VACINAS ORAIS E PARENTERAIS CONTRA *Flavobacterium columnare*: AVALIAÇÃO DA RESPOSTA IMUNE HUMORAL POR ELISA E DE SUA EFICIÊNCIA NA IMUNIZAÇÃO DE TILÁPIA DO NILO

CARLOS AUGUSTO GOMES LEAL

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Dissertação apresentada à Universidade Federal de Lavras como parte das exigências do Curso de Mestrado em Ciências Veterinárias, para a obtenção do título de "Mestre".

Orientador

Prof. Dr. Henrique César Pereira Figueiredo

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À força maior que conduz o Universo pela vida e por sempre me manter forte. À minha família, amigos e todos que sempre torceram por mim.

DEDICO.

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RESUMO

LEAL, Carlos Augusto Gomes. **Vacinas orais e parenterais contra** *Flavobacterium columnare*: avaliação da resposta imune humoral por ELISA e de sua eficiência na imunização de tilápia do Nilo. 2009. 42 p. Dissertação (Mestrado em Ciências Veterinárias) – Universidade Federal de Lavras, Lavras, MG.*

Flavobacterium columnare é um importante patógeno que acomete a maioria das espécies de peixe de água doce. Essa bactéria ocasiona surtos que cursam com altos índices de mortalidade em pisciculturas em todo o mundo. O uso de vacinas tem se destacado como uma potencial alternativa para a prevenção e controle dessa doença. Devido à facilidade de administração, baixo custo e estresse mínimo dos peixes, as vacinas orais são a alternativa mais promissora para imunização de peixes. Micropartículas de alginato incorporadas com antígenos vacinais é um dos veículos orais promissores para a imunização de peixes. Os objetivos do presente trabalho foram avaliar por ELISA o efeito de vacinas orais e parenterais contra *F. columnare* na resposta imune humoral e sua eficiência *in vivo* para alevinos de tilápia do Nilo. Alevinos de tilápia do Nilo foram imunizados com células de *F. columnare* inativadas por via oral, i.m., i.p., por banho e incorporada em micropartículas de alginato administradas por via oral. Para a detecção de anticorpos específicos contra a bactéria, um protocolo de ELISA sanduíche foi desenvolvido. Os animais foram desafiados com a amostra patogênica BZ-1 para determinação da porcentagem relativa de sobrevivência. Respostas imunes significantes foram induzidas pela bacterina administrada por via i.p. e i.m. ($P < 0.05$). Contudo, nenhum dos métodos de imunização promoveu PRS significativa em relação ao grupo controle ($P < 0.05$). Apesar dos níveis significativos de anticorpos, esses não conferiram proteção aos alevinos nos ensaios de infecção experimental. Esses dados sugerem que avaliação sorológica isolada pode fornecer inferências não confiáveis sobre a eficiência *in vivo* das vacinas contra *F. columnare* em tilápia do Nilo.

Palavras-chave: *Flavobacterium columnare*, tilápia do Nilo, vacinas, ELISA, proteção.

*Orientador: Dr. Henrique César Pereira Figueiredo.

ABSTRACT

LEAL, Carlos Augusto Gomes Leal. **Oral and parenteral vaccines against** *Flavobacterium columnare***:** evaluation of humoral immune response by ELISA and *in vivo* efficiency in Nile tilapia. 2009. 42 p. Master (Dissertation Post-Graduation in Veterinary Science) – Federal University of Lavras, Lavras, MG[*].

Flavobacterium columnare is a major bacterial pathogen for almost all freshwater fish species. It is responsible for outbreaks in fish farms worldwide, causing high mortality rates. Fish vaccination is a potential alternative for prevention and control of disease. Because of its easy application, low cost and minimum stress to fish, oral vaccines are a reasonable alternative to fish immunization. Alginate microparticles have been widely used as controlled release system, including for fish vaccination. The objectives of this work were to evaluate the capacity of oral and parenteral vaccines against *F. columnare* to induce humoral response, *in vivo* efficiency and the relation between antibodies levels and protection to Nile tilapia fingerlings. The fingerlings were immunized with bacterin by i.p., i.m. oral and immersion routes as well as orally with alginate microparticles incorporating formalin-killed bacteria. A sandwich ELISA was developed to detect specific antibodies against *F. columnare*. The animals were challenged with pathogenic strain BZ-1 to determine the relative percentage of survival. Significant humoral response were induced by bacterin administered by i.p. and i.m. ($P < 0.05$). However, no vaccine preparations were effective to protect fish against *F. columnare* infection ($P < 0.05$). In spite of high antibody levels, there was no relation between immunoglobulin titers and resistance to columnaris for Nile tilapia fingerlings. This data suggest that the use of serological analysis as the unique method to determine vaccine efficiency against *F. columnare* infection in Nile tilapia can result in imprecise determination of the usefulness of these products *in vivo*.

Keywords: *Flavobacterium columnare*, Nile tilapia, vaccines, ELISA, protection.

[*]Advisor: Dr. Henrique César Pereira Figueiredo

1 INTRODUÇÃO

Atualmente a aqüicultura é o ramo da produção animal que mais cresce em todo mundo, com uma evolução na produção total de menos de um milhão de toneladas nos anos 50 para aproximadamente 60 milhões de toneladas em 2004. Segundo dados da Food and Agriculture Organization - FAO (2006), a aqüicultura já atende metade da demanda mundial por peixes e outros animais aquáticos, sendo ainda satisfatoriamente capaz de atender a crescente procura por esse tipo de alimento. Nas últimas décadas houve um incremento significativo na produção aquícola em todo o mundo, com crescimento médio de 8,8%. Dentre esses, destaque para a América Latina e o Caribe, onde houve uma expansão média de 21,3 %. A produção de peixes responde, atualmente, por 47,4% do volume total produzido e 53,9% da receita total obtida na aqüicultura mundial.

O Brasil detém a 3º maior produção aquícola da América Latina, sendo expoente na produção de camarões e tilápias. De acordo com o último levantamento da FAO, o país encontra-se na 7º posição como produtor mundial de tilápias (FAO, 2006). Os cultivos de tilápias vêm apresentando crescimento médio anual de 11,5% em todo mundo, com produção bruta inferior apenas à de carpas e salmonídeos (El-Sayed, 1999). A indústria brasileira de tilápia cresce vigorosamente, com posição de destaque entre os países americanos.

Condições climáticas favoráveis e vastos reservatórios hídricos corroboram para a expansão significativa da produção nacional (Fitzsimmons, 2000). Se 2% do potencial hídrico do país fosse utilizado para o cultivo de peixes em tanques-rede, o Brasil estaria entre os maiores produtores mundiais. O estabelecimento da cadeia produtiva da tilápia pode propiciar o desenvolvimento de agroindústrias de processamento, geração de milhares de empregos, maior produção de alimentos, geração de renda e intercâmbio de tecnologias, incrementando o agronegócio nacional (Vera-Calderon & Ferreira, 2004).

O consumo "*per capita*" de pescado "*in natura*" no Brasil é pequeno (5-10 kg/hab/ano) em relação a outros países do mundo, como, por exemplo, nos países asiáticos (FAO, 2006). Somente cerca de 10% da população utilizam o pescado em sua alimentação. O hábito de consumir peixes e derivados varia de acordo com a região, sendo de 21% no Norte e Nordeste e 2% na região sul.

O emprego de sistemas de cultivo intensivos, como os tanques-rede, tem sido o principal impulsionador da produção comercial de peixes no país. Esses adotam altas densidades de estocagem e manejo intenso dos animais, que otimizam a produtividade das fazendas e produção por área cultivada. Porém, essas características criam condições favoráveis para a ocorrência de doenças infecciosas, sendo os problemas sanitários um dos maiores empecilhos para esse tipo de atividade no Brasil e no mundo. Nesse contexto, a columnariose tem se destacado por ser uma doença de distribuição mundial e que acomete a maioria das espécies de peixes de água doce. Altamente impactante, é a segunda enfermidade que mais causa prejuízos à indústria americana do "catfish" (Arias et al., 2004). No Brasil não existem dados sobre seu impacto na produção de peixes, porém, surtos da doença são freqüentemente observados nas pisciculturas nacionais, principalmente em larviculturas (Figueiredo et al., 2005).

Flavobacterium columnare é o agente etiológico dessa doença. Fenotipicamente essa bactéria é caracterizada como um bastonete longo, Gram negativo, móvel, forma colônias filamentosas de coloração amarelada (produz um pigmento denominado flexirubina) e não cresce em meios de cultura convencionais. Os sintomas clínicos da doença são caracterizados por erosão da pele e nadadeiras. Com a evolução do quadro (24-48 horas após o contágio), ocorre o acometimento de tecidos adjacentes, causando necrose muscular e destruição total das nadadeiras (Decostere et al., 1999b; Bader et al., 2003; Olivares-Fuster et al., 2007b).

A ocorrência dessa doença está associada a fatores predisponentes que aumentam a susceptibilidade dos animais ao agente etiológico. Amostras com alta virulência podem infectar peixes sadios, porém, o estresse oriundo da manipulação grosseira, transporte prolongado, baixa qualidade de água, lesões mecânicas etc. propiciam o desencadeamento de surtos. Adesinas, a enzima condroitina AC-liase e proteases são os principais fatores de virulência associados às infecções por *F. columnare* (Thomas-Jinu & Goodwin, 2004; Suomalainen et al., 2005a, b). Sua aderência às superfícies corpóreas (pele, brânquias e nadadeiras) tem sido descrita como uma etapa fundamental na patogênese da doença (Decostere et al., 1999a, b). Devido à ampla distribuição mundial desses microorganismos, variabilidades genéticas e fenotípicas entre amostras de diferentes origens geográficas têm sido descritas na literatura (Olivares-Fuster et al., 2007a, b). Através da comparação dos ácidos graxos metil esterificados (FAME), os isolados brasileiros de *F. columnare* mostraram-se diferentes de norte-americanos e israelenses, com possível implicação no padrão antigênico das diferentes amostras (Figueiredo et al., 2005). O diagnóstico é realizado pelo isolamento da bactéria em meios de cultura especiais e posteriormente a identificação por técnicas bioquímicas ou moleculares (Bader & Shotts, 1998; Bader et al., 2003; Darwish et al., 2004). O tratamento da columnariose é realizado principalmente pela administração de antibióticos por via oral e banhos com substancias desinfetantes (Thomas-Jinu & Goodwin, 2004).

O uso de vacinas como método imunoprofilático em aqüicultura tem aumentado significativamente em todo o mundo, seguindo a tendência de expansão da atividade. Vacinas contra os principais patógenos para peixes têm sido desenvolvidas (Gudding et al., 1999; Klesius et al., 2004; Vanderberg, 2004; Tonheim et al., 2008). Contudo, a vacinologia em peixes é ainda uma área prematura, tanto cientificamente como na capacidade de transferência de tecnologia para as indústrias (Gudding et al., 1999).

Os peixes podem ser imunizados por via injetável, preferencialmente por injeção intraperitoneal (i.p.); por imersão, em que os animais são imersos em solução aquosa contendo os antígenos vacinais; ou pela administração oral das vacinas. Esses métodos apresentam diferentes vantagens e desvantagens quanto à eficiência da imunização, praticidade, efeitos colaterais e relação custo – benefício (Gudding et al., 1999). O método de administração afeta diretamente a imunidade protetora induzida pela vacinação. As vacinas injetáveis são as que induzem respostas inatas e adaptativas de maior intensidade e duração. Por outro lado, métodos não injetáveis, como a imersão, permitem a imunização de milhares de peixes em um período reduzido de tempo (Klesius et al., 2004), contudo, grandes quantidades de vacina são necessárias (Vanderberg, 2004).

Em virtude da praticidade do processo e ausência de estresse, as vacinas orais têm se tornado uma alternativa viável para imunização dos peixes. Apesar disso, os níveis de resposta atingidos por essa via quando utilizada como única forma de imunização são baixos (Romalde et al., 2004) e até casos de imunossupressão foram observados (Vanderberg, 2004). Resultados positivos têm sido obtidos com o uso de veículos orais na imunização dos peixes. Dentre esses, o alginato tem se mostrado uma alternativa promissora para a síntese de veículos orais de liberação controlada, devido a seu baixo custo, características físico-químicas ideais para esse tipo de procedimento e por não necessitar de processos excessivamente laboriosos para sua produção, como verificados para outros veículos (Rodrigues et al., 2006). Romalde et al. (2004) avaliaram o uso de micropartículas de alginato como veiculo oral para a imunização de peixes, verificando seu efeito como método único de vacinação e como reforço ("booster") para doses primárias via i.p. ou imersão. Esse tipo de modelo vacinal foi eficiente em conferir proteção contra infecções causadas pela bactéria *Lactococcus garvieae*, em ensaios de infecção experimental.

Os objetivos do presente trabalho foram avaliar por ELISA a resposta imune humoral induzida por vacinas parenterais e orais contra *Flavobacterium*

columnare e sua eficiência para tilápia do Nilo (*Oreochromis niloticus*). Os dados foram organizados no formato de artigo cientifico como descrito a seguir.

2 Oral and parenteral vaccines against *F. columnare*: evaluation of humoral immune response by ELISA and *in vivo* efficiency in Nile tilapia

(Preparado de acordo com as normas da revista "Aquaculture International")

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Abstract

Flavobacterium columnare is a major bacterial pathogen for almost all freshwater fish species. It is responsible for outbreaks in fish farms worldwide, causing high mortality rates. Fish vaccination is a potential approach for prevention and control of disease, with oral vaccines suitable for fish because of their easier application, low cost and minimum stress to fish. Alginate microparticles have been widely used as controlled release systems, including for fish vaccination. The aim of this study was to evaluate the capacity of oral and parenteral vaccines against *F. columnare* to induce a humoral response, as well as the *in vivo* efficiency and the relation between antibody levels and protection in Nile tilapia fingerlings. The fingerlings were immunized with bacterin by intraperitoneal (i.p.), intramuscular (i.m.), oral and immersion routes, as well as orally with alginate microparticles containing formalin-killed bacteria. A sandwich ELISA was developed to detect specific antibodies against *F. columnare*. The animals were challenged with pathogenic strain BZ-1 to determine the relative percentage of survival. A significant humoral response was induced by bacterin administered by i.p. and i.m. routes ($P<0.05$). However, none of the vaccine preparations were effective in protecting fish against *F. columnare* infection ($P<0.05$). In

spite of high antibody levels, there was no relation between immunoglobulin titers and resistance to columnaris for Nile tilapia fingerlings. These data suggest that use of serological analysis as the only method to determine vaccine efficiency against *F. columnare* infection in Nile tilapia can lead to imprecise results for the usefulness of these products *in vivo*.

Keywords: *Flavobacterium columnare,* Nile tilapia, vaccines, ELISA, protection.

Introduction

Nile tilapia (*Oreochromis niloticus* L.) is an important freshwater fish for which worldwide cultivation has steadily increased over the last few decades. Brazil is currently ranked seventh in the world for tilapia production (FAO, 2006) and bacterial disease outbreaks are a major problem for Brazilian tilapia production (Figueiredo et al., 2005; Mian et al., 2008).

Flavobacterium columnare causes columnaris disease, one of the principal bacterial illnesses in freshwater fish species (Shoemaker et al., 2008). Distributed worldwide in aquatic environments, this pathogen is a Gram-negative, thin, rod-shaped bacterium that shows characteristic rhizoid yellow colonies in specific culture medium (Schneck & Caslake, 2006). The disease has been described in many commercially important and native fish species in Brazil, including Nile tilapia, piracanjuba (*Brycon orbignyanus*), pacu (*Piaractus mesopotamicus*), tambaqui (*Colossoma macropomum*) and cascudo (*Hypostomus plecostomus*) (Figueiredo et al., 2005; Pilarski et al., 2008). Clinical signs of columnaris are severe gill necrosis, skin epithelial erosion and ulcers in the area between the head and dorsal fin (Schneck & Caslake, 2006). The main treatments for columnaris are oral antibiotic therapy and bathing in

surface-active substances (Thomas-Jinu & Goodwin, 2004). Immunoprophylaxis, by stimulation of non-specific and specific immunity with vaccines, usually has a positive effect (Gudding et al., 1999). However, only a few satisfactory results have been obtained for vaccination against columnaris, with large variations according to the fish species evaluated, the immunization route and preparation, and the life stage of the animals (Grabowski et al., 2004).

Oral vaccine administration is considered a promising method for fish immunization because it is non-stressful and easy to administer. However, this method was reported to confer a very limited level of immune protection (Vanderberg, 2004). The main problem with this type of vaccine is that the antigens must be protected in some way so that they reach the lower gut intact, where they can stimulate an immune response (Romalde et al., 2004). Encapsulation in biodegradable and pH-sensitive enteric polymers is one alternative for antigen protection (Vanderberg, 2004). Alginate microparticles have been used as carriers in controlled release systems for fish vaccination. Positive results were obtained using this technology for common carp and rainbow vaccination against vibriosis (Joosten et al., 1997) and lactococcosis (Romalde et al., 2004), respectively. Rodrigues et al. (2006) developed a method for producing *Aeromonas hydrophila* bacterin entrapped in alginate microparticles for

vaccination of Nile tilapia. The physicochemical characteristics of the system confer resistance to acid pH and allow content release in an alkaline environment, thus matching the physiology of the stomach and intestine of this fish species.

The aim of the present study was use a sandwich ELISA to investigate the specific humoral immune response induced in Nile tilapia by parenteral and alginate microparticles incorporating vaccines against *F. columnare*. We also assessed the efficiency of vaccination *in vivo* and the relation between antibody titers and resistance to infection.

Material and Methods

Bacteria and growth conditions

The pathogenic *F. columnare* strain BZ-1, previously isolated from Nile tilapia in Brazil (Figueiredo et al., 2005), was used to prepare the different parenteral vaccines and alginate microparticles containing bacterin. The isolate was stored at –80 °C until use. For bacterin preparation and experimental infection, the strain was thawed and grown on modified Hsu-Shotts (MSH) agar (Bullock et al., 1986) containing 2 mg L^{-1} tryptone at 26 °C for 48 h.

Formalin-killed bacteria

After growth on MHS for 48 h, one colony was selected, transferred to 150 mL of MHS broth and cultivated at 26 °C for 42 h. The

optical density at 600 nm (OD_{600}) was 0.476, corresponding to 5.1×10^{12} CFU. mL^{-1} as determined by direct plate counts. Bacterial cells were harvested by centrifugation at 3000 *g* at 4 °C for 40 min. The cells were resuspended in phosphate-buffered saline (PBS) with 0.2% formalin and incubated at 4 °C for 24h. The formalin-killed bacteria (FKB) were washed three times by centrifugation and resuspended in PBS. To evaluate bacterial inactivation, an aliquot of FKB was plated onto MHS and incubated at 26 °C for 48 h to check the sterility.

Preparation of alginate microparticles containing FKB

Alginate microparticles particles were prepared using the emulsion approach established by Rodrigues et al. (2006). Briefly, aliquots of 7 mL of FKB (corresponding to 99.6 mg of cells, dry weight basis) containing 3.5% (w/v) sodium alginate were mixed with 63 mL of corn oil containing 0.2% (v/v) Span 80 at stirring rate of 2000 rpm for 5 min. The emulsion obtained was mixed with 70 mL of a gelification solution (containing 5 g of $CaCl_2{\cdot}2H_2O$ dissolved in 33.87 mL of distilled water, 33.87 mL of ethanol and 2.26 mL of acetic acid) for 10 min at 2000 rpm. The emulsion was disrupted by the addition of 150 mL of aqueous 0.05 M $CaCl_2$. The particles obtained were recovered by filtration and washed with acetone and then deionized water. The overall process temperature was maintained at 25 °C and the particles were

stored at 4 °C. Alginate microparticles without FKB (empty) were prepared in the same way and used as a control. All experiments were performed in a jacketed round-bottom glass tank with internal diameter of 5 cm and height of 13 cm. Stirring was carried out using a Q-251D2 mechanical stirrer (Quimis, Brazil) with a three-tilted-blade propeller (diameter 4 cm). Before use, the microparticles were evaluated to determine the rate of incorporation and FKB release in acid and basic pH. The results were approximately 100%, <2% and >90%, respectively. The final concentration of FKB incorporated in the alginate microparticles was 2.65×10^{11} $CFU.mL^{-1}$ vaccine. The size of synthesized particles ranged from 27 to 37 µm.

Food preparation for oral immunization

Commercial feed (VITAFISH 32% PB, Matsuda, Brazil) was mixed with microparticles containing FKB, empty microparticles or FKB, according to the experimental groups evaluated. The mixtures were prepared daily and incubated for 30 min at 30 °C for incorporation in the feed pellets. Taking into account that the amount of feed provided was 5% of body weight per day, the amount of oral preparation included in the ration was adjusted so that each fish would receive 2.65×10^{10} cfu day^{-1}. Throughout the experimental period, the fish were monitored to ensure that feed intake was regular.

Fish and immunization experiments

Nile tilapia fingerlings were acquired from a commercial hatchery at a weight ranging from 11.25 to 18.2 g (15.7±2.2) for use in the *in vivo* assays. Each experimental group comprised 20 fingerlings, which were divided in two 57-L aquaria supplied with flow-through dechlorinated tap water (0.5 L h^{-1}). Ten fish from each group were used for serological analyses and other 10 for challenge assays. Fish were maintained on a 12 h:12 h light/dark period at a water temperature of 26 °C and were fed with VITAFISH 32% PB (Matsuda, Brazil) twice a day at a rate of 5% of live weight.

Two experiments were performed to evaluate the effectiveness of the different vaccination methods and preparations as described by Romalde et al. (2004) with some modifications. Before vaccination, fish were anesthetized by immersion in an aqueous solution of 100 mg L^{-1} benzocaine. First the vaccines were tested as a single-dose immunization procedure. Group I was immunized intraperitoneally (i.p.) with a single injection of 0.1 mL of FKB at a dose of 4.29×10^8 cfu per fish (OD_{600} 0.276). Group II was immunized intramuscularly (i.m.) with a single injection of 0.1 mL of FKB at the same dose as for group I. Group III

immunized by immersion. Briefly, 500 mL of FKB (OO_{600} 0.476) was mixed with 4500 mL of pathogen-free water to yield a final FKB concentration of 5.1×10^{11} cfu mL^{-1}. After anesthetization as previously described, fish were immersed in a 10-L bucket containing the vaccine and supplied with aeration. After 30 min they were returned to their aquaria. Group IV was vaccinated with alginate microparticles containing FKB. Groups V and VI were vaccinated with empty alginate microparticles and oral FKB, respectively. Group VII was injected i.p. with 0.1 mL of PBS (negative control). Group VIII was neither vaccinated nor challenged (experimental conditions control). All oral vaccinations consisted of seven consecutive days of treatment. After 3 weeks, ten fish from each group were anesthetized and blood was collected by caudal venepuncture with a 22-gauge needle. These were incubated for 1 h at room temperature and overnight at 4 °C. The serum was stored at –20 °C for later analysis by sandwich ELISA. The other 10 fish from each group were challenged 21 days after immunization with strain BZ-1 as described below. Protection was evaluated by determining the relative percentage of survival (RPS) (Romalde et al., 2004).

Based on the results of the first experiment, a second experiment was carried out to verify the efficiency of the oral vaccine as booster immunization. Groups A and B were i.p immunized. Group C was

immunized with alginate microparticles containing FKB. Group D was immunized with 0.1 mL of PBS (negative control). Groups A and C were given a booster vaccine 1 month later using alginate microparticles containing FKB. At same time, Group E was i.p. immunized. Groups B and D were not given a booster vaccine. Group F was neither vaccinated nor challenged (experimental conditions control). The number of fish per group and the vaccine protocols and doses were as described for the first experiment. Blood collection and fish challenges were carried out 3 weeks after booster immunization.

Sandwich ELISA

F. columnare was cultured in MHS broth for 48 h and harvested as described above. The bacterial cells were washed three times, resuspended in PBS in the same volume and sonicated on ice at maximum power for 1 min. The total protein content of the supernatant was measured using a NanoDrop 1000 spectrophotometer (Thermo Scientific, USA) and bovine serum albumin as standard. Tilapia antibodies were collected, precipitated and dialyzed according to Al-Harbi et al. (2000). Polyclonal anti-tilapia immunoglobulin antibodies were produced in rabbits as previously described (Hudson & Hay, 1989). The sandwich ELISA was standardized by block titration of antigen (sonicated *F. columnare* cells), tilapia serum, rabbit polyclonal anti-fish antibodies and

peroxidase-conjugated mouse anti-IgG. The optimum antigen concentration was 400 ng per well. The optimum dilutions for tilapia serum, rabbit polyclonal anti-fish immunoglobulin and peroxidase-conjugated mouse anti-IgG were 1:400, 1:400 and 1:24 000, respectively. ELISA 96-well microplates (Costar, USA) were coated with 100 µL of antigen in 0.05 M sodium carbonate buffer, pH 9.6, for 1 h at 25 °C. The plates were washed three with PBS containing 0.05% Tween 20 (PBST; Amresco, USA) and then 200 µL of blocking solution (5% powdered skim milk) was added. After incubation for 1 h at 37 °C the plates were washed five times with PBST. Tilapia serum samples were diluted in PBST with 1% powdered skim milk and 100 µL of sample solution was added to each well. All sera were tested in duplicate. After incubation for 1 h at 25 °C the plates were washed three times with PBST. Then 100 µL of rabbit polyclonal serum diluted in PBST was added to each well and incubated at 37 °C for 30 min. After washing with PBST, 100 µL of peroxidase-conjugated mouse anti- rabbit IgG (Sigma-Aldrich lot. 075K4781, USA) was added to each well. After incubation for 15 min at 37 °C and three washes, 100 µL of *ortho*-phenylenediamine solution (0.4 mg mL^{-1}) in citric acid buffer, pH 5.0, was added to each well. After 30 min of incubation the reaction was stopped by addition of 50 µL of 3 N sulfuric acid. The absorbance of the plates was read using a microplate

reader (BioTek, USA) at 490 nm. Results are reported as optical density (OD). Positive and negative controls were used to verify the test reproducibility.

Experimental infection

All experimental infections were performed by immersion. Strain BZ-1 was thawed and grown on MHS as described above. One colony was inoculated in 150 mL of modified MHS broth and incubated at 26 °C under low agitation (150 rpm). The growth was monitored until OD_{600} was between 0.200 and 0.230, corresponding to a bacterial concentration of approximately of 10^8 cfu mL^{-1}. Challenges were carried out in a 10-L bucket containing 100 mL of bacterial innoculum and 9900 mL of pathogen-free water at 26 °C to yield a bacterial concentration of 10^6 cfu mL^{-1}. Each fish group was individually anesthetized as described above and then challenged for 15 min in the 10-L bucket. An additional fish group used as an experimental infection control was immersed in a 10-L bucket containing 100 mL of sterile MHS broth and 9900 mL of pathogen-free water for 15 min. These fish were returned to their aquarium without a bacterial innoculum. Samples of liver, kidney and subcutaneous tissue were collected aseptically from fish that died to reisolate the bacteria. Since reisolation of *F. columnare* is difficult, PCR

with specific primers was performed to confirm infection in the same tissues according to the protocol described by Darwish et al. (2004).

Statistical analysis

Antibody responses among treatment groups were analyzed by one-way ANOVA followed Student's *t*-test. Differences in mortality observed among fish groups were assessed by the χ^2 test. Probabilities lower than 0.05 ($P<0.05$) were considered significant. All statistical analysis was performed using SAS® statistical software STAT Version 6.12 (SAS Institute Inc., USA).

Results

Sandwich ELISA

The sandwich ELISA successfully detected specific antibodies against *F. columnare* in immunized Nile tilapia. The method was highly reproducible, as verified by similar inter-day results for both positive and negative controls. The mean OD results for the first vaccine experiment are listed in Table 1. The OD ranged from 0.040 to 0.552. In the first experiment, only immunization by parenteral FKB injection (i.p. and i.m.) induced specific humoral responses ($P<0.05$). The i.p. immunized group had the highest antibody titers ($P<0.05$), followed by the i.m. group ($P<0.05$). Fish immunized by immersion or oral preparations did not

show significant differences compared to the non-immunized control group.

In the second experiment, booster immunization with alginate microparticles was not efficient in inducing a specific immune response (Table 2). At 3 weeks after revaccination, there was no difference in mean OD between reimmunized groups A and C and the control group ($P<0.05$). Despite the high antibody levels induced by i.p. immunization verified in the first experiment, this response was maintained for only a short period. At 51 days post-vaccination, group B (i.p. immunized) did not exhibit a significant difference compared to the control group ($P<0.05$). The fish i.p. vaccinated 30 days later showed a high mean OD that was significantly different from the other groups ($P<0.05$).

Table 1 ELISA results, mortality and relative percentage of survival (RPS) for the first vaccination experiment.

Group	Vaccine preparation[1]	Delivery route	ELISA (mean OD±SD)	Mortality (%)	RPS (%)
I	FKB	Intraperitoneal	0.358±0.133[a]	90	0
II	FKB	Intramuscular	0.184±0.123[b]	90	0
III	FKB	Immersion	0.050±0.008[c]	100	0
IV	AM	Oral	0.050±0.004[c]	90	0
V	EAM	Oral	0.060±0.004[c]	90	0
VI	FKB	Oral	0.062±0.006[c]	100	0
VII	PBS	Intraperitoneal	0.055±0.008[c]	90	0
VIII	–	–	–	0	100

[1]FKB, formalin-killed bacteria; AM, alginate microparticles containing *F. columnare* bacterin; EAM, empty alginate microparticles.

[2]OD values followed by different letters are significantly different ($P<0.05$).

Table 2: ELISA results, mortality and relative percentage of survival (RPS) for the second vaccination experiment.

Group	Vaccine preparation[1]		ELISA	Mortality	RPS
	First vaccine	Booster	(mean OD±SD)[2]	(%)	(%)
A	FKB	AM	0.062±0.006[a]	100	0
B	FKB	NI	0.058±0.008[a]	90	0
C	AM	AM	0.057±0.006[a]	100	0
D	PBS	NI	0.055±0.011[a]	90	0
E	NI	FKB	0.266±0.071[b]	100	0
F	–	–	–	0	100

[1]FKB, formalin-killed bacteria; AM, alginate microparticles containing *F. columnare* bacterin; NI, not immunized.

[2]OD values followed by different letters are significantly different ($P<0.05$).

Fish challenge and vaccine efficiency

No signs of disease or mortalities were observed in the control groups for both experiments (Tables 1 and 2). Challenges with *F. columnare* strain BZ-1 resulted in clinical signs of illness 12 h post-infection. This included apathy, skin and fin erosion, followed by dermal and muscle necrosis. High mortality rates were verified for the strain evaluated, which is hypervirulent to Nile tilapia. The first mortalities were observed 16 h post-infection. *F. columnare* was reisolated from kidney and liver of all moribund and dead fish and confirmed by species-specific

PCR. Despite the variation in antibody response induced by different vaccines, there were no significant differences in mortality rates between immunized and non-immunized groups ($P<0.05$) in both experiments.

Discussion

In the present study, Nile tilapia fingerlings were immunized with oral and parenteral vaccines against *F. columnare* to evaluate vaccine effectiveness. This is usually determined as immunoglobulin levels measured by ELISA or agglutination and survival of a challenge with the virulent pathogen (Shelby et al., 2004). Previous studies evaluated the efficiency and humoral immune response induced by different vaccine preparations against *F. columnare* infection (Shelby et al., 2001; Shoemaker et al., 2003; Grabowski et al., 2004; Zhang et al., 2006). The sandwich ELISA developed here was useful for monitoring the specific humoral response in Nile tilapia. Similar performances have been obtained in previous studies (Shoemaker et al., 2003; Grabowski et al., 2004). Despite the use of similar methods and the same fish species, we observed lower OD values compared to a previous study by Grabowski et al. (2004), who evaluated the antibody response induced by *F. columnare* vaccines in Nile tilapia juveniles using an indirect ELISA. Differences in the protocols used, mainly the absence of blocking solution, could explain this disparity. This probably increased the background levels in the tests

by allowing non-specific binding of other substances present in fish serum (Shelby et al., 2001). The OD levels observed here are in accordance with previous reports for the response in channel catfish exposed to the same bacteria. However, i.m. vaccination induced higher OD levels than other delivery routes in catfish (Shoemaker et al., 2003), in contrast to the i.p. route observed in the present study.

FKB have been widely used as antigens for fish vaccination, mainly against diseases caused by Gram-negative organisms. For some illnesses, an acceptable level of protection can only be achieved by immunization with adjuvant bacterin delivered by injection (Gudding et al., 1999). Grabowski et al. (2004) reported that formalin-killed *F. columnare* administered i.p. and by immersion could not stimulate a specific immune response in Nile tilapia without Freund's complete adjuvant (FCA). Although the vaccine–FCA combination was more efficient due to the induction of high immune response, it is very stressful for fish and produces many adverse reactions, being less useful in practice. We demonstrated that FKB administered by i.p. and i.m. routes can induce significant antibody levels in fingerlings compared to non-immunized animals. However, similar to the results of Grabowski et al. (2004), immersion did not stimulate the immune system in our experiments. This might be due to low antigen amounts in the immersion

preparation or low antigen uptake (Bader et al., 2003; Grabowski et al., 2004).

Oral immunization is one of the best alternatives to fish vaccination, since it is less time-consuming, labor-intensive and stressful, and allows mass vaccination of fish of any size (Romalde et al., 2004; Vanderberg, 2004). Good fish protection results have been achieved with alginate microparticles used as a delivery system for vaccination against *Lactococcus garviae* (Romalde et al., 2004) and *Vibrio anguilarum* (Joosten et al., 1997) in rainbow trout and common carp, respectively. No data on the effectiveness of oral vaccination of Nile tilapia against *F. columnare* have been reported to date. Although the particles are stable at pH values similar to that in the gastrointestinal tract of *O. niloticus*, no significant *in vitro* and *in vivo* responses were observed for tilapia fingerlings. This could be due to problems during microparticle production, such as low chemical and mechanical stability during particle synthesis. Low antigenicity of released FKB on oral administration or failure of antigen uptake in the gut mucosa can also be possible explanation by these results. Another possible explanation is the low stability of alginate microparticles in the tilapia gut. Despite *in vitro* simulation of gut conditions (Rodrigues et al., 2006), particle behavior *in vivo* after administration was not evaluated in the present study.

Degradation of antigens caused by acidic pH and the proteolytic activity of stomach enzymes, as well failure of FKB release in the intestine, are possibilities. In future work, changes in the methodology for vaccine production need to be considered to solve these possible problems.

Although high immune responses were induced by some vaccine preparations, no positive RPS results were observed after experimental infection. There was no direct correlation between levels of the antibody specific to FKB and resistance to *F. columnare* infection. A direct relation between antibody titers and survival rates was observed for Nile tilapia immunized with *S. agalactiae* vaccine (Pasnik et al., 2005) and similar results were reported for hybrid striped bass vaccinated against *S. iniae* (Shelby et al., 2004), both Gram-positive pathogens associated with systemic infections. Our data suggest that the humoral immune response against whole *F. columnare* cells was not sufficient to block infection. The main virulence factors involved in columnaris pathogenesis are the release of exoenzymes, such as proteases and condroitin AC-lyase, which degrade protein and connective tissues, respectively (Schneck & Caslake, 2006). The bacteria do not show classical systemic infection and only sometimes induce septicemia in the advanced stages of the disease. These characteristics of *F. columnare* give three possible explanations for the poor vaccine efficiency in the present study. First, the bacteria initiate

infection by spreading from the dermis to muscle, with bloodstream infection causing septicemia only occurring in the terminal stage of the disease, independent of specific neutralizing antibodies; thus, external lesions are extensive in this stage and the host cannot recover. Second, formalin-killed bacteria do not contain extracellular products released during microorganism growth. Specific antibodies against these substances could be necessary to block the pathogenesis of the disease, mainly in the initial stage when dermis and muscle necrosis occurs. Finally, the challenges were performed 3 weeks after immunization. The peak of antibody concentration in plasma is reached at 21 days after immunization for Nile tilapia, however, in the surface mucus this occurs only after six weeks (Grabowski et al., 2004). It is possible that experimental infection after this period could improve the results, since high immunoglobulin levels capable of neutralizing the pathogen should then be present in the mucus.

In conclusion, inactivated *F. columnare* cells administered parenterally induced a significant humoral immune response in Nile tilapia fingerlings. Alginate microparticles containing FKB were not efficient in stimulating the immune response in the conditions evaluated in the present work. The oral and parenteral vaccines against *F. columnare* did not induce protection in tilapia fingerlings. There was no

relation between antibody titers and resistance of Nile tilapia fingerlings to *F. columnare* infection.

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Competing interests statement

The authors declare no competing financial interests.

3 CONCLUSÃO

A administração de células inativadas de *F. columnare* por vias parenterais induziu resposta imune humoral em alevinos de tilápia do Nilo. As micropartículas de alginato incorporadas com células de *F. columnare* inativadas não foram eficientes na estimulação de resposta imune nas condições avaliadas no presente trabalho. As vacinas orais e parenterais contra *F. columnare* não conferiram proteção aos peixes. Não houve relação entre os títulos de anticorpos e resistência a infecção causada por *F. columnare* em alevinos de tilápia do Nilo.

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