOS response genes and in particular *yneA* (van der Veen & Abee, 2010).

In the current study, established microcolonies with a few cellular layers were observed in single biofilm architecture of *L. monocytogenes* (Figures 15 and 16). The

type of strain and other environmental factors such as growth medium and temperature (15°C in current study versus 37°C in most other studies) are likely to markedly affect the architecture (mono-layer versus organized network of cells) and extracellular substances secretion by the pathogen (Renier et al., 2011). Also the initial level of inoculation has a great impact on biofilm formation. In the current study a low initial inoculum level (10^3 CFU/cm^2) was used to mimic the real conditions in a food processing plant where low numbers of *Listeria* cells could have escaped the sanitizing program to colonize surfaces, form biofilms under static nutrient conditions and subsequently encounter dry conditions.

Considering the growth conditions (15°C, 48 h and batch nutrient conditions) in this study, the SEM pictures (Figures 15 and 16) correspond to previous observations where unstructured biofilm with a few cellular layers was reported in *L. monocytogenes* under static conditions at 37°C (Rieu et al., 2008). However, since the initial inoculation level was lower and due to the low incubation temperature of 15°C the biofilm formation was in an earlier stage in the current study (early stationary phase versus late stationary phase in other studies: Rieu et al., 2008), the micrographs may not directly compare to other studies. Nevertheless, the existence of presumptive extracellular substances (Figure 16C) indicates that a more organized structure may develop if enough time is allowed for the formation of the mature biofilm at 15°C.

In the present study, the growth kinetics and biofilm structure of *L. monocytogenes* and the three Gram-negative food spoilage bacteria in single or dual-species biofilm were characterized and then followed by a study of the desiccation survival of *L. monocytogenes* and its biofilm co-cultures.

The desiccation survivor counts ($Log_{10}N/N_0$) were fitted by four available inactivation models which are commonly used in predictive food microbiology. The models with lag phase were excluded from further analysis as no lag time was observed in any of the survivor curves. Based on the statistical analysis, the double-Weibull distribution was selected as the model that could best describe the survival kinetics during the desiccation period (43% RH, 15°C, 21 d) for all biofilm treatments (Table 5). The double-Weibull model was fitted to individual desiccation survivor curves for each

strain in mono- and binary-culture biofilms to allow for estimation of a mean±standard error for each of the model parameters (Table 6).

The α parameter is defined as the logarithmic proportion of subpopulation 1 to subpopulation 2 ($Log_{10}N_{01}/N_{02}$) and therefore, the graphic difference between $Log_{10}(N_0)$ and the logarithm of the population where the inflection is seen gives the α (Coroller et al., 2006). The model assumes that the microbial population consists of two subsets where subpopulation 1 is more sensitive to the specific stress than subpopulation 2 (Coroller et al., 2006). In theory, the α can be any value from negative infinity to positive infinity; however, in practice the negative α values would not give an obvious inflection point on the curves (Coroller et al., 2006). In this case the model allows fitting of a linear shape (curved) with various *P* values (Coroller et al., 2006).

The parameter *P* is the shape factor that yields a linear curve if equal to 1, convex shape if less than 1 or concave shape if above 1. The various possible combinations of the α and *P* parameters permit the model to describe different inactivation kinetics perfectly. Essentially, biphasic curves with a nonlinear decrease, sigmoid curves, concave, linear or convex curves, biphasic and linear curves with a tail are all possible forms of inactivation kinetics that can be described using various combinations of the α and *P* parameters (Coroller et al., 2006). The $\boldsymbol{\delta}$ values show the decimal reduction times for the two subpopulations although in most cases the $\boldsymbol{\delta}_2$ solely shows the tail on the graphs (Coroller et al., 2006).

The statistical analysis of the estimated model parameters demonstrated that *P. fluorescens* and *Sh. putrefaciens* did not have any significant ($P>0.05$) influence on the α and *P* parameters for *L. monocytogenes*; however, the Gram-negative bacteria significantly ($P<0.05$) increased the listerial $\boldsymbol{\delta}_1$ and conversely decreased the $\boldsymbol{\delta}_2$ (Table 6 and 7). The Gram-negative bacteria also significantly ($P<0.05$) increased the overall reduction of the pathogen [$\Delta Log_{10}(N/N_0)$]. *S. liquefaciens* similar to the other Gram-negative strains significantly ($P<0.05$) increased the total loss of viable cells and reduced the $\boldsymbol{\delta}_2$ for *L. monocytogenes* during the desiccation, though the bacterium had no ($P>0.05$) impact on the shape factor (P), α and $\boldsymbol{\delta}_1$. *L. monocytogenes* on the other hand,

did not significantly (P>0.05) affect the shape factor, α and $\boldsymbol{\delta}_1$ obtained for *P. fluorescens* nevertheless it significantly (P<0.05) decreased the $\boldsymbol{\delta}_2$. Conversely the total loss of viable cells was significantly (P<0.05) increased in presence of *L. monocytogenes*. The impact on α and $\boldsymbol{\delta}_1$ on *S. liquefaciens* was quite similar to what was observed for *P. fluorescens*; however, the pathogen significantly (P<0.05) increased the shape factor observed for *S. liquefaciens*. No obvious influence (P>0.05) was observed on the $\boldsymbol{\delta}_2$ and absolute reduction of *S. liquefaciens* when grown in binary biofilms with *L. monocytogenes*. The Gram-positive pathogen had significant (P<0.05) positive influence on $\boldsymbol{\delta}_1$ and $\boldsymbol{\delta}_2$ in *Sh. putrefaciens*; nevertheless it did not statistically (P>0.05) change the α, shape factor and absolute reduction of the Gram-negative food spoilage bacterium (Tables 6 and 7).

Apparent from Tables 6 and 7 was the similarity of the α parameter among all biofilms. This means that neither the Gram-negative strains nor *L. monocytogenes* significantly (P>0.05) affected the resistance of each other to the matric stress. Although the growth models indicated significant (P<0.05) positive or negative influences of various test strains on the growth kinetics of each other in binary biofilms (Tables 3 and 4), each bacterium had no effect on the resistance of their biofilm partners to dry conditions (43% RH).

In contrast to the α parameter, all Gram-negative strains significantly (P<0.05) increased the absolute reduction in the number of *Listeria* cells during desiccation at 43% RH. This indicates that although the growth of diverse strains in a joint binary biofilm had no effect on the cellular resistance, the presence of Gram-negative strains significantly (P<0.05) affected the overall reduction in *L. monocytogenes* populations on SS coupons. This means that under the matric stress conditions (43% RH) the population of *L. monocytogenes* in co-culture with the food spoilage strains will be reduced more than when subjected to the same conditions as a single strain. This confirms the fact that in a static biofilm with limited source of nutrients, the competition for nutrients greatly affects the growth kinetics and subsequently the overall reduction during the desiccation period.

The HPLC analysis had already indicated the presence of major osmolytes including glycine betaine, carnitine, proline and trehalose (0.29±0.02, 0.06±0.00, 2.00 and 0.07 mg/ml, respectively) in the growth and desiccation medium (TSB+1% glucose) used in the present study (Huang, 2011). The essential role of compatible solutes and the mechanisms by which the *Listeria* cells cope with the matric or ionic stresses have previously been elucidated (Sleator et al., 1999; Wemekamp-Kamphuis et al., 2002; Angelidis & Smith, 2003; Dreux et al., 2008; Ells & Truelstrup Hansen, 2011; Huang, 2011). Therefore, it can be postulated that the presence of competitor strains during the growth and desiccation period afterwards, may have limited the osmolytes and other nutrients availability to the Gram-positive pathogen and as a result increased the absolute reduction while having no influence on the cellular resistance as compared to the control.

The increase in first decimal reduction time for subpopulation 1 ($\boldsymbol{\delta}_1$) in binary-culture biofilms consisting of *L. monocytogenes* with either *P. fluorescens* or *Sh. putrefaciens* as compared to the control appeared to contradict to observed increase in overall reduction discussed above. The higher $\boldsymbol{\delta}_1$ in binary biofilms, although very marginal, meant an increase in the time required for the decimal reduction of the subpopulation 1 (Figures 6 and 8) but, this does not necessarily mean an increase in the resistance of *Listeria* cells. Having a closer look at the binary biofilm structures of *L. monocytogenes* with either *P. fluorescens* or *Sh. putrefaciens* (Figures 17, 18, 21 and 22), it is obvious that there are expansive spaces and presumptive EPS in the biofilm matrix that can entrap the more water molecules than the mono-layer of cells observed in mono-culture *L. monocytogenes* biofilms, and therefore provide conditions under which the *Listeria* cells survived the matric stress better. Other researchers have also reported the putative role of EPS where Roberson and colleagues (1992) provided evidence in support of the correlation between desiccation and production of extracellular polysaccharides (EPS) by *Pseudomonas* species isolated from soil.

This condition may, however, be unstable because when the cells encounter starvation over the time, they may begin to consume the extracellular substances that they created during the growth, as a source of nutrient (Takhistov & George, 2004) and consequently demolish the 3D structure of the biofilm. This phenomenon may finally

lead to an increase in the rate of water evaporation from the substratum and a reduction in the $\boldsymbol{\delta}_2$ and increase in the $\Delta Log_{10}(N/N_0)$ as was observed for *Listeria* binary-culture biofilms as compared to the control (Tables 6 and 7).

The domination of *P. fluorescens* over *L. monocytogenes* during the growth (Tables 3 and 4, and Figure 3) and more organized biofilm architecture of the *Pseudomonas* with extracellular substances (Figures 9 and 10) as compared to the few cellular layers in mono-species biofilm of *L. monocytogenes* (Figures 15 and 16) may be reason for the observed phenomenon where the *Pseudomonas* strain greatly increased the $\boldsymbol{\delta}_1$ in *L. monocytogenes* while the *Listeria* had no meaningful ($P>0.05$) impact on that of the *Pseudomonas* strain (Table 6 and 7). However, over the time, the presumptive 3D structure destruction due to the desiccation challenge and energy starvation may have accelerated the evaporation rate and subsequently reduced the $\boldsymbol{\delta}_2$ and increased the $\Delta Log_{10}(N/N_0)$ in *Pseudomonas* binary biofilm as compared to the control (Table 6 and 7).

The kinetics of survival in the binary-culture *Sh. putrefaciens* and *L. monocytogenes* biofilm was largely similar to that of the controls, although since *Shewanella* survivors in single or binary biofilms dropped below the detection limit, estimated values for the $\boldsymbol{\delta}_2$ and $\Delta Log_{10}(N/N_0)$ parameters may not reflect the real conditions (Table 6).

S. liquefaciens as compared to the *Pseudomonas* and *Shewanella* did not form a dense cellular layer in neither the mono- nor the binary-culture biofilms with *L. monocytogenes* (Figures 11 and 19) and therefore, it is very likely that the similar $\boldsymbol{\delta}_1$ value found for *L. monocytogenes* in binary biofilm with *S. liquefaciens* and the control, could be due to the architectural design of the dual-species biofilm. Although the *Serratia* biofilm may have been able to retain the water, its effect was unable to significantly ($P>0.05$) increase the $\boldsymbol{\delta}_1$ for *Listeria*. The $\boldsymbol{\delta}_2$ and $\Delta Log_{10}(N/N_0)$ followed the same pattern as was observed for the *Pseudomonas* and *Shewanella* binary biofilms, in which the presumptive lack of osmolytes and biofilm demolition due to the desiccation may be the main factors in the observed phenomenon (Table 6 and 7). *L. monocytogenes* similarly had positive and negative influence on the $\boldsymbol{\delta}_2$ and $\Delta Log_{10}(N/N_0)$ in *S. liquefaciens*,

respectively, although this was not statistically significant (P>0.05). Also, the innate resistance of *Serratia* to the matric stress (low value of α and high value of $\boldsymbol{\delta}_1$ parameters, Table 6) may be the reasons to the similar behaviour of *S. liquefaciens* in mono- and dual-species biofilm with *L. monocytogenes* (Table 7).

The desiccation survival and/or hyper-osmotic tolerance of mono-species biofilms of *L. monocytogenes* and the other three Gram-negative food spoilage bacteria has been the subject of a few studies. In Gram-negative bacteria including *E. coli,* the synthesis or uptake of compatible solutes such as trehalose, which is governed by the alternative sigma (σ) factors, were also found to constitute a universal stress response in the bacterium (Ramos et al., 2001). Other strategies include changes such as in membrane permeability due to alteration in fatty acid composition of the cells (Ramos et al., 2001) or the reversible increase in cis-trans isomerization of monosaturated fatty acids that maintains the liquid crystalline phase of membranes during matric or ionic stress (Junker & Ramos, 1999). Similarly the *P. fluorescens* gene expression and sigma factors have been recognized to be involved in environmental fitness and desiccation tolerance of the food spoilage microorganism. The sigma factors rpoS (Stockwell et al., 2009) and algU (Schnider-Keel et al., 2001) were determined to be crucial factors in enhanced resistance of *P. fluorescens* to high osmolarity and dry conditions. However, in spite of these survival mechanisms *P. fluorescens* exhibited a lower desiccation resistance in the current study as compared to *L. monocytogenes* and therefore survivor numbers fell much faster ($\boldsymbol{\delta}_1$=0.35±0.08 d) as compared to *L. monocytogenes* ($\boldsymbol{\delta}_1$=3.06±0.19 d).

The interactive effects of desiccation and high salinity on the growth of *S. liquefaciens* have been investigated under simulated Mars conditions. While high salt concentrations (>10%) were inhibitory to the survival and replication of the cells in dry conditions, lower salt media (<10%) proved to have either neutral or positive effect on the bacterial survival and/or growth (Berry et al., 2010). In food systems, the capability of bacterium to grow in low water activity conditions was also demonstrated by Losantos and others (2000) where thirty strains of *S. liquefaciens* and *Proteus vulgaris* were isolated from spoiled dry-cured hams. *S. liquefaciens* was identified as the only isolate that could grow down to an a_w level of 0.949. The *S. liquefaciens* continued to grow

during the salting step until it finally became inhibited by the drop in a_w-values developing during the post salting and drying steps of the ham manufacturing (Losantos et al., 2000). The evidence of ability to become osmo-adapted and relative high desiccation tolerance of this Gram-negative bacterium is in agreement with the findings in the current study.

Although little is known about desiccation survival of *S. liquefaciens* in food systems, evaluation of the behaviours of its counterparts can help in better understanding of the survival of this Gram-negative food spoilage bacterium under matric stress. In this regard, *Salmonella* spp. can be good candidate; hence *Serratia* and *Salmonella* are from a same microbial family, i.e., Enterobacteriacea and additionally both species demonstrate enhanced tolerances to desiccation conditions. As discussed previously in the literature review (2.5.2), several food-borne outbreaks have been reported due to consumption of dry foods contaminated with *Salmonella* spp. (Hiramatsu et al., 2005). A study by Garmiri and others (2008) showed that the outer membrane polysaccharides play a key role in resistance of *Salmonella* spp. to the dry conditions. The authors demonstrated that the strains that lack the O polysaccharide (OPS) are less resistant to desiccation (Garmiri et al., 2008). They discussed the innate feature of the Enterobacteriacea family to express extracellular cellulose and thin aggregative fimbriae is the key element in enhanced survival of *Salmonella* Typhimurium to dry conditions (White et al., 2006; Garmiri et al., 2008). The water-binding capacity of exopolysacharides (lipopolysaccharide O chain polysaccharide in that study) was suggested as an important factor in reduction of the evaporation process and creation of hydrated microenvironment around the microbial cells that leads to the enhanced resistance of *Salmonella* cells to matric stress (Chaplin, 2007; Garmiri et al., 2008).

Other studies have also investigated the desiccation survival in other members of the Enterobacteriacea family. Barron and Forsythe (2007) studied desiccation resistance and survival time of select microbial species from the Enterobacteriacea family in dehydrated powdered infant formula over a 2.5-year period. They classified the Enterobacteriacea members to three sub-groups based on their survival time under the dry conditions. Some strains of *Cronobacter* including *C. freundii*, *C. koseri*, and also

Enterobacter cloacae faded after 6 months followed by *Salmonella* Enteritidis, *Klebsiella pneumoniae*, and *E. coli* that persisted less than 15 months. Among these family members only *Escherichia vulneris*, *Pantoea* spp. and *K. oxytoca* were recovered over 2 years followed by some capsulated strains of *Entro. sakazakii* that were still alive at the end of the 2.5-year experimental period (Barron and Forsythe, 2007). The authors suggested that the increased resistance of *Entero. sakazakii* to desiccated conditions could be due to the formation of extracellular polysaccharide that provides protection against matric stress in the pathogen (Lehner et al., 2005; Barron and Forsythe, 2007).These findings can justify the observed desiccation tolerance of *S. liquefaciens* (as a member of Enterobacteriacea family) in the current study where an aggregate of all above mentioned features of this bacterial family, i.e., production of extracellular polysaccharide, fimbriae and/or cellulose was evident in SEM micrographs taken of *S. liquefaciens* (Figures 12, 19 and 20).

The other Gram-negative food spoilage bacterium, *Sh. putrefaciens* has only been investigated for its salt tolerance response and hyper-osmotic adaptation but not for its desiccation survival. The sub-lethal concentrations of NaCl or cold stress were demonstrated to induce the synthesis of several polypeptides and proteins that then helped in cross-protection to the subsequent more intensive salt stress (Leblanc et al., 2003). Nevertheless, similar to *P. fluorescens*, the bacterium exhibited a relatively low tolerance to the low RH stress in the current study (high α and low $\boldsymbol{\delta}_1$ values of 8.63±0.34 and 0.04 ±0.00 d, respectively, which were much higher or lower than those of mono-species *L. monocytogenes,* respectively, Table 6).

To the best of our knowledge, this work was the first study that systematically assessed the biofilm formation by *L. monocytogenes* and any of the selected food spoilage Gram-negative bacteria including *P. fluorescens*, *S. liquefaciens* and *Sh. putrefaciens* in single or dual-species communities. Both quantitative and qualitative techniques were employed to characterize and assess the interaction of selected bacteria species on the growth kinetics of each another. These results were subsequently used to explain the mechanism of survival during the desiccation at 43% RH and 15°C. Although the desiccation survival or its closely related subject, hyper-osmotic stress, had previously been investigated for mono-culture biofilms or planktonic cells of the selected

microorganisms, it was the first time that the matric stress response in dual-species biofilms of *L. monocytogenes* and each of the selected food spoilage Gram-negative bacteria was investigated. It is hoped that the findings of current study will help in the future devising of strategies to effectively eliminate the *L. monocytogenes* hazards from the food-chain and ultimately improve food safety.

CHAPTER 6 CONCLUSION AND FUTURE WORK

Using the Logistic model with lag phase it was determined that growth in binary biofilms on SS coupons (100% RH, 15°C, 48 h) caused a significant ($P<0.05$) shift in growth kinetics of *L. monocytogenes* and its partner Gram-negative food spoilage bacteria (*P. fluorescens*, *S. liquefaciens* or *Sh. putrefaciens*) in the soiled food grade stainless steel model system. In this mutual relationship *P. fluorescens* and *S. liquefaciens* significantly ($P<0.05$) reduced the MPD of *L. monocytogenes* while *Sh. putrefaciens* ($P<0.05$) increased MPD of the pathogen. On the other hand, *L. monocytogenes* negatively ($P<0.05$) affected the MPD of *Sh. putrefaciens* while the impact was insignificant ($P>0.05$) or positive ($P<0.05$) on *S. liquefaciens* and *P. fluorescens*, respectively. The maximum specific growth rate (μ_{max}) of each strain in binary-culture biofilms was also affected by the partner strain as compared to when grown in mono-culture biofilms. Where *L. monocytogenes* significantly ($P<0.05$) either reduced the μ_{max} of *P. fluorescens* and *S. liquefaciens* or exerted no change ($P>0.05$) on that of *Sh. putrefaciens* as compared to the mono-culture biofilms, *P. fluorescens* and *S. liquefaciens* or *Sh. putrefaciens* were found to ($P<0.05$) reduce or increase that of *L. monocytogenes*, respectively, as compared to single biofilm made by the bacterium. The initial level of inoculation was similar between all strains ($\cong 10^3$ CFU/cm^2) although the model estimates for N_0 were slightly decreased due to a slight population drop during the initial 12 h, also observed as being the lag phase for several of the bacteria.

The 'Jameson Effect' was suggested as the key determinants in suppression of *L. monocytogenes* by *P. fluorescens* and *S. liquefaciens*. However, it could not be used to describe the domination of *L. monocytogenes* on *Sh. putrefaciens*. Other factors such as high volume of EPS production by the *Pseudomonas*, lag phase in growth of the *Listeria*, competition for nutrients and inhibition by end metabolites may have caused *P. fluorescens* and *S. liquefaciens* to outcompete *L. monocytogenes*.

The listerial suppression was also supported by the SEM and epifluorescence pictures taken of the biofilms formed on SS coupons under the same conditions as the quantitative assays. The SEM micrographs demonstrated the honeycomb-like chambers

in both mono- and binary-culture biofilms of *P. fluorescens* and *S. liquefaciens* with more presumptive EPS being observed in the *Pseudomonas* biofilms. Thick layers of *Sh. putrefaciens* in both single species and dual-species biofilms with *L. monocytogenes* were observed in SEM pictures. The established microcolonies with a few cellular layers were also observed in single-species biofilm architecture of *L. monocytogenes*. The specific *L. monocytogenes* strain (Lm 568) and other environmental factors such as growth medium and temperature (15°C in current study to reflect the temperature in food processing plants as opposed to 37°C in most other studies) may have affected the biofilm architecture (mono-layer versus organized network of cells) and EPS production by the pathogen. The epifluorescence images displayed the formation of single species microcolonies in binary biofilms and also the greater populations of *P. fluorescens* and *S. liquefaciens* than *L. monocytogenes* in binary-culture biofilms. As could be expected, the populations of *Sh. putrefaciens* and *L. monocytogenes* were almost evenly distributed, thus confirming the quantitative biofilm formation results.

Survivor counts and microscopic assessments were used to explain the mechanism of desiccation survival for the different strains. The demographic profile of the two subpopulations (α) with different tolerances towards the matric stress did not change between the mono- and binary-culture biofilms and this meant that the resistance of microbial cells seems not be influenced by presence of the competitor strain. However, the $\boldsymbol{\delta}_1$ and $\boldsymbol{\delta}_2$ were greatly affected by presence of the co-cultures. All the Gram-negative strains significantly ($P<0.05$) reduced the $\boldsymbol{\delta}_2$ of *L. monocytogenes* in binary-culture biofilms as compared to the single species biofilm while conversely *P. fluorescens* and *Sh. putrefaciens* greatly increased the $\boldsymbol{\delta}_1$ of the pathogen ($P<0.05$). *S. liquefaciens* did not make any significant ($P>0.05$) change to the $\boldsymbol{\delta}_1$ of *L. monocytogenes*. The Gram-positive pathogen similarly did not alter the $\boldsymbol{\delta}_1$ of *S. liquefaciens* and *P. fluorescens* ($P>0.05$), although an increase was observed in both $\boldsymbol{\delta}_1$ and $\boldsymbol{\delta}_2$ associated with *Sh. putrefaciens* when co-cultured in biofilms with the pathogen ($P<0.05$). The pathogen negatively affected the $\boldsymbol{\delta}_2$ of *P. fluorescens* ($P<0.05$) whereas it was neutral ($P>0.05$) to that of *S. liquefaciens*.

Although the Gram-negative strains did not impact ($P>0.05$) the proportion between desiccation sensitive and resistant subpopulations of *L. monocytogenes* cells (i.e., no change in the α parameter) when conditions shifted from single to binary species biofilms, their presence increased the absolute reduction of cells as compared to the control ($P<0.05$). This phenomenon proved that the presence of competitor strains affects the desiccation survival of the *Listeria* cells, as the Gram-negative strains increased the absolute loss of the pathogen cells during the desiccation. *L. monocytogenes* also increased the $\Delta Log_{10}(N/N_0)$ for *P. fluorescens* whereas it had no effect on the reduction of *S. liquefaciens* and *Sh. putrefaciens.* Energy starvation, loss of compatible solutes, density of cellular layers, EPS matrix and integrity of the biofilm structure before and after desiccation and its role in retention of water were all deemed to be involved in the observed phenomena in current study, since their putative role in desiccation survival of *L. monocytogenes* or other bacterial biofilms has previously been confirmed in other studies. However, future work should be carried out to verify the role of the above mentioned parameters in matric stress tolerance of *L. monocytogenes* and the selected Gram-negative food-spoilage bacteria in mono- or binary-culture biofilms.

Overall, the presence of the Gram-negative food spoilage bacteria, *P. fluorescens* and *S. liquefaciens* but not *Sh. putrefaciens*, greatly reduced the MPD during the growth on SS coupons (100% RH, 15°C and 48 h) and subsequently increased the overall reduction in *L. monocytogenes* numbers during the desiccation period (43% RH, 15°C and 21d). However, no change in the proportion of desiccation resistant and sensitive ppopulations (the α parameter) of *Listeria* cells was observed in presence of the competitor Gram-negative strains. Therefore, the co-culture strains appeared to affect the desiccation survival of *L. monocytogenes* due to competition during growth and biofilm formation, however, other factors such as availability of osmolytes, thickness of formed biofilm on the substratum, the gel matrix and integrity of the biofilm structure before and after the stress may be responsible for the observed phenomena. In fact, the presence of presumptive EPS in binary-culture biofilms made by *P. fluorescens* and *S. liquefaciens* in partnership with *L. monocytogenes* appeared to cause an increase in $\boldsymbol{\delta}_1$ –values (i.e., time to the first log reduction of the sensitive sub-population), which meant that the

desiccation resistance of the pathogen became elevated in the initial phases of the desiccation period.

The evaluation of the presumptive role of these factors should be considered in future work. Appropriate HPLC techniques and/or radioactive labeling techniques could be used to measure the uptake and accumulation of important osmolytes, i.e., glycine betaine, carnitine, proline and trehalose during the growth and subsequent desiccation of mono- or binary-culture biofilms. The SEM method may also be employed to image the changes taking place in biofilm structure during the exposure to the matric stress. The images may provide better insight into the possible role of EPS and cell density on the desiccation survival of different bacteria. The CLSM in cooperation with suitable fluorescence labeling techniques may also be utilized to determine the depth, thickness and spatial arrangement of various bacteria in multi-species biofilms. The changes in material composition of biofilms during growth and desiccation afterwards could also be monitored using the non-destructive FT-IR microscopy technique.

It is hoped that an improved understanding of the behaviour of *L. monocytogenes* and competitor Gram-negative food-spoilage bacteria during formation of mono- and binary-culture biofilm and survival during subsequent exposure to the matric stress will help in devising strategies to effectively eliminate the presence of *L. monocytogenes* and/or food spoilage bacteria from the food contact surfaces and ultimately assist in removal of the pathogen from the food chain to improve food safety for the benefit of consumers, the food industry and public institutions in the food inspection and health care area.

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APPENDIX A

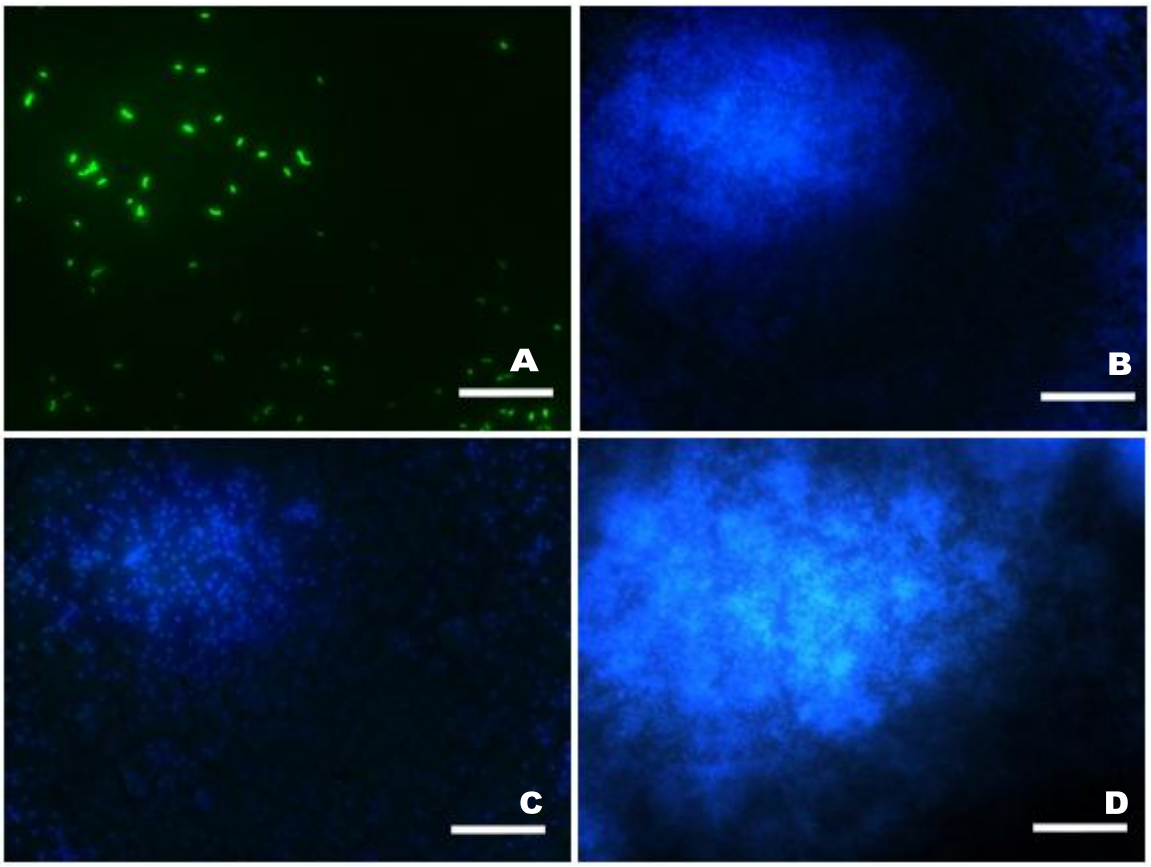

Figure 33. Epifluorescence micrographs taken of mono-culture biofilms of *L. monocytogenes* (A), *P. fluorescens* (B), *S. liquefaciens* (C) and *Sh. putrefaciens* (D) (Magnification × 1000). The biofilms were formed on SS coupons (48 h, 100% RH & 15°C) and were fluorescently labeled using pNF8 plasmid for *L. monocytogenes* (A) or the fluorescent nucleic acid dye DAPI for the other bacteria species (B, C and D) (scale bar, 10 μm).

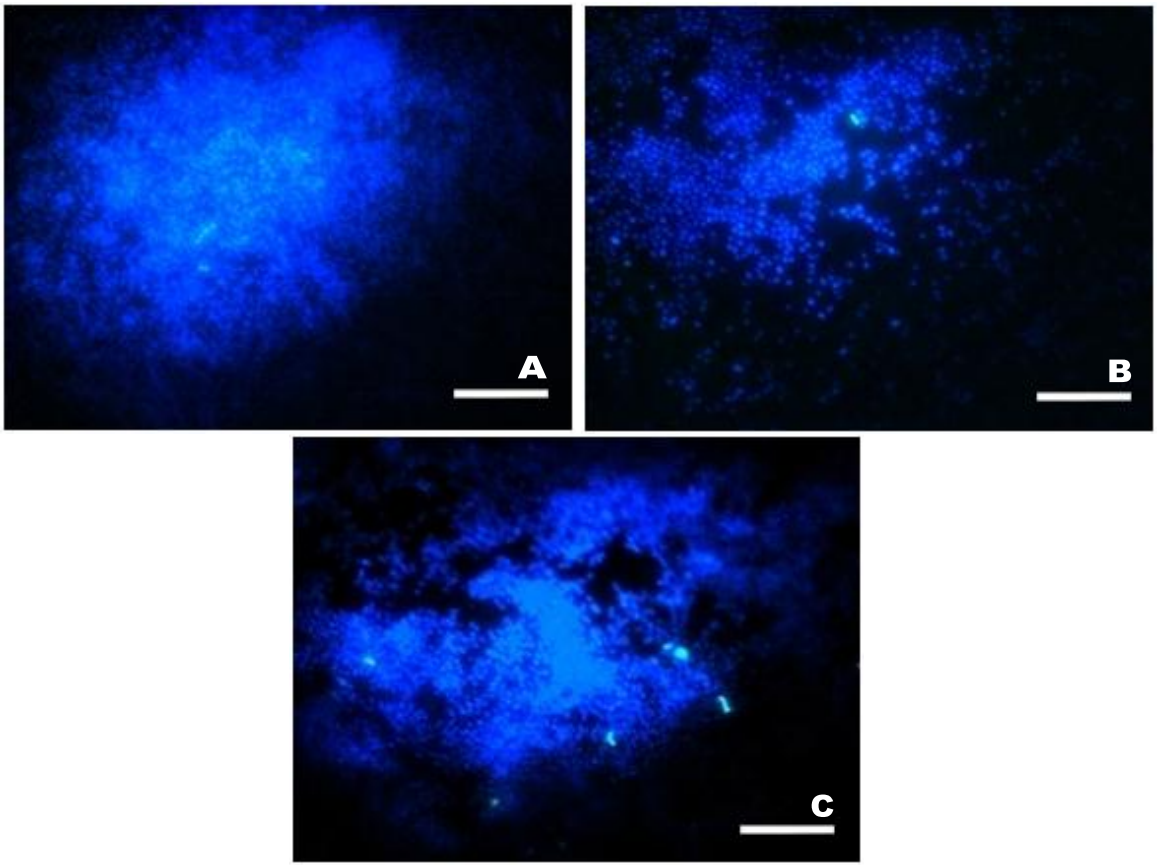

Figure 34. Epifluorescence micrographs taken of binary-culture biofilms of *L. monocytogenes* and *P. fluorescens* (A), *L. monocytogenes* and *S. liquefaciens* (B) or *L. monocytogenes* and *Sh. putrefaciens* (C) (Magnification × 1000). The biofilms were formed on SS coupons (48 h, 100% RH & 15°C) and were fluorescently labeled using pNF8 plasmid for *L. monocytogenes* and DAPI (a fluorescent nucleic acid dye) for the other bacteria species (scale bar, 10 μm).

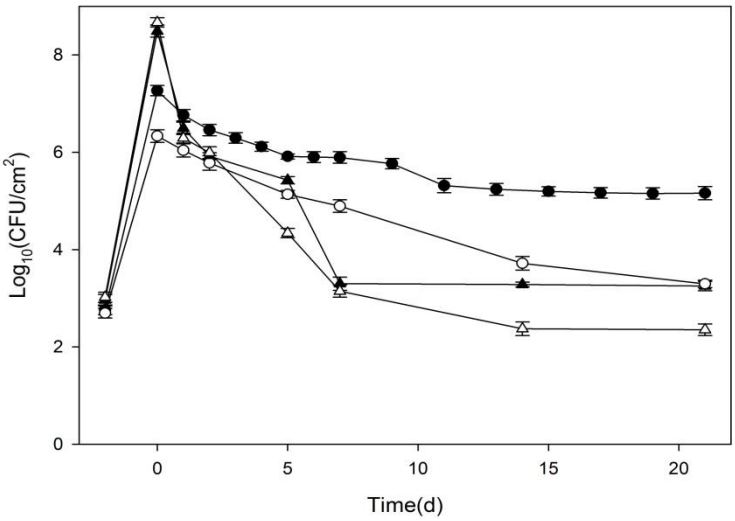

Figure 35. Desiccation survival of *L. monocytogenes* and *P. fluorescens* in mono- or binary-culture biofilms. The SS coupons carrying previously formed biofilms (100% RH, 15 °C, 48 h, started on day -2) were desiccated (43% RH, 15 °C, 21 d) and survivors were enumerated on general or selective agars (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* mono-culture biofilm, (▲) represents *P. fluorescens* mono-culture biofilm, (○) is *L. monocytogenes* in binary-culture biofilm with *P. fluorescens* and (Δ) represents *P. fluorescens* in binary-culture biofilm with *L. monocytogenes*.

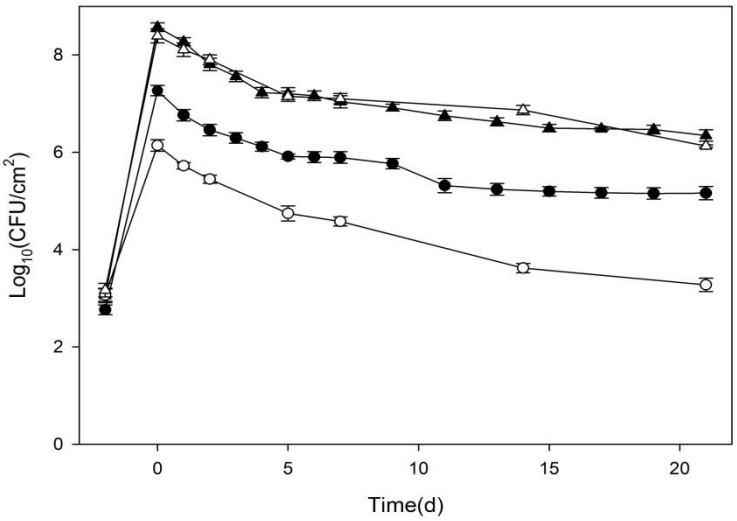

Figure 36. Desiccation survival of *L. monocytogenes* and *S. liquefaciens* in mono- or binary-culture biofilms. The SS coupons carrying previously formed biofilms (100% RH, 15 °C, 48 h, started on day -2) were desiccated (43% RH, 15 °C, 21 d) and survivors were enumerated on general or selective agars (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* mono-culture biofilm, (▲) represents *S. liquefaciens* mono-culture biofilm, (○) is *L. monocytogenes* in binary-culture biofilm with *S. liquefaciens* and (Δ) represents *S. liquefaciens* in binary-culture biofilm with *L. monocytogenes*.

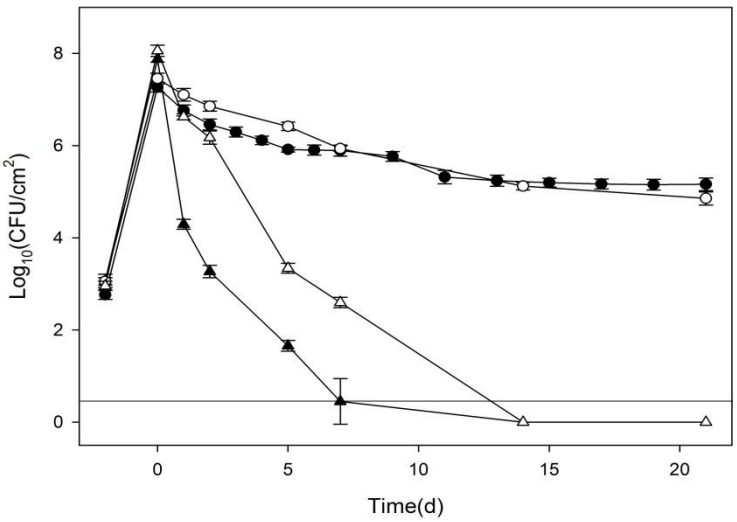

Figure 37. Desiccation survival of *L. monocytogenes* and *Sh. putrefaciens* in mono- or binary-culture biofilms. The SS coupons carrying previously formed biofilms (100% RH, 15 °C, 48 h, started on day -2) were desiccated (43% RH, 15 °C, 21 d) and survivors were enumerated on general or selective agars (n=6, ±SD, representing two independent experiments). (●) denotes *L. monocytogenes* mono-culture biofilm, (▲) represents *Sh. putrefaciens* mono-culture biofilm, (○) is *L. monocytogenes* in binary-culture biofilm with *Sh. putrefaciens* and (Δ) represents *Sh. putrefaciens* in binary-culture biofilm with *L. monocytogenes*. 0.6 $Log_{10}(CFU/cm^2)$ is the detection limit.

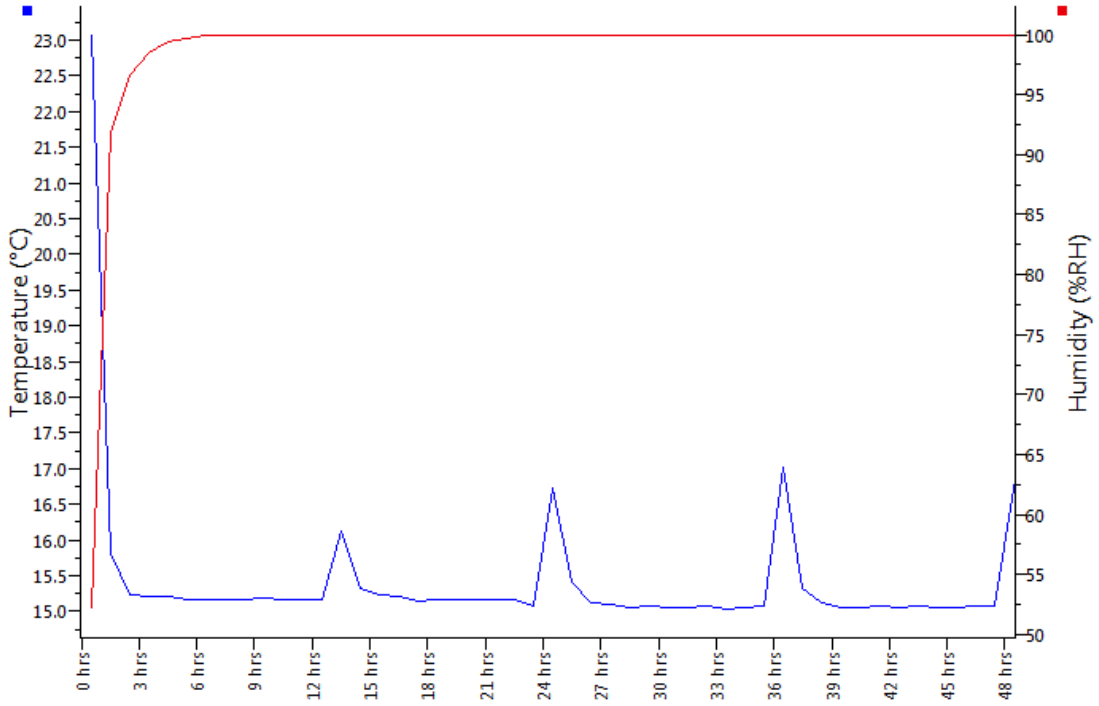

Figure 38. Temperature and humidity profile during biofilms formation on SS coupons. The temperature, humidity and time were monitored using a Tinytag data logger. The red line represents humidity (%RH) and blue line denotes temperature (°C) changes during 48 h of experiment. The spikes in blue line show temporary increase in temperature due to sampling.

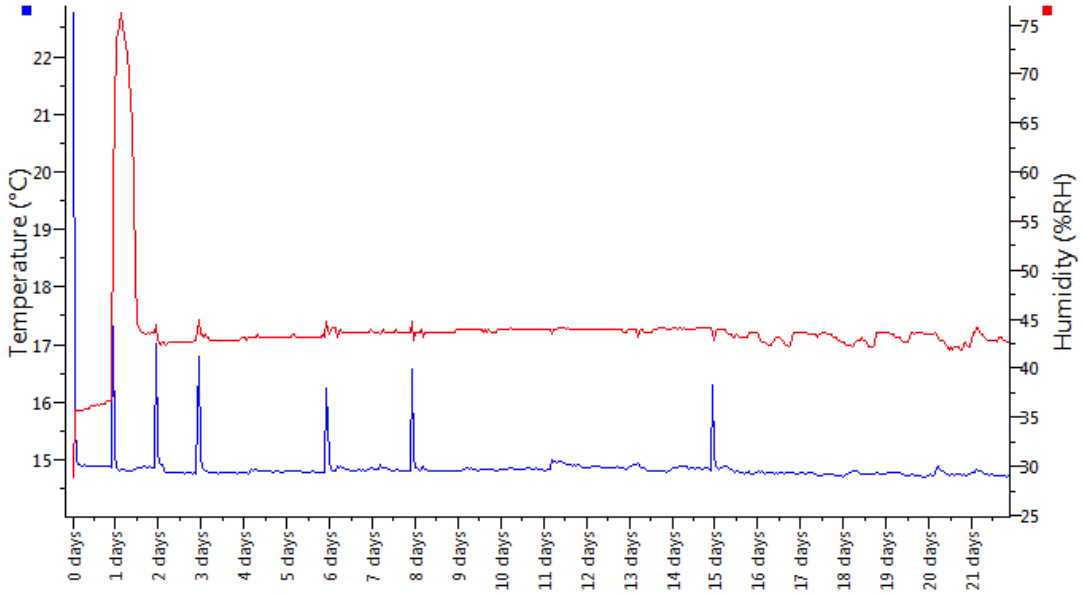

Figure 39. Temperature and humidity profile during biofilms desiccation on SS coupons. The temperature, humidity and time were monitored using a Tinytag data logger. The red line represents humidity (%RH) and blue line denotes temperature (°C) changes during 21 d of experiment. The spikes in blue line show temporary increase in temperature due to sampling.

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