1st International Conference on *Cronobacter* (*Enterobacter sakazakii*)

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University College Dublin, IRELAND

Presentation Abstracts

Keynote lecture

John J. Farmer III, Scientist Director, US Public Health Service (Retired) **SGM Speaker**

Cronobacter (Enterobacter sakazakii)* -- Reflections on the First 50 Years; Challenges and Unresolved Issues for the Next 50

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* Dedicated to the memory of Dr. Riichi Sakazaki (August 21, 1920 - January 11, 2002) for whom this organism is named; Dr. Don J. Brenner, Frances Brenner, Richard Fanning, Arnold J. Steigerwalt, and the late Mary Alyce Fife-Asbury all of whom were involved with the original CDC studies that led to this organism being recognized as a separate species; and to Dr. Harry Muytjens for his pioneering work in uncovering its ecology and epidemiology in cases of neonatal meningitis and the important role of powdered infant formula.

2008 marked the 50th anniversary of the first documentation case of neonatal meningitis caused by the organism now known as *Cronobacter* (*Enterobacter sakazakii)*. The case occurred in 1958 and was reported by Urmenyi and White-Franklin in 1961. In this presentation I will give some of the early history of Esak, and share my personal perspectives on these first 50 years. In the second part I will outline some of the challenges and unresolved issues for this organism -- its ecology, epidemiology, microbiology, and its role in human disease.

John J. Farmer III, Scientist Director, United States Public Health Service (Retired). Silver Hill Associates, 1781 Silver Hill Road, Stone Mountain, GA 30087, USA

SESSION 1: TAXONOMY & IDENTIFICATION

Dr Carol Iversen, University College Dublin, Ireland

- **What is (and isn't) *Cronobacter*?**

Enterobacter sakazakii is an opportunistic pathogen that can cause meningitis, necrotising enterocolitis, and bacteraemia infants. It was first designated as a species in 1980 by Farmer *et al*. and several outbreaks in NICUs have been linked to contaminated powdered infant formula. The organism is therefore of concern to infant food manufacturers as well as clinical microbiologists and food safety regulators. In 2008 the taxonomy of *E. sakazakii* was updated using a polyphasic approach based on extensive geno- and phenotypic evaluations. This resulted in the description of five novel species and the proposal that these be incorporated into a new genus, *Cronobacter*, which is contaxic with *E. sakazakii*. The isolation of *Cronobacter* is complicated by the existence of closely related species, *Enterobacter pulveris*, *E. helveticus* and *E. turicensis*. These species share similar characteristics to *Cronobacter* and occur in the same ecological niches including infant foods. However, no health risk has been attributed to these organisms.

Carol Iversen[1,2,3], Niall Mullane[1], Barbara McCardell[4], Ben D. Tall[4], Angelika Lehner[2], Eva Bidlas[3], John Marugg[3], Ilse Cleenwerk[5], Séamus Fanning[1], Roger Stephan[2], Han Joosten[3]. [1]Centre for Food Safety and Food-borne Zoonomics, UCD Veterinary Sciences Centre, University College Dublin, Belfield, Dublin 4, Ireland. [2]Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zürich, CH-8057, Zürich, Switzerland. [3]Quality and Safety Department, Nestlé Research Centre, Vers-chez-les-Blanc, CH-1000 Lausanne, Switzerland. [4] Centre for Food Safety & Applied Nutrition, US Food and Drug Administration, Laurel, MD 20708, USA. [5]BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie – Universiteit Gent, K.L. Ledeganckstraat 35, B-9000 Gent, Belgium.

Dr Ben Tall, Virulence Mechanisms Branch, CFSAN, FDS, USA

- **Phenotypic diversity among *Cronobacter* spp. (*Enterobacter sakazakii)***

One hundred sixty-five isolates of *Cronobacter* spp. (formerly known as *Enterobacter sakazakii*) from clinical samples, foods, food processing areas and isolates of unknown origin were phenotypically characterized by API20E; VITEK 2.0 Compact GN (VITEK), and Biolog Microlog3 microbial identification analytical systems; and by Biolog Phenotypic Microarray (PM) analysis. Also assessed were other phenotypic traits such as expression of rugosity, colony pigmentation, α-glucosidase activity, antibiotic susceptibility, Congo Red (CR) and Calcofluor (CAL) binding and growth on two chromogenic media. API20E analysis identified all isolates as *E. sakazakii* and VITEK analysis correctly identified 159 of 160 isolates (99.4 %) as *E. sakazakii*. In contrast, only 23 (60.5%) of a subset of 38 *Cronobacter* isolates were correctly identified by the Microlog ID system. Antibiotic susceptibility of 46 clinical isolates showed that all isolates were resistant to Ampicillin, Cefazolin, Amoxicillin, Cefalotin, and Cefpodoxime while a few isolates were resistant to Cefoxitin, Imipenem, Ertapenem, Nitrofuratoin, and Cefuroxime. Comparative PM analysis of 95 isolates demonstrated the metabolic diversity among the *Cronobacter* spp. Based on utilization of 89 carbon sources by known *Cronobacter* spp. isolates, the different species could be distinguished from one another and from *Enterobacter cloacae*. Approximately 53 (32%) of the isolates expressed the rugose colony phenotype when grown at 30°C or 37°C. Over 132 (80%) of the isolates produced yellow pigmented colonies when grown at 30°C, whereas the remaining 33 isolates produced white colonies. All isolates produced typical blue-grey to blue-black *E. sakazakii*-like colonies on ESPM agar, whereas, 152 (92%) of the isolates produced typical blue-green *Cronobacter*-like colonies on Druggan-Forsythe-Iversen (DFI) medium when both media were incubated at 37ºC for 24 h. Thirteen isolates which gave atypical colony phenotypes on DFI showed variable α-glucosidase activity. However, PCR analysis detected the presence of both α-*gluA* and α-*gluB* genes in these isolates. Lastly, CR- and CAL- binding studies showed that over 140 (87 %) of the isolates expressed both curli and cellulose when grown at 37°C, yet only 21 (13%) of the isolates expressed both structures at 23°C. Tn5 mutagenesis studies revealed two CAL-binding negative and one CR-binding negative mutant. Sequences around the Tn5 insertions in the CAL-binding negative mutants were similar to a RadA ATP-dependent serine protease gene and to an aminotransferase gene. The sequence associated with the CR-binding defective mutant, was found to be similar to an arginine repressor gene. PM analysis showed a variety of altered phenotypes such as different carbon utilization patterns, different osmolarity and acid-base tolerance phenotypes among these mutants. These results demonstrate that *Cronobacter* spp. display numerous phenotypes which may have significant implications, not only in understanding bacterial diversity, but in understanding survival strategies of the pathogen in confronting multiple stresses in the food processing environment and in hosts.

B. D. Tall[1], M. H. Kothary[1], L. Hu[1], A. R. Datta[1], S. K. Curtis[1], L. Carter[1], V. Sathyamoorthy[1.], M. L. Kotewicz[1], E. W. Brown[2], K. R. O'Neill[2], G. Ziobro[2], L. Restaino[3], C. Iversen[4], S. Fanning[4], the JIFSAN, HACU and WIP Student Task Forces[1] and B. A. McCardell[1]. [1]U. S. FDA, Laurel, MD 20708, [2]College Park, MD 20740, [3]R & F Laboratories, Downers Grove, IL 60515, and [4]Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland.*

Prof Peter Kuhnert, University of Bern, Switzerland **SGM Speaker**

- **Multilocus sequence analysis (MLSA) of *Cronobacter* and related taxa**

Genetic similarity as determined by DNA-DNA hybridization is still considered the 'gold standard' method to determine relatedness between bacterial species. Nevertheless, it is very time consuming and cumbersome to perform and requires cross-hybridization between representatives of a species and related taxa. Moreover, variation between experiments, techniques and laboratories make exchange and comparison of data difficult. Whole genome sequence comparisons could be an alternative to DNA-DNA hybridization however, data handling and the open question as to what genes should be used for defining genome similarity cannot be neglected. Therefore, for taxonomic purposes investigating as many isolates of a species as possible in order to respect the biodiversity of taxa, a few representative genes indicative for genetic similarity between isolates is the optimal way to go. Recently, we showed that the three genes *recN*, *rpoA*, and *thdF* can be used to estimate whole genome similarity of representatives of the family *Pasteurellaceae* [Kuhnert & Korczak (2006) Int.J.Syst.Evol.Microbiol 152: 2537-2548] In the presented work multilocus sequence analysis (MLSA) based on *recN*, *rpoA* and *thdF* genes was done on more than 30 species of the family *Enterobacteriaceae* with a focus on *Cronobacter* and the related genus *Enterobacter*. The sequences provided valuable data for phylogenetic, taxonomic and diagnostic purposes. Phylogenetic analysis showed that the genus *Cronobacter* forms a homogenous cluster related to recently described species of *Enterobacter*, but distant to other species of this genus. Combining sequence information on all three genes is highly representative for the species' %GC-content used as taxonomic marker. Sequence similarity of the three genes and even of *recN* alone can also be used to extrapolate genetic similarities between species of *Enterobacteriaceae*, being an alternative to DNA-DNA hybridization. Finally, the *rpoA* gene sequence, which is the easiest one to determine, provides a powerful diagnostic tool to identify and differentiate pathogens of this family. The comparative analysis gives important insights into the phylogeny and genetic relatedness of the family *Enterobacteriaceae* and will serve as a basis for further studies and clarifications on the taxonomy of this large and heterogeneous family.

Peter Kuhnert. Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Laenggass-Str. 122, CH-3001 Bern, Switzerland.

Prof Séamus Fanning, University College Dublin, Ireland

- **Molecular identification methods for *Cronobacter* spp.**

Historically the ancestry of the genus *Enterobacter* can best be described as nebulus and confusing. In the 1970's and 1980's considerable movement of species, originally assigned to this genus occurred, and these re-designations arose because of initial misplacements, based on older phenotypic and morphological approaches to describing taxonomy. Currently the genus *Enterobacter* comprises a large and heterogenous group of organisms within the *Enterobacteriaceae* family being accounted for by 16 distinct species. *Enterobacter sakazakii* (*E. sakazakii*) is one of these species and the only member of the genus recognised as a food-borne pathogen. Following a revision of *Enterobacter* taxonomy, a new genus *Cronobacter* was devised which is synonymous with *E. sakazakii. Cronobacter* consists of a least five distinct species and an additional genomospecies, *Cronobacter sakazakii* (*C. sakazakii*), *C. dublinensis, C. malonaticus, C. muytjensii, C. turicensis* and *C.* genomospecies I. A further three sub-species of *C. dublinensis* are also recognised. Correct identification of these organisms is important in order to improve our understanding of the broader epidemiology of the members of this new genus. In recent years there have been rapid improvements in the provision of microbiologically-based culture approaches to isolate and identify these organisms. A number of molecular identification methods have also been proposed, however the recent recognition of multiple species that share less than 70% DNA-DNA similarity has important implications for the sensitivity and specificity of these methods. In this paper, three examples of the application of molecular-based detection strategies for the identification of *Cronobacter* will be presented. These will include strategies to identify the genus, specific targets that are thought to be related to pathogenicity and the development of a molecular-based approach to begin to define the O-serotypes of *C. sakazakii.* Although by no means complete, these examples will illustrate some of the current and future challenges to enable a more refined and reliable molecular-based approach to the identification of all *Cronobacter* spp.

The development of appropriate molecular methods will facilitate not only a rapid identification of an isolate, but in addition complement the more traditional microbiological-based methods.

B. Healy, A. Lehner[¶], N. Mullane, S. Cooney, S. O'Brien, C. Iversen, R. Stephan[¶] & S. Fanning, Centres for Food Safety & Food-borne Zoonomics, UCD Veterinary Sciences Centre, University College Dublin, Belfield, Dublin 4. Ireland and Institute of Food Safety & Hygiene[¶], VetSuisse Faculty, University of Zurich, CH-8057 Zurich, Switzerland.

SESSION 2: ISOLATION METHODS

Dr Patrick Druggan, Thermofisher, UK **SGM Speaker**

- Culture media for isolation and detection of *Cronobacter* species

In 2001 a pre-term infant died of meningitis caused by *Enterobacter sakazakii* (*Cronobacter* spp.). Infant formula milk (IFM) was implicated as a potential source of the infection. The Food and Drugs Administration (FDA) independently develop a method for enumeration of this emerging pathogen in IFM using culture collections from national bodies that have later been shown to be poorly defined. This method was introduced in 2002 and has regulatory standing for the import of IFM and skimmed milk powder in to the USA and a number of other countries. The FDA method is a modification of the procedure for the detection of *Enterobacteriaceae,* with the addition of yellow pigmentation of colonies for presumptive identification of *Cronobacter* spp. It should be remembered that the FDA method was developed in a short time due to a public health concern, and this would have put a time constraint and significant pressure on those working on *Enterobacter sakazakii* (*Cronobacter*) to get a working method in the field as soon as possible. The FDA method has been shown to have a sensitivity of around 50 % and a specificity of around 70 %. Only 75 % of *Cronobacter* strains phenotypically express yellow pigmentation, and the low specificity of the method coupled with the recommendation that only five presumptive *Enterobacteriaceae* colonies are tested from Violet Red Bile Glucose Agar (VRBGA) may explain the poor sensitivity of the method. Assuming the prevalence of *Cronobacter* spp. in IFM is around 2 %, the FDA method will fail to detect around 50 % of batches contaminated with *Cronobacter*, while around 95 % of rejected batches will not contain this organism. This high rate of failure has lead many stakeholders to question the usefulness of the FDA method. This presentation reviews developments in culture media since the release of the FDA method in 2002, with specific emphasis on media that have improved the specificity of methods for *Cronobacter* spp. The unique phenotypic traits of this emerging pathogen that aid and hinder design of methods is discussed.

Patrick Druggan, Oxoid Ltd., Thermo Fisher Scientific, Basingstoke, Hampshire RG24 8PW, United Kingdom.

Dr Han Joosten, Nestlé Research Centre, Switzerland

- **Development of a CEN-ISO horizontal standard method for detection of *Cronobacter***

The availability of a reliable and internationally accepted reference method for detection of *Cronobacter* in powdered infant formula is an essential tool to verify compliance with regulatory requirements by public health authorities and manufacturers. ISO-TS 22964:2006 was developed as a temporary solution for this purpose, but shortly after being issued it was decided to prepare a full-fledged horizontal CEN-ISO standard. A summary will be given of the work done thus far, in particular with respect to the modifications that are envisaged to address the main shortcomings of TS 22964:

- the scope will be extended to all types of powdered infant formula (incl. soy- based) and infant formula ingredients.
- it will take into account the latest taxonomical revisions (e.g. definition of the genus Cronobacter and phenotypically related species)
- it will no longer use yellow pigment production as a confirmation criterion
- the enrichment broth (mLST) and chromogenic isolation agar (ESIA^{TM}) are too selective and need to be replaced by media that will also allow the detection of strains that are very susceptible to commonly used inhibitors of gram positive microorganisms.
- the main performance characteristics of the new standard will be determined

Based on the results obtained during an extensive comparative/collaborative trial a method based on the utilisation of Cronobacter Screening Broth (CSB) in combination with modified DFI agar appears to be the most suitable procedure to be adopted in the new standard.

Han Joosten. Quality and Safety Department, Nestlé Research Centre, Vers-chez-les-Blanc, CH-1000 Lausanne, Switzerland.

Prof Keith Lampel, Director Division of Microbiology, FDA, USA

- **Development of an FDA/AOAC standard method for detection of *Cronobacter***

Although the number of incidences of illnesses caused by the ingestion of the bacterial pathogen *Cronobacter* (*Enterobacter sakazakii*) has not been as dramatic as other foodborne pathogens, a need remains for a robust isolation method to recover this microbe from powdered infant formula (PIF). The current method described on the FDA website was developed in response to one such incident. Although *C. sakazakii* was a rather novel pathogen in an unusual food matrix, a method was devised quickly and applied to PIF samples. Unfortunately, this method requires multiple steps and at least 3-4 days for complete analysis of PIF for isolation and confirmation of *C. sakazakii* from the formula sample. The revised method, however, includes both a bacteriological enrichment and isolation protocol as well as the integration of a PCR-based assay. As for the bacteriological application, one-step enrichment is followed by plating on chromogenic agar(s) for presumptive identification of *C. sakazakii*. Suspected colonies are confirmed by either biochemical analysis or a real-time PCR-based assay. Therefore, isolation and identification of *E. sakazakii* from PIF is markedly improved and can be accomplished in 24-28 hrs.

WORKSHOP A: TAXONOMY, IDENTIFICATION & ISOLATION

Dr Matthias Kiehne, BIOTECON GmbH, Germany

- **Rapid detection of Enterobacteriaceae including identification of *E. sakazakii***

BIOTECON Diagnostics has developed a real-time PCR system for the detection of all Enterobacteriaceae with a simultaneous identification of *E. sakazakii* by parallel detection with differently labelled probes. The test is designed to run on all relevant real-time PCR instruments and comprises all necessary reagents. The method is thoroughly validated for the use in infant formulae with and without probiotic bacteria as well as raw materials and environmental samples. During the validation of the method it was recognized, that most infant formula products contain a background of inactive or non-cultivatable Enterobacteriaceae which leads to positive results in the PCR but cannot be confirmed by cultural methods. To overcome this discrepancy, Reagent D is applied in advance to the DNA extraction, a reagent which inactivates DNA from dead cells for PCR. The kit also includes all necessary controls such as positive and negative and an internal positive control to eliminate false negative results due to inhibition or failures. UNG is used to prevent false positive results by carry-over contamination with "old" amplificates.
The presentation will give an overview about the method, its application and results from validation and routine use.

Dr Mike Kotewicz, US FDA, CFSAN, OARSA, Laurel, MD 20708, USA.

- **Optical maps of subgroups of *Cronobacter* (formerly *Enterobacter sakazakii)* show large chromosomal regions of homology and differences among isolates**

Optical maps are whole genome restriction fragment maps made by spreading bacterial chromosomes on derivatized glass slides. The chromosomes are digested with a restriction enzyme and optically scanned with an automated CCD camera across nearly full-length chromosomes. Multiple scans create assemblies of contiguous restriction fragments and these are assembled into a complete whole genome map. Alignment software allows the whole chromosome maps of different isolates to be compared and referenced to sequenced *in silico* genome maps. These barcode-like whole genome scans and alignments can be used to measure similarities between different isolates as well as identify the chromosomal positions and sizes of deletions, insertions and replacements from 2 kb to >100,000 kb. The genomes of isolates representative

of the five *Cronobacter* type-species and sub-species groups (formerly *Enterobacter sakazakii*) were optically mapped. These included isolates from *Cronobacter* subgroup 1, *C. sakazakii*; subgroup 2, *C. turicensis*; subgroup 3, *C. muytjensii*; and subgroup 4, *C. dublinensis*. An isolate from subgroup 2a, *C. genomospecies* 1 and several clinical and powdered infant formula isolates were also optically mapped. Alignments of the maps to each other and to the *in silico* map of the sequenced reference group 1 strain from the 2001 Tennessee *Enterobacter sakazakii* outbreak, ATCC BAA894, were performed. The optical map of an independent isolate from the implicated powdered infant formula was indistinguishable from the *in silico* map of the sequenced outbreak strain BAA894. Isolates from within groups 1 and 3 show large regions of chromosomal homology and large differences between isolates within a group. There are fewer homologies between groups. We detailed chromosomal changes including prophage insertions, deletions and large replacements between isolates. These results support the taxonomic scheme proposed by Iversen *et al.* in that the genus *Cronobacter* is composed of several species and a number of sub-species with considerable sequence and genomic diversity.

M.L. Kotewicz and B.D. Tall. U. S. FDA, CFSAN, OARSA, Laurel, MD 20708

SESSION 3: PUBLIC HEALTH

Prof Jeff Farber, Health Canada

- ***Cronobacter sakazakii* advice, policy and research in Canada**

Although Canada has not had many reported cases of *Cronobacter sakazakii*, Health Canada has been actively studying this organism since 1998. In 2002, as a result of an outbreak in Tennessee in the USA, Health Canada issued an advisory to inform Health Professionals in Canada what measures they could take to reduce the risk to infants, of consuming powdered-infant formula (PIF). After reviewing the situation at the national level and due to health concerns with powdered formulae and its international trade, in 2003, Health Canada raised this issue at the international level by proposing to revise the Code of Practice for Powdered Formulae for Infants and Young Children at the Codex Alimentarius Committee of Food Hygiene. Canada volunteered to chair the Working Group that would be developing the Code. Because of the high level of interest in this issue, the Code was completed in four years, which is a relatively short time considering the complexity and politics behind this issue. The Code has contributed to a big improvement in the hygienic conditions in plants manufacturing PIF, resulting in a lower level of product contamination with *C. sakazakii*. Canada has produced a document detailing Good Manufacturing Practices (GMPs) for Infant Formula in Canada. The purpose of this text is to establish and document the current GMPs for the production and quality control of infant formula products made for distribution in Canada. Health Canada uses the GMPs as a basis on which to assess the manufacturing information received in pre-market notifications for new or changed infant formulas. Health Canada does have microbiological criteria for *C. sakazakii* in PIF; however, they are currently being revised to be more in line with recent Codex thinking. At present, unfortunately, there are no active or passive surveillance systems for *C. sakazakii*, in Canada, although this has been discussed. Health Canada has recently adapted and condensed FAO/WHO guidelines to develop a draft guidance document on the preparation and handling of PIF in home and hospitals/care settings, which outline requirements for parents, caregivers, and staff in hospitals and day-care centers. The guidance document can be used to educate parents, caregivers and staff in hospitals and day-care centres, on the potential hazards associated with PIF. Health Canada's Bureau of Microbial Hazards conducts research focussed at examining the ecology, biology and pathogenesis of the organism. Some of the research projects include specific aspects of molecular typing, virulence studies involving animal models, as well as in-vitro tissue culture work to examine adhesion and invasion. Collaborative research is also being done with the National Research Council, using NMR and mass spectroscopy to reveal the structure of the O-polysaccharide of the various *Cronobacter* species.

J. M. Farber and F. Pagotto, Health Canada, Bureau of Microbial Hazards, Food Directorate, Ottawa, Ontario, Canada.

Dr Cliodhna Foley-Nolan, *safe*food, Dublin, Ireland

- **Neonatal health**

The paper will focus on key epidemiological data available in Ireland on Neonatal health (birth rates; perinatal mortality rates; birth weight statistics; breastfeeding rates and gestational age/ prematurity rates). It will also detail the process of a communication initiative on safe powdered infant formula bottle feeding (multiagency multidisciplinary collaboration; clear culturally appropriate language; focus group testing and communication networks)

Dr Lorraine Kyne, University College Dublin, Ireland

- ***Cronobacter* and the elderly**

Cronobacter is a gram-negative, rod-shaped organism that is associated with rare but life-threatening cases of meningitis, necrotizing enterocolitis and sepsis in neonates. Individual cases of infection in adults have been reported, usually in patients with serious underlying disease. Its tropism for the central nervous system in neonates and infants has not been seen among adult cases. In adults it has been associated with pneumonia, splenic abscesses, bacteraemia, osteomyelitis, urosepsis and wound infections. Recently, it has been implicated in cases of aspiration pneumonia in older stroke patients with dysphagia. A possible link with thickening agents containing maize starch used by patients with an abnormal swallow has been postulated but not proven. Dr. Kyne will review the current knowledge regarding *Cronobacter* infection in older adults, outline the potential risk to the elderly and describe current research in this area.

Dr Wayne Anderson, Food Safety Authority of Ireland

- **Security of the Irish infant formula food chain**

The security of the Irish infant formula supply chain is vital for the health of infants and for the health of the Irish economy. The health of infants is of paramount importance and tolerance, by consumers, of food safety problems in this sector is lower than for the general population. Infant formula manufacturing is a major part of the Irish food industry, supplying around 12% of the world's infant formula. European food law in this area is comprehensive and prescriptive and compliance is a complex undertaking. The European Regulation on the General Principles of Food Law clearly places the onus on the food industry to ensure the food that is placed on the market is safe. The Authorities are required to verify that manufacturers are compliant with the food law and this is done via direct inspection and surveillance of the food chain. These, so called Official Controls, are also subject to scrutiny by the European Commission to ensure harmonisation across the European Union. However, there is also another equally important step in the infant formula food chain that is not regulated in any way but cannot be ignored. The correct use of infant formula by parents and caregivers is crucial for the safety of infants. It is not easy to dispel the common perception that powdered infant formula is sterile and therefore the Authorities must take extra care to define best practice and actively educate those who prepare infant formula. This presentation will focus on the microbiological controls used by infant formula manufactures in Ireland. It will look at the legislative framework and the operation of Official Controls in Ireland. Finally it will outline the work that has been done to ensure that Irish consumers utilise the product safely.

SESSION 4: ANALYSING THE RISK OF CRONOBACTER

Dr Anna Bowen, Centers for Disease Control and Prevention, USA

- ***Cronobacter* infections in infants**

Cronobacter sakazakii is a rare cause of bloodstream and central nervous system infections and has been associated with necrotizing enterocolitis among infants. Reported outcomes are often severe: seizures; brain abscess; hydrocephalus; developmental delay; and death in as many as 40%–80% of cases. We will review characteristics of infant cases to better define risk categories.

Dr Martine Reij, Wageningen University, The Netherlands **SGM Speaker**

Calculating risk associated with *Cronobacter*

Risk is defined as a function of the probability that an adverse health effect will occur and the severity of such event. For *Cronobacter* consumption of powdered infant formula (PIF) is a documented factor contributing to the risk this organism poses to infants, specifically to neonates and infants less than 2 months of age. When calculating the probability of infection, the prevalence and concentration in PIF are of utmost importance, but data are scarce. This paper attempts to describe the heterogeneity of contamination and its importance for the resulting risk. Data on the resulting risk, i.e. the number of patients and the severity of their illnesses have been described in a large number of publications. Here we present a preliminary estimate of the disability adjusted life years lost (DALYs) due to *Cronobacter* infections for the Dutch situation.

M.W. Reij, I. Jongenburger, E. Gkogka, L.G.M. Gorris, M.H. Zwietering. Laboratory of Food Microbiology, Wageningen University, Wageningen, the Netherlands.

Prof Francis Butler, University College Dublin, Ireland

- **Analysing the risk of *Cronobacter* : Risk Analysis in Ireland**

There is mounting evidence that *E. sakazakii* can enter into powdered infant formula during the manufacturing process. Therefore it is important that manufacturers monitor the level of contamination in powdered infant formula on an ongoing basis and have in place a robust system of process control to monitor and control the pathogen. This presentation describes ongoing work to develop possible sampling strategies / statistical process control that would be feasible within the operational environment of a powdered infant formula manufacturing facility and to consider the outcomes from different strategies in terms of pathogen reduction in the final product leaving the factory. Irish surveillance data has been evaluated using a simple risk assessment model (The WHO / FAO model and a simple risk assessment model developed by the project) using different scenarios in terms of sampling plans / control strategies within the process environment.

F. Butler. School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland

Dr Mary Smith, University of Georgia, USA

- ***Enterobacter sakazakii* invades brain and liver of 1-2 day old neonatal mice**

Premature or very-low-birth-weight infants who are exposed to *E. sakazakii* through oral feedings with contaminated infant formula can develop illnesses such as necrotizing enterocolitis, bacteremia, sepsis, meningitis and/or death. Our goal is to develop an animal model susceptible to *E. sakazakii* infection. The objectives of this study were to (1) determine which of three mouse strains (CD-1, BALB/C, C57BL/6) was most susceptible to *E. sakazakii* strain MNW2, (2) test *E. sakazakii* isolates for virulence in the neonatal mouse model, and 3) to determine whether age of neonate affects susceptibility to *E. sakazakii*. Neonatal mice were treated orally with reconstituted powdered infant formula inoculated with *E. sakazakii* strain MNW2 by oral gavage. One week after treatment, mice were sacrificed and brains, livers, and ceca were excised and *E. sakazakii* isolated. Of the three strains of mice treated with *E. sakazakii*, CD-1 mice were most susceptible to invasion in the tissues examined. CD-1 neonates were then used for the remainder of the experiments. Two strains of *E. sakazakii*, MNW2 (food isolate) and SK81 (clinical isolate) were administered to neonatal mice at 3-4 days old. The clinical isolate was more invasive than was the food isolate. The brain of younger neonates (1-2-day old) was more susceptible to invasion with *E. sakazakii* than older neonates (5-6-day old) after treatment with 3 log CFU of neonates (16% vs 0%, respectively) and this trend continued at the highest dose tested 10 log CFU (28% vs 5%, respectively). Overall, there was more isolation of *E. sakazakii* from 1-2 day-old mice than 5-6 day-old mice, with total *E. sakazakii* isolations of 79.1%, 53.2%, 61.3% in brains, livers, and ceca, respectively. In conclusion, CD-1 neonatal mice are susceptible to *E. sakazakii* infection after oral exposure. Invasion of neonatal brain and liver tissues shows a dose- and age-dependent response.

Dr Franco Pagotto, Health Canada

- **Pathogenesis of *Cronobacter*: enterotoxin production, adherence and invasion of the blood-brain barrier**

Although *Cronobacter sakazakii* has been implicated in outbreaks causing meningitis and enteritis, we do not know what factor(s) play a role in the transfer of the organism across the blood-brain-barrier in humans, or whether those factors are present in all strains of *C. sakazakii.* This study assessed whether strains from clinical, food, and environmental sources differed in their ability to adhere and/or invade human brain microvascular endothelial cells.
Adhesion and invasion of 30 *Cronobacter* isolates (10 each from clinical, environmental, and food sources) to human blood-brain-barrier cells was done using a modified gentamicin protection assay. A transposon mutant library was screened in the same manner to identify isogenic mutants showing increased and/or decreased adherence and invasion. The 30 strains were tested for enterotoxin production *in vitro* using a Vero cell assay.
All strains adhered to endothelial cells, and all but 2 clinical strains were able to invade. Interestingly, 70% of clinical strains were positive or indeterminate for capsule production, as compared to 40% and 30% for food and environmental isolates, respectively. Enterotoxin production varied amongst the 30 strains tested. Of the 6 most virulent strains, 5 were of food and one was of environmental origin. SDS-PAGE revealed a distinct protein band present at 66 kDa, the reported molecular weight of the enterotoxin. N-terminal sequencing was done on the excised protein.
There did not appear to be any direct correlation between the source of *Cronobacter* strains and their adherent or invasive abilities. Investigation into the transposon insertion sites in the genomes of non-adherent and non-invasive mutants are underway and should help shed some light on the identity of the factor(s). Capsule formation may be important in the blood-brain barrier pathogenesis.

F. Pagotto, Health Canada, Bureau of Microbial Hazards, Food Directorate, Ottawa, Ontario, Canada.

Dr Sarah Cahill, FAO, Rome, Italy

- **FAO/WHO risk assessments on *Enterobacter sakazakii* (*Cronobacter* spp.) in powdered infant formula and follow-up formula.**

Enterobacter sakazakii (*Cronobacter* spp.) is one of the primary pathogens of concern with regard to powdered infant formula. Although its' identification as a pathogen of concern is relatively recent, the severity of the illness which it causes, particularly in neonates and infants less than two months of age, and the relatively high mortality rates, stimulated the international community to react quickly to facilitate the implementation of protective and preventive actions to minimize the exposure of infants and young children to this hazard. Such actions have included the adoption of *a Code of Hygienic Practice for Powdered Formulae for Infants and Young Children* by the Codex Alimentarius Commission, the international food standards body and the development of *Guidelines for the Safe Preparation, Storage and Handling of Powdered Infant Formula* by WHO and FAO. A critical aspect to their development was the availability of a sound scientific foundation. While this was based on the work of, and data generated by scientists, regulators, the medical profession, care providers, industry and others, FAO and WHO facilitated a process of expert consultation and risk assessment with the aim of converting the available knowledge and information into scientific advice for risk managers, both at the international and national levels. Looking at both production practices and preparation and use, the difficulties of eliminating such a pathogen from a non-sterile product and the important role that preparation practices have in terms of both contamination and amplification of the pathogen were highlighted. Great diversity exists in terms of our knowledge base, the use of the product and the preparation practices as we move around the world. However, it is clear that both the producer and the end-user of this product need to be aware of the risks and the measures each needs to take to control them. Though, in many situations, the challenges to be overcome, particularly by the end-user in implementing such control measures, highlighted the need for the product to be as safe as possible before entering the market place. One of the important outputs of this work was the development of a web-based user-friendly risk assessment model which allows users to compare the impact of a range of risk management options, such as different sampling plans and practices

that could be employed in the preparation and use of powdered formula. The aim of this is to make risk assessment more accessible as a decision support tool to risk managers. This paper will highlight some of the significant outputs and challenges of the FAO/WHO risk assessment work, and its contribution to risk management.

Sarah M. Cahill, Peter Karim BenEmbarek and Maria de Lourdes Costarrica. Food and Agriculture Organization of the United Nations, Viale delle Terme di Caracalla, Rome, Italy.

WORKSHOP B: FAO/WHO RISK ASSESSMENT MODEL

Mr Greg Paoli, Canada - Demonstration of the FAO/WHO risk assessment model

SESSION 5: OCCURRENCE & SURVEILLANCE 1

Prof Larry Beuchat, University of Georgia, USA **SfAM Lecture**

- ***Cronobacter* in food and strategies for control**

Enterobacter sakazakii (Cronobacter), or at least strains identified as this bacterium using previous taxonomic and classification schemes, has been isolated from a wide range of environmental samples and from several foods of animal and plant origin. While infections caused by *E. sakazakii* have predominantly involved neonates and infants, its presence on or in foods other than powdered infant formula raises concern about the safety risks of these foods to older immunocompromised consumers. We have done a series of studies to better understand the survival and growth characteristics of *E. sakazakii* in infant formula, infant cereal, fresh-cut produce, and juices made from produce. Over a 12-month storage period, the organism survived better in dried formula and cereal at low a_w (0.25 - 0.30) than at high a_w (0.69 - 0.82) and at 4ºC compared to 30ºC. *E. sakazakii* grows in formulae and cereals reconstituted with water or milk and held at 12 - 30ºC. The composition of formulae or cereals does not markedly affect the rate of growth. *E. sakazakii* grows well on fresh-cut apple, cantaloupe, watermelon, cabbage, carrot, cucumber, lettuce, and tomato at 25ºC and on some types of produce at 12ºC. Treatment of produce inoculated with *E. sakazakii* with sanitizers such as chlorine, chlorine dioxide, and a peroxyacetic acid-based solution causes reductions of 1.6 - 5.4 log CFU/apple, tomato, or lettuce leaf. Cells of *E. sakazakii* in biofilms formed on stainless steel and enteral feeding tubes or dried on the surface of stainless steel have increased resistance to disinfectants. Death of cells in biofilms is affected by atmospheric relative humidity. Observations from these studies have contributed to a better understanding of the behavior of *E. sakazakii* in and on foods and on food-contact surfaces, thereby enabling the development of more effective strategies for its control.

Larry R. Beuchat. Center for Food Safety and, Department of Food Science and Technology, University of Georgia, 1109 Experiment Street, Griffin, Georgia 30223-1797, USA.

Dr Angelika Lehner, University of Zürich, Switzerland **SGM Speaker**

- **Highlighting environmental reservoir aspects for *Cronobacter* species**

Members of the recently proposed genus *Cronobacter* (*Enterobacter sakazakii*) are responsible for cases of severe illness with high fatality rates in neonates and newborns caused mainly by the ingestion of contaminated powder-based infant formula milk (IFM). Physiological features suggest an environmental origin of *Cronobacter*, but the question of possible ways of entry of these organisms into the IFM production facilities and the natural habitat is still under debate. Within the current lecture, a combination of literature and own research data will be presented, focusing on several biological sources (animal, human, insects, plants, protists) that could serve as vectors for contamination. In a recent screening, no evidence was found that humans as asymptomatic carriers could be a source of contamination. In addition, literature data confirm, that the raw material milk can also be ruled out as primary source of entry of these organisms. IFM factory surveillance data revealed the presence of *Cronobacter* spp. in plant-derived supplements added to the final product. Own experimental data on the hypothesis of a possible plant association of these organisms strongly support plants as a possible natural habitat for these organisms.

Moreover, preliminary results provide evidence for an interaction between *Cronobacter* spp. and *Acantamoeba* which are part of the natural plant associated environment. The presented data further substantiate the hypothesis of an environmental/plant-associated reservoir of *Cronobacter* spp.

Angelika Lehner. Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zurich, Winterthurerstrasse 272, 8057 Zurich, Switzerland.

Dr Norma Binsztein, Malbran Institute, Argentina

- ***Cronobacter* spp. isolates in Argentina: characterization and subtyping by Pulsed-Field Gel Electrophoresis (PFGE)**

Cronobacter spp. (formerly *Enterobacter sakazakii*), a known contaminant of powdered infant formula (PIF), has been involved in cases of neonatal meningitis, necrotizing enterocolitis and sepsis in different countries. We have isolated *Cronobacter* spp. from three different brands of imported milk formula. The objectives of this work were to evaluate the use of PFGE for *Cronobacter* spp. subtyping, to establish the genetic relationships between the isolates recovered and determine possible common sources of contamination. The isolation and identification of *Cronobacter* spp. was carried out according to the official method of USDA/FDA 2002 in PIF samples from eight batches of three different brands (A, B and C) between 2005 and 2008. One of the batches from brand A had been widely distributed in the country and, therefore, more PIF cans were analyzed than from the rest of the batches; 20 colonies of brand A, recovered in 2005, were selected for this study. From the other two brands, we analyzed 1 isolate from brand B (2006) and 2 from brand C (2007 and 2008). The 23 isolates were characterized by biochemical tests, antimicrobial susceptibility following CLSI recommendations for *Enterobacteriaceae* and subtyped by PFGE, applying the PulseNet protocol for *Shigella sonnei* with enzymes *Xba*I and *Spe*I. The twenty-three isolates studied showed two different biochemical patterns compatible with *C. sakazakii* subsp. *sakazakii* and *C. sakazakii* subsp. *malonaticus*. They were all susceptible to 11 antimicrobial agents tested. When analyzed by PFGE, the isolates showed 8 different profiles with *Xba*I. Among the 20 isolates from brand A, six different patterns were identified, five of them in samples from a single batch. The isolate from brand B showed a PFGE pattern indistinguishable from one of the patterns found in brand A; while the two strains from brand C showed two distinct PFGE profiles with more than seven bands difference. The isolates that showed identical *Xba*I-PFGE profiles were analyzed with the second enzyme, *Spe*I, which confirmed the genetic relationships among them. The *Cronobacter* spp. isolates studied were genetically diverse, even those recovered from a single batch of the same brand (A), suggesting that different sources could be implicated in the contamination of the infant formula. These results also underline the importance of analyzing more than one colony from each can of PIF, since different strains of *Cronobacter* spp. may be found in the same sample. On the other hand, the same *Cronobacter* spp. subtype was found in two different brands, suggesting a possible common source of contamination between the two companies. The use of a standardized PFGE protocol, currently under development by PulseNet, will be a very useful tool to determine the traceability and the possible contamination sources during the manufacturing of the formula. Furthermore, the creation of National and International Databases of genetic profiles will allow to know and to compare the circulating subtypes between the isolates of human and food origin in different countries.

R. Terragno[1], A. Salve[1], M. Pichel[1], S. Brengi[1], S. Epszteyn[2] and N. Binsztein[1]. [1]INEI-ANLIS "Carlos G. Malbran", Buenos Aires, ARGENTINA, [2]Dirección General de Higiene y Seguridad Alimentaria-GBA, Buenos Aires, ARGENTINA.

Mr Brendan Healy, University College Dublin, Ireland

- **Microarray analysis of *Cronobacter* species**

To better understand the genetic relationship between the different *Cronobacter* species and among strains within the same species, we have undertaken microarray-based comparative genomic indexing (CGI) that measures the presence or absence of genes within different *Cronobacter* strains. We examined 78 *Cronobacter* strains (60 *C. sakazakii*, 8 *C. malonaticus*, 5 *C. dublinensis*, 2 *C. muytjensii*, 1 *C. turicensis*, 1 *C.* genomospecies 1, and 1 *Cronobacter sp.*) representing clinical and environmental isolates from various geographical locations. The CGI

analysis allowed the assessment of gene content for 274 genes from the recently sequenced *C. sakazakii* strain ATCC BAA-894 for each *Cronobacter* strain. Despite the limited number of genes examined, a hierarchical clustering of the CGI data demonstrated that the species mostly differentiated as clusters. Indeed, the 5 *C. dublinensis* strains and 2 *C. muytjensii* strains formed distinct species clusters. Moreover, all of the *C. sakazakii* and 3 *C. malonaticus* strains formed a large cluster. The other 5 *C. malonaticus* strains formed a subcluster within a larger cluster that also contained *C. turicensis*, *C.* genomospecies 1, and an unknown *Cronobacter sp.* We observed that the *C. sakazakii* and 3 of 8 *C. malonaticus* strains were distinguished from the other strains by the presence of 10 fimbrial related genes. Capsule and/or LPS related glycosyltransferases distinguished several of the *C. sakazakii* strains from each other. In the future, examination of *Cronobacter* strains by a whole genome array would identify additional distinguishing genomic features and should allow the design of simplified molecular techniques to identify these bacteria at the species level.

C. Parker[1], B. Healy[2], S. Huynh[1], C. Iversen[2], S. O'Brien[2], S. Cooney[2], R. Mandrell[1], S. Fanning[2]
[1]Produce Safety and Microbiology Research Unit, USDA/ARS, WRRC, Albany, CA, [2]Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin.

SESSION 6: OCCURRENCE & SURVEILLANCE 2

Dr Jean-Louis Cordier, Nestlé Nutrition, Switzerland

- **Control in manufacturing facilities**

The control of *Enterobacter sakazakii* in manufacturing facilities is based on the implementation of preventive measures. While extensive killing is achieved during heat-treatment of liquid infant formulae, recontamination can occur during further processing, i.e. after the drying and up to the filling of the finished products. Prevention is thus focused on ensuring that added dry-mix ingredients are of the appropriate quality and fulfil the same stringent requirements as the finished products. Preventing post-process contamination is ensured through the implementation of particularly strict measures aiming at minimising the presence of Enterobacteriaceae, including *E. sakazakii*, in the processing environment. These measures will be discussed during the presentation and examples of the impact provided.

Jean-Louis Cordier, Nestlé Nutrition Operations/Quality Management, Avenue Reller 22 ; CH-1800 Vevey, Switzerland.

Prof Steve Forsythe, Nottingham Trent University, UK

- **Desiccation and persistence of *Cronobacter* species in infant formula**

Cronobacter is a newly described genus which includes opportunistic pathogens formerly known as '*Enterobacter sakazakii*'. These organisms have been isolated from a wide variety of sources, including powdered infant milk formula. This review focuses on the desiccation survival of *Cronobacter*, and its relevance to vehicles of infection. Our studies have shown that the organism can survive for long periods of time (>2 years) in the desiccated state, and can be recovered from a large number of powdered foods in addition to powdered infant formula. Due to its persistence, on reconstitution in the absence of other bacterial competition, it may rapidly multiply and present a risk to the immunocompromised. Currently neonates are recognised as a vulnerable group to *Cronobacter* infections; however the elderly may also be susceptible. It is expected that an improved understanding of the nature of *Cronobacter* persistence may aid in improved control measures and eliminate the bacterium from the critical food production environments.

T. Osaili[a] and S. Forsythe[b]. [a] Department of Nutrition and Food Technology, Jordan University of Science and Technology, Irbid, 22110, Jordan. [b] School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham, UK. NG11 8NS.

Dr Geraldine Duffy, Teagasc, Ireland

- **Sources and survival of *Cronobacter* in the farm to fork chain**

The aim of this study was to establish if food production animals and the wider farm and food environment were playing a role in the transmission of *Cronobacter* (*Enterobacter sakazakii*). A wide range of samples (n=518) were collected at dairy farms, meat abattoirs, retail food stores and domestic environs. Samples were examined for the pathogen using an adapted ISO /DTS 22964 cultural protocol and confirmed by Real Time PCR targeting *dna*G on the MMS operon. *Cronobacter* was not recovered from cattle faeces, farm soil or trough water but was recovered from a variety of other sample types including cattle feed (n = 10), pork and beef cuts (n = 4), beef burgers and beef mince (n = 4), green vegetables (n=2), organic breakfast cereals (n = 9) and domestic vacuum cleaner dust (n = 1). To further investigate the potential transmission of *Cronobacter* by cattle, the pathogen was inoculated into bovine faeces, and into laboratory models of the bovine rumen and abomasum and survival of the pathogen monitored over time. While the pathogen survived in faecal material for up 120 days under typical farm environmental conditions it was not recoverable from the abomasum model indicating that it would not survive passage thorough the bovine digestive system and is not zoonotic. The role of non-dairy ingredients and the factory and home environment should be addressed as potential sources of *Cronobacter* for infant formula.

Dr Niall Mullane, Danone Baby, Ireland

- **Best Practice in infant formula manufacture**

Adequate measures must be taken in order to guarantee control of processes and quality of subsequent product. Minimising the level of *Cronobacter* contamination in the environment of powdered infant formula manufacturing plants is a fundamental control strategy which serves to decrease the contamination pressure that an environment poses to its process. This approach, achieved through zoning and adapted dry cleaning, demonstrates good manufacturing practice and is a key risk reduction strategy. All processes at some stage will pose a risk of producing substandard product. Therefore process conditions should be continually monitored to provide the right information, at the earliest possible phase of production, to reduce the risk of contamination and minimise quality deviations in advance. Monitoring a process involves sampling product contact surfaces for the presence of *Enterobacteriaceae*, an indicator organism providing valuable information on the hygienic status and microbial ecology of a process. Ideally, Best Practice in Infant Formula Manufacture for the exclusion of *Cronobacter* from product begins with a robust process and facility. Older facilities, not of ideal design, can successfully manage and control *Cronobacter* through operator training, effective cleaning, surveillance and management of problematic sites. Identification, awareness and control of problematic sites within process is the overriding approach for control of *Cronobacter* powdered infant formula manufacture.

WORKSHOP C: OCCURRENCE & SURVEILLANCE

Ms Edita Custic, London Metropolitan University, UK

- **Biofilm formation by *Cronobacter* on infant feeding bottles and teats**

Cronobacter (*Enterobacter sakazakii*), a relatively rare cause of neonatal infections, can form biofilms on different materials. Biofilms withstand nutrient deprivation, disinfectants, pH change and antibiotics to a greater extent than planktonic cells. The extent to which biofilm formation by *Cronobacter* strains NCIMB 5920 and 8272 occurs on feeding bottles (polycarbonate) and teats (silicone) was examined to determine the need for additional hygiene precautions. Infant feeding bottles and teats were cut in 10x10x0.5mm pieces and sterilised. Separate suspensions containing 10^4 cfu/ml of *Cronobacter* strains 5920 and 8272 were prepared in infant formula milk (IFM). Silicone and polycarbonate pieces were added to the suspensions and incubated at 22°C for three days. The materials were then washed twice with phosphate buffered saline (PBS) and vortexed at maximum speed in PBS with anti-bumping granules for one minute to dislodge biofilm cells. Both PBS and IFM were diluted and plated on try tone soya agar. Data were analysed using the independent t-test. Planktonic cell numbers from both strains of *Cronobacter* were higher than those of biofilms. There was a significant difference between the strains forming biofilms on silicone and polycarbonate surfaces. Strain 5920 showed greater variability than strain 8272, with

a maximum of log 6.88 cfu/ml biofilm-forming cells on silicone surfaces. Strain 8272 was less variable; maximum cell numbers forming biofilms were log 6.36 cfu/ml on polycarbonate surfaces. Previous research showed that when grown in IFM, *Cronobacter* adheres strongly to different surfaces used for infant feeding preparation units and equipment. Our research showed that both strains 8272 and 5920 adhered and formed biofilms on polycarbonate and silicone from infant feeding bottles. Strain 5920 adhered and formed biofilm more readily on silicone, while strain 8272 adhered and formed biofilm more readily on polycarbonate. However, existing hygiene recommendations for sterilising feeding bottles should suffice.

Dr Stephen O'Brien, University College Dublin, Ireland

- **Holistic approach to identification of *Cronobacter* spp.**

Accurate, rapid and reliable identification of microorganisms is paramount to many food production and clinical identification processes. Traditionally samples were sub-cultured for morphological and microscopy analysis. Today we have an abundance of rapid methods for identification of microorganisms; consisting of both phenotypic and molecular approaches. This study investigates the use of biochemical profiling, molecular subtyping and gene sequencing to characterize a group of *Cronobacter* spp. The accurate identification of *Cronobacter* is vital to help prevent its transmission into foods and identify infections amongst patients. The results of this study demonstrate that adopting both phenotypic and molecular approaches can prove complimentary in the characterization of *Cronobacter* spp.

B. Healy, S. O'Brien, C. Iversen and S. Fanning. Centres for Food Safety & Food-borne Zoonomics. UCD Veterinary Sciences Centre, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland.

Mr Humid Ibrahim, UAE

- **Incidence of *Cronobacter sakazakii* in powdered-milk-based infant formula in Dubai (UAE)**

As an emerging pathogen *Cronobacter* (the name *Cronobacter* will be used interchangeably with *Enterobacter sakazakii* throughout the text), received much attention after April 2002 when the US - FDA issued an alert to health care professionals regarding the risk associated with *Cronobacter* infection among neonates fed milk based powdered infant formulas. *Cronobacter* infection was rare but, life threatening cause of neonatal meningitis, sepsis and severe neurological impairment. In general fatality rate varies from 40-80% among newborns diagnosed with this type of severe infection. The United Arab Emirates, like other Gulf countries largely depends on imported foods from around 100 countries around the world. Part of it consumed locally and the majority 75% re-exported to other countries in the Middle East and Africa. Surveys were conducted annually though the period 2003 to 2008 comprising a total of 735 samples with a total of 31 positive isolates for the organism. Isolates were maintained in Cryobank system and subjected to further confirmation and characterization studies. The organism was found to be resistant to 4 antibiotics out of 21 tested. Those are Cefoxitin, Cephalothin, Ampicillin and Amoxicillin. Generation times of the organism were calculated at different temperatures, 25, 30 and 45°C and was found to be 36, 24.6 and 20.4 minutes respectively. Survival of the organism in dry powder was studied and the population showed stability in counts during the 34 weeks experimental period with minimal fluctuations resulting from inoculums differences. Growth at different pH was studied and the organism was shown to be inhibited at pH 2.5, 3.5 and was able to grow at, 4.5, 5.5, 6.5, 7.5 and 8.5. Susceptibility of the organism to different disinfectants that are used in food manufacturing premises as well as for cleaning infant feeding bottles were studied and the organism was completely destructed by all of 5 types used in the experiment. The risk of infection form an infant formula sold in Dubai was calculated and described to be very minor as well as risk was decreasing on annual basis from 2003 up to now and that is due to awareness of connected parties, major changes in code hygiene and infant formula processing development in reaction to the problem.

SESSION 7: OCCURRENCE & SURVEILLANCE 3

Dr Norrakiah Abdullah Sani, National University of Malaysia (UKM),

- **Isolation, growth and survival characteristics of *Cronobacter sakazakii* and *C. muytjensii* in powdered infant formula milk in Malaysia**

Enterobacter sakazakii previously known as 'yellow-pigmented *E. cloacae'* has been reclassified as a new genus *Cronobacter* based on taxonomic analysis and geno-and phenotypic evaluation. This pathogenic organism has been associated with rare form of infant meningitis and necrotizing entercolitis (NEC) with high mortality rate (40-80%). Some cases have been linked to the consumption of contaminated powdered infant formula milk (IFM). The objectives of this study were to determine the presence of *Cronobacter* spp. and their growth and survival characteristics in powdered IFM. A total of 91 powdered IFM samples were tested for the presence of *Cronobacter* using chromogenic medium (Druggan–Forsythe–Iversen agar, DFI formulation). All the suspected isolates from DFI agar (blue-green colonies) were confirmed as *C. sakazakii* using phenotyping. Presumptive isolates were also identified as *C. sakazakii* using molecular assay such Real-Time PCR (RT-PCR) and 16S ribosomal DNA (rDNA) sequencing. *C. sakazakii* strains were isolated from 11% of IFM samples on the Malaysian market. Other Enterobacteriaceae associated with NEC such as *Escherichia coli*, *Klebsiella pneumoniae*, *E. cloacae* and *Salmonella* spp. were also present in the IFM samples. The growth characteristics of three isolated *C. sakazakii* strains and *C. muytjensii* type strain ATCC 51329 at 4, 10, 25, 37, 45 and 50°C showed that *C. sakazakii* did not grow at proper refrigeration temperature of 4°C but had a doubling time of 29.92 min at room temperature (25°C) in reconstituted IFM. Three isolated *C. sakazakii* strains and *C. muytjensii* were also used to determine the heat resistance of this organism at 52, 54, 56, 58 and 60°C in reconstituted dried IFM. The average *D*-values of 39.01, 18.89, 4.56, 2.99 and 1.89 min were obtained at each temperature respectively. The overall calculated *Z*-value was 5.91°C. This study shows that high ambient temperature and lack of refrigeration of reconstituted IFM will increase the opportunity for *Cronobacter* to multiply and possibly cause infection to the high risk group, i.e. infants and neonates. It also showed that molecular identification methods, in comparison to the biochemical tests which are still in use in many laboratories, give more accurate and sensitive identification results.

Masomeh Ghassem[a], Uma Priya Kupusamy[b], Norizan Jaafar[b], Abdul Salam Babji[a], Stephen J. Forsythe[c], Norrakiah Abdullah Sani[a]. [a] Food Science Programme, School of Chemical Sciences and Food Technology, Universiti Kebangsaan Malaysia, 43600, Bangi, Selangor, Malaysia
[b] Chemistry Department of Malaysia, Jalan Sultan, 46661, Petaling Jaya, Selangor, Malaysia
[c] School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, United Kingdom

Prof Kunho Seo, Konkuk University, Seoul, Korea

- ***Cronobacter* (*Enterobacter sakazakii*) research and surveillance in Korea**

Although no outbreak has been reported, the safety of powdered infant formula (PIF) and baby foods has been an important issue in Korea since numerous outbreaks associated with *E. sakazakii* occurred around the world in 2004. Two surveillance studies for *E. sakazakii* in PIF and baby foods were conducted by Korea Food and Drug Administration (KFDA).
In the first surveillance study, the strains of *E. sakazakii* were isolated from 7 out of 85 PIF samples (5.33%) at levels of 0.36∼2.86 MPN/100 g using FDA Most Probable Number method. In baby foods monitored monthly basis, *E. sakazakii* and *B. cereus* were isolated from 6 (6%) and 23 (23%) out of 100 samples, respectively. In the second surveillance study, *E. sakazakii* were positive in 16 samples (45%) out of 36 heat processed ground cereals (Sunsik: Korean foods for monks). The contamination levels in the Sunsik foods were from 0.72 MPN/100g to over 110 MPN/100g. A modified FDA's provisional MPN method has been used as standard method for *E. sakazakii* in KFDA and National Veterinary Research Quarantine Service (NVQRS). Two commercial selective media, *E. sakazakii* chromogenic agar (Oxoid, UK) and *E. sakazakii* media (Neogen, USA), are included in the standard methods used by the two government agencies. Real-time PCR methods have been recommended for improving the current culture-based detection method. A zero tolerance policy for PIF and baby foods by the KFDA and NVQRS along with the HACCP and GMP implementation in the food industries have significantly improved the microbial quality of the infant foods in Korea.

Kun-Ho Seo. Department of Public Health, College of Veterinary Medicine, Konkuk University, Seoul, 143-701, Korea

Ms Sharon Edelson Mammel, Division of Plant and Dairy Food Safety, FDA, USA

- **Thermal and Acid Resistance of *Enterobacter sakazakii* (*Cronobacter*).**

Thermal processing and acidified environments are common methods utilized by food processors to reduce and eliminate potential pathogens from food products. To utilize these methods, the thermal and acid sensitivity and/or resistance of the bacteria must be determined. We ascertained the thermal resistance of the organism in infant formula employing a heating coil. The strains utilized came from several sources such as infant formula and clinical isolates. Our studies determined that *Enterobacter sakazakii* (*Cronobacter*) is not a particularly heat resistant microorganism. Results from a separate set of heating trials indicated that heating rehydrated infant formula at ≥ 70°C for even a few seconds will result in a substantial inactivation of *E. sakazakii*. *E. sakazakii'*s inducible acid resistance was studied in Brain Heart Infusion (BHI) broth. Prior exposure by growth in a moderately acidic environment increases the acid resistance of *E. sakazakii,* indicating that the organism possesses one or more systems for inducible pH-dependent stationary phase acid tolerance.

Dr Peter Ben Embarek, WHO, Geneva, Switzerland

- A world perspective on *Cronobacter* research and surveillance

Dr Ben Embarek will summarise the current status and needs in relation to continued research on Cronobacter and surveillance for these organisms across the globe seen from the perspective of WHO and FAO.