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CONTROLE BACTERIANO NA ECLOSÃO E ENRIQUECIMENTO DE *Artemia* sp. PARA SUA APLICAÇÃO NA ALIMENTAÇÃO DE PÓS-LARVAS DE *Litopenaeus vannamei*

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Resumo

O presente trabalho teve por princípio avaliar os efeitos antibacterianos de diferentes suplementos na eclosão e no enriquecimento de *Artemia* sp. Os suplementos foram adicionados na água utilizada para eclosão de cistos de *Artemia* capsulados e descapsulados e à água de enriquecimento de metanáuplios de *Artemia*. O experimento de eclosão consistiu no acréscimo da diatomácea *Chaetoceros calcitrans*, de probiótico comercial (*Bacillus* spp.), do antimicrobiano Florfenicol e controle sem adição de agentes. O experimento de enriquecimento foi realizado pela aplicação de *C. calcitrans*, de probiótico comercial e de emulsão comercial rica em DHA/EPA à água de cultivo de metanáuplios e controle constituído por náuplios recém eclodidos. Os metanáuplios foram oferecidos para os estágios de PL_7 a PL_{19} de *Litopenaeus vannamei*. A carga de *Vibrio* spp. da água de eclosão e náuplios recém eclodidos foram quantificadas no final do período de eclosão. A quantificação de *Vibrio* spp de pós larvas, metanáuplios, água de cultivo das pós larvas e enriquecimento de *Artemia* também foi realizada. As colônias presuntivas de *Vibrio* oriundas de náuplios recém eclodidos foram identificadas. Além disso, *Vibrio* spp. presente em náuplios submetidos ao congelamento e colônias de *Bacillus* spp. em amostras de *Artemia* enriquecida e pós-larvas do tratamento Probiótico foram quantificadas. Avaliando dos resultados gerais do estudo, o processo de descapsulação não demonstrou ser eficiente na redução da carga de *Vibrio* spp. nos náuplios e na água de todos os tratamentos. A adição de *C. calcitrans* na água de eclosão de *Artemia* provou ser uma alternativa eficaz para em alternativa a utilização de antibióticos. A utilização de probiótico deve ser também considerado para controlar a carga de *Vibrio* spp em náuplios de *Artemia*. No entanto, a utilização de suplementos para o processo de enriquecimento de *Artemia* pode favorecer o aumento da carga bacteriana e outros procedimentos para o seu controle deve ser avaliada.

Palavras chave: *Artemia, Vibrio,* eclosão, enriquecimento, *Litopenaeus vannamei*.

Abstract

This study aimed to evaluate the antibacterial effects of different supplements on *Artemia* sp. hatching and enrichment. The supplements were added to the water used for *Artemia* hatching of capsulated and decapsulated cysts and for *Artemia* enrichment water. The experiment consisted in the addition of the diatom *Chaetoceros calcitrans*, a commercial probiotic (*Bacillus* spp.), antimicrobial Florfenicol to the hatching water and control without supplements. The enrichment experiment was performed by the application of *C. calcitrans*, probiotic and commercial emulsion DHA / EPA rich to enrichment water and control constituted by newly hatched nauplii. The enriched *Artemia* were offered for the PL_7 to PL_{19} of *Litopenaeus vannamei* stages. The *Vibrio* spp. load of hatching water and newly hatched nauplii were quantified at the end of hatching. The *Vibrio* spp. quantification of postlarvae, enriched *Artemia* and water of enrichment and postlarvae rearing was also performed. The *Vibrio* presumptive colonies isolated from newly hatched nauplii were identified. Furthermore, *Vibrio* spp. present in nauplii subjected to freezing and *Bacillus* spp. colonies of *Artemia* and postlarvae of Probiotic treatment were quantified. Assessing the overall results of the study, the decapsulation process did not shown to be effective in reducing the *Vibrio* spp load of nauplii and water in all treatments. The *C. calcitrans* addition in *Artemia* hatching water has proven to be an effective alternative to antibiotic use. The probiotic use must also be considered to control *Vibrio* spp. load in *Artemia* nauplii. However, the supplements use to *Artemia* enrichment process may promote a bacterial increase and other procedures for its control must be evaluated.

Keywords: *Artemia*, *Vibrio*, hatching, enrichment, *Litopenaeus vannamei*.

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1. Introdução

O camarão branco *Litopenaeus vannamei* é vastamente cultivado em diversas partes do mundo. Seu crescimento rápido e capacidade de cultivo com altas densidades fazem dessa espécie uma boa escolha para estratégias semi-intensivas e intensivas de aquicultura (WILLIAMS et al., 1996; PONCE-PALAFOX et al., 1997).

A maioria das fazendas de camarão marinho utilizam pós-larvas produzidas em laboratórios especializados. Apesar da tecnologia em larga escala da produção de pós-larvas já se encontrar bem desenvolvida, a intensificação das atividades de carcinicultura têm aumentado a ocorrência de doenças, as quais são consideradas como a maior causa de mortalidade nas larviculturas de camarão prejudicando a produção e o desempenho das pós-larvas no sistema de engorda (MORIARTY, 1998; GOMEZ-GIL et al., 2000; COSTA, 2006).

Em geral as larviculturas utilizam diversos tipos de alimentos, vivos e inertes, na tentativa de suprir as necessidades nutricionais das diferentes fases larvais. Apesar dos avanços tecnológicos na produção de rações específicas para larvicultura, o uso de alimento vivo ainda se faz necessário. Entre os alimentos vivos que podem ser utilizados, os principais são as microalgas (ex. *Chaetoceros* sp., *Tetraselmis* sp.) e os microcrustáceos conhecidos popularmente como artêmia (*Artemia* sp.). Ambos possuem alto valor nutritivo, suprindo as exigências nutricionais das larvas de crustáceos (SORGELOOS e LÉGER, 1992).

As vantagens da utilização dos náuplios de artêmia são inúmeras, tais como seu tamanho adequado, mobilidade, aceitação pelo predador, alto teor protéico, atração organoléptica, carapaça quitinosa fina, facilidade de armazenamento dos cistos e praticidade na sua utilização (LÉGER et al., 1987). As artêmias ainda podem ser

oferecidas vivas (recém eclodidas ou enriquecidas), congeladas ou na forma de cistos descapsulados.

O cultivo intensivo de larvas sofre por grandes mortalidades, as quais podem ser atribuídas a bactérias introduzidas no sistema de cultivo com alimentos vivos (NICOLAS et al., 1989; KESKIN et al., 1994). A alta carga orgânica associada com a produção intensiva de culturas seletivas de alimentos vivos induz a um crescimento proporcional de bactérias oportunistas que podem ser patogênicas para as larvas (SKJERMO e VADSTEIN, 1999). A desinfecção, embora benéfica, pode não prevenir a re-colonização dos alimentos vivos em um curto período de tempo (MUNRO et al., 1999).

Os antibióticos têm desempenhado um importante papel no combate de doenças de animais aquáticos cultivados. Embora uma grande variedade e número de quimioterápicos tenham sido desenvolvidos e aplicados na aqüicultura, os utilizados para a carcinicultura são limitados, pois o uso em larga escala de antibióticos tende a selecionar cepas de bactérias resistentes aos mesmos. Dessa maneira a aplicação de probióticos no cultivo de organismos aquáticos está crescendo com a demanda por mais práticas de aqüicultura sustentáveis (BOYD e MASSAAUT, 1999; ESIOBU et al., 2002; CHYTHANYA et al., 1999; ROQUE e GOMES-GIL, 2003; HOLMSTRÖM et al., 2003).

Outro método de controle bacteriano pode ser relacionado ao uso de microalgas. Segundo Kellan e Walker (1989) e Olsen et al.(2000), algumas microalgas parecem ser naturalmente bacteriostáticas ou bactericidas.

Com base nessas informações faz-se necessário a investigação de métodos para manipular e controlar a contaminação bacteriológica em alimentos vivos,

especificamente na eclosão e enriquecimento de artêmia, no intuito de reduzir a carga de bactérias nos cultivos de larvas de interesse.

2. Revisão de literatura

O camarão *Litopenaeus vannamei* é amplamente cultivado em países ocidentais onde 90% da produção total de camarões são desta espécie (WURMANN et al., 2004). Entre as atividades de aqüicultura que mais vêm se destacando encontra-se a carcinicultura.

Atualmente, o *L. Vannamei* também é a espécie o mais cultivada no Brasil, respondendo por mais de 95% da produção nacional. A escolha desta espécie para o cultivo foi devido principalmente a sua fácil adaptação às condições climáticas, tecnologias bem desenvolvidas, boa produtividade, grande aceitação no mercado internacional, como também maior adaptação em cativeiro (SIQUEIRA et al, 2009; ABCC, 2004; RODRIGUES, 2005).

Em 2008, a contribuição do cultivo de peneídeos para a produção mundial de crustáceos atingiu 73,3%. Além disso, o camarão continua a ser a maior commodity, única em termos de valor, contabilizando15% do valor total dos produtos da pesca comercializados a nível internacional em 2008 (FAO, 2010).

Aumentos nos cultivos de camarão têm desencadeado uma crescente demanda na produção de pós-larvas (NAEGEL e RODRÍGUEZ-ASTUDILLO, 2004). Em larviculturas comerciais, a artêmia está entre os alimentos naturais mais completos as exigências nutricionais de peixes e camarões, sendo adotado como alimento padrão (SORGELOOS et al., 1998; SORGELOOS et al., 2001).

O consumo de cistos de *Artemia* sp. no Brasil é centrado em sua quase totalidade (>95%) nos laboratórios de produção de larvas de camarão marinho. Em 2001, o Brasil produziu 7.915 bilhões de pós-larvas de *L. vannamei* e foram necessárias quatro

toneladas de cistos de *Artemia* sp. para cada bilhão de pós-larvas produzidas (YFLAAR e OLIVERA, 2003).

Por mais de 30 anos o uso de náuplios de artêmia como alimento vivo durante os estágios de desenvolvimento de Misis e pós-larval, tem sido uma prática comum em larviculturas de camarão, por causa de suas vantagens nutricionais e operacionais, além de serem amplamente reconhecidos como os melhores alimentos vivos armazenáveis avaliados (COOK e MURPHY, 1966; KUNGVANKIJ et al., 1986; LAVENS e SORGELOOS, 1986).

As pós-larvas são morfologicamente iguais a um camarão adulto e apresentam hábito bentônico. Nesta fase, em seu habitat natural apresentam hábito alimentar omnívoro, e em condições de cativeiro alimentam-se de ração e biomassa de *Artemia* adulta (TREECE e FOX, 1993). O passo inicial para o cultivo de larvas de organismos aquáticos ocorreu com a descoberta, por Seale (1933), de que náuplios de *Artemia* (Leach 1819, Crustacea, Branchiopoda, Anostraca, Artemiidae), constituem uma excelente fonte de alimento para larvas (SORGELOOS, 1980).

De acordo com Léger et al., (1986) e Sorgeloos et al., (1988), o náuplio de artêmia tem se apresentado como um dos melhores alimentos para a maioria de organismos de cultivo, pois é considerado como vetor para promover o crescimento, carregar drogas para aplicações terapêuticas (AGUILAR-ÁGUILA et al., 1994; OZKIZILCIK e CHU, 1994a; DIXON et al., 1995; TOURAKI et al., 1996; BURBOA-ZAZUETA, 1997), bem como para realizar um controle biológico em sistemas de aqüicultura (OESTMAN et al., 1995). Além disso, pode ser utilizado de diferentes formas durante uma larvicultura de camarões. Estas podem ser oferecidas como cistos descapsulados ou como náuplios congelados (SMITH et al., 1992).

O escudo exterior duro do cisto de artêmia, a camada alveolar, pode ser completamente removida através de um processo químico denominado descapsulação (SORGELOOS et al., 1983). Os cistos descapsulados podem então serem utilizados imediatamente ou desidratados em uma solução de salmoura para armazenamento, ou podem ainda serem submetidos a um processo de secagem para armazenamento a longo prazo. As vantagens da descapsulação incluem a desinfecção dos cistos, melhoria da eclosão dos cistos para obtenção de náuplios, maior conteúdo energético dos náuplios, e a isenção de risco da larva sofrer obstrução intestinal durante a ingestão do cisto (BENGTSON et al., 1991).

A artêmia congelada geralmente é utilizada em caráter de urgência, porém seu uso contínuo não só causa a deterioração da qualidade da água, mas também o costume da larva se alimentar de artêmia morta e a mesma pode não voltar a capturar presas móveis (SMITH et al., 1992). Contudo, Soares et al. (2006) em estudo realizado com *Farfantepenaeus paulensis*, indicaram que a artêmia congelada é recomendada apenas para pós-larvas com um a dez dias de idade (PL1-PL10).

Atualmente, a maioria dos cistos de *Artemia* encontrada no mercado é produzida no lago Great Salt Lake, cidade de Salt Lake, Estado de Utah, Estados Unidos (GSL), esses microcrustáceos possuem como habitat natural ecossistemas aquáticos continentais, sendo, portanto, supostamente inadequados para a alimentação de organismos marinhos (PONTES e ANDREATTA, 2003). Para suprir a deficiência de ácidos graxos altamente insaturados, característica dos cistos tipo água doce, desenvolveu-se a técnica de bioencapsulação (WATANABE et al., 1980), também conhecida como enriquecimento.

O valor nutricional dos náuplios de artêmia pode ser aumentado pela técnica de enriquecimento. A técnica explora o fato de que a artêmia é um organismo filtrador não

seletivo no segundo estágio (Instar II ou metanáuplio) de desenvolvimento, que ocorre após oito horas depois da eclosão (SORGELOOS et al., 2001). Isto permite a introdução de substâncias desejáveis nos metanáuplios, como ácidos graxos, probióticos, etc.

A maioria das bactérias associadas aos cistos de artêmia podem ser eliminadas por tratamento químico (SORGELOOS et al., 1977; GÓMEZ GIL, 1993; MERCHIE et al., 1997), no entanto larviculturas de camarão são relacionadas a mortalidades larvais com a presença de bactérias, especialmente no estágio de Zoea III, onde normalmente elas começam a serem alimentadas com artêmia (LÓPEZ-TORRES e LIZÁRRAGA-PARTIDA, 2001).

Duan et al., (1995) indicaram que bactérias produzem substâncias orgânicas que se desenvolvem em filmes em superfícies expostas à água do mar. Estes filmes são compostos por polissacarídeos, principalmente glicose e galactose (RODRÍGUEZ e BHOSLE, 1991), que podem proteger a bactéria contra lavagens e cloração das paredes dos tanques. Essas afirmações, em conjunto com a limpeza deficiente dos tanques de cultivo, podem explicar como espécies de *Vibrio* são encontradas e mantidas durante as operações de cultivo.

Espécies de *Vibrio* começam a ser dominantes depois de 24 horas, provavelmente por que durante a eclosão, os cistos de artêmia são rompidos e uma substância de reserva orgânica, glicerol, é excretada para a água de cultivo (SORGELOOS et al., 1986). Glicerol é um substrato orgânico que é utilizado eficientemente por espécies de *Vibrio* (BIANCHI, 1976).

A preocupação com os efeitos do uso de antibiótico contra enfermidades ocorrentes no ambiente aquático tem sido crescente nos últimos anos. Os antibióticos podem ser aplicados diretamente na água ou incorporados em alimentos vivos (HIRSCH et al., 1999;. SHAO, 2001). Em ambos os casos, estas substâncias ou seus

metabólitos, eventualmente, podem atingir o meio ambiente e causar efeitos adversos sobre os organismos selvagens (FERREIRA et al., 2007).

Estudos realizados apontaram que dentre os antibióticos e antimicrobianos mais comuns de uso na aquicultura, encontram-se compostos da classe das tetraciclinas, fluoroquinolonas, sulfonamidas e anfenicóis como o tianfenicol e o florfenicol (LALUMERA et al.,2004; DIETZE et al., 2005; CHRISTENSEN et al., 2006; LYLE-FRITCH et al., 2006).

Análises preliminares de uma variedade de produtos do mar têm revelado traços de cloranfenicol e nitrofuranos, que são antibióticos de amplo espectro de utilização e que apresentam alta taxa de risco de toxicidade para seres humanos. O cloranfenicol pode causar doenças potencialmente fatais como anemia e leucemia, enquanto que os nitrofuranos são carcinogênicos. O uso destes antibióticos na produção de alimentos para animais é proibido há mais de uma década na maioria dos países (GLOBAL AQUACULTURE ALLIANCE, 2002). Recentemente, a disseminação do Florfenicol tem crescido rapidamente por sua eficácia e pelo fato de que, mesmo tendo estruturas semelhantes ao cloranfenicol, não existem estudos que associem seu uso ao aparecimento de anemia (PEZZA, 2006). Segundo Roiha et al (2010), o microcrustáceo artêmia pode ser utilizado como vetor de partículas de florfenicol para estágios larvais de organismos aquáticos.

Além das vantagens da bioencapsulação, as artêmias quando ainda encistadas podem passar por um protocolo de desinfecção que é frequentemente realizado para reduzir a carga bacteriana antes da sua adição em tanques de cultivo. O protocolo de descontaminação dos náuplios geralmente envolve um ou mais agentes antimicrobianos, como desinfetantes, formaldeído, hipoclorito de sódio, peróxido de hidrogênio ou ozônio (GILMOUR et al., 1975; BENAVENTE e GATESOUPE, 1988; GOMEZ-GIL

et al., 1994; HAMEED e BALASUBRAMANIAN, 2000; GATESOUPE, 2002; TOLOMEI et al., 2004; GIMENEZ et al., 2006; SMITH e RITAR, 2006).

Um estudo realizado por Hoj et al., (2009), demonstrou que depois do tratamento de náuplios de *Artemia* com formalina, Virkon S (desinfetante), e uma mistura de antibióticos, a abundância total de bactérias cultiváveis foi reduzida para todos os náuplios de artêmia enriquecidos. Métodos moleculares mostraram, contudo, que o DNA de *Vibrio* spp. foi enriquecido depois do estágio de desinfecção. Isso demonstra que o DNA do *Vibrio* foi melhor protegido do que o DNA de outros gêneros de bactérias, sugerindo que as células do gênero *Vibrio* foram mais resistentes ao tratamento do que outras populações bacterianas. Uma alta incidência de genes multiresistentes a antibióticos na população de *Vibrio* seria uma possível explicação para esta observação. Vários autores têm relatado a existência de *Vibrios* multiresistentes a antibióticos, o que pode ser atribuído a genes presentes em plasmídios localizados no cromossomo bacteriano (MOLINA-AJA et al., 2002; AKINBOWALE et al., 2006; ROWE-MAGNUS et al., 2006; NEELA et al., 2007; LE ROUX et al., 2009).

Uma vez que os cistos de artêmia podem ser eclodidos em larviculturas sem condições estéreis, o enriquecimento alimentar de náuplios de artêmia permite a manipulação de sua composição bioquímica. Inoculando o trato digestivo dos organismos alvo a serem cultivados com bactérias probióticas, através do fornecimento de artêmia enriquecida, pode ter um efeito positivo pela melhora das propriedades da microflora indígena das larvas cultivadas. Esse efeito positivo dos probióticos pode ser atribuído a sua habilidade de competir com outras bactérias ou produzir micronutrientes importantes para o desenvolvimento das larvas (SUGITA et al., 1991; HAVENNAR et al., 1992; RINGØ et al., 1992; GATESOUPE, 1994; AUSTIN et al., 1995).

As propriedades quantitativas e qualitativas da microflora bacteriana dos alimentos vivos podem ser ajustadas para evitar efeitos negativos de uma sobrecarga de bactérias (BENAVENTE E GATESOUPE, 1988; NICOLAS et al., 1989; SKJERMO e VADSTEIN, 1993; KESKIN et al., 1994), e ao mesmo tempo realizar uma colonização bem sucedida do intestino de larvas de interesse (MUNRO et al., 1999). A incubação em curto prazo dos organismos do alimento vivo em uma suspensão bacteriana constituída por uma ou várias cepas probióticas é um possível caminho para substituir bactérias oportunistas por outras bactérias menos agressivas (REITAN et al., 1993).

Bactérias com várias características têm sido incorporadas em metanáuplios de artêmia por desafios orais (CHAIR et al., 1994; GRISEZ et al., 1996). Esta rota tem sido utilizada para vacinar alevinos de peixes e juvenis de carpas (CAMPBELL et al., 1993; JOOSTEN et al., 1995). Alguns estudos sugerem que as infecções bacterianas são iniciadas através da rota oral em larvas de peneídeos e suas pós-larvas (LAVILLA-PITOGO, 1990), portanto a adição de probióticos via alimentação pode ser um eficiente método para introduzir cepas probióticas.

O estudo da interação bacteriana com crustáceos como artêmia e camarões peneídeos é importante, pois presume-se que as bactérias proporcionam direta ou indiretamente elementos nutricionais como vitaminas, aminoácidos essenciais, ácidos graxos, poliaminas e enzimas (AUSTIN, 1988; BERGH, 1995; GRIFFITH, 1995; GOROSPE et al., 1996; VERSCHUERE et al., 2000). Douillet (1987) demonstrou que alimentos secos como farelo de arroz desengordurado, farelo de soja, *Spirulina*, e levedura Fleischmann resultaram em uma baixa ou nenhuma sobrevivência de larvas de artêmia, em condições axênicas. No entanto os mesmos alimentos resultaram em mais de 60% de sobrevivência larval, quando as culturas axênicas foram inoculadas com uma microflora selecionada.

As microalgas também compõem um papel importante ou até mesmo vital no cultivo de animais aquáticos como moluscos, camarões e peixes e têm um interesse estratégico na aqüicultura (MULLER-FEUGA, 1977). Elas são necessárias na alimentação na segunda fase de desenvolvimento larval de camarões peneídeos (Zoea), e em combinação com o zooplâncton para a terceira fase (Misis). A alimentação de larvas de interesse consiste em uma combinação de microalgas e estágios iniciais de desenvolvimento de *Artemia sp*., bem como rações comerciais específicas para cada fase de desenvolvimento. Os principais gêneros de microalgas utilizados são *Skeletonema, Chaetoceros, Tetraselmis, Chlorella, e Isochrysis* (MULLER-FEUGA, 2000). Dentre essas linhagens de microalgas, a espécie *C. calcitrans* é uma das mais adequadas para alimentar artêmia por causa de seu tamanho apropriado, digestibilidade, ausência de toxinas, e valor nutricional (KHOI et al., 2009).

Os benefícios nutricionais das algas e seu potencial como agentes biocontroladores na aquicultura têm sido reconhecidos. Kogure et al. (1979), por exemplo, demonstraram que espécies de *Pseudomonas* e *Vibrio* em cultura mista foram inibidos pela Bacillariophyta marinha *Skeletonema costatum.* Do mesmo modo, Austin et al. (1992) observaram que vários patógenos foram inibidos pelo sobrenadante e extratos de células de *Tetraselmis suecica.*

Os efeitos biológicos já descritos, para as microalgas abrangem atividades imunomoduloras, antivirais, antitumorais, antibacterianas e anticoagulantes, entre outras. Essas atividades benéficas são atribuídas às macromoléculas de polissacarídeos encontrados nas microalgas como material de reserva (BOHN e BeMILLER, 1995; SPOLAORE, 2006). Além disso, as microalgas quando participam do processo de enriquecimento de artêmia podem melhorar o valor nutritivo desse alimento vivo

através do fornecimento de ácidos graxos altamente insaturados (HUFAs) (LÉGER et al.,1986).

Como os náuplios de artêmia oriundos de fontes continentais não possuem ácidos graxos poliinsaturados de cadeia longa, a sua suplementação com emulsões lipídicas, ou microalgas com alto teor de ácidos graxos poliinsaturados através do processo de enriquecimento é recomendada (SORGELOOS et al., 2001).

Do ponto de vista energético, os lipídeos constituem a mais rica classe de nutrientes, por serem importantes fornecedores de energia metabólica, e possuírem valores superiores de energia bruta. Além de atuarem como fonte de ácidos graxos e outras classes de lipídeos essenciais, como os fosfolipídeos, também são importantes na absorção das vitaminas lipossolúveis A, D, E e K, constituem parte da estrutura da membrana celular, além de serem precursores de hormônios esteróides (TACON, 1987), desempenhando importante papel no metabolismo intermediário e na reprodução de organismos aquáticos (CAMARA, 1994).

O uso de lipossomos como produtos de enriquecimento podem fornecer diferentes vantagens e possibilidades (HONTORIA et al., 1994; OZKIZILCIK e CHU 1994b; TOURAKI et al., 1995; TONHEIM et al., 2000). Os lipossomas são partículas discretas com um tamanho adequado para os náuplios filtrarem. É possível encapsular substâncias hidrossolúveis na fase aquosa entre a bicamada lipídica destas vesículas, bem como moléculas hidrofóbicas na fracção de hidrocarbonetos da cadeia dos fosfolipídios. Além disso, os lipídios polares que formam os lipossomas também podem ser facilmente introduzidos nas cadeias tróficas utilizadas na larvicultura (MONROIG et al., 2003).

Estas características permitem o encapsulamento em vesículas lipídicas de diferentes nutrientes de especial importância para o bom desenvolvimento das larvas

marinhas. Alguns exemplos de tais nutrientes são as vitaminas solúveis (MERCHIE et al., 1997) e fosfolípideos (COUTTEAU et al., 1997).

De acordo com Tolomei et al. (2004), a prática corrente para a realização do enriquecimento de artêmia durante seu processo de crescimento pode envolver a combinação de diatomáceas e emulsões comerciais (ex. Selco, INVE®). O enriquecimento de artêmia com preparações comerciais como Super Selco, DHA Selco, DHA Protein Selco, DC DHA Selco, Algamac 3050 e óleos marinhos que são ricos em HUFAs, aprimora o valor nutricional para o desenvolvimento larval (HAI et al, 2011). Ainda, segundo Tolomei et AL. (2004), componentes do A1 DHA Selco conseguem inibir o crescimento de bactérias durante o enriquecimento assim como outros tipos de emulsões avaliadas comercialmente.

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4. Artigo científico

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Vibrio spp. control in *Artemia* hatching and enrichment for *Litopenaeus vannamei* postlarvae feeding

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Abstract

The bacterial load in *Artemia* hatching and enrichment were evaluated in two experiments (I): newly hatched *Artemia* nauplii were exposed to *Chaetoceros calcitrans* microalgae, commercial probiotic (*Bacillus* spp.) and Florfenicol antibiotic added to hatching water of decapsulated and capsulated cysts. Presumptive *Vibrio* counts were recorded in hatching water and *Artemia*. Bacterial isolates from *Artemia* were identified. Additionally, *Artemia* nauplii were frozen and the presuntive *Vibrio* load evaluated after 48 hours. (II): *Artemia* metanauplius were enriched with *C. calcitrans*, commercial probiotic and emulsion DHA / EPA rich. Newly hatched nauplii represented the

Control. *Artemia* from the treatments were offered to *Litopenaeus vannamei* postlarvae (PL_7 to PL_{19}). Presuntive *Vibrio* were quantified in *Artemia*, postlarvae and rearing water. Results indicated that adding *C. calcitrans* in *Artemia* hatching water is an effective alternative to antibiotics but probiotic must be also considered to control the *Vibrio* spp. load in *Artemia* nauplii. The enrichment supplements increased the bacterial load in *Artemia* but they did not affect *Vibrio* concentration in postlarvae.

Keywords: *Artemia*, *Vibrio*, hatching, enrichment, *Litopenaeus vannamei*.

Introduction

In fish and shellfish hatcheries the widespread use of *Artemia* as live food is due to their positive characteristics such as high protein content and ability to produce storable cysts (Léger, Bengton, Sorgeloos, Simpson & Beck 1987). The *Artemia* nutritional value can be further increased by the enrichment process (bioencapsulation). The enrichment technique exploits the fact that *Artemia* is a non-selective filter feeder organism in its second stage of development (instar II or metanauplius), which occurs eight hours after hatching (Campbell, Adams, Tatner, Chair & Sorgeloos 1993; Dixon , Vanpoucke, Chair, Dehasque, Nelis, De Leenheer & Sorgeloos 1995; Sorgeloos, Dhert & Candevra 2001). This feature also allows the use of *Artemia* in diseases control through the bioencapsulation of antimicrobial agents.

However, *Artemia* nauplii has been also reported as vector of pathogenic bacteria in shrimp hatcheries (López-Torres & Lizárraga-Partida 2001). The bacterial load associated with *Artemia* includes *Vibrio* spp. which are related to high mortality in penaeid shrimp rearings worldwide (Lightner & Lewis 1975; Baticados, Lavilla-Pitogo,

Cruz-Lacierda, dela Pena & Sunaz 1990, Gomez-Gil, Thompson, Thompson, Garcia-Gasca, Roque & Swings 2004).

Frequent and inappropriate use of antibiotics can induce the selection and proliferation of resistant bacterial strains. Therefore, alternative prophylactic measures to reduce *Vibrio* spp. spreading should be adopted as they are more cost-effective and less dependent on the chemicals use (Witte, Klare & Werner 1999; Planas & Cunha 1999).

In this context, this study evaluated different prophylactic methods to control the bacterial load in *Artemia* hatching and enrichment. *Litopenaeus vannamei* postlarvae fed with *Artemia* were also investigated.

Materials and methods

Experiment I

The experiment evaluated three supplements to control *Vibrio* spp. during the hatching of *Artemia* cysts INVE® (INVE, Belgium, www.inve.be) Great Salt Lake (GSL-USA). A sequence of two trials tested capsulated and decapsulated cysts. A 12% sodium hypochlorite solution was used to decapsulate the cysts. According to the type of supplement four treatments were established: Antibiotic, Probiotic, Microalgae and Control. All supplements were applied directly into the hatching water. Antibiotic treatment received 300 mg L^{-1} dose of Florfenicol (Roiha, Samuelsen & Otterlei 2010). A commercial probiotic consisting of *Bacillus subtilis*, *B. pumilus* and *B. licheniformis* (2×10^5 CFU mL^{-1}) was used in Probiotic treatment. Microalgae treatment received *Chaetoceros calcitrans* (8×10^5 cells mL^{-1}) collected during exponential growth phase. Only seawater was used in Control.

Artemia cysts (1g L^{-1}) were stocked in cylindrical-conical tanks filled with 20 L seawater previously disinfected with 15 ppm chlorine. Temperature, salinity, dissolved oxigen and pH were maintained at 29.9 ± 0.3 ºC, 27.7 ± 0.1 g L^{-1}, 5.7 ± 0.1 mg L^{-1} e 8.1 ± 0.03 respectively, as recommended by Van Stappen (1996). The *Artemia* hatching tanks were illuminated (2000 lux) and aerated continuously. Three replicates were used for each treatment. After 24 h incubation, the hatched nauplli were harvested and counted. The nauplii hatching efficiency (HE) was calculated using the formula: HE = (mean number of nauplii hatched mL^{-1} x water volume) / grams of cysts added.

Water and *Artemia* samples of all treatments were collected to quantify presumptive *Vibrio* colony forming units (CFU) using the agar Thiosulfate Bile Sucrose (TCBS agar; Himedia Laboratories Corporate Office, Mumbai, India, www.himediaslab.com). Water samples were serially diluted (1/10) in sterile saline solution (2.5% NaCl). Aliquots of 0.1 mL from three dilutions were spread plated on TCBS agar and incubated for 24 h at 30°C. After incubation the total colony forming units were enumerated.

The samples of *Artemia* were aseptically macerated, diluted and plated using the same methodology described for the water analysis. Additionally, samples of different *Artemia* treatments were frozen at a temperature of -25°C for 48 h and then the bacterial load was also determined. This analysis determined the temperature influence on the bacteria viability, since commercial hatcheries occasionally use *Artemia* in the frozen form.

From the nauplii samples, different bacterial morphotypes grown on TCBS agar were isolated and subjected to presumptive identification tests for the *Vibrio* genus (detection of glucose fermentation and oxidase). Further the morphotypes were identified through their biochemical profiles presented in the commercial bacterial

identification kit (API 20 E - Biomerieux, Marcy l'Etoile, France, www.biomerieux.com).

Experiment II

***Artemia* enrichment**

Vibrio concentration was evaluated in *Artemia* enriched with three different supplements: *C. calcitrans* (Microalgae treatment), commercial probiotic (Probiotic treatment) and a commercial emulsion rich in fatty acids (Selco treatment). Control was represented by newly hatched nauplii with no added supplements. Three replicates were used for each treatment.

Capsulated *Artemia* cysts (GSL, INVE®) were hatched under the same conditions of the Control in the first experiment. The newly hatched nauplii were washed with chlorine disinfected sea water and stocked (100 - 300 nauplii mL^{-1}) in 5 L containers filled with sea water and the different supplements. *Artemia* were submitted to enrichment process during 28 h under constant aeration.

Microalgae treatment was enriched with 8 x 10^5 cells mL^{-1} of *Chaetoceros calcitrans* and Probiotic treatments with 2 x 10^5 CFU mL^{-1} of commercial probiotic (*Bacillus subtilis*, *B. pumilus* and *B. licheniformis*). Selco treatment received 0.3 g L^{-1} every 12 h (Merchie, Lavens, Radull, Nelis, De Leenheer & Sorgeloos 1995) of commercial emulsion rich in docosahexaenoic and eicosapentaenoic fatty acids besides substances to provide *Vibrio* control.

The *Artemia* hatching and enrichment processes were repeated during 12 days for *L. vannamei* postlarvae feeding. Average water temperature, salinity, pH and dissolved oxygen were maintained between 27-31°C, 28-30 g L^{-1}, 8.5 to 9.0 and 4.69 to 6.65 mg L^{-1} respectively.

129

130 ***Litopenaeus vannamei* postlarvae rearing**

131 Shrimp postlarvae in PL_7 stage (7 days in the postlarval stage), with wet weight
132 mean of 0.843 mg, were randomly stocked in 12 plastic containers (10 L) at the density
133 of 50 PL L^{-1}. Water was previously desinfected with chlorine (15 ppm) for 24 h, and
134 then neutralized with ascorbic acid.

135 Enriched *Artemia* from the four treatments (Microalgae, Probiotic, Selco and
136 Control) were offered for postlarvae feeding during 12 days (three replicates each).
137 Shrimp were fed twice daily (07:00 and 19:00 h) at an initial rate of 5 nauplii mL^{-1}
138 reaching 12 nauplii mL^{-1} at the end of rearing according to the larvae consumption.

139 The postlarvae were maintained under constant aeration at a temperature of 27-
140 28°C. Daily, 50% of water was exchange in the experimental units. Temperature,
141 dissolved oxygen, pH and salinity were monitored daily by a multiparameter sensor
142 (YSI 556).

143

144 **Bacteria analysis**

145 Presumptive *Vibrio* spp. were quantified in samples of enriched *Artemia*, water
146 of *Artemia* enrichment, postlarvae and water of postlarvae rearing.

147 The procedure for *Vibrio* analysis in water and *Artemia* samples followed the
148 methodology presented in the experiment I. The PL samples were weighted, macerated
149 and diluted (1/10) and 0.1 mL of three dilutions were plated in TCBS agar, incubated
150 and enumerated as described.

151 Additionally, the colonization of bacteria from commercial probiotic, was
152 verified by the quantification of *Bacillus* CFU in enriched *Artemia* and postlarvae from
153 Probiotic treatment. The samples were weighted, macerated and diluted (1/10) and 0.1

mL of three dilutions were plated in MYP agar (Mannitol Egg Yolk Agar polymyxin, Himedia®), incubated and enumerated.

Data analysis

The hatching efficiency data were submitted to ANOVA and Tukey's test. The Student's T-test was used to compare the bacterial count of *Artemia* natural biomass and frozen biomass. Bacterial counts data were submitted to ANOVA and Fisher tests. Differences were considered at the 5% significance level. All analyzes were performed using Statistica 7.0.

Results

Experiment I

The hatching efficiency was similiar between treatments and the mean (± SE) ranged from $1.9 \pm 0.1 \times 10^5$ to 2.7×10^5 nauplii g^{-1}.

The decapsulation process did not shown to be effective in reducing the presumptive *Vibrio* spp. load in nauplii and water in all treatments (Table 1).

The number of bacterial colonies in water was significantly lower in Antibiotic followed by Microalgae treatment of capsulated cysts (Table 1). Only the Probiotic treatment did not significantly differ from the Control.

A similar pattern was observed in the decapsulated cysts analysis, where the significant lower *Vibrio* counts in water were observed in Antibiotic and Microalgae (Table 1). However, the Microalgae did not differ significantly from the other treatments.

Insert Table 1

In the trial with capsulated cysts, *Vibrio* spp. load in *Artemia* was significantly lower in the supplemented treatments (Table 1). However, *Artemia* from decapsulated cysts showed significantly lower contamination in Antibiotic and Probiotic treatments (Table 1). The Microalgae did not differ from the other treatments.

The freezing nauplii process during 48 hours resulted in a reduction of 70.33 to 99.75% of the *Vibrio* spp. load (Table 2).

Insert Table 2

Of the 43 bacterial colony morphotypes isolated from nauplii of all treatments (capsulated cysts) 54% was identified as *Vibrio alginolyticus* and 36% as Gram-negative cocci oxidase-negative. *Vibrio parahaemolyticus*, *Pasteurella multocida*, *Aeromonas hydrophila*, *Aeromonas salmonicida* and *Ochrobactrum anthropi* each represented 2% of the total isolates. *V. parahaemolyticus* and *O. anthropi* were resistant to the Florfenicol.

From the nauplii of decapsulated cysts were obtained 31 isolates. Of these, 42% were identified as *V. alginolyticus*, 42% as Gram-negative cocci oxidase-negative and 16% as Gram positive isolates.

Experiment II

The *Vibrio* spp. concentrations in the *Artemia* enrichment water were significantly lower in the Control (newly hatched nauplii) and did not differ among other treatments (Table 3). In *Artemia* the lowest values of bacterial colonies were observed in Control. However, the Control did not differ from Microalgae and Probiotic

treatments. Selco presented the highest level of contamination differing significantly from Control.

The *Vibrio* spp. concentrations in the postlarvae rearing water from Probiotic treatment was significantly lower than Control (Table 3). No significant differences were observed in the number of bacterial colonies in postlarvae among treatments (Table 3).

Insert Table 3

Bacillus quantification (± SE) in metanauplius and postlarvae from Probiotic treatment was $8.7 \pm 4.2 \times 10^5$ and $1.4 \pm 0.8 \times 10^6$ CFU g^{-1} respectively.

In the postlarvae rearing water, the mean values (± SE) of temperature (28.7 ± 0.04°C), OD (5.5 ± 0.1 mg L^{-1}), pH (8.50 ± 0.04) and salinity (29.5 ± 0.06 g L^{-1}) did not differ among treatments.

Discussion

Bacterial enumeration in TCBS medium shows that *Artemia* hold a high number of bacteria. According to Verdonck, Grisez, Sweetman, Minkoff, Sorgeloos, Ollevier and Swings (1994), *Artemia* are usually highly contaminated with bacteria (> 10^7 CFU per gram) and mostly identified as *Vibrio* spp. Moreover it is necessary to control these bacterial loads before the use of *Artemia* in culture systems.

The use of Florfenicol resulted in the lowest *Vibrio* spp. load among the supplements tested in *Artemia* hatchery. The Florfenicol has been authorized in several countries for aquaculture activities (FAO 2005). In Brazil, it is the only antibiotic registered for this purpose in the Ministry of Agriculture, Livestock and Supply

(MAPA) (Schering Plough 2009). In fish farming, Florfenicol has potent activity against a broad range of pathogens (Samuelsen, Ervik & Bergh 2003), including microorganisms resistant to other antibiotics (Nordmo, Varma, Brokken & Sutherland 1994; Rangdale, Richards & Alderman 1997; Bruun, Schmidt, Madsen & Dalsgaard 2000; Thyssen & Ollevier 2001; Vue, Schmidt, Stehly & Gingerich 2002; Samuelsen & Bergh 2004). Our findings suggested that the Florfenicol dose (300 mg L^{-1}) was efficient in reducing *Vibrio* counts but some potentially pathogenic strains of *Vibrio* (*V. alginolyticus* and *V. parahaemolyticus*) remained in *Artemia* nauplii. Nevertheless, proliferation of these resistant bacteria could make possible infections more difficult to treat in hatchery systems.

The Microalgae treatment was the second most efficient in reducing *Vibrio* load in water and nauplii (capsulated). In accordance, Tolomei, Burke, Crear and Carson (2004), recommended the genus *Chaetoceros* to sanitize the external surface of *Artemia*. This effect may be related to the bacteriostatic or bactericidal microalgae activity (Kellam & Walker 1989; Olsen, Olsen, Attramadal, Christie, Birkbeck, Skjermo & Vadstein 2000). The microalgae antibacterial activity has been detected in microalgae extracts (Duff & Bruce 1966; Austin & Day 1990, Austin, Baudet & Stobie 1992; Tendencia & dela Peña 2003) and it may be related to the associated microflora, antimicrobial proteins, fatty acids and oxygen free radicals produced by microalgae cells (Marshall, Salas, Oda & Hallegraef 2005; Makridis, Alves Costa & Dinis 2006; Kokou, Ferreira, Tsigenopoulos, Makridis, Kotoulas, Magoulas & Divanach 2007).

Despite newly hatched *Artemia* nauplii are unable to bioencapsulate, probiotic bacteria can be active in the gills and body surface by competing with other bacteria for adhesion sites (Gatesoupe 1991; Verschuere, Rombaut, Sorgeloos & Verstraete 2000).

253 The present study has shown that probiotics reduced the *Vibrio* load in *Artemia* from
254 capsulated and decapsulated cysts.

255 Freezing *Artemia* for 48 h sharply reduced the *Vibrio* counts, but most values
256 were still over 10^7 CFU. However, it has been suggested that *Vibrio* spp. are capable of
257 entering into a viable but non-culturable (VBNC) state when exposed to low
258 temperatures (Jiang & Chai 1996, Johnson & Brown 2002). Eventually, when
259 temperature rise bacterial cells are able to emerge from the VBNC state and become
260 culturable on bacteriological media. Thus, offering short-term frozen stored *Artemia* to
261 shrimp larvae may not reduce the potential of *Vibrio* spp. contamination, as cold-
262 induced death of bacteria only occur after several days to weeks (Oliver 1981).

263 The sodium hypochlorite used in the decapsulation process is able to totally
264 decontaminate *Artemia* cysts, but they can be quickly recolonized during the rupture
265 stage before hatching (Sorgeloos *et al*. 2001). At this stage, the organic substrate
266 glycerol is released from the cysts and offers an ideal culture medium for *Vibrio* spp. In
267 this study the decapsulation process did not effective reduced *Vibrio* concentration, but
268 decreased bacterial species in nauplii and may be regarded as an auxiliary prophylactic
269 treatment.

270 Hoj, Bourne and Hall (2009) characterized the bacterial community present in
271 *Artemia*, with more than half of *Vibrio* isolates being identified as *V. alginolyticus*.
272 López-Torres and Lizárraga-Partida (2001) observed that even when *Artemia* cysts were
273 hatched under sterile conditions *V. alginolyticus* was the dominant species. These
274 authors suggested that *V. alginolyticus* and *Vibrio* spp. isolated from *Artemia* hatching
275 tanks were associated with those isolated from tanks with zoea, mysis and postlarvae,
276 indicating that these *Vibrio* spp. remain associated with different development phases of
277 shrimp. Buglione, Vieira, Mouriño, Pedrotti, Jatoba and Martins (2010) observed that *V.*

alginolyticus strain caused high mortality of *L.vannamei* larvae. The *V. alginolyticus* virulence is correlated to enzyme collagenase activity, which can cause softening of shrimp muscle tissue (Brauer, Leyva, Alvarado & Sandez 2003; Yishan, Jiaming, Jichang & Zaohe 2011).

The presence of *V. parahaemolyticus* in samples of shrimp submitted to antibiotic treatment was reported by Verschuere *et al*. (2000). Likewise this bacteria was identified in *Artemia* from Antibiotic treatment. According to Gomez-Gil *et al*. (2004), *V. parahaemolyticus* attacks mainly shrimps at juvenile and adult stage.

Ochrobactrum anthropi was also resistant to the Florfenicol treatment. This species has been isolated from cryopreserved *Penaeus monodon* spermatophores (Nimrat, Bart, Keatsaksit & Vuthiphandchai 2008) and water samples from mangrove receiving shrimp farm effluents (Sousa 2006). However, this species has not been related to shrimp diseases in the literature.

Pasteurella multocida is also a bacteria usually not associated with disease in shrimp. However, Buglione *et al*. (2010) observed that after an experimental infection, this species increased mortality in *L. vannamei* larvae.

Aeromonas spp. compose the normal microflora of wild and reared crustaceans and can be considered an opportunistic pathogen (Lightner 1993). This genus has been associated to the soft shell syndrome in *Penaeus monodon* (Baticados, Coleso & Duremdez 1986; Uddin, Zafar Noman & Sharmin 2008).

The *Artemia* enrichment process increased bacterial load in water and *Artemia* specially in Selco treatment. This could be explained by the organic input from supplements addition and *Artemia* excretion, which allows a sudden increase of opportunistic bacteria (Igarashi, Sugita & Deguchi 1989; Skjermo & Vadstein, 1993; Verschuere, Dhont, Sorgeloos & Verstraete 1997; Olsen *et al*. 2000). Hoj *et al*. (2009)

and Haché & Plant (2011) also observed high bacterial abundance in *Artemia* enriched with microalgae and lipid emulsions in combination.

The bacterial load in postlarvae and rearing water were not associated with the concentrations of *Vibrio* observed in newly hatched nauplii (Control) and enriched *Artemia*. The daily renewal (50%) of postlarvae rearing water probably reduced the abundance of *Vibrio* spp. Krishnika and Ramasamy (2012) also recorded a significant reduction of *Vibrio* after the water exchange of *Artemia* rearing tanks. Silva, Soares, Calazans, Vogeley, Valle, Soares and Peixoto (2011), observed a presumptive *Vibrio* load of 7.9 x 10^7 CFU g^{-1} in *L.vannamei* postlarvae (PL_{10}) fed newly hatched *Artemia* nauplii. In the present study this load was slightly lower for PL_{19} (0.17 x 10^7 CFU g^{-1}).

Overall results of this study indicated that adding *C. calcitrans* in *Artemia* hatching water is an effective alternative to antibiotics. Additionally, the use of probiotic must be also considered to control the *Vibrio* spp. load in *Artemia* nauplii. The enrichment supplements increased the bacterial load in *Artemia* but they did not affect *Vibrio* concentration in postlarvae.

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Table 1. Average values (± SE) of presumptive *Vibrio* count in *Artemia* hatching water and *Artemia* nauplii (capsulated and decapsulated cysts) from different treatments.

	Vibrio count			
	Capsulated cysts		Decapsulated cysts	
	Water (10^5 CFU mL^{-1})	*Artemia* (10^7 CFU g^{-1})	Water (10^5 CFU mL^{-1})	*Artemia* (10^7 CFU g^{-1})
Control	620.0 ± 450.0^{c}	120.0 ± 90.0^{b}	200.0 ± 150.0^{b}	45.0 ± 24.0^{b}
Antibiotic	0.008 ± 0.007^{a}	0.02 ± 0.007^{a}	7.50 ± 2.90^{a}	3.10 ± 0.70^{a}
Microalgae	37.0 ± 21.0^{b}	0.40 ± 0.30^{a}	44.0 ± 1.90^{ab}	29.0 ± 10.0^{ab}
Probiotic	590.0 ± 230.0^{c}	15.0 ± 15.0^{a}	320.0 ± 190.0^{b}	15.0 ± 9.0^{a}

Different superscript letters in the same column indicate significant differences between treatments ($P < 0.05$).

Table 2. Average values (± SE) of presumptive *Vibrio* count in *Artemia* nauplii (capsulated and decapsulated cysts) from different treatments, before and after freezing.

	Vibrio count			
	Capsulated cysts		Decapsulated cysts	
	Before (10^7 CFU g^{-1})	After (10^7 CFU g^{-1})	Before (10^7 CFU g^{-1})	After (10^7 CFU g^{-1})
Control	120.0 ± 90.0^a	25.0 ± 23.0^b	45.0 ± 24.0^a	2.60 ± 1.00^b
Antibiotic	0.02 ± 0.007^a	0.00005 ± 0.00003^b	3.10 ± 0.70^a	0.92 ± 0.43^b
Microalgae	0.40 ± 0.30^a	1.30 ± 1.10^a	29.0 ± 10.0^a	6.00 ± 1.60^b
Probiotic	15.0 ± 15.0^a	1.40 ± 0.90^b	15.0 ± 9.0^a	2.50 ± 0.70^b

Different superscript letters in a row indicate significant differences ($P < 0.05$) between before and after freezing.

Table 3. Average values (± SE) of presumptive *Vibrio* count in *Artemia* rearing water, *Artemia*, *Litopenaeus vannamei* postlarvae (PL) and postlarvae rearing water from different treatments.

	Vibrio count			
	Artemia Water (10^6 CFU mL^{-1})	*Artemia* (10^7 CFU g^{-1})	PL Water (10^6 CFU mL^{-1})	PL (10^7 CFU g^{-1})
Control	3.4 ± 3.0^{a}	$6,5 \pm 3,0^{a}$	0.25 ± 0.00^{b}	0.17 ± 0.07
Selco	100.0 ± 30.0^{b}	160.0 ± 140.0^{b}	0.11 ± 0.03^{ab}	0.87 ± 0.86
Microalgae	17.0 ± 5.0^{b}	40.0 ± 7.0^{ab}	0.15 ± 0.09^{ab}	0.04 ± 0.02
Probiotic	67.0 ± 23.0^{b}	23.0 ± 2.0^{ab}	0.05 ± 0.02^{a}	0.06 ± 0.02

Different superscript letters in the same column indicate significant differences between treatments ($P < 0.05$).

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