Removal of *Cronobacter sakazakii* and *Listeria monocytogenes* biofilms using enzymes

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Food Technology

at Massy University, Manawatū

New Zealand

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2019

Abstract

C. sakazakii and *L. monocytogenes* are pathogens that may occur in dairy manufacturing plants with the potential cause serious diseases in neonates if dairy products containing these bacteria are consumed. Control of these pathogens is through cleaning with chemicals such as caustics and acids. Enzymes have been considered as alternative cleaning agent as they are more environmentally friendly compared with traditional chemical-based cleaning. However, the use of enzymes to remove biofilms of these pathogens has not been well studied. The objectives of this study were (1) to explore the biofilm formation of *C. sakazakii* and *L. monocytogenes* on hydrophilic and hydrophobic surfaces; (2) test the efficacy of α-amylase, proteases and endoglucanase on removal of these biofilms. The first part of this study focused on screening for the robust strains that can form strong biofilm in reconstituted infant milk formula using a microtiter plate assay and stainless steel coupons. The second part evaluated the efficacy of enzymes on the removal of biofilms by impedance detection and epifluorescence microscopy.

The results showed that 12 of 14 strains of *C. sakazakii* strains and half of the *L. monocytogenes* strains formed strong biofilm. The ability to form biofilm varied with the origin of the isolates with clinical isolates of *C. sakazakii* and food isolates of *L. monocytogenes* forming strong biofilm on microtiter plates and stainless steel surfaces.

For the evaluation of enzyme efficacy, the biofilms were treated with α-amylase, proteases and endoglucanase at 85°C, pH 3 for 15 min. The results from the microtiter

plate assay based on the absorbance at 550 nm showed that the enzymes especially proteases and endoglucanase were effective in removing biofilms. The effectiveness of cleaning by enzymes demonstrated by plate counting and impedance detection was supported by epifluorescence microscopy results. In conclusion, these results demonstrated the efficacy of enzymes on removal of biofilms of *C. sakazakii* and *L. monocytogenes*. Compared with traditional method of cleaning, enzymes are more effective in removing extracellular polymeric substance of biofilms.

Acknowledgments

I would like to thank my main supervisor, Professor Steve Flint, School of Food and Advanced Technology, Massey University, who provided me expert guidance, encouragement and much useful discussion and kept reminding me to “critical thinking”, which always keep me passionate about my project and microbiology. I always appreciate learning from him.

I am also thankful to my enzyme sponsor Dr Steve Yannone, Founder of CinderBio, who provided me much information and documents about the enzymes.

Special thanks to Dr Siti Norbaizura Md Zain who shared the knowledge about *C. sakazakii* and laboratory experience with me.

I would like to thank Ann-Marie Jackson, Kylie Evans and Haoran Wang for their assistance and help in the microbiology laboratory.

Finally, I would like to thank my families for their support and my beloved partner Yufan Wang who is always willing to discuss my project with me and inspire me to perform at my best.

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This work has been presented in part in the following presentations at scientific conferences:

He, Y., Flint, S. H. (2018). Removal of *Cronobacter sakazakii* and *Listeria monocytogenes* biofilms using enzymes. *New Zealand Microbiological Society conference*, Dunedin, November 2018.

Abbreviations

a_w	water activity
BHI	brain heart infusion
CDC	Centers for Disease Control and Prevention
CFU/mL	colony forming units per milliliter
CIP	Clean in Place
EFSA	European Food Safety Authority
ESR	Environmental and Scientific Research
EPS	extracellular polymeric substance
g	gram(s)
h	hour(s)
ICMSF	International Commission on Microbiological Specification for Foods
IMF	infant milk formula
LPS	lipopolysaccharide
LST	lysostaphin
LTS	long-term survival
min	minutes(s)
MRSA	methicillin- resistant *S. aureus*
NB	nutrient broth
NEC	necrotizing enterocolitis
O.D	optical density
PBS	phosphate-buffered saline

PIMF	powdered infant milk formula
PTFE	polytetrafluoroethylene
PVC	polyvinyl chloride
PW	peptone water
RTE	ready to eat
SEM	scanning electron micrograph
TSA	tryptic soy agar
TSB	tryptic soy broth
UHT	Ultra-High Temperature

Chapter 1. Introduction

Biofilm formation has been widely studied in many areas. Biofilms may be useful in waste water treatment and acetic acid production (Flint, 1998). However, in the food industry, biofilm is the main factor in surface-to-food cross contamination, resulting in both economic and public health consequences. From a medical perspective, biofilms may be present on different medical implants including catheters and contact lenses resulting illness and costs associated with treatment (Simões, Simões & Vieira, 2010).

In a dairy manufacturing plant, the bacteria released from biofilms have been recognized as a major source of microbial contamination of dairy products (Flint, Bremer & Brooks, 1997). There are many reasons why biofilms are likely to develop in dairy manufacturing environments. The nutrients of dairy products are the natural media for the biofilm formation. The biofilm formation in pasteurisers is widely reported with the potential of contamination of all dairy products (Srey, Jahid & Ha, 2013; Flint, Bremer & Brooks, 1997). *C. sakazakii* and *L. monocytogenes* are the both emerging pathogens that can form biofilm resulting in the occasional contamination of dairy products. *C. sakazakii* may be present in powdered infant milk formula (PIMF) as it can survive in a desiccated environment for many years (Chenu & Cox, 2009). *L. monocytogenes* is widely distributed in the general environment and is occasionally found in a dairy manufacturing environment (Møretrø & Langsrud, 2004). The biofilms of these pathogens particularly *C. sakazakii* biofilm are poorly understood, the traditional chemical cleaning methods fail

to provide satisfactory results and there are few reports exploring the cleaning effectiveness of alternative methods for *C. sakazakii* and *L. monocytogenes* biofilms.

One alternative cleaning method used for the removal of biofilms is enzymatic cleaning. α-amylase has been recognized as most widely used in the food industry due to its nature of thermo-stability (Molobela, Cloete & Beukes, 2010). Proteases were the most efficient in removing biofilms formed by microorganisms isolated from dairy products (*Streptococcus thermophilus*, *Lactobacillus lactis* and *Lactobacillus bulgaricus*) (Craigen, Dashiff & Kadouri, 2011; Augustin, Ali-Vehmas & Atroshi, 2004). However, endoglucanase, as an enzyme hydrolysing polysaccharide of the extracellular matrix of biofilm, has been poorly studied and not applied in the food industry (Robleto et al., 2012).

The objectives of this study were to (1) investigate the adhesion and the biofilm formation of *C. sakazakii* and *L. monocytogenes* on a hydrophobic surface (microtiter plates) and a hydrophilic surface (stainless steel coupons) and (2) analyse the efficacy of α-amylase, proteases and endoglucanase provided by CinderBio in removing biofilms developed by *C. sakazakii* and *L. monocytogenes* on microtiter plates and stainless steel coupons compared with traditional chemical-based cleaning methods. This study aims to develop an awareness of biofilm formation in the dairy environment and to provide recommendations to food-processing plants, concerning the removal of *C. sakazakii* and *L. monocytogenes* biofilms which may adhere on equipment and food packaging.

Chapter 2. Literature review

2.0 Introduction

Microbiological contamination results in product contamination and economic loss in the food industry (Brooks & Flint, 2008, Srey, Jahid & Ha, 2013). Most of this contamination is not related to food safety. However, in New Zealand, around $88.8 million was costed as a result of foodborne disease in the year of 2000 (Brooks & Flint, 2008). The Centers for Disease Control and Prevention (CDC) reported approximately 1000 foodborne disease outbreaks in 2011 (CDC, 2011) and there were 48 million illnesses, resulting in 128,000 hospitalizations and 3000 death annually in United States from 1996 to 2010 (Scallan et al., 2011). These serious cases of foodborne infections are often associated with biofilms, a major source of microbiological contamination (Srey, Jahid & Ha, 2013).

Since the first report of biofilms (Zobell, 1943), concern has grown in many areas such as food, medical and environmental fields (Poulsen, 1999, Mattila-Sandholm & Wirtanen, 1992, Flint, Bremer & Brooks, 1997, Shi & Zhu, 2009, Satpathy, Sen, Pattanaik & Raut, 2016). Microorganisms commonly attach to wet surfaces (food surfaces, equipment and processing environment), multiply and develop a slimy matrix composed of extracellular polymeric substance (EPS) to form biofilm (Srey, Jahid & Ha, 2013, Johansen, Falholt & Gram, 1997). Biofilms resist physical barriers and environmental stresses such as antibiotics, UV light, starvation, osmotic stress, heat and detergents (Lehner et al., 2005).

Cronobacter sakazakii belongs to the family of *Enterobacteriaceae*. It is recognized as an emerging pathogen and that has caused concern through the contamination of powdered infant milk formula. *C. sakazakii* has been associated with sporadic cases of

illness such as meningitis, necrotizing enterocolitis (NEC) and septicaemia in infants (Chenu & Cox, 2009).

Listeria monocytogenes is known as an important foodborne pathogen in human disease (Johnson, Doyle & Cassens, 1990) and some strains of this species can form biofilms. *L. monocytogenes* is widely distributed in the environment causing a serious disease: listeriosis. Newborn infants infected with listeriosis are likely to have meningitis and septicaemia and in pregnant women, it can cause abortion (Bemrach, Sanaa, Cassin, Griffiths & Cerf, 1998).

To overcome the hygiene problems related to microbial biofilms, enzyme cleaning methods for biofilm removal have been proposed. Enzymes have the ability to break down EPS associated with biofilms for an effective clean of surfaces (Lequette, Boels, Clarisse & Faille, 2010). However, due to the heterogeneity of the extracellular polysaccharides in the biofilm, a combination of enzymes may be required for the effective degradation of bacterial biofilm (Johansen, Falholt & Gram, 1997).

This review describes the features of two foodborne pathogens (*C. sakazakii* and *L. monocytogenes*) and the biofilms formed by *C. sakazakii* and *L. monocytogenes* causing the potential problem of microbial contamination in food manufacturing plants especially dairy. The current methods as well as alternative cleaning strategies used to control biofilms are discussed. Enzymatic treatment has been considered an important alternative cleaning method for use in the food industry. α-amylase, proteases and endoglucanase

that are used to treat *C. sakazakii* and *L. monocytogenes* biofilms in this study have been discussed in detail.

2.1 *Cronobacter sakazakii* (*C. sakazakii*)

Urmenyi and Franklin (1961) reported the first two known cases of terminal neonatal meningitis caused by an atypical 'yellow pigmented *Enterobacter cloacae*'. In 1980, the organism responsible was given species status, as *Enterobacter sakazakii*, based on the difference between *E. sakazakii* and *E. cloacae* in DNA-DNA hybridization, biochemical reactions, production of yellow-pigmented colonies and antibiotic susceptibility patterns (Nazarowec-White & Farber, 1997). Iversen et al (2008) reclassified *Enterobacter sakazakii* as the new genus *Cronobacter*.

2.1.1 Characteristics of *C. sakazakii*

C. sakazakii causes life-threating neonatal meningitis and sepsis. It belongs to the family of *Enterobacteriaceae* (Du, Lu & Wang, 2012). This microorganism is a Gram-negative, facultative anaerobic, rod-shaped and non-spore forming. It is motile with a peritrichous flagella. The colonies of *C. sakazakii* can be recognized as typical yellow pigmented colonies on Tryptic Soy Agar (TSA).

The temperature range for the growth of *C. sakazakii* is 6 – 45°C with an optimum range of 37 – 43°C. Some strains are able to grow at 47°C and slowly at refrigeration temperatures (4°C) (Cheu & Cox, 2009). This microorganism is resistant to desiccation and heat treatment. It can survive for at least 2 years in desiccated infant formula and

other powdered food products at low a_w and grow rapidly in reconstitution (Holý & Forsythe, 2014).

The microorganism produces viscous capsular material which can protect them against phagocytosis from the immune system or rapid drying leading to death and facilitate the microorganism forming biofilm (Nazarowec-White & Farber, 1997; Holý & Forsythe, 2014). Iversen and Forsythe (2004) found that after 2 years storage in desiccation, four of five strains of *C. sakazakii* were capsulated when recovered from reconstituted infant milk formula (IMF) and all strains recovered after 2.5 years formed capsular material. Therefore, the capsule of some *C. sakazkaii* strains plays an important role in storage in desiccated conditions. Iversen, Lane and Forsythe (2004) indicated that the strains of *C. sakazakii* with capsular material might form denser biofilms compared with non-capsule strains.

2.1.2 Morphological colony types

The bacterium can produce two morphological colony types when freshly isolated for purity (Nazarowec-White & Farber, 1997). One morphological colony type is described as being dry or mucoid, scallop edge shaped and hard to remove with a wire loop. The reason for these characteristics may be the production of a heteropolysaccharide (Holý & Forsythe, 2014). Another colony type is smooth and easily removed with a wire loop. Cells that produce mucoid colonies may revert to typical smooth colonies on subculturing from stock cultures (Gurlter, Kornacki & Beuchat, 2005). This distinct phenotypical

characterisation has been more thoroughly described by Iversen and Forsythe (2003) as being “matt” or “glossy”.

2.1.3 Sources of contamination – foods, clinical and environment

C. sakazakii has been isolated from a wide range of foods, clinical and environmental sources (Table 1) (Iversen & Forsythe, 2003; Chenu & Cox, 2009; Friedemann, 2007; Holý & Forsythe, 2013). The foods contaminated by this microorganism include cheese, kefir, tofu, sour tea, fermented bread, cured meats, minced beef and sausage meat. Muytjens and Kollée (1998) found that 20 of 141 breast milk substitute powders were contaminated by *C. sakazakii*. Unlike commercial available liquid feeds, dried milk powder is not sterile, therefore, cases of *C. sakazakii* contamination in dried milk products have occurred (Iversen & Forsythe, 2003).

This microorganism has been responsible for clinical illness mostly in neonates and infants and was isolated from a wide range of clinical sources, including urine, blood, inflamed appendix, bone marrow, cerebrospinal fluid, respiratory tracts and hospital environment (Iversen & Forsythe, 2003). It has also been isolated from a doctor’s stethoscope and from nursery food preparation equipment (Farmer et al., 1980).

Although neonate infections of *C. sakazakii* are perceived as being associated with infant milk formula and milk powder, it has been isolated from a diverse range of environments and more reservoirs are being investigated such as water, soil and plant material (Drudy et al., 2006). In addition, the contamination sources may be rats and flies (Jaradat et al.,

2014). Kandhai et al. (2004a) examined the environment of nine factories producing milk powder and isolated *C. sakazakii* from eight of these. Despite the occurrence in the dairy industry environment, the microorganism has been isolated from household vacuum cleaner bags, broom bristles, a room heater and rinsed beer-mugs in open-air-restaurants (Schindler & Metz, 1990). However, it could not be isolated from mud, rotting wood, bird dung and grain (Muytjens & Kollée, 1990), which may be due to the less competition from other microorganisms in these heavily contaminated samples.

Table 1. Sources of *C. sakazakii*

Sources	Details	References
Foods	Infant milk formula	Iversen and Forsythe (2003); Weir (2002); Jaradat et al. (2014); Osaili and Forsythe (2009)
	Cheese	Iversen and Forsythe (2003); Heperkan, Dalkilic-Kaya and Juneja. (2017)
	Sour tea	Tamura et al. (1995)
	Tofu	Osaili and Forsythe (2009)
	Fermented bread	Gassem (1999)
	Sausage meat	Leclercq, Wanrgue and Baylac (2002)

	Cured meat	Watanabe and Esaki (1994)
Clinical	Urine	Drudy et al. (2006)
	Blood	Drudy et al. (2006)
	Inflamed appendix	Iversen and Forsythe (2003); Drudy et al. (2006)
	Respiratory tracts	Iversen and Forsythe (2003); Drudy et al. (2006)
	Cerebrospinal fluid	Iversen and Forsythe (2003)
	Hospital air	Masaki et al. (2001); Drudy et al. (2006)
Environmental	Water, soil	Lampel and Chen (2009)
	Household vacuum cleaner bags	Jaradat et al. (2014)
	Broom bristles	Schindler and Metz (1990)
	Room heater	Schindler and Metz (1990)
	Rinsed beer-mugs	Schindler and Metz (1990)

2.1.4 Survival and antibiotic resistance

C. sakazakii has an unusual ability to survive at low water activities and a low storage temperature (4°C) (Friedemann, 2007). Beuchat et al. (2009) determined the effects of a_w and storage temperature on the survival characteristics of the pathogen in four commercially manufactured milk-based and two soybean-based powdered infant formulas. The study indicated that *C. sakazakii* can survive in powdered infant formula for long periods of time. Survival was influenced by a_w and temperature but not formula composition. The pathogen did not grow in powdered infant milk formula stored at 4°C however after reconstitution it was detected by enrichment in 72 h (Beuchat et al., 2009). *C. sakazakii* also has been found to survive in dry infant cereals and grow in reconstituted infant cereals.

Stock and Wiedemann (2002) reported that *C. sakazakii* is naturally resistant to all macrolides, clindamycin, fusidic acid, rifampicin, streptogramins and fosfomycin. It is susceptible to some antibiotics, including gentamicin, ampicillin and cefotaxamine (Dennison & Morris, 2002). The combination of ampicillin and gentamicin is recommended for the treatment of *C. sakazakii* meningitis (Willis & Robinson, 1988). The antibiotic resistance of different *C. sakazakii* strains varies. Nazarowec-White and Farber (1999) found 62.5% food and 88.9% clinical strains and one reference strain only showed resistance to cephalothin and sulphisoxazole. The other strains isolated from environment were susceptible to all agents. Kuzina, Peloquin, Vacek and Miller (2001)

found that *C. sakazakii* isolated from the guts of Mexican fruits flies had resistance to ampicillin, cephalothin and penicillin.

2.1.5 Disease caused by *C. sakazakii*

C. sakazakii is considered to be an opportunistic pathogen that may cause life-threating NEC, meningitis, bacteraemia and septicaemia in infants (Drudy et al., 2006; Cheu & Cox, 2009; Holý & Forsythe, 2014; Nazarowec-White & Farber, 1996; Jaradat, Mousa, Elbetieha, Nabulsi & Tall, 2014). Premature and low-birth-weight infants and infants < 28 days of age are at high risk compared with older infants however there have been a few reports of *C. sakazakii* infection in adults and it is not life-threating (Holý & Forsythe, 2014; Drudy et al., 2006). The clinical manifestation of *Cronobacter* infection includes meningitis leading to brain abscess, cyst formation, ventriculitis and hydrocephalus as well as intestinal necrosis and pneumatosis intestinalis (Nazarowec-white & Farber, 1997).

In infants, meningitis is the most frequently reported issue in neonatal *C. sakazakii* infections. In the U.S, one per 100,000 infants was reported to have meningitical *Cronobacter* infection and the incidence rate increased 9.4 per 100,000 infants of low birth weight (< 1.5 kg) with mortality rates as high as 80% being reported. The survivors often suffer from severe irreversible neurological disorders (Cheu & Cox, 2009). Biering et al. (1989) reported that infants infected with *Cronobacter* meningitis had consumed *C. sakazakii* contaminated reconstituted IMF before falling ill. In a recent study, there were few microorganisms observed on the majority of the PIMF packages. However, extrinsic

contamination of opened PIMF cans or utensils of formula preparation has been reported (Teramoto et al., 2010; Jaradat et al., 2009). In 2002, *C. sakazakii* was classified as a severe hazard for restricted populations by International Commission on Microbiological Specification for Foods (ICMSF).

2.1.6 Biofilm formation by *C. sakazakii*

A biofilm is a population of microbial cells attached and growing on surfaces with associated EPS (Oh, Chen & Kang, 2007; Lehner et al., 2005). Biofilm can form on abiotic and biotic surfaces and food-processing environment, potentially acting as the chronic source of food spoilage or transmission of diseases (Aparna & Yadav, 2008). Biofilms play an important role in pathogen persistence and physiology. Biofilms provide a defence barrier and protect the embedded cells against various environmental stress including nutrient shortages, acids, heat, osmotic stress, antibiotics and bacteriophages.

The formation of biofilms is a dynamic and complex process. Initially, flagella of cells enable bacteria to attach on surfaces. This process is reversible and many bacteria can be removed from surfaces (Srey, Jahid & Ha, 2013). The change from reversible to irreversible attachment is due to the production of extracellular polymers. The biofilm is difficult to remove at this phase unless using high shear force and chemicals. The microorganisms aggregate and multiply to become a microcolony with the production of EPS. The surrounding planktonic cells may be involved as a result of communications between cells (Chmielewski & Frank, 2003). The biofilm grows as a flat or mushroom-

shaped structure. The biofilm cells may release when the surrounding environment is nutrient limited (Srey, Jahid & Ha, 2013; McLean, Whiteley, Stickler & Fuqua, 1997).

C. sakazakii is able to adhere on silicon, stainless steel, polycarbonate and polyvinyl chloride (PVC) and form biofilms (Kim, Ryu & Beuchat, 2006). The biofilm of *C. sakazakii* has been extensively studied at the genetic level (Lehner et al., 2005; Hartmann et al., 2010; Grimm et al., 2008). Several studies show that *C. sakazakii* biofilm has the ability to survive in a food-processing environment such as milk powder factories (Oh, Chen & Kang, 2007). The EPS of *C. sakazakii* biofilm has the resistance to the desiccation however the strains isolated from IMF processing facilities are diverse with both EPS inducible and non-inducible strains. In a study by Grimm et al (2008), cellulose was identified as a component of the extracellular matrix of the biofilm. The cells produce heterpolysaccharide which consists of acetate, glucuronic acid, glucose, fructose and galactose (Shimada, Nakata & Nakamura, 1997). The capsule or slime layer is formed due to the production of exopolysaccharide. In addition, the incorporation of water into biofilm structure through hydrogen bonding causes the hydration of EPS (Grimm et al., 2008).

2.2 *Listeria monocytogenes* (*L. monocytogenes*)

Listeria monocytogenes was originally described by Murray, Webb and Swann. (1926) who named it *Bacterium monocytogenes* because the characteristic monocytosis was found in infected laboratory rabbits and guinea pigs. It was given present name by Pirie in 1940 (Gray & Killinger, 1966).

2.2.1 Characteristics of *L. monocytogenes*

L. monocytogenes is a Gram-positive, facultatively anaerobic, non-spore forming rod (WHO/FAO, 2004). It is 1-2 µm in length and 0.5 µm wide with peritrichous flagella which gives a characteristic tumbling motility (Figure 1) (Low and Donachie, 1997). When the bacteria are grown at 20 – 25°C, flagella are produced and assembled on the cell surface however, the production of flagella is reduced at 37°C (Farber & Peterkin, 1991). The bacterial colonies are smooth, slightly flattened and milky white by reflected light. When illuminated by obliquely transmitted light, the colonies demonstrated blue-green sheen (Low and Donachie, 1997; Farber & Peterkin, 1991).

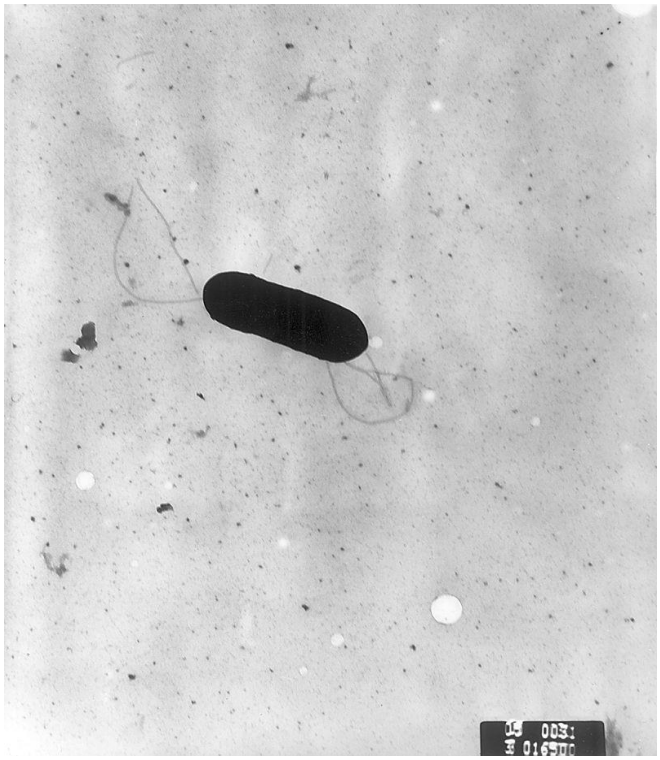


Figure 1. Electron micrograph of a flagella *L. monocytogenes* bacterium (White, 2002)

This microorganism is psychrotrophic and can grow at 3 – 45°C and at a wide pH range (pH 4 – 9). The optimum temperature of growth is 30 – 37°C (Møretrø & Langsrud, 2004; Nowak, Cruz, Palmer, Fletcher & Flint, 2015). Due to the psychrotrophic nature of the microorganism, *L. monocytogenes* widely occurs in refrigerated, ready-to-eat (RTE) food products that have been contaminated during processing and packaging (Freitag, Port & Miner, 2009; Gombas, Chen, Clavero & Scott, 2003).

2.2.2 Sources – meat products, seafood and environment

L. monocytogenes can be isolated from a variety of different foods and is widespread in nature. Amtsberg et al. (1970) isolated *L. monocytogenes* from muscle or spleen of 2 out of 207 slaughter cattle. In addition, the microorganism has been found in brain, spleen, kidney, liver and lymph nodes of pigs and lambs which died from natural listeria infection (Slivko, 1958). 3% of the healthy animals and 44% of the sick cattle were isolated *L. monocytogenes*, most commonly from the spleen and less commonly from the lungs (Johnson, Doyle & Cassens, 1990). In a Swiss study, Breer and Schopfer (1988) succeeded in isolating *L. monocytogenes* from 18% of poultry, which was the highest incidence of *L. monocytogenes* in meat samples. Several investigators have reported *L. monocytogenes* isolated in deli meat which may be contaminated during food processing (Johnson, Doyle & Cassens, 1990; Gombas, Chen, Clavero & Scott, 2003).

L. monocytogenes and other *Listeria* spp. have been isolated from freshwater samples and coastal seawater subject to pollution or contamination from industrial, human or animal sources (Embarek, 1994). Motes (1991) found three of the four water samples positive

for *L. monocytogenes* and two of the four effluent samples from sewage treatment plants contained *Listeria* (one *L. innocua* and three *L. monocytogenes*). The 52% of samples of seawater surrounding a salmon processing plant contained *Listeria*. (Jørgensen & Huss, 1998). In fresh and frozen fish and fish-based seafood, the prevalence of *L. monocytogenes* was 4% – 12% which was lower than the prevalence in raw meat (4 – 60%) and fresh poultry (23 – 60%) (Farber & Peterkin, 1991). In a survey of frozen seafood, an incidence of 27% was observed (Weagant et al., 1988). It is interesting that the prevalence of *L. monocytogenes* in tropical fresh fish and seafood is low (0 – 2%) (Jørgensen & Huss, 1998).

L. monocytogenes occurs widely in agricultural, aquacultural and food processing environments. A high prevalence has been observed in soils with high moisture and soils recently irrigated and cultivated (Strawn et al., 2013). Many studies have shown that *L. monocytogenes* is widely distributed in food-processing environments (Ferreira, Wiedmann, Teixeira & Stasiewicz, 2014; Tompkin, 2002; Buchanan, Gorris, Hayman, Jackson & Whiting, 2017). *L. monocytogenes* can grow on raw materials or equipment and can persist due to ineffective cleaning and sanitation, poor condition of food equipment or insufficient controls of movement of people (Buchanan et al., 2016). It also has been found in retail (Wang, Ray, Hammons & Oliver, 2015) and home environments (Evans & Redmond, 2015).

2.2.3 Survival and persistence

Many strains of *L. monocytogenes* can survive in a number of environmental conditions such as high salt or acidity in food as well as low humidity or low oxygen in food environments (Buchanan et al., 2016). The microorganism is able to survive at low temperatures between – 0.5°C and 9.3°C. The growth and survival are influenced by inhibiting parameters of the particular food or food environment, including competitive microflora. Due to the ecological and physiological traits of *L. monocytogenes*, it can colonize food plant environments, survive hurdles in processing and storage and multiply in food products (Zeng et al., 2014). Temperature abuse during transport and retail sale may allow the microorganism grow.

L. monocytogenes has been found to persist for decades in food processing plants with specific strains being isolated repeatedly (Ferreira et al., 2014). Some strains may survive in cracks of surfaces, seals and gaskets that are hard to clean and disinfect. The possible mechanism for the persistence in food environments is the formation of persister cells (Wen et al., 2011; Buchanan et al., 2016). Persister cells may allow the microorganism to survive under environmental stress and represent a long-term survival (LTS) state, with the changes of the cellular morphology from bacilli to cocci during the transition to the LTS phase (Knudsen, Ng & Gram, 2013). However, the mechanism triggering this morphological change to increase tolerance is unknown.

2.2.4 Disease associated with *L. monocytogenes* – Listeriosis

This bacterium is a foodborne pathogen causing a serious disease: listeriosis. It is estimated that 99% of human listeriosis results from the consumption of RTE foods contaminated by *L. monocytogenes* such as deli meat, cheese, smoked fish and seafood (Allen et al., 2014). Listeriosis rarely infects the healthy people however those with a weakened immune system including the elderly, the pregnant women and infants may be infected. The clinical symptoms of adult listeriosis are endocarditis, primary bacteremia and central nervous system infections and usually presents symptoms such as headache, vomiting, fever and malaise (Farber & Peterkin, 1991). Neonates infected with listeriosis are likely to have meningitis and septicaemia and in pregnant women, *L. monocytogenes* can cause abortion (Bemrach et al., 1998).

Although listeriosis accounts for only 0.02% of all foodborne illness, the mortality rate is as high as 20% (Tompkin, 2002). In a recent summary of listeriosis cases in 2013 from 27 countries of the European Union, the rate of listeriosis infection has increased 8.6% compared to 2012 with 99.1% of the cases hospitalized (EFSA, 2015). In the U.S, listeriosis outbreaks attributed to the consumption of contaminated RTE foods have decreased between 1998 and 2008. However, since 2010, the U.S has experienced a number of listeriosis outbreaks due to some foods considered to be “moderate risk” including fruits and vegetables as well as ice cream (Cartwright et al., 2013). In England and Wales, from 1983 to 2001, as shown in Figure 2, the number of human listeriosis cases was more than 100 with approximately 2 incidences per million population in

pregnant and non-pregnant women and during the year of 1987 – 1989, the number of human listeriosis cases was highest (around 280 cases) (McLauchlin, Mitchell, Smerdon & Jewell, 2004).

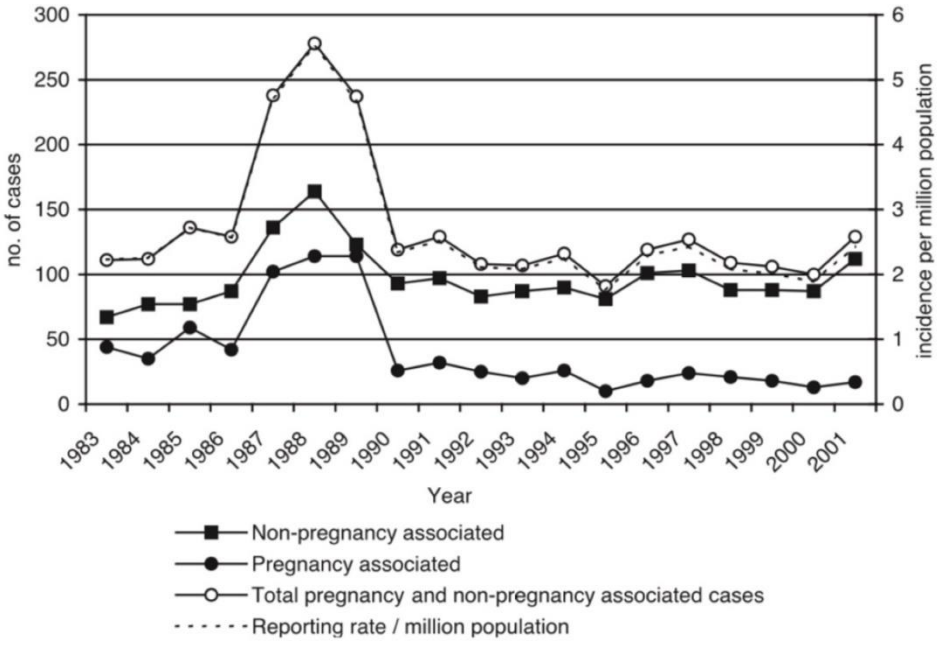


Figure 2. Annual totals and incidence of reported human listeriosis in England and Wales by year of specimen, 1993 – 2001 (McLauchlin, Mitchell, Smerdon & Jewell, 2004)

2.2.5 *L. monocytogenes* biofilm

In the food industry, *L. monocytogenes* is able to attach on many different types of surfaces including rubber, stainless steel, polymers and glass and form biofilms. Cunliffe et al. (1999) investigated the adhesion of *L. monocytogenes* biofilms on different to surfaces and found that adhesion was weakest on the hydrophilic surfaces. However, in

the other studies of biofilm formation by *L. monocytogenes* on hydrophilic (stainless steel) and hydrophobic surfaces (polytetrafluoroethylene, PTFE) was examined (Chavant et al., 2002). They concluded that the surface which biofilm formed faster was a hydrophilic substratum (stainless steel) and the bacterium attached poorly to hydrophobic substrata such as PTFE. In addition, there are many factors influencing the ability of biofilm to form on surfaces. Ronner and Wang (1993) found that nutrient limitation decreased *L. monocytogenes* adhesion and biofilm formation. Kim and Frank (1995) tested the effects of different sugars on the biofilm formation of *L. monocytogenes* to stainless steel after 12 days incubation. Trehalose and mannose had an ability to simulate the biofilm formation while, there was no effect of cellobiose and fructose to biofilm formation.

L. monocytogenes was observed to be able to form multi-layer biofilms (Møretrø & Langsrud, 2004; Marsh, Luo & Wang, 2003; Chavant et al., 2002). The cells in the biofilm stack upon each other around and between holes and the extracellular material anchors the cells in their positions (Marsh, Luo & Wang, 2003). There is a mass of microcolonies in the slime layer with vertical and horizontal channels which allows liquid flow and dispersion of nutrients (Møretrø & Langsrud, 2004).

The biofilm of *L. monocytogenes* in the food industry usually contains multi-species communities because several types of bacteria attached on the food-processing surfaces multiply and form multi-species biofilm. *Pseudomonas* spp. or *Staphylococcus* spp. with *L. monocytogenes* are the most frequently isolated from surfaces in the food industry (Mettler & Carpentier, 1998; Wirtanen et al., 2000; Sundheim et al., 1992). The

population of *L. monocytogenes* in multi-species biofilm was reported to account for 1 – 10% of the total biofilm population (Norwood & Gilmour, 2000). *Pseudomonas* spp. and *Flavobacterium* spp. were found to have the effect of simulating adhesion and biofilm formation of *L. monocytogenes* on a glass surface (Bremer et al., 2001; Sasahara & Zottola, 1993).

With regard to *L. monocytogenes* in the food industry, the resistance of biofilm-cells to disinfectants is important. The bacteria cells in suspension tested with most disinfectants are reduced significantly (Best et al., 1990; Norwood & Gilmour, 2000) however, the biofilm cells attached on the surfaces are less susceptible to disinfectants compared with their free-living counterparts (Møretrø & Langsrud, 2004). In addition, *L. monocytogenes* in thick biofilm was more resistant than *L. monocytogenes* in thin biofilm to sodium hypochlorite and dodecylbenzene sulphonic acid (Lee & Frank, 1991; Frank & Koffi, 1990). *L. monocytogenes* multi-species biofilms are more resistant than the biofilm only composed of *L. monocytogenes* (Elias & Banin, 2012).

2.3 Biofilm control strategies in the food industry

Several control measures have been proposed for *L. monocytogenes* biofilms. With the variety of organic and inorganic materials used in a food manufacturing environment, a variety of different chemical agents are used including alkaline solutions and surfactants used to emulsify fats and denature proteins on contact surfaces. Midelet and Carpentier (2004) suggested that regular cleaning and disinfecting were used to prevent the biofilm formation in order to not allow bacteria (reversible attachment) to attach on contact

surfaces. In addition, there were three strategies suggested by Meyer (2003). The first was to disinfect “in time” before biofilm formation. The second was using harsh cleaners and disinfectants (sodium hydroxide, nitric acid, peracetic acid/hydrogen peroxide sanitizer) to clean biofilms. The last was selecting surface materials which inhibit the attachment of microbes. Some researchers have suggested the incorporation of antimicrobial products in surfaces material (Park, Daeschel & Zhao, 2004) by modifying the properties of materials or by coating with antimicrobials. Silver coating surface material is effective in inhibiting biofilm (Choi et al. 2013). Zeraik and Nitschke (2010) reported that the bacterial attachment showed a significant decrease on hydrophobic surfaces however, the surfaces that are frequently cleaned with the surfactant became more hydrophilic. There have been many strategies considered to inhibit biofilm formation and contribute to the reduction of bacterial attachment.

2.3.1 Traditional cleaning method – Clean in Place (CIP)

Clean-in-Place (CIP) is a process that allows a complete system to be cleaned without dismantling or the manual involvement of the operator. It includes spraying and jetting surfaces or the circulation of cleaning solutions (conventional alkaline, acid chemicals) through the equipment under conditions of high turbulence and flow velocity (Romney, 1990; Srey, Jahid & Ha, 2013). CIP is effective in cleaning contact surfaces and milk fouling formed on the inside surface of heat exchangers. Therefore, CIP has been widely used in the food industry especially the dairy industry. However, there are various factors that can influence the effectiveness of CIP including the nature and thickness of the

fouling layer, composition and concentration of chemicals, cleaning time, cleaning temperature, degree of turbulence and the properties of surfaces (Bremer, Fillery & McQuillan, 2006; Srey, Jahid & Ha, 2013; Boulange-Petermann et al., 2004).

Although Parkar, Flint and Brooks (2003) concluded that conventional CIP is effective in cleaning biofilms of thermophilic bacilli, Bremer, Fillery and McQuillan (2006) studied laboratory scale CIP on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms (*Streptococcus thermophilus* and *Bacillus* species) and found that the use of caustic and nitric acid can remove the bacterial biofilms on surfaces in a dairy manufacturing plant however there was no cell number decrease after the treatment of stainless steel tubes with sanitizer. Exner, Tuschewitzki and Scharnagel (1987) found that the biofilm cells can be destroyed by chemical agents however the EPS matrix of biofilm was unaffected. Eide, Homleid and Mattsson (2003) investigated the life cycle of four CIP methods (alkaline/acid cleaning with hot water, enzyme cleaning with acid chemical disinfection, one-phase alkaline cleaning with acid chemical disinfection and cold nitric acid at pH 2) on the biofilm removal. It was found that enzyme-based cleaning was the best recommended alternative CIP method. Enzyme-based control has become a noteworthy alternative in the food industry (Lequette, Boels, Clarisse & Faille, 2010; Johansen, Falholt & Gram, 1997; Thallinger, Prasetyo, Nyanhongo & Guebitz, 2013) while the conventional chemical-based CIP is not providing satisfactory hygienic results (Lequette, Boels, Clarisse & Faille, 2010) and the use of chemicals may cause environmental issues (Srey, Jahid & Ha, 2013).

2.3.2 Enzymatic control

Due to the ineffectiveness of conventional cleaning methods and the environmental impact of traditional cleaners, enzymatic treatment has been considered to be an important alternative method for biofilm removal in the food industry (Johansen, Falholt & Gram, 1997). Enzymes are proteins that can catalyse on a specific chemical molecule. However, EPS is a heterogenic matrix, so a combination of enzymes is commonly required in order to degrade the complex (Simões, Simões & Vieira, 2010). Simões, Simões and Vieira (2010) showed that the effectiveness of enzymes on biofilm removal may be different due to the various species of bacteria. The enzyme-based detergents have been used synergistically to enhance the disinfectant efficacy. Augustin, Ali-Vehmas and Atroshi (2004) investigated the enzymatic cleaning products on the biofilms formed by microorganisms isolated from dairy products (*Lactobacillus bulgaricus, Lactobacillus lactis,* and *S. thermophilus*). The result showed proteolytic enzymes reduced the biofilm in milk and *S. thermophilus* was the most sensitive to removal by enzyme treatment. Craigen, Dashiff and Kadouri (2011) found that a mixture of proteases and amylases was successful to remove biofilm. Proteases can hydrolyze the proteins in biofilm and amylases can break the carbohydrates involved in the complex (Molobela, Cloete & Beukes, 2010). Compared to other 1,4-glycosidic bond cleaving amylases, α-amylase is most widely used due to its thermo-stability. However, the use of enzymes in biofilm control is still limited in the food industry due to the higher cost compared with the low price of chemicals. The enzyme-based detergent and enzymes used in biofilm removal

have been mostly patent-protected. The low commercial accessibility of different enzyme activities limits their current use (Johansen, Falholt & Gram, 1997). The next part will cover α-amylase, protease and endoglucanase that are used to clean *C. sakazakii* and *L. monocytogenes* biofilms in this study.

2.3.2.1 α-amylase

α-amylase inhibits biofilm and hydrolyzes the α-1,4 glycosidic linkage (Thallinger, Prasetyo, Nyanhongo and Guebitz, 2012). Many commercial α-amylases are obtained from biotic sources. α-amylase isolated from *Bacillus subtilis* has the ability to inhibit 90% of the *S. aureus* biofilm by degrading the biofilm EPS matrix (Craigen, Dashiff, & Kadouri, 2011). Kalpana, Aarthy, & Pandian (2012) used the extracellular α-amylase from *Bacillus subtilis* S8-18 of marine origin against the biofilm formation of methicillin-resistant *S. aureus* (MRSA), *Vibrio cholerae* and *Pseudomonas aeruginosa* ATCC10145. α-amylase resulted in 51.81 – 73.07% of biofilm degradation for these pathogens. The enzyme was also effective in the removal of mature biofilm by disrupting EPS. Watters, Burton, Kirui and Millenbaugh (2016) developed an *in vitro S. aureus* biofilm model containing 10% human plasma to test the anti-biofilm activity. It was found that α-amylase was active against five methicillin-resistant *S. aureus* strains by reducing biomass up to 97% after 2 hours treatment. α-amylase may be effective to remove biofilms and a promising strategy to be applied in the food and medical fields.

2.3.2.2 Proteolytic enzymes

Proteases are enzymes that can hydrolyze specific peptide bonds between amino acid residues (Thallinger, Prasetyo, Nyanhongo and Guebitz, 2012). The enzymes vary with the differences in structure, target substrate, reaction mechanism and physicochemical characteristics (Longhi, Scoarughi, Poggiali, & Cellini, 2008).

2.3.2.2.1 Subtilisins

Subtilisins are the mostly used in the food and paper industries. They can attack the peptide bonds of the protein structure. The proteinaceous adhesins play an important role in the biofilm attachment on surfaces. Subtilisins are able to break proteinaceous adhesins, which allows the biofilm structure to be dispersed (Leroy, Delbarre, Ghillebaert, Compere & Combes, 2008). These enzymes are commonly obtained from strains of *Bacillus* sp. Mitrofanova et al. (2017) used subtilisin-like protease acquired from *B. pumilus* 3-19 to remove biofilm formed by *Serratia*, an opportunistic pathogen. The result demonstrated that the subtilisin-like protease reduced the biomass of *S. marcescens* by 70%. Subtilisins were found to be successful in removing the biofilm of *Pseudoalteromonas* sp and *L. monocytogens* (Leroy, Delbarre, Ghillebaert, Compere & Combes, 2008; Thallinger, Prasetyo, Nyanhongo and Guebitz, 2012).

2.3.2.2.2 Lysostaphin

Lysostaphin (LST) is a metalloendopeptidase isolated from *S. simulans*. LST hydrolyzes the pentaglycine interpetide bonds of the peptidoglycan layer which is a primary defense

of cells against antibiotics and other antibacterial agents (Thallinger, Prasetyo, Nyanhongo and Guebitz, 2012). However, this enzyme is mainly used to treat some *S. aureus* strains. Cetto-Vigoder et al. (2016) found that the free form of LST can be active against *S. aureus* isolated from bovine mastitis and a high reduction of biofilm biomass and cell death in *S. aureus* 4181 were shown after 4 hours (h) treatment with 0.4µg/ml of LST.

2.3.2.2.3 Protease K

Protease K is another widely used serine-protease, obtained from *Tritirachium album*. In the food industry, this enzyme is used to remove a wide range of food-associated biofilm (Ramírez, Smid, Abee & Groot, 2015). A high reduction of total cell numbers and biofilm formation was found in food-spoilage *Lactobacillus plantarum* by protease K treatment (Ramírez et al., 2015). Fagerlund, Langsrud, Heir, Mikkelsen and Møretrø. (2016) investigated the cleaning effectiveness of several antibacterial enzymes, including protease K and trypsin on biofilm formed by eight Staphylococci strains. The results showed that the biofilms of *S. lentus*, *S. cohnii* and *S. saprophyticus* were sensitive to protease K. In addition, Nguyen and Burrows (2014) used >25µg/ml protease K to treat the biofilm of *L. monocytogenes* within 5 min, resulting in a complete dispersion of the *L. monocytogenes* biofilm attached on polystyrene and stainless steel. Some researchers also used bromelain and papain to remove biofilm of *L. monocytogenes*. Unlike other proteases, protease K can be used to remove multilayer biofilm (Banar et al., 2016).

2.3.2.3 Endoglucanase

Endoglucanase is a hydrolyzing cellulose enzyme. Some species belonging to the Enterobacteriaceae, including *Cronobacter* spp., *Enterobacter* spp. and *Klebisiella* spp. produce cellulose as a crucial component of the bacterial extracellular matrix (Robleto et al., 2012). Endoglucanase is able to hydrolyze β-(1,4) glycosidic bonds to remove biofilms (Torres, Negro, Fuente & Blanco, 2012). However, reports using endoglucanase to remove biofilms formed by foodborne pathogens are very limited. Endoglucanase has not been applied in the food industry.

2.4 Summary and Conclusions

C. sakazakii and *L. monocytogenes* are emerging foodborne pathogens that can form biofilm in the food industry. Due to the ability to survive in a desiccated environment, *C. sakazakii* widely occurs in PIMF and rapidly grows following reconstitution, causing serious diseases (NEC, meningitis, bacteraemia and septicaemia) in infants. *L. monocytogenes* is widely distributed in the environment, including dairy manufacturing environments and from there can contaminate products such as cheese. The biofilm formed by *C. sakazakii* and *L. monocytogenes* is a source of contamination of dairy products post pasteurisation. The control of biofilms of these bacteria is important.

Five factors are involved in the control of biofilm development in food processing plant: (1) the type of microorganisms (2) the type of product being processed (3) disinfection in time before biofilm formation (4) the operating conditions (including time, temperature and concentration of cleaners) (5) surface modifications to reduce bacterial attachment.

Traditional cleaning methods in the food industry still rely on chemicals including sodium hydroxide, nitric acid, sanitisers and detergents. However, these cleaning chemicals fail to provide satisfactory hygienic results and are not environmental friendly.

New techniques for bacteria control and biofilm removal focus on enzymatic treatment. In recent studies, α-amylase and proteases have been reported to be widely used in the food industry (Thallinger, Prasetyo, Nyanhongo and Guebitz, 2012). The emphasis on enzymatic control of biofilms has been on *Streptococcus* species. Other bacteria (*C. sakazakii* and *L. monocytogenes*) have not been studied in such detail although the pathogens form biofilms of concern in food manufacture, leading to the contamination of food products. In addition, there are no detailed studies about the effectiveness of endoglucanase on the biofilm removal.

Future research is needed to explore the mechanism of enzymes that remove biofilms on genetic level, and to develop new enzymes that will inhibit or slow the biofilm formation on food processing surfaces. Due to the high cost and low commercial accessibility of enzymes, a re-usable system could be developed to enable enzymes more cost effective.

Chapter 3. Materials and methods

3.1 Strains and growth media

A total 26 strains from the dairy industry (Table 2), AsureQuality (Table 3), Environmental and Scientific Research (ESR) (Table 4), Plant and Food Research Institute (Table 5), Hills Laboratories (Table 6) and National Collection of Type Cultures England (Table 7) were used in this study.

Table 2. Strains of *C. sakazakii* from the dairy industry and clinical sources used in this study

Bacteria	Strains	Sources
C. sakazakii	4.10C	Dried infant milk formula, S. Edelson-Mammel, FDA
C. sakazakii	607	Clinical, FDA
C. sakazakii	LCDC-648	Clinical J. M. Farber, Health Canada
C. sakazakii	576736-3D	Environment isolate
C. sakazakii	578349-7E	Environment isolate
C. sakazakii	3465-4A	Environment isolate

Table 3. Strains of *C. sakazakii* from AsureQuality used in this study

Bacteria	Strains	Source
C. sakazakii	A1	Environmental isolate
C. sakazakii	A7	Environmental isolate
C. sakazakii	NZRM50	Not known

C. sakazakii	NZRM2029	Not known

Table 4. Strains of *C. sakazakii* from ESR used in this study

Bacteria	Strains	Source
C. sakazakii	ERL1068	Not known
C. sakazakii	ERL54390	Not known
C. sakazakii	ERL073901	Not known
C. sakazakii	ERL104073	Not known

Table 5. Strains of *L. monocytogenes* from Plant and Food Research Institute used in this study

Bacteria	Strains	Source
L. monocytogenes	08A05	Fresh produce
L. monocytogenes	08A06	Fresh produce
L. monocytogenes	08A07	Fresh produce
L. monocytogenes	08A09	Fresh produce
L. monocytogenes	08A10	Fresh produce

Table 6. Strains of *L. monocytogenes* from Hills Laboratories used in this study

Bacteria	Strains	Source
L. monocytogenes	H1	NZ food industry sample
L. monocytogenes	H2	NZ food industry sample

L. monocytogenes	H3	NZ food industry sample
L. monocytogenes	H4	NZ food industry sample
L. monocytogenes	H7	NZ food industry sample
L. monocytogenes	H8	NZ food industry sample

Table 7. Strains of *L. monocytogenes* from National Collection of Type Cultures England used in this study

Bacteria	Strains	Source
L. monocytogenes	NCTC7973	Mammal guinea pig mesenteric lymph node

The media used in this study were nutrient broth (NB) (Merck Germany), brain heart infusion (BHI) (Merck, Germany), tryptic soy broth (TSB) (Becton Dickinson & Company, France), TSA (Becton Dickinson and Company, France), IMF (Karicare, Danone Nutricia New Zealand Ltd) at concentrations recommended by the manufacturers. Artificial media were autoclaved for 15 min at 121°C, while IMF was reconstituted and autoclaved for 15 min at 111°C in order to prevent browning.

3.2 Maintenance of *C. sakazakii* and *L. monocytogenes* cultures

Cultures were maintained for long-term storage by freezing at – 80°C using Cryobeads (Mast Group Ltd, UK). For temporary storage, active cultures were maintained on TSA

at 4°C. However, a new series of cultures was initiated from the frozen stock on a biweekly basis.

3.3 Culture preparation

Pure cultures of *C. sakazakii* and *L. monocytogenes* were grown on TSA for 24 h and a loop of pure colony from the agar was picked and put it into 9 mL fresh TSB and incubated for 18 – 24 h at 37°C.

3.4 Screening methods for biofilm formation

The following methods were used to screen the robust strains which can form strong biofilms at the start of the study.

3.4.1 Microtiter plate assay

The biofilm formation assay was performed based on Moltz and Martin (2004) to screen the different strains of *C. sakazakii* and *L. monocytogenes* for their ability to attach to surfaces and form biofilm. 230 µL of fresh sterilized reconstituted IMF were put into 96-well microtiter plates (Falcon, USA) and 24-hour cultures of 20 µL *C. sakazakii* and *L. monocytogenes* strains were added to wells of the microtiter plates in triplicate. Three wells contained sterilized reconstituted IMF to serve as negative controls. The plates were incubated at 37°C for 24 h.

Following incubation, 250 µL sterilized water were used to wash and remove loosely attached cells. After washing, the remaining cells were fixed with 250 µL methanol for 15 min and the plate was emptied and allowed to dry for 1 h. 0.5% crystal violet solution

(250 µL) were added to each well for 10 min to stain the bacterial cells. Staining was followed by five distilled water washes and the plate was dried for 30 min. 250 µL of 98% ethanol were added to solubilize the stain from the biofilms and left 30 min. The absorbance at 595 nm was determined using a microtiter plate reader (Spectrostar Nano, New Zealand).

3.4.2 Biofilm formation score

The biofilm formation assay was used to determine the ability of biofilm formation of each strain and the biofilm formation score was used to measure the biofilm relative to the cell growth in the microtiter plate. The adherence capabilities were classified into three categories: strongly, weakly and none adherent based on the O. Ds of biofilm cells.

The threshold value (O. Dc) was defined as the twice O. Ds above the mean O. D of the negative control. The strains were classified into three categories: O. D ≤ O.Dc = none biofilm former, O. Dc < O. D ≤ 2×O.Dc = weak biofilm former and O. D > 2 × O. Dc = strong biofilm former (Zain, 2008). The O. D_c was defined as 1.0 in this study. All tests were done in triplicate and the results were the mean with standard deviation.

Table 8. Quantitative classification of biofilm formation

Strong	Weak	None
O. D > 2.0	1.0 < O. D ≤ 2.0	O.D. ≤ 1.0

3.4.3 Stainless steel coupon trials

The microtiter plate assay is a good screening tool for biofilm formation however it does not reflect the main surface used in the food industry (stainless steel). The next series of trials look at the formation of biofilm on stainless steel.

3.4.3.1 Stainless steel coupons preparation

Stainless steel coupons (type 304; surface area, $4cm^2$) were washed once by 1 mol/L sodium hydroxide for 30 min at ambient temperature, then rinsed by distilled water three times and immersed in acetone for 10 min to remove grease associated with the manufacturing process. The coupons were rinsed by deionized water three times and placed in 50 mL beaker to autoclave at 121°C for 15 min.

3.4.3.2 Inoculum preparation

Each strain of *C. sakazakii* and *L. monocytogenes* was pre-cultured in 9 mL TSB at 37°C for 18-24 h. Following incubation, the cultures were recovered by centrifugation (8, 000 × g for 10 min) and washed with sterile phosphate-buffered saline (PBS), pH 7.2 twice and resuspended in 10 mL TSB.

3.4.3.3 Biofilm development

The biofilm formation on stainless steel coupons was performed by the modified method of Johansen, Falholt and Gram (1997). *C. sakazakii* and *L. monocytogenes* strains were inoculated (approximately 10^4 CFU/ mL) in TSB. The 1.5 mL suspensions of *C. sakazakii*

and *L. monocytogenes* strains were added to 24-well microtiter plates with one sterile stainless steel coupon. The coupons were incubated for 30 min at ambient temperature to prompt bacterial attachment. The coupons were transferred using sterile forceps to 1.5 mL sterile reconstituted IMF and incubated for 24 h at 37°C. Following incubation, the coupons were removed and rinsed in 50 mL sterile distilled water three times to remove unattached cells.

3.4.3.4 Plate counting

For enumeration of *C. sakazakii* and *L. monocytogenes*, the rinsed coupons were placed in sterile bottles (25 mL) containing 5 mL of 0.1% peptone water (PW) and 15 g of washed glass beads (ThermoFisher, United Kingdom). The bottles were vortexed for 1 min to remove the bacteria from coupons. After vortex, serial 10-fold dilutions were made in 0.1% PW and the bacteria enumerated by drop plating 10 µL of the diluents on TSA. The plates were incubated at 37°C for 24 h and the colonies counted and calculated as log CFU/ cm^2. Triplicate samples were prepared for each treatment.

3.4.4 The annular rotating reactor

In the previous trails of biofilm development, biofilms were grown with no fluid shear (static state) however, in dairy plants, biofilm might be grown under fluid shear conditions (dynamic state). Buckingham et al. (2007) reported that biofilm produced with high shear and turbulent flow was more stable than that grow under low shear counterparts. Therefore, the annular rotating reactor (Biosurface technologies Ltd) was used to develop

biofilms. The annular reactor contains a Teflon holder which fits three stainless steel coupons and a magnetic stir bar in the base to generate a fluid shear on the coupon surfaces (Figure 3). This reactor was autoclaved before being placed on a magnetic stirrer.

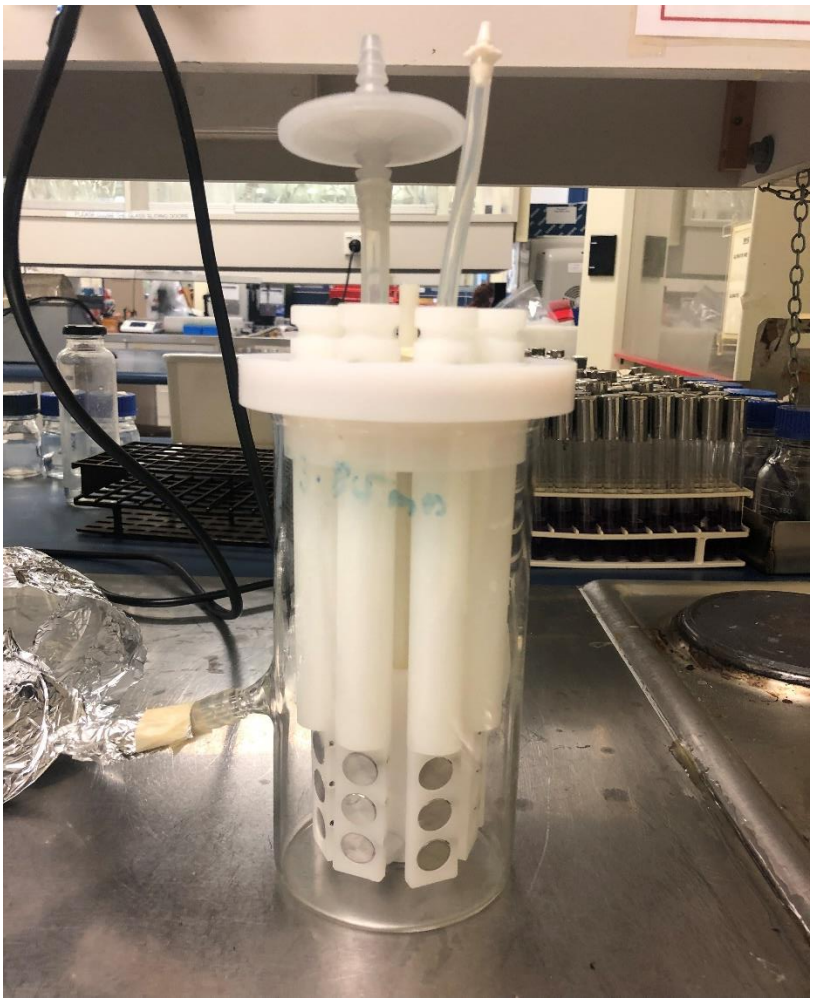


Figure 3. The annular rotating with stainless steel coupons used in this study

4mL inoculum from overnight culture of *C. sakazakii* and *L. monocytogenes* in TSB were inoculated into biofilm reactor containing 400 mL sterile reconstituted IMF at 37°C for 24 h with a rotating speed of 150 rpm to develop biofilm. Following incubation, stainless steel coupons were removed from Teflon holder and washed three times with sterilized

water. For the enumeration of biofilm cells, the procedures were similar to section 3.4.3.4 using plate counting assessment.

3.5 Removal of biofilms using chemical and enzymes

3.5.1 Chemical preparation

10 g of sodium hydroxide pellets were dissolved into 1000 mL deionized water to make 1% sodium hydroxide solution and autoclaved.

3.5.2 Enzymes preparation

The enzymes used in this study were two proteases, α-amylase and endoglucanase (CinderBio, USA). These enzymes are novel enzymes that act at high temperature (85°C) and low pH (pH 3) in a phosphate citric acid buffer. The trisodium phosphate (20mM) and citric acid (40mM) were dissolved in 1000 mL deionized water and autoclaved. 1 mL enzyme solutions were diluted 100 times in sterile 20mM phosphate buffer containing citric acid (at pH 3). The enzymes solutions were placed in water bath at 85°C for future use.

3.5.3 Biofilm removal from microtiter plates

The method of using enzymes to remove biofilms was performed based on Lequette et al. (2010). Sterile reconstituted IMF (230 µL) and bacterial suspension (20 µL) were added to each well of microtiter plates and incubated at 37°C overnight. The liquid was discarded and the plate was washed with 250 µL sterile distilled water for three times.

250 µL of 85°C enzyme solutions and 1% sodium solutions were added (85°C water used as a control) into each well and the microtiter plate was incubated at 85°C for 20 min, after which the solution was gently pipetted out. The microtiter plate was washed three times in sterile distilled water. The biofilms were treated as follows:

a. Detection of biomass

   The remaining cells were fixed by 250 µL methanol for 15 min and allowed to dry. The wells of microtiter plates were stained with 250 µL 0.5% crystal violet solution for 30 min and washed three times with distilled water. 250 µL 98% ethanol was used to solubilize the stain from the remaining cells. Plates were read at 595 nm using a plate reader. Triplicate samples were prepared for each treatment.

b. Detection of viable cell numbers

   The biofilms attached on microtiter plate were swabbed and placed in sterilized bottles (25 mL) containing 5 mL of 0.1% PW and 15 g of washed glass beads. The bottles were vortexed for 1 min. Serial 10-fold dilutions were made in 0.1% PW and the bacteria enumerated by drop plating 10 µL of the diluents on TSA. The plates were incubated at 37°C for 24 h and the colonies calculated as log CFU/ cm^2.

3.5.4 Treatment for biofilm removal from stainless steel coupons

The procedures for biofilm development were similar to 3.4.3.1, 3.4.3.2 and 3.4.3.3. For the biofilm removal assay, the rinsed stainless steel coupons were immersed in 10 mL

enzyme solutions at 85°C for 20 min (pH 3) and three coupons were used as a control with 85°C water added. The coupons were treated as follows:

a. Detection of biomass

   The coupons treated with enzymes were placed in 24-wells plates and 1.5 mL methanol were added into each coupon for 15 min. The liquid was pipetted out and air dried for 2 h. 0.5% crystal violet solutions (250µL) were used to stain viable cells for 20 min and 98% ethanol (250µL) solubilized the cells from surfaces of stainless steel coupons for 30 min.

b. Detection of viable cell numbers.

   See section of 3.4.3.4.

3.6 Microbiological analysis

3.6.1 Epifluorescence microscopy

To determine the effectiveness of enzymes in removing biofilms formed by *C. sakazakii* and *L. monocytogenes*, samples of stainless steel coupons were analysed by epifluorescence microscopy based on the method of Flint (1998) and Zain (2008).

10 mg of fluorochrome acridine orange were dissolved in 0.1 M sterilized phosphate buffer (pH 7.2) and filtered through a 0.2 µm Sartorius filter. The coupons supporting biofilms were washed three times by deionized water and 1% formalin was used to fix cells at ambient temperature for at least 2 min and then the coupons were immersed in acridine orange for 2 min at ambient temperature, following washed three times in sterile

deionized water and air dried. Each coupon was mounted on glass slides and observed using an Olympus microscope B × 53 with a FITC light excitation filter block and photographed using CellsSens Dimension software.

3.6.2 Impedance detection

Impedance microbiology is a rapid method for quantifying bacteria and has been applied to the detection of biofilms (Flint & Brooks, 2001). The BacTrac^{TM} 4300 (SyLab, purkersdorf-Vienna, Austria) instrument (Figure 4) was used to enumerate numbers of *C. sakazakii* and *L. monocytogenes* directly on stainless steel coupons. Compared to the conventional plate counting method, this method is time-saving and easy-to-use (Wang, Palmer & Flint, 2016).

Figure 4. BacTrac 4300^{TM} used in this study

3.6.2.1 Calibration of BacTrac^{TM} 4300

The method is a modification from a published method (Flint & Brooks, 2001; Wang, Palmer & Flint, 2016). The BacTrac^{TM} 4300 is based on the two simultaneous impedance detection systems to enumerate microorganisms – M value and E value. M value is the changes of impedance in the growth medium and E value is the changes in the ionic layers on the electrode surfaces (Flint & Brooks, 2001).

The overnight cultures in TSB at 37°C of *C. sakazakii* and *L. monocytogenes* strains were serially diluted in sterile 0.1% PW (from 10^{-1} to 10^{-9}). A 10 µL sample was taken from each dilution for drop plating. The plating was done in triplicate for each dilution and the

plates were incubated at 37°C for 24 h. A 20 µL of each dilution was inoculated into each vial of the BacTrac^{TM} 4300 containing 10 mL TSB and the vials were incubated at 37°C for 24 h. The vials were prepared in triplicate. The changes of impedance and the time taken for this impedance change to reach a 3% threshold was detected by software incorporated into the BacTrac^{TM}. The calibration equation of the time for impedance change against colony numbers from plates was determined by using the Microsoft Excel Programme. The number of viable cells of *C. sakazakii* and *L. monocytogenes* was calculated via the calibration equation.

3.6.2.2 Detection of biofilm cells of *C. sakazakii* and *L. monocytogenes* after cleaning

The method of biofilm formation is detailed in 3.4.3.1, 3.4.3.2 and 3.4.3.3. The stainless steel coupons were immersed by 10 mL enzyme solution at 85°C for 15 min after rinsing three times with deionized water. The coupons were transferred into vials of the BacTrac^{TM} containing 10 mL TSB. The viable biofilm cells were enumerated using the impedance method described above.

3.7 Statistical analysis

All samples in this study were carried out in triplicate and the quantitative data were reported as means with standard deviations. All data were analysed using Microsoft Excel software and Origin 8.5. SPSS 18.0 software was used for analysis of variance using Tukey's test with a critical probability of $P < 0.05$.

Chapter 4. Preliminary results of biofilm assessment

Before assessment of the efficacy of enzymes on removal of biofilms, it was necessary to screen strains of *C. sakazakii* and *L. monocytogenes* for those that can form strong biofilms. For this, a microtiter plate assay was used for the initial screen followed by a test to confirm biofilm formation on stainless steel.

4.1 Microtiter plate assay

4.1.1 *C. sakazakii* biofilm screening tests

The initial trial using the microtiter plate assay was done on all 14 strains of *C. sakazakii*. The strains were inoculated into artificial media (NB) and the tests performed as described in 3.4.1. However, at 595 nm, the absorbance values showed little growth on microtiter plate surfaces (Appendices Figure 37 – 41).

Using IMF as the growth media biofilm formation occurred in the microtiter plate assay. The results from the biofilm formation of the *C. sakazakii* strains isolated from the dairy industry and clinical sources as shown in Figure 5 and other sources as shown in Figures 6 and 7.

Three strains (4.10C, 607 and LCDC 648, one from dried infant milk formula and two clinical isolates) produced the largest biofilms with the O.D scores of 3.48, 3.48 and 3.28, significantly higher than the O.D scores of the three other strains (three environmental dairy isolates) at 3.12, 2.74 and 1.84 ($P < 0.05$) (Figure 5).

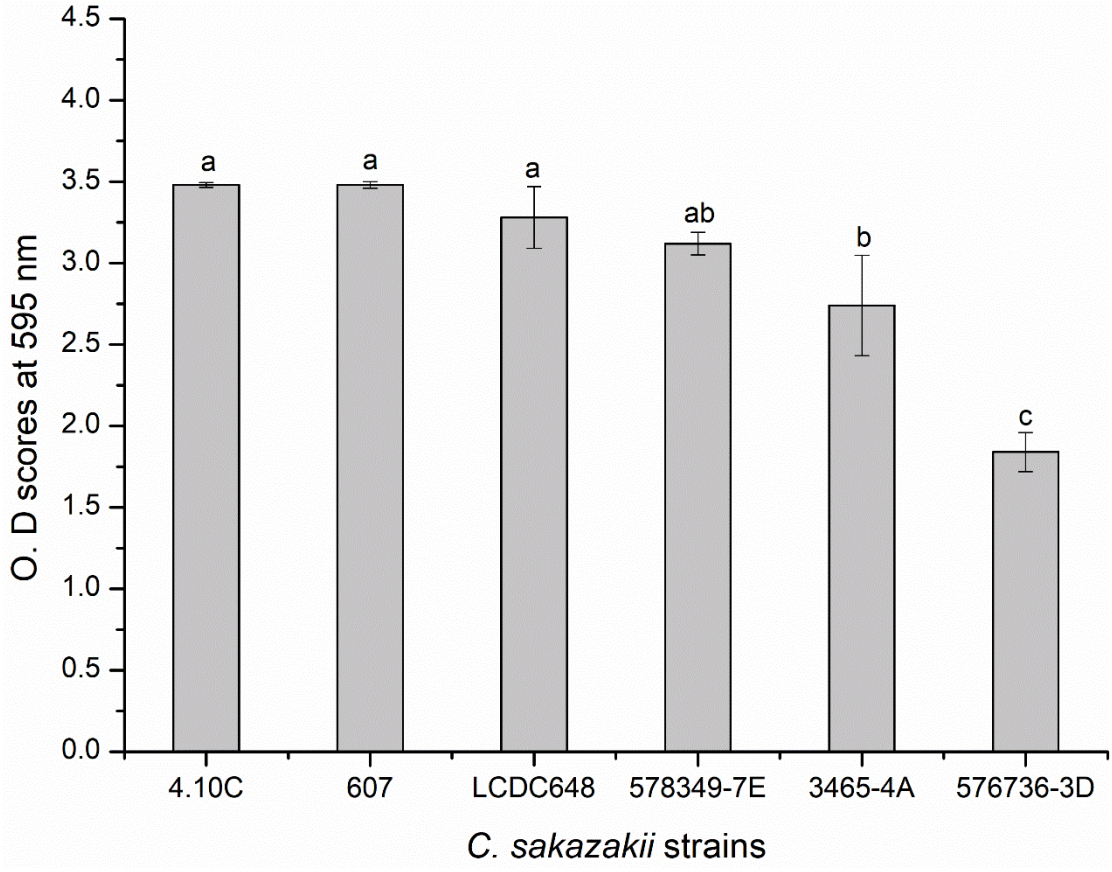


Figure 5. The O.D scores of biofilms of *C. sakazakii* strains isolated from the dairy industry and clinical sources at 595nm and grown in IMF using a microtiter plate assay. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

Three of the isolates from AssureQuality produced strong biofilms with O.D scores of 3.07, 3.07 and 2.97 with one strain producing a weaker biofilm at an O.D of 0.93 (Figure 6).

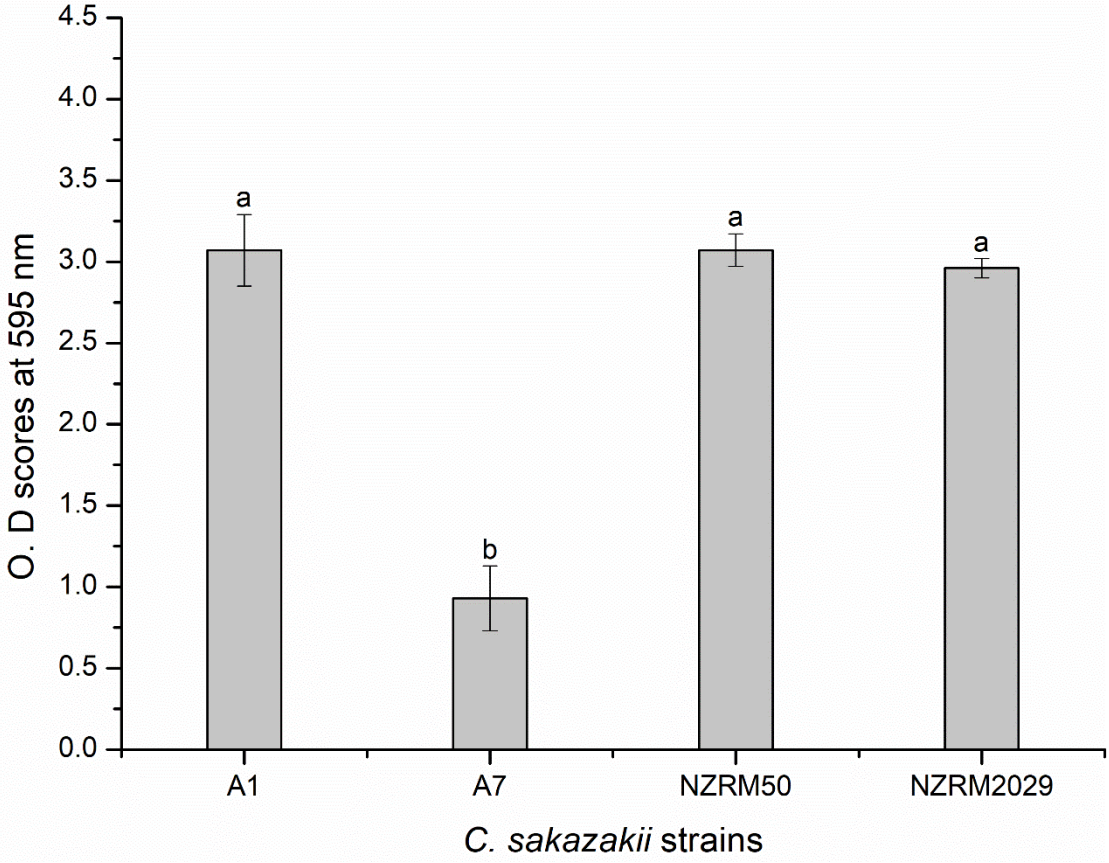


Figure 6. The O.D scores of biofilms of the other *C. sakazakii* strains from AsureQuality at 595 nm and grown in IMF using a microtiter plate assay. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

All isolates from ESR produced strong biofilms with O.D scores close to 3.0 (Figure 7).

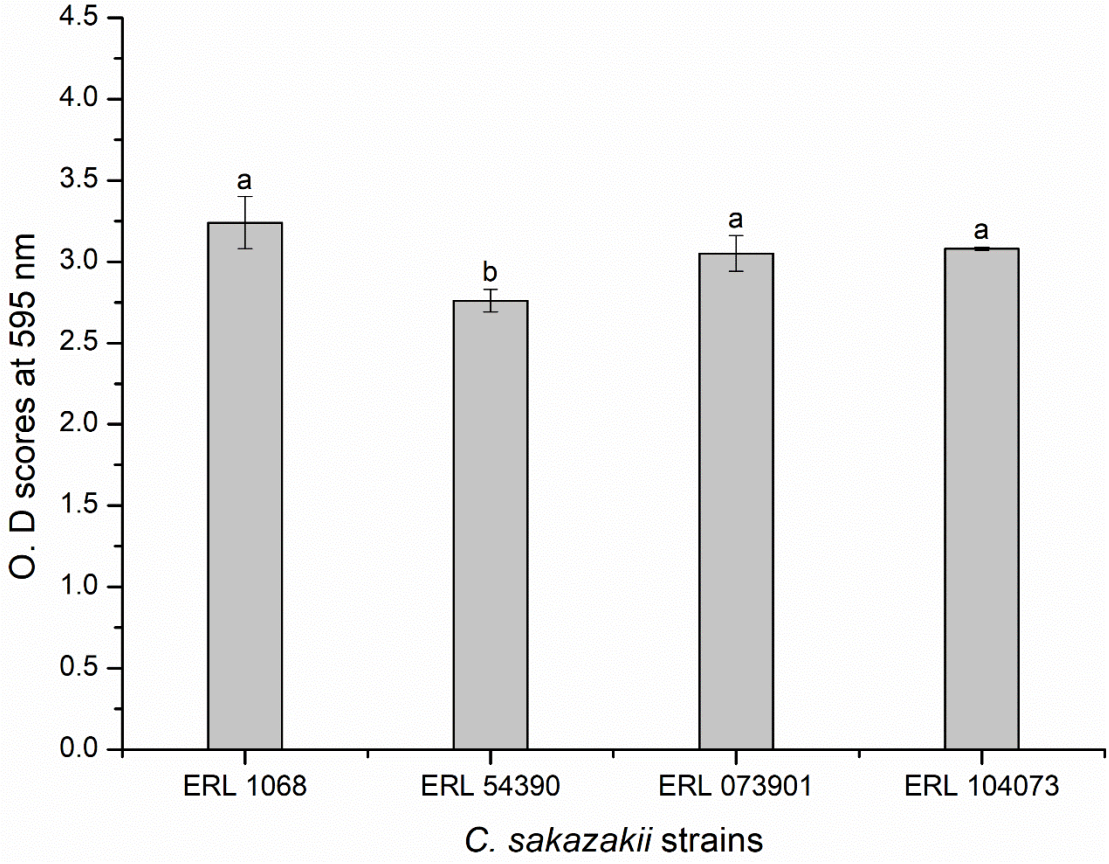


Figure 7. The O.D scores of biofilms of *C. sakazakii* strains from ESR at 595 nm and grown in IMF using a microtiter plate assay. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

4.1.2 *L. monocytogenes* biofilm screening tests

L monocytogenes isolates produced poor biofilm in laboratory media (BHI) (Figures 40 and 41) but strong in IMF. Twelve strains of *L monocytogenes* from a variety of sources were tested for biofilm formation using the microtiter plate assay. Figure 8 shows that the O.D scores for five strains of *L. monocytogenes* (fresh produce isolates) were lower than the O.D scores for *C. sakazakii* strains (both grown in IMF). The O.D scores of 08A05, 08A09 and 08A10 were significantly higher than the O.D scores of the other strains ($P < 0.05$) (Figure 8).

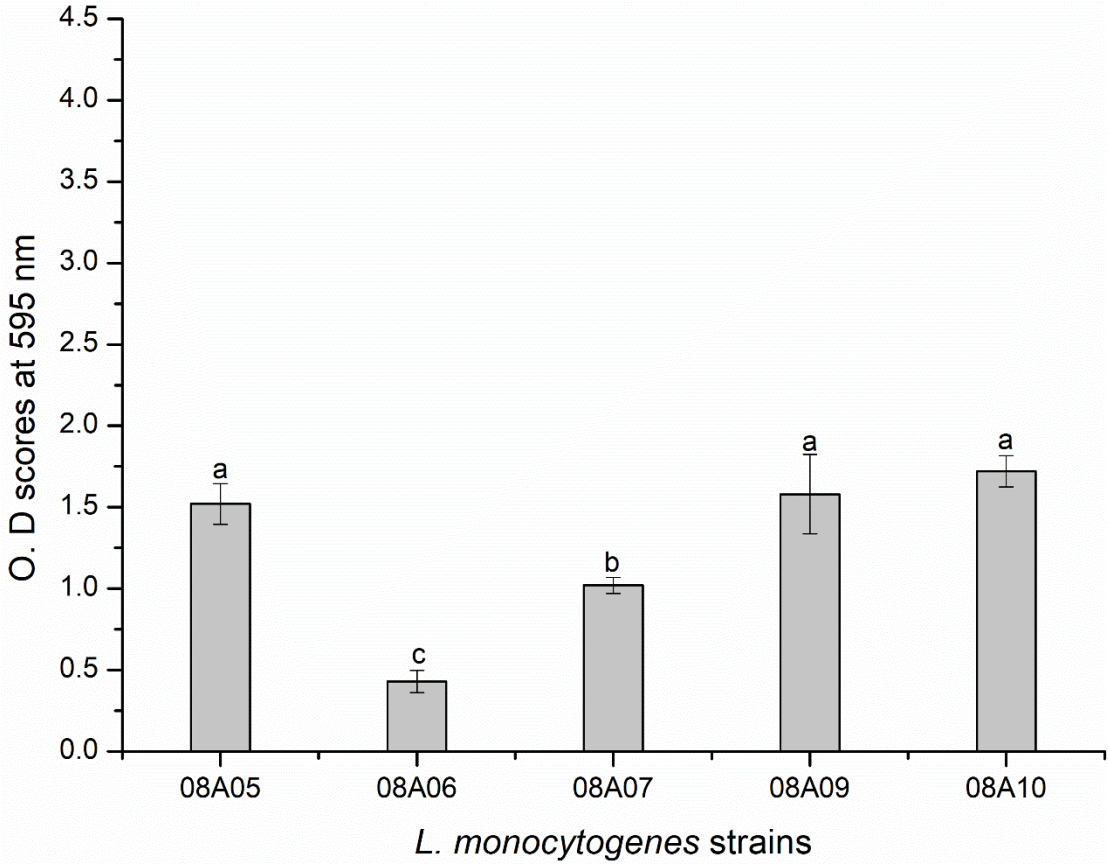


Figure 8. The O.D scores of biofilms of *L. monocytogenes* strains from Plant and Food research Institute at 595 nm and grown in IMF using a microtiter plate assay. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

Figure 9 shows that the O.D scores of seven strains of *L. monocytogenes* (NZ food industry sample isolates and one a guinea pig mesenteric lymph node isolate) grown in IMF were higher than fresh produce isolates. However, there were no significant differences in the O.D scores at 595nm for seven *L. monocytogenes* strains.

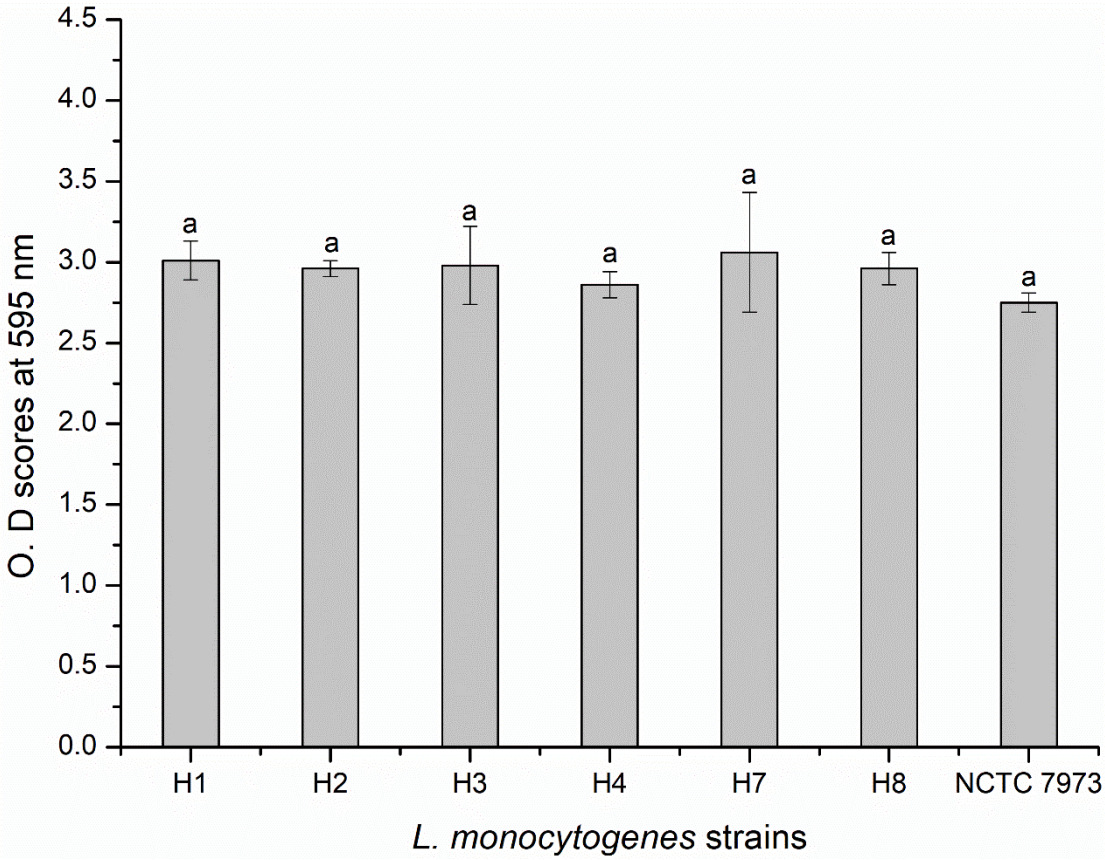


Figure 9. The O. D scores of biofilms of *L. monocytogenes* strains from Hills Laboratories and National Collection of Type Cultures England at 595 nm and grown in IMF using a microtiter plate assay. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

4.1.3 Summary of biofilm screening tests

For *C. sakazakii* strains, 12 strains (4.10C, 607, LCDC648, 578349-7E, 3465-4A, A1, NZRM50, NZRM 2029, ERL 1068, ERL 543901, ERL 104073 and ERL 073901) can form strong biofilms on microtiter plates. One strain can form weak biofilm (576736-3D) and one strain did not produce biofilm (A7) (Table 9). Therefore, 12 strains of *C. sakazakii* that can produce strong biofilm were used in the future research.

Table 9. Summary of *C. sakazakii* biofilm screening using the microtiter plate method

Strong biofilm	Weak biofilm	No biofilm
4.10C, 607, LCDC 648, 578349-7E, 3465-4A, A1, NZRM 50, NZRM 2029, ERL 1068, ERL 543901, ERL 104073, ERL 073901	576736-3D	A7

For *L. monocytogenes* strains, 7 strains (H1, H2, H3, H4, H7, H8 and NCTC 7973) produced strong biofilm, four strains (08A05, 08A07, 08A09, 08A10) produced weak biofilm while one strain (08A06) did not form biofilm (Table 10). Therefore, 7 strains of *L. monocytogenes* that can form strong biofilm were selected for future studies.

Table 10. Summary of *L. monocytogenes* biofilm screening using the microtiter plate method

Strong biofilm	Weak biofilm	No biofilm
H1, H2, H3, H4, H7, H8, NCTC 7973	08A05, 08A07, 08A09, 08A10	08A06

4.2 Detection of biofilm cells number on stainless steel

4.2.1 *C. sakazakii* biofilm formation

Strain 607 produced the strongest biofilm with approximately 8.04 log CFU/cm^2. Strains 4.10C, LCDC 648, 578349-7E and 3465-4A also produced strong biofilm of around 7.0 log CFU/ cm^2. All these strains were from dairy and clinical origin (Figure 10). Strains provided by AssureQuality and ESR also produced strong biofilms on stainless steel with approximately 7.0 log CFU/cm^2 (Figures 11 and 12).

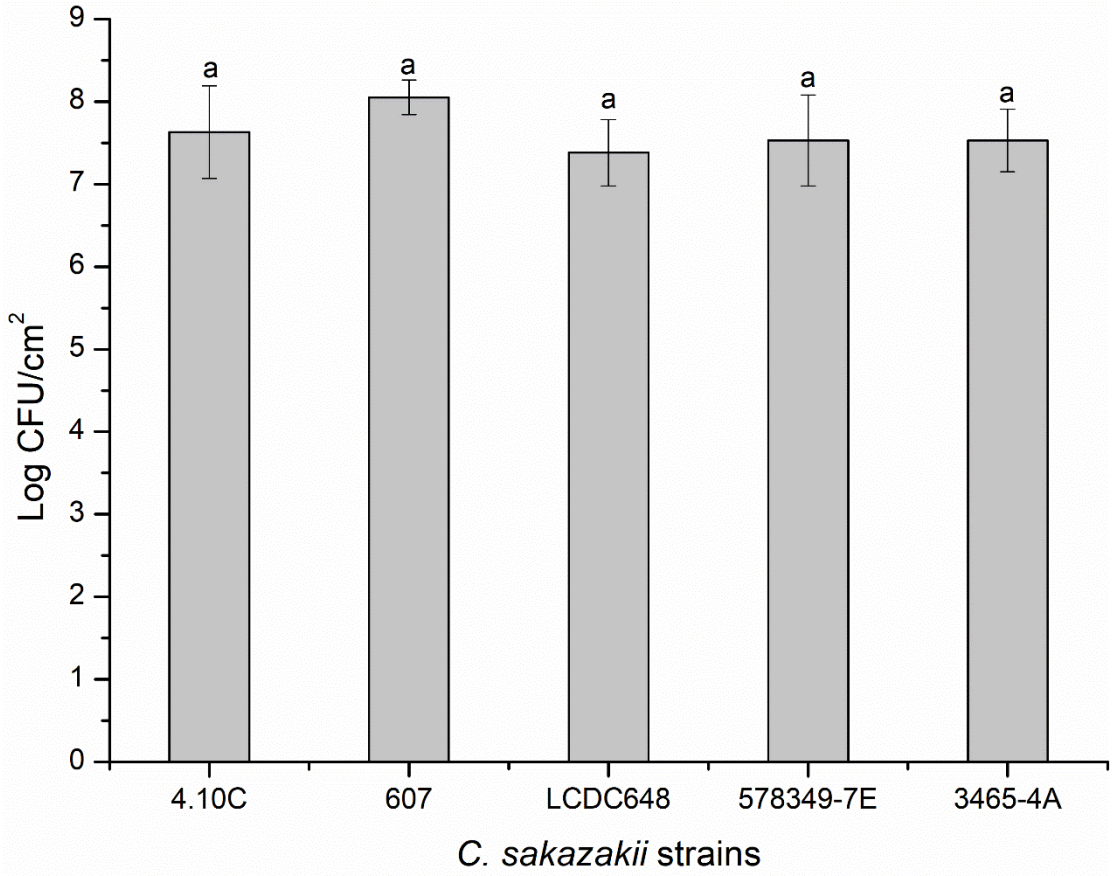


Figure 10. Total biofilm bacteria cells number of *C. sakazakii* strains isolated from dairy industry and clinical sources in IMF determined by drop plating. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

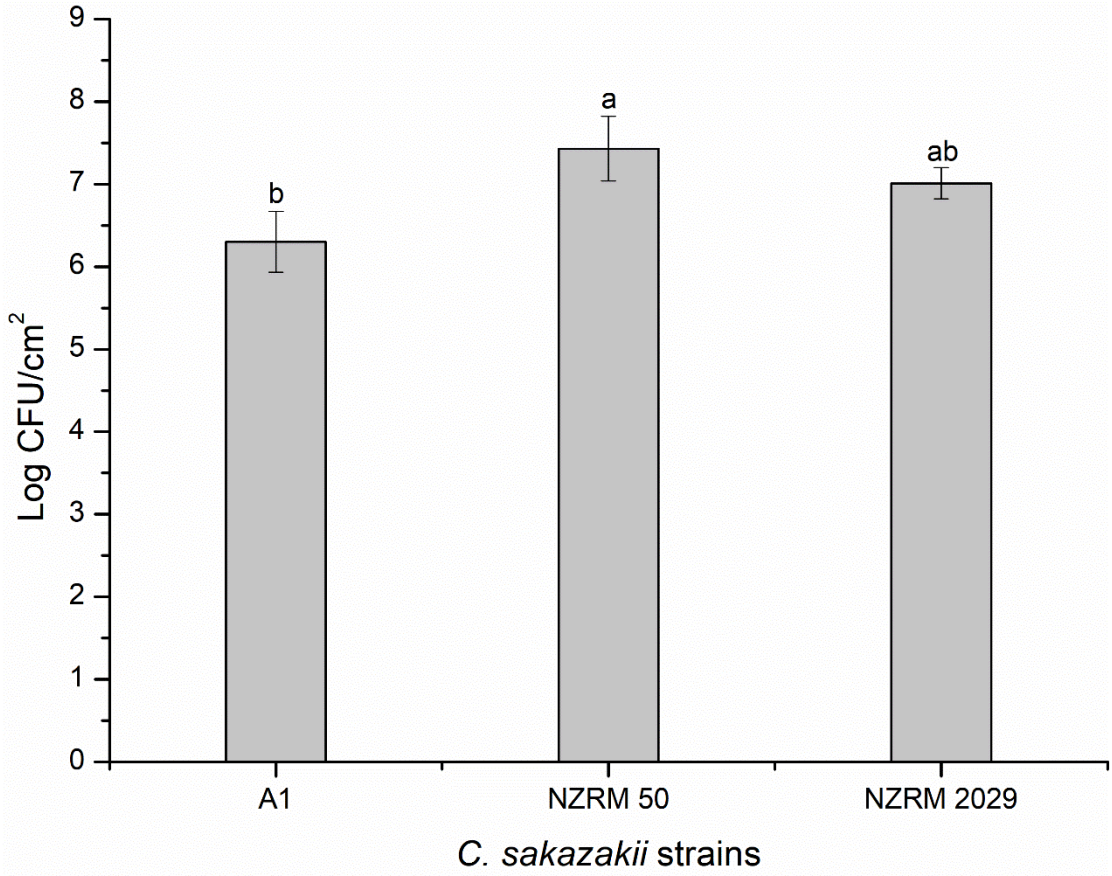


Figure 11. Total biofilm bacteria cells number of *C. sakazakii* strains from AsureQuality in IMF determined by drop plating. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

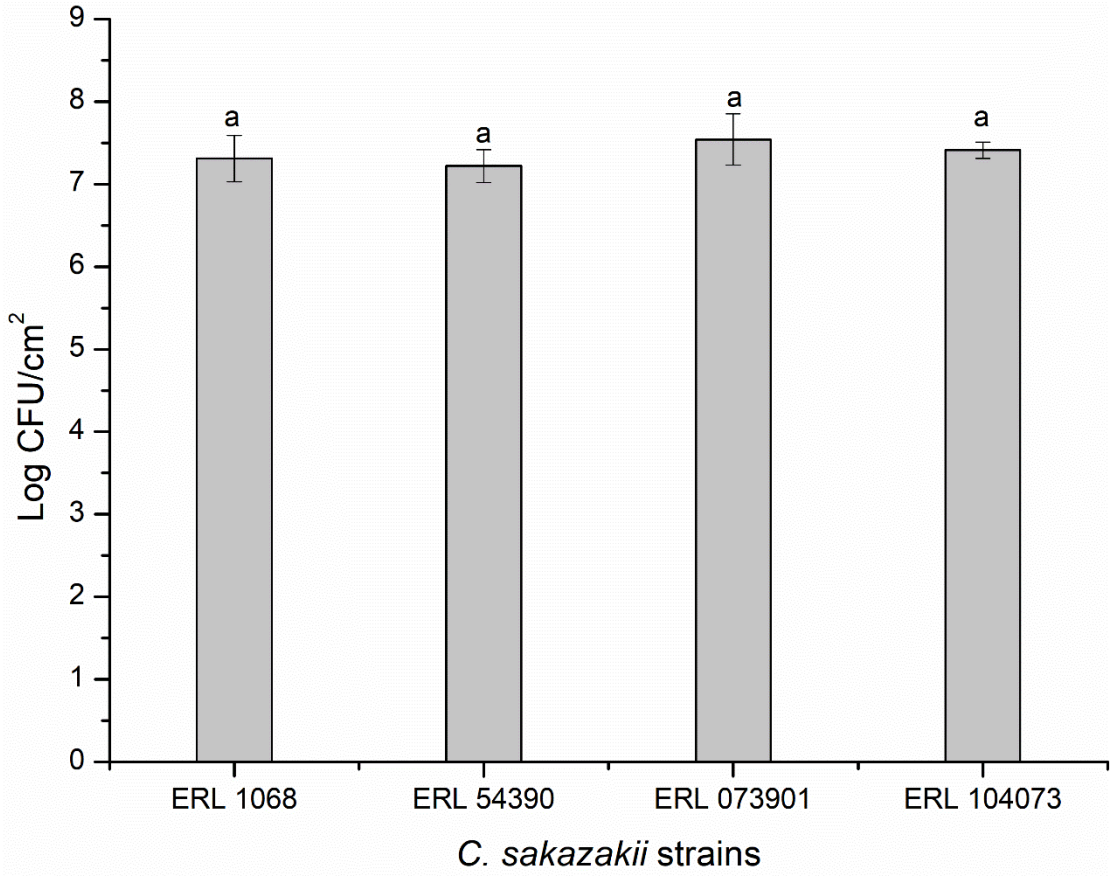


Figure 12. Total biofilm bacteria cells number of *C. sakazakii* strains from ESR in IMF determined by drop plating. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

4.2.2 *L. monocytogenes* biofilm formation

All the *L. monocytogens* strains that showed good biofilm in the screening assay produced biofilms of around 6.0 CFU/cm^2 or greater on stainless steel. Interestingly, the reference isolate, originally from a clinical source produced the weakest biofilm of all isolates tested on stainless steel (Figures 13).

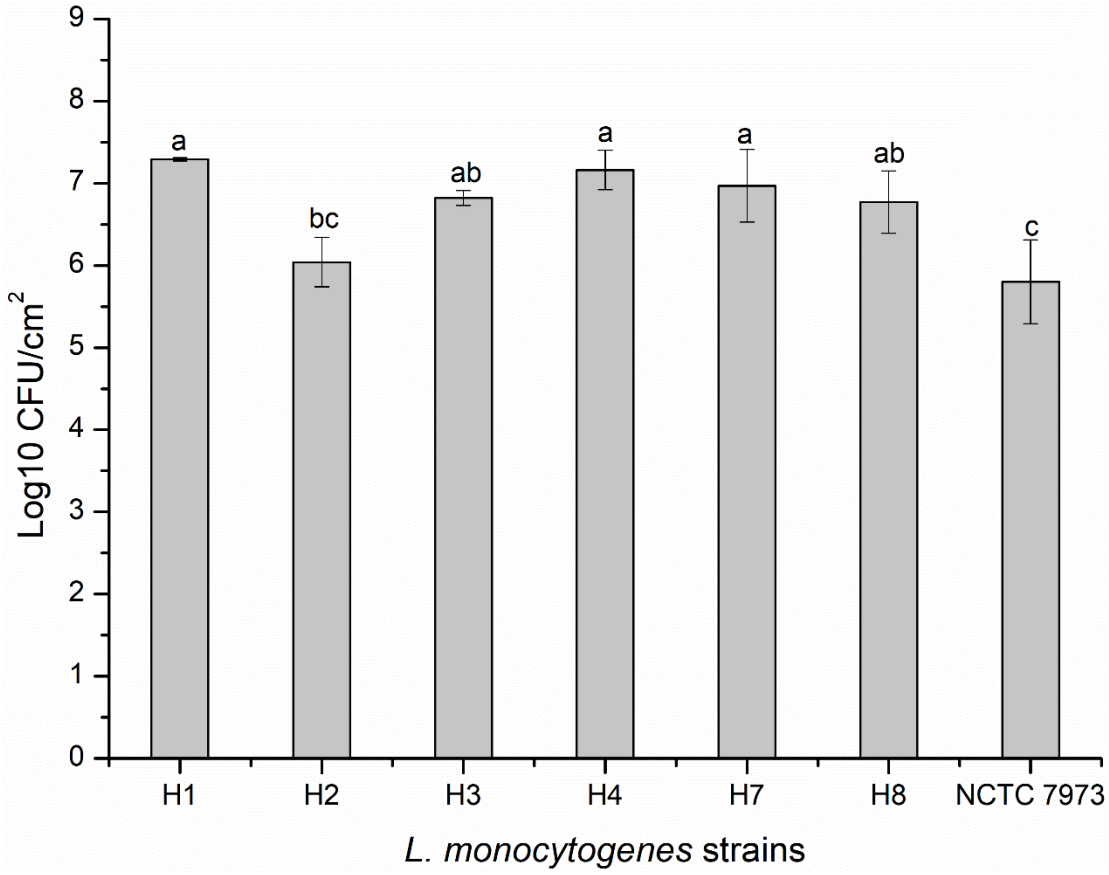


Figure 13. Total biofilm bacteria cells number of *L. monocytogenes* strains from Hills Laboratories and National Collection of Type Cultures England in IMF determined by drop plating. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

4.3 Comparison between the biofilm cells number grown under static or fluid conditions

Figures 14 – 16 show that a significant increase in *C. sakazakii* cells number was observed when they were grown in an annular rotating reactor compared with cells number in static condition ($P < 0.05$). However, the biofilm cells of *L. monocytogenes* incubated in the annular rotating reactor did not show a difference compared with the *L. monocytogenes* cells number grown in 24-wells microtiter plates (Figure 17).

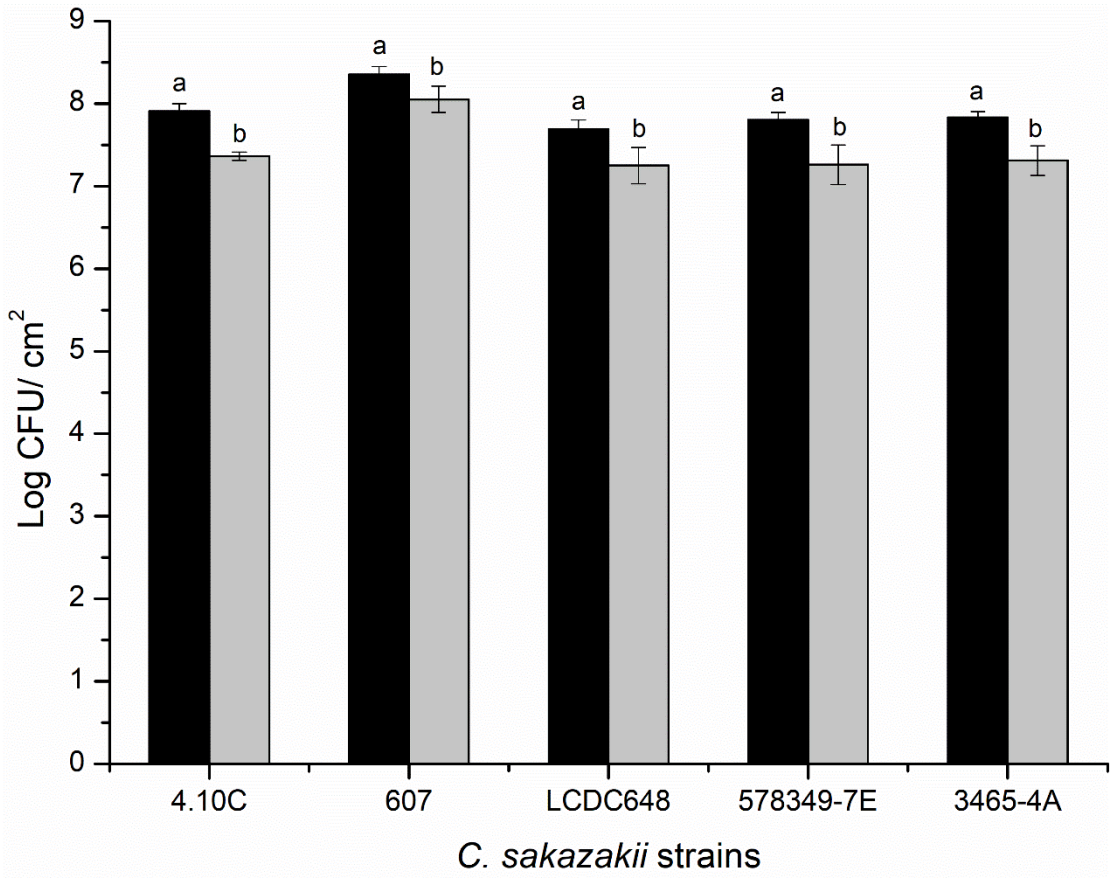


Figure 14. The number of cells of *C. sakazakii* strains isolated from dairy industry and clinical sources recovered in IMF under two conditions: black bar (fluid condition); grey bar (static condition). Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

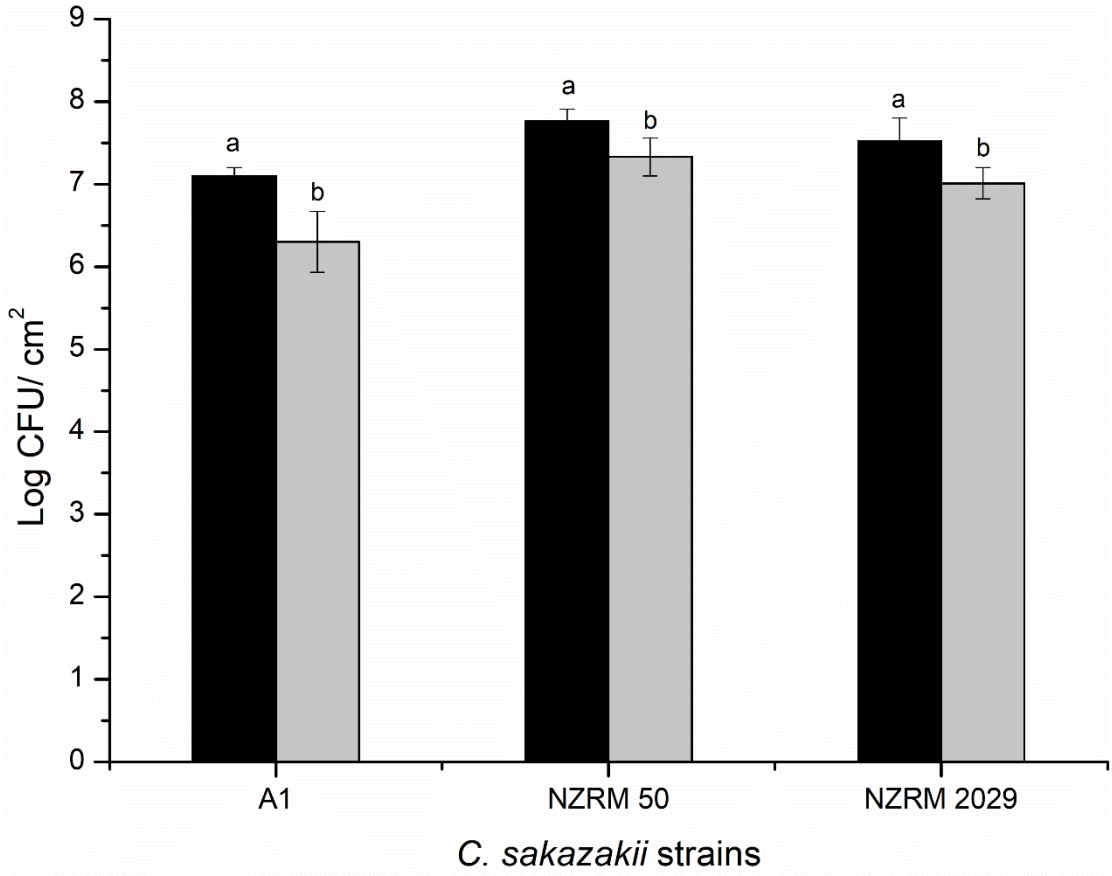


Figure 15. The number of cells of *C. sakazakii* strains from AsureQuality recovered in IMF under two conditions: black bar (fluid condition); grey bar (static condition). Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

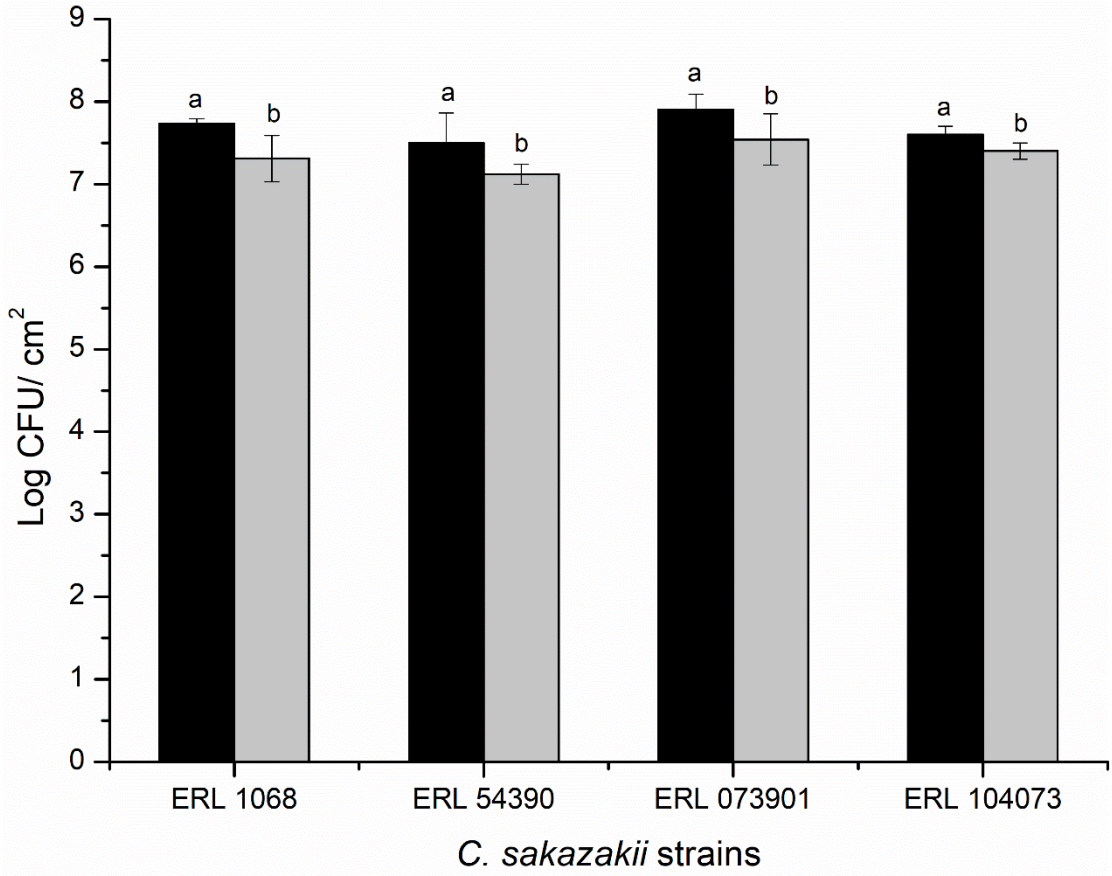


Figure 16. The number of cells of *C. sakazakii* strains from ESR recovered in IMF under two conditions: black bar (fluid condition); grey bar (static condition). Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

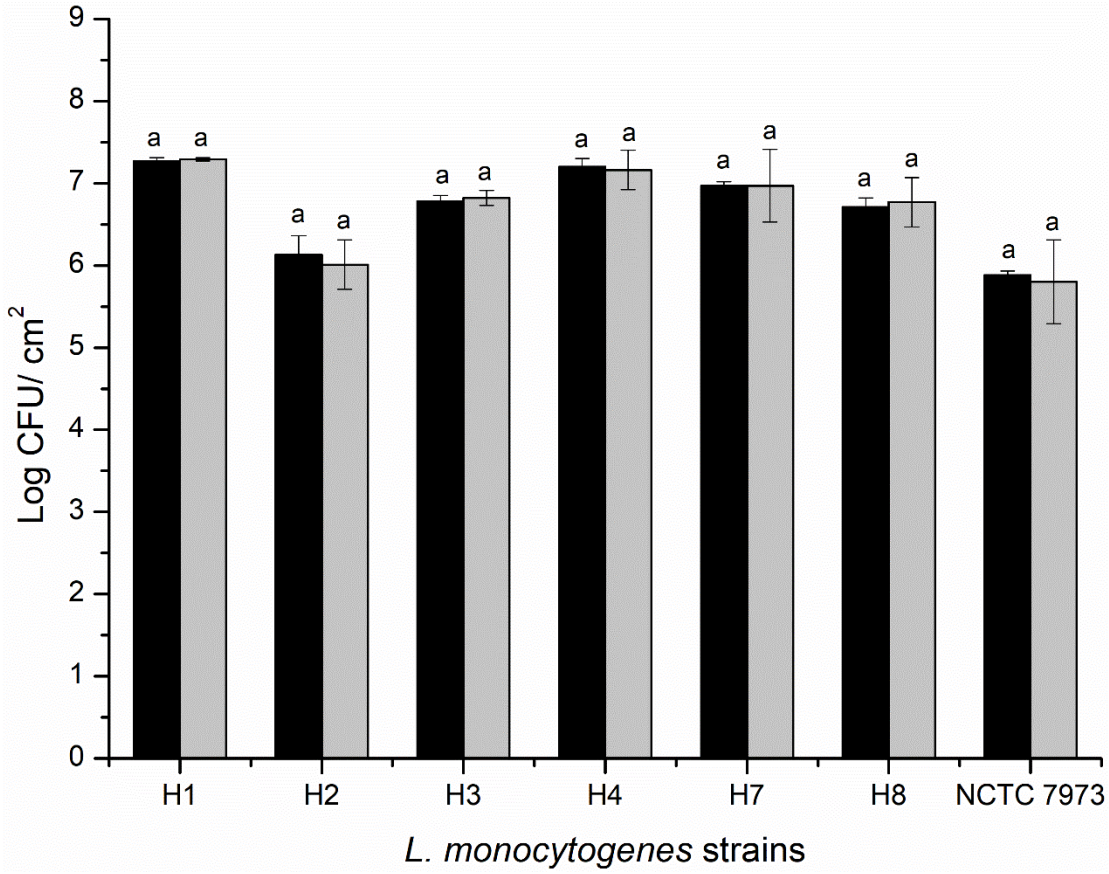


Figure 17. The number of cells of *L. monocytogenes* strains from Hills Laboratories and National Collection of Type Cultures England recovered in IMF under two conditions: black bar (fluid condition); grey bar (static condition). Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

4.4 Conclusions

- The components of milk are important in the formation of *C. sakazakii* biofilm.
- IMF supports the formation of biofilm of *C. sakazakii* strains from both dried milk and clinical sources.
- *L. monocytogenes* strains isolated from NZ food samples form better biofilms than *L. monocytogenes* from other sources.
- Dynamic conditions produce better biofilm of *C. sakazakii* than static conditions.
- *L. monocytogenes* is not influenced by dynamic or static conditions.

Chapter 5. Enzymatic removal of biofilms produced by *C. sakazakii* and *L. monocytogenes*

This chapter describes the results of *C. sakazakii* and *L. monocytogenes* biofilm removal using enzymes provided by CinderBio and comparisons with traditional cleaning (1% sodium hydroxide solution) by detection of biomass and counting viable microbial cells.

5.1 Microtiter plate assay for the evaluation of enzyme efficacy for biofilm removal

5.1.1 Detection of biomass

5.1.1.1 Effect of different cleaning methods on biofilm of *C. sakazakii*

The biofilm formed by 4.10C, 607 and LCDC 648 strains (one from dried infant milk formula, two clinical isolates) on microtiter plates was the highest compared with the other two strains with an O.D value of 3.48 before cleaning. The O.D value of 578349-7E and 3465-4A strains (environmental dairy isolates) was 3.12 and 2.74 absorbance, respectively. However, after enzyme and caustic treatment, the O.D values for both were significantly decreased by 1 absorbance unit ($P < 0.05$) and after buffer treatment (control), the O.D values were decreased by 0.5 absorbance units.

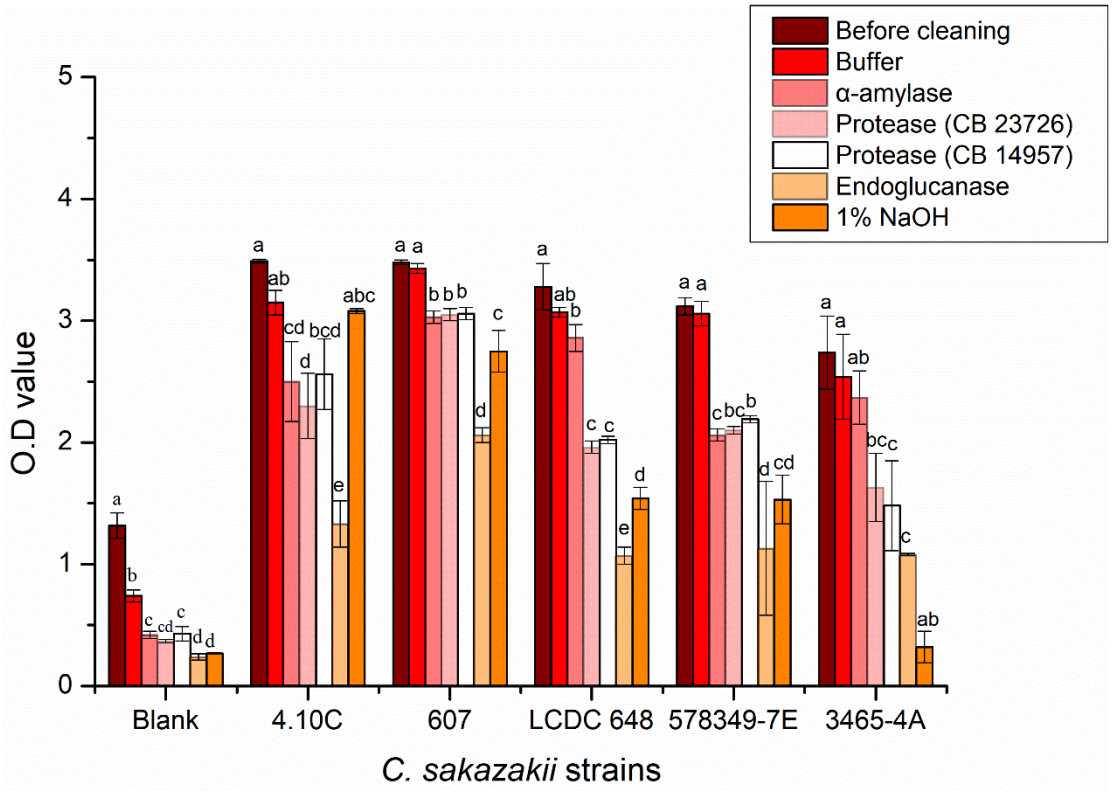


Figure 18. The O.D value of biofilms of *C. sakazakii* strains isolated from the dairy industry and clinical sources at 595 nm grown in IMF using a microtiter plate assay and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

The A1 strain was isolated from environment however the sources of NZRM 50 and NZRM 2029 were unknown. Before cleaning, the O.D value of three strains was similar (3.06, 3.08 and 2.97). The O.D value of A1 and NZRM 2029 was significantly decreased by 2 absorbance units after cleaning with buffer (control), enzymes and 1% NaOH solution ($P < 0.05$), however there were no significant differences in the O.D value before cleaning and buffer control for strain NZRM 50.

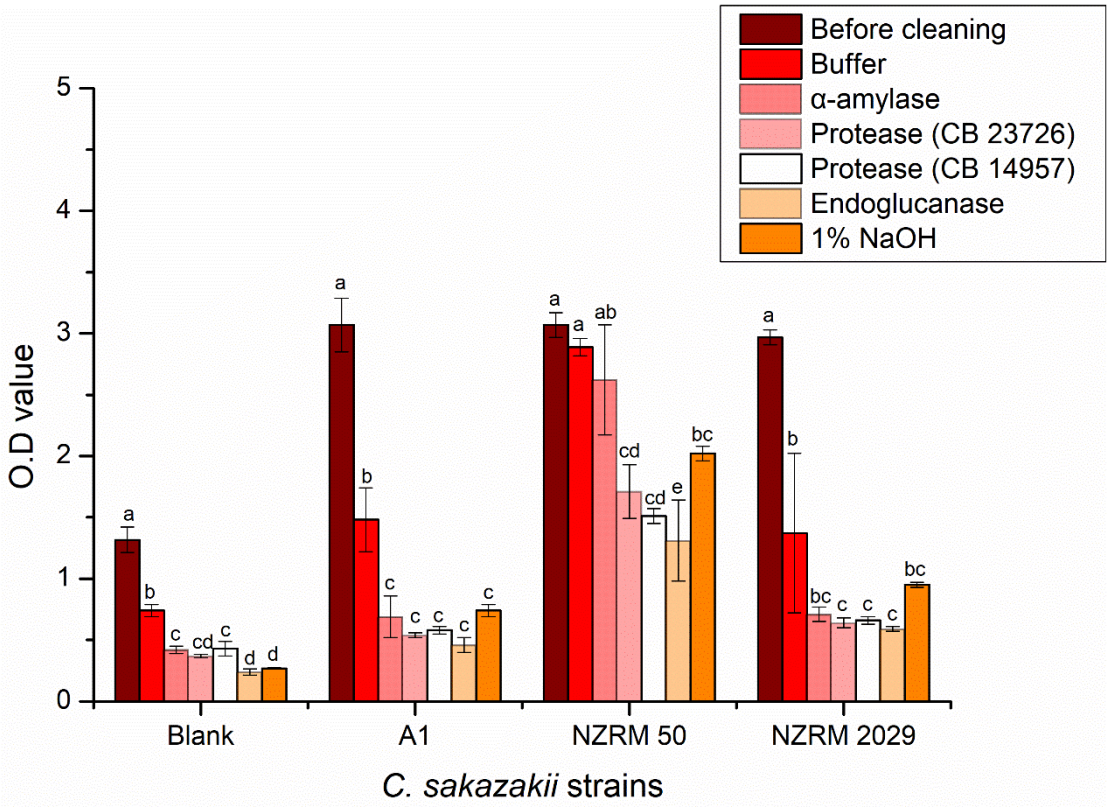


Figure 19. The O.D value at 595 nm of biofilms of *C. sakazakii* strains from AsureQuality in IMF using a microtiter plate assay and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

The biomass of ERL 1068 attached on microtiter plates was the highest, at 3.24 absorbance before cleaning and ERL 54390 was the most sensitive to cleaners (enzymes and caustic solution) decreasing by 2 absorbance units ($P < 0.05$). The O.D value of ERL 104073 was decreased by 1 absorbance units after cleaning (Figure 20).

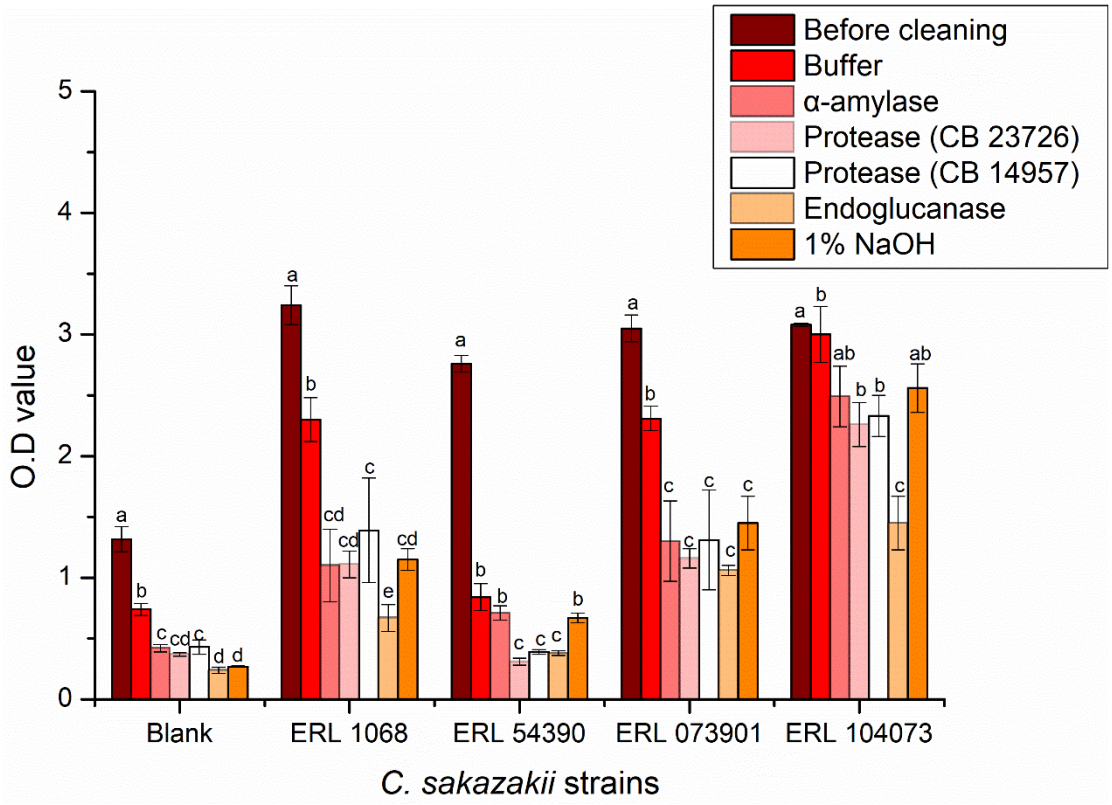


Figure 20. The O.D value at 595 nm of biofilms of *C. sakazakii* strains from ESR in IMF using a microtiter plate assay and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

5.1.1.2 Effect of different cleaning methods on biofilm of *L. monocytogenes*

Before cleaning, the biomass of the biofilm on microtiter plates was high with the O.D values of 3.01, 2.98, 2.98, 2.89, 3.07, 3.0 and 2.75. The *L. monocytogenes* biofilm cells on the microtiter plates were susceptible to the treatment with all cleaners, with decreases of about 2 absorbance units with endoglucanase showing the largest reduction ($P < 0.05$).

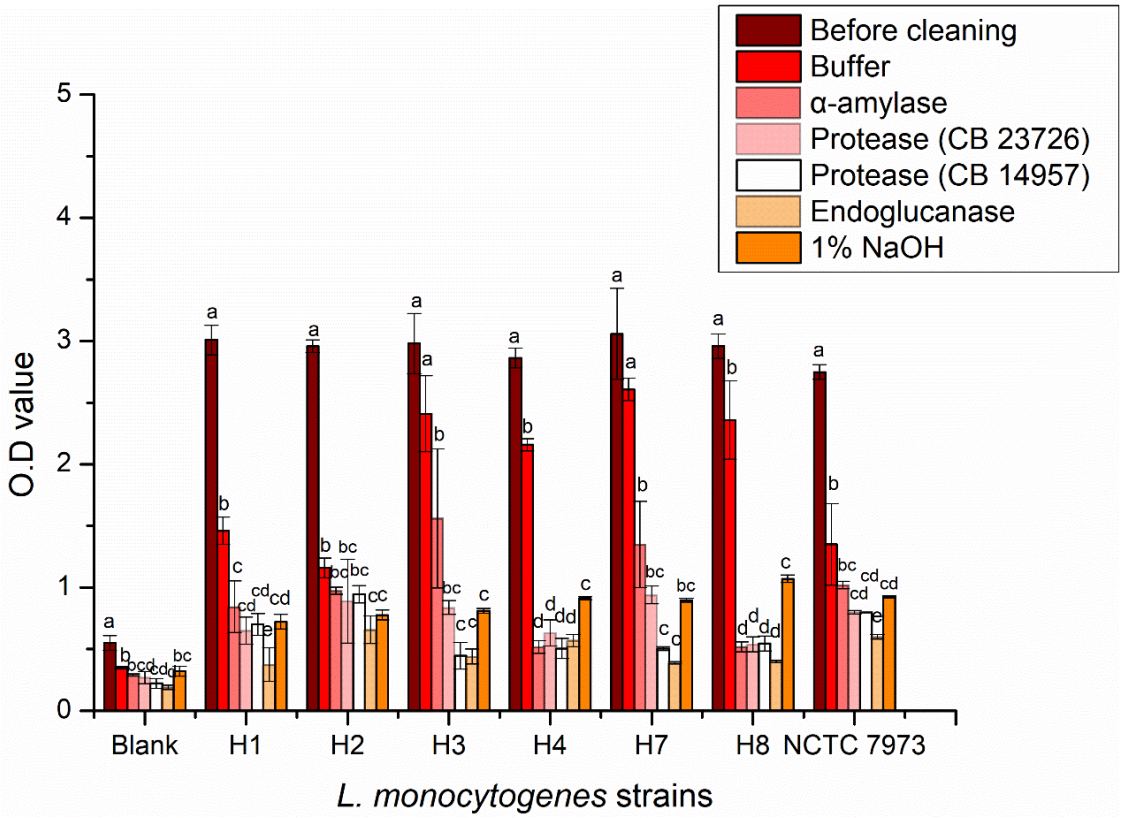


Figure 21. The O. D value at 595 nm of biofilms of *L. monocytogenes* strains from Hills Laboratories and National Collection of Type Cultures England in IMF using a microtiter plate assay and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

5.1.2 Detection of viable biofilm cells numbers attached on microtiter plates

Table 11 shows the total viable biofilm cells number on microtiter plates recovered from reconstituted IMF before and after cleaning. The number of biofilm cells of 607 strain on microtiter plates was the highest among all *C. sakazakii* strains with 8.18 log CFU/ cm^2 and the A1 strain showed the lowest number of biofilm cells (6.57 log CFU/ cm^2) on microtiter plates before cleaning. After all cleaning treatments, no viable cells were detected on plate (<10^3 log CFU/ cm^2).

Table 12 shows the number of biofilm cells of *L. monocytogenes* strains grown in IMF recovered from microtiter plates. The number of biofilm cells of *L. monocytogenes* attached on microtiter plates was lower than the number of *C. sakazakii*. The H1 strain produced the most biofilm cells attached on microtiter plates with 7.47 log CFU/ cm^2. After buffer, enzymatic and caustic treatment, the biofilm cells were less than the detectable limit at < 10^3 log CFU/ cm^2.

Table 11. Total viable biofilm cells number of *C. sakazakii* using a microtiter plate assay in IMF and exposed to different cleaners.

Strains no.	Before cleaning (log CFU/ cm^2)	Buffer (log CFU/ cm^2)	α-amylase (log CFU/ cm^2)	Protease (CB 23726) (log CFU/ cm^2)	Protease (CB 14957) (log CFU/ cm^2)	Endoglucanase (log CFU/ cm^2)	1% NaOH (log CFU/ cm^2)
4.10C	7.36±0.39	< 3	< 3	< 3	< 3	< 3	< 3
607	8.18±0.07	< 3	< 3	< 3	< 3	< 3	< 3
LCDC 648	7.10±0.10	< 3	< 3	< 3	< 3	< 3	< 3
578349-7E	7.33±0.15	< 3	< 3	< 3	< 3	< 3	< 3
3465-4A	7.48±0.24	< 3	< 3	< 3	< 3	< 3	< 3
A1	6.57±0.32	< 3	< 3	< 3	< 3	< 3	< 3
NZRM 50	7.54±0.16	< 3	< 3	< 3	< 3	< 3	< 3
NZRM 2029	7.18±0.16	< 3	< 3	< 3	< 3	< 3	< 3
ERL 1068	7.73±0.05	< 3	< 3	< 3	< 3	< 3	< 3

ERL 54390	7.61±0.10	< 3	< 3	< 3	< 3	< 3	< 3
ERL 073901	7.80±0.20	< 3	< 3	< 3	< 3	< 3	< 3
ERL 104073	7.66±0.05	< 3	< 3	< 3	< 3	< 3	< 3

Table 12. Total viable biofilm cells number of *L. monocytogenes* using a microtiter plate assay in IMF and exposed to different cleaners.

Strains no.	Before cleaning (log CFU/ cm^2)	Buffer (log CFU/ cm^2)	α-amylase (log CFU/ cm^2)	Protease (CB 23726) (log CFU/ cm^2)	Protease (CB 14957) (log CFU/ cm^2)	Endoglucanase (log CFU/ cm^2)	1% NaOH (log CFU/ cm^2)
H1	7.47±0.06	< 3	< 3	< 3	< 3	< 3	< 3
H2	6.26±0.15	< 3	< 3	< 3	< 3	< 3	< 3
H3	7.1±0.1	< 3	< 3	< 3	< 3	< 3	< 3
H4	7.21±0.11	< 3	< 3	< 3	< 3	< 3	< 3
H7	7.08±0.07	< 3	< 3	< 3	< 3	< 3	< 3

H8	6.87±0.14	< 3	< 3	< 3	< 3	< 3	< 3
NCTC 7973	6.20±0.11	< 3	< 3	< 3	< 3	< 3	< 3

5.2 The efficacy of enzyme cleaning for the removal of biofilms on stainless steel coupons in a static condition

5.2.1 Detection of biomass

5.2.1.1 Effect of different cleaning method on biofilm of *C. sakazakii*

The biomass of the biofilm of *C. sakazakii* isolated from the dairy industry and clinical sources on stainless steel coupons was lower than the biomass on microtiter plates. Strain 607 produced the highest O.D value (3.06 absorbance) compared with the other *C. sakazakii* strains before cleaning. However, all five strains from the dairy industry were sensitive to all cleaners with a significant reduction of 2.5 absorbance units ($P < 0.05$).

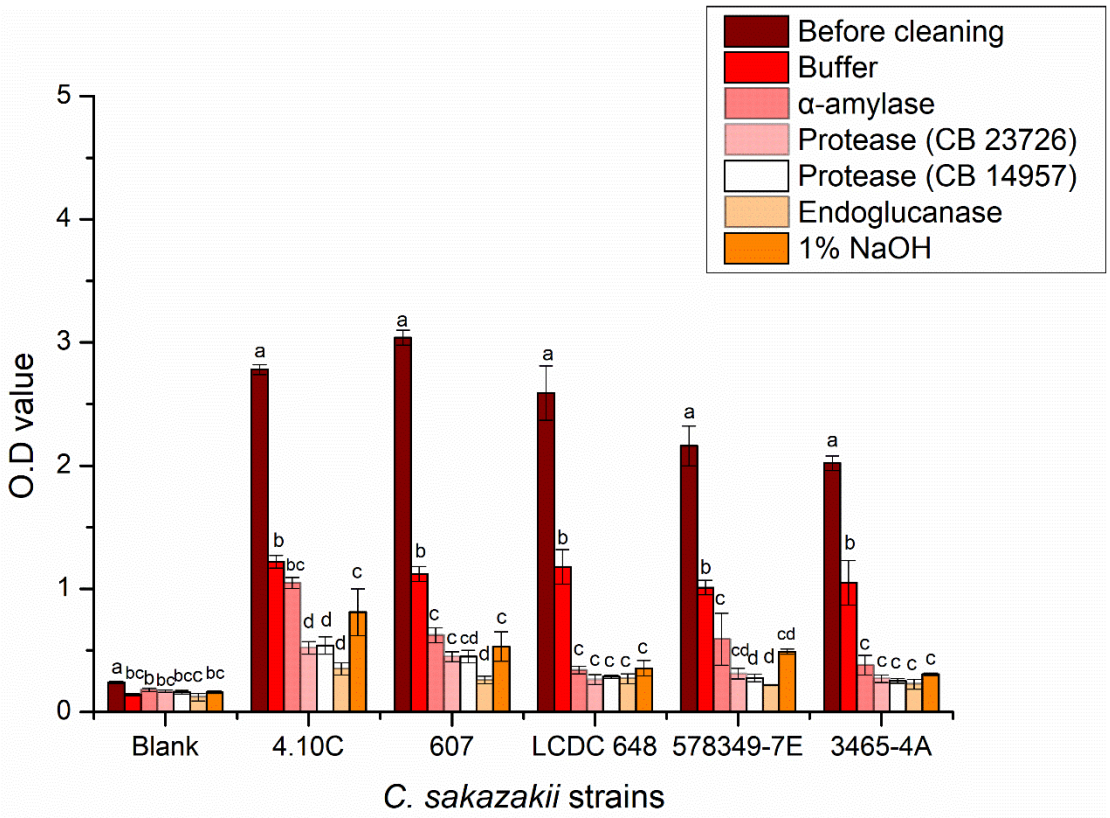


Figure 22. The O. D value at 595 nm of biofilms of *C. sakazakii* strains from the dairy industry and clinical sources in IMF using stainless steel coupons and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

The following figure shows that in IMF, the biomass of three strains (A1, NZRM 50 and NZRM 2029) attached on stainless steel coupons was similar with were 2.09, 2.1 and 2.04 before cleaning. However, after buffer, enzymes and caustic treatment, the biomass was decreased by approximately 2 absorbance units. Endoglucanase was still the most effective enzyme in removing biofilms ($P < 0.05$).

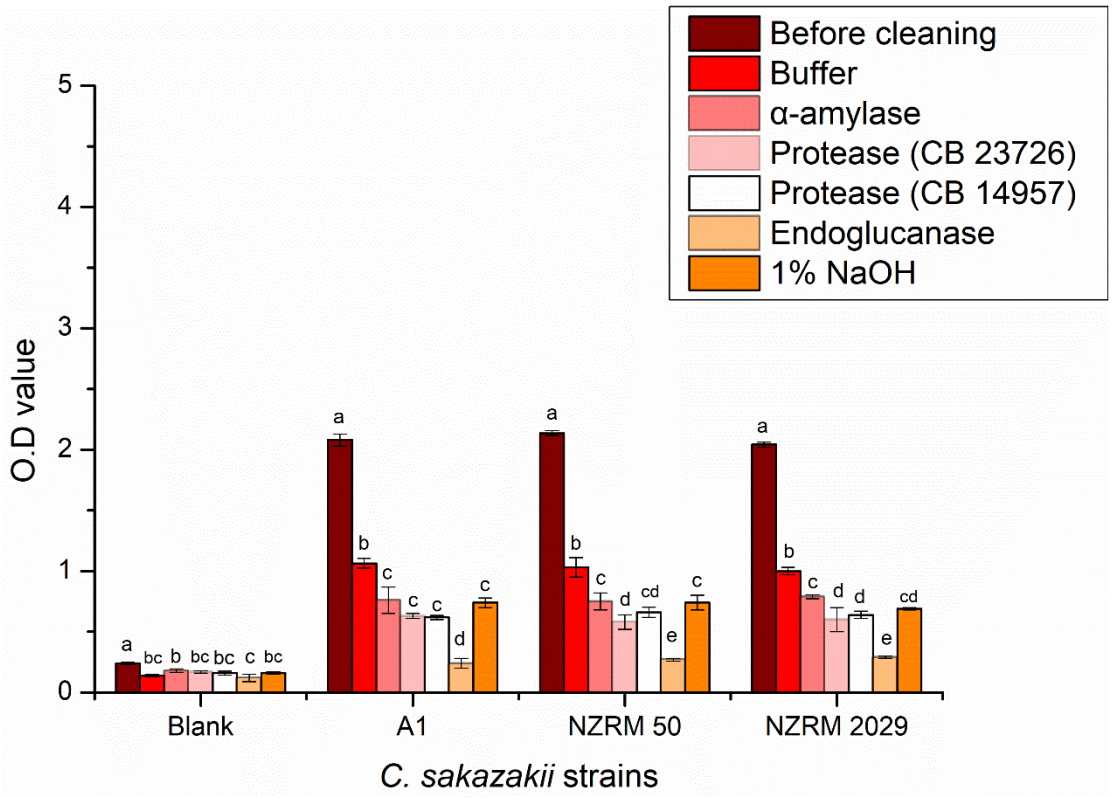


Figure 23. The O.D value at 595 nm of biofilms of *C. sakazakii* strains from AsureQuality in IMF using stainless steel coupons and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

Figure 24 shows the O.D value of the biomass of four *C. sakazakii* strains before and after cleaning. The biomass of biofilm of ERL 54390 on stainless steel coupons was the highest. After the treatment of cleaners, the O.D value was reduced more than 1 absorbance units ($P < 0.05$). The biomass treated with endoglucanase decreased the most.

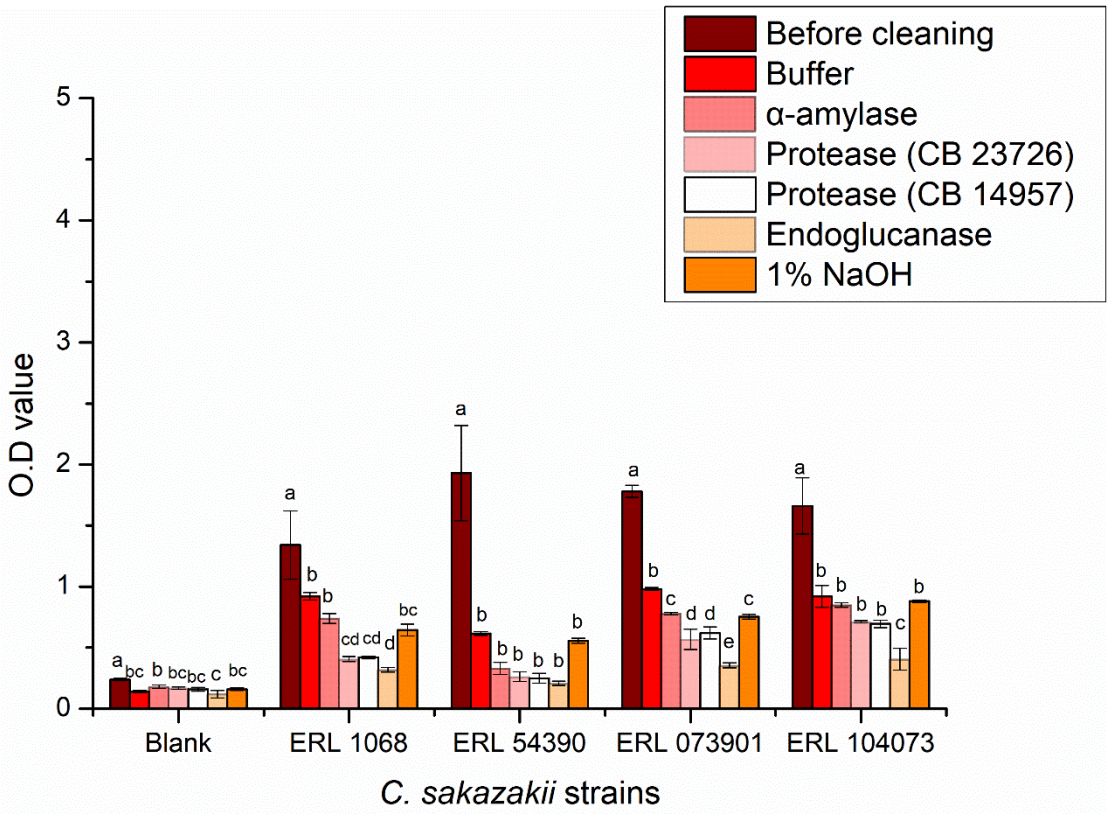


Figure 24. The O.D value at 595 nm of biofilms of *C. sakazakii* strains from ESR in IMF using stainless steel coupons and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

5.2.1.2 Effect of different cleaning method on biofilm of *L. monocytogenes*

For *L. monocytogenes* strains, the biomass of biofilm on stainless steel coupons was much lower than the biomass of *C. sakazakii* strains. The O.D value of seven strains ranges from about 1.37 to 0.96 before cleaning. The biomass attached on stainless steel coupons showed a significant reduction after treatment of cleaners, particularly with endoglucanase showing the larges reduction ($P < 0.05$).

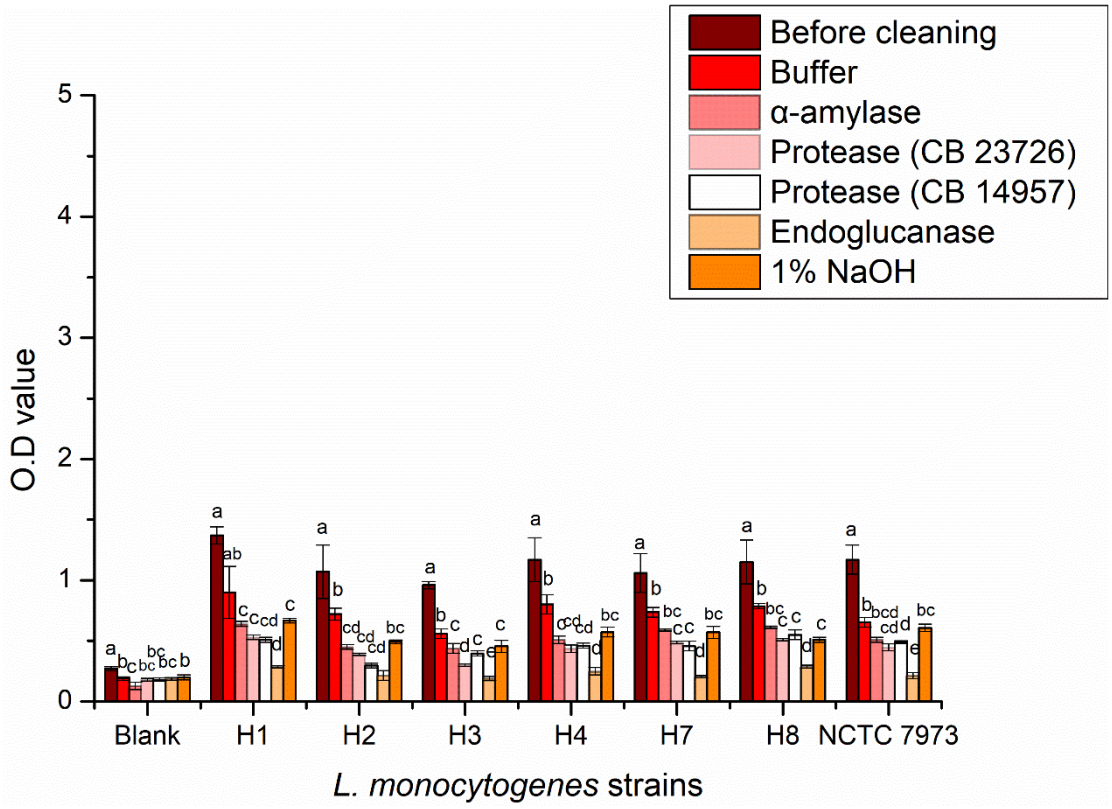


Figure 25. The O. D value at 595 nm of biofilms of *L. monocytogenes* strains from Hills Laboratories and National Collection of Type Cultures England in IMF using stainless steel coupons and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

5.2.2 Detection of viable biofilm cells number on stainless steel coupons

Tables 13 and 14 show the total biofilm cells number of *C. sakazakii* and *L. monocytogenes* attached on stainless steel coupons in IMF in a static condition. Before cleaning, the total viable biofilm cells of *C. sakazakii* and *L. monocytogenes* attached on stainless steel coupons ranged between 5.81 – 8.05 log CFU/ cm^2. After cleaning, no colonies forming on plate were detected (< 10^3 log CFU/ cm^2).

Table 13. Total viable biofilm cells number of *C. sakazakii* using stainless steel coupons in IMF and exposed to different cleaners

Strains no.	Before cleaning (log CFU/cm^2)	Buffer (log CFU/cm^2)	α-amylase (log CFU/cm^2)	Protease (CB 23726) (log CFU/cm^2)	Protease (CB 14957) (log CFU/cm^2)	Endoglucanase (log CFU/cm^2)	1% NaOH (log CFU/cm^2)
4.10C	7.36 ± 0.05	< 3	< 3	< 3	< 3	< 3	< 3
607	8.05 ± 0.16	< 3	< 3	< 3	< 3	< 3	< 3
LCDC 648	7.25 ± 0.22	< 3	< 3	< 3	< 3	< 3	< 3
578349-7E	7.26 ± 0.24	< 3	< 3	< 3	< 3	< 3	< 3
3465-4A	7.31 ± 0.18	< 3	< 3	< 3	< 3	< 3	< 3
A1	6.30 ± 0.37	< 3	< 3	< 3	< 3	< 3	< 3
NZRM 50	7.33 ± 0.23	< 3	< 3	< 3	< 3	< 3	< 3
NZRM 2029	7.01 ± 0.19	< 3	< 3	< 3	< 3	< 3	< 3
ERL 1068	7.31 ± 0.29	< 3	< 3	< 3	< 3	< 3	< 3

ERL 54390	7.12 ± 0.12	< 3	< 3	< 3	< 3	< 3	< 3
ERL 073901	7.55 ± 0.31	< 3	< 3	< 3	< 3	< 3	< 3
ERL 104073	7.40 ± 0.10	< 3	< 3	< 3	< 3	< 3	< 3

Table 14. Total viable biofilm cells number of *L. monocytogenes* using stainless steel coupons in IMF and exposed to different cleaners

Strains no.	Before cleaning (log CFU/cm^2)	Buffer (log CFU/cm^2)	α-amylase (log CFU/cm^2)	Protease (CB 23726) (log CFU/cm^2)	Protease (CB 14957) (log CFU/cm^2)	Endoglucanase (log CFU/cm^2)	1% NaOH (log CFU/cm^2)
H1	7.29 ± 0.02	< 3	< 3	< 3	< 3	< 3	< 3
H2	6.04 ± 0.30	< 3	< 3	< 3	< 3	< 3	< 3
H3	6.82 ± 0.09	< 3	< 3	< 3	< 3	< 3	< 3
H4	7.16 ± 0.24	< 3	< 3	< 3	< 3	< 3	< 3
H7	6.97 ± 0.44	< 3	< 3	< 3	< 3	< 3	< 3

H8	6.77 ± 0.38	< 3	< 3	< 3	< 3	< 3	< 3
NCTC 7973	5.81 ± 0.52	< 3	< 3	< 3	< 3	< 3	< 3

5.3 The efficacy of enzyme cleaning for the removal of biofilms on stainless steel coupons in a fluid condition

5.3.1 Detection of biomass

The biomass of biofilm of *C. sakazakii* strains on stainless steel coupons under a fluid condition was higher than the biomass grown in a static condition with cleaning reducing the biomass by approximately 1 absorbance unit ($P < 0.05$) (Figures 26, 27 and 28).

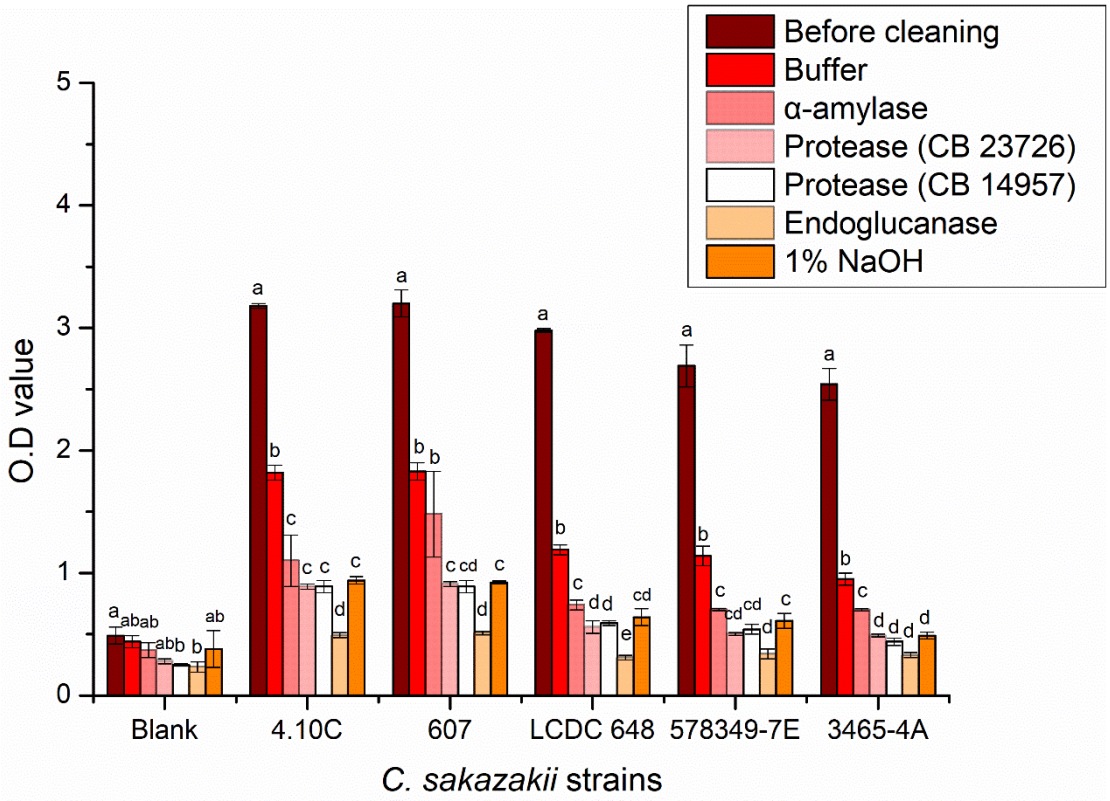


Figure 26. The O. D value at 595 nm of biofilms of *C. sakazakii* strains from the dairy industry in IMF using stainless steel coupons in fluid condition and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

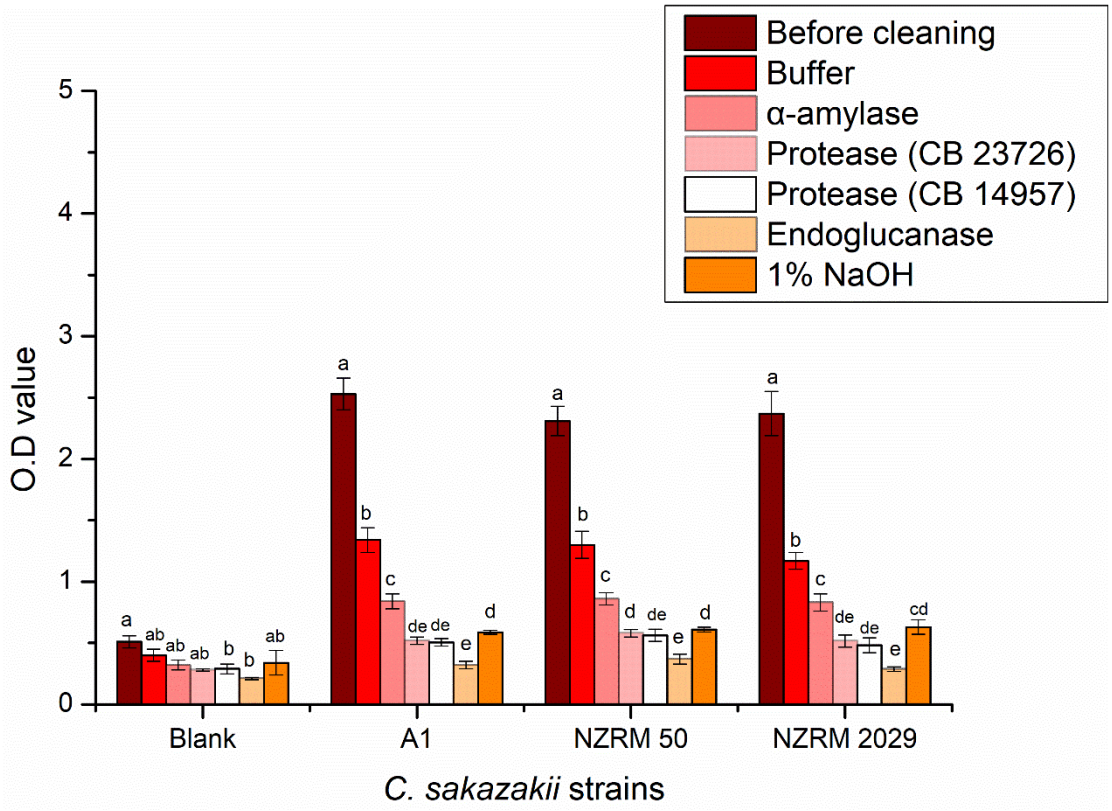


Figure 27. The O. D value at 595 nm of biofilms of *C. sakazakii* strains from AsureQuality in IMF using stainless steel coupons in fluid condition and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

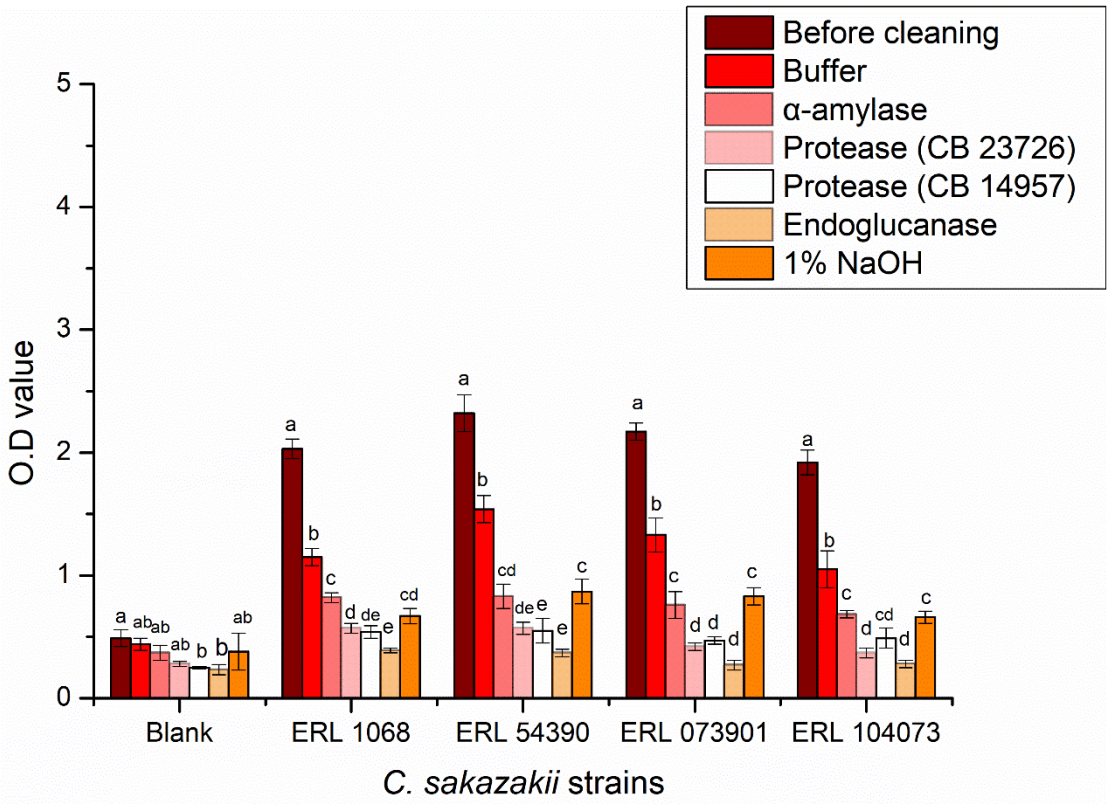


Figure 28. The O. D value at 595 nm of biofilms of *C. sakazakii* strains from ESR in IMF using stainless steel coupons in fluid condition and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

The biomass of *L. monocytogenes* strains attached on stainless steel coupons was lower than the biomass of *C. sakazakii* strains. A significant decrease was seen after cleaning for seven *L. monocytogenes* strains ($P < 0.05$). The biomass treated with endoglucanase showed the lowest O. D value compared with other enzymes. Therefore, the endoglucanase was the preferred enzyme for cleaning.

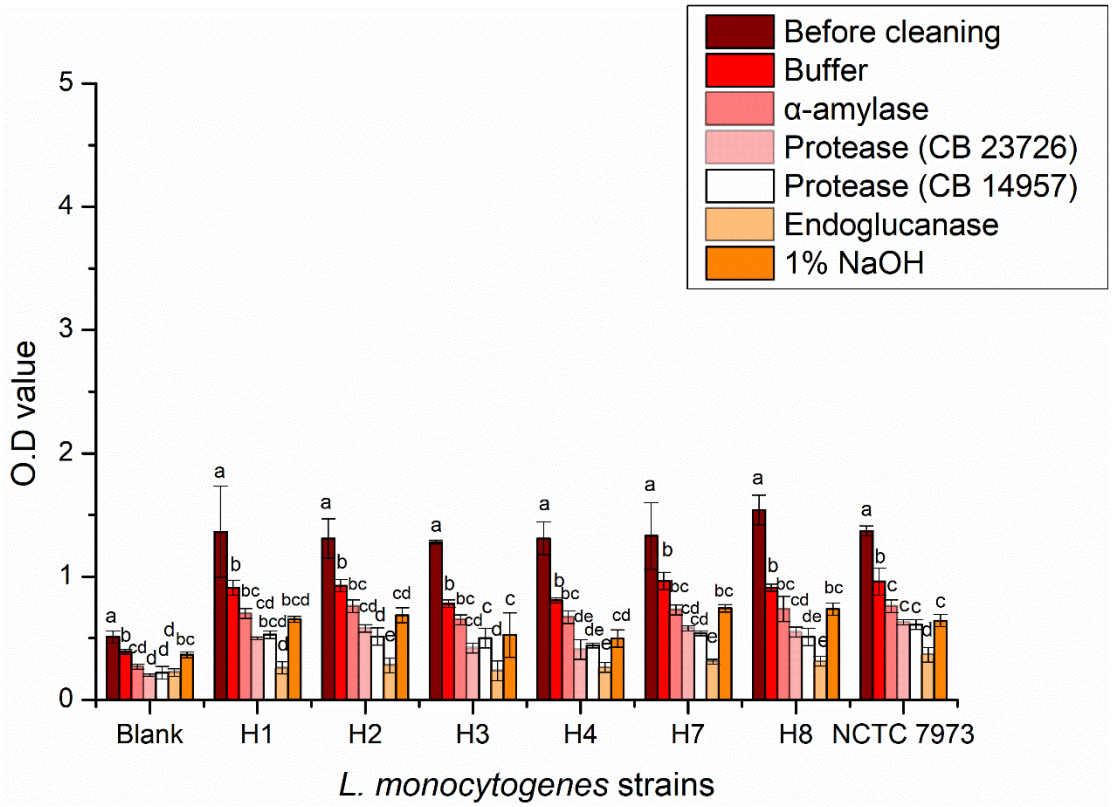


Figure 29. The O. D value at 595 nm of biofilms of *L. monocytogenes* strains from Hills Laboratories and National Collection of Type Cultures England in IMF using stainless steel coupons in fluid condition and exposed to different cleaners. Results are the mean and standard deviation from three replicates. Different letters indicate statistically significant differences, $P < 0.05$ (Tukey's test).

5.3.2 Detection of viable biofilm cells number on stainless steel coupons

As discussed in 4.3, the cells number of *C. sakazakii* grown under a fluid condition was higher than the biofilm cells grown in a static condition. However, there was no difference in biofilm cells number for *L. monocytogenes* grown in a fluid and static condition. The number of *C. sakazakii* biofilm cells on stainless steel coupons released into IMF reached 7.10 – 8.35 log CFU/ cm^2. The biofilm cells showed a decrease after the treatment with buffer, enzymes and caustic solution to < 3 log CFU/ cm^2. The biofilm cells of *L. monocytogenes* attached on stainless steel coupons were low at 5.88 – 7.27 log CFU/cm^2. The cleaning effect using buffer, enzymes and caustic solution was similar with *C. sakazakii* strains at < 3 log CFU/ cm^2.

Table 15. Total viable biofilm cells number of *C. sakazakii* using stainless steel coupons in IMF under fluid condition and exposed to different cleaners.

Strains no.	Before cleaning (log CFU/cm^2)	Buffer (log CFU/cm^2)	α-amylase (log CFU/cm^2)	Protease (CB 23726) (log CFU/cm^2)	Protease (CB 14957) (log CFU/cm^2)	Endoglucanase (log CFU/cm^2)	1% NaOH (log CFU/cm^2)
4.10C	7.91 ± 0.09	< 3	< 3	< 3	< 3	< 3	< 3
607	8.35 ± 0.10	< 3	< 3	< 3	< 3	< 3	< 3
LCDC 648	7.69 ± 0.11	< 3	< 3	< 3	< 3	< 3	< 3
578349-7E	7.80 ± 0.09	< 3	< 3	< 3	< 3	< 3	< 3
3465-4A	7.83 ± 0.07	< 3	< 3	< 3	< 3	< 3	< 3
A1	7.10 ± 0.10	< 3	< 3	< 3	< 3	< 3	< 3
NZRM 50	7.76 ± 0.15	< 3	< 3	< 3	< 3	< 3	< 3
NZRM 2029	7.52 ± 0.28	< 3	< 3	< 3	< 3	< 3	< 3
ERL 1068	7.73 ± 0.06	< 3	< 3	< 3	< 3	< 3	< 3

ERL 54390	7.50 ± 0.36	< 3	< 3	< 3	< 3	< 3	< 3
ERL 073901	7.91 ± 0.19	< 3	< 3	< 3	< 3	< 3	< 3
ERL 104073	7.61 ± 0.11	< 3	< 3	< 3	< 3	< 3	< 3

Table 16. Total viable biofilm cells number of *L. monocytogenes* using stainless steel coupons in IMF under fluid condition and exposed to different cleaners.

Strains no.	Before cleaning (log CFU/cm^2)	Buffer (log CFU/cm^2)	α-amylase (log CFU/cm^2)	Protease (CB 23726) (log CFU/cm^2)	Protease (CB 14957) (log CFU/cm^2)	Endoglucanase (log CFU/cm^2)	1% NaOH (log CFU/cm^2)
H1	7.27 ± 0.04	< 3	< 3	< 3	< 3	< 3	< 3
H2	6.13 ± 0.23	< 3	< 3	< 3	< 3	< 3	< 3
H3	6.78 ± 0.07	< 3	< 3	< 3	< 3	< 3	< 3
H4	7.20 ± 0.10	< 3	< 3	< 3	< 3	< 3	< 3
H7	6.97 ± 0.05	< 3	< 3	< 3	< 3	< 3	< 3

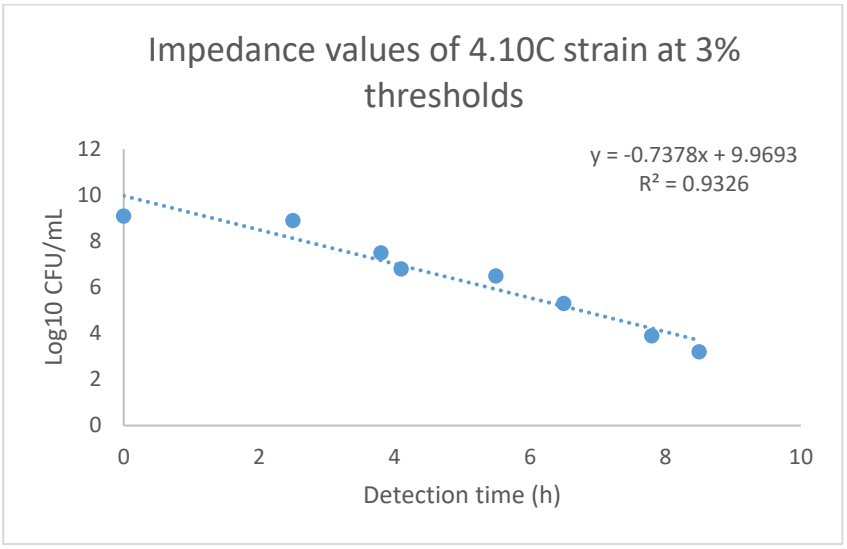
H8	6.71 ± 0.11	< 3	< 3	< 3	< 3	< 3	< 3
NCTC 7973	5.88 ± 0.05	< 3	< 3	< 3	< 3	< 3	< 3

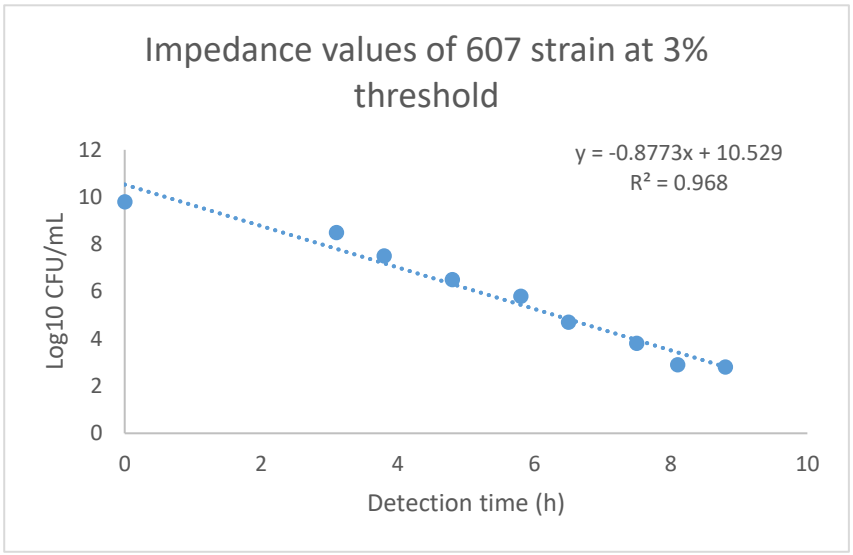
5.4 Biofilm enumeration

5.4.1 Impedance detection

5.4.1.1 Calibration curves from impedance microbiology

Figure 30 shows the impedance calibration curves for three *C. sakazakii* strains (4.10C, 607 and LCDC 648) isolated from dried infant milk formula, clinical and environment respectively. Due to the limited time, only three strains of *C. sakazakii* isolated from different sources were selected. Based on the results of impedance detection, the M-value showed reproducible growth curves while the curves of E-value were out of shape and not reproducible. Therefore, the M-value was used in this study to establish calibration equations. The calibration equations showed a good correlation ($R^2 > 0.9$). After buffer and enzymatic treatment for all three *C. sakazakii* strains, the M-value did not reach the 3% threshold which means the viable cells were less than the minimum cells number detected by using the impedance system. Therefore, the viable cells of 4.10C, 607 and LCDC 648 strains on stainless steel coupons were less than 3.2, 2.8 and 1.8 log CFU/ coupon. Figure 31 shows the calibration curves of two *L. monocytogenes*, one (H1) isolated from food samples and one (NCTC 7973) isolated from a guinea pig mesenteric lymph node. The correlation coefficients were good ($R^2 > 0.9$). After cleaning, the M-value did not reach 3% threshold indicating no viable cells remaining (< 2.1 and 2.3 log CFU/ coupon).





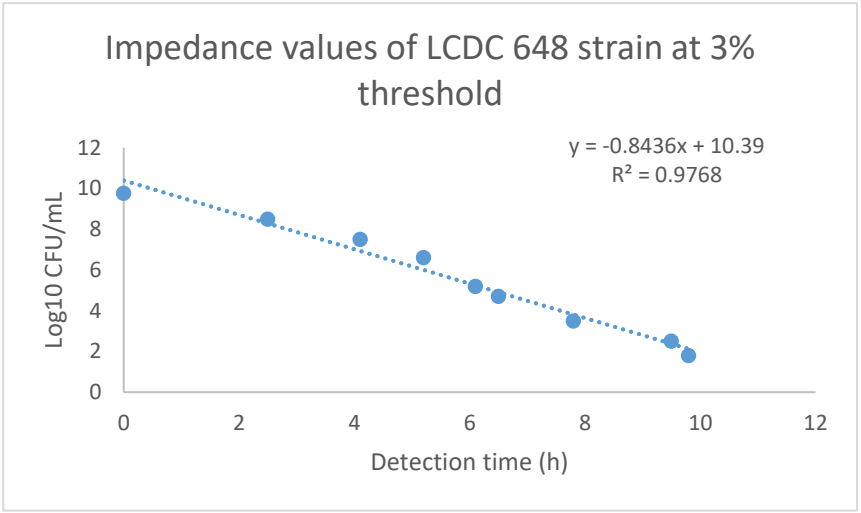
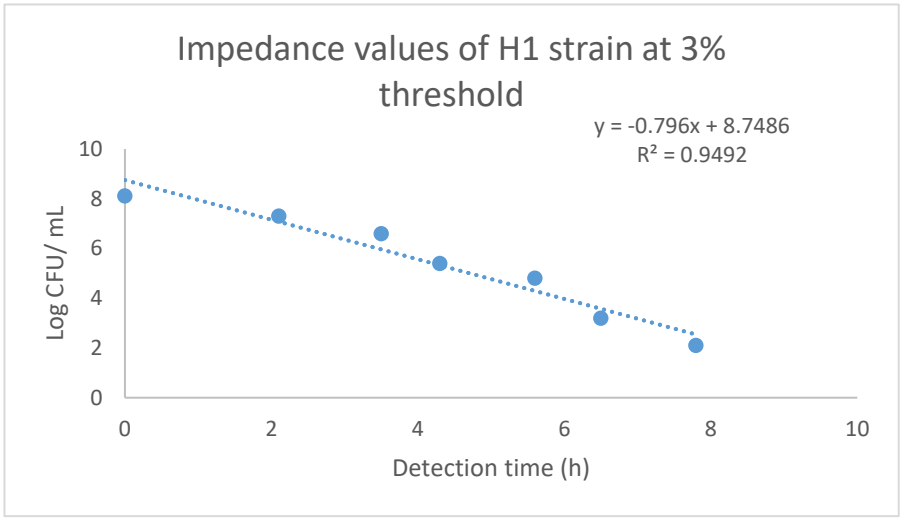


Figure 30. The calibration curves of three *C. sakazakii* strains to determine the reproducibility of BacTrac^{TM} 4300 detection time.



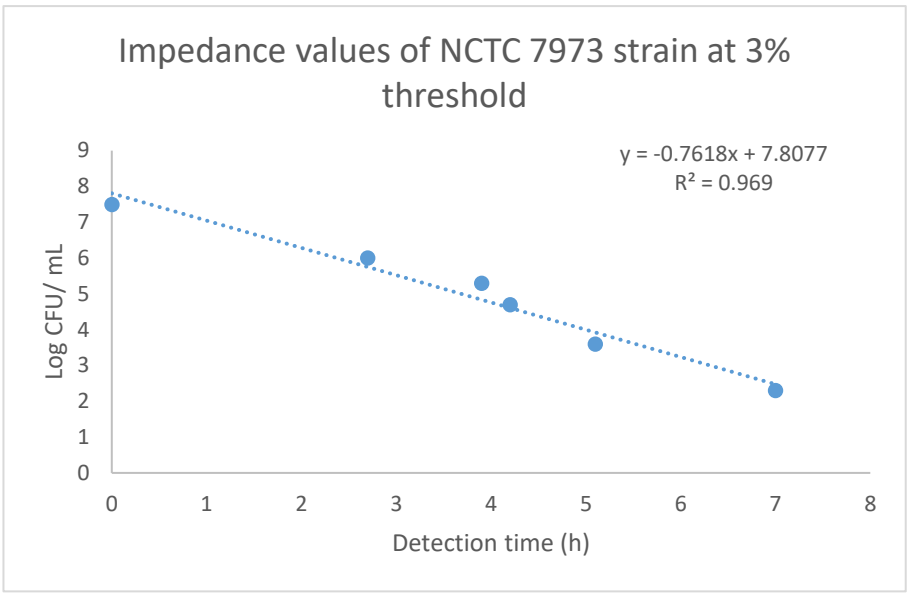


Figure 31. The calibration curves of two *L. monocytogenes* strains to determine the reproducibility of BacTrac™ 4300 detection time.

5.4.2 Epifluorescence microscopy

Figures 32 – 34 show epifluorescence microscope images of the biofilm of three *C. sakazakii* strains on stainless steel coupons before and after cleaning with buffer and enzymes. There were still some cells remaining on stainless steel coupons after buffer treatment. However, after the treatment of α-amylase, proteases and endoglucanase, a reduction in biofilm cells was observed based on three fields observed. There were almost no cells were seen on the stainless steel surfaces after using proteases and endoglucanase, which in line with the previous results that the O.D values were the lowest after cleaning with proteases and endoglucanase.

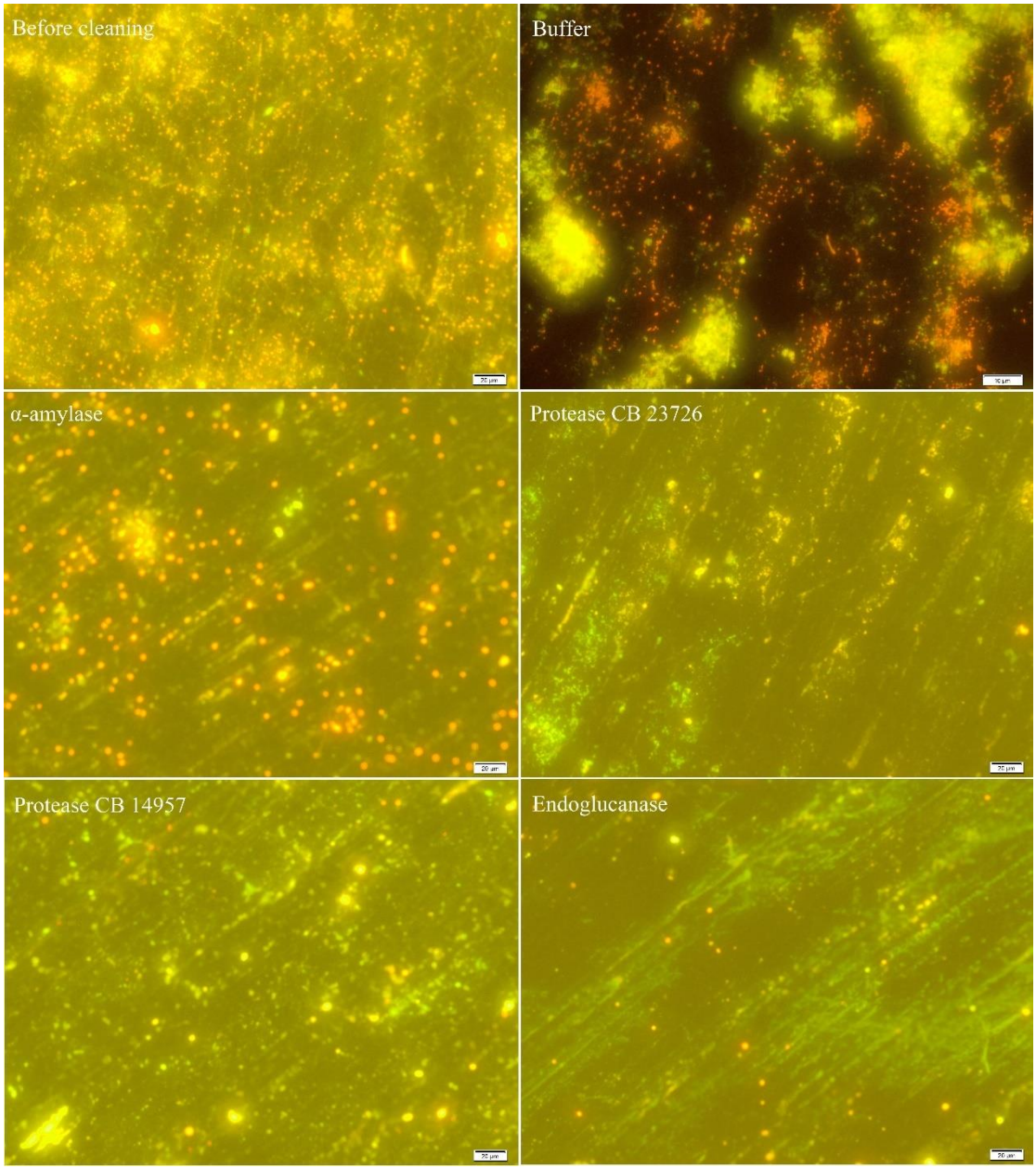


Figure 32. The epifluorescence micrograph of viable biofilm cells (orange spots) of 4.10C strain of *C. sakazakii* on stainless steel coupons surfaces. The images show biofilm treatment using buffer, α-amylase, proteases and endoglucanase (from left to right).

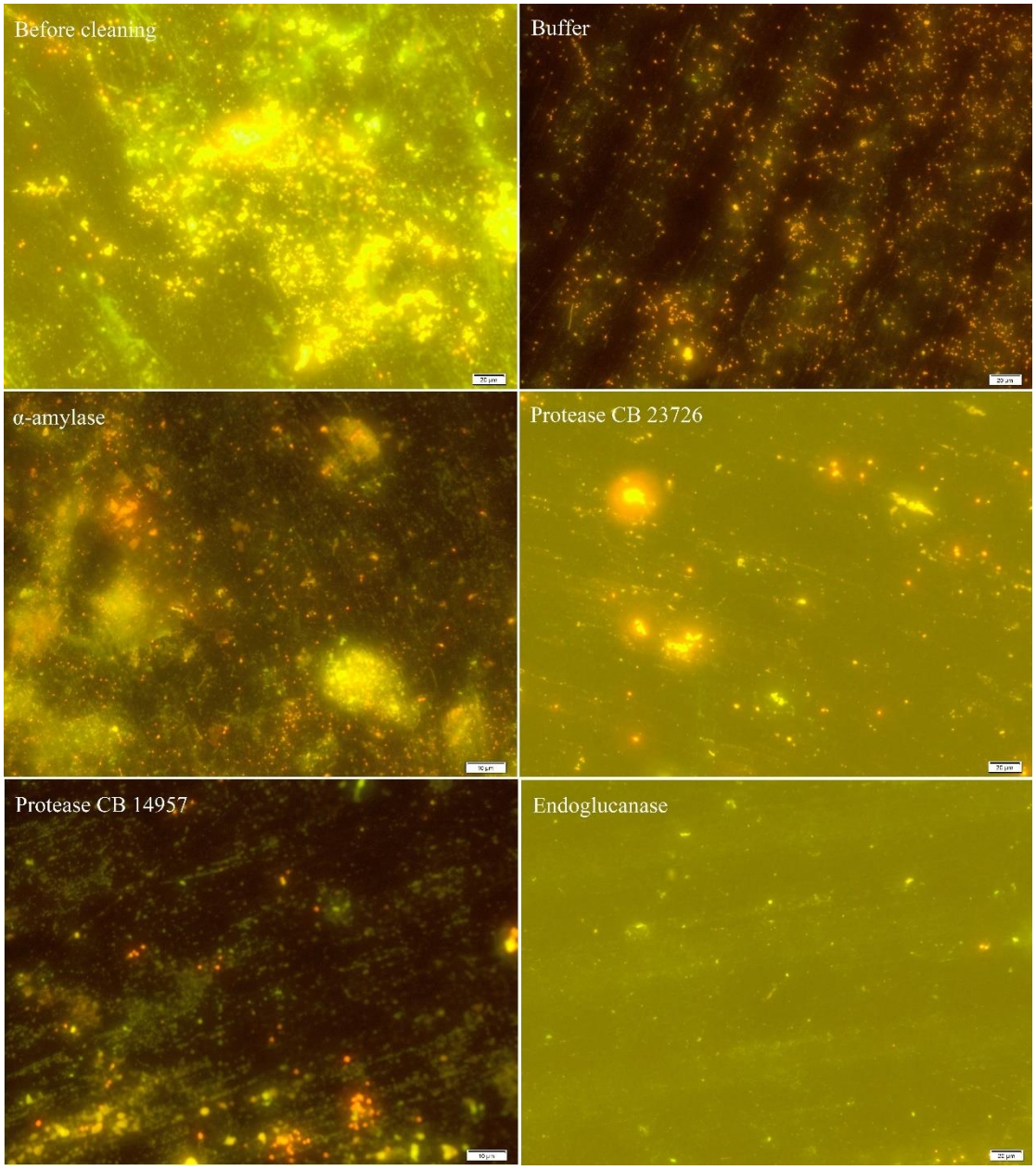


Figure 33. The epifluorescence micrograph of viable biofilm cells (orange spots) of 607 strain of *C. sakazakii* on stainless steel coupons surfaces. The images show biofilm treatment using buffer, α-amylase, proteases and endoglucanase (from left to right).

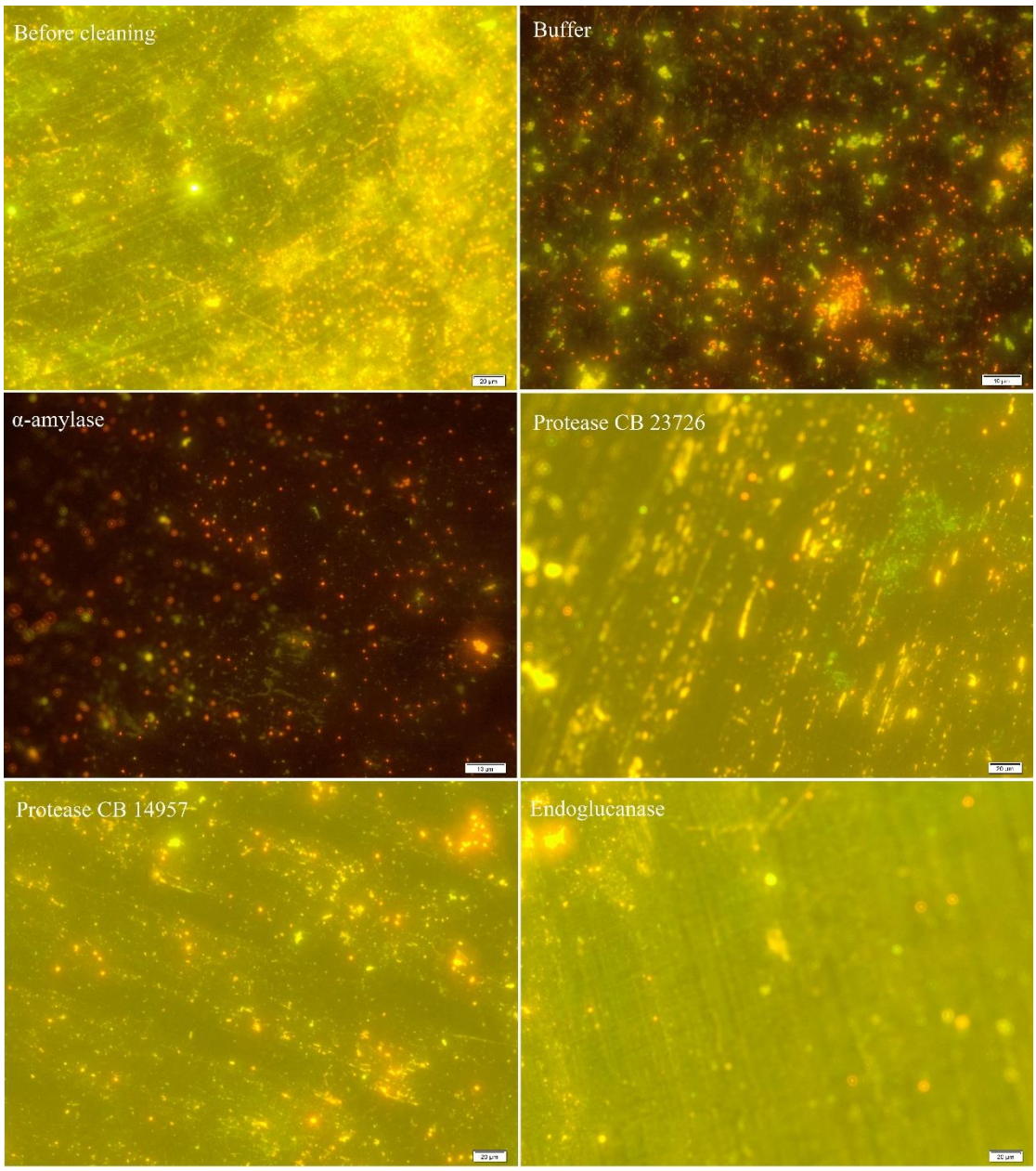


Figure 34. The epifluorescence micrograph of viable biofilm cells (orange spots) of LCDC 648 strain of *C. sakazakii* on stainless steel coupons surfaces. The images show biofilm treatment using buffer, α-amylase, proteases and endoglucanase (from left to right).

Figures 35 – 36 show the epifluorescence microscopic images of viable cells of two *L. monocytogenes* strains attached on stainless steel coupons before and after treatment with buffer and enzymes. After buffer treatment there were less cells on the stainless steel surface than for the *C. sakazakii* strains, which is in line with the previous results for the O.D value of *L. monocytogenes* biomass attached on stainless steel. After enzymatic treatment, the number of cells decreased especially after using protease and endoglucanase.

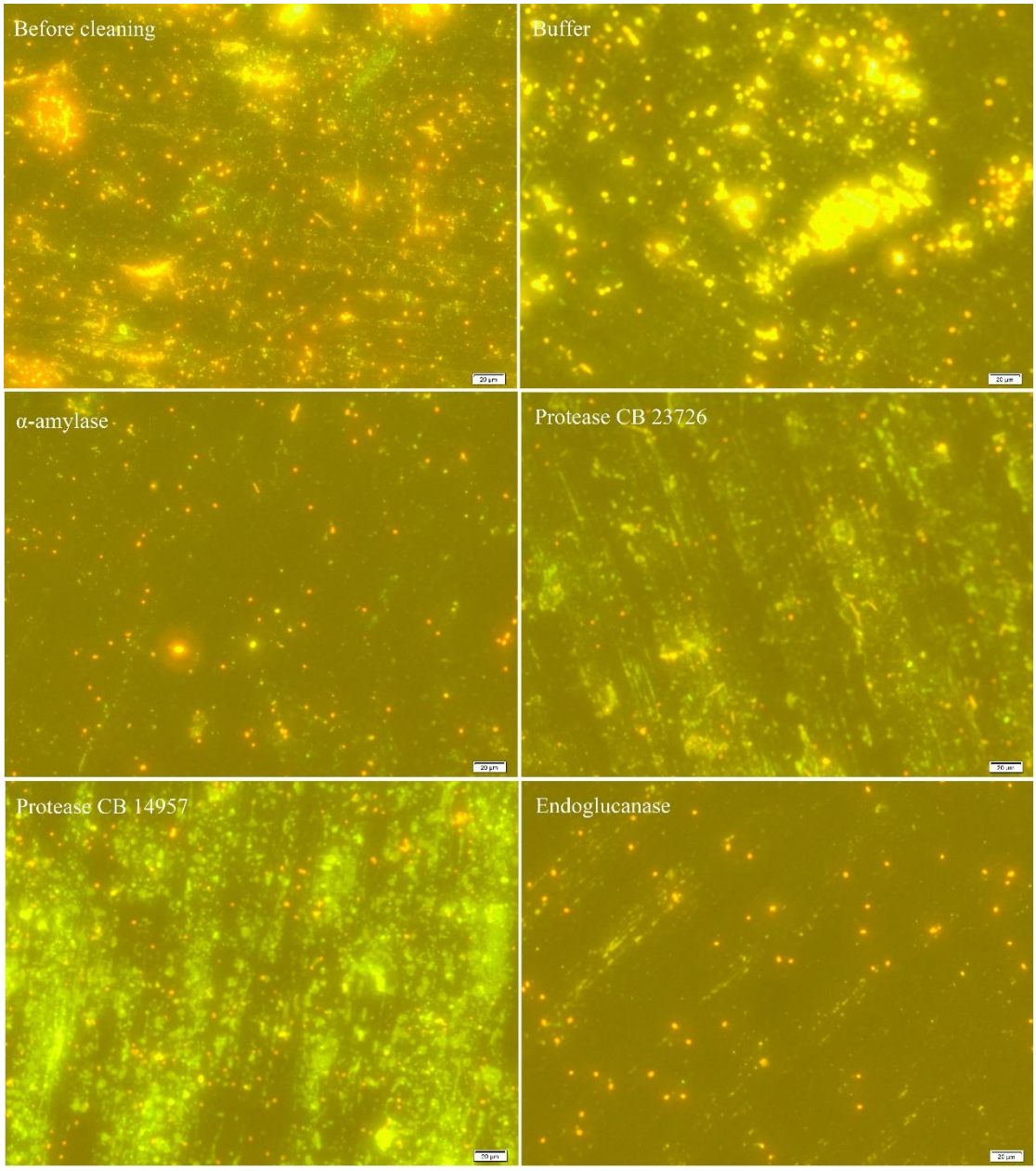


Figure 35. The epifluorescence micrograph of viable biofilm cells (orange spots) of H1 strain of *L. monocytogenes* on stainless steel coupons surfaces. The images show biofilm treatment using buffer, α-amylase, proteases and endoglucanase (from left to right).

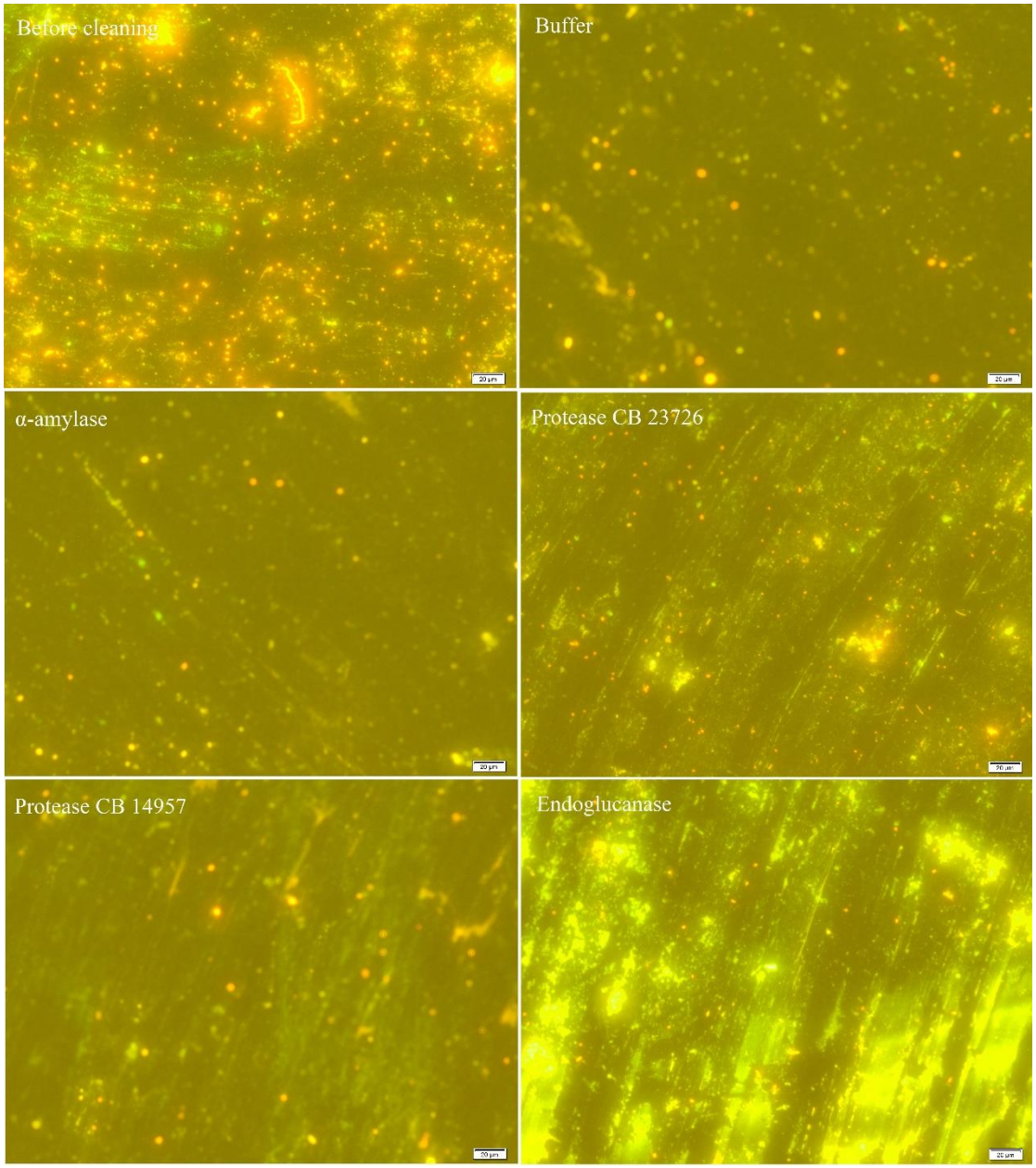


Figure 36. The epifluorescence micrograph of viable biofilm cells (orange spots) of NCTC 7973 strain of *L. monocytogenes* on stainless steel coupons surfaces. The images show biofilm treatment using buffer, α-amylase, proteases and endoglucanase (from left to right).

5.5 Conclusions

- Biofilm of *C. sakazakii* and *L. monocytogenes* form better on microtiter plates than stainless steel.
- The enzymes provided by CinderBio, especially endoglucanase, are effective in reducing *C. sakazakii* and *L. monocytogenes* biofilms.
- Endoglucanase is the most effective in removing biomass from biofilms of both these bacteria.
- Where the reduction in biofilm is seen with enzymatic treatment, enzymes are recommended as they are environmentally friendly and are safer to handle than caustic.

Chapter 6. Discussion

6.1 Preliminary results discussion

6.1.1 Media

6.1.2 Surface material

6.1.3 Static and dynamic incubation

6.2 Enzymatic treatment for removal biofilms

6.3 Impedance detection and epifluorescence microscope

6.4 Prevention of *C. sakazakii* and *L. monocytogenes* biofilms in the food industry

6.5 Limitations and recommendations for future work

This chapter discusses the external factors that affect biofilm formation of *C. sakazakii* and *L. monocytogenes* and the basic mechanism of enzymes (α-amylase, proteases and endoglucanase) in removing biofilms. The recommendations for prevention of *C. sakazakii* and *L. monocytogenes* biofilms in the food industry are discussed as well.

6.1 Preliminary results discussion

Many factors can influence biofilms formation including nutrients, incubation time, temperature, a_w, oxygen, properties of the isolates and the properties of contact surfaces (Arroyo, Condón & Pagán, 2009; Nowak et al., 2015; Moltz & Martin, 2004). Although some researchers have studied the influence of physiological and environmental factors on *L. monocytogenes* biofilm formation, studies on the influence of external factors on *C. sakazakii* biofilm are limited. From the preliminary results, there were three factors that appeared to affect the development of *C. sakazakii* and *L. monocytogenes* biofilms (media, surface material and static and dynamic incubation).

6.1.1 Media

In the initial microtiter plate assay, *C. sakazakii* strains were incubated in NB at 37°C for 24 h and *L. monocytogenes* strains were grown in BHI at 37°C for 24 h. However, the results showed that at 595 nm, there was little biomass on the wells of microtiter plates (Appendices Figure 37 – 41). When *C. sakazakii* and *L. monocytogenes* were incubated in sterile reconstituted IMF, biomass levels in the microtiter plates showed biofilm formation. This was particularly noticeable for *C. sakazakii* strains, where the reconstituted IMF became very thick and sticky after incubation at 37°C for 24 h. This phenomenon was considered to be due to the production of capsular polysaccharide, which has been reported in previous studies (Oh, Chen & Kang, 2007). Reconstituted IMF was the most effective media for *C. sakazakii* biofilm formation in the present trails.

In this study, 77.8% *C. sakazakii* strains produced biofilm at normal IMF concentration while only 16.7% *C. sakazakii* strains produced biofilm in artificial media. Clinical isolates of *C. sakazakii* produced the most biomass and viable cells (Chapter 4 Figures 5 and 10) in reconstituted IMF, suggesting that biofilm formation of *C. sakazakii* in IMF may be more hazardous where clinical isolates are present and reflects the potential hygienic problem of infant feeding tubes. Farmer et al. (1980) demonstrated that *C. sakazakii* strains isolated from clinical sources were distinctive in terms of colony morphology with a wrinkled appearance and leathery morphology. This colony morphology was due to the production of cellulose. The ability of *C. sakazakii* to produce cellulose may contribute to the important role played in biofilm-related infections (Grimm et al., 2008). The high biomass and viable cells produced by isolates obtained from dried infant milk formula (Chapter 4 Figures 5 and 10) show the potential risk of biofilm formation by *C. sakazakii* in the dairy industry.

L. monocytogenes produced less biomass and biofilm cells compared with *C. sakazakii*. *L. monocytogenes* growing in a food-processing environment may be exposed different levels of nutrients which depends on the location of plant. It is likely that this microorganism grows in reduced nutrient environments. This is in line with a previous report of Nowak et al. (2015) that the biofilm formation of *L. monocytogenes* was enhanced in nutrient-poor media compared with nutrient-rich media. The inaccessibility of nutrients may cause stress in *L. monocytogenes* cells thus prompting the biofilm formation. However, in the current study, the biomass of *L. monocytogenes* grown in reconstituted IMF was higher than the biomass in BHI. This might be due to the strains isolated from food samples, which could be suitable for nutrient rich environments. It is important to note that in the food processing environment, *L. monocytogenes* is more likely to exit as a multi-species biofilm with a complex bacterial community (Djordjevic,

Wiedmann & McLandsborough, 2002). This is also likely to influence biofilm formation both positively and negatively depending on the nature of the overall microbial population.

6.1.2 Surface material

Biomass and viable biofilm cells were higher on PVC microtiter plates than stainless steel for all *C. sakazakii* and *L. monocytogenes* strains which could be ascribed to the difference in the hydrophobicity of the cells and substrates. Djordjevic, Wiedmann and McLandsborough (2002) observed that *L. monocytogenes* was more likely to attach on a hydrophobic surface (PVC) than a hydrophilic surface (stainless steel) after 2 h of incubation. Kim, Ryu and Beuchat (2006) found that in most cases, higher biomass of *C. sakazakii* adhered on infant feeding tubes than stainless steel. Sommer, Martin-Rouas and Mettler (1999) proposed that the surface characteristics of bacteria including hydrophobicity, charge and surface appendages may influence the adhesion process. The hydrophobicity of the cell wall may be related to the differences in adhesion to hydrophobic and hydrophilic surfaces. In terms of a hydrophobic surface, hydrophobic interactions may be the dominant force for the adhesion to surfaces. For hydrophilic surfaces, the surface charge interactions may be more important than other factors. Due to the difference in hydrophobicity of PVC and stainless steel, we can assume that the hydrophobic nature of the cells promotes adhesion on PVC.

6.1.3 Static and fluid incubation

Biofilm formation of *C. sakazakii* and *L. monocytogenes* was tested in static and fluid conditions since both microorganisms are facultatively anaerobic. As shown in Chapter 4, Figures 14 – 17, the viable cells of *C. sakazakii* in the fluid condition were higher than in static condition however no difference in viable *L. monocytogenes* cells was observed in static and fluid conditions. It was assumed that oxygen presence had a noticeable effect

on biofilm formation by *C. sakazakii* but a minor effect on *L. monocytogenes* biofilm development. A possible reason was that IMF was the most effective media for *C. sakazakii* biofilm formation and the presence of oxygen in reconstituted IMF is able to cross cell membranes and enter cells to increase the formation of EPS. For *L. monocytogenes*, oxygen may be not the major factor for biofilm formation which in line with a previous report (Nowak et al., 2015).

6.2 Enzymatic treatment for removal biofilms

In order to select the most appropriate enzymes for biofilm removal, the components of the EPS should be known. Grimm et al (2008) indicated that cellulose was the major structural component of *C. sakazakii* EPS. Compared with biofilms of other microorganisms, the EPS of *C. sakazakii* was highly hydrated with the incorporation of water into structure to prevent desiccation from environment (Lehner et al., 2005). The structure of the EPS of *L. monocytogenes* reported is three-dimensional and the biofilms are composed of cells stacked upon each other around (Møretrø & Langsrud, 2004).

In the present study, endoglucanase and proteases were the methods for removing biofilms of *C. sakazakii* and *L. monocytogenes* from hydrophobic and hydrophilic surfaces. α-amylase was less effective for biofilm degradation. Molobela, Cloete and Beukes (2010) indicated that enzymes had the ability to remove biofilms by degrading the physical integrity of the EPS. The reason for the efficiency of proteases and endoglucanase most likely reflects the major components of the EPS. The degradation of proteins and cellulose in the EPS through hydrolysis of the protein and cellulose molecules, converting to small units that can be transported by cell membranes and then be metabolized is in agreement with a previous study (Molobela, Cloete & Beukes, 2010). Although there are no reports regarding the cellulose in the EPS of *L. monocytogenes* biofilm, it can be assumed that cellulose was also an essential component of the EPS of

L. monocytogenes biofilm based on the results in this study. Due to the complex polysaccharide structure and a greater range of polysaccharide bond linkages in EPS, it could be difficult for α-amylase to be effective.

The cleaning effectiveness of chemical solutions was similar to α-amylase (Figures 18 – 21). Alkaline solutions can induce the swelling of the EPS network providing access for the chemicals to remove biofilms as effectively as α-amylase treatment. However, as the cleaning efficacy of NaOH is not as effective as the proteases and endoglucanase in terms of biomass removal. Although the cells were killed by NaOH, the EPS still remained on the surfaces whereas with the enzyme treatment, much of the EPS was removed. Residual EPS may cause issues through providing a conditioning layer for further biofilm development.

6.3 Impedance detection and epifluorescence microscopy

The results showed that the viable biofilm cells detected by using the impedance measuring system were similar to the plate counts following recovery from surfaces using beads-beating. The impedance method is a time-saving and simple alternative to enumeration by plate counting. Successful use of this method depends upon a reliable calibration curve normally based on planktonic cells. The recovery of injured cells that cannot be counted on plates may result in a difference in results between plate counting and impedance results. There are also some questions around the efficacy in detecting biofilm cells, particularly if the metabolism of the biofilm cells is different to the planktonic cells.

The use of epifluorescence microscopy allows visualisation of the cells on a surface, whether or not they are alive. It also has the advantage in visualising any residual biofilm matrix. The microscopic images confirmed the plate count and impedance results for cell inactivation following treatment and the assumption that the biofilm cells are inactivated

under acidic conditions while the EPS remains. It is important to select a number of areas to observe in order to obtain a representative result for a sample. In addition, some other substances such as milk fouling will be stained and may make interpretation of results difficult.

6.4 Prevention of *C. sakazakii* and *L. monocytogenes* biofilms in the food industry

In the food industry, there is food debris everywhere which could prompt the accumulation of microorganisms and enhance biofilm formation. Therefore, regular cleaning of the contact surfaces is required to prevent the microbial contamination of food products (Srey, Jahid & Ha, 2013). As *C. sakazakii* and *L. monocytogenes* may occur in dairy products and environment, it is important to consider the hygienic plan and sanitation procedures for equipment and the food processing environment (Brooks & Flint, 2008). Particular care is needed for the control of *C. sakazakii* to prevent contamination of powdered IMF. Dairy manufacturing equipment, including pasteurizer and evaporators are cleaned every 6 – 12 h and extra stringent precautions are taken to ensure the environment is clean (Flint, Bremer & Brooks, 1997; Srey, Jahid & Ha, 2013). *L. monocytogenes* biofilm is a problem in slicing and dicing machines used for the preparation of sliced meat. Such equipment is required to be designed without dead spaces in order to be easily cleaned. In addition, since biofilm formation is a time-dependant process, it is important to immediately clean the contact surfaces after production. If the cleaning procedures are followed, it is impossible for bacteria to colonize and form thick biofilm (Møretrø & Langsrud, 2004). However, for non-contact surfaces such as walls, windows and floors, these areas are more likely to accumulate microorganisms because they are less frequently cleaned. The incidence of *L. monocytogenes* reported from non-contact surfaces is much higher than contact surfaces in dairy processing plants (Johnson, Doyle & Cassens, 1990).

The prevention of *C. sakazakii* and *L. monocytogenes* biofilm formation is also related to the contact surface material. Stevens and Holah (1993) reported that generally stainless steel is easier to clean than other hydrophobic surfaces. Stainless steel is found to be easier clean and disinfect to control *C. sakazakii* and *L. monocytogenes* in laboratories studies (Møretrø & Langsrud, 2004; Grimm et al., 2008; Srey, Jahid & Ha, 2013). As discussed above, stainless steel is widely used in food processing equipment and work benches.

6.5 Limitations and recommendations for future work

Although enzymes used in this study were effective for biofilm removal, there are still some issues in applying these enzymes for use in dairy manufacturing plant. These issues include concerns over residual enzyme activity that may act on the food product causing flavour issues. This problem can be avoided by ensuring thorough rinsing of the plant to remove residual enzyme and avoiding the temperatures and pH needed for the enzyme activity.

The cost of enzymes compared with other cleaners is a concern and the industry needs to be convinced of the cost effectiveness in terms of improvements in product quality, in order to justify the expense. The enzymes used in this study are novel in that they are active in acidic and hot conditions. This may enable the re-use of these enzymes as the conditions are not suitable for microbial growth. This re-use for subsequent cleans will make enzyme treatment more cost effective than a single use system. However, for the manufacture of some products such as whey protein manufacture, 85°C is the temperature that will denature whey protein and acids could have a negative effect on the sensory quality if residual acid is left in the manufacturing plant. Another concern is the effectiveness of enzymes on a variable EPS biofilm. The heterogeneity of the EPS in the biofilm can be overcome by using a combination of enzymes could be more effective in removing biofilms. Future study is recommended in the following areas:

1. Testing a combination of enzymes (α-amylase with two proteases and endoglucanase with two proteases) for biofilm removal.

2. Do some trials in an industry scale CIP system and explore the potential for a re-use strategy to make this treatment cost effective for the industry.

3. Trial enzymes for the removal of other contaminations, in particular thermophilic biofilms, from dairy manufacturing plant surfaces.

4. Trial these enzymes on multispecies biofilms.

Chapter 7. Final Summary and Conclusions

In this study, biofilm development by *C. sakazakii* and *L. monocytogenes* in reconstituted IMF formed the base material for trials using enzyme cleaners. In the present trial, the ability of *C. sakazakii* and *L. monocytogenes* isolates to form biofilm was determined. The clinical and dried infant milk formula isolates formed strong biofilms while other isolates from different sources varied in their ability to form biofilm. In addition, the nutrients of media, surface material and dynamic and static conditions were identified to affect the biofilm formation.

The effectiveness of alternative cleaning methods (enzymatic cleaning) as well as traditional chemical-based cleaning to control biofilms were determined based on culture growth, impedance assay and microscopic images. Before cleaning, the number of *C. sakazakii* biofilm cells reached 8 log CFU/ cm^2 with stained biomass level of O. D 3.48. The density of *L. monocytogenes* biofilm cells was less than *C. sakazakii*, at 7 log CFU/ cm^2. A reduction of biofilm cells was observed by plate counting leaving < 3 log CFU/ cm^2 after cleaning. The residual biomass, following treatment with α-amylase was similar to the biomass left following a traditional caustic clean. The biomass treated with proteases and endoglucanase was lower decreasing by 2 absorbance units after cleaning. Therefore, it can be concluded that the endoglucanase and proteases were most effective in removing biofilms based on the O.D value. Using epifluorescence microscopy, a decrease in viable cells on stainless steel coupons was observed as well after cleaning with proteases and endoglucanase, which could confirm that enzymes especially proteases and endoglucanase were effective cleaners for the removal of *C. sakazakii* and *L. monocytogenes* biofilms.

In conclusion, the formation of biofilms of *C. sakazakii* and *L. monocytogenes* is a concern for dairy manufacturing plant. The nutrients of growth environment, surface

material and oxygen presence were the factors of affecting biofilm formation. The cleaning trials demonstrated that proteases and endoglucanase have the potential to improve present chemical-based cleaning methods used in dairy manufacturing plant.

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Appendices

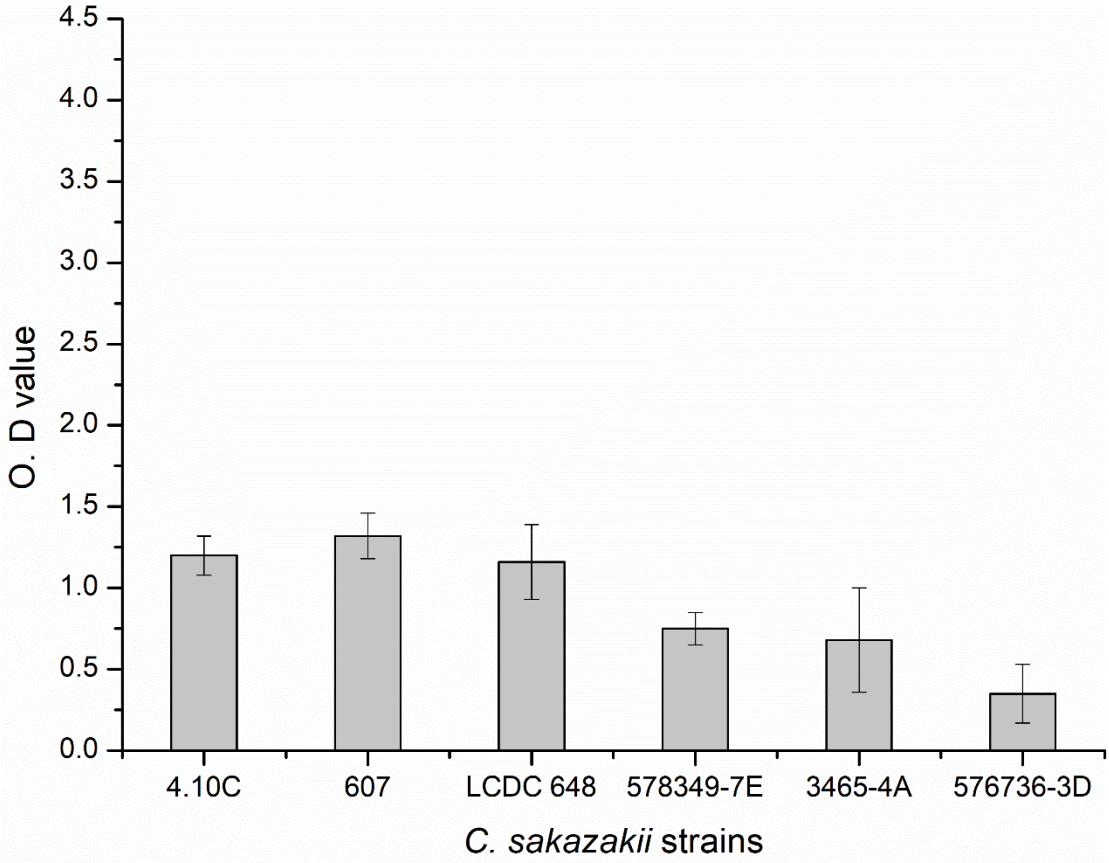

Figure 37. The O. D values of biofilms of *C. sakazakii* strains isolated from the dairy industry and clinical sources at 595 nm and grown in NB using a microtiter plate assay. Results are the mean and standard deviation from three replicates.

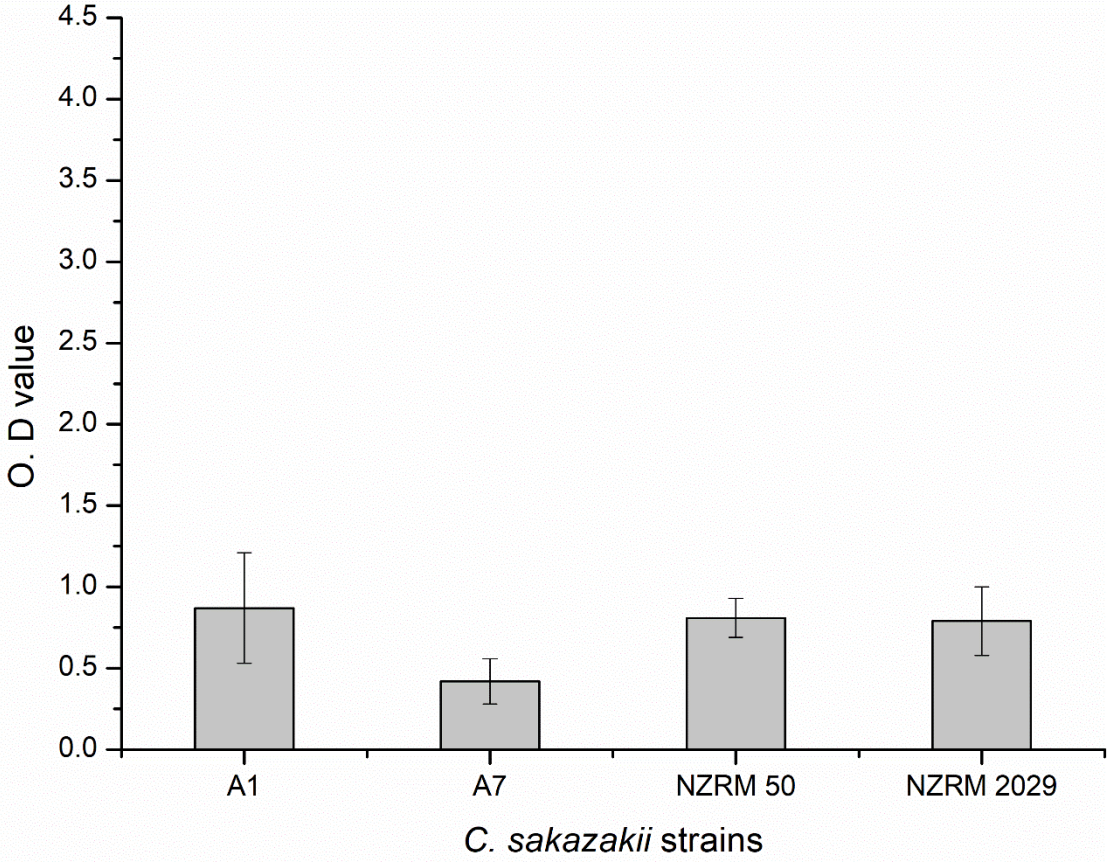

Figure 38. The O. D values of biofilms of *C. sakazakii* strains isolated from the AsureQuality at 595 nm and grown in NB using a microtiter plate assay. Results are the mean and standard deviation from three replicates.

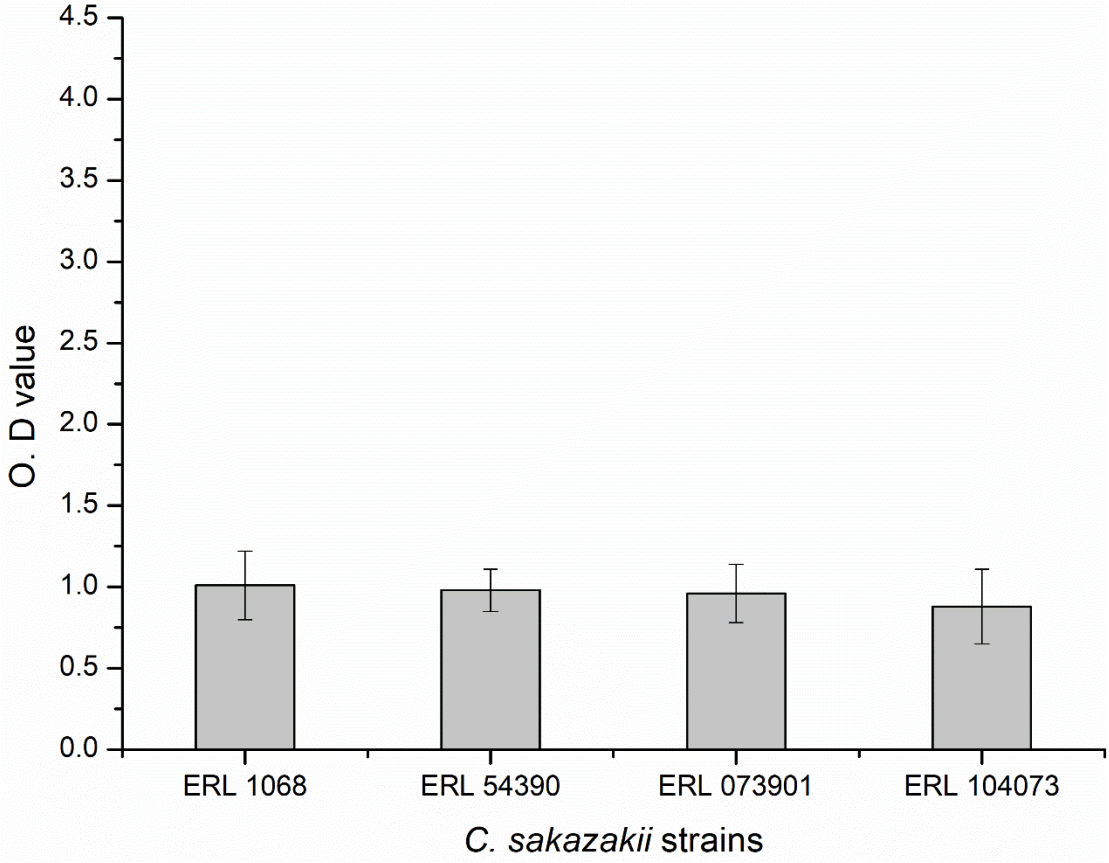

Figure 39. The O. D values of biofilms of *C. sakazakii* strains from ESR at 595 nm and grown in NB using a microtiter plate assay. Results are the mean and standard deviation from three replicates.

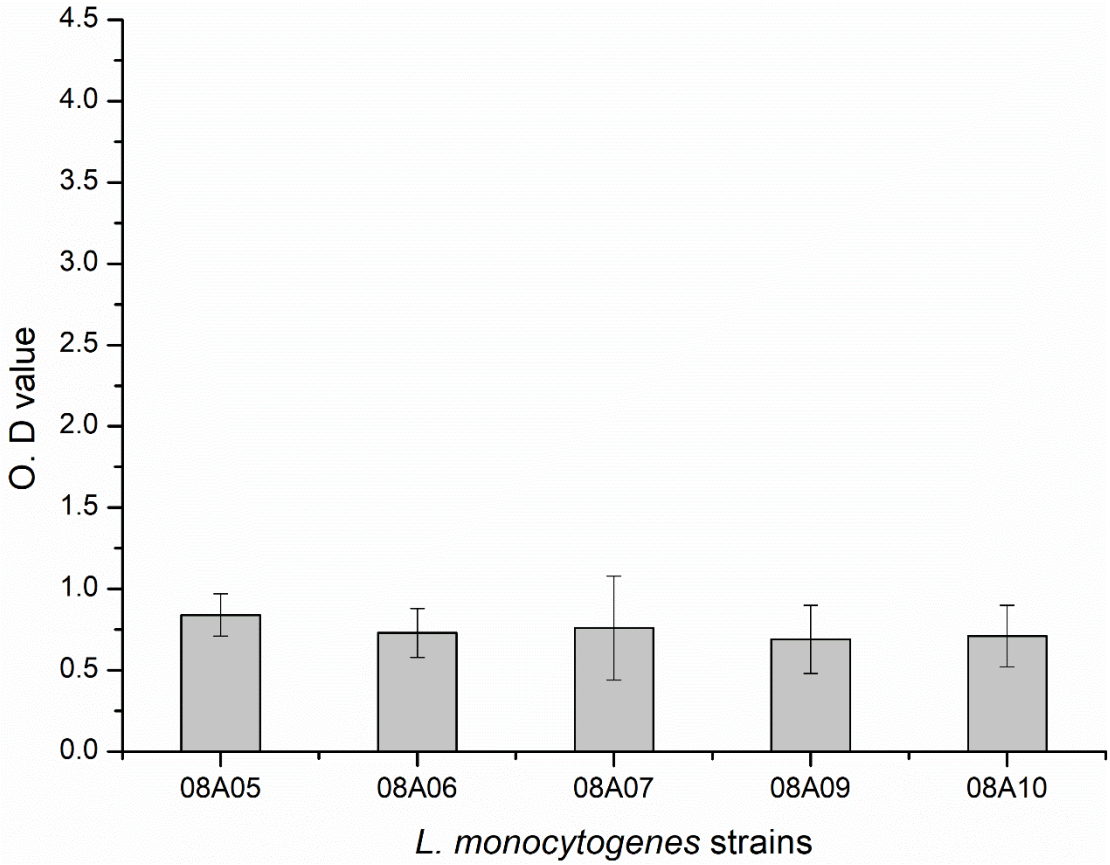

Figure 40. The O. D values of biofilms of *L. monocytogenes* strains from Plant and Food research Institute at 595 nm and grown in BHI using a microtiter plate assay. Results are the mean and standard deviation from three replicates.

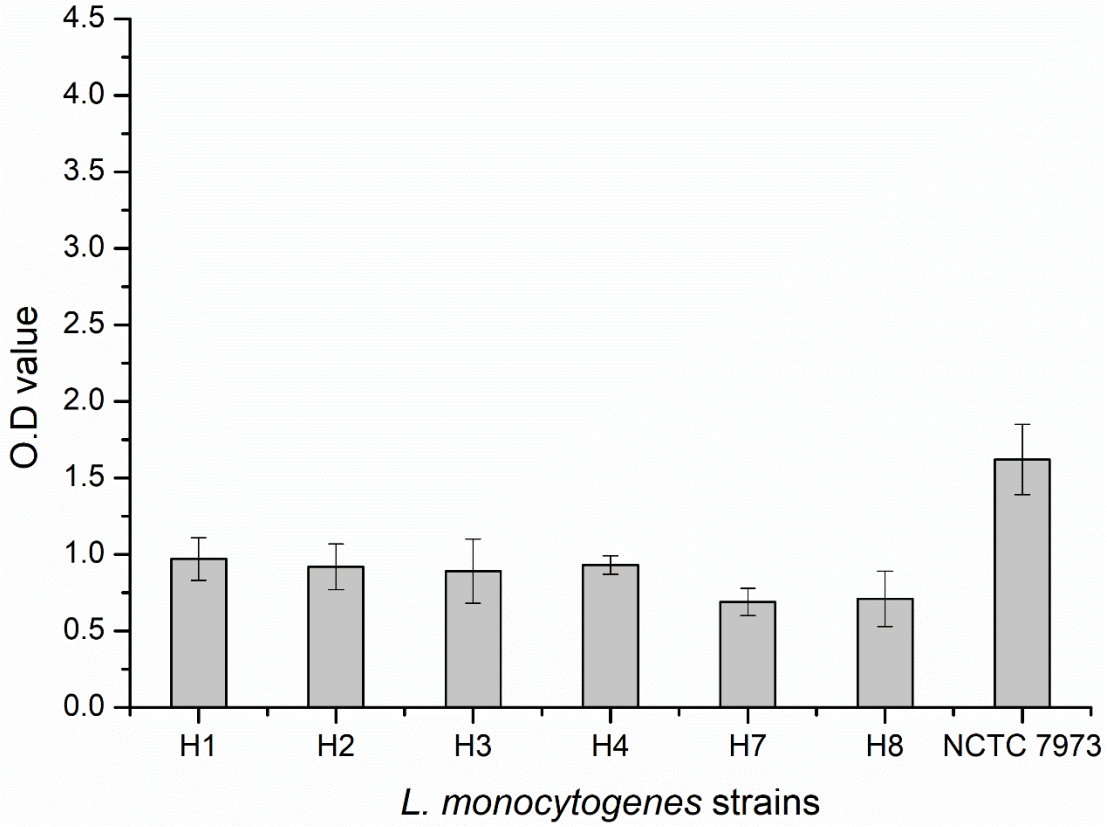

Figure 41. The O. D values of biofilms of *L. monocytogenes* strains from Hills Laboratories and National Collection of Type Cultures England at 595 nm and grown in BHI using a microtiter plate assay. Results are the mean and standard deviation from three replicates.