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an Animal Model for Enterobacter sakazakii**

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Ecology, Biological Characterization
and
Development of an Animal Model for *Enterobacter sakazakii*

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Abstract

Enterobacter sakazakii is an emerging foodborne pathogen which has been linked to a number of outbreaks associated with the consumption of powdered infant formula, especially in low-birth-weight and premature infants. Very little research has focused on molecular characterization of this organism and the mechanism(s) by which it causes disease in humans.

In this study, a collection of 260 isolates of *E. sakazakii* were assessed by phenotypic and genotypic tests. The use of 16S rDNA analysis showed an 82% identity amongst the strains tested. Interestingly, 14 strains originally identified as *E. sakazakii* by phenotypic characterization, were found to be other species or genera. Among the molecular typing methods, pulsed-field gel electrophoresis was found to be more discriminatory than automated ribotyping.

Furthermore, the theory that expressed human breast milk, if contaminated with *E. sakazakii*, would support *E. sakazakii* growth at 10, 23 and 37°C, was investigated. It was found that the intrinsically ascribed antimicrobial properties of breast milk do not appear to inhibit the growth of this foodborne pathogen *in vitro*.

Lastly, we assessed six animal species to find an animal model that would be well suited to conduct further studies on the virulence of *E. sakazakii* infection. Young (chicks, gerbils, guinea pigs, pigs, rabbits) and neonatal (gerbils, rats) animals were orally challenged with *E. sakazakii* at a level of 10^9 cells. Of all the animal models tested, it appears that the neonatal gerbil may be most suitable for further studies.

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Legend

A - Adenine

ADA – American Dietetic Association

ADRIA – Animal Disease Research Institute

ANN – Artificial Neural Networks

ATCC – American Type Culture Collection

BBB – blood-brain barrier

BM – human breast milk

BMEC – bone marrow endothelial cells

BMF – human breast milk with fortifiers

BPW – buffered peptone water

C – Cytosine

CDC – Centers for Disease Control and Prevention

CDD – Charged Coupled Device

CFIA – Canadian Food Inspection Agency

cfu – colony forming units

CHEF - Contour-clamped homogeneous electric field

c.i. – confidence interval

CLSI – Clinical and Laboratory Standards Institute

CSF – cerebrospinal fluid

c.v. – coefficient of variation

DFI – Druggan-Forsythe-Iversen

dNTP – deoxynucleotide triphosphate

dsDNA – double-stranded DNA

ESPGHAN – European Society for Pediatric Gastroenterology Hepatology and Nutrition

ESPM – Enterobacter sakazakii plating media

FAO – Food and Agriculture Organization of the United Nations

G - Guanine

GI – gastrointestinal tract

GT – generation time

h – hour(s)

HIV – Human Immunodeficiency Viruses

i.n. – intranasal

i.p. – intraperitoneal

ICMSF – International Commission for Microbiological Specifications for Foods

ILSI – International Life Sciences Institute

ISO – International Standards Organization

kb – kilo bases

LBW – low birth weight

LT – lag time

MID – minimum infectious dose

mLST – modified lauryl sulfate tryptose

min - minutes

n.a – non applicable

NCBI – National Centre of Biotechnology Information

NEC – necrotizing enterocolitis

O.D. – optical density

p.i. – post inoculation

PCR – polymerase chain reaction

PFGE – pulsed-field gel electrophoresis

PIF – powdered infant formula

rDNA – ribosomal DNA

RFLP – restriction fragment length polymorphism

s – second(s)

SDS – sodium dodecyl sulfate

tDNA – transfer DNA

TE – Tris-EDTA

TNTC – too numerous to count

TSA – tryptic soy agar

TSB – trypticase soy broth

UPGMA – Unweighted Pair Group Method with Arithmetic mean

USFDA – United States Food and Drug Administration

VIT – Vermicon identification technology

WASP – Whitley Automatic Scientific Spiral Plater

WHO – World Health Organization

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Chapter 1: Introduction

The information presented in this introduction was used in the following publication currently in print: Food Microbiology: Fundamentals and Frontiers, Chapter: *Enterobacter sakazakii*. Third Edition, 2007. Editors: M.P. Doyle; L.R. Beuchat and T. J. Monteville.

ASM Press)

Background and Taxonomy

The first report of a “yellow-pigmented coliform” as the causative agent in a case of septicemia in an infant dates back to 1929 (98), other cases associated to a yellow-pigmented coliform were described in England (1961) and Denmark (1965) (58, 116). However, it was only in 1980 that *Enterobacter sakazakii* was distinguished from *Enterobacter cloacae* and became a new species (27). In the early 1980s, *E. sakazakii* was isolated from powdered milk, but the first documented link of this bacterium to powdered infant formula (PIF) as a causative factor of infection was reported by Clark *et al.* in 1990 (17). To-date, there have been approximately 80 reported cases of *E. sakazakii* infections resulting in 19 deaths, described in 11 different countries and often associated with neonates and children from three days to four years of age. Only nine cases of adult infections due to *E. sakazakii* have been documented (22, 41, 56, 68).

E. sakazakii belongs to the genus *Enterobacter*, and as most species in this genus, it is considered an opportunistic pathogen. *E. sakazakii* is Gram-negative, oxidase-negative, non-sporeforming, non-acid-fast, straight, rod-shaped bacteria, having dimensions of 0.3–1.0 × 1.0–6.0 µm. It is motile by peritrichous flagella, non-halophilic, facultative anaerobic, and grows over a wide range of temperatures (6 - 45°C) (52, 59, 91). The main reasons that lead to the differentiation of *E. sakazakii* from *E. cloacae* in 1980s was the low percentage of DNA-DNA homology (32-49%), biochemical profiles, antimicrobial susceptibility and yellow pigment production (13, 14, 27).

The major biochemical differences among *E. sakazakii* and other *Enterobacter* species, with

minor exceptions, are the former's inability to ferment D-sorbitol, its positive α -glucosidase activity, and yellow pigment production. Recently, an Ad hoc group working towards an International Standards Organization (ISO) method for isolation/identification of *E. sakazakii* from food products has suggested withdrawing the yellow pigment formation tests suitable for *E. sakazakii* identification (46). Colonies of *E. sakazakii* on standard laboratory growth media such as tryptic soy agar (TSA) measure 2 – 3 mm and 1 – 1.5 mm in diameter at 36°C and 25°C, respectively, after overnight incubation. *E. sakazakii* produces colonies with distinct morphologies; one colony type is described as being dry, matt and leathery or rubbery, retracting to the agar when touched and having little biomass adhering to an inoculation loop. The second type is moist and glossy, and easy to remove from the agar with a loop (38).

Symptoms, At-Risk Populations and Foodborne Outbreaks

E. sakazakii has been mainly associated with necrotizing enterocolitis (NEC), septicemia and meningitis. Neurological sequelae are commonly reported and include brain abscess and infarction, ventricle compartmentalization due to necrosis of brain tissue and liquefaction of white cerebral matter, cranial cystic changes, as well as hemorrhagic and non-hemorrhagic intercerebral infarctions leading to cystic encephalomalacia (15, 65, 110, 120). While *E. sakazakii* can cause disease in all age groups, it has been reported to be a particular risk to infants less than 1-year old (30). Among infants, the immunocompromised, neonates younger than 28 days or with low-birth-weight (LBW, weighing less than 2500 g at birth) are believed to be the greatest at-risk group (108). Most recently, in a literature review of *E. sakazakii* reported cases, Bowen *et al.* (12) observed that of the 43 cases they reviewed, the

median age at *E. sakazakii* infection onset was 6 days in the group of infants with meningitis and 35 days in the group with bacteremia. Furthermore, they observed that 30 of 32 (94%) infants with meningitis, but only 2 of 11 (18%) infants with bacteremia, were <28 days old when infection was detected (12). Furthermore, Sondheimer *et al.* (108) found that premature or term neonates secrete less gastric acid than older infants, a potentially important factor contributing to the increased survival of *E. sakazakii* during its passage through the stomach and then onto the intestine. An additional issue is in developing countries where, due to the high number of HIV-positive mothers, a greater number of infants may be solely fed PIF in order to prevent the documented transmission of the virus from HIV-positive mothers to infants through breast feeding (30), possibly resulting in an increased vulnerability to *E. sakazakii* infection.

Cases of neonatal and infant infections associated with *E. sakazakii* have been reported and further described in many regions of the world including Belgium, Canada, Denmark, France, Germany, Greece, Iceland, Israel, The Netherlands, Spain, Portugal United Kingdom, New Zealand, Brazil and 11 states in the USA (12). Most of the reported infections have occurred in developed nations. The lack of reports from developing countries may be due to deficiency of awareness and of technical expertise to isolate it, or identifying its clinical manifestations, rather than the absence of illness related to this microorganism. Because of the general lack of knowledge concerning *E. sakazakii*, combined with poor existing methodologies, there are likely to be a number of underreported or misdiagnosed cases, even in developed countries where the alertness has been increasing in the past decades. Cases within adult populations have been reported, although with a much smaller

incidence, and often associated with prior immunodeficiency conditions (22, 25, 41, 56, 68, 100). Recently, Gosney *et al.* (35) reported the presence of *E. sakazakii* in the mouths of 7 out of 203 stroke patients investigated in the United Kingdom.

Niches and Reservoirs

The natural habitat of *E. sakazakii* remains unknown. However, it has been isolated from environmental samples such as water, dust, soil, plant materials and even household vacuum cleaner bags, indicating that its ecological niche is quite diverse and that similar to other members of the *Enterobacteriaceae* family, its reservoir is likely an environmental source. It has also been found in food products such as powdered milk, cheese products, cereal, chocolate, potato flour, pasta, minced beef, sausage meat, vegetables, herbs and spices (27, 38, 50, 60, 61, 69, 83, 118). Vectors and sources of contamination such as flies and rodents (39, 61, 67, 84) have been also reported. In the clinical setting, *E. sakazakii* has been recovered from samples of CSF, blood, stool, urine and other specimens from infected or colonized patients (8, 32, 41, 64, 65, 70, 82, 86, 88, 101, 103, 107, 117, 120). During a 7-month survey, Farmer (27) isolated *E. sakazakii* from the respiratory tract of 29 patients in one hospital. Nazarowec-White and Farber (89), using ribotyping, have shown the persistence of *E. sakazakii* in a clinical setting over an 11-year period.

E. sakazakii was isolated for the first time from an unopened can of dried milk by Farmer *et al.* in 1980 (27). This event was followed by the largest published survey of PIF conducted by Muytjens *et al.* (87) in which *Enterobacteriaceae* were isolated from 52.5% of 141 PIF cans from 35 countries, with *E. sakazakii* being detected in 20 of 141 (14.2%) samples from

13 of the 35 countries. More than two decades later, Leuschner *et al.* (75) could still isolate *E. sakazakii* from 8 of 58 (13.8%) samples of PIF from 11 countries. Iversen *et al.* (50) not only surveyed 82 samples of PIF, but also 404 other food products for the presence of *Enterobacteriaceae*, finding *E. sakazakii* in two of the formulas, 5 of 49 (10.2%) dried-infant foods, 3 of 72 (4.1%) milk powders, 2 of 62 (3.2%) cheese products, and various dry food ingredients, including 40 of 122 (37.8%) herbs and spices. Other studies have reported the isolation of *E. sakazakii* from other food products such as raw lettuce from restaurants in Spain, as well as cheese, minced beef, sausage meat and vegetables (20, 69, 104, 109).

Although isolated from a variety of food commodities, the only well documented food product that has been associated with *E. sakazakii* infection in neonates is PIF (94, 99, 106, 117). In some of these cases, *E. sakazakii* was isolated from unopened cans of PIF in concentrations ranging from 0.36 to 66 cfu/100g (51, 75, 87). In these cases, the identical molecular fingerprinting patterns were observed for the clinical isolates from patients and food isolates from unopened cans of PIF of the same lot as the PIF cans from which the infant had been fed, suggesting a causal link between consumption of PIF and infant infection (8, 17, 85, 106, 119).

Powdered Infant Formula, Human Breast Milk and *Enterobacter sakazakii*

PIF was introduced as a replacement for human breast milk (BM) almost 100 years ago. The commercially available powdered form constitutes over 80% of the infant formula used worldwide, having advantages as compared to the commercially available liquid form, both in terms of cost and storage. However, while the liquid form is sterile, the powder may

contain low levels of microorganisms. Current processing technology is unable to completely eliminate microbial contamination in PIF without affecting its organoleptic and nutritional requirements. A number of newer technologies, such as irradiation, combined with other potential hurdles, were suggested as being potential candidates in the future (30). Two recent studies reported the effects of high-pressure processing (200, 400 and 600 MPa) and gamma irradiation on *E. sakazakii* artificially inoculated in reconstituted and dehydrated PIF, respectively (34, 71). *E. sakazakii* strains (approximately 10^7 cfu/ml) were reduced up to 6.84-logs, varying among the strains tested, when processed in reconstituted PIF at 600 MPa. The reconstituted PIF, as compared to trypticase soy broth (TSB), was observed to have a protective effect on the bacteria when processed at 400 MPa (34). As for the gamma irradiation treatment, *E. sakazakii* (approximately 10^8 cfu/ml) was reduced 3-logs in number when treated in dry PIF with 3.0 kGy, while no bacteria were recovered from the PIF after a treatment of 5.0 kGy (71). However, both studies stated that further nutritional and sensory evaluation needed to be done before even considering the use of the described technologies in the industry.

Breast feeding is advantageous because BM has been reported to contain compounds with immunological properties, for instance, soluble and cellular factors such as immunoglobulin, lactoferrin, oligosaccharides, fatty acids, hormones and growth factors, as well as maternal leukocytes and cytokines. These compounds provide protection to the neonate while its immune system is still immature, and also act in synergy with the gastrointestinal tract (GI), enhancing antimicrobial properties (28, 45). However, it has also been suggested that some

of these BM components will need *in vivo* environments to be active, thus often making it difficult for *in vitro* evaluation of their antimicrobial effects (93).

Human breast milk fortifiers are powdered or liquid supplements added to expressed BM when the nutritional requirements of infants are not satisfied by BM alone. These can include thickening agents such as starches or cereals that increase the viscosity of the milk for feeding infants with gastro-oesophageal reflux, or supplements to complete nutritional requirements. To-date, *E. sakazakii* has not been isolated from human breast milk fortifiers. However, studies comparing bacterial growth in fortified breast milk (BMF) and BM alone have been reported. Jocson *et al.* (57) demonstrated an increase of 1-log in total bacterial counts in BMF when compared to BM, after 72h storage at 4 or 26°C. They hypothesized that nutrient fortification and storage duration may change some of the host defense properties of human milk, allowing bacterial proliferation to occur. Similarly, Chan (16) demonstrated a greater increase of total bacteria in BMF as compared to BM only, and suggested that the high iron content in the breast milk fortifier formulations could play a role in enhancing the growth of bacteria in the BMF due to the inhibition of lactoferrin in high-iron environments. Lactoferrin is a well known iron-binding glycoprotein found in BM and is known to have antibacterial properties.

Guidelines for preparing and/or manipulating PIF, BM and BMF in the household and health care settings for the management of *E. sakazakii* growth have been developed by groups such as the American Dietetic Association (7), the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) (1), the USFDA (114), Health

Canada (42) and the New Zealand Ministry of Health (92). Most guidelines recommend that, when nutritionally appropriate, sterile liquid formula products should be used over non-sterile PIF. In order to evaluate current recommendations regarding “hang times” (i.e., the amount of time a formula is kept at room temperature in the feeding bag and accompanying lines during enteral tube feeding), Telang *et al.* (111) evaluated the growth of resident aerobic mesophilic flora on artificially inoculated (10^2 or 10^3 cfu/ml of *E. sakazakii* ATCC 29544 (clinical isolate)) BM, BMF and PIF maintained at 22°C. In that study, *E. sakazakii* populations increased less than 1-log over a 6-h period. Kandhai *et al.* (59) investigated the growth of *E. sakazakii* in PIF using a clinical, an environmental, and a food isolate, along with strain ATCC 29544, and found that their lag times varied from 83.3 ± 18.7 h at 10°C to 1.73 ± 0.43 h at 37°C. They further observed that, 1) cells harvested from PIF at different phases of growth did not exhibit significant differences in either specific growth rate or lag time; 2) there were no differences in lag time among the four *E. sakazakii* strains studied, and 3) that lag times were surprisingly short even after the cells had spent 3 to 10 days in dry PIF.

Characterization of *Enterobacter sakazakii*

Considered an emerging opportunistic pathogen, much remains unknown about *E. sakazakii*. However, within the past decade, advances have been made in the molecular characterization of this organism using amplification and sequencing of the 16S rDNA (53, 72), pulsed-field gel electrophoresis (PFGE), ribotyping (89), and plasmid typing (17). DNA-based-techniques have been used by many groups to isolate, identify or differentiate strains of this emerging *Enterobacter* species. Clementino *et al.* (18) were one of the first research groups

to use tRNA intergenic spacer (tDNA-PCR) and 16S-23S internal transcribed spacer (ITS-PCR) for the characterization of *E. cloacae*. In their study, specific and reproducible patterns were obtained for *E. sakazakii* strain ATCC 29004. However, the study aimed at differentiating *E. cloacae*, and only a single *E. sakazakii* strain was used in their analyses. Following the study by Clementino *et al.* (18), Keyser *et al.* (63) published the first PCR-based application for *E. sakazakii*, in which they used the full length 16S rRNA gene from an *E. sakazakii* type strain to develop a detection system for upflow anaerobic sludge blankets. Subsequently, Lehner *et al.* (72) re-evaluated the work by Keyser *et al.* (63), and generated more 16S rDNA sequences which led to the description of two lineages. Later, Iversen *et al.* (53) used partial 16S rDNA, along with *hsp60* sequences, to investigate the phylogenetic relationship among 126 *E. sakazakii* strains. Their study reinforced the polyphyletic nature of *Enterobacter* spp., as did a previous study based on *gyrB* gene (21). Iversen *et al.* (53) identified four main clusters and reported that a substantial amount of taxonomic heterogeneity existed within the species. They were able to group most strains in one cluster, and hypothesized that the other three clusters could possibly represent new lineages within the species. Equally important, their work demonstrated that current methods based on biochemical profiling (i.e., API20E[®] or ID32E[®]) did not always correlate with the 16S rDNA-based approach. Liu *et al.* (76) and Malorny *et al.* (77), have used PCR approaches to isolate *E. sakazakii* from food products. Mohan and Venkitanarayanan (81) also applied sequence-based techniques to isolate *E. sakazakii* from food samples, i.e., they developed a PCR assay directed against the *ompA* gene of *E. sakazakii*. Lehner *et al.* (73) used two methods to try and differentiate *E. sakazakii* strains from other *Enterobacteriaceae*. They used an α -glucosidase encoding gene-specific PCR, as well as the vermicon identification

technology (VIT). With the latter technique, consisting of probing rDNA of viable bacteria under a VIT-adapted fluorescence microscope, they reported a detection limit of 10^3 cfu/ml. Iversen *et al.* (54) used Artificial Neural Networks (ANN) to analyze 16S rDNA and biochemical data of 189 *E. sakazakii* strains, which were among 282 *Enterobacteriaceae* strains. They emphasized the efficiency of ANN to reduce the dimensionality and complexity of analysis. With this computational data analysis, they were able to differentiate 99.3% of the *E. sakazakii* strains from the other closely related species tested. Alpha-glucosidase enzyme expression was found to be 98.7% and 100% correlated to 16S rDNA and phenotypic data, respectively. In addition, they failed to find one single test that could be used to differentiate *E. sakazakii* from other species, but rather suggested that a number of tests should be used to improve the likelihood of correct species identification (Table 1).

Table 1. Biochemical tests for the differentiation of *E. sakazakii*

Biochemical test ^a	<i>E. sakazakii</i> (%) ^b	Other <i>Enterobacteriaceae</i> (%)
α-glucosidase	100	0
Arginine dehydrogenase	97	67
Citrate	99	80
D-saccharic acid	0	33
Dulcitol	8	28
Glucose-1-phosphate	0	83
Glucose-6-phosphate	0	82
Lipase	96	4
Methyl red	5	57
Ornithine decarboxylase	91	74
Pyruvate	3	50
Raffinose	100	63
Sucrose	100	47
Voges-Proskauer	96	44
Yellow-pigment	98	28

^a Table modified from Iversen *et al.* (54).

^b Results in percentage for 189 *E. sakazakii* and 54 *Enterobacteriaceae* strains tested.

Virulence Factors and Pathogenesis

E. sakazakii infections in newborns were at one time suspected of occurring via passage of the organism through the mother's birth canal, similar to newborn infections caused by other pathogens transmitted from mother to child (82, 112). This hypothesis lost favour when *E. sakazakii* infections in neonates born by Caesarean section were reported (4, 85, 88), and when neonates testing negative for bacterial infection at birth, later developed infections. To-date, the host and environmental factors needed to be present in order to cause infection in neonates remain unclear. Keller *et al.* (62) have suggested that the virulence factors of *E. sakazakii* (exotoxins, aerobactin, and hemagglutinin) may be similar to *E. cloacae*. Our laboratory was the first to describe a putative mechanism of pathogenesis for *E. sakazakii*, demonstrating the organism's potential for producing cytotoxins and/or enterotoxins, using Vero, CHO and Y-1 cell lines, as well as suckling mice assay (97). The dose-response for an *E. sakazakii* infection also remains uncertain. Studies done on suckling mice suggest that a large number of cells may be required to cause infection in healthy neonates (97). Iversen *et al.* (48) hypothesized a minimum infectious dose of 1000 cfu, based on a comparison with *E. coli* O157:H7 and *Listeria monocytogenes* serotype 4b. In another study, Iversen *et al.* (53) suggested that, similar to the pneumococci, *Haemophilus* and meningococci, major pathogens associated with meningitis in children, *E. sakazakii* could also present a host developmental dependence on access to the central nervous system. In addition, the authors suggested that due to paracellular and transcellular mechanisms that may induce permeability of the blood/brain barrier, the choroids plexus may be the most likely entry site. Willis and Robinson (120) described two cases of *E. sakazakii* infections involving cerebral infarctions followed by the development of cystic lesions, symptoms similar to those caused by

Citrobacter koseri (former *C. diversus*). Based on previous work by Foreman *et al.* (31) describing possible cyst formation involving a sequence of vasculitis, necrosis and liquefaction of the cerebral white matter possibly misdiagnosed as abscesses, Willis and Robinson (120) suggested a similar ability of *E. sakazakii* and *C. koseri* to induce a cascade of events leading to a high rate of cyst formation. Farmer *et al.* (27) had previously shown that *C. koseri* is 50% related to *E. sakazakii* by DNA-DNA hybridization, while Iversen *et al.* (53) compared *Citrobacter* species and *E. sakazakii* 16S rDNA sequences and found that some *E. sakazakii* strains were 97.8% similar to the *C. koseri* assessed.

Rationale

Enterobacter sakazakii is an emerging foodborne pathogen that has increasingly raised interest among the scientific community, health care providers and the food industry since the early 1980s, when it was accepted as a new species. Nevertheless, there is still a lack of information concerning its natural habitat, mechanisms of virulence and pathogenicity, as well as its dose response for human infections. In 2002, the International Commission for Microbiological Specifications for Foods (ICMSF) (44) classified *E. sakazakii* as a severe hazard for restricted populations, causing life-threatening or substantial chronic sequelae or illness of long duration, with the high risk populations being newborns and immunocompromised infants. In 2004, the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) jointly held an expert meeting on *E. sakazakii* and other microorganisms of concern in PIF (30), aiming to gather information for the revision of the Recommended International Code of Hygienic Practice for Foods for Infants and Children (29). The FAO/WHO expert meeting of 2004 further agreed on a list of

recommendations to the scientific community and infant formula manufacturers. They recommended a focus on better understanding of *E. sakazakii* and potentially other microorganisms that could be found in infant formula. Specifically, they recommended; 1) the use of internationally validated detection and molecular typing methods for *E. sakazakii*; 2) the investigation and reporting of sources and vehicles of infection by *E. sakazakii* including the establishment of a laboratory-based network (e.g., PulseNet™); and 3) a better understanding of the ecology, taxonomy, virulence and other characteristics of this emerging pathogen. This project aims to address some of the FAO/WHO recommendations which were reinforced during their second meeting held in February 2006. Thus, the present study aimed to 1) generate phenotypic and genotypic characterization for 260 *E. sakazakii* strains using 16S rDNA sequences, PFGE and ribotyping methods, thereby increasing our *E. sakazakii* typing database and contributing to a prospective laboratory-based network; 2) provide a better understanding of *E. sakazakii* growth and survival in reconstituted PIF, BM and BMF, in order to support regulatory decision-making and policy development; and 3) develop a suitable animal model for the study of *E. sakazakii* pathogenesis which would be important for future studies of virulence amongst *E. sakazakii* strains and permit dose-response studies.

Objectives and Hypothesis

The objectives of this study are:

1. Phenotypic characterization of a collection of *E. sakazakii* strains by the observation of colony pigmentation, α -glucosidase activity and biochemical profiles.
2. Genotypic characterization of a collection of *E. sakazakii* strains by 16S rDNA sequencing, ribotyping and PFGE typing.
3. Assessment of the growth of *E. sakazakii* in PIF, BM and BMF.
4. Development of a non-primate animal model suitable for the investigation of *E. sakazakii* pathogenesis.

The hypotheses of this study are:

1. *E. sakazakii* strains may vary in phenotype, and therefore current methods of identification may be inaccurate, possibly generating false positive/negative results.
2. *E. sakazakii* strains from different sources may have different growth profiles, and antimicrobial properties of BM may inhibit their growth.
3. An animal model will closely mimic the symptoms of *E. sakazakii* infection in humans and will be suitable for future dose response and virulence studies.

Chapter 2: Phenotypic and Genotypic Characterization of *Enterobacter sakazakii*

The information presented in this chapter was presented in oral and poster forms at the 2005 Federal Food Safety and Nutrition Research Meeting (Ottawa, Canada); in oral and poster forms at the 2005 Health Canada Science Symposium (Ottawa, Canada) and as a poster form at the 2006 International Association for Food Protection Meeting (Calgary, Canada)

Introduction

Little is known about the ecology, taxonomy and virulence of *E. sakazakii*. Standard identification procedures for this bacterium consist of the observation of yellow pigmentation of colonies, positive α -glucosidase activity and identification by biochemical tests such as API20E[®] and ID32E[®]. Genotypic characterization, including ribotyping, pulsed-field gel electrophoresis (PFGE) and 16S rDNA sequencing methods have proven to be useful for epidemiological studies among laboratories and health care institutions. Although in the past decade advances have been made in the molecular characterization of *E. sakazakii* (18, 53, 63, 72, 89), and in the development of molecular-based techniques for the identification of this organism (76, 77, 105), much research still remains to be done in this field. A better understanding of the mechanism(s) of pathogenesis, and faster and more accurate methods for isolation and identification are major goals that the scientific community and the regulatory agencies are currently pursuing in order to be able to set policies and reduce the numbers of infections caused by this bacterium.

In this project, work was focused on the characterization of a large number of *E. sakazakii* strains isolated from environmental, food and clinical sources worldwide. Strains were characterized by phenotypic methods currently recognized for the identification of *E. sakazakii*, such as the above mentioned biochemical tests API20E[®] and ID32E[®], α -glucosidase activity and colony pigmentation, with the addition of the vancomycin resistance test. Secondly, the strains were typed with molecular methods presently being used in epidemiological investigations of other foodborne pathogens such as *E. coli* O157:H7, *Salmonella* spp., *L. monocytogenes* and *Shigella* spp., namely ribotyping and PFGE (96).

Furthermore, two PCR-based methods were also used for the characterization of these strains, the Dupont BAX[®] PCR system and the amplification and sequencing of a 1 kb 16S rDNA fragment.

Biochemical Profile

Biochemical characterization was done with the commercially available tests API20E[®] and ID32E[®]. These systems consist of 20 and 32 biochemical tests, respectively, and aim at the genus and species identification of non-fermenting Gram-negative bacteria. The API[®] system has been used as a diagnosis tool for identification of microorganisms, including foodborne bacteria, for more than 20 years. However, recent reports of characterization of *E. sakazakii* strains by both API[®] systems and PCR-based techniques (24, 53, 54), have suggested that biochemical and molecular techniques do not always render similar results. To-date, many methods of isolations/identification of *E. sakazakii* from PIF and other food products have been proposed (37, 47, 61, 75, 95). However, only one method is currently accepted as being official by the United States Food and Drugs Administration (FDA) (115), and in this method, API20E[®] is the final confirmation step for the identification of *E. sakazakii*.

Colony Morphology and Pigment Production

Prior to being submitted to the API[®] tests, presumptive identification of *E. sakazakii* consists of observing yellow pigment formation on tryptic soy agar (TSA) media after incubation at 25 or 37°C for periods varying from 24 to 72 h. Previous studies have suggested that the *E. sakazakii* pigment formation is more intense at 25°C than at 37°C, and more so in the

presence of light (37). Pigment formation has also been described to be unstable upon subculturing and to vary amongst strains (38). In the present study, the results of *E. sakazakii* yellow pigment formation on TSA were compared to biochemical profiles and to the genotypic characterization of 260 *E. sakazakii* strains.

Vancomycin Resistance

E. sakazakii has been previously described to be resistant to vancomycin (37) and a few groups have proposed the use of a selective broth enrichment consisting of a modified version of the lauryl sulfate tryptose broth (mLST), made up of LST containing 10 µg/ml of vancomycin and a higher concentration of NaCl (34 g/L). The mLST was recommended for the isolation of *E. sakazakii* from food products (37, 47, 76). However, it has been suggested by other groups that some *E. sakazakii* strains could not survive the selectiveness of the mLST (Larry Restaino, personal communication), either due to the antimicrobials or to the high salt content. Thus, the resistance of 260 *E. sakazakii* strains to vancomycin was included as a part of their phenotypic characterization.

16S rDNA Sequence Analysis

Ribosomal DNA (rDNA) consists of sequences encoding ribosomal RNA (rRNA), which regulate amplification and transcription initiation and contain transcribed and non-transcribed spacer segments. Because it is highly conserved, the sequencing of 16S rRNA is a powerful tool for phylogenetic and evolutionary studies. The use of 16S rDNA as a technique for the identification of bacteria may be an alternative when phenotypic

characterization methods fail to identify an organism. Reports have previously described the use of the 16S rDNA for phylogenetic classification of *E. sakazakii* (53, 54, 72). *E. sakazakii* presumptive colonies isolated from a food product have also undergone PCR amplification with specific primers targeting the *E. sakazakii* 16S rDNA (76, 77). In this study, we amplified and sequenced 1 kb of the 16S rDNA of 260 *E. sakazakii* strains to compare and evaluate the potential of using this method as an identification tool.

Pulsed-Field Gel Electrophoresis (PFGE)

PFGE is a molecular typing method largely used for epidemiological studies of outbreaks and sporadic cases linked to foodborne pathogens. PFGE is the method used for PulseNet™, an American network of public health and food regulatory agency laboratories coordinated by the Centers for Disease Control (CDC), which is now being replicated in different ways in Canada, Europe, the Asia Pacific region, and Latin America. The laboratory members of PulseNet™ perform standardized PFGE “fingerprinting” of pathogenic foodborne bacteria (e.g., *E. coli* O157:H7, *Salmonella* spp., *Shigella* spp., *Listeria* spp., or *Campylobacter* spp.), which are submitted to a database made available for rapid comparison to “fingerprints” already in the database, in order to try and make a “match” between, for example, food and clinical isolates.

The PFGE technique consists of periodic electrical pulses during the electrophoresis of an agarose gel loaded with fragmented DNA. These pulses lead to a better separation of large DNA fragments (>20 kb) which would not be separated in a constant electric field. In this method, intact bacterial cells are embedded in agarose plugs and the DNA is extracted *in*

situ, minimizing DNA shearing before enzymatic digestion by selected restriction enzymes, which results in large DNA fragments being visualized in the agarose gel. Amongst the advantages of the PFGE method is its high discriminatory power, which varies amongst bacterial species, and which is based on the small likelihood that unrelated bacterial strains will have the same pulsotype. Some disadvantages of PFGE are that it can be costly and time-consuming. However, it still is considered the “gold standard” for the molecular typing of foodborne pathogens. In the present study, 260 *E. sakazakii* isolates were typed according to the CDC PulseNet™ protocol (36).

Automated Ribotyping

Ribotyping consists of the use of nucleic acid probes to recognize ribosomal genes, which are highly conserved in microorganisms. The Southern blot technique is used in this method, allowing characterization of strains by their restriction fragment length polymorphism (RFLP) associated with rDNA operon(s). The ribosomal operons are comprised of 23S, 16S and 5S rRNA, and tend to vary within strains allowing one to be able to distinguish between different strains. In automated ribotyping, the bacterial genomic DNA is fragmented with restriction enzymes, electrophoresed in an agarose gel and transferred to a nitrocellulose membrane which is hybridized with a DNA probe against rRNA and visualized using a chemiluminescent agent. Once hybridized, the fragments of bacterial DNA containing a ribosomal gene are labelled, creating a chemiluminescent banding pattern. The banding pattern of DNA fragments corresponding to rRNA is the ribotype, which can be compared to a database for bacterial identification and epidemiological studies. In this study, 260 *E. sakazakii* strains were ribotyped by the Dupont automated RiboPrinter System® (11).

BAX[®] PCR System

The Bax[®] PCR System is an automated system for bacterial detection based on PCR technology. Bacterial DNA isolated from a food sample is treated and added to a pre-made PCR reaction tablet containing primers targeting specific DNA regions of the investigated bacteria. This system analyzes the fluorescence of the DNA melting curve, providing a more definitive result than solely endpoint measurements. A fluorescent dye confers specificity for the detection system producing a signal when excited by light in the presence of double-stranded DNA (dsDNA), which is detected before the melting point, at which dsDNA denatures into single-stranded fragments. This point varies with the length of the fragment and with the guanine and cytosine content. The data obtained from the tested samples are compared to a known target melting curve profile, generating a correlation coefficient. The Bax[®] PCR System for detection of *E. sakazakii* was used for the identification of 260 *E. sakazakii* strains previously submitted to the Bureau of Microbial Hazards of Health Canada as being *E. sakazakii* using other techniques.

Material and Methods

Phenotypic Characterization

The 260 *E. sakazakii* strains assessed in this study were part of the strain collection of the Bureau of Microbial Hazards of Health Canada. These strains were isolated from food or environmental samples, and identified as *E. sakazakii* at the Health Canada laboratory; or else submitted as *E. sakazakii* by other laboratories around the world. All strains were kept at

-80°C in TSB (Oxoid, Ottawa, Canada) containing 40% glycerol until time of use. For phenotypic characterization, strains were submitted for biochemical evaluation using the ID 32E[®] and API 20E[®] kits, colony pigmentation was observed after growth on TSA, and α -glucosidase activity was confirmed after growth on *E. sakazakii* specific chromogenic media (Druggan-Forsythe-Iversen agar media (DFI; Oxoid, Basingstoke, United Kingdom) (51) and *E. sakazakii* plating media (ESPM; R&F Laboratories, Wisconsin, United States) (102)).

Biochemical Profile

The identification of the strains using the biochemical tests API 20E[®] and ID 32E[®] (Biomérieux, Quebec, Canada) was done by suspending an isolated fresh colony from a TSA (Oxoid, Ottawa, Canada) plate into 0.85% saline solution, followed by inoculation of each well of the test, which was incubated at 37°C for 24 h. The biochemical profile of the strains was determined based on the colour-coded results observed in each well of the test, which indicates positive or negative results according to the ability of the given strain to use the substrate in the well. The colour-coded results are calculated as an identification number, which can be submitted to the API[®] Internet database (i.e., Apiweb) generating the identification of the strain by matching the profile to the database and finding the correspondent bacterial genus and species, as well as the percentage of tests that have matched to the species assigned.

Colony Pigment Production and α -glucosidase activity

For the evaluation of yellow-pigment production, each strain was streaked from the -80°C frozen stock onto TSA agar media, grown at 37°C overnight, followed by observation and classification of the colony-pigment as “very yellow”, “yellow”, “pale yellow” or “white”.

DFI and ESPM, two commercially available specific *E. sakazakii* chromogenic agar media containing the substrate 5-bromo-4-chloro-3-indolyl- α ,_D-glucopyranoside (X α Glc), were used to monitor α -glucosidase activity. The reaction of the former enzyme with the latter substrate results in an indigo pigment forming green and blue colonies on DFI and ESPM, respectively. Each strain was streaked from the -80°C frozen stock onto DFI and ESPM and classified as α -glucosidase positive when forming “green” or “blue” colonies, respectively. All observations were performed after overnight incubation of the inoculated media at 37°C.

The observation of the yellow pigment produced by the 260 strains, as well as the reading of the chromogenic media results, were performed under the same environment (e.g., same observer, same laboratory, same lighting conditions) in order to minimize as much as possible subjective analysis. A second and third observer was occasionally used to confirm findings.

Vancomycin Resistance

All 260 strains were tested for resistance to 30 μ g of vancomycin, using the disk diffusion antimicrobial susceptibility test. As stipulated by the Clinical and Laboratory Standards Institute (CLSI), strains were grown from a -80°C frozen stock onto a TSA plate overnight at

37°C, diluted using ISO-Sensitest broth (Oxoid, Ottawa, Canada) to obtain a McFarland Standard reading of 0.5, and swabbed onto a Müller-Hinton (MH) (Oxoid, Ottawa, Canada) agar plate. For each strain tested, the pre-inoculated plates contained 30 µg discs of vancomycin (Oxoid, Ottawa, Canada) in duplicate. Plates were incubated overnight at 37°C, the diameter of inhibition measured and the strains classified as susceptible or resistant to vancomycin. Culti-loops (Remel) of *Staphylococcus aureus* subsp. *aureus* (ATCC 25923) were used as positive controls.

Genotypic Characterization

For the genotypic profile, strains were assessed using 16S rDNA sequencing, PFGE and automated ribotyping typing.

16S rDNA Sequence Analysis

For 16S rDNA sequencing, *E. sakazakii* genomic DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, Wisconsin, United States). DNA concentration was measured with a Nanodrop spectrophotometer (NanoDrop Technologies, Delaware, United States) and 1 µg of the template DNA was added to a 50 µl PCR reaction mix made up of 41 µl of molecular biology grade water, 5 µl of 10 X PCR buffer (Invitrogen, Burlington, Canada), 1.5 µl of 50 mM MgCl (Invitrogen), 1 µl of 10 mM dNTP (Roche Diagnostics), 0.5 µl of Taq Polymerase (Invitrogen), and 0.5 µl (10 µM) of both forward and reverse primers (Sigma-Genosys, Texas, United States). The PCR reaction consisted of 1 min of denaturation at 94°C, followed by 45 cycles of 45 seconds (s) of denaturation at 94°C,

45 s of annealing varying from 55 to 62°C depending on the strain, and 2 min of elongation at 72°C; the reaction ended with one cycle of 10 min elongation at 72°C and the tubes were held in the thermocycler (Eppendorf, New York, United States) at 4°C at the end of the reaction. The target DNA segment amplified was 1,000 base pairs (1 kb) and the primers were designed by the Primer Designer software package (Scientific and Educational Software, North Carolina, United States), based on the reference strain ATCC 29544 (genbank accession number AB004746). The forward primer's sequence was FP-SakF: 5'-GGCCTAACACATGCAAGTCG-3' and the reverse primer, RP-SakR: 5'-GTATTCACCGTGGCATTCTG-3'. After amplification, 1 µl of the PCR product was added to an electrophoresis reaction in a 1% agarose gel at a constant pulse of 100V, followed by gel staining with ethidium bromide (1 mg/liter, Bio-Rad) and image capturing using the Gel-Doc 1000 system (Bio-Rad). The PCR products were sequenced by a third party company (DNA Landmarks, Montreal, Canada). Forward and reverse sequences were aligned with the TeraTerm 2.3 software (Pro) software and blasted against the National Center for Biotechnology Information (NCBI) nucleotide public database. The 16S rDNA sequences were analyzed with the BioNumerics version 3.5 software (Applied Maths) using the neighbour-joining method.

Pulsed-Field Gel Electrophoresis (PFGE)

For PFGE analysis, *E. sakazakii* were grown at 37°C overnight onto TSA plates, re-suspended into TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) to reach an optical density (O.D.) of 1.3±0.5 at 610 nm and lysed with a 10 mg/ml lysozyme solution for 10 min at 37°C. Plugs were made (1.2% SeaKem golden agarose, 10% SDS and 20 mg/ml

Proteinase K) with the bacterial suspension previously reacted with the lysozyme solution, and lysed in solution (50 mM Tris pH 8.0, 50mM EDTA, pH 8.0, 1% Sarkosyl, 0.15 mg/ml proteinase K) at 54°C for 2 h with constant agitation. After lysis, plugs were washed (twice in water and four times in TE buffer, 50-54°C), and a 1-mm slice was digested at 37°C with the restriction enzyme XbaI (Roche Diagnostics, Mississauga Canada) in its corresponding enzyme buffer. Restriction fragments were separated in an agarose gel (1% SeaKem Gold agarose in 0.5× Tris-Borate EDTA buffer) in a contour-clamped homogeneous electric field (CHEF)-MAPPER apparatus (Bio-Rad Laboratories, California, United States) set for 6 V/cm at 14°C for 22 h, using initial and final pulse times of 4 and 40 s, respectively. The DNA of *Salmonella* Braenderup strain H9812 digested with XbaI was used as a fragment size marker. The gels were stained using ethidium bromide (1 mg/liter; Bio-Rad Laboratories) for 30 min, destained in distilled water for 60 min, photographed using a Gel-Doc 1000 system (Bio-Rad Laboratories) and the images imported to the BioNumerics version 3.5 software (Applied Maths) for data analysis.

Automated Ribotyping

For the ribotyping analysis of *E. sakazakii* strains, an automated RiboPrinter[®] system (DuPont Qualicon, Delaware, United States) operated with proprietary reagents and membrane processing kits including all chemicals necessary for the technique, such as reagents for cell lysis, deproteination, restriction digestion and hybridization. For this analysis, *E. sakazakii* strains were grown overnight at 37°C on TSA media, re-suspended in 40 µl of TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) and heated at 80°C for 20 min to kill cells and denature enzymes. Heat treatment was followed by lysis using 5 µl of

lysostaphin and 5 μ l of N-acetyl muramidase, and samples were loaded to the RiboPrinter[®]. Once in the machine, samples were treated with the various enzymes contained in the DNA Prep Pack (DuPont Qualicon). This pack consists of 2 lyophilized tablets; 1) a peptidase which reacts with the lysed cells to degrade proteins and membranes, and removes DNA inhibitors before the digestion step, and 2) a second tablet which is a restriction buffer used in the DNA digestion step. Also included in the Pack are two dyes, a plain loading buffer and a loading buffer with the addition of a restricted marker and an empty well, reserved for the manual addition of the restriction enzyme EcoRI (DuPont Qualicon). The digestion process taking place in the RiboPrinter[®] consists of 15 min of lysostaphin and N-acetyl muramidase reaction, followed by 25 min of croma pepsidase reaction, and ending with 20 min of DNA digestion and heat inactivation. Samples are then automatically loaded in the gel and electrophoresis occurs at 35 V for 2 h and 15 min, until all fragments were transferred out of gel onto the nitrocellulose membrane located at its end. The next phase consists of membrane transfer to a chamber followed by membrane wash. Firstly, the membrane is hydrated with water, treated with sodium hydroxide for denaturation of membrane bound dsDNA fragments, then incubated with hybridization buffer and probe, followed by a blocking step and finishing off with the chemiluminescence reaction comprised of an alkaline phosphatase conjugate, sodium bicarbonate wash and a substrate (all reagents contained in the Membrane Processing Pack; DuPont Qualicon). The final phase is the detection of the ribotyping probe bound single-stranded fragments of rDNA with a Charged Coupled Device (CDD) camera, where chemiluminescence is emitted if a probe has bound to a single strand rDNA fragment in the presence of the substrate. The chemiluminescence is amplified to a detectable level by the CCD camera and a digital image is captured. When the test is complete, the membrane is

ejected and the digital image is passed to the computer where identification and characterization take place against a pre-defined database which includes previous samples run. The RiboPrinter[®] data was imported to BioNumerics version 3.5 software (Applied Maths) for analysis and comparison to the other characterization methods assessed.

Automated Ribotyping and PFGE Fingerprint Analysis

Fingerprint analysis was performed using BioNumerics, when comparisons of DNA fragment patterns and cluster analysis were determined. The Dice coefficient correlation was used to estimate the level of similarity among DNA fragment patterns, and cluster analysis of the fingerprints was performed with the unweighted-pair group method using arithmetic averages (UPGMA). The DNA fragments patterns of all samples tested by PFGE and automated ribotyping were analyzed with a tolerance of 1.5% (CDC standard) for both optimization and position tolerance.

BAX[®] PCR System

All strains were assessed with the Dupont BAX[®] PCR system. Bacteria were grown on TSA plates overnight at 37°C, re-suspended in brain heart infusion (BHI; Oxoid, Ottawa, Canada) broth, incubated for 3 h at 37°C, reaching an O.D. of 1.2 ± 0.5 at 660 nm, and 5 µl of the suspension was added to 200 µl of lysis buffer and heated for 20 min at 37°C, followed by 10 min at 95°C. After cooling for approximately 5 min in a cooling block, 50 µl of the lysed suspension was transferred to PCR tubes containing the *E. sakazakii* BAX[®] PCR mix. After the PCR reaction, the sample was identified as “negative”, “positive”, “intermediate” or

“error”. All samples identified as error or intermediate during this study were repeated as per the manufacturers’ protocol.

Results and Discussion

Biochemical Profile

Of the 260 *E. sakazakii* strains tested by API 20E[®], 67% (173/260 strains) were identified as *E. sakazakii*, matching the API[®] Internet database as excellent (14%, 36/260 strains), very good (16%, 42/260) or good (37%, 95/260) identification of *E. sakazakii* (Table 2). The remaining 33% (87/260) of the strains tested were not identified as *E. sakazakii*, they were given classifications such as acceptable identification (5 strains), excellent (47 strains), very good (4 strains) or good (1 strain) identification of the genus *Enterobacter*, doubtful (8 strains) or unacceptable (3 strains) profile, low discrimination (5 strains) or identification not valid (1 strain). In a few cases, the strains originally submitted as *E. sakazakii* were identified as other bacteria, namely, *E. cloacae* (3 strains), *Escherichia hermannii* (2 strains) and *Pantoea* spp. (1 strain).

When the same 260 strains were tested with the biochemical profiling test ID 32E[®], a higher percentage was identified as *E. sakazakii* compared to the results obtained from the API 20E[®]. Here, 78% (203/260) of the strains were identified as *E. sakazakii*, being classified as excellent (71%, 184/260) and very good (7%, 19/260) identification (Table 3). A very high incidence of doubtful profile (14%, 36/260) was observed with this test. Other designations (8%, 21/260) such as acceptable identification (2 strains), excellent (2 strains) and very good (1 strain) identification of the genus *Enterobacter*, unacceptable profile (9 strains), low discrimination (1 strain), among others were obtained, or strains were identified as other bacteria including *E. cloacae* (4 strains), *Citrobacter amalonaticus / farmeri* (1 strain) and *Escherichia hermannii* (1 strain).

Table 2. Classification of *E. sakazakii* strains according to API 20E[®] biochemical test

Profile Classification	Number of strains	% of strains
Excellent identification, 99.9%, <i>E. sakazakii</i>	36	14%
Very good identification, 99.- 99.9 %, <i>E. sakazakii</i>	42	16%
Good identification, 96.7 - 98.4 %, <i>E. sakazakii</i>	95	37%
Other classifications	87	33%

Table 3. Classification of *E. sakazakii* strains according to ID 32E[®] biochemical test

Profile Classification	Number of strains	% of strains
Excellent identification, 99.9%, <i>E. sakazakii</i>	184	71%
Very good identification, 99.9%, <i>E. sakazakii</i>	19	7%
Doubtful profile, 99.9%, <i>E. sakazakii</i>	36	14%
Other classifications	21	8%

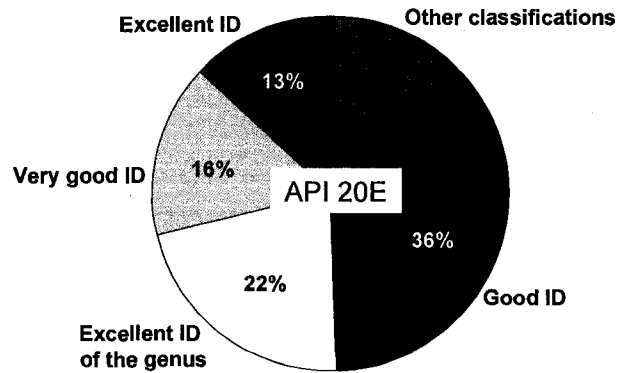
Of the 184 strains that were found by ID 32E[®] to be excellent identification of *E. sakazakii*, 23 were also identified as excellent by API 20E[®]. The remaining isolates were classified as very good (29 strains) or good (69 strains) identification of *E. sakazakii*, or as excellent identification of the genus *Enterobacter* (49 strains), doubtful profile (7 strains), acceptable identification (4 strains), low discrimination (1 strain), unacceptable profile (1 strain), or as *E. cloacae* (1 strain) by API 20E[®]. Figure 1 illustrates the correlation detailed above, as well as the relationship among strains identified as very good *E. sakazakii* ID and doubtful profile by ID 32E[®], as well as their respective classification by API 20E[®].

In February 2006, an ad hoc group meeting was held by the ISO organization (46) in order to gather information for ongoing technical guidance for the isolation and identification of *E. sakazakii* from food and environmental samples. During this meeting, a presentation on the BioMerieux miniaturized biochemical tests (i.e., API 20E[®] and ID 32E[®] tests) was given, during which it was suggested that the identification of *E. sakazakii* by these tests should only be valid when the results are excellent, very good or good identification of the species. Any of the other classifications (e.g., excellent, very good or good identification of the genus, doubtful profile, acceptable identification, etc.) should not be considered conclusive results. In these instances, the API 20E[®] and ID 32E[®] tests should be repeated or additional tests done for identification of the microorganism in question. The analysis of the biochemical profile results of the 260 strains in this present study was performed according to the procedures outlined in the BioMerieux meeting (46).

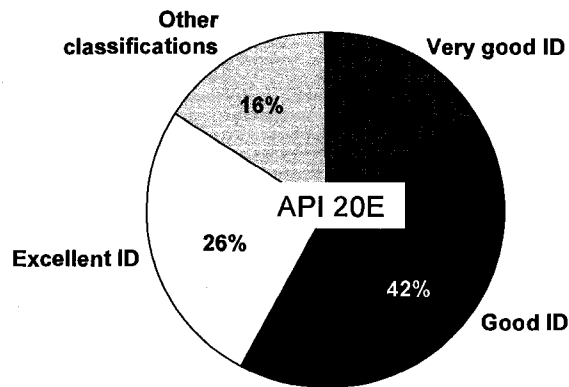
Figure 1. Correlation of biochemical tests API 20E[®] and ID 32E[®] results.

A) API 20E[®] classification of the 184 strains identified as excellent *E. sakazakii* ID by ID 32E[®]; B) API 20E[®] classification of the 29 strains classified by ID 32E[®] as very good *E. sakazakii* ID; and C) API 20E[®] classification of the 37 strains classified by ID 32E[®] as doubtful profile.

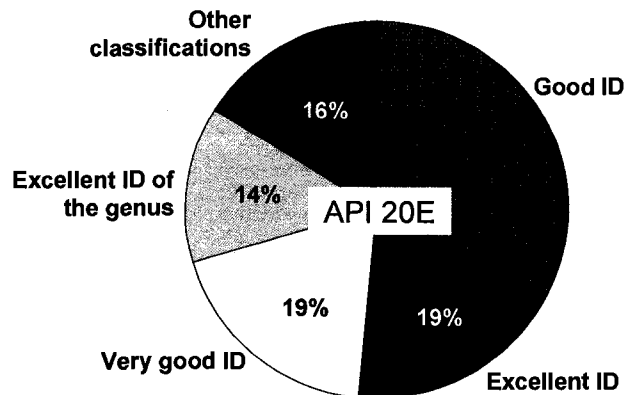
A) 184 strains classified by ID 32E® as *E. sakazakii* excellent ID



B) 29 strains classified by ID 32E® as very good *E. sakazakii* ID



C) 37 strains classified by ID 32E® as doubtful profile

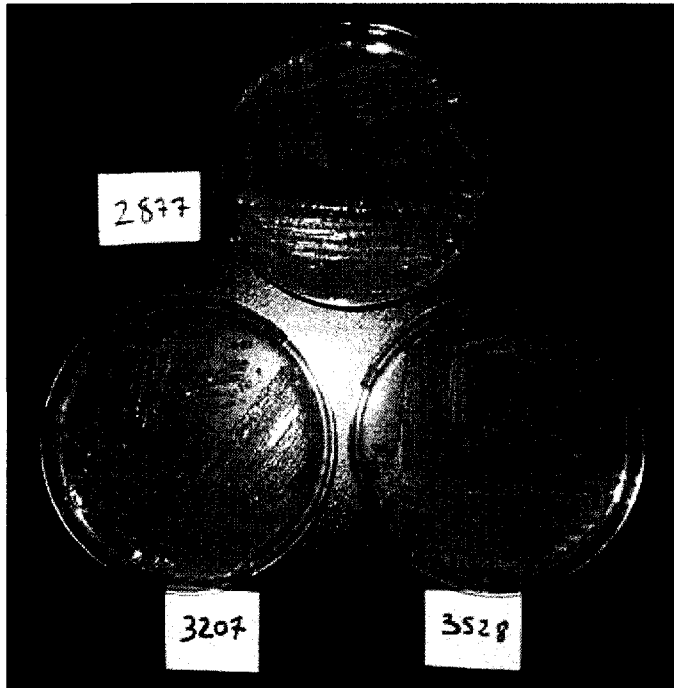


Colony Pigment Production and α -glucosidase activity

After overnight incubation at 37°C, the colonies streaked onto TSA plates were observed and classified as “very yellow”, “yellow”, “pale yellow” and “white” (Figure 2). Those classified as “very yellow” were colonies that produced a yellow pigment brighter than that commonly observed in the majority of *E. sakazakii* colonies studied in this laboratory. The largest portion of the 260 strains was considered to produce “yellow” pigmented colonies, known to be characteristic of *E. sakazakii*. The “pale yellow” pigmented colonies were attributed to those strains in which almost no color was observed when grown on TSA plates, but that when compared to white colonies of other *Enterobacter* species (e.g., *E. cloacae*) showed a pale quality of yellow pigment. The colonies considered “white”, were those that were identical to *E. cloacae* colonies, i.e., deprived of yellow pigment. The 260 presumptive *E. sakazakii* strains were firstly classified according to pigment production and subsequently submitted to genotypic and biochemical analysis. Thus, the classification by colour preceded the confirmation of *E. sakazakii* strains.

Figure 2: Illustration of yellow pigmentation variability among *E. sakazakii* strains

Examples of pale yellow (strain 2877), yellow (strains 3528, 2878) and white (strain 3207, 2879) colony pigmentation formed by *E. sakazakii* are shown.



According to the above classification, 80% (209/260) of the strains assessed produced “yellow” colonies, 11% (28/260) were classified as “white”, 7% (17/260) as “pale yellow” and 2% (6/260) “very yellow” colonies.

Colonies grown on ESPM were classified as “blue” or not blue, with the second category being subdivided in different observations such as, “blue/grey”, “yellow” or “white”. Colonies grown on DFI were classified as “green” or not green, also presenting sub-categories for the presumptive α -glucosidase-negative strains, namely, “pale green”, “white with a green centre” or “white”. If a strain gave “white” colonies on these media, or “white with green centre” on DFI and “yellow” on ESPM, it was interpreted as being negative for α -glucosidase activity, demonstrating its inability to hydrolyze the X α Glc substrate present in both media, and therefore unable to produce blue or green pigmented colonies. “Blue” colonies on ESPM and “green” colonies on DFI were considered as positive for α -glucosidase activity. However, the strains forming “blue/grey” and “pale green” colonies, on ESPM and DFI, respectively, were assumed to possess a lower level of α -glucosidase activity. This assumption was not investigated any further during the present study.

Amongst the 260 strains grown on DFI, 84% (217/260) formed “green” colonies, while 6% (16/260) formed “pale green” colonies, and the same number (6%, 16/260) formed “white” colonies, 1% (2/260) formed “white colonies with green centre”, and 3% (9/260) were unable to grow. The results observed when these strains were grown on ESPM, consisted of 84% (219/260) strains forming “blue” colonies, 10% (25/260) “blue/grey”, 2% (5/260)

“white”, and 2% (5/260) “yellow” colonies, with 2% (6/260) observed unable to grow. Interestingly, some of the strains that did not grow on DFI and/or ESPM were identified as *E. sakazakii* by BAX[®] PCR (strains 2873, 3214, 3271, 3276, 3280, 3282 and 3283) or 16S rDNA sequences (strains 2849, 2852, 2873, 3214, 3271, 3276, 3280, 3282, 3283 and 3644). Some of these same strains were classified as *E. sakazakii* according to their biochemical profile as follows; for API 20E[®], 1) very good ID (strain 3214), and 2) good ID (strains 3276 and 3280); while for ID 32E[®], 1) excellent ID (strains 3214, 3280, 3282 and 3283), and 2) very good ID (2852 and 2873).

Comparing the results of both chromogenic media, 203/219 (93%) strains that formed blue colonies on ESPM, also formed green colonies on DFI. The remaining 16 strains (7%) formed “pale green” (12 strains) and “white” (3 strains) colonies, or did not grow (1 strain) on DFI. When the 25 strains that formed “blue/grey” colonies on ESPM were streaked onto DFI, they formed “green” (14 strains), “white” (6 strains) or “pale green” (3 strains) colonies, or did not grow (2 strains). The 5 strains that formed “yellow” colonies on ESPM, formed “white” colonies on DFI; and of the five strains that grew as “white” colonies on ESPM, four were also “white” and one did not grow on DFI. Of the five strains that did not grow on ESPM, four also did not grow on DFI, but one formed pale-green colonies.

Correlating two features currently considered as being presumptively-positive for *E. sakazakii*, that is, yellow-pigment production and α -glucosidase activity, it was noted that 209 strains that formed “yellow” colonies on TSA were classified as “green” (183/209, 88%), “pale green” (16/209, 8%), “white” (6/209, 3%), “white with a green center” (1/209),

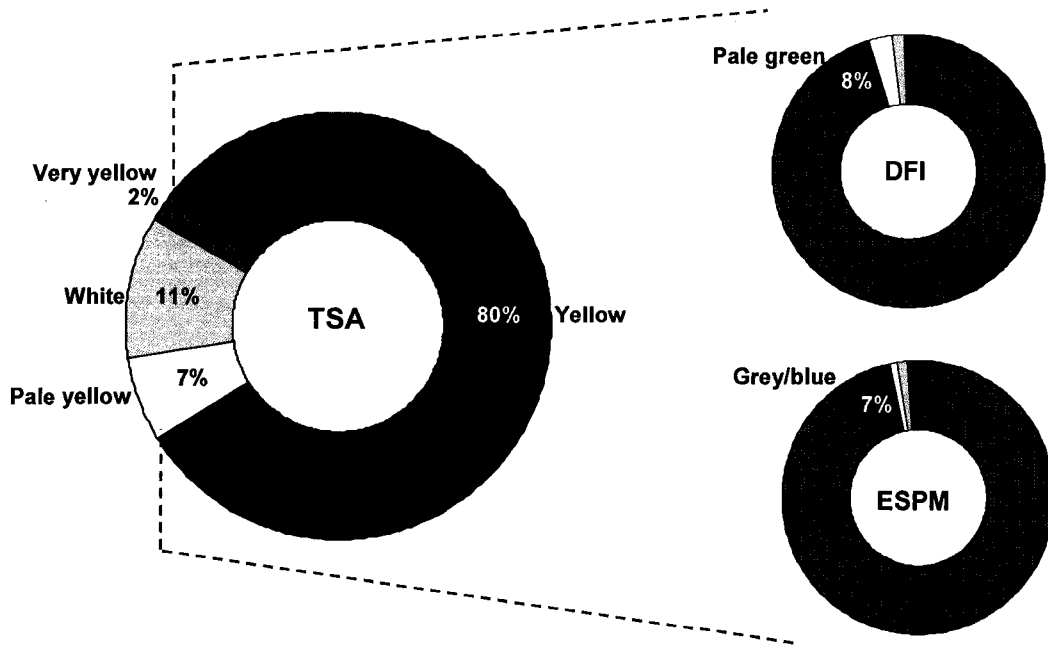
or were unable to grow (3/209) on DFI. When grown on ESPM, 174 of those 183 strains formed “blue” colonies, while 9 presented “blue/grey” colonies (Figure 3).

When the strains (n=28) that currently would not be considered as a presumptive *E. sakazakii* due to the lack of yellow pigment were examined, they were found to react with the substrate X α Glc present in the chromogenic media and therefore, were considered α -glucosidase positive. From these observations, 50% of the colonies that were “white” on TSA (14/28) formed “green” colonies on DFI, and of those, 11 also formed “blue” colonies on ESPM, whereas the remaining three strains formed “blue/grey” colonies on ESPM (Figure 3, Table 4).

Figure 3. *E. sakazakii* yellow pigment production on TSA as compared to reaction to indicator of α -glucosidase activity on DFI and ESPM

A) Results of chromogenic reaction on Druggan-Forsythe-Iversen (DFI) and *E. sakazakii* chromogenic plating medium (ESPM) of yellow pigmented strains on tryptic soy agar (TSA), n = 260; B) Results of chromogenic reaction of strains forming white colonies on TSA, n = 260

A) Chromogenic reaction of strains forming yellow pigmented colonies on TSA



B) Chromogenic reaction of strains forming white pigmented colonies on TSA

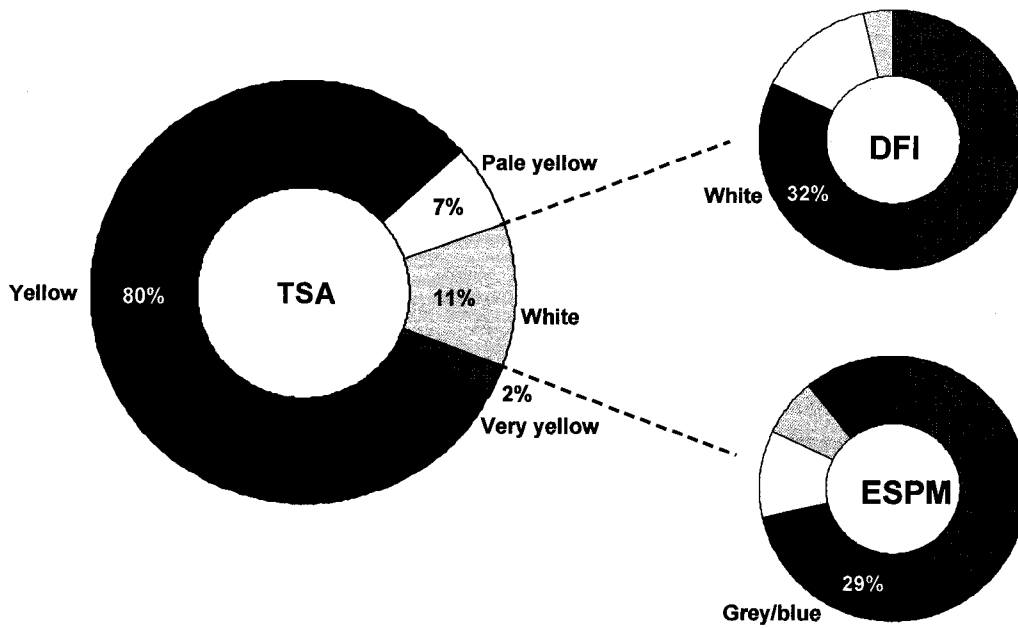


Table 4. Comparison of biochemical profiles to colony morphology on TSA and chromogenic media

Correlation of lack of yellow pigment production on tryptic soy agar (TSA), reaction for presumptive α -glucosidase activity on chromogenic media (Druggan-Forsythe-Iversen (DFI) and *E. sakazakii* chromogenic plating medium (ESPM)); and biochemical tests (API 20E[®] and ID 32E[®])

Strain ID	Source	TSA	DFI	ESPM	API 20E [®]	ID 32E [®]
3197	Unknown	White	Green	Blue	Excellent ID	Very Good ID
3207	Unknown				Excellent ID	Very Good ID
3200	Environmental				Very good ID	Excellent ID
3205	Food				Very good ID	Excellent ID
3204	Clinical				Good ID	Excellent ID
3206	Unknown				Good ID	Excellent ID
3213	Unknown				Good ID	Excellent ID
3219	Unknown				Good ID	Excellent ID
3221	Unknown				Good ID	Doubtful profile
3425	Unknown				Good ID	Excellent ID
3641	Food				Very good ID of the genus	Excellent ID
3223	Unknown			Doubtful profile	Excellent ID	
3224	Unknown			Excellent ID	Doubtful profile	
3225	Unknown			Good ID	Excellent ID	
						Blue/grey

The remaining 9% (23/260) of the strains that were classified as “pale yellow” colonies (17 strains), formed “green” colonies on DFI (14 strains), of which 12 formed “blue”, and two formed “blue/green” colonies on ESPM. The other three strains were unable to grow on DFI, and produced “blue/grey” colonies (1 strain) or did not grow (2 strains) on ESPM. Only six strains formed “very yellow” colonies on TSA, and when grown on the chromogenic media, formed “green” and “blue” colonies.

Interestingly, the two features often present in current isolation methods as characteristic of presumptive *E. sakazakii* colonies, i.e., α -glucosidase activity and yellow pigment production, were observed in many cases as being not congruent within the same strain. This could suggest that, 1) some strains identified as *E. sakazakii* due to the yellow pigment production may be unable to produce the α -glucosidase enzyme; or, 2) other *Enterobacter* spp. form yellow-pigmented colonies, and are mistakenly identified as *E. sakazakii* (51, 102), but are α -glucosidase negative as are many *Enterobacter* spp.; or, 3) some *E. sakazakii* isolates lacking yellow pigment production harbour α -glucosidase activity. The latter may be currently identified as negative *E. sakazakii*, generating therefore false-negative results. All 14 strains forming “white” colonies on TSA, and that also formed “green” and “blue” or “blue/grey” colonies on DFI and ESPM, respectively; were identified as *E. sakazakii* in 80% of the cases by both API 20E[®] and ID 32E[®] (Table 4). Furthermore, the 16S rDNA sequences and the BAX[®] PCR test of these strains also confirmed them as *E. sakazakii*, suggesting that hypothesis #3 may be correct. The ad hoc ISO group in charge of gathering information on methodology for the isolation and identification of *E. sakazakii* from food and environmental samples (46), have reported similar results, criticizing the validity and use

of yellow pigmentation as a criterion for presumptive identification of this bacterium. In their report of the February-2006 meeting (46), they suggested the exclusion of this trait from present and future ISO document(s).

Vancomycin Resistance

The CLSI for the vancomycin antimicrobial susceptibility disc test describes a diameter of inhibition zone denoting resistance and susceptibility for enterococci (<14 mm and >17 mm), other species of Gram-positive bacteria (<9 mm and >12 mm) and *S. pneumoniae* (non-applicable and >17 mm). Consequently, in this present study, it was not possible to compare the diameter of inhibition of *E. sakazakii* in the presence of the 30 µg vancomycin disc to a CLSI *E. sakazakii* standard. The selective enrichment broth mLST has been described for the isolation of *E. sakazakii* from food and environmental samples (37, 47, 76), and was employed in this study (Chapter 3) to isolate *E. sakazakii* from fecal and organs samples of previously inoculated animals. Therefore, it was of great importance to ensure that vancomycin, one of the selective components of the broth, would not reduce the chances of *E. sakazakii* isolation when mLST was used. For 47% (121/260) of the strains tested, no inhibition zone was observed. For 21% (55/260) of the strains, average inhibition zones of 8 mm (17%; 43/260), 9 mm (9%; 24/260) 10 mm (4%; 10/260), and 12 mm (2%; 4/260) were observed. Strains 2891, 2849 and 3644 were the exceptions, demonstrating inhibition zones of 14, 18 and 20 mm, respectively. Based on the CLSI parameters for vancomycin antimicrobial susceptibility disc test available for enterococci, all *E. sakazakii* strains tested, except 3644, 2891 and 2849, are considered resistant to 30 µg of vancomycin. In addition,

according to the standard for other Gram-positive species, 98% of the strains tested would also be resistant to this antibiotic at the concentration tested.

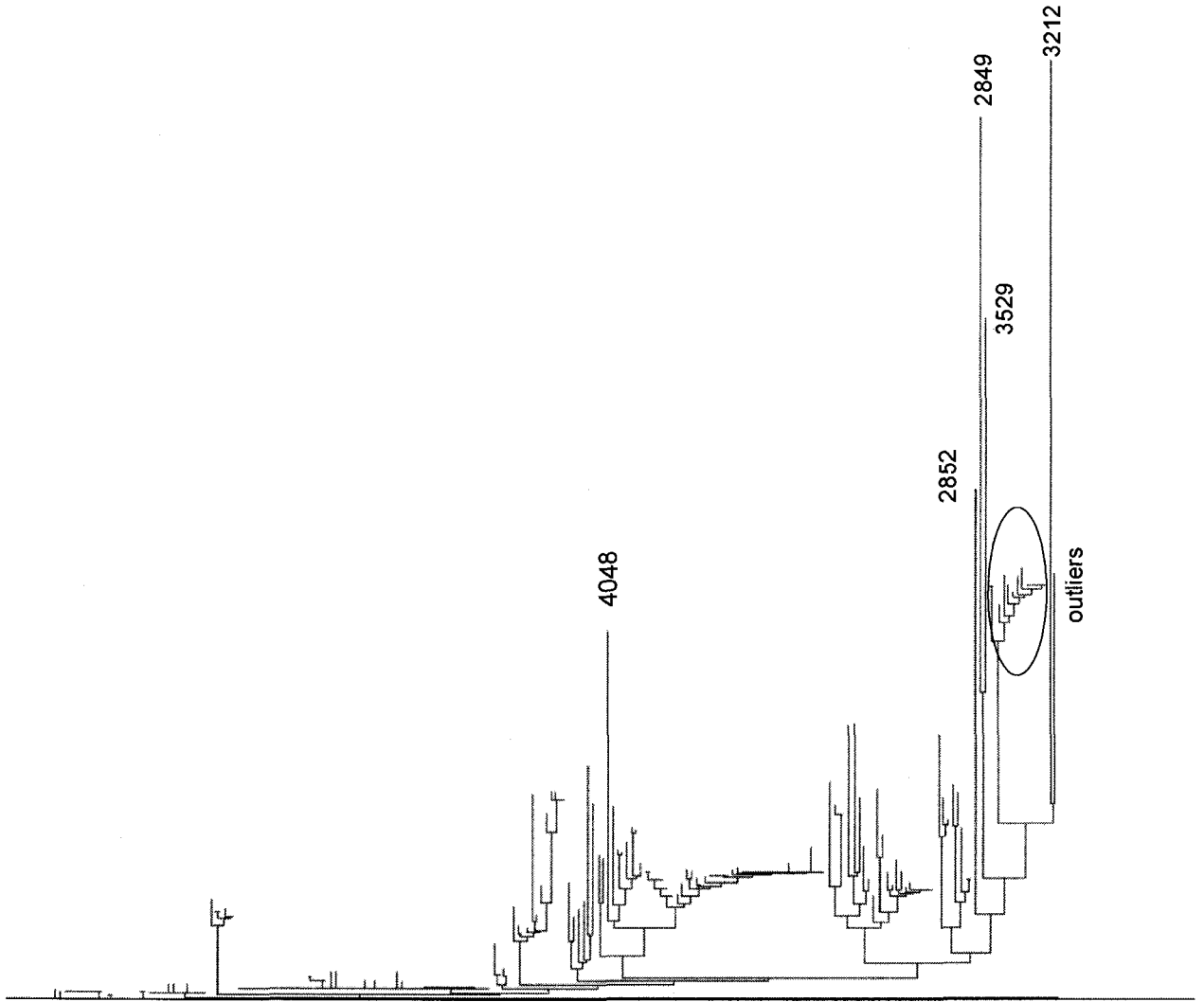
While strain 2891 was identified as *E. sakazakii* by other tests performed during this study, strains 2849 and 3644 had been classified earlier in the study as species other than *E. sakazakii*. The two latter strains were negative by the *E. sakazakii* Bax[®] PCR test, white on TSA, negative for *E. sakazakii* by both API 20E[®] and ID 32E[®], and did not grow on either ESPM or DFI media. According to their ribotypes, strains 2849 and 3644 were closely related to *S. warneri* and *E. faecium*, respectively.

16S rDNA Sequence Analysis

Among the 260 strains that had 1 kb of 16S rDNA amplified, sequenced, and blasted against the NCBI nucleotides database, 14 were identified as species other than *E. sakazakii* (e.g., *Pantoea* spp., *Averyella dalhousiensis*, and other species of *Enterobacter*). As expected, these 14 strains grouped quite differently when analyzed within the 260 strains by the BioNumerics software, and clustered by sequence relationship as illustrated in dendrogram form using the neighbor-joining method (Figure 4).

Figure 4. Dendrogram revealing the identity of the 16S rDNA sequences of 260 *E. sakazakii* strains.

The sequences were clustered by the neighboring-joining method showing 82% identity baseline.



The 260 strains were grouped into 96 clusters with the largest one containing 50 strains. The identity amongst them was 82%, with strains 3212, 2849, 2852, 3529 and 4048 being the most distinct, in this order. Among these, strains 3212 and 4048 were identified as *E. sakazakii* by BAX[®] PCR, API 20E[®] and ID 32E[®], forming yellow, blue and green colonies on TSA, ESPM and DFI, respectively, and therefore differences observed in 16S rDNA sequences could have been due to small evolutionary changes. However, strains 2849, 2852 and 3529 were not identified as *E. sakazakii* by API 20E[®] and ID 32E[®], formed white colonies or were unable to grow on the agar media evaluated, were *E. sakazakii* BAX[®] PCR negative and, by ribotyping, were closely related to *S. warneri*, *S. epidermidis* and *E. cloacae*, respectively. For these three strains, sequences were expected to be unrelated to the average *E. sakazakii* sequences.

Iversen *et al.* (53) have previously reported a comparative 16S rDNA sequence analysis of 126 *E. sakazakii* strains, for which they used the Micro-Seq 500 16S rDNA bacterial sequencing kit (Applied Biosystems), sequencing 500 bp of these strains' 16S rDNA. Based on their analysis of the 16S rDNA partial sequences, they grouped the 126 strains into four clusters. The bigger cluster, "cluster 1", contained 110 strains, with a 0.1 to 1.2% difference from the *E. sakazakii* type strain NCTC 11467, while "cluster 2" included 9 strains and exhibited 1.6 to 1.9% sequence divergence from the type strain. Interestingly, "cluster 3" (containing 5 strains) and "cluster 4" (containing 2 strains), included strains that had been identified as *E. sakazakii* by API 20E[®] and ID 32E[®] tests, but that were more closely related to other species of *Enterobacter* (e.g., *Enterobacter pyrinus*, *Enterobacter hormaechei*) or to *Citrobacter koseri* by 16S rDNA sequencing. This present study is in agreement with the

results of Iversen *et al.* (53), since we also noted that a discrepancy existed in the identification of *E. sakazakii* by biochemical profiling using API 20E[®] and ID 32E[®], and 16S rDNA sequences analysis (Table 5).

Table 5. Phenotypic classification of strains identified by 16S rDNA as being species other than *E. sakazakii*®

Strain	16S rDNA	BAX® PCR	Source ^e	API 20E	ID 32E	TSA ^b	DFI ^c	ESPM ^d
2868	<i>E. asburiae</i>	+	F	ND ^a	ND	ND	ND	ND
2879	<i>E. hormaechei</i> subsp. <i>steigerwaltii</i>	+	E	Good ID	Unacceptable profile	W ^f	W	B/G ^h
3172	<i>Enterobacter</i> spp.	-	F	Good ID	<i>E. cloacae</i>	W	W	Y
3173	<i>E. hormaechei</i>	-	F	<i>E. cloacae</i>	<i>E. cloacae</i>	W	W	Y
3174	<i>E. hormaechei</i> subsp. <i>steigerwaltii</i>	-	F	Doubtful profile	<i>E. cloacae</i>	W	W	Y
3175	<i>E. hormaechei</i> subsp. <i>steigerwaltii</i>	-	F	Doubtful profile	<i>E. cloacae</i>	W	W	B/G
3229	<i>E. cloacae</i> / <i>hormaechei</i>	+	C	<i>E. cloacae</i>	<i>E. amnigenus</i>	W	W	Y
3244	<i>E. cowani</i>	-	E	<i>Escherichia hermannii</i>	<i>Escherichia hermannii</i>	Y ^g	W	W
3248	<i>Averyella delhousiensis</i>	-	C	Excellent ID of the genus	<i>E. amnigenus</i>	W	W	B/G
3256	<i>Enterobacter</i> spp.	-	U	Low discrimination	<i>Citrobacter amalonaticus/farmeri</i>	Y	W	W
3287	<i>E. hormaechei</i> subsp. <i>steigerwaltii</i>	-	F	Very good ID of the genus	Doubtful profile	W	W	B/G
3643	<i>Pantoea</i> spp.	-	F	<i>E. cloacae</i>	<i>E. asburiae</i>	Y	W	Y
4042	<i>E. hormaechei</i>	+	E	Very good ID of the genus	<i>E. amnigenus</i>	W	W	B/G
4046	<i>Enterobacter</i> spp.	+	F	Good ID	Unacceptable profile	Y	G	B ⁱ

^a ND; not done.

^b TSA; tryptic soy agar, typical *E. sakazakii* form yellow-pigmented colonies.

^c DFI; Druggan-Forsythe-Iversen agar media, typical *E. sakazakii* form green colonies.

^d ESPM; *Enterobacter sakazakii* plating media, typical *E. sakazakii* form blue colonies.

^e Source; F, food isolate; C, clinical isolate; E, environmental isolate; U, unknown source

^f W, white colony.

^g Y, yellow colony.

^h B/G, blue/grey colony.

ⁱ B, blue colony.

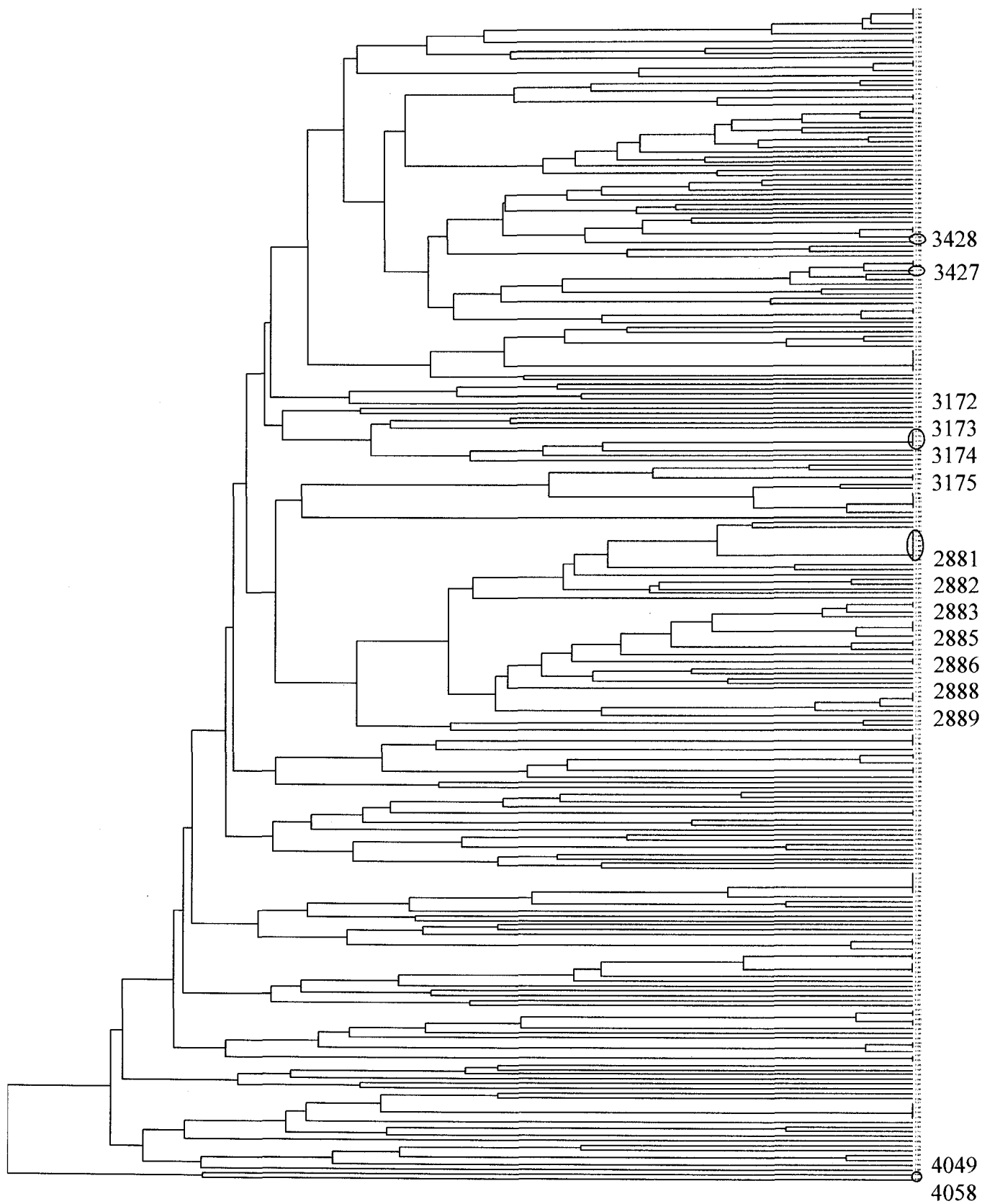
Pulsed-Field Gel Electrophoresis

Analysis of 240 PFGE fingerprints were performed, since 14 of the initial 260 strains were eliminated by the 16S rDNA analysis as being other bacterial species, and 6 strains had DNA degradation during the PFGE procedure, and were not interpretable. The 240 pulsotypes were grouped by the Dice coefficient correlation, and their cluster analysis was performed with the unweighted-pair group method using arithmetic averages (UPGMA), which generated a similarity base of 45% (Figures 5 and 6). The fingerprints were grouped into 188 different clusters, with the largest one containing 7 strains. Interestingly, some of the strains (2881, 2882, 2885, 2888 and 2889) in this larger cluster were also grouped by ribotyping, however, by ribotype analysis, strains 3208, 3214 (90% similar by PFGE to the former five strains), and 4055 and 4065 (85% similar by PFGE to the previous 7 strains), were also in the same cluster. However, strains 2883 and 2886 belonged to the same cluster by pulsotyping, but not by ribotyping.

Strains 3427 and 3428 originating from the same clinical case were indistinguishable by ribotyping, but were only 70% similar by PFGE. Strain 3430, isolated from a colonized infant during the same period and hospital as the two latter strains, and was found to be 95% similar to the two isolates by both typing methods. Among all 240 strains analyzed, two food isolates (4049 and 4058) were found to be the least similar by PFGE typing (4049 and 4058).

Figure 5. Dendrogram revealing cluster analysis of 240 *E. sakazakii* strains from various sources by PFGE.

The percentage of similarity among strains was determined using the Dice coefficient, and the clustering was performed by UPGMA.



3428

3427

3172

3173

3174

3175

2881

2882

2883

2885

2886

2888

2889

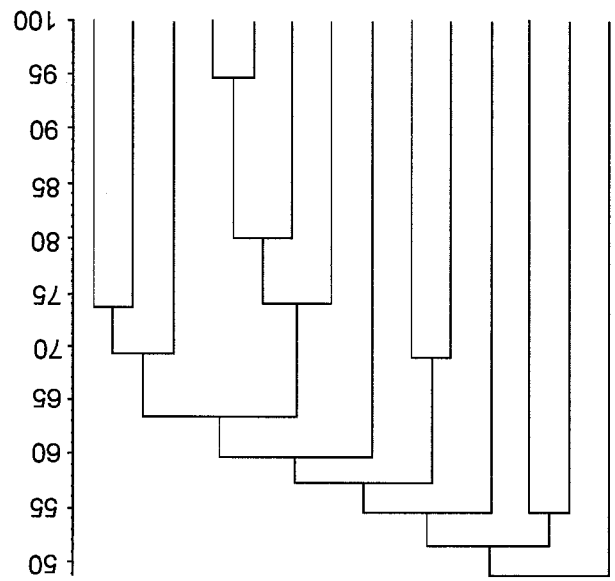
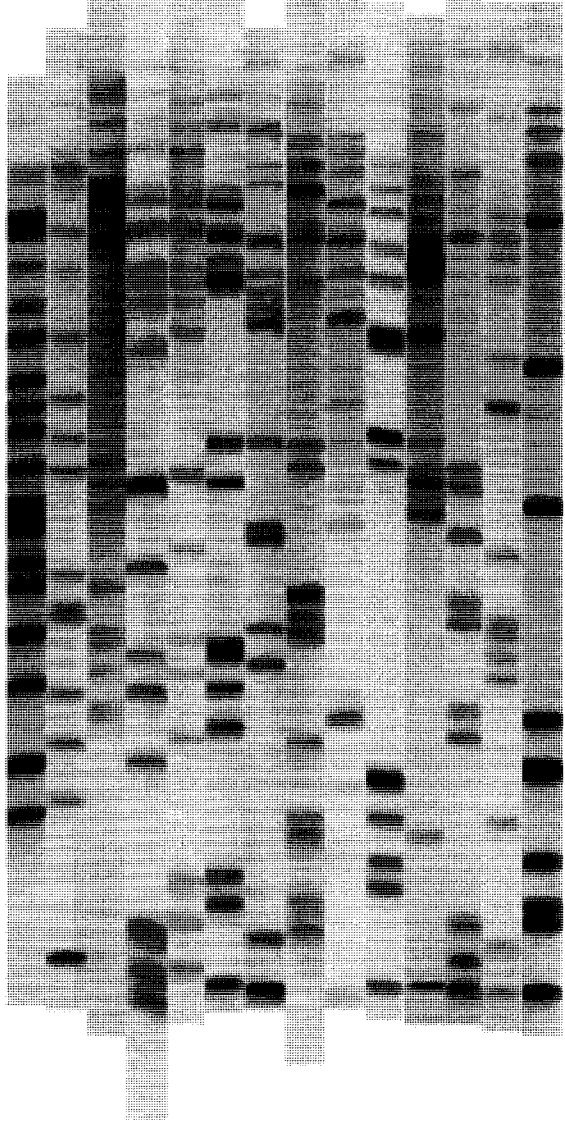
4049

4058

Figure 6. Image captured from an agarose gel after PFGE.

The dendrogram represents the cluster analysis of 14 of the *E. sakazakii* strains analyzed during this project.

3173
3232
3404
3524
3526
3411
3226
3440
3387
3395
2883
4043
4058
3168



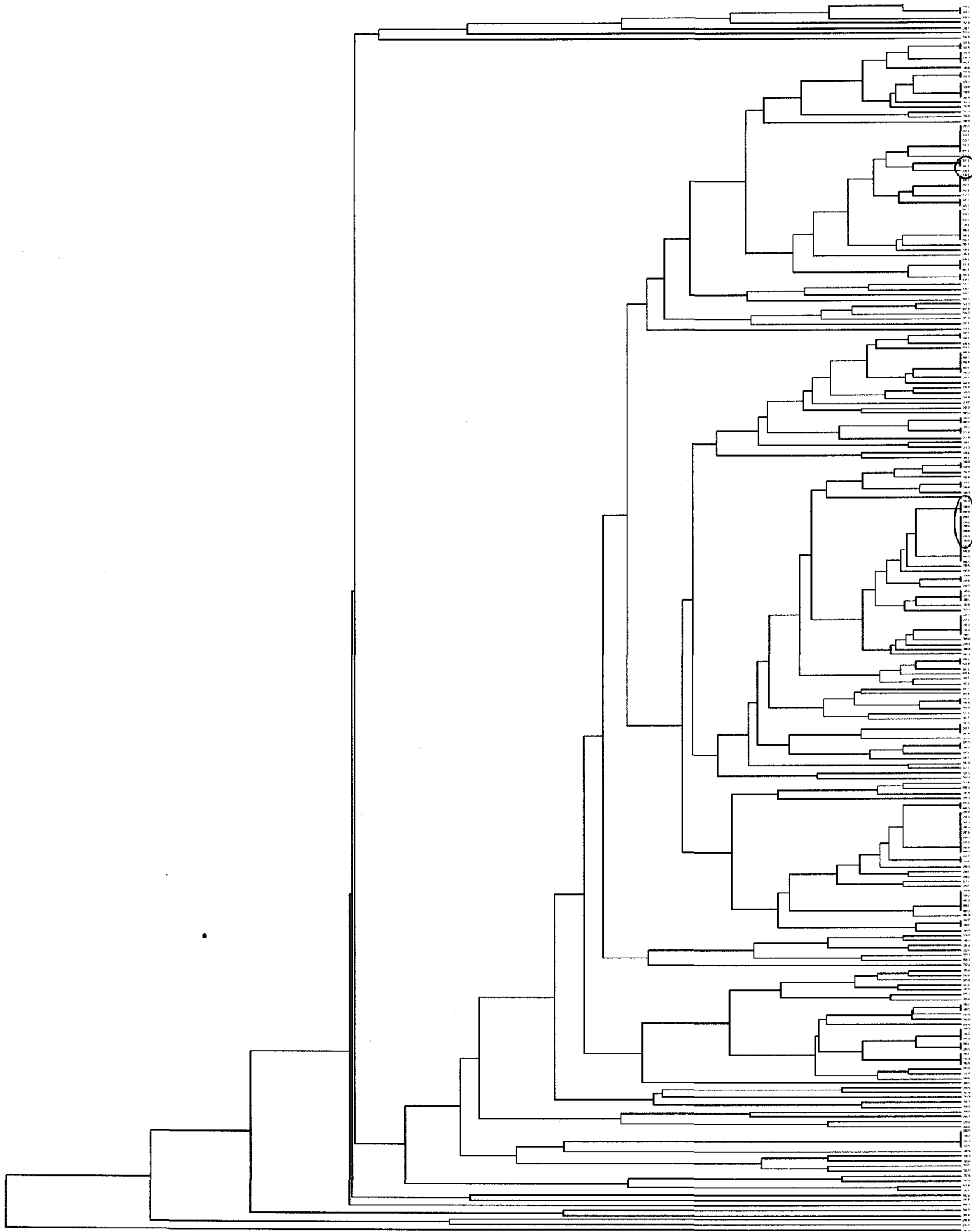
Automated Ribotyping

Fingerprints generated by the automated RiboPrinter[®] were analyzed for 240 strains. From the initial 260 strains, the DNA of 6 strains had degraded during ribotyping, and 14 strains identified as non-*E. sakazakii* by 16S rDNA were excluded in the analysis. Interestingly, the 6 strains that showed DNA degradation during ribotyping were not the same 6 that had DNA degradation during the PFGE procedure. The 240 ribotypes were analyzed using the same method as PFGE (UPGMA), generating a base similarity of 20%, with 158 clusters. The largest cluster contained 10 strains (Figures 7 and 8). Interestingly, not all 10 strains sharing the largest cluster originated from the same source. Of the 10 strains, 5 (2881, 2882, 2885, 2888 and 2889, also grouped by PFGE) were submitted by the CDC in 2002 but are of unknown origin, while strains 3208 and 3214 were submitted by the FDA, isolated from a food manufacturing site. Strains 4055, 4065 (soy flour) and 4048 (dried sodium caseinate) were isolated by the same laboratory. Strains 3427 (reconstituted PIF) and 3428 (blood), originating from a clinical case in 1999, and previously shown to be epidemiologically related, grouped together in a cluster with two other strains (3396 and 3428), which were environmental isolates submitted by the same laboratory, but isolated from different locations in Malaysia and France, respectively.

While PFGE was observed to be a more discriminatory typing method, ribotyping and 16S rDNA sequencing seemed to group the strains in a similar fashion.

Figure 7. Dendrogram revealing cluster analysis of 240 *E. sakazakii* strains from various sources by automated ribotyping.

The percentage of similarity among strains was determined using the Dice coefficient, and the clustering was performed by UPGMA.



3427
3428
3405
3396

2881
2882
2885
2888
2889
3208
3214
4048
4055
4065

Figure 8. Image captured from a nitrocellulose membrane after an automated ribotyping test.

The dendrogram represents the cluster analysis of 14 of the *E. sakazakii* strains analyzed during this project.

BAX[®] PCR System

Of the 260 strains tested by the BAX[®] PCR System (Dupont Qualicon), only 16 were negative for *E. sakazakii*. Interestingly, all these 16 strains were also identified as species other than *E. sakazakii* by automated ribotyping, and formed white (11 strains) or yellow (5 strains) colonies on TSA plates. These strains produced some white (13 strains) colonies on DFI and yellow (4 strains), white (3 strains) or blue/grey (6 strains) colonies on ESPM, suggesting the presumptive absence of α -glucosidase activity. The remaining three strains were unable to grow on ESPM or DFI. From the biochemical profiles, 2/16 strains were identified as *E. sakazakii*, one by API 20E[®] and one by ID 32E[®]. Interestingly, 9 of the 16 strains that were *E. sakazakii* negative by the BAX[®] PCR, were also identified as other species by 16S rDNA sequencing. Therefore, five strains identified as being other species by 16S rDNA sequencing were positively identified as *E. sakazakii* by the BAX[®] PCR method (Table 5). In contrast, 7 strains identified as *E. sakazakii* by 16S rDNA sequencing, were negative in the BAX[®] PCR test (Table 6).

Table 6. Strains identified as *E. sakazakii* negative by the BAX[®] PCR system

Isolate #	Source	Bax	Ribotyping	16S rDNA	API 20E [®]	ID 32E [®]	DFI	ESPM	TSA
2849	C ^a	- ^e	<i>S. warneri</i>	<i>E. sakazakii</i>	Unacceptable profile	Unacceptable profile	NG ^f	NG	W
2852	C	-	<i>S. epidermidis</i>	<i>E. sakazakii</i>	Unacceptable profile	Very good ID	NG	NG	W
2869	F ^b	-	<i>Vibrio vulnificus</i>	<i>E. sakazakii</i>	Low discrimination	Unacceptable profile	W ^g	W	Y
3172	F	-	<i>E. cloacae</i>	<i>Enterobacter</i> spp.	Good ID	<i>E. cloacae</i>	W	Y ^h	W
3173	F	-	<i>E. cloacae</i>	<i>E. hormaechei</i>	<i>E. cloacae</i>	<i>E. cloacae</i>	W	Y	W
3174	F	-	<i>E. cloacae</i>	<i>E. hormaechei</i> subsp. <i>steigerwaltii</i>	Doubtful profile	<i>E. Cloacae</i>	W	Y	W
3175	F	-	<i>E. cloacae</i>	<i>E. hormaechei</i> subsp. <i>steigerwaltii</i>	Doubtful profile	<i>E. cloacae</i>	W	B/G ⁱ	W
3244	E ^c	-	<i>Klebsiella</i> spp.	<i>E. cowani</i>	<i>Escherichia hermannii</i>	<i>Escherichia hermannii</i>	W	W	Y
3248	C	-	<i>S. thermophilus</i>	<i>Averyella</i> <i>dalhousiensis</i>	Excellent ID of the genus	Acceptable ID	W	B/G	W
3256	U	-	<i>E. hermannii</i>	<i>Enterobacter</i> spp.	Low discrimination	<i>Citrobacter</i> <i>amalonaticus/farmeri</i>	W	W	Y
3257	U ^d	-	<i>Bacillus</i> spp.	<i>E. sakazakii</i>	<i>Escherichia hermannii</i>	<i>Escherichia hermannii</i>	W	B/G	Y
3287	F	-	<i>E. cloacae</i>	<i>E. hormaechei</i> subsp. <i>steigerwaltii</i>	Very good ID of the genus	Doubtful profile	W	B/G	W
3436	F	-	No similarity	<i>E. sakazakii</i>	Doubtful profile	Low discrimination	W	B/G	W
3529	C	-	<i>E. cloacae</i>	<i>E. sakazakii</i>	Excellent ID of the genus	Excellent ID of the genus	W	B/G	W
3643	F	-	<i>E. cloacae</i>	<i>Pantoea</i> spp.	<i>E. cloacae</i>	Very good ID of the genus	W	Y	Y
3644	F	-	<i>E. faecium</i>	<i>E. sakazakii</i>	ID not valid	Acceptable ID	NG	NG	W

^a C; clinical isolate.

^b F; food isolate.

^c E; environmental isolate.

^d U; unknown source.

^e -; BAX[®] PCR negative for *E. sakazakii*.

^f NG; no growth.

^g W; white colonies.

^h Y; yellow colonies.

ⁱ B/G; blue/grey colonies.

Conclusion

Based on the phenotypic characterization of 260 *E. sakazakii* food, environmental and clinical isolates by API 20E[®], ID 32E[®], the production of yellow pigment and presumptive α -glucosidase; as well as on their genotypic characterization by PFGE, ribotyping, BAX[®] PCR and 16S rDNA sequencing, it is possible to draw some conclusions regarding the diversity of the strain set analyzed.

The identification of presumptive *E. sakazakii* isolates solely based on phenotypic characteristics (i.e., yellow pigmentation and biochemical tests) may lead to false-negative/positive results. In the present study, the identification of putative *E. sakazakii* strains by API 20E[®] and ID 32E[®] biochemical profiling very often was not consistent to their identification by the gene-based techniques used, i.e., BAX[®] PCR and 16S rDNA sequencing (Tables 5 and 6). However, as previously indicated in the introductory Chapter of this Thesis, after using Artificial Neural Networks to compare *E. sakazakii* 16S rDNA sequences with biochemical profiling, Iversen *et al.* (54) suggested that some biochemical tests (Table 1) would be more suitable than others for the differentiation of *E. sakazakii* from other *Enterobacteriaceae*. In addition, the yellow pigment was observed to not be a consistent characteristic ubiquitous in *E. sakazakii* strains, and, as previously suggested by other studies (46, 54), it should not be included for the identification of presumptive *E. sakazakii* in future guidelines and policies.

Gene-based techniques may be very useful for the detection of bacterial species that sometimes cannot be identified by phenotypic-based methods. For example, out of a total of

260 strains, 14 and 16 were not identified as *E. sakazakii* by 16S rDNA sequencing and BAX[®]PCR, respectively (Tables 5 and 6), although some of them were presumptively positive for *E. sakazakii* by phenotypic tests. The analysis of 16S rDNA sequences showed an 82% identity amongst the 260 strains tested. As previously recognized, this method is not discriminatory enough to be used as a subtyping method for epidemiological studies. However, it is an established powerful phylogenetic method and it has potential as an identification tool, as recently suggested by a few groups currently working with *E. sakazakii* (72, 76, 77).

Although the number of human cases of *E. sakazakii* are not as high as other foodborne pathogens (i.e., *E. coli*, *Salmonella*, *Listeria*, etc.), the fact that it appears to be an emerging opportunistic pathogen which affects a very fragile population, i.e., neonates, enhances the concern and the need for better understanding of this organism. The molecular typing data generated in this study indicates that PFGE is more discriminatory than automated ribotyping. However, both typing methods successfully grouped strains previously known to be epidemiologically linked/isolated from the same source (Figures 5 and 7). In general, due to its higher discriminatory power, PFGE would be the method of choice for molecular typing of *E. sakazakii* in an outbreak situation. Ribotyping would also be a good complimentary molecular method for this organism. In terms of identification, 16S rDNA sequencing is the definitive method of choice. However, for those laboratories who cannot, for whatever reason, do sequencing, a combination of phenotypic and PCR methods is recommended.

Chapter 3: Growth and Survival of *Enterobacter sakazakii* in Breast Milk With and Without Fortifiers as Compared to Powdered Infant Formula

The information presented in this chapter was presented as a poster at the 2005 Federal Food Safety and Nutrition Research Meeting (Ottawa, Canada), at the 2005 Health Canada Science Symposium (Ottawa, Canada) and at the 2006 International Association for Food Protection Meeting (Calgary, Canada). A manuscript of this work is ready and will be submitted for publication to Applied and Environmental Microbiology

Introduction

The association of *E. sakazakii* with outbreaks due to the consumption of powdered infant formula (PIF) (8, 17, 85, 106, 119), has led to the identification of high-risk populations that include newborns which have an immature immune system facilitating opportunistic infections (30). Furthermore, premature infants have more permeable gastrointestinal tracts due to a delay in feeding and the absence of natural resident gut microbiota, which in full-term infants are known to protect against pathogenic invading microorganisms (40). Equally, it is possible that the lower acidity of the newborn stomach, especially those of premature infants, represents an additional factor contributing to the survival and infection of neonates with this pathogen (30).

PIF is used as an alternative to human breast milk (BM) in providing newborns with nutritional needs either in addition to BM, or on its own when breast feeding is not possible. Although it has been previously demonstrated that *E. sakazakii* does not survive pasteurization temperatures (91), some dry PIF ingredients are added after the thermal treatment step. Thus, the presence of microorganisms is probably due to post-heat-treatment contamination from the processing environment, equipment or product mishandling. Currently, there is a push to reduce the risk of bacterial contamination of this product (30), and the behavior of *E. sakazakii* cells in dry products may be a key element to be considered in the evaluation of potential treatments for inactivating *E. sakazakii* and possibly other pathogens (30) found in PIF.

E. sakazakii has been isolated from unopened cans of PIF in concentrations ranging from 0.36 to 66 cfu/100g (51, 75, 87), and has been linked to infections in newborns. Identical molecular fingerprinting of clinical isolates from patients and of food isolates from unopened cans of the same lot as the PIF cans from which the infant had been fed, suggest a causal link between consumption of PIF and infant infection (8, 17, 85, 106, 119).

Based on the current guidelines for manipulation of PIF, BM and fortified breast milk (BMF) in the hospital settings (7), handling and fortification of BM may take place in the same environment where PIF is re-hydrated. In addition, given that in outbreak situations *E. sakazakii* has been isolated from utensils and environmental samples of hospital milk kitchens (4, 8, 9, 17, 55), we hypothesized that BM could become contaminated with *E. sakazakii* during manipulation in the hospital setting. Thus, we attempted to compare the survival and growth of *E. sakazakii* in BM and BMF at 10, 23 and 37°C, as compared to growth in PIF. Growth rates, generation (GT) and lag times (LT) were calculated. The temperatures used for this study were chosen to represent 1) the optimum temperature of growth for *E. sakazakii* (37°C), as well as temperatures close to which premature newborns are kept (30-35°C), 2) room temperature in North America (23°C), at which bottles of reconstituted PIF and of expressed BM are kept during infant feeding, and 3) an abusive refrigeration temperature of 10°C, higher than the recommended refrigeration temperature (4°C), but which may more closely represent reality. The growth of 9 different strains of *E. sakazakii* from three geographically diverse isolation sources, i.e., environmental, food and clinical settings, were assessed to determine if differences, if any, existed.

Material and Methods

Inoculum preparation

Nine *E. sakazakii* isolates were used in this study (Table 7). All strains were stored at -80°C in trypticase soy broth (TSB, Oxoid) containing 40% glycerol until time of use. Strains were streaked on TSA from the frozen stock, grown overnight, and an isolated colony was transferred to TSB for growth at 37°C for 12 h. After the period of growth, cultures previously assessed to contain approximately 10^9 cfu/ml of *E. sakazakii* by plate count, were serially diluted in peptone water to a concentration of 10 cfu/ml followed by inoculation into the growth media being tested. Samples at time zero of each growth curve were surface-spread onto TSA and DFI media to verify the bacterial load of the initial inoculum.

Table 7. Sources of *E. sakazakii* isolates used in the growth study

Isolate identification #	Source type	Source (donated from)
2855	Clinical	Hospital for Sick Children, Toronto, Canada (Health Canada)
3428	Clinical	Blood sample (Hadassah University Hospital, Dept. of Clin. Micro. & Infec. Dis., Jerusalem)
3295	Clinical	Blood sample (CDC, Atlanta, USA)
3198	Environmental	Food manufacturing plant (FDA, Maryland, USA)
3396	Environmental	(Nestle Research Center, Lausanne, Switzerland – isolated in Malaysia)
3403	Environmental	(Nestle Research Center, Lausanne, Switzerland - isolated in The Netherlands)
2871	Food	Powdered infant formula (Health Canada)
3434	Food	Lactose from infant food manufacturer (Center for Food Safety/University of Georgia, USA)
3267	Food	Powdered infant formula (Dept. of Medical Micro. Nijmegen, The Netherlands)

Growth media

Growth media were prepared and inoculated with approximately 10-100 cfu/ml of *E. sakazakii* grown overnight in TSB. Three variations of growth media were used in this study: 1) PIF, of which cans from 5 different lots of the product Similac Advance Neosure[®] were prepared with sterile water according to the manufacturer's instructions immediately before the start of each growth curve; 2) BM, donated by the Hospital for Sick Children in agreement with the Research Ethics Committee board of the Hospital. Mature BM samples were collected by the mothers (n=53) after being instructed on hygienic practices, at 8 weeks post partum and always, for standardization purposes, at the same time of the day. Sodium ascorbate (1%, Sigma) was added to each breast milk sample to prevent oxidation of vitamins (e.g., folate), and 20 ml samples were stored in sealed sterile plastic tubes at -80°C until the time of the experiment. Samples were thawed prior to use, and a single sample of BM was used as the medium for a single growth curve; and, 3) human milk fortifiers (Enfamil Human Milk Fortifier[®], 0.71g/envelope) were dissolved in BM samples to make BMF.

Growth study

A schematic of the growth study is shown in Figure 9. Briefly, a single preparation of growth medium (PIF, BM or BMF) was used for an individual growth curve. The medium of study was inoculated with an *E. sakazakii* culture diluted in peptone water (Oxoid) to approximately 10-100 cfu/ml and incubated at 10, 23 or 37°C; a non-inoculated negative control was included at all times. Growth curves at 10°C were kept in the incubator for up to

24 days, and sampled daily. Samples incubated at 23°C were sampled every 2 h and those at 37°C were sampled hourly for 24 h. For each sampling time, 1 ml samples were diluted in peptone water and plated in duplicate for bacterial plate counts. Inoculation for enumeration was done manually or using a spiral plater (WASP2, Don Whitley Scientific Limited) on tryptic soy agar (TSA, Oxoid) and Druggan-Forsythe-Iversen (DFI, Oxoid) (51) with overnight incubation at 37°C. Table 8 summarizes the number of growth curves assessed for each combination of temperature and growth medium.

Table 8: Summary of estimates for mean lag time and mean generation time parameters

Parameters calculated for three *E. sakazakii* isolate sources in three growth media at 10, 23 and 37°C

10°C				
Growth medium ^a	Isolate source	Growth curves with growth ^b	Lag time parameter (h) Est (s.e.) ^c [95% c.i.] ^d	Generation time parameter (h) Est (s.e.) [95% c.i.]
BM	Clinical	3	131.9 (10.45) [106.3, 157.5]	11.97 (3.816) [2.63, 21.31]
	Environmental	3	58.6 (29.65) [-14.0, 131.1]	27.08 (4.537) [15.98, 38.18]
	Food	3	102.9 (22.35) [48.3, 157.6]	17.03 (7.682) [-1.77, 35.83]
BMF	Clinical	1	192.2 (400.43) [-788, 1172]	59.67 (120.46) [-235, 354]
	Environmental	0	n.a. ^e	n.a.
	Food	0	n.a.	n.a.
PIF	Clinical	2	126.5 (20.57) [76.2, 176.8]	29.53 (3.402) [21.21, 37.85]
	Environmental	2	59.1 (11.23) [31.6, 86.5]	18.26 (1.014) [15.78, 20.74]
	Food	0	n.a.	n.a.
23°C				
Growth medium	Isolate source	Growth curves	Lag time parameter (h) Est (s.e.) [95% c.i.]	Generation time parameter (h) Est (s.e.) [95% c.i.]
BM	Clinical	5	2.87 (0.751) [1.36, 4.37]	0.94 (0.042) [0.85, 1.02]
	Environmental	5	3.84 (0.494) [2.85, 4.83]	0.84 (0.040) [0.76, 0.93]
	Food	3	4.86 (0.507) [3.85, 5.88]	0.75 (0.044) [0.67, 0.84]
BMF	Clinical	4	3.91 (0.665) [2.58, 5.25]	0.83 (0.049) [0.73, 0.93]
	Environmental	5	4.09 (0.674) [2.74, 5.44]	0.96 (0.056) [0.85, 1.07]
	Food	5	4.25 (0.940) [2.36, 6.13]	0.93 (0.058) [0.82, 1.05]
PIF	Clinical	5	4.49 (0.442) [3.60, 5.37]	0.71 (0.036) [0.64, 0.79]
	Environmental	6	3.86 (0.429) [3.00, 4.72]	0.71 (0.030) [0.65, 0.77]
	Food	6	4.67 (0.440) [3.78, 5.55]	0.71 (0.035) [0.64, 0.78]
37°C				
Growth medium	Isolate source	Growth curves	Lag time parameter (h) Est (s.e.) [95% c.i.]	Generation time parameter (h) Est (s.e.) [95% c.i.]
BM	Clinical	6	1.22 (0.701) [-0.18, 2.62]	0.51 (0.032) [0.45, 0.58]
	Environmental	3	2.19 (0.331) [1.53, 2.86]	0.42 (0.029) [0.36, 0.48]
	Food	3	3.22 (0.345) [2.53, 3.91]	0.33 (0.034) [0.26, 0.40]
BMF	Clinical	3	2.27 (0.538) [1.19, 3.34]	0.41 (0.039) [0.33, 0.49]
	Environmental	4	2.44 (0.588) [1.26, 3.62]	0.54 (0.047) [0.44, 0.63]
	Food	2	2.60 (0.905) [0.79, 4.41]	0.51 (0.053) [0.40, 0.61]
PIF	Clinical	6	2.84 (0.201) [2.44, 3.24]	0.29 (0.023) [0.25, 0.34]
	Environmental	6	2.22 (0.220) [1.78, 2.66]	0.29 (8.57×10 ⁻³) [0.27, 0.31]
	Food	5	3.02 (0.219) [2.58, 3.46]	0.29 (0.021) [0.24, 0.33]

^a Growth medium; BM, human breast milk; BMF, fortified human breast milk; PIF, reconstituted powdered infant formula.

^b At 10°C, *E. sakazakii* did not grow in all cases, and thus the LT and GT could not be estimated.

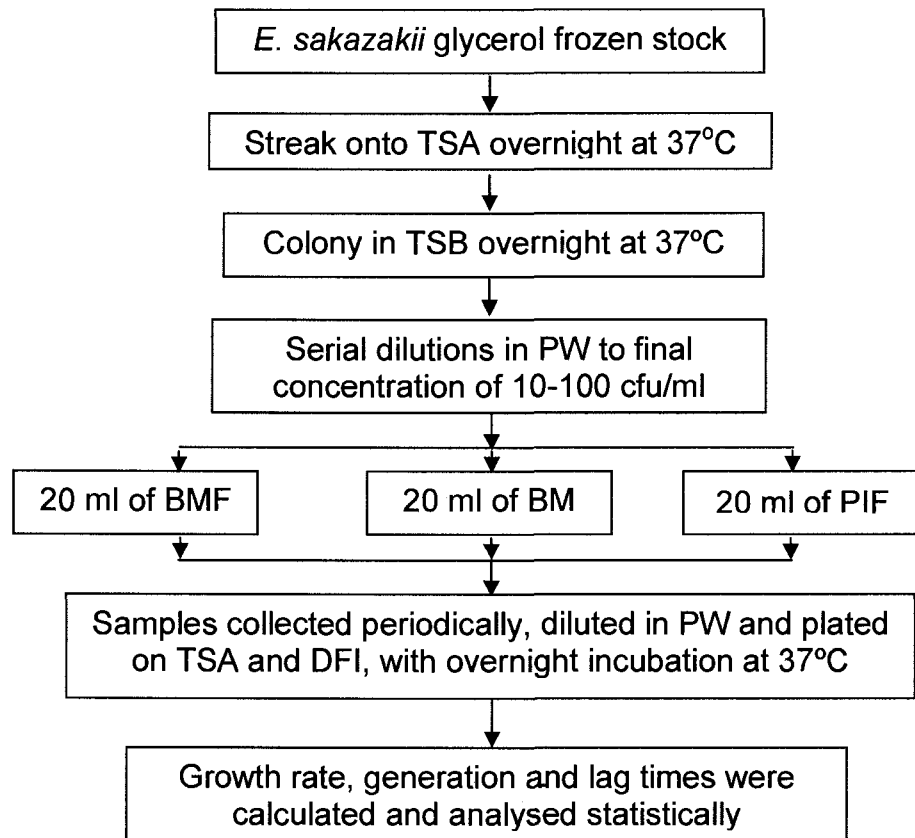
^c c.i.; Confidence interval.

^d s.e.; Standard error.

^e n.a.; Data not available due to the impossibility of fitting the modified Gompertz model to the raw data.

Figure 9. Schematic of the method used to study the growth of *E. sakazakii* in PIF, BM and BMF.

***E. sakazakii* growth in reconstituted powdered infant formula (PIF), human breast milk (BM) and human breast milk with fortifiers (BMF) was assessed at 10, 23 and 37°C.**



Statistical methods

Statistical analyses were done in two steps, first, the modified Gompertz model (mGompertz) was fit to each individual growth curve in order to generate a collection of GTs and LTs which were then analyzed by a mixed effects method. This method consisted of modeling the GTs and LTs with respect to temperature, growth media, isolate source and replicate growth curves. For comparison purposes, the 3-segment model (2) and the Baranyi model (2, 3, 6) were also fit to the individual growth curves. Furthermore, for growth curves at 10°C, the hypothesis of growth versus no growth was tested by fitting the mGompertz model with the additional constraint that when the GT approached zero, it was assumed that no growth took place. Data collected from plate counts were transferred to SAS[®] 8.02.

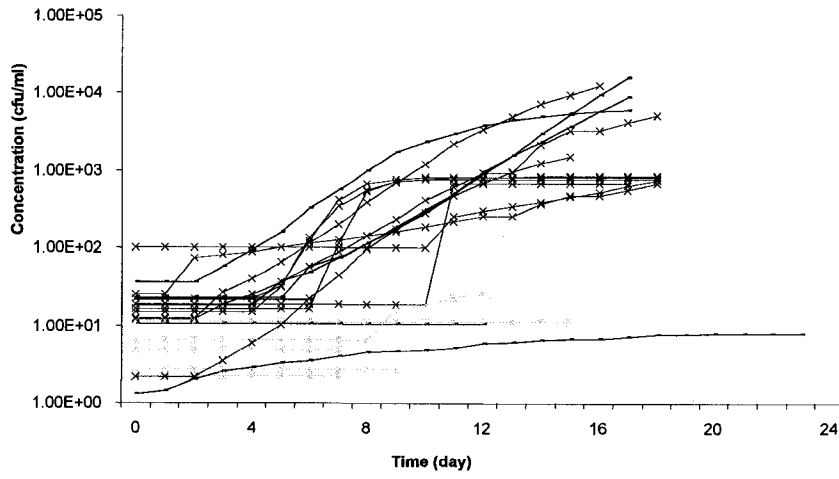
Final data set

A total of 112 individual growth curves were constructed in a completely randomized design. From these, 105 were retained for statistical analysis since 7 were rejected due to background growth or insufficient enumeration points. Figure 10 illustrates all growth curves and Table 8 summarizes the number of growth curves assessed for every combination of growth medium and temperature, as well as the average LTs and GTs generated by the statistical analysis of the model fit to the collection of growth curves.

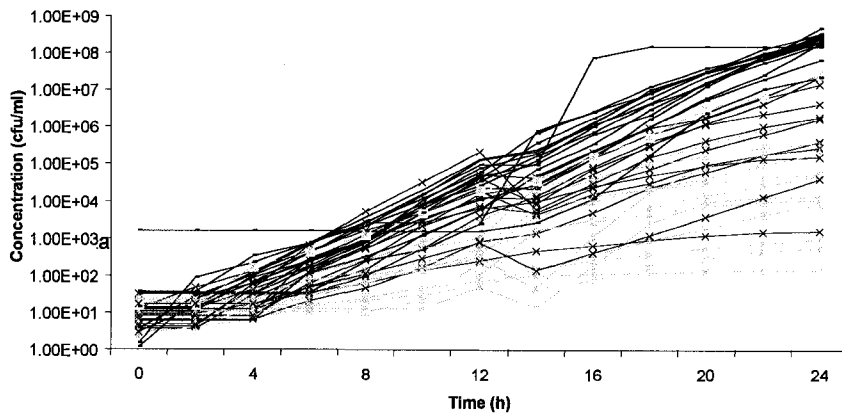
Figure 10. The modified Gompertz model fitted to all growth curves of *E. sakazakii* isolates in all media.

Where: red, human breast milk; yellow, human breast milk with fortifiers and blue, reconstituted powdered infant formula. A) 10°C; B) 23°C and C) 37°C.

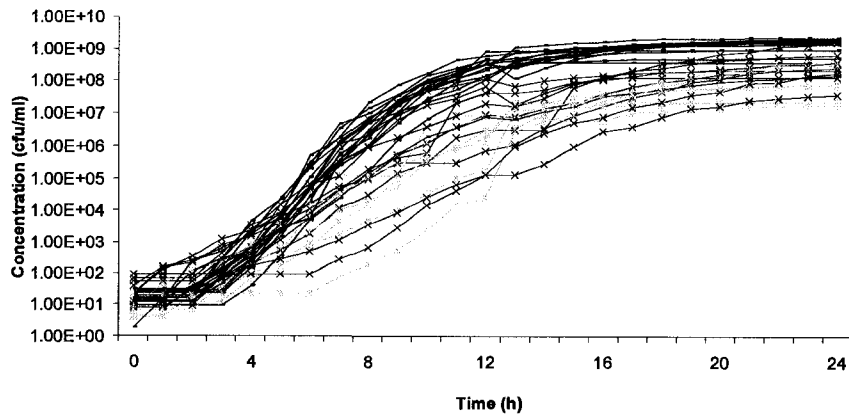
A) Collection of growth curves at 10°C



B) Collection of growth curves at 23°C



C) Collection of growth curves at 37°C



Results

Growth

The variability in concentration of *E. sakazakii* among growth curves was observed to increase with time for growth at 23 or 37°C (Figure 10B). Among the 23 curves obtained at 10°C (Figure 10A), 9 curves showed a decline or no growth. After a short LT, the 44 curves done at 23°C demonstrated the growth of *E. sakazakii* through most or all of the observation period. The 38 curves at 37°C showed a short lag LT for *E. sakazakii*, with the organism reaching a maximum population density soon after 10-12 h (Figure 10C).

The GT and LT parameters calculated for each growth curve are presented in Table 8 where GTs for *E. sakazakii* had increasingly smaller coefficients of variation (c.v.) for the growth curves in the order, 37°C<23°C<10°C. The GTs showed smaller c.v. for growth curves in BM and PIF than in BMF, while c.v. of the LTs were smaller among growth curves in PIF than in BM or BMF. The GTs and LTs had c.v. of the same size among growth curves from all isolate sources.

Generation time

GTs were estimated using the mGompertz model fit to the raw data obtained by plate counts, calculated for a total of 91 growth curves. At 10°C, *E. sakazakii* growth occurred in 14 of the 23 (61%) trials. When growth occurred, GTs were long, ranging between 11.97 h [2.63, 21.31] 95% c.i., and 29.53 h [21.21, 37.85] 95% c.i. (Table 8). The GTs differed at 10°C among the various medium-isolate source combinations, indicating a shorter GT for some

isolates than others in BM, and a longer GT for a single clinical isolate in BMF. In all cases, growth occurred at 23°C and 37°C. GTs were 0.42 h longer (0.42 h [0.36, 0.48 h] 95% c.i.) at 23°C than at 37°C for all isolates in all media and were 0.16 h shorter (0.16 h [0.13, 0.20] 95% c.i.) in PIF than in BM and BMF. At 23°C and 37°C, the GTs for the growth of *E. sakazakii* in BMF were slightly longer than in BM ($p=0.04$) (BMF–BM=0.06 h [0.004, 0.12] h 95% c.i.). Averaged among temperatures and growth media, the GTs of the isolates were not significantly different ($p=0.53$), but strains isolated from different isolate sources had significantly different GTs when grown in PIF, BMF or BM ($p<0.001$). At 10°C, GTs for *E. sakazakii* ranged from a mean value of 11.97 h for clinical isolates in BM to 59.67 h for a single clinical isolate in BMF, but no growth was observed for any of the environmental or food isolates cultured in BMF. The average GTs among all temperatures was longer for environmental and food isolates in BMF than in BM, while for the clinical isolates, the average GT was longer in BM. For all isolate sources, the average GT was shorter when *E. sakazakii* was growing in PIF than in BM or BMF.

Lag time

In the same way that GT parameters were calculated for the data collected from the *E. sakazakii* growth curves, LTs were derived from 77 growth curves using the mGompertz model fit to the *E. sakazakii* plate counts observations. In some cases, the LT could not be calculated, for reasons related to experimental data or to statistics analysis, i.e., the growth curve fitting algorithm failed to converge, a standard error could not be estimated for the LT, or there was a lack of plate count data at early observation times.

When growth did occur at 10°C, LTs were long, with significant differences among media and among isolate sources (Table 8). The LTs for *E. sakazakii* in BM were significantly longer than in PIF, with clinical strains having longer LTs than environmental or food isolates.

As compared to 37°C, longer LTs were observed at 23°C in all media ($p=5\times 10^{-4}$), with differences in LTs among different isolate sources ($p=0.009$) in the relationship $\text{food}_{\text{LT}} > \text{clinical}_{\text{LT}} > \text{environmental}_{\text{LT}}$. LTs for *E. sakazakii* growing at 23°C were 1.6 h (± 0.41) [0.84, 2.5] h (95% c.i.) longer than for growth curves at 37°C. Mean differences in LTs between food and environmental isolates were significant at the 5% level.

Discussion and Conclusions

In the context of this study, *E. sakazakii* was observed to grow in all media studied at 23°C and 37°C, and in BM and PIF at 10°C (Figure 10). At 10°C, considered an “abusive refrigeration temperature”, growth of *E. sakazakii* with long LTs and GTs was observed in PIF and BM. Refrigeration temperatures, not surprisingly, are the most appropriate for storage of hospital and home prepared formula prior to consumption.

While growth was only observed in 1/7 trials performed in BMF at 10°C, *E. sakazakii* cells could still be recovered up to 12 days after inoculation. The microorganism survived at 10°C in all media for a minimum of 12 days, with GTs ranging from 11.97 ± 3.82 h to 27.08 ± 4.54 h and 18.26 ± 1.01 h to 29.53 ± 3.40 h in BM and PIF, respectively. Clearly, this reaffirms the need for aseptic and proper handling of PIF to control *E. sakazakii*. The American Dietetic

Association (ADA) storage recommendations of 2-4°C for 24 or 48 h for PIF and BM/BMF (7), respectively, are supported by the data from this study.

The growth in PIF yielded higher population densities than those in BM or BMF at all temperatures (Figure 10). At 23°C, *E. sakazakii* populations in BMF were lower than those observed in the other two media (Figure 10B), whereas at 37°C, no important differences in maximum population density were found between BM and BMF (Figure 10C). The reduced population density in BMF as compared to BM at 23°C and the absence of growth of *E. sakazakii* at 10°C in BMF could possibly be related to the nutrient content of the fortifiers, which may affect the growth of *E. sakazakii*. Chan (16) previously reported the effects of powdered human milk fortifiers on the growth of *Staphylococcus aureus*, *Escherichia coli*, Group B *Streptococci* and *E. sakazakii* using the paper filter method (i.e., a disk of filter paper soaked in breast milk with and without fortifiers and placed on the surface of an agar media previously swabbed with bacteria), as well as assessing the growth inhibiting properties of BM or BMF added to a liquid bacterial culture. Chan noted that while BM alone and BM supplemented with Similac Human Milk Fortifier[®] (0.35 mg of iron, 1.8 g of carbohydrates and 0.36 g of fat when added to 100 ml of BM) inhibited bacterial growth, BM containing Enfamil Human Milk Fortifier[®] (1.44 g of iron, <0.4 g of carbohydrates and 1.0 g of fat when added to 100 ml of BM) did not. Our data clearly showed that BM fortified with Enfamil Human Milk Fortifier[®] had some inhibitory effect(s) on the growth of *E. sakazakii* when compared to BM. Jocson *et al.* (57) suggested that nutrient fortification and storage duration may change some of the host defense properties of BM, thereby allowing bacterial proliferation to occur. It is possible that the time of collection of BM in our study,

as well as its storage, may have contributed to the growth of *E. sakazakii* observed in BM. The BM samples used herein were collected from complete expression of a single breast 8 weeks post-partum in order to ensure mature BM samples were obtained prior to involution of the mammary gland. It has been demonstrated that mature BM chemical composition may change during the course of lactation, although not as markedly as in the early weeks (i.e., colostrum and transitional milk) (80). Equally, for standardization purposes, the mothers were requested to express the BM at the same time of the day, since BM may vary in its fat and fat soluble components (e.g., vitamins A, E and zinc) within this period (26). Furthermore, sodium ascorbate (1%) was added to each BM sample to prevent oxidation of vitamins (e.g., folate), and samples were frozen at -80°C until time of use. The protection of vitamins by sodium ascorbate may have played a role in the extent of *E. sakazakii* growth observed in our study. Moreover, although storage at -80°C is known to be better in terms of protecting antimicrobial host defense than -20°C, frozen storage of the samples may have led to a loss of antimicrobial and host defense properties which are characteristic of BM, and thus may have facilitated the growth of *E. sakazakii* (57).

The average LTs among food isolates of *E. sakazakii* was longer in BM than in BMF at 23 and 37°C, while clinical and environmental isolates had longer LTs in BMF at both temperatures. Interestingly, the average GTs of the clinical isolates were longer in BM than in BMF at 23 and 37°C, as compared to the environmental and food isolates, whose GTs were shorter in BM than in BMF. This indicates that isolates from different sources may have varying LTs, depending on the environment from which they originate (Table 8).

Guidelines on storage and “hang time” (i.e., the amount of time a formula is kept at room temperature in the feeding bag and accompanying lines during enteral tube feeding) for PIF and BM with and without fortifiers have been issued by the ADA (7). The ADA recommends 4 h as the maximum time for BM (fortified or not) to be kept at room temperature (25°C) before and during feeding (7). In fact, based on the data generated in the present study, under normal conditions, an extremely short or no LT would need to exist in order for there to be significant growth of *E. sakazakii* during the recommended maximum 4 h “hang time” at room temperature. However, pre-term infants who are kept in incubators at higher temperatures (30-35°C), depending on their weight, may have their feed exposed to higher temperatures during the feeding period. For those cases, further evaluations of the most adequate “hang time” would be important, considering that the LTs obtained in our study for *E. sakazakii* growth at 37°C in the three media assessed ranged from 1.22 (± 0.70) to 3.02 (± 0.22) h.

Telang *et al.* (111) reported that 10^2 to 10^3 cfu/ml of *E. sakazakii* (ATCC 29544) inoculated into reconstituted PIF, BM and BMF and kept at 23°C for 6 h did not show a significant increase in numbers. However, extrapolating the GTs at 23°C from our study to the initial inoculum used by Telang *et al.* (111) in BM, BMF or PIF, an increase of approximately 2-logs of *E. sakazakii* after 6 h of growth would be expected in the absence of a lag time (Figure 10B). However, it is possible that the cells used in the Telang study had a longer LT, since they prepared the inoculum from 24-48 h stationary cultures of *E. sakazakii*.

In a recent study, Kandhai *et al.* (59) investigated the lag time, specific growth rate, and maximum population density of *E. sakazakii*. They used a clinical, environmental, and food isolate, along with the ATCC 29544 strain, and came up with optimal temperatures of growth and growth rates of 39.4°C and 2.31/h, respectively. Their estimated LT varied from 83.3±18.7 h at 10°C to 1.73±0.43 h at 37°C, while in our study (Table 8), the estimated LT for *E. sakazakii* grown in PIF ranged from 59.1±11.23 h to 126.5±20.57 h at 10°C and from 2.22±0.22 h to 3.02 ±0.22 h at 37°C. They further noted that 1) cells harvested from PIF at different phases of growth did not exhibit significant differences in either specific growth rate or LT, 2) there was no difference in LTs among the 4 *E. sakazakii* strains studied, and 3) that LTs were surprisingly short given that the cells had spent 3 to 10 days in dry PIF.

A dose response is not currently available for *E. sakazakii* infections in humans. Iversen *et al.* (48) hypothesized that a total of 1000 cfu of *E. sakazakii* could theoretically cause illness in humans. However, this was based on the minimum infectious doses currently assumed for *Neisseria meningitidis*, *E. coli* O157:H7 and *L. monocytogenes* serovar 4b. They further suggested that based on the average initial number of 0.36 - 66 cfu/100 g of *E. sakazakii* reported in PIF cans (87, 90) and the recommended average portion of 18 g of powder reconstituted for one single infant feeding, 14 generations would be needed to reach a level of 1000 cfu. Under these assumptions, our generation time data suggests that while in PIF, 4 h (at 37°C) and 9 h (at 23°C), would be necessary for this proposed minimum infectious dose of 1000 cfu to be reached; in BM and BMF, 7 and 7.5 h, respectively, would be needed at 37°C, and 13 h at 23°C (in both BM and BMF) if the same initial microbial load was present (Figure 10, Table 8).

In conclusion, we have shown for the first time that *E. sakazakii* can grow in BM and in BMF. While BM has never been reported to naturally harbour *E. sakazakii*, once contaminated, it could support its growth. In fact, in a case of *E. sakazakii* meningitis recently described in Brazil, contaminated BM was suspected (5). In addition, Stoll *et al.* (110) described another case of *E. sakazakii* infection in a premature infant that had been fed only sterile ready-to-use infant formula and BM. Our study also reinforces the importance of good hygienic practices in hospitals and in the household when preparing, handling or storing prepared reconstituted powdered infant formula, and when expressing human breast milk, with or without the addition of fortifiers.

**Chapter 4: Development of a Non-Primate Animal Model for *Enterobacter sakazakii*
infection**

The information presented in this chapter was presented as a poster at the 93rd Annual Meeting of the International Association for Food Protection (August 13-16, 2006. Calgary, Canada)

Introduction

Enterobacter species are known as opportunistic pathogens which generally cause infection in debilitated patients with an underlying illness, patients on immunosuppressive medication or those taking antimicrobial agents, all factors that may facilitate the colonization of *Enterobacter* spp. in humans (10). A member of the genus *Enterobacter*, *Enterobacter sakazakii* has been associated with infant gastroenteritis, bacteremia and meningitis, linked in many cases to the consumption of reconstituted powdered infant formula (8, 9, 17, 87, 94, 106, 117, 119). It is possible that the immature neonatal immune system may increase the risk of acquiring an *E. sakazakii* infection. However, it is not known exactly what host and environmental factors need to be present in order to cause infection in neonates.

In a first attempt to investigate the pathogenesis of *E. sakazakii*, (previously conducted in this laboratory), Vero, CHO and Y-1 cell lines were infected with *E. sakazakii* supernatant free of bacterial cells, and suckling mice were inoculated orally and via i.p. with up to 10^8 cells of *E. sakazakii*. Interestingly, not all *E. sakazakii* isolates tested demonstrated cytopathic effects against the cell lines and some of the strains that tested positive in the enterotoxin assay done with suckling mice, did not cause any cytopathic effects in the cell lines tested (97). It is possible that the results obtained in these experiments indicate that; 1) there may be differences in receptor-toxin binding in addition to a complex regulation system; 2) *E. sakazakii* may produce more than one cytotoxin or; 3) perhaps horizontal gene transfer events involving the transfer of genes encoding for cytotoxin(s) (and/or for related genes), occurs only in certain isolates (97). These results may also explain why some infants are reported to be colonized by *E. sakazakii*, but do not develop any symptoms of infection.

An *in vitro* assay described by Iversen *et al.* (49) suggests that *E. sakazakii* is toxic to N2a neuroblastoma cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) cell proliferation assay. They theorized that protease, phosphatase, and lipase activities may contribute to host cell death during an *E. sakazakii* infection. Recently, *in vitro* assays have shown the ability of *E. sakazakii* to adhere to epithelial cells (19, 78). Not surprisingly, as observed for most bacterial pathogens, this bacterium seems to be able to adhere to cells, an important first step in pathogenesis. Collado *et al.* (19), demonstrated the adherence of *E. sakazakii* type strain (ATCC 29544) to immobilized human intestinal mucus and its displacement by bifidobacteria. Furthermore, Mange *et al.* (78), described the adhesive properties of 50 *E. sakazakii* strains, at different phases of growth, to human epithelial (Hep-2 and Caco-2) and brain microvascular (HBMEC) cell lines. They observed two distinctive adherence patterns, diffuse and localized clusters of adhesion to the three cell lines. *E. sakazakii* adherence to the cell lines increased with a higher multiplicity of infection (MOI) and was maximal at the late bacterial exponential growth phase. Interestingly, adhesion to the epithelial and endothelial cells was suggested to be mostly non-fimbrial based. Hurrell *et al.* (43) initiated a comparative investigation into a range of potential virulence factors such as attachment and invasion in CaCo-2 cell lines, macrophage uptake (using human macrophage-like cell line U937), serum resistance studies, as well as the potential role of motility and capsule formation. They compared the virulence of various *Enterobacteriaceae* species to *E. sakazakii*, and found that the latter strains had different potential for invading cells (ranging from 5×10^4 to 2.5×10^5 cfu/well) and were all less invasive than *S. typhimurium* and *C. freundii*. As for macrophage survival, two *E. sakazakii*

strains had similar survival rates to *C. freundii* (approximately 30% of the inoculum was found intracellular within 24 h). In the serum resistance assay, capsule formation and motility tests, Hurrell *et al.* (43) reported that the *E. sakazakii* strains tested were diverse, in that they were all motile, but serum resistance and capsule formation were not consistent phenotypes.

The mechanism(s) that *E. sakazakii* uses to reach the brain and cause meningitis has not yet been well investigated. However, Sondheimer *et al.* (108) showed that 2-week-old infants have more acidic gastric pH (ranging from 2.9 to 5.2) than 1-week-old infants (ranging from pH 4.6 to 5.8), while Dinsmore *et al.* (23) and Mehall *et al.* (79) demonstrated in the neonatal rabbit model that less acidic conditions in the gut facilitated the translocation of *E. cloacae* from the intestine to the mesenteric lymph nodes, spleen and cecum. Since most of the *E. sakazakii* infections involve newborns, it is possible that the lower acidity in the neonatal gastrointestinal (GI) tract plays a role in the colonization and translocation of this organism. Once *E. sakazakii* makes its way past the GI tract to the blood stream, it likely attaches to endothelial cells and then somehow crosses through the blood brain barrier (BBB) to infect the meninges and brain.

The objective of this study was to find a suitable animal model to study the pathogenesis of *E. sakazakii*, allowing further investigation of mechanism(s) of virulence, as well as to provide a means to extrapolate from observations of animal models to the human neonate infected with *E. sakazakii*. Initially, young animals (pigs, chicks, rabbits, guinea pigs and gerbils) were orally challenged with three *E. sakazakii* isolates which were previously

phenotypically and genotypically characterized. Furthermore, in an attempt to more closely simulate human infection in young neonates, 1 to 2-day old neonatal rats and gerbils were also challenged.

Material and Methods

Inoculum Preparation

Three *E. sakazakii* strains, two clinical and a food isolate, were used for the animal challenge studies (Table 9). These strains were chosen by a sub-committee from the International Life Sciences Institute North America (ILSI-NA), the funding agency for this project. The strain selection was based on the phenotypic and genotypic characterization of the *E. sakazakii* strains as presented in Chapter 1 of this study.

For the inoculum preparation, the three strains were grown from a frozen stock made of tryptone soy broth (TSB) containing 40% glycerol, onto tryptic soy agar (TSA) overnight, and then a pure colony was inoculated into freshly reconstituted powdered infant formula (PIF) for oral challenge of young animals. TSB was used to grow the strains for the oral challenge of neonatal animals and the intraperitoneal (i.p.) inoculation of both neonatal and young animals. Bacterial cultures were grown for 12 h to obtain a concentration of 10^9 cfu/ml and dilutions in peptone water were surface-spread onto TSA and Druggan-Forsythe-Iversen agar (DFI) media for colony enumeration. All PIF used for this study was previously tested to confirm the absence of *E. sakazakii*.

For the oral challenge of young animals, *E. sakazakii* cultures grown in PIF were aliquoted in 1 ml amounts and kept on ice until the time of use. For the oral challenge of the neonatal animals, TSB cultures of *E. sakazakii* were aliquoted in 1 ml samples, centrifuged at 14,500 × g for 5 min, the supernatant removed, and the pellets re-suspended in 10 µl freshly prepared reconstituted PIF. For the challenge via i.p., and for the oral challenge of the 1-day old chicks, aliquots of 1 ml of *E. sakazakii* cultures were pelleted as described above, and re-suspended in 85% sterile saline solution.

All samples were kept on ice until the time of use. The time between sample preparation and animal inoculation never exceeded 1 h.

Table 9. Sources of *E. sakazakii* isolates used for the animal study

Isolate #	Type	Source
2855	Clinical	Sick Kids Hospital, Toronto, Canada (Health Canada)
3290	Clinical	CSF isolate (Tennessee (TN), USA)
2871	Food	Powdered infant formula (Health Canada)

Animal Challenge

This animal study was approved by the Animal Ethics Committee of the Animal Disease Research Institute (ADRI) and Canadian Food Inspection Agency (CFIA).

Young pigs, chicks, New Zealand white rabbits, guinea pigs, neonatal Sprague-Dawley rats, young and neonatal Mongolian gerbils were used for this study. All animals were anesthetized with isoflurane prior to inoculation. Young animals received oral inoculation via a 1-ml feeding syringe, while the infant models were orally inoculated via a P-20 Pipetman. The i.p. inoculations were given with a 1-ml syringe. After the observation period, young animals were euthanized with pentobarbital, while the neonates were euthanized by CO₂ inhalation, according to the animal care committee guidelines. All post-mortem procedures for the organs' collection were assisted by a double-blinded pathologist from the CFIA animal facilities, who participated in the visual examination of the organs for abnormalities. Every phase of the animals' challenge study was performed by the student as part of the project for her Masters of Science Degree. While at the CFIA animal facilities, the student was monitored at all times by animal care technicians during the inoculation and euthanasia procedures, and by an animal pathologist during the post mortem examinations.

Young Animals

The young pigs, chicks and guinea pigs used in this study were bred in the ADRI/CFIA facilities (Ottawa, Canada). Young rabbits and gerbils were purchased from Charles Rivers Laboratories at Saint-Constant (Quebec, Canada) and Wilmington (Massachusetts, USA),

respectively. Young animals, for the purpose of this study, were recently weaned from the mothers, and were from 3-to-5 weeks of age depending on the species. The young animals received from the Charles Rivers laboratory were maintained in opaque polypropylene cages and supplied with food, water and environmental conditions to satisfy their needs for a week, allowing for recovery from the change of location and adaptation to the new environment, prior to being challenged.

Sixteen young animals of each species were studied at a time and were divided into four groups of four animals (each group was maintained in a separate cage). Each group was inoculated orally with 1 ml of PIF containing 10^9 cells of one single *E. sakazakii* isolate (Table 9). One of the four groups of animals was kept as a negative control, receiving 1 ml of reconstituted PIF free of bacteria. Animals were observed up to 14 days post-inoculation (p.i.), with two animals of each group being euthanized on day 7 p.i. After euthanasia, organs (brain, heart, liver, spleen, kidney, the mesentery and intestines) were collected, examined for any pathological abnormalities and microbiologically analyzed for the presence of *E. sakazakii*. Animal feces were also collected for microbiological analysis throughout the period of observation.

Neonatal Animals

Pregnant rats and gerbils were purchased from Charles Rivers Laboratories at Saint-Constant (Quebec, Canada) and Wilmington (Massachusetts, USA), respectively. The female rats were 14-16 days pregnant, while the gerbils were 20-21 days pregnant at the time of shipping. Both species had their litters 7-8 days after arrival.

The rat and gerbil litter sizes varied from 10 to 14 pups, and 6 to 9 pups, respectively. The infants were left with their mothers and all neonatal animals of the same litter (except two that were kept as negative controls), were orally inoculated with 10 µl of PIF containing 10^9 cells of a single *E. sakazakii* strain at the age of 2-days old (Table 9). Negative control groups inoculated with reconstituted PIF free of bacteria were included at all times, and all animals were marked with a permanent pen, corresponding to the strain they were inoculated with. In parallel to the oral inoculation, five neonatal gerbils (three tests, two controls) were inoculated i.p. with 10^9 cells of *E. sakazakii* food isolate 2871.

While all neonatal rats were observed for 7 days p.i., neonatal gerbils were divided into two groups, the first being euthanized on day 2 p.i. and the other on day 7 p.i.. Organs (brain, heart, liver, spleen, kidney and intestines) were collected post-euthanasia, examined for pathological abnormalities and microbiological analysis done to verify the presence of *E. sakazakii*.

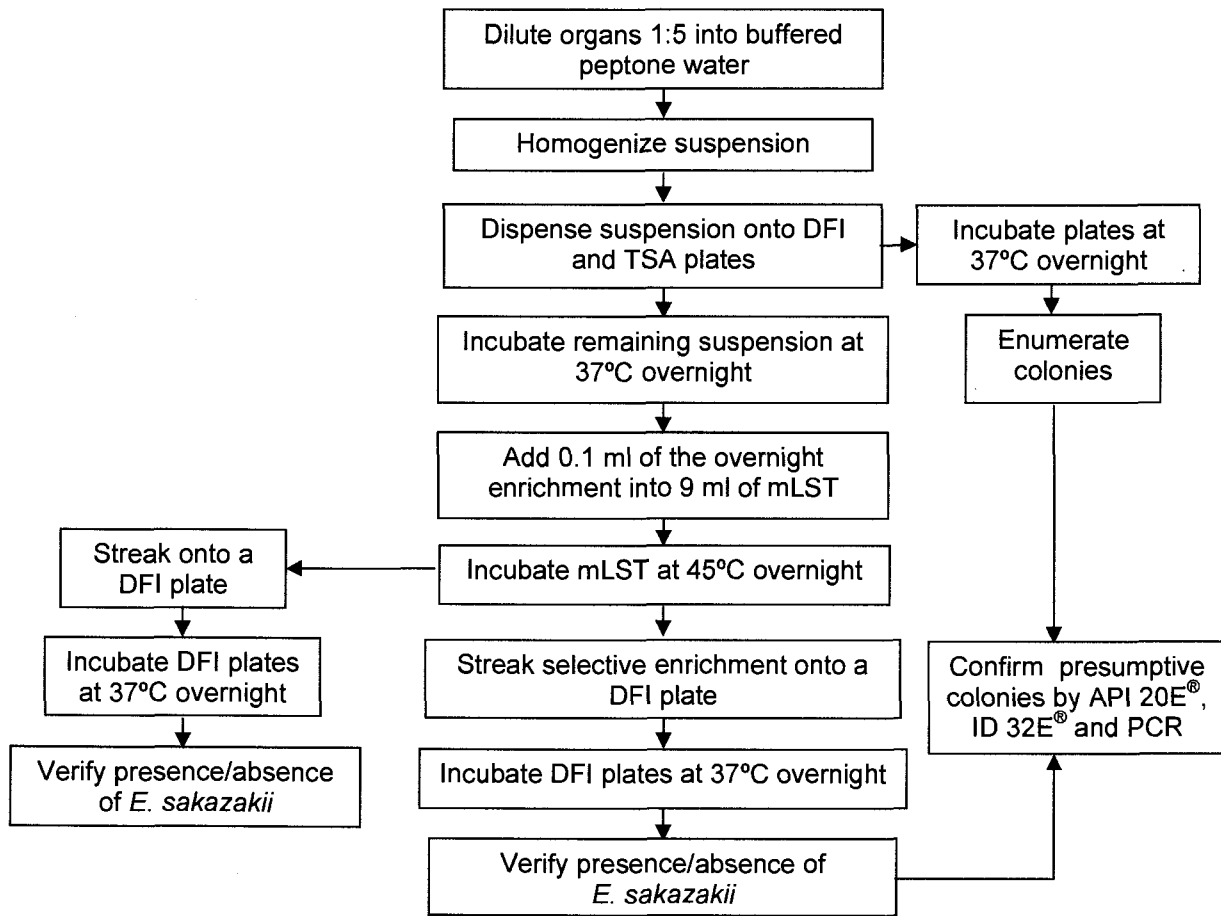
Microbiological Analysis of Animal Organs and Feces

Organs

A schematic of the steps followed for the organ analysis is shown in Figure 11. All organs were kept at -20°C until the time of analysis, then diluted 1:5 with sterile buffered peptone water (BPW, Oxoid), homogenized and dispensed onto TSA and DFI plates, surface-spread and incubated at 37°C overnight for colony enumeration. The remaining suspension was incubated at 37°C overnight to recover potentially damaged *E. sakazakii* cells, 0.1 ml of

which was transferred to 9 ml of modified lauryl sulphate (mLST) broth and incubated overnight at 45°C. The selective enrichment was streaked onto a DFI plate and incubated at 37°C overnight for verification of the presence/absence of *E. sakazakii* in the organs.

Figure 11. Flow chart of the protocol used for analyzing fecal samples and animal organs for the presence of *E. sakazakii*.



Fecal samples

The same method followed for the organ analysis was used for the fecal samples (Figure 11). However, after the initial dilution and homogenization in BPW, the suspension was streaked onto DFI for verification of the presence/absence of *E. sakazakii*, instead of surface spreading for colony enumeration. All fecal samples were kept at -20°C until the time of analysis.

Confirmation tests for presumptive *E. sakazakii* colonies

Yellow colonies on TSA and green colonies on DFI were treated as presumptively-positive for *E. sakazakii* colonies and were confirmed by API 20E[®] and ID 32E[®] tests, as well as by 16S rDNA PCR, with primers targeting a specific region of the *E. sakazakii* 16S rDNA, as previously described by Lehner *et al.* (72).

Results

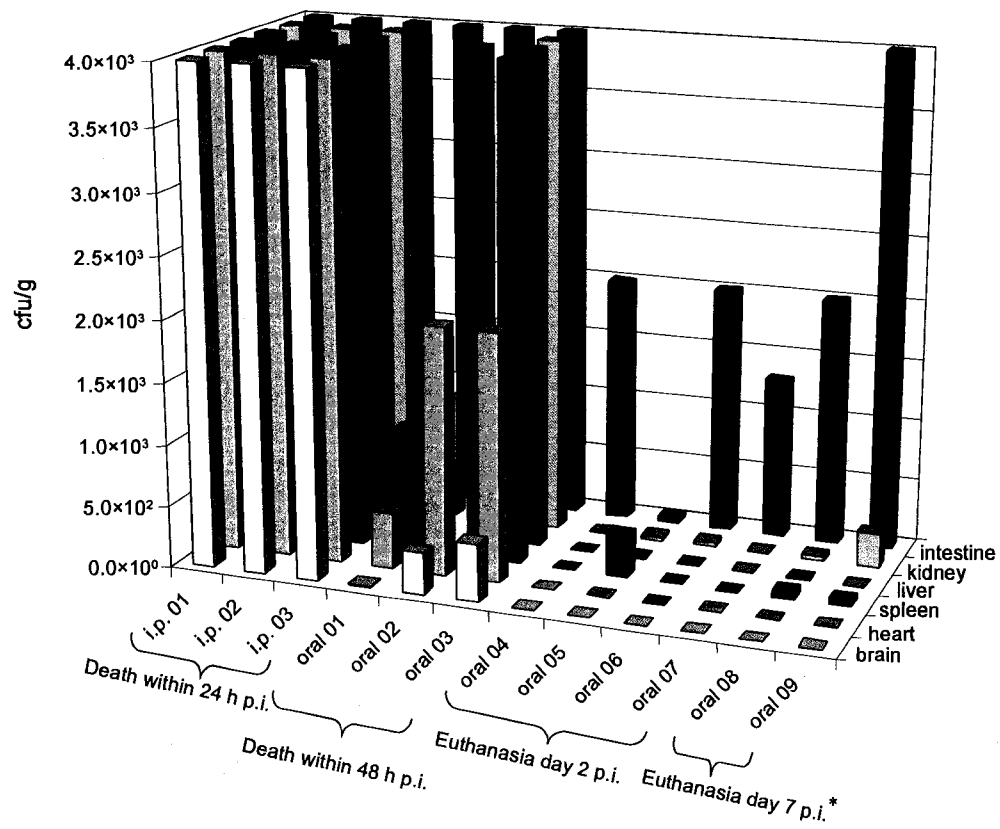
None of the young animals challenged with *E. sakazakii* via the oral or i.p. route showed any symptoms of infection. While some of the organs collected from young gerbils and chicks after euthanasia were positive for *E. sakazakii*, none of the organs collected from pigs, rabbits or guinea pigs harboured *E. sakazakii*. Among the neonatal models used in this study, the rats appeared to be healthy until day 7 p.i., when they were euthanized, and none of their organs contained *E. sakazakii*. However, among the 26 infant gerbils challenged in this study, six died within 48 h p.i., three that had been challenged via the oral route and three by i.p. injection *E. sakazakii* was isolated from the dead animals' organs, as well as from the organs collected after euthanasia of the remaining 20 infant gerbils (Table 10, Figure 12). *E. sakazakii* was not isolated from any of the young or neonatal animals kept as negative controls, or from any of the controls which died during the course of this experiment.

Of the 14 infant gerbils inoculated with *E. sakazakii* food isolate 2871, 9 received an oral inoculation. Among these, three died within 48 h p.i. and contained higher *E. sakazakii* levels in all organs examined, when compared to organs of animals euthanized on days 2 or 7 p.i. (Table 10, Figure 12). The three neonatal gerbils which died within 48 h p.i. contained 1.6×10^1 , 3.3×10^2 and 4.6×10^2 cfu/g of *E. sakazakii* in their brains, while only one of the other six infant gerbils which survived until euthanasia contained *E. sakazakii* in the brain (3.0 cfu/g). The hearts of the three former infant gerbils contained 4.5×10^2 , 2.0×10^3 and 2.0×10^3 cfu/g of *E. sakazakii*, while among the euthanized animals, only one heart was positive for *E. sakazakii* (5.0 cfu/g). The three neonatal gerbils which died within 48 h contained *E. sakazakii* in the spleen at levels at least 3-times higher than in the latter six

gerbils. All infant gerbils (9/9) inoculated orally contained *E. sakazakii* in their kidneys (at levels ranging from 30 to $>3.0 \times 10^3$ cfu/g), as well as in the intestines ($\geq 1.0 \times 10^3$ cfu/g).

All infant gerbils inoculated i.p. with *E. sakazakii* food isolate 2871 died within 24 h p.i., and contained greater than 1.0×10^3 *E. sakazakii* cells/g in all organs examined (Table 10, Figure 12).

Figure 12. The presence of *E. sakazakii* in the organs of neonatal gerbils after oral and intraperitoneal (i.p.) inoculation with 10^9 cells of strain 2871.



* p.i., post-inoculation

Table 10. The presence of *E. sakazakii* in the organs of neonatal gerbils after oral and i.p. inoculation with 10^9 cells of strain 2871

Time of death/euthanasia	Mode of inoculation	Neonatal gerbil number	Brain (cfu/g)	Heart (cfu/g)	Spleen (cfu/g)	Liver (cfu/g)	Kidney (cfu/g)	Intestine (cfu/g)
24 h p.i. ^a (death)	i.p. ^d	01	TNTC ^e	TNTC	TNTC	TNTC	TNTC	TNTC
	i.p.	02	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
	i.p.	03	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC
48 h p.i. (death)	Oral	04	2×10^1	5×10^2	1×10^3	1×10^3	1×10^3	TNTC
	Oral	05	3×10^2	2×10^3	NC ^b	TNTC	NC	TNTC
	Oral	06	5×10^2	2×10^3	TNTC	TNTC	TNTC	TNTC
48 h p.i. (euthanasia)	Oral	07	NG ^c	NG	1.0×10^0	NG	5.0×10^0	2×10^3
	Oral	08	NG	NG	3.4×10^2	2.0×10^0	3×10^1	4×10^1
	Oral	09	NG	NG	1.4×10^1	1.0×10^0	3×10^1	2×10^3
	Oral	10	3.0×10^1	5.0×10^0	NG	NG	5.0×10^0	1×10^3
7 days p.i. (euthanasia)	Oral	11	NG	NG	6×10^1	3.0×10^0	3×10^1	2×10^3
	Oral	12	NG	NG	5×10^1	NG	3×10^2	TNTC

^a p.i.; Post inoculation.

^b NC; Not collected.

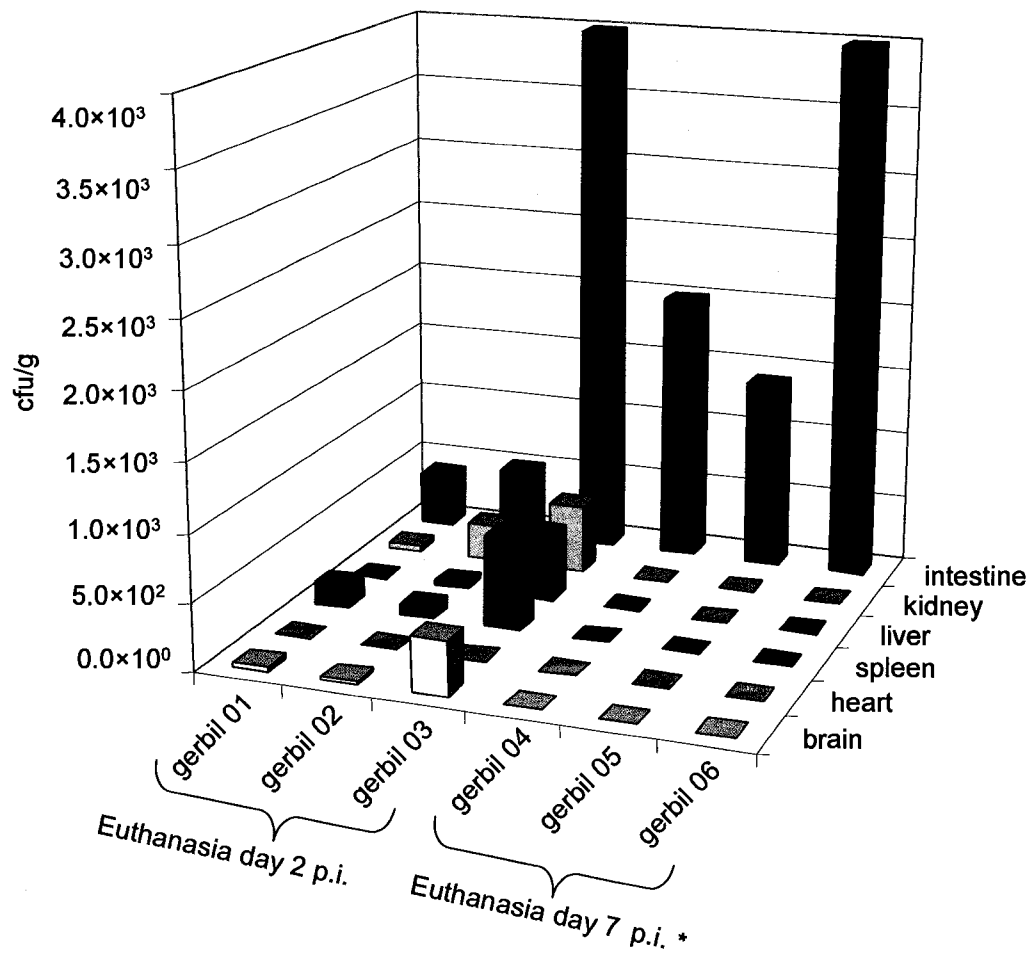
^c NG; No growth.

^d i.p. ; Intraperitoneal.

^e TNTC; Too numerous to count.

Six infant gerbils were challenged with the *E. sakazakii* clinical isolate 3290 via oral inoculation, of which the bacteria could be recovered from all organs (except the liver and heart of one animal) of the three animals euthanized on day 2 p.i. Similar to infant gerbils challenged with food isolate 2871, the intestine was the organ found to contain the highest levels of *E. sakazakii*. After the intestine, *E. sakazakii* was recovered at greatest concentrations from the spleen, followed by the kidney, liver and the brain (Table 11, Figure 13). However, of the animals euthanized on day 7 p.i., with the exception of the intestine, which contained more than 1.4×10^3 cfu/g of *E. sakazakii*, most of the other organs were negative for *E. sakazakii*. The exceptions were a single kidney that contained low cell counts (3.0 cfu/g), and the kidneys from a second animal which were positive for *E. sakazakii* only upon selective enrichment in mLST. An important difference was observed among organs collected from animals on day 2 and day 7 p.i from the neonatal animals inoculated with clinical strain 3290. Interestingly, most of the organs from the neonatal gerbils euthanized on day 2 p.i. contained *E. sakazakii*, while only the intestines and the spleen from one of the three gerbils euthanized on day 7 p.i., were positive for this bacterium (Table 11, Figure 13). However, this did not occur with animals inoculated with the other two isolates of *E. sakazakii* (Figures 12 and 14). Among the 6 infant gerbils inoculated with *E. sakazakii* clinical isolate 2855, only one was positive for the organism in the brain after microbiological analysis. Interestingly, all the other organs, with exception of the heart and the kidney of one animal euthanized on day 7 p.i., contained *E. sakazakii* (Table 12, Figure 14). The intestine, similar to observations of all other neonatal animals challenged, had higher *E. sakazakii* levels. The spleen, liver and heart also contained relatively high counts (10^2 cfu/g).

Figure 13. The presence of *E. sakazakii* in the organs of neonatal gerbils after oral inoculation with 10^9 cells of strain 3290.



* p.i., post-inoculation

Table 11. The presence of *E. sakazakii* in the organs of neonatal gerbils after oral inoculation with 10^9 cells of strain 3290

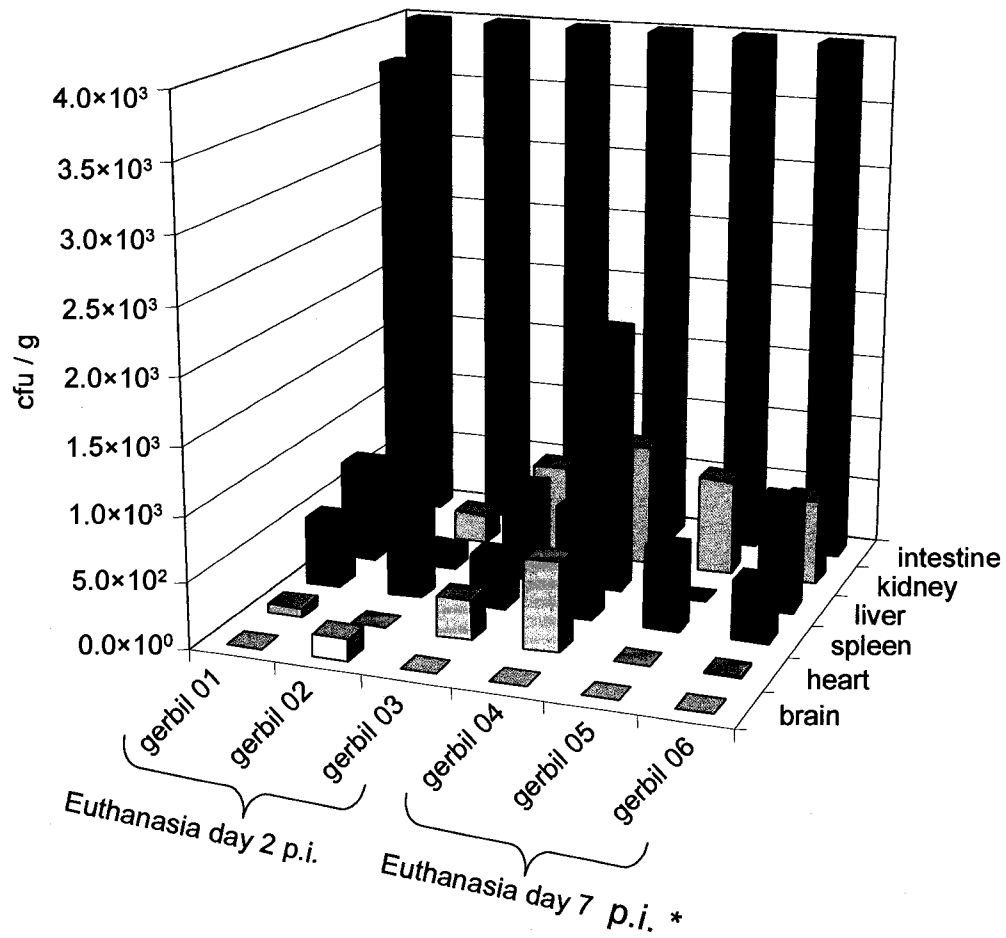
Time of euthanasia	Neonatal gerbil number	Brain (cfu/g)	Heart (cfu/g)	Spleen (cfu/g)	Liver (cfu/g)	Kidney (cfu/g)	Intestine (cfu/g)
2 days p.i.	01	3×10^1	NG ^a	2×10^2	NG	4×10^1	TNTC ^b
	02	2×10^1	5	8×10^1	4×10^1	3×10^2	5×10^2
	03	4×10^2	8	7×10^2	5×10^2	5×10^2	TNTC
7 days p.i.	04	NG	NG	NG	NG	+ ^c	2×10^3
	05	NG	NG	NG	NG	3	1×10^3
	06	NG	NG	NG	NG	NG	TNTC

^a NG; No growth.

^b TNTC; Too numerous to count.

^c +; Positive upon selective enrichment.

Figure 14: The presence of *E. sakazakii* in the organs of neonatal gerbils after oral inoculation with 10^9 cells of strain 2855



* p.i.; post inoculation

Table 12. The presence of *E. sakazakii* in the organs of neonatal gerbils after oral inoculation with 10^9 cells of strain 2855

Organ		Brain (cfu/g)	Heart (cfu/g)	Spleen (cfu/g)	Liver (cfu/g)	Kidney (cfu/g)	Intestine (cfu/g)
Animals euthanized on day 2 p.i.	Infant gerbil 01	NG ^a	7×10^1	5×10^2	8×10^2	6×10^2	TNTC ^b
	Infant gerbil 02	2×10^2	5	TNTC	1×10^2	2×10^2	TNTC
	Infant gerbil 03	NG	3×10^2	4×10^2	7×10^2	7×10^2	TNTC
Animals euthanized on day 7 p.i.	Infant gerbil 04	NG	7×10^2	9×10^2	2×10^3	9×10^2	TNTC
	Infant gerbil 05	NG	NG	6×10^2	NG	7×10^2	TNTC
	Infant gerbil 06	NG	1×10^1	5×10^2	9×10^2	7×10^2	TNTC

^a NG; No growth.

^b TNTC; Too numerous to count.

In the young gerbil model, important strain differences were observed in the levels of *E. sakazakii* isolated from the various organs (Figure 12, 13 and 14). Clinical isolate 3290 was isolated more often from the animal organs than the other two isolates, and also in higher concentrations ($>3.0 \times 10^3$ cfu/g) from the brains of the two animals euthanized on day 7 p.i.. Isolate 2871 was isolated only upon enrichment from the brains collected within the same period, and from one of two brains collected on day 14 p.i. (5.0×10^1 cfu/g). Only one out of four animals inoculated with strain 2855, had a brain positive for *E. sakazakii* (2.5×10^1 cfu/g, on day 7 p.i.). Next to the brain, the highest levels of strain 3290 were found in the liver (10^3 cfu/g), but none of the livers from the animals challenged with strain 2855 were positive, and only two of four livers from animals inoculated with isolate 2871, were positive on day 7 p.i. upon selective enrichment. Of the 12 young gerbil hearts collected for analysis, *E. sakazakii* was isolated from two of the animals challenged with strain 3290 on day 7 p.i., and from one inoculated with strain 2871, but in the latter case, only after selective enrichment. As for the spleens, four out of 12 were positive, i.e., those inoculated with *E. sakazakii* strains 2855 (1 spleen), 3290 (2 spleens) and 2871 (1 spleen). Only the kidneys of animals challenged with isolate 3290 were found to contain *E. sakazakii* in numbers ranging from 8.5×10^1 to 3.0×10^2 cfu/g, on day 7 p.i. With the exception of the brain of one young gerbil inoculated with strain 2871 that contained 5.0×10^1 cfu/g on day 14 p.i., and the intestine of one young gerbil inoculated with strain 2855 that was positive for *E. sakazakii* upon selective enrichment, all other organs of all animals were negative for *E. sakazakii* on day 14 p.i. (Figure 14).

Interestingly, none of the intestines of the four young gerbils inoculated with isolate 3290 were positive for *E. sakazakii*, while the intestines of animals inoculated with isolates 2855

(2/4) and 2871 (3/4) were positive for *E. sakazakii* after selective enrichment. However, their fecal samples were positive until day 7 p.i.

Similar to the findings in young gerbils, the *E. sakazakii* strain that was most often recovered from young chicks' organs was clinical strain 3290 (Figure 13). This strain was isolated after selective enrichment (mLST) from the liver, pro-ventriculus, gizzard and intestine of one chick on day 7 p.i.. Only one organ was positive upon direct plating, the brain of a chick inoculated with strain 2871 (5 cfu/g) on day 14 p.i.. The same strain was isolated after selective enrichment from the heart and gizzard of a chick, on day 7 and 14 p.i., respectively. Clinical isolate 2855 was only isolated from the gizzard of one chick after selective enrichment.

Table 13. The recovery of *E. sakazakii* in the organs of young gerbils on days 7 and 14 post-inoculation

Time of euthanasia	Animal (<i>E. sakazakii</i> strain)	Brain (cfu/g)	Heart (cfu/g)	Spleen (cfu/g)	Liver (cfu/g)	Kidney (cfu/g)	Intestine (cfu/g)
day 7 p.i. ^a	Gerbil 01 (strain 2855)	2.5×10 ¹	NG	NG	NG	NG	+
	Gerbil 02 (strain 2855)	NG ^b	NG	1.6×10 ²	NG	NG	+
	Gerbil 03 (strain 2871)	+ ^c	NG	NG	+	NG	+
	Gerbil 04 (strain 2871)	+	+	+	+	NG	+
	Gerbil 05 (strain 3290)	>3.0×10 ³	5.0×10 ²	3.3×10 ²	1.6×10 ³	3.0×10 ²	NG
	Gerbil 06 (strain 3290)	>3.0×10 ³	1.0×10 ¹	9.0×10 ¹	1.1×10 ³	8.5×10 ¹	NG
day 14 p.i.	Gerbil 07 (strain 2855)	NG	NG	NG	NG	NG	NG
	Gerbil 08 (strain 2855)	NG	NG	NG	NG	NG	NG
	Gerbil 09 (strain 2871)	5.0×10 ¹	NG	NG	NG	NG	NG
	Gerbil 10 (strain 2871)	NG	NG	NG	NG	NG	+
	Gerbil 11 (strain 3290)	NG	NG	NG	NG	NG	NG
	Gerbil 12 (strain 3290)	NG	NG	NG	NG	NG	NG

^a p.i.; Post inoculation.

^b NG, No growth.

^c +; Positive upon selective enrichment.

Table 14. The recovery of *E. sakazakii* in the organs of 1-day old chicks on days 7 and 14 post-inoculation

Time of euthanasia	Animal (<i>E. sakazakii</i> strain)	Brain (cfu/g)	Heart (cfu/g)	Pro-ventriculus (cfu/g)	Liver (cfu/g)	Gizzard (cfu/g)	Intestine (cfu/g)
day 7 p.i. ^a	Chick 01 (strain 2855)	NG ^b	NG	NG	NG	+	NG
	Chick 02 (strain 2855)	NG	NG	NG	NG	NG	NG
	Chick 03 (strain 2871)	NG	NG	NG	NG	+	NG
	Chick 04 (strain 2871)	NG	NG	NG	NG	NG	NG
	Chick 05 (strain 3290)	NG	NG	+ ^c	+	+	+
	Chick 06 (strain 3290)	NG	NG	+	+	NG	NG
day 14 p.i.	Chick 07 (strain 2855)	NG	NG	NG	NG	NG	NG
	Chick 08 (strain 2855)	NG	NG	NG	NG	NG	NG
	Chick 09 (strain 2871)	5×10 ⁰	+	NG	+	NG	NG
	Chick 10 (strain 2871)	NG	NG	NG	NG	NG	NG
	Chick 11 (strain 3290)	NG	NG	NG	NG	NG	NG
	Chick 12 (strain 3290)	NG	NG	NG	NG	NG	NG

^a p.i.; Post inoculation.

^b NG; No growth.

^c +; Positive upon selective enrichment.

Discussion and Conclusion

Due to a lack of available animal models for the study of *E. sakazakii* pathogenesis, this study was designed to screen several non-primate species in order to identify the most suitable model that could be used to extrapolate results to human infections. From the results obtained in this study, young pigs, rabbits and guinea pigs were not seen as good candidates to mimic the infectious process seen in human neonates. However, young chicks and gerbils were seen as potentially useful models, while neonatal gerbils seem to be the most promising. Interestingly, neonatal rats are currently used as models for neonatal meningitis studies, and another group successfully conducted a study of *E. sakazakii* infections (113). However under the conditions of this study, neonatal rats did not seem to be suitable for investigating the pathogenesis of *E. sakazakii*.

Infant Sprague-Dawley rats have been used for more than three decades as animal models of neonatal bacterial meningitis. These animals are recognized models for meningitis caused by *E. coli* K1, and *Citrobacter* species (33, 65, 121), and are often inoculated on day 2 of age via the oral, intranasal (i.n.), i.p. or cardiac route. It appears that, similar to observations of human infant clinical cases, *Citrobacter* meningitis in these animal models is age dependent, wherein 5-day old rats are resistant to infection as compared to 2-day old rats (65). Neonatal rats inoculated both i.n. (10^5 cfu) and i.p. (10^5 cfu) with *Citrobacter koseri* have been reported to develop bacteremia, meningitis and death within 24-72 h, with some of the animals surviving up to day 8 p.i. (65); at this point the brain abscesses were more advanced than early on in infection. Both *Citrobacter* and *E. coli* K1 meningitis appear to be hematogenous, since there is a correlation between the levels of bacteremia and the

developments of meningitis (121). The infection of the meninges caused by these two pathogens seem to occur more often (in both human infants and neonatal rat model), when the levels of bacteria in the blood are $>10^3$ cfu/ml (65, 121). This type of data is currently not available for *E. sakazakii* infections. Due to the similarities that exist among *E. sakazakii* and the latter organisms which also cause neonatal meningitis, some of the mechanisms of pathogenesis established for *Citrobacter* and *E. coli* K1, may be similar in *E. sakazakii*.

To-date, no data on dose-response models for *E. sakazakii* in humans are available. Iversen *et al.* (48) hypothesized a minimum infectious dose of 1000 cfu, based on a comparison with *E. coli* O157:H7 and *L. monocytogenes* serotype 4b. A previous study conducted in this laboratory suggested that a large number of *E. sakazakii* cells (10^8 cfu) were required to cause infection in a suckling mice model (97). Extrapolating from that data, a high dose of *E. sakazakii* may also be required to cause infection in human neonates. Interestingly, two of the clinical *E. sakazakii* isolates tested in that study, one being positive and the other negative in the *in vitro* assay using cell lines, had the lowest minimum lethal i.p. doses, and were non-lethal at oral doses. This could indicate that some *E. sakazakii* strains may lack the virulence factors that would allow them to survive hostile conditions, such as the colonization and translocation of the intestinal tract. Nevertheless, epidemiological data of *E. sakazakii* neonatal infections linked to consumption of PIF indicate low concentrations of the bacteria initially present in PIF can lead to illness. For example, Simons *et al.* (106) reported *E. sakazakii* and *E. cloacae* populations of 8 cfu/100g and 48 cfu/100 g, respectively, in powdered formula linked to neonatal infection cases. In those cases, the strains isolated from the infants and from the powdered formula had similar plasmid and multilocus enzyme

profiles (17). Thus, in some cases, it seems that low levels of *E. sakazakii* in PIF can lead to infection in neonates (17, 106). However, another important point to consider would be the possibility of infant formula being mishandled during preparation or storage, thus facilitating the growth of *E. sakazakii* to potentially high levels. Extrapolating from the work done to-date on animal models (97), it appears that high levels of the organism are necessary to cause illness.

In the present study, all animals were challenged with the same dose (10^9 cfu). Although none of the young gerbils died or presented visible symptoms of infection, many of them contained *E. sakazakii* cells in their organs (Table 13). However, infant gerbils (6/26) died within 48 p.i., and *E. sakazakii* was isolated from all the infant gerbils inoculated. Because *E. sakazakii* was isolated from all infant gerbils when using a high dose, further investigations are warranted to see if lower doses can also cause infection in infant gerbils. Furthermore, additional studies with this model would be interesting regarding the different indicators and end-points that could be considered during investigations of *E. sakazakii* pathogenesis.

The food isolate (2871) and clinical isolate (2855) used in this study have previously been used by Pagotto *et al.* (97) to assess *E. sakazakii* pathogenesis by the suckling mouse assay. They observed that food isolate 2871 (MNW2 in Pagotto *et al.* (97)) and clinical isolate 2855 (SK81 in Pagotto *et al.* (97)) were the only isolates, out of the 18 tested, that caused death of the mice when administered orally. While isolate 2871 was the only one to cause the death of neonatal gerbils in the present study after oral inoculation with 10^9 cells (Table 10), in the study by Pagotto *et al.* (97), 3 of 4 and 1 of 4 neonatal mice died after oral inoculation with

this same isolate at concentrations of 10^8 and 10^7 cfu, respectively. Moreover, strain 2855 was among the five isolates that caused the death of suckling mice when inoculated via the i.p. route at a lower dose (10^7 cfu). However, in the study by Pagotto *et al.* (97), isolate 2871 and 2855 did not test positive in the suckling mice enterotoxin assay, which is based on the ratio of intestine-to-carcass weight. In our study, strain 2871 was isolated more often than strain 2855 from the young gerbils on day 7 p.i., but only after selective enrichment. Interestingly, clinical isolate 3290, not included in the study by Pagotto *et al.* (97), was found more often in the organs of the young chicks, and in higher numbers from the organs of the young gerbils, as compared to strains 2871 and 2855. While there were no major difference in the levels of the three strains in the intestines and brains of neonatal gerbils, strain 2855 was observed more often and in higher numbers in the spleens, livers, hearts and kidneys.

Among the features that allow *E. coli* K1 to survive hostile environments, are the K1 capsular polysaccharide, O-lipopolysaccharides (O-LPS) and outer membrane protein A (OmpA). It has been shown that the deletion of *E. coli ompA* results in significant reduction of bacteremia in neonatal rat models (121). Interestingly, it has recently been demonstrated that the *ompA* of *E. sakazakii* had a high degree of homology to the *ompA* genes of other gram-negative *Enterobacteriaceae* (81).

Townsend *et al.* (113) orally challenged neonatal rats with 10^8 cells of *E. sakazakii* with or without a dosing with LPS. They found cerebrospinal fluid (CSF) to be positive for *E. sakazakii* in 25% of the animals challenged with the bacteria only, and for 40% of the animals in which *E. sakazakii* inoculation was preceded by a LPS dose. Interestingly, despite

all putative similarities between *E. sakazakii*, *C. koseri* and *E. coli* K1, and in contrast to the results obtained by Townsend *et al.* (113), in the present study *E. sakazakii* was not isolated from any of the neonatal rats orally inoculated with 10^9 cells, nor did the animals present any symptom of infection. Although the technique used for the oral inoculation of the neonatal rats in the present study was very similar to that used by Townsend *et al.* (113), the microbiological methods used to analyze the organs may have been approached differently. In addition, the *E. sakazakii* strains chosen for each of the studies could also have caused a difference in the results.

Since 1996, however, gerbils have been a well established model for *Helicobacter pylori* infection (66), and have also been used as a model for *Streptococcus pneumoniae* meningitis. However, this is the first time that gerbils have been used as a model for Gram-negative neonatal meningitis. From the results obtained in this study, the neonatal gerbil model seems to be a suitable model for studies of *E. sakazakii* infection. Furthermore, the young gerbils, perhaps with some further investigations, may also give useful information, since *E. sakazakii* caused infection and was recovered from the organs of both young and neonatal gerbils. Interestingly, *E. sakazakii* could not be recovered from some of the intestines (7/12) of the young gerbils, even when their fecal specimens were positive for the bacteria up to day 7 p.i. We theorize that this could be due to the background microbiota present in the intestine, which may compete with *E. sakazakii* for growth on the laboratory media. Moreover, since *E. sakazakii* was recovered from the intestines of 5/12 young gerbils, it is possible that variability exists amongst the young animals intestinal microbiota. This variability should be less important in the neonatal gerbils, since they are only fed by the

mother and have been less exposed to the environment. Further studies using the neonatal gerbil model will be important in order to investigate if differences among *E. sakazakii* isolates could be consistently identified in challenge studies, as well as to improve the protocol used herein.

The causes of the differences in numbers of *E. sakazakii* cells isolated from the organs of the different animal models in this study remain unclear. Factors such as previous exposure of young gerbils to *Enterobacter* spp. from the environment, may have played a role in the clearing of *E. sakazakii* cells. However, the neonatal gerbils were inoculated at day 2 of age and most likely did not live long enough to develop any immunocompetent cells. In order to better clarify the reason for the recovery of different levels of *E. sakazakii* from the organs of neonatal gerbils, aspects such as amount of mother's milk being fed to the neonates or the timing of inoculation in relation to the feeding time, could be further assessed.

General Discussion and Future Work

During the 2004 expert meeting on *E. sakazakii* and other microorganisms of concern in powdered infant formula (30) held jointly by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), a list of recommendations to the scientific community and infant formula manufacturers was released. This present research project addressed some of those research needs and has contributed towards the use of internationally validated detection, identification and molecular typing methods for *E. sakazakii*.

Other relevant findings of this thesis work are; 1) *E. sakazakii* is able to survive and grow in human breast milk (whether fortified or not), at 10, 23 and 37°C, as well as it does in reconstituted powdered infant formula at 23 and 37°C, and; 2) the development of an *in-vivo* model to allow a better understanding of the virulence factor(s) and the mechanism(s) of pathogenesis used by this emerging opportunistic pathogen to cause infection in human neonates.

The research design in this thesis consisted of the largest set of *E. sakazakii* strains ever typed by internationally validated molecular methods by one laboratory. The extensive phenotypic and genotypic characterization of 260 *E. sakazakii* strains is of great importance to increase our understanding of this microorganism. A critical assessment of the methods currently being used for the isolation and identification of *E. sakazakii* was also done during this project.

The results obtained by PFGE and automated ribotyping, techniques internationally recognized for epidemiological studies, showed a vast diversity among the 260 *E. sakazakii* strains assessed. Between the two typing methods, PFGE was found to be most discriminatory, grouping the strains into 188 clusters while ribotyping grouped the same strains into 158 clusters. Thus, PFGE would be the recommended method for typing *E. sakazakii* strains during outbreaks or sporadic cases, where links need to be made between clinical isolates and either environmental or food isolates. PFGE could also be applied for environmental control in hospitals or food manufacturing sites when contamination of food products is suspected. Ribotyping could be a useful alternative and complementary method for molecular typing of *E. sakazakii*.

A laboratory-based electronic network for molecular typing of *E. sakazakii* is not currently available. The database established during this study, which will be published for the information of the scientific and communities working with *E. sakazakii*, represents the potential beginning of work towards an active *E. sakazakii* network among laboratories. This will hopefully lead to an improvement in the identification and control of *E. sakazakii* infections.

As for all of the identification methods assessed herein, the sequencing of 1 kb of the 16S rDNA which resulted in 82% identity among strains, was found to be the most accurate technique. Only 4 of the 260 strains identified as *E. sakazakii* gave less than 90% identity, while the 14 strains that were recognized as other bacterial species varied from 82 to 91% identity to *E. sakazakii*. These results demonstrate that amongst a population of 246 *E.*

sakazakii strains assessed, 98% had a high identity and thus *E. sakazakii* could likely be identified by only using a PCR-based technique targeting a specific region of its 16S rDNA. This gene-based technique was more accurate for the identification of *E. sakazakii* than the phenotypic techniques currently being used for identification, indicating that it would potentially be valuable to add a gene-based technique to the suite of *E. sakazakii* identification methods.

The presumptive tests, i.e., yellow-pigment production and α -glucosidase activity, presently accepted for the initial identification of *E. sakazakii*, as well as the biochemical tests API 20E[®] and ID 32E[®] which are used to confirm presumptive *E. sakazakii* colonies, were often found to provide false-positive or false-negative results during this study. Only 77 and 66% of the strains tested herein were identified as *E. sakazakii* by ID 32E[®] and API 20E[®], respectively, while the PCR-based methods, 16S rDNA sequencing and BAX[®]PCR, identified 94 and 95% of the strains, respectively. Among the presumptive tests assessed, 28 of the strains tested formed white colonies on TSA, and of these, 14 were identified as *E. sakazakii* by ID 32E[®] or API 20E[®]. On DFI and ESPM agars, out of the 260 strains assessed, 229 (88%) and 234 (90%), respectively, were observed to be presumptively positive for α -glucosidase activity. As previously suggested by Iversen *et al.* (54), testing for α -glucosidase activity may be the most accurate of the biochemical tests for the identification of *E. sakazakii*. Therefore, the PCR approach targeting the *E. sakazakii* α -glucosidase gene, as proposed by Lehner *et al.* (74), is an interesting gene-based technique to be considered for the identification of this organism.

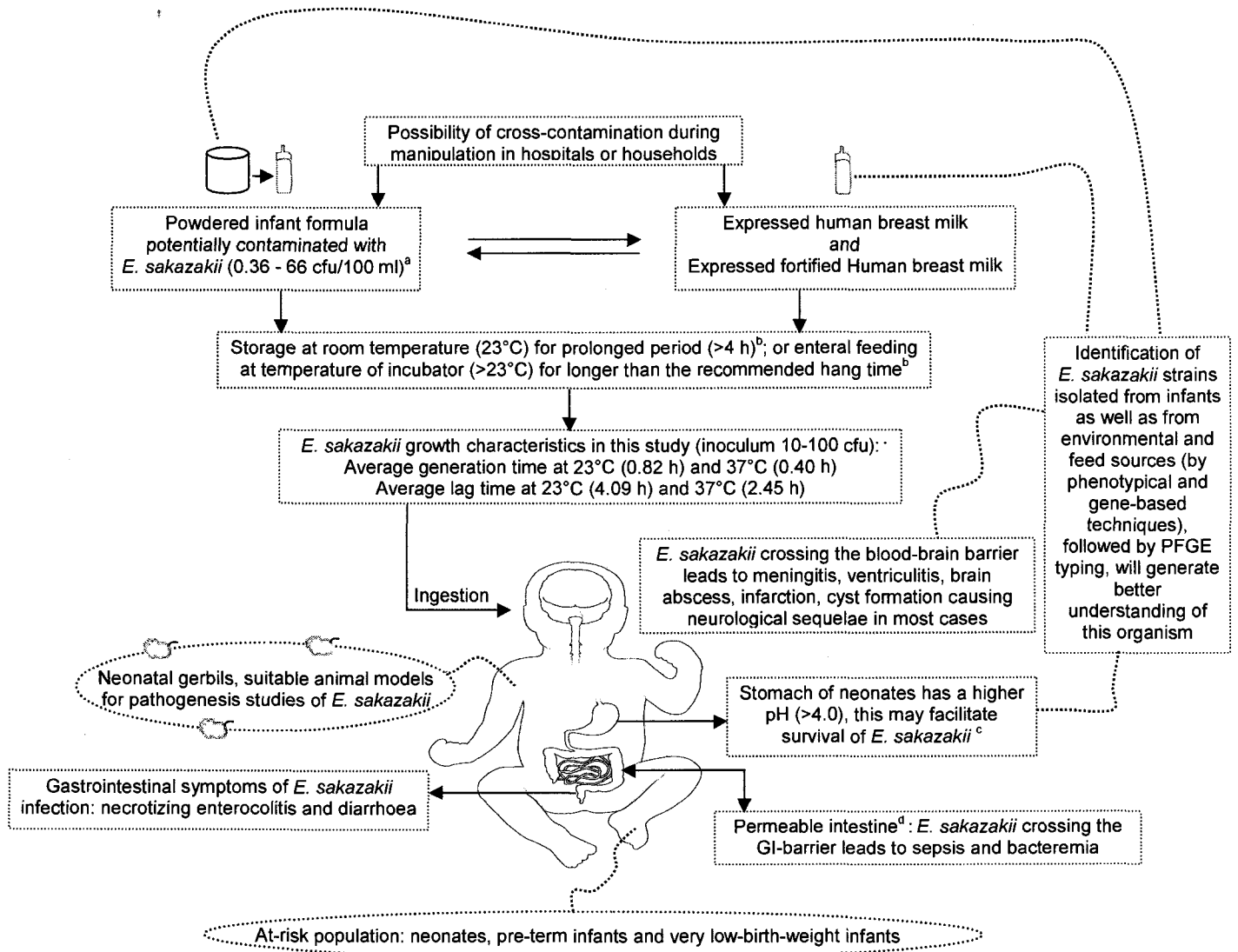
The ability of bacteria to grow in different media at different temperatures is an important feature to be investigated when studying its ecology. In the specific case of *E. sakazakii*, it was important to see if it could grow in infant feed, other than PIF, such as breast milk, and fortified breast milk, using the different temperatures that these foods are stored, handled and fed to the infants. Since it was observed that all 9 *E. sakazakii* isolates from different sources grew at 23 or 37°C in PIF, BM and BMF with no significant differences, it emphasizes the enormous significance of using proper hygienic practices in hospitals and in other environments where breast milk and infant formula are prepared. This is especially important given that in the case of cross-contamination, an infant could be infected through contaminated breast milk or fortified breast milk. Interestingly, at 10°C, *E. sakazakii* did not grow in BMF in most of the trials in this study (Table 8, Figure 10) and had long lag times (ranging from average 2.4 to 5.5 days) when grown in PIF and BM. The ADA guidelines for the storage of infant feed at refrigeration temperatures recommend that one should not exceed periods of 24 or 48 h for PIF and BM/BMF, respectively (7). Our data on the growth of *E. sakazakii* at 10°C supports the time frame recommended by the ADA guideline.

The lack of knowledge of the mechanism(s) of pathogenesis of *E. sakazakii* impedes the development of better treatment methods for infected infants, as well as a means of illness prevention. In addition, the current failure to determine the minimum infectious dose needed for this organism to cause disease, limits the establishment of effective policies and regulations for *E. sakazakii* in powdered infant formula, including its preparation in hospitals and households. In view of this, part of this research aimed to develop an animal model for future studies of *E. sakazakii* pathogenesis, and to gain a better understanding of its

minimum infectious dose. The findings presented herein suggest that the neonatal gerbil is a suitable model for *E. sakazakii* infections, since similar to the situation in human neonates, the organism was isolated from the brain of gerbils and in some cases caused their death. Further work is needed in order to investigate the best indicators and timing post-challenge to address knowledge gaps in the area of pathogenesis, dose-response and virulence differences amongst strains.

The research community has been joining efforts to find solutions for many of the unanswered questions concerning *E. sakazakii*. This field of study comprises a new area that has been brought to public attention only a little more than two decades ago. The large amount of work done to increase awareness of this organism in the past 10 years is illustrated by the number of recent research publications (77 of the 116 documents published on *E. sakazakii* date from the past six years), as well as the increase in the number of scientific and industry meetings dedicated to this bacterium. This research work adds to the growing body of scientific knowledge that is starting to accumulate on this bacterium. Figure 15 summarizes the extensive findings reported elsewhere and in this research project, and illustrates the integration of our results with previously published work.

Figure 15. Proposed model for the pathogenesis of *E. sakazakii* infection.



^a The average concentration of *E. sakazakii* in powdered infant formula according to Iversen *et al.* (51), Leuschner *et al.* (75) and Muytjens *et al.* (87).

^b Recommended storage and enteral feeding hanging time according to the American Dietetic Association (7).

^c Due to a higher pH in the gastrointestinal tract, newborns may be more susceptible to pathogenic bacteria, as suggested by Sondheimer *et al.* (108), Dinsmore *et al.* (23) and Mehall *et al.* (79).

^d Absence of natural microbiota may increase permeability of the gastrointestinal tract of newborns, facilitating bacterial translocation into the blood stream (40).

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