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

Food Institutional Research Measure

Final Report

The use of Marine derived antibacterial agents to combat the prevalence of *Salmonella* in pork *products*.

DAFM Project Reference No 11/F/009

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End Date: 30/04/2018

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Basic/Fundamental → Applied → Pre Commercial

1	2	3	4*	5	6	7

Priority Area (s)	**SUPPLY CHAIN SAFETY & INTEGRITY**

Key words: *(max 4) Salmonella, Pseudovibrio, Marine antimicrobials,*

1. Rationale for Undertaking the Research

Ireland has a large number of commercial pig production unit's pork consumption worldwide growing steadily on an annual basis. Thus, there is an ongoing requirement to maintain and indeed improve the quality and safety of the meat. It is widely recognized that pathogens such as *Salmonella* can be transmitted through the food chain and can be a major source of human illness, with infections due to *Salmonella* being a major cause of food-borne gastroenteritis. Serovar Typhimurium and serovar Enteritidis are the serotypes most commonly associated with cases of food-borne salmonellosis in humans.

Consumption of pork products containing salmonellae is one of the leading causes of food-borne illness each year, with serovar Typhimurium remaining the serovar most commonly isolated from pigs in Ireland and elsewhere. A variety of different strategies have previously been investigated to control *Salmonella* in the pig industry. These have included the addition of organic acid to pig feed or water and the administration of either probiotics or prebiotics to the pigs. While the addition of probiotics and acids to the feed have been shown to have a positive effect on decreasing the fecal excretion of Salmonella, these effects are restricted to specific age groups. In addition, while the use of vaccines can reduce the prevalence of *Salmonella* in pigs, however the cost effectiveness of such an approach must be questionable.

This project aimed to develop natural antimicrobial compounds, which could potentially be encapsulated in nanosomes and used as an anti-*Salmonella* based pig feed additive as a low-cost solution, to reduce *Salmonella* levels in the Irish pig herd. These antimicrobial compounds being targeted were derived from three marine derived *Pseudovibrio* isolates previously identified by our group, which displayed potent anti-salmonella activity. The project planned to characterize the antimicrobial activities of these isolates, following genome analysis, heterologous expression and to perform structural elucidation of the bioactive compounds, following heterologous expression of the genes encoding the bioactivities.

2. Research Approach

Genomic based approaches were employed to sequence the genomes of a number of bioactive *Pseudovibrio* genomes were sequenced at in-house at the Teagasc Next Generation Sequencing Centre in Moorepark using the Illumina (MiSeq) and 454 (GS-FLX) platforms. Sequences from the *Pseudovibrio* strains were then assembled and annotated using appropriate sequence assembly programs. antiSMASH was then employed to identify putative Biosynthetic Gene Clusters (BGCs) present in the genomes of these isolates. This genome analysis indicated the presence of at least 1 Polyketide Synthase (PKS) and 1 non ribosomal peptide synthase (NRPS) gene cluster among the *Pseudovibrio* isolates. The BGC's identified potentially encoded Bacteriocin, Homoserine Lactone (Hserlactone), Non-Ribosomal Peptide Synthetase (NRPS), NRPS/PKS hybrid clusters (NRPS-PKS), trans-AT polyketide synthases (trans-AT), Polyketide synthases (PKS), Siderophore and Terpenes. While these BGC's were highly similar among the *Pseudovibrio* isolates, they showed little similarity to BGC's from other bacterial species, suggesting a high potential for novel product biosynthesis amongst our *Pseudovibrio* isolates.

Further comparative analysis was undertaken to identify BGCs in our *Pseudovibrio* strains that might encode anti *Salmonella* activity by including publically available genome sequences of 11 other *Pseudovibrio* strains in order to ascertain the abundance and distribution of gene clusters possessing putative secondary metabolite biosynthetic capabilities among members of the genus. This allowed us to identify a large number of putative small molecule BGCs biosynthetic gene clusters, and in particular identifies strain AD2 as a potential prolific source of bioactive natural products of diverse function.

A range of software tools were employed, including:
- *Regulatory Sequence Analysis Tools (RSAT): (http://rsat.ulb.ac.be/rsat)*
- *Phisite: http://www.phisite.org/main/index.php?nav=tools&nav_sel=hunter*
- *BACC: http://www.bacpp.bioinfoucs.com/home*

to identify potential regulatory regions upstream from these BGCs, but it was not possible to unambiguously and definitively identify these potential regulatory regions.

Attempts were also undertaken to generate Pseudovibrio mutants that did not produce tropodithietic acid (TDA), and subsequently screen these mutants for anti-*Salmonella* activity. However, the *Pseudovibrio* strains were completely recalcitrant to genetic manipulation. The overall concept was to generate mutant strains that Transposon mutagenesis involving the use of a series of new broad-host-range mini-Tn5-vectors, termed pBAMDs, for generating saturated mutagenesis libraries in gene function studies in gram negative bacteria were employed, without success. We did develop a screen to identify *Pseudovibrio* TDA mutants has been developed, we were unable to generate mutants via the mini-Tn5-vector system.

Screens were conducted to determine whether *Pseudovibrio* strains Ad2, W64, W74 and WM33, displayed antimicrobial activity against other food pathogens/food spoilage microorganisms. This involved deferred antagonism tests using a range of indicator organisms with widespread activities being identified. Anti-*Salmonella* extracts were obtained from three isolates and the bioactive compound identified as tropodithietic acid (TDA). TDA has a strong inhibitory effect on a broad spectrum of bacterial pathogens including *Salmonella typhimurium* strain LT2, *Salmonella typhimurium* strain C5369 and *Salmonella enterica* subsp. *arizone*.

Growth experiments were undertaken to identify culture conditions under which anti – *Salmonella* activity could be obtained in extracts which did not contain TDA, to determine the physiological factors that affect TDA production. This revelated that TDA production was affected by conditions of phosphate limitation. This was subsequently exploited in growth experiments using modified carbohydrate-mineral medium containing a surplus of phosphate in order to suppress TDA production in *Pseudovibrio* isolates AD2, WM33, W64 and W74 and examine secondary metabolite production in these strains at different growth phases. Reverse transcription (RT)-PCR was employed to monitor expression of the *tda* biosynthetic genes which is co-ordinately regulated upon transition from log to stationary phase growth.

Purification methods were developed in an attempt to isolate the antimicrobials produced by the different *Pseudovibrio* strains using *Pseudovibrio* AD2 vs. *Salmonella* LT2 co-extracts, with crude extracts being concentrated using a C-18 SPE column but unfortunately no inhibitory zones were observed. The crude extracts were also passed through a 3KDa filter to separate the proteins based on size. we hypothesized that the size of the protein would be approximately 4 KDa based on predictions made by AntiSmash. However, no inhibitory activity was observed in either fraction. Colony mass spectrometry was also carried out on all *Pseudovibrio* strains in search of the production of antimicrobials of the predicted mass. Low pH solvents were utilised in an attempt to extract the putative bacteriocins from the cell wall. While, in general, masses consistent with the size of the predicted antimicrobial were not detected (this is more likely to reflect their chemical structure rather than non-production), in the case of the AD2 isolate a small peak was seen at 3965Da, which may be significant.

With respect to chemical-based analysis that was undertaken during the project, extraction of the secondary metabolites from the bacterial cell free supernatants was performed and a gradient HPLC method was developed to separate the various components both in the culture medium and bacteria-cultured samples. Using this HPLC method, cell-free supernatants from *Pseudivibrio* sp. grown at 28°C overnight from eight different strains were analyzed for the separation and detection of novel metabolites, with tropodithietic acid (TDA) being found to be the major component of the bacteria in a number of strains.

Mass spectrometry (MS) methods were also optimised for the detection and structural characterisation of various secondary metabolites from the cultured *Pseudovibrio* strains. Electrospray mass spectra data were recorded both on a negative and positive ionisation mode for a mass range m/z 100 to 1000. Collision induced dissociation (CID) or tandem mass spectrometry (MS/MS) of the targeted analytes was achieved using 12 eV to 20 eV collision energy depending on m/z values of the targeted molecular ions. In the full scan mass spectra, $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions were observed in positive ion mode, while deprotonated $[M-H]^-$ ions were predominant in negative ion mode. Accurate masses of the intact ions and their fragment ions were determined using a lockspray reference of a known standard leucine-enkelphine (555.2693 Da).

LC-MS/MS was employed to characterise thirteen different metabolites that were detected in the ethyl acetate fraction of *Pseudovibrio* sp. W64. The identified metabolites included a new secondary metabolite, i.e., a methyl ester of TDA, several cholanic acid derivatives, amino diols and triols, which have not been previously reported from this microorganism.

3. Research Achievements/Results

A number of *Pseudovibrio* strains previously isolated from the marine sponges *Axinella dissimilis*, *Polymastia boletiformis* and *Haliclona simulans* were screened for anti-*Salmonella* activity using deferred antagonism/disc diffusion assays. A number of strains showed anti-*Salmonella* activity including *Pseudovibrio* strains Ad2, W64, W74 and WM33. In addition screens were also conducted to determine whether these Pseudovibrio strains, displayed antimicrobial activity against other food pathogens/food spoilage microorganisms/clinical pathogens, including *Lactococcus lactis* HP, *Lactobacillus bulgaricus* DPC5383, *Listeria*

monocytogenes L028, *Listeria monocytogenes* 10403S and *Listeria innocua* , *Cl. perfringens*, *C. difficile, C. ramosum*, *C. symbiosum* and *C. boltae.* Deferred antagonism assays indicated Cl. Perfringens, C. difficile, *L. innocua*, *C. ramosum*, *C. symbiosum* and *C. boltae* as being sensitive.
Deferred antagonism assays were also conducted using the TDA resistant isolates, B98C31, B98C32, B98C36 and B98C38 as indicator strains in order to ascertain environmentally relevant culture conditions which may influence the bioactivity of our *Pseudovibrio* isolates, in a TDA independent manner.

Non-TDA related bioactivity was observed against the TDA resistant sponge isolate, B98C38 only upon co-culture of *Pseudovibrio* sp. W64 + AD13 or W64 + AD5. Bioactivity decreased when decreasing volumes of W64 were mixed in co-culture with *Pseudovibrio* sp. AD5 or AD13, indicating that W64 was the strain responsible for the production of the inhibitory compound. Non-TDA related bioactivity was also examined using *Photobacterium damselae*, *Vibrio aestuarianus*, and *V. splendidus* as indicator strains given their importance as pathogens of fish and shellfish. Bioactivity was observed against *V. aestuarianus*, in all the *Pseudovibrio* isolates (with the exception of strain AD2).

Comparative genomic analysis of ten sequenced genomes of *Pseudovibrio* strains isolated from marine sponges from the west coast of Ireland, and two publicly available genomes was performed. Homogeneity was apparent in terms of both the orthologous genes and the metabolic features shared amongst the 12 strains. The analysis also indicated a large diversity of both metabolic features and systems for interacting with the sponge host.
We further interrogated the genomes of 21 Pseudovibrio strains, including Pseudovibrio strains that had shown anti-*Salmonella* activity, for the presence, diversity and distribution of biosynthetic gene clusters (BGCs). A low level of similarity shared between the BGCs identified in the *Pseudovibrio* genomes and those reported in the MIBiG database indicated the potential novelty of the metabolites produced by the pathways.
We then attempted to identify the redundancy of NGCs within the genus and their similarity with known and characterized biosynthetic clusters. To achieve this, we performed global alignments of amino acid sequences of proteins identified in proteins predicted by antiSMASH that were involved in the biosynthesis of secondary metabolites and constructed similarity networks. This revealed that with the bacteriocin, terpene, nonribosomal peptide synthase (NRPS) and polyketide synthase (PKS) families that overall identity within the genus was considerably high (>75%) suggesting a key role for these metabolites in interactions between the *Pseudovibrio* strains and their hosts. However significant differences were observed amongst the different strains particularly with respect to BGCs encoding aryl polyene-like molecules which were only found in 2 of the 21 strains. Derivatives of aromatic polyenes isolated from marine microorganisms have been shown to have antibacterial activity.
A hybrid *trans-AT* PK-NRPS gene cluster was observed in *P. axinellae* AD2. Trans-AT PKS systems are known to be involved in the biosynthesis of many pharmacologically important polyketides such as the antibiotic mupirocin. Phylogenetic analysis revealed that the KS domains of this *trans-AT* PK-NRPS BGC in AD2 formed independent branches. A subsequent antiSMASH comparison revealed 61% similarity between this *trans-AT* PK-NRPS BGC in strain AD2 and a gene cluster from *Xenorhabdus bovienii*, which is well known for its ability to

produce bioactive secondary metabolites. Despite the fact that some gene clusters were shared among many of the Pseudovibrio strains and in particular the NRPS, a more detailed analysis of the domain organisation of these BGCs revealed a certain degree of specificity. Many of the NRPS clusters differed in their organisation, structure and function of their associated domains, indicating a potential for variation in the final structures of the resulting products. This NRPS product diversity primarily derives from the substrate incorporated at the adenylation (A) domain of each module. Thus, analysis of this predicted A domain binding in the NRPS clusters, highlighted the potential for the production of a number of structurally diverse novel products from members of the *Pseudovibrio* genus.

Mass spectrometry methods were developed for the rapid screening and identification of novel bioactive compounds from Pseudovibrio sp. Based on high-resolution mass spectrometric analysis, thirteen metabolites were detected from the ethyl acetate fraction from *Pseudovibrio* sp. W64. Among the thirteen metabolites, a methyl ester of TDA, a number of cholic acid derivatives, and amino diols and triols were characterised.
These metabolites were specifically identified as 3,7,12-trihydroxychol-5-enoylglycine, glycocholic acid, 3,7,12-trihydroxy-5-cholenoic acid, Palmitamide, Hydroxyl steramide, Cholic acid, Glycochenodeoxycholic acid, Stearamide, Chenodeoxycholic acid, Eicosanamide, Hydroxyl behenamide, Behenamide, Hydroxyl lignoceramide, as well as a novel TDA methyl ester.
In addition, a mechanism of fragmentation of these compounds was proposed. This was in fact the first time that the mechanism of fragmentation of these compounds on low collision induced dissociation (CID or MS/MS ions) had been proposed which assisted in their structural elucidation. In addition, HPLC/MS/MS proved to be an efficient tool for rapid screening and characterisation of these molecules, with minimum sample preparation steps, without involving actual purification. However, further scale-up studies to isolate pure compounds and their absolute characterisation using NMR spectroscopy holds an encouraging scope to discover novel molecules from this bacterium. Finally, this work highlighted the fact that a combination of analytical techniques such as liquid chromatography, UV-detector and mass spectrometry, can achieve the identification of metabolites in a complex mixture, when supported by background knowledge and literature.

4. Impact of the Research

The emerging antibiotic resistance among pathogenic microorganisms is a matter of great concern for the food industry. Food is an essential item for survival but also a vector of foodborne pathogens, which can pose great threats to human health as well as the food industry. Use of antibiotics to tackle these pathogens has protected the human health; however, continuous pressure of the use of such antibiotics among bacteria would lead to the development for antibiotic resistance. Marine ecological niches have been established as “promising” sources for new antimicrobials to combat antibiotic-resistant strains of pathogenic microorganisms. In this task, the discovery of a unique analogue of potent antibacterial tropodithietic acid (TDA), i.e. methyl ester of TDA in *Pseudovibrio* W64 strain, may serve as an agent to combat foodborne pathogens in food processing industries.

4(a) Summary of Research Outcomes

(i) Collaborative links developed during this research

- The post-doc, Dr. Alka Choudhary, recruited in this research task was offered a permanent position (Assistant Professor) at National Institute of Pharmaceutical Education and Research (NIPER), Guwahti, India (http://www.niperguwahati.ac.in/profile/ALKA.pdf).

- The team members (UCC and Teagasc) involved in this task were able to subsequently secure a FIRM funding of ~€750 K on a research project (FIRM 15/F/698) titled "Seaweed-Microbe Interactions to enhance bioactive yields for food applications".

- Teagasc Ashtown was also able to secure ~€574K through Horizon2020 Funded projects:

- EU HDHL JPI BIOCAR-FOOD: Extraction and characterization of BIOactives and CARBohydrates from seaweeds and seagrasses FOR FOOD-related applications (17/RD/SUSFOOD2/ERA-NET/1) - €305.9K.

- EU RDF: Interreg Atlantic Area. ENHANCEMICROALGAE: High added-value industrial opportunities for microalgae in the Atlantic Area (EAPA 338/2016) - €267.9K.

- Teagasc Ashtown was also able to secure €8M 'Meat Technology Ireland' and €5M Prepared Consumer Food fundings from Enterprise Ireland and Agri-Food industries. These have helped to procure numerous modern equipment and hire post-doctoral researchers, and thereby greatly enhancing the research infrastructure and skilled personnel.

- UCC obtained Horizon 2020 funding in the Marine Biotechnology area for the project *Marine Biodiversity as Sustainable Resource of Disease-Suppressive Microbes and Bioprotectants for Aquaculture and Crop Diseases.* [MARBLES] to perform multi-omics analyses to assess microbiome composition, metabolic potential and bioactivity, linking global biodiversity patterns to overall microbial and BGC diversity in marine sponge and Atlantic Salmon samples.

- UCC obtained funding in in the Marine Biotechnology area under the ERA.NET BlueBio initiative for two projects
  A) *From Sustainable Resources to novel marine nutraceuticals for the management of Metabolic Syndrome* [SuReMetS] with the objectives of isolating novel enzymes from bacteria and metagenomic libraries from bacteria on fish and macroalgae surfaces, with biotechnological applications in the enzyme assisted extraction procedures to generate novel nutraceuticals, which can be used in the management of metabolic syndrome.

B) *Marine Innovation using Novel Enzymes for waste Reduction and Valorisation of Algal biomass* [Minerva] with the objectives of isolating novel enzymes from bacteria and metagenomic libraries from decaying macroalgae, with biotechnological applications in the enzyme assisted extraction procedures to generate bioactive fractions from these macroalgae.

(ii) Outcomes where new products, technologies and processes were developed and/or adopted

(iii) Outcomes with economic potential

(iv) Outcomes with national/ policy/social/environmental potential

4 (b) Summary of Research Outputs

(i) Peer-reviewed publications, International Journal/Book chapters.

1. Crowley, S.P., O' Gara, F., Orla O'Sullivan, O., Paul D. Cotter, P.D. and Dobson, A.D.W. (2014) Marine *Pseudovibrio* sp. as a Novel Source of Antimicrobials. Marine Drugs 12, 5916-29.
2. Harrington, C., Reen, J.F., Mooij, M.J., Stewart, F., Chabot, J-B., Antonio Fernandez Guerra, A.F., Glockner, F.O., Nielsen, K.F., Gram, L., Dobson, A.D.W., Adams, C. and O'Gara, F. (2014). Characterisation of non-autoinducing tropodithietic acid (TDA) production from marine sponge *Pseudovibrio* species. Marine Drugs 12, 5960-5978.
3. Romano, S., Fernandez-Guerra, A., Glöckner, F., Crowley S.P, O'Sullivan, O., Cotter, P., Reen, J.F., Adams, C., Dobson, A.D.W. and O'Gara, F. (2016) Comparative genomic analysis reveals a diverse repertoire of genes involved in prokaryote-eukaryote interactions within the *Pseudovibrio* genus. Frontiers in Microbiology. 7:387.
4. Choudhary, A., Naughton, L.M., Montanchez, I., Dobson, A.D.W., Rai, D.K. (2017). Current status and future prospects of marine natural products (MNPs) as antimicrobials. Marine Drugs 15, 272.
5. Naughton, L.M., Romano, S., O'Gara, F. and Dobson, A.D.W. (2017). Identification of secondary metabolite gene clusters in the *Pseudovibrio* genus reveals a promising potential toward the discovery of novel bioactive compounds. Frontiers in Microbiology 8: 1494.
6. Choudhary, A., Naughton, L.M., Dobson, A.D.W., Rai, D.K (2018). High-performance liquid chromatography/electrospray ionisation mass spectrometric characterisation of metabolites produced by *Pseudovibrio* sp. W64, a marine sponge derived bacterium isolated from Irish waters. Rapid Communications in Mass Spectrometry. 63, 1737-1745.

(ii) Popular non-scientific publications and abstracts including those presented at conferences

- A poster presentation was given by Susan Crowley at the EMBL-EBI-Wellcome Trust Centre, Hinxton, Cambridge, UK entitled " Marine *Pseudovibrio* sp. as a Novel Source of Antimicrobials"
- A presentation was given by Paul Cotter entitled "Modulation of the Gut Microbiota", at the Food Nutrition & Agriculture Economic Conference in London, UK.
- A presentation was given by Alan Dobson entitled "Metagenomic and genomic approaches to exploit the biodiversity of coastal and deep sea sponges"; at the International conference "Drugs from the Sea", in Eilat, Israel, 9-14th February.
- A presentation was given by Alan Dobson entitled "Marine bacteria as a source of novel bioactive agents for use in strategies against foodborne pathogens and in food processing applications", at the NutraMara Conference - "Harnessing Marine Bioresources for Innovations in the Food Industry", Royal Dublin Society, Dublin, 29-30th June 2015. http://www.nutramara.ie/nutramara-conference-2015/.
- Presentation by Alan Dobson entitled "Mining our oceans for novel bioactive molecules with biotechnological applications", at the Society for Applied Microbiology- Winter Meeting, London, 19th January, 2016. http://www.sfam.org.uk/en/events/index.cfm/Winter_meeting#tabs1
- Invited Presentation by Paul Cotter "Modulation of the Gut Microbiota using Bacteriocin-producing strains" at the International Scientific Conference on Probiotics and Prebiotics Budapest, Hungary June 2017.
- Invited Presentation by Paul Cotter "Bioengineered Bacteriocins" at the Bacteriocin Symposium - International Scientific Conference on Probiotics and Prebiotics in Budapest, Hungary June 2017.
- Invited Presentation by Alan Dobson "Metagenomic strategies for the discovery of novel enzymes with potential biotechnological applications from marine ecosystems" at the Symposium: "La Biotechnologia y sus Fronteras". Universidad Autonoma del Estado de Morelos, Centro de Investigacion en Biotechnologia, Curnevaca, Morelos, Mexico, 31st October 2016.

(iii) National Report

(iv) Workshops/seminars at which results were presented

(v) Intellectual Property applications/licences/patents

(vi) Other

5. Scientists trained by Project

Total Number of PhD theses: __1__

Total Number of Masters theses: __0__

6. Permanent Researchers

Institution Name	Number of Permanent staff contributing to project	Total Time contribution (person years)
Teagasc Moorepark	Professor Paul Cotter	
UCC Microbiology	Professor Alan Dobson Professor Fergal O'Gara	
Teagasc Ashtown	Dr Dilip Rai	
Total		

7. Researchers Funded by DAFM

Type of Researcher	Number	Total Time contribution (person years)
Post Doctorates	4	
PhD students	1	
Masters students		
Temporary researchers		
Other		
Total		

8. Involvement in Agri Food Graduate Development Programme

None.

9. Project Expenditure

Total expenditure of the project:	€450,849.41
Total Award by DAFM:	€490,392.50
Other sources of funding including benefit in kind and/or cash contribution(specify):	€0

Breakdown of Total Expenditure (€)

Category	UCC	Teagasc Moorepark	Teagasc Ashtown	Total
Contract staff				
Temporary staff			63,641.03	63,641.03
Post doctorates	117,620.56	27,394.76		145,015.32
Post graduates		44,000.00		44,000.00
Consumables	45,005.54	29,284.49	13,673.69	87,963.72
Travel and subsistence	3,899.00	1,305.40	983.00	6187.40
Sub total	166,525.10	101,984.65	78,297.72	346,807.47
Durable equipment				
Other				
Overheads	49,957.23	30,595.40	23,489.31	104,041.94
Total	**216,482.33**	**132,580.05**	**101,787.03**	**450,849.41**

10. Leveraging

- The team members (UCC and Teagasc) involved in this task were able to subsequently secure a FIRM funding of ~€750 K on a research project (FIRM 15/F/698) titled "Seaweed-Microbe Interactions to enhance bioactive yields for food applications".

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