UNIVERSIDADE DE PASSO FUNDO
FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOEXPERIMENTAÇÃO

OLIGODEOXINUCLEOTÍDEOS CpGs PROTEGEM JUNDIÁS (*Rhamdia quelen*) DO DESAFIO POR *Aeromonas hydrophila*

DISSERTAÇÃO DE MESTRADO

Raíssa Canova

Passo Fundo, RS, Brasil
2016

OLIGODEOXINUCLEOTÍDEOS CpGs PROTEGEM JUNDIÁS (*Rhamdia quelen*) DO DESAFIO POR *Aeromonas hydrophila*

Raíssa Canova

Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Bioexperimentação, Área de Concentração em Bioexperimentação, da Faculdade de Agronomia e Medicina Veterinária da Universidade de Passo Fundo (UPF), como requisito parcial para a obtenção do grau de **Mestra em Bioexperimentação**

Orientador: Prof. Luiz Carlos Kreutz

Passo Fundo, RS, Brasil
2016

UNIVERSIDADE DE PASSO FUNDO
FACULDADE DE AGRONOMIA E MEDICINA VETERINÁRIA
PROGRAMA DE MESTRADO EM BIOEXPERIMENTAÇÃO

A comissão examinadora, abaixo assinada, aprova a Dissertação de Mestrado

OLIGODEOXINUCLEOTÍDEOS CpGs PROTEGEM JUNDIÁS (*Rhamdia quelen*) DO DESAFIO POR *Aeromonas hydrophila*

Elaborada por
Raíssa Canova

Como requisito parcial para a obtenção do grau de
Mestra em Bioexperimentação

Comissão Examinadora

Luiz Carlos Kreutz, PhD, UPF
(Orientador/Presidente)

Rafael Frandoloso, Dr, UPF

Deniz Anziliero, Dr, IMED

Passo Fundo, RS, Brasil
2016

CIP – Catalogação na Publicação

C227o Canova, Raíssa

Oligodeoxinucleotídeos CpGs protegem Jundiás (*Rhamdia quelen*) do desafio por *Aeromonas hydrophila* / Raíssa Canova. – 2016.

47 f. : il. ; 30 cm.

Orientadora: Prof. Luiz Carlos Kreutz.

Dissertação (Mestrado em Bioexperimentação) – Universidade de Passo Fundo, 2016.

Catalogação: Bibliotecária Schirlei T. da Silva Vaz - CRB 10/1364

AGRADECIMENTOS

A realização deste trabalho não seria possível sem o apoio e colaboração de inúmeras pessoas. Gostaria de agradecer a cada um que de alguma forma contribuiu nessa caminhada, seja nos momentos de alegria, que serviram de incentivo nesta trajetória ou nos momentos difíceis, que permitiram crescimento pessoal e profissional.

Primeiramente gostaria de agradecer ao meu orientador, Prof. Dr. Luiz Carlos Kreutz pela excelente orientação, pela disponibilidade, confiança, pelos conselhos e principalmente pela amizade que foi construída nesses dois anos. Muito obrigada!

Ao Prof. Dr. Rafael Frandoloso pelas contribuições para realização deste trabalho.

A Dra. Michela Miani Colli pelas contribuições científicas, por todos muffins, brownies e cupcakes, pelos inúmeros conselhos pessoais e profissionais, pelas indicações de seriados, filmes e livros e pela amizade, a qual tenho muito apreço e carinho.

Aos funcionários do setor de piscicultura do CEPAGRO, sempre disponíveis e atenciosos.

Aos colegas do Laboratório de Microbiologia e Imunologia Avançada por todas as experiências trocadas e pela amizade.

Aos meus amigos que sempre me apoiaram em todas minhas decisões e entenderam minha ausência em alguns momentos. Em especial, a Mayara Biazus por sempre ter me ajudado e me apoiado em toda a caminhada.

Agradeço muito a minha família, por todo apoio e confiança e que nunca desacreditaram em mim, mesmo nas dificuldades.

MUITO OBRIGADA A TODOS!

A realização deste trabalho somente foi possível com o apoio financeiro do CNPq e pela concessão de bolsa da CAPES.

EPÍGRAFE

Alea Jacta Est!

Júlio César

ÍNDICE

LISTA DE FIGURAS

3. CAPÍTULO 1

LISTA DE ABREVIATURAS

BHI	*Brain Heart Infusion*
BSA	Albumina Sérica Bovina
CDs	Células Dendríticas
CpG	Citosina-fosfato-guanina
DNA	Ácido Desoxirribonucléico
FAO	Organização das Nações Unidas para Alimentação e Agricultura
FCA	Adjuvante completo de Freund
g	Gramas
h	Horas
i.p	Intraperitoneal
IL-1β	Interleucina um beta
IL-6	Interleucina seis
IL-12	Interleucina doze
INF-α	Interferon alfa
LB	Luria-Bertani
LPS	Lipopolissacarídeo
mg/l	Miligrama por litro
ml	Mililitro
MYD 88	Proteína de domínio 88
ODN	Oligodeoxinucleotídeo
PAMP	Padrões Moleculares Associados ao Antígeno
PBS	Solução salina fosfatada
PCR	Reação em cadeia da polimerase
PRR	Receptores de reconhecimento padrão
RNA	Ácido Ribonucléico
Rpm	Rotação por minuto
TLR	Receptor do tipo *Toll*
TNF-α	Fator de Necrose Tumoral alfa
UFC	Unidades Formadoras de Colônias
UPF	Universidade de Passo Fundo
µg	Micrograma

RESUMO

Dissertação de Mestrado
Programa de Pós-Graduação em Bioexperimentação
Universidade de Passo Fundo
OLIGODEOXINUCLEOTÍDEOS CpGs PROTEGEM JUNDIÁS (*Rhamdia quelen*) DO DESAFIO POR *Aeromonas hydrophila*
Autor (a): Raíssa Canova
Orientador: Luiz Carlos Kreutz
Passo Fundo, 05 de agosto de 2016

Neste estudo nós descrevemos a utilização de diferentes CpGs ODNs na modulação do sistema imune natural de jundiás (*Rhamdia quelen*). Os CpGs ODNs 1668, 2102, 2133 e 2143 foram selecionados devido a suas características e efeitos imunológicos já avaliados em outras espécies de vertebrados, incluindo peixes. Para avaliar o efeito protetor dos CpGs ODNs, grupos de peixes foram inoculados intraperitonealmente (i.p.) com cada um dos CpGs ODNs e posteriormente inoculados i.p. com *Aeromonas hydrophila* (2×10^8 UFC/peixe). O efeito imunoprotetor foi avaliado mensurando-se bacteremia e mortalidade nos peixes inoculados. No primeiro experimento, os jundiás foram divididos em 5 grupos (40 peixes/grupo) e cada peixe recebeu um inóculo de 0.1ml de um CpG ODN (0.5μg/peixe) e o grupo controle recebeu 0.1ml de PBS. Após 24 horas da administração dos CpGs ODNs ou de PBS, os peixes foram desafiados com *A. hydrophila* (2×10^8 UFC/peixe; 0,1 ml). E, 24 h após a inoculação com *A. hydrophila*, amostras de sangue de pelo menos 10 peixes foram coletadas visando a recuperação bacteriana. Os demais peixes inoculados (n=30) foram monitorados e o número de peixes mortos diariamente foi anotado por 7 dias. Neste experimento, os peixes inoculados com CpG ODN 1668, 2102 e 2133 apresentam bacteremia significativamente menor ($p<0.05$) do que o grupo inoculado com CpG ODN 2143 ou grupo controle. No grupo de peixes inoculados com CpG ODN 1668, o percentual de peixes com bacteremia foi de apenas 50% enquanto que no grupo inoculado com CpG ODN 2102 o percentual de peixes com bacteremia foi de 92%. Nos demais grupos o percentual de peixes com bacteremia foi de 100%. Além disso, os peixes inoculados com CpG ODN 1668 apresentam taxa de sobrevivência de 95%, que foi significativamente superior ($p < 0.05$) em relação aos demais grupos, inclusive do grupo controle. Os demais experimentos foram feitos utilizando-se o CpG ODN 1668. E, para avaliar o efeito da dose do CpG ODN 1668, quatro grupos de peixes (10 peixes/grupo) foram inoculados com CpG ODN nas doses 0.004μg, 0.02μg, 0.1μg e 0.5μg por peixe, respectivamente. O grupo controle foi inoculado com PBS e o desafio com *A. hydrophila* e a coleta de sangue foi feita conforme descrito acima. Nesse experimento, os peixes inoculados com as doses de 0.1μg/peixe e 0.5μg/peixe apresentam bacteremia significativamente menor ($p < 0.05$) do que os peixes do grupo controle. Nestes dois grupos o percentual de peixes com bacteremia foi de 45% enquanto que no grupo controle o percentual de peixes com bacteremia foi de 88,7%. Para avaliar o efeito do tempo de administração do CpG ODN na bacteremia, o CpG ODN 1668 (0.5μg/peixe) foi administrado nos tempos de 7 dias, 96, 48 ou 24 h antes do desafio com *A. hydrophila* (2×10^8 UFC/peixe). O grupo controle recebeu PBS antes do desafio. A detecção da bactéria no sangue foi feita conforme descrito acima. Neste experimento, os peixes inoculados com CpG ODN 1668 96 h antes do desafio apresentam bacteremia significativamente menor ($p<0.05$) em relação aos demais grupos. Além disso, nos peixes inoculados com CpG ODN 96h antes do desafio, o percentual de peixes com bacteremia foi de 80% enquanto que nos demais grupos o percentual de bacteremia foi de 100%. Com este estudo nós concluímos que a administração intraperitoneal de CpGs ODNs em jundiás estimula mecanismos que conferem proteção ao desafio por

Aeromonas hydrophila, diminuindo a bacteremia e aumentando a taxa de sobrevivência dos peixes. Nossos dados sugerem que o efeito protetor do CpG ODN nos jundiás é dependente da sequência de nucleotídeos, da dose e do tempo em que o CpG ODN foi administrado aos peixes.

Palavras-chave: CpG ODN, jundiá, *Aeromonas hydrophila*, bacteremia, sistema imune.

ABSTRACT

Master's Dissertation
Programa de Pós-Graduação em Bioexperimentação
Universidade de Passo Fundo
CpGs OLIGODEOXYNUCLEOTIDES PROTECT SILVER CATFISH (*Rhamdia quelen*) FROM *Aeromonas hydrophila* CHALLENGE
Author: Raíssa Canova
Advisor: Luiz Carlos Kreutz
Passo Fundo, august 5th, 2016

In this study we evaluated the effect of different CpGs ODN on their ability to modulate the innate immune system of silver catfish (*Rhamdia quelen*). The CpGs ODNs 1668, 2102, 2133 and 2143 were selected based on their characteristics and because their immunological effect has already been reported in other vertebrate species, including fish. To evaluate the protecting effect of CpGs ODNs, groups of fish were intraperitoneally inoculated (i.p.) with each CpGs ODNs and 24h after they were i.p. inoculated with *Aeromonas hydrophila* (2×10^8 UFC/fish). The protective effect was evaluated by recovering the bacteria from blood and fish mortality. In the first experiment, silver catfish were divided in 5 groups (40 fish/group); each fish was inoculated with 0.1ml of CpG ODN (0.5μg/fish) and fish from the control group were inoculated with 0.1 ml of PBS. Then, 24h after CpGs ODNs or PBS inoculation, all fish were inoculated with *A. hydrophila* (2×10^8 CFU/fish; 0,1 ml). And, 24 h post-challenge with *A. hydrophila*, blood samples were collected from at least 10 fish *per* group aiming to detect bacteremia. The remaining fish (n=30) were monitored and the number of death fish was daily annotated for up to seven days. In this experiment, fish inoculated with CpG ODN 1668, 2102 and 2133 had significantly lower ($p<0.05$) bacteremia than fish from the CpG ODN 2143 or control group. In the CpG ODN 1668 group, the percentile of fish with bacteremia was only 50% whereas in the CpG ODN 2102 group, the percentile of fish with bacteremia was 92%. In the other groups the percentile of fish with bacteremia was 100%. In addition, in the group inoculated with CpG ODN 1668, the survival (95%) and significantly higher ($p<0.05$) when compared to the other groups. Thus, the remaining experiments were carried out using CpG ODN 1668. And, to evaluate the effect of the dose of CpG ODN 1668, 4 groups of fish (10 fish/group) were inoculated with 0.004μg, 0.02μg, 0.1μg or 0.5μg/fish, respectively. Control group received PBS; bacteria challenge and recovery from blood was performed as described above. Here, fish inoculated with 0.1μg and 0.5μg of CpG ODN had significantly lower ($p<0.05$) bacteremia than fish from the control group. In this 2 groups, the percentile of fish with bacteremia was 45% compared to 88,7% of fish with bacteremia in the control group. To evaluate the effect of time of CpG ODN administration on the level of bacteremia, CpG ODN 1668 (0.5 μg/fish) was inoculated at 7 days, 96, 48 or 24h prior to *A. hydrophila* challenge (2×10^8 CFU/fish). The control group was inoculated with PBS prior to challenge and bacteria recovery from blood was carried out as indicated above. In this experiment, fish inoculated with CpG ODN 1668 at 96h prior to challenge had significantly ($p<0.05$) lower bacteremia compared to the other groups. In addition, in the fish inoculated

with CpG ODN at 96h prior to challenge, the percentile of fish with bacteremia was 80% whereas in the remaining groups the percentile of fish with bacteremia was 100%. Thus, with these studies we concluded that i.p. inoculation of CpGs ODNs stimulates protection against challenge with *A. hydrophila* in silver catfish, reducing bacteremia and increasing fish survival rate. Our data suggest that the protective effect of CpG ODN depends on the nucleotide sequence, dose and time of administration prior to fish challenge.

Keywords: CpG ODN, silver catfish, *Aeromonas hydrophila*, bacteremia, immune system

1. INTRODUÇÃO

Os peixes e seus produtos representam uma fonte valiosa de proteínas e micronutrientes necessários para uma alimentação balanceada. Em 2009, 17% da proteína animal ingerida foi proveniente do pescado (1). A crescente demanda pela carne de pescado exige a intensificação do cultivo, que predispõe a doenças causadas principalmente por bactérias e vírus, as quais impõem altas perdas econômicas e podem se tornar um fator limitante para a produção de peixes (2). Evitar e controlar o surgimento de doenças infecto contagiosas é um dos principais desafios da aquicultura moderna (3).

No intuito de evitar perdas econômicas relacionadas a problemas sanitários, diferentes drogas de uso veterinário têm sido utilizadas visando o tratamento ou prevenção de doenças (1). O uso indiscriminado de antibióticos não é uma medida aceitável e, em alguns países, não é mais permitido (3). A vacinação representa uma das principais medidas profiláticas e no Brasil existe apenas uma vacina comercialmente disponível que protege contra infecções causadas pelo *Streptococcus agalactiae* (Aquavac® Strep AS, MSD). No entanto, a vacinação em peixes representa um grande desafio, visto que as vacinas disponíveis utilizam adjuvantes tradicionais, e são administradas pela via intraperitoneal, podendo causar aderência das vísceras (4).A modulação do sistema imune inato através da administração de moléculas imunoestimulantes representa uma alternativa no controle de patógenos (5); moléculas com propriedades imunoestimulantes são encontradas principalmente em plantas medicinais, ervas e micro-organismos (1,5). Entre as moléculas obtidas de micro-organismos, encontram-se oligodeoxinucleotídeos (ODN) compostos por sequências de citosina-fosfato-guanina (CpG) não metiladas presentes no DNA, os quais se constituem em padrões moleculares associados aos patógenos (*Pathogen associated molecular pattern - PAMPs*) (7). Os CpGs ODNs são reconhecidos pelos receptores similares ao *Toll* (*Toll-like receptor* – TLR) tipo 9, um tipo de receptor de reconhecimento padrão (*Pattern recognition receptor- PRR*), presente nos endossomos de macrófagos, monócitos, células dendríticas e linfócitos B (8,9). As propriedades imunoestimulantes dos CpGs ODNs foram reportadas anteriormente em diferentes espécies de peixes (5,7,10,11).

O jundiá (*Rhamdia quelen*) é um bagre de hábito noturno pertencente à família Heptapteridae e comumente encontrado em rios e lagos da América do Sul (12), que tem sido utilizado em mono ou policultivo na região sul do Brasil (13) e como modelo para estudos do

efeito de pesticidas agrícolas sobre os mecanismos de defesa natural e adquirido (14–16). Recentemente, demonstramos que jundiás vacinados com CpGs ODNs + albumina sérica bovina (BSA) produziram anticorpos anti-BSA em níveis similares àqueles produzidos em jundiás vacinados com adjuvantes clássicos (17). A estimulação do sistema imune específico (linfócitos B e T) depende fundamentalmente da estimulação das células do sistema imune natural (macrófagos e células dendríticas) pelos antígenos. Nesse contexto, além do potencial uso como adjuvante vacinal, preconiza-se que CpGs ODNs poderiam também ter um efeito imunoestimulante sobre o sistema imune inato. Até o momento, não há estudos sobre os efeitos de CpGs ODNs no sistema imune natural de jundiás.

Nesse trabalho, nosso objetivo foi avaliar se CpGs ODNs possuem capacidade de estimular o sistema imune natural e proteger jundiás desafiados pela inoculação intraperitoneal de *Aeromonas hydrophila*. Os resultados obtidos estão descritos no capítulo um o qual se constitui em um artigo científico intitulado "**Oligodeoxynucleotides CpGs increase silver catfish (*Rhamdia quelen*) resistance to *Aeromonas hydrophila* challenge**" submetido para publicação no periódico *Aquaculture*.

2. REVISÃO DE LITERATURA

A aquicultura apresentou um rápido crescimento nas últimas décadas (3). Da mesma maneira que outras cadeias produtivas onde os animais são criados em cativeiro, os peixes também são suscetíveis a doenças infecto contagiosas, causadas por diferentes micro-organismos. A contaminação da água por produtos químicos e tóxicos, estresse, baixa qualidade da água e das instalações, excesso de matéria orgânica na água, oxigênio dissolvido abaixo das concentrações ideais, entre outros fatores, influenciam na imunidade do peixe, deixando-os suscetíveis a micro-organismos que normalmente seriam avirulentos (18,19). A *Aeromonas hydrophila* habita ambientes aquáticos e é considerada uma bactéria emergente e, principalmente em peixes imunossuprimidos, causa septicemia hemorrágica, necrose nas nadadeiras e cauda, levando a morte entre dois e dez dias após o início dos sinais clínicos (20). Nesse sentido, o desenvolvimento sustentável da piscicultura depende da implementação de medidas de prevenção de micro-organismos (21).

Os antibióticos constituem uma das principais ferramentas para controlar, tratar e muitas vezes prevenir doenças na criação de animais de produção, incluindo na aquicultura. Prevenindo processos essenciais como síntese da parede celular, proliferação de DNA e síntese de proteínas (22), os antibióticos tornaram-se uma ferramenta estratégica para manter os animais livres de bactérias patogênicas. A maioria dos antibióticos são administrados pela via oral, com exceção em peixes reprodutores, em que a administração é pela via intraperitoneal. Todos os antibióticos utilizados são legalmente aprovados pelo órgão governamental de medicina veterinária, variando conforme o país em que se aplica(23). Nos EUA, os antibióticos aprovados pela FDA para uso na aquicultura são: oxitetraciclina, florfenicol, sulfadimetoxina/ormetropin. Infelizmente, o uso em larga escala de antibióticos pode levar à seleção de bactérias resistentes (24). Com o objetivo de combater bactérias patogênicas sem desenvolver bactérias resistentes, métodos alternativos devem ser investigados (22).

A vacinação é uma forma eficaz de proteção contra microrganismos patogênicos, mas na aquicultura ainda está em fase de desenvolvimento. Estudos recentes já demonstraram a eficiência das vacinas contra alguns vírus de peixes(25). As vacinas visam estimular o sistema imunológico adaptativo a montar uma resposta contra um agente patogênico ou contra partes específicas desse agente, ou seja, as estruturas altamente imunogênicas(18). Nesse contexto, o desenvolvimento de vacinas são uma alternativa para conferir proteção aos peixes utilizando

microrganismos inteiros ou apenas partes do microrganismo, as quais conferem imunidade para o hospedeiro. Assim, proteínas de membrana, proteínas excretadas durante o crescimento da bactéria e demais proteínas, são alternativas para o desenvolvimento de vacinas, seja vacinas recombinantes, vacinas de DNA ou vacinas de micropartículas não sendo necessária a utilização do microrganismo inteiro(3).

A utilização de moléculas com potencial imunomodulador e/ou imunoestimulante (estimulantes naturais) representam uma alternativa importante e ainda pouco explorada para o controle de doenças na piscicultura comercial. As ervas medicinais tem sido amplamente exploradas como imunoestimulantes em peixes, por induzirem poucos efeitos colaterais e por serem facilmente degradadas no ambiente aquático, além de apresentar efeitos benéficos na imunidade inata e adaptativa dos peixes, proporcionando proteção contra agentes patogênicos (26). Entre as principais características das moléculas imunoestimulantes é a presença, em sua arquitetura, de sequências repetidas de formas moleculares individuais, tais como a glucose em β-glucanas, (deoxi) riboses em DNA/RNA, ácidos graxos em lipopolissacarídeo (LPS) bacteriano e certas lipoproteínas, que estimulam o sistema imune dos vertebrados (27).

Os micro-organismos possuem moléculas específicas que os diferenciam de outras células. Essas moléculas são conhecidas como PAMPS. Os CpGs ODNs são um tipo de PAMP que possuem dinucleotídeos citosina-fosfato-guanina não metilados encontrados no DNA de muitos micro-organismos e são reconhecidos pelos receptores de reconhecimento padrão (PRR, *pattern recognition receptor*) das células do hospedeiro (28,29). Os receptores TLR são um tipo de PRR e são encontrados tanto na membrana citoplasmática como em vesículas intracelulares das células imunológicas. Entre esses receptores, o TLR-9 é responsável pelo reconhecimento de CpGs ODNs (30). O TLR-9 é expresso constitutivamente no retículo endoplasmático ou nos endossomos das células dendríticas, macrófagos, monócitos e linfócitos B (6). A entrada do CpG dentro da célula pode ser feita a partir de duas vias. Uma dessas vias utiliza a enzima de classe III fosfadilinositol kinase (PI3K) que permite internalização do CpG em uma vesícula endossomática contendo o TLR-9; a outra via é através da fagocitose (31). A interação entre o CpG e o TLR-9 leva a uma cascata de sinalização ativando a proteína MYD88 a qual estimula os receptores de interleucina 1 (TIR) e o complexo IRAK-TRAF6 que são receptores para o fator de necrose tumoral (TNF) e estes ativam as proteínas kinase JNK1/2, P38 e o inibidor de NF-κB/IKK culminando na ativação do NF-κB e da proteína ativadora 1 (AP1). A AP1 e NF-κB migram até o núcleo da célula e

estimulam o DNA a iniciar a expressão de genes como: TNF, IL-1, CCL2, CXCL8, E-selectina, IFN, etc. culminando no recrutamento de neutrófilos, macrófagos, células dendríticas (9,31), essenciais para o início da resposta inflamatória. Vários CpGs ODNs apresentam propriedades imunoestimulantes em diversas espécies de peixes em que foram testados, incluindo truta arco-íris (*Oncorhynchus mykiss*) (32), salmão do Atlântico (*Salmo salar L.*) (33), carpa comum (*Cyprinus carpio L.*) (10), falso-alabote-Japonês (*Paralichthys olivaceus*) (34), carpa-capim (*Ctenopharyngodon idellus*) (35), bagre Americano (*Ictalurus punctatus*) (36) e rodovalho (*Scophthalmus maximus*) (7). Sabe-se que assim como em mamíferos, o TLR-9 é a única forma de promover a cascata de ativação, uma vez que quando utilizados peixes com deleção do gene do TLR-9, não ocorreu a expressão de genes inflamatórios (9).

O jundiá (*Rhamdia quelen*), membro da família dos Heptapteridae, é um dos peixes mais comuns dos rios naturais e lagoas artificiais do sul da América do Sul (12). O aumento da criação de jundiás em cativeiro levou ao aumento da incidência de surtos de Aeromonose, doença causada pela bactéria *Aeromonas hydrophila* (13). Em estudos anteriores realizados com jundiás, demonstra-se que a exposição a agroquímicos como a atrazina e o glifosato aumenta a suscetibilidade dos peixes a infecções causadas pela *A. hydrophila* alterando a atividade fagocítica e parâmetros imunológicos (14). Ainda, foi demonstrado que a utilização da β-glucana como aditivo alimentar melhora a atividade hemolítica natural do sistema do complemento, reduzindo os níveis de bacteremia e aumentando a resistência dos jundiás ao desafio por *A. hydrophila* (37).

Os CpGs ODNs ainda não estão comercialmente disponíveis para uso como adjuvantes ou imunoestimulantes. Ainda assim, representam uma alternativa para diminuir o uso de quimioterápicos na criação de peixes. Os CpGs ODNs demonstram capacidade em induzir a migração e proliferação de leucócitos em peixes (11) além de aumentar a expressão de genes pró-inflamatórios envolvidos na destruição de micro-organismos (38). Nesse contexto, a ausência de estudos sobre os efeitos imunoestimulantes dos CpGs ODNs em jundiás deixa uma lacuna importante no que diz respeito a prevenção e controle de micro-organismos uma vez que os CpGs ODNs quando administrados aos peixes, não possuem efeitos colaterais indesejáveis, além de serem biologicamente seguros (6).

3. CAPÍTULO 1

Oligodeoxynucleotides CpGs increase silver catfish (*Rhamdia quelen*) resistance to *Aeromonas hydrophila* challenge.

Raíssa Canova[1], Karina Schreiner Kirsten[1], Lucas de Figueiredo Soveral[2], Rafael Frandoloso[1], Luiz Carlos Kreutz*

(Artigo submetido ao periódico *Aquaculture* -2016)

[1] Programa de Pós-Graduação em Bioexperimentação, Universidade de Passo Fundo.

[2] Curso de Medicina Veterinária, Faculdade de Agronomia e Medicina Veterinária, Universidade de Passo Fundo.

*Autor correspondente: Luiz Carlos Kreutz, Universidade de Passo Fundo, Faculdade de Agronomia e Medicina Veterinária, Campus I, Bairro São José. CEP 99052-100.

Telefone +55 54 3316 8444; Fax +55 54 3316 8163. E-mail: lckreutz@upf.br.

Oligodeoxynucleotides CpGs increase silver catfish (*Rhamdia quelen*) resistance to *Aeromonas hydrophila* challenge.

Raíssa Canova, Karina Schreiner Kirsten, Lucas de Figueiredo Soveral, Rafael Frandoloso, Luiz Carlos Kreutz*

Universidade de Passo Fundo (UPF), Faculdade de Agronomia e Medicina Veterinária (FAMV), Laboratório de Microbiologia e Imunologia Avançada – Prédio G3. Campus I, Bairro São José, BR 282, km 171. CEP 99052-900 Passo Fundo, RS, Brazil. Tel. +55 54 33168444; Fax. +55 54 3316 8163.

* Corresponding author. E-mail: lckreutz@upf.br (L.C. Kreutz)

Abstract

Immune modulation stands up as a strategic management procedures that might overcome the challenges of water-borne pathogens. Synthetic oligodeoxynucleotides (ODNs) containing cytidine-phosphate-guanosine (CpG) motifs are known to activate vertebrate innate immune cells and have been evaluated in several fish species but, to our knowledge, not yet in silver catfish. In this study we analyzed the effect of selected ODN CpGs on their ability to enhance the resistance of silver catfish to a lethal infection with *Aeromonas hydrophila*. We found that silver catfish pretreated with some ODN CpGs had lower bacterial counts in the blood and the percentile of bacteremic fish was lower compared to control fish. In addition, the survival rate of ODN CpG-treated fish was higher following challenge with *A. hydrophila*. The effect of ODN CpG 1668, which provided higher protection against challenging bacteria, was dose and time dependent. Our results demonstrate that ODN CpG can induce disease resistance in silver catfish.

Key words: fish; ODN CpG; disease resistance; *Aeromonas hydrophila*; immunostimulant; immunomodulation.

1. Introduction

Fish farming in continental water increased steadily in the last decades and is becoming an important worldwide economic activity. According to recent projections, and aiming to attend to global demands, fish production should be improved to 100 M ton/year and at least one fifth of this volume should come from Brazil (FAO, 2014; Valladão et al., 2016). Intensive fish farming, however, comes along with several challenges such as outbreaks of infectious diseases that accounts for losses of up to twenty percent each year (FAO, 2014). Most commonly diagnosed infectious diseases of fish are caused by bacteria (Brudeseth et al., 2013) and current therapeutic approaches based on the use of antibiotics do not suffice to keep infections under control (Van Muiswinkel, 2008). Furthermore, widely and improperly use of antibiotics in aquaculture drives the emergence of antibiotic-resistant bacteria and might lead to food and environmental contamination (Cabello, 2006) with deleterious effects on public health. In this scenario, the use of specific vaccines (Brudeseth et al., 2013; Plant and Lapatra, 2011; Tafalla et al., 2013) or immune modulating molecules (Bairwa et al. et al., 2012; Newaj-Fyzul and Austin, 2015; Vallejos-Vidal et al., 2016) should be explored aiming to improve fish ability to cope with these water-borne challenging pathogens.

Molecules with immune modulating properties have been obtained mostly from herbs, plants and microorganism (Tafalla et al., 2013; Vallejos-Vidal et al., 2016; Van Hai, 2015) and their ability to improve defense mechanisms rely on the interaction with immune cells. Different microorganisms share structures named pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors (PRRs) found on the surface and intracellular compartment of immune cells (Pietretti and Wiegertjes, 2014). Bacterial DNA, for instance, contains unmethylated cytosine-phosphate-guanine (CpG) dinucleotides

that might serve as PAMPs to stimulate the immune system of vertebrates (Carrington and Secombes, 2006). Synthetic oligodeoxynucleotides (ODNs) containing CpGs motifs (CpG ODNs) mimic bacterial DNA and have been used to modulate the innate and acquired immune response of fish and mammals (Carrington and Secombes, 2006; Klinman et al., 2009). The immune modulating effects of CpG ODNs are mediated by interaction with the PRR Toll-like receptor 9 (TLR9) expressed on macrophages, dendritic cells and B lymphocytes (Rutz et al., 2004). The intraperitoneal injection of CpG ODNs, for instance, improved the production of lysozyme and phagocytic activity in carps (*Cyprinus carpio*) (Tassakka and Sakai, 2003), the production of IL-1β in rainbow trout (*Oncorhunchus mykiss*) (Jørgensen et al., 2001b) and provided protection to bacterial challenge in olive flounder (*Paralichthys olivaceus)* and turbot (*Scophthalmus maximus*) (Lee et al., 2003; Liu et al., 2010a, 2010b). The immune modulating properties of CpG ODNs are related to the sequence and structure characteristics and, most importantly, to the species in which they are evaluated. Therefore, their effects should be evaluated in each economically important fish species to ascertain that they shall be used to improve defense mechanisms.

Silver catfish is an omnivorous fish commonly found in lakes and rivers from Mexico to the southern part of South America (Schulz and Leuchtenberger, 2006) and is suitable to farming alone or comingled with other fish species (Da Silva et al., 2008). However, outbreaks of *A. hydrophila*-induced hemorrhagic septicemia (Barcellos et al., 2008) is of major concern to fish farmers and researchers, mainly under stressful situations caused by management practices, decay in water quality or contamination with immunotoxic agrichemical. In a previous work we found that the resistance of silver catfish to *A. hydrophila* challenge could be improved by β-glucan enriched diet (Di Domenico et al., in press) and that β-glucan or CpG ODNs, could be used as adjuvant to potentiate antibody

production to a model antigen (Pavan et al., 2016). In the present study, build up on our previous work, our main goal was to investigate whether the intraperitoneal administration of specific ODN CpGs could protect silver catfish against *A. hydrophila* challenge. We found that ODN CpG protection is dose and time dependent and might be exploited as important immune modulating PAMPs in this important fish species.

2. Materials and Methods

2.1 Fish

Juvenile mixed sex silver catfish (30±10g) were used throughout the experiment. For all experiments fish were first acclimatized for at least seven days in the experimental tanks fed with continuously running water and protected from direct sunlight exposure. Fish density was always smaller than 1 gr of fish/L of water. The level of dissolved oxygen varied from 5.0 to 7.0 mg/L and the pH ranged from 7.0 to 7.2. Water hardness and alkalinity were both 45 ± 5 mg $CaCO_3$/L and total ammonia was below 0.6 mg/L. Prior to and during the experiments fish were fed commercial pelleted food (30% crude protein, Supra, Brazil). In addition, prior to each experiment, randomly selected fish were scrutinized for disease sign and blood sampling to ascertain they harbor no specific bacterial pathogens in their tissues. All sampling and inoculation procedures were carried out with anesthetized fish (Eugenol, 50 mg/L^{-1}).The study was approved by the Institutional Ethical and Animal Welfare Committee (protocol number 011/2012).

2.2 Bacterial isolate and growing conditions

The *A. hydrophila* isolate used in this study was isolated from a case of hemorrhagic septicemia; the isolate was biochemically characterized in our laboratory and the identity of

the isolate was ascertain by polymerase chain reaction (PCR) using primers A.hemF (5´CTTCTACCTCAACGTCAACC3´) e A.hemR (5´GAATCCCTTGTAGCTGAGTG3´) which are specific for the hemolysine gene of *A. hydrophila* (GenBank: EU009398.1). PCR product were then analyzed by electrophoresis on 1% agarose gel and verified by DNA sequencing.

For fish inoculation, the bacteria inoculum was prepared by cultivating in Luria Broth (LB) at 37ºC under constant shaking (200 rpm) until reaching optical density (OD) of 0.5 at 600 nm. The bacterial was washed three times with PBS (pH 7.2) by centrifuging at 4000x g (4ºC). The bacterial pellet was then suspended in PBS and the OD adjusted to 0.5 at 600 nm that corresponded to approximately 2 x 10^9 colony forming units (UFC)/ml.

2.3 CpG ODNs

The immunological properties and nucleotide sequence of the CpG ODNs used in our study were previously reported (Carrington and Secombes, 2006). We selected ODN CpGs identified as 1668, 2102, 2133 and 2143 that were constructed on a phosphorothioate backbone (Invivogen, France).

2.4 Bacteria recovery from silver catfish blood

Blood samples were collected under aseptic conditions from fish anesthetized with an overdose of Eugenol (100 mg/L^{-1}) and stored on ice up to the time of plating that was done usually within 2h. Blood aliquots (0.1 and 0.01 ml) were plated on brain heart infusion (BHI) agar plates and incubated at 37ºC for 24 h. The colonies were counted and the identity of the bacteria was confirmed by PCR as indicated above.

2.5 Effect of CpGs ODNs on bacteremia and fish survival

In this experiment fish were assigned randomly to 5 groups (50 fish/group). Fish from the control group were inoculated intraperitoneally (i.p.) with 0.1 ml of PBS. Fish from the other groups were inoculated i.p. with 0.5μg of CpG 1668 (group 1668) or 0.5μg/fish of CpGs 2102, 2133 or 2143, to each respectively group. Twenty four h after ODN CpG administration, all fish were inoculated i.p. with *A. hydrophila* (100μl, containing 2 x 10^8 CFU/fish) corresponding to the LD_{50} for silver catfish (Kreutz et al., 2010). Then, 24h after bacteria inoculation, 10 fish from each group were captured and sampled aiming to recover bacteria from blood as described above. The remaining fish were monitored and daily fish mortality was annotated up to seven days after challenging.

2.6 The effect of ODN CpG 1668 regarding the dose and time of administration on bacteria recovery from blood

Because in the first experiment fish survival was higher in the group inoculated with ODN CpG 1668, this CpG was used in the next experiments. To evaluate the effect of dose on bacteria recovery from challenged fish, 50 fish were equally allocated to five groups (10 fish/group) and inoculated i.p. with 0.1 ml PBS (control group) or with CpG 1668 at 0.004μg, 0.02μ, 0.1μg or 0.5μg/fish in 0.1 ml PBS, respectively. Bacteria challenge and recovery from blood were carried out as described above using all fish from the experiment. To examine the effect of time of inoculation on bacteria recovery from blood, 5 groups of fish (10 fish/group) were used. Fish from the control group were inoculated i.p. with 0.1 ml of PBS. The other four groups were inoculated i.p. with (0.5 μg/fish) and challenged by i.p. injection of *A. hydrophila*, as described above, after 24, 48, 96 and 168 h of CpG inoculation. Bacteria

challenge and recovery from blood were carried out as described above using all fish from the experiment.

2.7 Statistical analysis

The data were evaluated by the Shapiro-Wilk's test and found to have normal distribution. Differences amongst treatment were analyzed by Anova, followed by Tukey's post-test, as stated on figure legends, and plotted using GraphPadPrism Software v5 (GraphPad Software, Inc, USA). Results are reported as the mean ± standard error of the mean (SEM) and *p* values of 0.05 or smaller were considered to be significant.

3. Results

3.1 Analysis of ODN CpGs with protective effect against *A. hydrophila* in silver catfish

Aiming to select ODN CpGs with immunoprotective effect of silver catfish, we examined the effect of ODN CpGs 1668, 2102, 2133 and 2143 on their capacity to protect fish against challenge with pathogenic *A. hydrophila*. Silver catfish were injected i.p. with each of the four ODN at 0.5 μg/fish or with PBS (control) and challenged with *A. hydrophila* at 24 h post-ODN administration. Bacterial recovery from blood (bacteremia) was determined at 24 h post-infection and survival rate was monitored up to 7 days post-infection. The number of bacteria recovered from control fish and from fish inoculated with ODN CpG 2143 was significantly higher ($p < 0.05$) when compared with fish inoculated with ODN CpGs 2102, 2133 and 1668 (Figure 1). Interestingly, 100% of fish from the control group and from fish inoculated with ODN CpGs 2133 and 2143 were bacteremic; in contrast, only 50% of fish inoculated with ODN CpG 1668 were bacteremic. The survival rate (Figure 2) was significantly higher ($p<0.001$) in fish inoculated with ODN CpGs 1668, 2143 and 2102 (95% for CpG 1668 and 75% for CpGs 2143 and 2102). In contrast, fish inoculated with ODN CpG

2133 had survival rate (23.3%) significantly lower ($p < 0.001$) than control fish (40% survival rate). Because ODN CpG 1668 protected 50% of fish from bacteremia and provided the higher survival rate, this ODN CpG was used in the remaining experiments.

3.2 The protective effect of ODN CpG 1668 in relation to dose and time of administration

To evaluate the effect of dose on protection against *A. hydrophila* challenge, fish were inoculated with different dose of ODN CpG (0.004μg, 0.02μg, 0.1μg e 0.5μg/fish) and 24h later challenged with *A. hydrophila*. Bacteria recovery data indicated that the higher doses (0.1μg and 0.5μg/fish) had significantly lower ($p<0.05$) bacteria in blood than the other groups (Figure 3). In addition, in this experiment, recovery of bacteria from the blood was achieved in only 45% of fish inoculated with 0.1μg and 0.5μg whereas 88.7% of fish from the control group had bacteremia.

To evaluate whether the effect of ODN CpG 1668 on protecting silver catfish against *A. hydrophila* bacteremia is dependent on the time of administration prior to challenge, we inoculated four groups of fish with ODN CpGs 1668 (0.5μg/fish) and challenge them with *A. hydrophila* after 24h, 48h, 96h and 168h. In this experiment, fish inoculated with ODN CpG 1668 96h prior to challenge had significantly ($p<0.05$) lower bacteremia than the other groups (Figure 4) and a lower percentile of fish (80%) had bacteremia whereas in the other groups 100% of fish were bacteremic.

4. Discussion

Consumer demand for fish products are on the rise at the same speed as the natural resources dwindle steadily. Thus, the shortfall in supply of fish and related product relies on the aquaculture industry which has experienced an unusual expansion in the last decade (FAO, 2014) altogether with several challenges related mostly to outbreaks of infectious

diseases (Brudeseth et al., 2013; Plant and Lapatra, 2011). In this scenario, the use of vaccines and immune modulating molecules should be explored aiming to reduce economic losses imposed by pathogens and assure the efficiency of fish farming. Bacterial DNA and synthetic ODN CpGs, which mimic bacterial DNA, have been used to modulate the immune system of fish (Carrington and Secombes, 2006; Klinman et al., 2009). These PAMPs interact with specific receptors leading to the proliferation and differentiation of B and T lymphocytes, natural killer (NK) cells, monocytes, macrophages and dendritic cells (Rutz et al., 2004). Because the immunological effects of ODN CpGs varies according to the nucleotide sequence and fish species, their use should be recommended only after evaluating their effect in each economically important fish species to ascertain that they can be widely used.

In a previous study we demonstrated that ODNs CpGs are potent adjuvants when mixed to bovine serum albumin (BSA) rivaling with Freund's complete and incomplete adjuvants in boosting the production of specific antibodies (Pavan et al., 2016). Here, we selected four ODN CpGs to evaluate their effectiveness to improve innate immune defenses on silver catfish challenged with *A. hydrophila*. The mean number of bacteria recovered from the blood of challenged fish was similar amongst fish inoculated with ODN CpGs 1668, 2102 and 2133; however, the percentile of bacteremic fish was lower in the group inoculated with ODN CpG 1668 (50%) compared to the percentile of bacteremic fish from the group inoculated with ODN CpG 2102 (92%) and 2133 (100%). Furthermore, in fish inoculated with ODN CpGs 1668 the survival rate (93.3%) was significantly higher ($p<0.001$) when compared to the other groups. Even though the efficacy of ODN CpG might depend on the fish species (Rankin et al., 2001), similar results were observed with olive flounder (*Paralichthys olivaceus*) and Atlantic salmon (*Salmo salar*) (Bridle et al., 2003; Lee et al., 2003) suggesting that regardless of the phylogenetic differences amongst these species, ODN CpG 1668 induces similar immune modulating signals that conferred protection upon

challenge with pathogenic bacteria. In addition to ODN CpG nucleotide sequence and fish species, other experimental factors, such as dose and time of administration prior to challenge, have been reported as important variables that might affect immune-modulating capability (Rankin et al., 2001; Tafalla et al., 2013). In our study, a lower percentile of bacteremic fish and a significantly ($p<0.05$) lower number of bacteria were observed in fish inoculated with the higher doses (0.1 and 0.5 μg/fish) of ODN CpG 1668. In a similar study, the survival rate of olive flounder injected with 0.25μg or 0.5μg of ODN CpG 1668 48 h prior to challenge with *Edwarsiella tarda* by immersion was similar (Lee et al., 2003). Furthermore, Atlantic salmon treated with ODN CpG 1668 (50μg/fish) had higher survival rate than control fish (Bridle et al., 2003). Unfortunately, the effect of dose of ODN CpG 1668 on fish resistance to challenge, measured as bacteremic fish or survival rate, is scarce and hamper further comparison but it seems that even small doses (e.g. 0.5 μg/fish) provides enough protection to challenge. A similar dose effect has been reported with different ODN CpG in other fish species (Kang and Kim, 2014; Liu et al., 2010a, 2010b). Because ODN CpG are potent adjuvants and their effects are mediated by cytokines released from stimulated cells, the identification of the lowest most effective immune-modulating dose is relevant aiming to prevent over stimulation with a subsequent cytokine storm that could be harmful to fish (Klinman et al., 2009).

The effect of time of ODN CpG inoculation prior to challenge has also poorly reported. In our study, most experiments were performed challenging fish 24 h after ODN CpGs inoculation, similarly to prior studies reported by other researchers (Bridle et al., 2003; Kang and Kim, 2014; Liu et al., 2010a, 2010b; Strandskog et al., 2008; Tassakka and Sakai, 2003). However, when we performed a study aiming to evaluate the time-dependent effect we found that a lower percentile of bacteremic fish and lower number of bacteria were found in the group of fish challenged 96 h after ODN CpG 1668 administration. In this experiment

(Figure 4), however, 100% of fish inoculated with ODN CpG 1668 24 h prior to challenge were bacteremic in contrast to only 50% and 45% in the first and second experiments (Figure 1 and Figure 3). We have no explanation for these findings. However, it was the last experiment of the season, in middle autumn, and water temperature were lower (data not shown) than when we started the experiments (summer). Thus, we hypothesize that lower water temperature could have affected innate immune response leading to a higher percentile of bacteremic fish. In the other hand, because each experiment was done separately, using a new batch of fish and a new batch of bacteria, we assume that at this particular experiment either fish were naturally more resistant to *A. hydrophila* or the bacteria inoculum could be slightly higher. In a similar study, the percentile of bacteremic turbots inoculated with ODN CpG 205 48 h prior to challenge with *E. tarda* was lower compared to turbot inoculated 12 and 24 h prior to challenge (Liu et al., 2010a), but in Japanese flounder, the same researchers found no difference on the percentile of bacteremic fish treated with ODN CpG 205 at 12, 24 or 48 h prior to challenge with *E. tarda* (Liu et al., 2010b). ODN CpG 1668 inoculated into Atlantic salmon 6 days prior to challenge with *Neoparamoeba pemaquidensis*, the etiologic agent of amoebic gilt disease, provided higher protection compared to non-treated control fish (Bridle et al., 2003). Furthermore, i*n vitro* studies indicated that the effect of ODN CpG 1668 on the respiratory burst of head kidney phagocytes of olive flounder peaked at 3 and 7 days post injection (Lee et al., 2003) with a significant decline at 5 days post injection, and was also affected by dose. Thus, again, based on our study and on published data, finding the ideal lapse time between ODN CpG injections prior to a potential challenge becomes relevant to achieve better results.

Because ODN CpG improves innate and adaptive immune response, the mechanisms behind their immune stimulating effect are being intensively investigated. ODN CpG is efficiently taken up by immune cells via non-specific endocytosis (Häcker et al., 1998) and, at

endosomal compartment, interact with TLR-9 triggering the expression of cytokines central to innate and adaptive immune response (Rutz et al., 2004). In rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon, i.p. inoculation of ODN CpG 1668 stimulates the expression of the pleiotropic cytokine IL-1β and the production of antibodies (Jørgensen et al., 2001a). The i.p. inoculation of different types of ODN CpG (type A, B or C, and poly I:C) in Atlantic salmon augmented the production of several cytokines mainly IFN α1/α2, IFN-γ and the IFN-stimulated gene (ISG) Mx gene, CXCL10 and IL-1β (Strandskog et al., 2008). The inoculation of ODN CpG 205 in turbot (*Scophthalmus maximus*) enhanced the respiratory burst activity of head kidney macrophage and serum bactericidal activity and, in vitro, induced the proliferation of peripheral leukocytes (Liu et al., 2010a). Similarly, in Japanese flounder, a combination of four ODN CpGs activated head kidney macrophage and enhanced the complement mediated bactericidal activity of serum (Liu et al., 2010b). Grass carp (*Ctenopharyngodon idellus*), common carp (*Cyprinus carpio* L.) and olive flounder inoculated i.p. with ODN CpG had improved macrophage activation and production of superoxide anion (Lee et al., 2003; Meng et al., 2003; Tassakka and Sakai, 2003). These studies strengthen the hypothesis that ODN CpGs effects are mediated by different mechanism according to their type and nucleotide sequence and fish species in which they are evaluated. Unfortunately, most ODN CpGs have been tested in a single fish species hampering our understanding on their mechanisms of action (Carrington and Secombes, 2006) .

In conclusion, we demonstrated that synthetic ODN CpGs are effective *in vivo* immune stimulators for silver catfish enhancing the protection against i.p. challenge with pathogenic *A. hydrophila*.

Acknowledgements

This work was carried out using financial support from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, grant nº476317/2012-6. Raíssa Canova (01589073029) and Karina S. Kirsten (01837123071) were Master Students with CAPES fellowships. Lucas de Figueiredo Soveral is an undergraduate student of veterinary medicine with a FAPERGS fellowship (0413-2551/15-5).

References

Bairwa et al., Jakhar, K., Satyanarayana, Y., Reddy, D., 2012. Animal and plant originated immunostimulants used in aquaculture 2, 397–400.

Barcellos, L.J.G., Kreutz, L.C., Rodrigues, L.B., Ruschel, L., Costa, A., Ritter, F., Bedin, A.C., Bolognesi, L., 2008. *Aeromonas hydrophila* em *Rhamdia quelen* : aspectos macro e microscópico das lesões e perfil de resistência a antimicrobianos *. Bol. do Inst. Pesca 34, 355–363.

Bridle, A.R., Butler, R., Nowak, B.F., 2003. Immunostimulatory CpG oligodeoxynucleotides increase resistance against amoebic gill disease in Atlantic salmon, *Salmo salar* L. J. Fish Dis. 26, 367–371.

Brudeseth, B.E., Wiulsrød, R., Fredriksen, B.N., Lindmo, K., Løkling, K.E., Bordevik, M., Steine, N., Klevan, A., Gravningen, K., 2013. Status and future perspectives of vaccines for industrialised fin-fish farming. Fish Shellfish Immunol. 35, 1759–1768.

Cabello, F.C., 2006. Heavy use of prophylactic antibiotics in aquaculture: A growing problem for human and animal health and for the environment. Environ. Microbiol. 8, 1137–1144.

Carrington, A.C., Secombes, C.J., 2006. A review of CpGs and their relevance to aquaculture. Vet. Immunol. Immunopathol. 112, 87–101.

Da Silva, L.B., Barcellos, L.J.G., Quevedo, R.M., De Souza, S.M.G., Kessler, A.D.M., Kreutz, L.C., Ritter, F., Finco, J.A., Bedin, A.C., 2008. Introduction of jundia *Rhamdia quelen* (Quoy & Gaimard) and Nile tilapia *Oreochromis niloticus* (Linnaeus) increases the productivity of carp polyculture in southern Brazil. Aquac. Res. 39, 542–551.

FAO, 2014. The state of world fisheries and aquaculture, Food and Agriculture Oraganization of the United Nations. FAO, Rome, Italy.

Häcker, H., Mischak, H., Miethke, T., Liptay, S., Schmid, R., Sparwasser, T., Heeg, K., Lipford, G.B., Wagner, H., 1998. CpG-DNA-specific activation of antigen-presenting cells requires stress kinase activity and is preceded by non-specific endocytosis and endosomal maturation. EMBO J. 17, 6230–6240.

Jørgensen, J.B., Johansen, a, Stenersen, B., Sommer, a I., 2001a. CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (*Salmo salar* L.) leucocytes to produce supernatants with antiviral activity. Dev. Comp. Immunol. 25, 313–21.

Jørgensen, J.B., Zou, J., Johansen, a, Secombes, C.J., 2001b. Immunostimulatory CpG oligodeoxynucleotides stimulate expression of IL-1beta and interferon-like cytokines in rainbow trout macrophages via a chloroquine-sensitive mechanism. Fish Shellfish Immunol. 11, 673–82.

Kang, Y.J., Kim, K.H., 2014. Therapeutic potential of CpG-ODN 1668 against scuticociliatosis in olive flounder (*Paralichthys olivaceus*). Aquaculture 430, 17–20.

Klinman, D.M., Klaschik, S., Sato, T., Tross, D., 2009. CpG oligonucleotides as adjuvants for

vaccines targeting infectious diseases. Adv. Drug Deliv. Rev. 61, 248–255.

Kreutz, L.C., Barcellos, L.J.G., Marteninghe, A., Dos Santos, E.D., Zanatta, R., 2010. Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings (*Rhamdia quelen*) to *Aeromonas hydrophila* challenge. Fish Shellfish Immunol. 29, 694–7.

Lee, C.H., Jeong, H. Do, Chung, J.K., Lee, H.H., Kim, K.H., 2003. CpG motif in synthetic ODN primes respiratory burst of olive flounder *Paralichthys olivaceus* phagocytes and enhances protection against Edwardsiella tarda. Dis. Aquat. Organ. 56, 43–48.

Liu, C., Sun, Y., Hu, Y., Sun, L., 2010a. Identification and analysis of a CpG motif that protects turbot (*Scophthalmus maximus*) against bacterial challenge and enhances vaccine-induced specific immunity. Vaccine 28, 4153–61.

Liu, C., Sun, Y., Hu, Y., Sun, L., 2010b. Identification and analysis of the immune effects of CpG motifs that protect Japanese flounder (*Paralichthys olivaceus*) against bacterial infection. Fish Shellfish Immunol. 29, 279–85.

Meng, Z., Shao, J., Xiang, L., 2003. CpG oligodeoxynucleotides activate grass carp (*Ctenopharyngodon idellus*) macrophages. Dev. Comp. Immunol. 27, 313–321.

Newaj-Fyzul, A., Austin, B., 2015. Probiotics, immunostimulants, plant products and oral vaccines, and their role as feed supplements in the control of bacterial fish diseases. J. Fish Dis. 38, 937–955.

Pavan, T.R., Di Domenico, J., Kirsten, K.S., Nied, C.O., Frandoloso, R., Kreutz, L.C., 2016. Antibody response in silver catfish (*Rhamdia quelen*) immunized with a model antigen

associated with different adjuvants. Brazilian J. Med. Biol. Res. 49, 1–5.

Pietretti, D., Wiegertjes, G.F., 2014. Ligand specificities of Toll-like receptors in fish: Indications from infection studies. Dev. Comp. Immunol. 43, 205–222.

Plant, K.P., Lapatra, S.E., 2011. Advances in fish vaccine delivery. Dev. Comp. Immunol. 35, 1256–62.

Rankin, R., Pontarollo, R., Ioannou, X., Krieg, a M., Hecker, R., Babiuk, L. a, van Drunen Littel-van den Hurk, S., 2001. CpG motif identification for veterinary and laboratory species demonstrates that sequence recognition is highly conserved. Antisense Nucleic Acid Drug Dev. 11, 333–340.

Rutz, M., Metzger, J., Gellert, T., Luppa, P., Lipford, G.B., Wagner, H., Bauer, S., 2004. Toll-like receptor 9 binds single-stranded CpG-DNA in a sequence- and pH-dependent manner. Eur. J. Immunol. 34, 2541–50.

Schulz, U.H., Leuchtenberger, C., 2006. Activity patterns of South American silver catfish (*Rhamdia quelen*). Brazilian J. Biol. 66, 565–574.

Strandskog, G., Skjaeveland, I., Ellingsen, T., Jørgensen, J.B., 2008. Double-stranded RNA- and CpG DNA-induced immune responses in Atlantic salmon: comparison and synergies. Vaccine 26, 4704–15.

Tafalla, C., Bøgwald, J., Dalmo, R. a, 2013. Adjuvants and immunostimulants in fish vaccines: Current knowledge and future perspectives. Fish Shellfish Immunol. 1–11.

Tassakka, A.C.M. a. R., Sakai, M., 2003. The in vitro effect of CpG-ODNs on the innate immune response of common carp, *Cyprinus carpio* L. Aquaculture 220, 27–36.

Valladão, G.M.R., Gallani, S.U., Pilarski, F., 2016. South American fish for continental

aquaculture. Rev. Aquac. 1–19.

Vallejos-Vidal, E., Reyes-López, F., Teles, M., MacKenzie, S., 2016. The response of fish to immunostimulant diets. Fish Shellfish Immunol. 56, 34–69.

Van Hai, N., 2015. The use of medicinal plants as immunostimulants in aquaculture: A review. Aquaculture 446, 88–96.

Van Muiswinkel, W.B., 2008. A history of fish immunology and vaccination I. The early days. Fish Shellfish Immunol. 25, 397–408.

Figure 1. Bacteria recovering from infected fish. Silver catfish were injected intraperitoneally with different ODN CpGs (0.5μg/fish) or PBS and challenged with *Aeromonas hydrophila* (2 x 10^8 CFU/fish) 24 h post injection. Bacteria recovery from blood was evaluated 24h post challenge. The dada are represented as the mean ± SEM of the natural logarithm of the number of bacterial colonies (x+1) isolated from each fish (n = 10). Significant differences ($p<0.05$) are represented by small letters and the percentile of bacteremic fish in each group is indicated inside the columns. Differences amongst treatments were analyzed by ANOVA followed by Tukey's multiple comparisons test.

Figure 2. Survival rate of silver catfish inoculated with different ODN CpGs (0.5μg/fish) of PBS and challenged with *Aeromonas hydrophila* (2 x 10^8 CFU/fish) 24 h post injection. Fish were monitored twice a day up to the seventh day when the experiment was finished. All groups consisted of 40 fish and the data is represented as daily survival rate ± SEM. Differences amongst groups are represented by different small letters ($p<0.001$).

Figure 3. The effect of the dose of ODN CpG 1668 on bacteria spread in infected fish. Fish were inoculated with different doses of ODN CpG (0.004μg, 0.02μg, 0.1μg and 0.5μg/fish) or PBS and challenged with *Aeromonas hydrophila* (2 x 10^8 CFU/fish) 24 h post injection. Bacteria recovery from blood was evaluated 24h post challenge. The dada are represented as the mean ± SEM of the natural logarithm of the number of bacterial colonies (x+1) isolated from each fish (n = 10). Significant differences ($p<0.05$) are represented by small letters and the percentile of bacteremic fish in each group is indicated. Differences amongst treatments were analyzed by ANOVA followed by Tukey's multiple comparisons test.

Figure 4. The effect of time of administration of ODN CpG 1668 on fish bacteremia. ODN CpG 1668 (0.5μg/fish) was injected at different time points (168h, 96h, 48h and 24h) prior to challenge with *Aeromonas hydrophila* (2 x 10^8 CFU/fish). Blood samples were collected 24h post challenge to evaluate bacteremia. The dada are represented as the mean ± SEM of the natural logarithm of the number of bacterial colonies (x+1) isolated from each fish (n = 10). Significant differences ($p<0.05$) are represented by small letters and the percentile of bacteremic fish in each group is indicated. Differences amongst treatments were analyzed by ANOVA followed by Tukey's multiple comparisons test.

Figura 1.

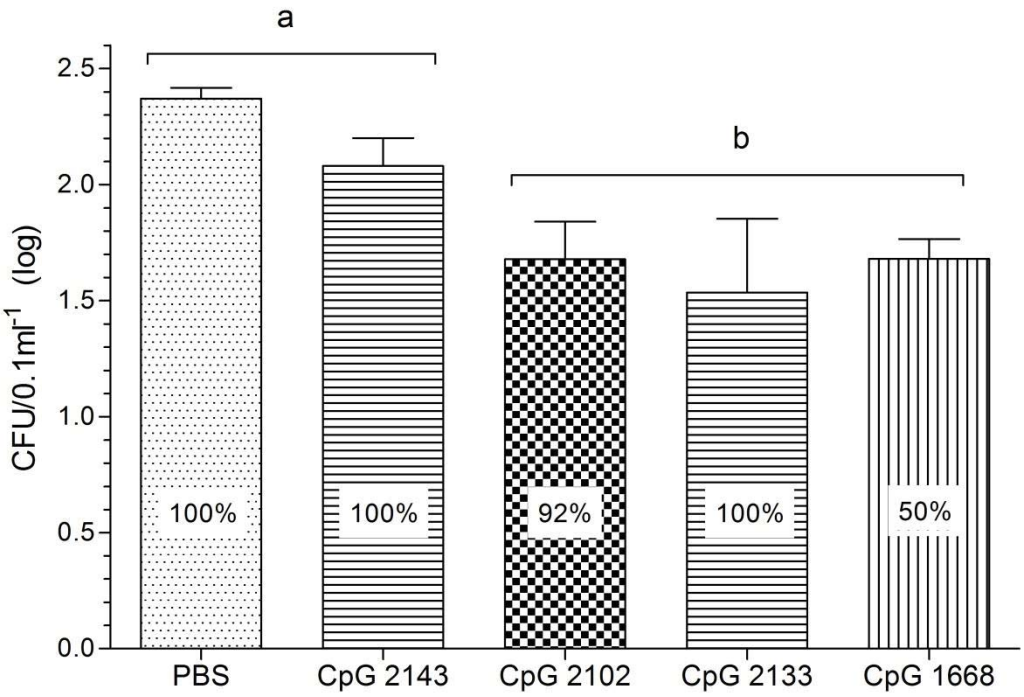

a
b
2.5
2.0
1.5
1.0
0.5
0.0
CFU/0.1ml-1 (log)
100%
100%
92%
100%
50%
PBS
CpG 2143
CpG 2102
CpG 2133
CpG 1668

Figura 2.

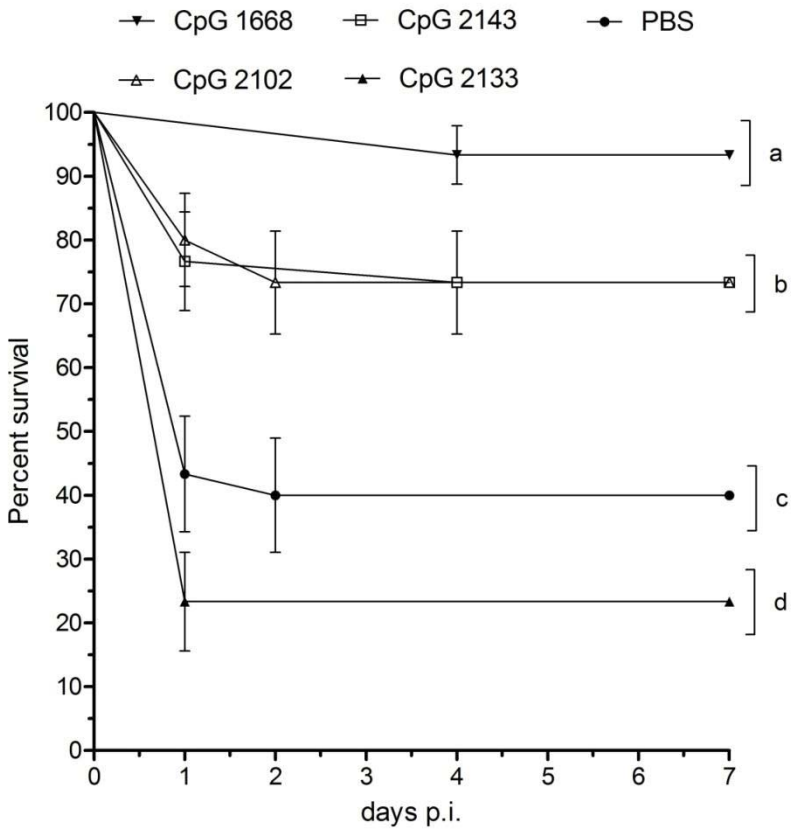

CpG 1668
CpG 2143
PBS
CpG 2102
CpG 2133
Percent survival
days p.i.
a
b
c
d

Figura 3.

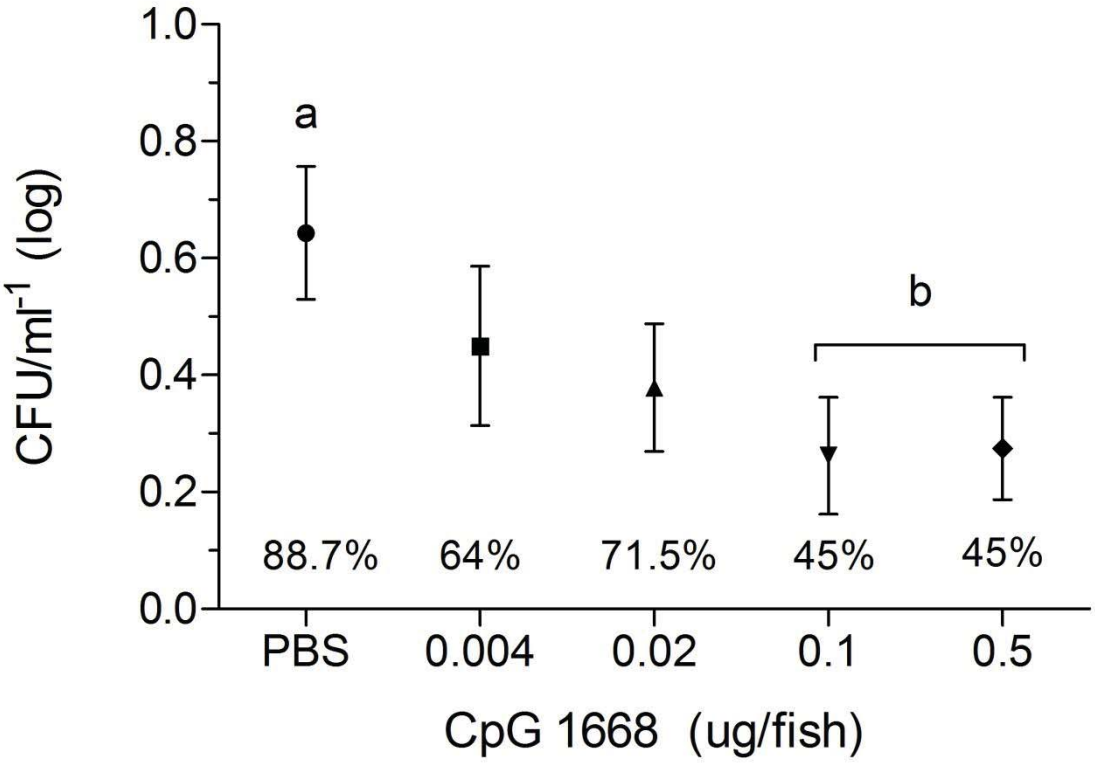
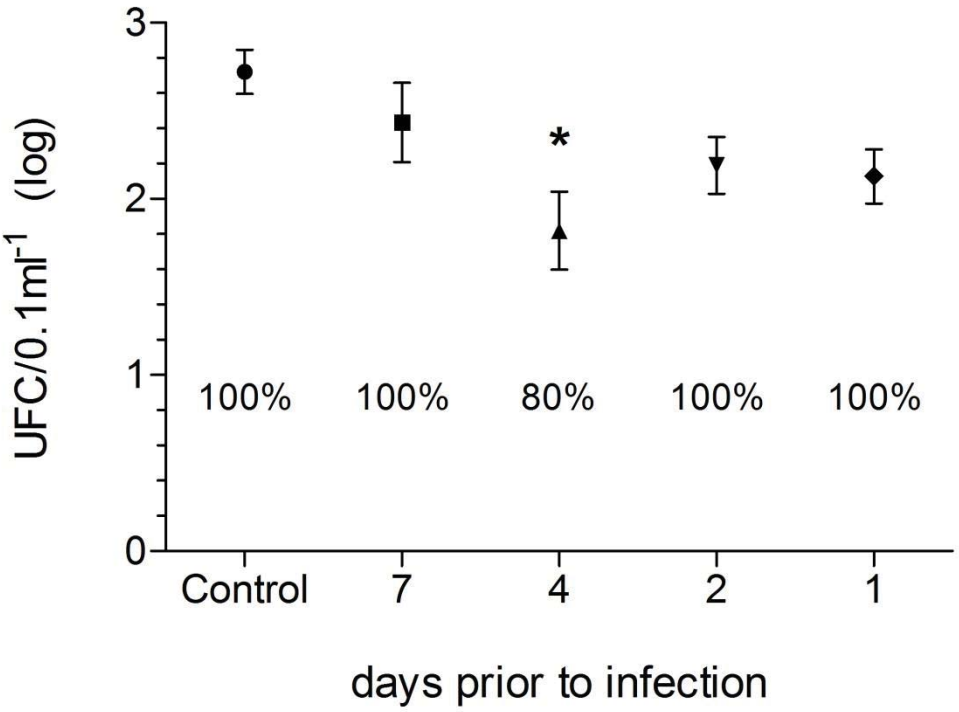

1.0
0.8
0.6
0.4
0.2
0.0
CFU/ml-1 (log)
a
b
88.7%
64%
71.5%
45%
45%
PBS
0.004
0.02
0.1
0.5
CpG 1668 (ug/fish)

Figura 4.


3
2
1
0
UFC/0.1ml-1 (log)
*
100%
100%
80%
100%
100%
Control
7
4
2
1
days prior to infection

4. CONCLUSÕES

Neste estudo, nós concluímos que a administração via intraperitoneal de CpGs ODNs em jundiás (*Rhamdia quelen*) estimula proteção ao desafio por *Aeromonas hydrophila*, diminuindo a bacteremia e aumentando a taxa de sobrevivência dos peixes. Nossos dados sugerem que o efeito protetor do CpG ODN nos jundiás é dependente da dose e do tempo em que o CpG ODN foi administrado aos peixes.

A alta densidade nos tanques e o manejo intensivo afetam negativamente a saúde dos peixes. O estresse, a baixa qualidade da água e das instalações predispõem os peixes a imunossupressão e consequentemente a doenças. A utilização de vacinas e fármacos para prevenir e tratar doenças é uma realidade. No entanto, o uso contínuo de quimioterápicos leva a resistência dos micro-organismos e ainda não há vacinas para todas as doenças de peixes. Aqui nós demonstramos que o uso de imunoestimulantes, como os CpGs ODNs, são uma alternativa de medida profilática conferindo proteção a micro-organismos patogênicos e permitindo o desenvolvimento sustentável da aquicultura.

5. CONSIDERAÇÕES FINAIS

A criação de peixes em cativeiro tornou-se uma atividade lucrativa onde uma ou mais espécies de peixes são criadas em conjunto e com uma alta densidade populacional, proporcionando condições para que micro-organismos patogênicos se estabeleçam e causem doenças. A demanda populacional e o consumo da carne de pescado têm aumentado nos últimos anos e consequentemente, a preocupação com o fornecimento de um produto livre de contaminantes e com alta qualidade nutricional tornou imprescindível o controle de infecções. O desenvolvimento de vacinas e novos adjuvantes e o uso de imunoestimulantes são alternativas viáveis para o desenvolvimento de uma aquicultura livre de patógenos.

Neste estudo, foi avaliado o efeito de CpGs ODNs no sistema imune de jundiás (*Rhamdia quelen*) e sua capacidade em conferir proteção a jundiás desafiados com *Aeromonas hydrophila.* Ainda que os CpGs ODNs não estejam disponíveis comercialmente, futuramente eles representam uma alternativa para uso em sistemas de criação de peixes em cativeiro, prevenindo doenças. Este é o primeiro trabalho demonstrando o efeito de CpGs ODNs no sistema imune de jundiás.

Por fim, nossa perspectiva é avaliar a expressão de genes imunológicos em jundiás inoculados com CpG ODN e verificar os mecanismos pelos quais os CpGs ODNs modulam a resposta imune.

6. REFERÊNCIAS BIBLIOGRÁFICAS

1. Reverter M, Bontemps N, Lecchini D, Banaigs B, Sasal P. Use of plant extracts in fish aquaculture as an alternative to chemotherapy : Current status and future perspectives. Aquaculture. 2014;433:50–61.

2. Gillund F, Dalmo R, Tonheim TC, Seternes T, Myhr AI. DNA vaccination in aquaculture - Expert judgments of impacts on environment and fish health. Aquaculture. 2008;284(1-4):25–34.

3. Evensen Ø, Leong J-AC. DNA vaccines against viral diseases of farmed fish. Fish Shellfish Immunol. 2013;35(6):1751–8.

4. Bridle AR, Butler R, Nowak BF. Immunostimulatory CpG oligodeoxynucleotides increase resistance against amoebic gill disease in Atlantic salmon, *Salmo salar L*. J Fish Dis. 2003;26(6):367–71.

5. Carrington AC, Secombes CJ. A review of CpGs and their relevance to aquaculture. Vet Immunol Immunopathol. 2006;112:87–101.

6. Liu CS, Sun Y, Hu YH, Sun L. Identification and analysis of the immune effects of CpG motifs that protect Japanese flounder (*Paralichthys olivaceus*) against bacterial infection. Fish Shellfish Immunol. 2010;29(2):279–85.

7. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature. 1995;546–9.

8. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000;408(6813):740–5.

9. Citra A, Tassakka MAR, Sakai M. CpG oligodeoxynucleotides enhance the non-specific immune responses on carp, *Cyprinus carpio*. Aquaculture. 2002;209:1–10.

10. Carrington AC, Collet B, Holland JW, Secombes CJ. CpG oligodeoxynucleotides stimulate immune cell proliferation but not specific antibody production in rainbow trout (*Oncorhynchus mykiss*). Vet Immunol Immunopathol. 2004;101:211–22.

11. Gomes LDC, Golombieski JI, Gomes ARC, Baldisserotto B. Biologia do jundiá *Rhamdia quelen* (Teleostei, Pimelodidae). Ciência Rural. 2000;30:179–85.

12. Barcellos LJG, Kreutz LC, Rodrigues LB, Ruschel L, Costa A, Ritter F, et al. *Aeromonas hydrophila* em *Rhamdia quelen* : aspectos macro e microscópico das lesões e perfil de resistência a antimicrobiano. B Inst Pesca. 2008;34(3):355–63.

13. Kreutz LC, Gil Barcellos LJ, Marteninghe A, Davi dos Santos E, Zanatta R. Exposure to sublethal concentration of glyphosate or atrazine-based herbicides alters the phagocytic function and increases the susceptibility of silver catfish fingerlings

(*Rhamdia quelen*) to *Aeromonas hydrophila* challenge. Fish Shellfish Immunol. 2010;29(4):694–7.

14. Kreutz LC, Gil Barcellos LJ, de Faria Valle S, de Oliveira Silva T, Anziliero D, Davi dos Santos E, et al. Altered hematological and immunological parameters in silver catfish (*Rhamdia quelen*) following short term exposure to sublethal concentration of glyphosate. Fish Shellfish Immunol. 2011;30(1):51–7.

15. Kreutz LC, Pavan TR, Alves a. G, Correia a. G, Barriquel B, dos Santos ED, et al. Increased immunoglobulin production in silver catfish (*Rhamdia quelen*) exposed to agrichemicals. Brazilian J Med Biol Res. 2014;47:499–504.

16. Pavan T, Di Domenico J, Kirsten K, Nied C, Frandoloso R, Kreutz LC. Antibody response in silver catfish (*Rhamdia quelen*) immunized with a model antigen associated to different adjuvants. Brazilian J Med Biol Res. 2016, *in press*

17. Evensen Ø. Development in fish vaccinology with focus on delivery methodologies , adjuvants and formulations. Options Méditerranéennes. 2009;177–86.

18. Gudding R, Van Muiswinkel WB. A history of fish vaccination: Science-based disease prevention in aquaculture. Fish Shellfish Immunol. 2013;35(6):1683–8.

19. Singh V, Somvanshi P, Rathore G, Kapoor D, Mishra BN. Gene cloning, expression and homology modeling of hemolysin gene from *Aeromonas hydrophila*. Protein Expr Purif. 2009;65(1):1–7.

20. Brudeseth BE, Wiulsrød R, Fredriksen BN, Lindmo K, Løkling KE, Bordevik M, et al. Status and future perspectives of vaccines for industrialised fin-fish farming. Fish Shellfish Immunol. 2013;35(6):1759–68.

21. Weihua Chu, Shuxin Zhou WZ& XZ. Quorum quenching bacteria *Bacillus* sp. QSI-1 protect zebrafish (*Danio rerio*) from *Aeromonas hydrophila* infection. Nature Scientific Reports. 2014;1–6

22. Goossens H, Ferech M, Vander Stichele R, Elseviers M. Outpatient antibiotic use in Europe and association with resistance: A cross-national database study. Lancet. 2005;365(9459):579–87.

23. Harikrishnan R, Balasundaram C, Heo MS. Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. Aquaculture. 2011;317(1-4):1–15.

24. Ringø E, Olsen RE, Vecino JLG, Wadsworth S, Song SK. Use of immunostimulants and nucleotides in aquaculture : a review. 2012;2(1):1–22.

25. Krieg AM. CpG Motifs in bacterial DNA and their immune effects. Annu Rev Immunol. 2002;20(1):709–60.

26. Harandi AM, Medaglini D, Shattock RJ. Vaccine adjuvants: A priority for vaccine research. Vaccine. 2010;28:2363–6.

27. Bauer S, Kirschning CJ, Häcker H, Redecke V, Hausmann S, Akira S, et al. Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. Proc Natl Acad Sci. 2001;98(16):9237–42.

28. Jørgensen JB, Zou J, Johansen a, Secombes CJ. Immunostimulatory CpG oligodeoxynucleotides stimulate expression of IL-1beta and interferon-like cytokines in rainbow trout macrophages via a chloroquine-sensitive mechanism. Fish Shellfish Immunol. 2001;11(8):673–82.

29. Jorgensen JB, Johansen a., Stenersen B, Sommer a. I. CpG oligodeoxynucleotides and plasmid DNA stimulate Atlantic salmon (*Salmo salar L.*) leucocytes to produce supernatants with antiviral activity. Dev Comp Immunol. 2001;25:313–21.

30. Lee CH, Jeong H Do, Chung JK, Lee HH, Kim KH. CpG motif in synthetic ODN primes respiratory burst of olive flounder *Paralichthys olivaceus* phagocytes and enhances protection against *Edwardsiella tarda*. Dis Aquat Organ. 2003;56(1):43–8.

31. Meng Z, Shao J, Xiang L. CpG oligodeoxynucleotides activate grass carp (*Ctenopharyngodon idellus*) macrophages. Dev Comp Immunol. 2003;27(4):313–21.

32. Oumouna M, Jaso-Friedmann L, Evans DL. Activation of nonspecific cytotoxic cells (NCC) with synthetic oligodeoxynucleotides and bacterial genomic DNA: Binding, specificity and identification of unique immunostimulatory motifs. Dev Comp Immunol. 2002;26(3):257–69.

33. Di Domenico J, Canova R, Soveral L, Nied C, Costa M, Frandoloso R, et al. Immunomodulatory effects of dietary β-glucan in silver catfish (*Rhamdia quelen*). Pesqui Veterinária Bras. 2016, *In press*.

34. Tassakka ACMAR, Sakai M. Expression of immune-related genes in the common carp (*Cyprinus carpio L.*) after stimulation by CpG oligodeoxynucleotides. Aquaculture. 2004;242(1-4):1–12.