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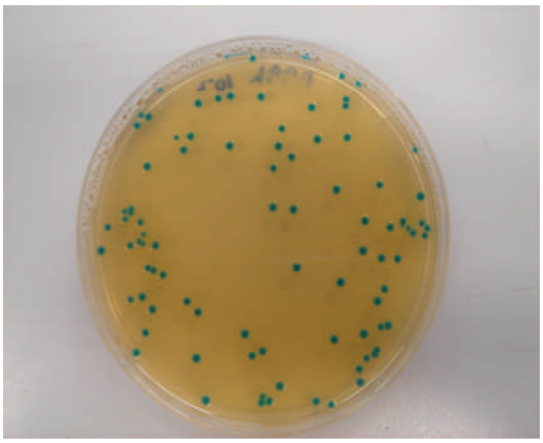
# Detection and surveillance of *enterobacter sakazakii (cronobacter* spp.) along the infant formula food chain



## Key external stakeholders:

Infant milk formula industry, Food Safety Authority of Ireland

## Practical implications for stakeholders:

*Cronobacter* spp. is a key food safety issue for the infant formula sector. Apart from an obligation to meet the regulatory microbiological criteria for this pathogen, the sector would be severely damaged by any food safety scare affecting infants consuming these products. This study has focused on transmission sources and survival characteristics of *Cronobacter* spp. The study highlighted that *Cronobacter* can occur widely in the environment and are particularly associated and adapted to survive in dry environs.

## Main results:

- *Cronobacter* spp. are not 'ubiquitous' in the environment and would be best described as 'widespread but infrequent' as it appears they have found a particular niche in dry environments
- Dry ingredients added to milk powder may have a role in transmission of *Cronobacter* spp.
- *Cronobacter* spp. are resilient, surviving the time/temperature profile experienced during spray-drying, in soil, in rumen fluid, in inulin and lecithin (ingredients in infant formula manufacture)
- An adaptive tolerance response to sub-lethal heat that confers increased heat resistance can be induced. However, the increased heat tolerance was not transferred to increased survival potential in a dry environment. Changes in the ratio of saturated to unsaturated fatty acids in the cell membrane appear to be responsible for this adaptation.

## Opportunity / Benefit:

This project has generated knowledge about the transmission and survival of *Cronobacter* in the farm to fork chain which will underpin risk management of this pathogen

## Collaborating Institutions:

University College Dublin, UCD
Food Safety Authority of Ireland, FSAI

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## 1. Project background:

*Enterobacter sakazakii* (renamed *Cronobacter*) has emerged as a rare cause of life-threatening illness resulting in meningitis, septicaemia and enterocolitis, particularly in neonates, premature infants, low birth weight infants and immunocompromised infants. The presence of this organism in dry infant milk formula products and the potential for improper handling of formulas has been implicated in several clinical cases. The overall objective of this study was to reduce the microbiological risks related to powdered infant milk formula, contaminated with *E. sakazakii*.

## 2. Questions addressed by the project:

- Are the microbiological media in current (2006) use suitable for isolation of *Cronobacter* spp. under all conditions?
- Are *Cronobacter* spp. ubiquitous in the environment?
- Will *Cronobacter* spp. survive under different conditions and in different matrices?
- Can *Cronobacter* spp. exhibit an adaptive response to heat? If so, what is the mechanism of this adaptation and will the increased survival potential be transferred?
- Is rpoS important in survival of *Cronobacter* spp.?

## 3. The experimental studies:

Studies were conducted on:

*Sampling for occurrence of Cronobacter*
Samples from pilot processing plants, households, milk powder processing environment, farm environment, and bovine faeces, animal feed, and foods sold at retail processing environment, were examined for *Cronbacter.*

*Survival of Cronobacter spp. under different conditions*

- During spray drying: Four strains of *Cronobacter* spp. were inoculated into 35% reconstituted skim milk at $10^7$ and $10^2$ cfu/g dry wt.
- In faeces at different temperatures: Faecal samples were inoculated with *Cronobacter* and survival on the soil and in containers stored outdoors was examined over time.
- In inulin and lecithin: inulin and lecithin were inoculated with isolates of *Cronobacter sakazakii*. Samples were stored and examined for *Cronobacter sakazakii*..
- D-values: The thermo-tolerance of the five strains was investigated in reconstituted Infant Milk Formula at 55, 60 and 65°C.
- In rumen fluid and simulated gastric juice: models of the bovine abomasum and rumen were inoculated with *Cronobacter* strains and survival was examined over time in these environs using an adapted ISO /DTS 22964 culture protocol.

*Adaptation of Cronobacter*
The impact of adaptation on survival of *Cronobacter* spp. was examined by adapting cells to a sub-lethal heat treatment of 46°C for 30 min prior to lethal stress at 52°C. The mechanism of adaptation was further investigated using flow cytometry. Unlike the traditional indirect plate count method, flow cytometry can provide direct information on the metabolic and physiological status of bacterial cells and can be used to compare adapted and unadapted cells.



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*Stress response*
RpoS is a protein that is involved in the stress response of some bacteria. Strain 823, whose genome has been sequenced, was found to possess an amber mutation (a termination code) at amino acid position 201, resulting in the strain expressing a truncated form of RpoS. The other strains in this study did not contain this amber mutation (i.e. strains 532, 784 and 796). Apart from this one nucleotide difference, both strains possess identical *rpoS* sequences. This study aimed to determine the effect of RpoS on stress tolerance of *Cronobacter* spp., and also to determine if the truncated RpoS produced by strain 823 still retained a function in stress tolerance of the strain.

## 4. Main Results

*Sampling for occurrence of Cronobacter*
Of all the diverse samples examined only 2 (0.57%) were positive. However, of the 43 samples from a milk powder processing environment, 12 (28%) were positive. The results showed that occurrence was high in a powder processing plant and in dry matrices but occurrence was relatively low in other environments. The results indicate that *Cronobacter* spp. have an ecological niche in dry environments. However, the term 'ubiquitous' does not accurately describe their occurrence as this implies that they are *omni-present*. The term '**widespread but infrequent**' better describes their occurrence. Furthermore, it was shown that the FDA and ISO methods in use in 2006 did not recover *att* strains, especially in conditions where there was a large number of competing micro flora. Since 2006, the FDA and ISO methods have been improved with use of modified media.

*Survival of Cronobacter spp. under different conditions*
- During spray drying all strains survived the process and were detected in the powders with a low inoculum and enumerated in all the powders with the high inoculum for at least 12 weeks.
- In faeces, *Cronobacter* survived 105 days in sealed containers and was detectable after 112 days in soil.
- In inulin and lecithin three of four strains were still detectable in both matrices after 338 days storage. Higher numbers of the environmental strains were recoverable after 338 days than the clinical strains.
- The thermo-tolerance of a clinically derived type strain, NCTC 11467$^{T}$ and a mutant strain were shown to be significantly more thermo-tolerant than other strains in reconstituted Infant Milk Formula at 55, 60 and 65°C..
- In rumen fluid and simulated gastric juice there was no significant changes in the number of *Cronobacter* in rumen fluid over a 24 h period but it was undetectable after 30 min incubation in the model abomasum.

*Adaptation of Cronobacter*
The results showed that survival of *Cronobacter* spp. at 52°C was greater in milk-grown cells than in broth-grown cells. They also showed that the survival potential of heat stressed cells was increased if cells were adapted to a sub-lethal heat treatment of 46°C for 30 min prior to lethal stress at 52°C. The acquired survival potential following adaptation was not transferred to survival in a dry environment or to survival during reconstitution of artificially contaminated milk powder by conventional or microwave heat. The ratio of membrane unsaturated to saturated fatty acids decreased, possibly resulting in a more rigid membrane in adapted cells. The alterations in the ratio of fatty acids in the membrane may explain the adaptation.

The mechanism of adaptation was further investigated using flow cytometry. Unlike the traditional indirect plate count method, flow cytometry can provide direct information on the metabolic and physiological status of bacterial cells and can be used to compare adapted and unadapted cells. The flow cytometry studies showed that indicators of metabolic activity, such as Fluorescein diacetate (FDA), Carboxy-fluorescein diacetate succinimidyl 3,3'-dihexylocarbocyanine iodide [CFDAse, DiOC6(3)] and Hydroethidide (HE), showed increased intensity in adapted cells (46ºC for 30 min) compared to unadapted cells. In addition, Reactive Oxygen Species, an indicator of cell death, were increased in un-adapted cells.

*Stress response*
Strain 532 was found to have a greater tolerance to acid at low pH (pH 3, pH 3.5) than strain 823, indicating that the truncated RpoS had lost some of its function. To investigate this further, the *rpoS* gene of strain 823 was completely disrupted (by gene knock-out, by means of homologous recombination, involving double cross-over of DNA flanking the gene of interest) resulting in a mutant strain 823*ΔrpoS*. The acid tolerance of wild-type strain 823 was compared with that of the strain 823*ΔrpoS*, at pH 3.5 over 4.5 hours, using stationary phase cells. There was an approximate 2 log-cycles difference in survival after 3 h, the wild-type


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strain surviving better. These results indicate that *rpoS* plays a role in acid tolerance of *Cronobacter* spp., and that the truncated *rpoS* strain 823 still appears to retain some function in stress tolerance.

## 5. Opportunities / benefits

Knowledge about the transmission and survival of *Cronobacter* in the farm to fork chain will underpin risk management of this pathogen

## 6. Dissemination

The scientific knowledge generated in the project was disseminated via peer publications, popular publication, conferences and workshops

### Main publications:

Arku, B., Fanning, S. and Jordan, K (2011). Heat Adaptation and Survival of *Cronobacter* spp. (Formerly *Enterobacter sakazakii*). *Foodborne Pathogens and Disease*, 8(9): 975-981.

Walsh, D., Molloy, C., Carroll, J., Cagney, C., O'Brien, S., Fanning S., Iversen, C. and Duffy, G. (2011). Survival characteristics of environmental and clinically derived strains of *Cronobacter sakazakii* in infant milk formula (IMF) and ingredients. *J Appl Microbiol.* 110(3):697-703.

Arku B, Fanning S, **Jordan** K. (2011). Flow cytometry to assess biochemical pathways in heat-stressed *Cronobacter* spp. (formerly Enterobacter sakazakii). *J Appl Microbiol*.;111(3):616-24

**Popular**

Arku, B. Fox, E., Mullane, N., Fanning, S. and Jordan, K.N. (2007). Survival of *Cronobacter spp.* during spray-drying. Proceedings of the IDF World Dairy Symposium in Dublin, Oct. 2007.

Jordan. K, and Duffy, G (2007). *Enterobacter sakazakii, an emerging pathogen.* T-Research 1st International Conference on *Cronobacter* spp. held at UCD in January 2009.

## 7. Compiled by:

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