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

January 22nd & 23rd, 2009
University College Dublin, IRELAND

Poster Abstracts

P01 Detection and frequency of *Cronobacter* (*Enterobacter sakazakii*) in different categories of ready-to-eat foods other than infant formula.

Andreas Baumgartner[1], Marius Grand[1], Marianne Liniger[1] and Carol Iversen[2]

[1]Federal Office of Public Health, Section of Microbiological and Biotechnological Risks, 3003 Bern, Switzerland. [2]Centre for Food Safety, UCD Veterinary Sciences Centre, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland.

Cronobacter is an opportunistic pathogen mainly associated with infections in neonates, however adult infections have also been reported. As information about the ecology of *Cronobacter* is still incomplete, the occurrence of these organisms in foods other than infant formula needs to be addressed. In the present work samples of commercially available ready-to-eat foods were screened for the presence of *Cronobacter* and other Enterobacteriaceae. As it has been reported that *Cronobacter* can persist in food production environments, further samples of products that were found positive for the presence of *Cronobacter* were analyzed and isolated strains compared using pulse-field gel electrophoresis (PFGE) to detect possible persisting contamination of production plants or retail areas. *Cronobacter* was isolated from 14/23 samples of sprouts and fresh herbs/salads (60.9%), 7/26 samples of spices and dried herbs (26.9%) and 3/42 confectionery samples (7.1%). In cases where repeat samples were available, foods positive for *Cronobacter* were retested twice. In total, 54 *Cronobacter* isolates from 24 foods were recovered and fingerprint patterns generated using PFGE. Identical PFGE-profiles were generated for *Cronobacter* isolates from five samples of two confectionery products obtained from a particular bakery shop over a period of 11 months. This may indicate a persistent contamination of the production site. For all other isolates, no clustering by phylogenetic analysis of PFGE-profiles was observed, indicating the sporadic nature of *Cronobacter* in ready-to-eat foods. Enterobacterial counts varied from a maximum value of 2.9 x 107 CFU / g (in dill) to a minimum value of < 10 CFU / g (in confectionery and dried herbs/ spices). There was no correlation between Enterobacterial count and the presence of *Cronobacter*. The study showed that *Cronobacter* may be regularly imported into private households.

P02 Comparison of virulence risk factors in *Cronobacter* and novel *Enterobacter* species

Carol Iversen[1,2], Angelika Lehner[1], Brendan Healy[2], Séamus Fanning[2] and Roger Stephan[1]

[1]Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zürich, CH-8057, Zürich, Switzerland. [2]Centre for Food Safety, UCD Veterinary Sciences Centre, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland.

Ingestion of infant formula containing *Cronobacter* (*Enterobacter sakazakii*) has been associated with rare cases of neonatal infections. *Cronobacter* spp. are noted for their desiccation resistance and production of exopolysaccharide (EPS), factors which may allow them to persist in dry environments. Some isolates can attach and invade epithelial cells and interact with macrophages *in vitro*. Three other recently described species (*E. helveticus, E. turicensis* and *E. pulveris*) are found in the same environmental niches as *Cronobacter*, including infant formula factories. However there has been no association of these species with neonatal illness. In this study, factors that may contribute to the infection of infants via infant formula are investigated in these novel species in comparison to *Cronobacter* isolates. Strains comprised 12 *Cronobacter* (representative of different geno- and phenotypes), 22 isolates from the novel *Enterobacter* spp. and 5 other

Enterobacteriaceae. Biochemical and phenotypic assays were performed using conventional methods and included motility, siderophore, lactose fermentation, DNAse, esterase, gelatinase, mucinase, albuminase, elastase and haemolysin activity. Production of exopolysaccharide was scored semi-quantitatively based on colony size. Selected isolates were subjected to in vitro assays to determine the relative propensity to attach and invade human epithelial cells, persist in macrophages (24 and 48 h) and survive compliment mediated cytotoxity. The relative desiccation tolerance in dried milk powder was also determined over 6 months. The *Enterobacter* species lacked DNAse and siderophore activity. *E. helveticus* and *E. turicensis* were sensitive to compliment-mediated lysis and lacked gelatinase activity. Although the *Enterobacter* species were able to attach to epithelial cells they were less invasive than *Cronobacter* isolates, they were also unable to persist in macrophages. Although the production of EPS was variable among these species, *Enterobacter* isolates appear to be as desiccation resistant as *Cronobacter* isolates. Although the novel *Enterobacter* spp. appear to be adapted to exist in the same environments as *Cronobacter*, and are present at similar levels in infant foods, they appear to lack mechanisms involved in mammalian cell invasion and maintenance of systemic infection.

P03 Genotyping of *Cronobacter* species from infant formula production environments

Carol Iversen[1], Angelika Lehner[1] Claudia Feer[2], Karl Gschwend[2] and Roger Stephan[1]

[1]Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zürich, CH-8057, Zürich, Switzerland. [2]QA and Food Safety Department, Hochdorf Nutritec AG, CH-6280 Hochdorf, Switzerland.

Cronobacter (*Enterobacter sakazakii*) has been associated with rare cases of meningitis, bacteraemia and necrotizing enterocolitis in neonates. Infant formula has been identified as a possible route of ingestion and therefore manufacturers are keen to address this potential hazard within production facilities. *Cronobacter* spp. have been found in various foods, factories and home environments and appear to be normally innocuous ubiquitous organisms. However a clear contamination route for infant formula has not been established. In this study factory sites were surveyed for the presence and distribution of *Cronobacter* species. Samples were taken from finished products, raw materials and various manufacturing environments across two factory sites between 1/5-30/10/2007. Isolated *Cronobacter* spp. were genotyped using both PFGE and RAPD techniques and similarities between isolates from different locations and sample types were assessed. Over the course of the study a total of 153 *Cronobacter* isolates were obtained. These represented 71 PFGE pulsetypes, with 24 pulsetypes containing multiple isolates. The greatest diversity of PFGE pulsetypes occurred among the environmental and ingredient isolates, while a limited number appeared to be responsible for end product contamination. The RAPD profiles were less discriminatory than PFGE patterns. Examples of all recently proposed *Cronobacter* species were found during this study. Clonal isolates present in products could be matched to indistinguishable pulsetypes in raw materials and in the environment, however no strains were identified that were present in both the environment and raw materials. *Cronobacter* spp. are ubiquitous organisms found in factory environments and dried food materials. In this study raw materials are implicated as a source of contamination of infant food products. However it is not possible to conclude whether isolates found in the environment originated from or contaminated the products.

P04 The taxonomy and identification of *Enterobacter sakazakii*: a proposed new genus *Cronobacter*

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] and 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.

The organism described as *Enterobacter sakazakii* is an opportunistic pathogen that can cause infections in neonates. When the species was defined in 1980 it was suggested that it might consist of multiple species. DNA-DNA hybridizations gave no clear generic assignment with 53-54% relatedness to species in two different genera, *Enterobacter* and *Citrobacter*. In this study, independent molecular methods were employed to clarify the taxonomic relationship of 210 strains and amendments to the classification of these organisms are proposed. Full length 16S rRNA sequence analysis, automated ribotyping, f-AFLP analysis and DNA-DNA hybridization were performed as previously described. PCR amplification assays targeting the *dna*G and *glu*A genes, biochemical galleries (API20E and ID32E), and α-glucosidase assays were also performed as described. Strains identified as *E. sakazakii* were divided into separate groups on the basis of their 16S rDNA sequences, ribopatterns and f-AFLP fingerprints. DNA-DNA hybridizations revealed six genomospecies. The phenotypic profiles of the species were determined and biochemical markers identified. All *Cronobacter* strains expressed α-glucosidase activity and were positively identified by dnaG RT-PCR and α-glucosidase PCR, with no false positive strains. The API20E test system identified 70% of the strains as *E. sakazakii* however 7/102 strains were mis-identified, as they appeared to belong to other *Enterobacter* species using 16S rDNA sequence analysis and ribotyping. Using the ID32E gallery with version 3.0 of the apiweb™ database, 189/210 *Cronobacter* strains (90%) were identified to the species level (as *E. sakazakii*) with 'good-excellent' identification. The nearest significant taxon for all but one of the remaining strains (20/21) was *E. sakazakii* and none of the other Enterobacteriaceae were misidentified as *E. sakazakii* with this gallery. This study clarifies the taxonomy and identification of *E. sakazakii* and proposes a novel genus, *Cronobacter*.

P05 Development of a screening method for the isolation of *Cronobacter*.

Carol Iversen[1], Patrick Druggan[2], Han Joosten[3], Sandra Schumacher[4], Angelika Lehner[4], Claudia Feer[5], Karl Gschwend[5] and Roger Stephan[4]

[1]Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland. [2]Oxoid Ltd., Thermo Fisher Scientific, Basingstoke, Hampshire RG24 8PW, United Kingdom. [3]Quality and Safety Department, Nestlé Research Centre, Vers-chez-les-Blanc, CH-1000 Lausanne, Switzerland. [4]Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zürich, CH-8057, Zürich, Switzerland. [5]QA and Food Safety Department, Hochdorf Nutritec AG, CH-6280 Hochdorf, Switzerland.

It has been reported that some strains of *Cronobacter* do not grow in selective enrichment media commonly used for the isolation of Enterobacteriaceae and that fermentation of sucrose in conjunction with metabolism of 5-bromo-4-chloro-3-indolyl-a-D-glucoside are useful differential tests for *Cronobacter*. Therefore a differential broth has been developed that enables samples to be screened for potential *Cronobacter* contamination without incorporating selective agents that may affect the recovery of sensitive strains. The *Cronobacter* screening broth (CSB) comprises a non-selective base media into which a niche fermentable carbohydrate (sucrose) and a Gram-positive inhibitor (vancomycin) were added to detect the presence of presumptive *Cronobacter*. Inclusivity and exclusivity of CSB was assessed using 329 strains including 229 target *Cronobacter* isolates. The broth was further tested using spike and naturally contaminated samples. A parallel comparison with the current FDA BAM method and the ISO/TS 22964 methods for raw materials, line/end products, and factory environment samples was also undertaken. After 24 h at 37°C all *Cronobacter* strains tested were able to grow in CSB and ferment the sucrose. The Sensitivity and Negative Predict Value (NPV) of CSB in conjunction with chromogenic agar was 100%. The Specificity was 94% and the Positive Predictive Value (PPV) was 97.4%. The CSB screening method was able to detect *Cronobacter* in spiked and in naturally contaminated samples. Potentially, this screening method can enable the decision to release uncontaminated product after 48 h. As CSB is a differential rather than selective enrichment broth, all *Cronobacter* strains are able to grow in CSB. This differential screening broth is complementary to any agar medium that incorporates a test for metabolism of alpha-glucopyranoside. The numbers of positive samples found from ingredients and the environment are in line with previous findings that *Cronobacter* spp. are ubiquitous environmental organisms that can be isolated from various food products as well as from households.

P06 Development and application of a novel Peptide Nucleic Acid probe for the specific detection of *Cronobacter* (*Enterobacter sakazakii*) in powdered infant formula

C. Almeida[1,2], N. F. Azevedo[1,2], C. Iversen[3] , S. Fanning[3], C. W. Keevil[2] and M. J. Vieira[1]

[1]IBB - Institute for Biotechnology and Bioengineering, Centre of Biological Engineering, Universidade do Minho, Campus de Gualtar 4710-057, Braga, Portugal. [2]Environmental Healthcare Unit, School of Biological Sciences, University of Southampton, Basset Crescent East, Southampton SO16 7PX, United Kingdom. [3]Centres for Food Safety & Food-borne Zoonomics, UCD Veterinary Sciences Centre, University College Dublin, Belfield, Dublin 4, Ireland.

Cronobacter spp. are causative agents of meningitis, septicemia and necrotizing enterocolitis in neonates and immunocompromised infants. Recently, contaminated powdered infant formula (PIF) has been reported as a source of these infections. In order to minimize the risk of infection, the development of a rapid, sensitive and specific method for the early detection of this bacterium in infant formula is of the utmost importance. Fluorescence *in situ* hybridization (FISH), a technique that allows direct visualization of whole cells, has been combined with specific peptide nucleic acid (PNA) probes, a new synthetic molecule with a better hybridization performance than DNA probes. In this work, a new FISH method for the detection of *Cronobacter* spp. using a novel PNA probe is reported. This PNA-FISH method was then adapted for the detection of this bacterium in PIF. The PNA-FISH procedure using the *Cronobacter* probe proved to be a reliable method for the detection of this pathogen in PIF samples and an alternative to existing molecular methods. It presented high specificity and sensitivity, detected less than 1 CFU per 10g of *Cronobacter* in infant formula and provided detection in less than 12 hours. Direct visualization of bacterial cells was possible and the method was simple and easy to use, without any special equipment apart from an epifluorescence microscope. The samples can be also analysed by flow cytometry.

P07 Application of pulsed-field gel electrophoresis to characterize and control the transmission of *Enterobacter sakazakii* in an infant formula processing facility

Niall Mullane[1], Paul Whyte[1], Patrick Wall[2], Teresa Quinn[1] and Séamus Fanning[1]

[1]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. [2]School of Public Health and Population Sciences, University College Dublin, Belfield, Dublin 4, Ireland.

Enterobacter sakazakii (*Cronobacter*) has been associated with life-threatening infections in premature low birth weight infants. Since the first reported case of *E. sakazakii* neonatal meningitis published in 1961 several outbreaks of infection have been documented. *Enterobacter sakazakii* is an ubiquitous organism and although its exact mode of transmission has not been fully elucidated, powdered infant formula (PIF) has been epidemiologically implicated in several clinical cases. *E. sakazakii* contamination of PIF and its processing environment were evaluated during monitoring conducted between April 2005 and March 2006 in a PIF processing facility producing 17,000 tonnes of base powder and 22 million 900 g cans of PIF per year. All isolates were cultured on selective medium, biotyped and checked by 'real-time' PCR to confirm identification. PFGE macrorestriction profiles using XbaI were generated in each case. A dendrogram was produced using the BioNumerics software programme. All isolates cultured were confirmed by conventional and molecular methods as *E. sakazakii*. PFGE analysis revealed the existence of several clusters of indistinguishable DNA profiles within the PIF production facility. These isolates were mapped to specific problem areas. This information would aid the development of more powerful targeted intervention measures for its exclusion form the PIF processing chain. Since PIF is not a sterile product, and to reduce the risk of infection, microbiological control of *E. sakazakii* in the production environment is essential. This study identified of problem areas within the production environment that required careful intervention. In addition PFGE was applied to further characterise the strains recovered and to establish any potential dissemination routes. Controlling *E. sakazakii* will improve PIF food safety and reduce the risk of infection among vulnerable infants.

P08 Development of Multiple-Locus Variable Number Tandem Repeat Analysis for the molecular subtyping of *Enterobacter sakazakii*

Niall Mullane[1,2], Mary Ryan[2], Carol Iversen[1,2], Mary Murphy[3], Peadar O'Gaora[4], Teresa Quinn[1,2], Paul Whyte[1,2], Patrick Wall[5] and Séamus Fanning[1,2]

[1]Centre for Food Safety, [2]School of Agriculture, Food Science and Veterinary Medicine,[4] Conway Institute of Biomolecular and Biomedical Research,[5] School of Public Health and Population Sciences, University College Dublin, Belfield, Dublin 4, Ireland. [3] Veterinary Food Safety Laboratory and Cork County Council, Inniscara, County Cork, Ireland.

Enterobacter sakazakii is an emerging opportunistic pathogen that is the cause of rare cases of meningitis, necrotising enterocolitis and bacteremia in infants. The tandem repeat DNA motifs were identified in the genome sequence of *E. sakazakii* ATCC BAA-894. Four variable number tandem repeat (VNTR) loci selected and amplified individually by PCR with fluorescent labelling. PCR products were electrophoresed on a ABI 377 DNA analyser and size values were assigned to the GeneFlo™ TAMRA labelled ladder. A four-digit allele string based on the number of repeats was assigned to all isolates in the order ESTR-1, ESTR-2, ESTR-3, and ESTR-4. Allele strings were imported into Bionumerics and a minimum-spanning tree (MST) was generated. The genetic relationships of the analysed isolates were deduced by the construction of a MST. Forty-nine unique allele strings were observed among these isolates. A number of branched clusters were visible but no association could be established between isolate type, geographical origin, or biogroup. The discriminatory index was comparable to pulsed-field gel electrophoresis (PFGE). Molecular typing remains an important tool for surveillance, outbreak investigation and tracing of bacteria through the food chain. Although this method has less discriminatory power than PFGE, it could be potentially used to rapidly monitor outbreaks and trace this organism in food production facilities. It is realistic to assume that the inclusion of additional polymorphic loci would further increase the discriminatory power of the method.

P09 Molecular analysis of the *Enterobacter sakazakii* O-antigen gene locus

Niall Mullane[1,2], Brendan Healy[1,2], Peadar O'Gaora[3], Jarlath Nally[2], Carol Iversen[1,2,4], Paul Whyte[1,2], Patrick Wall[5] and Séamus Fanning[1,2]

[1]Centres for Food Safety and Food-borne Zoonomics, UCD Veterinary Sciences Centre, [2]School of Agriculture, Food Science & Veterinary Medicine, [3]Conway Institute of Biomolecular & Biomedical Research, [5]School of Public Health & Population Sciences, University College Dublin, Belfield, Dublin 4, Ireland and [4]Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zürich, CH-8057, Zürich, Switzerland.

Nucleotide polymorphism associated with the O-antigen encoding locus, *rfb*, in *Enterobacter sakazakii* was determined by PCR-RFLP analysis. Based on the analysis of these DNA profiles, 12 unique banding patterns were detected amongst a collection of 62 strains from diverse origins. Two common profiles were identified and designated as serotypes O:1 and O:2. DNA sequencing of the 12,500-bp region, flanked by *galF* and *gnd*, identified 11 open reading frames, all with the same transcriptional direction. Analysis of the proximal region of both sequences demonstrated remarkable heterogeneity. A polymerase chain reaction (PCR) assay targeting genes specific for the two prominent serotypes was developed, and applied for the identification of these strains recovered from food, environmental and clinical samples.

P10 Comparison of the ISO/TS 22964 procedure with a new one-broth strategy for *Cronobacter* spp. detection in environmental and product samples

Nicole Giezendanner[1], Patrick Druggan[2], Carol Iversen[3], Peter Stephens [2] and Roger Stephan[1]

[1]Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zürich, CH-8057, Zürich, Switzerland. [2]Oxoid Ltd., Thermo Fisher Scientific, Basingstoke, Hampshire, RG24 8PW, UK.[3] Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland.

The current ISO standard method for detection of Enterobacteriaceae (21528-1:2004) includes enrichment in EE broth, which has been shown to be inhibitory to some members of this family, notably *Cronobacter* spp. A shortened procedure omitting the EE broth has been proposed (Joosten et al. 2008), however competition from Gram-positive flora may be detrimental to the effective recovery of low levels of target organisms in some sample matrices. In a recently finished study we investigated novel cost effective modifications, designed to improve ISO 21528-1:2004 for the detection of Enterobacteriaceae by a one broth strategy. Supplementation of buffered peptone water (BPW) with 8-hydroxyquinoline, ammonium iron(III) citrate, sodium deoxycholate and sodium pyruvate (BPW-S) improved the recovery of Enterobacteriaceae from artificially and naturally contaminated samples. The Enterobacteriaceae strains chosen included 16 *Cronobacter* spp. strains, and especially 9 strains that had been previously found to be particularly sensitive to selective agents used in microbiological growth media. In this study we are comparing the ISO/TS 22964 procedure, the *Cronobacter* screening broth (CSB) method (Iversen et al. 2008) as well as the new one broth strategy (BPW-S) for *Cronobacter* spp. detection in different samples from a PIF factory site. Samples divided into 55 finished products (PIF), and 10 supplementary food samples. 10g sample sizes were used, and dilution in BPW and BPW-S was at 1/10. All samples were pre-enriched for 24 h at 37°C. For the one broth strategy, the enriched samples (BPW-S) were streaked directly onto the modified version of Chromogenic *E. sakazakii* agar - DFI formulation (mDFI; Oxoid Ltd., Basingstoke, UK).

P11 Identification of proteins involved in osmotic stress response in *Cronobacter* spp. by proteomics

Kathrin Riedel[1], Roger Stephan[2] and Angelika Lehner[2]

[1]Department of Microbiology, Institute of Plant Biology, and [2]Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zürich, CH-8057, Zürich, Switzerland.

Cronobacter sakazakii is considered an opportunistic foodborne pathogen, causing rare but significant illness especially in neonates. It has been proposed that the organism is relatively resistant to osmotic and dry stress compared to other species of the Enterobacteriaceae group. To understand the mechanisms involved in osmotic stress response, 2-DE protein analysis coupled to MALDI-TOF MS was employed to investigate changes in the protein profiles of *C. sakazakii* cells in response to two different types of osmotic stress (physical desiccation and growth in hyper-osmotic media). In total, 80 differentially expressed protein spots corresponding to 53 different protein species were identified. Affiliation of proteins to functional categories revealed that a considerable part of the differentially expressed proteins from desiccated and hyper-osmotic grown samples belonged to the same functional category but were regulated in opposite directions. The most striking effects observed for both types of osmotic stress in *C. sakazakii* were a significant down-regulation of the motility apparatus and the formation of filamentous cells. Our data show that the protein pattern of NaCl-grown cultures reflect more or less a general down-regulation of central metabolic pathways, whereas adaptation of (non-growing) cells in a desiccated state represents an accumulation of proteins that serve some structural or protective role.

P12 International survey for *Cronobacter* and related organisms in infant foods and formulas

Jessica Chap[a], Philip Jackson[a], Rosana Siqueira[b], Nelma Gaspar[c], Célia Quintas[c], Jon Park[d], Tareq Osmaili[e], Reyad Shaker[e], Ziad Jaradat[f], Sri Harminda P. Hartantyo[g], Norrakiah Abdullah Sani[g], Sri Estuningsih[h], and Steve Forsythe[a]

[a] School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham, UK. NG11 8NS. [b] Food Technology Institute, 2880 Brazil Ave, Campinas 13070-178, Brazil. [c] University of the Algarve, Engenharia Alimentar, Campus da Penha, 8000-118 Faro, Portugal. [d] Department of Food Science and Biotechnology, College of Engineering, Kyungwon University, Songnam, Kyonggi-do 461-701, South Korea. [e] Department of Nutrition and Food Technology, Faculty of Agriculture, Jordan University of Science and Technology, P.O. Box (3030), Irbid-22110, Jordan. [f] Department of Biotechnology and Genetic Engineering, Jordan University of Science and Technology, P. O Box 3030, Irbid-22110, Jordan. [g] Food Science Programme, School of Chemical Sciences and Food Technology, Faculty of Science and Technology, National University of Malaysia,

43600 UKM Bangi, Selangor, Malaysia. [h] *Faculty of Veterinary Medicine, Bogor Agricultural University, Indonesia.*

In response to the FAO/WHO (2008) call for microbiological data concerning follow up formula, 8 laboratories in Brazil, Indonesia, Jordan, Korea, Malaysia, Portugal, and UK participated in surveying for *Cronobacter* and related organisms in their local infant formula and foods. The products were categorised according to their principle ingredients, and reconstitution instructions were collated. A total of 262 products were analysed using a standardised procedure of pre-enrichment of a 25g sample in 225 ml Buffered Peptone Water (BPW), followed by enrichment in Enterobacteriaceae Enrichment (EE) broth, plating on chromogenic *Cronobacter* agar; Druggan-Forsythe-Iversen agar (DFI formulation) and presumptive identification with ID32E. Presumptive *Cronobacter* isolates were identified using 16S DNA sequence analysis. Aerobic plate counts (APC) of the products were also determined. Thirteen samples had APC $>10^5$ cfu/g, 3 of which contained probiotic cultures. *Cronobacter* spp. were isolated from 36 products; 3/89 (3%) follow on formulas (as defined by Codex Alimentarius Commission), and 24/170 (14%) infant foods and drinks. *Cronobacter* spp. were less prevalent in follow up formula, than other foods given to infants over the same age range. Nevertheless, the later products are less likely to be subject to temperature abuse with an increase in bacterial load and risk of infection. A range of other bacteria were also isolated, including *Acinetobacter baumanii*, *Enterobacter.cloacae*, *Klebsiella pneumonia*, *Citrobacter freundii*, *Serratia ficaria* and *Pseudomonas aeruginosa*. There was significant variation in the reconstitution instructions for follow up formulas including the using water at temperatures which would enable bacterial growth. Additionally, the definition of follow up formula varied between countries.

P13 *Cronobacter* (*Enterobacter*) *sakazakii*, Enterobacteriaceae and aerobic plate count in raw and pasteurized milk

Norrakiah Abdullah Sani, Noorzatul Shuhada Md Zaini and Lim Yen Yi
Food Science Programme, School of Chemical Sciences and Food Technology, Faculty of Science and Technology, National University of Malaysia, 43600, UKM Bangi, Selangor, Malaysia.

This study was carried out to detect the presence of *Cronobacter* spp. *(Enterobacter sakazakii*), other Enterobacteriaceae and the general microbial population in raw and pasteurized milk. Total plate count (TPC) was carried out on the samples obtained from restaurant (KJG), market (FS1) and farms (ST and BL). *Brilliance Enterobacter sakazakii* chromogenic agar (DFI formulation) was used in isolating and detecting Enterobacteriaceae and *Cronobacter* spp.. Biochemical tests (Microgen GN-ID and Rapid One System) were used for the confirmation of presumptive *Cronobacter* isolates. Overall, results for raw milk at first and second sampling, ST first sampling contained the highest microbial load (9.4 log cfu/mL) while FS1 second sample contained the lowest microbial load at 6.4 log cfu/mL. For pasteurized milk at first and second sampling, KJG second sampling contained the highest microbial load at 7.4 log cfu/mL while FS1 first sampling contained the lowest microbial load at 5.3 log cfu/ml The TPC of pasteurized milk samples did not comply with the microbiological standards (< 100,000 cfu/ml) set by both FDA and Malaysian Food Act 1983. *Cronobacter* was detected in 63% (n=16) of raw milk samples and 13% (n=16) of pasteurized milk samples. One strain that was identified as *'E. sakazakii'* using Microgen GN-ID profile was also identified as *'E. sakazakii'* using Rapid One System profile but with a higher % of probability. Enterobacteriaceae and other bacteria such as *E. asburiae*, *Proteus vulgaris*, and *Xanthomonas malthophilia* were also identified in the raw milk and pasteurized milk samples.

P14 Colonisation of enteral feeding tubes by *Cronobacter* spp. and other Enterobacteriaceae

Edward Hurrell, Eva Kucerova, Michael Loughlin and Steve Forsythe

School of Biomedical and Natural Sciences, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, United Kingdom.

Powdered infant formula has come under considerable attention with regard to its microbial safety. In addition to reconstitution with water >70°C, advice has been given that feeds should be used within 3 hours of preparation to reduce microbial risks. Nevertheless this overlooks the issue of the enteral feeding tubes which can be in place for more than 48 hours, and may act as *loci* for

bacterial attachment and not limited to *Cronobacter* spp. Twenty-three strains were selected for analysis. These were *Cronobacter* spp., *Enterobacter cloacae, Citrobacter freundii, C. koseri, Pantoea* spp*., Esch. coli, Esch. hermanii, Esch. vulneris, Klebsiella oxytoca, K. pneumoniae, Hafnia alvei,* and *Acinetobacter baumanii*. Five types of enteral feeding tubes were investigated. These varied in composition, including anti-microbial silver-impregnation, and diameter. Bacterial colonisation was determined using impedance microbiology. All organisms formed biofilms which could not be removed by vigorous washing. The biofilm cell densities varied, for example, on PVC tubing the biofilms were 103, 105 and 107 cfu/cm, for *H. alvei*, *Cr. sakazakii* ATCC 12868 and *Esch. vulneris* respectively. *Cr. sakazakii* NCTC 11467T formed less dense biofilms than the other strains of *Cr. sakazakii*. The biofilm on the 'antibacterial' tubing was not significantly lower than that of ordinary enteral feeding tubes. Enteral feeding tubes may act as *loci* for bacterial attachment, and need to be considered with regard to risk of neonatal infections.

P15 Comparative virulence of *Cronobacter* and *Enterobacter* species with *Citrobacter koseri* and *Escherichia coli* k1.

Juncal Caubilla-Barron, Mike Loughlin, Stacy Townsend, Edward Hurrell and Steve Forsythe

School of Biomedical and Natural Sciences, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, United Kingdom.

A number of Enterobacteriaceae are associated with neonatal infections, of particular interest are *Escherichia coli* K1, *Cronobacter* spp., and *Citrobacter koseri* which may cause infant meningitis. Other organisms of concern include *Enterobacter cloacae*, *E. hormaechei*, and *Salmonella* serovars. 29 strains of *Cronobacter* spp., *Ent. cloacae, Ent. hormaechei, Cit. freundii, Cit. koserii, Salmonella serovars and E. coli* K1 was studied. The virulence potential was assessed using attachment and invasion assays of human intestinal cell lines, macrophage survival, and invasion of human and rat brain cell lines. *Ent. cloacae* showed the highest attachment (3.5% inoculum) to human intestinal cells, but *Salm*. Enteritidis had the highest invasion rate (0.4% inoculum). One *Cr. sakazakii* strain (associated with a fatal neonatal meningitis case) showed significantly greater invasion rates (0.25%) of intestinal cells than other *Cronobacter* strains (0.05-0.2%) and other Enterobacteriaceae, except *Salmonella* Enteritidis. The survival of *Cronobacter* in macrophages varied considerably, with *Cr. sakazakii* persisting longer than other *Cronobacter* species. Similarly, *Cit. koseri* persisted in macrophages for up to 48 hours. *Cit. koseri* showed the highest invasion of rat brain capillary endothelial cells (2.5% inoculum), the second highest was the meningitic strain of *Cr. sakazakii* (1.6%). *Cit. freundii, Ent. cloacae*, and *Ent. hormaechei* showed similar rates for all assays as the non-meningitic strains of *Cronobacter*. The virulence potential of *Cronobacter* isolates varies according to species. Clinical outcome of infection as yet cannot be predicted using in vitro tissue culture assays. Discrepancies will occur as the immune status, and neonate exposure is unknown.

P16 *Cronobacter* (*Enterobacter sakazakii*) and the risk to infant health.

Stephen O'Brien[1], Brendan Healy[1], Carmen Negredo[1], Wayne Anderson[2], Séamus Fanning[1] and Carol Iversen[1]

[1]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. [2] Food Safety Authority of Ireland, Lower Abbey Street, Dublin 1, Ireland.

Cronobacter spp. are considered emerging opportunistic pathogens and the aetiological agents in life-threatening infections amongst infants. The risk posed by contaminated infant formula for consumption by neonates is clear, however the risks associated with powdered follow-on formulae intended for consumption by older infants is now also under consideration. The aim of this work was to test commercially available milk and non-milk based infant drinks intended for consumption by infants over six months of age for the presence of *Cronobacter* spp. A review of the published cases of *Cronobacter* infection in children was also undertaken in order to assess the incidence and case fatality rate in infants compared to neonates. A total of 470 samples comprising 31 different products from 18 brand names belonging to seven companies were tested for the presence of *Cronobacter* species. No milk or soy based infant formula powders were found to contain *Cronobacter* species. However, two cereal-based infant drinks were positive for *Cronobacter*

sakazakii. A review of the published cases spanning the past 48 years did not reveal any fatalities attributable to *Cronobacter* spp. in children over three months. The low incidence of *Cronobacter* in infant powdered drinks, lack of fatal *Cronobacter* infections in infants greater than three months and low incidence of *Cronobacter*-related reported illness in this age group indicated that ingestion of these products currently present a low risk for the intended consumers.

P17 Evaluation of a new onestep enrichment for the isolation of *Cronobacter* spp. from powdered milk formula in association with a chromogenic medium.

Stephen O'Brien[1], Orla Condell[1], Brendan Healy[1], Antoine Vimont[2], David Mosticone[2], Pascal Montes[2], Séamus Fanning[1] and Carol Iversen[1]

[1] Centres for Food Safety & Food-borne Zoonomics, Veterinary Sciences Centre, School of Agriculture, University College Dublin, Belfield, Dublin 4, Ireland. [2] bioMérieux Industry F-69280 Marcy l'Étoile, France.

The aim of the present study was to evaluate a new one-step enrichment protocol for the isolation of *Cronobacter* spp. (*Enterobacter sakazakii*) from powdered infant formula. This protocol consists of a combined pre-enrichment/enrichment broth (*Cronobacter* Enrichment Broth, bioMérieux Marcy l'Etoile, France) used in conjunction with selective-differential agar ChromID Sakazakii (bioMérieux Marcy l'Etoile, France) to facilitate a shortened two-day cultural method for detection of *Cronobacter*. The *Cronobacter* Enrichment Broth (CEB) was evaluated using powdered infant formula (PIF) spiked with low concentrations of 10 lyophilized strains, representative of the genus *Cronobacter*. The isolation of strains was compared in parallel with the current ISO/TS 22964 and a recently proposed differential screening broth method for the detection of *Cronobacter*. All of the *Cronobacter* strains were recovered using the CEB whereas one strain was not detected using either the current ISO/TS 22964 or the differential screening broth method. Counts of the cell concentration after enrichment showed a significantly higher bacterial concentration in the CEB than in the other enrichment broths. There was no difference in the cell concentration for cultures grown in CEB at 37 and 41.5°C. *Cronobacter* was recovered from both 1/10 (50 g:450 ml) and 1/5.5 (100 g:450 ml) "sample to broth" ratios. No significant difference was observed between the cell counts obtained from either ratio. This study found CEB to be significantly better at recovering *Cronobacter* spp. from PIF than the other enrichment media tested. Overall, the use of CEB with a differential plating media such as ChromID Sakazakii facilitates the rapid release (40-48 h) of PIF.

P18 Characterization of *Cronobacter* recovered from dried milk and related products

Brendan Healy, Stephen O'Brien, Carmen Negredo, Carol Iversen and Séamus 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.

Cronobacter is a recently proposed genus consisting of six genomospecies that encompass the organisms previously identified as *Enterobacter sakazakii*. Infant formula has been identified as one route of transmission for infection in infants. However, the primary reservoirs for subsequent contamination of foods with *Cronobacter* remain undefined due to the ubiquitous nature of these organisms. More recently, infections in adults have been reported, especially amongst the elderly and patients who are immunocompromised. The aim of this study was to identify and characterize *Cronobacter* isolated from dried-milk and related products available in an Egyptian food market. In total sixteen *Cronobacter* isolates were identified and characterized using pheno- and genotyping experiments. Real-time PCR confirmed the detection of *Cronobacter*. Antibiotic susceptibilities were obtained for each of the strains. Phenotype profiles were generated based on key biochemical distinguishing tests. Pulsed-field gel electrophoresis and repetitive sequence based PCR analysis provided molecular subtyping information. Sequencing of the recN gene was used to differentiate between the recently described species of *Cronobacter*. Phenotype and genotype analysis should be applied to further characterize *Cronobacter* and prevent its transmission into food products.

P19 Comparison of Biolog GN2, BBL Crystal ENF, RapID ONE and Rapid 32E identification systems for the identification of *Cronobacter* (*Enterobacter sakazakii*).

Brendan Healy[1], Angelika Lehner[2], Jadwiga Goetz[3], Séamus Fanning[1], Roger Stephan[2], Han Joosten[3] and Carol Iversen[1]

[1] Centre for Food Safety, UCD Veterinary Sciences Centre, School of Agriculture, Food Science and Veterinary Medicine, 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.

Cronobacter is a newly proposed genus to encompass the different species referred to as *Enterobacter sakazakii.* These organisms have been associated with rare cases of neonatal infections. The correct identification of these species is important to infant food manufacturers as well as clinicians. The separate species within *Cronobacter* are as yet not represented in many identification databases. Three other recently described *Enterobacter* species, *E. helveticus, E. turicensis* and *E. pulveris,* that are found in the same environmental niches as *Cronobacter* and which represent a challenge to current isolation and conformation schemes for *Cronobacter* are also as yet not identifiable using many commercial systems. This study compares the performance of several identification systems for the confirmation of *Cronobacter* isolates and investigates the potential of the galleries to distinguish the different novel species. Strains comprised 60 *Cronobacter* isolates, covering the phenotypic and genetic variability of this genus, and 30 other Enterobacteriacae. Biolog GN2, BBL Crystal, RapID ONE and rapid 32E galleries were inoculated and incubated according to the manufacturer's instructions. Biolog GN2 Microplates and BBL Crystal galleries read spectrophotometrically using the prescribed automated analyzers. The RapID ONE and rapid 32E galleries were read manually. The Biolog GN2 Microplates gave the best performance for the identification of *Cronobacter* species, also there is more potential for the database associated with this gallery to be amended in order to distinguish the different species. Amendments to the respective databases used for the interpretation of the results obtained from commercial galleries may improve the accuracy of these kits for identification of *Cronobacter* species. Clinicians and food manufacturers need to be aware of the limitations of some biochemical based identification systems for the confirmation of *Cronobacter* isolates.

P20 Evaluation of an automated repetitive sequence–based PCR system for subtyping *Enterobacter sakazakii*

Brendan Healy[1], Niall Mullane[1], V. Collin[2], Sandrine Mailler[2], Carol Iversen[3], Sonia Chatellier[2], Michéle Storrs[4] and Séamus Fanning[1]

[1]Centre for Food Safety. School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Belfield, Dublin 4, Ireland, [2]bioMérieux, La Balme Les Grottes, France and, [3]Institute for Food Safety & Hygiene, Vetsuisse Faculty, University of Zürich, CH-8057 Zürich, Switzerland. [4]bioMérieux, Marcy l'Etoile, France.

Enterobacter sakazakii is regarded as a ubiquitous organism that can be isolated from a wide range of foods and environments. Infection in at-risk infants has been epidemiologically linked to the consumption of contaminated powdered infant formula. Preventing the dissemination of this pathogen in a powdered infant formula manufacturing facility is an important step in ensuring consumer confidence in a given brand together with the protection of the health status of a vulnerable population. In this study we report the application of a repetitive sequence–based PCR typing method to subtype a previously well-characterized collection of *E. sakazakii* isolates of diverse origin. While both methods successfully discriminated between the collection of isolates, repetitive sequence–based PCR identified 65 types, whereas pulsed-field gel electrophoresis identified 110 types showing ≥95% similarity. The method was quick and easy to perform, and our data demonstrated the utility and value of this approach to monitor in-process contamination, which could potentially contribute to a reduction in the transmission of *E. sakazakii.*

P21 Current epidemiological aspects of *Cronobacter* infections

Miriam Friedemann

Federal Institute for Risk Assessment, Diedersdorfer Weg 1, Berlin, Germany.

Cronobacter spp. (*Enterobacter sakazakii*) is a very rare cause of invasive neonatal infection. Public health relevance is attributed to the severe, complicated and often fatal course of neonatal *Cronobacter*-infections. The aim of this study was to elucidate current epidemiological information on *Cronobacter*-infections between 2000 and 2008 like lethality, sources of neonatal *Cronobacter*-infections other than contaminated infant formula and information about epidemiological reporting systems and food contaminations concerning *Cronobacter* spp. The data about neonatal *Cronobacter*-infections between 2000 and 2008 has been extracted from publications in scientific journals and from international (EWRS, FAO, RASFF, WHO) and national epidemiological reporting systems. Statistical analysis was carried out with SAS 9.1. Between 2000 and 2008, hundred and one cases of neonatal *Cronobacter*-infection have been reported. Detailed data have been available for sixty cases. The overall lethality of these neonatal *Cronobacter*-infections was 28.3%. The lethality of *Cronobacter*-meningitis, *Cronobacter*- bacteraemia / septicaemia and -necrotizing enterocolitis / hemorrhagic colitis) was calculated 76.5% ($p<0.0001$), 5.9% ($p=0.33$) and 23.5% ($p<0.05$), respectively. Univariate analysis of risk factors (gender, gestation age, birth weight, day of onset, region) revealed the minor gestation age ($p<0.0001$), minor birth weight ($p=0.0002$) and the European region ($p<0.0001$) as significant risk factor for letal outcome in neonatal *Cronobacter*-meningitis. In the bivariate linear regression model, the European region ($p<0.0001$) and minor gestation age ($p<0.01$) remain as significant risk factors for death in *Cronobacter*-meningitis. Nine cases of invasive *Cronobacter*-infections in neonates and infants, not fed with powdered infant formula, have been reported between 2000 and 2008. Among the five cases with detailed information, four are healthcare related infections. Neonatal *Cronobacter*-infection, based on clinical, epidemiological or microbiological criteria, should be reported, e.g. in Brazil, England and Wales, France, Hungary, Minnesota (USA), Northern Ireland, Norway, The Netherlands and New Zealand. In other countries, e.g. in Germany and Switzerland, (food-borne) related outbreaks are notifiable to local authorities. Between 2000 and 2008, the lethality of *Cronobacter*-meningitis in neonates has remained very high. Neonatal *Cronobacter*-infections are mainly associated with contaminated infant formula and the preparation equipment. Other sources for infection, e.g. the hospital environment should be considered in *Cronobacter*-infections. Neonatal *Cronobacter*-infections should be recorded in centralized (continental) reporting systems.

P22 Growth of Enterobacteriaceae in milk

Shane Cooney, Brendan Healy, Stephen O'Brien, Séamus Fanning, Carol Iversen.

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.

Cronobacter (*Enterobacter sakazakii*) have been established as opportunistic pathogens associated with ingestion of contaminated reconstituted powdered infant formula (PIF). *Cronobacter* can be aetiological agents of meningitis, bacteremia and necrotizing enterocolitis in neonates. *Enterobacter* spp., such as *E. aerogenes, E. cloacae* and *E. hormaechei*, have also been implicated as causal organisms in outbreaks within neonatal intensive care units (NICUs). It is possible that other members of the Enterobacteriaceae that also contaminate infant formula could share risk factors similar to *Cronobacter* and cause cases of neonatal illness. Knowledge of the prevalence and risk factors associated with Enterobacteriaceae strains present in infant formula would contribute to improved detection and control paradigms for the safety of infants. The doubling time for *Enterobacter* spp. ranged from 22-37 min. The mean doubling time for the majority of *Cronobacter* spp. commonly found in PIF was 25 minutes, ranging from 22-29 min. Other more rarely isolated *Cronobacter* spp. showed longer doubling times (41-48 min). This may be relevant to the low occurrence of these species in clinical and food samples. For *Klebsiella* spp., *Salmonella* Typhimurium and *Citrobacter* spp. the doubling times were 23 minutes, 26 minutes and 35.5 min respectively. The ability to ferment lactose did not correspond directly to the growth rates obtained for individual species. *Cronobacter* and *Salmonella* are two of the greatest concerns in PIF and the growth of these organisms in IFM is an important factor in progression of disease. This study showed that other Enterobacteriaceae also had similar growth rates in IFM regardless of their

ability to ferment lactose. This included *Klebsiella pneumoniae* and *oxytoca* as well as *Enterobacter hormaechei* which have both been previously associated with neonatal infections such as necrotising enterocolitis, bacteremia and meningitis (particularly in NICUs). This work indicates that contamination of IFM, either from endogenous organisms or poor hygiene during preparation, can lead to proliferation of potential pathogens increasing the risk of infection in neonates.

P23 Examination of cattle faeces, dairy farm and food environs for the presence of *Cronobacter* (*E. sakazakii*)

Catherine Molloy[1], Claire Cagney[1], Stephen O'Brien[2], Séamus Fanning[2] and Geraldine Duffy[1]

[1] Teagasc Ashtown Food Research Centre. Ashtown, Dublin 15, Ireland. [2]Centre for Food Safety, School of Agriculture, Food Science and Veterinary Medicine, University College Dublin, Ireland.

Cronobacter (formally *Enterobacter sakazakii*) has been linked to illness in infants from contaminated powdered infant formula but there is limited information on the environmental sources and potential transmission routes of this pathogen. The aim of this study was to establish if food production animals (cattle, pigs), and the wider farm environment were playing a role in the transmission of *Cronobacter* and to assess the risk of cross contamination in the home where infant formula is prepared, from the presence of the pathogen on other foods and the general domestic environment. A wide range of samples (n=518) were collected at dairy farms, meat abattoirs, retail food stores and domestic environs and examined for the pathogen using an adapted ISO /DTS 22964 cultural protocol. Presumptive *Cronobacter* colonies were confirmed by Real Time PCR targeting the dnaG 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) as well as organic breakfast cereals (n = 9) and domestic vacuum cleaner dust (n = 1). This study indicates that *Cronobacter* is present in a range of sample types but has a particular association with dry environs.

P24 Rapid detection of Enterobacteriaceae including identification of *E. sakazakii* (*Cronobacter*)

Cordt Grönewald, Matthias Kiehne, Kornelia Berghof-Jäger

BIOTECON Diagnostics GmbH, Hermannswerder Haus 17, 14473 Potsdam, Germany.

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 labeled 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.

P25 Bacteriocidal preparation of powdered infant formula

Juncal Caubilla-Barron, Eva Kucerova, Michael Loughlin, Emma Hartnett, Greg Paoli and Steve Forsythe

School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham, UK. NG11 8NS.

The reconstitution of powdered infant formula with water at a raised temperature as a means of reducing the bacterial load was assessed using *Salmonella*, *Cronobacter*, other *Enterobacteriaceae* and *Acinetobacter* spp. These organisms were chosen as they are categorised by FAO/WHO (2004 & 2006) as either 'Clear evidence of causality' or 'Causality plausible, but not yet demonstrated' with respect to causing infant infections through contaminated formula. Thermal susceptibility (D and z values) were calculated for the different studied strains, different reconstitution scenarios were analysed, and different temperature treatments were chosen to reflect current practices. All of this information was gathered and analysed using the 'E. sakazakii' Risk Model (JEMRA). In total, 30 strains from 10 bacterial genera were analysed for growth range (4-44°C) and thermal susceptibility (D and Z values) in various types of infant formula. Temperature treatments were chosen to reflect current practices, and compared with the recommended reconstitution temperature of 70°C. Growth rates were determined using impedance microbiology, and thermal treatment using conventional enumeration of cultures following treatment in a capillary coil waterbath. Data was collated and analysed using the 'E. sakazakii' Risk Model (JEMRA) using defined scenarios. *Salmonella* serovars, *Citrobacter* spp. and *Serratia* spp. were unable to grow at 44°C, unlike *Cr. sakazakii, C. muytjensii*, *Klebsiella* spp., and *Enterobacter* spp. The most thermotolerant organisms were *Salmonella* serovars and *Cr. sakazakii*, ie. D_{55} ~30 min which was in the order of ten-fold greater than the other *Enterobacteriaceae*. The greater thermoresistance of *Salmonella* serovars and *Cr. sakazakii*, combined with persistence in the desiccated state, are basic physiological traits resulting in the greater exposure of infants to these two pathogens. This work was funded by the UK Food Standards Agency, and supported by the Infant and Dietectic Food Association, Oxoid ThermoFisher and LabM.

P26 The misidentification of *Enterobacter hormaechei* as *Cronobacter* spp. (*E. sakazakii*) in strains from Aldova et al. (1983) and Muytjens et al. (1988), and a neonatal intensive care unit outbreak.

Stacy M. Townsend, Edward Hurrell, Juncal Caubilla-Barron, Eva Kucerova, and Stephen J. Forsythe

School of Science and Technology, Nottingham Trent University, Clifton Lane, Nottingham, UK. NG11 8NS.

The accurate identification of hospital isolates is crucial for due recognition of the importance of a particular pathogen, and its control. Due to the inherent inaccuracies of isolate identification based on biochemical profiling, we undertook a review of our culture collection using 16S sequence analysis. 16S DNA sequence analysis was undertaken for strains previous described as '*E. sakazakii'* in the literature by Aldova *et al*. (1983), Muytjens *et al*. (1988), Poirel et al. (2007) and a collection of 7 *Cronobacter* spp. '*E. sakazakii'* strains from a more recent neonatal intensive care unit outbreak (NICU). One strain referred to by Aldova *et al*. (1983) was *Enterobacter hormaechei* subsp. *hormaechei*, another strain from Muytjens *et al*. (1988) was *E. hormaechei* subsp. *steigerwaltii* and not *Cronobacter* spp. (*E. sakazakii*). The *QnrA*-mediated quinolone resistant *E. sakaz*akii from Poirel *et al.* 2007) was *E. hormaechei*. None of the NICU isolates were *Cronobacter* spp. (*E. sakazakii*). Six strains were *E. hormaechei* subsp. *hormaechei*, and the remaining isolate was *E. hormaechei* subsp. *steigerwaltii*. *E. hormaechei* is of increasing importance as a cause of neonatal infections, and often possesses extended β-lactamase activity. It is important to recognise that a number of organisms other than *Cronobacter* spp. can be the causative agents of neonatal infections, and that robust identification methods are adopted by both infant formula manufacturers as well as clinical laboratories.

P27 Isolation and characterisation of *Cronobacter* from infant food

Orla Condell, Brendan Healy, Stephen O'Brien, Shane Cooney, Séamus Fanning, Carol Iversen

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.

Cronobacter are a causative agent of illness in premature and low birth weight neonates and transmission has been associated with infant formula. In this study *Cronobacter* isolated from baby food samples were assessed for their virulence potential. A total of 20 different baby foods were

tested for the presence of Enterobacteriaceae using the ISO TS 22964. Isolates were identified using ID32E (bioMerieux) and confirmed as *Cronobacter* by rt-PCR. The *Cronobacter* isolates were speciated using biochemical tests and 16S rRNA sequencing and were typed using PFGE. The virulence characteristics of the isolates were assessed using a macrophage survival assay and PCR assays to detect selected virulence markers. The antibiotic resistance profiles were also determined. Other Enterobacteriaceae isolates from infant foods were used for comparative purposes, including a *Salmonella* Ealing isolate previously implicated in an outbreak of neonatal infection. Of the *Cronobacter* isolates obtained, 4 were found to be *Cronobacter turicensis* and 3 were *Cronobacter sakazakii*. Using PFGE, the *C. turicensis* isolates clustered together but were distinct from E866, the type strain of this species. The *C. sakazakii* isolates also clustered together within one band difference, however were also distinct from the type strain of this species (ATCC 29544). All isolates were resistant to erythromycin but sensitive to the other antibiotics tested with the exception of one isolate that was resistant to cephalothin. All the *Cronobacter* isolates were positive for the presence of *ompA*, *tolC* and *phoP*. However using these primer sets, only the *C. turicensis* isolates were positive for *acrA* and only the *C. sakazakii* isolates were positive for phoQ. Persistence in macrophages was evident at 24 h after phagocytosis for all the *Cronobacter* isolates. However at 48 h none of the *C. sakazakii* isolates were recovered in contrast to continued persistence of the *C. turicensis* isolates. In comparison *Salmonella* Ealing isolated from infant formula in 1985 showed proliferation at 24 h and persistence up to 72 h. In conclusion, Enterobacteriaceae including *Cronobacter* spp. can be isolated from baby foods. These *Cronobacter* strains may harbor potential virulence genes and posses the ability to persist in macrophages. However, few cases of *Cronobacter* infections have been reported in children older than 3 months and no epidemiological evidence exists to suggest that ingestion of baby foods containing low levels of Enterobacteriaceae are a health concern.

P29 Evaluation of the ISO/IDF draft standard method for the detection of *Enterobacter sakazakii* in milk powder products and powder infant formulas

Véronique Deperrois, Sylviane Derzelle, Véronique Maladen, Nicole Soudrie and Nathalie Gnanou-Besse

Unité Composition et Hygiène des Produits Alimentaires, Agence Française de Sécurité Sanitaire des Aliments, Laboratoire d 'Etudes et de Recherches sur la Qualité des Aliments et des Procédés agro-alimentaires.

*Enterobacter sakazaki*i is the causative agent of rare but severe neonatal infections, linked to the consumption of contaminated powdered infant food formulas. As a result of the growing recognition of *E. sakazakii* as an emergent pathogen, the International Dairy Federation (IDF) and the International Standard Organisation (ISO) have standardized a reference method project for its detection in infant food formulas factories products and environmental samples. The objectives of this study were to assess the applicability of the ISO/IDF draft standard, and to compare several chromogenic selective media for *E. sakazakii*. We evaluated three chromogenic media based on α-glucoside activity, i.e. ESIA™ (AES Laboratoires), DFI™ (Oxoid) and COMPASS™ (Biokar diagnostics) and a selective medium for Enterobacteriaceae, VRBG (AES Laboratoires). Thirty-eight suspected naturally contaminated samples from infant formulae factories were analysed, and also twenty-eight *E. sakazakii* strains and sixteen other Enterobacteriaceae strains were tested. We showed that the ISO/IDF method is sensitive, selective and applicable to the analysis of powder infant food formula but may still be improved. (i) Observation of yellow pigmentation for suspected *E. sakazakii* colonies on tryptone soy agar (TSA) at 25°C should be excluded. (ii) Another chromogenic agar than the one prescribed (ESIA) in the ISO/IDF method can be successfully used for the isolation of *E. sakazakii*.

P30 Incidence of *Cronobacter sakazakii* in powdered-milk-based infant formula in Dubai (United Arab Emirates).

Hamid Mohamed Ibrahim

Dubai Municipality, United Arab Emirates (U. A. E.)

As an emerging pathogen *C. sakazakii*, received much attention after April 2002 when the US - FDA issued an alert to health care professionals regarding the risk associated with *C. sakazakii*

infection among neonates fed milk based powdered infant formulas. *C. sakazakii* 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.

P31 Complete inactivation and removal of attached cells of *Cronobacter* spp. using Hydrofem electrolysed water ('Anolyte').

Edward M. Fox[1], Edmond O'Reilly[2] and Kieran N. Jordan[1].

[1] Teagasc, Moorepark Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland and [2] Trustwater Ltd., Gurtnafleur Business Park, Clonmel, Co. Tipperary, Ireland.

Cronobacter spp. comprise a family of bacteria, many of which are pathogens associated with meningitis, septicemia, and necrotizing enterocolitis, primarily among low birth-weight and premature infants. Powdered infant formula has been epidemiologically linked with infection. Attachment and biofilm formation by *Cronobacter* spp. in the production of PIF may present a source of recontamination of final product. The aim of this work was to demonstrate the effectiveness of Hydrofem electrolysed water (or 'Anolyte') for inactivation and removal of attached cells of *Cronobacter* spp. fifteen strains (including clinical isolates from different sources) were shown to attach to stainless steel coupons when incubated in Nutrient Broth for 3 days. Inactivation of planktonic cells was tested by direct inoculation into 'Anolyte', or by spraying culture that was spresd on surfaces. Generally, the number of attached cells was about 3.5 log CFU/cm2, although one strain attached at a rate of 5.6 log CFU/cm2. A subsequent 5 minute 'Anolyte' treatment was sufficient to completely remove and inactivate 10 of these strains. Inoculation of cells into 'Anolyte' caused immediate and complete inactivation. Cultures spread on different surfaces, including glass, a rough surface ceramic tile, a smooth surface ceramic tile and a laboratory bench were completely inactivated by spraying the surface with 'Anolyte' (with a residence time of 2 min.) In conclusion, 'Anolyte' has the potential to contribute to the overall strategy for control of *Cronobacter* spp. on surfaces.

P33 Heat adaptation improves the survival potential of *Cronobacter* spp. to heat stress, but not dry stress.

Benedict Arku[1], Séamus Fanning[2] and Kieran Jordan[1]

[1]Teagasc, Moorepark Food Research Centre, Fermoy, Cork, Ireland and [2]Centre for Food Safety, UCD Veterinary Sciences Centre, University College Dublin, Belfield, Dublin 4, Ireland.

For the vast majority of the population, *Cronobacter* spp. (formally *Enterobacter sakazakii*) are of little significance. However, for a particularly vulnerable group of low birthweight and immunocompromised infants, this pathogen can cause a rare and potentially fatal illness with symptoms including neonatal meningitis, septicaemia and enterocolitis. The mortality rate is about 30 – 80%, and for those that survive the consequences are severe. Most bacteria have been

shown to exhibit an adaptive response, where exposure to a mild stress confers resistance to severe stress either with the same or additional stressors. The survival of *Cronobacter* spp. at 52°C after an adaptive period of 30 min. at 46°C was investigated for milk and broth-grown cells. Adapted and unadapted cells were inoculated into dry powder and survival was monitored over time. The effect of reconstitution on *Cronobacter* spp. survival was also monitored. On adaptation, survival after 5 min. at 52°C of milk-grown cells was increased by about 1.5-log whereas survival of broth-grown cells was increased by about 3-log. While adaptation increased the survival potential of heat stress, it did not increase the survival potential in dry stress or during reconstitution of deliberately contaminated powder using microwave heating or conventional heating. Since adaptation increased the growth potential of *Cronobacter* spp. in the presence of the membrane active antibiotic ampicillin (but not in the presence of RNA polymerase/protein inhibitors rifampicin or tetracycline), it is likely that membrane changes during adaptation are responsible for the increased survival. Adaptation of *Cronobacter* spp. to heat does not increase the survival potential in powder or during reconstitution.

P34 Assessment of growth of *Enterobacter sakazakii* (*Cronobacter* spp) in reconstituted powdered infant food by using three chromogenic media

Alfonsina Fiore and Laura Toti

Department of Veterinary Public Health and Food Safety, Istituto Superiore di Sanità, viale Regina Elena, Rome, Italy.

Enterobacter sakazakii (*Cronobacter* spp), is an emerging pathogen associated to neonatal cases of necrotizing enterocolitis and meningitis particularly related to consumption of contaminated powdered infant formula. In order to guarantee the safety of foods for infants and to reduce the risks of contamination by *E. sakazakii* (*Cronobacter* spp) it is necessary to introduce rigorous control measures at industrial, legislative and domestic levels; particularly, correct information regarding preparation, handling, and storage are required. The aim of this work was to assess how the growth of *E. sakazakii* (*Cronobacter* spp) in reconstituted powdered infant formula was affected by time, temperature and composition by comparing three commercial chromogenic media. Three different powdered infant food (infant milk powder, powdered infant milk for special medical purposes and powdered infant food manufactured from wheat) purchased from local markets were tested for presence of Enterobacteriaceae by ISO 21528-1:2000. Samples negative for Enterobacteriaceae were reconstituted according to instructions of manufacturer and inoculated with a 4-strain mixture of *E. sakazakii* (*Cronobacter* spp) at populations of ca. 850cfu/g, ca. 85cfu/g (to reproduce naturally contaminated products) and ca. 0,75cfu/g of powdered food, respectively. Each reconstituted and inoculated food was stored at: 4°C (that inhibit growth of *E. sakazakii*), 15°C (abuse temperature), room temperature, ca. 23-25°C (to simulate the wrong custom to hold unused reconstituted food without refrigeration). Populations were monitored at moment of inoculum (D0) and at 4 h, 24 h, 48 h, and 72 h by using, at the same time, three commercial chromogenic media: ESIA (Biolife), Compass agar (Biokar Diagnostic), Agar ChromoID Sakazakii-ESPM (Biomerieux). The data of this study indicate that all reconstituted infant food can support growth of *E. sakazakii* (*Cronobacter* spp) at three different storage temperatures; *E. sakazakii* (*Cronobacter* spp) survives for up 72 h a 4°C and increases of one log; the hang time for reconstituted infant food held at room temperature should be no longer than 4 h; all chromogenic media had a good performance.

P35 Incidence and growth characteristics of *Cronobacter sakazakii* isolated from powdered infant formula (PIF) milk on sale in Abu Dhabi

Yousef Alsaadi and Farouq Hashim

Special Microbiology Unit Laboratories Division, Abu Dhabi Food Control Authority, PO Box 52150, Abu Dhabi – United Arab Emirates (U. A. E.)

Cronobacter sakazakii is an emerging pathogen associated with life-threatening neonatal infections resulting from the consumption of contaminated powdered infant formula (PIF) milk. *C. sakazakii* has been found in manufacturing equipment, which is a potential source of contamination for powdered infant formula (PIF) after pasteurization. This study was conducted to determine the risk of using infant formula for *C. sakazakii* infection and to identify any potential risks if the product is

mishandled. Stores in Abu Dhabi were surveyed to determine the market availability of PIF and packaging details were recorded. Random samples were analyzed for *C. sakazakii* and aerobic colony count (ACC). There were 18 different companies exporting their products for sale in Abu Dhabi. The most common method of labelling was direct printing onto the can without any paper. This was observed on 91% of the samples studied. All of the products used bilingual labelling and most of them (91%) were using Arabic and English. Nine samples (4.5%) were found to be contaminated with *C. sakazakii. C. sakazakii* contamination did not correlate with ACC results, which ranged between <10 CFU/g and 5.6 X 103 CFU/g. The disc diffusion method in addition to the Becton Dickinson Phoenix system were used to determine the antimicrobial susceptibility of *C. sakazakii* isolates. The *C. sakazakii* isolates were resistant to the following antibiotics: cephalothin, cefoxitin, ampicillin and amoxicillin/ clavulanate. This could have an influence on treatment. Growth curves were determined using PIF reconstituted at 23°C and 37°C, the average generation times were 73 min and 25 min respectively. *C. sakazakii* takes a short time to double at 37°C, a common ambient kitchen temperature in the Gulf. D-values were determined using the survival curve method at temperatures of 50°C, 60°C, 70°C and 80°C. They were 909; 0.87; 0.32 and 0.15 min respectively. *C. sakazakii* was significantly tolerant when exposed to 50°C, the recommended reconstitution temperature. This study concluded that there is a risk, especially if PIF milk is mishandled and therefore *C. sakazakii* should be included in the Gulf Standard for PIF milk and also correct labelling of cans and instructions should be included in the standard.

P36 Pathogenesis of *Cronobacter*: enterotoxin production, adherence and invasion of the blood-brain barrier.

Franco Pagotto, Jeffrey M. Farber, Graeme Ferguson and Amy Louise Commodore

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

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.

P37 Development of a non-primate animal model for *Cronobacter* species.

Franco Pagotto, Raquel Lenati, Karine Hébert, Min Lin, Luciana Esper and Jeffrey M. Farber

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

The mechanism(s) by which *Cronobacter* species cause disease in humans and its minimum infectious dose (MID) remain unknown. As such, it is difficult for regulatory agencies to set policies and for industry to develop control measures for this organism. Herein, we assessed six animal species to find a model that better mimicked human pathogenesis and clinical manifestations of *Cronobacter* infection. A number of animal models, including pigs (6.3–7.2 kg; 5 weeks), chicks (1 day), rabbits (2.7–3.0 kg; 2 months), guinea pigs (300–400 g; 3–4 months) and young gerbils (40–50g; 1–2 months), were used. Animals were challenged orally with 109 colony-forming-units

of clinical, environmental or food isolates, and followed for up to 14 days. Blood, fecal specimens and organs (brain, heart, liver, spleen, mesentery, kidney, and intestines) were examined for the presence of *Cronobacter* spp. None of the young animals presented clinical symptoms observed in *Cronobacter* infections seen in humans. Some neonatal (6/36) gerbils died within 48 h of infection. While *Cronobacter* spp. was isolated from fecal samples of all animals challenged, it was only recovered from tissues, including the brain, of gerbils (young and neonatal). Interesting, clinical isolates had the least invasive capabilities, with environmental strains present in larger numbers in organs such as the brain. The data indicated that the probability of positive tissues such as brain, kidney, liver and spleen increases as intestinal concentrations increased. Statistically speaking, no differences among-isolate source (i.e., clinical versus food versus environmental) were observed. Gerbils may be a suitable animal model to evaluate the virulence of *Cronobacter* strains. In addition, an animal model for the study of *Cronobacter* pathogenesis will be useful for future policy development. Currently, the neonatal gerbil model is being refined.

P38 Phenotypic and genotypic characterization of clinical, environmental and food isolates of *Cronobacter* species

Franco Pagotto, Karine Hébert, Kevin Tyler and Jeffrey M. Farber

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

The purpose of this study was to perform phenotypic and genotypic characterization on over 250 strains of *Cronobacter* species from around the world. Traditional methods of identification include observation of yellow colony pigmentation on tryptic soy agar (TSA), testing for positive α-glucosidase activity, and a variety of biochemical assays. Traditional phenotypic and genotypic [ribotyping, pulsed-field gel electrophoresis (PFGE), 16S rDNA sequencing, BAX® PCR] methods were performed. The use of 16S rDNA analysis showed 82% identity amongst 260 strains tested, grouping them in 96 clusters, with 50 strains in the largest one (containing *C. sakazakii* and *C. malonaticus*). *C. muytjensii* were able to be separated from other strains, including *C. dublinensis.* Molecular typing using XbaI-based PFGE was shown to be more discriminatory than automated ribotyping. PFGE analyses of 240 strains (14 eliminated by 16S rDNA as being non-*Cronobacter*, 6 strains having degradation during PFGE) generated 188 clusters, the largest containing 7 strains. Ribotyping, using the restriction endonuclease EcoRI, grouped the same strains in 158 clusters. From the 260 strains tested, 16 were negative for *E. sakazakii* by BAX® PCR; among these, 7 were identified as *E. sakazakii* by the 16S rDNA sequencing. Furthermore, amongst the 14 outliers from the 16S rDNA sequences, 4 were identified as *E. sakazakii* by API 20E®, 4 produced yellow pigment on TSA and 6 had α-glucosidase activity on DFI and/or ESPM. Of the 260 isolates tested, only 66% were identified as being positive for *E. sakazakii* by API 20E® and 77% by ID 32E®. While 80% (n=209) of the strains tested presented yellow pigment on TSA, 11% (n=28) formed white colonies, with 2- and 7% showing a strong and pale yellow colonies, respectively. Amongst those white colonies on TSA, 14 were green/blue on DFI/ESPM media. Combining molecular characterization to phenotypic assays will increase the confidence in the development of rapid diagnostic tools for identifying and characterizing this emerging foodborne pathogen.

P39 Genotypic and phenotypic characterisation of a collection of *Enterobacter sakazakii* isolates

Rabeb Beennour Miled, Annaelle Kerouanton, Thomas Meheut, Pierre Colin, Nathalie Gnanou-Besse

Agence française de sécurité sanitaire des aliments Laboratoire d'Etudes et de Recherches sur la Qualité des Aliments et sur les Procédés agro-alimentaires (AFSSA) LERQAP, 23 avenue du Général de Gaulle , 94706 Maisons Alfort cedex, France.

Enterobacter sakazakii has been identified as the causative agent of serious neonatal infections, associated with high mortality rate. In most cases, powdered infant formula (PIF) has been identified as the source of infection. Recently, strains commonly referred as *E. sakazakii* were proposed to be classified in a new genus, *Cronobacter*. Since knowledge on this pathogen is still incomplete, it is recommended to type all isolates in order to favour epidemiological investigation and evaluate strain variability. In order to characterise a collection of isolates from various origins, we used a Pulse-Field Gel Electrophoresis technique (PFGE) developed in our laboratory. Relevant biochemical tests were also performed to classify strains into the different *Cronobacter* species. In

order to compare growth characteristics, growth curves for each strain were determined in non selective brain heart infusion broth, at 25°C and 37°C, by measuring optical density using a spectrophotometer. Growth rate was estimated from the slope of the tangential line in mid-exponential phase. Results showed that PFGE technique seemed not to be suitable for establishing taxonomic relationships within *Cronobacter* genus, since we had difficulties to obtain coherent clustering of *E. sakazakii* strains using PFGE patterns. The majority of the strains belonged to *Cronobacter sakazakii*, which seemed to be the most commonly species distributed in environment of PIF production plants. Growth rates were homogenous within the genus, and no significant differences were observed between strains, according to their origin or species. This study did not allow to establish potential links between genotypic and phenotypic profiles. The high genetic heterogeneity observed could be opposed to the high homogeneity in growth behaviour, which is capital information for quantitative risk assessment studies. In order to have a more precise idea of the pathogen behaviour, further studies should be performed on lag phase distribution among the isolates.

P40 Impact of sample pooling on *Enterobacter sakazakii* growth and detection

Nathalie Gnanou-Besse, Rabeb Beennour Miled, Nicolas Baudouin, Pierre Colin

Agence française de sécurité sanitaire des aliments Laboratoire d'Etudes et de Recherches sur la Qualité des Aliments et sur les Procédés agro-alimentaires (AFSSA) LERQAP, 23 avenue du Général de Gaulle , 94706 Maisons Alfort cedex, France.

Enterobacter sakazakii has been identified as the causative agent of serious neonatal infections, associated with high mortality rate. In most cases, the source of infection has been powdered infant formula (PIF). The standardised method for detection of *E. sakazakii* from PIF (ISO/TDS 22964; IDG/DRM 210) involves preenrichment in buffered peptone water (BPW), followed by selective enrichment and plating on chromogenic agar ESIA. In order to reduce analysis cost and heaviness, common practice in industries consist in pooling samples at constant dilution rate, in order to realise single first enrichment. Consequences on *E. sakazakii* growth and detection are not established. In order to evaluate pooling impact on enrichment, growth curves for 3 *E. sakazakii* strains were determined at various initial contamination levels and powder quantity, in first age PIF decimally diluted in BPW and incubated for 24 hours at 37°C. Growth of the pathogen was monitored by direct plating on ESIA agar or using a recently developed sensitive enumeration method, based on membrane filtration followed by transfer of the filter on the selective agar. Results showed that pooling had a negative impact on *E. sakazakii* maximal population attained, whereas no clear effect was observed on growth beginning. Variance analysis revealed a significant impact of powder quantity but no effect of initial contamination level on maximal population attained. A mean comparison student t-test revealed a significant impact of powder quantity for a same pathogen concentration, and no impact of bacterial concentration for a same powder quantity. The impact of pooling on the behaviour of *E. sakazakii* undergoing enrichment culturing in BPW, mainly for maximal population attained, suggests strong bacterial interactions with the PIF background microflora. This may affect detection method performances. Impact of background flora on pooling results should be confirmed with further studies, in order to better explain this phenomenon.

P41 Express design and manufacturing of *E. sakazakii* TaqMan Pathogen Detection Kit by Applied Biosystems

Cristina Rodriquez

Ordesa, Spain.

E. sakazakii (now re-named *Cronobacter*) is a gram-negative rod-shaped bacterium which is a rare cause of invasive infection with high mortality rates in neonates. Powdered milk-based infant formulas have been associated with the *E. sakazakii*-related outbreaks in premature or other immunocompromised infants. In general, the reported case-fatality rate varies from 40-80 % among newborns diagnosed with this type of severe infection. The type of meningitis caused by *E. sakazakii* may lead to cerebral abscess or infarction with cyst formation and severe neurologic impairment. The conventional method approved by FDA requires 5 days for detection. To improve sensitivity and shorten detection time Applied Markets Division has designed a TaqMan Pathogen

Detection Kit for *E. sakazakii*. Working on alternatives to 16S-based TaqMan design, we selected RnaseP gene as a good TaqMan target based on literature and our previous experience. However, there is no RnaseP sequence for *E. sakazakii* in the public databases. Based on Bioinformatics analysis, we selected conserved regions across *Enterobacter* species to design primers to amplify, and sequence RnaseP gene in ATCC *E. sakazakii* strains. Using the new sequence information we designed a real-time PCR assay to specifically detect *E. sakazakii*. The assay was tested with four target strains and a panel of antitargets including *Salmonella enterica*, *Shigella flexneri, Escherichia coli, Enterobacter nimipressuralis, Enterobacter dissolvens, Enterobacter cloacae, Enterobacter aerogenes, Citrobacter freundi and Listeria monocytogenes*.

P42 Validation study of an *E.sakazakii* TaqMan(R) RT-PCR kit with Infant Formula

Nuria Queralt

Applied Biosystems, Spain.

Cronobacter sakazakii is an emerging pathogen involved in sporadic situations of serious infections in just born babies of up to 4-6 weeks of age. It is an opportunistic pathogen that can cause meningitis, necrotizing enterocolitis and sepsis in premature or immuno-compromised babies. *C sakazakii* is a ubiquitous micro-organism and its control in food processing plants requires strict hygiene measures. Even when it is possible to pasteurise recontamination can occur on the manufacturing and packaging phases. The current reference analytical method for the *Cronobacter sakazakii* detection (technique ISO 22964) requires a minimum of 4 days. There is a need to develop new analytical methods that allow better time to results which also offer the same or better specificity and sensitivity than the reference method. The PCR technique allows the rapid detection of micro-organisms by using specific DNA primers. In order to validate the PCR based analysis technique we analysed 3 different infant formulas artificially inoculated with *C. sakazakii* following the ISO 22964 method and the new TaqMan® PCR *Enterobacter Sakazakii* amplification/detection kit of Applied Biosystems in parallel. We carried out 36 analyses (12 samples per validation) tested on 3 different days. Each group of analyses had 3 negative control samples (not inoculated) and 9 contaminated samples (artificially inoculated). Of the 27 artificially inoculated samples, 3 of them were not detected by either of the 2 evaluated techniques (ISO 22964 or Real-Time PCR) and for this reason they were considered to be negative. The results obtained in the 3 validations show that the ISO 22964 technique and the Real Time PCR detect the same 24 positive samples; the advantage of the second technique is that the results are obtained in approximately 27 hours when the ISO 22964 technique requires a minimum of 4 days (7 including biochemical confirmation).

P43 Genetic analysis of biofilm formation in *Cronobacter sakazakii*

Isabel Hartmann[1], Paula Carranza[2], Roger Stephan[1], Kathrin Riedel[2] and Angelika Lehner[1]

[1] Institute for Food Safety and Hygiene, University of Zürich, Winterthurerstrasse 272, 8057 Zürich, Switzerland, [2] Department of Microbiology, Institute of Plant Biology, University of Zürich, 8057 Zürich, Switzerland.

Cronobacter spp. are opportunistic foodborne pathogens that can cause severe disease such as meningitis, sepsis and necrotizing enterocolitis in neonates. Mortality is high, and survivors often suffer irreversible brain damage. In a number of outbreaks, the most likely source of infection was found to be powdered infant formula. The organisms' desiccation resistance and ability to form biofilms on different surfaces could contribute to their survival in formula powder and persistence in the hospital and production environment. So far, nothing is known about the genetic basis of biofilm formation and little about typical biofilm structure in these organisms. To genetically investigate biofilm development of *Cronobacter* spp., a random transposon mutant library of strain *C. sakazakii* ES5 was screened for altered biofilm formation. Identified mutations affected genes involved in flagellum structure and biogenesis, cellulose biosynthesis, c-d-GMP-signalling and basic cellular processes. Furthermore, a considerable proportion of identified mutations were located in poorly or uncharacterized genes. Interestingly, mutations were found in two uncharacterized genes localized next to each other in the genome. These mutants display a distinctive phenotype on congo red agar, which could indicate a role of this locus in extracellular matrix composition or regulation. Additionally, biofilm architecture was characterized by growing wildtype and mutant

biofilms in a flow chamber system and subsequent investigation by CLSM. A model of Gram-negative biofilm formation developed in *Pseudomonas aeruginosa* seems to also largely apply to *C. sakazakii* biofilms. However, the number of uncharacterized genes found in this study illustrates that many questions remain to be answered.

P44 Elucidating the white phenotype in *Cronboacter sakazakii*

Sophia Johler, Roger Stephan and Angelika Lehner

Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zürich, CH-8057, Zürich, Switzerland.

Yellow pigmentation is one of the physiological features that have been used as criteria for the identification of *Cronobacter* spp., although only 70-80% of its strains express this feature. Within a recent study, the carotenogenic nature of the pigment produced in a *Cronobacter* spp. strain was elucidated by molecular and chemical means but so far, nothing is known about the molecular basis of the white phenotype or regulatory elements possibly involved in the expression of the pigment. To gain insight in the pigment production of *Cronobacter* spp. on the genetic level, a random transposon mutant library of strain *C. sakazakii* Es5 was screened for diminished pigment production. In addition, eight white wild type (wt) strains of *Cronobacter* spp. were analyzed by PCR and Dot Blot for the presence of the seven known pigment operon genes. By screening the transposon library build up with 10000 mutants, 35 white mutants were found. Mutations identified included the genes crtE, crtX, crtY, crtI, and crtB of the carotenoid operon, general regulators, genes involved in the sugar pathway and precursors for carotenoids, genes associated with basic cellular processes and a large proportion of poorly characterized or uncharacterized genes. With respect to the eight white wt strains we demonstrated the absence of all genes of the pigment operon for Cs1.7, whereas the presence of all genes of the pigment operon was disclosed for the remaining seven white strains. Some of the mutants as well as the white wt strain Cs1.7 show that the absence of one or several genes of the pigment operon can lead to loss of pigment production. Our results also prove a crucial role of regulatory elements in the expression of the white phenotype of *Cronobacter* spp. Selected white mutants will now be further characterized by phenotypic means and their performance regarding persistence and fitness in view of environmental stress factors.

P45 Alpha glucosidase activity and yellow pigmentation in *Cronobacter* spp. and recently described new *Enterobacter* spp.

Britta Stoop[1], Roger Stephan[1], Thomas Rattei[2] and Angelika Lehner[1]

[1] *Institute for Food Safety and Hygiene, Vetsuisse Faculty, University of Zürich, CH-8057, Zürich, Switzerland.* [2]*Genome Oriented Bioinformatics, Technical University Munich, Freising, Germany.*

Recently, three novel *Enterobacter* species (*Enterobacter helveticus, Enterobacter turicensis, Enterobacter pulveris*), exploiting the same niche as *Cronobacter* spp. (IFM, production environment), have been described. By employing the currently available cultural detection methods for *Cronobacter* spp., theses species may lead to false positive results, due to their alpha glucosidase activity and the yellow pigment production. The aim of this project is to characterize both features on the genetic level, not having sequenced genomes for these new species. Bacterial Artificial Chromosome (BAC) libraries were constructed for the type strains of the newly described *Enterobacter* spp. Screening of transformants for alpha-glucosidase activity was performed in microplates containing LB broth supplemented with 4-methylumbelliferyl-α-D-glucoside. The pigment expression of the *E. coli* transformants was assessed on LB agar after 48 hrs of exposition to natural light at room temperature. Screening for alpha-glucosidase activity resulted in 5 (*E. helveticus*), one (*E. pulveris*) and one (*E. turicensis*) positive transformants. Individual library testing for pigmentation revealed 3 (*E. helveticus*), 10 (*E. turicensis*) and 6 (*E. pulveris*) candidates. So far, sequencing of two candidate clone inserts - BAC3B1 (alpha-glucosidase) and BAC20A1 (yellow pigmentation) both originating from the *E. helveticus* library has been completed and the annotation is currently ongoing. Sequencing and annotation of the remaining 4 BAC clones (from 2 libraries) is work in progress. Sequence comparisons among these novel *Enterobacter* spp. and relative to the *Cronobacter* spp. will be performed in order to design a multiplex PCR approach enabling a clear discrimination between these organisms in the infant formula production line.

P46 Antimicrobial Peptides from fermented bovine milk

Bhagat Singh and Renu Singh

Department of Microbiology, Institute of Applied Medicines and Research, Duhai, Ghaziabad Utter Pradesh, India.

With the recent restriction of antibiotic use in food producing animals the greater concerns about antibiotic resistant bacteria in both the animal and human population and the desire to reduce food borne pathogen levels, an increasing need to develop effective but human and animal compatible, antibiotic alternatives for the medical industry has arisen. Recently, natural proteins have been identified that possess these attributes. *In vitro*, these proteins upon degradation by digestive enzymes or microbial enzymes have been shown to release antimicrobial peptides (AMPs), which exhibit unique mechanism for killing bacteria compared with current antibiotics. These AMPs selectively binds to the outer lipid membrane of the bacterium and form blisters and pores, which eventually result in lyses of the cell and cellular death. To study antimicrobial activity of peptides five strains of *Lactobacillus helveticus* obtained from NCDC, NDRI, Karnal, were screened for antimicrobial activity exerted by the individual strains in the whey supernatant when grown in bovine skim milk. The cell free extract of different strains of *L. helveticus* showed variable activity in case of both Gram's positive, Gram's negative pathogens and spoilage organisms. *L. helveticus* NCDC-288 was found to be more effective than that of the rest strains. The agar well assay inhibition zone diameter for Gram's positive *Bacillus cereus, Streptococcus agalactiae and Staphylococcus aureus* was 3.2, 3.0 and 2.4cm respectively. The supernatant also showed good activity against Gram's negative pathogens. It showed more than 2.2 inhibition zones against *Escherichia coli, Enterobacter faeciua, Pseudomonnas fragi, Serratia marcesens* and *Salmonella* typhi. The factor responsible for the antimicrobial activity was found to be peptide in nature. To achieve maximum antimicrobial activity in terms of the peptide content, conditions were optimized. Purification of the desired peptide was carried out using gel filtration, ion exchange chromatography and HPLC. In addition to the antimicrobial activity these peptides also exhibited anti-oxidative activity. Moreover the ACE inhibitory activity in these peptides also has been demonstrated. This observation has been supported with analytical data indicating relatively higher valine content responsible for properties. The peptide thus obtained may be beneficial to explore the possibility for treatment against microbial infection and cardiovascular risk including hypertension.

P47 Effect of Different Factors on *Enterobacter sakazakii* Heat and PEF Resistance

Cristina Arroyo, Rafael Pagán and Santiago Condón

Unidad de Tecnología de los Alimentos, Facultad de Veterinaria de Zaragoza, Universidad de Zaragoza C/ Miguel Servet, nº 177, 50013, Zaragoza, Spain.

Due to the physiological characteristics of *Enterobacter sakazakii* and the current tendency to produce minimally processed foods it is foreseeable an increase in the sanitary importance of this species. A deeper knowledge of the environmental factors affecting the efficacy of currently available food preservation technologies, i.e. heat treatments, and alternative processes, such as Pulsed Electric Fields (PEF), is required in order to improve their lethal effect. However, present knowledge about the influence of these factors on *E. sakazakii* heat resistance is limited and there is almost no data available about their effect on its PEF resistance. The aim of this work was to study the influence of time (up to 120 h) and temperature (10, 20, 30 and 37 ºC) of growth, and pH (4.0, 5.0, 6.0 and 7.0) and water activity (0.27, 0.54, 0.81 and 1.08 g sucrose/ml) of the treatment medium on *E. sakazakii* heat and PEF resistance. *E. sakazakii* CECT 858 (ATCC 29544) was grown to stationary phase, and its resistance to both technologies was measured by the multipoint method. A mixing method (thermorresistometer TR-SC) was used for the determination of microbial heat resistance, and exponential waveform pulse equipment was used for PEF-resistance determinations. Entering into stationary growth phase triggered an increase in resistance against both technologies. Longer incubation times (up to 120 h) resulted in a gradual increase of resistance to heat but not to PEF. Decimal reduction time values increased exponentially with growth temperature up to 37 ºC, however PEF resistance was not influenced by this factor. *E. sakazakii* showed maximum heat resistance at pH close to neutrality (pH 6.0). On the contrary, PEF resistance was higher at the lower pH (pH 4.0). A decrease in the water activity of the treatment medium had the same protective effect on both technologies and resulted in cells

that were nine times more heat resistant and eleven times more PEF resistant. Our results demonstrate the importance of the studied factors on *E. sakazakii* heat and PEF resistance, which should be taken into account when designing heat and PEF based hygienization processes.

P48 Inactivation of *Cronobacter* spp (*Enterobacter sakazakii*) in infant formula using lactic acid and copper sulphate

Murad A. Al-Holy[1], Luis F. Castro[2], Hamzah M. Al-Qadiri[3] and Barbara A. Rasco[2]

[1]Department of Clinical Nutrition and Dietetics, Faculty of Allied Health Sciences, Hashemite University, Zarqa-Jordan. [2]School of Food Science, Box 646376, Washington State University, Pullman, WA, USA. [3]Department of Nutrition and Food Technology, Faculty of Agriculture, University of Jordan, Amman, Jordan.

Enterobacter sakazakii (*Cronobacter* spp) is an opportunistic pathogen that poses a potential health risk to neonates. It is a known contaminant of infant formula milk (IFM), and has been associated with cases of necrotizing enterocolitis and infant meningitis. The use of natural antimicrobials has not been fully explored as an option for the control of the pathogen. The purpose of this study was to investigate the use of lactic acid and copper sulfate, as natural antimicrobials against *E. sakazakii* in IFM. Re-hydrated infant formula milk (RIFM) and powdered infant formula milk (PIFM) were inoculated with a five-strain mixture of *E. sakazakii*, and the samples were treated with copper and lactic acid, alone and in combination. The use of a copper sulfate at a concentration of 50 ppm and lactic acid at 0.2% v/v had a slight but remarkable effect on the growth of *E. sakazakii*, the combination of both agents resulted in a complete inhibition of growth in PIFM after 2 hours. In RIFM, the combination resulted in a 3-log reduction after 2 hours, and complete inhibition of growth after 6 hours. The results indicate that the combination of lactic acid and copper sulfate could be used for the control of *E. sakazakii* contamination in IFM. The use of small doses of natural antimicrobials can be beneficial to both the food industry and the consumers. It could also improve food safety, and reduce the need for artificial chemical preservatives.

P49 A comparative study between overlay method and selective-differential media for recovery of stressed *Enterobacter sakazakii* cells from infant formula

Murad A. Al-Holy[1], Mengshi Lin[2], Hamzah M. Al-Qadiri[3] and Barbara A. Rasco[4]

[1]Department of Clinical Nutrition and Dietetics, Faculty of Allied Health Sciences, Hashemite University, P.O Box: 150459, Zarqa-Jordan. [2]Food Science Program, 256 William Stringer Wing, Eckles Hall, University of Missouri Columbia, Missouri 65211, USA. [3]Department of Nutrition and Food Technology, Faculty of Agriculture, The University of Jordan, Amman-Jordan. [4]Department of Food Science and Human Nutrition, Box 646376, Washington State University, Pullman, WA 99164, USA.

This study compares the performance of different selective-differential media with the overlay method for recovery of stressed cells of *Enterobacter sakazakii* from infant formula milk (IFM). Five different selective-differential media were used in this study: OK medium, violet red bile agar (VRBA), Druggan-Forsythe-Iversen agar (DFI), Enterobacteriaceae enrichment (EE) agar, and fecal coliform agar (FCA). Tryptic soy agar supplemented with 0.1% sodium pyruvate (TSAP) was used as a control. The overlay method involved applying a thin layer (8 ml) of each of the selective media onto TSAP after spreading a sample onto TSAP. Reconstituted IFM was inoculated by *ca* 1×10^7 CFU/ml of a mixture of four strains of *E. sakazakii* and subjected to different stress conditions: heat (55 °C for 10 min), a freeze thaw cycle (–20 °C for 24h, thawed at room temperature, frozen again at –20 °C, and thawed), acidic pH (pH 3.56 for 15 min), alkaline pH (pH 11.04 for 15 min), and desiccation (*E. sakazakii* was inoculated onto powdered IFM at a level of *ca* 1×10^6 CFU/g, held at 21 °C, water activity of the inoculated product was 0.29 and examined at 0, 15, and 30 d). No major differences were noticed between the control (TSAP) and the overlay methods. However, the overlay method recovered significantly higher numbers of stressed *E. sakazakii* cells compared to selective-differential media. Also, the selective-differential media exhibited some variability in terms of their capabilities to recover stressed cells of *E. sakazakii*. Among all the examined selective-differential media, DFI performed better for recovering stressed *E. sakazakii* cells. This

study suggests that the overlay method may serve as a potential alternative to direct selective plating for best recovery of *E. sakazakii* from IFM.

P50 Modelling of thermal inactivation of *Cronobacter sakazakii* type strain, ATCC 29544, at 60˚C in reconstituted infant formula milk.

Jenna M. Warby, Francis Butler, B. Tiwari, Séamus Fanning, Ciara Walsh and Amalia Scannell

College of Life Sciences,School of Agriculture, Food Science & Veterinary Medicine, UCD Agriculture and Food Science Centre, UCD, Dublin, Belfield, Dublin 4, Ireland.

C. sakazakii, an emerging food pathogen associated with neonatal meningitis, has been found to be more resistant to high temperature treatments in dairy products, than many other Enterobacteriaceae. The objective of this research was to compare the applicability of the primary models for describing the thermal inactivation *of C. sakazakii* in IMF. *C. sakazakii* strain, 29544, was used to assess heat resistance, using rubber sealed thin walled glass tubes. Stationary cells were suspended in IMF, divided into 1ml aliquots and heated at 60˚C for up to 180 minutes. Samples were removed from the water-bath at one min intervals, cooled on ice and duplicate samples were plated on Tryptone Soya Agar (Oxoid, Basingstoke England) using the spread plate technique. Duplicate experiments were carried out on three separate occasions. Nonlinear microbial survival models (Weibull, modified Weibull and mixed Weibull distributions), which cover a wide range of known inactivation curvatures for vegetative cells, were fitted to the inactivation data and evaluated. Fitted models were also validated by comparing the predicted model parameters with the experimental values. Based on statistical indices and model characteristics, each model parameter reflected a survival characteristic, and all the models were flexible. The Weibull, the modified Weibull and the Mixed Weibull model fitted well to the experimental values ($R2>0.79$; RMSE <1.06). The isothermal inactivation of *C. sakazakii* in media was better described by three nonlinear kinetic models, the Weibull-type, the modified Weibull and the mixed Weibull models. Analytical results showed that root mean square error values (RMSE) of the models to be 0.74, 0.33 and 0.77 respectively, at 60°C. The distinct tailing of strain 29544 at 60°C was attributed to the presence of a heat-resistant subpopulation. The Mixed Weibull model conveniently accounts for the frequently observed nonlinearity of semi-logarithmic survivor curves, and the classical first-order approach is a special case of the Weibull model. The characteristic 4 log reduction values were exponentially correlated to the temperature and were found to decrease from 8.4 min to 0.6 min for mixed weibull model. Results of this study indicate that the mixed weibull model adequately describes the non linear inactivation of *C. sakazakii* in media and the bacterial resistance towards temperature.

P51 Validation of D-value determination methodology to be used to screen environmental and clinical *Enterobacter sakazakii*.

Jenna M. Warby, Niall Mullane, Carmel McNamee, F. Byrne, Ciara Walsh, Séamus Fanning, J. Lyng and Amalia Scannell

College of Life Sciences,School of Agriculture, Food Science & Veterinary Medicine, UCD Agriculture and Food Science Centre, UCD, Dublin, Belfield, Dublin 4, Ireland.

The use of D-values as a means to evaluate the thermal resistance of bacteria is widespread in the food industry, but the methods used to determine them may vary resulting in different published D-values for the same bacterial strains in similar media. The aim of this study was to determine whether the use of a thin walled glass tube could be used to calculate D-values of *Staphylococcus aureus* and to validate the method by comparison with peer reviewed values in published literature. Bacterial cultures of *S. aureus* were grown in Tryptone Soya Broth (TSB) at 37°C for 18-24 h, inoculated to a level of 10^7 cfu/ml in infant formula milk and was added as 1 ml aliquots into a series of thin walled (ca 0.15mm) glass tubes (9.6 cm long, internal diameter 0.6 mm), and sealed with a rubber bung. The inoculated tubes were immersed into a temperature controlled water bath. Initial experiments were carried out at 55, 60 and 65ºC. Duplicate tubes were prepared for each time point, and a minimum of three independent experiments were performed. Serial dilution was carried out in Ringers (Oxoid) solution and microbial enumeration done using spread plate technique on TSA. The method was also used to determine D-values of *Enterobacter sakazakii* strain NCTC8155 at 56 and 60ºC where the effect of overlaying with VRBGA (Oxoid) to

inhibit *Bacillus* spore outgrowth was also investigated. Using the thin walled glass tubes, the D-values for *S. aureus* were calculated as, 2.3, 1.5 and 0.1 min for 55, 60 and 65°C respectively. These values fall well within the range of published values for this microbe in reconstituted milk products. D-values of *E. sakazakii* NCTC8155 at 56°C was 8.1 min (which falls between 5.1 and 10.5 min previously reported), and at 60°C was 0.98 min (compared to 1.1min reported in literature). There was no difference in *E. sakazakii* counts obtained by direct plating on TSA or on TSA overlaid with VRBGA, however the latter method eliminated the risk of *Bacillus* outgrowth, particularly when testing at temperatures less than 60°C. The use of the thin walled tubes gave more rapid heat-up and cool down times than conventional TDK tubes. D-values obtained using these tubes are similar to published values. This method is suitable for the screening of clinical and environmental *E. sakazakii* strains.

P52 Osmo-tolerance of *Enterobacter sakazakii* strains using Phenotype Micro-Array analysis

Jenna M. Warby, Ciara Walsh, Séamus Fanning and Amalia Scannell

College of Life Sciences,School of Agriculture, Food Science & Veterinary Medicine, UCD Agriculture and Food Science Centre, UCD, Dublin, Belfield, Dublin 4, Ireland.

E. sakazakii is a food-borne pathogen associated with a rare form of neonatal meningitis, sepsis and necrotizing enterocolitis (NEC), resulting in a high mortality rate (40%-80%) in newborns and infants. The mode of transmission associated with *E. sakazakii* is generally powdered infant milk formula, which has a water activity (aw) of ca 0.2. The lowest aw at which proliferation of most pathogens is limited, occurs at 0.8 however, bacteria have been shown to survive in conditions of much lower moisture availability. This study seeks to assess the ability of 12 *E. sakazakii* strains to grow in a range different osmolytes. The effect of water activity on the growth of twelve strains of *E. sakazakii* was investigated using multiple osmotic solutes arranged in a commercial high-throughput phenotype micro-array plate 9 (Biolog, Inc). Stationary phase cells were suspended in the IF-10a base inoculating solution and transferred in 100µl aliquots to individual wells. Tetrazolium dye was used as a measure of the respiratory activity of the cells, an indicator of metabolic activity. The experiment was conducted at 37°C for 48 hours. The results for respiratory activity were plotted against time to produce the growth profiles for each strain. Analysis of the 12 strains revealed considerable variation in growth in different osmolytes at varying concentrations. Notable, *E. sakazakii* strains were most severely inhibited by sodium benzoate (20 -200 mM), followed by urea (2- 7 %) and sodium formate (1- 6 %). The strains were least inhibited by sodium sulfate (2-5 %), ethylene glycol (5 -20 %), sodium phosphate pH 7 and ammonium sulphate (20 –200 mM). An inverse relationship was observed between the remaining osmolytes (e.g. NaCl, 1 -10 %)and respiratory activity. Of the 12 *E sakazakii* strains, NCTC08155 was the most resistant isolate followed by, E789, ES104 This study also found E901P, E787 and ATCC 29544 to the most sensitivity to low aw. This study shows that while *E. sakazakii* is relatively resistant to osmotic stress when compared with other strains of the Enterobacteriaceae, this capacity depends largely on the strain used and the osmolytes against which it is tested.

P53 Thermal death kinetics of *Enterobacter sakazakii* at 60ºC in Reconstituted infant formula milk

Jenna Warby, Ciara Walsh, Séamus Fanning and Amalia Scannell

College of Life Sciences,School of Agriculture, Food Science & Veterinary Medicine, UCD Agriculture and Food Science Centre, UCD, Dublin, Belfield, Dublin 4, Ireland.

E. sakazakii, a unique species defined in 1980, has been implicated as the causative agent in a rare but severe form of neonatal meningitis, with Infant Formula Milk (IFM) being implicated as the mode of transmission. Despite the high temperatures (72 – 200ºC) used during the production of IFM, *E. sakazakii*, may still be recovered at low levels in the finished product. This study aims to compare the D-values of a number of *Ent sakazakii* strains at 60ºC. Ten *E. sakazakii* strains (7 clinical, 2 environmental and 1 food isolate) were used to assess heat resistance using rubber sealed thin walled glass tubes. Stationary cells were suspended in IFM, divided into 1 ml aliquots and heated at 60°C for up to 180 minutes. Duplicate tubes for each time point were removed from the water-bath at one min intervals, cooled on ice and samples were plated on Tryptone Soya Agar

(Oxoid, Basingstoke, England) using spread plate technique. The duplicate experiments were carried out on three separate occasions. Results, Log cfu/ml, were graphed against time and D-values (time required for a 1 log reduction to occur) were calculated using the graphical regions demonstrating first order kinetics. The resultant D-values ranged from 0.71 to 2.51 min. This is comparable with those previously reported (2.5 min) for 60ºC. Previous literature has stated that *E. sakazakii* strains possessing a KT protein tend to have a higher D-value than those without. However, no significant difference ($p>0.05$) was observed between the KT+ strains and KT- strains, in this study. Extensive tailing of curves was observed in this study which may be explained by cell exposure to increasing temperatures during testing (heat-up times), resulting in increased resistance to subsequent thermal conditions. Such heat resistance therefore, poses a greater risk to the health of infants should reconstitution of IFM not follow WHO recommendations, using water at 70ºC. For this reason, a thorough understanding of the thermal death kinetics of *E. sakazakii* in IFM is essential.

P54 Thermal resistance, growth and inactivation of *Enterobacter sakazakii* in powdered and reconstituted infant formula

Murad A. Al-Holy[1], Hamzah A. Al-Qadiri[2], Mahmoud, M. Abu-Ghoush[1], Barbara A. Rasco[3]

[1] Department of Clinical Nutrition and Dietetics, Faculty of Allied Health Sciences, Hashemite University, P.O Box: 150459, Zarqa-Jordan. [2] Department of Nutrition and Food Technology, Faculty of Agriculture, University of Jordan, Amman-Jordan. [3] Department of Food Science and Human Nutrition, Box 646376, Washington State University, Pullman, WA 99164-6373

Enterobacter sakazakii has recently been recognized as an opportunistic foodborne pathogen. Dry infant formula has been implicated as the mode of transmission for this microorganism, which may cause a severe form of neonatal meningitis and high fatality rate among infants. The objectives of this study were to investigate the heat resistance, growth pattern and inactivation under room and refrigeration temperatures storage of dry and reconstituted infant formula milk (IFM). *E. sakazakii* strains (8 strains) showed a wide variability in heat resistance at different temperatures (55, 60, and 63 ^{o}C). The *D*-values at 55 ^{o}C ranged from 1.51-14.83 min, at 60 ^{o}C from 0.17 to 2.71 min and at 63 ^{o}C from 0.05 min to 0.88 min. The calculated *z*-values for the studied *E. sakazakii* strains ranged from 3.76-10.11 ^{o}C. A household microwave was used to heat 60 ml portions of reconstituted IFM. The reconstituted IFM was inoculated with 1×10^{5} CFU/ml of a cocktail of four heat-resistant strains of *E. sakazakii*. Heating reconstituted IFM from 20-30 s was not effective in reducing *E. sakazakii*. However, heating for 40-50 s was effective in eradicating all inoculated *E. sakazakii*. Additionally, storing powdered IFM for 15 days at refrigeration resulted in at least a 1 log unit reduction in inoculated *E. sakazakii* strains. Whereas storing reconstituted IFM at refrigeration for two weeks resulted in more than 2 log units reduction in *E. sakazakii*. However, keeping reconstituted IFM at room temperature resulted in a very sharp increase in *E. sakazakii* count. *Lactobacillus acidophilus* was examined for its antimicrobial activity against *E. sakazakii*. However, no antimicrobial effect for *Lactobacillus acidophilus* was observed.

P55 Biofilm formation by *Cronobacter* on infant feeding bottles and teats

Edita Custic, Sumeet Kaur and Jane P. Sutherland

Microbiology Research Unit, Department of Human Sciences, London Metropolitan University, 166 220 Holloway Road, London N7 8DB, UK.

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 22ºC for three days. The materials were then washed twice with phosphate buffered saline (PBS) and vortexed at maximum speed in PBS with antibumping granules for one minute to dislodge biofilm cells. Both PBS and IFM were diluted and plated on tryptone 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.

P56 Effect of the sample size on the detection of *Cronobacter sakazakii* in infant formula.

José Juan Rodriguez-Jerez, Dora Isela Salas-Vázquez, Fabián González-Rivas & Mercedes Marín De Mateo

Departamento de Ciencia Animal y de los Alimentos, Facultad de Veterinaria, Campus UAB, 08193 Bellaterra (Barcelona), Spain.

According to current legislation in Europe, is compulsory an analysis of 30 samples by lot if Enterobacteria are detected in infant formula. However, this amount of samples are very time consuming and expensive. Consequently, a number of laboratories may perform a composite of samples, increasing the amount of product, with a risk of no detection of the pathogen. The aim of our work was to compare different protocols to determine the detection level when the quantity of sample is higher than 25g, using different contamination levels. A total of 180 samples were analyzed, according to ISO-TS-22964-2006 method. Three different wild *Cronobacter sakazakii* strains have been used to inoculate the samples at different contamination levels. All the study has been performed by duplicates and positive and negative controls in each analysis used. Series of 30 samples has been studied according to current legislation. Our results shown equal results, when compositing of two samples (50g) is used and when only a sample is analyzed (25g). In any case, if the number of samples is higher than 5 (more than 125g), *Cronobacter sakazakii* is detected when the contamination level is lower than 100 ufc. However, when a composite of 5 samples was performed results are different, according to the volume of enrichment broth and the pre-enrichment inoculum. On this case, an increment in the volume of enrichment broth linked to an increase of the pre-enrichment inoculums used let us to detect all expected positive samples. All composites with a low level of contamination were not detected if the pre-enrichment volume was not equivalent to the 5 grouped samples (0,5 ml). When we make a dry or wet composite and only 0,1ml of pre-enrichment has been inoculated into enrichment medium, a group of each 5 samples has been not detected. However, if we increased level of inoculums to 0,5 ml, we obtain a detection of 100% of samples. Currently, if a composite of samples is performed, is necessary to consider a modification of the protocol to assure a nice detection of the pathogen. If the composite is higher than 2 samples, detection is not assured, if the laboratory cannot increase the volume of pre-enrichment broth in equal proportion than the recommended in the ISO protocol.

P57 Detection of *Cronobacter spp.* with different standard methods

Sha Zhu, Miryam Fischer-Reinhard and Matthias Fischer

C*entral Laboratories Friedrichsdorf, Bahnstrasse 14-30, Friedrichsdorf, D-61381, Germany.*

Cronobacter (C.) spp. is an important opportunistic pathogen which has been isolated from a variety of foods and is thought to be common in the environment. Occasionally found as contaminant of powdered infant formula (PIF), this organism is associated in neonatal and infant cases of meningitis, septicemia and necrotizing enterocolitis. This has brought the organism into the focus of food microbiology over the recent years and a number of standard methods based on chromogenic agars and molecular biological methods have been developed. The topic of the current investigations was to compare the different approaches in terms of sensitivity and specificity. This has been done especially against the background of the species *Enterobacter sakazakii* recently being renamed to *Cronobacter spp.* and showing a broader diversity now. We

have included several *Cronobacter* species in the study to investigate differences in the detection ability of standard methods.

P58 Characterization of plasmids possessed by clinical strains of *Cronobacter* species (formerly *Enterobacter sakazakii*) and the development of a PCR assay for the presence of two common plasmids.

Lan Hu, V. Sathyamoorthy, J. Kim and Ben D. Tall

Centre for Food Safety & Applied Nutrition, US Food and Drug Administration, Laurel, MD 20708, USA.

Cronobacter species, formerly *Enterobacter sakazakii* (Cs) are emerging food-borne pathogens that cause sepsis, meningitis, and necrotizing enterocolitis in neonates and infants. However, little is known about the pathogenicity of these organisms. Strain BAA-894 has been shown to carry two plasmids, pESA-3 and pESA-2 (molecular sizes ~131 Kb and 31 Kb, respectively). Plasmid-specific genes include an ompT protein encoded on the larger plasmid and a VirB-Type IV secretion system (T4SS) encoded on the smaller plasmid. OmpT belongs to a family of highly homologous outer membrane proteases, known as omptins, which play a role in the virulence of *Salmonella, Shigella* and *Yersinia* spp. T4SS gene clusters in *Brucella* spp. and *Helicobacter pylori* have been shown to play a role in these organisms' ability to persist intracellularly within host cells. It is reasonable to predict that ompT and VirB would be good plasmid markers. Plasmid profile analysis of 45 clinical isolates was performed. Plasmid DNAs were extracted using a QIAprep spin Miniprep kit (Qiagen), and then separated by agarose gel electrophoresis (AGE). Results from AGE analysis showed that one strain did not possess any plasmid. However based on size, 12 different plasmid profiles were observed among the strains. A common plasmid band was observed among plasmid positive strains. To identify which plasmid(s) was being carried, we performed polymerase chain reaction (PCR) analysis with primers targeting ompT and VirB11. Forty-two strains were found to possess the ompT gene, while 30 strains were found to possess the VirB11 gene. Also, 27 strains were positive for both plasmid targets. Interestingly, no strains found to possess one or both plasmids by AGE, were PCR negative. The strain negative by AGE analysis was also negative by PCR analysis. In summary, our data suggests that most clinical strains possess both plasmids and that the presence of the plasmids can be determined by using PCR analysis. These data also suggest that some strains carry plasmids other than pESA 3 or pESA-2. The role of these plasmids in the pathogenesis of Cs is currently under investigation.

P59 Development of a new test panel for identification of both Gram-negative and Gram-positive bacteria

Amalia Franco-Buff, Vanessa Gomez, Eric Olender, Peter Gadzinski and Barry Bochner.

Biolog Inc., 21124 Cabot Blvd., Hayward, CA, 94545, USA.

The objective of this project was to simplify biochemical/phenotypic testing of bacteria by developing a single test panel that could be used to identify both Gram-negative and Gram-positive bacteria. This has been impossible in the past because bacterial genera are quite diverse and no one has found universal culture and chromogenic testing chemistries that would work with a broad range of genera. We employed phenotype MicroArrays to study, in detail, the metabolic and chemical sensitivity properties of diverse bacteria. From this effort we succeeded in developing assay conditions and a tetrazolium-based colorimetric chemistry that can be used to fingerprint and identify more than 1,000 species, including Gram-negative enteric, non-fermenter and fastidious species as well as important Gram-positive species such as *Bacillus, Corynebacterium, Staphylococcus, Streptococcus, Lactobacillus,* and *Nocardia*. Using computer-assisted analysis we selected from the Phenotype MicroArray assays a final set of 94 tests that are taxonomically informative. The 71 carbon source utilization assays include sugars, carbohydrates, sugar alcohols, hexose-phosphates, hexuronic acids, D- and L-amino acids, carboxylic acids, and other types of naturally occurring biochemicals. The 23 chemical sensitivity assays test the sensitivity to potentially inhibitory conditions. The conditions include high salt (8% NaCl), high lactic acid (1%), low pH (5.0), and sensitivity to other taxonomically useful agents such as antibiotics, cations, anions, oxidizers, and detergents. Because the panel identifies both Gram-negative and Gram-positives, a bacterial isolate can be identified without a Gram-stain or any other pre-tests. The

one-minute set up procedure consists of preparing a cell suspension from agar-grown cells, and inoculating 100 µl into each well of the panel. The system recommends 33° C for incubation, which permits identification of many more species. In addition to simplicity, a major advantage over identification based on 16s-rDNA gene sequence is that you simultaneously gain detailed information about the properties and growth phenotypes of the bacterium that you have identified.

P60 Optical maps of subgroups of *Cronobacter* (formerly *Enterobacter sakazakii)* show large chromosomal regions of homology and differences among isolates

Mike L. Kotewicz and Ben D. Tall.

U. S. FDA, CFSAN, OARSA, Laurel, MD 20708 USA.

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.

POSTER PRIZE AWARDS WILL BE AT THE END OF WORKSHOP C

Undergraduate and postgraduate students, as well as postdocs within their first year after graduation, are eligible to enter the poster competition. We are grateful to the following for providing the prizes;

1st Prize (€500) – Technopath
2nd Prize (€250) - Food Safety Authority of Ireland
3rd Prize (€100) - ***safe*food**