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BIOFILM FORMATION OF
***ENTEROBACTER SAKAZAKII* ON THREE**
DIFFERENT MATERIALS OF INFANT
FEEDING TUBE

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BIOFILM FORMATION OF
***ENTEROBACTER SAKAZAKII* ON THREE DIFFERENT MATERIALS**
OF INFANT FEEDING TUBE

A THESIS PRESENTED IN PARTIAL FULLFILLMENT OF THE REQUIREMENT S
FOR THE DEGREE OF MASTER OF TECHNOLOGY IN FOOD MICROBIOLOGY
AT MASSEY UNIVERSITY, PALMERSTON NORTH, NEW ZEALAND

SITI NORBAIZURA BINTI MD ZAIN

2009

IN LOVING MEMORIES OF MY BELOVED DAD

MD ZAIN BIN ABDULLAH

(1955 - 2003)

YOU ARE ALWAYS IN MY HEART

ABSTRACT

The aim of this study was to observe biofilm formation by *Enterobacter sakazakii* (*E. Sakazakii*) from different clinical, dairy and environmental origins on three infant feeding tubes made of different materials. Infant formula milk was selected as the medium for *E. sakazakii* growth.

Seventeen isolates from different origins were retrieved and tested for purity, using a plating method and biochemical tests to eliminate the non *E. sakazakii* strains from this study. A method to rapidly and accurately detect viable cells of *E. sakazakii* on infant feeding tube surfaces using of the BacTrac® 4000 microbiological growth analyser was developed. The sources of errors such as from cleaning, operation and handling procedures were assessed prior to experimental runs.

The strength of biofilm formation by different isolates of *E. sakazakii* on plastic surfaces was scrutinised using a microtiter plate assay. The results from the microtitre plate assay were based on the absorbance at 550 nm of crystal violet stained films and showed that all the clinical isolates were able to attach and form strong biofilms on the plate. Some environmental isolates formed strong or weak biofilms and some did not produce biofilm at all. However, dairy isolates formed both strong and weak biofilms in the microtitre plate when incubated in 10% reconstituted infant formula milk.

The further studies were to quantify biofilm formation by three isolates of different origin on three different materials of infant feeding tubes using a batch system. Tubing pieces were incubated with infant formula milk inoculated with *E. sakazakii* cells at approximately $8 \log \text{CFU mL}^{-1}$ and the biofilm formation was assessed at three time intervals: 4, 12 and 24 hours. Biofilm formation on the

tubing by clinical isolates was also observed using epifluorescence microscopy and the scanning electron microscope.

E. sakazakii from clinical, dairy and environmental isolates were able to form biofilm on three different materials of infant feeding tubes. The results showed that the initial attachment at 4 h on silicone tubing was low compared with the other two tubes. The scanning electron micrographs showed the surface characteristics of each tubing and the biofilm formation by *E. sakazakii* clinical isolates after 4, 12 and 24 hours. Silicone tubing appeared to be the best choice for premature babies that need feeding using feeding tubes, as it was slow to become colonised compared with the PVC and polyurethane tubing.

LIST OF PRESENTATIONS

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

1. Zain, S. N. M, Flint, S. H., Brooks, J. D. (2008). Assessment of *Enterobacter sakazakii* growth on three types of infant feeding tubes. *14th World Congress of Food Science and Technology*, Shanghai, China, October 2008.
2. Zain, S. N. M, Flint, S. H., Brooks, J. D. (2008). Differences in attachment of *Enterobacter sakazakii* in reconstituted infant formula with respect to origin. *New Zealand Microbiological Society conference*, Christchurch, November 2008.

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Chapter one

INTRODUCTION

This thesis is part of a programme of research at Massey University, Palmerston North, New Zealand, studying the biofilm formation by *E. sakazakii* on three different materials of infant feeding tubes.

E. sakazakii is recognised as an emerging opportunistic pathogen that has caused much concern in the food industry and also in the medical field. This bacterium is unique in that it is ubiquitous in the environment and for the most part affects only a small subset of the population, i.e. premature infants and infants under 1 year of age. Outbreaks do occur among adults but are underreported.

The intestinal tract of newborn infants is microbiologically sterile and the neonate rapidly acquires its microbial flora during passage through the vagina at birth, through contact with the environment and through feeding. The stomach of newborns, especially premature babies, is less acidic than adults; this may be a possible factor in the survival of an infection with *E. sakazakii* in infants. Powdered infant formula is used as an alternative to human breast milk in providing newborns with nutritional needs, either in addition to breast milk, or on its own when breast feeding is not possible.

Powdered infant formulas have been implicated as the cause of a number of infectious *E. sakazakii* outbreaks presenting as meningitis, necrotizing enterocolitis (NEC) and sepsis. *E. sakazakii* is ubiquitous in nature; however only powdered infant formula and preparation equipment have been linked to *E. sakazakii* outbreaks among infants. It also survives the drying process very well and can be found in dry food products such as skimmed milk powder, lactose, starch, lecithin, banana powder, all ingredients that can be added to powdered infant formula. Since *E. sakazakii* affects the youngest and

most vulnerable segment of our population, the issue has been raised to the highest levels of attention and effort.

E. sakazakii is reportedly capable of forming biofilms on most surfaces. These bacterial biofilms colonise a wide variety of substances including medical devices, wastewater treatment systems and tissues. Film formation is influenced by the surrounding environmental conditions of each individual situation. Growth on medical implants including gastrostomy tubes has been shown to lead to adverse conditions associated with patient health and tube deterioration and eventually leads to device removal. Contributing factors to biofilm development included nutrient formulas, antibiotics and various medications such as acid reducing drugs, duration of tube implantation and surface characteristics of the tubing.

The focus of this study was to define the ability of *E. sakazakii* to attach and form biofilm on hydrophobic surfaces, e.g. plastic surfaces of a microtitre plate. Furthermore, the biofilm formation of *E. sakazakii* was tested on three different materials of infant feeding tubes. This study aimed to develop a greater awareness of biofilm development by *E. sakazakii* in the tubes used to feed the infants and to provide recommendations to hospitals concerning the tubing selection, temperature manipulation and control selection during infant feeding treatment.

E. SAKAZAKII ASSOCIATION WITH FATAL OUTBREAKS AMONG NEONATES

A description of the bacterium and biofilm formation ability, infant formula and feeding tube and the current concerns regarding this issue.

A REVIEW OF THE LITERATURE

2.0 Introduction

2.1 *Enterobacter sakazakii* (*E. sakazakii*)

2.1.1 Genotype and phenotype

2.1.2 Sources- clinical, food and environment

2.1.3 Characteristics of *E. sakazakii*- capsulated or non-capsulated

2.1.4 Survival and growth characteristics

2.1.5 Virulence factors and pathogenicity

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2.2 Infant formula

2.2.1 Composition

2.2.2 Sterility of powdered infant formula

2.3 Infant feeding tube

2.3.1 Feeding tubes

2.3.2 Methods of feeding /delivery

2.3.3 Material of the tubes- polyvinyl chloride, polyurethane and silicone

2.3.4 Importance of microbial colonisation of infant feeding tubes

2.4 Conclusion

Chapter 2

Literature review

2.0 Introduction

An incident in New Zealand in 2004 linked powdered infant formula to the death of a premature infant after contracting *E. Sakazakii* meningitis. A follow-up investigation in the neonatal intensive care unit (NICU) found that four other infants had been infected with the organism. Powdered infant formula was confirmed as the source. *E. sakazakii* invasive disease was made notifiable in New Zealand in 2005 (Ministry of Health, New Zealand, 2005).

2.1 *Enterobacter sakazakii* (*E. sakazakii*)

E. sakazakii is a Gram-negative facultative anaerobe and belongs to the Enterobacteriaceae family. It is straight and rod-shaped, 0.3 to 10 µm long by 1.0 to 6.0 µm wide. It is motile by peritrichous flagella and grows over a wide temperature range (6 to 45°C). It is an oxidase-negative, non-sporeforming, non-halophilic and non-acid fast bacterium (Figure 2.1).

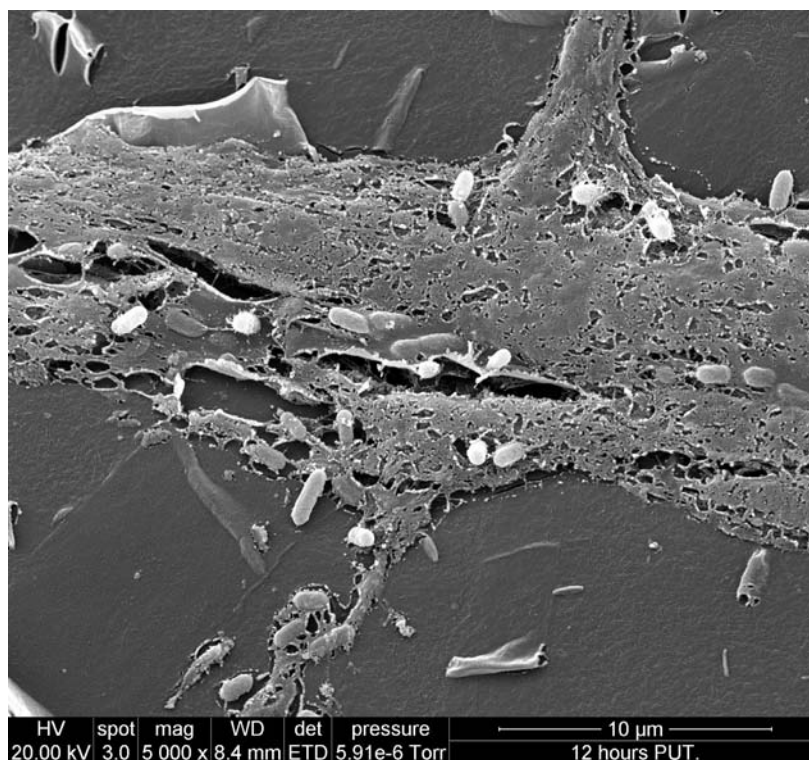


Figure 2.1: Scanning electron micrograph of *E. sakazakii* cells that attached to polyurethane infant feeding tube after 12 hours incubation in 10% reconstituted infant formula.

2.1.1 Genotype and phenotype

E. sakazakii was originally known as yellow-pigmented *Enterobacter cloacae* (*E. Cloacae*) and was described as a new species in 1980 (Farmer et al., 1980). This bacterium possesses biochemical reactions similar to *E. cloacae*, but was distinguished based on differences in DNA, yellow pigment production, some biochemical traits and antibiotic susceptibilities. The DNA-DNA hybridization studies by Farmer and colleagues (1980) showed that there is no clear generic assignment for *E. sakazakii* as it was 53-54% related to *Enterobacter* and *Citrobacter* species. Comparisons with type strains of these two genera showed that *E. sakazakii* was 41% related to *C. freundii* and 51% related to *E. cloacae*. *E. sakazakii* was assigned to the *Enterobacter* genus as it has a similar phenotype to *E. cloacae* (Table 2.1).

Most (~75%) *E. sakazakii* strains form yellow-pigmented colonies that range from bright to pale yellow. Gurtler et al. (2005) noted that the bacterium produced greater pigment at temperatures less than 36°C, with optimum pigment production at 25°C. Nazarowec-White and Farber (1997) also stated that *E. sakazakii* produced a stronger yellow pigment at 25°C compared with 36°C. The yellow pigment produced by *E. sakazakii* is a protection mechanism from UV rays in sunlight (Mullane et al., 2006). The only other species of *Enterobacter* that may produce a yellow pigment is *E. agglomerans*.

Table 2.1: Biochemical differentiation of opportunistic *Enterobacter* species

Test	Reaction ^a				
	<i>E. sakazakii</i>	<i>E. cloacae</i>	<i>E. aerogenes</i>	<i>E. agglomerans</i>	<i>E. gergoviae</i>
Lysine decarboxylase	-	-	+	-	+
Arginine dihydrolase	+	+	-	-	-
Ornithine decarboxylase	+	+	+	-	+
KCN, growth in	+	+	+	V	-
Fermentation of:					
Sucrose	+	+	+	(+)	+
Dulcitol					
Adonitol	-	(-)	-	(-)	-
D-sorbitol	-	(-)	+	-	-
Raffinose	-	+	+	V	-
α-methyl-D-glucoside	+	+	+	V	+
D-arabitol					
	+	(+)	+	-	-
	-	(-)	+	-	+
Yellow pigment	+	-	-	(+)	-

^a Where + represents 90-100% positive;

(+): 75-89% positive;

v : 25-74% positive;

(-) : 10-24% positive;

- : 0-9% positive

(Nazarowec-White and Farber, 1997)

2.1.2 Sources - clinical, food and environment

E. sakazakii is responsible for clinical illness mostly in infants and has been isolated from spinal fluid, blood, respiratory tract, the intestines and skin or wounds of clinically infected patients. It has also been isolated from bone marrow, eye, ear and a breast abscess in patients without clinical infection (Farmer et al., 1980). Clinically important disease is normally associated with food, particularly infant formula.

A variety of different foods have been found to contain *E. sakazakii*. Muijtjens et al., (1988) isolated twenty *E. sakazakii* from 141 samples of milk based infant formulae. Other foods that have been found containing this organism include cheese, fermented bread, tofu, sour tea, cured meats, sausage meat, minced beef, vegetables, grains, herbs and spices (Iversen and Forsythe, 2003). It has also been isolated from UHT milk where contamination is most likely occurring from the processing plant environment following UHT processing (Skladal et al., 1993).

E. sakazakii is widespread in the general environment and has been isolated from processing plant environments (Skladal et al., 1993). This bacterium also has been isolated from floor drains, air, a vacuum canister, broom bristles, a room heater and electrical control box, transition socks, a clean-in-place (CIP) valve, a floor dryer, floor and condensate in a dry product processing environment in the United States (Gurtler et al., 2005). Interestingly, Muijtjens and Kollee, (1990) could not isolate this bacterium from surface water, soil, mud, rotting wood, grain, bird dung, rodents, domestic animals, cattle and raw cow's milk. This may be due to the sensitivity of the bacterium to competition from other micro-organisms in these heavily contaminated samples. Kandhai et al., (2004) isolated *E. sakazakii* from almost all environments examined, including milk powder manufacturing facilities and household vacuum cleaners (Table 2.2).

Table 2.2: Environmental samples tested for the presence of *E. sakazakii*

	Number of samples analysed	Number of samples positive for <i>E. sakazakii</i> (%)	95 % Confident limit
Origin:			
Milk powder factory	23	2 (9%)	0.01-0.27
Milk powder factory	26	9 (35%)	0.19-0.53
Milk powder factory	11	1 (9%)	0.002-0.404
Milk powder factory	8	2 (25%)	0.03-0.64
Chocolate factory	8	2 (25%)	0.03-0.64
Cereal factory	9	4 (44%)	0.15-0.78
Potato flour factory	15	4 (27%)	0.08-0.53
Pasta factory	26	6 (23%)	0.10-0.42
Spice factory	5	0	0-0.52
Households	16	5 (31%)	0.12-0.57

(Kandhai et al., 2004)

2.1.3 Characteristic of E. sakazakii – capsulated or non-capsulated

Some strains from these organisms are capsulated and this may give these strains a selective advantage by protecting them from the environment. Interestingly, Iversen et al., (2004) found that during the first 18 months of desiccated storage of *E. sakazakii*, there was no correlation between capsulation and recovery of viable organisms. However, after 2 years, four of five strains recovered were capsulated, and the only strains recoverable after 2.5 years were the capsulated strains. Therefore, capsulation may play an important role in recovery after extended periods. Fewer sublethally injured cells were generated after the desiccation of capsulated strains of *E. sakazakii* than non-capsulated species, which may explain why there was no prolonged lag time when desiccated *E. sakazakii* was recovered from infant formula after a period of 3 to 10 days. Furthermore, the formation of an extracellular polysaccharide could provide protection to *E. sakazakii* against physical and environmental stress. Iversen et al., (2004) suggested that the capsule produced by *E. sakazakii* might increase its ability to attach to surfaces and form biofilms. In addition, a capsulated strain formed denser biofilms compared with a noncapsulated type strain.

2.1.4 Survival and growth characteristics

E. sakazakii has several distinctive characteristics such as biofilm formation, adherence to hydrophilic and hydrophobic surfaces, production of extracellular polysaccharides and cell-to-cell signalling with other bacteria, which may help promote environmental persistence and the formation of a physical barrier to protect cells from environmental stress such as heat, dry conditions, antibiotics, osmotic stress and UV light (Lehner et al., 2005; Kim et al., 2008a).

Kim et al., (2006) found that *E. sakazakii* can produce biofilm on many surfaces including silicone, latex, polycarbonate, glass, polyvinyl chloride and stainless steel. The cells produce exopolysaccharide that forms a capsule or slime layer surrounding the cells. This capsule is biologically significant in that it serves as a

safeguard against phagocytosis or rapid drying leading to death. *E. sakazakii* produces heteropolysaccharides composed of galactose, fructose, glucose, glucuronic acid and acetate (Shimada et al., 1997). Breeuwer et al., (2003) reported that *E. sakazakii* was more resistant than other Enterobacteriaceae to dry environments, such as found in milk powder factories, over a 46-day period. Production of exopolysaccharide by *E. sakazakii* may enhance its ability to survive in low water activity (a_w) environments.

2.1.5 Virulence factors and pathogenicity

The pathogenesis of *E. sakazakii* has been extensively studied at a molecular level. *Enterobacter* species all possess endotoxin as a typical fever inducing, virulence factor of Gram-negative bacteria. Another virulence factor of *E. sakazakii* is enterotoxin. Pagotto et al., (2003) reported that four of 18 strains tested in their study were found positive for enterotoxin production. They investigated the *E. sakazakii* virulence factors using the suckling mouse assay with orally and intraperitoneal injections of *E. sakazakii* from clinical and food sources. Pathogenesis in neonates frequently involves bacteremia and/or sepsis, cerebrospinal fluid (CSF) infection and meningitis, brain abscess and infarction, ventricle compartmentalization due to necrosis of brain tissue and liquefaction of white cerebral matter, cranial cystic changes, fluid collection and dilated ventricles and haemorrhagic and non-haemorrhagic intercerebral infarctions leading to cystic encephalomalacia (softening of the brain) and has been associated with necrotizing enterocolitis.

2.1.6 Disease associated with E. sakazakii

E. sakazakii is now considered to be an opportunistic pathogen implicated in food-borne diseases. It is an occasional contaminant of powdered infant formulae that can cause a rare, but life-threatening form of neonatal meningitis, bacteraemia, necrotizing enterocolitis (NEC) and necrotizing meningoencephalitis after ingestion (Biering et al., 1989; Iversen and Forsythe, 2003; Iversen and Forsythe, 2004; Gurtler et al., 2005).

The incidence of all three forms of *E. sakazakii* invasive infection appears to be higher among infants than older age groups. Meningitis, an acute inflammation of the meninges surrounding the brain and the spinal chord, frequently results in mortality. Meningitis is the most frequently reported condition in neonatal *E. sakazakii* infections, resulting in *ca.* 90% of the cases leading to brain abscesses. The onset of *E. sakazakii* septicaemia and meningitis is sometimes without causal explanation in neonates and infants. Low birth weight (i.e. under 2.5 kg) has been identified as a contributor to higher risk of contracting illness caused by *Enterobacter* species. Mortality is predictably high, with up to 92% mortality reported in cases involving *E. sakazakii*. Meningital *E. sakazakii* infection has been reported as arising between the fourth and fifth day after birth and can be fatal within a few hours to several days following the first clinical signs.

Necrotizing enterocolitis (NEC) is the most common important gastrointestinal illness in the premature newborn due to the immature nature of the intestinal tract and it can be caused by a variety of bacterial pathogens. The pathogenesis is associated with neonatal intestinal ischaemia, microbial colonisation of the gut and excess protein substrate in the intestinal lumen. The latter is associated with oral formula feeding. Both meningitis and NEC caused by *E. sakazakii* have a high mortality rate of 40-80% and 55%, respectively. Bacterial contamination in powdered infant formula, notably *E. sakazakii*, can cause fatal outcomes in newborns. Several publications implicated contaminated infant milk formula as the source of neonatal infection (Muytjens et al., 1983; Van Acker et al., 2001; Forsythe, 2005). Bar-Oz et al., (2001) implied that rehydrated infant formula milk as well as the equipment and utensils used to prepare the formulae in hospitals were potential sources of contamination for neonatal infections. Rapid growth of *E. sakazakii* in contaminated formula might account for nosocomial neonatal infections associated with the pathogen.

2.1.7 Hazard identification

The following summaries are adapted from a meeting conducted by the WHO at Geneva in 2004 regarding *E. sakazakii* and other microorganisms in powdered infant formula.

a. Category A organisms - clear evidence of causality

Enterobacter sakazakii and *Salmonella enterica* are in category “A” because both are well-established causes of illness in infants (e.g. systemic infection, necrotizing enterocolitis and severe diarrhoea) and they have been found in powdered infant formula. Contaminated powdered infant formula has been convincingly shown, both epidemiologically and microbiologically, to be the vehicle and source of infection in infants.

b. Category B organisms - Causality plausible, but not yet demonstrated

Other Enterobacteriaceae are in category “B” because they are well-established causes of illness in infants (e.g. systemic infection, NEC and severe diarrhoea) and have been found in powdered infant formula, but contaminated powdered infant formula has not been convincingly shown, either epidemiologically or microbiologically, to be the vehicle and source of infection in infants. These organisms include, for example: *Pantoea agglomerans* and *Escherichia vulneris*.

c. Category C organisms - Causality less plausible or not yet demonstrated

Other microorganisms are in category “C” either because, despite causing illness in infants (e.g. systemic infection, NEC and severe diarrhoea), they have not been identified in powdered infant formula or although having been identified in powdered infant formula, they have not been implicated as causing such illness in infants. These organisms include *Bacillus cereus*, *Clostridium botulinum*, *C. perfringens* and *Staphylococcus aureus*.

2.2 Infant formula

E. sakazakii was found in 2.5 to 14% of powdered infant formula milk at concentration of 0.36 to 66.0 CFU/100g (Muytjens et al., 1988). Infant formula comes in three forms in the market:

- a. **Powder** - the least expensive of the infant formula. It must be mixed with water before feeding. This might not be a sterile product.
- b. **Liquid concentrate** - must be mixed with an equal amount of water.
- c. **Ready to feed** - the most expensive form of formula that requires no mixing. This is a sterile product.

2.2.1 Composition

Powdered infant formula is used as an alternative to human breast milk in providing newborns with nutritional needs either in addition to breast milk, or on its own when breast feeding is not possible. Dried infant formula milk has a low water activity (a_w) of 0.2. Breeuwer et al., (2003) reported that *E. sakazakii* is resistant to osmotic and dry stress, such as found in infant formula powder. Lin and Beuchat, (2007) also observed that the bacterium can survive in infant cereal at $a_w = 0.30$ to 0.69 for up to one year. IFM has high total solids, fat and sugar contents, which may further protect the microorganism from heat.

Milk or milk proteins have been shown to either enhance or inhibit the growth of bacteria on abiotic surfaces (Nonnecke and Smith, 1984; Gomes et al., 1998). Infant formula milk (IFM) may provide the nutrients that *E. sakazakii* requires for pre-attachment to stainless steel or feeding tubes to survive and grow (Van Acker et al., 2001).

2.2.2 Sterility of infant formula milk powder

Powdered infant formula preparations are not manufactured as sterile products. Powdered milk based infant formulas are heat-treated during processing, but unlike liquid formula products, they are not subjected to high temperatures for sufficient time to make the final packaged product commercially sterile. Farmer et al., (1980) noted that a strain that would now be defined as *E. sakazakii* was isolated from dried milk in 1960. Therefore, *E. sakazakii* has been present in dried milk products for many decades. Reconstituted infant formula milk is nutritious and can support bacterial growth when given favourable conditions of water availability, time and temperature. Therefore, once rehydrated, the only remaining barriers to increased bacterial growth and risk of infection are time and temperature. Cells grown in the infant formula milk may have a greater ability to attach than those grown in other media (Iversen et al., 2004; Oh et al., 2007). Infant formula milks are protein-rich media that contain casein and whey protein. Nutrients and other components play a major role in the attachment of microorganisms to surfaces of various materials Rolandelli (2005).

Dry infant formula is manufactured according to three process types:

- a. **Wet-mix process:** all ingredients are handled in a liquid phase and heat-treated (critical control point [CCP]), e.g. pasteurized or sterilized and then dried.
- b. **Dry-mix process:** individual ingredients are prepared, heat-treated as appropriate, dried and then dry-blended.
- c. **Combined process:** some of the ingredients are processed according to (a), in order to produce a base powder to which the rest of the ingredients are added according to (b).

Two main routes through which *E. sakazakii* can enter the reconstituted infant formula are known:

a. **Intrinsic contamination**

Either through contaminated ingredients added after drying or from processing environment following drying and before packing.

b. **Extrinsic contamination**

Through external contamination of the formula during reconstitution and handling.

For example, through poorly cleaned utensils during preparation.

The role of powdered milk formula in the development of NEC should not be underestimated. Milk formula can serve not only as an ideal substrate for bacterial growth but also as a source of possible pathogens, as most formula products are intrinsically contaminated.

E. sakazakii has been detected in 14.2% of powdered infant formulas originating from 35 countries (Muytjens et al., 1988). Iversen et al., (2004) reported that 2.4% of the powdered infant formula milk (IFM) produced in nine countries contained *E. sakazakii*. Nazarowec-White and Farber, (1997) examined infant formula milk powder manufactured by five companies (48 cans per company) and detected this pathogen at an average population of 0.36 colony-forming-units per gram in 8 cans. This is similar to the value of 8 cells/100g estimated by Simmons et al., (1989) for an open can of powdered milk formula used during the time of an outbreak in a neonatal intensive care unit (NICU). Estuningsih et al., (2006) found that 13.5% of ten samples from Indonesian manufacturers yielded *E. sakazakii*. Meanwhile, Witthuhn et al., (2007) managed to isolate four *E. sakazakii* strains from 22 products in South African food products (18%) which were milk with lactose free infant starter milk formula, full cream instant milk powder, soy infant formula and infant cereal. Shaker et al., (2007) found 17.4% of infant food and infant formula milk were positive for *E. sakazakii*.

The frequency of intrinsic *E. sakazakii* contamination in powdered infant formula is of concern, even though intrinsic concentration levels of *E. sakazakii* appear to be typically very low. In a study of the prevalence of *E. sakazakii* contamination in 141 powdered infant formulas, twenty were found culture-positive, yet all met the microbiological specifications for coliform counts in powdered infant formula (<3 CFU/g) of the current CODEX code (Muytjens et al., 1988; Van Acker et al., 2001).

In an attempt to lower the risk of infant formula causing neonatal *E. sakazakii* infections, interim guidelines were issued in the U.S. (Himelright et al., 2002) for proper procedures for preparing, feeding and storing powdered infant formula in health care facilities.

2.3 Infant feeding tubes

Frequently, premature babies are placed in Neonatal Intensive Care Units (NICU) and they are unable to get as many calories as they need through regular feeding from a bottle. The nurses will use a small feeding tube (e. g. 5-French) to deliver infant formula milk (Fig 2.2). The tubes are either placed into the babies' stomach through the mouth or by the nose. If an infant is able to take some milk from the bottle, the nurse will just give the rest through the feeding tube. The feeding tubes should not be painful as they are taped in place so they would not move around and cause friction. However, if they are in place for a long time they can cause erosions in the stomach or nose against which they rub, and so should be changed routinely to avoid this.

PROXIMAL

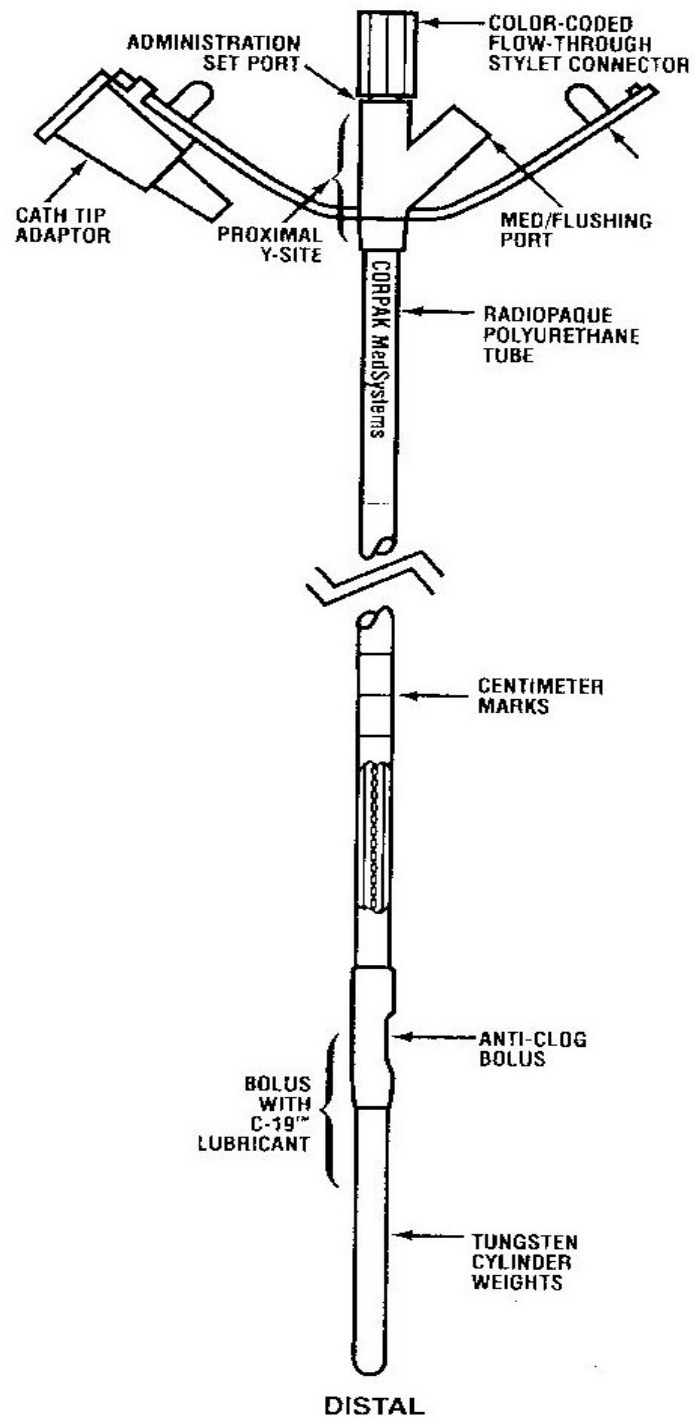


Figure 2.2: The diagram of standard feeding tube made from polyurethane (Guenter & Silkroski, 2001).

2.3.1 Feeding tubes

A feeding tube is a medical device used to provide nutrition to patients who cannot obtain nutrition by swallowing. A variety of feeding tubes are used in medical practice. They are usually made of polyurethane or silicone. The diameter of a feeding tube is measured in French units (each French unit equals 0.33 millimeters). They are classified by site of insertion and intended use.

1. Less than 3 - 4 weeks
 - a. aspiration risk or delayed gastric emptying: nasoduodenal or nasojejunal tube
 - b. no aspiration risk or gastric problems: nasogastric tube
2. Greater than 3 - 4 weeks
 - a. aspiration risk or delayed gastric emptying: jejunostomy or combined gastrostomy-jejunostomy
 - b. no aspiration risk or gastric problems: gastrostomy tube

2.3.2 Methods of feeding / delivery

The choice of a method of delivery is based on the location of the feeding tube tip, the patient's tolerance to tube feedings and pre-existing medical conditions and caretaker and lifestyle needs. Information about various methods of delivery are presented in Table 2.3

Table 2.3: Taken from Rolandelli (2007)

Several options for feeding using different types of feeding tubes

Number	Methods of delivery
1	<p>Continuous tube feedings:</p> <ol style="list-style-type: none"> 1. Tube feedings are administered via infusion pump delivering constant volume per unit time 2. Preferred for the following patients with: <ul style="list-style-type: none"> • Poor glycemic control • Refeeding syndrome • Jejunostomy • Intubation due to respiratory failure • Poor tolerance to intermittent gravity drip feedings
2	<p>Intermittent gravity drip tube feedings:</p> <ol style="list-style-type: none"> 1. Administration of 240 – 720 mL of enteral formula over 20 - 60 minutes 2. Number of feedings per day is dependent upon formula requirements 3. Preferred for patients requiring long-term nutritional support 4. Allow patients to be mobile, without confinement to an infusion pump 5. Contraindicated with jejunal feeding tubes
3	<p>Bolus feeding:</p> <ol style="list-style-type: none"> 1. Administration of 240 - 480 mL of enteral formula over 10 - 20 minutes 2. Number of feedings per day is dependent upon formula requirements 3. Preferred for patients requiring long-term nutritional support 4. Allow patients to be mobile, without confinement to an infusion pump 5. Contraindicated with jejunal feeding tubes

	6. Reserved for the patient on a stable tube feeding regimen
4	Cyclic tube feedings: <ol style="list-style-type: none">1. Tube feedings are administrated via an infusion pump over a specified period of time2. Preferred for patients requiring supplement tube feedings3. Recommended for patients with jejunal tube feedings, to allow time off of the infusion pump

Regardless of the method of delivery, patients receiving enteral nutrition should be routinely monitored for tolerance. There are several parameters that should be monitored during initiation, advancement and duration of therapy. These parameters include nausea, vomiting, gastric residual volumes, bowel pattern, frequency and number of bowel movements, findings on an abdominal examination and biochemical markers. It was originally thought that patients receiving enteral nutrition should remain in a semi recumbent position at a 30 to 45 degree angle to minimize gastroesophageal reflux.

2.3.3 Material of the tubes – Polyvinylchloride (PVC), polyurethane and silicone

Table 2.4 Taken from (Guenter and Silkroski, 2001)

The tube characteristic and its nursing consideration

Characteristic	Nursing Considerations
Material	
Polyvinylchloride (PVC)	Easy to insert and check residuals. Uncomfortable to patient and stiffens more with time. Standard nasogastric tubes for suction are made of these materials.
Polyurethane	Adequate stiffness for insertion and checking residuals. Good patient comfort and durability. Most feeding tubes are made of his material.
Silicone	Too soft for insertion and checking residual. Excellent patient comfort but damages easily.

Some examples of the types of materials that infant feeding tubes are composed of are shown in Table 2.4. All medical devices such as catheters, pacemakers, prosthetics heart valves, joint replacements and percutaneous endoscopic gastrostomy (PEG) can be colonised by microorganisms which form a biofilm consisting of a mono or multilayer of cells embedded within a matrix of extracellular polymeric material. Trevisani et al., (2005) noted that fungal colonisation plays an important role in inducing deterioration of silicone tubes and the use of polyurethane tubes as been recommended to prevent the problems posed by fungus colonisation. Surprisingly, silicone enteral feeding devices have been found to be significantly impaired after an 8-week immersion in commercial liquid feeding formulas. It has been suggested that silicone softening could be caused by soaking of the medium-chain triglycerides oil contained in the enteral formulas (Trudel et al., 1998). A recent study investigating 18 silicone gastrostomy devices found that all devices exhibited biofilm growth. Twenty-four bacterial species were identified and they were strongly suspected to have played a significant role in device failure (Dautle et al., 2003). The gastrostomy tube is an ideal incubator as it has high humidity, a temperature close to 36 - 37°C and regular provision of fresh culture medium.

Sartori et al., (2003) also reported that fungal colonisation on silicone as one of a factor of deterioration. Fungi use alkanes as a carbon source and a component used in this context by many fungi is epoxidized oil, a plasticizer-stabilizer also used in silicone tubes.

Silicone catheters deteriorated and had to be removed and replaced more frequently than polyurethane catheters (25/69 vs 36/228, $P=0.0005$) (Sartori et al., 2003). Moreover, tube deterioration occurred significantly earlier in the 25 silicone catheters than in the 36 polyurethane catheters. However, the same trial reported some interesting differences between the two materials.

The median survival of the tubing was 916 days for the polyurethane PEG catheter and 354 days for the silicone catheter. The PEG catheter had a clear advantage over the silicone catheter when the survival of bacteria on the catheters was compared 400 days after placement.

Roberts and Davidson, (1986) found that *Aspergillus fischeri* and *Aspergillus niger* were able to utilize the stearamide component of the PVC film / tubing as a carbon source. Growth of fungi on the PVC film strongly suggested that the film could be utilized as a nitrogen source too. In summary, the whole PVC film was found to be susceptible to fungal attack.

2.3.4 Importance of microbial colonisation of infant feeding tubes

Enteral feeding tubes can act as foci for pathogenic bacteria. Mehall et al., (2002b) reported that 71 of 125 tubes from 50 neonates had bacterial counts of > 1,000 CFU per tube. During the study, seven neonates developed NEC. All were fed formula contaminated with greater than 100,000 CFU of Gram-negative bacteria per tube. Mehall et al., (2002a) also showed that enteral feeding tubes can act as reservoirs for nosocomial antibiotic-resistant pathogens.

Antibiotic-resistant nosocomial bacteria can be harboured in enteral feeding tubes Mehal et al., (2002a). Moreover, the passage from a clinically infected patient to a feeding tube in another patient also was shown to occur. The bacteria were trafficked in both directions between patients with contaminated feeding tubes and those with clinical infections.

2.4 Conclusions

Powdered infant formula milk (PIFM) is used as an alternative to human breast milk in providing newborns with nutritional needs either in addition to breast milk, or on its own when breast feeding is not feasible. Because PIFM contains an abundance of nutrients to potentially support the growth of *E. sakazakii*, appropriate temperature control and hygiene of reconstituted formula are critical to inhibiting multiplication and minimizing the risk of illness.

Despite the fact that formulas are exposed to heat treatment during processing, *E. sakazakii* was still isolated from these products. Post-processing contamination of the infant formula from food production environments may be responsible for the presence of this pathogen in infant formula (Shaker et al., 2007). The presence of *E. sakazakii* in PIFM can be due to post-pasteurisation contamination during mixing with non-heated ingredients such as minerals or vitamins or during filling and packing (Breeuwer et al., 2003).

In order to help reduce the risk of infant illness, reconstituted infant formula at ambient temperature is generally held no longer than 4h in neonatal intensive care units to minimize the potential for growth of *E. sakazakii*. Longer storage of reconstituted formula should be at $\leq 4^{\circ}\text{C}$.

The following are guidelines for the preparation and handling of infant formula (Himelright et al., 2002);

- Formula products should be selected based on nutritional needs; alternatives to powdered forms should be chosen when possible.
- Trained personnel should prepare powdered formula using aseptic techniques in a designated preparation room.
- Manufacturer's instructions should be followed; product should be refrigerated if not fed immediately and discarded if not used within 24 h after preparation.
- The administration or "hang" time for continuous enteral feeding should not exceed 4 h.

-
- Written hospital guidelines should be available in the event of a product recall, including notification of health care providers, a system for reporting, follow-up of specific formula products used and retention of recall records.

Although these guidelines will help in reducing the risk of infant infection due to *E. sakazakii*, growth in the infant feeding tubes is a concern. Although the tubes are rinsed after feeding, they remain in the infant for many feeds. Residual nutrients on the tube surface are likely to provide nutrients for any microbes attached to the tubing surface, allowing growth between feeds and during subsequent feeds. Microbial colonisation of the tubing does occur but the effect of different materials used for feeding tubes on colonisation with *E. sakazakii* is not clear. We do not know whether there is any difference in the ability of *E. sakazakii* strains from different sources to colonise infant formula tubing.

MATERIALS AND METHODS

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Chapter 3

Materials and methods

3.1 Source of isolates

The cultures used in this study originated from clinical, dairy and environment isolates retrieved from Fonterra (Table 3.1), AsureQuality (Table 3.2) and Auckland University of Technology (AUT) stock culture (Table 3.3).

Table 3.1

Ten isolates from Fonterra and their origin:

Nomenclature	Strain	Source
F1	4.10 C	Dried infant formula, S. Edelson-Mammel, FDA
F2	SK 90	Clinical J. M. Farber, Health Canada
F3	3465-4 A	Environment isolate
F4	3465-6 B	Environment isolate
F5	4648-4 C	Environment isolate
F6	576736-3 D	Environment isolate
F7	578349-7 E	Environment isolate
F8	607	Clinical, FDA
F9	LCDC-648	Clinical J. M. Farber, Health Canada
F10	EWFAKRS 11 NN 1493	Clinical J. M. Farber, Health Canada

Table 3.2

Ten isolates from AsureQuality and their origin

Nomenclature	API Profile	Source
A1	3427 4767 251	Environment isolate
A2	3427 4767 250	Environment isolate
A3	3427 6763 250	Environment isolate
A4	3407 4763 050	Environment isolate
A5	3407 4767 050	Environment isolate
A6	3427 4763 251	Environment isolate
A7	3427 6767 051	Environment isolate
A8	3427 6377 150	Environment isolate

Table 3.3

Two isolates from AUT and their origin:

Nomenclature	Strain	Source
E1	NCTC 8155	Dried milk
E2	CDC 4562/70	Clinical, Child's throat

3.2 Maintenance of *E. sakazakii* culture

Stock cultures were received on agar slants or freeze dried. These were grown to active cultures as described below. Where cultures were going to be needed for a significant period of time (some months) active cultures were stored in glycerol at -80°C. For temporary storage, *E. sakazakii* was maintained on tryptic-soy agar slants at 4°C. A new series of cultures was initiated from the frozen stock on a biweekly basis.

3.3 Culture preparation

For the routine preparation of cultures, all isolates were cultured in Tryptic soy broth (TSB) (Becton, Dickinson & Company, USA) and on Tryptic soy agar (TSA) (Merck, Germany). Cultures were grown aerobically at 37°C for 18-24 h.

3.4 Screening method for strain purity and characterisation

The following methods were used to ensure the purity and of the isolates obtained at the start of the study.

3.4.1 TSA and skim milk plate count agar plating

The bacterium was isolated on the TSA and skim milk plate count agar (SMPCA) to obtain a pure culture. Typical *E. sakazakii* colonies are yellow pigmented colonies. Freshly isolated *E. sakazakii* may produce colonies with two distinct morphologies (Farmer et al., 1980). One colony type is described as being dry or mucoid, crenated (notched or scalloped) and leathery or rubbery when touched with wire loop, i.e., very little biomass adheres to the loop and the colony snaps back to the agar when touched. A second colony morphology has been described as smooth and more amenable to removal of cells from colonies with a wire loop (Gurtler et al., 2005).

On agar plates it may form two colony types (glossy and matt) depending upon media and strain. Growth on TSA at 25°C produces a non-diffusible, yellow pigment (Iversen and Forsythe, 2003). *E. sakazakii* will grow on non selective media (e.g. TSA) commonly used in enteric bacteriology. On TSA, colonies 2-3 mm in diameter form within 24 h at 36°C, and if grown at 25°C, the colonies will typically be 1-1.5 mm in diameter.

3.4.2 Gram staining

The isolates were Gram stained and observed microscopically for Gram-negative rod shaped cells, typical of *E. sakazakii*.

3.4.3 *API 20E*

The identification of the isolates was confirmed using the API 20E biochemical identification kit (bioMérieux, Chemin de l'Orme, France) according to the manufacturer's instructions. The API 20E biochemical profiles were calculated using the API 20E V4.1 (Biomereux, <https://apiweb.biomerieux.com>)

3.4.4 *Capsule staining procedure (Anthony's method)*

The extracellular gelatinous material synthesized by certain bacteria is known as capsule. Capsules are polysaccharides, polypeptides or lipoproteins. They render physical and physiological protection to the cell.

Anthony's method was chosen to observe the typical capsules of *E. sakazakii* (Anthony, 1931). The *E. sakazakii* culture was smeared on two clean microscope slides. The smear was not heat fixed. One drop of 1% crystal violet was placed onto the smear. The slide was held in an angular position above the sink and 10 drops of 20% copper sulphate (CuSO₄) solution were gently dripped onto the smear. The slide was blotted dry and the smear was observed under the microscope using 1000x magnification. If the capsule and cells appeared dark, the smear was washed again with few more drops of CuSO₄.

3.5 *Microtitre plate assay*

A Microtitre plate assay based on (Oh and Kang, 2005; Oh et al., 2007) study was used to screen the different strains of *E. sakazakii* for their ability to attach to surfaces and form biofilm. A 96-well flat-bottomed polystyrene microplate (Microtest™ 96, 35 3072, Becton, Dickinson & Company, USA) was filled with 230 µL of 10% reconstituted infant formula milk (Anmum, Fonterra). Wells were filled with milk only as negative controls. Overnight *E. sakazakii* culture (20 µL) was added into the test wells and the plates were incubated aerobically for 24 h at 30°C. The contents of the plates were removed by inverting the plates, and then the wells washed three times with 300 µl of sterile distilled water. The remaining

attached *E. sakazakii* were fixed with 250 µl of methanol per well for fifteen minutes. The liquid was discarded and the plates air-dried. The microplate wells were stained with 250 µl of 0.05% (w/v) crystal violet for five minutes. The excess stain was rinsed off by placing the microplate under running distilled water. After the microplate air-dried, the dye bound to the adherent cells was resolubilized with 250 µl of 33% (v/v) glacial acetic acid per well. The OD of each well was measured at 550 nm using an automatic 96-well microplate reader (ELx 808-Ultramicroplate reader, Bio-tek Instruments, INC, Winooski, VT, USA) (Appendix 1).

3.6 Enumeration of *E. sakazakii* using impedance detection with the BacTrac® 4000

3.6.1 Operation of the BacTrac® 4000

Impedance microbiology is selected to enumerate numbers of *E. sakazakii* using BacTrac® 4000 instrument. BacTrac® 4000 (Minitrac, Sy-Lab, Purkers dorf, Austria) (Appendix 2) is operated by setting the temperature of incubation and the threshold level for detection prior to each experiment. Basically, the BacTrac® 4000 has built-in algorithms, which determine when the rate of conductance/capacitance change is greater than the preset threshold. The threshold point in the BacTrac® 4000 for this experiment was at 3% of the electrodal value (time taken to reach threshold level of impedance change, measured as capacitance change at the electrode).

3.6.2 Cleaning procedure

First the cleaning procedure was assessed and this method is based on Zain et al., (2008). It is critical to ensure that the BacTrac® 4000 tubes are properly cleaned before each experiment. Two sets of cleaning procedures of BacTrac® 4000 tubes were done to determine the reliability of cleaning the tubes. Twenty tubes were soaked with 95% ethanol for 24 hours and the surfaces of electrodes were swabbed several times before being rinsed with distilled water five times. The cleaned tubes were autoclaved at 121°C for 15 minutes. TSB solution were filled into the tubes

and incubated for 24 hours at 30°C. These controls were used to determine the level of cleanliness achieved, assessed on the stability of the baseline reading.

3.6.3 Handling error

The accuracy of pipetting was determined. Amounts of 5 mL, 1 mL and 0.1 mL of 10% reconstituted infant formula milk were pipetted into a 10 mL beaker and the weight was recorded. This practise was repeated 10 times at the same volume and same balance (ED224S, Sartorius, AG, Germany). The standard deviation errors were calculated.

3.6.4 Calibration of BacTrac® 4000

This method is a modification from a published method (Flint and Brooks, 2001; Zain et al., 2008). A single colony of *E. sakazakii* from pure culture was inoculated into 10 mL Tryptic Soy Broth (TSB) and incubated at 30°C for 24 h. After incubation, the culture was agitated on a vortex mixer (Talboys, Henry Troemner LLC, USA) to make the suspension homogeneous before taking 1 mL of culture and transferring to another bottle containing 9 mL TSB to make a 10^{-1} dilution. The process was repeated to provide a series of 10-fold dilutions down to 10^{-9} . A 0.1 mL volume was taken from each dilution and transferred onto TSA for surface plating. The plating was done in triplicate for each dilution and the plates were incubated at 30°C for 24 h. Simultaneously, 1 mL from each of the dilutions was transferred into a BacTrac® 4000 cell in duplicate (limited by the capacity of the machine) and incubated at 30°C for 24 h to determine the detection time for the growth of the *E. sakazakii* cells. Using the software incorporated into the BacTrac® 4000, conductance/capacitance changes were calculated to obtain calibration curves so that the initial microbial loads in the samples could be deduced. The calibration curve was determined by manually plotting the $\text{Log}_{10} \text{CFU mL}^{-1}$ versus detection time in hour.

3.6.5 Plate counts

Plate counts were carried out by the spread plate method. This method was chosen to minimise the heat applied to organisms (pour plate method), for optimum exposure to oxygen and for ease of counting. Spread plates were prepared by

applying 0.1 mL of the required dilution to the plate and spreading with a sterile glass spreader. Where possible, counts were made using plates containing between 30 and 300 colony forming units.

3.7 *Biofilm development*

3.7.1 *Tube preparation*

Three different types of infant feeding tube (Appendix 3), each composed of different materials, silicone, polyurethane and polyvinyl chloride (PVC), each with an internal diameter of 5-French (0.33 mm) were used in this study. To assay for biofilm formation on the tubing surfaces, feeding tube segments were cut to give identical surface area (2 cm²) based on the outer diameter measurements specified by the manufacturers. The tubing segments were sliced longitudinally to ensure ready access to all surfaces of the tubing. The tubing segments were cleaned and sterilized by immersing into 95% ethanol for 10 min, rinsed three times with 10 mL volumes of phosphate-buffered saline (PBS, pH 7.15) and autoclaved at 121.1°C for 15 min.

3.7.2 *Inoculum preparation*

One loop of selected strain was inoculated into 10 mL TSB and incubated at 37°C for 18 - 24 h prior to experimental work on the next day. The cultures were centrifuged at 4000 x g for 15 minutes to collect the cell pellets from the solution and washed by reconstituting in PBS.

Centrifugation and resuspension in PBS was repeated three more times to thoroughly remove all culture media. The initial inoculums were approximately 8 log CFU mL⁻¹.

3.7.3 *Inoculation media – 10% reconstituted IFM*

The inoculation media used for the trial, comprised 10% reconstituted infant formula milk, prepared by adding 10 g of skim milk powder (Anmum, Fonterra) into 90 mL

of distilled water and mixed to reconstitute. The pH of the milk sample was adjusted if required to achieve $\text{pH } 7.0 \pm 0.1$ using 1M KOH or 1M HCl, then autoclaved at 121.1°C for 15 min. *E. sakazakii* cell pellets were transferred into the sterile 10% reconstituted infant formula milk sample and mixed using a vortex mixer for one minute.

3.7.4 Incubation

A 5 mL volume of the inoculated 10% reconstituted infant formula milk was pipetted into each sterile screw-capped bottle containing the tube samples (PVC, polyurethane and silicone) and incubated in an aerobic shaker at 25°C and speed at 50 rpm for 24 h. Each test was done in duplicate. The samples of milk (containing planktonic cells) and tubing (containing biofilm cells) were taken every 4, 12 and 24 hours. One mL of each milk sample was pipetted into a BacTrac® 4000 tube containing 9 mL of TSB and incubated at 30°C 24 h for planktonic cells counts. Each sample of tube was washed five times with sterile distilled water and inserted into a BacTrac® 4000 tube containing 10 mL of TSB and incubated in the BacTrac® 4000 microbiological growth analyser at 30°C for 24 h. The number of viable cells recorded using the BacTrac® 4000 algorithm.

3.8 Detection methods

3.8.1 Epifluorescence microscopy

To determine the total number of cells colonising a surface, samples of tubing were analysed by epifluorescence microscopy. The fluorochrome acridine orange (BD) (10mg) was dissolved in 100 mL of sterile phosphate buffer pH 7.2 and filtered through a 0.2 mm Sartorius filter.

Samples were prepared for epifluorescence microscopy as follows. The specimens (samples supporting biofilms) were washed five times in sterile deionised water and then fixed with 1% formalin (BDH) at ambient temperature for at least 2 min. Samples were exposed to acridine orange for 2 mins at ambient temperature, washed five times in sterile deionised water, air dried, mounted on glass microscope slides using epoxy resin and observed under ultraviolet (UV) light using a Leitz Ortholux II microscope with an H2 incident light excitation filter block (Ernst Leitz Wetzlar,

GmbH, Wetzlar, Germany) and photographed using PJC 1600 film (Kodak) (Flint, 1998).

3.8.2 *Capacitance detection*

The procedures were similar to section 3.6.4 and 3.7.4 using impedance microbiology assessment.

3.8.3 *Scanning Electron Microscopy (SEM)*

Tube segments for microscopy were cut into 0.835 x 2 mm samples. Samples fixed in 3% Glutaraldehyde and 2% Formaldehyde in 0.1M Phosphate buffer separately, pH 7.2, for approximately 3 hours, buffer washed 3 times, dehydrated in an Ethanol series, 25%, 50%, 75%, 95%, and two changes of 100%, [15 minutes each and 1 hour in the last 100% step]. They were then Critical Point dried, using liquid CO₂ as the critical point fluid in a Polaron E300 critical point drier.

Dry samples were mounted on to aluminium specimen stubs with double sided tape and conductive silver paint, and sputter coated with gold in a Bal-Tec SCD050 sputter coater. Samples were studied using a FEI Quanta 200 SEM (Hopcroft, D., personal communication, 2008).

3.9 *Accuracy and reproducibility*

Throughout this study, care was taken to ensure accurate and reproducible results. All tests were carried out in duplicate and, where appropriate, quantitative data were reported with standard deviations. Equipment (e.g. the BacTrac® 4000 microbiological growth analyser) was calibrated on a regular basis. Replicates were limited by the capacity of the BacTrac® 4000 microbiological growth analyser.

Errors for incubation temperatures were +/- 0.5°C and for pH were +/- 0.1 (readability of the pH meter).

3.10 *Data analysis*

All data analysis was done using Microsoft Excel computer software.

IDENTIFICATION AND ATTACHMENT OF *ENTEROBACTER SAKAZAKII* - PRELIMINARY RESULTS

- 4.1 Introduction - biofilms
- 4.2 Results and discussion
 - 4.2.1 Isolation and identification of *E. sakazakii*
 - 4.2.2 Morphology and biochemical profiles
 - 4.2.3 Inoculation media for biofilm study - 10% reconstituted IFM
 - 4.2.4 Microtitre plate assay
- 4.3 Conclusions

Chapter 4

Preliminary results of biofilm assessment

4.1 Introduction- biofilms

A biofilm is a population of microbial cells growing on a surface and enclosed in an amorphous extracellular matrix (Donlan, 2002; Donlan and Costerton, 2002).

Biofilm growth is the predominant form of microbial growth in most environments. It can consist of either single or multiple species populations (O'Toole et al., 2000). The formation of biofilms represents a protected mode of microbial growth, which confers survival advantages in many hostile environments, in particular, protection from environmental stresses and availability of nutrients. As a result, biofilms exist on almost every solid-liquid interface in nature and play vital roles in ecosystem function (Costerton, 1995).

Biofilms can be formed by all types of microorganisms, under suitable conditions (Poulsen, 1999). Some bacteria have a greater tendency than others to form a biofilm. The bacteria most commonly associated with biofilms are *Pseudomonas*, *Enterobacter*, *Flavobacterium*, *Alcaligenes*, *Staphylococcus* and *Bacillus* (Poulsen, 1999).

The first and most critical stage in biofilm development is adhesion of the bacteria to the substrate. This is a time dependent process and can be roughly divided into two phases: the reversible and the irreversible. The reversible phase mainly includes various long distance interactions (electrostatic, hydrophobic interaction and van der Waals forces). The interactions in the irreversible phase are various short range forces including dipole-dipole, hydrophobic, ion-dipole, ion-ion, covalent bonds and hydrogen interaction (Hood and Zottola, 1995; Kumar and Anand, 1998).

As the biofilm grows, there is an increase in microorganisms encapsulated in the polymer matrix. Diffusion through this matrix becomes a major factor when determining the structure of the biofilm. The production of exopolysaccharides (EPS) by biofilm bacteria serves many functions. These include: facilitation of the initial attachments of bacteria to a surface; formation and maintenance of microcolony and biofilm structure; enhanced biofilm resistance to environmental stress and antimicrobial agents.

Biofilm formation can have different consequences in different environments (e.g. cooling water system, food industry, medical and paper industries). Different specialist groups, for example, microbiologists, engineers, ecologists and chemists, have a special interest in biofilm formation and microbial growth in the industries and in the medical world. Biofilms can form everywhere; in toilets, sinks, industrial water systems and on medical devices. Biofilm can cause energy loss and blockage of pipes, cooling towers and water reservoirs.

E. sakazakii has been shown to form biofilms on latex, polycarbonate, silicone rubber and glass (Iversen et al., 2003; Iversen et al., 2004). Capsule formation does vary between *E. sakazakii* strains, and capsule formation correlates with biofilms with higher cell counts (up to 10^4 CFU cm²). Those strains that produce capsules are likely to be the most important in colonising manufacturing plant and nasogastric tubing used for infant feeding. Barron and Forsythe, (2007) showed that some capsulated *E. sakazakii* strains survived in dehydrated powdered infant formula for at least 2.5 years. *E. sakazakii* could be present as attached cells or as cells in biofilms on the abiotic surfaces in processing plants and preparation kitchens, thereby representing a source of contamination.

Biofilm formation may be determined in several different ways, but most frequently it is performed by using a test tube (Christensen et al., 1982; Christensen et al., 1985) in which the bacterial film lining the culture tube is stained with cationic dye and visually scaled, or by the Microtitre plate test (Christensen et al., 1985) in which the optical density (O.D.) of the stained bacterial film is determined spectrophotometrically.

Our aims were to test the biofilm production by 17 different *E. sakazakii* strains on a plastic surface and to determine the influence of the growth medium and IFM on biofilm formation.

The first objective of this study was to confirm that the *E. sakazakii* can attach and grow biofilm on plastic surfaces (Microtitre plate assay).

4.2 Results and discussion

4.2.1 Isolation and Identification

Twenty isolates were received from Fonterra, AsureQuality and AUT for this study. The isolates were taken for purity testing before proceeding with further biofilm experiments. Preliminary confirmation of identification of the 20 isolates received was based on colony morphology on TSA and SMPCA (Figure 4.1 and 4.2). The basic characteristic of this bacterium on TSA is a yellow pigmented colony, mucoid or matte. The yellow pigment is a defining feature used as an indicator to differentiate *E. sakazakii* from *E. cloacae*; the latter forms white, mucoid colonies on TSA plates.

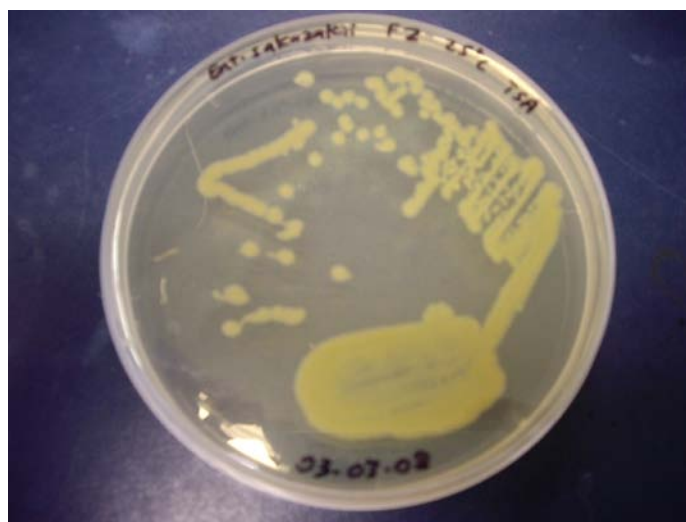


Figure 4.1: The growth of *E. sakazakii* on tryptic soy agar (TSA) after 48 hours of incubation at 25°C. The colonies were mucoid, smooth and amenable to removal of cells from colonies with a wire loop. The colonies, with diameter 1- 1.5 mm, produced non-diffusible yellow pigment.

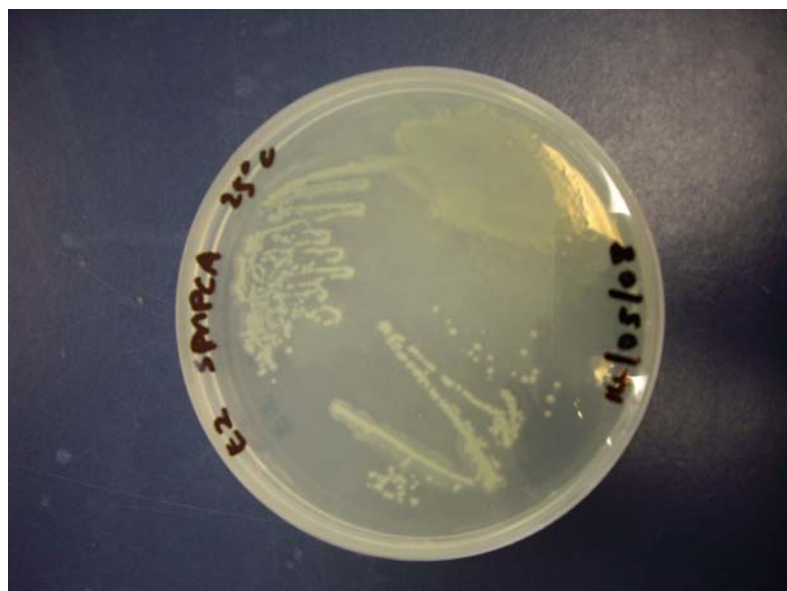
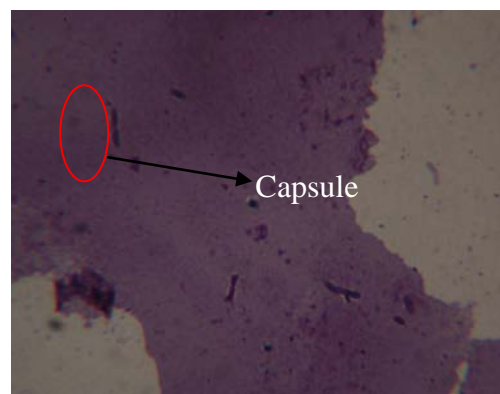


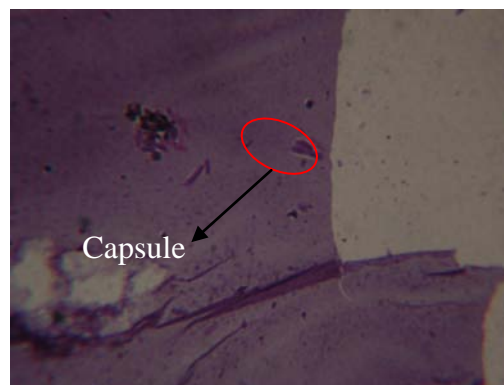
Figure 4.2: The growth of *E. sakazakii* isolate on skim milk plate count agar after 48 hours of incubation at 25°C. The colony formations were small, mucoid and rubbery when touched with a wire loop. It also produced non-diffusible yellow pigmented glossy colonies.

Eight isolates from AsureQuality were inoculated on TSA and SMPCA, however strains A4 and A5 were heavily contaminated. Strain A3 produced white colonies on TSA and strain A7 produced yellow and white colonies on TSA and SMPCA (data not shown). All the Fonterra and AUT isolates produced typical colonies.

Isolates with typical colony appearance were observed microscopically and found to have an appearance typical of *E. sakazakii* - that is rod-shaped, Gram-negative and motile with some clumping. Capsule staining showed one strain, F8, to produce a capsule visible under microscopy (Fig. 4.3a and 4.3b). Capsules were not visible on other strains.



(a)



(b)

Figure 4.3: The capsule staining (a) and (b) showed that F8 formed a capsule surrounding its cells (unstained area).

The API 20E test confirmed that ten isolates from Fonterra, five isolates from AsureQuality and two isolates from AUT were positive (97.3% to 99.9%) for *E. sakazakii*.

A summary of the isolates tested is given in Tables 4.1 – 4.2.

Table 4.1 Ten isolates from Fonterra and their origin:

Nomenclature	Strain	Source	Identity
F1	4.10 C	Dried infant formula, S. Edelson-Mammel, FDA	Pure culture
F2	SK 90	Clinical J. M. Farber, Health Canada	Pure culture
F3	3465-4 A	Environment isolate	Pure culture
F4	3465-6 B	Environment isolate	Pure culture
F5	4648-4 C	Environment isolate	Pure culture
F6	576736-3 D	Environment isolate	Pure culture
F7	578349-7 E	Environment isolate	Pure culture
F8	607	Clinical, FDA	Pure culture
F9	LCDC-648	Clinical J. M. Farber, Health Canada	Pure culture
F10	EWFAKRS 11 NN 1493	Clinical J. M. Farber, Health Canada	Pure culture

Table 4.2 Eight isolates from AsureQuality and their origin

Nomenclature	API Profile	Source	Identity
A1	3427 4767 251	Environment isolate	Pure culture
A2	3427 4767 250	Environment isolate	Pure culture
A3	3427 6763 250	Environment isolate	Contaminated
A4	3407 4763 050	Environment isolate	Contaminated
A5	3407 4767 050	Environment isolate	Contaminated
A6	3427 4763 251	Environment isolate	Pure culture
A7	3427 6767 051	Environment isolate	Pure culture
A8	3427 6377 150	Environment isolate	Pure culture

Table 4.3 Two isolates from AUT and their identification

Nomenclature	Strain	Source	Identity
E1	NCTC 8155	Dried milk	Pure culture
E2	CDC 4562/70	Clinical, Child's throat	Pure culture

4.2.2 10% reconstituted infant formula milk (IFM)

In order to prepare IFM for biofilm tests on specific strains, the IFM had to be sterilised. Two samples of 10% reconstituted infant formula milk were subjected to heat treatment for sterilization and one sample was the control (non heat treated). The first IFM sample was autoclaved at 121°C for 15 minutes and left to cool in the autoclave. Another milk sample was autoclaved at 121° for 15 minutes and immediately cooled in ice water. Browning was visible in the IFM that was autoclaved and allowed to cool in the autoclave, while the sample that was cooled rapidly after autoclaving was only slightly brown compared with the control sample (Fig 4.4). A rapidly cooled autoclaved IFM treatment was selected for further experiments to ensure a sterile medium with minimal damage.

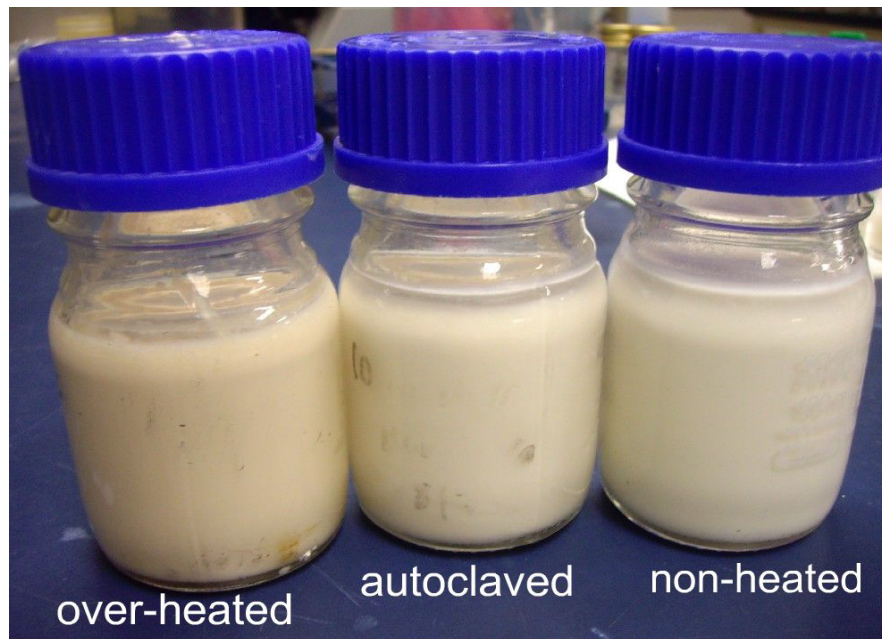


Figure 4.4: The results from heat treatment of the 10% reconstituted IFM. The left image is of the milk autoclaved at 121°C for 15 minutes and cooled in the autoclave. The middle image shows the milk autoclaved at 121°C for 15 minutes and subsequently cooled in ice water. The image on the right is the unheated control.

4.2.3 *Microtitre plate assay*

An initial trial using the microtitre plate assay was done on the 10 isolates from Fonterra. The isolates were inoculated into TSB and were processed according the method described in Chapter 3, section 3.5. However, the O.D. reading at 550 nm gave very low levels of attachment, which indicated slow growth or no growth at all (data not shown). This procedure was repeated twice and still there was no attachment or biofilm formation.

Oh et al., (2007) studied biofilm formation by *E. sakazakii* in artificial medium and IFM. Based on the results from 72 strains that they studied: 62 strains formed no biofilm, 9 formed weak biofilm and 1 strong biofilm when incubated in TSB. However, when they substituted the TSB with reconstituted infant milk formula with normal concentration (1 D dilution), biofilm was produced: 16 strains formed no biofilm, 30 formed weak biofilms and 26 formed strong biofilms. The IFM was further diluted twofold (2 D), fourfold (4 D) and eightfold (8 D) and *E. sakazakii* strains managed to produced biofilm when incubated in the solutions.

Thus, for this study, the inoculation medium was substituted with sterilised 10% IFM (1D dilution) for the microtitre plate assay. The milk became very sticky and viscous after 24 h of incubation at 37°C, especially for strain F8. This finding is likely to be due to the exopolysaccharide capsule noted in the microscopic observations. Similar observations have been made for other strains producing capsules (Iversen et al., 2004).

At 550 nm wavelength, the absorbance showed that all 10 isolates from Fonterra attached and produced biofilm in 10% reconstituted IFM. IFM appeared to enhance the growth and attachment of *E. sakazakii* on such hydrophobic surfaces (microtitre plate). This is similar to the results of Oh et al., (2008), where IFM was inoculated with *E. sakazakii* to observe biofilm formation using the microtitre plate assay. The five environmental isolates from AsureQuality and two isolates from AUT also showed some attachment and biofilm formation on microtitre plate surfaces.

Reconstituted IFM was the more effective medium for biofilm formation than artificial medium (TSB). Using the microtitre plate assay and IFM, ten strains produced strong biofilms; (one dairy, five clinical and four environmental isolates), two produced weak biofilms; (one dairy and one environmental isolates), and five environmental isolates did not produce biofilms at all, according to the interpretation given in Table 4.4.

An example of the microtitre plate assay is given in Fig 4.5 and summaries of the results (means and standard deviations) are given in Figures 4.6, 4.7, and 4.8 for the dairy, clinical and environmental isolates respectively. As all clinical isolates formed biofilms, this may be an indication of their potential pathogenicity. The effect of IFM on biofilm development suggests that a conditioning layer with milk components is important for the attachment and biofilm growth of *E. sakazakii*, though an alternative explanation is that the IFM provides some essential nutrient missing from the TSB.

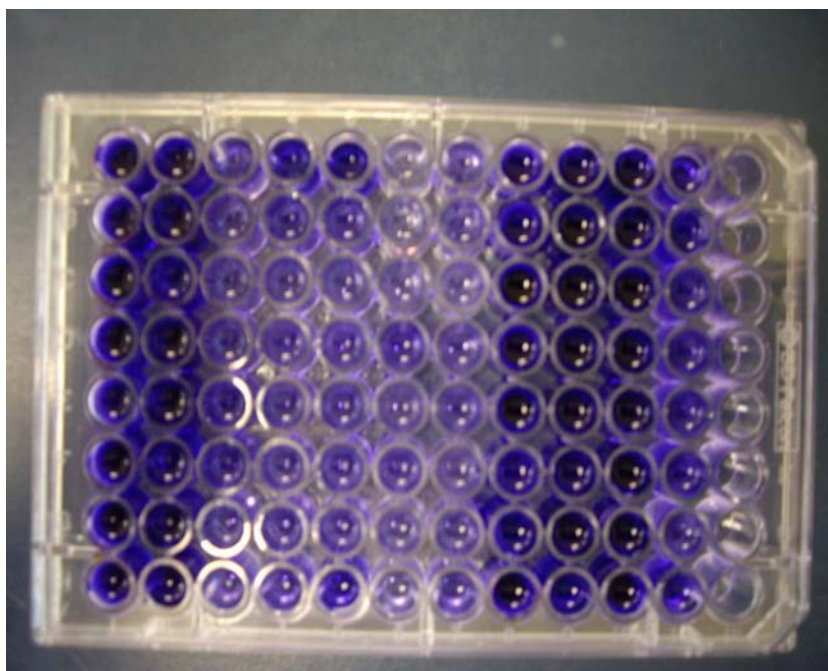


Figure 4.5: The *E. sakazakii* isolates were incubated for 24 h and then the liquid was discarded. The remaining cells attached on the well were stained with 0.1% crystal violet which was resolubilized with 33% acetic acid for absorbance (O.D.) reading at 550 nm.

Table 4.4: The score based on the biofilm formation

Score (O.D.)	Biofilm formation by <i>E. sakazakii</i>
O.D. < 1.0	None
1.0 < O.D. < 2.0	Weak
O.D. > 2.0	Strong

For the purposes of comparative analysis of test results, adherence capabilities of tested strains were classified into three categories, i.e., non adherent, weakly or strongly adherent, based upon the O.Ds of bacterial films (Oh et al., 2007). The cutoff O.D. (O.Dc) was defined as three SDs above the mean O.D. of the negative control. Strains were classified as follows: O.D. < O.Dc = no biofilm former, O.Dc < O.D. < (2x O.Dc) = weak biofilm former and O.D. > (2 x O.Dc) = strong biofilm former. All tests were carried out in triplicate and the results were averaged. The O.Dc was determined as 1.0 for this study. The weak and strong biofilm producers were classified as biofilm producers.

Table 4.5: Summary of the biofilm score

Strong biofilm	Weak biofilm	No biofilm
10 one dairy isolate five clinical isolates four environmental isolates	2 one dairy isolate one environmental isolate	5 environmental isolates

Table 4.5 shows the microtiter plate assay results based on the O.D. of strains that produced biofilms. Strains were classified as strong, weak or non-biofilm producers. Ten strains (E2, F1, F2, F8, F9, F10, A2, A6, A7 and A8) produced strong biofilms, 2 strains (A1 and E1) produced weak biofilms and 5 strains (F3, F4, F5, F6 and F7) did not produce biofilm at all in this study.

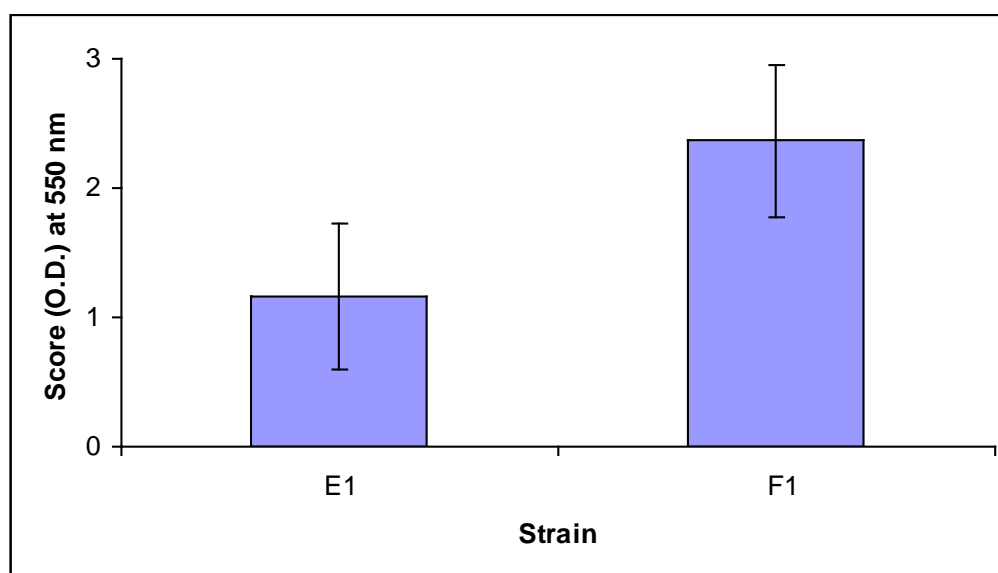


Figure 4.6: The O.D. scores of the dairy isolate biofilms at 550 nm absorbance using the microtitre plate assay. The mean and standard deviation for E1 were 1.16 and 0.56 respectively while the mean and standard deviations for F1 were 2.37 and 0.59 respectively.

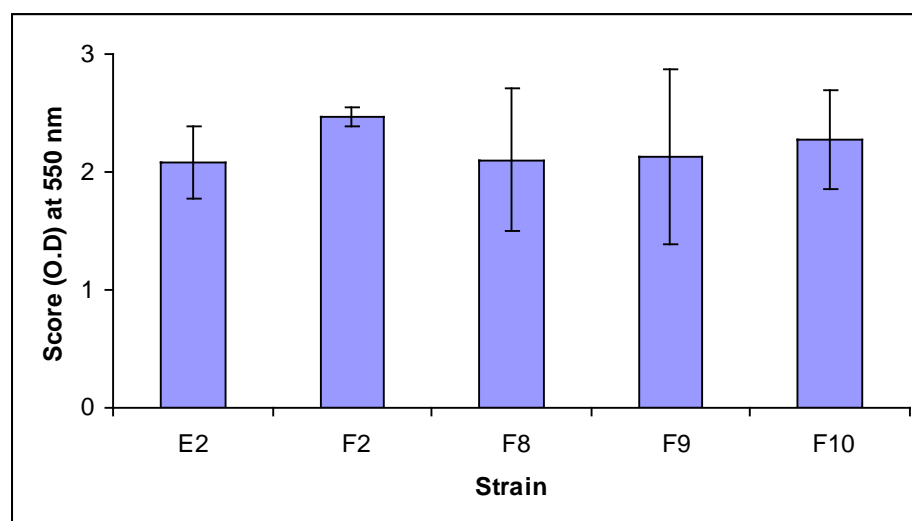


Figure 4.7: The O.D. scores of clinical isolate biofilms at 550 nm absorbance using the microtitre plate assay. The means for E2, F2, F8, F9 and F10 were 2.08, 2.47, 2.10, 2.13 and 2.28 respectively. The standard deviations for E2, F2, F8, F9 and F10 were 0.31, 0.08, 0.60, 0.74 and 0.42 respectively.

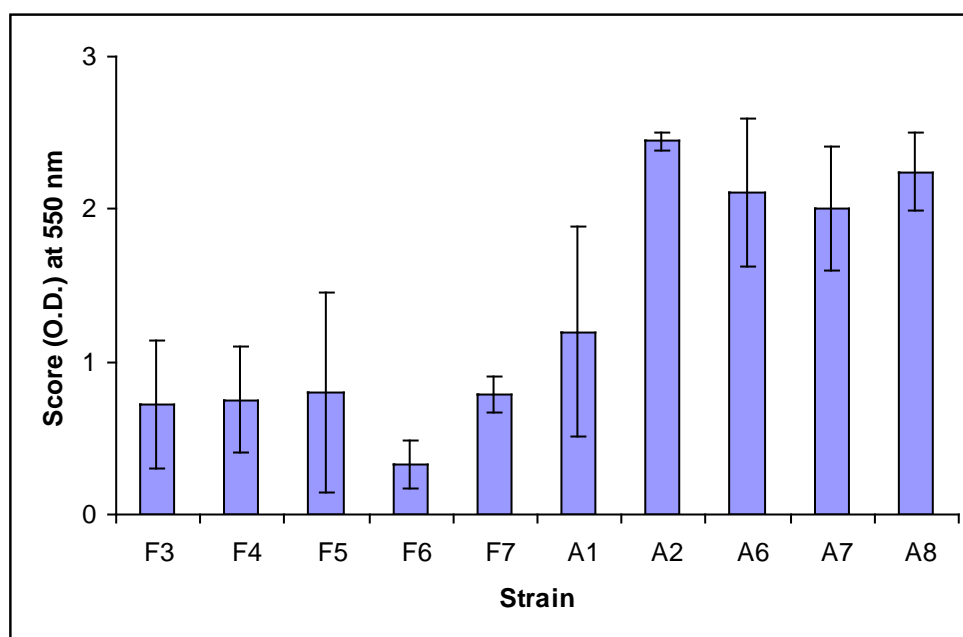


Figure 4.8: The O.D. scores of environmental isolate biofilms at 550 nm absorbance using the microtitre plate assay. The means for F3, F4, F5, F6, F7, A1, A2, A6, A7 and A8 were 0.72, 0.75, 0.79, 0.33, 0.79, 1.20, 2.45, 2.11, 2.00 and 2.25 respectively. The standard deviations for F3, F4, F5, F6, F7, A1, A2, A6, A7 and A8 were 0.42, 0.35, 0.66, 0.15, 0.12, 0.69, 0.06, 0.48 and 0.41 respectively.

4.3 Conclusions

- Seventeen of the 20 isolates provided for this trial were confirmed as *Enterobacter sakazakii*.
- Attachment and biofilm growth, determined using the microtitre plate assay, will occur in IFM but not in TSB, suggesting the importance of milk components in biofilm formation by *E. sakazakii*.
- All the clinical strains attached and formed strong biofilms when inoculated in IFM and this may be a reflection of their potential pathogenicity.
- Three strains (F1, F8 and A6) were chosen for further detailed study of biofilm formation on three different materials of infant feeding tube.

BIOFILM FORMATION BY *ENTEROBACTER* *SAKAZAKII* ON INFANT FEEDING TUBES

- 5.1 Introduction
- 5.2 Procedures
- 5.3 Results and discussion
 - 5.3.1 Cleaning protocol
 - 5.3.2 Handling error
 - 5.3.3 Calibration curves from impedance microbiology
 - 5.3.3.1 Repeatability of BacTrac® 4000 measurements
 - 5.3.4 Biofilm enumerations
 - 5.3.4.1 Dairy strain
 - 5.3.4.2 Clinical strain
 - 5.3.4.3 Environment strain
 - 5.3.5 General discussion of the results
 - 5.3.6 SEM images of the tubing surface (control)
 - 5.3.7 SEM images of F8 biofilm on PVC, polyurethane and silicone at different time of incubation
- 5.4 Conclusions

Chapter 5

Biofilm formation on infant feeding tubes

5.1 Introduction

Powdered infant formula is used as an alternative to human breast milk in providing newborns with nutritional needs either in addition to breast milk, or on its own when breast feeding is not possible. The reconstituted IFM is supplied to the babies using infant feeding tubes (nasogastric tubing). However, several problems arise from this feeding method including clogging, leaking and biofilm formation on the tubing itself. This study is focused on the latter problem associated with *E. sakazakii* colonisation of the tubing surface that can lead to sickness of the neonate.

Three different types of tubing materials that are used in the Neonatal Intensive Care Units (NICU) were selected for this study to investigate whether tubing composition affected biofilm formation by *E. sakazakii* using dairy, clinical and environmental isolates. Polyvinylchloride (PVC), polyurethane and silicone materials were chosen and they have different hydrophobicity, glass transition temperature and surface features.

Impedance microbiology was selected to enumerate the numbers of *E. sakazakii* cells that formed biofilm on three types of infant feeding tubes in this study. This has been used previously as a convenient and reproducible method to estimate viable cells colonising a surface (Foster, 1996; Flint and Brooks, 2001; Iversen et al., 2004). The basic principle of impedance microbiology is defined below:

When bacteria grow in the medium, they modify the composition of the medium by producing metabolic products which change the ionic content, resulting in conductivity changes in the growth medium. There are also changes at the

electrode interface (capacitance). The rates of change of conductivity and capacitance are proportional to the concentration of viable microorganisms. Impedance microbiology depends on detecting the decrease in the electrical impedance of the growth medium as the microorganisms metabolize and multiply. Impedance can simply be defined as the resistance to flow of an alternating current as it passes through a conducting material. It is made up of two components: conductance and capacitance. Microbial metabolism usually results in an increase in both conductance and capacitance causing a decrease in impedance. The electrical properties of the cells *per se* are not involved in the measurements. Only the broth (where the cells are immersed) and the electrode-electrolyte interface electrical characteristics play a role in these temporal changes. The BacTrac® 4000 system is capable of detecting all cells capable of metabolism, provided that growth results in a change in the electrical characteristics of the medium and the electrode interface.

After the microbial population reaches a certain threshold level (10^6 to 10^7 cells per millilitre for bacteria, 10^4 to 10^5 for yeast), an exponential change is observed in the conductance and capacitance signals. After the threshold is passed, the impedance change with time may be proportional to the number of viable cells. The time required before a microbial population produces this exponential change is inversely proportional to the initial cell concentration and the growth kinetics of the microorganisms in a given medium and is defined as detection time (DT). The impedance system needs to be calibrated to the specific organisms used in the investigation.

In this trial, F1, F8 and A6 strains of *E. sakazakii* from dairy, clinical and environment sources respectively, were chosen for this study as they formed strong biofilm in the microtitre plate screening assays (Chapter 3, section 3.5). One aspect of this study was to learn whether the origin of the strains may determine their ability to form biofilm on infant feeding tubes in a selected time interval.

In this chapter, the surface characteristics of each tube were observed and the biofilm formation of *E. sakazakii* was measured using impedance microbiology.

5.2 Procedures

A batch system rather than a flowing system was chosen for biofilm growth because it was easier to do the sampling for quantification and microscopic observation on the tubing. It was also necessary to reduce the amount of IFM used in the experiment and avoid potential complications through blockages over 24 hours of experiments. Contamination risk and additional manipulations that would have been needed for the microscopy work if a flowing system had been used were additional reasons for not choosing the flowing system as the incubation method.

The surface area of the tubing which was cut into half was calculated to gain the exact measurement for the biofilm determination. The tubing is a cylinder shape and the surface area was determined using the following formulae;

$$A: 2\pi r (r + h)$$

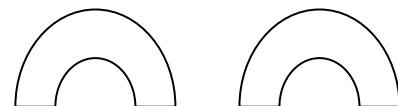
For the outer surface; $(2\pi r_1 \times h)/2$

The inside surface; $(2\pi r_2 \times h) /2$

The two sides surface; $[\text{wall thickness} \times h] \times 2$



The two edges surface; $[\pi r_1^2 / 2 - \pi r_2^2 / 2] \times 2$



The entire surface area was taken into account in estimating the biofilm formation by the organism.

To gauge the wall thickness of the tubing, a micrometer eyepiece and stage were used. The eyepiece scale was calibrated first using the stage micrometer to get the resolution of the microscope (Appendix 4).

To quantify the biofilm formation on the tubing, the methods from Chapter 3, section 3.7.1 until 3.7.4 were used. The tubing samples and samples of the surrounding media, were taken at three time intervals - 4, 12 and 24 h. The samples were washed with sterile distilled water five times and incubated in TSB the BacTrac® 4000 impedance monitor for 24 to 48 h for capacitance detection to estimate viable cells colonising the surface. The detection time, measured as the time required to reach the 3% threshold of the E-value (electrode value or capacitance) in the BacTrac® 4000 was inserted into the equation generated from the calibration curve prepared earlier for each strain to estimate the number of viable cells.

In order to view the biofilm formation on the tubing, the tubing samples were prepared for scanning electron microscopy (Chapter 3, section 3.8.3).

5.3 *Results and discussions*

Table 5.1: List of repetitions of biofilm experimental runs

Run	Description
26 33	Biofilm experiment with F1 strain on PVC, polyurethane and silicone tube pieces in 10% reconstituted IFM
27 31 34	Biofilm experiment with F8 strain on PVC, polyurethane and silicone tube pieces in 10% reconstituted IFM
28 41	Biofilm experiment with A6 strain on silicone tube pieces in 10% reconstituted IFM
30	Biofilm experiment with F1 strain on polyurethane and silicone tube pieces in 10% reconstituted IFM
32 40	Biofilm experiment with A6 strain on PVC and polyurethane tube pieces in 10% reconstituted IFM
37	Biofilm experiment with A6 strain on PVC, polyurethane and silicone tube pieces in 10% reconstituted IFM

38	Biofilm experiment with F1 strain on PVC tube pieces in 10% reconstituted IFM
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Table 5.1 showed the list of repetition of the same experiments carried out on three different tubing materials to obtain the best three results. There were duplicate sample on each time treatment (4, 12 and 24 h) for each tubing materials. The mean values from duplicate sample were averaged out.

5.3.1 *Cleaning protocol*

The cleaning procedure used for the tubes at the start of the trial was validated using the method described in Chapter 3. Twenty control tubes as described in Chapter 3, section 3.6.2 tests did not show any growth in the BacTrac® 4000 impedance system indicating that the tubes were sterile after the cleaning process.

5.3.2 *Handling errors*

The pipetting techniques from 10 repeated practises of pipetting 5 mL, 1 mL and 0.1 mL sample produced a standard deviation – 0.05%, 0.10% and 0.81% respectively. Thus the human operating error contributed very little to the overall experimental data.

5.3.3 Calibration curves from impedance microbiology

A series of calibration curves were prepared for each of the *E. sakazakii* isolates using impedance microbiology. The reproducibility of this detection method was determined using the F8 clinical isolate.

The initial plan was to observe the biofilm formation of 17 isolates on three types of infant feeding tube in this study. However, due to time constraints, only three isolates, each from different origin, were selected. These isolates produced strong biofilm based on the screening assay using the microtiter plate assay. The three calibration curves (Figure 5.1) from impedance microbiology were generated as a standard base for each strain before the biofilm experiments preceded.

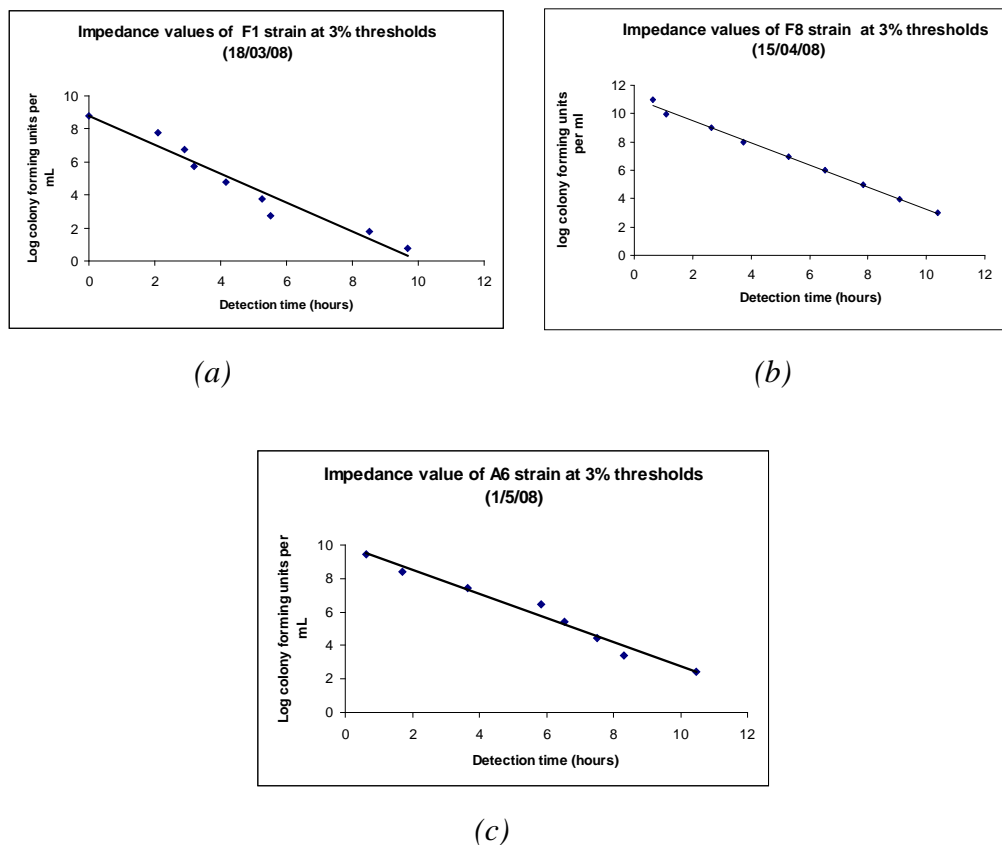
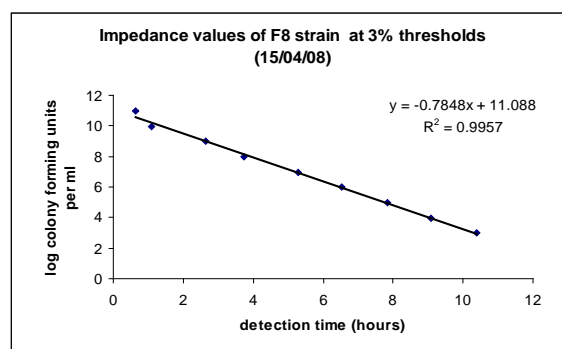
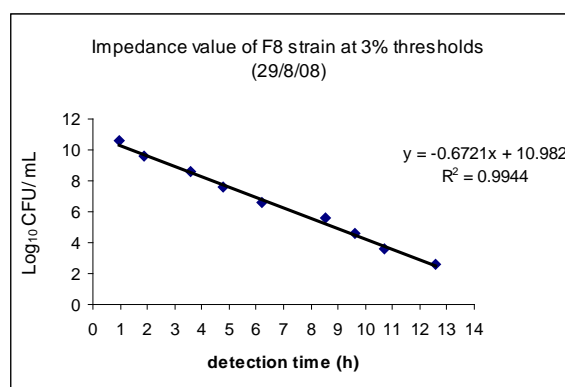


Figure 5.1: The individual calibration curves for each of the *E. sakazakii* strains used in the trial, generated by the BacTrac® 4000 impedance system using 3% thresholds for the E-value to measure the detection time. The image (a) shows the F1 strain regression calibration curve ($r^2 = 0.9489$), (b) shows the F8 strain regression calibration curve ($r^2 = 0.9957$) and (c) shows the A6 strain regression calibration curve ($r^2 = 0.9781$).

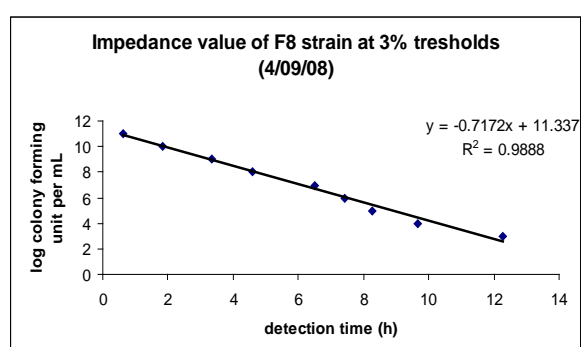
5.3.3.1 Reproducibility of BacTrac® 4000 measurements



(a)

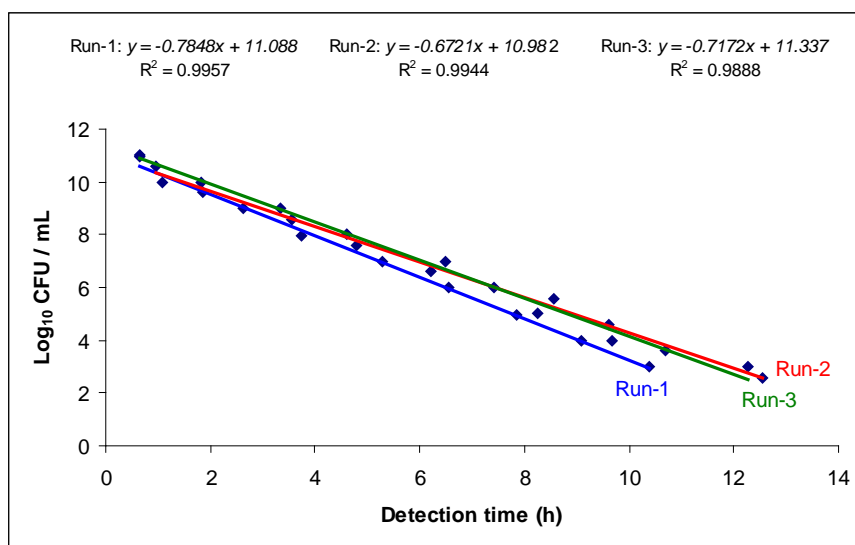


(b)

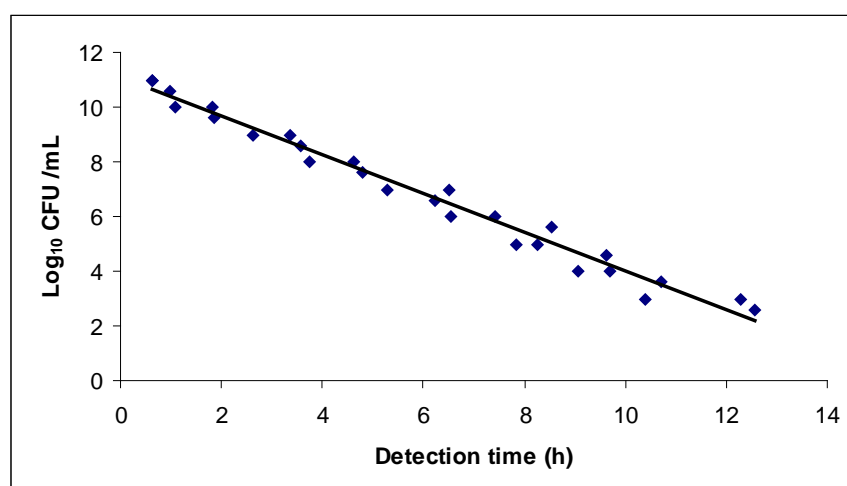


(c)

Figure 5.2: The regression calibration curve generated for F8 strain to determine the reproducibility of BacTrac® 4000 detection time.



(a)



(b)

Figure 5.3: The reproducibility of BacTrac® 4000 calibration of F8 strain at 3% threshold detection time. The figure shows that the BacTrac® 4000 produced regression $r^2 = 0.9782$ from three replicates.(a) shows the regression lines for the three individual replicates (b) shows the combined regression for the three replicates.

Impedance measurement is an accepted rapid microbiological method of analysis used for a variety of applications (Flint and Brooks, 2001). It is particularly useful for biofilm studies where it may be difficult to remove cells from the substrate. The impedance system cuts times needed to obtain results using the standard plate enumeration.

The calibration curves generated for each strain used in this trial produced regression co-efficients between 0.94 to 0.99 showing good reliability when compared with the plate count assay.

5.3.4 Biofilm enumerations

5.3.4.1 Dairy isolate (F1)

5.3.4.1.1 PVC tube

a) Planktonic cells

The numbers of planktonic F1 cells in the experiment using PVC tubing measured at 4 h for run 26 showed a large difference compared with runs 33 and 38. We postulated that there was temperature control failure during the incubation in the aerobic shaker which led to the death of the *E. sakazakii* cells from the initial inocula (10^8 CFU mL⁻¹) in the IFM in early incubation state. The planktonic cells, released from the tubing in the surrounding media, were approximately 5.7 log CFU mL⁻¹ compared with the other two runs which had approximately 7 log CFU mL⁻¹ (Fig. 5.4). However, the cell numbers increased so that by 12 h of incubation the numbers of cells preleased from run 26 was similar to that measured for runs 33 and 38. No change was noted from 12 - 24 h incubation.

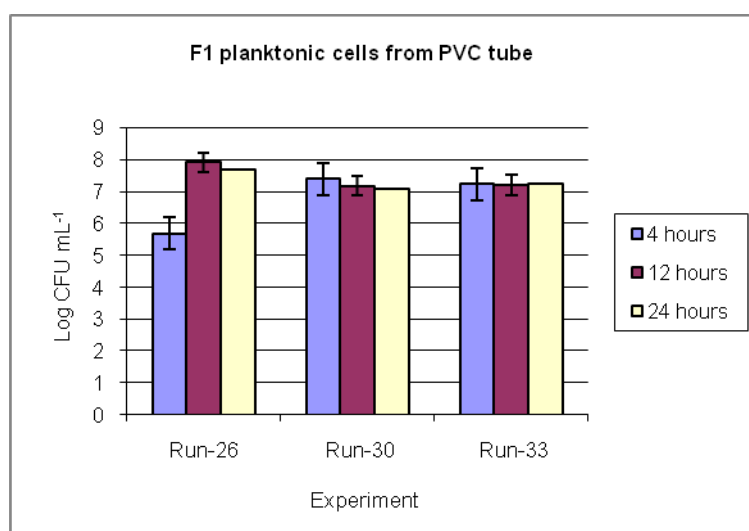


Figure 5.4: The numbers of F1 planktonic cells after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

b) Biofilms

The initial attachment at 4 h for run 33 showed very low cell numbers at approximately 1 log CFU cm⁻² (Fig 5.5). However the cell numbers increased rapidly so that after 12 h of incubation cell numbers were approximately 6 log CFU cm⁻². A slight decrease was seen after 24 h of incubation for run 33. For run 38, the initial attachment was quite high but decreased slightly after 12 h of incubation and regained higher cell numbers after 24 h of incubation.

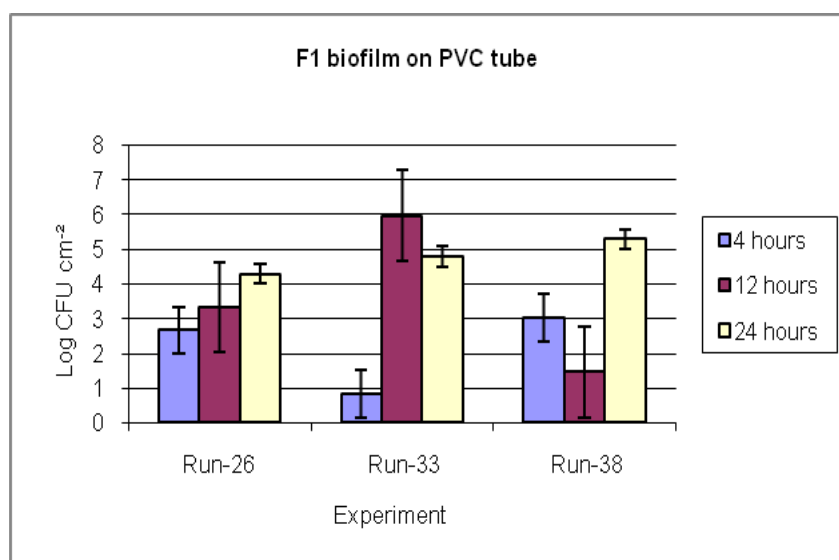


Figure 5.5: The numbers F1 cells attached and forming biofilms on PVC tubing surfaces at 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviations were determined on duplicate samples for each experimental run.

5.3.4.1.2 Polyurethane

a) *Planktonic cells*

The numbers of planktonic cells released into 10% reconstituted IFM was similar for the three experimental runs (Fig 5.6). The inoculum concentration was approximately 8 log CFU per mL⁻¹ (Chapter 3, section 3.7.2) and the number of planktonic cells determined by impedance microbiology was approximately 7 log CFU mL⁻¹ following 24 h of incubation. There was a slight decrease in cell numbers after 24 h incubation for run-26. Again, we postulated that there was a temperature fluctuation in the aerobic shaker during the incubation as the shaker was shared with other lab users. This was not observed in the other runs.

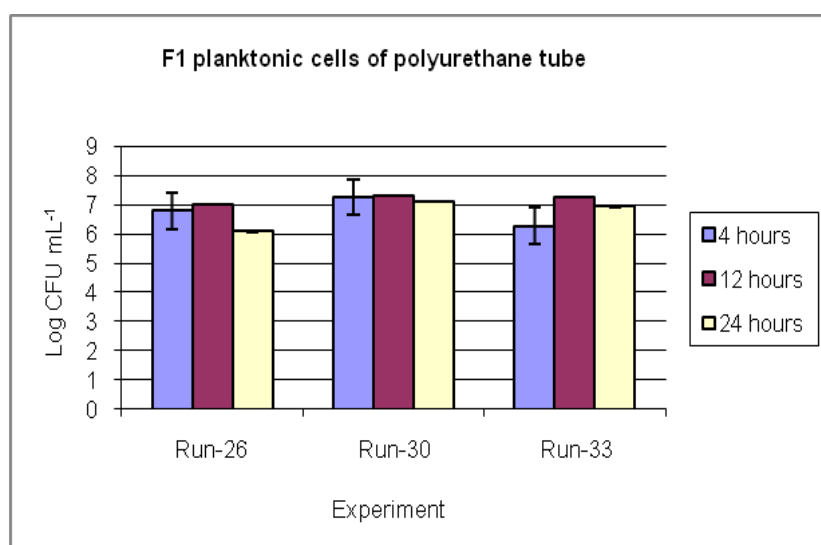


Figure 5.6: The numbers of planktonic cells after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

b) Biofilms

The biofilm formation for run 26 was low at 4 h. Note that the planktonic cells at 4 h were also low. This is most likely due to temperature fluctuation in this experiment. The initial attachment of the F1 isolate measured on polyurethane tubing at 4 h was approximately 2 - 3 log CFU cm⁻² for the three replicate runs (Fig 5.7). At 12 h the biofilms showed a 1 log CFU cm⁻² increase in cell numbers for runs 26 and 30 but not in run 38. The F1 isolate reached over 4 log CFU cm⁻² after 24 h incubation for each of the three experimental runs.

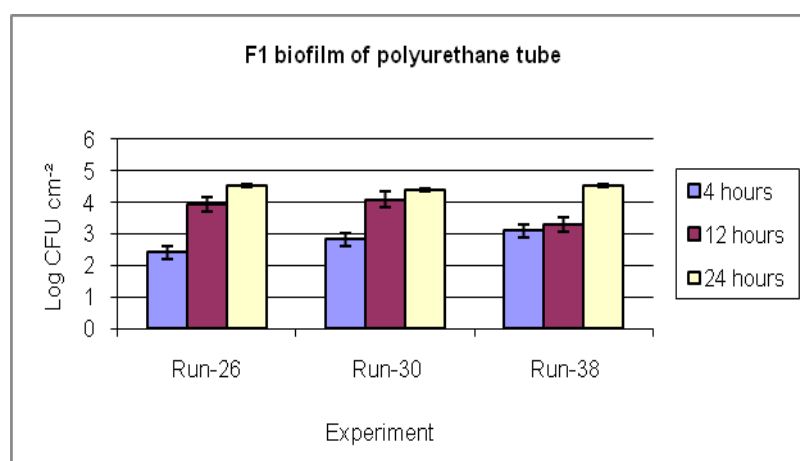


Figure 5.7: The numbers F1 cells attached and forming biofilm on polyurethane tubing surfaces at 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined using duplicate samples for each experimental run.

5.3.4.1.3 *Silicone*

a) *Planktonic cells*

The F1 planktonic cells recovered from 10% reconstituted IFM at 4, 12 and 24 h were approximately 7 log CFU mL⁻¹ for each of the three experimental runs (Fig 5.8). The planktonic cells showed a slight decrease after 12 h for run-26 but increased again after 24 h incubation.

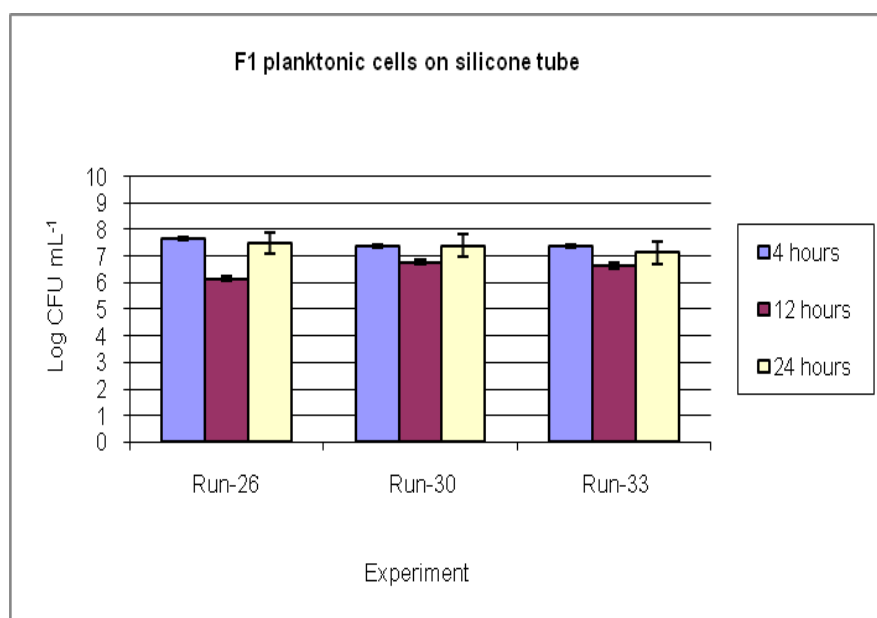


Figure 5.8: The numbers of F1 planktonic cells detected after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

b) Biofilms

The initial attachment of F1 cells to silicone tube was very low after 4 h incubation at 25°C (Fig 5.9). The numbers increased after 12 h and continued to increase until 24 h of incubation. However, the F1 planktonic cells in run 26 decreased slightly at 24 h (about 1 log CFU cm⁻²).

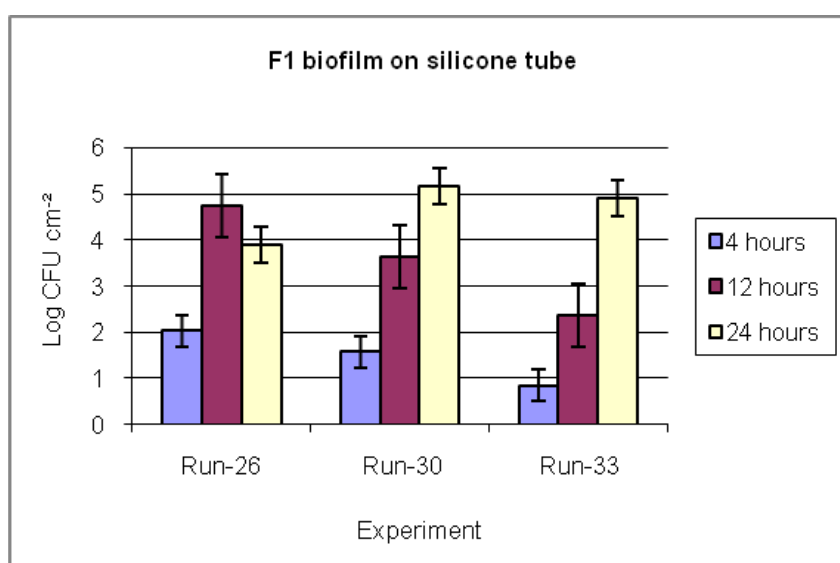


Figure 5.9: The numbers F1 cells attached and formed biofilm on silicone tubing surfaces at 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

The following figure (5.10) shows the comparison of cell numbers that formed biofilm on three types of infant feeding tubes- PVC, polyurethane and silicone. The F1 cell attachment on silicone tube was the lowest among the three types of tubing at 4 h of incubation. However, further incubation showed that the cells numbers increased and reached similar numbers on all the three types of tubing.

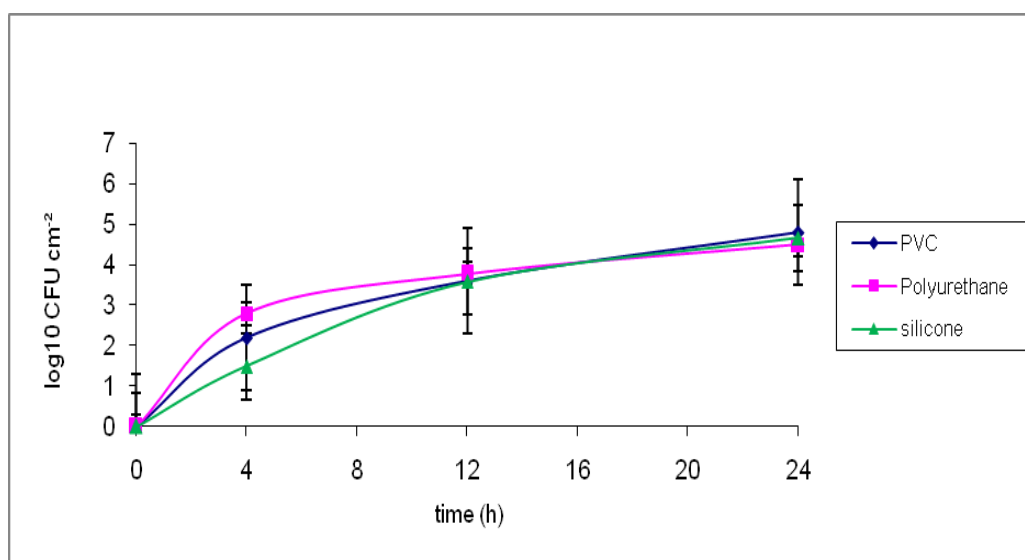


Figure 5.10: The numbers of F1 cells forming biofilm on three types of infant feeding tubes tube at three time intervals - 4, 12 and 24 h. The mean and standard deviation were calculated from replicate samples incubated in the 10% reconstituted IFM at 25°C.

5.3.4.2 Clinical isolate (F8)

5.3.4.2.1 PVC

a) *Planktonic cells*

The numbers of F8 planktonic cells increased from 8 log CFU mL⁻¹ to 10 log CFU mL⁻¹ for the experiments on PVC tubing and remained approximately the same in the three experimental runs during 24 h of incubation in 10% reconstituted IFM at 25°C (Fig 5.11).

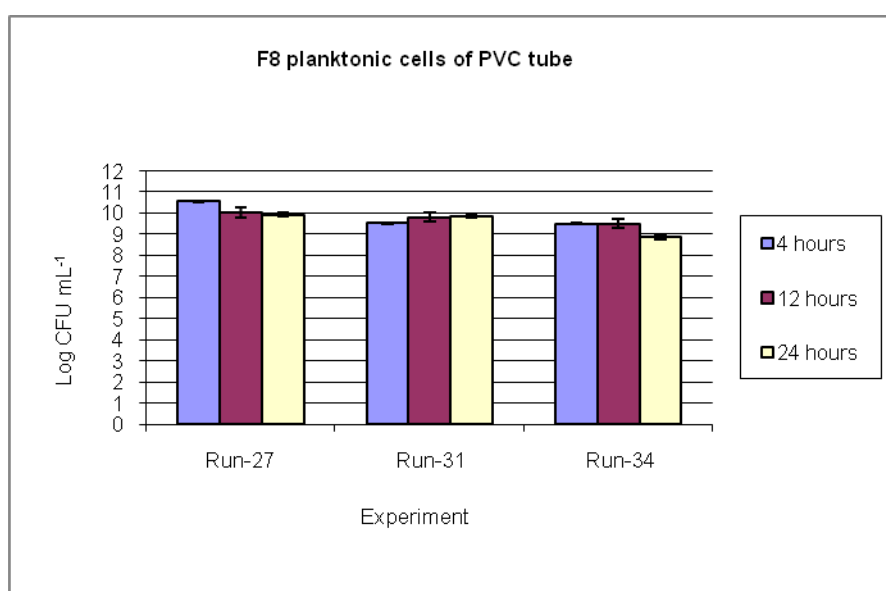


Figure 5.11: The numbers of F8 planktonic cells after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

b) Biofilms

The numbers of F8 cells attached to PVC tube after 4 h incubation were high and the numbers of cells decreased during 12 h of incubation for run 27 and run 31 (Fig 5.12). However, for run 34, the cell numbers were slightly increased after 12 h and decreased after 24 h of incubation at 25°C.

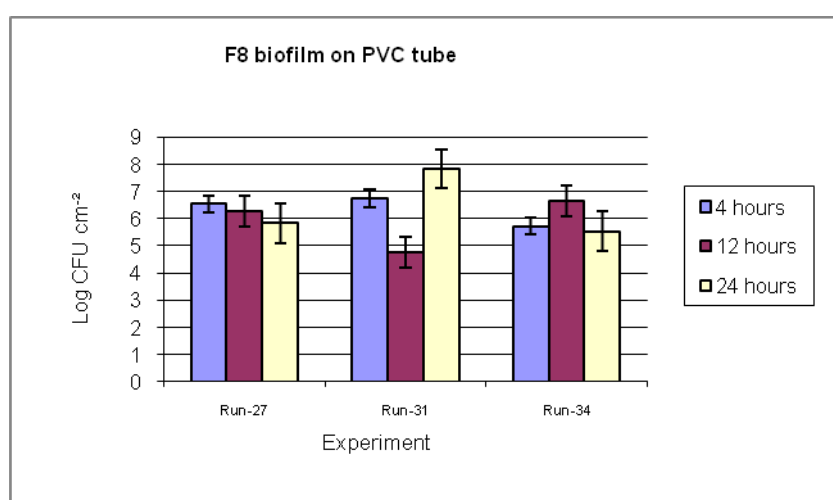


Figure 5.12: The numbers F8 cells attached and forming biofilm on PVC tubing surfaces at 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

5.3.4.2.2 Polyurethane

a) *Planktonic cells*

The numbers of F8 planktonic cells increased from 8 log CFU mL⁻¹ to 10 log CFU mL⁻¹ in experiments on polyurethane tubing and remained constant in the three experimental runs during 24 h of incubation in 10% reconstituted IFM at 25°C (Fig 5.13).

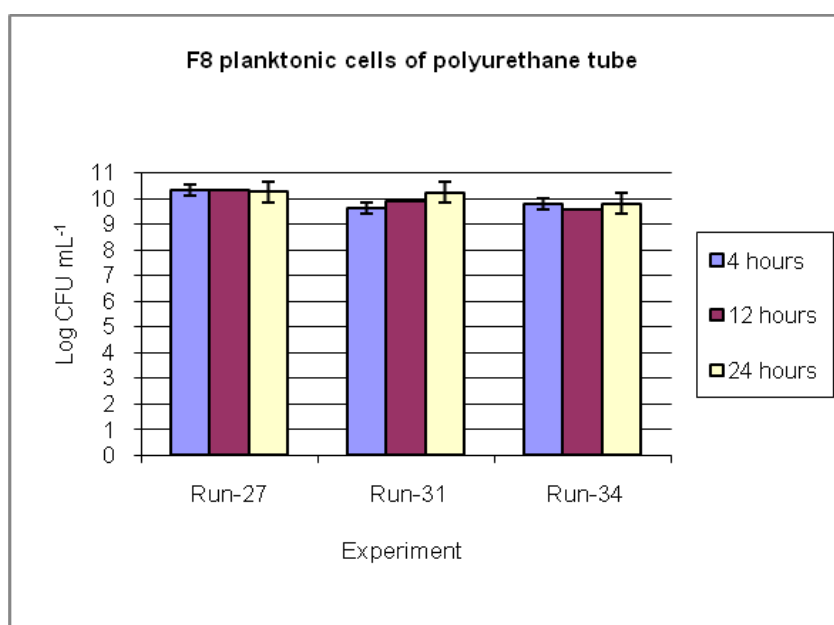


Figure 5.13: The numbers of F8 planktonic cells after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

b) Biofilms

The experiment investigating F8 biofilm formation on polyurethane showed different patterns between runs (Fig 5.14). Run 27 showed a decrease in cell numbers with time of incubation with the final count being about 1 log CFU cm⁻² less than the initial attachment after 24 h incubation. Run 34 showed a low initial attachment after 4 h of incubation and a large increase after 12 h of incubation. However, the cell numbers decreased slightly after 24 h of incubation. Run 39 showed a low initial attachment at 4 h and the cell numbers increased with time until 24 h of incubation in 10% reconstituted IFM at 25°C.

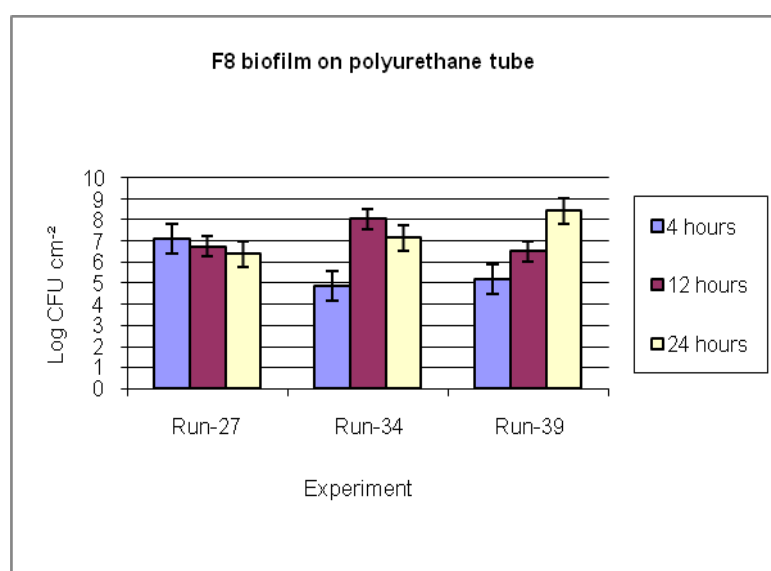


Figure 5.14: The numbers F8 cells attached and formed biofilm on polyurethane tubing surfaces after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

5.3.4.2.3 Silicone

a) *Planktonic cells*

The numbers of F8 planktonic cells increased from 8 log CFU mL⁻¹ to 10 log CFU mL⁻¹ on silicone tubing and remained constant in the three experimental runs during 24 h of incubation in 10% reconstituted IFM at 25°C (Fig 5.15). However, there were no data for run 34 at 4 h of incubation as the capacitance detection showed an unacceptable value. The *E. sakazakii* curve based on the growth monitor showed irregularity from standard *E. sakazakii* growth curve. Thus, the value from BacTrac® 4000 was discarded.

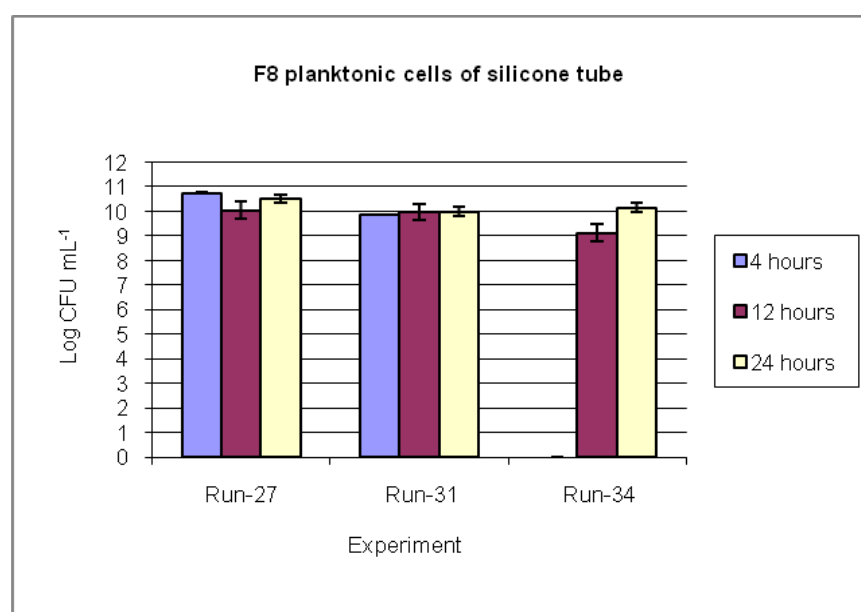


Figure 5.15: The numbers of F8 planktonic cells after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run. The BacTrac® 4000 produced no data in Run 34 at 4 h of incubation.

b) Biofilms

The attachment of F8 cells on silicone tubing after 4 h incubation was high at approximately 5 log CFU cm⁻² and the cell numbers increased to 7 log CFU cm⁻² with time until up to 24 h of incubation in 10% reconstituted IFM at 25°C (Fig 5.16).

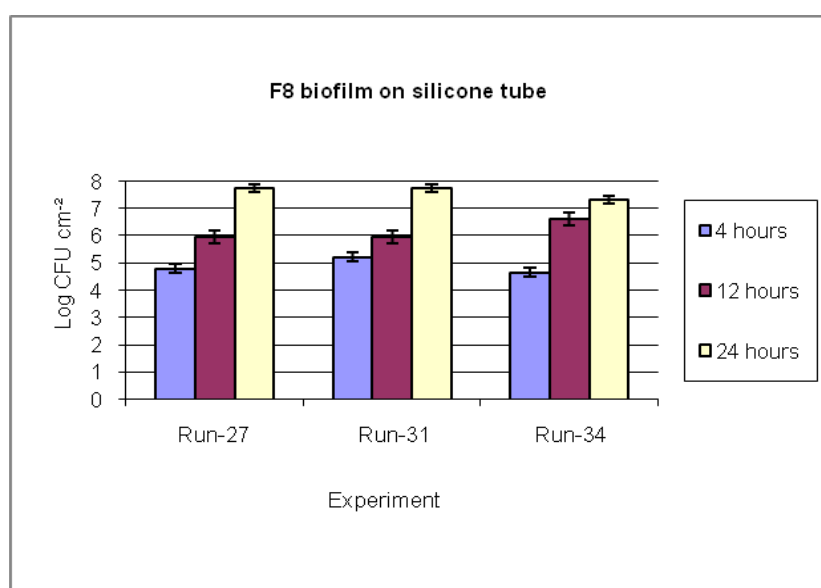


Figure 5.16: The numbers F8 cells attached and forming biofilm on silicone tubing surfaces after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

The figure below (Fig 5.17) shows the comparison between biofilm on three types of infant feeding tubes - PVC, polyurethane and silicone. The attachment of F8 cells on silicone tube was the lowest among the three types of tubing tested at 4 h of incubation. However, further incubation showed that the cells numbers on polyurethane and silicone tubing increased. Meanwhile, F8 cell numbers on PVC were decreased slightly after 12 h of incubation but increased again after 24 h of incubation in 10% reconstituted IFM at 25°C.

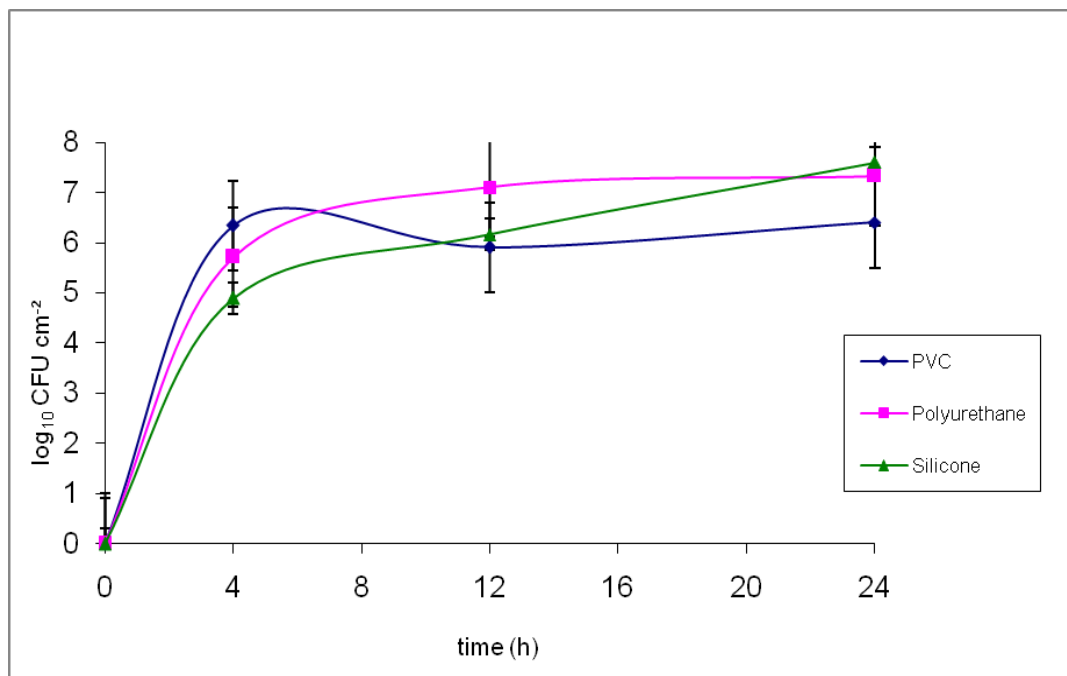


Figure 5.17: The numbers of F8 cells forming biofilm on three types of infant feeding tubes tube at three time intervals - 4, 12 and 24 h. The mean and standard deviation were calculated from duplicate samples incubated in the 10% reconstituted IFM at 25°C.

5.3.4.3 Environmental isolate (A6)

5.3.4.3.1 PVC

a) Planktonic cells

The number of A6 planktonic cells increased from 8 log CFU mL⁻¹ to 9 log CFU mL⁻¹ in experiments using PVC tubing and this number remained constant in the three experimental runs during 24 h of incubation in 10% reconstituted IFM at 25°C (Fig 5.18).

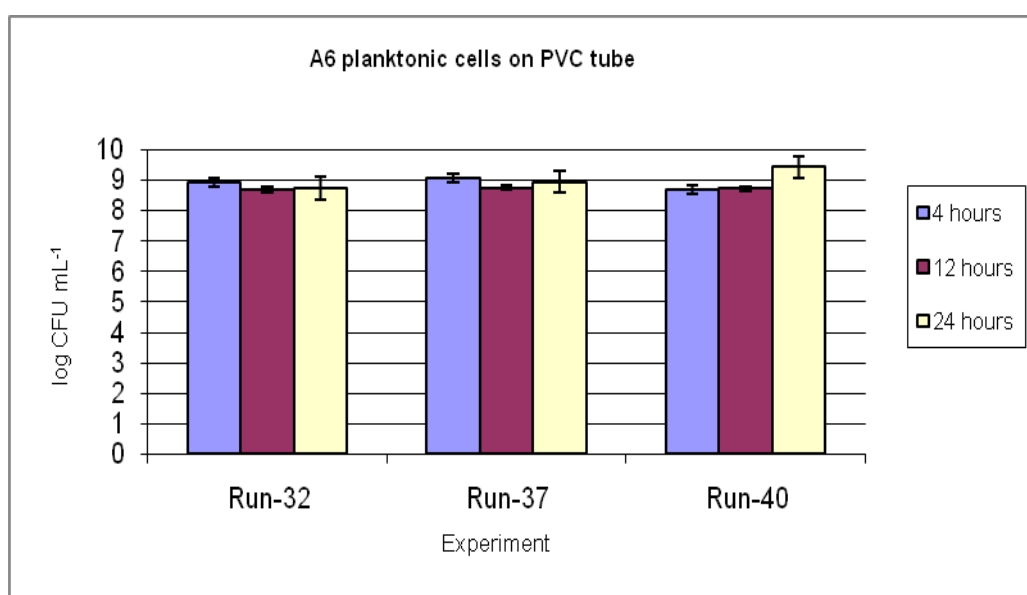


Figure 5.18: The numbers of A6 planktonic cells after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

b) Biofilm

The A6 cell numbers attached and forming biofilm on PVC tubing was quite high after the 4 h of incubation for run 32 with approximately 6.5 log CFU cm⁻² (Fig 5.19). The cell numbers increased after 12 h of incubation. Run 37 showed a different pattern of biofilm development compared with run-32. The number of A6 cells attached was low after 4 h but increased up to 24 h of incubation reaching 6.2 log CFU cm⁻². For run 40, the initial attachment of A6 cells was similar to run 37 but the cell numbers increased by about 1 log CFU cm⁻² after 12 h of incubation. After 24 h, the cell numbers decreased slightly in 10% reconstituted IFM at 25°C.

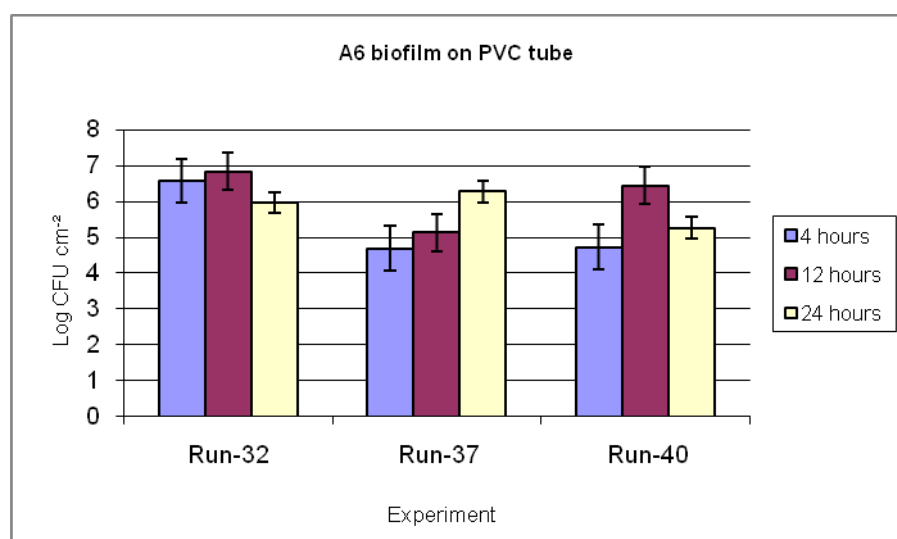


Figure 5.19: The numbers of A6 cells attached and forming biofilm on PVC tubing surfaces after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

5.3.4.3.2 Polyurethane

a) Planktonic cells

The number of A6 planktonic cells increased from 8 log CFU mL⁻¹ to 10 log CFU mL⁻¹ on polyurethane tubing and remained constant in the three experimental runs during 24 h of incubation in 10% reconstituted IFM at 25°C (Fig 5.20).

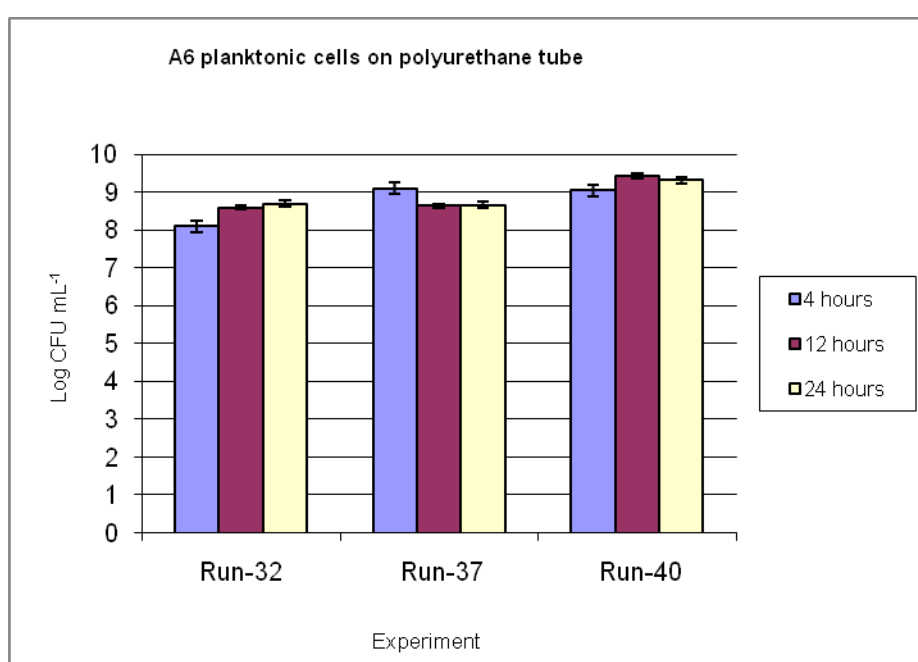


Figure 5.20: The numbers of A6 planktonic cells after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

b) Biofilm

For run 32, the number of A6 cells attached to the tubing was quite high at approximately $5.2 \log \text{CFU cm}^{-2}$ after 4 h incubation (Fig 5.21). Meanwhile, for run 37 the initial cell attachment at 4 h was quite high and increased up to $7 \log \text{CFU cm}^{-2}$ during 12 h of incubation. In run 40 the initial cell attachment was relatively low after 4 h incubation, but the cell numbers increased continuously until 24 h of incubation in 10% reconstituted IFM at 25°C .

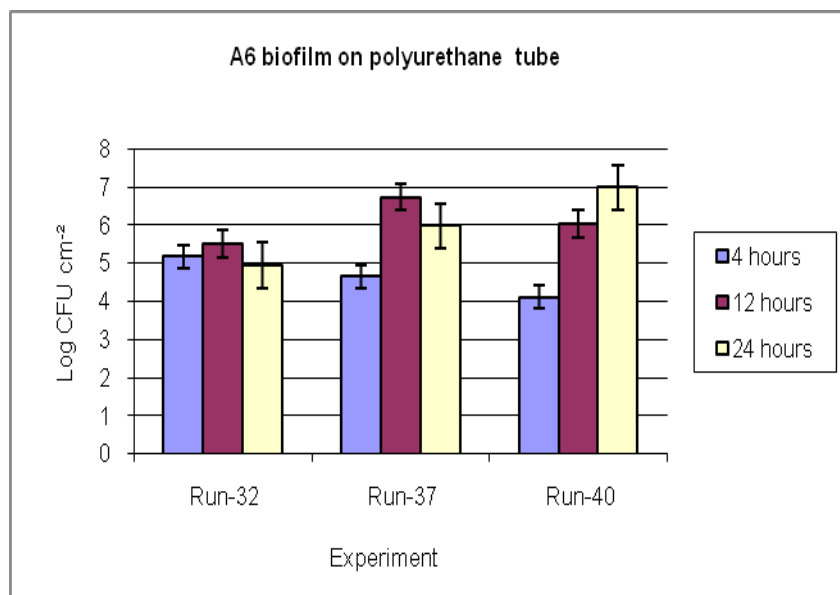


Figure 5.21: The numbers A6 cells attached and forming biofilm on polyurethane tubing surfaces at 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

5.3.4.3.3 *Silicone*

a) *Planktonic cells*

The A6 planktonic cells for run 28 were lower than the initial inocula (8 log CFU mL⁻¹) after 4 h of incubation and the cell counts increased slightly during 12 h of incubation (8.2 log CFU mL⁻¹) (Fig 5.22). After 24 h of incubation, the cell counts were approximately 9.1 log CFU mL⁻¹. The low cell numbers of A6 planktonic cells at 4 h was probably because there was a temperature fluctuation in the aerobic shaker that affected the cell growth in 10% reconstituted IFM as discussed in the previous run. The planktonic cells in run 37 and run 41 were quite high at approximately 9 log CFU mL⁻¹ after 4 h incubation and the cell numbers remained constant until 24 h of incubation in 10% reconstituted IFM at 25°C.

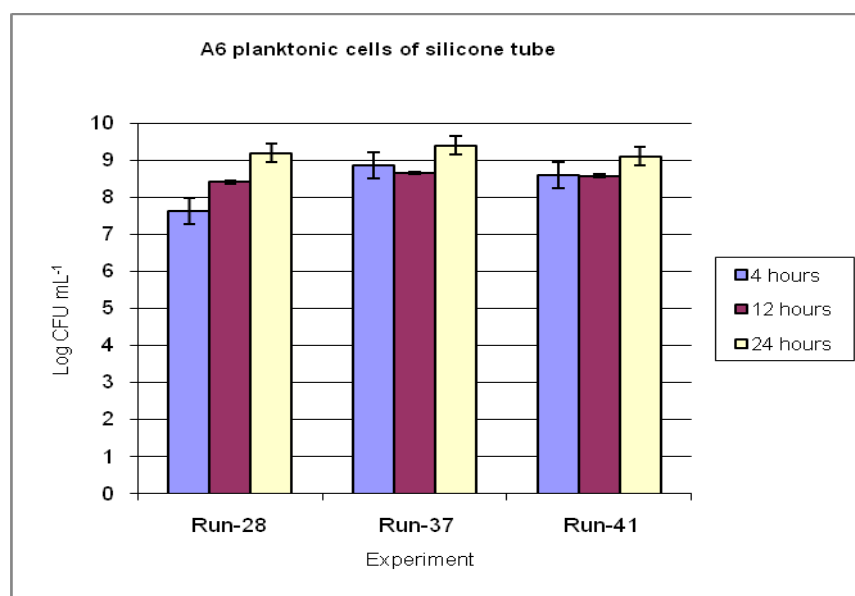


Figure 5.22: The numbers of A6 planktonic cells after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

b) Biofilm

The initial attachment of A6 cells on silicone tube was low for runs 26 and 37 at approximately 4 log CFU cm⁻² (Fig 5.23). After 12 h, the cell numbers increased by almost 1.5 log for run 26 and 3 log for run 37. The cell counts increased for the latter run after 24 h of incubation. For run 41, the initial attachment on silicone tubing was quite high at approximately 5.5 log CFU per cm⁻² after 4 h of incubation and the numbers increased until 24 h of incubation in 10% reconstituted IFM at 25°C.

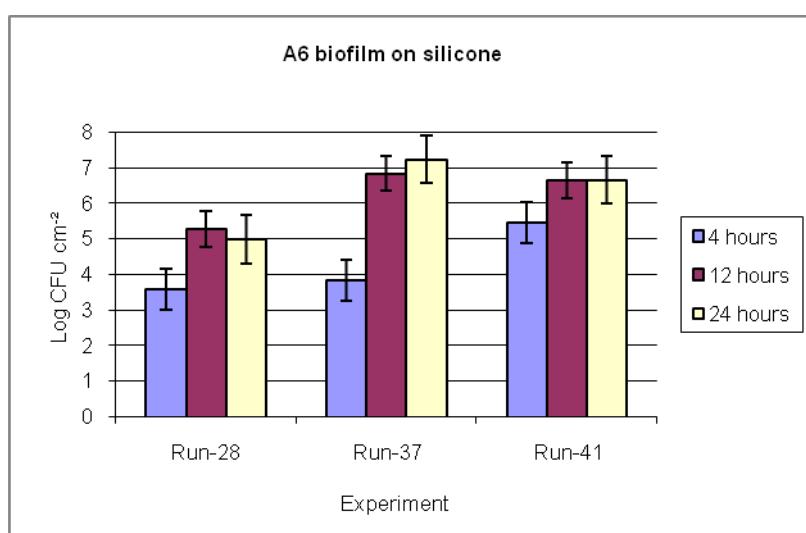


Figure 5.23: The numbers A6 cells attached and forming biofilm on silicone tubing surfaces after 4, 12 and 24 h of incubation in 10% reconstituted infant formula milk. The mean and standard deviation were determined by duplicate samples for each experimental run.

The following figure (Fig 5.24) shows the comparison of cell numbers that formed in the biofilm on three types of infant feeding tubes: PVC, polyurethane and silicone. The attachment of A6 on silicone tube after 4 h was the lowest among the three types of tubing. However, further incubation showed that the cell numbers increased to reach similar numbers for all three types of tubing.

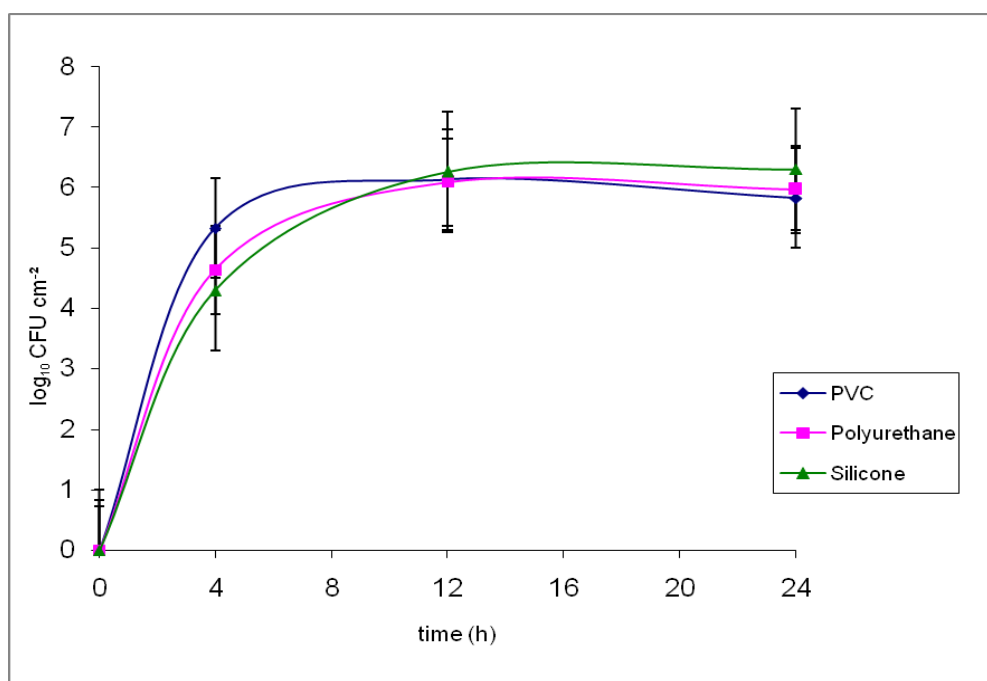


Figure 5.24: The numbers of A6 cells forming biofilm on three types of infant feeding tubes tube at three time intervals- 4, 12 and 24 h. The mean and standard deviation were calculated from duplicate samples incubated in the 10% reconstituted IFM at 25°C.

5.3.5 General discussion of results

To observe the biofilm formation in 10% reconstituted IFM on infant feeding tube, the temperature was initially set at 37°C for incubation at 4, 12 and 24 h in the aerobic shaker. Several publications (Nazarowec-White and Farber, 1997; Breeuwer et al., 2003; Iversen et al., 2004; Kandhai et al., 2006) reported that the doubling time of *E. sakazakii* in rich media such as brain heart infusion and infant formula milk at 37°C is around 20 minutes. However, in our several initial trials (data not shown) no growth was detected on tubing surfaces incubated at 37°C in IFM. When the temperature was altered to 25°C for tubing incubation in 10% reconstituted IFM, bacteria were detected on tubing samples. Kim et al., (2006) reported that *E. sakazakii* managed to form biofilm at 25°C but not at 12°C on stainless steel and feeding tubes when incubated in IFM.

Biofilm growth of *E. sakazakii* appears to be limited by temperature while planktonic growth can occur in much wider temperature range. There are major physical and biological differences between cells grown in suspension and those in a biofilm. Some of the unique physiological characteristics of biofilm cells have been correlated with differential gene expression. The best example is the production and secretion of polymers by biofilm cells. Bacteria possess a sophisticated mechanism for adapting to changing environments by switching between planktonic and biofilm growth. It should be noted that for most modelling purposes, biological reactions of biofilm bacteria are assumed to be identical to those of planktonic bacteria and differences between biofilm and suspended systems are assumed to result from the substantial mass transfer and diffusional resistances encountered by chemicals within the biofilm (Palmer et al., 2007).

An example of such metabolic flexibility is the bacterial response to oligotrophic growth conditions, i.e. those which offer little to sustain life. Biofilms form mainly in nutrient-rich environments, because polymer production and secretion, which initiate and maintain biofilm formation, are energy-intensive processes. This does not mean however that biofilms are not found in oligotrophic environments. If nutrient conditions improve temporarily, planktonic starved cells will produce

exopolysaccharides and attach to surfaces, forming food reserves in the biofilm. When oligotrophic conditions return, cells in the biofilm will persist on the entrapped nutrients and exopolysaccharides (Donlan and Costerton, 2002).

Another important difference between suspended and biofilm cells is the relative hydrophobicity of bacteria growing in biofilms. Hydrophobic forces are thought to aid in the initial attachment of bacteria to surfaces by overcoming the electrostatic repulsion present between negatively charged cells and surfaces. Although conclusive work remains to be done, recent studies suggest that bacteria may alter their surface hydrophobicity in response to environmental changes, thereby promoting or discouraging biofilm development. Cells that detach from biofilms were shown to be significantly more hydrophilic than bacteria remaining in the biofilm (Kumar and Anand, 1998; Donlan, 2002). This may reflect substances produced by cells during biofilm growth.

In this study, the milk became very thick and sticky over 12 h to 24 h of incubation at 25°C for all three isolates probably due to polysaccharide production. There were some difficulties when trying to withdraw a 1 mL sample of planktonic cells from the inoculation media as the milk was very thick. The tips ends of the pipette were cut to increase the orifice and thus make it easier to pipette 1 mL sample for the enumeration of planktonic cells.

For the biofilm enumeration, the entire surface area of the tubing was taken into account to obtain the exact count of cells per unit area that formed biofilm on the tubing surface. The diameter and the wall thickness of the tubing were measured for surface area calculation. This measurement was important, as the cells are likely to attach to every surface including the edges of the tubing.

Epifluorescence microscopy (Chapter 3, section 3.8.1) on samples taken after 4 h of incubation showed that the tubes (PVC, polyurethane and silicone) were stained lightly on both surfaces and no obvious patches/clumps of the F1, F8 and A6 strains were seen. After 12 h to 24 h of incubation, there were heavy patches/clumps of cells on both surfaces (internal and external) especially towards

the edges of the PVC, polyurethane and silicone tubing. Similar observations were seen for all three strains tested. However, there were some difficulties in observing cell colonisation of the tubing surfaces using epifluorescence microscopy since the surfaces are not flat. This is why it was not possible to obtain good images of biofilm on the tubing surface using epifluorescence microscopy. We were afraid that further preparation to make the tubing flat might destruct the biofilm formation.

5.3.6 SEM images of the tubing surfaces (control)

Using SEM, the PVC tubing surface, before inoculation, appeared smooth with a few small cracks and crevices (Fig 5.25). Meanwhile, the polyurethane tubing surface appeared quite rough with cracks in flaky shapes. This distinctive feature could be seen for the entire tubing surface. The silicone tubing appeared smooth with a number of pits. The pits sometimes contained crystallised features that look like small colonies under scanning electron microscopy observation.

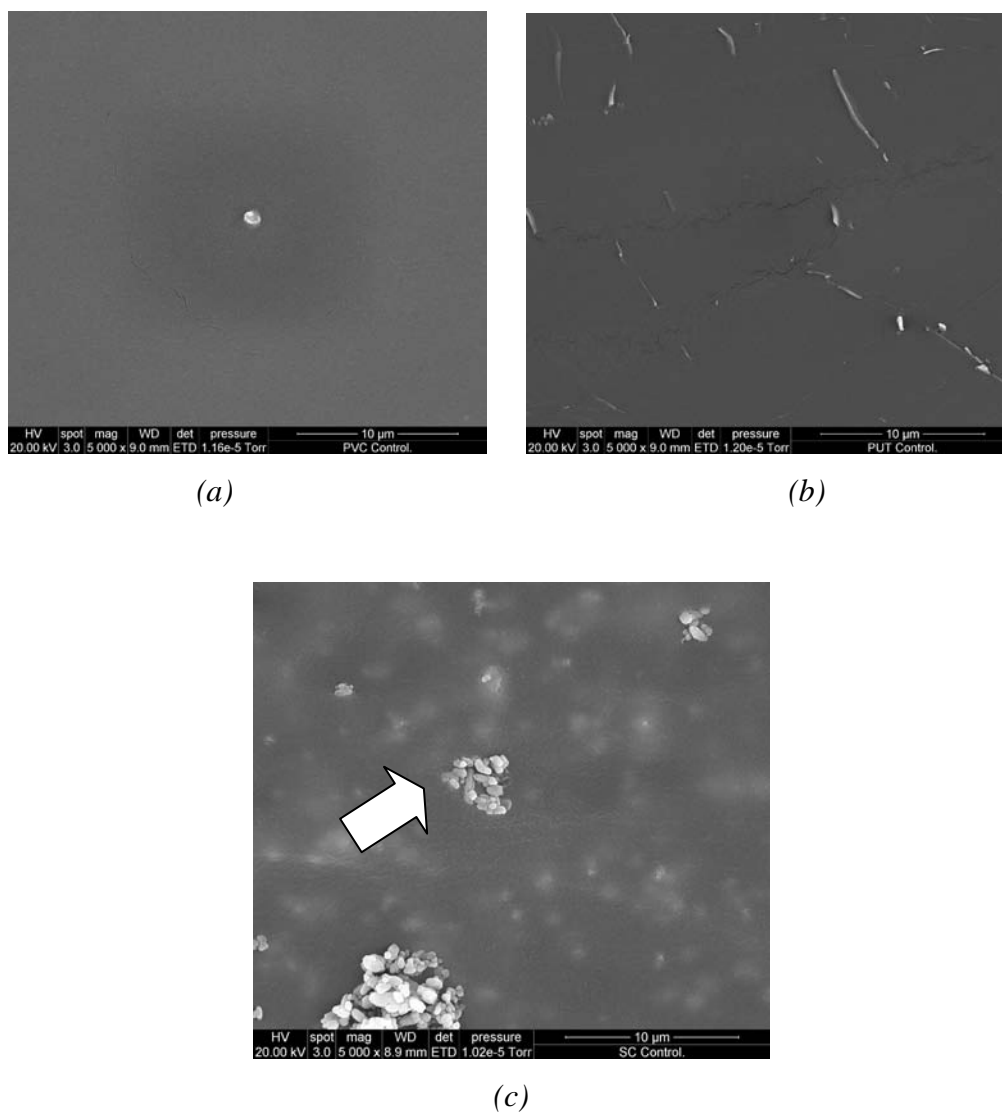


Figure 5.25: The scanning electron micrograph of infant feeding tube surfaces that acted as control in this study. The image (a) shows PVC surface, (b) polyurethane surface and (c) silicone surface. The arrow shows the image of surface properties of silicone tubing.

5.3.7 SEM images of F8 biofilm on PVC, polyurethane and silicone after different incubation periods

5.3.7.1 After 4 h of incubation

The three images made at 4 h incubation show that the F8 strain attached and formed biofilm on PVC and polyurethane tubes (5.26). However, silicone tube after 4 h incubation appeared similar to the control tubing, with no evidence of bacterial colonisation.

Based on the biofilm cell counts obtained by impedance microbiology, about 4 to 6 log CFU cm⁻² of F8 cells colonised the surfaces of PVC and polyurethane tubing during 4 h of incubation. Colonisation of the silicone tubing was less than the other two types of tubing and this appears to be reflected in the SEM results.

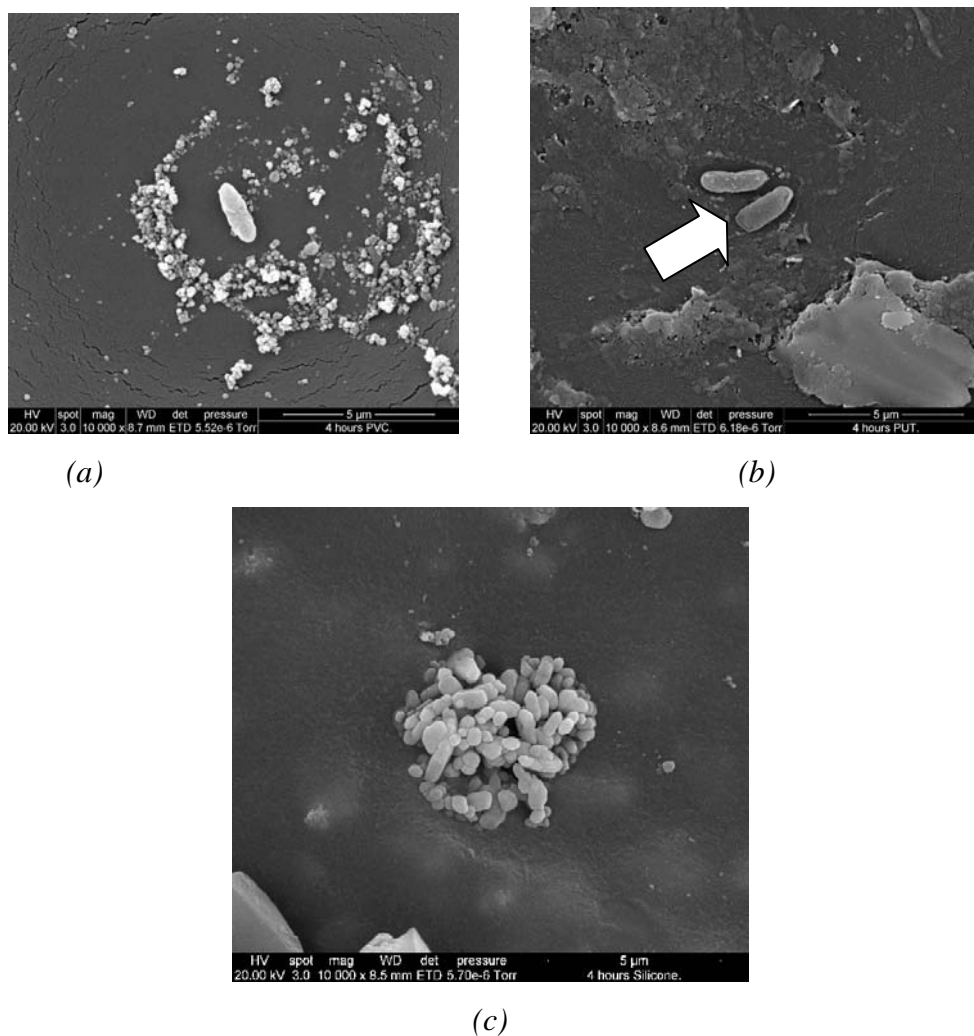


Figure 5.26: The images represent the attachment of strain F8 after 4 h of incubation. The three scanning electron micrographs show (a) PVC tubing, (b) polyurethane tubing and (c) silicone tubing. The arrow shows the image of a single *E. sakazakii* cell.

5.3.7.2 After 12 h of incubation

Figure 5.27 shows SEM images of all three tubing surfaces after 12 h incubation. F8 cells attached and formed a dense biofilm on polyurethane and silicone tubes. For the PVC tube, no *E. sakazakii* rod shaped cells could be found in the images. This may be a result of cells being covered by the milk proteins and globules on the tubing surface so they were not visible microscopically.

During this 12 h of incubation, more milk deposits were seen on the tubing surfaces and this is likely to play a role on surface colonisation by *E. sakazakii*.

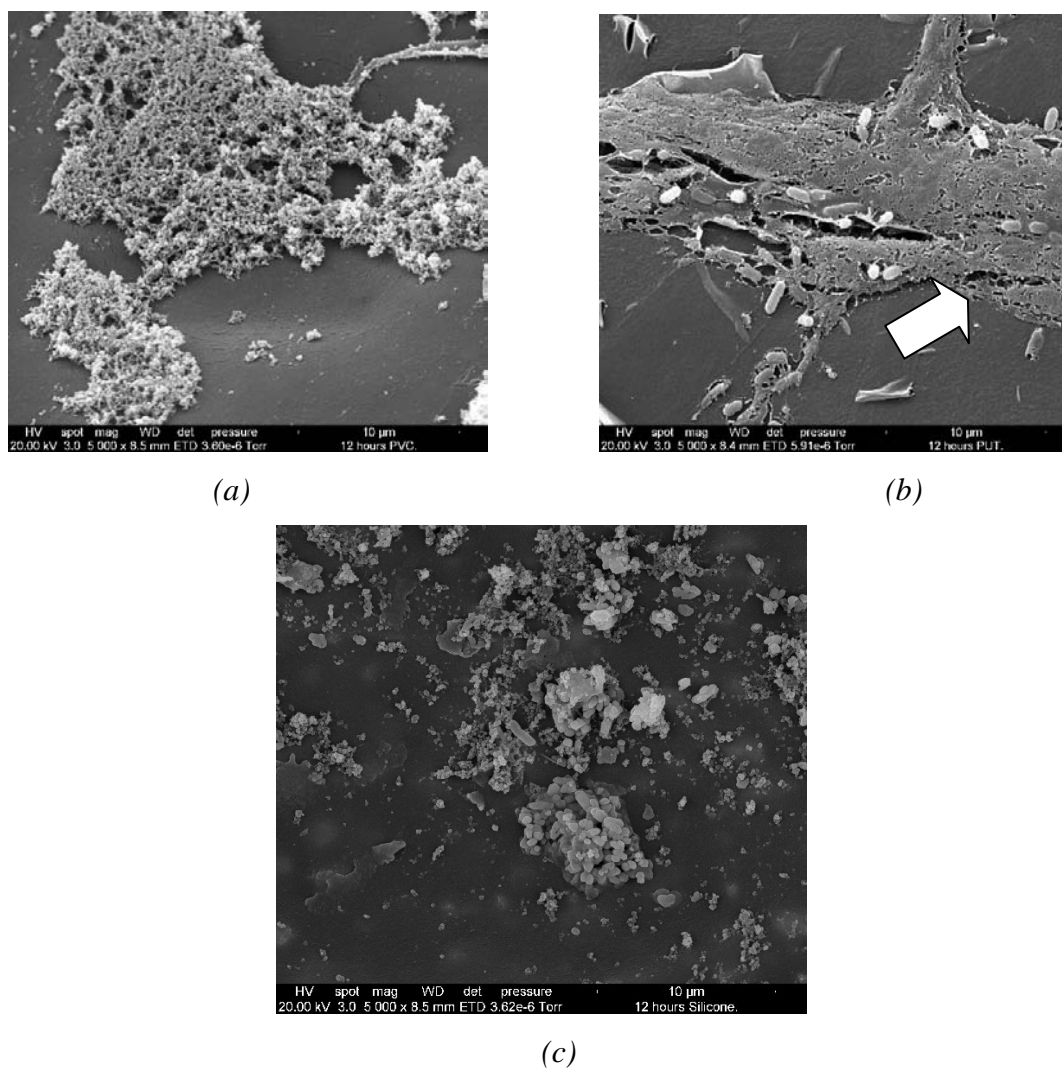


Figure 5.27: The images represent the biofilm formation by F8 strain at 12 h of incubation. The three scanning electron micrographs show (a) PVC tubing, (b) polyurethane tubing and (c) silicone tubing. The arrow shows the exopolysaccharides produced by *E. sakazakii*.

5.3.7.3 After 24 h of incubation

The three images shown below (Fig 5.28) were captured following 24 h incubation and show that F8 cells attached and formed biofilm on PVC and silicone tubes and these were associated with milk deposits and polysaccharide, the latter presumably produced by the cells colonising the tubing surface. Based on impedance microbiology counts, there were approximately 6 to 7 log CFU of F8 cells cm⁻² on the three types of tubing.

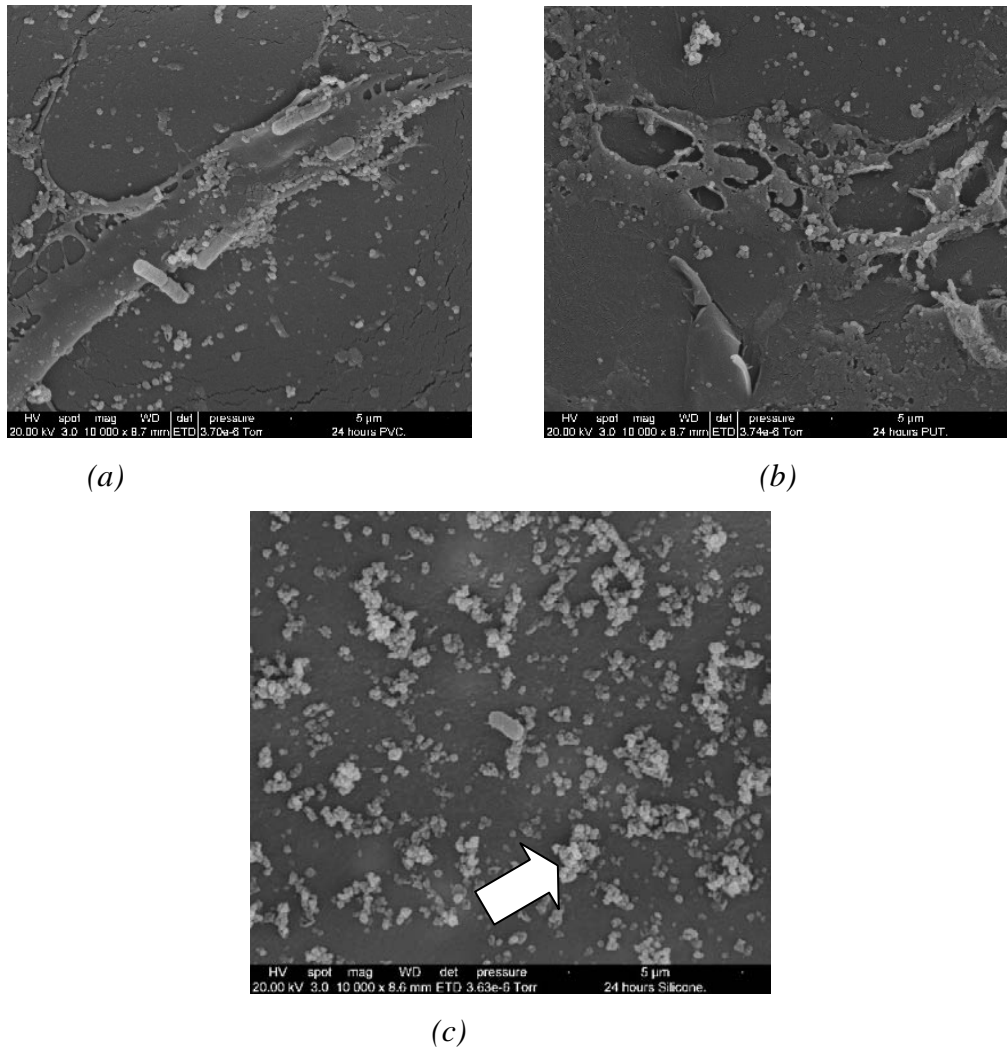


Figure 5.28: The images represent the biofilm formation by F8 strain at 24 h of incubation. The three scanning electron micrographs show (a) PVC tubing, (b) polyurethane tubing and (c) silicone tubing. The arrow shows the milk fat or globules.

5.4 Conclusion

The observation that *E. sakazakii* was unable to grow as a biofilm at 37°C in the 10% reconstituted IFM (based on the impedance detection by BacTrac® 4000 that unable to produce the acceptable values readings of *E. sakazakii* numbers) suggests that temperature has a major influence on biofilm formation of this organism in an IFM environment. We also experienced the death of *E. sakazakii* planktonic cells in several experimental runs when there was some variation of temperature in the aerobic shaker during incubation of the tubing in the 10% reconstituted IFM.

E. sakazakii F8, a clinical strain, was able to colonise all three types of feeding tube tested in this trial. Silicone tubing seemed more difficult to colonise, with lower cell numbers recorded after 4 h incubation. This suggests that of all three types of tubing tested, silicone tubing may be slower to colonise and therefore may be the preferred type of tubing to be used in infant feeding.

Note that in these experiments, the tubing was exposed to high numbers of *E. sakazakii*, much higher than would be experienced in the natural environment. Although all three types of tubing were well colonised by 24 h, in a neonatal feeding environment the conditions are different. Tubes are flushed with water after feeding, and the inoculum levels are low, so the time to develop a mature biofilm that threatens the health of the neonate is likely to be longer than the time course of these experiments.

The F8 strain (clinical isolates) produced high numbers of cells on all three tubes over a 24 h period. The milk deposits and polysaccharide material observed by SEM are both likely to contribute to the colonisation of the tubing surface.

Chapter 6

FINAL DISCUSSION AND CONCLUSIONS

This study was designed to test two hypotheses – (1) that isolates of *E. sakazakii* from different sources vary in their ability to form biofilm and (2) that different materials used to manufacture nasogastric tubing for infant feeding vary in their ability to support biofilm growth of *E. sakazakii*. The ability of individual isolates to form biofilm was determined using a microtitre plate screening assay that quickly determined that all clinical isolates readily formed biofilm, while isolates from other sources varied in their ability to form biofilm. The ability to colonise tubing surfaces was determined in a simple batch culture with the use of impedance to enumerate viable cells colonising the tubing. This determined that silicone tubing was slower to colonise than PVC or polyurethane tubing and on this basis should be the tubing of choice for infant feeding. All trials were done in infant formula milk (IFM).

Some of the *E. sakazakii* biofilms forming on the tubing after 24 hours were mature and thick, whereas others were thin and immature. Biofilms associated with PVC and polyurethane feeding tubes were visible under SEM with micro-organisms seen within crevices naturally present in the tubing surface.

E. sakazakii grows rapidly in reconstituted IFM, with inocula of 1 CFU mL⁻¹ stored at room temperature reaching levels of > 10⁷ CFU mL⁻¹ within 10 h (Nazarowec-White and Farber, 1997, Pagotto et al. 2003). Note that in the present study, a high inoculum (8 log CFU mL⁻¹) of *E. sakazakii* was used. This represented an extreme case with reconstituted infant formula milk heavily contaminated with *E. sakazakii* cells. Natural contamination would be much lower than this, but to ensure that a more rapid result was obtained, a heavy inoculum was used.

The batch system was selected instead of a flowing system in this study because of the small diameter (5-French) of the enteral feeding tubes and to reduce the amount of IFM needed for each trial. There was some concern that the small diameter of tubing might lead to clogging in a prolonged experiment.

The effect of temperature on the growth of *E. sakazakii* biofilm growth observed during these trials may be able to be manipulated in infant feeding to reduce the likelihood of biofilm growth and neonatal infection.

There were two other key findings from this study. Firstly, those isolates of *E. sakazakii* that cause clinical infections also tend to form strong biofilms, confirming the link between biofilm formation and disease. This may relate to the greater likelihood of these strains forming biofilm in feeding tubes producing a high inoculum and high chance of infection. Secondly, silicone tubing is slower to colonise than PVC or polyurethane tubing and, other things being equal, is likely to be the best tubing for neonate feeding to avoid *E. sakazakii* infection from biofilm growth.

Prospects for future research

1. Tubing sterilisation

Tubing sterilisation used in this study was achieved using heat treatment (autoclaved at 121°C for 15 minutes). Radiation or chemical treatment for sterilising the tubing should be compared with autoclaving as the heat treatment used in this trial may alter the surface structure of the tubing and this may effect biofilm growth.

2. Prolong incubation period

In neonatal feeding, tubing is often used for many days without changing, therefore observing biofilm growth over a longer period of time with a lower inoculum, may be a better model of actual biofilm growth during neonatal feeding. During this time, if tubing could be exposed to IFM, and rinsed between exposures to IFM, this would more closely replicate the real situation in a neonatal feeding programme.

3. Applying antimicrobial for surface treatment

There are many experimental treatments that could be applied to the tubing to delay biofilm growth. Further research should consider treatment of the surface of feeding tubing to inhibit the attachment and biofilm formation by *E. sakazakii*. (Kim et al., 2007; Kim et al., 2008b).

4. Preparation of infant formula

Steele et al, (2008) found that a centralised infant formula preparation room in the neonatal intensive care unit reduces the incidence of microbial contamination. Their study showed that the contamination is related to the initial preparation of the formula and therefore related to environmental contamination. This should be investigated further.

5. Tests

It is essential that detection methods for *E. sakazakii* are established that are robust and reliable to ensure the safety of IFM. Rapid tests for *E. sakazakii* and a method to rapidly screen isolates with ability to form strong biofilms may be useful tools in exploring the epidemiology of *E. sakazakii* infant infection.

6. Tubing design

Reducing the formation of biofilm must be a critical goal in the design and selection of biomaterials for new enteral feeding tubing. Determining why silicone tubing is slow to colonise than other materials of tubing may help in designing or selecting new materials for enteral feeding tubes.

APPENDICES

Appendix 1



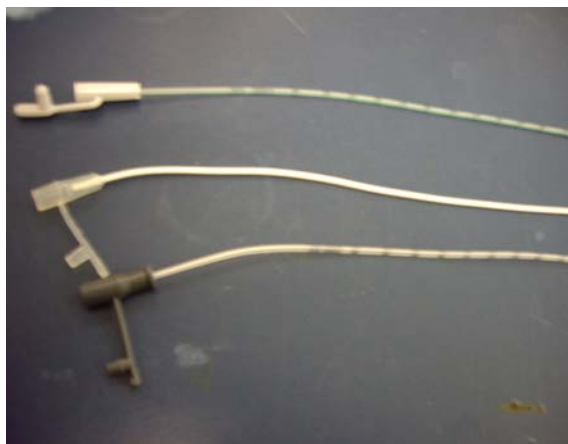
Figure A1 : The image of auto microtitre plate reader (EL_x 808-Ultramicroplate reader, Bio-tek Instruments, INC, Winooski, VT, USA) used in this study.

Appendix 2



Figure A2 : The image of BacTrac® 4000 microbiological growth analyser (Minitrac, Sy-Lab, Purkers dorf, Austria) used in this study.

Appendix 3



(a)



(b)

Figure A3: The image (a) showed the three different materials of infant feeding tubes (PVC, silicone and polyurethane) with 5-French diameter used in this study and image (b) showed the tubing was cut into 2 cm segment each.

Appendix 4

Objective:

Wall thickness of the three tubing determination

Materials:

- 1) Tubing- PVC, polyurethane and silicone
- 2) Micrometer eyepiece
- 3) Micrometer calibration slide
- 4) Light microscope

Methods A:

- 1) The micrometer eyepiece was placed in the place of the standard eyepiece of the microscope.
- 2) After placing the special eyepiece, it is necessary to calibrate the microscope with calibration slide.
- 3) The slide was placed on the stage and the top of the slide (the surface with the microscopic lines engraved on it) was pointed up.
- 4) The calibration slide was set to low power and the lines engraved on the surface of the calibration slide were focused.
- 5) The number of lines must be counted.
- 6) How much each line of the eyepiece measures was calculated. In other means, the distance between each line of the eyepiece must be determined. The equation below was used:

$$Y/X \times 10 \mu\text{m} = \text{measurement between 2 lines on the eyepiece}$$

Y= calibration slide lines,

X= eye piece lines

- 7) If X= 38 and Y=10 lines, by using the formula, we got $10/38 \times 10 \mu\text{m} = 2.63 \mu\text{m}$ which means that as looking through microscope at low power the space between each line on the micrometer eyepiece measures 2.63 μm .
- 8) Repeat the same practise with medium and high power magnification.

9) Now it is possible to use the microscope to observe and measure things. The calibration slide is taken out and the slide with tubing piece was placed on the stage for observation.

Method B:

1) One power (e.g. low power) is calibrated and then calculates the proportions rather than measuring the other power.

2) For example,

40X	160X	400X
2.63 μm	1.05 μm	0.26 μm

3) Each time the objective lens is zoomed, it increased the value of the proportion by 10-fold. If we have the number for low power (2.63 μm for 40X), use the formula below to find out /calculate the calibration for medium power;

$$\begin{aligned} &= \frac{\text{Medium power magnification} \times \text{calibration for low power}}{\text{Low power magnification}} \div 10 \\ &= \text{Calibration for medium power} \end{aligned}$$

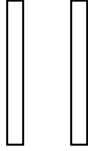
Results:

1. Lower power (40X) = $\frac{Y}{X} \times 10 \mu\text{m}$
= $\frac{10}{39} \times 10 \mu\text{m}$
= $2.56 \mu\text{m}$

2. Medium power (100X) = $\frac{(100 \times 2.56)}{40} \div 10$
= $0.64 \mu\text{m}$

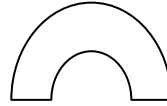
3. Silicone

A



= 43 ocular unit each

B



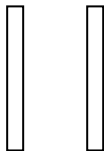
= 44 ocular unit

a. Wall thickness A = $43 \times 0.64 \mu\text{m}$
= $27.5 \mu\text{m}$
= 0.0028 cm

b. Wall thickness B = $44 \times 0.64 \mu\text{m}$
= $28.2 \mu\text{m}$
= 0.0028 cm

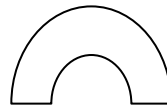
4. PVC

A



= 35 ocular unit each

B

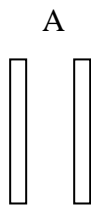


= 36 ocular unit

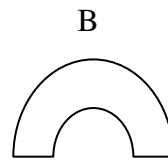
a. Wall thickness A = $35 \times 0.64 \mu\text{m}$
= $22.4 \mu\text{m}$
= 0.0022 cm

b. Wall thickness B = $36 \times 0.64 \mu\text{m}$
= $23.0 \mu\text{m}$
= 0.0023 cm

5. Polyurethane



= 40 ocular unit each



= 35 ocular unit

a. Wall thickness A = $40 \times 0.64 \mu\text{m}$
= $25.6 \mu\text{m}$
= 0.0026 cm

b. Wall thickness B = $35 \times 0.64 \mu\text{m}$
= $22.4 \mu\text{m}$
= 0.0022 cm

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