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Retention of viable microsporidial (*Loma salmonae*) spores within the blue mussel (*Mytilus edulis*): Use of an experimental laboratory model probing pathogen transfer within a multi-trophic aquaculture setting

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ABSTRACT

An *in vivo* rainbow trout (*Oncorhynchus mykiss*) branchial xenoma expression model was used to detect the presence of viable microsporidial spores (*Loma salmonae*) within aquarium-held blue mussels (*Mytilus edulis*) experimentally exposed to a suspension of semi-purified *L. salmonae* spores. In two separate studies, viscera from mussels exposed to spores for 4 hr were fed to trout that subsequently developed xenomas within their gills 6 weeks post-infection. The results from these studies provide proof of principle evidence that *L. salmonae* spores are captured from the water column by mussels, and remain viable within mussel tissue. However, mussel-fed fish yielded significantly fewer xenomas compared to reference fish exposed to an intraperitoneal injection of spores. A spore-transfer efficiency value was calculated (xenomas/spore dose) and revealed 0.03% of spores led to xenomas in IP injected fish, while 0.005% of spores exposed to mussels yielded xenomas in viscera-fed fish. Relative spore-transfer efficiency provided by mussel filtration and IP injection is therefore 16.6%. In a separate trial, we examined the influence of time, after mussels ingest spores for 4 hr, on xenoma development in trout. While 75% of fish became infected after 4 hr of exposure (similar to previous trials), only 10% became infected after 7 days, which indicates that blue mussels may act as a reservoir for infectious *L. salmonae* spores for at least 7 days. In a final pilot study, we aimed to determine whether the addition of mussels to *L. salmonae* contaminated water provides bioremediation. The percentage of trout that became infected was similar in fish exposed to contaminated water versus those exposed to contaminated water filtered by mussels. Interestingly, trout exposed to water filtered by mussels, developed higher mean xenoma counts which suggests that viable spores are expelled via mussel feces and pseudofeces, which can then be consumed by trout.

1. Introduction

Loma salmonae is the causative agent of Microsporidial Gill Disease of Salmon (MGDS). *Loma salmonae* is an intracellular pathogen which targets pillar cells, endothelial cells or leukocytes within salmon gills and cause the development of xenomas where spores develop and proliferate (Rodriguez-Tovar et al., 2002). The xenomas cause a characteristic chronic inflammatory branchitis (Kent and Speare, 2005) that is devastating to susceptible salmon species. One of the most susceptible species is the Chinook salmon (*Oncorhynchus tshawytscha*), which is an economically important Pacific salmon species often used in caged aquaculture (Constantine, 1999). Mortality rates for MGDS are nearly constant at ~12% in fish approaching market weight, but since the disease cannot be reliably treated or prevented, losses can be costly (e.g., $315,000 in direct and $1,470,000 in indirect costs; Constantine, 1999).

Since spores are inherently environmentally protected entities, it is difficult to manage and treat MGDS where it is endemic. One proposed mode of protection against MGDS is through the concept of integrated multi-trophic aquaculture (IMTA). Farming blue mussels (*Mytilus edulis*) and salmon together may improve disease dynamics in cultured systems. Blue mussels can filter particles ranging from 4.0 to 8.0 μm (Reid et al., 2010). Since *L. salmonae* spores range from ~2 to 6 μm (Bruno et al., 1995) they should be filtered by the mussels. Blue mussels have been shown to deactivate diseases that heavily impact salmon farming operations (i.e., Infectious Salmon Anaemia [ISA], Skår and Mortensen, 2007; Bacterial Kidney Disease [BKD], Paclibare et al., 1994). However, it appears that more environmentally protected pathogens remain infective within mussel tissue for a few weeks post-exposure. For example, *Toxoplasma gondii* remains infective within *Mytilus galloprovincialis* mussels up to 21 days post-exposure (Arkush et al., 2003). Similarly, *Cryptosporidium parvum* oocysts remain infective within *M. galloprovincialis* for up to 14 days post infection (Tamburrini and Pozio, 1999). Therefore, it would be beneficial to determine how mussels interact with *L. salmonae* spores and how that would translate to disease transfer to farmed salmon species.

In this study we looked to demonstrate whether *L. salmonae* spores are still viable within mussel tissue at least 4 hr following filtration. The

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primary objective was to compare infection rates in rainbow trout (*Oncorhynchus mykiss*) following ingestion of mussel tissue following 4 hr of filtration. Trout were used a model species since Chinook salmon experience severe clinical symptoms and heavy mortality when exposed to *L. salmonae* (see Speare et al., 1998 for information regarding using rainbow trout as a model for MGDS). We compared spore transfer efficiency between the mussel tissue and fish that had been intraperitoneally injected with viable spores. Our second objective was to determine how long viable spores remained within filtering mussels and the occurrence of infection up to 7 days following spore filtration. Thirdly, we observed whether mussels effectively filter *L. salmonae* spores from the water column, reducing the incidence of disease to fish exposed to previously filtered water.

2. Materials and methods

2.1. Experimental design and study animals

All experiments were completed in accordance with the guidelines of the Canadian Council on Animal Care (CCAC, Canadian Council on Animal Care, 2005). The juvenile rainbow trout (~25 g) used for this study were acquired from a certified pathogen-free commercial hatchery (Ocean Trout Farms Inc., Brookvale, PE, Canada) with no previous history of *L. salmonae*. Positive and negative control groups were utilized in each experiment to verify our disease model. Rainbow trout are resistant to *Loma salmonae* after one exposure and thus a positive disease outcome would verify that fish were previously unexposed to the pathogen (Rodriguez-Tover et al., 2006). Prior to experimentation, the trout where held in a 900 L circular fibreglass tank in a quarantine room. Fish were exposed to a set photoperiod in 11 ± 0.3°, well aerated water, with a flow-rate of 2 L min^{-1}. Fish were fed 2% of their body weight three times per week (Corey Aquafeeds, Fredericton, NB, Canada). When used for experimentation, fish were transferred to a separate room at least 1 week in advance. At this time, fish were fed dexamethasone (dex; Dexasone, Valeant, Montreal, QC, Canada) coated feed at a dose of 300 mg kg^{-1} food three times per week. Dex is an immunosuppressant that allows for approximately 5-fold higher infections in trout exposed to *L. salmonae* compared to trout not exposed to dex (Lovy et al., 2008). Water conditions were kept constant, with water temperature at 15 ± 0.3 °C but fish were randomly allocated into 70 L fibreglass tanks hooked up to a fresh water flow-through system.

Aquaculture-derived blue mussels from Prince Edward Island, Canada were obtained from a local commercial source. *L. salmonae* has not been detected in the waters used for commercial blue mussel culture. Mussels were placed in 11.3 L totes containing artificial seawater (17 ± 0.3 °C ranged 30–35 ppt; Instant Ocean®, United Pet Group Inc., Cincinnati, OH, USA). The mussels were allowed to acclimatize for 7 days preceding experimentation and were fed algae paste (Innovative Aquaculture Products Limited, Skerry Bay, Lasqueti Island, BC, Canada) three times per week, with daily water changes.

2.2. Preparation of L. salmonae spores

Rainbow trout heavily infected with *L. salmonae* xenomas (i.e., presence of white cysts filled with spores on gills) were euthanized by an overdose of benzocaine (120 mg L^{-1}; 4-Aminobenzoic acid ethyl ester ethyl 4-aminobenzoate, Sigma-Aldrich, Oakville, ON, Canada). The gills were immediately dissected from the fish, removed from the cartilaginous gill arches, and minced using a razor blade. Gill material was then ground in a glass tissue grinder (Wheaton Science Products; Millville, NJ, USA), re-suspended in equal volume of sterile saline (0.85%, 4 °C) and centrifuged at 1000 rpm for 10 min. The supernatant was poured off and the pellet was pressed through a Cellector (Homogenizer, BellCo Glass, Vineland, NJ, USA) metal screen and then filtered again through Nitex mesh (63 μm, Hoskin Scientific, Burnaby, BC, Canada), rinsed with 0.85% sterile saline and centrifuged at 1000 rpm for 10 min. This procedure was repeated, pouring off the supernatant each time. The surfactant Triton-X 100 (6%; (1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol Polyethylene glycol *tert*-octylphenyl ether, Sigma-Aldrich, Oakville, ON, Canada) was added to the pellet and vortexed for 30 sec. The pellet was centrifuged twice with equal volume of 0.85% sterile saline at 2000 rpm for 15 min. The supernatant was poured off and the pellet was resuspended in 0.85% sterile saline. A dilution was made, when required, with a small amount of spores (0.1 mL) and placed under a haemocytometer (40×; Fisher Scientific, Markham, ON, Canada) in order to determine the concentration of the spore mixture.

2.3. Trial 1: viability of spores following exposure to mussels

The objective of trial 1 was to determine whether mussels could transmit infective *L. salmonae* spores following a period of filtration. Seventy naïve rainbow trout were allocated into 4, 70 L circular tanks ($N = 20$ per tank with one tank of $N = 10$) and treated with dexamethasone as described in Section 2.1. Sixty blue mussels were obtained and acclimated as described above in 3 containers ($N = 20$ per container; A, B and C). After the acclimation period, the mussels in container A received 10 mL of purified spore material (7.32×10^6 spores mL^{-1}) and were allowed to filter the spore material for 4 hr. Mussels in containers B and C did not receive any spore material. After the exposure period all containers of mussels were humanely harvested. Container C was harvested first and the resulting mussel tissue and nectar was not exposed to any *L. salmonae* spores. The mussel tissue from container B was removed and promptly injected with 7.32×10^5 100μL^{-1} *L. salmonae* spores in saline to determine whether the mussels somehow deactivated *L. salmonae* upon contact. Mussel tissue and nectar was also removed from container A. The tissue from each of the mussel treatment groups was fed to 3 of the 4 tanks of rainbow trout (the tanks were referred to as Tanks A–C depending on the treatment received). The fourth tank containing 10 fish, Tank D, acted as a positive control to determine whether our spores were viable and able to cause heavy *L. salmonae* infections. The 10 fish were anaesthetized with benzocaine (60 mg L^{-1}) and intraperitoneally injected with 7.32×10^5 spores per 100 μL^{-1} saline. Fish were maintained as described in Section 2.1for a 6-week period in order to allow the infection to mature. Beginning at week 4 post exposure (PE), all fish were anaesthetized with benzocaine (60 mg L^{-1}) and non-lethally screened for the presence or absence of xenomas using a dissecting microscope (Stereo-MSA, Wild Leitz Canada Ltd., Willowdale, ON, Canada). At 6 week PE, all fish were euthanized via benzocaine overdose (120 mg L^{-1}). From each euthanized fish, the first left gill arch was dissected free for whole-mount observation and the numbers of xenomas were counted using a light microscope (40×, LM; Olympus BH2). The mean number of xenomas per fish was also enumerated, along with the transfer efficiency of the spores exposed to each fish: Transfer Efficiency = number of xenomas per fish/number of spores exposed ×100. The present and all proceeding data were analysed with MINITAB® software, version 16 (State College, PA, USA). For trial 1, one-way ANOVAs were conducted, and significant differences were reported at the $\alpha = 0.05$ level of probability. Bonferroni pair-wise comparisons were completed to determine where differences existed. Normality and homogeneity of variances were observed and suitable transformations were applied where data did not meet normality assumptions.

2.4. Trial 2: mussel tissue infectivity following 4 hr filtration of L. salmonae contaminated water

The objective of trial 2 was to further investigate the claim that *L. salmonae* spores still remain viable and infective following