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

# 15F731 - Isolation, characterisation and exploitation of natural anti-yeast agents and their application as consumer-friendly preservatives in food and beverages

## Final Report

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

## SUMMARY

This project ran from 2017 until 2021 and had significant scientific output. It originally set out to characterise both lactic acid bacteria and defensins/plant extracts for anti-yeast activity. The work was performed at both MTU with UCC partnering. A panel of typical spoilage yeasts from the food, feed and beverage sectors were chosen for testing including Kluyveromyces lactis, Zygosaccharomyces bailii, Zygosaccharomyces rouxii, Saccharomyces spp. and Debaryomyces hansenii. Typical food products spoiled by yeast include salad dressings (Debaryomyces), soft drinks (Zygosaccharomyces), dairy products (Kluyveromyces), and alcoholic beverages (Saccharomyces). In order to prevent the growth of such spoilage yeast, several methods of preservation have been adopted by food industries, ranging from chemical to physical preservation techniques. However, spoilage by yeast still occurs, hence this investigation. Briefly, the four technical objectives in the project were (a) to characterise bacterial or plant extracts with anti-yeast activity, (b) to identify, isolate and characterise the molecules involved (c) to apply them in food and beverage systems and (d) to synthesise the responsible molecules artificially and perform toxicity assays. While lactic acid bacteria did not show significant anti-yeast activity, a number of plant extracts showed considerable success. These included extracts of barley endosperm, potato tuber, radish seeds and white mustard seeds. Though other plant materials were also tested to perfect methodologies, the food-grade status of barley endosperm, potato tuber, radish seeds and white mustard seeds were considered an advantage. Synthetic versions of all peptides were made and used in various detailed testing, which included evaluating stability of the peptides to proteolytic digestion, heat degradation, susceptibility to pH variation and different salts concentrations and detailed data is shown in the published papers. Potential for cytotoxicity against mammalian cells was also evaluated (as described in the various publications) and no cytotoxic capacity was identified.

## KEYWORDS

Antiyeast, bio-preservation, antimicrobial peptides.

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| **ACRONYM** | ANTIYEAST |
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| **PUBLICATION DATE** | July 2022 |

# Section 1 - Research Approach & Results

**Start Date**

01 March 2017

**End Date**

31 December 2021

**Research Programme**

Food Institutional Research Measure

**TRL Scale**

TRL 6: Technology demonstrated in relevant environment

**NRPE Priority area**

Processing technologies and novel materials

**Total DAFM Award**

€421,200.00

**Total Project Expenditure**

€398,512.87

**Rationale for undertaking the Research**

Yeasts are capable of growing in a wide range of foods when conditions are favourable. Under such conditions the growth and metabolic activity of these agents in foods and beverages often results in extensive economic losses to the industry. Unlike moulds which tend to grow on surfaces, yeasts tend to grow within the food and drink matrices where they ferment the sugars under anaerobic conditions causing off-flavours and spoilage.

The majority of the work in the area of antimicrobial agents has been focused on bacteriocins, which are inhibitors of Gram-positive bacteria and a small number of these bacteriocins have reached commercialisation. Yeasts are not sensitive to the antimicrobial peptides like nisin (only one currently food grade) or MB-21 (developed by Unilever Home & Personal Care program).

There are very few research groups in the world concentrating on LAB with antiyeast activity. The majority of the work carried out in the area of antifungal (mould) area has been performed by this group. The research on plant-derived antimicrobial peptides and small proteins up to now focused on fundamental investigations on their mode of action. Moreover, the applications only concerned the medical field focussing on human yeast pathogens, like C. albicans, or the protection of plant crops via biotechnological applications. To our knowledge, the only research of applications of plant-AMPs in food products is limited to the activity against bacteria or filamentous fungi. The anti-yeast topic is calling for investigation in the food context.

**Methodology**

The four technical objectives in the project were (a) to characterise bacterial or plant extracts with anti-yeast activity, (b) to identify, isolate and characterise the molecules involved (c) to apply them in food and beverage systems and (d) to synthesise the responsible molecules artificially and perform toxicity assays. Methodology employed for the (a) objective mainly included antiyeast assays; some of the tests such as colony count assay, disc diffusion and soft-agar overlay were performed on agar plates, while the Minimum Inhibitory

Concentration values were calculated with broth microdilution methods. Antiyeast molecules were purified (b) from plant materials using classical extraction methods which include several steps as: cold buffer lysis, ammonium sulphate precipitation, heat denaturation, dialysis, and ion-exchange chromatography. In addition, antiyeast molecules were characterised using SDS-PAGE techniques, circular dichroism and a various free available bioinformatics tools (e.g., Robetta). Antiyeast molecules were tested for their potential as food and beverages bio-preservative (c) employing antiyeast assays as described for point (a) performed in foods materials and under conditions typical of the manufactory environment (pH variation, heat treatment and high salt concentrations). Finally, antiyeast molecules identified from natural material were chemically synthetised (d) and tested if safe for consumption; in this case the methodology employed include cytotoxicity assay on human gut cells and haemolytic test on mammalian red blood cells

**Project Results**

The discovery of novel antimicrobial molecules would benefit considerably different biotechnological fields, including medicine, agriculture and the food industry, on which this project focused. Specifically, studies were performed on LAB and plant-derived antimicrobial molecules for the reduction of food spoilage caused by yeast microorganisms. While lactic acid bacteria did not show significant anti-yeast activity, a number of plant extracts showed considerable success. In particular, extracts of barley endosperm, potato tuber, radish seeds and white mustard seeds showed antiyeast potential; in point of fact, several antiyeast small proteins and peptides were identified and isolated. Among the identified antiyeast molecules, a small protein from white mustard seeds called Sin a 1 possessed several suitable properties for a green food preservative agent. It is present in a relatively high abundance in seeds (0.82-2.94 mg/g). Its purification required only one step of chromatographic separation; it displayed potent in vitro activity against spoilage yeast strains, and it successfully inhibited Z. bailii growth in several beverage matrices including fruit juices (cranberry and apple), soft drinks (Fanta Orange) and wine. Like most plant defence-related proteins, Sin a 1 mode of action involved yeast membrane permeabilisation as it caused leakage of cytoplasmatic components. Moreover, the protein did not show cytotoxicity towards mammalian cells and displayed resistance to heat denaturation and insensitivity to pH variations and the presence of salts.

Subsequently, synthetic version of six antiyeast plant-endogenous peptides were made (Rs-AFP1 and Rs-AFP2 from radish, D-lp1 from barley, SN-1 from potatoes and Pn-AMP1 and Pn-AMP2 from Morning glory). In addition, two ultra-short peptides SinA-pepI and SinApepIII derived from fragments of the protein Sin a 1 isolated from mustard seeds were also chemically synthetised along with four rationally designed peptides (labelled as: KK-14 Dip KK-14 dKK-14

KK-14 (R10)) with sequences derived from a previously demonstrated antifungal peptide. All the synthetic peptides were used in various detailed testing, which included evaluating stability of the peptides to proteolytic digestion, heat degradation, susceptibility to pH variation and different salts concentrations. Potential for cytotoxicity against mammalian cells was also evaluated and no cytotoxic capacity was identified. The results indicated the peptides' suitability for testing as potential preservatives peptide in different beverages and food systems.

This project explored the possible use of plant-derived antimicrobial proteins as antiyeast agents to be incorporated in food and beverages to overcome the phenomenon of food loss and waste. Preservatives have been incorporated into foods for centuries; however, modern preservation techniques mainly consist of temperature control, vacuum packaging, water content reduction, acidification and chemical preservation. Some yeast species are known to survive the acidification methods, and the use of chemicals has become unappealing for many consumers, creating a demand for more naturally occurring forms of food preservatives that are generally considered healthier and safer. Throughout the following decades, food preservation will change and evolve, antimicrobial agents will continue to be needed, and plant antimicrobial proteins (either in their native form or chemically synthesised) could make their way into the market as plant-based alternatives.

# Section 2 – Research Outputs

**Summary of Project Findings**

The research conducted during this project represents a foundation for the future development of plant-derived small proteins and peptides as novel classes of bio-preservatives. Protocols described in the various publications (listed below) could be employed to discover and characterise other candidates with biotechnological potential. Moreover, the preliminary analysis carried out during the project highlights how the food industry could benefit from the applications of these types of molecules as alternatives to the current methods of preservation. Specifically, data presented throughout this project discussed the potential of antimicrobial plant proteins/peptides as conservative agents, whereas natural or synthetic. Many of the antiyeast proteins described had potent in vitro activity via membrane permeabilisation, low cytotoxic potential and high stability; thus, they are promising candidates for future industrial applications. The use of natural antimicrobials for food preservation, either as an ingredient or combined with traditional techniques, is not a new concept; spices and herbs have been used since ancient times to improve foods sensory characteristics and shelf life. However, there is a lack of knowledge on the large-scale application of these types of compounds for food preservation. Research involving plant antimicrobial peptides mainly had focused on the inhibition of food-borne pathogens and only a tiny number of bacteriocins (antimicrobial peptides from bacteria) have been regulated for modern bio-preservation techniques (e.g., the nisin peptide isolated from the bacterium

Lactococcus lactis). Although data on the applicability of antimicrobial plant peptides in the food industry are scarce to non-existent, the work discussed in this project sustains their potential as potential novel preservative agents; nevertheless, the work is here presented with a future prospective. More future research will be needed before plant antimicrobial peptides can be awarded the GRAS (generally recognised as safe) status and be considered as antimicrobial additives by the food industry.

**Summary of Staff Outputs**

| Research Output | Male | Female | Total Number |
|---|---|---|---|
| PhD Students | 0 | 2 | 2 |

**Summary of Academic Outputs**

| Research Outputs | Total Number | Details |
|---|---|---|
| Publications in Peer Reviewed Scientific Journals | 7 | These details have been provided in Section 3 of the report under 'Project Publications'. |
| Peer Reviewed Conference Papers | | 1. Mignone, G., Thery, T., Arendt, E.K., Coffey, A. (2018). Activity of synthetic peptide Pn-AMP1 against yeasts responsible of food spoilage. Proceedings of the 47th Annual Food Science and Technology Conference. Cork, Ireland. Dec 6-7, 2018 (Poster).<br>2. Shwaiki, L.N., Arendt, E.K., Lynch, K.M., Thery, T.L.C. (2018) Inhibitory Effect of Four Novel Synthetic Peptides on Food Spoilage Yeasts. Proceedings of 35th European Peptide Symposium. Dublin, Ireland. August 26-31, 2018 (Poster).<br>3. Shwaiki, L.N., Arendt, E.K., Lynch, K.M., Thery, T.L.C. (2018) Inhibitory Effect of Four Novel Synthetic Peptides on Food Spoilage Yeasts. Proceedings of 8th International Meeting on Antimicrobial Peptides. Edinburgh, UK. September 25, 2018 (Poster).<br>4. Shwaiki, L.N., Arendt, E.K., Lynch, K.M., Thery, T.L.C. (2018) Inhibitory Effect of Four Novel Synthetic Peptides on Food Spoilage Yeasts. Proceedings of the 47th Annual Food Science and Technology Conference. Cork, Ireland. Dec 6-7, 2018 (Oral Presentation).<br>5. Shwaiki, L.N., Lynch, K.M., Arendt, E.K.. (2019). Inhibitory effect of four novel synthetic peptides on food spoilage yeasts. FSAI Science Conference, Convention Centre Dublin, Ireland, August 2019 (Poster)<br>6. Mignone, G., Arendt, E.K., Coffey, A. Inhibitory activity of two synthetic plant-based peptides against food and beverage spoilage yeasts. Proceedings of 48th Annual Food Science and Technology Conference - 16th December 2019, Limerick, Ireland (Poster)<br>7. Mignone G., Shwaiki L., Arendt, E.K., Coffey, A. Antifungal and antiyeast activity of an extract obtained from white mustard seeds and its potential use as preservative in |

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| | | food industry. Proceedings of the 49th Annual Food Science and Technology Conference - 15th December 2020, Online (Poster) |
| PhD Theses | 2 | 1. Laila Shwaiki - A Study on the Application of Synthetic Antimicrobial Peptides Derived from Plants for the Reduction of Yeast Spoilage in Food. Submitted to UCC in February 2021.<br>2. Giulia Mignone - A study on plant-derived small proteins and peptides with antiyeast activity and preliminary investigations in food materials. Submitted to MTU in February 2022 |
| Manuscripts under review: | 3 | 1. Mignone, G. Shwaiki, L.N. & Coffey, A. "Inhibitory activity of two synthetic Pharabitis nil L. antimicrobial peptides against common spoilage yeasts" submitted to the journal "Applied food Research" ISSN: 2772-5022<br>2. Mignone, G. & Coffey, A. "Antiyeast activity by ultra-short synthetic peptides bioinspired to the mustard Napin protein Sin a 1" submitted to the journal "BBA – General Subjects" ISSN: 0304-4165<br>3. Mignone, G. & Coffey, A. "Design and production of a recombinant Sin a 1 derived protein in Escherichia coli and evaluation of its antiyeast activity" submitted to the journal "BBA – General Subjects" ISSN: 0304-4165 |

**Summary of other Project Outputs**

| Project Outputs | Details | Total No. |
|---|---|---|
| New Industry Collaborations Developed | Over the course of the project, informal discussions were had by the UCC partner with ABMauri (NL), ABInbev (Belgium) and Kerry group. | 3 |

**Potential Impact related to Policy, Practice and Other Impacts**

| Impact | Details |
|---|---|
| Environmental Sustainability | In general, the main scientific results obtained in this project adhered to the policies outlined in the project proposal (Thinking Green, - SHARP report, - Food Wise 2025 report, - JPI (Joint Programming Initiative - A HEALTHY DIET FOR A HEALTHY LIFE), and - Action Plan for Jobs/DFRI/NRPE/ SSAPRI/Strategy for Science, Technology, and Innovation). Several plant-derived compounds were evaluated and characterised for the biotechnological application as foods and beverages preservatives, the preliminary analysis conducted throughout the project highlighted as these molecules could be employed in an industrial setting as a greener alternative to the current chemical form of preservation for the reduction of food waste and loss caused by yeast microorganisms. |

**Dissemination Activities**

| Activity | Details |
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| Workshops at which results were presented | 1) The outcomes of the Antiyeast project were presented by the two partners in the project at the Nutri Event held at Cork Institute of Technology on Friday Dec 6th, 2019 in the council room and main reception area of the Admin Building. Invited to the event were Aisling Aherne, Kerry Group; Angela Buckley, Marigot Ltd.; Annalisa Segat, Kerry PLC; Bríd O'Riordan, Carbery Group; Brian Healy, Teagasc; Cal Flynn, Kerry Group; Catherine Stanton, Teagasc; Ciara Monks, Eurofins; Claire Walsh, Danone; Damian O'Kelly, Nutritics; Denis Kiely, Industrial Management Systems - Food Safety Consultancy; Denise Kingston, Glenilen Farm; Emma Gill, Pernod-Ricard; Emma Walsh, Pepsico; Eoin Murphy, Teagasc; Frankie Daly, Arrabawn Co-op; Helen Slattery, Teagasc; Heather Pratt, Nutribio; Jean Tierney, Kerry Group; Jia Hou, Nestle; Jonathan O'Regan, Nestle; Julia Scanlon, Atlantia; James Sugrue, Lee Strand Co-op; Kate Murphy, Southern Scientific; Kieran Kilcawley, Teagasc; Kevin Turner, Carbery Group; Kyle Crowley, Protein Works; Linda Giblin, Teagasc; Lydia Slattery, Eurofins; Mary Kelleher, Cloverhill Food Ingredients; Mairead Gilmore, ALS Life Sciences Ltd; Mary Naughton, Silver Pail Dairy; Michael O'Dwyer, Arrabawn; Nora O'Shea, Teagasc; Norann Galvin, Teagasc; Olivia McAuliffe, Teagasc; Olwen Maher, ALS Life Sciences Ltd; Orla O'Sullivan, Teagasc; Patrick Halley, C&C Group; Patricia Leamy, C&C Group; Paul Cotter, Teagasc; Rita Hickey, Teagasc; Sinead Fitzsimons, Dairygold; Tim Guinee, Teagasc; Ursula Leonard, Atlantia Food Trials; Yvonne Fitzpatrick, Pepsico. The event was opened by Senator Gerry Buttimer. |

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| | 2) Due to the COVID pandemic restrictions, an online event was organised in conjunction with the APC at UCC on Tuesday 1st June 2021 entitled: “Application of Synthetic Antimicrobial Peptides Derived from Plants for the Reduction of Yeast Spoilage in Food”.<br>Chair - Silvia Melgar<br>Audience: 60 people attending. |
| Media Events | A project website was set up in 2021: https://www.cit.ie/anti-yeast |

## Section 3 – Leveraging, Future Strategies & Reference

**Future Strategies**

The research conducted during this project represents a foundation for the future development of plant-derived small proteins and peptides as novel classes of bio-preservatives. Protocols described in various publications could be employed to discover and characterise other candidates with biotechnological potential. The approval of plant-derived antimicrobial peptides/proteins as bio-preservative by regulatory agencies such as the FDA (U.S. Food and Drug Administration) and EFSA (European Food Safety Authority) will rely on sufficient data on their cytotoxicity and behaviour in the gastrointestinal environment; thus, future objectives mainly regard the detailed exploration of the safety and toxicity of these plant-derived compounds. In general, the evaluation of a food additive involves the examination of the chemical structure and characteristics of the additive, including its specifications, impurities and the potential breakdown products in its intended use. Toxicological data from in vivo analysis and animal testing must be examined to identify and characterise possible health hazards; in addition, tests must be carried out using higher doses of the additive than would be used in food for humans and the data are then used to determine safe levels of intake for consumers.

**Project Publications**

Papers published:


1. Shwaiki, L. N. Arendt, E. K. and Lynch, K. M. and Thery, T.L.C. (2019) ‘Inhibitory effect of four novel synthetic peptides on food spoilage yeasts’, International Journal of Food Microbiology. Elsevier, 300, pp. 43–52. doi: 10.1016/J.IJFOODMICRO.2019.04.005.
2. Shwaiki, L. N., Arendt, E. K. and Lynch, K. M. (2020) ‘Anti-yeast activity and characterisation of synthetic radish peptides Rs-AFP1 and RsAFP2 against food spoilage yeast’, Food Control, 113, p. 107178. doi: 10.1016/j.foodcont.2020.107178.
3. Shwaiki, L. N., Arendt, E. K. and Lynch, K. M. (2020) ‘Study on the characterisation and application of synthetic peptide Snakin-1 derived from potato tubers – Action against food spoilage yeast’, Food Control. Elsevier Ltd, 118. doi: 10.1016/j.foodcont.2020.107362.
4. Shwaiki, L. N., Sahin, A. W. and Arendt, E. K. (2020) ‘Study on the Inhibitory Activity of a Synthetic Defensin Derived from Barley Endosperm against Common Food Spoilage Yeast’, Molecules. MDPI AG, 26(1), p. 165. doi: 10.3390/molecules26010165.
5. Shwaiki, L. N., Arendt, E. K. and Lynch, K. M. (2021) ‘Plant compounds for the potential reduction of food waste–a focus on antimicrobial peptides’, Critical Reviews in Food Science and Nutrition. Bellwether Publishing, Ltd. doi: 10.1080/10408398.2021.1873733.
6. Shwaiki, L. N., Lynch, K. M. and Arendt, E. K. (2021) ‘Future of antimicrobial peptides derived from plants in food application – a focus on synthetic peptides. Trends in Food Science and Technology. Elsevier. doi: 10.1016/j.tifs.2021.04.010.
7. Mignone, G. Shwaiki, L.N. Arendt, E.K. & Coffey, A. (2022). Isolation and characterisation of the antifungal activity of the mustard Napin protein Allergen Sin a 1. Biochemistry and Biophysics Reports (Elsevier). In Press. Reference BBREP-D-21-00478R1