DETECTION, IDENTIFICATION AND LIVE/DEAD DIFFERENTIATION

OF THE EMERGING PATHOGEN *ENTEROBACTER SAKAZAKII* FROM INFANT

FORMULA MILK AND THE PROCESSING ENVIRONMENT

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that it has not previously, in its entirety or in part, been submitted at any university for a degree.

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ABSTRACT

The World Health Organisation (WHO) estimates that at least 75% of infants receive infant formula milk (IFM) either entirely or in conjunction with breast milk during the first four months after birth. The presence of the emerging pathogen *Enterobacter sakazakii* in IFM has been associated with rare but fatal cases of neonatal infections and deaths. There is thus a need for accurate methods for the rapid detection of *E. sakazakii* in foods. At present, the methods used to detect and identify this micro-organism are inadequate, controversial and contradictory. The aim of this study was to determine the most suitable method for *E. sakazakii* detection after evaluation of the currently available methods. A further aim was to optimise a polymerase chain reaction (PCR) method for the detection of only viable *E. sakazakii* cells utilising the DNA-intercalating dyes ethidium monoazide (EMA) and propidium monoazide (PMA).

The Food and Drug Administration (FDA) method for *E. sakazakii* detection was utilised to select 50 isolates from IFM and 14 from the environment, regardless of colony appearance. These isolates were identified by sequencing a 1.5 kilobase (kb) fragment of the 16S ribosomal DNA (rDNA) and by using the National Centre for Biotechnological Information (NCBI) database to confirm the closet known relatives. Seven of the 50 (14%) IFM isolates and six of the 14 (43%) environmental isolates were identified as *E. sakazakii*. The methods that were evaluated for accuracy in detecting and identifying these *E. sakazakii* isolates included yellow pigment production on tryptone soy agar (TSA), chromogenic Druggan-Forsythe-Iversen (DFI) and *Enterobacter sakazakii* (ES) agars and PCR using six different species-specific primer pairs described in the literature.

The suitability of the FDA method was lowered by the low sensitivity, specificity and accuracy (87%, 71% and 74%, respectively) of using yellow pigment production for *E. sakazakii* identification. DFI and ES agars were shown to be sensitive, specific and accurate (100%, 98% and 98%, respectively) for the detection of *E. sakazakii*. The specificity of the PCR amplifications was found to vary between 8% and 92%, with Esakf and Esakr being the most accurate of the primer pairs evaluated.

The current FDA method for *E. sakazakii* detection requires revision in the light of the availability of more sensitive, specific and accurate detection methods. Based on the results obtained in this study, a new method is proposed for the detection of *E. sakazakii* in food and environmental samples. This proposed method replaces the culturing steps on violet red bile glucose agar (VRBGA) and TSA with culturing on

chromogenic DFI or ES agar.   For identification and confirmation of presumptive *E. sakazakii* isolates, the oxidase test, yellow pigment production and API biochemical profiling is replaced by DNA sequencing and/or species-specific PCR with the most accurate primer pair (Esakf and Esakr). The amendments to the current FDA method will reduce the time to detect *E. sakazakii* from approximately 7 days to 4 days and should prove to be more sensitive, specific and accurate for *E. sakazakii* detection.

In this study, a novel PCR-based method was developed which was shown to be capable of discriminating between viable and dead *E. sakazakii* cells.   This was achieved utilising the irreversible binding of bacterial DNA to photo-activated PMA or EMA in order to prevent PCR amplification from the dead cells. At concentrations of 50 and 100 µg.ml^{-1}, PMA completely inhibited PCR amplification from dead cells, while causing no significant inhibition of the PCR amplification from viable cells. EMA was equally effective in preventing PCR amplification from dead cells, however, it also inhibited PCR amplification from viable cells. PMA-PCR in particular, will be useful for assessing the efficacy of processing techniques, as well as for monitoring the resistance, survival strategies and stress responses of *E. sakazakii*. This will be an important step in the efforts to eliminate *E. sakazakii* from food and food production environments.

UITTREKSEL

Die Wêreld Gesondheidsorganisasie (WGO) beraam dat ten minste 75% van alle babas net baba formule melk (BFM) of BFM in kombinasie met moedersmelk in die eerste vier maande na geboorte kry. Die teenwoordigheid van die voortkomende patogeen *Enterobacter sakazakii* in BFM is al geassosieer met skaars maar noodlottige gevalle van neonatale infeksies en sterftes. Akkurate metodes word dus benodig vir die vinnige deteksie van *E. sakazakii* in voedsel. Die metodes wat huidiglik gebruik word vir die deteksie en identifikasie van hierdie mikroörganisme is onvoldoende, kontroversieël en teenstrydig. Die doel van hierdie studie was om die beste metode vir die deteksie van *E. sakazakii* te bepaal, na 'n evaluasie van die metodes wat huidiglik beskikbaar is. 'n Verdere doel was om 'n polimerase ketting reaksie (PKR) metode vir die deteksie van slegs lewensvatbare *E. sakazakii* selle te optimiseer deur gebruik te maak van die DNS-bindende kleurstowwe, etidium mono-asied (EMA) en propidium mono-asied (PMA).

Die Voedsel en Medisyne Administrasie (VMA) se metode vir *E. sakazakii* deteksie is gebruik om, ongeag van die kolonie kleur, 50 isolate vanuit BFM en 14 isolate vanuit die omgewing te kies. Hierdie isolate is geïdentifiseer deur die DNS volgorde van 'n 1.5 kilo-basis (kb) fragment van die 16S ribosomale DNS (rDNS) te bepaal en die Nationale Sentrum vir Biotegnologiese Informasie (NSBI) databasis te gebruik om die mees verwante spesie te bevestig. Sewe van die 50 (14%) BFM isolate en ses van die 14 (43%) omgewings isolate is geïdentifiseer as *E. sakazakii*. Die metodes wat geëvalueer is in terme van akkuraatheid vir deteksie en identifikasie van hierdie *E. sakazakii* isolate het PKR met ses verskillende spesie-spesifieke peiler pare soos beskryf in die literatuur, geel-pigment produksie op triptoon soja agar (TSA) en chromogeniese Druggan-Forsythe-Iversen (DFI) en *Enterobacter sakazakii* (ES) agars ingesluit. Die geskiktheid van die VMA metode is verlaag deur die lae sensitiwiteit, spesifisiteit en akkuraatheid (87%, 71% en 74% onderskeidelik) van geel pigment produksie vir *E. sakazakii* identifikasie. Chromogeniese DFI en ES agars was sensitief, spesifiek en akkuraat (100%, 98% en 98% onderskeidelik) vir die identifikasie van *E. sakazakii*. Die spesifisiteit van die PKR produkte het gewissel tussen 8% en 92%, en Esakf en Esakr is as die akkuraatste geëvalueerde peiler paar geidentifiseer.

Die huidige VMA metode vir *E. sakazakii* deteksie vereis hersiening aangesien meer sensitiewe, spesifieke en akkurate deteksiemetodes voortdurend beskikbaar word. 'n Nuwe metode, gebaseer op die resultate van hierdie studie, word voorgestel vir die deteksie van *E. sakazakii* in voedsel- en omgewingsmonsters. Die voorgestelde

metode vervang die kwekingsstap op violet rooi gal glukose agar (VRGGA) en TSA deur kweking op chromogeniese DFI of ES agars. Verder word die oksidase toets, geel pigment produksie en API biochemiese profiele van vermoeidelike *E. sakazakii* isolate vervang deur DNS volgorde bepaling en/of spesie-spesifieke PKR met die mees spesifieke peiler paar (Esakf and Esakf) vir die identifikasie en bevestiging van *E. sakazakii*. Die voorgestelde wysigings van die VMA metode sal die tydsduur van *E. sakazakii* identifikasie van 7 dae na 4 dae verminder, en behoort ook meer sensitief, spesifiek en akkuraat te wees vir die deteksie van *E. sakazakii*.

'n Nuwe PKR-gebaseerde metode wat tussen lewensvatbare en dooie *E. sakazakii* selle kan onderskei is in hierdie studie ontwikkel. Dit is bereik deur die onomkeerbare binding van bakteriële DNS aan lig-geaktiveerde EMA of PMA om die PKR amplifisering van dooie selle te voorkom. Konsentrasies van 50 en 100 µg.ml^{-1} PMA het PKR amplifikasie heeltemal geïnhibeer, terwyl geen inhibisie van lewensvatbare selle bespeur kon word nie. EMA was ook suksesvol in die voorkoming van die PKR amplifikasie van dooie selle, alhoewel daar ook 'n mate van DNS inhibisie was tydens die amplifikasie van lewensvatbare selle. PMA-PKR kan ook van nut wees vir die assessering van die doeltreffendheid van prosesseringstegnieke, en ook vir die waarneming van die weerstandigheid, oorlewingsstrategieë en stresresponse van *E. sakazakii*. Dit sal 'n belangrike stap wees in pogings om *E. sakazakii* van voedsel en voedsel produksieomgewings te elimineer.

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No problem can stand the assault of sustained thinking

— *Voltaire*

Dedicated to my parents

CONTENTS

Language and style used in this thesis are in accordance with the requirements of the *International Journal of Food Science and Technology*. This thesis represents a compilation of manuscripts where each chapter is an individual entity and some repetition between chapters has, therefore, been unavoidable.

CHAPTER 1

INTRODUCTION

Microbial foodborne diseases pose a considerable threat to human health and have become a growing concern to food legislators, food manufacturers and consumers worldwide. The risks associated with the microbial contamination of foodstuffs are increased by the globalisation of the food supply, as well as changes in the health, economic status and dietary patterns of the human population (WHO, 2002). The proportion of individuals that are highly susceptible to foodborne diseases is increasing due to ageing and HIV-associated infections. In developing countries, reduced immunity caused by malnutrition renders infants and children especially vulnerable to foodborne infections (WHO, 2002).

It is estimated that less than 35% of infants worldwide are exclusively breastfed during the first four months of their lives. Complementary feeding practices are often ill-timed, nutritionally inadequate and unsafe due to poor hygiene and improper handling (WHO, 2001). Newborns have immature immune systems and sterile gastro-intestinal tracts making them highly susceptible to infections (Newburg, 2005). Therefore, products like infant formula milk (IFM) require high levels of microbiological quality control during production, distribution and use (Iversen & Forsythe, 2003). The manufacture of sterile IFM is, however, not feasible using the current processing technology. Since product sterility is not mandatory under the prevailing microbiological specifications for powdered IFM, commercially available IFM products may occasionally contain low levels of pathogens (FAO/WHO, 2004).

Enterobacter sakazakii is an opportunistic foodborne pathogen, which has emerged as a public health concern due to its association with contaminated IFM (Iversen & Forsythe, 2003). The bacterium has been implicated as the cause of life-threatening cases of meningitis, sepsis and necrotising enterocolitis in infants (Biering *et al.*, 1989; Bar-oz *et al.*, 2001; Lai, 2001; Van Acker *et al.*, 2001). The prognosis of *E. sakazakii* infections is poor, with case mortality rates reported to be between 40 and 80% (Willis & Robinson, 1988). The predominant factors predisposing infants to *E. sakazakii* infections include premature birth, low birth weight (less than 2 500 g) and suppressed immunity (Bar-oz *et al.*, 2001; Block *et al.*, 2002; WHO, 2002; FAO/WHO, 2004). This is a particular problem in developing countries, which often have considerably higher proportions of infants that have a low birth weight, or that are born

to HIV-positive mothers, than developed countries (FAO/WHO, 2004). These infants are more likely to be susceptible to infections in general and may also specifically require IFM due to the risk of mother-to-child HIV transmission through breastfeeding (WHO, 2001; FAO/WHO, 2004).

Since low *E. sakazakii* numbers (1 cfu.100 g^{-1}) can have a severe impact on health, rapid detection of the pathogen has become an important subject in clinical research and food safety (Van Acker *et al.*, 2001). Nevertheless, there are still no standardised or official methods for direct isolation and detection of *E. sakazakii* from foods (Nazorowec-White *et al.*, 2003). The methods presently used for the detection and identification of *E. sakazakii* are inadequate and controversial, with ambiguous results being reported with different methods (Iversen *et al.*, 2004; Lehner *et al.*, 2004; Lehner *et al.*, 2006; Liu *et al.*, 2006; Hassan *et al.*, 2007). Inaccurate *E. sakazakii* detection results can have serious consequences for public health and for the food industry. False-negative results can lead to contaminated products being distributed to the public, while false-positive results can lead to financial losses for food manufacturers due to product rejections and recalls.

Despite the increased research interest in *E. sakazakii*, the threats it poses to human health are compounded by the lack of knowledge on this pathogen (FAO/WHO, 2004). Little is known, for instance, about the ecology, taxonomy, virulence and survival characteristics of *E. sakazakii*. Furthermore, there is very limited information on the prevalence and genetic diversity of this micro-organism in South Africa. A comprehensive understanding of *E. sakazakii* is imperative, given the substantial proportion of the South African population that are HIV-positive and immuno-compromised. The aim of this study was to evaluate and compare various methods for the isolation, detection and identification of *E. sakazakii* isolates derived from IFM and the processing environment. These included conventional culturing methods, culturing on selective chromogenic media, species-specific PCR using six different primer pairs described in the literature and 16S ribosomal DNA (rDNA) sequencing. A further aim was to optimise a novel PCR method for the detection of only viable *E. sakazakii* cells utilising the DNA-intercalating dyes ethidium monoazide (EMA) and propidium monoazide (PMA).

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CHAPTER 2

LITERATURE REVIEW

A.   Background

The World Health Organisation (WHO) recommends that infants be exclusively breastfed for the first 6 months of life for optimum growth and development, with supplementary foods being introduced to complement breast milk until at least 2 years of age (FAO/WHO, 2004). The percentages of infants being exclusively breastfed and the stages at which supplementary foods are introduced differ from country to country. In Scandinavian countries, 95% of infants are exclusively breastfed at birth and 75% at 6 months of age. In other European countries, however, these percentages are less than 30% and close to 0% at the same ages (FAO/WHO, 2004). When a mother cannot breastfeed due to physiological reasons, or chooses not to, it is vital that safe and nutritionally adequate breast milk substitutes are provided to satisfy the needs of the growing infant. Powdered infant formula milk (IFM) is the most frequently used infant formula product. It is, however, not sterile and can occasionally contain low levels of pathogens (INFOSAN, 2005).

One such foodborne pathogen, *Enterobacter sakazakii*, has emerged as a public health concern due to its association with contaminated powdered IFM (Iversen & Forsythe, 2003). The organism has been associated with sporadic cases or outbreaks of neonatal meningitis, necrotizing enterocolitis and sepsis (Urmenyi & Franklin, 1961; Bar-oz *et al.*, 2001; Van Acker *et al.*, 2001). A review of 48 cases of *E. sakazakii* infections occurring since 1961 revealed that at least 25 cases (52%) were linked to powdered IFM (FAO/WHO, 2004). However, considering the limitations of the current surveillance systems in most countries, it is likely that there is a significant under-reporting of *E. sakazakii* infections (INFOSAN, 2005).

Enterobacter sakazakii was recently ranked by the International Commission for Microbiological Specifications for Foods (ICMSF, 2002) as a 'severe hazard for restricted populations, life threatening or substantial chronic sequelae or long duration'. Consequently, it has the same ranking as well recognised foodborne and waterborne pathogens such as *Listeria monocytogenes*, *Clostridium botulinum* types A and B and *Cryptosporidium parvum* (Iversen & Forsythe, 2003). The presence of even low

numbers of *E. sakazakii* in IFM and the potential effects of this micro-organism on infected infants is a significant worldwide public health concern (FAO/WHO, 2004).

B.   The species *Enterobacter sakazakii*

Enterobacter sakazakii is a Gram-negative, facultative anaerobic, straight rod belonging to the genus *Enterobacter* and family *Enterobacteriaceae* (Nazarowec-White & Farber, 1997a). The cells have dimensions of approximately 3 µm by 1 µm, are non-sporulating and are motile by perithrichous flagella (Farmer *et al.*, 1980). The earliest recorded use of the name *E. sakazakii* was by Farmer *et al.* (1977) and Brenner *et al.* (1977), who derived the name to honour the Japanese microbiologist Riichi Sakazaki (Gurtler *et al.*, 2005). Prior to this, five other names were used, including the "Urmenyi and Franklin bacillus", "yellow coliform", "yellow *Enterobacter*", "pigmented *cloacae* A organism" and most notably "yellow-pigmented *Enterobacter cloacae*" (Gurtler *et al.*, 2005). *Enterobacter sakazakii* was designated as a new species by Farmer *et al.* (1980), based on differences from *E. cloacae* in DNA relatedness, yellow pigment production, biochemical reactions and antibiotic susceptibility.

Enterobacter sakazakii* can grow over a temperature range of 6° - 47°C, with optimum growth occurring at 39°C (Iversen & Forsythe, 2003). The bacterium has been described as moderately acid resistant due to its ability to survive at pH 3.5, but generally not below pH 3 (Edelson-Mammel *et al.*, 2006). Although little information exists on its resistance to alkaline conditions, growth at neutral pH values has been reported (Kim & Beuchat, 2005). *Enterobacter sakazakii* displays remarkable resistance to osmotic stress and drying, enabling it to survive in products with a low water activity (a_w) (Breeuwer *et al.*, 2003). It has been demonstrated that *E. sakazakii* can persist for up to two years in cans of dehydrated IFM (Edelson-Mammel *et al.*, 2005).

When grown on tryptic soy agar (TSA) at 25°C for 48 h*, E. sakazakii* colonies reach a diameter of 2-3 mm, and are typically bright yellow (Farmer *et al.*, 1980). Yellow pigment production is, however, less pronounced at 36°C and is unstable with repeated sub-culturing. Newly isolated strains of *E. sakazakii* may produce colonies with two distinct morphologies (Farmer *et al.*, 1980). One colony type is dry or mucoid, with scalloped edges, and is difficult to pick with an inoculation loop. The second colony type is typically smooth, often exhibiting little pigment production, and is easily removed with a loop. More recently, this differentiation has been described as "matt" or "glossy"

(Iversen & Forsythe, 2003). It is presently not known whether differences in virulence or other phenotypic traits exist between these two colony types (Nazorowec-White & Farber, 1997a). On sub-culturing, it has been observed that "matt" colonies may spontaneously revert to "glossy" colonies.

Biochemical reactions

Biochemical differentiation of *E. sakazakii* is based on the ability of the bacterium to ferment sucrose, raffinose, and α-methyl-D-glucoside, but usually not D-sorbitol, dulcitol, adonitol or D-arabinol (Farmer & Kelly, 1992). Of the biochemical traits, the inability to ferment D-sorbitol, as well the ability to produce an extracellular deoxyribonuclease, were traditionally considered to be the most significant differentiating characteristics of *E. sakazakii* (Farmer *et al.*, 1980). More recently, however, it has been demonstrated that some *E. sakazakii* strains are able to ferment D-sorbitol (Heuvelink *et al.*, 2001).

   After studying the enzymatic profiles of 226 *Enterobacter* strains (of which 129 were *E. sakazakii),* Muytjens *et al.* (1984) found two major differences between *E. sakazakii* and other related species. The absence of the enzyme phosphoamidase was unique to *E. sakazakii* and the activity of the α-glucosidase enzyme was demonstrated in all *E. sakazakii* strains, but not in other *Enterobacter* species. Consequently, the activity of α-glucosidase has become a defining characteristic for differentiation of *E. sakazakii* from other species in the *Enterobacteriaceae* family (Iversen *et al.*, 2006a). This biochemical feature has been used as a selective marker in the development of differential chromogenic media (Iversen *et al.*, 2004a), despite the recent finding that a small number of other *Enterobacteriaceae* do test positive for the α-glucosidase enzyme (Iversen *et al.*, 2006b).

Phylogeny and typing

On designation of *E. sakazakii* as a unique species, Farmer *et al.* (1980) described 15 different biogroups based on biochemical profiles, with the wild type biogroup 1 being the most common. However, a new biogroup of *E. sakazakii* (biogroup 16) has subsequently been introduced (Iversen *et al.*, 2006a). Based on partial 16S ribosomal DNA (rDNA) sequence analysis, it was shown that a relationship exists between the 16 *E. sakazakii* biogroups and the genotypes of the micro-organism (Iversen *et al.*, 2006a). Strains of *E. sakazakii* form at least four genetically and biochemically distinct clusters

and it is generally recognised that the species is genetically diverse and taxonomically complex.

In order to facilitate epidemiologic investigations and to identify vehicles of infection, it has been recommended that laboratories type all *E. sakazakii* isolates (Nazarowec-White & Farber, 1999). Methods that have been used to type these isolates include restriction endonuclease analysis (REA), ribotyping, plasmid typing, pulsed field gel electrophoresis (PFGE) and the random amplified polymorphic DNA (RAPD) technique (Biering *et al.*, 1989; Clark *et al.*, 1990; Nazarowec-White & Farber, 1999).

C.   Sources of *Enterobacter sakazakii*

Clinical sources

Krieg and Holt (1984) reported that *E. sakazakii* is more prevalent in foods and the environment than in clinical surroundings. Nonetheless, the micro-organism has been isolated from a range of clinical sources, including blood, cerebrospinal fluid, bone marrow, respiratory tracts (sputum, nose and throat), intestinal tracts, eyes, wounds, urine and faeces (Table 1). In the past, neonatal *E. sakazakii* infections were suspected to originate from maternal vaginal contamination during passing of the infant through the birth canal. Since infections have subsequently been found to occur in newborns delivered by Caesarean section, this hypothesis seems improbable (Urmenyi & Franklin, 1961; Muytjens *et al.*, 1983; Muytjens & Kollee, 1990; Bar-Oz *et al.*, 2001).

Apart from being isolated from a physician's stethoscope (Farmer *et al.*, 1980), *E. sakazakii* was also detected on spoons and on a blender used to prepare IFM in a hospital nursery (Bar-Oz *et al.*, 2001; Simmons *et al.*, 1989). Furthermore, epidemiological evidence obtained by pulsed field gel electrophoresis (PFGE) confirmed that a contaminated brush used for cleaning infant bottles was the source of three cases of infections in 1981 (Smeets *et al.*, 1998). It appears that *E. sakazakii* is capable of persisting for extended time periods in a clinical environment. Three isolates which were collected in the same hospital over 11 years had indistinguishable ribotype patterns and thus appeared to be the identical strain (Nazorowec-White & Farber, 1999).

Table 1 Sources of *Enterobacter sakazakii* (adapted from Iversen & Forsythe, 2003)

Source	Details	References
Neonates & infants	Meningitis	Urmenyi & Franklin (1961), Jøker *et al.* (1965), Kleiman *et al.* (1981), Muytjens *et al.* (1983), Arseni *et al.* (1987), Willis & Robinson (1988), Biering *et al.* (1989), Lecour *et al.* (1989), Simmons *et al.* (1989), Clark *et al.* (1990), Muytjens & Kollee (1990), Noriega *et al.* (1990), Gallagher & Ball (1991), Bar-Oz *et al.* (2001), Lai (2001).
	Bacteraemia	Monroe & Tift (1979), Clark *et al.* (1990), Bar-Oz *et al.* (2001).
	Necrotising enterocolitis	Van Acker *et al.* (2001).
	Wounds, appendicitis, conjunctivitis	Reina *et al.* (1989).
Adults	Bacteraemia	Jimenez & Gimenez (1982), Pribyl *et al.* (1985), Murray *et al.* (1990), Hawkins *et al.* (1991), Lai (2001), Dennison & Morris (2002).
Food & Drink	Infant formula milk powder	Farmer *et al.* (1980), Postupa & Aldová (1984), Block *et al.* (2002), Biering *et al.* (1989), Simmons *et al.* (1989), Muytjens *et al.* (1988), Bar-Oz *et al.* (2001), Heuvelink *et al.* (2001), Himelright *et al.* (2002).
	Milk powder	Postupa & Aldová (1984), Muytjens *et al.* (1988), Heuvelink *et al.* (2001).
	Water, pipelines & biofilm	Bartolucci *et al.* (1995), Al-Hadithi & Al-Edani (1995).
	Water springs	Mosso *et al.* (1994).
	Rice seed	Cottyn *et al.* (2001).
	Beer mugs	Schindler & Metz (1990).
	Cured meat	Watanabe & Esaki (1994).
	Fermented bread	Gassem (1999).
	Lettuce	Soriano *et al.* (2001).
	Tofu	No *et al.* (2002).
	Sour tea	Tamura *et al.* (1995).
	Cheese, minced beef, sausage, meat, vegetables	Leclercq *et al.* (2002).
Environ-mental	Hospital air	Masaki *et al.* (2001).
	Clinical material	Tuncer & Ozsan (1988).
	Factories & houses	Kandhai *et al.* (2004).
	Preparation equipment (blender, spoons)	Block *et al.* (2002), Clark *et al.* (1990), Smeets *et al.* (1998), Bar-Oz *et al.* (2001).
	Rats	Gakuya *et al.* (2001).
	Flies	Hamilton *et al.* (2003), Kuzina *et al.* (2001).
	Soil	Neelam *et al.* (1987).
	Grass silage	Van Os *et al.* (1996).
	Rhizosphere	Emilani *et al.* (2001).
	Sediment, wetlands	Espeland & Wetzel (2001).
	Crude oil	Assadi & Mathur (1991).
	Cutting fluids	Suliman *et al.* (1988).

Environmental and food sources

Enterobacter sakazakii, like most other members of the genus *Enterobacter*, has been shown to be ubiquitous in nature (Table 1). To date, the reservoir and mode of transmission of *E. sakazakii* has not been identified (Nazorowec-White & Farber, 1997a). It has been suggested that environmental and plant material is likely to be the primary source of *E. sakazakii* (Mossel & Struijk, 1995). More specifically, the main environmental sources may be soil, water and vegetables, with rodents and flies as secondary means of contamination (Iversen & Forsythe, 2003). *Enterobacter sakazakii* has been isolated from the digestive tract of the Mexican fruit fly, *Anastrpha ludens* (Kuzina *et al.*, 2001) and the stable fly, *Stomoxys calcitrans* (Hamilton *et al.*, 2003).

It appears that *E. sakazakii* is also a common inhabitant of food manufacturing facilities and households. In a survey of nine food factories (milk powder, chocolate, cereal, potato flour, spices and pasta) and 16 households, 23% of the factory samples and 31% of the household samples tested positive for *E. sakazakii* (Kandhai *et al.*, 2004). It has been reported that *E. sakazakii* occurs more commonly in the manufacturing environment than species of *Salmonella* (FAO/WHO, 2004).

Enterobacter sakazakii has been isolated from a wide variety of different food products including meat, vegetables, milk-based products, grains and fermented breads (Table 1). On evaluation of a variety of foods for the presence of *Enterobacteriaceae*, 2.4% of IFM samples, 10.2% of dried infant foods, 4.1% of milk powders, 3.2% of cheese products and 37.8% of the herb and spice samples tested positive for the presence of *E. sakazakii* (Iversen & Forsythye, 2004). Although studies of this kind illustrate the widespread occurrence of this micro-organism in foods, it is only powdered IFM that has been epidemiologically linked to outbreaks of *E. sakazakii* illnesses.

D.   Infant formula milk as a source of *Enterobacter sakazakii*

Manufacture of infant formula milk

Since the beginning of the 20th century, the production of IFM from cow's milk has shown a steady increase (Nazarowec-White & Farber, 1997a). Powdered IFM is formulated to mimic the nutritional profile of human breast milk rather than cow's milk (Breeuwer *et al.*, 2003). Since the two differ in composition, various modifications are made during processing, including reducing the levels of protein, minerals and fat, while increasing the levels of whey protein and carbohydrates in the milk (Nazorowec-White &

Farber, 1997b). In addition, the calcium to phosphorus ratio is increased and vitamins are added.

In most processing facilities, IFM is produced by combining milk with other essential ingredients (milk derivatives, carbohydrates, soy protein isolates, vitamins, minerals and additives) using either a "wet'" or "dry" blending method (Fig. 1) (FAO/WHO, 2004). The "wet" blending method involves combining all ingredients in a liquid phase, heat-treating the liquid, and then spray-drying to achieve a powdered product. In the "dry" blending method all ingredients are individually prepared and heat-treated prior to being combined in the dry form. Problems which limit the use of the latter method include difficulties in mixing, segregation of ingredients, as well as a higher probability of post-processing contamination (Nazarowec-White & Farber, 1997a,c). In some processing facilities, a combined "wet" and "dry" method is used, where the soluble ingredients are added during the liquid phase, followed by the less soluble ingredients being added to the spray-dried powder.   Since in-factory contamination most probably occurs at some point between spray drying and packaging, the risk of IFM contamination is dependent on the specific factory environment rather than solely the manufacturing processes (Gurtler *et al.*, 2005).

Presence of *Enterobacter sakazakii* in infant formula milk

Several investigations into outbreaks of *E. sakazakii* infections occurring in neonatal intensive care units have provided both statistical and microbiological evidence that has implicated IFM consumption as the cause of infection (Simmons *et al.*, 1989; Van Acker *et al.*, 2001; Himelright *et al.*, 2002). It is generally accepted that *E. sakazakii* does not survive the pasteurisation process applied during IFM manufacture, and that contamination probably occurs following heat treatment (Iversen & Forsythe, 2003; FAO/WHO, 2004).   *Enterobacter sakazakii* can gain entrance into powdered IFM by two routes (FAO/WHO, 2004) (Fig. 1). Intrinsic contamination may arise from the addition of contaminated ingredients after drying, or from the factory environment between drying and packaging. External contamination, on the other hand, may occur during reconstitution and handling, for instance when using poorly cleaned equipment or utensils.

Analyses of commercial powdered IFM products have revealed the prevalence of *E. sakazakii* at varying frequencies (*ca.* 0-18% of IFM products); but almost always at concentrations of *ca.* 1 cfu.100 g^{-1} (ICMSF, 2004; Edelson-Mammel *et al.*, 2005). In one of the most notable surveys of powdered IFM products obtained from 35 countries,

Raw ingredients[a]

"Wet" blending[a]

1. Pasteurisation of liquid skim milk (82°C, 20 s)
2. Pasteurisation of pre-mix (skim milk & fats) (80°C, 20 s)
3. Pasteurisation of total mixture (107°-110°C, 60 s)
4. Mixture concentrated (falling film evaporator)
5. Vitamins added
6. Spray-dried

"Dry" blending[a]

1. Pasteurised evaporated skim milk dry blended with other ingredients
2. Pasteurisation of total mixture (110°C, 60 s)
3. Spay-dried

Intrinsic contamination

Powdered infant formula

Reconstitution[a]

People[a]

Equipment[a]

External contamination

Biofilm[a] formation

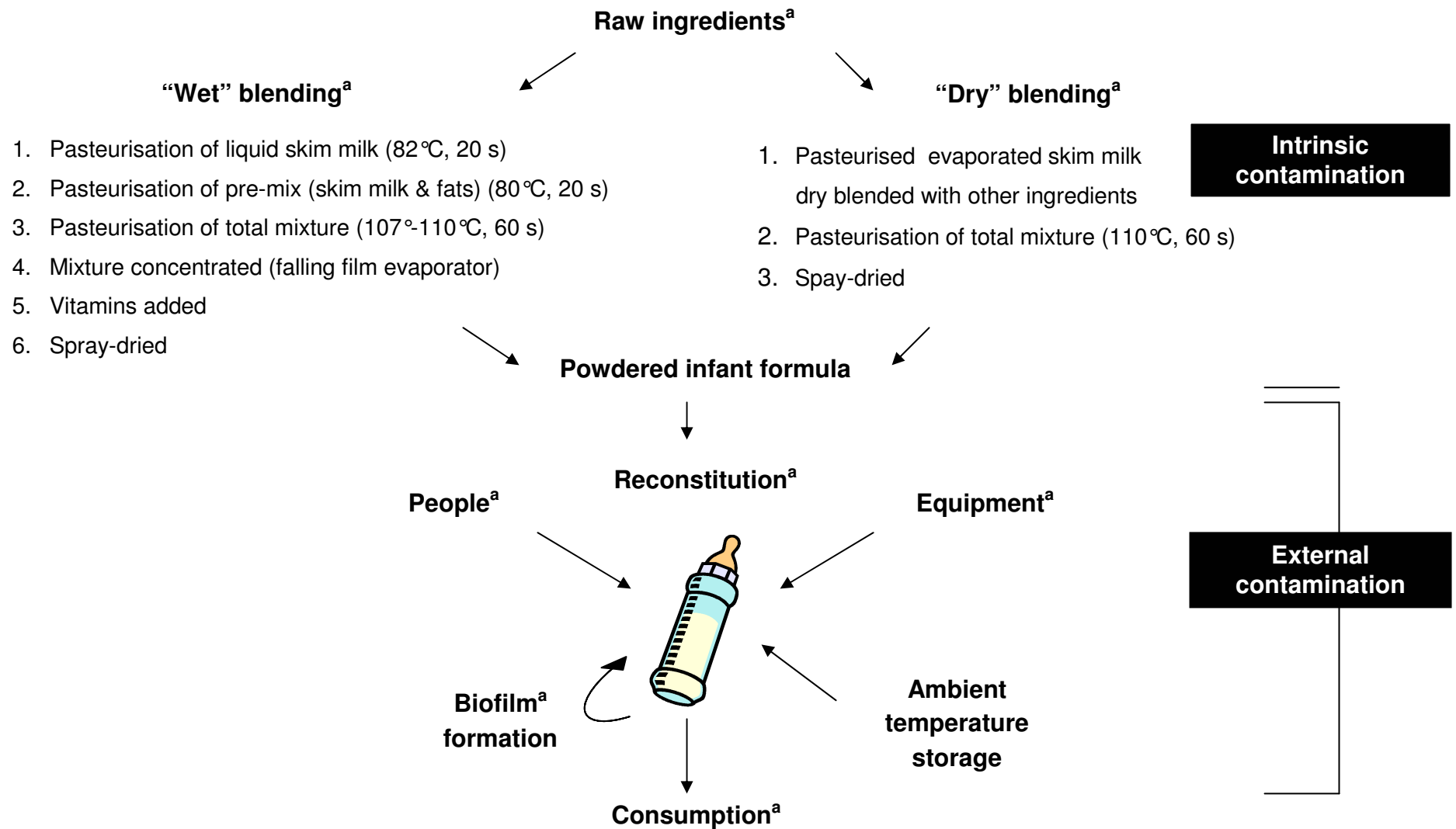**Ambient temperature storage**

Consumption[a]

Figure 1 Risk factors in the manufacture and preparation of powdered infant formula milk (IFM) (adapted from Forsythe, 2005).

[a]Potential sites for microbial contamination

E. sakazakii was isolated at low levels (0.36 cfu.100 g^{-1}) from 14.2% of the 141 samples tested (Muytjens *et al.*, 1988). All of these products, however, met the prevailing Codex Alimentarius Commission (CAC) microbiological specifications for coliform counts in powdered IFM (less than 3 cfu.g^{-1}) (Van Acker *et al.*, 2001; Muytjens *et al.*, 1988). Evaluation of commercial powdered IFM and baby foods available on the South African market revealed the presence of *E. sakazakii* in 18% of the products tested (Witthuhn *et al.*, 2006).

Reconstituted IFM is nutritious, and may allow rapid growth of bacteria when the prevailing water activity, the time for growth, and the temperature are favourable (Forsythe, 2005). Numerous studies have focused on the conditions promoting survival and growth of *E. sakazakii* in reconstituted IFM. Minimum growth temperatures of 5° - 8°C have been reported for strains of *E. sakazakii* (Nazarowec-White & Farber 1997c). Since it has been estimated that 20% of household refrigerators are maintained at temperatures above 10°C (Daniels, 1991), these refrigerators provide conditions at which the micro-organism may grow. At temperatures of 10°, 21° and 23°C, average doubling times for *E. sakazakii* in reconstituted IFM have been reported to be 4.98 h, 75 min and 40 min, respectively (Nazarowec-White & Farber 1997c; Iversen & Forsythe, 2003). Thus, even low levels of *E. sakazakii* in IFM can pose a health risk given the potential for rapid multiplication of the bacterium during the preparation and holding time (FAO/WHO, 2004). While optimum growth of *E. sakazakii* has been reported to occur at 37° - 43°C (Iversen *et al.*, 2004b), Iversen & Forsythe (2003) suggested that growth of the micro-organism at temperatures above 47°C is improbable. Since *E. sakazakii* does not appear to be particularly thermotolerant (Breeuwer *et al.*, 2003), the survival of the bacterium is unlikely if IFM is rehydrated with hot water (70°C) prior to consumption.

Regulatory aspects

Regulations governing the hygienic manufacture and preparation of IFM appear in the *Recommended International Code of Hygienic Practice for Foods for Infants and Children* (CAC, 1979). This code, adopted by the Codex Alimentarius Commission (CAC) in 1979, mandates adherence to good manufacturing practices (GMPs) and clear labeling, but contains no requirement for IFM to be sterile. Rather, the current CAC microbiological specifications stipulate allowable levels for mesophilic aerobic bacteria, coliforms and

salmonellae in powdered IFM. There is currently no CAC requirement to test specifically for *E. sakazakii* in IFM (FAO/WHO, 2004). The limit set for coliforms is a minimum of four of five control samples with less than 3 coliforms.g^{-1} and a maximum of one of five control samples with more than 3 but less than or equal to 20 coliforms.g^{-1}. These specifications do not provide a sufficient level of safety, as evident by outbreaks caused by IFM contaminated with *E. sakazakii* at levels below this limit (Van Acker *et al*., 2001).

In November 2004, a working group formed by the Codex Committee on Food Hygiene (CCFH) drafted a revised code of practice for IFM. To date, however, no consensus has been reached by the CAC on the proposed code of practice. It is expected that the CAC will adopt a revised code in 2009, containing standards for *Enterobacteriaceae* and, in specific for *E. sakazakii* (CAC, 2007). In addition, the revised code is likely to include validated testing methods, ideally including culturing and PCR methodologies. In the meantime, the European Union has officially introduced microbiological standards for *E. sakazakii* (negative in 30 x 10 g samples) in powdered IFM (EC, 2005).

## E.	*Enterobacter sakazakii* infections

History of outbreaks

The earliest accounts of infections caused by *E. sakazakii* originated in England in 1958 (Urmenyi & Franklin, 1961). Since then, additional cases of infection due to *E. sakazakii* have been reported in countries such as Canada, Belgium, Germany, Greece, Israel, The Netherlands, Spain and the United States of America (Iversen & Forsythe, 2003). At least 76 neonatal cases of *E. sakazakii* infection were reported to have occurred worldwide between 1958 and 2003 (Iversen & Forsythe, 2003). However, it is likely that the reported numbers underestimate the actual incidence of *E. sakazakii* infections, since many clinical laboratories do not test for *E. sakazakii* and official reporting systems have not been implemented by many countries (Farber, 2004). There is a lack of information on both the contamination of IFM distributed in developing countries, as well as the disease burden resulting from consumption of contaminated IFM in these countries (FAO/WHO, 2004). More recent reports of outbreaks of *E. sakazakii* infection have included the death of a

premature infant in July 2004 in New Zealand, as well as an outbreak (2 deaths, 4 diseased individuals and 9 infected individuals) in France between October and December of the same year (INFOSAN, 2005).

Characteristics of disease

Enterobacter sakazakii has been documented to cause sporadic and severe forms of meningitis (an acute inflammation of the membranes surrounding the brain and spinal chord) and septicemia (a disease caused by bacteria in the blood) in pre-term and full-term infants (Muytjens & Kollee, 1990; Himelright *et al.*, 2002). In addition, the micro-organism has been associated with several cases of necrotising enterocolitis (the most common gastro-intestinal disease in newborns), although it has never been established as the causative agent (Muytjens *et al.*, 1983; Van Acker *et al.*, 2001).   The manifestation of disease caused by *E. sakazakii* is severe, with mortality rates estimated at 40 - 80% and fatalities often occurring within days of infection (Nazorowec-White & Farber, 1997a). *Enterobacter sakazakii* affects the central nervous system (Gallagher & Ball, 1991) and survivors often suffer from severe neurological impairments, including hydrocephalus, quadriplegia and developmental delay (Lai, 2001).

In most cases, *E. sakazakii* infections are responsive to antibiotic therapy (FAO/WHO, 2004), and infections are traditionally treated with ampicillin in combination with chloramphenicol or gentamicin (Lai, 2001). Unfortunately, a number of authors have reported that *E. sakazakii* is becoming increasingly resistant to these antibiotics by means of transposable elements, and also to β-lactam antibiotics by the production of β-lactamase (Muytjens *et al.*, 1983; Pitout *et al.*, 1997; Lai, 2001).

Risk groups

Although *E. sakazakii* has caused illness in all age groups, a review of the reported cases of infections reveals that infants (children less than 1 year) appear to be at greatest risk (FAO/WHO, 2004) (Table 1). Within this group, neonates (less than 28 days), are particularly susceptible to *E. sakazakii* infections, especially those that are premature, low birth weight (less than 2 500 g) or immuno-compromised. Although only a few *E. sakazakii* infections have been reported in adults (Table 1), those that have been documented all involved immuno-compromised individuals (Hawkins *et al.*, 1991; Lai, 2001).

Human infants are born with an immature immune system and a gastro-intestinal tract that is devoid of micro-organisms (Newburg, 2005). The inability of infants to produce an effective immune response makes this group highly susceptible to infections. It has been suggested that infants are more vulnerable to *E. sakazakii* infection than adults because their stomachs, especially when premature, are notably less acidic than that of adults (FAO/WHO, 2004). This makes them less capable of naturally combating pathogens, and may allow prolonged survival of *E. sakazakii* in the body. The 2002 US FoodNet survey estimated that the rate of infant *E. sakazakii* infections is 1 per 100 000, while among low-birth-weight neonates, the rate was estimated to be 8.7 per 100 000 (FAO/WHO, 2004).

Of concern is the considerable threat that *E. sakazakii* poses to infants of HIV-positive mothers. Not only are these infants more likely to be susceptible to infection in general, but they may also specifically require IFM instead of breast milk, due to the risk of HIV-transmission from mother to child through breast milk (FAO/WHO, 2004). This is problematic in developing countries, which often have considerably higher proportions of infants that are low birth weight or of HIV-infected mothers than developed countries. These factors increase the demand and consumption of powdered IFM. The risks are further increased in developing countries with high ambient temperatures, especially when there is a lack of refrigeration facilities to store rehydrated IFM. Under such circumstances, it is likely that relatively rapid growth of *E. sakazakii* might occur following IFM reconstitution. According to FAO/WHO (2004), the relative risk of ingesting *E. sakazakii* in reconstituted IFM after 6 h and 10 h at 25°C increases by 30-fold and 30 0000-fold, respectively.

Pathogenicity and infectious dose

Enterobacter sakazakii is considered an opportunistic pathogen since it rarely causes disease in healthy individuals. However, little is known at the molecular level about the virulence factors involved in the pathogenesis of this micro-organism. Pagotto *et al.* (2003) evaluated 18 clinical and food isolates of *E. sakazakii* for enterotoxin production using a suckling mousse assay. Four of the 18 strains tested positive for enterotoxin production. All *E. sakazakii* strains were lethal to mice when doses of 10^8 cfu per mouse were administered by intraperitoneal injection, while two strains caused death in mice when administered orally. In addition, some *E. sakazakii* strains were shown to produce cytotoxic effects in mice. It was concluded that differences in virulence may exist between *E. sakazakii* strains, and some strains may be non-pathogenic. This is in

agreement with the reports of Block *et al.* (2002), who, after studying a small cluster of neonatal infections caused by an unusual biochemical variant of *E. sakazakii*, suggested that there may be several different *E. sakazakii* biotypes capable of causing human illness.   The antioxidant activity resulting from the production of bacterial pigments is reported to promote virulence in pathogenic bacteria and allow persistence in harsh environments (Liu *et al.*, 2005; Clauditz *et al.*, 2006).   Further research is required to elucidate the potential relationship between *E. sakazakii* virulence and yellow pigment production (Lehner *et al.*, 2006a).

In the more than 76 documented cases of *E. sakazakii* infections, no epidemiological evidence was obtained that could provide a value for the infectious dose (the amount of agent that must be consumed to produce symptoms of foodborne disease).   It has been estimated that 1000 cells is the infectious dose for *E. sakazakii*, since this is approximately the infectious dose of the pathogenic bacteria *Escherichia coli* O157, *Neisseria meningitidis*, and *Listeria monocytogenes* (Iversen & Forsythe, 2003).   The growth rate of *E. sakazakii* was used to calculate the time required for the bacterium to attain an infectious dose (1000 cells) at different temperatures, using an initial *E. sakazakii* concentration of 1 cfu.100 g^{-1} in contaminated IFM (Muytjens *et al.*,1988; Nazarowec-White & Farber, 1997a,b,c).   According to this simplistic model, reconstituted IFM at 8°, 21° and 37°C would require 9 days, 17.9 h and 7 h, respectively to achieve this infectious dose.

F.   Growth and death characteristics

Thermal tolerance of *Enterobacter sakazakii*

The thermal tolerance of *E. sakazakii* was investigated after the micro-organism was isolated from unopened cartons of ultra-heat treated (UHT) milk (Skladal *et al.*, 1993). Concerns over whether the micro-organism could survive pasteurisation, coupled with the limited information on its survival characteristics, resulted in a number of thermal inactivation studies (Table 2).   Nazarowec-White and Farber (1997b) reported that the D-values (the time required for a 10-fold reduction in the viable numbers of a micro-organism at a given temperature) for *E. sakazakii* in rehydrated IFM at 52° and 60°C were 54.8 min and 2.5 min, respectively (Table 2).   Extrapolation of this data to 72°C indicated that *E. sakazakii* is very thermotolerant, and that a 6 – 7 log viable cell reduction would require heating at 60°C for 15 – 17 min.

More recent thermal resistance studies, however, indicated that the thermal resistance of *E. sakazakii* is strain-dependent and that it is not likely to persist after pasteurisation (Breeuwer *et al.*, 2003; Edelson-Mammel & Buchanan, 2004; Nazarowec-White *et al.*, 1999). In the thermal tolerance studies carried out by Iversen *et al.* (2004b) in rehydrated powdered IFM, the D-values reported for a capsulated *E. sakazakii* strain were generally lower than those for the type strain (Table 2). However, the z-values (the temperature change required to reduce the D-value by one log cycle) for the capsulated and type strains were 5.7 and 5.8, respectively. It was calculated that high temperature short time (HTST) pasteurisation (72°C for 15 s) would theoretically result in a 21 log reduction of viable *E. sakazakii* cells (Iversen *et al.*, 2004b). In general, a 4 - 7 log reduction of micro-organisms is required for process control during pasteurisation. It is thus accepted that *E. sakazakii* would not be capable of surviving a commercial pasteurisation process and that product contamination probably occurs during drying, filling or reconstitution of IFM (Iversen & Forsythe, 2003).

A submerged vessel method was utilised to evaluate D_{58}-values of 12 strains of *E. sakazakii* in rehydrated IFM (Edelson-Mammel & Buchanan, 2004) (Table 2). An approximate 20-fold divergence in thermal tolerance was demonstrated between the least thermally resistant strain and the most thermally resistant strain. It was suggested that *E. sakazakii* strains can be divided into two distinctive phenotypes based on their different thermal resistance characteristics (Edelson-Mammel & Buchanan, 2004). Thermal tolerance studies conducted by Breeuwer *et al.* (2003) produced substantially lower D- and z-values (Table 2) than those determined in other studies (Edelson-Mammel & Buchanan, 2004; Nazarowec-White & Farber, 1997b). They concluded that *E. sakazakii* is not particularly thermotolerant, and that it is the remarkable osmotic and desiccation resistance of the micro-organism that allows its survival in IFM (Breeuwer *et al.*, 2003).

Osmotic and desiccation tolerance

The a_w of powdered IFM is *ca.* 0.2 and *E. sakazakii* possesses the ability to survive for extended periods in such dry conditions (Gurtler *et al.*, 2005). In fact, *E. sakazakii* has been shown to exhibit greater osmotic and desiccation tolerance than *E. coli*, species of *Salmonella* and other *Enterobacteriaceae* (Breeuwer *et al.*, 2003).

Bacteria are known to protect themselves from dehydration by a rapid intracellular

Table 2 Decimal reduction time (D-value) and z-value for *Enterobacter sakazakii* in powdered IFM

D-value (min)									z-value (ºC)	Reference
52ºC	53ºC	54ºC	56ºC	58ºC	60ºC	62ºC	65ºC	70ºC		
54.8 ± 4.70		23.7 ± 2.50	10.3 ± 0.70	4.2 ± 0.60	2.5 ± 2.00				5.80	Nazarowec-White & Farber, 1997b[a]
	8.30	6.40	1.10	0.27					3.10	Breeuwer *et al.*, 2003[b]
	20.20	7.10	2.40	0.34					3.60	
				0.40						
				0.48						
				0.50						Breeuwer *et al.*, 2003
				0.51						Edelson-Mammel & Buchanan, 2004[c]
		21.1 ± 2.70	9.9 ± 0.80	4.4 ± 0.4		0.6 ± 0.30		0.07	5.60	Edelson-Mammel & Buchanan, 2004[d]
		16.4 ± 0.67	5.1 ± 0.30	2.6 ± 0.48	1.1 ± 0.11	0.3 ± 0.12			5.8 ± 0.40	Iversen *et al.*, 2004b[e]
		11.7 ± 5.80	3.69 ± 0.06	3.8 ± 1.95	1.8 ± 0.82	0.2 ± 0.11			5.7 ± 0.12	Iversen *et al.*, 2004b[f]

[a] D-values of 10 strains (5 clinical isolates and 5 food isolates).

[b] D-values for 4 different strains determined in phosphate buffer.

[c] D-value at 58 °C for *E. sakazakii* ATCC 51329, the least heat resistant strain.

[d] D-values for *E. sakazakii* strain 607, the most heat resistant *E. sakazakii* strain.

[e] D- and z-values for *E. sakazakii* type strain.

[f] D- and z-values for *E. sakazakii* capsulated strain.

accumulation of ions (particularly K$^+$) and compatible solutes (such as trehalose, proline and glycine betaine). The mechanism involved in the ability of *E. sakazakii* to resist dehydration has been related to the accumulation of trehalose in the cells (Breeuwer *et al.*, 2003). Trehalose is a non-reducing disaccharide of glucose, which may play a crucial role in protecting bacteria from dehydration by stabilising proteins and phospholipid membranes (Potts 1994; Kempf & Bremer 1998; Welsh & Herbert, 1999).

The genetic basis of the dry stress resistance of *E. sakazakii* involves a genome-wide expression of functionally different gene clusters (Breeuwer *et al.*, 2004). These include four genes belonging to the cyclic AMP receptor protein regulon, six genes concerned with the stringent response, seven genes from the heat shock regulon and several genes involved in cell wall function and trehalose synthesis. These mechanisms may offer an explanation for the reports demonstrating the persistence of *E. sakazakii* in dehydrated IFM for at least two years (Edelson-Mammel *et al.*, 2005).

Acid tolerance

Enterobacter sakazakii has been described as a moderately acid resistant enteric bacterium (Edelson-Mammel *et al.*, 2006). The acid resistance of the bacterium is similar to salmonellae (Gorden & Small, 1993), but less than the documented acid resistant pathogens *L. monocytogenes* (Buchanan & Golden, 1998) and *E. coli* (Buchanan & Edelson, 1996, 1999). Acid resistance studies have indicated that *E. sakazakii* could endure exposure to pH 3.5 for more than 5 h (Edelson-Mammel *et al.*, 2006). At pH values below 3, however, its survival was found to be transitory, with substantial diversity in acid resistance existing among different strains.

In food products, *E. sakazakii* has been shown to grow in tomato (pH 4.4), watermelon (pH 5.0) and cantaloupe (pH 6.8) juices incubated at 25°C; however, it did not grow in strawberry juice (pH 3.6) or apple juice (pH 3.9) (Kim & Beuchat, 2005). *Enterobacter sakazakii* has also been found to survive in various fermented food products. These include Khamir, a fermented bread with a pH of *ca.* 3.9, and Sobia, a traditional fermented beverage with a pH range between 3.37 and 5.53 (Gassem, 1999, 2002).

Biofilm formation

The attachment of bacterial cells to surfaces can be followed by growth, the production of exopolysaccharides (EPS), and subsequent biofilm formation (Kim *et al.*, 2006).

Biofilms have been described as complex aggregations of cells attached to a surface, or to each other, and typically embedded in protective and adhesive polymeric substances excreted by the bacteria (Marshall, 1992). *Enterobacter sakazakii* has been reported to form biofilms on silicon, latex, stainless steel, polycarbonate, glass and polyvinyl chloride (PVC) (Iversen *et al.*, 2004b; Lehner *et al.*, 2005). The formation of biofilms by *E. sakazakii* may promote its persistence on equipment surfaces in factory and food preparation areas, as well as on infant feeding bottles and utensils (Kim *et al.*, 2006). Levels of *E. sakazakii* on silicon and latex from infant bottles have been reported to be as high as 10^4 bacteria.cm^{-2}. Thus ineffective cleaning of bottles and utensils could enable the bacterium to accumulate and serve as a source of infection (Forsythe, 2005).

Biofilm formation by *E. sakazakii* is enhanced by the production of a novel heteropolysaccharide, which comprises of 29 – 32% glucuronic acid, 23 – 30% D-glucose, 19 – 24% D-galactose, 13 – 22% D-fucose and 0 – 8% D-mannose (Harris & Oriel, 1989). The existence of bacteria within this matrix substantially increases their resistance to environmental stresses, detergents and antibiotics (Norwood & Gilmour, 2000; Frank *et al.*, 2003). It has been suggested that bacterial capsules formed by excretion of the EPS could promote survival of the organism in IFM for up to 24 months (Iversen & Forsythe, 2003).

Potential strategies for inactivation of *Enterobacter sakazakii*

Evaluation of potential treatments for the inactivation of microbial pathogens in IFM requires an understanding of the unique characteristics of vegetative cells in dry products (FAO/WHO, 2004). Very often, bacteria in a dehydrated state demonstrate increased heat resistance, and their survival may be enhanced when the a_w is very low (Edelson-Mammel *et al.*, 2005). Furthermore, powdered IFM is packaged in an inert atmosphere to prevent nutrient oxidation, which may foster the survival of dormant bacterial cells.

Since powdered IFM is not a sterile product, the inclusion of a lethal step during the preparation of powdered IFM, as well as a decrease in the holding time before and during feeding, is recommended to reduce the risks of *E. sakazakii* ingestion (FAO/WHO, 2004). A 4 log cfu.ml^{-1} reduction in *E. sakazakii* levels can be achieved by using water at 70°C for rehydration of powdered IFM (Edelson-Mammel & Buchanan, 2004). This approach may reduce or even eliminate *E. sakazakii* from reconstituted IFM. The effectiveness of microwave heating has also been assessed as a strategy to eliminate potential pathogens from foods. The mechanism of microbial killing is thought

to involve both thermal and non-thermal electromagnetic radiation effects (Najdovski *et al.*, 1991).   A greater than 4 log cfu.ml^{-1} reduction in *E. sakazakii* cells was accomplished by microwaving reconstituted IFM in infant bottles for 85 – 100 s to a temperature of 82° – 93°C (Kindle *et al.*, 1996).

The incorporation of antimicrobials into IFM has been investigated to reduce the risks of *E. sakazakii* infections.   Caprylic acid is a eight-carbon fatty acid that has received GRAS (generally recognised as safe) status due to its natural presence in breast and bovine milk (Nair *et al.*, 2004).   Monocaprylin, the monoglyceride ester of caprylic acid, reduced *E. sakazakii* levels by more than 5 log cfu.ml^{-1} when incorporated into reconstituted IFM.   However, it was concluded that the effects of monocaprylin on the sensory attributes of IFM requires further evaluation.

The efficacy of probiotic cultures in controlling *E. sakazakii* growth in rehydrated IFM has been evaluated (Lihono *et al.*, 2004).   *Enterococcus faecium* was found to be more inhibitory to *E. sakazakii* than *Lactobacillus acidophilus* or *Pediococcus acidilacticii*.   The inhibitory effect on *E. sakazakii* is thought to be due to the pH reduction in IFM resulting from the production of acid by the probiotic micro-organisms (Lihono *et al.*, 2004).

Based on current knowledge, sterilisation of IFM in its powdered form appears to be only possible using irradiation.   Unfortunately, due to the high doses required to inactivate *E. sakazakii* in the dry state, the application of this technology seems to be limited by the organoleptic deterioration of the product (FAO/WHO, 2004).   Other potential technologies for IFM sterilisation, such as ultra-high pressure and magnetic fields, are still at an early stage of development and are currently not suitable for dried foods.   Further research in this field is a priority, as is the need for a detection method which allows quantitative validation of the killing effect.

## G.	Isolation and identification of *E. sakazakii*

The United States Food and Drug Administration (FDA, 2002) has a recommended method for the isolation and enumeration of *E. sakazakii* from IFM, which is similar to those originally proposed by Muytjens *et al.* (1988) and Nazarowec-White and Farber (1997a,b,c) (Fig. 2).   These methods are all based on a most probable number (MPN) approach using a total of 333 g of product (3 x 100 g, 3 x 10 g, 3 x 1 g), followed by a series of culturing steps that may take up to seven days to produce results (Oh & Kang, 2004).   The culturing steps include pre-enrichment, enrichment in *Enterobacteriaceae*

enrichment (EE) broth, and isolation using selective violet red bile glucose agar (VRBGA). These protocols are only selective for *Enterobacteriaceae* and are not specific for *E. sakazakii* (Iversen & Forsythe, 2003). Consequently, five presumptive *E. sakazakii* colonies are chosen from VRBGA and are sub-cultured on TSA at 25°C for 48 – 72 h. Colonies are then selected for confirmation tests based on yellow pigment production, a trait reported to be typical of *E. sakazakii* (FDA, 2002; Nazarowec- White & Farber, 1997c). The FDA (2002) protocol was modified by Wyeth Nutrition (Fig. 2) to eliminate the MPN format and to test for the presence or absence of *E. sakazakii* cells, with a sensitivity of 0.365 cfu.100 g^{-1} (Donnelly, 2005).

Tests used to confirm the identification of *E. sakazakii* include the oxidase test (oxidase negative) and the API 20E biochemical identification system (Iversen & Forsythe, 2003). DNA-based technologies (Anon., 1996; Kandhai *et al.*, 2004) and α-glucosidase-activity tests have recently been utilised as additional means of confirming the identification of *E. sakazakii*. However, these methods have not been validated by the international organisations responsible for establishing microbiological standards for foods (FAO/WHO, 2004).

Isolation of *E. sakazakii* utilising conventional microbiological methods has a number of disadvantages.   This approach is time consuming and *E. sakazakii* may be outgrown by other members of the family *Enterobacteriaceae* during pre-enrichment and enrichment. This results in few *E. sakazakii* colonies being transferred to VRBGA and the reduced probability of selecting the bacterium for growth on TSA (Iversen *et al.*, 2004a; Iversen & Forsythe, 2004).   Furthermore, VRBGA contains selective and differential ingredients (crystal violet and bile salts no. 3) that can prevent resuscitation of injured *E. sakazakii* cells. Thus these factors might preclude the detection of *E. sakazakii* in powdered IFM and other foods (Gurtler & Beuchat, 2005). There is a great need for more rapid, reliable and specific methods for screening infant foods for *E. sakazakii* contamination (Iversen *et al.*, 2004a).

Phenotypic identification

Yellow pigment production

A trait of most *E. sakazakii* strains is the production of a non-diffusible yellow pigment when grown on TSA (FAO/WHO, 2004). This feature is used in various *E. sakazakii* detection methods to select presumptive-positive colonies for confirmation tests (Nazarowec-White & Farber, 1997a,b,c; Muytjens *et al.*, 1988; FDA, 2002).
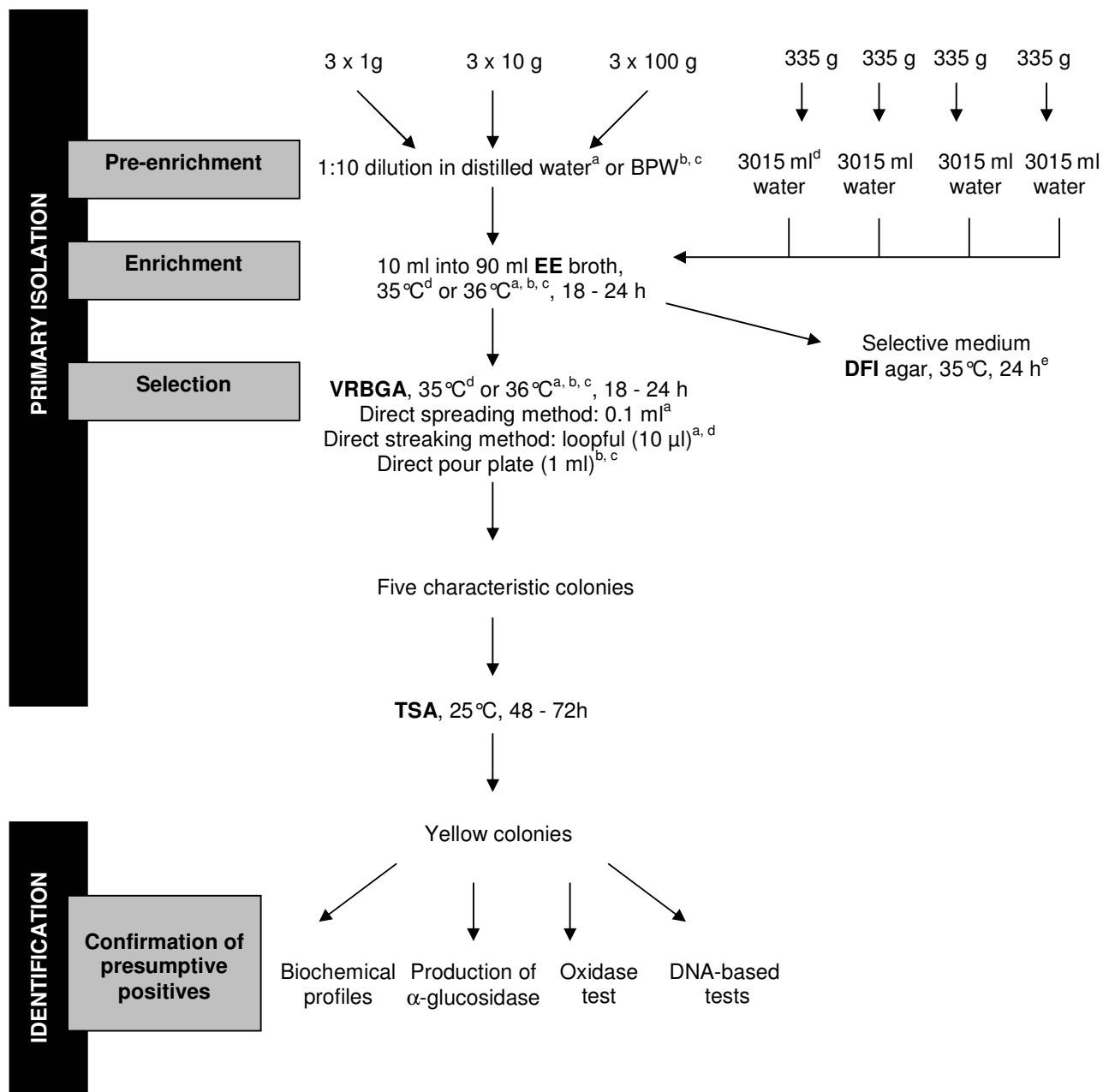
Figure 2 Procedures for isolation and identification of *Enterobacter sakazakii* (Adapted from Iversen & Forsythe, 2003). [a]FDA method (FDA, 2002); [b]Muytjens *et al.* (1988); [c]Nazorowec-White & Farber (1997a,b,c); [d]Wyeth Nutrition Method (Donnelly, 2005); [e]Iversen & Forsythe (2004).

Farmer *et al.* (1980) suggested that yellow pigment production should not be used alone as a differential criterion for identification of *E. sakazakii*, since yellow pigment production is not completely unique to *E. sakazakii*. In fact, this trait is frequently found in the closely-related genus *Pantoea,* which has also been isolated from reconstituted IFM (Muytjens *et al.*, 1988; Iversen & Forsythe, 2004). Identification based on pigment production is further hampered by the occurrence of white *E. sakazakii* strains (Block *et al.*, 2002), the occasional transient nature of the trait and the fact that pigment production is significantly more pronounced at 25°C than at higher temperatures (Farmer *et al.*, 1980).

α-glucosidase activity

The activity of the enzyme α-glucosidase has formed the basis for the development of numerous chromogenic and fluorogenic selective media (Iversen & Forsythe, 2004; Iversen *et al.*, 2004a; Oh & Kang, 2004; Iversen *et al.*, 2006b), which are recommended to serve as supplementary tests to confirm the identification of *E. sakazakii* (Fig. 2). Druggan-Forsythe-Iversen agar (DFI) is a selective differential chromogenic agar which was specifically formulated for selective detection of *E. sakazakii* in IFM (Iversen *et al.*, 2004a). A chromogen, 5-bromo-4-chloro-3-indolyl-α-D glucopyranoside (X-α-Glc), is incorporated in the medium to act as a differential agent by indicating α-glucosidase activity. *Enterobacter sakazakii* hydrolyses X-α-Glc to liberate the aglycone, 5-bromo-4-chloro-indolol (Iversen *et al.*, 2004a). This aglycone subsequently dimerises in the presence of oxygen to produce the pigment bromo-chloro-indigo, which is detected as blue-green colonies on the medium. In addition to the chromogen, DFI agar also contains sodium deoxycholate, a selective agent for *Enterobacteriaceae*, as well as a hydrogen sulphide indicator (sodium thiosulphate and ammonium iron citrate) to differentiate weak α-glucosidase, H_2S-positive micro-organisms (such as *Proteus vulgaris*) from *E. sakazakii* (Iversen *et al.*, 2004a).

Iversen *et al.* (2004a) compared the sensitivity of DFI agar with that of the current FDA method (Fig. 2) using 95 clinical and food isolates. All of the *E. sakazakii* strains evaluated were reportedly detected on DFI agar two days sooner than when using the FDA method. The specificity of the medium was also evaluated using 148 *Enterobacteriaceae* strains, excluding *E. sakazakii*. Only 19 strains representing three genera (*Escherichia, Pantoea* and *Citrobacter*) gave false-positive results on DFI agar,

in comparison with 31 of these strains producing false-positives using the FDA method (Iversen *et al.*, 2004a).

A selective fluorogenic medium known as Oh and Kang (OK) agar incorporates a fluorogen, 4-methyl-umbelliferyl α-D-glucoside (α-MUG), which serves as an indicator of α-glucosidase production by *E. sakazakii* (Oh & Kang, 2004). In this medium, bile salts no. 3 is the selective agent for enteric bacteria, while sodium thiosulphate and ferric citrate differentiate H_2S-producing *Enterobacteriaceae*. This fluorogen is also present in Leuscher, Baird, Donald and Cox (LBDC) agar developed by Leuscher *et al.* (2004) for presumptive detection of *E. sakazakii* in IFM. Detection with this medium is based on the formation of yellow-pigmented colonies by *E. sakazakii* that fluoresce under UV light when grown on nutrient agar supplemented with α-MUG. Colonies formed by other *Enterobacteriaceae* and non-*Enterobacteriaceae* reportedly do not fluoresce under UV light, even when the colonies produced are yellow pigmented (Leuscher *et al.*, 2004).

A major advantage of chromogenic and fluorogenic substrates is that strong, non-diffusible colours are produced in detection media, and thus even small positive colonies are observed in the presence of more abundant competitors (Iversen *et al.*, 2004a). Although the use of these media appear beneficial to decrease the time to detect presumptive-positive *E. sakazakii* isolates (Gurtler & Beuchat, 2005), their efficiency is lowered by the co-isolation of a small number of other *Enterobacteriaceae* that are also α-glucosidase positive (Iversen *et al.*, 2004c; Lehner *et al.*, 2006b). Although these methods seem to be useful for presumptive detection of *E. sakazakii*, or as a supplementary confirmation test (Muytjens, 1985), presumptive colonies produced on selective media require further species identification (Lehner *et al.*, 2006b).

Biochemical profiles

Biochemical profiles are frequently used as a confirmative test for presumptive-positive *E. sakazakii* isolates (Fig. 2) (Nazarowec-White & Farber, 1997a,b,c; Muytjens *et al.*, 1988; FDA, 2002). However, contradictory identification results have been reported to occur in different biochemical kits for the same bacterial strain (Iversen *et al.*, 2004a,c; Drudy *et al.*, 2006). Iversen *et al.* (2004a) compared different biochemical kits for identification of *E. sakazakii*, and reported that three strains which were identified as *E. sakazakii* by the API 20E system were identified as *Enterobacter cloacae*, *Enterobacter amnigenus* and *Enterobacter cloacae/gergoviae* with the ID32E system. Eight strains identified as *E. sakazakii* with the ID32E kit gave patterns consistent with *Pantoea* species with the API 20E kit (Iversen *et al.*, 2004a). Further biochemical

characterisation is therefore required to determine the traits most strongly associated with strains of *E. sakazakii*. Furthermore, there is a significant need for improvement in the current, mainly phenotypically based, approach for detecting and confirming presumptive *E. sakazakii* isolates (Gurtler *et al.*, 2005).

Molecular identification

To minimise the health risks associated with *E. sakazakii*, effective methods must be developed to confirm the results obtained with traditional microbiological methods. Such methods are required to rapidly and accurately detect and identify *E. sakazakii* isolates in IFM (Liu *et al.*, 2006). Molecular assays have become well established as valuable alternatives to traditional culturing methods, since they offer rapid, sensitive and specific identification of micro-organisms from a variety of sources (Malorney *et al.*, 2003, Lehner *et al.*, 2004). A number of molecular methods are utilised for rapid detection and/or identification of bacteria, most of which are based on the polymerase chain reaction (PCR). These include conventional PCR with species-specific primers, real-time PCR, PCR-ELISA and DNA sequencing. Despite the promise that these methods hold for microbial diagnostics, the acceptance of these techniques is hindered by the high investment costs and the lack of official standard regulations (Malorney *et al.*, 2003).

Species-specific PCR

Although PCR detection methods are widely used to identify micro-organisms, no validated PCR methods exist at present for the identification of *E. sakazakii*. The first PCR system published for the detection of *E. sakazakii* was developed by Keyser *et al.* (2003) based on a 16S rDNA sequence. Lehner *et al.* (2004) developed and evaluated an alternative PCR detection system for *E. sakazakii*, which was based on the 16S rDNA sequences of 13 *E. sakazakii* strains derived from different origins, as well as the type strain. This PCR system identified *E. sakazakii* isolates from both of the phylogenetically distinct groups within the species, as well as an *E. sakazakii* strain not detected using the Keyser *et al.* (2003) primers (Lehner *et al.*, 2004).

Several other PCR assays for the identification of *E. sakazakii* have recently been described. These assays employ the macromolecular synthesis operon (MMS), the α-glucosidase activity gene, the 16S – 23S rDNA internal transcribed spacer (ITS) region, or the 16S rDNA as the target sequence (Seo & Brackett, 2005; Lehner *et al.*,

2006b; Liu *et al.*, 2006; Hassan *et al.*, 2007). In addition, a commercial PCR system (BAX®, Oxoid) is available for the identification of *E. sakazakii* (Hassan *et al.*, 2007).

Unfortunately, it has been demonstrated that some PCR systems are non-specific, reacting with several other closely related species (Hassan *et al.*, 2007). While PCR is generally considered more accurate and reliable for bacterial identification than traditional culturing methods (Charteris *et al.*, 1997), these methods are highly dependant on the sequence of the primers utilised as well as the PCR conditions of the assays. Therefore, it is important that PCR systems be rigorously tested against closely related species to ensure that they are specific for the bacterium to be detected (Hassan *et al.*, 2007).

H.   Improvements in DNA-based diagnostics

PCR techniques have significantly improved the detection and identification of bacterial pathogens (Nogva *et al.*, 2000), and have found increasing application in food hygiene and control systems. Although there are numerous advantages of PCR methodologies, such as their specificity and sensitivity (Scheu *et al.*, 1998), the disadvantage of the techniques is their inability to differentiate between the DNA derived from viable and dead cells (Herman, 1997). As a result of the relatively long persistence of DNA in the environment after cell death, from a few days to three weeks (Josephson *et al.*, 1993; Masters *et al.*, 1994), DNA-based detection methods may detect DNA derived from viable and dead cells, and thus frequently tend to overestimate the number of viable cells present (Nocker *et al.*, 2006). The tendency of PCR to detect DNA from dead cells for long periods after cell death was demonstrated by Allmann *et al.* (1995). These researchers showed that heat-killed *Campylobacter jejuni* cells added to raw milk could be detected with PCR five weeks after inoculation. Since dead, viable but non-culturable, and culturable micro-organisms may be present in a food sample, it is necessary to be able to distinguish between these physiological bacterial states (Scheu *et al.*, 1998).

Fluorescent-staining techniques have been applied for the differential detection of viable and dead bacterial cells (Caron *et al.*, 1998) using fluorescence microscopy, fluorometery, or flow cytometry. Differential live/dead staining with fluorescent dyes is also the basis for the commercial LIVE/DEAD® BacLight™ viability kit developed by Molecular Probes (Eugene, Oregan, USA). This kit is based on cell membrane permeability and differentiation due to dye inclusion or exclusion (Haugland, 1999). The main disadvantage of most fluorescent-staining techniques is that all bacterial species

in a sample are differentially stained and no bacterial identification can be made (Maukonen *et al.*, 2006).

The detection of mRNA using reverse transcriptase PCR has also been used in an attempt to selectively detect viable bacterial cells (Novak & Juneja, 2001; Bentsink *et al.*, 2002). However, it is difficult to obtain reproducible and accurate viable and dead cell counts with mRNA as a target, due to its intrinsic instability (McKillip *et al.*, 1998; Sheridan *et al.*, 1998; Norton & Batt, 1999). In order to exploit the full potential of PCR in microbiological diagnostics, there is a great need for a methodology that allows PCR-discrimination of DNA derived from live and dead cells (Lee & Levin, 2006).

Recently, a novel technique using DNA-intercalating stains, such as ethidium monoazide (EMA) or propidium monoazide (PMA) (Nocker *et al.*, 2006), in combination with real-time PCR was reported to successfully distinguish and quantify viable and dead cells in complex samples (Nogva *et al.*, 2003; Rudi *et al.*, 2005a; Lee & Levin, 2006; Nocker & Camper, 2006; Nocker *et al.*, 2006; Wang & Levin, 2006). The basis for differentiation between viable and dead cells relies on cell membrane integrity, since live cells with intact membranes are able to exclude DNA-binding dyes that easily penetrate the compromised membranes of dead cells. Thus, EMA or PMA enters bacteria with damaged membranes and can be covalently linked to DNA by photo-activation (Nogva *et al.*, 2003). It was previously reported that DNA which is covalently bound with the intercalating dye cannot be PCR amplified (Nogva *et al.*, 2003; Rudi *et al.*, 2005a; Lee & Levin, 2006; Wang & Levin, 2006) and only DNA from viable cells can be detected. In a more recent publication, however, Nocker and Camper (2006), showed that EMA and PMA cross-linking actually renders the DNA insoluble; subsequently there is a loss of the insoluble DNA with cell debris during genomic DNA extraction. Furthermore, the same study verified that when subjecting a bacterial population comprised of both viable and dead cells to this treatment, there is a selective removal of DNA from dead cells (Nocker & Camper, 2006).

This method has been described as an easy-to-use alternative to both microscopic and flow cytometric discriminations between viable and dead cells (Nogva *et al.*, 2003; Rudi *et al.*, 2005a,b; Wang & Levin, 2006) and has to date been applied to a number of pathogenic bacteria including *Campylobacter jejuni* (Rudi *et al.*, 2005a), *Escherichia coli* 0157:H7 (Nogva *et al.*, 2003; Nocker & Camper, 2006, Nocker *et al.*, 2006), *Listeria monocytogenes* (Nogva *et al.*, 2003; Rudi *et al.*, 2005b), *Salmonella* spp. (Nogva *et al.*, 2003 ; Nocker & Camper, 2006, Nocker *et al.*, 2006), *Staphylococcus aureus* (Nocker *et al.*, 2006) and *Vibrio vulnificus* (Wang & Levin, 2006).

Conclusions

Newborn infants have immature immune systems and sterile gastro-intestinal tracts, which are rapidly colonised by micro-organisms through oral ingestion. Infants are therefore highly susceptible to infections, and food products designed for their consumption require high levels of microbiological quality control during production, distribution and use. The implication of powdered IFM as the primary source of the opportunistic bacterial pathogen, *E. sakazakii*, has become a growing concern to consumers, food manufacturers and legislators worldwide. Since it has been demonstrated that the current manufacturing technology does not allow for the production of sterile IFM, effective risk management strategies are required to address the presence of *E. sakazakii* in food products and to prevent contaminated products from being distributed.

The methods currently used for the detection and identification of *E. sakazakii* in IFM are inadequate and controversial. Conventional microbiological methods are based on morphological, physiological and biochemical characteristics. These methods are time consuming and they reflect only the portion of the bacterial genome expressed under specific cultivation conditions. Therefore, the conventional methods may underestimate the presence of *E. sakazakii* in IFM. Molecular methods, which are based on the composition of nucleic acids rather than on the products of their expression, are considered more reliable for bacterial detection and identification. To date, there are no standardised or official methods for the direct isolation of *E. sakazakii* from foods. Since low *E. sakazakii* levels can lead to fatalities, rapid and accurate detection, identification and typing methods are urgently required.

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CHAPTER 3

EVALUATION OF DIFFERENT METHODS FOR THE DETECTION AND IDENTIFICATION OF *ENTEROBACTER SAKAZAKII* ISOLATED FROM SOUTH AFRICAN INFANT FORMULA MILKS AND THE PROCESSING ENVIRONMENT

Abstract

Enterobacter sakazakii is an emerging pathogen associated with life-threatening neonatal infections resulting from the consumption of contaminated powdered infant formula milk (IFM). Accurate methods are required for rapid detection of this bacterium, since even low cell numbers have been reported to cause disease. The aim of this study was to evaluate various *E. sakazakii* detection methods in order to ascertain the most suitable method for detection and identification of this pathogen. Samples from IFM and the environment were evaluated for the presence of *E. sakazakii* using the isolation steps (pre-enrichment, enrichment and selection) described in the Food and Drug Administration (FDA) method for *E. sakazakii* detection. Sixty-four isolates (50 from IFM and 14 from the environment) were selected from tryptone soy agar (TSA), regardless of colony appearance, and these isolates were identified by 16S ribosomal DNA (rDNA) sequencing. Thereafter, different culture-dependent and culture-independent methods were evaluated to accurately detect and identify the *E. sakazakii* isolates. These methods included the assessment of yellow pigment production on TSA, typical colonies on chromogenic Druggan-Forsythe-Iversen (DFI) and Chromocult® *Enterobacter sakazakii* (ES) media and polymerase chain reaction (PCR) using six different species-specific primer pairs described in the literature. Identification of *E. sakazakii* using yellow pigment production was demonstrated to have a low sensitivity, specificity and accuracy (87%, 71% and 74%, respectively), which lowers the suitability of the FDA method. Chromogenic DFI and ES media were sensitive, specific and accurate (100%, 98% and 98%, respectively) for the detection of *E. sakazakii*. The specificity of the PCR amplifications ranged from 8% to 92%, emphasising the need for rigorous primer testing against closely related species. Of the primers evaluated, Esakf/Esakr were most suitable for *E. sakazakii* detection and identification. The detection limit of Esakf/Esakr was found to be 10^4 cfu.ml^{-1}. The current FDA method for *E. sakazakii* detection should be revised in the light of the availability of more sensitive, specific and accurate detection methods.

Introduction

Enterobacter sakazakii is a Gram-negative, motile, rod-shaped bacterium belonging to the genus *Enterobacter* and family *Enterobacteriaceae* (Nazorowec-White & Farber, 1997). Until its designation as a unique species in 1980, the bacterium was referred to as a "yellow-pigmented *Enterobacter cloacae*" (Farmer *et al.*, 1980). In recent years, *E. sakazakii* has become increasingly recognised as an emerging opportunistic pathogen and cause of infections in premature and immuno-compromised infants. Although rare, *E. sakazakii* infections are often life-threatening, and most frequently cause meningitis, sepsis and necrotizing enterocolitis. The symptoms of infection are severe and the prognosis is poor (Biering *et al.*, 1989; Bar-oz *et al.*, 2001; Lai, 2001; Van Acker *et al.*, 2001), with case mortality rates varying from 40 to 80% among infected infants (Willis & Robinson, 1988). A growing number of reports have epidemiologically implicated powdered IFM as the source and vehicle of *E. sakazakii* infections (Biering *et al.*, 1989; Van Acker *et al.*, 2001; Himelright *et al.*, 2002).

With the utilisation of the currently available technology, it is not possible to manufacture sterile powdered IFM (FAO/WHO, 2004). Thus, IFM products containing low levels of pathogens may occasionally be distributed in spite of them complying with the prevailing microbiological standards for powdered IFM. Analyses of commercial powdered IFM products have revealed the prevalence of *E. sakazakii* in 0 - 18% of the products tested, with concentrations almost always being <1 cfu.100 g^{-1} (Muytjens *et al.*, 1988; ICMSF, 2004; Iversen & Forsythe, 2004; Witthuhn *et al.*, 2006). Since even low *E. sakazakii* cell numbers pose a considerable health risk, accurate and sensitive methods are required for the rapid detection of this bacterium in foods.

At present, the methods used for detection and identification of *E. sakazakii* are inadequate and controversial. There has also been a lack of consistency between results obtained with different detection and identification methods (Hassan *et al.*, 2007; Lehner *et al.*, 2004; Lehner *et al.*, 2006). The current FDA detection method makes use of a series of culturing steps to isolate *E. sakazakii* from food matrices. These culturing steps include pre-enrichment, enrichment in *Enterobacteriaceae* enrichment (EE) broth, culturing on violet red bile glucose agar (VRBGA) and sub-culturing onto tryptone soy agar (TSA) (FDA, 2002). The FDA method utilises the production of yellow pigmented colonies on TSA as a criterion for the identification of *E. sakazakii*. However, yellow pigment production is not unique to *E. sakazakii* and the occurrence of white *E. sakazakii* strains has been documented (Farmer *et al.*, 1980; Block *et al.*, 2002;

Iversen *et al.*, 2006). The need for simplified and selective *E. sakazakii* detection methods has prompted the development of several chromogenic and fluorogenic media, which have been reported to be more rapid and selective for *E. sakazakii* than the FDA method (Iversen *et al.*, 2004a).

Various researchers (Lehner *et al.*, 2004; Witthuhn *et al.*, 2006) have suggested that species-specific PCR is a rapid and accurate alternative to traditional microbiological techniques for the detection and identification of *E. sakazakii*. The reliability and accuracy of PCR-based methods are, however, reliant on the primers chosen and the temperature cycling conditions utilised. It has been reported that certain PCR systems show a lack of specificity for *E. sakazakii* detection (Lehner *et al.*, 2004). The aim of this study was to evaluate and compare various methods for the detection and identification of *E. sakazakii* isolates derived from IFM products and the processing environment. These included the FDA culturing method, culturing on selective chromogenic Druggan-Forsythe-Iversen (DFI) and Chromocult® *Enterobacter sakazakii* (ES) media, species-specific PCR using six different primer pairs described in the literature, as well as 16S rDNA sequencing.

Materials and methods

A schematic representation of the methods utilised to isolate and identify *E. sakazakii* is provided in Figure 1. The figure shows the FDA (2002) method that was used to isolate pigmented and non-pigmented colonies on TSA. The *E. sakazakii* identification methods used in the FDA protocol, as well as the additional *E. sakazakii* identification methods that were evaluated, are depicted (Fig. 1).

Isolation of *Enterobacter sakazakii*

A total of 50 IFM samples and 14 environmental samples were evaluated for the presence of *E. sakazakii* and other micro-organisms. IFM products were obtained from an IFM manufacturer in South Africa, and samples were taken from the products according to the method described by Donelly (2005). Twenty cans of IFM were sampled at regular intervals per production lot and these cans were divided into four subgroups, with five cans per subgroup. The cans were surface sterilised with 70% ethanol (Sigma-Aldrich, Gauteng, South Africa) and were opened under laminar flow. A mass of 67 g from each of the five cans within the subgroups was transferred to a self-seal bag using a sterile spoon. This was repeated for all the subgroups in order to

obtain four 355 g composites. Each 335 g composite was added to 3.015 l of sterile distilled water (pre-warmed to 45℃) and after mixing it was incubated at 35℃ for 18 - 24 h (Donelly, 2005). For environmental sampling, a sterile cellulose sponge immersed in 10 ml neutralising buffer within a re-sealable polythene pouch (3M Microbiology, Minnesota, USA) was used to swab production areas, including floors, walls, preparation surfaces and equipment. The sponge swab was replaced in its pouch and transported to the laboratory under refrigeration conditions.

In accordance with the FDA method, a 10 ml aliquot of the pre-enrichment suspension or swab sample was added to 90 ml of EE broth (Oxoid, supplied by C.A. Milsch, Cape Town, South Africa), followed by incubation at 35℃ for 18 – 24 h (FDA, 2002). A loopful of the enrichment culture was streaked onto four quadrants of duplicate VRBGA (Oxoid) plates and after incubation at 35℃ for 18 - 24 h, growth was examined. A total of five colonies were picked from both VRBGA plates, regardless of the colony appearance. When more than five different colonies were observed on VRBGA, the colonies most representative of *E. sakazakii* (opaque purple to pink, sometimes with a purple halo and yellow centres) were picked. The five presumptive colonies were streaked onto TSA (Oxoid) and incubated at 25℃ for 48 - 72 h. Following incubation, both pigmented and non-pigmented colonies on TSA were selected for further investigation.

The cell morphology and purity of all isolates was evaluated by Gram-staining and microscopic examination. For long-term storage, the purified isolates were maintained in 20% (v/v) glycerol (Sigma-Aldrich) at -20℃. The *E. sakazakii* type strain DSM 4485[T] (ATCC 29544) and *E. sakazakii* 1039 (Department of Food Science Culture Collection, Stellenbosch University), isolated from a food processing environment (Keyser *et al.*, 2003), were used as positive controls to validate the detection and identification methods used.

DNA isolations

Single colonies from the purified isolates on TSA plates were transferred to 10 ml tryptone soy broth (TSB) (Oxoid), and the cells were grown overnight at 25℃. DNA was isolated according to the method described by Wang and Levin (2006). Cells were harvested from 250 µl aliquots by centrifugation (Eppendorf Centrifuge 5415D) at 8000 g for 5 min. Cell pellets were re-suspended in 250 µl distilled water. All samples were mixed with 250 µl double-strength TZ (2 x TZ) (Abolmatty *et al.*, 2000), which consisted of 4% (v/v) Triton X-100 (Merck) and 5 mg.ml^{-1} sodium azide (Merck) in 0.1 M Tris–HCl
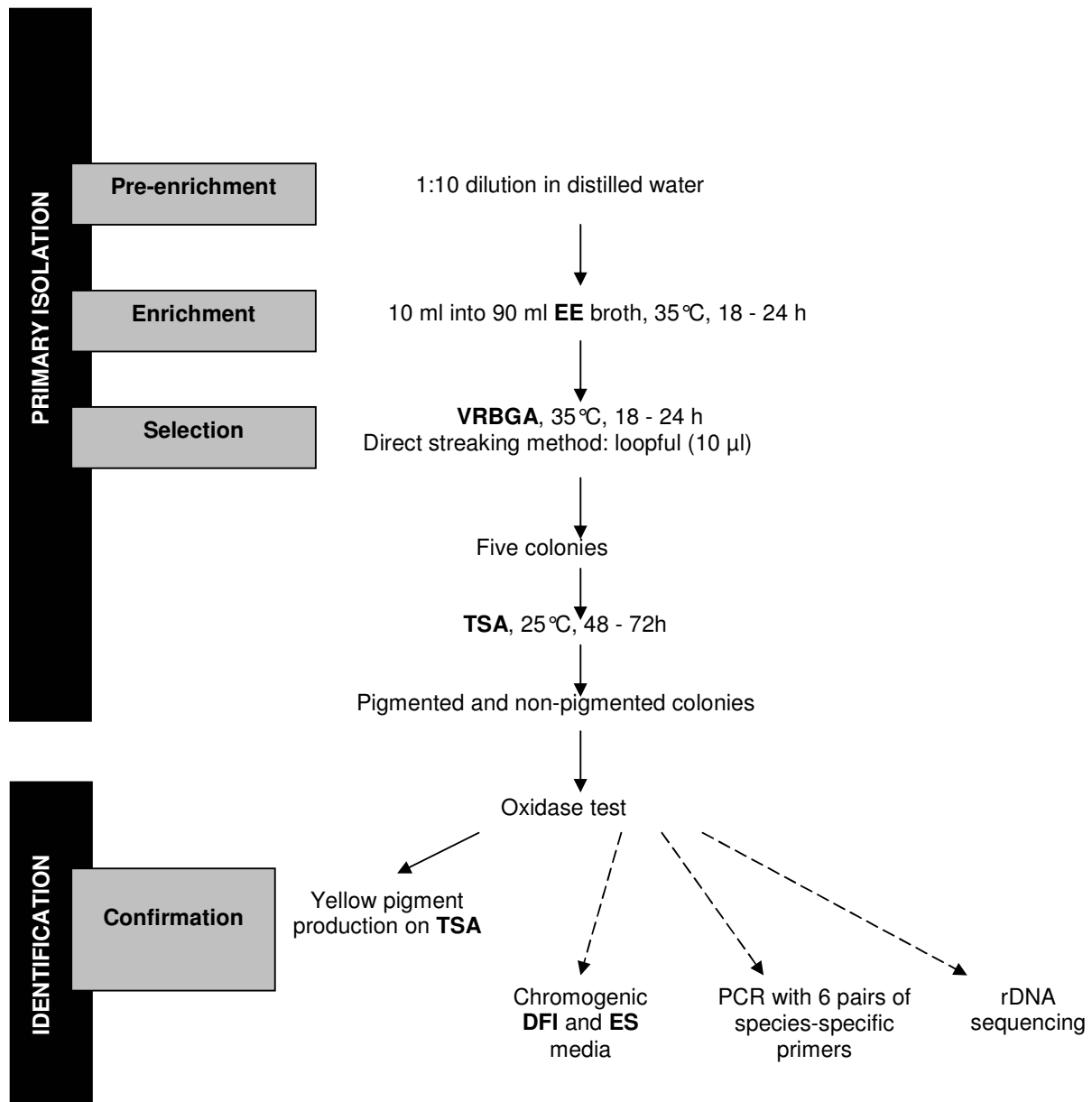
Figure 1 Schematic outline of the methods used to isolate and identify *E. sakazakii* and other micro-organisms from IFM and the processing environment. The FDA method (2002) used to isolate *E. sakazakii* is shown, as well as the different identification methods evaluated to confirm the presumptive *E. sakazakii* isolates. The solid arrows represent steps forming part of the FDA method, while the dashed arrows indicate additional *E. sakazakii* detection and identification methods evaluated.

(Fluka, supplied by Sigma-Aldrich) at pH 8. Sample tubes were heated in a boiling waterbath for 10 min to lyse the cells. Cell debris was pelleted by centrifugation (10 000 g, 5 min) and 200 µl of crude DNA from each sample was purified using a Micropure-EZ column (Millipore, supplied by Microsep, Cape Town, South Africa).

Identification of isolates by 16S rDNA sequencing

Isolates were identified using DNA sequence data of a 1.5 kilobase (kb) fragment of the 16S rDNA. PCR amplification of this fragment was performed using the primers F8 (5'- CAC GGA TCC AGA CTT TGA TYM TGG CTC AG -3') and R1512 (5'- GTG AAG CTT ACG GYT AGC TTG TTA CGA CTT -3') (Felske *et al.*, 1997). The PCR reaction mixture (50 µl total volume) contained 2 µl (400 nM) of each primer, 1 µl (5 U) *Taq* DNA polymerase (5 U.µl^{-1}, Super-Therm, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 5 µl 10 X buffer (with $MgCl_2$) (Super-Therm), 2 µl (0.4 mM) dNTPs (10 mM, AB gene, supplied by Southern Cross Biotechnologies), 2 µl 99% (v/v) DMSO (Merck) and 2 µl DNA template. Thermal cycling parameters were as follows: initial denaturation at 92°C for 3 min; 35 cycles of denaturation at 92°C for 30 s, annealing at 54°C for 30 s, elongation at 68°C for 60 s and final elongation at 72ºC for 7 min (Felske *et al.*, 1997).

PCR products were purified using Sigma Spin Post-Reaction Purification Columns (Sigma-Aldrich) according to the instructions of the manufacturer. Sequencing of the PCR products was carried out at the DNA Sequencing Facility at Stellenbosch University using the ABI 3100 Genetic Analyser (Applied Biosystems, Foster City, USA). The generated sequences were compared to sequences in the National Centre for Biotechnology Information (NCBI) database utilising the BLASTn search option to determine the closest recognised relatives (Altschul *et al.*, 1997).

Oxidase test and yellow pigment production

Oxidase tests were carried out on overnight cultures of the isolates growing on TSA (Oxoid). Oxidase reagent was freshly prepared before testing by adding 1 g *N, N, N',N'*-tetramethyl-p-phenylenediamine.2HCl (Sigma-Aldrich) to 100 ml distilled water. A sterile applicator was used to transfer a small amount of culture to a piece of Whatman no. 1 filter paper (Merck, Cape Town, South Africa), which was impregnated with oxidase reagent. Isolates producing a dark purple colour within 10 s of application were recorded as oxidase positive, while those giving no colour reaction were recorded as oxidase negative.

In accordance with the FDA method, the production of yellow pigmented colonies was investigated as a means of identifying presumptive positive *E. sakazakii* colonies. All the purified isolates were streaked onto TSA (Oxoid) and incubated at 25°C for 48 - 72 h (FDA, 2002). Following incubation, the isolates producing yellow pigmented colonies on TSA were recorded in order to assess the suitability of this criterion for *E. sakazakii* identification.

Chromogenic media

The selective chromogenic media evaluated and compared in this study were DFI agar (Iversen & Forsythe, 2004) and Chromocult® ES agar (Merck). DFI agar (pH 7.3) contained sodium deoxycholate (1.0 g.l^{-1}, Sigma-Aldrich), 5-bromo-4-chloro-3-indolyl α-D-glucopyranoside (0.1 g.l^{-1}, Glycosynth, Warrington, UK), sodium thiosulphate (1.0 g.l^{-1}, Sigma-Aldrich) and ammonium iron(III) citrate (1.0 g.l^{-1}, Sigma-Aldrich), dissolved in distilled water prior to addition of TSA (40 g.l^{-1}, Oxoid). Single colonies from the incubated TSA plates were streaked onto DFI and ES agar plates, followed by overnight incubation at 37°C for the DFI agar and 44°C for the ES agar. Colonies that were entirely blue-green on DFI agar, or entirely turquoise on ES agar, were considered positive for *E. sakazakii* (Iversen & Forsythe, 2004; Iversen *et al*., 2004a; Manafi & Lang, 2005).

PCR detection of *Enterobacter sakazakii*

Species-specific PCR for the detection of *E. sakazakii* was carried out using six different primer pairs (Table 1). The reaction mixtures for each PCR system were prepared as shown in Table 2. PCR amplification was carried out using a Mastercycler Personal (Eppendorf, Germany) using the cycling conditions described in the literature (Table 1). PCR amplifications using all six primer pairs were repeated in triplicate on all isolates to ensure the accuracy of the results obtained. All the amplified PCR products obtained with the different primers were separated on 1.5% (m/v) agarose gels, containing 0.02 µl.ml^{-1} ethidium bromide, in 0.5 X TBE electrophoresis buffer. The separated PCR fragments were visualised under an ultraviolet light (Vilber Lourmat, Marne La Vallee, France). The isolates were grouped based on the number of positive PCR results obtained with the six primer pairs (Table 3).

For the determination of the PCR detection limits, *E. sakazakii* (1039) was grown overnight in TSB (Oxoid) at 25°C. A 1 ml aliquot of the overnight cell suspension was

Table 1 Species-specific PCR primers evaluated for the detection of *Enterobacter sakazakii*

Primer name	Primer sequence (5' to 3')	Target	PCR cycling conditions	Amplicon size (bp)	Reference
Esak2	CCCGCATCTCTGCAGGATTCTC	16S rDNA	95°C - 2 min; 35 x (95°C - 35 sec, 61°C - 60 sec, 72°C - 60 sec); 72°C – 10 min.	900 bp	Keyser *et al*., 2003
Esak3	CTAATACCGCATAACGTCTACG	16S rDNA			
Esakf	GCTYTGCTGACGAGTVGGCGG	16S rDNA	94°C - 2 min; 29 x (94°C - 30 sec, 66°C - 60 sec; 72°C - 90 sec) ; 72°C - 5 min.	929 bp	Lehner *et al*., 2004
Esakr	ATCTCTGCAGGATTCTCTGG	16S rDNA			
EsAgf	TGAAAGCAATCGACAAGAAG	Gene responsible for α-glucosidase activity	94°C - 2 min; 29 x (94°C - 30 sec, 58°C - 60 sec, 72°C - 90 sec); 72°C - 5 min.	1680 bp	Lehner *et al*., 2006
EsAgr	ACTCATTACCCCTCCTGATG	Gene responsible for α-glucosidase activity			
SG-F	GGGTTGTCTGCGAAAGCGAA	ITS[a] sequence	94°C - 10 min; 30 x (94°C - 30 sec, 57°C - 60 sec, 72°C - 1 min); 72°C - 5 min.	282 bp	Liu *et al*., 2006
SG-R	GTCTTCGTGCTGCGAGTTTG	ITS[a] sequence			
SI-F	CAGGAGTTGAAGAGGTTTAACT	ITS[a] sequence	94°C - 10 min; 30 x (94 °C - 30 s, 57°C - 60 sec, 72°C - 1 min); 72°C - 5 min.	251 bp	Liu *et al*., 2006.
SI-R	GTGCTGCGAGTTTGAGAGACTC	ITS[a] sequence			
Saka-1	ACAGGGAGCAGCTTGCTGC	16S rDNA	95°C - 4 min; 30 x (95°C - 60 sec, 50°C - 60 sec, 72°C - 90 sec) ; 72°C - 4 min.	952 bp	Hassan *et al*., 2007
Saka-2b	TCCCGCATCTCTGCAGGA	16S rDNA			

[a]Internal transcriber spacer (ITS) sequence between 16S and 23S rDNA.

Table 2 PCR reaction mixtures utilising species-specific primers for the detection of *Enterobacter sakazakii*

Primer name	Total volume	Primer 1	Primer 2	*Taq* DNA polymerase[a]	Reaction buffer	MgCl$_2$	dNTPs[d]	Dimethyl Sulphoxide (DMSO)	DNA template	KCl	Tris-HCl
Stock solution concentration		(10 µM)	(10 µM)	(5 U.µl^{-1})	(10 X)	(25 mM)	(10 mM)	99%		(1M)	(1M)
Esak2/ Esak3	25 µl	1.25 µl (500 nM)	1.25 µl (500 nM)	0.1 µl (0.5 U)	2.5 µl[b]	2 µl (1.5 mM)	2.5 µl (1 mM)	-	2 µl	-	-
Esakf/ Esakr	50 µl	1 µl (200 nM)	1 µl (200 nM)	0.70 µl (3.5 U)	5 µl[b]	3 µl (1.5 mM)	2 µl (0.4 mM)	2 µl	4 µl	-	-
EsAgf/ EsAgr	50 µl	0.5 µl (100 nM)	0.5 µl (100 nM)	0.4 µl (2U)	5 µl[c]		2 µl (0.4 mM)	-	4 µl		
SG-F/ SG-R	50 µl	1.25 µl (250 nM)	1.25 µl (250 nM)	0.4 µl (2 U)	-	6 µl (3 mM)	4 µl (0.8 mM)	-	5 µl	2.5 µl (50mM)	0.5 µl (10 mM)
SI-F/ SI-R	50 µl	1.25 µl (250 nM)	1.25 µl (250 nM)	0.4 µl (2 U)	-	6 µl (3 mM)	4 µl (0.8 mM)	-	5 µl	2.5 µl (50mM)	0.5 µl (10 mM)
Saka1/ Saka2b	30 µl	1 µl (333 nM)	1 µl (333 nM)	0.2 µl (1U)	3 µl[b]	1.8 µl (1.5 mM)	0.6 µl (0.2 mM)	-	2.5 µl		

[a]Super-Therm, supplied by Southern Cross Biotechnologies.
[b]MgCl$_2$-free reaction buffer, Super-Therm.
[c]Reaction buffer containing MgCl$_2$, Super-Therm.
[d]AB gene, supplied by Southern Cross Biotechnologies.

centrifuged to harvest the cells and the pellet was re-suspended in 0.85% (m/v) NaCl (Merck). A dilution series (10^{-1} to 10^{-9}) of the re-suspended cell suspension was prepared in 0.85% (m/v) NaCl (Merck). For enumeration, duplicate spread plates were prepared on TSA (Oxoid). Following incubation of the plates at 25°C for 48 h, the cell numbers (cfu.ml^{-1}) of each dilution was calculated. DNA was extracted from each of the cell dilutions as previously described. PCR amplification was performed on the extracted DNA with each of the six primer pairs (Table 1) to determine the minimum number of cells that could be detected.

Validating detection and identification methods

The sensitivity, specificity and accuracy of yellow pigment production, selective chromogenic DFI and ES media and different PCR detection methods were determined by comparing the identification results obtained with these methods to those obtained with 16S rDNA sequencing. Sensitivity was defined as the number of true positives divided by the sum of true positives plus false negatives, expressed as a percentage (Table 4) (Greenhalgh, 1997). Sensitivity, also called the true positive rate, gives an indication of the efficiency of a test in detecting isolates that are truly *E. sakazakii*. Specificity was defined as the number of true negatives divided by the sum of false positives plus true negatives, expressed as a percentage (Table 4). Specificity, also called the true negative rate, gives an indication of the efficiency of a test in correctly excluding isolates that are not *E. sakazakii* (Greenhalgh, 1997).   Accuracy was defined as the number of true positives and true negatives as a proportion of all the results (Table 4), and thus shows the proportion of all the tests that gave the correct result (Greenhalgh, 1997).

Results and discussion

Isolation and identification of isolates from IFM and the processing environment

In order to assess the ability of different methods to accurately detect and identify *E. sakazakii*, the methods evaluated in this study were tested on isolates from South African IFM and the processing environment.   Members of *E. sakazakii* and other *Enterobacteriaceae* were isolated using the FDA method, but were selected from TSA regardless of colony pigmentation. After purification, the isolates were identified using 16S

rDNA sequencing. The identification results for a total of 64 isolates (50 from IFM and 14 from the processing environment) are presented in Table 3. Based on the 16S rDNA sequencing data, 52 of the 64 strains (81%) isolated from IFM and the processing environment were identified as species of *Enterobacter* (Table 3). Only 13 of the 64 strains (20%) were identified as *E. sakazakii* and this emphasises the need for accurate methods to identify *E. sakazakii* isolates following selection on VRBGA as part of the FDA method.

The isolates obtained from seven of the 50 IFM products (14%) evaluated in this study were confirmed as *E. sakazakii* by 16S rDNA sequencing (Table 3). This percentage is slightly lower than that reported by Witthuhn *et al*. (2006), who found that 18% of South African IFM and baby food products tested positive for the presence of *E. sakazakii*. However, in one of the most prominent studies on IFM products obtained from 35 countries, Muytjens *et al*. (1988) also found 14% of IFM products to be contaminated with low levels of *E. sakazakii*. It thus appears that the prevalence of *E. sakazakii* in South Africa is similar to that observed worldwide. However, given the considerable proportion of immuno-compromised individuals in South Africa, the risks that the pathogen poses in this and other developing countries should warrant cause for concern.

Other *Enterobacteriaceae* that were identified among the isolates obtained from the 50 IFM products included seven *Pantoea* spp. (14%), five *Erwinia* spp. (10%), five *Enterobacter hormaechei* strains (10%), three *Enterobacter cloacae* strains (6%), one *Enterobacter ludwigii* strain (2%) and 17 isolates identified as *Enterobacter* spp. (34%) (Table 3). Non-*Enterobacteriaceae* that were identified from the 50 IFM products included two *Pseudomonas* spp. (4%), two *Stenotrophomonas maltophilia* strains (4%) and one *Acinetobacter* species (2%) (Table 3).

From the 14 isolates obtained from the processing environment, six of these (43%) were confirmed to be *E. sakazakii* by 16S rDNA sequencing (Table 3). This high prevalence of *E. sakazakii* in the processing environment poses a major risk for contamination of IFM products. This again emphasises the problems posed by the fact that the reservoir and route by which the bacterium enters the processing environment remains unknown (Nazorowec-White & Farber, 1997). Other *Enterobacteriaceae* that were identified among the 14 environmental isolates included two *E. cloacae* strains (14%), one *Enterobacter helviticus* strain (7%), one *E. hormaechei* strain (7%) and 4 isolates identified as *Enterobacter* spp. (29%) (Table 3).

Evaluation of detection and identification methods

Oxidase test and yellow pigment production

The FDA method utilises the oxidase test and yellow pigment production on TSA as criteria for identification of presumptive *E. sakazakii* isolates. However, it has been documented that these methods are not sufficiently sensitive and specific to ensure the detection and correct identification of *E. sakazakii* in food and environmental samples (Iversen *et al.*, 2004a), Therefore, in this study the suitability of the oxidase test and yellow pigment production for *E. sakazakii* identification was evaluated on the isolates that were randomly selected from South African IFM and processing environments and which were identified by 16S rDNA sequencing.

A total of 62 of the 64 isolates obtained from IFM and the processing environment produced a negative result to the oxidase test (Table 3). Since the FDA method is intended to specifically isolate members of the oxidase-negative *Enterobacteriaceae* family (Krieg & Holt, 1984; Iversen & Forsythe, 2003), it was expected that the majority of the isolates would be oxidase negative. The two isolates that were found to be oxidase positive (F11 and F13) (Table 3) were both identified by 16S rDNA sequencing as members of the genus *Pseudomonas*, which typically give a positive result to the oxidase test (Holt *et al.*, 1994). All other *Enterobacteriaceae* isolates (including *Enterobacter*, *Erwinia* and *Pantoea*) as well as non-*Enterobacteriaceae* isolates (including *Acinetobacter* and *Stenotrophomonas*) gave negative oxidase reactions, which was consistent with the recognised oxidase status of these micro-organisms (Holt *et al.*, 1994; Mukhopadhyay *et al.*, 2003). For the purposes of isolating and identifying *E. sakazakii*, the inclusion of the oxidase test is useful for eliminating oxidase-positive organisms from further confirmatory testing. In this study, however, only 2 of 64 isolates (3%) would have been eliminated from further testing by means of this test (Table 3). Since the oxidase test does not provide a means to differentiate *E. sakazakii* form other oxidase-negative microbes, a more specific method for *E. sakazakii* identification is required to replace this step in the FDA method.

Although yellow pigment production is considered to be characteristic of *E. sakazakii* two of the 15 isolates (13%) identified as *E. sakazakii* by 16S rDNA sequencing did not produce yellow-pigmented colonies on TSA after incubation at 25°C (Table 3). Both of these non-pigmented *E. sakazakii* strains (E01 and E04) (Table 3) were isolated from the IFM processing environment. The fact that these isolates were not yellow pigmented

implies that they would not have been selected for further testing using the FDA method, and would thus have remained undetected. This percentage of non-pigmented *E. sakazakii* strains (13%) is greater than that reported by Iversen and Forsythe (2007), who found that 2% of *E. sakazakii* strains did not produce yellow-pigmented colonies on TSA at 25°C. These false-negative results when utilising yellow pigment production to identify *E. sakazakii* lowered the sensitivity of the method to 87% (Table 4).

The production of yellow-pigmented colonies was also not unique to *E. sakazakii*, as 15 of the 51 non-*E. sakazakii* isolates (29%) were found to produce yellow-pigmented colonies on TSA at 25°C (F04, F08, F14, F18, F19, F20, F21, F25, F33, F37, F43, F44, F45, F46 and E12) (Table 3). This high number of false-positive results reduced the specificity of *E. sakazakii* identification based on pigment production to 71% (Table 4). The yellow pigment producing non-*E. sakazakii* isolates included members of the genera *Acinetobacter* and *Pantoea*, as well as other species of *Enterobacter* (Table 3). This observation is consistent with previous reports of yellow-pigment production by *Acinetobacter* sp. (Leuscher *et al*., 2004), *Pantoea* sp. (Muytjens *et al*., 1988; Iversen & Forsythe, 2004) and *Enterobacter agglomerans* (Leuscher *et al*., 2004), which was recently renamed *Pantoea agglomerans* (Brenner & Farmer, 2005). Members of all of these genera have previously been isolated from IFM (Muytjens *et al*., 1988; Iversen & Forsythe, 2004; Leuscher *et al*., 2004) and could give false-positive based on yellow pigment production.

The accuracy, or the overall percentage of correct identification results, was 74% when utilising yellow pigment production to identify *E. sakazakii* (Table 4). Given the serious nature of *E. sakazakii* infections, this method is not sufficiently accurate to identify members of *E. sakazakii*. However, it has been reported that the production of pigments by bacteria promotes their virulence and persistence in harsh environments (Reverchon *et al*., 2002; Liu *et al*., 2005). Therefore, it may be of interest to investigate the correlation between yellow pigment production and the virulence of different strains of *E. sakazakii*.

Although the FDA method includes the utilisation of biochemical profiling for the final confirmation of presumptive-positive *E. sakazakii* isolates, the disadvantages associated with these tests have been published in the literature (Iversen *et al*., 2004a,b; Drudy *et al*., 2006). Since numerous reports have been made of contradictory identification results occurring with different biochemical kits for the same bacterial strain (Iversen *et al*., 2004a; Drudy *et al*., 2006), it was decided that these test methods would not be re-evaluated in this study. Rather, the focus would be on the evaluation of alternative methods that could

serve to replace these non-specific methods currently recommended for *E. sakazakii* detection.

Chromogenic media

Selective chromogenic media have been reported to be more specific for the detection of *E. sakazakii* than the culturing media currently used in the FDA method (Iversen *et al.*, 2004a). Isolates were detected and identified as *E. sakazakii* on the chromogenic media based on the production of characteristic blue-green colonies on DFI agar and as turquoise colonies on ES agar (Fig. 2). Both DFI and ES media gave positive results for all 15 isolates (including the two controls) (DSMZ 4485^T, 1039, E01, E02, E03, E04, E05, E06, F01, F03, F05, F06, F07, F09, F10) that were identified as *E. sakazakii* by 16S rDNA sequencing (Table 3). Since no non-characteristic colonies were obtained with these media for the isolates identified as *E. sakazakii*, the sensitivity of both DFI and ES media was 100% for the detection of *E. sakazakii* (Table 4). This was expected, since it has been reported that 100% of *E. sakazakii* strains test positive for the α-glucosidase enzyme, and thus would be able to hydrolyse the chromogen present in these media and produce characteristic coloured colonies (Iversen & Forsythe, 2004; Iversen *et al.*, 2004a).

The occurrence of one false-positive result (E12) (Table 3) from the 64 isolates tested, however, marginally reduced the specificity of both ES and DFI media to 98% (Table 4). Although all other non-*E. sakazakii* isolates produced white or yellow colonies on both chromogenic media, E12 produced blue-green colonies on DFI agar and turquoise colonies on ES agar. However, isolate was identified as *Enterobacter helviticus* by 16S rDNA sequencing (Table 3). *Enterobacter helviticus*, originally isolated from fruit powder, was recently introduced as a novel species (Stephan *et al.*, 2007). In agreement with the results obtained in this study, *E. helviticus* has been documented to produce yellow-pigmented colonies on TSA plates, although 16S rDNA sequencing revealed that these isolates were clearly distinct from *E. sakazakii* (Lehner *et al.*, 2006). Strains of this species have also been reported to produce colonies typical of *E. sakazakii* after incubation on DFI agar (Lehner *et al.*, 2006) and ES agar (Stephan *et al.*, 2007). Therefore, the efficiencyof chromogenic media such as DFI agar and ES agar is lowered by the occurrence of other species of *Enterobacter* that demonstrate α-glucosidase activity. In previous studies it has been suggested that some members of the species *Pantoea* gave false-positive results on

Table 3 The results obtained from conventional microbiological and molecular methods for the detection and identification of *Enterobacter sakazakii*

Isolate number	Source	Yellow pigment	Oxidase test	DFI agar	ES agar	Esakf Esakr	Esak2 Esak3	SG-R SG-F	SI-F SI-R	Saka1 Saka2b	EsAgF EsAgR	Identification	% Sequence similarity[b]	NCBI Accession number
		Phenotypic identification		**Chromogenic media**		**E. sakazakii specific PCR primers**						**DNA sequencing**[a]		
Controls (n = 2)														
DSMZ 4485[T]	Clinical	+	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	99%	EF088379.1
1039	Environ	+	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	98%	EF059824.1
Group 1 (n = 14)														
E01	Environ	-	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	98%	EF059831.1
E02	Environ	+	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	96%	AY803190.1
E03	Environ	+	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	97%	EF059856.1
E04	Environ	-	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	95%	EF059847.1
E05	Environ	+	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	98%	AY752939.1
E06	Environ	+	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	98%	EF059831.1
F01	Food	+	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	90%	EF059873.1
F02	Food	-	-	-	-	+	+	+	+	+	+	*Enterobacter* sp.	96%	AY689062.1
F03	Food	+	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	97%	EF059856.1
F04	Food	+	-	-	-	+	+	+	+	+	+	Uncultured *Pantoea* sp.	98%	EF072302.1
F05	Food	+	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	97%	EF059829.1
F06	Food	+	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	97%	EF059826.1
F07	Food	+	-	+	+	+	+	+	+	+	+	*Enterobacter sakazakii*	96%	EF059829.1
F08	Food	+	-	-	-	+	+	+	+	+	+	*Acinetobacter* sp.	97%	EF514909.1

Isolate number	Source	Phenotypic identification		Chromogenic media		*E. sakazakii* specific PCR primers						Identification	% Sequence similarity[b]	NCBI Accession number
		Yellow pigment	Oxidase test	DFI agar	ES agar	Esakf Esakr	Esak2 Esak3	SG-R SG-F	SI-F SI-R	Saka1 Saka2b	EsAgF EsAgR			
Group 2 (*n* = 2)														
F09	Food	+	-	+	+	+	-	+	+	+	+	*Enterobacter sakazakii*	97%	AM075208.1
F10	Food	+	-	+	+	+	-	+	+	+	+	*Enterobacter sakazakii*	97%	AM075208.1
Group 3 (*n* = 2)														
E07	Environ	-	-	-	-	-	-	+	+	+	+	*Enterobacter* sp.	98%	EF471901.1
F11	Food	-	+	-	-	-	-	+	+	+	+	*Pseudomonas fulva*	98%	AM184228.1
Group 4 (*n* = 1)														
F12	Food	-	-	-	-	+	-	-	+	+	+	*Enterobacter cloacae*	95%	AJ417459.1
Group 5 (*n* = 2)														
F13	Food	-	+	-	-	-	-	+	+	-	+	*Pseudomonas putida*	100%	EF204241.1
F14	Food	+	-	-	-	-	-	+	+	-	+	*Pantoea agglomerans*	96%	AF130953.1
Group 6 (*n* = 1)														
F15	Food	-	-	-	-	+	-	-	+	-	+	*Enterobacter* sp.	98%	EF419181.1
Group 7 (*n* = 13)														
E08	Environ	-	-	-	-	-	-	+	+	-	-	*Enterobacter cloacae*	99%	Y17665.1
E09	Environ	-	-	-	-	-	-	+	+	-	-	*Enterobacter cloacae*	97%	EF120473.1
E10	Environ	-	-	-	-	-	-	+	+	-	-	*Enterobacter* sp.	99%	DQ068769.1
F16	Food	-	-	-	-	-	-	+	+	-	-	*Enterobacter cloacae*	99%	Y17665.1
F17	Food	-	-	-	-	-	-	+	+	-	-	*Enterobacter cloacae*	99%	EF120473.1
F18	Food	+	-	-	-	-	-	+	+	-	-	*Enterobacter* sp.	99%	DQ855282.1

DNA sequencing[a]

Isolate number	Source	Phenotypic identification		Chromogenic media		E. sakazakii specific PCR primers						Identification	% Sequence similarity[b]	NCBI Accession number
		Yellow pigment	Oxidase test	DFI agar	ES agar	Esakf Esakr	Esak2 Esak3	SG-R SG-F	SI-F SI-R	Saka1 Saka2b	EsAgF EsAgR			
F19	Food	+	-	-	-	-	-	+	+	-	-	*Enterobacter* sp.	99%	DQ855282.1
F20	Food	+	-	-	-	-	-	+	+	-	-	*Enterobacter* sp.	99%	DQ855282.1
F21	Food	+	-	-	-	-	-	+	+	-	-	*Enterobacter* sp.	98%	AM396909.1
F22	Food	-	-	-	-	-	-	+	+	-	-	*Enterobacter* sp.	98%	DQ659161.1
F23	Food	-	-	-	-	-	-	+	+	-	-	*Enterobacter* sp.	98%	DQ659161.1
F24	Food	-	-	-	-	-	-	+	+	-	-	*Enterobacter* sp.	97%	AM184248.1
F25	Food	+	-	-	-	-	-	+	+	-	-	Uncultured *Pantoea* sp.	98%	EF072302.1

Group 8 (*n* = 25)

Isolate number	Source	Yellow pigment	Oxidase test	DFI agar	ES agar	Esakf Esakr	Esak2 Esak3	SG-R SG-F	SI-F SI-R	Saka1 Saka2b	EsAgF EsAgR	Identification	% Sequence similarity	NCBI Accession number
E11	Environ	-	-	-	-	-	-	-	+	-	-	*Enterobacter hormaechei*	99%	EF059890.1
E12	Environ	+	-	+	+	-	-	-	+	-	-	*Enterobacter helviticus*	97%	DQ273683.1
F26	Food	-	-	-	-	-	-	-	+	-	-	*Enterobacter hormaechei*	98%	EF210100.1
F27	Food	-	-	-	-	-	-	-	+	-	-	*Enterobacter hormaechei*	98%	EF210100.1
F28	Food	-	-	-	-	-	-	-	+	-	-	*Enterobacter hormaechei*	98%	AY995561.1
F29	Food	-	-	-	-	-	-	-	+	-	-	*Enterobacter hormaechei*	98%	AY995561.1
F30	Food	-	-	-	-	-	-	-	+	-	-	*Enterobacter hormaechei* subsp. *steigerwaltii*	97%	AJ853890.1
F31	Food	-	-	-	-	-	-	-	+	-	-	*Enterobacter* sp.	98%	AM184248.1
F32	Food	-	-	-	-	-	-	-	+	-	-	*Enterobacter* sp.	98%	AY297788.1
F33	Food	+	-	-	-	-	-	-	+	-	-	*Enterobacter* sp.	96%	AM184248.1
F34	Food	-	-	-	-	-	-	-	+	-	-	*Enterobacter* sp.	98%	AM184248.1
F35	Food	-	-	-	-	-	-	-	+	-	-	*Enterobacter* sp.	98%	AM184248.1

DNA sequencing[a]

Isolate number	Source	Phenotypic identification		Chromogenic media		E. sakazakii specific PCR primers						Identification	% Sequence similarity[b]	NCBI Accession number
		Yellow pigment	Oxidase test	DFI agar	ES agar	Esakf Esakr	Esak2 Esak3	SG-R SG-F	SI-F SI-R	Saka1 Saka2b	EsAgF EsAgR			
F36	Food	-	-	-	-	-	-	-	+	-	-	*Enterobacter* sp.	98%	AM184248.1
F37	Food	+	-	-	-	-	-	-	+	-	-	*Enterobacter* sp.	98%	AM184248.1
F38	Food	-	-	-	-	-	-	-	+	-	-	*Enterobacter* sp.	97%	AM184248.1
F39	Food	-	-	-	-	-	-	-	+	-	-	*Erwinia* sp.	98%	AY660693.1
F40	Food	-	-	-	-	-	-	-	+	-	-	*Erwinia* sp.	99%	EF088378.1
F41	Food	-	-	-	-	-	-	-	+	-	-	*Erwinia soli*	99%	EF540893.1
F42	Food	-	-	-	-	-	-	-	+	-	-	*Erwinia soli*	98%	EF540893.1
F43	Food	+	-	-	-	-	-	-	+	-	-	*Pantoea ananatis*	99%	DQ133545.1
F44	Food	+	-	-	-	-	-	-	+	-	-	*Pantoea ananatis*	99%	DQ133545.1
F45	Food	+	-	-	-	-	-	-	+	-	-	*Pantoea ananatis*	99%	DQ133545.1
F46	Food	+	-	-	-	-	-	-	+	-	-	*Pantoea* sp.	97%	DQ413253.1
F47	Food	-	-	-	-	-	-	-	+	-	-	*Stenotrophomonas maltophilia*	100%	AJ293472.1
F48	Food	-	-	-	-	-	-	-	+	-	-	*Stenotrophomonas maltophilia*	100%	AJ293472.1
Group 9 (*n* = 4)														
E13	Environ	-	-	-	-	-	-	-	-	-	-	*Enterobacter* sp.	98%	DQ659161.1
E14	Environ	-	-	-	-	-	-	-	-	-	-	*Enterobacter* sp.	97%	AB114268.1
F49	Food	-	-	-	-	-	-	-	-	-	-	*Enterobacter ludwigii*	97%	AM184235.1
F50	Food	-	-	-	-	-	-	-	-	-	-	*Erwinia* sp.	99%	AY660693.1

[a]Based on DNA sequencing of a 1.5 kb fragment of the 16S rRNA gene.

[b]Percentage similarity of the isolate to the closest relative in the NCBI database.

Environ - Environmental samples.

Table 4 Evaluation of various *Enterobacter sakazakii* detection methods compared with 16S rDNA sequencing results

	Detection method evaluated	*E. sakazakii* positive	*E. sakazakii* negative	Sensitivity	Specificity	Accuracy
	16S rDNA sequencing	15	51			
Phenotypic identification	**Yellow pigment**	28	38	87%	71%	74%
Selective media	**DFI agar**	16	50	100%	98%	98%
	ES agar	16	50	100%	98%	98%
PCR detection primer pairs	**Esakf/Esakr**	20	46	100%	90%	92%
	Esak2/Esak3	16	50	87%	94%	92%
	SG-F/SG-R	34	32	100%	63%	71%
	SI-F/SI-R	62	4	100%	8%	29%
	Saka1/saka2b	21	45	100%	88%	91%
	EsAgf/EsAgr	24	42	100%	82%	86%
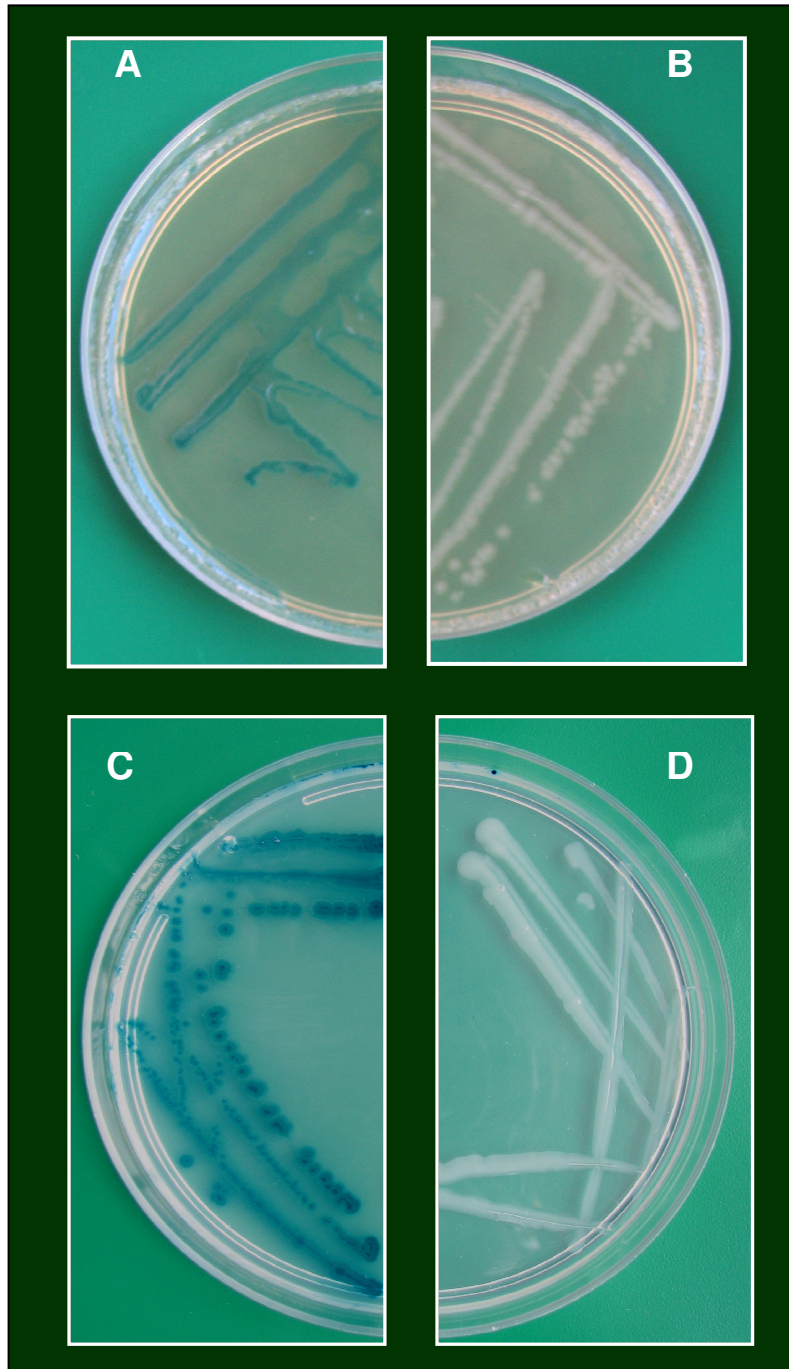
Figure 2 Detection and identification of *E. sakazakii* with selective chromogenic DFI and ES media: A - blue-green colonies typical of *E. sakazakii* on DFI agar; B - white colonies non-typical of *E. sakazakii* on DFI agar; C - turquoise colonies typical of *E. sakazakii* on ES agar; and D - white colonies non-typical of *E. sakazakii* on ES agar.

DFI agar (Iversen *et al.*, 2004a). In this study, however, all *Pantoea* species produced non-typical colonies on both DFI and ES media and thus were correctly identified as non-*E. sakazakii* strains.

The accuracy of both DFI agar and ES agar was 98% for the detection of *E. sakazakii*. The accuracy of the chromogenic media for detection and identification of *E. sakazakii* surpassed the accuracy of all other detection methods evaluated in this study. Thus, the growth of colonies characteristic of *E. sakazakii* on either DFI agar or ES agar appears to be the most suitable method for the detection and identification of *E. sakazakii*. However, due to the fact that these media were not 100% accurate, and that other researchers have reported slightly lower specificity results with chromogenic media (Iversen *et al.*, 2004a; Lehner *et al.*, 2006), it is recommended that these media are not used as the only means of identifying *E. sakazakii*.

Species-specific PCR

All six of the species-specific PCR systems evaluated in this study were demonstrated to be highly sensitive, but the specificity of the systems varied greatly (Table 4). Table 3, in which isolates are grouped according to the number of positive PCR results, clearly shows the inconsistencies that existed between the results obtained with different species-specific primer pairs. In comparison to 13 of the 64 isolates being identified as *E. sakazakii* by 16S rDNA sequencing, PCR detected 16 (Esak2/Esak3), 20 (Esakf/Esakr), 21 (Saka1/Saka2b), 24 (EsAgf/EsAgr), 34 (SG-F/SG-R) and 62 (SI-F/SI-R) of the 64 isolates as *E. sakazakii* (Table 3). The variation in the results obtained with the different primer pairs indicate that some of the primers cited in the literature are neither specific, nor suitable for *E. sakazakii* detection and identification.

With the exception of the primer pair Esak2/Esak3 (Keyser *et al.*, 2003), which demonstrated 87% sensitivity for the detection of *E. sakazakii*, all the other primer pairs evaluated (Esakf/Esakr, SG-F/SG-R, SI-F/SI-R, Saka1/Saka2b and EsAgf/EsAgr) had a sensitivity of 100%. The latter five primer pairs detected all of the 15 *E. sakazakii* isolates (including the two controls) (Table 3). The sensitivity of the primer pair Esak2/Esak3 (87%) (Table 4) was lowered due to the failure of these primers to produce positive detection results for two (F09 and F10) of the 15 *E. sakazakii* isolates (Table 3). F09 and F10 were isolated from IFM powder, and were both confirmed to be *E. sakazakii* (97% DNA

sequence similarity, NCBI accession number AM075208.1). The lack of sensitivity of the primers Esak2/Esak3 has previously been described by Lehner *et al.* (2004), who reported that they failed to detect one of 48 target *E. sakazakii* strains (ATCC 51329). This PCR system was the first published PCR system for detection of *E. sakazakii* and was based on the single *E. sakazakii* 16S rRNA gene sequence that was available in the NCBI database at the time. Since the development of Esak2/Esak3, a number of additional *E. sakazakii* 16S rRNA gene sequences have been added to the NCBI database, and it has been demonstrated that *E. sakazakii* is a diverse and genetically complex species.

Despite the fact that Esak2/Esak3 had the lowest sensitivity of the primers evaluated, this pair was demonstrated to be the most specific for *E. sakazakii* detection (94%) (Table 4). This indicates that, of the six *E. sakazakii* primer pairs evaluated, Esak2/Esak3 produced the lowest number of false-positive results. The three isolates that were incorrectly identified as *E. sakazakii* with Esak2/Esak3 (F02, F04 and F08) (Table 3) were most closely related to an *Enterobacter* sp. (NCBI Accession number AY689062.1), an uncultured *Pantoea* sp. (NCBI Accession number EF514909.1) and an *Acinetobacter* sp. (NCBI Accession number EF072302.1), respectively.

The species-specific primers Esakf/Esakr was shown to be 90% specific for *E. sakazakii* detection (Table 4). These primers produced false-positive results for five of the food isolates (F02, F04, F08, F12 and F15) (Table 3), which were confirmed to be most closely related to an *Enterobacter* sp. (NCBI Accession number AY689062.1), an uncultured *Pantoea* sp. (NCBI Accession number EF514909.1), an *Acinetobacter* sp. (NCBI Accession number EF072302.1), *Enterobacter cloacae* (NCBI Accession number AJ417459.1) and an *Enterobacter* sp. (NCBI Accession number EF419181.1), respectively.

The two species-specific primer pairs Saka1/Saka2b and EsAgf/EsAgr, were 88% and 82% specific for *E. sakazakii*, respectively (Table 4). Both primer pairs produced false-positive results for the isolates F02, F04, F08, E07, F11 and F12 (Table 3), which were identified as being most similar to an *Enterobacter* sp. (NCBI Accession number AY689062.1), an uncultured *Pantoea* sp. (NCBI Accession number EF514909.1), an *Acinetobacter* sp. (NCBI Accession number EF072302.1), an *Enterobacter* sp. (NCBI Accession number EF471901.1), *Pseudomonas fulva* (NCBI Accession number AM184228.1) and *Enterobacter cloacae* (NCBI Accession number AJ417459.1), respectively. In addition, the primer pair EsAgf/EsAgr gave false-positive results for the

isolates F13, F14 and F15 (Table 3), which were identified as being most similar to *Pseudomonas fulva* (NCBI Accession number EF204241.1), *Pantoea agglomerans* (NCBI Accession number AF130953.1) and an *Enterobacter* sp. (NCBI Accession number EF419181.1).

The species-specific primer pairs SG-F/SG-R and SI-F/SI-R showed the lowest specificity (63% and 8%, respectively) of the six primer pairs evaluated for the detection of *E. sakazakii* (Table 4). The primers SG-F/SG-R were most prone to producing false-positive results for members of the genera *Enterobacter*, *Pseudomonas* and *Pantoea* (Table 3). The primers SI-F/SI-R produced false-positive results for 47 of the 51 (92%) non-*E. sakazakii* isolates (Table 3). Both SG-F/SG-R and SI-F/SI-R were designed based on the sequencing and alignment of the 16S–23S rDNA internal transcribed spacer (ITS) of *E. sakazakii* (Liu *et al*., 2006). After evaluation on 23 *E. sakazakii* strains and 65 non-*E. sakazakii* strains, these primers were reported to be specific for the *E. sakazakii* detection. In this study, however, SG-F/SG-R and SI-F/SI-R were shown to be unsuitable for the specific detection of *E. sakazakii* due to the lack of specificity demonstrated by both of these primer pairs.

Overall, the two primer pairs that were established to be the most accurate for the detection of *E. sakazakii* were Esak2/Esak3 and Esakf/Esakr. Although both primer pairs demonstrated 90% accuracy (Table 4) for *E. sakazakii* detection, Esak2/Esak3 was more specific for *E. sakazakii* (94% specificity) than Esakf/Esakr (90% specificity) (Table 3). However, Esakf/Esakr was more sensitive for *E. sakazakii* detection (100% sensitivity) than Esak2/Esak3 (87% sensitivity) (Table 4). Due to the severe health threats that even low *E. sakazakii* cell concentrations pose to vulnerable individuals, it is imperative that detection methods for *E. sakazakii* do not produce false-negative results (indicated by a low sensitivity). There is a risk that if *E. sakazakii* remains undetected in IFM products due to the low sensitivity of a detection method, that the contaminated product may be distributed and consumed. For this reason, it is proposed that the primer pair Esakf/Esakr is more suitable for *E. sakazakii* detection than Esak2/Esak3.

Since foodborne pathogens such as *E. sakazakii* are often present in food products at low cell numbers, it is important that the systems designed for the isolation and identification of these pathogens have the lowest possible detection limits. The primer pair SI-F/SI-R had the lowest detection limit of the six primer pairs evaluated, giving positive results for cell concentrations as low as 10 cfu.ml^{-1}. However, given the low specificity of

this primer set, as well as the fact that this detection limit was considerably lower than the detection limit of 10^3 cfu.ml^{-1} normally associated with PCR systems (Olsen *et al.*, 1995), these results were suspected to be invalid. The detection limits of Esakf/Esakr and Esak2/Esak3, the two primer pairs demonstrated to be the most accurate for *E. sakazakii* detection, were 10^4 cfu.ml^{-1} and 10^6 cfu.ml^{-1}, respectively. The lower detection limit of Esakf/Esakr supports the former consensus that that this primer pair is more suitable for *E. sakazakii* detection than Esak2/Esak3. The detection limits of the primer pairs SG-F/SG-R, Saka1/Saka2b and EsAgf/EsAgr were found to be 10^6 cfu.ml^{-1}, 10^4 cfu.ml^{-1}, and 10^2 cfu.ml^{-1}, respectively. However, given that these primers were demonstrated to be less accurate than Esakf/Esakr, the latter primers were considered to be more suitable for *E. sakazakii* detection than the former primers.

Conclusions

The results of this study indicated inconsistencies between the results obtained using different *E. sakazakii* detection methods. The current FDA method for *E. sakazakii* detection not only proved to be a lengthy procedure, but this method was not sufficiently accurate to ensure the detection and correct identification of *E. sakazakii*. The use of the oxidase test to eliminate oxidase positive micro-organisms was time consuming and labour intensive, and this method had little bearing on identifying the potential pathogens. Furthermore, the sensitivity, specificity and accuracy of the FDA method were demonstrated to be lowered by the use of yellow pigment production as an identification criterion for *E. sakazakii*. The current FDA method thus needs to be revised and alternative identification methods need to be incorporated that allow more rapid and specific detection of *E. sakazakii*.

In this study, DFI and ES agar were identified as highly sensitive, specific and accurate means for detecting and identifying *E. sakazakii* isolated from both food and environmental samples. These media could be used as a first-step approach for detecting *E. sakazakii* colonies after enrichment. The large degree of variation in the specificity of the PCR primers evaluated emphasised the need for PCR applications to be rigorously tested against closely related species to validate the methods. Based on the results of this study, Esakf/Esakr were the most suitable for *E. sakazakii* detection and identification, with an overall accuracy of 92%. rDNA sequencing and/or the use of species-specific PCR

with Esakf/Esakr would prove useful as a means of confirming the identification of presumptive *E. sakazakii* colonies obtained on DFI and ES media. Overall, it was demonstrated that selective chromogenic media, as well as molecular methods such as DNA sequencing and PCR, should have an important place in methods recommended for the detection and identification of *E. sakazakii.*

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CHAPTER 4

NOVEL PCR DETECTION OF VIABLE *ENTEROBACTER SAKAZAKII* CELLS UTILISING PROPIDIUM MONOAZIDE AND ETHIDIUM BROMIDE MONOAZIDE

Abstract

Due to the persistence of DNA in the environment after bacterial cell death, DNA-based detection methods such as the polymerase chain reaction (PCR) are unable to distinguish between positive PCR signals arising from viable and dead bacterial targets. In order to ensure that PCR detection systems for foodborne pathogens are reliable, the discrimination between viable and dead bacteria is crucial. The detection of the emerging pathogen *Enterobacter sakazakii* is important due to its association with sporadic outbreaks of life-threatening infections, especially in low-birth weight, immuno-compromised infants. The aim of this study was to optimise a method to selectively detect only viable *E. sakazakii* cells in the presence of dead cells, utilising the DNA-intercalating dyes propidium monoazide (PMA) or ethidium bromide monazide (EMA) in combination with PCR. With this method, the basis for differentiation between viable and dead cells relies on the ability of these dyes to penetrate the compromised membranes of dead cells, while being unable to penetrate the intact membranes of viable cells. On penetration of the dead cells, EMA and PMA can be covalently linked to the bacterial DNA by photo-activation, resulting in the prevention of DNA amplification during the subsequent PCR reaction. At concentrations of 50 and 100 μg.ml^{-1}, PMA completely inhibited DNA amplification from dead cells, while causing no significant inhibition of the amplification from viable cells. PMA was also shown to be effective in allowing selective PCR detection of only viable cells in mixtures of varying ratios of viable and dead cells. EMA was equally effective in preventing amplification from dead cells, however, it also inhibited DNA amplification from viable cells. This study demonstrated the efficiency of PMA for viable/dead differentiation of *E. sakazakii*, as well as the lack of selectivity of EMA for this purpose. The PMA-PCR, in particular, will be useful for monitoring the resistance, survival strategies and stress responses of *E. sakazakii* in foods and the environment.

Introduction

The accurate detection of the emerging pathogen *Enterobacter sakazakii* is of importance to public health, due to its association with sporadic outbreaks of sepsis, meningitis and necrotising enterocolitis (Nazarowec-White & Farber 1997; Bar-Oz *et al.*, 2001; van Acker *et al.*, 2001). Of particular concern is that the population groups that are at the highest risk from *E. sakazakii* infections are low-birth weight or immuno-compromised neonates and infants (Forsythe, 2005), who are so susceptible to infections that even low concentrations of this bacterium may cause fatalities (Liu *et al.*, 2006). *Enterobacter sakazakii* has been detected in various food products and food production facilities (Postupa & Aldová, 1984; Soriano *et al.*, 2001; Kandhai *et al.*, 2004). However, to date, powdered infant formula milk (IFM) is the only food that has been epidemiologically linked to outbreaks of disease.

Since the discovery of the polymerase chain reaction (PCR) (Mullis *et al.*, 1986; Mullis & Faloona, 1987), there has been a progressive increase in the application of the technique for the detection of specific bacterial pathogens within mixed microbial populations (Nogva *et al.*, 2000). The attractiveness of PCR-based techniques includes their enhanced specificity and sensitivity over traditional culture techniques, as well as their ability to obtain results more rapidly (Olsen *et al.*, 1995). In spite of these advantages, the major disadvantage of conventional PCR is that it does not provide information related to the viability of the detected bacteria. In fact, the DNA derived from cells destroyed by heat, disinfectants and antibiotics may serve as a template for PCR amplification for many days following the loss of viability (Chaiyanan *et al.*, 2001; Nogva *et al.*, 2003; Rudi *et al.*, 2005). The possibility of false-positive PCR results limits the application of PCR for microbiological monitoring of foods (Wang & Levin, 2006).

Most food decontamination and preservation treatments are aimed at complete removal or inactivation of potential pathogens (Bolder, 1997). Since conventional PCR-based methodologies are not suitable for bacterial viability assessment, they cannot be used to assess the effectiveness of inactivation techniques. To circumvent this, a novel alternative has recently been developed, which allows selective detection of only viable bacteria in a sample. The novelty of the method lies in the use of specific DNA-intercalating dyes, capable of selectively modifying the DNA present in dead cells upon penetration through their compromised cell membranes. Since the dyes are unable to penetrate the intact membranes of viable cells, they cannot intercalate with the DNA from

these cells. Consequently, only DNA from viable cells is PCR-amplified and detected (Nogva *et al*., 2003). Although selective analysis of DNA from viable cells was first described by Nogva *et al*. (2003) for ethidium bromide monoazide (EMA), Nocker *et al*. (2006) recently reported a similar technique using propidium monoazide (PMA) as the DNA-intercalating dye. The aim of this study was to optimise and compare the PMA-PCR and EMA-PCR methods for selective detection of viable *E. sakazakii* cells in the presence of dead cells.

Materials and methods

Strain and culture conditions

Enterobacter sakazakii (1039, Department of Food Science Culture Collection, Stellenbosch University) was isolated from a food processing environment (Keyser *et al*., 2003). The strain was stored in 20% (v/v) glycerol (Sigma-Aldrich, Gauteng, South Africa) at -20°C prior to use. It was subsequently streaked onto tryptone soy agar (TSA) (Oxoid, supplied by C.A. Milsch, Cape Town, South Africa) and was incubated at 25°C for 24 h. Single colonies from the TSA plates were transferred to 10 ml tryptone soy broth (TSB) (Oxoid), and the cells were grown overnight at 25°C. The purity of the strain was confirmed after Gram-staining and microscopic examination.

Enterobacter sakazakii cell concentrations were determined spectrophotometrically. A 1 ml aliquot of the overnight cell suspension was centrifuged to harvest the cells and the pellet was re-suspended in 1.5% (m/v) NaCl (Merck, Cape Town, South Africa) (Wang & Levin, 2006). A dilution series (10^{-1} to 10^{-8}) of the re-suspended cell suspension was prepared in 1.5% (m/v) NaCl (Merck), and the optical density of each dilution was determined with a spectrophotometer (Beckman Coulter DU530, Beckman Instruments, Fullerton, USA) at 600 nm. Duplicate spread plates were prepared from the dilutions (10^{-1} to 10^{-8}) on TSA, followed by incubation at 25°C for 48 h. A standard curve was constructed of the cell counts (cfu.ml^{-1}) against the corresponding optical density values.

Conditions causing cell death

Two strategies were followed to kill cells prior to PMA- or EMA-PCR. Tubes containing 250 µl of a 10^7 cfu.ml^{-1} cell suspension were heated at 100°C in a waterbath for 5 min and

then placed on ice. Alternatively, 250 µl aliquots of a 10^7 cfu.ml^{-1} cell suspension were centrifuged and the cells were re-suspended in 70% (v/v) isopropanol (Merck) for 10 min (Nocker & Camper, 2006). The isopropanol was removed by harvesting the cells by centrifugation prior to re-suspension in 250 µl 1.5% (m/v) NaCl (Merck). The absence of viable cells was confirmed by spread plating 100 µl aliquots of heat- or isopropanol-treated cells on TSA plates as described.

Optimisation of PMA and EMA treatments

PMA (Biotium, Inc., California, USA) or EMA (Sigma-Aldrich) was added at final concentrations of 100, 50 or 10 µg.ml^{-1} to sample tubes containing 250 µl of either viable, heat-killed or isopropanol-killed cells (all derived from a 10^7 cfu.ml^{-1} cell suspension). Samples were incubated in the dark for 5 min to allow penetration of PMA or EMA into dead cells. Photo-induced cross-linking of PMA and EMA was achieved by exposing the samples for 1 min to a 750 W halogen light bulb (Osram, supplied by Starlight Electrical, Cape Town, South Africa), positioned 20 cm from the sample tubes. Sample tubes were placed on ice to avoid excessive heating. Each sample treatment was repeated in triplicate to ensure the reproducibility of the results. Control samples used in the study were 250 µl of viable, heat- and isopropanol-killed cells (all derived from a 10^7 cfu.ml^{-1} cell suspension) that were not exposed to treatment with PMA or EMA.

Mixtures of viable and dead cells

In order to determine the effectiveness of PMA and EMA in selectively allowing DNA amplification from viable cells when in the presence of dead cells, mixtures of viable and dead cells were evaluated. Following the heat-killing of a 10^7 cfu.ml^{-1} suspension of *E. sakazakii* as described, the dead cells were mixed with viable cells (10^7 cfu.ml^{-1}) in defined ratios. Mixtures were prepared such that viable cells represented 0, 25, 50, 75 or 100% of the total bacterial cell concentration. PMA and EMA treatments were carried out in triplicate on 250 µl of the cell mixtures using 100 µg.ml^{-1} PMA or EMA, 5 min penetration in the dark and 1 min light exposure. As controls, mixtures containing viable cells at 0, 25, 50, 75 or 100% of the total bacterial cell concentration were utilised without any PMA or EMA treatment.

DNA isolation

DNA was isolated from all PMA- and EMA-treated samples and from the controls according to the method of Wang and Levin (2006). Following light exposure, all PMA- and EMA-treated samples were centrifuged at 8000 g for 5 min (Eppendorf Centrifuge 5415D, Hamburg, Germany) and the pellets were washed with 250 µl 1.5% (m/v) NaCl (Merck) and then with 250 µl distilled water. Pellets were re-suspended in 250 µl distilled water. Control samples were also centrifuged to harvest the cells and the pellets were re-suspended in 250 µl distilled water. All samples were mixed with 250 µl of double-strength TZ (2 x TZ) (Abolmatty *et al.*, 2000), which consisted of 4% (v/v) Triton X-100 (Merck) and 5 mg.ml^{-1} sodium azide (Merck) in 0.1 M Tris–HCl (Fluka, supplied by Sigma-Aldrich) at pH 8.0. Tubes were heated in a boiling waterbath for 10 min to lyse the cells. Cell debris were pelleted by centrifugation at 10 000 g for 5 min and 200 µl of the crude DNA from each sample was purified using a Micropure-EZ column (Millipore, supplied by Microsep, Cape Town, South Africa).

PCR amplification

The *E. sakazakii*-specific primers Esakf (5' GCT YTG CTG ACG AGT GGC GG 3') and Esakr (5' ATC TCT GCA GGA TTC TCT GG 3') were utilised to amplify a fragment of the 16S ribosomal RNA (rRNA) gene of *E. sakazakii* (Lehner *et al.*, 2004). PCR amplification was performed in a total volume of 50 µl containing 1 µl (200 nM) of each primer (10 µM), 0.70 µl (3.5 U) *Taq* DNA polymerase (5 U.µl^{-1}, Super-Therm, supplied by Southern Cross Biotechnologies, Cape Town, South Africa), 5 µl 10 x reaction buffer (MgCl$_2$ free) (Super-Therm), 3 µl (1.5 mM) MgCl$_2$ (25 mM, Super-Therm), 2 µl (0.4 mM) of dNTPs (10 mM, AB gene, supplied by Southern Cross Biotechnologies), 2 µl 99% (v/v) dimethyl sulphoxide (DMSO) (Merck) and 4 µl of DNA template. Thermal cycling was carried out as follows: initial denaturation at 94°C for 2 min; followed by 29 cycles of denaturation at 94°C for 30 sec; primer annealing at 66°C for 1 min; and chain elongation at 72°C for 90 sec. Final elongation was performed at 72°C for 5 min. The PCR products were separated on a 1.5% (m/v) agarose gel, containing 0.02 µl.ml^{-1} ethidium bromide, in 0.5 x TBE electrophoresis buffer. The separated DNA fragments were visualised under an ultraviolet transilluminator (Vilber Lourmat, Marne La Vallee, France).

PCR product concentration and yield

PCR products were purified using Sigma Spin Post-Reaction Purification Columns (Sigma-Aldrich) according to the manufacturer's instructions. The concentration of the PCR products was fluorometrically determined using a DyNA Quant 200 fluorometer (Hoefer Pharmacia Biotech, San Francisco, USA). The fluorometer was calibrated with a standard 100 ng.ml^{-1} DNA solution, which was prepared by adding 2 µl of calf thymus DNA (100 µg.ml^{-1}, Sigma-Aldrich) to 2 ml assay solution (1 µg.ml^{-1} Hoechst 33258 dye (Sigma-Aldrich) in 1 x TNE buffer (0.2 M NaCl (Merck), 10mM Tris-HCl (Fluka), 1 mM EDTA (Merck), pH 7.4)). The DNA concentrations were determined by placing a cuvette containing 2 µl sample and 2 ml assay solution into the fluorometer, and recording the DNA concentrations.

In order to compare the effect that PMA and EMA treatments had on the concentration of the PCR product amplified from viable and dead cells, the PCR product concentration of each sample was compared to the PCR product concentration of a viable, untreated sample expected to contain the highest PCR product concentration (Nocker *et al.*, 2006). The PCR product yield (%) was calculated as follows: (PCR product concentration of sample in ng.ml^{-1} / PCR product concentration of viable, untreated control in ng.ml^{-1}) x 100.

Statistical analysis

All statistical analyses were performed using Statistica™ 7.1 (StatSoft, Inc., 2006). For optimisation of the PMA and EMA treatments on either 100% viable or 100% dead *E. sakazakii* cells, a three-way cross-classification analysis of variance (ANOVA) was performed. The factors were the treatment used (PMA, EMA or control receiving no treatment), the viability of the cells exposed to treatment (viable, heat-killed or isopropanol-killed) and the concentration of the PMA or EMA used for treatment (100, 50, 10 or 0 µg.ml^{-1}). Since the interaction of the PMA or EMA concentration with the other factors was deemed non-significant ($P > 0.05$), the statistical analysis was repeated using a two-way ANOVA, with factors being the treatment used and the viability of the cells. The relationship between the percentage of viable cells and the PCR product yields was investigated using a two-way ANOVA, with the factors being the percentage of viable and heat-killed cells and the treatment used. Differences were considered statistically

significant at a level of 5% ($P < 0.05$). Where interactions were non-significant ($P > 0.05$), main effects were interpreted directly. A Bonferroni multiple comparisons procedure was used when interactions were significant in order to interpret which interaction effects differed. Where residuals were not normally distributed, the interactions were further analysed using the non-parametric Bootstrap multiple comparisons procedure (Efron & Tibshirani, 1993).

Results and discussion

Optimisation of PMA and EMA treatments

Control samples

The PCR products (929 base pair (bp) in size) that were amplified from the control DNA samples, isolated from either 100% viable or 100% dead (heat- and isopropanol-killed) cells, are presented in Fig. 1A (lanes 1-3). Since these control samples received no PMA or EMA treatment, the results represent conventional PCR amplification. The amplification of prominent bands from the viable *E. sakazakii* cells (lane 1), as well as from the heat-killed (lane 2) and isopropanol-killed (lane 3) cells, indicate that conventional PCR systems amplify DNA from both viable and dead cells.

The PCR product yields obtained from the viable, heat- and isopropanol-killed control samples were 100%, 92% and 83%, respectively. Statistically, the yields from all three control samples differed significantly ($P < 0.05$) from one another (indicated by the subscripts a, b and c, Fig. 1B). Therefore, even when no PMA or EMA treatment was applied, the killing method alone led to a significant reduction in the PCR product yield in comparison to the yield from viable cells. The PCR product yield was significantly ($P < 0.05$) lower when isopropanol was used to kill the cells in comparison to when heating was used (Fig. 1B, lane 2 and 3, subscripts b and c). The significant difference in the PCR product yields between the two dead controls may have originated during the cell-killing treatments. A greater proportion of genomic DNA could have been destroyed during exposure of *E. sakazakii* cells to isopropanol than was destroyed during exposure to heat, providing less DNA template for PCR amplification. In spite of the significant differences between the PCR product yields from viable and dead controls, it was observed that PCR product yields of greater than 80% were obtained from both of the dead controls (Fig. 1B,

lanes 2 and 3) compared to the viable cell control (Fig. 1B, lane 1), for which the PCR product yield was defined as 100%. This demonstrates that a substantial amount of DNA must have persisted after the heat- or isopropanol-induced death of *E. sakazakii* cells, which served as a template for PCR amplification. It is the amplification of this persisting DNA from dead cells that may lead to false-positive conventional PCR results.

PMA and EMA treated samples

Successful amplification products were obtained from all PCR reactions containing DNA from 100% viable cells that were treated with PMA and EMA (Fig. 1A, lanes 4, 7, 10, 13, 16, 19). Since PMA and EMA are unable to penetrate the intact cell membrane of viable cells (Rudi *et al.*, 2005; Nocker *et al.*, 2006; Wang & Levin, 2006), successful PCR amplification products were expected from samples containing viable cells, regardless of whether they were untreated or treated with the intercalating dyes.   PMA and EMA can, however, penetrate the compromised cell membranes of dead cells. Thus, as expected, both PMA and EMA treatments inhibited DNA amplification from dead cells (Fig. 1A), independent of the killing method employed or the PMA and EMA concentrations added.

The PCR product yields obtained from the 100% viable cells (Fig. 1B, lanes marked V) were higher than those obtained from the dead cells (Fig. 1B, lanes marked H and I) at all concentrations (100, 50 or 10 $\mu g.ml^{-1}$) of PMA or EMA. When 100% viable cells were treated with 100, 50 and 10 $\mu g.ml^{-1}$ PMA (Fig. 1B, lanes 4, 7, 10) the PCR product yields were 95%, 96% and 97%, respectively.   These PCR product yields did not differ significantly ($P > 0.05$) from the yield obtained from the 100% viable control that received no PMA or EMA treatment (defined as a PCR product yield of 100%) (Fig. 1B, lane 1).

Treatment of 100% viable cells with 100, 50 and 10 $\mu g.ml^{-1}$ EMA (Fig. 1B, lanes 13, 16, 19) resulted in PCR product yields of 74%, 82% and 92%, respectively. In contrast with PMA, the PCR product yields from the 100% viable cells treated with EMA at all three concentrations were significantly ($P < 0.05$) lower than that of the 100% viable control (Fig. 1B, lane 1). The observation that the PCR product yields from the 100% viable cells treated with EMA (Fig. 1B, lanes 13, 16, 19) were significantly ($P < 0.05$) lower than those from the PMA-treated 100% viable cells (Fig. 1B, lanes 4, 7, 10) suggests that the intact membrane of viable *E. sakazakii* cells can effectively exclude PMA, while EMA appears to be capable of penetrating these cells. This explains why less PCR product was amplified

from viable cells after EMA treatment in comparison to after PMA treatment. For PMA- and EMA-PCR methods to be most effective, they should bring about complete inhibition of DNA amplification from dead cells, yet no significant inhibition of DNA amplification from viable cells. This study demonstrates that, in contrast to EMA, PMA does not significantly inhibit PCR amplification from viable *E. sakazakii* cells.

With PMA treatments of 100, 50 and 10 µg.ml^{-1}, the respective PCR product yields from 100% heat-killed cells (0%, 0% and 6%) (Fig. 1B, lanes 5, 8 and 11) and 100% isopropanol-killed cells (0%, 0% and 9%) (Fig. 1B, lanes 6, 9 and 12) were significantly ($P < 0.05$) lower than the yields from 100% viable cells (95%, 96% and 97%) receiving the same treatments (Fig. 1B, lanes 4, 7, 10). Similarly, treatments with EMA (100, 50 or 10 µg.ml^{-1}) also produced significantly ($P < 0.05$) lower PCR product yields from 100% heat-killed cells (0%, 0% and 5%) (Fig. 1B, lanes 14, 17 and 20) and isopropanol-killed cells (0%, 0% and 6%) (Fig. 1B, lanes 15, 18 and 21) than from 100% viable cells (95%, 96% and 97%) (Fig. 1B, lanes 4, 7, 10). These results illustrate the ability of PMA and EMA to differentiate between viable and dead *E. sakazakii* cells, and to prevent DNA amplification from dead cells.

Statistically, the aforementioned PCR product yields obtained from the 100% heat- and isopropanol-killed cells treated with PMA and EMA at all three concentrations (100, 50 and 10 µg.ml^{-1}) (Fig. 1B, lanes 5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 20 and 21) were significantly ($P < 0.05$) lower than the yields of the 100% heat- and isopropanol-killed controls (92% and 83%) receiving no PMA or EMA treatment (Fig. 1B, lanes 2 and 3). It is evident that, in comparison to the respective controls (which demonstrate the amplification of DNA that occurs from dead cells with conventional PCR), both PMA and EMA were capable of significantly reducing the PCR signal from dead *E. sakazakii* cells. This shows the potential of these DNA-intercalating dyes in PCR applications where detection of only viable bacteria is required.

At a given concentration of PMA or EMA (100 or 50 or 10 µg.ml^{-1}), the PCR product yields from the PMA- and EMA-treated heat-killed cells did not differ significantly ($P > 0.05$) from one another (Fig. 1B, comparing the PCR product yields of 0% and 0% in lanes 5 and 14; 0% and 0% in lanes 8 and 17; and 6% and 5% in lanes 11 and 20). Similar results were obtained with the PMA- and EMA-treated isopropanol-killed cells (Fig. 1B, comparing the PCR product yields of 0% and 0% in lanes 6 and 15; 0% and 0% in lanes 9 and 18; and 9% and 6% in lanes 12 and 21). Thus, both PMA and EMA

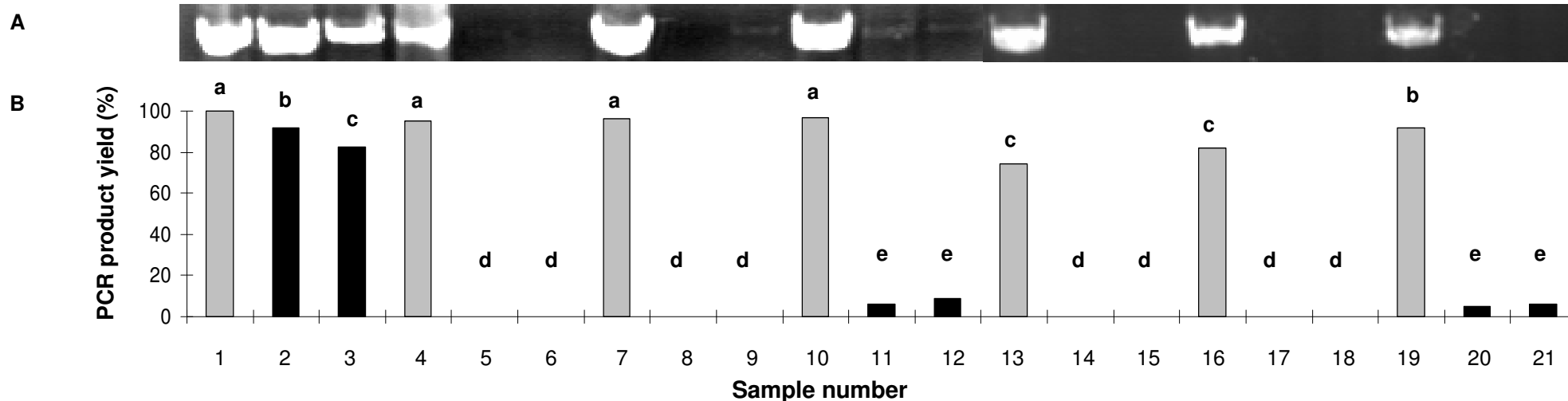|	Control			PMA									EMA								
Viable:dead cells	100:0	0:100	0:100	100:0	0:100	0:100	100:0	0:100	0:100	100:0	0:100	0:100	100:0	0:100	0:100	100:0	0:100	0:100	100:0	0:100	0:100
Viablity[1]	V	H	I	V	H	I	V	H	I	V	H	I	V	H	I	V	H	I	V	H	I
[PMA] in µg.ml^{-1}	0	0	0	100	100	100	50	50	50	10	10	10	0	0	0	0	0	0	0	0	0
[EMA] in µg.ml^{-1}	0	0	0	0	0	0	0	0	0	0	0	0	100	100	100	50	50	50	10	10	10

Figure 1 The effect of different concentrations of PMA and EMA on the PCR product yield obtained from viable (grey columns) and dead (heat- or isopropanol-killed) (black columns) *E. sakazakii* cells. (A) PCR products visualised on an agarose gel. (B) PCR product yields shown in relation to the yield obtained from the viable untreated control sample (Lane 1; PCR product yield defined as 100%). Lanes 1, 2, 3: control samples, receiving no EMA or PMA treatment; lanes 4, 5, 6: samples treated with 100 µg.ml^{-1} PMA; lanes 7, 8, 9: samples treated with 50 µg.ml^{-1} PMA; lanes 10, 11, 12: samples treated with 10 µg.ml^{-1} PMA; lanes 13, 14, 15: samples treated with 100 µg.ml^{-1} EMA; lanes 16, 17, 18: samples treated with 50 µg.ml^{-1} EMA; lanes 19, 20, 21: samples treated with 10 µg.ml^{-1} EMA. Statistically significant differences ($P < 0.05$) between PCR product yields are indicated by non-identical subscript letters, whereas non-significant differences ($P > 0.05$) are indicated by identical subscript letters.

[1]V = viable, H = heat-killed and I = isopropanol-killed.

significantly inhibited PCR amplification from heat- and isopropanol-killed cells. At the same PMA and EMA concentrations, there was with no statistical difference ($P < 0.05$) in the efficiency of the two dyes in inhibiting PCR amplification from dead cells.

The specific concentration of PMA and EMA, however, had a significant effect on the efficiency of the treatments in some cases. The PCR product yields of 0% from 100% dead cells treated with either 100 or 50 µg.ml^{-1} PMA (Fig. 1B, lanes 5, 6, 8, 9) or EMA (Fig. 1B, lanes 14, 15, 17, 18) were significantly ($P < 0.05$) lower than those from 100% dead cells treated with 10 µg.ml^{-1} PMA (6% and 9%) (Fig. 1B, lanes 11 and 12) or EMA (5% and 6%) (Fig. 1B, lanes 20 and 21). The percentage to which PCR amplification was inhibited by PMA and EMA treatments indicate that at concentrations of 100 or 50 µg.ml^{-1}, there was 100% inhibition of PCR amplification from heat- and isopropanol-killed cells by PMA (Fig. 1, lanes 5, 6, 8, 9) and EMA (Fig. 1B, lanes 14, 15, 17, 18). PMA and EMA treatments of 10 µg.ml^{-1} allowed PCR yields of up to 10% to be amplified from 100% dead cells (Fig. 1B, lanes 11, 12, 20, 21). This indicates that PMA or EMA concentrations of 100 or 50 µg.ml^{-1} were more successful in completely inhibiting PCR amplification from dead cells.

The results from the grouped analyses indicate that the most efficient overall treatment used for viable/dead discrimination of *E. sakazakii* was with PMA at a concentration of either 100 or 50 µg.ml^{-1}. Such a treatment would, according to the results of this study, not significantly inhibit DNA amplification from viable cells, while bringing about complete inhibition of DNA amplification from dead cells.

Effects of PMA and EMA on defined ratios of viable and dead cells

Controls

The PCR products (929 bp in size) amplified from the control cell mixtures are presented in Fig. 2A (lanes 1-5). When no PMA or EMA treatment was applied to the control cell mixtures containing viable cells at 0, 25, 50, 75 or 100% of the total cell concentration, there was no significant ($P > 0.05$) difference in the PCR product yields obtained (92, 94, 96, 97 and 100%, respectively) (Fig. 2B, lanes 1-5). Thus, the PCR product yields of all control samples exceeded the percentages of viable cells in the mixtures, demonstrating that amplification occurred from the DNA derived from the dead cells. The concentration of the PCR product obtained from the control sample containing a 100% dead cells and

that containing a 100% viable cells was not significantly different ($P > 0.05$), indicating that a large proportion of DNA can persist after cell death and serve as a template during PCR amplification.

PMA- and EMA-treated samples

The PCR product yields amplified from PMA- and EMA-treated cell mixtures are presented in Figure 2B (lanes 6-15). If PMA and EMA treatments were 100% effective in preventing DNA amplification from dead cells, it would be expected that the DNA yields would correspond directly with the percentage of viable cells in the sample. Thus, when viable cells constituted 0, 25, 50, 75 or 100% of the total cell concentration, PCR product yields of 0, 25, 50, 75 or 100%, respectively, would be expected. The PCR product yields for PMA-treated cell mixtures containing viable cells at 0, 25, 50, 75 and 100% of the total cell concentration were 0, 24, 51, 73 and 97% (Fig. 2B, lanes 6-10), respectively. Therefore, PMA treatments provided successful viable/dead differentiation, and accurately brought about selective amplification from the proportion of viable cells present. The corresponding PCR product yields from the EMA-treated cell mixtures were 0, 20, 39, 58, and 71%, respectively (Fig. 2A, lanes 11-15), which were considerably lower than expected. The consistently lower PCR product yields obtained from cell mixtures containing increasing numbers of viable cells is again indicative of the inhibitory effect that EMA had on PCR amplification from viable cells.

When cell mixtures contained 0% viable cells, there was a significant ($P > 0.05$) difference between the PCR product yields from the control sample receiving no treatment and the yields from the samples treated with PMA and EMA (Fig. 2B, comparing the PCR product yields of 92%, 0% and 0% in lanes 1, 6 and 11 respectively). Similarly, when cell mixtures contained 25% viable cells, there was a significant ($P > 0.05$) difference between the PCR product yields from the control sample and the yields from the samples treated with PMA and EMA (Fig. 2B, comparing the PCR product yields of 94%, 24% and 20% in lanes 2, 7 and 12 respectively). At these ratios of viable to dead cells (0:100 and 25:75), there was no significant ($P > 0.05$) difference between the PCR product yields obtained from the samples treated with PMA and those treated with EMA (Fig. 2B, comparing 0% and 0% in lanes 6 and 11; and comparing 24% and 20% in lanes 7 and 12). Thus, PMA

and EMA had similar efficiencies in preventing PCR amplification from dead cells when low concentrations of viable cells were present in the mixtures.

When cell mixtures contained 50% viable cells, there was a significant difference between the PCR product yields from the control samples (receiving no treatment) and the yields from the samples treated with PMA and EMA (Fig. 2B, comparing the PCR product yields of 96%, 51% and 39% in lanes 3, 8 and 13, respectively). Similarly, when cell mixtures contained 75% viable cells, there was a significant difference between the PCR product yields from the control samples and the yields from the samples treated with PMA and EMA (Fig. 2B, comparing the PCR product yields of 97%, 73% and 58% in lanes 4, 9 and 14, respectively). However, at these ratios of viable to dead cells (50:50 and 75:25), the PCR product yields obtained from PMA-treated cells were significantly ($P < 0.05$) higher than those obtained from EMA-treated cells (Fig. 2B, comparing 51% and 39% in lanes 8 and 13; and comparing 73% and 58% in lanes 9 and 14). When cell mixtures contained 100% viable cells, there was no significant difference ($P > 0.05$) between the PCR product yield of 100% from the control (Fig. 2B, lane 5) and the yield of 97% from the PMA-treated sample (Fig. 2B, lane 10). However, at this ratio of viable to dead cells (100:0), the PCR product yield of 71% from the EMA-treated sample (Fig. 2B, lane 15) was significantly ($P < 0.05$) lower than the yield of 100% from the control (Fig. 2B, lane 5) and the yield of 97% from the PMA-treated sample (Fig. 2B, lane 10).

Evaluation of the efficacy of PMA-PCR and EMA-PCR on mixtures of viable and dead cells demonstrated that PMA is effective in selectively allowing PCR amplification from viable cells when in the presence of DNA from dead cells. In contrast, EMA was shown to significantly inhibit PCR amplification from viable cells, as well as from dead cells. Although an intact cell membrane should be an effective barrier to EMA because of the charge of the molecule, various reports (Nocker & Camper, 2006; Nocker *et al.*, 2006) have also suggested that EMA may penetrate viable cells of some bacterial species. The first report of EMA inhibiting PCR amplification from viable cells was made for the bacterium *Anoxybacillus* (Rueckert *et al.*, 2005). Nocker *et al.* (2006) subsequently reported that although EMA and PMA were equally efficient in preventing PCR amplification from dead *Staphylococcus aureus*, *Listeria monocytogenes*, *Micrococcus luteus*, *Mycobacterium avium* and *Streptococcus sobrinus* cells, EMA also caused inhibition of PCR amplification from viable cells of these species. These studies indicate that EMA cannot be considered to be membrane impermeable for all bacterial species.

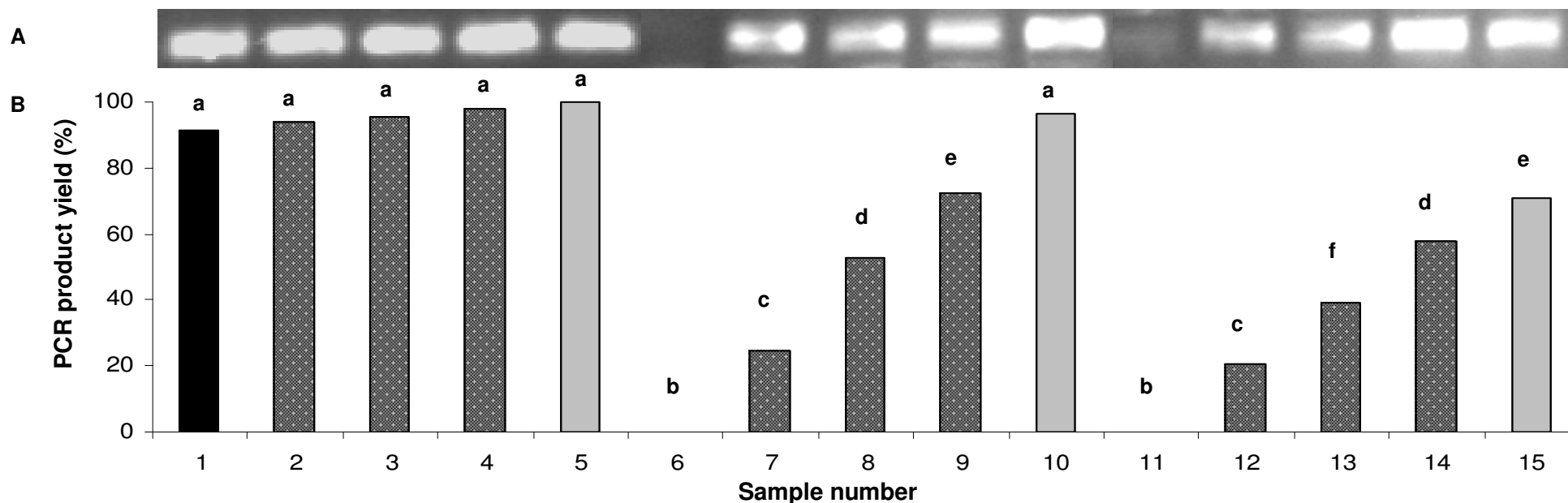|	Controls (No PMA or EMA treatment)					PMA (100 µg.ml^{-1})					EMA (100 µg.ml^{-1})				
Ratio viable: dead cells	0 : 100	25 : 75	50 : 50	75 : 25	100 : 0	0 : 100	25 : 75	50 : 50	75 : 25	100 : 0	0 : 100	25 : 75	50 : 50	75 : 25	100 : 0

Figure 2 The effect of no treatment and PMA and EMA treatments on PCR product yields of defined ratios of viable and heat-killed *E. sakazakii* cells. Samples containing only dead cells are indicated by black columns, those containing only viable cells are indicated by grey columns, and mixtures of dead and viable cells are indicated by dotted columns. (A) PCR amplification products from viable and dead cell mixtures visualised on an agarose gel. (B) PCR product yields of non-treated viable and dead cell mixtures (controls) shown in relation to the PCR product yields from PMA- and EMA-treated viable and dead cell mixtures. Statistically significant differences ($P < 0.05$) between PCR product yields are indicated by non-identical subscript letters, whereas non-significant differences ($P > 0.05$) are indicated by identical subscript letters.

PMA appears to have the advantage over EMA of not penetrating membranes of viable cells, yet effectively entering damaged or dead cells (Nocker & Camper, 2006). The enhanced selectivity of PMA is most likely due to the higher charge of the PMA molecule (PMA has two positive charges, while EMA has only one), and the greater impermeability through the intact cell membrane (Nocker *et al.*, 2006).

Conclusions

This is the first report on the development of a PCR-based technique for selectively amplifying a targeted DNA sequence from only viable *E. sakazakii* cells in the presence of dead cells. The ability to selectively amplify DNA from only viable and not dead cells shall significantly enhance the utility of PCR for rapidly determining and quantifying targeted viable micro-organisms of public health concern in food products, food processing facilities, and in environmental and clinical samples. This study demonstrated that the utility of EMA for viable/dead differentiation of *E. sakazakii* is hindered by the penetration of viable cells by the dye, and the resulting decreased PCR product yields. In contrast, the PMA-PCR method described appears highly effective for this purpose. The assays described in this study allow qualitative viable/dead differentiation in pure cultures of *E. sakazakii*. For future applications, however, the method should be adapted for quantitative viable/dead differentiation in mixed bacterial cultures by using quantitative real-time PCR. Such a method would aid in attaining a greater understanding of the resistance and survival strategies of *E. sakazakii* in foods and in the environment, as well as the stress responses of the pathogen to various inactivation and preservation treatments. This would be an important step in the efforts to eliminate it from food production environments.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

Enterobacter sakazakii is an emerging pathogen associated with life-threatening neonatal infections, predominantly through the consumption of contaminated powdered infant formula milk (IFM) (Nazorowec-White & Farber, 1997; Iversen & Forsythe, 2003). While *E. sakazakii* poses a considerable food safety threat worldwide, developing countries such as South Africa are often at the highest risk for foodborne infections (FAO/WHO, 2004). The risks posed by foodborne pathogens are increased by the fact that many individuals living in developing countries do not have access to adequate storage and refrigeration facilities and many are ignorant with regards to hygienic food handling practices. Furthermore, reduced immunity due to HIV-infections and malnutrition affect many individuals in developing countries, making them susceptible to foodborne infections (INFOSAN, 2005).

To date, there have been no official reports of *E. sakazakii* infections occurring in South Africa. However, considering the limitations of the current surveillance systems in most developing countries, official documentation may not always adequately reflect the true prevalence of foodborne diseases in these countries (FAO/WHO, 2004). Given the widespread use of IFM in South Africa and the considerable proportion of immuno-compromised individuals, the identification of *E. sakazakii* in South African IFM products (Witthuhn *et al.*, 2006) should warrant cause for concern.

At present, the monitoring of IFM products for the presence of *E. sakazakii*, and the assurance that these products are free of the pathogen, remains a great challenge for food manufacturers. This is largely due to the fact that there are no validated or official methods available for the detection of *E. sakazakii* (Nazorowec-White *et al.*, 2003). Furthermore, the prevailing microbiological food standards do not require the specific testing for *E. sakazakii*, nor do they specify allowable levels for the pathogen in food products (FAO/WHO, 2004; INFOSAN, 2005). The contradictory results obtained with different detection methods not only lead to financial losses to food manufacturers, but also lead to confusion regarding this pathogen. There is thus a need for an accurate, internationally validated method for the detection and identification of *E. sakazakii* in food products.

The current Food and Drug Administration (FDA, 2002) method for *E. sakazakii* detection requires revision, particularly with regard to the identification and confirmation of presumptive *E. sakazakii* isolates. This was demonstrated by the low sensitivity, specificity and overall accuracy of utilising yellow pigment production as a criterion for identification of *E. sakazakii*. More rapid, sensitive, specific and accurate selective chromogenic media have been developed for *E. sakazakii* detection (Iversen & Forsythe, 2004; Iversen *et al*., 2004), and their has recently been increased utilisation of molecular methods such as species-specific PCR and DNA sequencing for the identification of microbial pathogens (Olsen *et al*., 1995; Van Belkum *et al*., 2001)

Based on the results obtained in this study, a new method is proposed for the detection and identification of *E. sakazakii* in food and in environmental samples. In this proposed method (Fig. 1), the culturing steps on violet red bile glucose agar (VRBGA) and tryptone soy agar (TSA) used in the FDA method are replaced by culturing on selective chromogenic Druggan-Forsythe-Iversen (DFI) or *Enterobacter sakazakii* (ES) agar. For identification and confirmation of presumptive *E. sakazakii* isolates, it is proposed that the use of the oxidase test, yellow pigment production and API biochemical profiling be replaced by species-specific PCR and/or DNA sequencing. Species-specific PCR should be carried out with the primer pair Esakf and Esakr, which was demonstrated in this study to be the most suitable primer pair for the detection of *E. sakazakii*. This proposed method will not only reduce the time to detect *E. sakazakii* from approximately 7 days to 4 days, but it will also be more sensitive, specific and accurate than the current FDA method. With the availability of the more accurate method proposed for *E. sakazakii* detection, it is further recommended that specific microbiological standards for *E. sakazakii* be introduced into food regulations (Iversen & Forsythe, 2003). The recent introduction of microbiological standards for *E. sakazakii* in powdered IFM by the European Union (negative in 30 x 10g samples) (EC, 2005), could be useful in establishing similar specifications worldwide.

While the introduction of an accurate method for *E. sakazakii* detection will greatly aid in reducing the risks associated with the pathogen, efficient inactivation and preservation techniques are needed to reduce the prevalence of the bacterium in food products. In order to assess the effectiveness of such inactivation techniques, accurate methods are required to validate the killing effect of the treatments. The PMA-PCR methodology developed in this study will be particularly useful for assessing the efficacy of processing
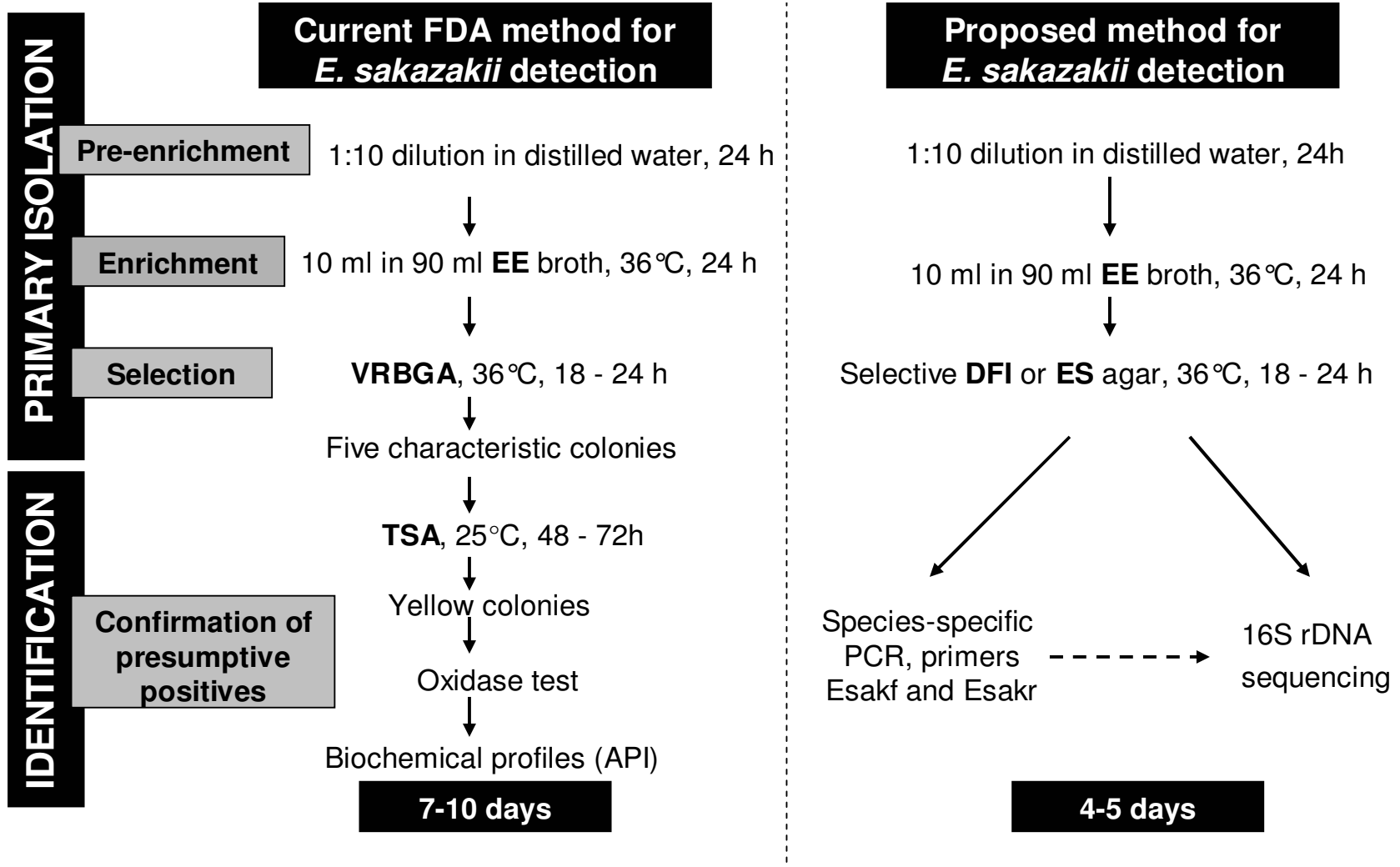
PRIMARY ISOLATION

IDENTIFICATION

Current FDA method for *E. sakazakii* detection

Pre-enrichment — 1:10 dilution in distilled water, 24 h

Enrichment — 10 ml in 90 ml **EE** broth, 36°C, 24 h

Selection — **VRBGA**, 36°C, 18 - 24 h

Five characteristic colonies

TSA, 25°C, 48 - 72h

Yellow colonies

Confirmation of presumptive positives — Oxidase test

Biochemical profiles (API)

7-10 days

Proposed method for *E. sakazakii* detection

1:10 dilution in distilled water, 24h

10 ml in 90 ml **EE** broth, 36°C, 24 h

Selective **DFI** or **ES** agar, 36°C, 18 - 24 h

Species-specific PCR, primers Esakf and Esakr

16S rDNA sequencing

4-5 days

Figure 1 Comparison of the FDA method and a proposed new method for *E. sakazakii* detection, based on the results obtained in this study.

techniques, such as irradiation or reconstitution of IFM with water at 70°C, on inactivating *E. sakazakii* in food products. The PMA-PCR method will greatly aid in monitoring the resistance, survival strategies and stress responses of *E. sakazakii*. This would be an important step in the efforts to eliminate *E. sakazakii* from food and food production environments.

Concluding remarks

Human infants are so susceptible to infection that products such as IFM must be manufactured and monitored according to strict food safety standards to ensure that they are safe for consumption. The presence of *E. sakazakii* in IFM constitutes a significant risk to infant health if conditions after product reconstitution allow the bacterium to multiply to infectious levels.

The FDA method presently recommended for the detection of *E. sakazakii* in IFM is inadequate and non-specific. A new method has been proposed for detection of this bacterium based on a comprehensive evaluation of a variety of currently available detection methods. This method is not only more sensitive, specific and accurate for *E. sakazakii* detection than the current FDA method, but it also considerably reduces the time taken to detect this pathogen. The introduction of more stringent microbiological standards, together with the use of a more accurate method for *E. sakazakii* detection are recommended to reduce the health risks currently posed by this pathogen.

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