An Roinn Talmhaíochta,
Bia agus Mara
Department of Agriculture,
Food and the Marine

13F423 - Systems microbiology applied to the reduction and control of bacterial transmission in the powdered infant formula (PIF) production environment – towards scientifically validated improvements in food safety

Final Report

This project was funded under the Department of Agriculture, Food and the Marine Competitive Funding Programme.

SUMMARY

Microbial safety of powdered infant formula (PIF) presents a significant challenge to producers and those with an interest in public health, alike. An earlier FAFM-sponsored research project targeted a bacterium of concern, Cronobacter sakazakii and which epidemiologically is associated with neonatal cases involving contaminated in PIF. Despite advances made in our understanding of the nature of this bacterium in PIF and its production environment, no information on the total microbiology or microbiome of the manufacturing niche was available. This the first study of its kind, using NGSbased approaches (16S rDNA sequencing, metagenomics and metatranscriptomics) to characterize PIF built production plant environment microbiome.

The following were the overarching objectives:

- To undertake an extensive sampling plan of two PIF production sites to establish the microbial resident populations;
- Using flow cytometry to describe the dynamics related to microbial populations changes;
- Describe the microbiome and metatranscriptome of selected microbial populations to identify changes in population structure and links to factors driving this process;
- Extend our understanding of the connections/networks between all of the above using a systems microbiologyapproach linked to risk analysis of the impact of the microbiome on pathogens of concern in PIF
- Provide whole genome sequences for a selected number of food-borne zoonotic pathogens cultured from the PIF production environment
- Provide a description of the bacterial microbiome of the two production sites
- Using a suitable laboratory-derived model, explore the bacterial signalling, by deep-level RNA-seq, of Cronobacter sakazakii SP291 in response to persistence in low-moisture conditions

Results obtained from the demonstrated the utility of FC in characterising the microorganisms colonising the built PIF production environment. These data were subsequently extended using matched environmental samples taken from two PIF production facilities and which were compared, and a core microbiome identified.

KEYWORDS

Cronobacter species; PIF contamination; low-moisture microbiology; microbiome

ACRONYM	SMART-pif
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COLLABORATORS, INSTITUTION	Prof Francis Butler, Dr Marta Martins & Dr Matthew McCusker, University College Dublin. Dr Kieran Jordan, Teagasc.
PUBLICATION DATE	December 2019.

Section 1 - Research Approach & Results

Start Date

01 December 2013

End Date

31 May 2018

Research Programme

Food Institutional Research Measure

TRL Scale

TRL 4: Technology validated in lab

NRPE Priority area

Diagnostics

Total DAFM Award

€585,795.42

Total Project Expenditure

€580,328.05

Rationale for undertaking the Research

Ireland's largest indigenous industry comprises both the agri-food and dairy sectors, and these are of strategic importance to the domestic Irish economy. Food Harvest 2020 sets out ambitious targets aiming to achieve a 50% increase in milk production within the next 7-year period, to 2020. Already there is a well-established track record of food safety collaboration between UCD-CFS/BioS/TFRC-M and Danone, which has marked the latter as International leaders. The rationale underpinning this research proposal is to maintain this collaboration and to extend it by continuing to develop scientifically sound approaches to limit the risk of food safety events during PIF production. If the stated aspirations of Food Harvest 2020 are to be realised, the increased demands placed on PIF manufacturing, may increase the risk of threats to the economic viability, through losses in brand confidence.

This project directly addresses the requirement to develop, validate and apply sound biological control strategies, designed to improve food safety and the quality of the product. The state-of-the-art element in this research avails of the risk assessment expertise uniquely available in Ireland, at UCD, linked to a description of the microbiome across the complete PIF food chain, to uncover the dynamic changes in microbial populations. These data would provide an unparalleled insight into the dynamics associated with a microbial colonisation within a PIF built production environment. It would offer the prospect of dissecting the interactions between those organisms identified, with a view to harnessing any antibacterial properties, as a natural means of control.

Methodology

The technical approach deployed to deliver this programme, included the design and implementation of a bespoke sampling plan at both factory locations. This was done in an effort to capture, temporal; geographical and any unintended shifts in the microbiomes at both sites. Samples included for study included environmental swabs at defined (GIS-referenced) locations, along with intermediate and final product.

Initially, flow cytometry (FC) was carried out to provide an early characterisation of both PIF production environments. This approach produced data on the microbial cell density in the environment and their live/dead ratio. These data also provided support for downstream NGS experiments.
Next generation sequencing (NGS) techniques including 16S rDNA-based sequencing; shotgun metagenomics and metatranscriptomic sequencing, were subsequently used to study the PIF plant environment microbiome and the viable microbiome in detail based on selected high-quality samples taken from scheduled monthly environment sampling visits. Another elemental part of the project also deployed whole (bacterial) genome sequencing (WGS) to study isolates of interest along with the application of deep-level RNA sequencing (RNA-seq) to explore the bacterial responses at the transcriptional level to low-moisture.
These technical approaches represent novel approaches to the study of the PIF production environment and crucially to address the question as to why Cronobacter sakazakii persists in this environment.

Project Results

This project applied FC and WGS-based strategies to describe the following:

- description of microbial colonisation dynamics of the PIF production environment, using FC to determine microbial cell density and the associated physiological status.
- characterisation of the metagenome of the PIF production environment, at both locations.
- characterisation of the metatranscriptome of the PIF production environment, using a set of 23 matched samples, to elaborate on the microbial species (bacteria; fungi; protozoae; viruses); the antimicrobial resistance genes (ARG) and signals associated with active metabolic pathways.
- WGS determination and subsequent bioinformatic characterisation.
- RNA-seq analysis of Cronobacter sakazakii SP291, to describe the genetic signalling supporting the persistent phenotype at low-moisture conditions.
- description of the 16S rDNA-based metegenetics.

To our knowledge, this is the first study using NGS-based approaches (16S rDNA sequencing, metagenomics and metatranscriptomics) to characterize PIF production plant environment microbiome.

Section 2 - Research Outputs

Summary of Project Findings

Collaborative links developed during this research included, links fostered between UCD and US-FDA and this has resulted in a very strong collaboration on subjects of mutual interest, that extend beyond Cronobacter sakazakii. Outcomes with national/ policy/social/environmental potential include the adoption of WGS-based methods devised and shared with DAFM-Backweston. This step will assist the National regulator with its programme of implantation of these protocols to protect public health.
In summary the research programme carried out during the lifetime of this grant, has contributed to an improved understanding of the role of Cronobacter species in the PIF production environment. The technical capacity now exists to study this and other bacteria of interest to public health, at great depth.

Summary of Staff Outputs

Research Output	Male	Female	Total Number
PhD Students	1	1	2
Post Doctorates	3	2	5

Summary of Academic Outputs

Research Outputs	Total Number	Details
Publications in Peer Reviewed Scientific Journals	5	These outputs refer to original peer-reviewed research papers.
Peer Reviewed Conference Papers	9	These consist of reviewed conference posters and invited oral presentations.
PhD Theses	2	Two Doctoral theses were completed and supported through this project.

Intellectual Property

None.

Summary of other Project Outputs

Project Outputs	Details	Total No.
New Technology	Protocols to implement 16S rDNA-based metagenetics; metagenomics; and metatranscriptomics have been made available to the industry and regulator.	1

Potential Impact related to Policy, Practice and Other Impacts

Impact	Details
Other	Adoption of these technologies will benefit existing food safety monitoring strategies

Dissemination Activities

Activity	Details
Workshops at which results were presented.	Aspects of this research programme have been presented at scientific meetings, both at home and abroad. In addition, an exit workshop was held with all partners prior to the completion of this report.

Section 3 - Leveraging, Future Strategies & Reference

Leveraging Metrics

None.

Future Strategies

SMART-pif formed the basis of a major application to Enterprise Ireland (EI) and which culminated in the largest award made by EI, in the amount of €1.7 million. This funded project, Sequencing Alliance for Food production Environments (SAFE) commenced the application of WGS-based methods to support food safety and quality efforts in the Irish food industry. It is now up to the food industry to embrace these technologies and include them as part of the food safety infrastructure.

Project Publications

Learned peer-reviewed original research papers:

1. Srikumar, S., Cao, Y., Yan, Q., Van Hoorde, K., Nguyen, S., Coonsy, S., Gopinath, G.R., Tall, B.D., Sivasankaran, S.K., Lehner, A., Stephan, R. and Fanning, S. RNA sequencing based transcriptional overview of xerotolerance in Cronobacter sakazakii SP291. Applied & Environmental Microbiology (2019) 85: doi: 10.1128/AEM.01993-18.
2. Jang, H., Woo, J., Lee, Y., Negrete, F., Finkelstein, S., Chase, H., Addy, N., Ewing-Peeples, L., Gilles Beaubrun, J.J., Patel, I., Gangiredla, J. Eshwar, A., Jaradat, Z.W., Seo, K., Shabarinath, S., Fanning, S., Stephan, R., Lehner, A., Tall, B.D. and Gopinath, G.R. Draft genomes of Cronobacter sakazakii strains isolated from dried spices bring unique insights into the diversity of plant-associated strains. Standards in Genomic Sciences (2018) 13: 35 doi: 10.1186/s40793-018-0339-6.
3. Cao, Y., Fanning, S., Proos, S., Jordan, K. and Srikumar, S. A review on the applications of next generation sequencing technologies as applied to food-related micrbiome studies. Frontiers in Microbiology (2017) 8: 1829. doi: 10.3389/fmicb.2017.01829.
4. Anvarian, A.H.P., Cao, Y., Srikumar, S., Fanning, S. and Jordan, K. Flow cytometric and 16S sequencing methodologies for monitoring the physiological status of the microbiome in powdered infant formula production. Frontiers in Microbiology (2016) 7: 968 doi: 10.3389/fmicb.2016.00968.
5. von Westerholt, F., Gonzales-Barron, U., Butler, F., 2016. Bayesian approach to estimating the uncertainty in the distribution of Cronobacter spp. in powdered infant formula arising from microbiological criteria test outcomes. Microbial Risk Assessment, 4(1), 36-42.

Poster presentations:

1. A 16S rDNA-based sequencing study to describe the environmental microbiome in powdered infant formula production sites in Ireland. Cau. Y., Srikumar, S., Naithani, A. and Fanning, S. Presented at Asset 2018 Belfast Summit on Global Food Integrity, Waterfront Hall, Belfast, Northern Ireland, 28th-31st May, 2018.
2. von Westerholt, F., Butler, F., April 2017 Use of a Bayesian inference model with multisampling for microbial pathogens. Conference Presentation: Q-Safe 2017, Syros, Greece.
3. von Westerholt, F., Butler, F., September 2017. Bayesian analysis of surveillance data for Cronobacter spp. in a dairy ingredient process plant. Conference Poster: ICPMF10 2017, Cordoba, Spain.
4. A 16S rDNA sequencing study of the microbiome in a powdered infant formula production site. Cao, Y., Srikumar, S., Anvarian, A., Jordan, K., Fanning, S. Presented at the FoodMicro 2016 Conference, Dublin, Ireland,19th-21st July 2016.
5. von Westerholt, F., Butler, F., July 2016. A Bayesian approach to assess microbiological criteria for Cronobacter spp. in infant formula. Conference Presentation: FoodMICRO 2016, Dublin Ireland.
6. Anvarian AHP, Cao Y, Srikumar S, Fanning S, Jordan K. July 2016. Development of a protocol for monitoring the physiological status of the microbiome in powdered infant formula production units using flow cytometric and metagenomic technique. FoodMICRO 2016, Dublin Ireland.
7. von Westerholt, F., Gonzales-Barron, U., Butler, F., July 2015. A Bayesian Approach to Interpreting Quality Control Data of Cronobacter spp. in Infant Powder Formula. Conference Presentation: IAFP 2015, Portland Oregon, USA.

Invited oral presentations:

1. Alive and well in low-moisture conditions- what can we learn about Cronobacter sakazakii using RNA sequencing and transposon-directed insertion site (TraDIS) sequencing. Presented at the US-FDA Virulence Mechanisms Branch, Division of Food & Environmental Microbiology, Centre for Food Safety & Applied Nutrition, Laurel, Maryland, United States of America 21st March 2017.
2. Living in a dry environment- bacteria style. Presented at the Annual Meeting of the International Association for Food Prorection (IAFP), Convention Centre, St. Louis, Missouri, United States of America, 1st August 2016.

Doctoral theses:

1. Systems microbiology applied to the reduction and control of bacterial transmission in the powdered infant formula (PIF) production environment. Y. Cao Ph.D. (UCD) graduated in September 2019; Supervisors: S. Fanning, M and K. Jordan (Teagasc-Moorepark).
2. Monitoring and control of Cronobacter spp. in dairy powder processing facilities. F. von Westerholt Ph.D. (UCD) graduated in September 2018; Supervisors: F. Butler, S. Fanning and E. Cummins.