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Dietary supplementation of garlic (*Allium sativum*) to prevent monogenean infection in aquaculture

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ABSTRACT

Development of an effective preventative treatment for managing infections by Monogenea (Platyhelminthes) in aquaculture remains elusive. Present treatment methods offer only temporary respite and are either labor intensive, harmful to fish welfare or environmentally destructive. This study used garlic (*Allium sativum*) supplemented feed to assess its potential, in relation to its allicin content (an active component of garlic), to prevent infection by *Neobenedenia* sp. (Monogenea: Capsalidae) on farmed barramundi, *Lates calcarifer*. Two garlic supplemented diets of different concentrations and a non-supplemented control diet were fed to *L. calcarifer* for 10 and 30 days prior to challenging fish with *Neobenedenia* sp. Long-term (30 days) supplementation with garlic significantly reduced infection success by up to 70% compared to controls and did not negatively affect palatability of the feed. Infection success was not influenced by short-term (10 days) supplementation suggesting that a delayed host response must occur to improve resistance to infection. Incorporation of garlic into a pressure-extruded pellet was found to be an effective method of delivery as only minimal leaching of allicin from the diet occurred (<3% of allicin detected) during the interval of water contact between delivery and consumption. This study demonstrates that garlic extract administered as a dietary supplement is one of the most practical methods to prevent *Neobenedenia* sp. infection in mariculture.

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1. Introduction

Infectious disease has been identified as one of the greatest problems in the advancement of mariculture worldwide (Bondad-Reantaso et al., 2005). Parasites are important pathogens in mariculture (Bondad-Reantaso et al., 2005, 1995; Seng, 1997), with capsalid skin flukes (Monogenea: Capsalidae) considered one of the most serious and chronic problems in finfish (Ogawa and Yokoyama, 1998; Ogawa et al., 1995).

At present, there are no methods to prevent monogenean infections (Whittington, 2012). Commercially employed management strategies are limited to recurring freshwater and chemical bath treatments that offer only temporary respite by removing parasites within the treatment area (Ellis and Watanabe, 1993; Seng, 1997; Mueller et al., 1992; Thoney and Hargis, 1991). Such treatments must occur at regular intervals as parasite life stages often exist outside the treatment area and can be resilient to treatment (Militz et al., 2013; Mueller et al., 1992). Furthermore, bathing treatments are labor-intensive, time consuming, weather dependent, environmentally deleterious and harmful to fish welfare (Wilkinson et al., 2006). Despite considerable industry expertise in bath treatments, mortalities still occur because of the low therapeutic indices of bath solutions, difficulties in calculating bath concentrations and associated handling stress (Williams et al., 2007; Yamamoto et al., 2011).

A preventative agent administered in-feed is a practical alternative to bathing applications, as no handling of livestock is required and the treatment is delivered directly to the host rather than to the host's environment (Dunn et al., 1990). However, there are no effective oral preventative treatments for monogenean management in aquaculture. While some synthetic antihelminthics (i.e. praziquantel) have been successful, practical application is seriously compromised by host toxicity, failure to prevent recurring infection and extremely low palatability (Hirazawa et al., 2004; Williams et al., 2007).

Research is now being directed towards the vast unexplored source of plant-based antimicrobials and immunostimulants for disease management, many of which are without the negative side effects associated with synthetic chemotherapy (Colorni et al., 1998; Hutson et al., 2012; Immanuel et al., 2009; Palavesam et al., 2006). The value of garlic, *Allium sativum*, extract for bacterial disease control and immunostimulation has previously been demonstrated for a number of cultured fishes (Aly and Mohamed, 2010; Nya and Austin, 2009; Sahu et al., 2007; Talpur and Ikhwanuddin, 2012). Moreover, only relatively short periods of supplementation (14 days) appear necessary to achieve the associated benefits, including increased immune cell activity and density (Nya and Austin, 2009; Sahu et al.,

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2007; Talpur and Ikhwanuddin, 2012). The observed antimicrobial and immunostimulant activity of garlic in teleosts is largely explained by the transient phytochemical allicin (diallyl thiosulfinate) and its derivatives (Nya et al., 2010). Despite garlic extract supplemented feeds conferring resistance to a number of bacterial pathogens, dietary supplementation for parasite management in aquaculture has not been demonstrated.

The marine metazoan ectoparasites *Neobenedenia* spp. are of critical concern to aquaculture because they have direct life cycles with short generation times (Hirazawa et al., 2010) and low host specificity (Whittington and Horton, 1996). Within Australasia, *Neobenedenia* spp. are responsible for major stock losses in several commercially important cultured fishes, including yellowtail (*Seriola quinqueradiata*), amberjack (*Seriola dumerili*), tiger puffer (*Takifugu rubripes*), Japanese flounder (*Paralichthys olivaceus*), red sea bream (*Pagrus major*), cobia (*Rachycentron canadum*), chub mackerel (*Scomber japonicas*), and barramundi (*Lates calcarifer*) (see Deveney et al., 2001; Hirazawa et al., 2004; Ogawa and Yokoyama, 1998; Ogawa et al., 2006). The aim of this study was to examine the effects of dietary supplementation with allicin-containing garlic extract as a preventative treatment against monogenean infection. This was done using *Neobenedenia* sp. which is known to infect farmed *L. calcarifer*. The palatability of the feed and the propensity for allicin to leach from feed pellets in seawater were also examined to determine the practicality of garlic extract enhanced feed in commercial applications.

2. Materials and methods

2.1. Parasite culture

Embryonated *Neobenedenia* sp. eggs were initially collected from an inland, marine *L. calcarifer* farm in Queensland, Australia. Eggs were maintained in seawater (35‰) until the onset of hatching. Newly hatched (<6 h post-hatch) oncomiracidia were introduced to healthy, naïve *L. calcarifer* maintained in 20 L non-renewal aquaria (35‰, 25 °C) from which the successive generations of *Neobenedenia* sp. eggs were collected and incubated until hatching.

Adult parasites were identified as *Neobenedenia* morphologically. The species of *Neobenedenia* investigated in this study is presently unidentified given the absence of diagnostic criteria to differentiate between geographical/host isolates and species pending a revision of the genus (Whittington, 2012). Representative adult specimens were accessioned in the Australian Helminth Collection, South Australian Museum, Australia (SAMA AHC 35461).

2.2. Garlic extract

Garlic (variety Glen Large) grown in Queensland, Australia, was homogenized with H_2O at a ratio of 5 mL per 1 g for 60 s. The homogenate was allowed to stand for 5 min without agitation before being filtered through a 90 μm screen and Whatman 1 filter in succession to produce the garlic extract.

Determination of the garlic extract's allicin content was done through the use of high performance liquid chromatography (HPLC) using an Applied Biosystems SPERI-5 C18 250 mm × 4.6 mm column (5 μm bead size) with a methanol and water (50/50) mobile phase run at a flow rate of 0.5 mL min^{-1}. An injection volume of 100 μL was used and peaks were detected at 254 nm and recorded by a Varian Pro Star UV–visible recorder. An aqueous solution of pure (>98%) allicin was created using previous methods (Lawson and Wang, 2001) and used as the HPLC standard for allicin quantification. The concentration of allicin present in the garlic extract was determined to be 0.68 μL L^{-1}.

2.3. Diet formulation

Commercially available *L. calcarifer* feed (Ridley AgriProducts) was ground, sieved to 1 mm and dried at 60 °C for 24 h before being mixed with pre-gelatinized starch binder at 5% (d/w). Proximate composition of this basal diet was 45% protein, 20% lipid, and 2.5% fiber. Garlic extract was incorporated into the diets directly to formulate two diets (50 mL L^{-1} and 150 mL L^{-1}) and a control diet without extract. Chilled H_2O was used in feed formulation to offset any heat production from the pelleter. The resulting diets were then transferred to a pelleter (La Monferrina) with the control diet pelleted first to ensure against the possibility of garlic contamination. Diets were dried in a temperature controlled room (20 °C) under constant air flow for 20 h until 10% moisture content. After drying, the diets were stored in air tight containers at −20 °C.

2.4. Leaching

Immediately following formulation, the 150 mL kg^{-1} garlic extract diet was examined for the propensity of allicin leaching in seawater. Ten grams of the 150 mL kg^{-1} diet was exposed to 10 mL of synthetic seawater (35‰) for 3.4 s, 30 s, and 10 min. The periods of seawater exposure corresponded to the average consumption time of the feed pellets by *L. calcarifer* (3.4 s), the upper limit of time taken to consume a pellet (30 s) and the time required for degradation of pellet shape (10 min). After the exposure period, the seawater solution was immediately filtered through a 0.2 μm filter before neat injection into HPLC using the previously defined HPLC method. This methodology was repeated for three replicates at each exposure time in addition to three replicates of a seawater blank (control) with no feed exposure.

A dichloromethane extraction of the diet was also performed by homogenizing 10 g of feed (w/w) with 30 mL of dichloromethane for 2 min. The solution was then centrifuged at 5300 rpm for 5 min until the feed-dichloromethane solution segregated into separate layers. The dichloromethane layer was removed, filtered and rotary evaporated at 20 °C under vacuum (300 mbar) until evaporation of the dichloromethane. The resulting solution was reconstituted in 5 mL of H_2O before neat injection into HPLC for allicin detection.

2.5. Host challenge

Eighty-four hatchery-reared, freshwater *L. calcarifer* (12.4 ± 0.4 g) from a commercial farm in Queensland, Australia were acclimated to

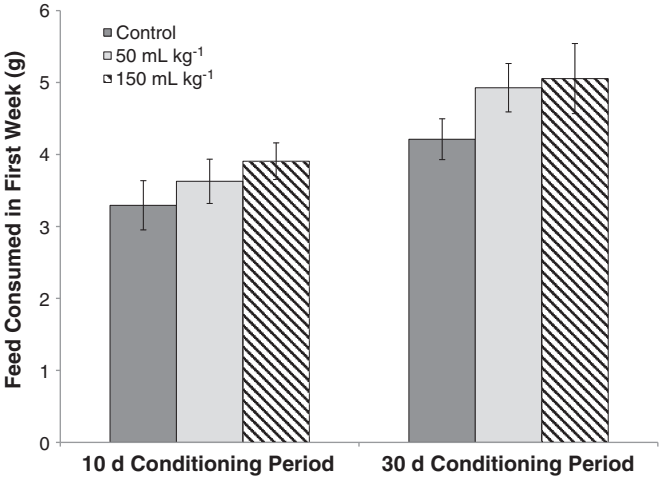

Fig. 1. Mean (±SE) quantity of feed consumed by *Lates calcarifer* for each of the garlic extract experimental diets within the first 7 days of introducing the experimental diets.

seawater and individually maintained in 15 L aquaria in a seawater recirculating system. Hatchery records indicated that all experimental fish were of good health and disease free. Fish were fed a daily ration of the commercial diet used in the formulation of the experimental diets equivalent to approximately 1–2% of their body weight for 7 days to acclimate them to experimental conditions.

L. calcarifer were randomly assigned to six experimental groups based on dietary treatment and period of conditioning: the two dietary treatments (50 mL kg^{-1} and 150 mL kg^{-1}) and the control diet fed over 10 or 30 days. Fish were fed to satiation twice daily, one pellet at a time. Satiation was defined as the point at which a feed pellet would sink to the base of the aquarium and not be consumed within 30 s. After 30 s the uneaten pellet was removed and the average weight of one pellet was omitted from the quantity of feed delivered. Temperature (26.7 ± 0.1 °C) and salinity (35.9 ± 0.1‰) of the system were recorded at each feeding for the duration of the trial.

At the end of the conditioning period (10 or 30 days) fish were challenged with 15 *Neobenedenia* sp. oncomiracidia. To facilitate infection, aeration and water flow to individual aquaria were suspended for 1 h (Hirazawa et al., 2010). Following challenge, the dietary trial resumed and continued for another 5 days to allow oncomiracidia the opportunity to attach to their host and commence development. On day six post-challenge, fish were freshwater bathed to remove parasites as detailed in Militz et al. (2013). Infection success was expressed as the number of *Neobenedenia* sp. recruits collected to the number of oncomiracidia introduced to each aquarium. Infection intensity and prevalence were determined as per Bush et al. (1997).

2.6. Statistical analyses

Values for each parameter measured were expressed as the arithmetic mean ± standard error (SE). The measures of *Neobenedenia* sp. infection success for the 10 and 30 day duration dietary trials were expressed as percentages and independently analyzed using permutational multivariate analysis of variance (PERMANOVA) based on Euclidean distances with PRIMER 6 (version 6.1.13) and PERMANOVA+ (version 1.0.3) statistical packages. To determine the presence and nature of the association between oncomiracidia infection success of *L. calcarifer* and the quantity of garlic extract consumed, a Pearson's correlation test was run. For all statistical tests, significance was accepted at $p < 0.05$.

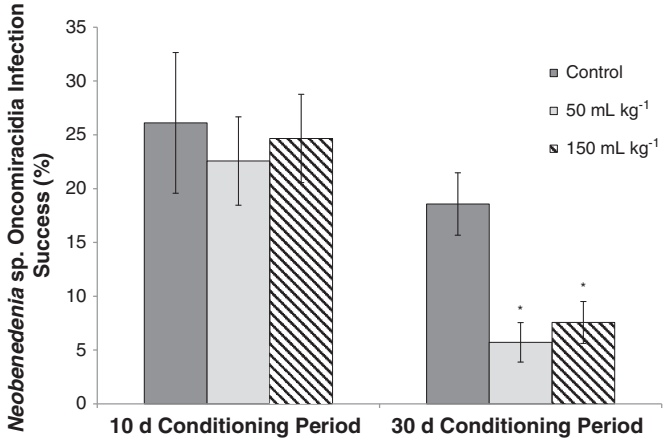

Fig. 2. Mean (±SE) percent *Neobenedenia* sp. oncomiracidia infection success of *Lates calcarifer* fed the control and garlic supplemented diets over a 10 or 30 day conditioning period before parasite challenge. * Denotes significant difference from control ($p < 0.05$).

Table 1
Prevalence and intensity of *Neobenedenia* sp. infecting *Lates calcarifer* fed diets of varying garlic extract concentrations for 30 days prior to challenge.

Diet	*n*	Prevalence (%)	$\bar{X}$ intensity (range)
Control	14	100	2.8 (1–7)
Garlic 50 mL kg^{-1}	14	50	1.7 (0–3)
Garlic 150 mL kg^{-1}	14	64	1.8 (0–3)

3. Results

3.1. Feed acceptance

Dietary supplementation with garlic extract had no significant effect on the palatability of the commercial-feed based diets in either the 30 day ($p > 0.05$) or the 10 day ($p > 0.05$) study although a positive trend in acceptance was noticeable, increasing with garlic extract concentration in both trials (Fig. 1).

3.2. Infection success

Dietary supplementation with garlic extract 30 days prior to challenge significantly reduced *Neobenedenia* sp. oncomiracidia infection success of *L. calcarifer* (Fig. 2; $p < 0.05$). Inclusion of garlic extract in the diet (at either 50 or 150 mL kg^{-1}) reduced oncomiracidia success to less than 10% (5.7 ± 1.8% and 7.5 ± 1.9%, respectively). In comparison, oncomiracidia were nearly three times more successful in infecting *L. calcarifer* fed the control diet (18.6 ± 2.9%) which did not contain allicin. The prevalence of infection was 100% in control fish, while only 50% and 64% of fish in the 50 and 150 mL kg^{-1} garlic supplemented treatments were infected, respectively (Table 1). The mean intensity of infection was also greater among control fish (2.8, *Neobenedenia* sp.) compared to fish fed diets containing allicin (1.7–1.8, Table 1).

The 10 day conditioning period with garlic extract did not lead to a difference in *Neobenedenia* sp. oncomiracidia infection success of *L. calcarifer* (Fig. 2; $p > 0.05$). All three diets (control, 50 and 150 mL kg^{-1} garlic extract) demonstrated comparable infection intensity (4.3, 4.0 and 3.7, respectively) and prevalence (all ≥ 91%; Table 2).

Oncomiracidia infection success did not correlate with the total garlic extract quantity ingested for either the 10 day ($p > 0.05$) or the 30 day ($p > 0.05$) conditioning period trial.

3.3. Dietary leaching

Leaching of allicin from the experimental diets was minimal with less than 3% of the allicin detected with the dichloromethane extraction (5.0 μL kg^{-1} w/w) leaching from the feed for any length of seawater exposure (Fig. 3). No allicin was detected in the seawater blank runs.

4. Discussion

The results of this study reinforce the growing view that dietary supplementation of garlic extract is beneficial to fish health by conferring protection against pathogens (Aly and Mohamed, 2010; Nya and Austin, 2009; Nya et al., 2010; Sahu et al., 2007). Feeding garlic extract to *L. calcarifer* for a period of 30 days significantly reduced infection prevalence (36–50% less) and intensity (50% less) in fish challenged

Table 2
Prevalence and intensity of *Neobenedenia* sp. infecting *Lates calcarifer* fed three diets of varying garlic extract concentrations for 10 days prior to challenge.

Diet	*n*	Prevalence (%)	$\bar{X}$ intensity (range)
Control	11	91	4.3 (0–10)
Garlic 50 mL kg^{-1}	12	92	4.0 (0–6)
Garlic 150 mL kg^{-1}	12	100	3.7 (1–7)

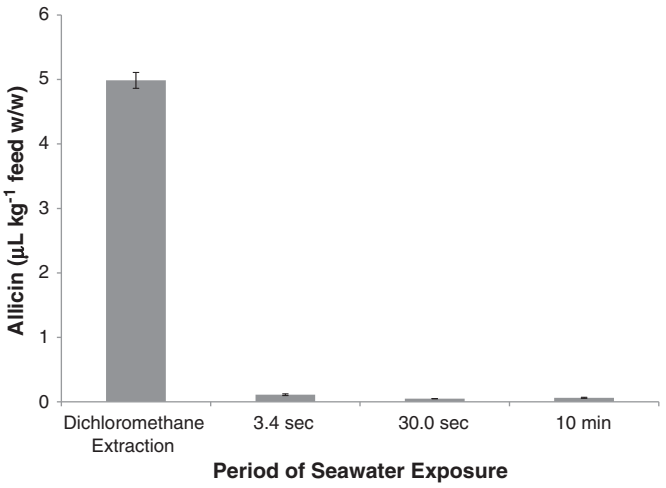

Fig. 3. Mean (±SE) concentration of allicin leaching from the 150 mL kg^{-1} garlic extract experimental diet following varying periods of seawater exposure in comparison to the concentration of allicin extracted with dichloromethane at the time of sampling.

with *Neobenedenia* sp. Both garlic diets conferred similar degrees of resistance to *Neobenedenia* sp. and fall within the range of garlic concentrations (0.5–30 g kg^{-1}) shown to effectively prevent bacteria associated mortalities in cultured fishes (Aly and Mohamed, 2010; Nya and Austin, 2009; Sahu et al., 2007; Talpur and Ikhwanuddin, 2012).

The premise of garlic's mode of action in preventing pathogens in fishes is not well understood. However, both crude garlic and pure allicin extract dietary supplements have been shown to enhance the teleost immune system by conferring direct antimicrobial activity to serum, increasing the number of leucocytes and by increasing phagocytic, lysozyme, and anti-protease activities (Aly and Mohamed, 2010; Nya and Austin, 2009; Nya et al., 2010; Sahu et al., 2007; Talpur and Ikhwanuddin, 2012). Of these immunoregulatory responses to garlic, lysozyme activity, a component of teleost mucus, has specifically been associated with innate immunity to monogeneans (Jones, 2001). The necessary concentration of allicin that must be incorporated into a feed to facilitate increased lysozyme activity was determined by Nya et al. (2010) for rainbow trout (*Oncorhynchus mykiss*) to be ≤5 µL kg^{-1} over a 14 day conditioning period. In comparison, the initial allicin concentrations of the 50 and 150 mL kg^{-1} garlic extract diets used in this study over the 30 day conditioning period were substantially higher (44 and 133 µL kg^{-1} feed w/w) and administered for twice as long. Enhanced lysozyme production in *L. calcarifer* has likewise been reported following the administration of garlic enriched diets (Talpur and Ikhwanuddin, 2012) and likely explains the reduced infection observed in this study. The fact that individual *L. calcarifer* demonstrated a poor correlation between garlic extract consumption and infection success suggests that as with most chemical compounds (Sharp et al., 2004), host response is not only dependent on concentration and/or exposure time but also on an individual's capacity to respond.

The period of garlic extract conditioning necessary to reduce infection success of *Neobenedenia* sp. to juvenile *L. calcarifer* was determined to be greater than 10 days. This is because *L. calcarifer* did not demonstrate reduced infection by *Neobenedenia* sp. after consuming garlic for 10 days but a clear reduction in infection occurred after a 30 day conditioning period. The delayed resistance to *Neobenedenia* sp. by *L. calcarifer* fed garlic supplemented feeds is likely a result of the time required for immunostimulation to occur in response to extract ingestion and obtain a level at which the protective effect can be observed. Sahu et al. (2007) demonstrated that when supplementing juvenile carp, *Labeo rohita*, for 20 and 40 days, several immunological indices continually increased (e.g. leucocyte density, superoxide anion production and antimicrobial activity of the serum) with longer periods of conditioning. Thus, it can be assumed that a similar graded response to garlic supplementation occurred with the *L. calcarifer* in this study and that the degree of immunostimulation following 10 day supplementation with garlic was insufficient to reduce infection success of *Neobenedenia* sp. While the up regulation of the immune system of *L. calcarifer* following garlic extract supplementation has been demonstrated to occur in as little as 14 days (Talpur and Ikhwanuddin, 2012), to the authors' best knowledge there are no studies examining the immunoregulatory response or pathogen resistance associated with garlic conditioning periods over a shorter time span. This study would suggest that the minimal conditioning period of garlic supplemented diets necessary to provide a broad spectrum preventative effect would need to be greater than 10 days.

It was confirmed, via dichloromethane extraction, that a portion of the initial allicin content was retained through the feed formulation process and was present in the feed at the time of consumption. Leaching of allicin from the 150 mL kg^{-1} feed was negligible (Fig. 3). The retention of >99% of available allicin in the feed matrix, despite immersion in seawater in excess of 10 min, indicates that the reduced infection success of *Neobenedenia* sp. oncomiracidia was entirely attributed to the ingestion of the garlic extract as opposed to its presence in the water column, which can only impair parasitic *Neobenedenia* sp. survival at high concentrations (≥10 mL L^{-1}, Militz et al., 2013). The low solubility of allicin in H_2O despite a high affinity for lipids, which were known to be present in the commercial feed, confirms the observed results (Lawson et al., 1991). Oral therapeutants with minimal leaching are ideal in commercial applications as they facilitate the ease at which farmers can administer a required dose accurately and avoid potential for environmental contamination.

In relation to other feed additives assessed for preventing *Neobenedenia* spp., garlic extract shows superior efficacy to a wide range of antibiotics (including oxytetracycline, florfenicol, ampicillin, erythromycin and sulfamonomethoxine), all of which had negligible effects in preventing *Neobenedenia girellae* infection in *S. dumerili* (Ohno et al., 2009). The proportion of successful *Neobenedenia* sp. recruits in response to 30 day garlic supplementation (33–40%) in this study was also comparable to the proportion of adult *N. girellae* surviving treatment (20–70%) with oral delivery of the synthetic antihelminthic praziquantel (Yamamoto et al., 2011). However, praziquantel does not prevent immediate re-infection (Yamamoto et al., 2011) and continual admission is not feasible based on potential for host toxicity and palatability issues surrounding its use in commercial feed preparations (Hirazawa et al., 2004; Williams et al., 2007). Oral admission of garlic is without these negative ramifications. The present study showed that garlic supplemented feeds did not affect palatability following dietary transition from a commercial feed to the experimental garlic diets. Additionally, Talpur and Ikhwanuddin (2012) found that garlic supplemented diets significantly improved the specific growth rate (SGR) and food conversion ratio (FCR) of *L. calcarifer* compared to a control diet. Thus, garlic possesses a range of attributes making it the ideal dietary supplement for managing *Neobenedenia* sp. in mariculture.

5. Conclusion

Measures to prevent or reduce the intensity of *Neobenedenia* spp. infections would substantially assist in on-farm disease management. This study clearly shows that garlic extract administered as a dietary supplement is the most practical preventative method currently known for *Neobenedenia* sp. infection in mariculture. Oral treatments are directly applicable in a diversity of aquaculture systems where chemical or freshwater bathing is impractical and garlic can be fed continuously in contrast to praziquantel treatments (Hirazawa et al., 2004; Williams et al., 2007). Confirming histological immunoregulatory changes at the host parasite interface following garlic extract supplementation would help to clarify garlic's mechanism of action following

ingestion and allow for further development of an already effective control measure for *Neobenedenia* spp. and other potential pathogens and parasites.

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