

Probiotic bacteria for hatchery production of Greenshell™  
mussels, *Perna canaliculus*

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**Certificate of Authorship / Originality**

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

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## LIST OF ABBREVIATIONS

AB	Antibiotic
AD	Antimicrobial drug
ARB	Antibiotic resistant bacteria
ATCC	American Type Culture Collection
BLIS	Bacteriocin-like inhibitory substance
CFU	Colony forming unit
DO	Dissolved oxygen
EDTA	Ethylenediaminetetraacetic acid
ETOH	Ethanol
EU	European Union
GACL	Glenhaven Aquaculture Centre Limited
GIT	Gastrointestinal tract
GSM	Greenshell™ mussel
H+E	Haematoxylin eosin
LAB	Lactic acid bacteria
MB	Marine broth
MIC	Minimum inhibitory concentration
TSA	Tryptone soy agar
TSB	Tryptone soy broth
MEPD	Minimum effective pathogenic dose
MH	Mueller-Hinton
NZ	New Zealand
ONPG	<i>o</i> -Nitrophenyl- $\beta$ -D-galactopyranoside
R & D	Research and development
RDP	Ribosomal Database Project II
TCBS	Thiosulphate citrate bile sucrose
TCD	Tissue culture dish
US	United States of America
UV	Ultraviolet
vs	Versus

## ABSTRACT

The Greenshell™ mussel (GSM), *Perna canaliculus*, industry in New Zealand (NZ) is the largest aquaculture sector in the country. In 2006, the export earnings were valued at US\$145 million which represented 65% of NZ aquaculture earnings. Historically, and at present, GSM production involves the capture of wild mussels on ropes followed by on-growing of these animals to market size (approximately 14 months). However, hatchery production of GSM has been developed in recent years. Hatchery production will alleviate the seasonal uncertainties of current techniques and allow the benefits of selective breeding programs. To date, efforts to produce commercial quantities of GSM in hatcheries have been hampered by unreliable larval rearing. These problems were often alleviated by antibiotic use, which implied bacterial pathogens as the cause. Yet, the ongoing use of antibiotics is not sustainable because of increasing legislative restrictions on their use and the possible emergence of antibiotic resistant bacteria. Hence, the identification and use of novel probiotics was investigated as an alternative.

Because of a lack of previous work, it was necessary to investigate the bacterial pathogenesis of GSM larvae in the initial stages and, hence, to determine the cause of disease against which the probiotics would be active. Twenty-two bacterial strains, isolated from compromised larvae, were screened for larval toxicity using a larval bioassay. Two strains were identified as potential pathogens. Sequencing of the 16S rRNA gene identified *Vibrio splendidus* and *Vibrio* sp. DO1, a *Vibrio coralliilyticus/neptunius*-like isolate, as pathogens of GSM larvae. These strains had the

ability to cause 83 and 75% GSM larval mortality *in vitro* respectively, at a concentration  $10^2$  CFU ml<sup>-1</sup>. Histopathology indicated the route of infection was via the digestive system. Using healthy larvae as target hosts, Koch's postulates were confirmed for the two isolates.

Although two bacterial pathogens were identified, the successful design and implementation of protective measures in the hatchery still required an understanding of the dynamics of the infection process. Developing an *in situ* experimental model for infection was therefore paramount. The minimum effective pathogenic dose (MEPD) of *V. splendidus* ( $10^5$  CFU ml<sup>-1</sup>) and *Vibrio* sp. DO1 ( $10^6$  CFU ml<sup>-1</sup>) was demonstrated for GSM larvae during hatchery production. In a flow-through water hatchery system, larvae given 1-2 hours of static water exposure with these pathogen doses, after which flow-through processes resumed, averaged 58% and 69% cumulative mortality, respectively, on the fourth day following pathogen exposure. Larvae exposed to a dosage one order of magnitude greater than the MEPD, had higher mortalities of 73% and 96% for *V. splendidus* and *Vibrio* sp. DO1 respectively. These four levels of mortality were significantly greater than those of the non-exposed control larvae, averaging 23% in the experiments involving *V. splendidus* and 35% with *Vibrio* sp. DO1. Experiments were repeated four times to establish reproducibility. The infection models were reproducible and provided a tool to assess measures for the protection of GSM larvae against infection in the hatchery environment.

A bioassay was developed to screen and select bacterial strains as potential probiotics for GSM larvae. Sixty-nine isolates originating from a GSM hatchery environment were tested for probiotic activity in larval pathogen-challenge bioassays conducted in tissue culture dishes (TCDs). *Vibrio* sp. DO1 and *V. splendidus* were the tested pathogens. Forty of the tested isolates afforded larval survival significantly greater than pathogen controls ( $p < 0.05$ ). The bioassay technique achieved a 58% success rate in searching for putative probiotics and highlighted the benefit of including the host animal in the first stage of the screening procedure. The time of inoculation of putative probiotic strains prior to pathogen challenge influenced the outcome of the assay. A pre-exposure period of 20 hours revealed a greater number of potential probiotics than a two-hour pre-exposure period. Pilot challenge tests, under normal hatchery conditions, confirmed the usefulness of the TCD screening method in recognising effective probiotics.

Following hatchery pilot trials, two probiotic strains were chosen for further study, namely strains 0444 and 0536. Sequencing of the 16S rRNA gene and phylogenetic analysis identified the strains as *Alteromonas macleodii* 0444 and *Neptunomonas* sp. 0536. Both probiotics were evaluated separately in a GSM hatchery facility during routine larval rearing and when the larvae were challenged with a high and low pathogenic dose of *Vibrio* sp. DO1 and *V. splendidus*. In all experiments, probiotic application significantly improved larval survival, if administered prior to pathogen exposure. Across all experiments, larvae that were exposed to the high and low dosages of pathogens averaged 14% and 36% survival respectively on the fourth day following pathogen exposure. If the probiotics were administered prior to pathogen challenge, larval

survival averaged 50% and 66% respectively. Non-inoculated control larvae and larvae administered the probiotic alone demonstrated 67% and 79% survival respectively. In a repeat experiment, these benefits were reproduced, with the exception of *A. macleodii* 0444 trialled against *V. splendidus*. *Neptunomonas* sp. 0536 appeared to suppress naturally occurring vibrios in the culture environment of healthy GSM larvae. This was the first time *A. macleodii* and *Neptunomonas* sp. were demonstrated as probiotic bacteria.

Many studies document probiotic application in aquaculture under conditions of pathogen attack, yet few describe the use of probiotics during routine production. The effects of administering the probiotic, *A. macleodii* 0444, during routine GSM larvae production, were compared against larvae from the same cohort that were not treated with the probiotic. The probiotic was administered daily for the first 11 days of the larval period and was provided at two concentrations,  $10^7$  CFU ml<sup>-1</sup> and  $10^8$  CFU ml<sup>-1</sup>. Measures of larval swimming activity, gut colouration, lipid levels, larval survival, larval size and settlement success were recorded. There were minimal differences in all parameters between larvae provided the probiotic and control larvae. Probiotic treated larvae consumed more food and had higher lipid levels at the end of the larval period, but these were not statistically significant. All treatments completed the larval phase and settled successfully after metamorphosis. Survival at the end of the larval period was 37.2%, 38.8%, and 34.8% for control,  $10^7$  CFU ml<sup>-1</sup> and  $10^8$  CFU ml<sup>-1</sup> treatments respectively. The probiotic was still detected in larvae seven days after the final addition to the tanks.

Animals were further grown in the field at a commercial farm. The probiotic was not detected in mussels at four months after leaving the hatchery.

Combination use of the two probiotics, *A. macleodii* 0444 and *Neptunomonas* sp. 0536, was investigated to determine whether additive protection against pathogen attack with *Vibrio* sp. DO1 and *V. splendidus* was afforded to GSM larvae. The effects of combination administration were compared with larvae administered each probiotic as single strains and non-inoculated larvae. Additionally, two concentrations were tested for each probiotic, both singly and in combination,  $10^7$  and  $10^8$  CFU ml<sup>-1</sup>. Larvae were administered probiotics daily for the first six days, challenged with pathogens on the third day and then reared until settlement (day 19). Although protection against pathogen attack was observed in combination treatments, when compared with single-strain administration, additive protection was not apparent. Administration of  $10^8$  CFU ml<sup>-1</sup> levels of probiotics, both singly and in combination, afforded larval survival slightly better than  $10^7$  CFU ml<sup>-1</sup> levels, although this was rarely statistically significant. On the other hand, the higher levels of probiotic led to smaller larvae and lower feed rates for the majority of the 19-day trial. At the end of the study, larval sizes were smaller in the treatment applied a combination of probiotics at  $10^8$  CFU ml<sup>-1</sup> than those of the other treatments. Additionally, towards the end of the larval period, feed consumption in the combination  $10^8$  CFU ml<sup>-1</sup> treatment was similar to that witnessed in the other probiotic treatments one day previously. This suggested that either the larvae were compromised or they were growing slower. Despite a lack of additive protection against a single strain pathogen attack being demonstrated, the potential benefit of multi-strain probiotics, as



prophylactic measures against every-day microbial encounters in larviculture, would remain. Although  $10^8$  CFU ml<sup>-1</sup> levels appeared to protect against pathogen attack slightly better, they were also potentially detrimental to normal larval rearing when administered in combination. Following the successful completion of the larval period and pathogen protection afforded with a combination of probiotics at  $10^7$  CFU ml<sup>-1</sup>, this level was recommended as the best concentration of each probiotic where combination administration would be applied.

The work presented in this thesis supports the use of *A. macleodii* 0444 and *Neptunomonas* sp. 0536 in the routine rearing of GSM larvae. The ability to produce settled juvenile mussels, equal in numbers to those produced in normal healthy conditions, plus the benefits against pathogen attack led to the recommendation of their use on a routine prophylactic basis in GSM larval rearing. Their use for this purpose is intended in the near future. A provisional patent has been prepared and will be submitted shortly. It is anticipated that future work will continue with these probiotic strains to determine their potential benefit for other aquaculture species.