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13F462 - Natural peptides to enhance food quality and safety.

Final Report

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

SUMMARY

The Overall Objective of PeptideProtectants project is the development of novel antimicrobial combinations to control food pathogens and spoilage organisms associated with the dairy foods, meat, bread and beer.

Some of the main results are the following: (1) New approaches for the quantification of bacteriocins (by ELISA and/or HPLC) in different materials; (2) In order to target food related pathogens, a range of effective concentrations of bacteriocins with other food grade antimicrobial/food preservatives have been established; (3) A food grade approach has been used to obtain a collection of novel nisin-variant producer strains, with very effective activity against spoilage and disease-causing bacteria, which can be used as starters. By using this food grade approach, the strains are not regarded as being genetically modified; (4) An affordable media using milk-based substrates for the production of bacteriocins at a semi-industrial scale has been developed; (5) Food trials with different bacteriocins and bacteriocin producing strains have been completed.

The utilization of novel bacteriocins and defensins, combinations with other antimicrobials or the direct application of the bacteriocin-producing strains in foods, are viable solutions to the development of natural food biopreservatives, by providing better activity against food spoilage and pathogenic bacteria. Through this research: (1) Food production companies can enhance the food safety and increase the shelf-life of the final products while protecting the health of the consumers; (2) Food preservatives suppliers will be able to offer other products (novel bacteriocins/defensins and bacteriocin-producing strains) to increase the safety of food products; (3) Policy makers can consider the use of other bacteriocins other than Nisin A and defensins in order to properly regulate their use in foods. The data generated within the project can contribute to this process.

KEYWORDS

Food preservation, Food security, Antimicrobials, Bacteriocins, Defensins, Upscaling.

ACRONYM	PeptideProtectants
PROJECT COORDINATOR, INSTITUTION	Prof Paul Cotter, Teagasc.
EMAIL	Click or tap here to enter text.
COLLABORATORS, INSTITUTION	Prof Colin Hill, Dr Des Field & Prof Elke Arendt, University College Cork. Prof Ursula Bond, Trinity College Dublin. Dr Mary Rea, Dr Sheila Morgan, Dr Andre Brodkorb, Dr Paula O'Connor, Prof Paul Ross, Teagasc.
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Section 1 - Research Approach & Results

Start Date

01 March 2014

End Date

31 December 2018

Research Programme

Food Institutional Research Measure

TRL Scale

TRL 3: Experimental Proof of Concept

NRPE Priority area

Sustainable Food Production and Processing

Total DAFM Award

€997,139.00

Total Project Expenditure

€908,596.61

Rationale for undertaking the Research

The options available to food processors wishing to employ natural food preservatives for safety applications or the prevention of food spoilage are limited. This is despite the fact that chemical preservatives have become increasingly unpopular with consumers. Furthermore, processing changes in response to the consumers' desire for minimally processed foods or foods with reduced salt or calorie levels can lead to microbial safety/spoilage issues. Paradoxically, the need to enhance safety remains a key market driver for new food antimicrobials. The CBG are Internationally regarded as leaders in the natural food antimicrobial field and, to address the deficiencies of Nisin, have developed variants, Nisin V and Nisin 29, that are active over a wider pH range, display enhanced antimicrobial activity against Gram positive pathogens and Gram-negative targets. Another novel bacteriocin Bactofencin LS1 exhibits significant antimicrobial activity against several microbial targets. Finally, the CBG own the technology associated with Lacticin 3147. This project also addressed food spoilage issues in bread and beer, both classically produced from yeast-based fermentations. The products of yeast-based fermentations can be contaminated with exogenous microbes, leading to food and beer spoilage which can amount to product lost and/or recall. For beers, the most likely contaminants are LABs while moulds, as well as by LABs, are common contaminants of breads. The use of defensins to prevent microbial contamination has broader applications in the food and beverage industries and could lead to prolonged shelf-life of food and beverages.

Methodology

The methodology followed has been the following: (a) Literature search and bioinformatic analysis of defensins from plant, insects, fungi and humans to identify a set of defensins with anti-fungal and/or antibacterial activity; (b) The generation of purified bacteriocins and defensins for investigation of their antifungal and antibacterial activity against common food pathogens and spoilage organisms; (c) Undertaking studies to identify the combinations of antimicrobials, both bacteriocins and defensins, which together possess greatest activity; (d) Insertion of the DNA encoding for the modified defensin or bacteriocin into Saccharomyces cerevisiae strains; (e)

The scaled-up production of CBG antimicrobials to (i) facilitate antimicrobial activity studies and (ii) lead to production of peptides on an industrial scale / pre commercial scale; (f) Food trials to optimise the application of CBG peptides and defensins (alone and in combination) and to demonstrate to industry the value of employing these peptides in food situations for the control of both food spoilage and pathogenic microorganisms.

Project Results

In this project a methodology for the purification and concentration of nisin and nisin derivatives from commercially produced products and from bacterial fermentates has been developed. Additionally, an ELISA method and a HPLC method have been successfully developed for quantification of nisin and bactofencin LS1. Based in this methodology, a range of effective concentrations of nisin peptide with other food grade antimicrobial/food preservatives have been established to target food related pathogens including L. monocytogenes and E. coli. Also, a collection of strains converted to nisin variant producers using food grade approaches were generated. Nisin variants that effectively inhibit organisms possessing nisin resistance proteins (including food-related and pathogenic microbes e.g. Enterococcus, Streptococcus, Staphylococcus) have been generated. An affordable media using milk-based substrates, mainly whey permeate, for the upscale production of bacteriocins has been developed. Also, bacteriocin-containing powders have been generated using different drying techniques and different carriers. These powders have been used to carry out several food trials with different bacteriocins in cottage cheese, a chicken juice model and fresh salmon against several common food-borne pathogenic bacteria. Each food system tested gave different results, but in general it can be concluded that bacteriocins and/or bacteriocin-producing strains can be used to increase the safety, food security as well as the economic value of food systems tested. Finally, the legal status of the antimicrobial peptides used in this project has been established and further steps towards commercialization have been made clear.

The activity of eleven recombinant lyophilised peptides was studied against common fungal contaminants of cereal based products, Fusariumculmorum, Aspergillusniger, Penicillium expansum and Penicillium roqueforti species. Eight defensins show clearly partial or total inhibition of fungal growth. Human Beta Defensin 3 (HBD3) appears to be the peptide with the strongest potency of inhibition. It's stability in salty solutions, and its thermal stability, makes it a good candidate for food applications. A modified S. pastorianus strain, expressing the defensin HBD-3, has been used efficiently to extend the shelf-life of bread. In addition, synthetic antimicrobial peptides can have a protective role towards fungal contamination of chilled dough or several beverages such as beer. However, for more specific applications in baking or for larger scale, the use of S. cerevisiae strains expressing a high amount of defensins is required. In this project, the effect of synthetic peptides and non-commonly used strains on the quality of the bread or beer was also studied. Microscopy and rheometer analysis revealed similar abilities to ferment baking dough between S. pastorianus and a control bakers' yeast, despite differences in structural homogeneity. The supplementation with defensin KT43C did not affect the activity of the yeast strains in fermentation of dough or brewing wort.

Section 2 - Research Outputs

Summary of Project Findings

In this project several improvements have been made in the bacteriocin field:
First of all, a methodology for the purification and concentration of nisin and nisin derivatives from commercially produced products and from bacterial fermentates has been developed. An ELISA method and a HPLC method have been successfully developed for quantification of nisin and bactofencin LS1. Based in this methodology, a range of effective concentrations of nisin peptide with other food grade antimicrobial/food preservatives have been established to target food related pathogens including L. monocytogenes and E. coli. Also, a collection of strains converted to nisin variant producers using food grade approaches were generated. Nisin variants that effectively inhibit organisms possessing nisin resistance proteins have been generated. An affordable and environment friendly media using milk-based substrates, mainly whey permeate, for the upscale production of bacteriocins has been developed. In general, it can be concluded that bacteriocins and/or bacteriocin-producing strains can be used to increase the safety, food security as well as the economic value of food systems tested. Finally, the legal status of the antimicrobial peptides used in this project has been established and further steps towards commercialization have been made clear.
Through this research:

- Food production companies can enhance the food safety and increase the shelf-life of the final products while protecting the health of the consumers.
- Food preservatives suppliers will be able to offer other products (novel bacteriocins and bacteriocin-producing strains) to increase the safety of food products.
- Policy makers can consider the use of other bacteriocins other than Nisin A in order to properly regulate their use in foods. The data generated within the project can contribute to this process.

Summary of Staff Outputs

Research Output	Male	Female	Total Number
Post Doctorates	1	2	3
PhD Students	2	0	2

Summary of Academic Outputs

Research Outputs	Total Number	Details
Publications in Peer Reviewed Scientific Journals	18	1. Field D, Daly K, O'Connor PM, Cotter PD, Hill C, Ross RP. 2015. Efficacies of nisin A and nisin V semi-purified preparations alone and in combination with plant essential oils for controlling Listeria monocytogenes. Appl. Environ Microbiol. 81:2762–2769. doi:10.1128/AEM.00070-15. 2. Egan, K., Field, D., Rea, M.C., Ross, R.P., Hill, C., and Cotter, P.D.(2016). Bacteriocins: Novel Solutions to Age Old Spore-Related Problems? Frontiers in Microbiology 7. Doi: 10.3389/fmicb.2016.00461. 3. Alicia Campion, Ruth Morrissey, Des Field, Paul D. Cotter, Colin Hill and R. Paul Ross. (2017) Use of enhanced nisin derivatives in combination with food-grade oils or citric acid to control

Cronobacter sakazakii and Escherichia coli O157:H7. Food Microbiology 65: 245-263.
4. Kraszewska, J., Beckett, M.C., James, T.C., Bond, U. (2016). Comparative Analysis of the Antimicrobial Activities of Plant Defensin-Like and Ultrashort Peptides against Food-Spoiling Bacteria. Appl Environ Microbiol. 82:4288-4298. doi: 10.1128/AEM.00558-16.
5. Thibaut Thery, James C. Tharappel, Joanna Kraszewska, Michael Beckett, Ursula Bond and Elke K. Arendt (2016). Antifungal activity of the Human β-defensin 3 and potential applications in cereal-based products. Innovative Food Science & Emerging Technologies, 38: 160-168. doi: 10.1016/j.ifset.2016.09.018
6. Gough, R., Gómez-Sala, B., Rea, M.C., Miao, S., Hill, C. andBrodkorb, A. (2017) A simple method for the purification of nisin. Probiotics and Antimicrobial Proteins, 9:363-369. doi: 10.1007/s12602-017-9287-5.
7. Smith, M., Draper, L., Hazelhoff, P.J., Cotter, PD., Ross, R.P., Hill, C.(2016) A bioengineered derivative, M21A, in combination with food grade Additives eradicates biofilms of Listeria monocytogenes. Front. Microbiol. 7: 1939 doi.org/10.3389/fmicb.2016.01939.
8. Kevin Egan, Paul Ross and Colin Hill (2017) Bacteriocins: Antibiotics in the age of the Microbiome. (2017). Emerging topics in Life Science, 1: 55-63.
9. Thibaut Thery, Elke K. Arendt (2018) Antifungal activity of synthetic cow pea defensin Cp-thionin II and its application in dough. Food Microbiology, 73:111-121.
10. Thibaut Thery, Yvonne O'Callaghan, Nora O'Brien, Elke K. Arendt. Optimisation of the antifungal potency of the amidated peptide HOrn-Orn-Trp-Trp-NH2 against food contaminants. International Journal of Food Microbiology, 265: 40-48.
11. Egan K, Kelleher P, Field D, Rea MC, Ross RP, Cotter PD, Hill C. (2017) Genome Sequence of Geobacillus stearothermophilus DSM 458, an Antimicrobial-Producing Thermophilic Bacterium, Isolated from a Sugar Beet Factory. Genome Announcements 2017, 5(43).
12. Field D, Baghou I, Rea M, Gardiner G, Ross R, Hill C (2017) Nisin in Combination with Cinnamaldehyde and EDTA to Control Growth of Escherichia coli Strains of Swine Origin. Antibiotics 2017, 6(4):35.
13. Suda S, Field D, Barron N(2017) Antimicrobial Peptide Production and Purification. Methods in molecular biology (Clifton, NJ) 2017, 1485:401-410.
14. Mathur H, Field D, Rea MC, Cotter PD, Hill C, Ross RP (2017) Bacteriocin-Antimicrobial Synergy: A Medical and Food Perspective. Frontiers in microbiology 2017, 8:1205.
15. Campion A, Morrissey R, Field D, Cotter PD, Hill C, Ross RP (2017) Use of enhanced nisin derivatives in combination with food-grade

		oils or citric acid to control Cronobacter sakazakii and Escherichia coli O157:H7. Food microbiology 2017, 65:254-263. 16. Aspri M, O'Connor PM, Field D, Cotter PD, Ross P, Hill C, Papademas P (2017) Application of bacteriocin-producing Enterococcus faecium isolated from donkey milk, in the bio-control of Listeria monocytogenes in fresh whey cheese. International Dairy Journal 2017, 73:1-9. 17. Mathur H, Field D, Rea MC, Cotter PD, Hill C, Ross RP (2018) Fighting biofilms with antibiotics and other groups of bacteriocins. npj Biofilms and Microbiomes 2018, 4(1):9. 18. Field D, Ross RP, Hill C (2018) Developing bacteriocins of lactic acid bacteria into next generation bio preservatives. Current Opinion in Food Science 2018, 20:1-6.DOI:10.1016/j.cofs.2018.02.004
PhD Theses	2	1. Kevin Egan (UCC Microbiology): Title: Discovery and evaluation of novel and characterised bacteriocins for future applications. Thesis presented in October 2018. 2. Thibaut Thery (UCC Nutrition): Title: Fundamental study of natural and synthetic antimicrobial peptides as novel preservatives against fungal food spoilage Thesis presented in May 2019.

Intellectual Property

Not applicable

Summary of other Project Outputs

Project Outputs	Details	Total No.
New Processes	New approaches for the quantification of bacteriocins (by ELISA and/or HPLC) in different materials. An ELISA method was developed during this project to quantify the Nisin and bactofencin concentrations. An HPLC method was developed for the quantification of Nisin	2
New Processes	A food grade approach has been developed to obtain a collection of novel nisin-variant producer strains, with very effective activity against spoilage and disease-causing bacteria, which can be used as starters. By using this food grade approach, the strains are not regarded as being genetically modified.	1
New Processes	An affordable media using milk-based substrates for the production of bacteriocins at a semi-industrial scale has been developed	1

Potential Impact related to Policy, Practice and Other Impacts

Impact	Details
Industry	Food production companies and all industries working in the field of food preservation can benefit from the results of this project as they can assure and improve the microbial quality and safety status of their food. Nutritional and functional ingredient manufacturer/suppliers could also benefit from this project as they will be able to offer other products such as bacteriocins and bacteriocin producing strains, to increase the safety of food products
Other	Policy makers: During this project, several dossiers have been prepared and sent to different entities: A dossier was submitted to the Environmental Protection Agency (EPA) with all nisin variant to confirm the non-GMM status of these strains. As a result of this consultation, EPA confirmed the non-GMM status of the of nine derivative strains of nisin A producing Lactococcus lactis strains. This information is very valuable as it confirms that the method for obtaining this nisin variants with enhanced antimicrobial activity against spoilage and food-borne microorganisms is safe and a first step to place this bacteriocins in the market. A dossier was submitted to the European Commission (EC) to get an opinion about the regulatory status of nisin V as a food additive. The objective of sending this dossier to the EC was for the EC to confirm nisin V complies with the specifications of nisin detailed in Commission Regulation 231/2012 and can thus be used in future as a food additive in the EU. The result of this consultation was that the variant Nisin V does not comply with the specifications of E234 nisin and the safety of the nisin variants need to be confirmed before placing them in the market. This information is very valuable as now we know which route we need to take to place the nisin variants into the market.
Environmental sustainability	Bacteriocin fermentation is influenced by many factors such as the strain, composition of the nutrient broth, pH, temperature and aeration. For several bacteriocins, including nisin A, nisin V, lacticin 3147 and bactofensin, production is growth associated and high cell concentration is required for high bacteriocin titres. For these reason, in order to obtain a high concentration of bacteriocin, the first step is to optimize the fermentation media used to grow the bacteriocin-producing strain. Commercial media, such as MRS and LM17, are described as the best media for bacteriocin production. However, such media are too expensive for an economical production process. In this context, whey permeate is a by-product of the cheese industry and may serve as an inexpensive medium for bacteriocin production. Furthermore, the high chemical oxygen demand (COD) of whey permeate makes its disposal a pollution problem. Therefore, the uses of whey permeate as fermentation feedstock has long been of industrial interest. In this project, a low cost media based on whey permeate for the production of high concentrations of bacteriocins has been developed.

Dissemination Activities

Activity	Details
Media Events	Project highlighted in GUT Reaction (Newsletter of the Alimentary Pharmabiotic Centre) Issue 7, January 2014. http://www.ucc.ie/research/apc/content/news_events/newsletters.html
Other	• Poster presentation in the congress "Exploring the Microbiome-Immune System Interface". Cork (Ireland). 1-2 September. • Gómez-Sala, B., P.M. O´Connor, A. Brodkorb, P.D. Cotter, C. Hill, R.P. Ross, M.C. Rea. 2016. Generation of polyclonal antibodies against nisin V from Lactococcus lactis NZ9800.

Knowledge Transfer Activities

Identify knowledge outputs generated during this project.	• New approaches were developed to facilitate the quantification of bacteriocins (by ELISA and/or HPLC) in different materials. • A food grade approach to obtain novel nisin-variant producer strains. The EPA confirmed the non-GMM status of 9 strains obtained using this approach. • An affordable and environmental friendly medium using milk-based substrates, mainly whey permeate, for the production of bacteriocins at a semi-industrial scale
Identify any knowledge transfer activities executed within the project.	During the lifetime of the project, a number of companies were approached in relation to the production of the bacteriocins and their application in food systems. This did not result in direction commercialisation but it is hoped that this can still happen in the future.
List any impacts resulting from the knowledge transferred during the project.	The knowledge generated in this project has helped to increase the knowhow in the bacteriocin field and will help in the future to approach new industries as well as to apply to new projects in this field.

Section 3 - Leveraging, Future Strategies & Reference

Leveraging Metrics

Type of Funding	Funding €	Summary
Additional Staff	€0.00	As reported in previous PPRs, the following additional funding were leveraged: • Teagasc provided funding for two years from Oct 2016 for a MSc student (Gwynn Halley). • The APC Microbiome Institute funded a student for 2 months during summer 2016 (Sarah O'Donnell). • A student from UCC Masters in Food Science Course joined the project in May 2016 for 6 months and prepared a thesis for submission to UCC in September 2016 (Scott Landers Cribben). Title of the thesis in partial fulfilment for the degree of Masters in Food Microbiology: Optimisation of growth medium for the production of bactofensin A by Lactobacillus salivarius DPC 6502.
EU R&I programmes	€3,400,000.00	Prof Cotter is coordinating a H2020 IA focusing on the microbiomes and the food chain. Applications include the use of the bacteriocins to enhance the safety of the food chain. Total funding = €10.9 mill. €3.4 secured by Irish RPOs.
Other		Funding from an international Food Company to investigate the possibility of converting their Nisin producing strains to produce a Nisin bioengineering peptide

Future Strategies

The research from the project will contribute to the future identification and application of bacteriocins and defensins (both natural and bioengineered). In the food industry as well as the broader food chain. This knowledge could also be applied to bacteriocin produced by probiotics and other gut microbes.

Project Publications

- Project highlighted in GUT Reaction (Newsletter of the Alimentary Pharmabiotic Centre) Issue 7, January 2014. http://www.ucc.ie/research/apc/content/news_events/newsletters.html

1. Field D, Daly K, O'Connor PM, Cotter PD, Hill C, Ross RP. 2015. Efficacies of nisin A and nisin V semi-purified preparations alone and in combination with plant essential oils for controlling Listeria monocytogenes. Appl Environ Microbiol 81:2762–2769. doi:10.1128/AEM.00070-15
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17. Mathur H, Field D, Rea MC, Cotter PD, Hill C, Ross RP (2018) Fighting biofilms with lantibiotics and other groups ofbacteriocins. npj Biofilms and Microbiomes 2018, 4(1):9.
18. Field D, Ross RP, Hill C (2018) Developing bacteriocins of lactic acid bacteria into next generation biopreservatives. Current Opinion in Food Science 2018, 20:1-6.DOI:10.1016/j.cofs.2018.02.004.

19. Kevin Egan won the CommBeBizBioeconomy photo competition for his entry “It’s what’s inside that counts”. http://apc.ucc.ie/whats-inside-counts/

Congress

1. Gómez-Sala, B., P.M. O´Connor, A. Brodkorb, P.D. Cotter, C. Hill, R.P. Ross, M.C. Rea. 2016. Generation of polyclonal antibodies against nisin V from Lactococcus lactis NZ9800. Exploring the Microbiome-Immune System Interface. Cork (Ireland). 1-2 September.