

UNIVERSIDADE FEDERAL DO RIO GRANDE
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

MARIA ANGÉLICA DOS REIS GARRIDO PEREIRA

**USO DO PROBIÓTICO *Bacillus* spp. NA LARVICULTURA DO BIJUPIRÁ
Rachycentron canadum EM SISTEMA DE RECIRCULAÇÃO DE ÁGUA**

RIO GRANDE, RS

2011

UNIVERSIDADE FEDERAL DO RIO GRANDE
PROGRAMA DE PÓS-GRADUAÇÃO EM AQUICULTURA

USO DO PROBIÓTICO *Bacillus* spp. NA LARVICULTURA DO BIJUPIRÁ
***Rachycentron canadum* EM SISTEMA DE RECIRCULAÇÃO DE ÁGUA**

MARIA ANGÉLICA DOS REIS GARRIDO PEREIRA

Dissertação apresentada como parte dos requisitos para obtenção do grau de mestre em Aqüicultura no Programa de Pós-Graduação em Aqüicultura da Universidade Federal do Rio Grande.

Orientador: Prof. Dr. Luís André Sampaio

Co-orientador: PhD. Michael Schwarz

RIO GRANDE, RS, BRASIL

JANEIRO, 2011

AGRADECIMENTOS

Ao Prof. Dr. Luís André Sampaio pela orientação, pela oportunidade de trabalhar na Virginia Tech, pela ajuda e ensinamentos.

Ao PhD. Michael Schwarz, querido Mike, por todo suporte técnico, científico, e pessoal na estadia em Hampton, EUA.

Ao Prof. Dr. Luis Alberto Romano pela amizade e ajuda acadêmica. Ainda devo mais de quinze mil bacalhaus!

À equipe VSAREC, principalmente Prof. Michael Jahncke, Brendan C. Delbos e Helen Sutton, mas também Abigail Villalba, Dan Kauffman e Bob Lane.

Ao MSc. Ricardo Rodrigues pela ajuda gigantesca no pré-projeto, projeto, experimento, amostragem, análises estatísticas, chateações, enfim, por ter sido um grande colega e amigo (meu 'co-co-orientador').

Aos colegas e amigos da FURG que diretamente ou indiretamente me ajudaram nesta caminhada acadêmica e de farras: André, Andréa, Alessandro Del Duca, Alinca, Ana Velloso, Augusto, Beck, Cami Dal, Carlinhos, Cassiê, Cíntia, Cláudio, Dariano, Diego, Diogão, Eduardo Martins, Faby, Fê Rosa, Gabi David Canabarro, Gabriel, Getúlio, Iuri, Jana, Kassio, Kotoko, Leo, Luciano Jensen, Manuel, Martita, Midori, Miguel, Okamoto, Prof. Klebinho, Prof. Luciano, Prof. Mano, Prof. Mineiro, Prof. Paulo Abreu, Prof. Tesser, Renatão, Sabrina, Shakirita, Shay, Shei, Tati Fito, Verônica, Vikaláctica, Vivi. Em ordem alfabética. Perdoem-me se esqueci de citar alguém.

Aos professores do Programa de Pós-Graduação em Aquicultura.

Aos funcionários da Estação Marinha de Aquicultura.

Às minhas queridas amigas do triângulo, Fefê e Dani, e Ju pelo apoio. Distante fisicamente, mas sempre presente no coração.

Ao William, por todos os momentos compartilhados, pelo companheirismo, calma e paciência durante todo esse tempo.

Aos meus pais, Sônia e Gilberto, por todo apoio que tenho recebido, por todo amor. Essa vitória é principalmente para vocês.

Aos meus irmãos, Malonta, Ti e Céci, muito obrigada por tudo.

À minha família, pela união inabalável: tias, tios, primas, primos, avós.

Principalmente, obrigado Ao Pai Celestial. Sem ele nada seria possível.

ÍNDICE

Resumo	v
Abstract	vi
Introdução geral	1
Artigo anexo	11
“Probiotic effects of <i>Bacillus</i> spp. on cobia <i>Rachycentron canadum</i> larvae reared in a recirculating aquaculture system”	
Abstract	13
1. Introduction	14
2. Material and methods	17
3. Results	21
4. Discussion	22
5. Conclusion	27
Acknowledgements	27
References	27
Tables	35
Figures	36

RESUMO

O bijupirá (*Rachycentron canadum*) é um peixe marinho que apresenta bom potencial para seu cultivo. Muitas pesquisas demonstram os efeitos benéficos do probiótico *Bacillus* spp. na aquicultura, porém não há estudos sobre os efeitos de probióticos em sistemas de recirculação de água (SRA). Este trabalho analisa os efeitos do probiótico *Bacillus* spp. na performance de larvas de bijupirá criadas em SRA. O experimento foi realizado na Universidade Virginia Tech (EUA). As larvas foram estocadas em dois SRA independentes entre si - tratamento probiótico (T_P) e tratamento controle (T_C) - na densidade de 15 larvas/L, durante 26 dias após a eclosão. Um dos sistemas (T_P) recebeu a adição de probiótico comercial composto por *B. subtilis*, *B. licheniformis* e *B. pumilus* diretamente na água e via alimento vivo, conforme o fabricante. As análises de microbiologia e qualidade de água foram feitas diariamente por medição de parâmetros físico e químicos e plaqueamento em meio de cultura para contagem de *Bacillus* totais e *Vibrio* totais. Ao final do experimento foram realizados biometria e contagem total dos animais, teste de resistência ao estresse salino e fixação de larvas para imunohistoquímica do timo para avaliar a expressão dos complexos multirreceptores de antígenos CD3 e CD4 de linfócitos T e morfometria do rim cefálico. A sobrevivência foi similar e de 14,9±2,4% e 15,1±0,9% para T_P e T_C respectivamente. O peso final foi de 170±10 mg para T_P e 160±10 mg T_C, não havendo diferença estatística entre os tratamentos. Na microbiologia da água não foram encontradas diferenças significativas entre os tratamentos na contagem de vibrios totais (T_P: 3.366±522 UFC/mL; T_C: 4.964±902 UFC/mL). Na contagem de *Bacillus* totais os tratamentos diferiram estatisticamente, sendo maior a concentração no T_P pela adição dos *Bacillus* spp. (901±88 UFC/mL e 61±24 UFC/mL). O teste de estresse por salinidade demonstrou uma maior resistência ao estresse salino pelas larvas do tratamento probiótico. A análise imunohistoquímica mostrou uma maior expressão de CD4 no timo em PT (T_P: 43,7 ± 6,16% CD4 células/mm²; T_C: 25,4 ± 3,5% CD4 células/mm²), o que pode indicar uma melhor resposta imune contra patógenos. A análise morfométrica do rim cefálico indica que este órgão é maior no T_P (T_P: 0,97 ± 0,33 milímetros; T_C: 0,69 ± 0,22 milímetros), provavelmente devido a um aumento na quantidade de linfócitos B. Estes resultados indicam que o probiótico utilizado em um sistema de recirculação de água é efetivo quanto à imunoestimulação e aumenta a resistência ao estresse salino.

Palavras-chave: microbiologia, teste de estresse, imunohistoquímica, piscicultura marinha

ABSTRACT

Cobia (Rachycentron canadum) is a marine finfish with good potential for mariculture. The aim of this study was to analyze the effects of probiotic *Bacillus* spp. on the performance of cobia larvae reared in a recirculating aquaculture system (RAS). Many studies have demonstrated the beneficial effects of probiotic *Bacillus* spp. in aquaculture, but there are no studies on the effects of probiotics in RAS, requiring research on their effectiveness, since in open systems the beneficial effects are already known. The trial was conducted at Virginia Tech VSAREC (USA). Larvae were stocked into two independent RAS - probiotic treatment and control - at a density of 15 larvae L⁻¹, for 26 days after hatching. One of the systems (Probiotic treatment) received the addition of a commercial probiotic consisting of *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* directly into the water and by live feed, according to the manufacturer. At the end of the experiment we performed biometrics, larvae counts, stress test and larvae were fixed for morphometric analysis of head kidney and for immunohistochemistry of thymus to evaluate the phenotypic expression of complex antigens CD3 and CD4 in T-lymphocytes. Survival and final weight had no significant difference between groups. The analysis of water microbiology had no significant differences between treatments in total counts of *Vibrio*, but in the total count of *Bacillus* treatments differed statistically, with a higher concentration in Probiotic treatment because of the addition of *Bacillus* spp. in this group. The salinity stress test was expressed by stress sensitivity index (SSI), which demonstrated a greater resistance to salt stress by larvae of probiotic treatment. Immunohistochemical analysis showed a higher expression of CD4 in Probiotic treatment, which may indicate better immune response against pathogens. The morphometric analysis of head kidney indicates that this organ is larger in Probiotic treatment, probably due to an increase in the quantity of

B-lymphocytes. These results indicate that *Bacillus* spp. probiotics used in RAS may stimulate the immune system and increase the general stress resistance of cobia larvae.

Keywords: stress test, immunohistochemistry, marine fish farming

INTRODUÇÃO GERAL

O bijupirá *Rachycentron canadum*, internacionalmente conhecido por cobia, é a única espécie da família Rachycentridae. É um peixe migratório e pelágico que ocorre em todos os mares tropicais e subtropicais, com exceção da parte central e oriental do Oceano Pacífico (Shaffer & Nakamura 1989). Os indivíduos desta espécie habitam águas costeiras e plataformas continentais, podendo ser encontrados em baías e estuários, assim como em profundidades de até 1.200 m (Shaffer & Nakamura 1989). Espécimes já foram coletados em águas de salinidade entre 22 e 44 (Kaiser & Holt 2005), e têm sido cultivados com sucesso em sistemas com salinidade entre 5 e 30 (Resley et al. 2006).

Este peixe geralmente é encontrado sozinho ou em pequenos cardumes, e é comum estarem perto de alguma estrutura flutuante ou mesmo na coluna d'água (Kaiser & Holt 2005). São carnívoros oportunistas que se alimentam de várias espécies de peixes, caranguejos, camarões e lulas. O corpo do bijupirá é alongado e pode chegar a 2 metros de comprimento e 60 kg, sendo que as fêmeas crescem mais rápido que os machos (FAO 2007). São amarronzados dorsalmente, possuem a superfície ventral branca e apresentam listras que variam de claro a escuro nas laterais do corpo. A longevidade é de aproximadamente 15 anos (Shaffer & Nakamura 1989).

Por não ser um peixe abundante no meio natural, o bijupirá não é um pescado muito presente no comércio. Segundo a FAO (2010) a produção mundial do cultivo da espécie no ano de 2009 foi de 24.860 t. É considerado um excelente alimento, mas devido à baixa oferta do produto muitos consumidores desconhecem este peixe (Kaiser & Holt 2005).

O bijupirá é uma das espécies de peixes marinhos com bom potencial para a aquicultura. Entre seus atributos estão a facilidade de desova (Liao et al. 2004), taxas de crescimento de até 6 kg/ano (Fraser & Davies 2009), altas taxas de sobrevivência em tanques e gaiolas (estágio pós-larvicultura), adaptabilidade ao cativeiro (Arnold et al. 2002) e à ração (McLean et al. 2009). A espécie também demonstra alta resistência a doenças (Holt et al. 2007), boa resposta à vacinação (Lin et al. 2006), aos probióticos (Benetti et al. 2008) e aos imunostimulantes (Leaño et al. 2003), e tolera ampla faixa de salinidade (Resley et al. 2006). Além disso, possui alta qualidade como pescado e demanda de mercado crescente (Faulk & Holt 2005).

Devido a essas características favoráveis à criação, a produção de bijupirá tem crescido desde o início dos anos 1990 (Liao et al. 2004). A espécie tem sido cultivada com sucesso na Ásia, sendo que aproximadamente 80% da sua produção ocorrem em tanques-rede em alto-mar em Taiwan (Liao et al. 2004). Vietnã, China, Indonésia, Filipinas, Irã e La Reunion já produzem bijupirá em tanques-rede e recentemente os EUA, Brasil, Belize, República Dominicana, Martinica e México têm produzido e desenvolvido pesquisas (Benetti et al. 2010). Em Taiwan, os peixes são frequentemente criados em viveiros desde a eclosão até 30 g, quando são transferidos para viveiros maiores ou tanques cobertos para crescimento. Após este período os peixes são criados em tanques *offshore* até o final da fase de engorda, chegando ao peso de mercado de 6-8 Kg (para exportação) ou 8-10 Kg (para consumo interno em Taiwan) (Liao et al. 2004).

O uso de sistemas de recirculação de água (SRA) durante a larvicultura e primeiros estágios de juvenil propicia aos produtores um controle ambiental maior, permitindo a produção de alevinos durante todo ano e expandindo a produção para regiões mais frias (Faulk et al. 2007). Segundo D'Orbcastel et al. (2009), um SRA na aquicultura é uma combinação de processos como remoção de sólidos (filtração mecânica, decantação), controle de gases (oxigênio, gás carbônico) e processos biológicos (nitrificação da amônia por biofiltro, desinfecção por UV).

Por propiciar um cultivo com maior biossegurança e manter a qualidade da água em níveis adequados, o SRA pode proporcionar maiores taxas de sobrevivência (Ridha & Cruz 2001). O aumento da produtividade, juntamente com o tratamento contínuo e reuso da água, faz com que o SRA seja um sistema eficiente tanto para o produtor quanto para o meio-ambiente (D'Orbcastel et al. 2009).

O cultivo do bijupirá é relativamente recente, quando comparado a outras culturas já estabelecidas de peixes marinhos, como salmão do Atlântico (*Salmo salar*), robalo europeu (*Dicentrarchus labrax*), pargo (*Pagrus auratus*), linguados (Pleuronectiformes) e outros. Por isso ainda há limitações significantes na sua produção, como a produção viável de ovos e juvenis em quantidade, a ausência de dietas específicas e o mercado ainda em desenvolvimento (Holt et al. 2007).

Ainda não está definido o pacote tecnológico do cultivo do bijupirá, porém muitos estudos têm sido realizados no Brasil e no exterior, principalmente nas áreas de nutrição, reprodução e fisiologia, como digestibilidade de nutrientes (Zhou et al. 2004), alimentação larval (Faulk & Holt 2005), exigências nutricionais (Chou et al. 2001, Fraser & Davies 2009), reprodução em cativeiro (Weirich et al. 2006, Faulk & Holt

2008), efeitos da salinidade (Denson et al. 2003), nitrogenados (Feeley et al. 2007, Rodrigues et al. 2007) e fisiologia do estresse (Trushenski et al. 2010).

As pesquisas na área de imunologia são limitadas nesta espécie, sendo o único trabalho publicado nesta área sobre eficácia de vacinas em bijupirás (Lin et al. 2006).

Pesquisas acerca do desenvolvimento e funcionamento do sistema imune de peixes teleósteos tem tido cada vez mais importância (Press, 1999), uma vez que o uso de quimioterápicos nas criações está sendo condenado (Gatesoupe 1999, Vine et al. 2006, Kesacordi-Watson et al. 2008, Wang et al. 2008). A intensificação da aquicultura no mundo, em resposta à crescente demanda por pescado, levou a um aumento do uso inadequado de antibióticos e produtos químicos nas criações, resultando em problemas na segurança alimentar e resistência bacteriana (Decamp & Moriarty 2007).

Muitas estratégias alternativas ao uso profilático de antibióticos no controle de doenças foram propostas: instalação de manejos de biossegurança, vacinações efetivas, estimulação imunológica não específica, assim como o uso de probióticos (Ringø et al. 2010). Gatesoupe (1999) define probiótico como microorganismos vivos, que ao serem oferecidos como suplemento alimentar, melhoram a saúde geral do hospedeiro.

O uso de probióticos na produção de animais domésticos é conhecidamente vantajoso e estes produtos têm grande potencial para a aquicultura marinha (Hansen & Olafsen 1999). Em sistemas aquáticos os probióticos podem atuar de várias formas: excluindo bactérias patogênicas por competição, melhorando a qualidade da água e a condição nutricional e estimulando o sistema imune do organismo cultivado (Chen & Chen 2001, Decamp & Moriarty 2006, Gatesoupe 1999, Kesarcodi-Watson et al. 2008, Picchiatti et al. 2009a, Velmurugan & Rajagopal 2009).

Os probióticos comumente usados em aquicultura abrangem vários taxa, como bactérias lácticas, bacilares, nitrificantes e também leveduras (Decamp & Moriarty 2007). Em caso de bactérias, preferencialmente os probióticos são compostos por organismos que formam esporos e, portanto, capazes de permanecer viáveis por muito tempo no ambiente (Kesarcodi-Watson et al. 2008). Dentre as bactérias formadoras de esporos utilizadas como probiótico na aquicultura destaca-se o gênero *Bacillus*.

Espécies de *Bacillus* formadoras de esporos estão presentes nos sedimentos marinhos e são naturalmente ingeridas por animais aquáticos, sendo presentes em sua microflora intestinal. Os *Bacillus* são usados como probióticos por sua ação inibitória contra *Vibrio* e por sua ação no manejo de qualidade de água, degradando a matéria orgânica (Gatesoupe 1999, Chen & Chen 2001 e Decamp & Moriarty 2006). Eles

também possuem habilidades na competição por locais de adesão, produzem bacteriocinas (peptídeos antimicrobianos) e promovem imunoestimulação sistêmica (Kesarcodi-Watson et al. 2008).

A adição de cepas selecionadas de *Bacillus* mostrou potencial de aprimorar a produção, diminuindo a abundância de *Vibrio* na água e melhorando a taxa de crescimento de *Paralichthys olivaceus* (Decamp et al. 2006 e Decamp et al. 2007). O uso de *Lactobacillus* spp como probiótico na larvicultura de *Sparus aurata*, administrado através do alimento vivo, promoveu aumento na sobrevivência e no crescimento comparado aos grupos controle (Suzer et al. 2008).

A administração de probióticos durante a larvicultura propicia benefícios, uma vez que as formas larvais da maioria dos peixes são liberadas no ambiente externo começando a alimentação antes mesmo do trato digestório e o sistema imunológico estarem completamente desenvolvidos (Picchietti et al. 2007).

Além disso, larvas de peixes marinhos normalmente não se alimentam de dietas artificiais durante as fases iniciais de desenvolvimento, sendo necessário o uso de alimento vivo. A dieta, seja ela composta por alimento inerte ou vivo, pode auxiliar no transporte de microorganismos benéficos. Isso permite que a comunidade bacteriana do hospedeiro seja modificada desde o início da larvicultura, através da adição de probióticos (Picchietti al. 2009a)

A modulação do sistema imune é um dos benefícios mais importantes dos probióticos. Sua potência abrange tanto a imunidade local (linfócitos associados à mucosa intestinal) quanto a sistêmica (Nayak 2010). A estimulação da imunidade inata de peixes gera um aumento da resposta imunológica rápida, ou seja, da imunidade humoral. Este tipo de resposta, mediada por componentes inatos, pode ser benéfico para os peixes sob as condições de cultivo, em particular na maricultura. Grandes perdas podem ser observadas durante a larvicultura marinha, sendo essa mortalidade muitas vezes atribuída à infecções por bactérias oportunistas presentes no sistema de cultivo (Magnadóttir 2006).

A resposta imune é mediada principalmente pelos órgãos linfóides, que nos peixes teleósteos incluem o rim cefálico, timo, baço e tecidos linfóides associados à mucosa (Zapata et al. 2006). Nos peixes, o rim cefálico atua equivalentemente à medula óssea de mamíferos, desempenhando um papel importante na função imunológica, sendo o local para a maturação de linfócitos B e antígenos de captura (Laing et al.

2006). Já o timo é o órgão linfóide predominante, atuando como o local para a produção, diferenciação e maturação de linfócitos T (Patel et al. 2009).

O timo desempenha um papel fundamental no desenvolvimento do sistema imune adaptativo (Picchiatti et al. 2009b). O complexo CD3 é um co-receptor que serve como identificador geral de células T em peixes (Øvergård et al. 2009). Uma das principais subclasses de linfócitos T são as chamadas células T auxiliares (ou T-helper), que expressam o co-receptor CD4 (Buonocore et al. 2008). Este tipo de linfócito atua estimulando a expressão e a secreção de citocinas, produzindo respostas via anticorpo, através dos linfócitos B, ou fazendo ativação macrofágica (Buonocore et al. 2008).

Evidências demonstram que a administração suplementar de probióticos estimula a imunidade de larvas e diminui a transcrição de genes pró-inflamatórios, além de causar um aumento da quantidade de células-T (Picchiatti et al. 2009a), eritrócitos e leucócitos em geral (Irianto & Austin 2002) Em teoria, o aumento ou diminuição dos co-receptores de linfócitos T, CD3 e CD4, pode estar associado à velocidade de desenvolvimento do tecido linfóide e/ou à reatividade dos linfócitos em questão (Miceli & Parnes 1993).

Como a larvicultura de peixes marinhos é um grande gargalo na produção, pode-se utilizar sistemas total ou parcialmente fechados a fim de reduzir os riscos de contaminação e influências ambientais, que são dois grandes problemas na aquicultura (Lyndon, 1999).

Em sistemas abertos, como viveiros e tanques, probióticos são acrescentados a cada renovação de água e seus efeitos são comprovadamente benéficos. Em sistemas fechados de recirculação, contudo, não há muitos estudos sobre os efeitos da adição de probióticos, principalmente porque a ação dos aparatos de recirculação sobre a abundância de microorganismos na água de cultivo é pouco conhecido. Chen & Chen (2001) e Taoka et al. (2006) relataram o uso de probióticos em SRA, mas nestes trabalhos não há menção dos componentes do sistema.

Além da ausência de informação acerca do uso de probióticos em sistemas de recirculação, não existem estudos publicados sobre o uso de probióticos no cultivo do bijupirá. O objetivo deste estudo foi analisar os efeitos do probiótico *Bacillus* spp. na larvicultura do bijupirá em sistema de recirculação de água.

REFERÊNCIAS BIBLIOGRÁFICAS

- ARNOLD, CR, JB KAISER, GJ HOLT. 2002. Spawning of Cobia *Rachycentron canadum* in Captivity. *J. World Aquacult. Soc.*, 33:205-208.
- BENETTI DD, R ORHUNM, B SARDENBERG, B O'HANLON, A WELCH, R HOENIG, I ZINK, JÁ RIVERA, B DENLINGER, D BACCOAT, K PALMER, F CAVALIN. 2008. Advances in hatchery and grow-out technology of cobia *Rachycentron canadum* (Linnaeus). *Aquacult. Res.*, 39:701-711.
- BENETTI, DD, B O'HANLON, JA RIVERA, AW WELCH, C MAXEY, MR ORHUN. 2010. Growth rates of cobia (*Rachycentron canadum*) cultured in open ocean submerged cages in the Caribbean. *Aquaculture*, 302:195-201.
- BUONOCORE, F, E RANDELLI, D CASANI, L GUERRA, S PICCHIETTI, S COSTANTINI, AM FACCHIANO, J ZOU, CJ SECOMBES, G SCAPIGLIATI. 2008. A CD4 homologue in sea bass (*Dicentrarchus labrax*): Molecular characterization and structural analysis. *Mol. Immunol.*, 45:3168–3177.
- CHEN, CC, CHEN SN. 2001. Water Quality Management with *Bacillus* spp. in the High-Density Culture of Red-Parrot Fish: *Cichlasoma citrinellum* X *C. Synspilum*. *N. Am. J. Aquacult.*, 63:66–73.
- CHOU, R, MS SU, HY CHEN. 2001. Optimal dietary protein and lipid levels for juvenile cobia (*Rachycentron canadum*). *Aquaculture*, 193, 81-89.
- D'ORBICASTEL, ER, JP BLANCHETON, A BELAUD. 2009. Water quality and rainbow trout performance in a Danish Model Farm recirculating system: Comparison with a flow through system. *Aquacult. Eng.*, 40:135-143.
- DECAMP, O, MORIARTY, D J W. 2006. Probiotics as alternative to antimicrobials: limitations and potential. *J. World Aquacult. Soc.*, 37:60-62.
- DECAMP, O, MORIARTY, D J W. 2007. Aquaculture species profit from probiotics. *Feed Mix* vol. 15, nº 1.
- DENSON, M R, KR STUART, TIJ SMITH, CR WEIRLCH, A SEGARS. 2003. Effects of Salinity on Growth, Survival, and Selected Hematological Parameters of Juvenile Cobia *Rachycentron canadum*. *J. World Aquacult. Soc.*, 34:496-504.
- FAO. 2007. Cultured Aquatic Species Information Programme. *Rachycentron canadum*. Cultured Aquatic Species Information Programme. Text by J.B. Kaiser, J.G. Holt. In: FAO Fisheries and Aquaculture Department [online]. Rome. Updated

- 23 May 2007. [Cited 14 December 2010].
http://www.fao.org/fishery/culturedspecies/Rachycentron_canadum/en
- FAO. 2010. Global Aquaculture Production (online query). Disponível em:
<<http://www.fao.org/fishery/statistics/global-aquaculture-production/en>>.
Acesso em: 20/10/2010.
- FAULK, C, HOLT, G. 2008. Biochemical composition and quality of captive-spawned coxia *Rachycentron canadum* eggs. *Aquaculture*, 279, 70-76.
- FAULK, CK, HOLT, GJ. 2005. Advances in rearing coxia *Rachycentron canadum* larvae in recirculating aquaculture systems: Live prey enrichment and greenwater culture. *Aquaculture*, 249:231-243.
- FAULK, CK, JB KAISER, GJ HOLT. 2007. Growth and survival of larval and juvenile coxia *Rachycentron canadum* in a recirculating raceway system. *Aquaculture*, 270:149-157.
- FEELEY, MW, DD BENETTI, JS AULT. 2007. Elevated oxygen uptake and high rates of nitrogen excretion in early life stages of the coxia *Rachycentron canadum* (L.), a fast-growing subtropical fish. *J. Fish Biol.*, 71:1662-1678.
- FRASER, TWK, DAVIES, SJ. 2009. Nutritional requirements of coxia, *Rachycentron canadum* (Linnaeus): a review. *Aquacult. Res.*, 40:1219-1234.
- GATESOUBE, FJ. 1999. The use of probiotics in aquaculture. *Aquaculture*, 180:147–165
- HANSEN, GH, OLAFSEN, JA. 1999. Bacterial Interactions in Early Life Stages of Marine Cold Water Fish. *Microb. Ecol.*, 38:1–26.
- HOLT, GJ, CK FAULK, MH SCHWARZ. 2007. A review of the larviculture of coxia *Rachycentron canadum*, a warm water marine fish. *Aquaculture*, 268:181-187.
- IRIANTO A & AUSTIN B. 2002 Probiotics in aquaculture. *J. Fish Dis.*, 25: 633–642.
- KAISER, JB, HOLT, GJ. 2005. Species Profile – Coxia. *Southern Regional Aquaculture Center Publication*, 7202.
- KESARCODI-WATSON, A, H KASPAR, MJ LATEGAN, L GIBSON. 2008. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture*, 274:1-14.
- LAING, K J, J J ZOU, M K PURCELL, R PHILLIPS, C J SECOMBES, J D HANSEN. 2006. Evolution of the CD4 family: Teleost fish possess two divergent forms of CD4 in Addition to Lymphocyte Activation Gene-3. *J. Immunol.*, 177: 3939-3951.

- LEAÑO, E M, J J GUO, S L CHANG, I C LIAO. 2003. Levamisole enhances non-specific immune response of cobia, *Rachycentron canadum*, fingerlings. *J. Fish. Soc. Taiwan*, 30:321-330.
- LIAO, IC, HM SU, EY CHANG. 2001. Techniques in finfish larviculture in Taiwan. *Aquaculture*, 200:1-31.
- LIAO, IC, TS HUANG, WS TSAI, CM HSUEH, SL CHANG, EM LEAÑO. 2004. Cobia culture in Taiwan: current status and problems. *Aquaculture*, 237:155-165.
- LIN, JHY, TH CHEN, MS CHEN, HE CHEN, RL CHOU, TI CHEN, MS SU, HL YANG. 2006. Vaccination with three inactivated pathogens of cobia (*Rachycentron canadum*) stimulates protective immunity. *Aquaculture*, 255:125–132.
- LYNDON, A.R. 1999. Fish Growth in Marine Culture Systems: A Challenge for Biotechnology. *Mar. Biotechnol.*, 1:376–379.
- MAGNADÓTTIR, B. 2006. Innate immunity of fish (overview). *Fish shellfish immunol.*, 20:137-151.
- MCLEAN, E, G SALZE, MH SCHWARZ & SR CRAIG. 2009. Cobia cultivation. In: BURNELL, G & G ALLAN (eds.) *New Technologies in aquaculture: Improving production efficiency, quality and environmental management*. CRC Press, Estados Unidos, Chap. 25:804-821.
- MICELI MC, PARNES JR. 1993. Role of CD4 and CD8 in T cell activation and differentiation. *Adv. Immunol.* 53:59–122.
- NAYAK, SK. 2010. Probiotics and immunity: A fish perspective. *Fish Shellfish Immunol.*, doi: 10.1016/j.fsi.2010.02.017.
- OLAFSEN, JA. 2001. Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture*, 200:223-247.
- ØVERGÅRD, AC, I HORDVIK, AH NERLAND, G EIKELAND, S PATEL. 2009. Cloning and expression analysis of Atlantic halibut (*Hippoglossus hippoglossus*) CD3 genes. *Fish Shellfish Immunol.*, 27:707–713
- PATEL, S, E SØRHUS, IU FIKSDAL, PG ESPEDAL, Ø BERGH, OM RØDSETH, C MORTON, AH NERLAND. 2009. Ontogeny of lymphoid organs and development of IgM-bearing cells in Atlantic halibut (*Hippoglossus hippoglossus* L.). *Fish Shellfish Immunol.*, 26:385–395.

- PICCHIETTI, S, M MAZZINI, AR TADDEI, R RENNA, AM FAUSTO, V MULERO, O CARNEVALI, A CRESCI, L ABELLI, 2007. Effects of administration of probiotic strains on GALT of larval gilthead seabream: immunohistochemical and ultrastructural studies. *Fish Shellfish Immunol.*, 22:57-67.
- PICCHIETTI, S, AM FAUSTO, E RANDELLI, O CARNEVALI, AR TADDEI, F BUONOCORE, G SCAPGLIATI, L ABELLI. 2009a. Early treatment with *LactoBacillus delbrueckii* strain induces an increase in intestinal T-cells and granulocytes and modulates immune-related genes of larval *Dicentrarchus labrax* (L.). *Fish Shellfish Immunol.*, 26:368-376.
- PICCHIETTI, S, L GUERRA, F BUONOCORE, E RANDELLI, AM FAUSTO, L ABELLI. 2009b. Lymphocyte differentiation in sea bass thymus: CD4 and CD8- α gene expression studies. *Fish Shellfish Immunol.*, 27:50–56.
- PRESS, C. 1999. The morphology of the immune system in teleost fishes. *Fish Shellfish Immunol.*, 9:309-318.
- RESLEY M, K WEBBJR, G HOLT. 2006. Growth and survival of juvenile cobia, *Rachycentron canadum*, at different salinities in a recirculating aquaculture system. *Aquaculture*, 253:398–407.
- RIDHA, MT, CRUZ, EM. 2001. Effect of biofilter media on water quality and biological performance of the Nile tilapia *Oreochromis niloticus* L. reared in a simple recirculating system. *Aquacult. Eng.*, 24:157-166.
- RINGØ, E, RE OLSEN, TØ GIFSTAD, RA DALMO, H AMLUND, GI HEMRE, AM BAKKE. 2010. Prebiotics in aquaculture: a review. *Aquacult. Nut.*, 16:117-136.
- RODRIGUES, R, M SCHWARZ, B DELBOS, L SAMPAIO. 2007. Acute toxicity and sublethal effects of ammonia and nitrite for juvenile cobia *Rachycentron canadum*. *Aquaculture*, 271:553-557.
- SHAFFER, RS, NAKAMURA, EL. 1989. Synopsis of Biological Data on the Cobia *Rachycentron canadum* (Pisces: Rachycentridae). NOAA Technical Report NMFS 82.
- SUZER, C, D ÇOBAN, HO KAMACI, S SAKA, K FIRAT, O OTGUOGLU, H KÜÇÜKSARI. 2008. *Lactobacillus* spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: Effects on growth performance and digestive enzyme activities. *Aquaculture*, 280:140-145.
- TAOKA, Y, H MAEDA, J JO, M JEON, SC BAI, W LEE, K YUGE, S KOSHIO, 2006. Growth, stress tolerance and non-specific immune response of Japanese

- flounder *Paralichthys olivaceus* to probiotics in a closed recirculating system. *Fisheries Sci.*, 72, 310-321.
- TRUSHENSKI, J, M SCHWARZ, R TAKEUCHI, B DELBOS, LA SAMPAIO. 2010. Physiological responses of cobia *Rachycentron canadum* following exposure to low water and air exposure stress challenges. *Aquaculture*, 307:173-177.
- VELMURUGAN, S, RAJAGOPAL, S. 2009. Beneficial uses of probiotics in mass scale production of marine ornamental fish. *Afr. J. Microbiol. Res.*, 3:185-190.
- VERSCHUERE, L, G ROMBAUT, P SORGELOOS, W VERSTRAETE. 2000. Probiotic Bacteria as Biological Control Agents in Aquaculture. *Microbiol. Mol. Biol. Rev.*, december, 655–671.
- VINE, N G, WD LEUKES, H KAISER. 2006. Probiotics in marine larviculture. *FEMS Microbiol. Rev.*, 30:404-427.
- WANG, Y, J LI, J LIN. 2008. Probiotics in aquaculture: Challenges and outlook. *Aquaculture*, 281:1-4.
- WEIRICH, CR, AD STOKES, TIJ SMITH, WE JENKINS, MR DENSON. 2006. Outdoor Tank and Pond Spawning of Cobia in Coastal South Carolina. *Aquaculture*, 18:1-17.
- ZAPATA, A, B DIEZ, T CEJALVO, CG FRÍAZ, A CORTÉS. 2006. Ontogeny of the immune system of fish. *Fish Shellfish Immunol.*, 20:126-136.
- ZHOU, QC, BP TAN, KS MAI, YJ LIU. (2004). Apparent digestibility of selected feed ingredients for juvenile cobia *Rachycentron canadum*. *Aquaculture*, 241:441-451.

ARTIGO ANEXO

**Efeitos do probiótico *Bacillus* spp. na larvicultura do bijupirá
Rachycentron canadum em sistema de recirculação de água**

Co-autores: Michael Schwarz, Brendan C. Delbos, Ricardo V. Rodrigues, Luis Alberto Romano, Luís André Sampaio

Segundo as normas da revista *Aquaculture* (fator de impacto 1.925).

Probiotic effects of *Bacillus* spp. on cobia *Rachycentron canadum* larvae reared in a recirculating aquaculture system

M. Angélica Garrido-Pereira^a, Michael H. Schwarz^b, Brendan C. Delbos^b, Ricardo V. Rodrigues^a, Luis A. Romano^c, Luís A. Sampaio^{a,*}

^a *Universidade Federal do Rio Grande — FURG, Instituto de Oceanografia, Laboratório de Piscicultura Estuarina e Marinha, CP 474, Rio Grande, RS, 96201-900, Brazil*

^b *Virginia Tech — Virginia Seafood Agricultural Research and Extension Center, 102 S. King Street, Hampton, VA, 23669, USA*

^c *Universidade Federal do Rio Grande – FURG, Instituto de Oceanografia. Laboratório de Patologia de Organismos Aquáticos, CP 474, Rio Grande, RS, 96201-900, Brazil*

*Corresponding author. Tel.: +55 53 32368131, Fax: + 55 53 32368042

E-mail address: sampaio@mikrus.com.br (LA Sampaio)

Abstract

Cobia (Rachycentron canadum) is a marine finfish with good potential for mariculture. The aim of this study was to analyze the effects of probiotic *Bacillus* spp. on the performance of cobia larvae reared in a recirculating aquaculture system (RAS). Many studies have demonstrated the beneficial effects of probiotic *Bacillus* spp. in aquaculture, but there are no studies on the effects of probiotics in RAS, requiring research on their effectiveness, since in open systems the beneficial effects are already known. The trial was conducted at Virginia Tech VSAREC (USA). Larvae were stocked into two independent RAS - probiotic treatment and control - at a density of 15 larvae L⁻¹, for 26 days after hatching. One of the systems (Probiotic treatment) received the addition of a commercial probiotic consisting of *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* directly into the water and by live feed, according to the manufacturer. At the end of the experiment we performed biometrics, larvae counts, stress test and larvae were fixed for morphometric analysis of head kidney and for immunohistochemistry of thymus to evaluate the phenotypic expression of complex antigens CD3 and CD4 in T-lymphocytes. Survival and final weight had no significant difference between groups. The analysis of water microbiology had no significant differences between treatments in total counts of *Vibrio*, but in the total count of *Bacillus* treatments differed statistically, with a higher concentration in Probiotic treatment because of the addition of *Bacillus* spp. in this group. The salinity stress test was expressed by stress sensitivity index (SSI), which demonstrated a greater resistance to salt stress by larvae of probiotic treatment. Immunohistochemical analysis showed a higher expression of CD4 in Probiotic treatment, which may indicate better immune response against pathogens. The morphometric analysis of head kidney indicates that this organ is larger in Probiotic treatment, probably due to an increase in the quantity of

B-lymphocytes. These results indicate that *Bacillus* spp. probiotics used in RAS may stimulate the immune system and increase the general stress resistance of cobia larvae.

Keywords: stress test, immunohistochemistry, marine fish farming

1. Introduction

Cobia (*Rachycentron canadum*), a marine finfish, is the only species in the Rachycentridae family (Kaiser & Holt 2005). It has an emerging global potential for mariculture, due to its extraordinary growth rate (Fraser & Davies 2009) and overall aquaculture performance (Holt et al. 2007, Benetti et al. 2008,). Moreover, cobia have demonstrated the capacity for induced and natural tank spawning and high fecundity rates (Arnold et al. 2002, Liao et al. 2004), disease resistance (Holt et al. 2007), and adaptability to commercially available aquafeeds (McLean et al. 2009).

Aquaculture environments can be stressful, triggering high mortality, especially during larval rearing (Avella et al. 2010). Due to the interaction of microbes and their effects on animals and their environment under typical intensive larviculture production protocols, diseases caused by opportunistic pathogens like *Vibrio anguillarum* (Hansen and Olafsen 1999), *Photobacterium damsela* (Lin et al. 2006), *Streptococcus iniae* (Yanong and Francis-Floyd 2010) and many others (McLean et al. 2008), are usually only treated from a clinical pathology perspective (Moriarty, 1998). Often, the diseases are treated rather than the underlying causes, resulting in unnecessary drug use that may have potentially negative environmental impacts (Gatesoupe 1999, Vine et al. 2006, Wang et al. 2008, Kesacordi-Watson et al. 2008, Velmurugan and Rajagopal 2009).

Probiotics can be defined as live microbial feed supplements, which are administered in such a way as to survive after entering the gastrointestinal tract. This can be beneficial to the host animal by improving its intestinal microbial balance and in

turn its health (Gatesoupe 1999). In aquaculture, probiotics are inoculated into the rearing water to improve culture conditions or incorporated into the feed (Taoka et al. 2006). The influence of the microbial community of the rearing water on the gastrointestinal flora of the cultured animal is widely recognized (Gatesoupe 1999, Verschuere et al. 2000).

Probiotic bacteria are commonly used in the animal industry to prevent the occurrence of pathogenic microorganisms, thus minimizing the use of drugs to treat diseases (Gatesoupe 1999, Vine et al. 2006, Kesacordi-Watson et al. 2008, Wang et al. 2008). They have been found to be effective in the control of pathogens in aquaculture (Moriarty 1998, Silvi et al. 2008). The roles and effects of probiotics in aquaculture have been examined over the past decade (Avella et al. 2010), in an ongoing search for alternative preventive health strategies (Picchietti et al. 2007).

Several positive effects of probiotics in fish culture have been demonstrated, such as higher survival (Velmurugan & Rajagopal 2009), faster growth (Venkat et al. 2004, Aly et al. 2008), improved stress tolerance (Rollo et al. 2006, Taoka et al. 2006), immune system enhancement (Picchietti et al. 2007, Aly et al. 2008, Tinh et al. 2008, Silvi et al. 2008, Picchietti et al. 2009a), and general welfare (Silvi et al. 2008).

The effects of probiotics on immune stimulatory functions include the enhancement of non-specific immune responses (Taoka et al. 2006) and increase of T-cells (Picchietti et al. 2009a). Some immunohistochemical markers can be used to study leukocyte populations, like the T-cell co-receptors: CD3 and CD4. These receptors are transmembrane glycoproteins, they belong to the Ig superfamily and are essential for cell-mediated immunity (Laing et al. 2006). The CD4 co-receptor occurs in T-helper cells, which play a central role in the immune system, stimulating the proliferation of B

lymphocytes and generating clones of memory cells for both cellular and humoral immunity (Romano 2010).

Bacillus are gram-positive, spore-forming bacteria, used commercially as probiotics (Chen & Chen 2001, Decamp & Moriarty 2006). *Bacillus* preparations are resistant and have a long lasting shelf life and so they can be stored long-term in a dehydrated form. Moreover, the beneficial roles of *Bacillus* spores applications in the aquaculture field are well established (Gatesoupe, 1999, Avella et al. 2010).

Since marine fish larviculture is still a significant bottleneck toward industrial expansion for many species, fully or partially closed, recirculating, onshore culture units can be used to maximize biosecurity, reduce diseases and seasonal environmental influences, important constraints on aquaculture in many areas (Lyndon, 1999). Marine fish larvae can be reared in recirculating aquaculture systems (RAS), but little is known on the effects of the equipment (i.e. filters and sterilizers) used to treat water in a RAS on the probiotic bacteria. Chen & Chen (2001) and Taoka et al. (2006) reported the use of probiotics in RAS, but they did not mention the system components.

Stress tests are useful tools to evaluate fry quality. They are based on the same principle of exposing the organisms to a short but extreme stressful situation in which the physiological condition of the animals determines their chances to survive (Dhert et al. 1992b).

Thus, this study aims to examine the effects of a probiotic composed of three *Bacillus* strains on survival, growth, resistance to a salinity stress challenge, and immunostimulation in cobia larvae reared in a RAS.

2. Material and methods

2.1 Experimental design

The experiment was conducted at Virginia Tech's Virginia Seafood Agricultural Research and Extension Center (VSAREC) (Hampton, VA, USA). Two independent RAS were used in this experiment, each comprised of 3 tanks (300 L each), a circulation pump (1/3 HP, AmpMaster 5600/4700, Pensacola, FL, USA), fluidized-bed biofilter (Kaldness biological filtration media, R&B Aquatics, Waring, TX, USA), bubble bead filter (BBF-XS4000, Aquaculture Systems Technologies, New Orleans, LA, USA), 50 µm bag filter (X100 Convertible Filter Housing, FSI Filter Specialists, Michigan City, IN, USA), UV sterilizer (025080, Emperor Aquatics, Pottstown, PA, USA), immersion titanium heaters, protein skimmer (TF300, Top Fathom, Hudsonville, MI, USA) for processing/removal of dissolved organics and small suspended solids, and a diffusion aeration/degassing system. The two systems were maintained completely independent of each other to ensure that no probiotics from the treatment system contaminated the control system.

2.2 Larviculture

Fertilized cobia eggs were air transported from TroutLodge Marine Farms (Vero Beach, FL, USA) to the VSAREC. Eggs were maintained in an incubator until hatching, and then newly hatched larvae were stocked into the experimental tanks at a density of 15 larvae/L.

Larvae were produced according to standard larviculture production protocols (McLean et al. 2009). L-type rotifers were enriched for 6 h with Protein Selco Plus (INVE, Salt Lake City, UT, USA) and were fed from 2 through 9 dph, coinciding with algal paste additions (Instant Algae Nanno 3600, Reed Mariculture, Campbell, CA, USA) in a concentration of 10^6 cells/mL. Newly hatched AF *Artemia* (INVE, Salt Lake

City, UT, USA) were fed from 7 through 10 dph and enriched GSL *Artemia* (INVE, Salt Lake City, UT, USA) were fed from 10 through 23 dph. *Artemia* were enriched for 24 h with DC DHA Selco (INVE, Salt Lake City, UT, USA). Co-feeding of larvae with Otohime Marine Larvae Weaning Diets (Reed Mariculture, Campbell, CA, USA) began on 15 dph, weaning protocols began on 21 dph, and all fish were fully weaned and *Artemia* discontinued by 23 dph.

Flow rate in the RAS was maintained at 60 L/hour from stocking through 9 dph. On 10 dph flow increased to 90 L/hour; 12 dph flow was increased to 120 L/hour; 13 dph flow increased to 180 L/hour; 16 dph flow was increased to 240 L/hour and on 18 dph flow increased to 300 L/hour and remained there for the rest of the study.

At the end of the experiment (26 dph), 150 weanlings were randomly sampled from each tank to determine final individual weight. All remaining larvae from the six experimental tanks were counted to determine survival.

2.3 Probiotic strain

An experimental *Bacillus* mix provided by INVE Technologies (Belgium) was used. It is composed of *Bacillus subtilis*, *Bacillus pumilus* and *Bacillus licheniformis* (1×10^{10} CFU/g). Each tank in Probiotic treatment received the probiotic daily dose of 5 g/m³ directly into the water; equally divided into four applications. Live feeds for this treatment were also enriched with the probiotic, adding 0.5 g of commercial probiotic per liter enrichment medium 4 hours before harvesting, according to the manufacturers specifications.

2.4 Water Quality

Temperature, salinity, pH, dissolved oxygen, alkalinity, total ammonia–nitrogen (TA-N), nitrite-nitrogen (NO₂-N), and nitrate (NO₃-N) of each experimental tank were measured daily before the first feeding. Temperature and salinity were measured with

an YSI Model 30 (Yellow Springs Instruments, Yellow Springs, OH, USA). Dissolved oxygen concentration was measured with an YSI Model 550A meter (Yellow Springs Instruments, Yellow Springs, OH, USA) and pH was measured with an YSI Model pH100 meter (Yellow Springs Instruments, Yellow Springs, OH, USA). Alkalinity was determined using the Hach digital titration method 8203. TAN, NO₂-N, and NO₃-N were determined via colorimetric assays, methods 10031, 8153, and 8039 using a D/R 2010 spectrophotometer (Hach, Loveland, CO, USA). The photoperiod was maintained at 24 h light using fluorescent light banks.

2.5 Microbiology of Water

Water samples for microbial analysis were taken daily for abundance analysis of total *Vibrio* and total *Bacillus*. Total *Bacillus* and Total *Vibrio* abundance were determined by plating water samples from selected tanks. The culture medium TSA 2% NaCl (Trypticase™ Soy Agar, Difco Laboratories, Detroit, MI, USA) was used for total *Bacillus* abundance after sample pasteurization (Miskin et. al. 1998). For Total *Vibrio*, the samples were plated on TCBS agar (thiosulfate citrate bile salt sucrose agar; Difco Laboratories, Detroit, MI, USA).

The samples were plated in petri dishes mechanically through a WASP Spiral Plater (Don Whitley Scientific Limited, Frederick, MD, USA). Abundance was expressed in Colony-Forming Units per mL (CFU/mL) according to Banwart (1989).

2.6 Stress challenge

At the end of the experimental period, a stress challenge was carried out in triplicate for each treatment, using 10 larvae per tank (n= 30 per treatment), according to Dhert et al. (1992b). Fish from the Probiotic and Control treatment were transferred with nets to 2L-beakers containing water at salinity 60, and survival was then monitored every 5 minutes for 1 hour. The stress sensitivity index is the sum of the cumulative

mortalities in the consequent time intervals: the higher the numeric value of the index, the more the larvae are stressed.

2.7 Histology and immunohistochemistry analysis

Larvae were sampled at 26 dph for histology and immunohistochemical analysis. For morphometric analysis of head kidney, 30 larvae from each group were euthanized with tricaine methanesulfonate (MS-222, Finquel®; Argent Chemical Laboratories, Inc., Redmond, Washington, DC, USA). They were transferred to vials containing 10% buffered formalin and stored until processing. Then larvae were dehydrated in a graded series of ethanol, embedded with Paraplast®Plus (Paraplast®Tissue Embedding Media, Leica Microsystems Inc., Bannockburn, IL, USA). Serial longitudinal sections of 5 µm were made until the whole head kidney was sectioned. Resulting sections were subsequently stained with haematoxylin and eosin. Length and height of the head kidney was measured using an optical microscope Olympus B201 with an ocular micrometer (Carl Zeiss, Thornwood, NY, USA) (Romano et al. 1996).

An additional 30 larvae from each group were fixed in Bouin solution and processed according to Prophet et al. (1992) for the immunohistochemistry evaluation of the thymus. Immunohistochemistry was performed by ABC peroxidase (ABC, Vectastain Elite, Vector) as described by Hsu et al. (1981). Serial sections of 5 µm were made and they were incubated with a monoclonal anti CD3 and anti CD4 (Dako, Argentina). Following rinses (0.1% diaminobenzidine solution), sections were dehydrated, mounted and examined under bright-field illumination. The evaluation of CD3 and CD4 receptors were calculated through quantitative analysis of the percentage of phenotypic expression per square millimeter of tissue (Romano, 1996). The phenotypic expression of CD3 and CD4 receptors were quantified using the software Bioscan OPTIMAS® 6.1 (Weibel, 1979).

2.8 Statistical analysis

All data were compared with Student's T test, using the software Statistica ® 7.0. The analyses were performed with significance level of $P < 0.05$. Percent data were analyzed after they were transformed on arc-sine, but only original data are presented. Results are shown as mean \pm SD.

3. Results

There were no differences ($P > 0.05$) in water quality between treatments: measured values for TAN, NO₂-N, NO₃-N, temperature, alkalinity, pH, DO, and salinity were: TAN: 0.13 \pm 0.04 mg/L; NO₂-N: 0.05 \pm 0.02 mg/L; NO₃-N: 2.96 \pm 0.76 mg/L; temperature: 27.9 \pm 0.5 °C; alkalinity: 166 \pm 19 mg/L; pH: 7.95 \pm 0.1; DO: 6.38 \pm 0.18 mg/L; salinity was reduced from 35 to 22 from day 1 to 13 dph.

There were no significant differences ($P > 0.05$) on survival and final weight between treatments (Tab. 1). In the analysis of water microbiology Total *Vibrio* had no statistical difference. Nevertheless, total count of *Bacillus* in the Probiotic treatment differed statistically; with a higher concentration in the Probiotic treatment ($P < 0.05$) (Tab. 2). Variations in the abundance of microorganisms (CFU/mL) throughout the experiment are shown in Fig. 1 and Fig. 2.

INSERT TABLE 1

INSERT TABLE 2

INSERT FIGURE 1

INSERT FIGURE 2

In the stress challenge, larvae of the Probiotic treatment had a better Stress Sensitivity Index than the control ($P < 0.01$) (Fig. 3).

INSERT FIGURE 3

Morphometric analysis showed that the larvae of the Probiotic treatment have larger head kidneys than larvae of the control treatment (Probiotic: 0.97 ± 0.33 mm; Control: 0.69 ± 0.22 mm) ($P<0.01$) (Fig. 4).

INSERT FIGURE 4

The expression of CD3 in thymic tissue showed no statistical differences ($P>0.05$) between groups (Probiotic: 63.9 ± 5.4 % CD3 cell/mm²; Control: 65.5 ± 5.0 % CD3 cell/mm²) (Fig. 5, Fig. 6a and Fig. 6b). However CD4 expression was significantly higher ($P<0.05$) in the Probiotic treatment than in the control (Probiotic: 43.7 ± 6.2 % CD4 cell/mm²; Control: 25.4 ± 3.5 % CD4 cell/mm²) (Fig. 7, Fig. 8a and Fig. 8b).

INSERT FIGURE 5

INSERT FIGURE 6a

INSERT FIGURE 6b

INSERT FIGURE 7

INSERT FIGURE 8a

INSERT FIGURE 8b

4. Discussion

The use of *Bacillus* species as a probiotic in aquaculture is common (Gatesoupe, 1999). For example, it has been shown that *Bacillus subtilis* can improve the immune conditions, growth and/or survival of *Tilapia nilotica* (*Oreochromis niloticus*) (Aly et al., 2008), catfish (*Ictalurus punctatus*) (Queiroz and Boyd, 1998) and shrimp (*Penaeus monodon*) (Rengpipat et al. 2000), and can inhibit the growth of pathogenic *Vibrio* strains (Decamp et al. 2008).

Throughout the experimental period all water quality parameters were maintained within suitable ranges for cobia larviculture (Holt et al. 2007). However, the accidental temperature increase observed 6 dph (temperature reached 29.4 °C) due to a

broken air conditioning system, led to an increase in bacterial concentrations in both treatments. Marine bacteria, such as *Vibrio* sp. and some kinds of *Bacillus* have higher growth rates at higher temperatures (Stanley and Morita 1968). Although the temperature rise was similar for both treatments, the abundance of *Vibrio* in the Control treatment was much higher than in the Probiotic treatment. This may indicate an inhibitory effect of circulating *Bacillus* on *Vibrio* loads, as reported by Moriarty (1998), Gatesoupe (1999), Chen and Chen (2001), Vaseeharan and Ramasamy (2003), among others.

Probiotics have been used in organisms raised in closed or RAS (McIntosh et al., 2000; Taoka et al., 2006). These systems utilize water more efficiently with multiple production cycles per year (Ridha and Cruz, 2001), due to consistent and optimal environmental conditions such as temperature. In RAS, the water may be colonized by opportunistic pathogens due to the high load of organic matter generally associated with minimal water exchange in RAS (Sharrer et al., 2005). In these situations, water disinfection is beneficial (Liltved et al., 1995). Thus, the frequency of probiotic addition in closed-culture systems equipped with UV must be determined and would likely differ from that recommended by the manufacturers for open systems (Garrido-Pereira et al. 2010). UV radiation has been successfully used for the elimination of pathogenic bacteria and viruses in seawater in closed recirculating water systems (Liltved et al. 1995; Sharrer et al. 2005; Sharrer and Summerfelt 2007). However, the time necessary for this radiation to affect probiotics is unknown (McIntosh et al. 2000; Taoka et al. 2006).

Due to the addition of commercial *Bacillus* strains into Probiotic treatment, total *Bacillus* counts were higher in this group. The abundance of *Bacillus* increased until 5 dph; thereafter it decreased, reaching levels similar to the Control treatment. It can be a

result of the higher flow rate through the mechanical filters and/or the UV sterilizer. The tank water exchange rate with the RAS is increased as fish grow larger to maintain homogeneous fish and live feed distribution (Holt et al. 2007). This practice makes water recirculate fast in the system filters, which can remove probiotics mechanically, and it also increases the flow of water into UV sterilizer. UV radiation can cause lethal effects, like disturbance of metabolic functions or generate mutations in essential genes, resulting in the death of the microorganism. Sublethal effects, on the other hand, do not cause cell death but can negatively influence bacterial growth and metabolism (Moran and Zepp 2000, Summerfelt 2003).

The probiotic showed no effects on growth and survival of fish in this trial, probably due to the low abundance of *Bacillus*. In open aquaculture systems such as ponds and tanks, probiotics are added after each water exchange. However, in RAS the appropriate frequency of adding probiotics has not been determined, mostly because the effect of UV radiation on probiotics in water is unclear. The addition of *Bacillus subtilis* and *Bacillus licheniformis* to closed culture systems with UV lamps must be carried out at least every 6 h, because after this period, the bacteria suffer lethal effects of the UV radiation (Garrido-Pereira et al. 2010). As the use of UV radiation in RAS is an effective way to disinfect the water, it can hamper the positive effects of added probiotic bacteria.

Larvae reared with probiotic during this trial showed better resistance to the stress test, suggesting that larvae supplemented with *Bacillus* spp. are stronger and have better quality than those reared without probiotic. The rationale for this test is that when tested organisms are exposed to different treatments they will first undergo some physiological changes, which will inevitably affect their resistance, before the impact on growth and eventually survival may be noticed (Dhert et al. 1992b). As survival during

larval rearing is influenced by many parameters it should not be considered as the sole indicator in determining larval quality, since survival does not reveal the actual condition of the fish (Dhert et al. 1992a).

The immune system of teleost fish is different from mammals: bone marrow, lymph nodes and Peyer's patches are absent (Rombout et al. 2005). Instead of bone marrow as the primary site for hemopoiesis, teleosts use the head kidney, which has no renal function and lacks nephrons (Zwollo et al. 2005). At the same time, the head kidney also serves as a secondary lymphoid organ – a lymph node analogue, important in the induction and elaboration of immune responses (Press and Evensen 1999). Next to the thymus as primary T cell organ, head kidney is considered the primary B cell organ (Rombout et al. 2005). However, young fish do not possess a mature specific immune system and depend on nonspecific cellular defense mechanisms to resist microbial infections (Raa 1996). Probiotics can be added at this stage and enhance local and systemic immunity (Nayak 2010).

The thymus plays a pivotal role in the development of the adaptive immune system (Picchiatti et al. 2009b), and the CD3 complex is a co-receptor that serves as a marker for general T-cell identification in fish (Øvergård et al. 2009). One of the major subclasses of T cells is CD4-expressing cells called T helper (Buonocore et al. 2008). These types of lymphocytes stimulate the expression and secretion of cytokines that produce antibody responses or lead to macrophage activation (Buonocore et al. 2008).

According to Conrad et al. (2007), CD3 and CD4 co-receptors are evolutionarily conserved from fish to mammals and can be used in immunity research in species with less well-characterized immune systems, such as coho. Immunohistochemistry analysis confirmed CD3 and CD4 thymocytes at 26 dph in coho, suggesting that critical events of differentiation and selection of T-lymphocytes could occur before weaning in this

species. There was no increase in the number of T lymphocytes in general, but there was an increase of T helper type in the Probiotic treatment. This demonstrates the immunostimulation of T helper achieved by *Bacillus* spp. in the Probiotic treatment, since the expression of CD4 was higher in this group (Fig. 7).

Irianto and Austin (2002) reported that probiotic addition in feed led to a stimulation of cellular rather than humeral immunity, increasing the number of lymphocytes, macrophages and erythrocytes. Nayak (2010), in turn, showed that probiotics can enhance immune responses in head kidney. Morphometric analysis showed that Probiotic treatment larvae had a larger head kidney. As the kidney is an organ that plays hematopoietic and lymphoid function, it can be inferred that this enlargement is due an increase of B lymphocytes, since immunostimulatory effect occurred on T helper in thymus, and the T helper stimulates B cells (Romano 2010). Further work will be carried out to elucidate which type of cells in head kidney are stimulated by probiotics.

Olafsen (2001) describes the potential use of live feed (*Artemia* and rotifers) cultured with selected bacterial strains to improve growth and survival of fish larvae. Assuming the harmful effects of RAS filters and sterilizers on probiotic bacteria are not fully understood, the ingestion of live feed containing probiotics may have been crucial to the effects on stress and immunity observed for cobia in this trial. Therefore, the relative importance of probiotic added in the water or to the food should be evaluated.

5. Conclusions

The probiotic bacteria (*B. subtilis*, *B. pumilus* and *B. licheniformis*) tested in the present work improved stress tolerance and immunity of cobia larvae reared in RAS, although it had no noticeable effect on survival and growth.

Acknowledgements

The authors wish to acknowledge the support of this project from the Virginia Tech – VSAREC and the International Initiative for Sustainable and Biosecure Aquafarming (IISBA). M. Angélica Garrido-Pereira and Ricardo V. Rodrigues are supported by Brazilian CNPq. L.A. Sampaio is a research fellow of Brazilian CNPq (# 308013/2009-3). Partial fundings for this study were provided by MCT/CNPq/CT-Agronegócio/MPA (Edital 036/2009, # 559741/ 2009-0) and EMBRAPA.

References

- Aly, S.M., Ahmed, Y.A., Ghareeb, A.A., Mohamed, M.F., 2008. Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections. Fish Shellfish Immun. 25, 128-136.
- Arnold, C.R., Kaiser, J.B., Holt, G.J., 2002. Spawning of cobia *Rachycentron canadum* in captivity. J. World Aquacult. Soc. 33, 205-208.
- Avella, M.A., Gioacchini, G., Decamp O., Makridis P., Bracciatelli C., Carnevali, O., 2010. Application of multi-species of *Bacillus* in sea bream larviculture. Aquaculture 305, 12–19.
- Banwart, G.J., 1989. Basic Food Microbiology. New York, AVI Book, 2nd edition, 773 pp.

- Benetti, D.D., Orhunm, R., Sardenberg, B., O'hanlon, B., Welch, A., Hoenig, R., Zink, I., Rivera, J.Á., Denlinger, B., Bacoat, D., Palmer, K., Cavalin, F., 2008. Advances in hatchery and grow-out technology of Cobia *Rachycentron canadum* (Linnaeus). *Aquaculture Res.* 39, 701-711.
- Buonocore, F., Randelli, E., Casani, D., Guerra, L., Picchiatti, S., Costantini S., Facchiano, A.M., Zouc, J., Secombes, C.J., Scapigliati, G., 2008. A CD4 homologue in sea bass (*Dicentrarchus labrax*): molecular characterization and structural analysis. *Mol. Immunol.* 45, 3168–3177.
- Chen, C.C., Chen, S.N., 2001. Water quality management with *Bacillus* spp. in the high-density culture of red-parrot fish: *Cichlasoma citrinellum* x *C. synspilum*. *N. Am. J. Aquacult.* 63, 66-73.
- Conrad, M.L., Davis, W.C., Koop, B.F., 2007. TCR and CD3 antibody cross-reactivity in 44 species. *Cytometry A*, 71, 925-933.
- Decamp, O., Moriarty, D.J.W., 2006. Probiotics as alternative to antimicrobials: Limitations and potential. *J. World Aquacult. Soc.* 37, 60-62.
- Decamp, O., Moriarty, D.J.W., Lavens, P., 2008. Probiotics for shrimp larviculture: review of field data from Asia and Latin America. *Aquacult. Res.* 39, 334-338.
- Dhert, P., Lavens, P., Sorgeloos, P., 1992a. A simple test for quality evaluation of cultured fry of marine fish. *Med. Fac. Landbouww Univ. Gent.* 57/4B, 2135-2142.
- Dhert, P., Lavens, P., Sorgeloos, P., 1992b. Stress evaluation: a tool for quality control of hatchery-produced shrimp and fish fry. *Aquacult. Europe* 17, 6-10.
- Fraser, T.W.K., Davies, S.J., 2009. Nutritional requirements of cobia, *Rachycentron canadum* (Linnaeus): a review. *Aquacult. Res.* 40, 1219-1234.

- Garrido-Pereira, M.A.R., Rocha, A.F., Braga, A.L, Sampaio, L.A., Abreu, P.C., 2010. Efeito da exposição à radiação ultravioleta “C” na taxa de respiração e na abundância de probióticos adicionados em água marinha. 4th Congress of Brazilian Society of Aquaculture and Aquatic Biology, 12-15 September 2010, Recife/Pernambuco, Brazil. Abstract.
- Gatesoupe, F.J., 1999. The use of probiotics in aquaculture. *Aquaculture* 180, 147–165.
- Hansen, G.H., Olafsen, J.A., 1999. Bacterial Interactions in Early Life Stages of Marine Cold Water Fish. *Microb. Ecol.* 38, 1–26.
- Holt, G.J., Faulk, C.K., Schwarz, M.H., 2007. A review of the larviculture of cobia *Rachycentron canadum*, a warm water marine fish. *Aquaculture* 268, 181-187.
- Hsu, S.M., Raine, L., Fanger, H., 1981. Use of avidin-biotin peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabelled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29, 577-580.
- Irianto, A., Austin, B., 2002. Probiotics in aquaculture. *J. Fish Dis.*, 25, 633–642.
- Kaiser, J.B., Holt, G.J., 2005. Species Profile – Cobia. Southern Regional Aquaculture Center Publication n° 7202.
- Kesarcodi-Watson, A., Kaspar, H., Lategan, M.J., Gibson, L., 2008. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture* 274, 1-14.
- Laing, K.J., Zou, J.J., Purcell, M.K., Phillips, R., Secombes, C.J., Hansen, J.D., 2006. Evolution of the CD4 family: Teleost fish possess two divergent forms of CD4 in Addition to Lymphocyte Activation Gene-3. *J. Immunol.* 177, 3939-3951.
- Liao, I.C., Huang, T.S., Tsai W.S., Hsueh, C.M., Chang, S.L., Leño. E.M., 2004. Cobia culture in Taiwan: current status and problems. *Aquaculture* 237, 155-165.

- Liltved, H., Hektoen, H., Efraimsen, H., 1995. Inactivation of bacterial and viral fish pathogens by ozonation or UV radiation in water of different salinity. *Aquacult. Eng.* 14, 107-122.
- Lin, J.H.Y., Chen, T.H., Chen, M.S., Chen, H.E., Chou, R.L., Chen, T.I., Su, M.S., Yang, H.L., 2006. Vaccination with three inactivated pathogens of Cobia (*Rachycentron canadum*) stimulates protective immunity. *Aquaculture* 255, 125–132.
- Lopez, M., Li, N., Kataria, J., Russel, M., Neu, J., 2008. Live and ultraviolet-inactivated *Lactobacillus rhamnosus* GG decrease flagellin-induced interleukin-8 production in Caco-2 Cells¹⁻³. *J. Nutr.* 138, 2264-2268.
- Lyndon, A.R., 1999. Fish Growth in Marine Culture Systems: A Challenge for Biotechnology. *Mar. Biotechnol.* 1, 376–379.
- McIntosh, D., Samocha, T.M., Jones, E.R., Lawrence, A.L., Mckee, D.A., Horowitz, S., Horowitz, A., 2000. The effect of a commercial bacterial supplement on the high-density culturing off *Litopenaeus vannamei* with a low-protein diet in an outdoor tank system and no water exchange. *Aquacult. Eng.* 21, 215-227.
- McLean, E., Salze, G., Craig, S.R., 2008. Parasites, diseases and deformities of Cobia. *Ribarstvo* 66, 1-16.
- McLean, E., Salze, G., Schwarz, M.H., Craig S.R., 2009. Cobia cultivation. In: Burnell, G., Allan, G. (Eds.), *New Technologies in Aquaculture: Improving production efficiency, quality and environmental management*, Woodhead Publishing Limited, Cambridge, United Kingdom, 1191 pp.
- Miskin, I., Rhodes, G., Lawlor, K., Saunded, J.R., Pickup, R.K., 1998. Bacteria in post-glacial freshwater sediments. *Microbiol.* 144, 2427-2439.

- Moran, M.A., Zepp, R.G., 2000. UV radiation effects on microbes and microbial processes. In: Kirchman, D.L. (Ed.), *Microbial ecology of the oceans*. Wiley-Liss, USA, pp. 201-228.
- Moriarty, D., 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture* 164, 351-358.
- Nayak, S.K., 2010. Probiotics and immunity: a fish perspective. *Fish Shellfish Immunol.* 29, 2-14.
- Olafsen, J.A., 2001. Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture* 200, 223-247.
- Ouwehand, A.C., Tölkö, S., Kulmala, J., Salminen, S., Salminen, E., 2000. Adhesion of inactivated probiotic strains to intestinal mucus. *Lett. Appl. Microbiol.* 31, 82-86.
- Øvergård, A.C., Hordvik, I., Nerland, A.H., Eikeland, G., Patel, S., 2009. Cloning and expression analysis of Atlantic halibut (*Hippoglossus hippoglossus*) CD3 genes. *Fish Shellfish Immunol.* 27, 707–713.
- Picchiatti, S., Fausto, A.M., Randelli, E., Carnevali, O., Taddei, A.R., Buonocore, F., Scapigliati, G., Abelli, L., 2009a. Early treatment with *Lactobacillus delbrueckii* strain induces an increase in intestinal T-cells and granulocytes and modulates immune-related genes of larval *Dicentrarchus labrax* (L.). *Fish Shellfish Immun.* 26, 368-376.
- Picchiatti, S., Guerra, L., Buonocore, F., Randelli, E., Fausto, A.M., Abelli, L., 2009b. Lymphocyte differentiation in sea bass thymus: CD4 and CD8-a gene expression studies. *Fish Shellfish Immunol.* 27, 50–56.
- Picchiatti, S., Mazzini, M., Taddei, A.R., Renna, R., Fausto, A.M., Mulero, V., Carnevali, O., Cresci, A., Abelli, L., 2007. Effects of administration of

- probiotic strains on GALT of larval gilthead seabream: immunohistochemical and ultrastructural studies. *Fish Shellfish Immunol.* 22, 57–67.
- Press, C.Mc.L., Evensen, Ø., 1999. The morphology of the immune system in teleost fishes. *Fish Shellfish Immunol.* 9, 309-318.
- Prophet, E.B., Millis. B., Arrington, J.B., Sobin, L.H., 1992. Laboratory methods in histotechnology. Washington, Armed Forces Institute of Pathology. USA.
- Queiroz, F., Boyd, C., 1998. Effects of a bacterial inoculum in channel catfish ponds. *J. World Aquaculture Soc.* 29, 67-73.
- Raa, J., 1996. The use of immunostimulatory substances in fish and shellfish farming. *Rev. Fish Sci.* 4, 229–288.
- Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasaveta, P., 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* S11). *Aquaculture* 191, 271-288.
- Ridha, M.T., Cruz, E.M., 2001. Effect of biofilter media on water quality and biological performance of the Nile tilapia *Oreochromis niloticus* L. reared in a simple recirculating system. *Aquacult. Eng.* 24, 157-166.
- Rollo, A., Sulpizio, R., Nardi, M., Silvi, S., Orpianesi, C., Caggiano, M., Cresci, A., Carnevali, O., 2006. Live microbial feed supplement in aquaculture for improvement of stress tolerance. *Fish Physiol. Biochem.* 32, 167–177.
- Romano, L.A., 2010. El sistema Imune Inespecífico de los Peces. In: *Patología e Sanidade de organismos Aquáticos*. Silva–Souza, A.T., Perez Lizama, M.A., Takemoto, R.M., ABRAPOA. 211-222.
- Romano, L.A., Ferder, M.D., Stella, I.Y., Inserra, F., Ferder, L.I., 1996. High correlation in renal tissue between computed image analysis and classical morphometric analysis. *J. Histotechnol.* 19, 121-123.

- Rombout, J.H.W., Huttenhuis, H.B.T., Picchietti, S., Scapigliati, G., 2005. Phylogeny and ontogeny of fish leucocytes. *Fish Shellfish Immunol.* 19, 441-455.
- Sharrer, M.J., Summerfelt, S.T., 2007. Ozonation followed by ultraviolet irradiation provides effective bacteria inactivation in a freshwater recirculating system. *Aquacult. Eng.* 37, 180-191.
- Sharrer, M.J., Summerfelt, S.T., Bullock, G.L., Gleason, L.E., Taeuber, J., 2005. Inactivation of bacteria using ultraviolet irradiation in a recirculating salmonid culture system. *Aquacult. Eng.* 33, 135-149.
- Silvi, S., Nardi, M., Sulpizio, R., Orpianesi, C., Caggiano, M., Carnevali, O., Cresci, A., 2008. Effects of addition of *Lactobacillus delbrueckii* subsp *delbrueckii* on gut microbiota composition and contribution to the well-being of the European sea bass (*Dicentrarchus labrax* L.). *Microb. Ecol. Health* 20, 53–59.
- Stanley, S.R.Y., Morita, R.Y., 1968. Salinity effect on the maximal growth temperature of some bacteria isolated from marine environments. *J Bacteriol.* 95, 169–173.
- Summerfelt, S.T., 2003. Ozonation and UV irradiation – an introduction and examples of current applications. *Aquacult. Eng.* 28, 21-36.
- Taoka, Y., Maeda, H., Jo, J., Jeon, M., Bai, S.C., Lee, W., Yuge, K., Koshio, S., 2006. Growth, stress tolerance and non-specific immune response of Japanese flounder *Paralichthys olivaceus* to probiotics in a closed recirculating system. *Fisheries Sci.* 72, 310-321.
- Tinh, N.T.N., Dierckens, K., Sorgeloos, P., Bossier, P., 2008. A review of the functionality of probiotics in the larviculture food chain. *Mar. Biotechnol.* 10, 1-12.

- Vaseeharan, B., Ramasamy, P., 2003. Control of pathogenic *Vibrio* spp. By *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. Lett. Appl. Microbiol. 36, 83-87.
- Velmurugan, S., Rajagopal, S., 2009. Beneficial uses of probiotics in mass scale production of marine ornamental fish. Afr. J. Microbiol. Res. 3, 185-190.
- Venkat, H.K., Sahu, N.P., Jain, K.K., 2004. Effect of feeding *Lactobacillus*-based probiotics on the gut microflora, growth and survival of postlarvae of *Macrobrachium rosenbergii* (de Man). Aquaculture Res. 35, 501-507.
- Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic Bacteria as Biological Control Agents in Aquaculture. Microbiology and Molecular Biology Reviews December, 655–671.
- Vine, N.G., Leukes, W.D., Kaiser, H., 2006. Probiotics in marine larviculture. Microbiol. Rev. 30, 404–427.
- Wang, Y., Li, J., Lin, J., 2008. Probiotics in aquaculture: Challenges and outlook. Aquaculture 281, 1-4.
- Weibel, E.R., 1979. Stereological Methods: Practical Methods for Biological Morphometry, Vol 1. Academic Press, London, 415 pp.
- Yanong, R.P.E., Francis-Floyd, R., 2010. Streptococcal infections of fish. University of Florida, Institute of Food and Agricultural Sciences - Fisheries and Aquatic Sciences Department Circular, 57.
- Zwollo, P., Cole, S., Bromage, E., Kaattari, S., 2005. B Cell heterogeneity in the teleost kidney: evidence for a maturation gradient from anterior to posterior kidney. J. Immunol. 174, 6608-6616.

Tables:

Tab. 1 – Survival and final weigh (average \pm SD) of cobia larvae reared with or without probiotic ($P > 0.05$).

	Probiotic	Control
Survival (%)	14.9 \pm 2.4	15.1 \pm 0.9
Final Weight (mg)	170 \pm 10	160 \pm 10

Tab. 2 – Water microbiology between Probiotic and Control treatments.

	Probiotic	Control
Total <i>Vibrio</i> (mL⁻¹)	3366 \pm 522 CFU	4964 \pm 902 CFU
Total <i>Bacillus</i> (mL⁻¹)	901 \pm 88 CFU ^a	61 \pm 24 CFU ^b

Different letters indicate significant difference ($P < 0.05$) after the Test of Student.

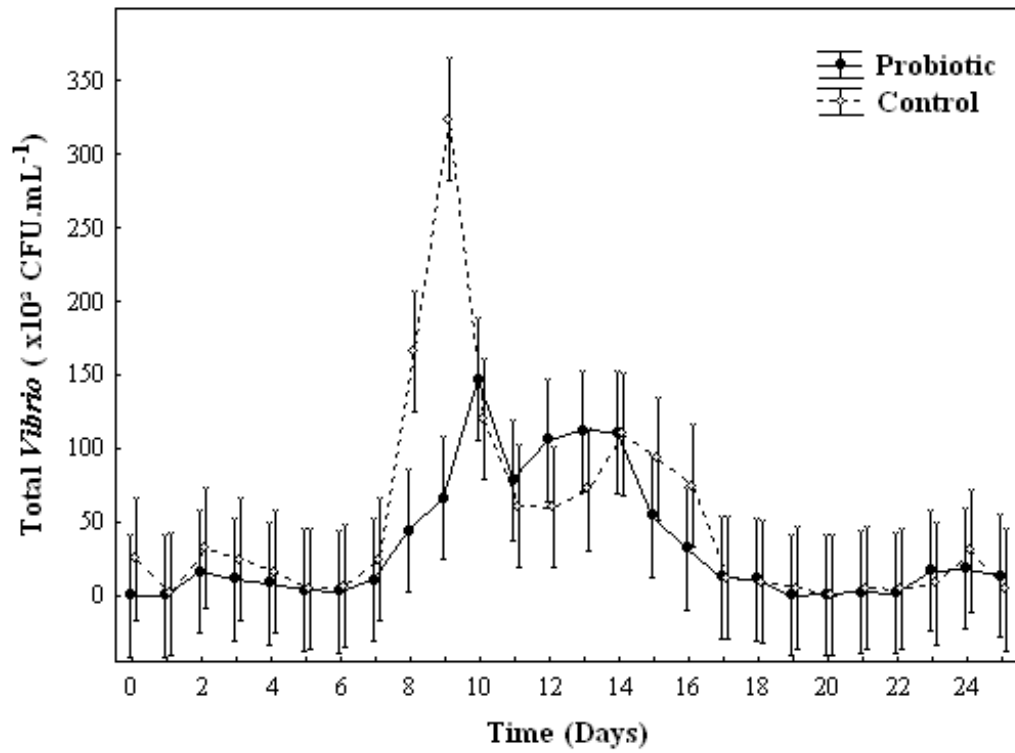


Figure 1 - Mean abundance ($10^2 \text{ CFU} \cdot \text{mL}^{-1}$) of total *Vibrio* in water samples from Probiotic and Control treatments during the experimental period.

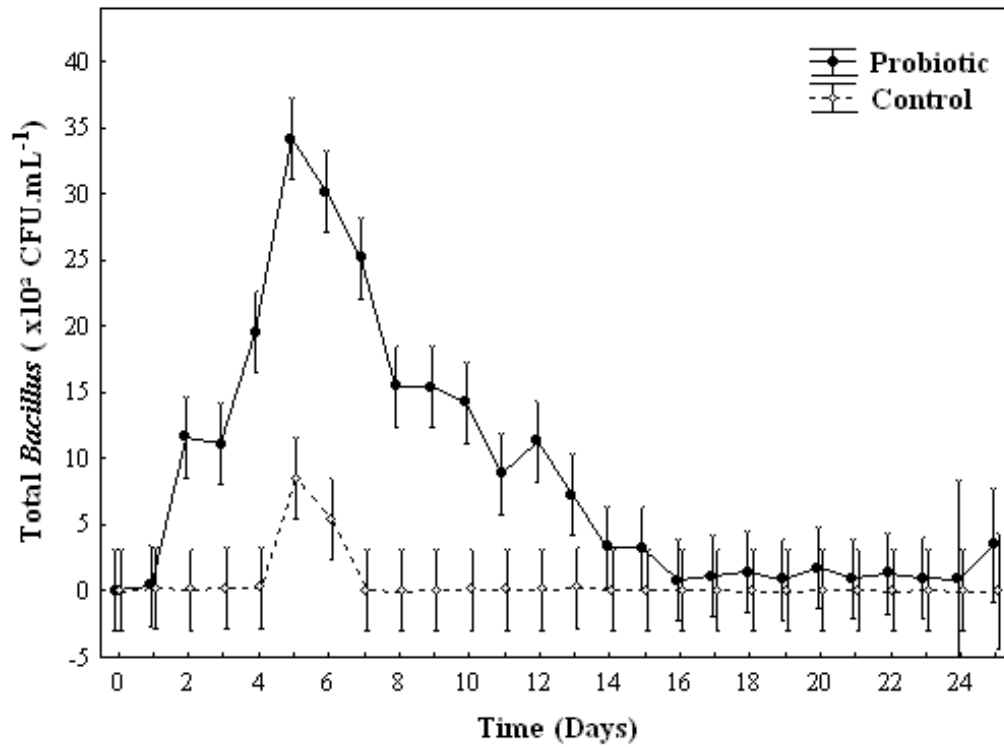


Figure 2 - Mean abundance ($10^2 \text{ CFU} \cdot \text{mL}^{-1}$) of total *Bacillus* in water samples from Probiotic and Control treatments during the experimental period.

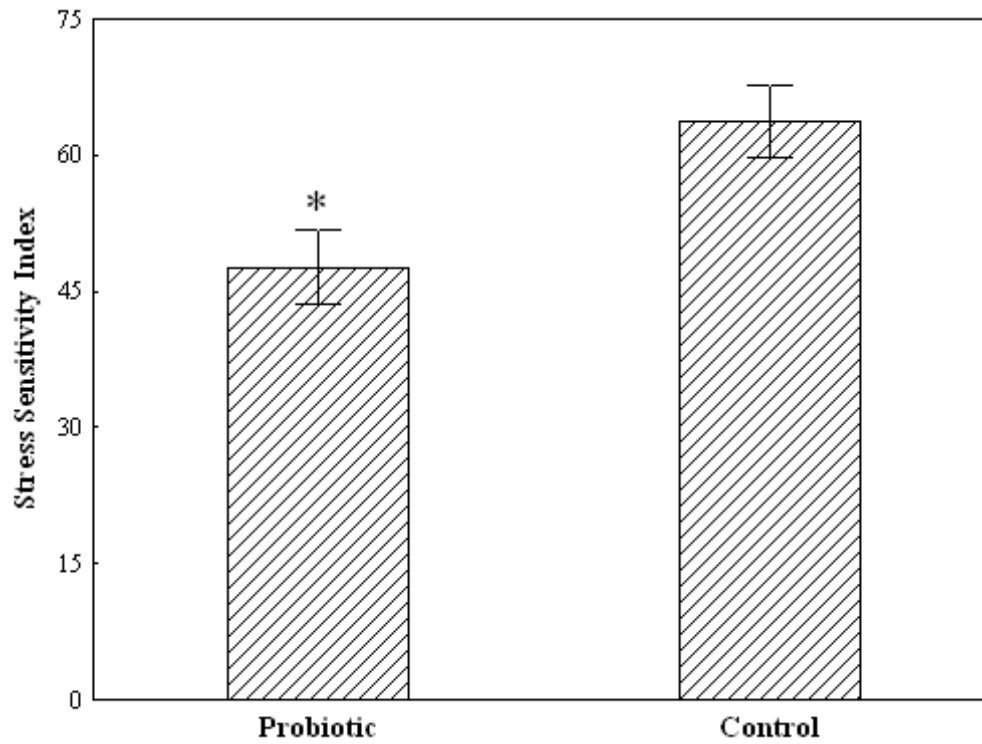


Figure 3 – Stress Sensitivity Index of cobia larvae reared with or without probiotic at the end of experimental period. Vertical bars represent one standard deviation and asterisk shows significantly statistic difference ($P < 0.05$).

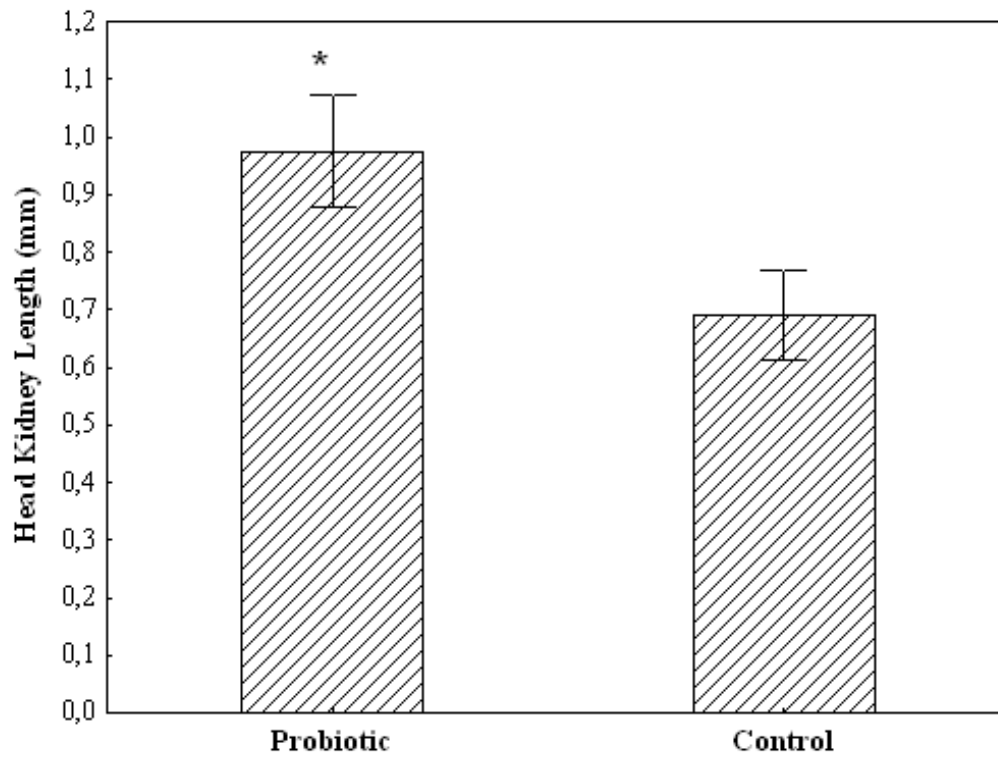


Figure 4 - Morphometric analysis of head kidney of cobia larvae reared with or without probiotic. Vertical bars represent one standard deviation and asterisk shows significantly statistic difference ($P < 0.01$).

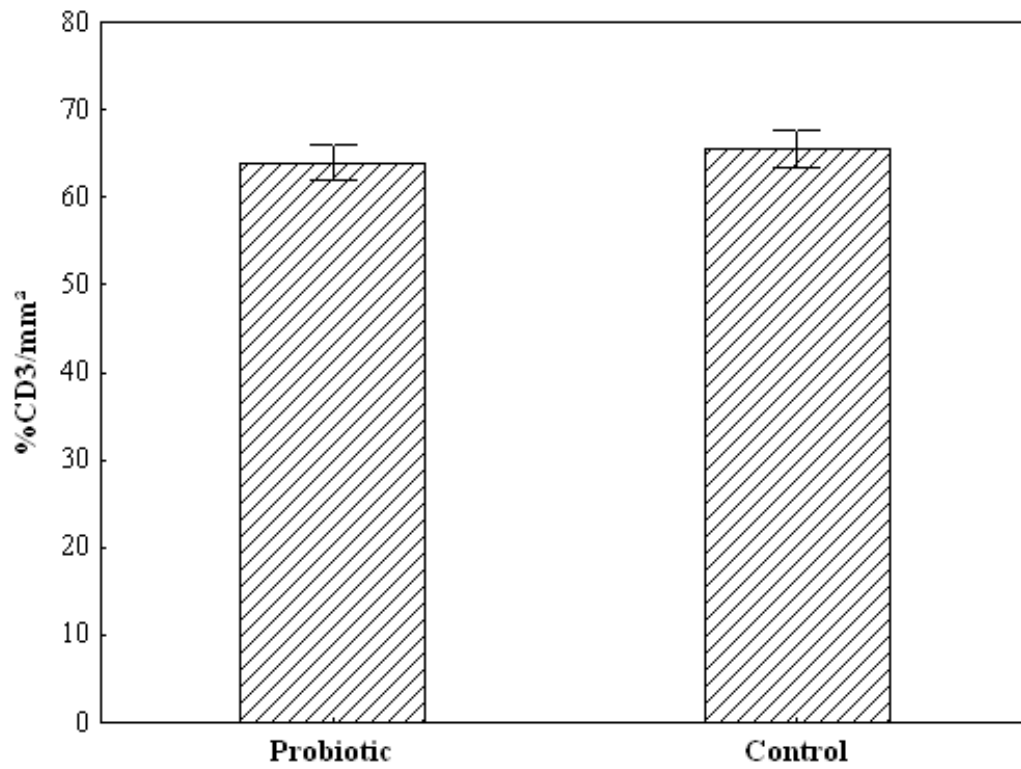


Figure 5 - CD3 expression (%/mm²) in thymus of cobia larvae reared with or without probiotic. Vertical bars represent one standard deviation. No significant statistical difference ($P>0.05$).

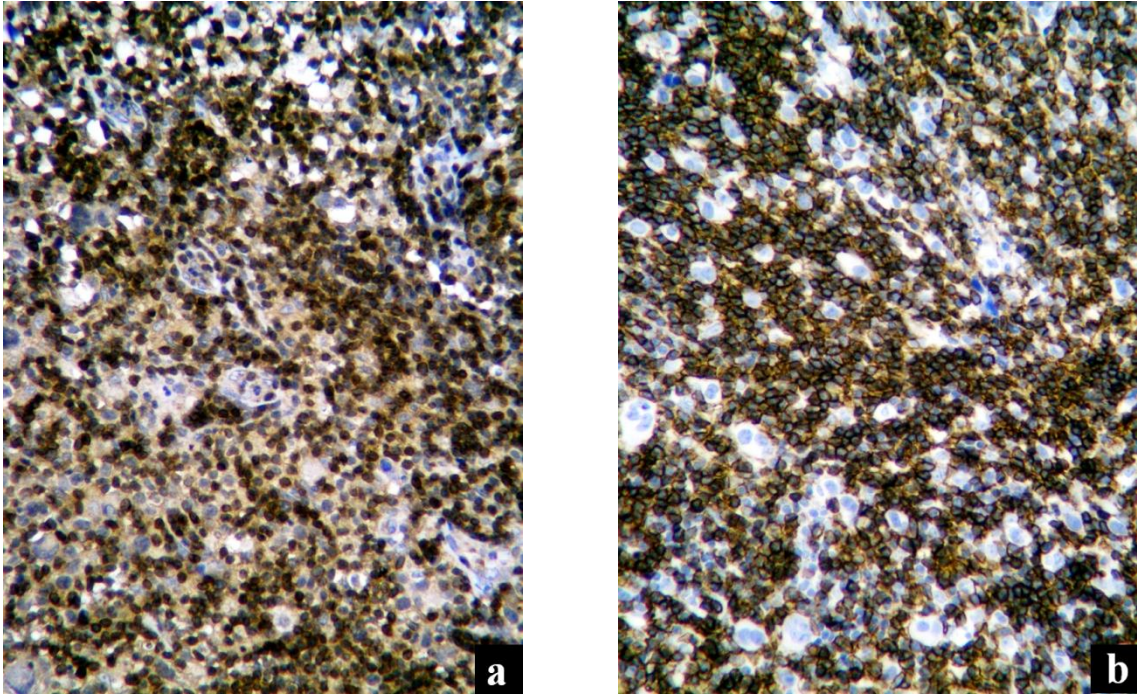


Figure 6. Immunohistochemistry of thymus, marking T-cell co-receptor CD3 in Control treatment (a) and Probiotic treatment (b) (brown cells). This receptor is a general T-lymphocyte marker, and it had no statistical difference between treatments (Probiotic: 63.9 ± 5.4 % CD3 cell/mm²; Control: 65.5 ± 5.0 % CD3 cell/mm²) ($P > 0.05$), showing that probiotics did not increased the number of T-cells in thymus.

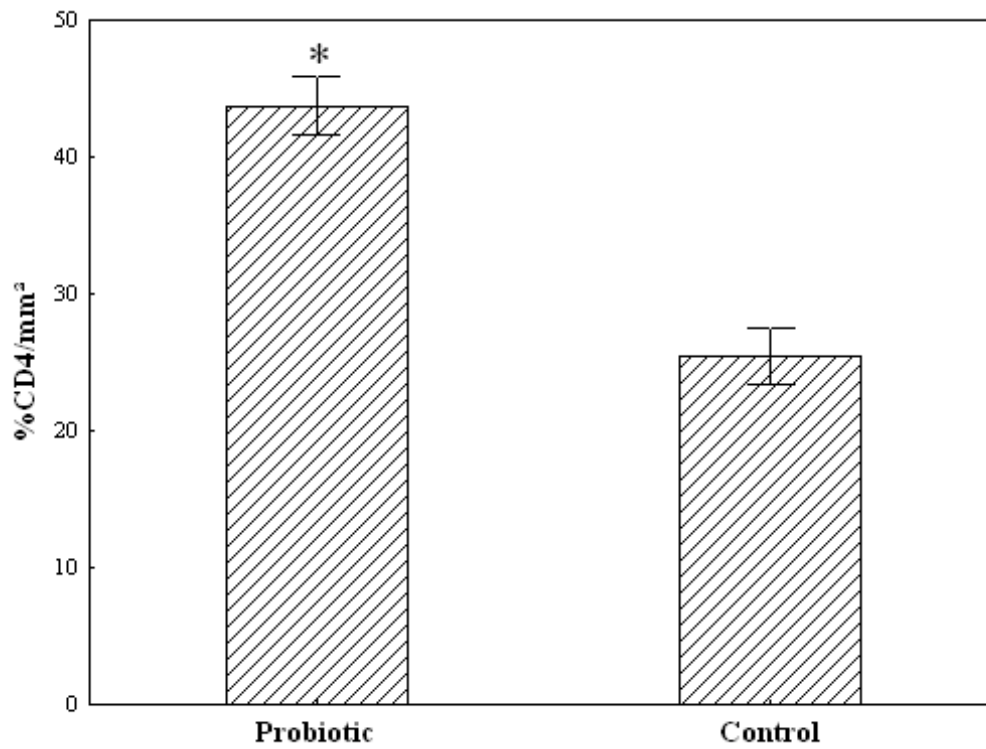


Figure 7 - CD4 expression (%/mm²) in thymus of cobia larvae reared with or without probiotic. Vertical bars represent one standard deviation and asterisk shows significantly statistical difference ($P < 0.01$).

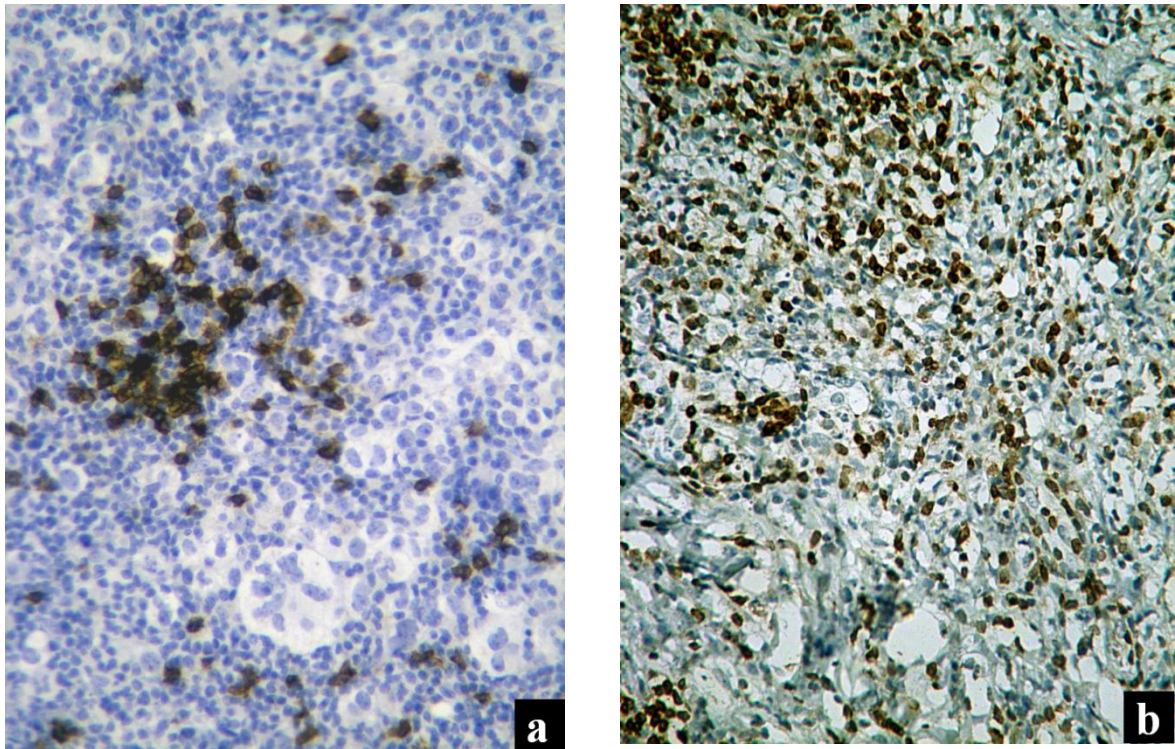


Figure 8. Immunohistochemistry of thymus, marking T-cell co-receptor CD4 in Control treatment (a) and Probiotic treatment (b) (brown cells). This receptor is mostly T-helper marker, a subclass of T-lymphocytes, that stimulates the expression and secretion of cytokines that produce antibody responses or lead to macrophage activation. CD4 expression was significantly higher ($P<0.05$) in the Probiotic treatment than in the control (Probiotic: 43.7 ± 6.16 % CD4 cell/mm²; Control: 25.4 ± 3.5 % CD4 cell/mm²).