Future Fisheries
VETERINARY SERVICE

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Assessment of frozen uncooked imported prawns for antimicrobial-resistant microorganisms of aquaculture and public health significance and residues of Ag-vet chemicals

FRDC Project No 2017-091 report
February 2018

Version 1.0 23 January 2018

ISBN 978-1-64316-657-5

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FRDC Project 2017-091

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In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

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Acronyms

AST	Antibiotic sensitivity Testing
CLSI	Clinical Laboratory Standards Institute
APFA	Australian Prawn Farmers Association
CFU	Colony Forming Unit
DAWR	Australian Government, Department of Agriculture and Water Resources
ECOFFs	Epidemiological Cut-off Values
EUCAST	European Committee for Antimicrobial Susceptibility Testing
IRA	Import Risk Analysis
IGB	Inspector General of Biosecurity
IIGB	Interim Inspector General of Biosecurity
LC-MS	Liquid Chromatography-Mass Spectrometry
MALDI-TOF	Matrix Assisted Laser Desorption/Ionisation- mass spectrometer
MIC	Minimum inhibitory concentration
NARM	National Antimicrobial Resistance Monitoring System
OCVO	Office of the Chief Veterinary Officer
OIE	Office International des Epizooties World Organisation for Animal Health
PCR	Polymerase Chain Reaction
rDNA	Ribosomal Deoxy Nucleic Acid
WTOSPS	World Trade Organisation Sanitary and Phytosanitary Agreement
TSV	Taura Syndrome Virus
UNK	Unidentified bacteria
WHO	World Health Organisation
WSSV	White Spot Syndrome Virus
WSD	White Spot Disease
WTO	World Trade Organisation
YHV	Yellowhead Virus

Glossary

Litopenaeus vannamei	Pacific white shrimp
Peneaus monodon	Black tiger prawn

Tables

Executive Summary

What the report is about

As part of the response to the outbreak of White Spot Disease (WSD) in prawn farms along the Logan River, Southern Queensland in late November 2016, uncooked prawns were purchased by Dr Matt Landos (Future Fisheries Veterinary Service Pty Ltd) from various retail outlets in northern NSW and south east Queensland. These retail prawn samples were tested by qPCR for White Spot Syndrome Virus (WSSV) under FRDC project 2016/066. This project (2017-091) utilised residual sample material which had been held at -20^{o}C at Future Fisheries Veterinary Service, East Ballina NSW, since collection during December 2016 and January 2017. Residual samples which were labelled as imported product either by signage at the retail outlet, or on product packaging were tested for:

1) Presence of bacteria that were of significance to aquaculture and public health (tested at the University of Adelaide)

2) Presence of resistance to a range of antimicrobials (tested at the University of Adelaide)

3) Presence of a suite of antimicrobials and other Ag-vet chemicals (tested at Queensland Government Chemical Residue Laboratory)

Thirty six (36) imported prawn commodities were provided. The University of Adelaide research Team worked-up testing and analysis protocols.

There were a range of live bacteria (Appendix 1) recovered from imported prawn commodities. Some were identified as potential aquaculture pathogens and others of potential public health importance. Further testing did not identify any significant antibiotic resistance in the cultured bacteria from uncooked imported prawns.

Feed stock powders (VS 300W-B1 and Scan Viron) are commonly used as top-coat supplements on prawn feeds in Asia. These two powders were initially tested at University of Adelaide for antimicrobial properties by disk diffusion assay. *Gram-positive* and *Gram-negative* bacteria were utilised and both powders possessed *significant antimicrobial properties*. The powders were then sent to the Queensland Government Chemical Residue Laboratory for forensic analysis of the identity of the putative antimicrobial substances. This laboratory identified them as containing enrofloxain and ceftriaxone. Both are not permitted for any use on food animals in Australia. Neither compound is presently listed as part of the National Residue Survey on imported foods into Australia.

Thirty five (35) prawn commodities were tested for residues of a wide range of antimicrobials and agricultural chemicals (Appendix 2).

Nine of the thirty five commodities were found to contain residue detections above the Limit of Reporting (LOR). Chemicals identified in these name commodities included the antibiotics: oxytetracycline; chlortetracycline; doxycycline; trimethoprim; sulphamethoxazole and the herbicide, diuron.

There are no Codex Maximum Residue Limits established for any of these chemicals in the commodity, *Litopenaeus vannamei*, in which they were identified. Hence the 'permissible' residue level is considered to be below the limit of detection of the laboratory. The acceptable daily intake of these chemicals has not been established for this commodity type. Results will be forwarded to the Commonwealth National Residue Survey and the Food Standards Australia and New Zealand. These commodities containing residues appear to be non-compliant to Australian guidelines.

Background

Antimicrobial resistance (AMR) is a globally emerging trend. Micro-organisms are acquiring or developing resistance to antimicrobials. Broadly, increased use of antimicrobials has promoted this emergence. Antimicrobials are a cornerstone of production animal and public health advancement. Micro-organisms are now understood to have multiple pathways through which they can acquire/share antimicrobial resistance genes. Australia has relatively low levels of AMR on a global scale, in part due to its tight regulation of antimicrobial use on animals destined for human consumption. One pathway for transmission of AMR to humans is via consumption of animals carrying AMR genes. The trade of such animal commodities is recognised to be a risk for movement of AMR.

The Australian prawn farming industry has low volumes of antimicrobial use, with any use confined to hatcheries and specifically targeted for the control of bacterial diseases such as *Vibriosis* sp. under guidance of a registered Veterinarian. This limited use, whilst infrequent, can be essential for reliable output of seedstock from an individual hatchery.

A range of antimicrobials, including many not permitted for use in prawns based on Australian legislation/regulation, are utilised in prawn farming in SE Asia in response to disease outbreaks (Chi, et al., 2017). The identity of exact compounds in use is not well described. Hence screening for the presence of their residues in imported prawn commodities is difficult, without prior knowledge of their identity. The risk of importation of AMR into Australia via uncooked prawn commodities has not been assessed.

The Australian prawn farming industry has remained free from OIE listed internationally reportable bacterial diseases. Notwithstanding that since 2015, there have been some outbreaks of limited geographic extent of *Penaeus Monodon Mortality Syndrome* in association with the molecular detection of PirA B toxin DNA, which bears some similarities to the OIE listed condition, Acute Hepatopancreatic Necrosis Disease (AHPND). The potential for live aquatic bacterial pathogens to be present on imported prawn commodities which are intended for human consumption has not been the subject of any substantial prior research. Given the now understood pathway of potential dissemination of uncooked prawn commodities into waterways via disposal (berley)/use (bait) of uncooked prawns there is a clear risk entry pathway for introducing new pathogens, or pathogens which have differing virulence factors that could cause significant disease particularly in aquaculture enterprises which are farming susceptible species.

Objectives

1. Define the identity of bacteria and their status with respect to phenotypic and genotypic antimicrobial resistance associated with imported frozen uncooked prawn commodities.
2. Identify the chemical compositions of two feed stock powders (suspected antimicrobials) which are being added to prawn feed in Asian countries.
3. Quantify the type and level of antibiotic and agricultural chemical residue in a range of imported prawn commodities purchased at Australian retail outlets.
4. Discuss the implications of study's findings in respect of biosecurity controls and how they can contribute to protection of the productivity of the prawn farming industry, other aquaculture industries and protection of human health.

Methodology

1) Define the identity of bacteria and their status with respect to phenotypic and genotypic antimicrobial resistance associated with imported frozen uncooked prawn commodities.
    a. University of Adelaide, Australian Centre for Antimicrobial Resistance Ecology were sent 36 imported frozen uncooked prawn commodities.

    b. Samples from each commodity were cultured on a range of agars to identify and quantify the numbers and types of micro-organisms present.

    c. Up to 10 isolates per commodity were identified by use of MALDI-TOF mass spectroscopy to determine if they were of aquaculture or public health significance.

    d. Isolates of aquaculture or public health significance were screened for antimicrobial resistance by Minimum Inhibitory Concentration (MIC) and by real-time Polymerase Chain Reaction (PCR).

2) Identify the chemical compositions of two feed stock powders (suspected antimicrobials) which are being added to prawn feed in Asian countries.
    a. Feed stock powders were tested for antimicrobial properties by disk diffusion assay. *Gram-positive* and *Gram-negative* bacteria were utilised.

    b. Feed stock powders were examined by LC-MS to determine identity against laboratory standards.

3) Quantify the type and level of antibiotic and agricultural chemical residue in a range of imported prawn commodities purchased at Australian retail outlets.
    a. Queensland Government Chemical Residue Laboratory were sent 35 imported prawn samples to be examined by LC-MS using the modification of in-house method AB046 for the 58 different antimicrobial compounds and 159 different pesticides (Appendix 2), including those identified in Objective 2.

Results/key findings

One hundred and fifty four (154) species of live microbes were detected in association with frozen uncooked imported prawn commodities.

Seventy four (74) of these species were able to be identified by MALDI-TOF mass spectrometry. With the remainder, identification was attempted through grouping by colony morphology and 16S rDNA PCR and rDNA sequencing.

Forty seven (47) species of bacteria were identified to the species level (See Table 1). Five of these species were considered to be associated with aquaculture and eleven of these species were considered to be environmental organisms with potential as opportunistic human pathogens

The potential aquaculture pathogens, *Lactococcus garviae*, *Pseudomonas putida*, *Carnobacterium maltaromaticum* were detected.

Implications for relevant stakeholders

No bacterial pathogens of significance for prawn farming were detected on the frozen uncooked commodities.

No antimicrobial resistance was identified within the bacterial species which were isolated from the commodities, suggesting these commodities posed no risk for introduction of AMR into prawn farms.

Of those bacteria identified, there were species present which could be of significance to other species in aquaculture, such as finfish. *Lactococcus garviae* is a recognised and significant pathogen of Yellowtail Kingfish and Rainbow Trout (Meyburgh, et al., 2017). *Pseudomonas putida* and *Carnobacterium maltaromaticum* have been described to be associated with fish diseases (Altinok, et al., 2006) (Smolowitz, et al., 1998) (Loch, et al., 2011) . *Carnobacterium maltaromaticum* has also been associated with meningoencephalitis in wild salmon shark strandings in California (Schaffer, et al., 2013).

The identification of two powders which were in use on Asian prawn feed, as potent antimicrobials, which are listed by the World Health Organisation (WHO) as the highest priority, Critically Important[1], antibiotics is of serious concern. Such use is contraindicated and is of global concern as there are currently no alternatives for them to treat many serious human infections.

The National Residue Program should consider adding these compounds to the screening program for imported seafood.

FSANZ should consider whether the residues detected should lead to a restriction of trade, actions against the importer, product recalls and the implementation of an enhanced surveillance program.

It should be recognised that this is a small snapshot of commodities which are being imported, and may not be representative of the disease status, AMR status or residue status of all imported material.

Recommendations

14. The National Residue Survey should consider adding the two antibiotics identified in Asian stock feed powders to the routine screening program for imported seafood.

15. FSANZ should consider whether the residues detected should lead to a restriction of trade, actions against the importer, product recalls and the implementation of an enhanced surveillance program.

16. Imported finfish should be subjected to a similar research study to better appreciate the risks associated with this import entry pathway for pathogens, AMR and food safety risks such as chemical residues.

17. Data from this project should be considered within the Commonwealth review of the Import Risk associated with imported uncooked prawn commodities.

18. Biosecurity Australia should consider improved sanitary measures such as cooking prawns which could eliminate the entry of potential aquaculture and wild fishery pathogens on uncooked prawns which continue to be diverted from human consumption and used as bait by

[1] http://www.who.int/foodsafety/publications/cia2017.pdf

recreational fishers. Such a measure would also discourage anglers from using prawns which were destined for human consumption, into use as angling bait.

Keywords

***Litopenaeus vannamei*; Pacific White Shrimp; Antimicrobial resistance; Antibiotic residue; pesticide residue; Antibiotic;; imported frozen commodity prawn; *Penaeus monodon*; Black Tiger Prawn**

Introduction

Antimicrobial resistance is a globally emerging trend, whereby, micro-organisms acquire or develop resistance to antimicrobials. Antimicrobials have been a cornerstone of production animal and public health advancement since the first discovery of the efficacy of penicillin to control some bacterial infections. Micro-organisms are now understood to have multiple pathways through which they can acquire/share antimicrobial resistance genes. Australia has relatively low levels of anti-microbial resistance (AMR) on a global scale, in part due to its tight regulation of antimicrobial use on animals destined for human consumption.

The Australian prawn farming industry has very low volumes of antimicrobial use with any of use confined to hatcheries and specifically targeted for the control of bacterial diseases such as Vibriosis. This limited use, whilst infrequent, can be essential for reliable output of seedstock from an individual hatchery.

A range of antimicrobials, including many not permitted for use in prawns based on Australian legislation/regulation, are utilised in prawn farming in SE Asia in response to disease outbreaks. The identity of exact compounds in use is not well described.

One of the pathways for transmission of AMR to humans is via the use of antimicrobials in animals destined for human consumption. The trade of such animal commodities is recognised to be a risk for movement of AMR.

The risk of importation of AMR into Australia via uncooked prawn commodities has not been assessed.

Given the now understood pathway of potential dissemination of uncooked prawn commodities into waterways via disposal (berley)/use (bait) of uncooked prawns the entry and establishment of AMR could impact on prawn hatchery performance, where juvenile stages are particularly sensitive to bacterial diseases such as Vibriosis. Should AMR enter hatcheries via the use of wild broodstock, water or aerosol pathways it could contribute to great challenges in maintaining reliable hatchery production for the entire prawn farming sector, for it could render the currently available antimicrobials useless. Endemic bacteria still intermittently are associated with significant mortality particularly in the hatchery phase of production.

The Australian prawn farming industry has remained free from OIE listed internationally reportable bacterial diseases. Since 2015 there have been some outbreaks of limited geographic extent of *Penaeus Monodon Mortality Syndrome* in association with the molecular detection of PirA B toxin DNA. The potential for live aquatic pathogens to be present on imported prawn commodities which are intended for human consumption has not been the subject of prior research. The aforementioned release pathway risks introducing new pathogens, or pathogens which have differing virulence factors that could cause significant disease particularly in aquaculture enterprises.

Objectives

1. Define the identity of bacteria and their status with respect to phenotypic and genotypic antimicrobial resistance associated with imported frozen uncooked prawn commodities.
2. Identify the chemical compositions of two feed stock powders (suspected antimicrobials) which are being added to prawn feed in Asian countries.
3. Quantify the type and level of antibiotic and agricultural chemical residue in a range of imported prawn commodities purchased at Australian retail outlets.
4. Discuss the implications of study's findings in respect of biosecurity controls and how they can contribute to protection of the productivity of the prawn farming industry, other aquaculture industries and protection of human health.

Methods

Bacterial identification

Ten randomly selected frozen prawns per commodity (n=36) were thawed and removed aseptically from the commodity. Utilising a *stomacher* and a *constant weight*, commodity samples were added to peptone water, homogenised and inoculated onto the test agars a-d identified below. Samples were plated at a serial dilution and accurate viable bacterial cell numbers calculated as colony forming units per ml (CFU/ml). A total viable count was performed on plate count agar (incubated at 37^{o}C) and TCBS marine agar (incubated at 25^{o}C), with the first dilution a weight/volume dilution and subsequent serial dilutions volume/volume.

a) Rich Media

b) MacConkey Agar

c) Chromogenic agar

d) TCBS marine agar

Colonies were assessed for growth, enumerated and grouped into colony types within a commodity (data is provided in the **Appendix 3**). Each colony type was given a reference number and stocked (preserved in glycerol).

All colony types were analysed for identification using the MALDI-TOF mass spectrometer (*Biotyper*, Bruker).

Any isolates that were not able to be identified were initially classified as "unknown". Such isolates could result from the absence of a significant "hit" on the *Biotyper* database, indicating a strong correlation to the colony being tested. Any "unknowns" will be grouped into distinct, colony types. From each colony type one isolate will be chosen for further analysis using 16S rDNA PCR and DNA sequencing to provide the bacterial identification.

Bacteria from the initial isolations were assessed for their potential aquaculture and public health significance. These isolates were chosen for Antibiotic Sensitivityy Testing (AST; using custom designed *TREK diagnostics Sensititre Veterinary* panels).

Antibiotic Sensitivity Testing (AST)

AST was performed by broth microdilution using Veterinary Reference Card panels (Sensititre®, Trek Diagnostics, East Grinstead, UK). Inoculation and incubation were as by the manufacturers' guidelines. In addition, in-house broth microdilution panels made according to Clinical and Laboratory Standards Institute (CLSI) standards. The antimicrobial concentration range for each agent is shown in Appendix 5, 6, 7.

The minimum inhibitory concentrations (MIC) were interpreted according to CLSI VET01S or the European Committee for Antimicrobial Susceptibility Testing (EUCAST) epidemiological cut-off values (ECOFFs) as indicated in Appendix 5, 6, 7. CLSI M100S breakpoints were used where animal species antimicrobial agent combinations were not available. Interpretation of the MICs were based on Clinical and Laboratory Standards Institute (Wayne, PA) interpretive criteria when available; otherwise European Committee on Antimicrobial Susceptibility Testing (EUCAST; Basel, Switzerland). The dual EUCAST/CLSI system was used in order that the results were able to be

completely internationally relevant i.e. there were two prevalence estimates: 1) EUCAST ECOFF for the percent non-wild, and 2) CLSI intermediate break point for the percent non-susceptible. Where no EUCAST or CLSI interpretative criteria were available, breakpoints were harmonised with those of the National Antimicrobial Resistance Monitoring System (NARMS), USA.

Identification of Asian feed stock powders

University of Adelaide tested feed stock powders for antimicrobial properties by disk diffusion assay. *Gram-positive* and *Gram-negative* bacteria were utilised.

Queensland Government Chemical Residue Laboratory tested feed stock powders by LC-MS to determine identity against laboratory standards.

Antibiotic and pesticide residue screening of imported prawn commodities

Queensland Government Chemical Residue Laboratory were sent 35 imported prawn samples to be examined by LC-MS using the modification of in-house method AB046) for the 58 different antimicrobial compounds and 159 different pesticides (Appendix 2), including those identified in Objective 2.

Samples were homogenised, then residues were extracted from a sub-sample using an acetonitrile/water mix which was centrifuged and the supernatant was divided into two portions. The first portion of the acetonitrile/water extract was concentrated and solvent exchanged using SPE (HLB) for Gas Chromatography determination. The second portion was defatted using hexane and cleaned-up using dispersive SPE (C18). This extract was used for the Liquid Chromatography determination of the majority of analytes listed in the report appendix. After the acetonitrile/water (supernatant) was removed from the original sub-sample, the remaining tissue plug was re-extracted using perchloric acid. This acid extract was cleaned-up using SPE (SCX). This extract was used for the determination of aminoglycosides.

The extracts were screened for the suite of analytes using a combination of chromatography systems. For LC compatible analytes, a Shimadzu LC-MSMS and a Thermo Fisher Q Exactive Orbitrap LC-MS system in ESI+ mode using reverse phase (C18) UPLC columns were used. The GC compatible analytes, OC pesticides and some of the OP/SP pesticides, were determined using a Shimadzu GC-MSMS with a DB5 equivalent column.

Where residues were detected, confirmation and quantification was performed using additional sub-samples and targeted extraction methods depending on the class of residue detected. In all cases identification of the positive detections was by LC-MSMS and quantitation was by comparison against matrix matched reference standards.

Results

Bacterial identification

Colonies were assessed for growth, enumerated and grouped into colony types within a commodity (data is provided in the **Appendix 3**). There were 154 colony types in total but it is worth noting that this is a total number across all the commodities and colony types are replicated between commodities. All 154 colony types were analysed for identification using the MALDI-TOF mass spectrometer (*Biotyper*, Bruker). There were 74 identifications made by the *Biotyper* (**Appendix 3**). All 154 tests did provide good quality data, However, there were 80 that were not able to be identified (classified as "unknown"); there was not a significant "hit" on the *Biotyper* database. These 80 "unknowns" were then grouped into distinct, colony types (there were 22 groups, one was chosen from each group - **Appendix 4**) and analysed, using 16S rDNA PCR and DNA sequencing to provide the bacterial identification. These identifications have been added to the complete list (**Appendix 3**).

A list of the aggregated bacterial identifications is given in **Table 1 below,** (with an indication of relevance of those bacteria with some significance to aquaculture or public health).

The major identifications as genus are shown in **Table 2** (numbers and percent). A one-look, qualitative overview of the bacteria identified per commodity is given **Table 3**. An assessment of the bacteria from this list that are of potential aquaculture and public health significant were chosen for Antibiotic Sensitively Testing (AST; using custom designed *TREK diagnostics Sensititre Veterinary* panels). This complete data set is provided in **Appendix 5, 6, 7.**

Table 1: A total aggregated list of bacterial isolate identifications

1. *Pseudomonas spp*	2. *Kocuria rhizophilia*
3. *Microbacterium maritypicum*	4. *Enterococcus faecalis*
5. *Chryseobacterium spp.*	6. *Enterococcus gilvus*
7. *Carnobacterium maltaromaticum*	8. *Enterococcus mundtii*
9. *Lactococcus garvieae*	10. *Sphingobacterium multivorum*
11. *Macrococcus caseolyticus*	12. *Enterococcus thailandicus*
13. *Staphylococcus sciuri*	14. *Psychrobacter sp.*
15. *Exiguobacterium sp*	16. *Exiguobacterium aurantiacum*
17. *Macrococcus brunensis*	18. *Brevundimonas diminuta*
19. *Macrococcus sp*	20. *Arthrobacter protophormiae*
21. *Enterococcus casseliflavus*	22. *Pseudomonas putida*
23. *Delftia acidovorans*	24. *Pseudomonas fluorescens*
25. *Carnobacterium gallinarum*	26. *Pseudomonas koreensis*
27. *Stenotrophomonas spp*	28. *Rothia marina*
29. *Psychrobacter arenosus*	30. *Psychrobacter maritimus*

31. *Microbacterium maritypicum*	32. *Bacillus vietnamensis*
33. *Brochothrix thermosphacta*	34. *Klebsiella pneumoniae*
35. *Bacillus pumilus*	36. *Pseudomonas brenneri*
37. *Pseudomonas gessardii*	38. *Lelliotta amnigena*
39. *Pseudomonas fragi*	40. *Pseudomonas extremorentalis*
41. *Pseudomonas tolaasii*	42. *Pseudomonas synxantha*
43. *Hafnia alvei*	44. *Pseudomonas lundensis*
45. *Pseudomonas libanensis*	46. *Pseudomonas taetrolens*
47. *Buttiauxella agretis*	

Blue – environment; and opportunistic human pathogen

Green – environmental

Red – spoilage

Purple – associated with aquaculture

Table 2. **Frequency of identifications, shown as number (% of total identifications)**

Frequent identifications; number (% of total).	
Pseudomonas sp.	8 (5.2)
Carnobacterium sp.	10 (6.5)
Microbacterium sp.	4 (2.6)
Lactococcus sp.	3 (2.1)
Chryseobacterium sp.	4 (2.6)
Psychrobacter sp.	5 (3.3)
Entercococcus sp.	11 (7.1)
Staphylococcus sp.	12 (7.8)
Macrococcus sp.	15 (9.7)
Other	25 (16.2)
Unknown	57 (37.0)

Table 3. A qualitative overview of the bacterial identifications per commodity. The bacterial species/genus identified across the first 24 commodities are shown. Unidentified bacteria are shown as UNK. Colour-code: *Pseudomonas* sp. (blue), *Carnobacterium* sp, (green), *Microbacterium* (brown), *Lactococcus* (pink), *Chryseobacterium* (orange), *Psychrobacter* sp. (pale blue), *Enterococcus* sp. (purple), *Staphylococcus* sp. (grey), *Macrococcus* sp. (yellow).

ML70	ML61	ML68	ML60	ML72	ML66
Pseudomonas spp	*Lactococcus garvieae*	*Staphylococcus sciuri*	*Macrococcus caseolyticus*	*Exiguobacterium sp*	*Enterococcus casseliflavus*
Microbacterium maritypicum	*Macrococcus caseolyticus*	UNK	UNK	*Macrococcus spp*	*Delftia acidovorans*
Carnobacterium maltaromaticum	*Exiguobacterium sp*	*Macrococcus caseolyticus*	*Lactococcus garvieae*	UNK	*Staphylococcus sciuri*
Chryseobacterium spp	UNK	*Lactococcus garvieae*	*Macrococcus brunensis*	*Carnobacterium maltaromaticum*	*Chryseobacterium spp*
UNK	*Microbacterium maritypicum*	*Chryseobacterium spp*			*Macrococcus caseolyticus*
					UNK
ML56	**ML71**	**ML67**	**ML69**	**NKE50**	**ML78**
Staphylococcus sciuri	*Macrococcus caseolyticus*	*Staphylococcus sciuri*	*Exiguobacterium aurantiacum*	*Staphylococcus sciuri*	UNK
Sphingobacterium multivorum	*Staphylococcus sciuri*	UNK	*Staphylococcus sciuri*	*Exiguobacterium aurantiacum*	*Arthrobacter protophormiae*
Kocuria rhizophilia	UNK	*Pseudomonas spp*	UNK	*Carnobacterium maltaromaticum*	*Staphylococcus sciuri*
UNK	*Enterococcus thailandicus*	*Macrococcus caseolyticus*	*Carnobacterium maltaromaticum*	*Enterococcus casseliflavus*	*Macrococcus caseolyticus*
Enterococcus faecalis		*Psychrobacter sp*		*Macrococcus caseolyticus*	*Arthrobacter protophormiae*
Enterococcus gilvus		*Carnobacterium maltaromaticum*		UNK	*Enterococcus faecalis*
Enterococcus mundtii		*Delftia acidovorans*		*Brevundimonas diminuta*	*Carnobacterium maltaromaticum*
		Enterococcus thailandicus			
VMC1	**ML59**	**NKE56**	**ML74**	**ML73**	**MLX**
Enterococcus faecalis	*Pseudomonas putida*	UNK	UNK	*Pseudomonas koreensis*	*Psychrobacter arenosus*
Psychrobacter sp	*Pseudomonas fluorescens*	*Pseudomonas fluorescens*		UNK	UNK

UNK	UNK	UNK		*Rothia marina*	*Carnobacterium maltaromaticum*
Carnobacterium maltaromaticum	*Macrococcus caseolyticus**	*Macrococcus brunensis*		*Stenotrophomonas spp*	
		Enterococcus casseliflavus			
		Carnobacterium gallinarum			
NKE62	**NKE3**	**ML62**	**ML77**	**VMC2**	**VMC3**
Psychrobacter maritimus	UNK	UNK	UNK	*Macrococcus caseolyticus*	*Pseudomonas spp*
	Carnobacterium maltaromaticum	*Bacillus vietnamensis*	*Pseudomonas gessardii*		*Klebsiella pneumoniae*
	Microbacterium maritypicum	*Pseudomonas fluorescens*	*Pseudomonas proteolytica*		UNK
		Brochothrix thermosphacta			*Macrococcus caseolyticus*
					Chryseobacterium spp

Antibiotic Sensitivity Testing (AST) and Antimicrobial Resistance Gene analysis

No antimicrobial resistance was identified in any of the isolates tested. This analysis included all the isolates which were considered to be of potential aquaculture or public health importance.

Due to the absence of detected AMR in the AST, samples were not analysed for presence of AMR genes.

Identification of Asian stock feed powders

VS 300W-B1 and Scan Viron were both found to exhibit potent antimicrobial properties in the disk diffusion assays.

Subsequent LC-MS testing revealed each contained an antimicrobial compound (Appendix 8- CRL Report Number 10064, 08/02/18).

VS 300W-B1: Contained Enrofloxacin (Fluoroquinolone)

Scan Viron: Contained Ceftriaxone (Third generation Cephalosporin)

Antibiotic and pesticide residue screening of imported prawn commodities

Nine of the 35 commodities were found to contain one or more residues with a total of 16 different residues detected. Chemicals identified included the antibiotics: oxytetracycline (0.004 - 0.016mg kg^{-1}); tetracycline (0.002mg kg^{-1}); chlortetracycline (0.004mg kg^{-1}); doxycycline (0.004-0.011mg kg^{-1}); trimethoprim (0.001mg kg^{-1}); sulphamethoxazole (trace - 0.053mg kg^{-1}) and the herbicide diuron (0.01 - 0.019mg kg^{-1}).

Complete list of results in Appendix 8.

Discussion and Implications

Bacterial identification

The pathway for uncooked prawn commodities imported for human consumption subsequently entering Australian waterways via their use or disposal when used as berley and/or bait, has been clearly established. Whilst efforts to encourage recreational anglers not to use product intended for human consumption may reduce this behaviour, it is almost certain that it will not be eliminated. The convenience of access to uncooked prawns at supermarkets, and their cheaper price point compared to bait prawns will continue to influence purchase of bait by recreational fishers.

Numerous breaches of labelling requirements have already been detected at supermarkets selling uncooked prawns in the delicatessen section, where the signage "Not to be used as bait" has not been present. It is worth noting that this signage was present on the bags of uncooked prawns being used in the Logan River by anglers. So it is clearly an inadequate measure to entirely mitigate the risk. Hence the risk pathway for release of pathogens, should the imported prawn commodities be carrying them, remains a significant threat to Australian aquatic biosecurity. This pathway remains the most likely explanation for the entry of White Spot Syndrome Virus into waterways leading to the 2016-2017 outbreak on the Logan River prawn farms.

The detection of live bacteria on imported frozen uncooked prawns is therefore also of significant concern, as the release pathway into Australian waters has not been meaningfully mitigated.

Of those bacteria identified, there were species present which could be of significance to other species in aquaculture, such as finfish. *Lactococcus garvieae* is a recognised and significant pathogen of Yellowtail Kingfish and Rainbow Trout (Meyburgh, et al., 2017). To date it has not caused significant disease in the emerging Australian Yellowtail Kingfish industry. However, it continues to cause major disease issues and economic costs in the Japanese yellowtail industry where the industry has been forced to vaccinate and use a range of antimicrobials on feed to control losses. The pathogen has appeared to evolve there such that previously effective vaccines are now are failing before fish are harvested. Introduction of new strains to Australia could be of biosecurity significance and poses a risk which has not been previously considered in relation to importation of uncooked prawn commodities. The pathogen is not exotic to Australia with isolations from trout reported in NSW, Victoria and Tasmania. The similarity of these isolates to those found on the prawn commodities was not explored within the context of this project. The organism is known to be possess a diversity of toxin and virulence genes, which are likely to vary between strains globally.

Pseudomonas putida and *Carnobacterium maltaromaticum* have been described to be associated with fish diseases (Altinok, et al., 2006) (Smolowitz, et al., 1998) (Loch, et al., 2011). *Carnobacterium maltaromaticum* has also been associated with meningoencephalitis in wild salmon shark strandings in California (Schaffer, et al., 2013). *C. maltaromaticum* has also been infrequently isolated from sick salmonids in Australia. Again, the similarity of these isolates to those found on the prawn commodities was not explored within the context of this project. However, the organism is known to be able to possess a diversity of potential toxin producing genes, so strain differences are a distinct possibility.

It is notable that all three of these identified aquaculture pathogens have been associated with disease outbreaks in farmed salmonids outside of Australia. Farmed salmonids represent the most valuable aquaculture sector in Australia. The success of the industry has been achieved through remaining free from some of the internationally important diseases of salmonids and controlling the strains of disease which are endemic. New strains of bacteria may render existing vaccines ineffective and pose

additional risks to the sustainability of the salmonid industry both in Tasmania and on the mainland of Australia.

The biosecurity barrier in relation to uncooked imported prawns could be improved through requiring all incoming prawns to be cooked. This would have a multiple benefits:

1) It would kill any virus or bacterial load in the tissues

2) It would make the product undesirable for anglers to use as bait. For anglers almost universally prefer to use uncooked prawns for bait. Cooking imported product would remove a significant amount of product from ending up being released into Australian waterways, when it was intended for human consumption only.

Antimicrobial resistance

The project failed to identify any evidence for significant AMR associated with live microbes isolated from the imported uncooked prawn commodities. The lack of isolations of common prawn associated microbes, suggests processing sanitation procedures have removed these organisms from superficial surface of commodities.

It is acknowledged that many microbes are not readily culturable, so there remains a potential for some AMR genes to have remained unidentified through this project. Modern microbiome analysis that also targets and screens for antimicrobial resistance genes in microbes that cannot be cultured or that are no longer viable would provide further information.

Identification of Asian stock feed powders

The identification of two powders which were in use on Asian prawn feed, as potent antimicrobials, which are listed by the World Health Organisation (WHO) as the highest priority, Critically Important[2], antibiotics is of serious concern.

The products were not labelled as antibiotics and did not provide any advice on appropriate use. Such use is contraindicated and is of **global** concern. The compounds identified are critically important for human treatments, as there are currently no alternatives for them to treat many serious human infections. Hence, development of resistance to these high level antibiotics, through irresponsible use in production animals, could be seriously detrimental to public health.

The National Residue Survey (NRS) should consider adding these compounds to the screening program for imported seafood as neither are currently listed (Current NRS Survey Seafood List: Appendix 10).

Antibiotic and pesticide residues

The detection of low levels of multiple antibiotics in imported prawns is cause for food safety concern. The prawn species, where able to be confirmed with appropriate bag labelling, was uniformly *Litopenaeus vannamei*. Codex does not list MRL's for any of the compounds detected in this project, for this particular species of animal. FSANZ similarly has not established Acceptable Daily Intake limits either.

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[2] http://www.who.int/foodsafety/publications/cia2017.pdf

Where MRL's are not established for a chemical in a food product, the acceptable limit is generally considered to be below the limit of laboratory detection. Hence these detections in 25% of the tested imported uncooked prawn commodities represent significant cause for concern. They also draw into question the sensitivity of the National Residue Survey which has not reported results of any similar magnitude.

It is important to note also that in 4 of the 35 commodities tested there were multiple different antibiotic residues present. Such mixtures may lead to unanticipated additive or synergistic impacts. In terms of appropriate food safety regulation, such mixtures need to be considered in aggregate, and not individually in isolation.

FSANZ should consider whether the residues detected should lead to a restriction of trade, actions against the importer, product recalls and the implementation of an enhanced surveillance program.

It should be recognised that this is a small snapshot of commodities which are being imported, and may not be representative of the microbial status, AMR status or residue status of all imported material.

Conclusions

1. Bacterial culture of imported uncooked prawns yielded results which are useful to inform Australian Biosecurity policy and risk frameworks.

2. Screening for AMR has provided some of the first data on the AMR status of imported seafood in Australia.

3. Stock feed powder analysis has revealed a serious misuse of the potent antibiotics, enrofloxacin and ceftriaxone, in Asian prawn farms.

4. Residue status of 25% of tested imported uncooked prawns revealed a range of antibiotic residues which do not meet current Australian food safety guidelines.

Recommendations

1. The National Residue Survey should consider adding the two antibiotics identified in Asian stock feed powders to the routine screening program for imported seafood.

2. FSANZ should consider whether the residues detected should lead to a restriction of trade, actions against the importer, product recalls and the implementation of an enhanced surveillance program.

3. Imported finfish should be subjected to a similar research study to better appreciate the risks associated with this import entry pathway for pathogens, AMR and food safety risks such as chemical residues.

4. Data from this project should be considered within the Commonwealth review of the Import Risk associated with imported uncooked prawn commodities.

5. Biosecurity Australia should consider improved sanitary measures such as cooking prawns which could eliminate the entry of potential aquaculture and wild fishery pathogens on uncooked prawns which continue to be diverted from human consumption and used as bait by recreational fishers. Such a measure would also discourage anglers from using prawns which were destined for human consumption, into use as angling bait.

Extension and Adoption

Results of testing have been distributed to the APFA Executive and R&D Committee.

Any further distribution of results will be at direction of APFA.

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Appendix 1 – Project staff

Field sample collection and Principal Investigator:

Matt Landos - Future Fisheries Veterinary Service Pty Ltd

Bacterial Identification and AMR analysis and Co-investigators: Darren Trott; Stephen Kidd; Tania Veltman – University of Adelaide

Stock feed powder identification and antibiotic and pesticide residue analysis and Co-investigators: Stephen Were; Cindy Giles – Queensland Government, Chemical Residue Laboratory.

Project administration: APFA

Appendix 2 – Chemical anolytes

Limits of Reporting (LOR) for Residues in Prawns (Modification of in-house method AB046)

Tetracyclines	
Analyte	**LOR (mg/Kg)**
Oxytetracycline	0.01
Tetracycline	0.01
Chlortetracycline	0.02
Doxycycline	0.01

Aminoglycosides	
Analyte	**LOR (mg/Kg)**
Gentamycin	0.05
Apramycin	0.05
Neomycin	0.05
Streptomycin	0.1
Dihydrostreptomycin	0.1

Beta Lactams	
Analyte	**LOR (mg/Kg)**
Amoxicillin	0.02
Ampicillin	0.01
Penicillin G.	0.02
Cloxacillin	0.02

Cephalosporins	
Analyte	**LOR (mg/Kg)**
Ceftiofur	0.2
Cephalonium	0.1
Cefuroxime	0.25
Ceftriaxone	0.1

Sulphonamides	
Analyte	**LOR (mg/Kg)**
Sulfadimidine	0.01
Sulfadoxine	0.02
Sulfafurazole	0.02
Sulfamerazine	0.02
Sulfamethoxazole	0.02
Sulfameter	0.02
Sulfamethoxypyridazine	0.02
Sulfapyridine	0.02
Sulfathiazole	0.02
Sulfatroxazole	0.02
Sulfadimethoxine	0.02
Sulfadiazine	0.01
Sulfachloropyridazine	0.02
Sulphaquinoxaline	0.02

Macrolides / Lincosamides / Triamilides	
Analyte	**LOR (mg/Kg)**
Avilamycin	0.05
Tulathromycin	0.2
Erythromycin	0.05
Lincomycin	0.05
Oleandomycin	0.05
Tilmicosin	0.05
Tylosin	0.1
Virginiamycin	0.05

Miscellaneous / other antibiotics	
Analyte	**LOR (mg/Kg)**
Trimethoprim	0.01
Chloramphenicol	0.01
Malachite Green	0.01

Leucomalachite green	0.01
Furazolidone	0.01

Quinolones	
Analyte	**LOR (mg/Kg)**
Danofloxacin	0.005
Norfloxacin	0.005
Ciprofloxacin	0.005
Difloxacin	0.005
Enrofloxacin	0.005
Flumequin	0.005
Ofloxacin (Levoflaxacin)	0.005
Lomefloxacin	0.005
Marbofloxacin	0.005
Moxifloxacin	0.005
Naladixic Acid	0.005
Orbifloxacin	0.005
Oxolinic Acid	0.005
Sarafloxacin	0.005
Gatifloxacin	0.005

Synthetic Pyrethroids	
Analyte	**LOR (mg/Kg)**
Bifenthrin	0.02
Cypermethrin	0.02
Cyfluthrin	0.02
Deltamethrin	0.02
Fenvalerate	0.02
Permethrin	0.02
Transfluthrin	0.02

OP Pesticides	
Analyte	**LOR (mg/Kg)**
Acephate	0.02
Azamethiphos	0.02
Azinphos-methyl	0.02
Cadusafos	0.02

OP Pesticides (cont)	
Analyte	**LOR (mg/Kg)**
Coumaphos	0.02
Demeton-S-methyl	0.02
Diazinon	0.02
Dichlorvos	0.02
Dimethoate	0.02
Disulfoton	0.02
Ethion	0.02
Ethoprophos	0.02
Fenamiphos	0.02
Fenchlorphos	0.05
Fenitrothion	0.02
Fenthion	0.02
Maldison (Malathion)	0.02
Methamidophos	0.02
Methacrifos	0.02
Methidathion	0.02
Mevinphos	0.02
Monocrotophos	0.02
Omethoate	0.02
Parathion	0.02
Parathion-methyl	0.02
Phorate	0.02
Phosmet	0.02
Profenofos	0.02
Pirimiphos-methyl	0.02
Prothiofos	0.02
Sulprofos	0.02
Temephos	0.02
Terbufos	0.02
Tetrachlorvinphos	0.02

Carbophenothion	0.02
Chlorfenvinphos	0.02
Chlorpyifos	0.02
Chlorpyifos-methyl	0.02

OC Pesticides	
Analyte	**LOR (mg/Kg)**
Aldrin	0.02
Dieldrin	0.02
cis-Chlordane	0.02
trans-Chlordane	0.02
p,p' DDD	0.02
p,p' DDE	0.02
p,p' DDT	0.02
o,p' DDT	0.02
Endosulfan-alpha	0.02
Endosulfan-beta	0.02
Endosulfan sulfate	0.02
BHC-alpha	0.02
BHC-beta	0.02
BHC-delta	0.02
Heptachlor	0.02
Heptachlor epoxide	0.02
Lindane	0.02
Methoxychlor	0.02

Fungicides	
Analyte	**LOR (mg/Kg)**
Azoxystrobin	0.02
Benalaxyl	0.02
Boscalid	0.02
Bitertanol	0.02
Carbendazin	0.02
Cyflufenamid	0.02
Cyprodinil	0.02
Dichlofluanid	0.02
Difenoconazole	0.02

Fungicides (cont.)	
Analyte	**LOR (mg/Kg)**
Flutriafol	0.02
Fluxapyroxad	0.02
Furalaxyl	0.02
Imazalil	0.02
Iprodione	0.02
Metalaxyl	0.02
Myclobutanil	0.02
Oxadixyl	0.02
Oxythioquinox	0.02
Penconazole	0.02
Penthiopyrad	0.02
Prochloraz	0.02
Procymidone	0.02
Propiconazole	0.02
Pyraclostrobin	0.02
Pyrimethanil	0.02
Quintozene	0.02
Quinoxyfen	0.02
Tebuconazole	0.02
Thiabendazole	0.02
Tolylfluanid	0.02
Triadimenol	0.02
Triadimefon	0.02
Trifloxystrobin	0.02
Vinclozolin	0.02

Dimethomorph	0.02
Fenarimol	0.02
Fenhexamid	0.02
Fludioxonil	0.02
Fluquinconazole	0.02
Flusilazole	0.02

Herbicides	
Analyte	**LOR (mg/Kg)**
Atrazine	0.02
Bromoxynil	0.02
Diflufenicam	0.02
Diuron	0.02
Haloxyfop methyl	0.02
Hexazinone	0.02
Imazapic	0.02
Linuron	0.02
Metolachlor	0.02
Metribuzin	0.02
Metsulfuron methyl	0.02
Oxyfluorfen	0.02
Pendimethalin	0.02
Propanil	0.02
Propyzamide	0.02
Tebuthiuron	0.02
Trifluralin	0.02

Other pesticides	
Analyte	**LOR (mg/Kg)**
Abamectin	0.02
Bifenazate	0.02
Bendiocarb	0.02
Buprofezin	0.02
Bupirimate	0.02
Carbaryl	0.02
Carbofuran	0.02
Carbosulfan	0.02

Other pesticides (cont.)	
Analyte	**LOR (mg/Kg)**
Forchlorfenuron	0.02
Furazolidone	0.02
Hexythiazox	0.02
Imidacloprid	0.02
Indoxacarb	0.02
Methiocarb	0.02
Methomyl	0.02
Methoxyfenozide	0.02
Oxamyl	0.02
Piperonyl butoxide	0.02
Pirimicarb	0.02
Propargite	0.02
Propoxur	0.02
Pyriproxyfen	0.02
Spinosad	0.02
Spirotetramat	0.02
Tebufenozide	0.02
Tebufenpyrad	0.02
Tetradifon	0.02
Thiacloprid	0.02
Thiamethoxam	0.02
Thiodicarb	0.02
Triflumuron	0.02
Triazophos	0.02

Chlorfenapyr	0.02
Clofentezine	0.02
Clothianidin	0.02
Diflubenzuron	0.02
Fenoxycarb	0.02
Fenpyroximate	0.02
Fipronil	0.02

Appendix 3: Data set of bacterial colony enumeration and identification.

		Nutrient Agar	Chrom ESBL	MacConkey	TCBS	
Commodity #	**Colony Ref #**	*CFU/ml*	*CFU/ml*	*CFU/ml*	*CFU/ml*	**isolate identification (Biotyper or 16S rDNA seq)**
ML70	ML70-01-001	n	10	no growth	no growth	*Pseudomonas spp*
	ML70-01-002	2.2x10^3	-			*Microbacterium maritypicum*
	ML70-01-003	1.4x10^3	-			*Microbacterium maritypicum*
	ML70-01-004	3.0x10^2	-			*Chryseobacterium spp.*
	ML70-01-005	1.5x10^3	-			Uncultured bacterium
	ML70-01-006	1.0x10^3	-			*Carnobacterium maltaromaticum*
ML61	ML61-02-001	n	no growth	120	no growth	*Lactococcus garvieae*
	ML61-02-002	n		50		*Macrococcus caseolyticus*
	ML61-02-003	2				*Exiguobacterium sp*
	ML61-02-004	3				UNK
	ML61-02-005	38				UNK
	ML61-02-006	24				*Microbacterium maritypicum*
	ML61-02-007	58				UNK
ML68	ML68-03-001	n	no growth	see NUT	10	*Staphylococcus sciuri*
	ML68-03-002	n				UNK
	ML68-03-003	3				*Macrococcus caseolyticus*
	ML68-03-004	n				UNK
	ML68-03-005	45.2x10^3				*Chryseobacterium spp.*
	ML68-03-006	78.0x10^3				*Lactococcus garvieae*
ML60	ML60-04-001	n	no growth	30	no growth	*Macrococcus caseolyticus*
	ML60-04-002	n		10		UNK
	ML60-04-003	10				*Macrococcus caseolyticus*
	ML60-04-004	11				Uncultured environmental isolate
	ML60-04-005	2				*Macrococcus brunensis*
	ML60-04-006	56				*Lactococcus garvieae*
ML72	ML72-05-001	2	no growth		no growth	*Exiguobacterium sp*
	ML72-05-002	4				*Macrococcus sp.*
	ML72-05-003	2.4x10^3				UNK
	ML72-05-004	4.5x10^3				*Carnobacterium maltaromaticum*
ML66	ML66-06-001	n	no growth		10	*Enterococcus casseliflavus*
	ML66-06-002	n		10		*Delftia acidovorans*
	ML66-06-003	n		60		*Staphylococcus sciuri*
	ML66-06-004	n		140		*Enterococcus casseliflavus*
	ML66-06-005	6				*Macrococcus caseolyticus*

	ML66-06-006	7				*Staphylococcus sciuri*
	ML66-06-007	11.0x10^3				UNK
	ML66-06-008	16.0x10^3				*Chryseobacterium spp.*
ML56	ML56-07-001	n			20	*Staphylococcus sciuri*
	ML56-07-002	24				*Staphylococcus sciuri*
	ML56-07-003	1.0x10^2				*Kocuria rhizophilia*
	ML56-07-004	2x10^3	no growth			Uncultured environmental isolate
	ML56-07-005	4x10^3				*Enterococcus faecalis*
	ML56-07-006	40x10^3				*Enterococcus gilvus*
	ML56-07-007	36x10^3				*Enterococcus mundtii*
	ML56-07-008	n		40		*Sphingobacterium multivorum*
ML71	ML71-08-001	7				*Macrococcus caseolyticus*
	ML71-08-002	3				*Staphylococcus sciuri*
	ML71-08-003	2				UNK
	ML71-08-004	56	no growth		no growth	NP
	ML71-08-005	n		30		*Staphylococcus sciuri*
	ML71-08-006	n		80		*Enterococcus thailandicus*
ML67	ML67-09-001	n			10	*Staphylococcus sciuri*
	ML67-09-002	n			10	UNK
	ML67-09-003	2				*Pseudomonas spp*
	ML67-09-004	2x10^2				*Macrococcus caseolyticus*
	ML67-09-005	10x10^2				*Psychrobacter sp.*
	ML67-09-006	56x10^2				*Carnobacterium maltaromaticum*
	ML67-09-007	n	70			*Delftia acidovorans*
	ML67-09-008	n	70			*Enterococcus thailandicus*
ML69	ML69-10-001	n			10	*Exiguobacterium aurantiacum*
	ML69-10-002	n			140	*Staphylococcus sciuri*
	ML69-10-003	n			50	UNK
	ML69-10-004	n			20	*Exiguobacterium aurantiacum*
	ML69-10-005	n	no growth		20	*Exiguobacterium aurantiacum*
	ML69-10-006	1x10^3				UNK
	ML69-10-007	96x10^3				UNK
	ML69-10-008	31x10^3				*Carnobacterium maltaromaticum*
NKE50	NKE50-11-001	n			210	*Staphylococcus sciuri*
	NKE50-11-002	n			10	*Exiguobacterium aurantiacum*
	NKE50-11-003	n			180	*Staphylococcus sciuri*
	NKE50-11-004	n			20	*Enterococcus casseliflavus*
	NKE50-11-005	13x10^3				*Macrococcus caseolyticus*
	NKE50-11-006	3x10^3	no growth			UNK
	NKE50-11-007	1x10^3				*Brevundimonas diminuta*
	NKE50-11-008	81x10^4				*Carnobacterium maltaromaticum*
	NKE50-11-009	n			40	*Exiguobacterium aurantiacum*
ML78	ML78-12-001	n			20	UNK

	ML78-12-002	n			70	*Arthrobacter protophormiae*
	ML78-12-003	n			40	*Staphylococcus sciuri*
	ML78-12-004	8x10^3				*Macrococcus caseolyticus*
	ML78-12-005	4x10^3				*Arthrobacter protophormiae*
	ML78-12-006	2x10^3				*Enterococcus faecalis*
	ML78-12-007	14x10^3				UNK
	ML78-12-008	2x10^4				*Carnobacterium maltaromaticum*
VMC1	VMC1-13-001	n			60	*Enterococcus faecalis*
	VMC1-13-002	n		10		*Psychrobacter sp.*
	VMC1-13-003	n		10		UNK
	VMC1-13-004	7x10^3				UNK
	VMC1-13-005	5x10^3				UNK
	VMC1-13-006	6x10^3				UNK
	VMC1-13-007	12x10^3				UNK
	VMC1-13-008	52.8x10^5				NP
	VMC1-13-009	33.6x10^5				*Carnobacterium maltaromaticum*
ML59	ML59-14-001	n	40			*Pseudomonas putida*
	ML59-14-002	n	10			*Pseudomonas fluorescens*
	ML59-14-003	4x10^3				UNK
	ML59-14-004	4x10^3				*Macrococcus caseolyticus*
	ML59-14-005	5x10^4			no growth	*Psychrobacter sp.*
	ML59-14-006	13x10^4				UNK
	ML59-14-007	43x10^4				UNK
	ML59-14-008	76x10^4				UNK
NKE56	NKE56-15-001	n	20			UNK
	NKE56-15-002	n	10			*Pseudomonas fluorescens*
	NKE56-15-003	1x10^4				UNK
	NKE56-15-004	1x10^4				*Macrococcus brunensis*
	NKE56-15-005	1x10^4			no growth	UNK
	NKE56-15-006	1x10^2				UNK
	NKE56-15-007	11x10^2				UNK
	NKE56-15-008	20x10^4				*Enterococcus casseliflavus*
	NKE56-15-009	22.8x10^5				*Carnobacterium gallinarum*
ML74	ML74-16-001	n	10			UNK
	ML74-16-002	40x10^3				Unknown baterium
	ML74-16-003	60x10^3				UNK
	ML74-16-004	20x10^3			no growth	UNK
	ML74-16-005	10x10^3				UNK
	ML74-16-006	10.4x10^5				UNK
ML73	ML73-17-001	n	70			*Pseudomonas koreensis*
	ML73-17-002	n	20			UNK
	ML73-17-003	2x10^3			no growth	UNK
	ML73-17-004	1x10^4				UNK

	ML73-17-005	3x10^3				*Stenotrophomonas spp*
	ML73-17-006	2x10^4				UNK
	ML73-17-007	1x10^4				*Rothia marina*
	ML73-17-008	87x10^4				UNK
MLX	MLX-18-001	14x10^2				*Psychrobacter arenosus*
	MLX-18-002	85x10^4			no growth	UNK
	MLX-18-003	81.6x10^5				*Carnobacterium maltaromaticum*
NKE62	NKE62-19-001	44	NG		no growth	*Psychrobacter maritimus*
NKE3	NKE3-20-001	2				UNK
	NKE3-20-002	26				UNK
	NKE3-20-003	1x10^4	no growth		no growth	UNK
	NKE3-20-004	4x10^3				*Microbacterium maritypicum*
	NKE3-20-005	16x10^4				*Carnobacterium maltaromaticum*
ML62	ML62-21-001	n	2200			UNK
	ML62-21-002	1				*Bacillus vietnamensis*
	ML62-21-003	5x10^3				*Pseudomonas fluorescens*
	ML62-21-004	68x10^4			no growth	UNK
	ML62-21-005	27x10^4				*Brochothrix thermosphacta*
	ML62-21-006	88x10^4				UNK
	ML62-21-007	3x10^4				*Brochothrix thermosphacta*
ML77	ML77-22-001	n		180		UNK
VMC2	VMC2-23-001	2x10^2				*Macrococcus caseolyticus*
	VMC2-23-002	17x10^2				UNK
	VMC2-23-003	88x10^4	no growth		no growth	UNK
	VMC2-23-004	12.4x10^5				UNK
VMC3	VMC3-24-001	n	220			*Pseudomonas sp.*
	VMC3-24-002	n		20		*Klebsiella pneumoniae*
	VMC3-24-003	n		30		UNK
	VMC3-24-004	17				*Macrococcus caseolyticus*
	VMC3-24-005	54x10^2				UNK
	VMC3-24-006	1x10^3			no growth	UNK
	VMC3-24-007	6x10^3				UNK
	VMC3-24-008	19x10^3				UNK
	VMC3-24-009	52				*Chryseobacterium spp*
NKE32	NKE32-25-001					*Bacillus pumilus*
	NKE32-25-002					*Pseudomonas brenneri*
	NKE32-25-003					*Pseudomonas gessardii*
	NKE32-25-004					*Lelliotta amnigena*
	NKE32-25-005					*Pseudomonas gessardii*
	NKE32-25-006					*Pseudomonas fragi*
	NKE32-25-007					*Carnobacterium maltaromaticum*
ML54	ML54-26-001					*Pseudomonas koreensis*

	ML54-26-002					*Pseudomonas extremorientalis*
	ML54-26-003					*Carnobacterium maltaromaticum*
	ML54-26-004					*Pseudomonas spp*
	ML54-26-005					*Pseudomonas brenneri*
NKE8	NKE8-27-001					*Pseudomonas spp*
	NKE8-27-002					*Pseudomonas extremorientalis*
	NKE8-27-003					*Pseudomonas tolaasii*
	NKE8-27-004					*Pseudomonas tolaasii*
	NKE8-27-005					*Pseudomonas synxantha*
	NKE8-27-006					*Hafnia alvei*
NKE7	NKE7-28-001					*Pseudomonas lundensis*
	NKE7-28-002					*Pseudomonas proteolytica*
	NKE7-28-003					*Carnobacterium maltaromaticum*
	NKE7-28-004					*Pseudomonas libanensis*
	NKE7-28-005					*Pseudomonas spp*
	NKE7-28-006					*Pseudomonas taetrolens*
ML38	ML38-29-001					*Pseudomonas brenneri*
	ML38-29-002					*Pseudomonas libanensis*
	ML38-29-003					*Bacillus cereus*
	ML38-29-004					*Macrococcus caseolyticus*
	ML38-29-005					*Pseudomonas fluorescens*
	ML38-29-006					*Pseudomonas gessardii*
NKE35	NKE35-30-001					*Pseudomonas gessardii*
	NKE35-30-002					*Pseudomonas spp*
	NKE35-30-003					*Serratia liquefaciens*
	NKE35-30-004					*Pseudomonas spp*
	NKE35-30-005					*Buttiauxella agrestis*
NKE61	NKE61-31-001					*Pseudomonas extremorientalis*
	NKE61-31-002					*Pseudomonas fluorescens*
	NKE61-31-003					UNK
	NKE61-31-004					*Pseudomonas fluorescens*
	NKE61-31-005					*Pseudomonas fragi*
NKE36	NKE36-32-001					*Pseudomonas fluorescens*
	NKE36-32-002					*Pseudomonas libanensis*
	NKE36-32-003					*Pseudomonas libanensis*
	NKE36-32-004					*Pseudomonas libanensis*
NKE41	NKE41-33-001					*Pseudomonas protegens*
	NKE41-33-002					*Pseudomonas lundensis*
	NKE41-33-003					*Pseudomonas gessardii*
	NKE41-33-004					*Pseudomonas gessardii*
ML75	ML75-34-001					*Pseudomonas fluorescens*
	ML75-34-002					*Pseudomonas proteolytica*
	ML75-34-003					*Pseudomonas extremorientalis*

	ML75-34-004					*Stenotrophomonas sp*
	ML75-34-005					*Pseudomonas libanensis*
ML53	ML53-35-001					*Pseudomonas koreensis*
	ML53-35-002					*Serratia liquefaciens*
	ML53-35-003					UNK
	ML53-35-004					UNK
	ML53-35-005					*Serratia liquefaciens*
	ML53-35-006					*Pseudomonas koreensis*
ML79a	ML79a-36-001					*Pseudomonas fluorescens*
	ML79a-36-002					*Pseudomonas lundensis*
	ML79a-36-003					*Pseudomonas rhodesiae*
	ML79a-36-004					*Pseudomonas extremorientalis*
	ML79a-36-005					*Pseudomonas rhodesiae*
	ML79a-36-006					*Pseudomonas extremorientalis*
	ML79a-36-007					*Pseudomonas extremorientalis*

N – no growth; NG no growth; UNK unknown

Appendix 4: The colony groups from the "unknown" *Biotyper* results.

16S rDNA sequencing was used to identify one bacterial stock from each group.

Group #	Colony Ref#	Bacterial ID
T1	ML70-01-004	*Chryseobacterium* sp.
	ML61-02-005	
	NKE3-20-003	
	ML62-21-004	
T2	ML70-01-005	Uncultured bacterium
	ML61-02-004	
	ML61-02-007	
	ML68-03-002	
	ML72-05-003	
	ML67-09-002	
	NKE50-11-006	
T3	ML68-03-005	*Chryseobacterium* sp.
T4	ML60-04-004	Uncultured environmental isolate
	ML78-12-007	
	VMC1-13-005	
	NKE56-15-007	
	MLX-18-002	
	NKE3-20-001	
T5	ML60-04-005	*Macrococcus brunensis*
T6	ML72-05-002	*Macrococcus* sp.
T7	ML66-06-008	*Chryseobacterium* sp.
	ML69-10-007	
	ML74-16-005	
T8	ML68-03-004	
	ML56-07-004	Uncultured environmental isolate
	ML78-12-001	
	ML73-17-004	
T9	ML67-09-005	*Psychrobacter* sp.
	VMC1-13-007	
T10	VMC1-13-002	*Psychrobacter* sp.
	VMC3-24-007	
T11	ML59-14-001	*Pseudomonas putida*
	NKE56-15-005	
T12	VMC1-13-004	
	ML59-14-005	*Psychrobacter* sp.
	ML74-16-003	
	VMC3-24-006	
T13	NKE56-15-002	*Pseudomonas fluorescens*
	ML73-17-002	
T14	ML71-08-003	
	ML69-10-006	
	ML59-14-003	
	NKE56-15-003	
	NKE56-15-004	*Macrococcus brunensis*
	ML73-17-003	
T15	ML66-06-007	
	ML71-08-004	
	VMC1-13-008	
	ML59-14-008	
	NKE56-15-009	*Carnobacterium gallinarum*
	ML74-16-006	
	ML73-17-008	
	VMC2-23-004	
	ML62-21-006	
	VMC3-24-008	

T16	ML60-04-002	
	ML74-16-002	unknnown
	VMC3-24-003	
T17	ML59-14-007	
	NKE56-15-006	
	ML73-17-007	*Rothia marina*
T18	VMC1-13-006	
	ML73-17-006	
	MLX-18-001	*Psychrobacter arenosus*
	NKE3-20-002	
	VMC2-23-003	
	VMC3-24-005	
T19	ML59-14-006	
	ML74-16-004	
	NKE62-19-001	*Psychrobacter maritimus*
T20	ML62-21-002	*Bacillus vietnamensis*
T21	ML69-10-003	
	VMC1-13-003	
	NKE56-15-001	
	ML74-16-001	
	ML62-21-001	
	ML77-22-001	
	VMC2-23-002	
	VMC3-24-001	*Pseudomonas* sp.
T22	VMC3-24-009	*Chryseobacterium* sp.

Appendix 5: Minimum inhibitory concentration (MIC) distribution of *Carnobacterium maltaromaticum*

Isolates identified in uncooked frozen prawns. (n=12)

	Number and percentage of isolates with MICs (mg/L) at: [a]															
Antimicrobial agent	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	**>2048**
Ciprofloxacin	11	0	1													
	91.7		8.3													
Chloramphenicol					11	1										
					91.7	8.3										
Daptomycin		4	5	2	0	1										
		33.3	41.7	16.7		8.3										
Erythromycin		12														
		100														
Gentamicin											12					
											100					
Kanamycin											12					
											100					
Lincomycin				7	4	1										
				58.3	33.3	8.3										
Linezolid			10	2												
			83.3	16.6												
Quinuprisrin-dalfopristin			12													
			100													
Streptomycin (high-level)													12			
													100			
Penicillin		12														
		100														
Nitrofurantoin					4	6	2									
					33.3	50.0	16.7									
Tetracycline				10				2*								

				83.3				16.7								
Vancomycin		2	5	4	1											
		16.7	41.7	33.3	1.2											
Tylosin tartrate		12														
		100														
Tigecycline	12															
	100															

[a] Unshaded areas indicate MIC range for each agent available on the Sensititre CMV3AGPF card. MICs > than highest concentration available are indicated in the shaded region
Vertical green lines indicate EUCAST epidemiological cut-off (ECOFF) values; CLSI susceptible (blue) and resistant (red) breakpoints (for closest Gram positive); NARMS breakpoints (dashes)

* Non-relevant antibiotic; bacteria contains an intrinsic resistance.

Appendix 6: Minimum inhibitory concentration (MIC) distribution of *Enterococcus facalis*

Isolates identified in uncooked frozen prawns. (n=3)

	Number and percentage of isolates with MICs (mg/L) at: [a]															
Antimicrobial agent	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	**>2048**
Ciprofloxacin					3											
					100											
Chloramphenicol							3									
							100									
Daptomycin					1	2										
					33.3	67.7										
Erythromycin			1	1	2											
			33.3	33.3	33.3											
Gentamicin											3					
											100					
Kanamycin											3					
											100					
Lincomycin							3*									
							100									
Linezolid					3											
					100											
Quinuprisrin-dalfopristin							3*									
							100									
Streptomycin (high-level)													3			
													100			
Penecillin						3										
						100										
Nitrofurantoin								3								
								100								
Tetracycline				3												

				100												
Vancomycin				2	1											
				67.7	33.3											
Tylosin tartrate					3											
					100											
Tigecycline	3															
	100															

[a] Unshaded areas indicate MIC range for each agent available on the Sensititre CMV3AGPF card. MICs > than highest concentration available are indicated in the shaded region
Vertical green lines indicate EUCAST epidemiological cut-off (ECOFF) values; CLSI susceptible (blue) and resistant (red) breakpoints (for Enterococcus); NARMS breakpoints (dashes)

* Non-relevant antibiotic; bacteria contains an intrinsic resistance.

Appendix 7: Minimum inhibitory concentration (MIC) distribution of *Lactococcus garvieae*

Isolates identified in uncooked frozen prawns. (n=3)

	Number and percentage of isolates with MICs (mg/L) at: [a]															
Antimicrobial agent	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**64**	**128**	**256**	**512**	**1024**	**2048**	**>2048**
Ciprofloxacin				1	2											
				33.3	67.7											
Chloramphenicol						2	1									
						67.7	33.3									
Daptomycin		3														
		100														
Erythromycin		3														
		100														
Gentamicin											3					
											100					
Kanamycin											3					
											100					
Lincomycin							3*									
							100									
Linezolid				1	2											
				33.3	67.7											
Quinuprisrin-dalfopristin						3*										
						100										
Streptomycin (high-level)													3			

													100			
Penecillin				3												
				100												
Nitrofurantoin								2	1							
								67.7	33.3							
Tetracycline				3												
				100												
Vancomycin			1	2												
			33.3	67.7												
Tylosin tartrate								3								
								100								
Tigecycline	3															
	100															

[a] Unshaded areas indicate MIC range for each agent available on the Sensititre CMV3AGPF card. MICs > than highest concentration available are indicated in the shaded region

Vertical green lines indicate EUCAST epidemiological cut-off (ECOFF) values; CLSI susceptible (blue) and resistant (red) breakpoints (for nearest available Gram Positive); NARMS breakpoints (dashes)

* Non-relevant antibiotic; bacteria contains an intrinsic resistance.

In addition, there was one Gram negative bacteria of significance that was tested (by AST); *Klebsiella pneumoniae.* It was tested against Augmentin (sensitive; S), Cefoxitin (S), Ceftriaxone (S), Chloroamphenicol (S), Ciprofloxacin (S), Gentamicin (S), Tetracycline (S), Trimethoprim (S).

Appendix 8: Stock feed powder analytical results

Report Number: 10064

Lab Reference Numbers	BQ17895-17896
Method	LC-MS

Lab Ref No:	Sample Descriptor:	Qualitative Result:
BQ17895	Scan Viron	Ceftriaxone identified
BQ17896	VS 300W B1	Enrofloxacin identified

Appendix 9: Antibiotic and pesticide residue results

Lab Ref No:	Sample Descriptor:	Chemical:	Result:	
BQ17948	VMC1	Oxytetracycline	0.004	* mg/Kg
BQ17949	VMC2	No residues detected		
BQ17950	VMC3	No residues detected		
BQ17951	NKe3	No residues detected		
BQ17952	NKe7	No residues detected		
BQ17953	NKe8	No residues detected		
BQ17954	NKe30	Diuron	0.018	mg/Kg
BQ17955	NKe32	No residues detected		
BQ17956	NKe35	Oxytetracycline	0.010	mg/Kg
BQ17957	NKe36	Chlortetracycline	0.004	* mg/Kg
		Oxytetracycline	0.007	* mg/Kg
BQ17958	NKe41	No residues detected		
BQ17959	NKe56	Diuron	0.019	mg/Kg
BQ17960	NKe62	Doxycycline	0.004	* mg/Kg
		Sulphamethoxazole	Trace	
BQ17961	ML38	Doxycycline	0.004	* mg/Kg

BQ17961	ML38	Oxytetracycline	0.12	mg/Kg
BQ17962	ML53	No residues detected		
BQ17963	ML54	No residues detected		
BQ17964	ML56	No residues detected		
BQ17965	ML59	No residues detected		
BQ17966	ML60	No residues detected		
BQ17967	ML61	No residues detected		
BQ17968	ML62	No residues detected		
BQ17969	ML66	Tetracycline	0.002	* mg/Kg
BQ17970	ML67	No residues detected		
BQ17971	ML68	No residues detected		
BQ17972	ML69	No residues detected		
BQ17973	ML70	No residues detected		
BQ17974	ML71	No residues detected		
BQ17975	ML72	No residues detected		
BQ17976	ML73	No residues detected		
BQ17977	ML75	Diuron	0.010	mg/Kg
		Doxycycline	0.011	mg/Kg
		Oxytetracycline	0.16	mg/Kg
		Sulphamethoxazole	0.053	mg/Kg
		Trimethoprim	0.001	mg/Kg
BQ17978	ML77	No residues detected		
BQ17979	ML78	No residues detected		
BQ17980	ML79	No residues detected		
BQ17981	ML79A	No residues detected		
BQ17982	Sample ID 76	No residues detected		

* Please note that for your information we have reported tetracycline class residues below our usual method limit of reporting. These residues have been confirmed but the uncertainty in the concentration reported will be quite high. 'Trace' means that the residue was detected and the presence and identity of the residue was confirmed, but the concentration was below the level at which we can quantify.

Results reported on an "as received" basis. Results pertain only to the samples submitted.

Appendix 10: National Residue Survey list of antimicrobials at 30/01/2018

The following compounds are screened for in imported seafood into Australia as part of the National Residue Survey.

Analyte Name	Level of Detection (mg/kg)	Level of Reporting (mg/kg)
amoxicillin	0.01	0.01
ampicillin	0.01	0.01
apramycin	0.25	0.25
avilamycin	0.1	0.1
benzyl G penicillin	0.01	0.01
ceftiofur	0.2	0.2
cefuroxime	0.05	0.05
cephalonium	0.05	0.05
chlortetracycline	0.05	0.05
cloxacillin	0.05	0.05
dihydrostreptomycin	0.1	0.1
doxycycline	0.05	0.05
erythromycin	0.1	0.1
gentamycin	0.1	0.1
lincomycin	0.1	0.1
neomycin	0.1	0.1
oleandomycin	0.2	0.2
oxytetracycline	0.1	0.1
streptomycin	0.1	0.1
sulfachloropyridazine	0.02	0.05
sulfadiazine	0.02	0.05

sulfadimethoxine	0.02	0.05
sulfadimidine	0.02	0.05
sulfadoxine	0.02	0.05
sulfafurazole	0.02	0.05
sulfamerazine	0.02	0.05
sulfamethoxazole	0.02	0.05
sulfamethoxydiazine	0.02	0.05
sulfamethoxypyridazine	0.02	0.05
sulfapyridine	0.02	0.05
sulfaquinoxaline	0.02	0.05
sulfathiazole	0.02	0.05
sulfatroxazole	0.02	0.05
tetracycline	0.1	0.1
tilmicosin	0.2	0.2
trimethoprim	0.02	0.05
tulathromycin	0.3	0.3
tylosin	0.1	0.1
virginiamycin	0.2	0.2