



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

15HDHL3 - Food fermentations for purpose: Health promotion and biopreservation Final Report

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SUMMARY

The LONGLIFE project involved Irish research partners (Teagasc and UCC) to develop innovative processes for transforming food substrates (milk, Teagasc and cereals, UCC) into fermentates, using novel strains of lactic acid bacteria (LAB) and yeasts to produce value added fermented liquids and powders, and long-fermented sourdough bread with improved health benefits, improved organoleptic qualities and extended shelf-life. The objective was to deliver new knowledge on the fate and function of food constituents and determinants of bio-accessibility, bioavailability and efficacy of food ingredients to advance fermented functional food design. The aims included identification and characterisation food grade cultures with exopolysaccharides (EPS), polyol, antimicrobial and antifungal producing properties. Bacterial strain screening was completed at both UCC and Teagasc. At Teagasc, yoghurt shelf-life studies were carried out with milk from cows fed on indoor diets, and outdoor grass-fed cows. Using previously isolated bacteriocin (antimicrobial peptide) producers chosen for application within dairy products; a nisin, lacticin 3147, pediocin, and enterocin producer were applied in fermentations with the goal of producing bacteriocin enriched supernatants, which could be concentrated to a powder form and added to pasteurised milk for extended shelf life. Another experiment applied a commercially available additive containing nisin, Nisaplin, to extend the shelf life of pasteurised milk. Nisaplin at a concentration of 5% was found to prevent the growth of milk spoilage organisms to below detectable levels at day 0, 7, and 13 of shelf life. Further experiments examined the effect of pure nisin A on the shelf life of pasteurised milk. Overall, bacteriocins may be used as an additive to extend the shelf life of pasteurised milk.

KEYWORDS

Dairy, Fermentation, Lactic Acid Bacteria

ACRONYM

LONGLIFE

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Section 1 - Research Approach & Results

Start Date

01 March 2016

End Date

31 December 2019

Research Programme

Food Institutional Research Measure

TRL Scale

TRL 6: Technology demonstrated in relevant environment

NRPE Priority area

Food for Health

Total DAFM Award

€667,000.00

Total Project Expenditure

€628,810.39

Rationale for undertaking the Research

LAB fermentation of milk enhances final product texture, sensory and health promoting qualities, due to the functional bioconversion of milk constituents. Additionally, lactose is metabolised during milk fermentation, thus mitigating a potentially problematic sugar in dairy for lactose intolerant consumers. Sourdough is a traditional technology where a mixture of flour and water is fermented with LAB and yeast to enhance flavour and leavening of bakery products. The microbes determine the sourdough characteristics via acid production, aroma and leavening properties (Hammes and Gaenzle, 1998). Besides effects on aroma, structure and shelf-life, sourdough may have important nutritional benefits (Poutanen et al., 2009). It has been shown to reduce starch digestibility (low GI), increase content of bioactive compounds (e.g., vitamins) and improve bioavailability of potentially beneficial bioactive compounds. The positive effects of sourdough are mainly due to the metabolic activities of LAB and acidity induced enzymatic changes in the cereal matrix. Selection of sourdough LAB based on functional properties may be considered as a tool to improve the technological, sensorial, and nutritional value of novel “personalised” food and beverage products.

Methodology

The methodologies across the project included bacterial strain isolation on selective media and culturing under aerobic and anaerobic conditions. Antibacterial isolates were identified by agar overlay antagonism assays (Sugrue et al. 2020) and antifungal/polyol producing isolates were determined using high performance liquid chromatography (HPLC). HPLC analysis was used for polyol identification using an Agilent 1260 high performance liquid chromatography system equipped with a refractive index detector (RID) and an ultraviolet-diode array detector (UV/DAD). The concentration of mannitol was determined over the RID (40 °C) by elution of the extract from a Rezex™ RPM-Monosaccharide Pb + column (300 × 7.8 mm, Phenomenex, USA) at 80 °C, equipped with a guard column (Carbo-Pb, 4 × 3.0 mm, Phenomenex, California, USA), using MiliQ-water at a flow rate of 0.6 mL/min and an injection volume of 20 µL. EPS producers were identified by phenotypic testing using a combination of loop-touch, ruthenium red, and sugar supplemented media. Strains were subject to whole genomic DNA extractions, 16s rRNA gene PCR and sanger sequencing (Genewiz, Leipzig, Germany) in addition to whole-genome shotgun sequencing with the Illumina MiSeq platform (Teagasc Sequencing Centre). Assembled genomes were aligned to reference genomes using Mauve, visualised using Artemis Genome Browser and subject to analysis using programs such as BAGEL4, antiSMASH (for prediction of antimicrobial encoding genes). Antimicrobial peptides were detected using matrix-assisted laser desorption ionization time of-flight mass spectrometry (MALDI-TOF MS) and purified from broth cultures using reverse-phase HPLC. Dairy prototype products were subject to an array of analyses including, microbiological plating, colour, texture, and compositional analysis in addition to head space solid phase micro extraction (HS-SPME) and gas chromatography-mass spectrometry (GCMS) for volatile compounds. For safety testing the lowest concentration completely inhibiting growth (MIC) value of 16 antibiotics was assessed using the VetMIC Lact-1 and Lact-2 MIC determination plates (National Veterinary Institute, Sweden).

Project Results

Teagasc:

- Artisanal yoghurts were produced using milks derived from a perennial ryegrass pasture (GRS) and total mixed ration diets (TMR) Pasture feeding increased the protein content of milk but led to a significantly lower lactose content compared with total mixed ration diets. A higher concentration of characteristic yoghurt flavour and aroma compounds were found in TMR yoghurt and increased off-flavour aldehyde compounds in GRS. These data illustrate a potentially negative effect of GRS derived milk on yoghurt flavour counterbalanced against an improvement in late-shelf-life texture and health.
- We investigated the effect of nisin addition on the quality of pasteurized whole milk. Nisaplin® and pure nisin A significantly reduced the total bacterial count in whole milk. These data demonstrate that nisin is effective at preventing bacterial spoilage of pasteurized milk, but only at relatively high concentrations.
- 799 isolates were recovered on LAB selective agar from a range of environmental sources. Five EPS producing strains capable of reliable growth in milk were used as adjunct cultures in small scale dairy fermentations. Each strain significantly improved the texture of the resulting yogurt. This work highlights the prevalence of EPS production among LAB and the advantage of using multiple screening methods for identification of EPS producing LAB.

- The demand for low calorie dairy products without sugar has created a need for alternative sweeteners. We isolated LAB from raw milk samples and screened them for their ability to produce mannitol. *Weissella paramesenteroides* LL677 emerged as the highest mannitol producing strain, generating 0.58 g/L mannitol. This study highlights the potential benefits of including a mannitol producing strain to reduce sugar content in dairy products.
- We examined the distribution of bacterial gene homologs of the antimicrobial peptide, actifensin. 29 new gene clusters were identified across the phylum Actinobacteria - separated into three distinct groups. Groups I and II share characteristic features of AfnA, while group III was found to be similar to eukaryotic inhibitor cysteine knot peptides. This data builds upon our previous knowledge of actifensin and show further links between actifensin and eukaryotic peptide structures. Using a combination of genome analysis and laboratory experimentation, we discovered shuffling of bacteriocin structural genes between *Streptococcus infantarius* and *Streptococcus gallolyticus* (gallocin D).

UCC:

We optimised wheat bran-based sourdough for the development of high-quality bakery products. Fermented wheat brans showed significantly higher contents of antifungal compounds compared to the unfermented wheat bran, thus indicating their ability to have antifungal activity. For the beverage prototypes, we fermented three different worts (barley, oat, wheat) with three different LAB strains after an enzyme treatment with amyloglucosidase and glucose-isomerase. *Leu. citreum* TR116 produced the highest concentration of 3phenyllactic acid (35 ppm) by the end of the fermentation. Considering the additional exopolysaccharides forming ability, *Leu. citreum* TR116 resulted to be the most promising sourdough-isolated LAB strain among the other bacteria studied in this work. In conclusion, enzymatic steps combined with mannitol-producing LAB represent a great novel technological strategy for future improvement in the field of sugar reduced beverages.

Section 2 - Research Outputs

Summary of Project Findings

The LONGLIFE project at the outset was conceptualised around novel strain and ingredient application to produce products with improved health and shelf-life properties. The data produced by the project is relevant to scientists both food and clinically based, the Agri-food sector, policy makers and to members of the public looking for alternatives. The outputs from LONGLIFE have demonstrated how the unique phenotypic traits of bacterial species and their application can be exploited for the benefit of the Irish Agri-Food sector and therefore add to the bank of ever-expanding scientific knowledge in the area of natural preservatives and health promoting bacteria to improve the food we eat. The methods and publications obtained from this work will be used to showcase the importance of exploiting untapped niches for the discovery of useful bacterial strains and molecules for the benefit of the Irish Agri-Food sector and will help in future funding submissions. Knowledge was gained with regard to the screening process in the optimization of future screens for LAB, and producers of EPS, polyols, and antimicrobials. Novel antimicrobial compounds will expand the research base of antimicrobials. Biobank has been created and characterized for antimicrobial, EPS, and polyol capability.

Summary of Staff Outputs

Research Output	Male	Female	Total Number
PhD Students	1	1	2

Summary of Academic Outputs

Research Outputs	Total Number	Details
Publications in Peer Reviewed Scientific Journals	7	<ol style="list-style-type: none"> Hill, D., Sugrue, I., Arendt, E., Hill, C., Stanton, C. and Ross, R.P., 2017. Recent advances in microbial fermentation for dairy and health. <i>F1000Research</i>, 6. Hill, D., Sugrue, I., Tobin, C., Hill, C., Stanton, C. and Ross, R.P., 2018. The <i>Lactobacillus casei</i> group: history and health related applications. <i>Frontiers in microbiology</i>, 9, p.2107. O'Callaghan, T.F., Sugrue, I., Hill, C., Ross, R.P. and Stanton, C., 2019. Nutritional aspects of raw milk: a beneficial or hazardous food choice. In <i>Raw milk</i> (pp. 127-148). Academic Press. Sugrue, I., Tobin, C., Ross, R.P., Stanton, C. and Hill, C., 2019. Foodborne pathogens and zoonotic diseases. In <i>Raw milk</i> (pp. 259-272). Academic Press. Sugrue, I., O'Connor, P.M., Hill, C., Stanton, C. and Ross, R.P., 2019. Actinomyces produces defensin-like bacteriocins (actifensins) with a highly degenerate structure and broad antimicrobial activity. <i>Journal of bacteriology</i>, 202(4), pp.e00529-19. Hill, D., O'Connor, P.M., Altermann, E., Day, L., Hill, C., Stanton, C. and Ross, R.P., 2020. Extensive bacteriocin gene shuffling in the <i>Streptococcus bovis</i>/<i>Streptococcus equinus</i> complex reveals gallocin D with activity against vancomycin resistant enterococci. <i>Scientific Reports</i>, 10(1), pp.1-11. Sahin, A.W., Coffey, A. and Zannini, E., 2021. Functionalisation of wheat and oat bran using single-strain fermentation and its impact on techno-functional and nutritional properties of biscuits. <i>European Food Research and Technology</i>, pp.1-13.

PhD Theses	2	<ol style="list-style-type: none"> 1. Ivan Sugrue, Thesis title 'An investigation into novel bacteriocin producers isolated for shelf-life extension of dairy products' 2. Daragh Hill, Thesis title 'Identification and characterisation of lactic acid bacterial secondary metabolites with functional and health benefits'
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Intellectual Property

Not applicable.

Summary of other Project Outputs

Project Outputs	Details
New Products	<ol style="list-style-type: none"> 1. Biobank Characterised for antimicrobial, EPS, and polyol capability at Teagasc. Total no: 799 2. Promising bacteria for the dairy industry Promising strains were identified to species level and GRAS/QPS strains were chosen for future applications. Total no: 4 3. Bacteriocin producer Novel bacteriocin identified, actifensin, with potential applications in the field of health. Total no: 1

Potential Impact related to Policy, Practice and Other Impacts

Impact	Details
Industry	Isolates generated within the project may be relevant to industry, for application in food fermentations.

Dissemination Activities

Activity	Details
Seminars at which results were presented	<ol style="list-style-type: none"> 1. Poster: Daragh Hill, Elke Arendt, Catherine Stanton, and R. Paul Ross, Techno-functional screen for exopolysaccharide producing lactic acid bacteria, Host-Bacteria Interaction Symposium 2018, Reading UK. 2. Poster: Ivan Sugrue, R. Paul Ross, Colin Hill, Catherine Stanton Bacteriocin production from isolates of milk, milk products and sheep faeces UCC, Microbiology Postgraduate Poster day, 5th December 2017 3. Poster: Ivan Sugrue, Paula M. O'Connor, Catherine Stanton, Colin Hill, R. Paul Ross, From zone to genome: Identification of a novel group of bacteriocins within the genus Actinomyces, Host-Bacteria Interaction Symposium 2018, Reading UK 4. Poster: Ivan Sugrue, Paula M. O'Connor, Catherine Stanton, Colin Hill, R. Paul Ross, From zone to genome: Identification of a novel group of bacteriocins within the genus Actinomyces, Microbiology Department Poster Day, UCC, Cork, 2018 5. Poster: "Defensin-like bacteriocins, a previously undiscovered group of class II bacteriocins" ISAPP-SFA (International Scientific Association for Probiotics and Prebiotics – Students and Fellows Association) Annual Meeting, Antwerp, Belgium, I Sugrue May 14, 2019 6. Oral presentation: Catherine Stanton, Lactic Acid bacteria in cheese for heart health, Microbiology Society Annual Meeting, March 2016 7. Oral presentation: Catherine Stanton, Microbial metabolite production by gut microbes and implications for host health, Chinese Lactic Acid Bacteria Conference, May 2016 8. Oral presentation: Catherine Stanton, EPS formation and potential health effects The Netherlands, May, 2016 9. Catherine Stanton, Chinese LAB Annual Conference, Invitation by Prof. Wei Chen, Jiangnan University, Wuxi, PR China., Jun-16

Knowledge Transfer Activities

Identify knowledge outputs generated during this project.

From task 1 the biobank of food grade lactic acid bacteria with GRAS/QPS status generated within the project may be relevant to industry, for application in food fermentations. Knowledge was gained with regard to the screening process in the optimisation of future screens for LAB, and producers of EPS, polyols, and antimicrobials.

The results outlined within task 2 will benefit the Irish Agri-food sector greatly. Promising strains were identified to species level and selected strains were chosen for applications in development of fermented dairy and cereal products. These cultures may be relevant to industry, for application in food and beverage fermentations in particular we have shown that both increased shelf-life and consumer demand for low-calorie and safe dairy products can be met through the utilization of GRAS bacterial strains as adjuncts thus increasing the valorisation of such products.

Within task 3, we have identified and characterised two unique antimicrobial peptides which add to the arsenal of viable alternatives to traditional antibiotics. The knowledge gleaned here will be relevant to scientists both food and clinically based, the Agri-food sector, policy makers and to members of the public looking for alternatives

Identify any knowledge transfer activities executed within the project.

Key knowledge outputs were passed onto potential next or end users during the project in a number of ways. Oral and poster presentations showcasing the findings of the project were made at several scientific conferences listed in section 2. The publications that arose from the project also served to disseminate the findings of the project. The JPI HDL JFA "Food processing for Health" website provides information to the public on Longlife including a final project report (<https://www.healthydietforhealthylife.eu/index.php/joint-actions/foodprocessing>). APC Microbiome Ireland posted about Longlife and a photo of the consortium at UCC brewery in their online news (<https://apc.ucc.ie/recent-funding-successes/>). Teagasc published a technology update for the public describing the outputs of Longlife available at https://www.teagasc.ie/media/website/publications/2020/6828_LONGLIFE_CatherineStanton_Food.pdf

List any impacts resulting from the knowledge transferred during the project.

A jointly funded Teagasc/AgRes NZ jointly funded Walsh Fellowship was secured. At AgRes, New Zealand, the post graduate student undertook application of exo-polysaccharide producing strains in yoghurts to determine their effect on texture. Product prototype development studies continued with the application of antimicrobial, polyol, and EPS producing strains in dairy fermentations, with the aim of producing products with extended shelf life, improved health qualities, and improved texture, respectively. The inclusion of a polyol producing strain aims to investigate if in situ production of mannitol has potential to produce a low calorific, sweetened fermented milk product for the dairy industry. Furthermore, both PhD students on the project have joined the community of Irish scientists in the area food science as post-doctoral researchers.

Section 3 – Leveraging, Future Strategies & Reference

Leveraging Metrics

Type of Funding Resource	Funding €	Summary
Additional Staff	€84,000.00	A Teagasc/AgRes NZ jointly funded Walsh Fellowship was secured entitled 'YOGHURTDRENKS ENRICHED IN MILK FERMENTATION PEPTIDES FOR GASTROINTESTINALHEALTH' (2017-2020).

Future Strategies

Future strategies include greater characterization of the novel antimicrobial compound produced by *Actinomyces ruminicola* which was identified. This novel antimicrobial compound could have use in the field of health and will expand the research base of antimicrobials. A selection of LAB starter culture with unique techno-functional properties able to deliver food and beverage products with improved nutritional properties in cereal and dairy are available for application at commercial scale as a result of this project.

Project Publications

- Hill, D., Sugrue, I., Arendt, E., Hill, C., Stanton, C. and Ross, R.P., 2017. Recent advances in microbial fermentation for dairy and health. *F1000Research*, 6.
- Hill, D., Sugrue, I., Tobin, C., Hill, C., Stanton, C. and Ross, R.P., 2018. The *Lactobacillus casei* group: history and health related applications. *Frontiers in microbiology*, 9, p.2107.
- O'Callaghan, T.F., Sugrue, I., Hill, C., Ross, R.P. and Stanton, C., 2019. Nutritional aspects of raw milk: a beneficial or hazardous food choice. In *Raw milk* (pp. 127-148). Academic Press.
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- Sahin, A.W., Coffey, A. and Zannini, E., 2021. Functionalisation of wheat and oat bran using single-strain fermentation and its impact on techno-functional and nutritional properties of biscuits. *European Food Research and Technology*, pp.1-13.