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

14F883 – Development of SPORE ANALYSUS CRITICAL CONTROL POINT (SACCP) charts for application in dairy manufacturing processes (SACCP)

Final Report

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

SUMMARY

The SACCP project identified the key problematic spore-forming bacteria in Irish powdered dairy ingredients, assessed existing detection systems, and evaluated novel process technologies to assess their efficacy in reducing spore numbers within dairy manufacturing processes.

The project had three core research themes that worked synergistically together to deliver the overall project brief. The first research thematic area focused on developing next generation sequencing techniques for the isolation and identification of spore forming bacteria in both dairy products and processes. As the food industry attempts to move away from classical microbiological approaches based on plating techniques these molecular microbiological approaches may form the basis for quality control laboratories in the dairy industry in the future.

The second thematic research area focused on the survival of highly heat resistant spore forming microbes in model dairy systems, with the aim of understanding the survival behaviors of spores in current conventional dairy processes. Additionally novel reduced thermal load approaches were evaluated as an innovative approach to maximise microbial inactivation while minimising deleterious effects on product quality associated with very high heat loads.

The third and final research areas focused on the development of a biosensor, to detect spores of target microbial species. An electrochemical or antibody based approach was used to design and fabricate miniaturised and disposable sensing chips. The sensor consisted of a three electrode cell, a working electrode, counter electrode and reference electrode. Through monitoring of the current that forms between the working electrode and the solution under investigation, important chemical information could be obtained such as presence or absence of specific bacteria.

KEYWORDS

Spores, thermal processes, dairy

ACRONYM	SACCP
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COLLABORATORS, INSTITUTION	Dr Paul Cotter, Teagasc Dr Karen Twomey, Tyndall National Institute Dr Martyn Pembleton, Tyndall National Institute
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Section 1 - Research Approach & Results

Start Date

01 April 2015

End Date

30 September 2019

Research Programme

Food Institutional Research Measure

TRL Scale

TRL 5: Technology validated in relevant environment

NRPE Priority area

Processing technologies and novel materials

Total DAFM Award

€624,729.10

Total Project Expenditure

€501,151.00

Rationale for undertaking the Research

In 2013 a large scale recall of dairy products and in particular infant milk formulae in the Chinese market highlighted the vulnerability of dairy processes to systematic microbiological failure when a putative pathogen contamination was detected in manufactured ingredients. The incident in question was further exacerbated by mis-identification of the contaminating organism during microbiological analysis, resulting in a food safety scare of international proportions and consequential reputational damage not alone for the company involved, but also at a national level. The underlying message from this incident was that the previously held tolerance of low-levels of spore-forming bacteria entering milk primarily at farm production level may no longer be taken for granted from a food safety perspective. The close DNA-DNA homology between spore-forming bacteria such as Clostridia species in particular highlighted the importance of correct identification during microbiological analysis, particularly since this class of microorganisms can produce toxins.

In contrast to the thermal lability of microorganisms in their vegetative state, the thermal resistance of bacterial spores highlighted the absence of appropriate thermal and/or non-thermal dairy processes capable of controlling or eliminating spores during most typical milk processes. As ‘low level’ spore forming bacterial contamination in milk was now deemed a greater potential hazard than heretofore, new risk assessment and risk management tools and processes were needed to provide greater monitoring and in-process controls during dairy ingredient production. Thus, the SACCP project concept was born in an effort to address this risk in the context of the Irish milk-processing sector.

Methodology

1. The project initially focused on industry standard bacterial identification/enumeration techniques, focusing in particular on the accuracy of the frequently used MYP (Mannitol Egg Yolk Polymyxin) agar as a means of detecting the presence of Bacillus cereus group bacteria. With presumptive B. cereus group colonies isolated and plated on BACARA agar (regarded as a more selective agar) followed by 16S rRNA sequencing.

2. Molecular microbiology approaches were used to provide greater insights into the true microbial communities present in industrial dairy plants. Whereby DNA sequencing was used to map and track innate and equipment derived microflora throughout the industrial process.
3. In parallel non thermal approaches (microfiltration and bactofugation) were assessed for their ability to remediate spore contamination in whey protein concentrate with studies identifying much higher reductions in spore numbers for filtration approaches, although significant by-products streams were also created.
4. The project has assessed sequential heating (80–90 °C) processes which are commonly applied to milk by the dairy industry as part of their microbiological control strategy, relative to a control high temperature heating process. This provided a benchmark on the impact on the survival of both thermally resistant microbial populations and thermally labile milk components during processing.
5. Separately microwave volumetric heating (MVH) was assessed as an innovative heating technology for inactivation of bacterial species using a combination of classical microbiology and flow cytometry approaches.
6. Electrodes were developed in Tyndall to detect glucose, which was selected as a model analyte. The techniques developed have then been utilised to address the key issue associated with the project namely the detection of Bacillus spores. With a new matrix developed based on the use of fragmented antibodies, which was shown to be capable of detecting bacillus spores directly.

Project Results

The greater accuracy of BACARA, over MYP, agar as a mean of detecting the presence of B. cereus group bacteria was demonstrated. Of 507 isolates presumed to be B. cereus group bacteria on the basis of growth on MYP, only 177 showed typical growth typical of B. cereus group bacteria on BACARA agar. 98 of the isolates that grew on BACARA were sequenced using 16S rRNA sequencing and 81 were tentatively identified as presumptive B. cereus group species. Molecular based assays are preferred as they identify the species or even strain present and determine if specific genes (such as toxin genes) are present or absent.

This project has demonstrated the higher survivability of spores and thermoduric species in concentrated dairy systems, based on the thermal survivability of cultures of Geobacillus Stearothermophilus spores in both static and dynamic models to elucidate the relationship between time/temperature combinations to understand the logarithmic destruction of spores in liquid medium. A clear relationship between processing temperature and dry matter content (10-30 % w/w) was observed relative to the survival of Geobacillus spores. Increasing dry matter contents requires higher thermal loads to achieve complete inactivation of highly heat resistant spores in dairy systems.

This project demonstrated the advantages of microfiltration compared to centrifugal separation (Bactofugation) relative to removal rates of spores in dairy streams.

However, the cost consideration both from a capital and operational perspective mean that for the vast majority of commercial process lines, centrifugal separation will remain the process of choice to meet finished powder quality requirements relative to spore forming bacteria.

The project determined if double pasteurisation each for 15 s, at 1 of 9 different temperature combinations from a 3 (first heat treatment) x 3 (second heat treatment) matrix of 80, 85 and 90˚C with an overnight storage between heat treatments, would achieve the desired microbial removal and result in an acceptable WPNI comparable to an industry high heat treatment. The results showed that the most severe heat treatment used (90 x 90 C) gave an acceptable WPNI of about 3.5, and although mesophilic spores were reasonably well reduced, thermophilic spores were not.

The project assessed the potential of microwave volumetric heating as an alternative direct heating technology for inactivation of heat resistant microbial groups. Microwave heating in general is an emerging technology when compared to well-established commercial heating technologies in the dairy industry but does offer potential benefits regarding transfer of thermal energy to the product without the need for an intermediate transfer surface or the introduction into the product of a heating medium. The results of this study indicate comparable

microbiological inactivation rates for microwave compared to tubular heating based on classic plate-counting methods and flow cytometry.

The project has developed a new matrix based on the use of fragmented antibodies which has been shown to be capable of detecting bacillus spores directly. Further development of the fragmented antibody approach could result in the development of at-line sensors for the detection of spores at various points in dairy processes.

Section 2 - Research Outputs

Summary of Project Findings

The SACCP project has determined that molecular based assays are preferred over classic plating techniques as they identify the species or even strain present and determine if specific genes (such as toxin genes) are present or absent. It is expected these methods will become the method of choice at industry level in the coming years.

The SACCP task has delivered one of the first Irish studies focused on a total supply chain tracking approach using the latest molecular microbiology techniques to understand the evolution of the microbial community and any inherent risks relative to the species identified therein. Heat resistant, spore forming, and environmentally contributed contaminants have been tracked with one industry collaborator, which has generated unique insights on the contribution of incoming milk and in-process microflora relative to potential food safety risks.

The SACCP project has demonstrated the higher survivability of spores and thermoduric species in concentrated dairy systems and to guarantee a specific microbial reduction therein, a higher time/temperature combination is required which could have significant implications for product quality. To overcome this challenge microfiltration and centrifugal separation have been assessed. However, while microfiltration is more efficient in terms of spore removal, capital and operational costs indicate that centrifugal separation will remain the process of choice to remove spore forming species.

Both sequential heating approaches and a reduced thermal load microwave heating technology have also been assessed as industrial feasible and scalable technologies, which provides similar food safety to classical heating technologies. However, no advantage over classical heating techniques was observed. The assessment of analytical techniques both classical/molecular microbiology and flow cytometry has allowed the project team to gain valuable insights relative to the efficacy of individual inactivation approaches, and their ability to provide better information regarding the evolution of microbial populations during processing and in finished products.

Summary of Staff Outputs

Research Output	Male	Female	Total Number
PhD Students	0	3	3

Summary of Academic Outputs

Research Outputs	Total Number	Details
PhD Theses	3	1. Thesis - Design, development and characterization of nanostructured electrochemical sensors. (2020) Juska, Vuslat B. 2. Thesis - Novel sequencing-based methods to characterise microbiomes, and in particular spore-forming bacteria, in the dairy processing chain. (2020) McHugh, Aoife J. 3. Thesis – Efficiency of novel thermal processes to reduce spore numbers within dairy manufacturing processes. (2019) Li, Fang

Publications in Peer Reviewed Scientific Journals	11	Teagasc – Paul Cotter (PI) 1. McHugh, A.J., Feehily, C., Hill, C., & Cotter, P.D. (2017). Detection and Enumeration of Spore-Forming Bacteria in Powdered Dairy Products. Front Microbiol, 8, 109. doi:10.3389/fmicb.2017.00109. 2. McHugh, A. J., Feehily, C., Tobin, J. T., Fenelon, M. A., Hill, C., & Cotter, P. D. (2018). Mesophilic Sporeformers Identified in Whey Powder by Using Shotgun Metagenomic Sequencing. Appl Environ Microbiol, 84(20). doi:10.1128/AEM.01305-18. 3. McHugh, A.J., Yap, M., Crispie, F., Feehily, C., Hill, C., Cotter, P.D. Microbiome-based environmental monitoring of a dairy processing facility highlights the challenges associated with low microbial-load samples (2021) npj Science of Food, 5 (1) DOI: 10.1038/s41538-021-00087-2. 4. McHugh, A.J., Feehily, C., Fenelon, M.A., Gleeson, D., Hill, C.,Cotter, P.D. Tracking the dairy microbiota from farm bulk tank to skimmed milk powder (2020). mSystems, 5 (2), DOI: 10.1128/mSystems.00226-20 Tyndall National Institute – Martyn Pemble (PI) 5. Buk V., *Pemble M. E., Twomey K., Fabrication and evaluation of a carbon quantum dot/gold nanoparticle nanohybrid material integrated onto planar micro gold electrodes for potential bioelectrochemical sensing applications, Electrochimica Acta 293 (2019) 307-317, https://doi.org/10.1016/j.electacta.2018.10.038. 6. Buk V., Pemble M. E., A highly sensitive glucose biosensorbased on a micro disk array electrode design modified withcarbon quantum dots and gold nanoparticles, Electrochimica Acta 298 (2019) 97-105, https://doi.org/10.1016/j.electacta.2018.12.068. 7. Juska V. B., Walcarius A., Pemble M. E., Cu NanodendriteFoams on Integrated Band Array Electrodes for the Non-Enzymatic Detection of Glucose, ACS Appl. Nano Mater.2019, 2, 5878-5889, DOI: 10.1021/acsanm.9b01325. 8. Juska V. B., Pemble M. E., A dual-enzyme, micro-band array biosensor based on the electrodeposition of carbonnanotubes embedded in chitosan and nanostructured Au-foams on microfabricated gold band electrodes, RSC, Analyst, DOI: 10.1039/c9an01664c. Teagasc – John Tobin (PI) 9. Li, F., Santillan-Urquiza, E., Cronin, U., O'Meara, E., McCarthy, W., Hogan, S.A., Wilkinson, M.G., Tobin, J.T. Assessment of the response of indigenous microflora and inoculated Bacillus licheniformis endospores in reconstituted skim milk to microwave and conventional heating systems by flow cytometry (2021). Journal of Dairy Science, 104 (9), pp. 9627-9644. DOI: 10.3168/jds.2020-19875 10. Li, F., Hunt, K., Buggy, A.K., Murphy, K.M., Ho, Q.T., O'Callaghan, T.F., Butler, F., Jordan, K., Tobin, J.T. The effects of sequential heat treatment on microbial reduction and

	spore inactivation during milk processing (2020). International Dairy Journal, 104, DOI: 10.1016/j.idairyj.2020.104648 11. Li, F., Hunt, K., Van Hoorde, K., Butler, F., Jordan, K., Tobin, J.T. Occurrence and identification of spore-forming bacteria in skim-milk powders (2019) International Dairy Journal, 97, pp. 176-184. DOI: 10.1016/j.idairyj.2019.05.004

Intellectual Property

N/A

Summary of other Project Outputs

Project Outputs	Details	Total Number
New Processes	The application of a microwave volumetric heating system for spore inactivation of dairy streams was developed.	1

Potential Impact related to Policy, Practice and Other Impacts

Impact	Details
Industry	Research linkages were established with a number of dairy processors including Arrabawn, Dairygold and Glanbia with a number of the publications in this project stemming from work that directly addressed industry issues/concerns.

Dissemination Activities

Activity	Details
Media Events	New sensor to make milk products safer and their production less wasteful (March 21, 2015) https://www.agriland.ie/farming-news/new-sensor-to-make-milk-productssafer-and-their-production-less-wasteful/
Seminars at which results were presented	• Tyndall Technology Days, 14th-15th October 2015, demonstrator (Buk V) • Annual Research Days, IADR (International Association for Dental Research),3rd-4th March 2016, Use of semiconductor processing technology for development of miniaturized devices for oral health monitoring Buk V., O`Mara P., O`Callaghan S., McKenna G., Twomey K. Poster presentation. • 8th Conference on Analytical Science Ireland (CASI), 14th-15th April 2016, A novel miniaturized biosensor for hydrogen peroxide sensing for use in agrifood applications Buk, V.; Kelly, P.; Pemble, M.; Twomey, K. Oral presentation. • Tyndall National Institute, Poster Competition, 29th July 2016, A New

	• Approach to the Sensing of Hydrogen Peroxide for Application in the Food Industry Buk, V.; Kelly, P.; Pemble, M.; Ogourtsov, V.; Twomey, K. Poster presentation • 68th Irish Universities Chemistry Research Colloquium, 23rd & 24th June2017, Novel miniaturized gold microelectrodes for electrochemical detection of hydrogen peroxide Buk, V.; Kelly, P.; Pemble, M.; Twomey, K. • 5th International Conference on Bio-Sensing Technology, 7-10 May 2017Development of carbon quantum dot-based nano-hybrid materials and their application as electrochemical biosensors Buk, V.; Pemble, M.; Twomey, K (Oral). • Aoife McHugh, 05/07/2017 Moorepark open day, DNA sequencing stand demonstrations. • Aoife McHugh Awarded FEMS YSMG award (reduced registration fee) to attend and present a poster at the 8th European Spore conference (16/04/18- • 19/04/18, Royal Holloway). Poster title "Utilisation of Shotgun Metagenomic Sequencing for the Investigation of Populations of Spore-formers in Dairy Powders" (Oral). • Aoife McHugh, 21/06/2018, Competed in ResearchFest final, 3 minute presentation to a general audience on the detection of spore forming bacteria in dairy, without slides, at the ResearchFest stage at Inspirefest in the Bord Gais energy theatre (Oral). • Aoife McHugh, 03/09/18-06/09/18, Awarded Microbiology Society TravelGrant (Competitively funded Microbiology Society Travel grant to 500 pounds sterling) to attend and present poster at FoodMicro, Berlin. Poster title "Utilisation of DNA Sequencing to Investigate the Dynamic Dairy Microbiota from Farm Bulk Tank Milk to Milk Powder" (Oral). • Aoife McHugh, 04/09/18, Awarded best poster abstract in topic A. Prize was a5 minute presentation on the main stage at FoodMicro 2018 on the poster, titled "Utilisation of DNA Sequencing to Investigate the Dynamic Dairy Microbiota from Farm Bulk Tank Milk to Milk Powder" (Oral).
Workshops at which results were presented	Workshops were held with industry stakeholders: • 24/09/2015 – Meeting with Arrabawn (in Moorepark) on spore reduction potential of bactofugation vs microfiltration – John Tobin, Brendan Kennedy, Fang Li • 05/05/2018 – Meeting with Dairygold on Bacterial Clarification (Clonmel Road) – John Tobin, Ian O'Loughlin, Gearoid Lane, George McLeod • 25/09/2018 – Meeting with Dairygold on Bacillus Licheniformis spores (Castlefarm) – John Tobin, Paul Cotter, Kieran O'Mahony, Deirdre Watkins • 15/07/2019 – Meeting with Glanbia (Belview) – John Tobin, Mark Fenelon, Noelle Brenna, Michael Cosgrove, Conor Feeney

Knowledge Transfer Activities

Identify knowledge outputs generated during this project.	The primary knowledge outputs from the SACCP projects revolve around the 9 peer reviewed publications which provide easy and instant access to the research outputs from the project. This repository of knowledge will be available to key stakeholders in the dairy sector both now and in the future.
Identify any knowledge transfer activities executed within the project.	Knowledge transfer activities include: 1. 11 Peer reviewed publications 2. 2 Presentations at conferences

	3. Meetings and collaborative initiatives with industry 4. 3 PhD theses 5. Publications in popular press
List any impacts resulting from the knowledge transferred during the project.	In general, the research outputs from this project provide greater insights into the survival of highly heat resistant microbial species in dairy process, addresses knowledge gaps surrounding existing and innovative heating technologies relative to thermal inactivation rate and also addresses state of the art identification and enumeration approaches based on molecular microbiology. The knowledge generated in this project directly impacts the dairy sector and ensures that Ireland is equipped to address food safety concerns in particular as they arise.

Section 3 - Leveraging, Future Strategies & Reference

Leveraging Metrics

Type of Funding Resource	Funding €	Summary
Other	€0.00	Currently no funds have been leveraged in this research area by the project coordinator.

Future Strategies

Currently there are no plans for securing funding by the project coordinator (John Tobin) in this research area. However, the microbial tracking approaches developed by Paul Cotter relative to the processing environment have been further exploited in both the EI funded DPTC project and also in the SFI funded VistaMilk program.

Project Publications

Teagasc – Paul Cotter (PI)

1. McHugh, A. J., Feehily, C., Hill, C., & Cotter, P. D. (2017). Detection and Enumeration of Spore-Forming Bacteria in Powdered Dairy Products. Front Microbiol, 8, 109. doi:10.3389/fmicb.2017.00109.
2. McHugh, A. J., Feehily, C., Tobin, J. T., Fenelon, M. A., Hill, C., & Cotter, P. D. (2018). Mesophilic Sporeformers Identified in Whey Powder by Using Shotgun Metagenomic Sequencing. Appl Environ Microbiol, 84(20). doi:10.1128/AEM.01305-18.
3. McHugh, A.J., Yap, M., Crispie, F., Feehily, C., Hill, C., Cotter, P.D. Microbiome-based environmental monitoring of a dairy processing facility highlights the challenges associated with low microbial-load samples (2021) npj Science of Food, 5 (1) DOI: 10.1038/s41538-021-00087-2.
4. McHugh, A.J., Feehily, C., Fenelon, M.A., Gleeson, D., Hill, C., Cotter, P.D. Tracking the dairy microbiota from farm bulk tank to skimmed milk powder (2020). mSystems, 5 (2), DOI: 10.1128/mSystems.00226-20.

Tyndall National Institute – Martyn Pemble (PI)

1. Buk V., Pemble M. E., A highly sensitive glucose biosensor based on a micro disk array electrode design modified with carbon quantum dots and gold nanoparticles, Electrochimica Acta 298 (2019) 97-105, https://doi.org/10.1016/j.electacta.2018.12.068
2. Buk V., *Pemble M. E., Twomey K., Fabrication and evaluation of a carbon quantum dot/goldnanoparticle nanohybrid material integrated onto planar micro gold electrodes for potential bioelectrochemical sensing applications, Electrochimica Acta 293 (2019) 307-317, https://doi.org/10.1016/j.electacta.2018.10.038

3. Juska V. B., Walcarius A., Pemble M. E., Cu Nanodendrite Foams on Integrated Band Array Electrodes forthe Non-Enzymatic Detection of Glucose, ACS Appl. Nano Mater. 2019, 2, 5878-5889, DOI:10.1021/acsanm.9b01325
4. Juska V. B., Pemble M. E., A dual-enzyme, micro-band array biosensor based on the electrodeposition of carbon nanotubes embedded in chitosan and nanostructured Au-foams on microfabricated gold band electrodes, RSC, Analyst, DOI: 10.1039/c9an01664c

Teagasc – John Tobin (PI)

1. Li, F., Hunt, K., Van Hoorde, K., Butler, F., Jordan, K., Tobin, J.T. Occurrence and identification of spore forming bacteria in skim-milk powders (2019) International Dairy Journal, 97, pp. 176-184. DOI: 10.1016/j.idairyj.2019.05.004
2. Li, F., Hunt, K., Buggy, A.K., Murphy, K.M., Ho, Q.T., O'Callaghan, T.F., Butler, F., Jordan, K., Tobin, J.T. The effects of sequential heat treatment on microbial reduction and spore inactivation during milk processing (2020). International Dairy Journal, 104, DOI: 10.1016/j.idairyj.2020.104648
3. Li, F., Santillan-Urquiza, E., Cronin, U., O'Meara, E., McCarthy, W., Hogan, S.A., Wilkinson, M.G., Tobin, J.T. Assessment of the response of indigenous microflora and inoculated Bacillus licheniformis endospores in reconstituted skim milk to microwave and conventional heating systems by flow cytometry (2021). Journal of Dairy Science, 104 (9), pp. 9627-9644. DOI: 10.3168/jds.2020-19875