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Development of a bacterial challenge test for gnotobiotic Nile tilapia *Oreochromis niloticus* larvae

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ABSTRACT: Gastrointestinal microbiota have an important impact on fish health and disease, stimulating interest in a better understanding of how these gastrointestinal microbial communities are composed and consequently affect host fitness. In this respect, probiotic microorganisms have been extensively used in recent aquaculture production. To study the use of probiotics in the treatment of infectious diseases, the establishment of a method of experimental infection to obtain consistent results for mortality and infection in challenge tests is important. In pathogen-screening tests, 4 candidate pathogenic bacteria strains (*Edwardsiella ictaluri* gly09, *E. ictaluri* gly10, *E. tarda* LMG2793 and *Streptococcus agalactiae* LMG15977) were individually tested on xenic Nile tilapia larvae. Only *Edwardsiella* strains delivered via *Artemia* nauplii, with or without additional pathogen delivery via the culture water, led to increased mortality in fish larvae. A gnotobiotic Nile tilapia larvae model system was developed to provide a research tool to investigate the effects and modes-of-action of probiotics under controlled conditions. A double disinfection procedure using hydrogen peroxide and sodium hypochlorite solution was applied to the fish eggs, which were subsequently incubated in a cocktail of antibiotic and antifungal agents. In the gnotobiotic challenge test, *E. ictaluri* gly09R was added to the model system via *Artemia* nauplii and culture water, resulting in a significant mortality of the gnotobiotic fish larvae. The developed gnotobiotic Nile tilapia model can be used as a tool to extend understanding of the mechanisms involved in host–microbe interactions and to evaluate new methods of disease control.

KEY WORDS: Gnotobiotic · Bacterial challenge test · *Oreochromis niloticus* · Edwardsiellosis · *Edwardsiella ictaluri* · Host–microbe interactions

INTRODUCTION

Tilapia *Oreochromis* spp. are an important group in freshwater fish aquaculture due in part to their ease of culture under a wide range of environmental conditions and their relative resistance to environmental stressors when compared to other cultured finfish species (Welker & Lim 2011). However, significant losses due to disease still occur, especially under intensive culture where tilapia can become infected with a wide range of viral, parasitic, fungal and, particularly, bacterial pathogens. Bacteria of the genus *Edwardsiella,* including *E. ictaluri* and *E. tarda*, are very important bacterial pathogens that cause severe economic losses in both freshwater and marine aquaculture in many countries (Ewing et al. 1965, Hawke et al. 1981, Evans et al. 2011). *E. ictaluri,* the causative agent of enteric septicaemia of catfish (ESC), also infects and causes mortality in Nile tilapia fingerlings (Hawke et al. 1981, Plumb & Sanchez 1983, Keskin et al. 2004, Nagai et al. 2008, Soto et al. 2012). *E. tarda,* which is a common bacterium in freshwater environments, infects and causes disease in red tilapia (Iregui et al. 2012).

In some cases, the use of antibiotics for the control of bacterial diseases in aquaculture has resulted in the

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development and spread of antibiotic resistance, leading to ineffective treatment for some diseases (Defoirdt et al. 2011). This has lead researchers to examine other mechanisms for the control of bacterial diseases in fish. As gastrointestinal (GI) bacteria play important roles in the nutrition and health of the host organism, various means of altering the intestinal bacteria to achieve favourable effects such as better resistance to pathogens, enhanced growth and immune stimulation of the host have been investigated in various species of fish and shrimp (Yousefian & Amiri 2009, Sihag & Sharma 2012). In this respect, the use of probiotics and prebiotics is considered to be a sustainable and effective alternative to antibiotics for disease control in aquaculture production (Gatesoupe 1999, Verschuere et al. 2000). In order to be able to discover more effective probiotic bacteria, a better understanding of the host–microbe interactions of several putative probiotic microorganisms through *in vivo* experiments is essential and still needed (Tinh et al. 2008).

The composition of the GI microbial community can be influenced by host genotype (Spor et al. 2011, Kostic et al. 2013), environmental conditions and stochastic factors (Verschuere et al. 1999, Fjellheim et al. 2012). This complicates the study of host–microbe interactions, as the structure of the GI microbial community of fish reared in conventional rearing systems is very dynamic, often causing problems in experiments such as lack of repeatability and reproducibility. Thus, a key strategy in studying host–microbe interactions is to first determine interactions under axenic conditions then further evaluate the effects of a single or defined populations of microbes or specified compounds added under gnotobiotic conditions (Gordon & Pesti 1971). The use of gnotobiotic organisms leads to an increased control of variables, enhanced reproducibility of results and more accurate experimental designs (Coates 1975, Marques et al. 2005) and thus can be an excellent tool to extend the understanding of the mechanisms involved in host–microbe interactions (Marques et al. 2006).

The aims of the present work were to (1) screen candidate pathogenic bacteria strains on xenic/conventional Nile tilapia *Oreochromis niloticus* larvae, (2) develop a standardized gnotobiotic Nile tilapia larvae culture system to facilitate the study of host–microbe interactions, and (3) develop a standardized bacterial challenge test for gnotobiotic Nile tilapia larvae enabling further studies on the mode-of-action of putative pre- and probiotics as new ways of disease control, especially in freshwater aquaculture. To our knowledge, this is the first study of a gnotobiotic food chain consisting of Nile tilapia larvae and *Artemia* nauplii.

MATERIALS AND METHODS

Experimental set-up for pathogen-screening test with xenic Nile tilapia larvae

Nile tilapia *Oreochromis niloticus* that ranged from 120 to 150 g wet weight and 21 to 23 cm total length were naturally bred in our laboratory. Larvae were collected from the mouths of brooding females 3 d after hatching (3 DAH) and pooled. Upon collection, the pooled larvae were acclimatized in a 30 l aquarium for 6 d at a mean (±SD) temperature of 27 ± 1°C prior to bacterial challenge. Candidate pathogenic bacteria strains were tested individually in triplicate 1.5 l aquaria, each of which contained 10 larvae. All aquaria were provided with gentle aeration and kept in a heated room (constant air temperature of 29 ± 1°C), and the water temperature was maintained at 26 ± 1°C. Filtered standard synthetic freshwater containing 96 mg l^{-1} $NaHCO_3$, 60 mg l^{-1} $CaSO_4 \cdot 2H_2O$, 60 mg l^{-1} $MgSO_4$ and 4 mg l^{-1} KCl (USEPA 2002) was used as fish culture water. To maintain the NH_4^+ and NO_2^- levels below 0.5 and 0.2 mg l^{-1}, respectively, 25% of the culture water was renewed every 3 d during the 12 d experimental period.

Bacterial strains and culture conditions

Streptococcus agalactiae LMG15977 and 2 strains of *Edwardsiella ictaluri,* referred to as gly09 and gly10, were tested in the first bacterial pathogen-screening test (Expt 1a). Another *Edwardsiella* species, *E. tarda* LMG2793, was tested along with the 2 *E. ictaluri* strains in the second pathogen-screening test (Expt 1b). Both *E. ictaluri* strains were obtained from the Laboratory of Aquaculture & Artemia Reference Center (Ghent University). These strains were isolated from ichthyophthriasis (white spot disease)-infected striped catfish *Pangasius hypophthalmus*. *S. agalactiae* LMG15977, which was previously isolated from Nile tilapia brain, was obtained from BCCM/LMG (Belgian Co-ordinated Collections of Micro-organisms, Laboratory of Microbiology, Ghent University). An isolate of *E. tarda,* LMG2793 was provided by the Laboratory of Veterinary Bacteriology and Mycology (Ghent University). This isolate was obtained from human faeces.

The strains were stored in brain heart infusion (BHI) broth (FLUKA, Sigma-Aldrich) supplemented with 20% (v/v) glycerol at −80°C. *Edwardsiella* strains were grown in BHI broth and incubated on a horizontal shaker at 160 rpm and 27°C. *Streptococ-*

cus agalactiae LMG15977 was grown in BHI broth and incubated on a horizontal shaker at 180 rpm and 37°C. The density of each bacterial culture was determined by measuring turbidity with a spectrophotometer (Genesys 20, Thermospectronic) at 550 nm and comparing to the McFarland standard (BioMérieux).

Bacterial challenge procedure with xenic Nile tilapia larvae

Fish were challenged daily over the experimental period using 3 challenge methods: (1) via culture water, (2) via axenic *Artemia* nauplii, and (3) via both culture water and axenic *Artemia* nauplii. For the culture water challenges, bacterial suspensions were harvested by centrifuging at 1000 × *g* for 10 min and washed twice in their respective culture medium then once in fish culture water. The density of bacterial suspension was determined by measuring turbidity with a spectrophotometer at 550 nm and comparison to the McFarland standard. Sufficient bacteria were added to achieve a density of 10^6 colony forming units (CFU) ml^{-1} in the tank.

For the challenge via axenic *Artemia* nauplii, axenic *Artemia* cysts were incubated and hatched following the procedure of Marques et al. (2004). Axenic *Artemia* nauplii were harvested after 24 h incubation at 27 ± 1°C, washed using filtered autoclaved synthetic freshwater and counted. Bacterial suspensions were harvested by centrifuging at 1000 × *g* for 10 min, washed twice using their respective culture medium and added to axenic *Artemia* culture (100 *Artemia* ml^{-1}) at a final bacteria density of 10^8 CFU ml^{-1}. Bacteria-loaded *Artemia* nauplii were harvested after 1 h incubation and washed twice using sterile saline solution (9 g l^{-1} NaCl), counted and added at a density of 20 *Artemia* per fish. The bacterial load of the *Artemia* nauplii in each treatment was determined by the plate count method. After rinsing and counting, subsamples of bacteria-loaded *Artemia* were homogenized according to the procedure described by Huys et al. (2001). Subsequently, 50 µl of the homogenized *Artemia* suspension was plated (Spiral plater™, Spiral Systems) on Lysogeny broth (LB) agar.

For the challenge via both *Artemia* nauplii and culture water, larvae received a waterborne challenge as well as a challenge via axenic *Artemia* administered at the same time. These challenges were conducted as described above. Mortality observation and dead fish removal were done twice daily.

Disinfection protocol to obtain axenic Nile tilapia larvae

Nile tilapia were naturally bred in our laboratory and 3 d post fertilization (3 DPF) eggs were collected and pooled following the procedures described above for the xenic pathogen-screening tests. Upon collection, eggs were put on a sterile nylon sieve (mesh size 300 µm) and washed 4 times with 250 ml 0.2 µm-filtered autoclaved standard synthetic freshwater (USEPA 2002) at 25 ± 1°C to remove loose bacteria. Unfertilized eggs, dead eggs or eggs with ruptured yolk were discarded prior to the disinfection procedure. A double disinfection procedure was applied for the remaining eggs at the eyed egg stage (3 DPF or stage 14 to 15) (Fujimura & Okada 2007). In the first disinfection procedure, eggs were immersed in diluted 30% hydrogen peroxide (MERCK-Schuchardt 386790) with a final active peroxide concentration of 2000 mg l^{-1} for 10 min at 25 ± 1°C. During disinfection, eggs were gently agitated to ensure that all eggs had equal contact with the disinfecting agent. Subsequently, the eggs were rinsed 4 times with 250 ml 0.2 µm-filtered autoclaved synthetic freshwater. Following the first disinfection, eggs were incubated in an axenic incubation medium which consisted of 0.2 µm-filtered autoclaved standard synthetic freshwater containing 10 mg l^{-1} each of ampicillin (Sigma-A0166), rifampicin (Sigma-83907), trimethoprim (Sigma-T7883) and gentamycin (Sigma-G1264), and the antifungal agents Amphotericin-B (Sigma-A9528) and Fluorescent Brightener 28 (Sigma-F3543) at concentrations of 0.5 mg l^{-1} and 25 mg l^{-1}, respectively.

The second disinfection procedure was done 24 h after the first. A 75 mg l^{-1} active chlorine solution was prepared using sodium hypochlorite technical grade (Sigma Aldrich 425044) and 0.2 µm-filtered autoclaved synthetic freshwater. Eggs were immersed in this solution for 2 min at 25 ± 1°C. The concentration of active chlorine in the sodium hypochlorite solution was determined 24 h prior to use by a standard iodine/thiosulfate titration method (Lavens & Sorgeloos 1996). Following disinfection, eggs were rinsed 4 times with 250 ml 0.2 µm-filtered autoclaved synthetic freshwater. All disinfection procedures were performed in a laminar flow hood.

Disinfected eggs were aseptically distributed to 500 ml sterile glass bottles containing 200 ml incubation medium and incubated at a density of 500 eggs l^{-1}. Fish were maintained in the axenic incubation medium throughout the non-feeding larval stage. Eggs of the control group underwent the

same incubation procedure; however, they were not surface disinfected or treated with antibiotic and antifungal agents. Each incubation bottle was equipped with 2 sterile 0.2 µm filters (Sartorius) for the aeration in- and outlet to provide gentle sterile aeration during the egg incubation and larval stage. Eggs hatched after 3 d incubation following the disinfection procedure. The effect of the disinfection procedure on egg hatching was evaluated using the egg hatching percentage, which was measured as the proportion of the total number of eggs incubated that hatched, regardless of their viability (Komar et al. 2004). The hatching percentage was determined 1 DAH.

Tests for axenity

Axenity was checked at several crucial steps during the experiments. After 24 h following the disinfection procedure, 5 eggs were aseptically sampled from each incubation bottle, individually homogenized and plated on LB medium + 15 g l^{-1} agar (LB agar; bacteriological grade, MP Biomedicals). In addition, 1 ml water from each incubation bottle was added into a sterile tube containing 9 ml LB broth (10%). After determination of the larval survival, the fish larvae from the axenic treatments were checked for bacterial contamination using the plate culture method. From each incubation bottle, 2 larvae were euthanized using 1 g l^{-1} benzocaine and 1 g l^{-1} benzalkonium chloride before being rinsed and homogenized in sterile saline solution (9 g l^{-1} NaCl). Subsequently, 50 µl of the homogenized fish suspension was plated (Spiral plater™, Spiral Systems) on LB agar plates. The inoculated plates were incubated at 27 ± 1°C for 96 h. At stocking of the larvae (6 DAH) and at the end of the gnotobiotic challenge test, the fish larvae from the axenic treatments were checked for bacterial contamination using the above-mentioned procedure.

Bacterial challenge procedure with gnotobiotic Nile tilapia larvae

Along with the antifungal agents (0.5 mg l^{-1} Amphotericin-B and 25 mg l^{-1} Fluorescent Brightener 28), a mixture of antibiotics containing ampicillin, rifampicin, kanamycin, trimethoprim and gentamycin (each at 10 mg l^{-1}) was used in the larvae medium throughout the gnotobiotic challenge tests. Therefore, it was necessary to identify antibiotic resistant mutants that could be used for challenge. We investigated *Edwardsiella ictaluri* gly09 resistance to the different antibiotics by inoculating an overnight *E. ictaluri* gly09 culture into sterile tubes containing BHI broth and 1 of the 5 antibiotics at a concentration of 10 mg l^{-1}. These cultures were incubated overnight on a horizontal shaker at 160 rpm at 27°C. Growth, determined by the presence of turbidity, was obtained in the presence of 10 mg l^{-1} ampicillin, trimethoprim and gentamycin, suggesting that *E. ictaluri* gly09 is intrinsically resistant to these antibiotics. No growth occurred in the presence of 10 mg l^{-1} rifampicin or kanamycin.

In order to obtain spontaneous rifampicin-resistant and kanamycin-resistant *Edwardsiella ictaluri* gly09 mutants, wild type *E. ictaluri* gly09 was cultured separately on BHI agar plates containing 2 mg l^{-1} of each antibiotic. Colonies appearing on the BHI-rifampicin and BHI-kanamycin plates after an incubation period ranging from 48 to 72 h at 27°C were harvested, mixed and transferred into several tubes containing BHI broth with 2 mg l^{-1} of both antibiotics. Cultures were incubated on a horizontal shaker at 160 rpm and 27°C overnight, after which time 1 ml samples were transferred into new tubes containing 9 ml BHI broth with a higher antibiotic concentration of 5 mg l^{-1}. Following overnight incubation, selected grown cultures (cultures with an absorbance level higher than 0.5 at a wavelength of 600 nm) were then transferred into new tubes containing 9 ml BHI broth with an even higher antibiotic concentration of 10 mg l^{-1}.

Cultures of *Edwardsiella ictaluri* gly09 that were resistant to 10 mg l^{-1} rifampicin and kanamycin were transferred and grown in BHI broth containing 10 mg l^{-1} ampicillin, rifampicin, kanamycin, trimethoprim and gentamycin. The resulting *E. ictaluri* strain (referred to as *E. ictaluri* gly09R) with multiple antibiotic resistance was used in gnotobiotic challenge tests. Axenic fish larvae were challenged with *E. ictaluri* gly09R via both axenic *Artemia* nauplii and culture water, following the procedures applied in the pathogen-screening test on xenic larvae.

The gnotobiotic challenge tests were done in triplicate (n = 3) in 500 ml sterile glass bottles containing 200 ml incubation medium with 10 fish per bottle. Each incubation bottle was equipped with 2 sterile 0.2 mm air filters (Sartorius) to provide gentle sterile aeration and a sterile septum for aseptic larval feeding during the experimental period. An unchallenged-axenic fish group was used as the control group for the gnotobiotic system. In order to verify that there was no effect of the culture system set-up on patho-

gen virulence, in Expt 2a, the *Edwardsiella ictaluri* gly09R strain was also tested on xenic larvae using the same culture system set-up but without the air 0.2 µm filtration or the use of both antibiotics and antifungal mixtures. To maintain conditions in the experimental system, 25% of the incubation medium with or without antimicrobials was replaced every 3 d.

The gnotobiotic challenge test was repeated twice (Expts 2a and 2b). In Expt 2a, *Edwardsiella ictaluri* gly09R was tested on both the xenic and gnotobiotic fish groups, while in Expt 2b, it was only tested on the gnotobiotic group as there were insufficient numbers of xenic larvae due to a poor hatching of the xenic eggs.

In order to determine larval bacterial load at the end of the challenge test, surviving larvae were killed using an overdose of benzocaine, surface-disinfected in a bath of benzalkonium chloride, rinsed and homogenized according to the procedure described by Huys et al. (2001). Subsequently, 50 ml of the homogenized larval suspension was plated (Spiral plater™, Spiral Systems) on BHI agar containing 10 mg l^{-1} ampicillin, rifampicin, kanamycin, trimethoprim and gentamycin. The experimental designs for bacterial challenge tests on Nile tilapia larvae were approved by the ethical committee of Ghent University under file number EC2012/070 for the xenic challenge tests (Expts 1a and 1b) and EC2013/69 for the gnotobiotic challenge tests (Expts 2a and 2b).

Statistical analysis

A chi-square test was used to detect significant differences in the hatching percentage between the xenic and the disinfected eggs. For comparison of the cumulative mortality of fish larvae, data were arcsine transformed before a 1-way analysis of variance using the general linear model of STATISTICA 7.0 (StatSoft 2004) was performed. A Tukey test was performed on the transformed data for multiple comparisons among means (Sokal & Rohlf 1955). All analyses were run at a minimum level of significance of 5%. Results are reported as mean ± standard deviation.

The relative percentage of survival (RPS), which is the larval survival after challenge when compared to control fishes (Amend 1981), was calculated to evaluate the efficacy of xenic and gnotobiotic bacterial challenge tests as RPS = [1 − (% mortality in challenged group/% mortality in control group)] × 100. The RPS values obtained in the challenge studies were analysed using a chi-square test.

RESULTS AND DISCUSSION

Pathogen-screening tests on xenic Nile tilapia larvae

Streptococcus sp. and *Edwardsiella* sp. are the common groups of bacteria reported to infect wild and farmed tilapia (Plumb & Hanson 2010). *E. tarda* infects and causes significant pathology in red tilapia (Iregui et al. 2012). In the last few years, different strains of *E. ictaluri*, which were already known as etiological agents of ESC, have also been reported as causative agents of morbidity and mortality in Nile tilapia fingerlings (Soto et al. 2012). In this study, 4 candidate pathogenic bacteria strains representing the 2 groups were screened for the development of a bacterial challenge test for gnotobiotic Nile tilapia larvae: *E. ictaluri* gly09, *E. ictaluri* gly10, *E. tarda* LMG2793 and *S. agalactiae* LMG15977.

In the first pathogen-screening test (Expt 1a), mortality was first observed in the group challenged with *Edwardsiella ictaluri* gly09 via *Artemia* nauplii and in the group challenged with *E. ictaluri* gly10 via the culture water 6 d after challenge (6 DAC). With respect to *E. ictaluri* gly09, there were no significant differences between treatments (challenge routes and control) until 11 DAC when significantly higher levels of mortality were seen in the groups challenged via *Artemia* and via both *Artemia* and culture water when compared to control and the groups challenged via culture water (Table 1). A similar result was observed for *E. ictaluri* gly10, with the exception that the group challenged via both *Artemia* and culture water had significantly higher mortalities than all other treatment groups and the control at 10 DAC. At 11 DAC, significantly higher levels of mortality were observed in the groups challenged via *Artemia* and via both *Artemia* and culture water when compared to the control and the group challenged via culture water (Table 1).

The pathogenicity of both *Edwardsiella ictaluri* strains for tilapia larvae was confirmed in Expt 1b (Table 2), where oral and waterborne/immersion exposure of larvae to either *E. ictaluri* gly09 or gly10 resulted in a significant mortalities of 93 ± 11 to 100 ± 0%, respectively when compared to the 40 ± 17% mortality seen in the control group at 9 DAC. The results of both pathogen-screening tests suggest that both *E. ictaluri* strains can cause significant larval mortalities when delivered orally through the feeding of pathogen-loaded *Artemia* nauplii, with or without additional pathogen delivery via the culture water; while lower or no significant mortality was

Table 1. Cumulative mortality (%, mean ± SD) of control and challenged (*Edwardsiella ictaluri* gly09, *E. ictaluri* gly10 and *Streptococcus agalactiae* LMG15977) xenic Nile tilapia larvae *Oreochromis niloticus* using different challenge methods in Expt 1a. Different letters within the same row denote significant differences ($p < 0.05$). Number of replicates = 3; initial number of fish larvae per replicate = 10. DAC: days after challenge

Time (DAC)	Control	*Edwardsiella ictaluri* gly09 via culture water	*Edwardsiella ictaluri* gly09 via *Artemia* nauplii	*Edwardsiella ictaluri* gly09 via *Artemia* and water	*Edwardsiella ictaluri* gly10 via culture water	*Edwardsiella ictaluri* gly10 via *Artemia* nauplii	*Edwardsiella ictaluri* gly10 via *Artemia* and water	*Streptococcus agalactiae* LMG15977 via culture water	*Streptococcus agalactiae* LMG15977 via *Artemia* nauplii	*Streptococcus agalactiae* LMG15977 via *Artemia* and water
6	0 ± 0^a	0 ± 0^a	4 ± 6^a	0 ± 0^a	4 ± 6^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	0 ± 0^a
7	0 ± 0^a	4 ± 6^a	7 ± 6^a	0 ± 0^a	4 ± 6^a	4 ± 6^a	0 ± 0^a	0 ± 0^a	0 ± 0^a	4 ± 6^a
8	0 ± 0^a	33 ± 29^a	30 ± 17^a	18 ± 6^a	18 ± 13^a	41 ± 28^a	33 ± 29^a	18 ± 23^a	4 ± 6^a	15 ± 17^a
9	26 ± 26^a	44 ± 22^a	56 ± 11^a	52 ± 6^a	41 ± 6^a	63 ± 28^a	74 ± 6^a	22 ± 29^a	26 ± 28^a	37 ± 26^a
10	37 ± 23^a	59 ± 6^a	74 ± 13^a	81 ± 6^a	48 ± 6^a	74 ± 17^a	85 ± 6^b	44 ± 22^a	48 ± 28^a	63 ± 17^a
11	48 ± 13^a	67 ± 0^a	81 ± 6^b	81 ± 6^b	70 ± 6^a	81 ± 13^b	89 ± 0^b	67 ± 19^a	70 ± 13^a	74 ± 6^a

Table 2. Cumulative mortality (%, mean ± SD) of control and challenged (*Edwardsiella ictaluri* gly09, *E. ictaluri* gly10 and *E. tarda* LMG2793) xenic Nile tilapia larvae *Oreochromis niloticus* via both *Artemia* nauplii and culture water in Expt 1b. Different letters within the same row denote significant differences ($p < 0.05$). Number of replicates = 3; initial number of fish larvae per replicate = 10. DAC = days after challenge

Time (DAC)	Control	*E. ictaluri* gly09	*E. ictaluri* gly10	*E. tarda* LMG2793
5	0 ± 0^a	3 ± 6^a	0 ± 0^a	0 ± 0^a
6	0 ± 0^a	17 ± 6^a	17 ± 21^a	3 ± 6^a
7	0 ± 0^a	37 ± 31^{ab}	47 ± 25^b	60 ± 17^b
8	27 ± 23^a	77 ± 6^b	100 ± 0^c	83 ± 15^b
9	40 ± 17^a	93 ± 11^b	100 ± 0^b	93 ± 11^b

observed when the fish were only exposed to the pathogens via the culture water. In the study by Soto et al. (2012), tilapia fingerlings (~15 g) immersed in 10^6 CFU ml^{-1} of tank water presented significant 100% mortality events at 8 d post challenge. The results of this current study suggest that bacterial infection via water culture (immersion challenge) in tilapia larvae culture is less likely than in adult culture.

Due to the emergent nature of edwardsiellosis in non-ictalurid fish, little is known regarding the dynamics of *Edwardsiella ictaluri* infection in Nile tilapia culture, especially during its larviculture stage. To our knowledge, this is the first report of *E. ictaluri*-induced mortality in Nile tilapia larviculture. In a further study, Soto et al. (2013) increased our understanding of the pathogenesis of *E. ictaluri* in Nile tilapia fingerlings, suggesting that the cutaneous and oral routes were the main ports of entry for the bacterium, which later spreads haematogenously throughout the fish body, with the spleen and head kidney as the main targets of infection. Their observations support the study by Li et al. (2012), which suggested that *E. ictaluri* gains entry through the intestinal epithelium, possibly using actin polymerization and receptor-mediated endocytosis as mechanisms of invasion.

Bullock & Herman (1985) suggested that *E. ictaluri* is more pathogenic than *E. tarda* to channel catfish *Ictalurus punctatus* fingerlings. In this study, a significantly higher larval mortality was observed in the group challenged with *E. tarda* LMG2793 via *Artemia* and culture water when compared to the control group, starting from 7 DAC and with a the final mortality of 93 ± 11% at 9 DAC (Table 2).

In both of the pathogen-screening tests, the first fish mortalities in the treatment group challenged with *Edwardsiella ictaluri* occurred at 5 to 6 DAC. However, the mortality from the challenges via both *Artemia* and culture water in Expt 1a differed when compared to Expt 1b, where significant differences in mortality following challenge occurred as early as 7 DAC. The differences in the incubation period until significant mortalities were observed could be caused by the presence of other microorganisms besides the tested pathogens or a difference in pathogen resistance between the different egg batches.

With respect to *Streptococcus agalactiae* LMG 15977, which was the only isolate actually isolated from tilapia culture, there were no significant differences between treatments (challenge routes) and the control group at 11 DAC. However, significantly lower mortalities were observed when compared to the groups challenged with *Edwardsiella ictaluri* strains via *Artemia* and via both *Artemia* and culture water (Table 1). It is possible that the pathogenicity of *S. agalactiae* in this experiment was affected by the water temperature, which was below the optimal growth temperature for *S. agalactiae* culture. A study

by Mereghetti et al. (2008) revealed that the transcription of some important virulence factors by human *S. agalactiae* increased at higher temperature (40°C compared with 30°C). Our results are also supported by Rodkhum et al. (2011), who evaluated the association between water temperature and susceptibility of Nile tilapia to *Streptococcus agalactiae* infection. In their study, Nile tilapia (100 g) were bath challenged with 10^6, 10^7 or 10^8 CFU ml^{-1} of *S. agalactiae* serotype Ia and maintained at different water temperatures (25, 30 or 33°C) for 1 wk. Cumulative mortality of tilapia was positively correlated to higher temperature, while no clinical signs of disease were exhibited at 25°C. These results indicate that the susceptibility of Nile tilapia to *S. agalactiae* infection is temperature dependent.

To study the use of pro- and/or prebiotics in the prevention and treatment of infectious diseases, establishing a method of experimental infection to obtain steady results in mortality and infection in challenge tests is important. The results of both pathogen-screening tests showed that all *Edwardsiella* strains tested in this study can cause significant mortality in tilapia larvae culture and can be used in the development of bacterial challenge tests with gnotobiotic Nile tilapia larvae, with the challenge route via both *Artemia* and culture water as an effective challenge procedure.

Egg disinfection protocol for axenic Nile tilapia larvae

The effect of the disinfection procedure on egg hatching was evaluated by measuring the hatching percentage at 1 DAH for the xenic and the disinfected eggs. There was no significant effect of the disinfection procedure on the egg hatching in both gnotobiotic experiments (mean ± SD): 32 ± 14 % or 23 ± 3 % and 20 ± 7 % or 11 ± 6 % for the disinfected or the xenic eggs of Expts 2a and 2b, respectively.

In order to obtain axenic larvae, artificial incubation of surface-disinfected eggs in axenic conditions until hatching is crucial. The efficiency of a tilapia egg incubation system/incubator depends on its type, size and shape, the developmental stages of the eggs, and the water quality and flow (El-Sayed 2006). It is important that eggs are kept in gentle motion imitating the natural incubation inside the mouths of female broodstock (Rana & Beveridge 1989). In this study, unlike in xenic incubators, egg incubation was done under static conditions with periodic water renewal using a series of autoclavable flat-bottom glass bottles. As suggested by Rana (1988) and El-Sayed (2006), in cases where eggs are not suspended in the water by a current, they quickly sink and clump. This may be the reason for the relatively low egg hatching of the non-disinfected eggs, ranging from 11 to 27 %, compared to hatching percentages of >60 % using down-welling round-bottomed incubators or up-welling conical containers reported previously (Rana 1988, Rana & Macintosh 1988). With respect to these design and operational requirements, there were several constraints on the egg incubation system design for this research on the gnotobiotic Nile tilapia larviculture. These included the need for a gentle motion of the eggs and the need for an axenic closed system with easy access for removal of non-developing embryos and also refreshment of sterile culture medium. For both gnotobiotic challenge tests, a higher ($p > 0.05$) egg hatching was observed in the disinfected egg group compared to the xenic group, indicating that the disinfection procedure improved egg hatching in our experiments.

In gnotobiotic studies with fish, the common method for obtaining axenic larvae is to collect fertilized eggs, disinfect them and then incubate them in a cocktail of disinfectants and antibiotics. Egg surface disinfection protocols using glutaraldehyde, at different concentrations and exposure times, have been used successfully to produce axenic/gnotobiotic larval marine fish, including turbot *Scophthalmus maximus*, Atlantic halibut *Hippoglossus hippoglossus*, European sea bass *Dicentrarchus labrax* and Atlantic cod *Gadus morhua* L. larvae (Munro et al. 1995, Verner-Jeffreys et al. 2003, Dierckens et al. 2009, Forberg et al. 2011). For the purposes of this study, we chose not to use glutaraldehyde due to its high diffusion rate over the range of water temperatures used for tilapia culture (Salvesen et al. 1997).

A protocol to obtain bacteria-free zebrafish *Danio rerio* for use in gnotobiotic studies has been established, in which fertilized embryos are surface-disinfected using 1000 mg l^{-1} povidone-iodine (PVP-I) solution for 2 min and 30 mg l^{-1} sodium hypochlorite solution for 20 min to 1 h (Pham et al. 2008). A higher sodium hypochlorite concentration of 100 mg l^{-1} for the disinfection of tropical marine red drum *Sciaenops ocellatus* eggs has been reported to increase larval survival, while disinfection using a 5 min exposure to 3 % hydrogen peroxide resulted in bacteria-free larvae (Douillet & Holt 1994). In terms of antifungal treatment, the use of hydrogen peroxide at various concentrations from 100 up to 6000 mg l^{-1} has been shown to also effectively control saprolegniasis in cultured fish eggs, including rainbow trout *Onco-*

rhynchus mykiss, common carp *Cyprinus carpio* and channel catfish *Ictalurus punctatus* (Marking et al. 1994, Schreier et al. 1996, Barnes et al. 1998, Rach et al. 1998, Small & Wolters 2003, Mitchell et al. 2009).

Samples of homogenized eggs that were taken 24 h after disinfection never resulted in growth on LB plates after 96 h incubation, indicating egg/larvae axenity following the egg disinfection procedure. Samples of the incubation medium or homogenized larvae at larval stocking also did not show any colonies on LB plates after 96 h incubation. This indicated that the application of the double disinfection protocol using hydrogen peroxide (2000 mg l^{-1} for 10 min) and sodium hypochlorite (75 mg active chlorine l^{-1} solution for 2 min), followed by incubation in medium containing antibiotics (10 mg l^{-1} ampicillin, rifampicin, trimethoprim and gentamycin) and antifungal products (0.5 mg l^{-1} Amphotericin-B and 25 mg l^{-1} Fluorescent Brightener 28) was effective in obtaining axenic Nile tilapia larvae.

To our knowledge, this is the first published protocol for generating axenic Nile tilapia larvae with continued exposure to antimicrobial agents throughout the gnotobiotic experiment. In the course of the experiment, fish samples were taken from the axenic control treatment for bacteriology. No bacteria were detected in the samples of the axenic fish at the end of Expt 2a, as verified by fish homogenate plating on the LB plates. A very low level of bacterial contamination was observed in the axenic group at the end of Expt 2b, as bacteria colonies grew on the LB plates after 96 h incubation at a density of <30 CFU per fish. This bacterial contamination might be related to the technical complexity of the system (Marques et al. 2006) where handlings and manipulations, such as the culture medium exchange and daily feeding during the experimental period, might have resulted in contamination.

Quantification of the bacteria load in *Artemia* nauplii and Nile tilapia larvae

In Expt 1a, incubation of axenic *Artemia* nauplii with *Edwardsiella ictaluri* gly09, *E. ictaluri* gly10 and *Streptococcus agalactiae* LMG15977 resulted in densities of 7.8 ± 0.2, 5.2 ± 0.1 and 6.5 ± 0.1 × 10^2 CFU per *Artemia* nauplius (ind.$^{-1}$) after 1 h incubation, respectively. Similar results were observed in Expt 1b, where incubation of axenic *Artemia* nauplii resulted in densities of 6.9 ± 0.7, 5.1 ± 0.4 and 7.7 ± 0.2 × 10^2 CFU ind.$^{-1}$ for *E. ictaluri* gly09, *E. ictaluri* gly10 and *E. tarda* LMG2793, respectively. In the challenge tests using the gnotobiotic system, the *E. ictaluri* gly09R loaded onto the *Artemia* nauplii resulted in densities of 4.5 ± 0.9 × 10^3 and 8.8 ± 0.5 × 10^2 CFU ind.$^{-1}$ after 1 h incubation in Expts 2a and 2b, respectively.

Bacterial colonization of *Artemia* nauplii could occur externally, via attachment to the body surfaces or internally by ingestion (Grisez et al. 1996). In this experiment, however, only instar I *Artemia* nauplii were used. The digestive system of instar I *Artemia* nauplii is not yet functional (mouth and anus are not open) so they do not take up food and thrive completely on their yolk reserves (Lavens & Sorgeloos 1996). Therefore, it is most likely that bacterial colonization occurred externally, via attachment to the body surfaces. It has been suggested that bacterial content decreases rapidly after the nauplii are removed from the bacterial suspension (Gomez-Gil et al. 1998). This decrease might be due to the removal of the external bacteria after the nauplii are washed and placed in new sterile water. The bacteria still detected are those colonizing the interior or firmly attached to the external surfaces. The differences in bacteria load of *Artemia* nauplii between treatments and between experiments may be related to differences in bacterial attachment on *Artemia* nauplii during incubation. Waltman et al. (1986a,b) suggested that although both *Edwardsiella ictaluri* and *E. tarda* lack chitinase and chitin-binding proteins, 100% and 56% of 100 strains of both *E. ictaluri* and *E. tarda*, respectively, degraded chondroitin sulphate, which is also an important structural component of *Artemia* nauplii (Forward & Rittschof 1999). At the end of the gnotobiotic challenge test, the densities of *E. ictaluri* gly09R inside the surviving fish was 7.9 ± 0.9 × 10^4 and 4.8 ± 0.6 × 10^3 CFU per fish in Expts 2a and 2b, respectively.

Mortality of the gnotobiotic challenged Nile tilapia larvae

In both gnotobiotic challenge tests, the mortality of the fish group after challenge with *Edwardsiella ictaluri* gly09R was not significantly different ($p > 0.05$) from the unchallenged (axenic or xenic) fish up to and including 9 DAC (Table 3). From 10 DAC onwards, significantly higher mortalities were observed in this challenged group ($p < 0.05$) compared to the unchallenged (axenic) group in both gnotobiotic challenge tests, with a mortality at 12 DAC of 57 ± 6% or 23 ± 6% (challenged versus axenic, Expt 2a) and 63 ± 11% or 23 ± 6% (chal-

Table 3. Cumulative mortality (%, mean ± SD) of xenic (not challenged or challenged), axenic and gnotobiotic Nile tilapia larvae *Oreochromis niloticus* challenged with antibiotic-resistant *Edwardsiella ictaluri* gly09R via *Artemia* nauplii and culture water in Expts 2a and 2b. Different letters within the same row within 1 experiment denote significant differences ($p < 0.05$). Number of replicates = 3; initial number of fish larvae per replicate = 10. DAC: days after challenge

Time	Expt 2a				Expt 2b	
(DAC)	Xenic	Xenic *E. ictaluri* gly09R	Axenic	Gnotobiotic *E. ictaluri* gly09R	Axenic	Gnotobiotic *E. ictaluri* gly09R
5	0 ± 0^{a}	3 ± 6^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
6	3 ± 6^{a}	3 ± 6^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
7	7 ± 6^{a}	3 ± 6^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
8	7 ± 6^{a}	10 ± 10^{a}	0 ± 0^{a}	0 ± 0^{a}	7 ± 6^{a}	10 ± 10^{a}
9	7 ± 6^{a}	10 ± 10^{a}	0 ± 0^{a}	3 ± 6^{a}	13 ± 6^{a}	23 ± 11^{a}
10	7 ± 6^{ab}	13 ± 6^{b}	0 ± 0^{a}	13 ± 6^{b}	20 ± 0^{a}	53 ± 15^{b}
11	7 ± 6^{a}	23 ± 6^{ab}	10 ± 10^{ab}	33 ± 15^{b}	20 ± 0^{a}	60 ± 10^{b}
12	13 ± 6^{a}	40 ± 10^{bc}	23 ± 6^{ab}	57 ± 6^{c}	23 ± 6^{a}	63 ± 11^{b}

lenged versus axenic, Expt 2b) (Table 3). Challenge with *E. ictaluri* gly09R under xenic conditions resulted in a mortality ($p < 0.05$) of 40 ± 10%, significantly higher than that of the xenic unchallenged group, but no significant differences were observed when compared to the gnotobiotic challenged group (12 DAC; Expt 2a).

In this laboratory test system, we relied on antibiotics to maintain gnotobiotic conditions. This resulted in some limitations, as resistant spontaneous mutants need to be isolated for use in challenge trials. Such spontaneous mutations, however, have been found in several Gram-negative bacteria, including *Edwardsiella* species (Ingham & Furneaux 2000, Sikorski & Nevo 2005, Thavasi et al. 2007). *E. ictaluri* strains have been reported which are naturally resistant to rifampicin, macrolides, lincosamides, streptogramins, glycopeptides, fusidic acid, oxacillin (Stock & Wiedemann 2001) and gentamycin (Reger et al. 1993); and also *E. ictaluri* isolates displaying acquired resistance to trimethoprim, streptomycin, oxytetracycline (Dung et al. 2008), kanamycin (Russo et al. 2009) and ampicillin (Russo 2011). In this study, the multiple antibiotic resistance of *E. ictaluri* gly09R resulted from both intrinsic mechanism (for ampicillin, trimethoprim and gentamycin) and spontaneous mutation (for rifampicin and kanamycin). This *E. ictaluri* gly09R strain was used in both gnotobiotic challenge tests (Expts 2a and 2b) and resulted in significant larvae mortality.

When introducing antibiotic resistance to a strain of bacteria, either via plasmids or by selecting spontaneous mutants, the acquired resistance represents an additional cost for the strain that can result in a longer generation time and altered metabolic activity (Andersson & Levin 1999, Levin et al. 2000, McDermott et al. 2006, Feng & Rise 2010). This implies that a bacterial strain with an introduced resistance may behave differently than the original strain. However, the *Edwardsiella ictaluri* gly09R strain used in this study resulted in significant mortalities in the gnotobiotic challenged groups compared to the unchallenged/axenic groups, indicating that the bacterial pathogenicity was preserved after the acquisition of antibiotic resistance.

There was no significant difference ($p > 0.05$) in mortality of the gnotobiotic group challenged with *Edwardsiella ictaluri* gly09R when compared to the xenic challenged group at 12 DAC (57 ± 6% vs. 40 ± 10%; Table 3). Similar results were also observed with respect to RPS: values of 67 and 73% ($p > 0.05$) were obtained for the gnotobiotic and xenic challenged groups, respectively. Several studies using gnotobiotic culture systems have suggested that the cellular and humoral defence systems of axenic animals appear to be underdeveloped, hence making them more susceptible to disease compared to xenic animals (Coates 1975, Marques et al. 2006). Unlike these previous studies, the results of the current study did not show that axenic Nile tilapia larvae are more susceptible to pathogen infection compared to xenic larvae, as indicated by the similar RPS values of the gnotobiotic and xenic challenged groups. More importantly, this study showed that the bacterial challenge test in a gnotobiotic system allows a similar/comparable result with a xenic/conventional open system, but without uncontrolled interference. In conclusion, the Nile tilapia gnotobiotic system developed here is a useful tool for extending understanding of the mechanisms involved in host–microbe interactions and evaluating new methods of disease control in aquaculture production.

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LITERATURE CITED

Amend DF (1981) Potency testing of fish vaccines. Dev Biol Stand 49:447–454

Andersson DI, Levin BR (1999) The biological cost of antibiotic resistance. Curr Opin Microbiol 2:489–493

Barnes ME, Ewing DE, Cordes RJ, Young GL (1998) Observations on hydrogen peroxide control of *Saprolegnia* spp. during rainbow trout egg incubation. Prog Fish-Cult 60:67–70

Bullock GL, Herman RL (1985) *Edwardsiella* infections of fishes. Fish Disease Leaflet 71. US Fish and Wildlife Service, Division of Fishery Research

Coates ME (1975) Gnotobiotic animals in research: their uses and limitations. Lab Anim 9:275–282

Defoirdt T, Sorgeloos P, Bossier P (2011) Alternatives to antibiotics for the control of bacterial disease in aquaculture. Curr Opin Microbiol 14:251–258

Dierckens K, Rekecki A, Laureau S, Sorgeloos P, Boon N, Van den Broeck W, Bossier P (2009) Development of a bacterial challenge test for gnotobiotic sea bass (*Dicentrarchus labrax*) larvae. Environ Microbiol 11:526–533

Douillet PA, Holt GJ (1994) Surface disinfection of red drum (*Sciaenops ocellatus* Linnaeus) eggs leading to bacteria-free larvae. J Exp Mar Biol Ecol 179:253–266

Dung TT, Haesebrouck F, Tuan NA, Sorgeloos P, Baele M, Decostere A (2008) Antimicrobial susceptibility pattern of *Edwardsiella ictaluri* isolates from natural outbreaks of bacillary necrosis of *Pangasianodon hypophthalmus* in Vietnam. Microb Drug Resist 14:311–316

El-Sayed AFM (2006) Tilapia culture. CABI Publishing, Wallingford

Evans JJ, Klesius PH, Plumb J, Shoemaker CA (2011) *Edwardsiella* septicaemias. In: Woo PTK, Bruno DW (eds) Fish diseases and disorders, Vol 3: bacterial, viral and fungal infections. CABI Publishing, Wallingford, p 512–534

Ewing WH, McWhorter AC, Escobar MR, Lubin AH (1965) *Edwardsiella*, a new genus of Enterobacteriaceae based on a new species, *E. tarda*. Int Bull Bacteriol Nomencl Taxon 15:33–38

Feng CY, Rise ML (2010) Characterization and expression analyses of anti-apoptotic Bcl-2-like genes NR-13, Mcl-1, Bcl-X1, and Bcl-X2 in Atlantic cod (*Gadus morhua*). Mol Immunol 47:763–784

Fjellheim AJ, Playfoot KJ, Skjermo J, Vadstein O (2012) Inter-individual variation in the dominant intestinal microbiota of reared Atlantic cod (*Gadus morhua* L.) larvae. Aquacult Res 43:1499–1508

Forberg T, Arukwe A, Vadstein O (2011) A protocol and cultivation system for gnotobiotic Atlantic cod larvae (*Gadus morhua* L.) as a tool to study host microbe interactions. Aquaculture 315:222–227

Forward RB, Rittschof D (1999) Brine shrimp larval photoresponses involved in diel vertical migration: Activation by fish mucus and modified amino sugars. Limnol Oceanogr 44:1904–1916

Fujimura K, Okada N (2007) Development of the embryo, larva and early juvenile of Nile tilapia *Oreochromis niloticus* (Pisces: Cichlidae) developmental staging system. Dev Growth Differ 49:301–324

Gatesoupe FJ (1999) The use of probiotics in aquaculture. Aquaculture 180:147–165

Gomez-Gil B, Herrera-Vega MA, Abreu-Grobois FA, Roque A (1998) Bioencapsulation of two different *Vibrio* species in nauplii of the brine shrimp (*Artemia franciscana*). Appl Environ Microbiol 64:2318–2322

Gordon HA, Pesti L (1971) The gnotobiotic animal as a tool in the study of host microbial relationships. Bacteriol Rev 35:390–429

Grisez L, Chair M, Sorgeloos P, Ollevier F (1996) Mode of infection and spread of *Vibrio anguillarum* in turbot *Scophthalmus maximus* larvae after oral challenge through live feed. Dis Aquat Org 26:181–187

Hawke JP, McWhorter AC, Stegerwalt AG, Brenner DJ (1981) *Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish. Int J Syst Bacteriol 31: 396–400

Huys L, Dhert P, Robles R, Ollevier F, Sorgeloos P, Swings J (2001) Search for beneficial bacterial strains for turbot (*Scophthalmus maximus* L.) larviculture. Aquaculture 193:25–37

Ingham CJ, Furneaux PA (2000) Mutations in the β subunit of the *Bacillus subtilis* RNA polymerase that confer both rifampicin resistance and hypersensitivity to NusG. Microbiology 146:3041–3049

Iregui CA, Guarin M, Tibata VM, Ferguson HW (2012) Novel brain lesions caused by *Edwardsiella tarda* in a red tilapia (*Oreochromis* spp.). J Vet Diagn Invest 24: 446–449

Keskin O, Secer S, Izgur M, Turkyilmaz S, Mkakosya R (2004) *Edwardsiella ictaluri* infection in rainbow trout (*Oncorhynchus mykiss*). Turk J Vet Anim Sci 28:649–653

Komar C, Turnbull JF, Roque A, Fajer E, Duncan NJ (2004) Effect of water treatment and aeration on the percentage hatch of demersal, adhesive eggs of the bullseye puffer (*Sphoeroides annulatus*). Aquaculture 229:147–158

Kostic AD, Howitt MR, Garrett WS (2013) Exploring host–microbiota interactions in animal models and humans. Genes Dev 27:701–718

Lavens P, Sorgeloos P (1996) Manual on the production and use of live food for aquaculture. FAO Fish Tech Paper No. 361. FAO, Rome

Levin BR, Perrot V, Walker N (2000) Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. Genetics 154:985–997

Li C, Zhang Y, Wang R, Lu J, Nandi S (2012) RNA-seq analysis of mucosal immune responses reveals signatures of intestinal barrier disruption and pathogen entry following *Edwardsiella ictaluri* infection in channel catfish, *Ictalurus punctatus*. Fish Shellfish Immunol 32:816–827

Marking LL, Rach JJ, Schreier TM (1994) Evaluation of antifungal agents for fish culture. Prog Fish-Cult 56:225–231

Marques A, François JM, Dhont J, Bossier P, Sorgeloos P (2004) Influence of yeast quality on performance of gnotobiotically grown *Artemia*. J Exp Mar Biol Ecol 310: 247–264

Marques A, Dinh T, Ioakeimidis C, Huys G and others (2005) Effects of bacteria on *Artemia franciscana* cultured in different gnotobiotic environments. Appl Environ Microbiol 71:4307–4317

Marques A, Ollevier F, Verstraete W, Sorgeloos P, Bossier P (2006) Gnotobiotically grown aquatic animals: opportunities to investigate host-microbe interactions. J Appl Microbiol 100:903–918

McDermott JR, Leslie FC, D'Amato M, Thompson DG, Grencis RK, McLaughlin JT (2006) Immune control of food intake: enteroendocrine cells are regulated by CD4+ T lymphocytes during small intestinal inflammation. Gut 55:492–497

Mereghetti L, Sitkiewicz I, Green NM, Musser JM (2008) Remodeling of the *Streptococcus agalactiae* transcriptome in response to growth temperature. PLoS ONE 3: e2785

Mitchell AJ, Radomski AA, Straus DL, Carter R (2009) The effect of hydrogen peroxide on the hatch rate of channel catfish eggs and on *Saprolegnia* spp. infesting channel catfish eggs. N Am J Aquaculture 71:276–280

Munro PD, Barbour A, Birkbeck TH (1995) Comparison of the growth and survival of larval turbot in the absence of culturable bacteria with those in the presence of *Vibrio anguillarum, Vibrio alginolyticus*, or a marine *Aeromonas* sp. Appl Environ Microbiol 61:4425–4428

Nagai T, Iwamoto E, Sakai T, Arima T and others (2008) Characterization of *Edwardsiella ictaluri* isolated from wild ayu *Plecoglossus altivelis* in Japan. Fish Pathol 43: 158–163

Pham LN, Kanther M, Semova I, Rawls JF (2008) Methods for generating and colonizing gnotobiotic zebrafish. Nat Protoc 3:1862–1875

Plumb JA, Hanson LA (2010) Tilapia bacterial diseases. In: Health maintenance and principal microbial diseases of cultured fishes. Wiley-Blackwell, Oxford, p 445–463

Plumb JA, Sanchez DJ (1983) Susceptibility of five species of fish to *Edwardsiella ictaluri.* J Fish Dis 6:261–266

Rach JJ, Gaikowski MP, Howe GE, Schreier TM (1998) Evaluation of the toxicity and efficacy of hydrogen peroxide treatments on eggs of warm- and coolwater fishes. Aquaculture 165:11–25

Rana KJ (1988) Reproductive biology and hatchery rearing of tilapia eggs and fry. In: Muir JF, Roberts RJ (eds) Recent advances in aquaculture. Croom Helm, London and Sydney and Timber press, Portland, OR, p 343–406

Rana KJ, Beveridge MCM (1989) Tilapias and their cultures. In: Coche A, Edwards D (eds) Selected aspects of warmwater fish culture. FAO, Rome, p 181

Rana KJ, Macintosh DJ (1988) A comparison of the quality of hatchery-reared *Oreochromis niloticus* and *O. mossambicus* fry. In: Pullin RSV, Bhukaswan T, Tonguthai K, Maclean JL (eds) Tilapia in aquaculture. Proc 2nd Int Symp Tilapia in Aquaculture. ICLARM, Manila, p 497–502

Reger PJ, Mockler DF, Miller MA (1993) Comparison of antimicrobial susceptibility, β-lactamase production, plasmid analysis and serum bactericidal activity in *Edwardsiella tarda, E. ictaluri* and *E. hoshinae.* J Med Microbiol 39: 273–281

Rodkhum C, Kayansamruaj P, Pirarat N (2011) Effect of water temperature on susceptibility to *Streptococcus agalactiae* serotype Ia infection in Nile tilapia (*Oreochromis niloticus*). Thai J Vet Med 41:309–314

Russo R (2011) An attenuated strain of *Edwardsiella ictaluri* is killed by channel catfish (*Ictalurus punctatus*) macrophages and confers protection in few days. Dyn Biochem Process Biotech Mol Biol 5:76–82

Russo R, Panangala VS, Wood RR, Klesius PH (2009) Chemical and electroporated transformation of *Edwardsiella ictaluri* using three different plasmids. FEMS Microbiol Lett 298:105–110

Salvesen I, Øie G, Vadstein O (1997) Surface disinfection of Atlantic halibut and turbot eggs with glutaraldehyde: Evaluation of concentrations and contact times. Aquacult Int 5:249–258

Schreier TM, Rach JJ, Howe GE (1996) Efficacy of formalin, hydrogen peroxide, and sodium chloride in fungal-infected rainbow trout eggs. Aquaculture 140:323–331

Sihag RC, Sharma P (2012) Probiotics: the new ecofriendly alternative measures of disease control for sustainable aquaculture. J Fish Aquat Sci 7:72–103

Sikorski J, Nevo E (2005) Adaptation and incipient sympatric speciation of *Bacillus simplex* under microclimatic contrast at 'Evolution Canyons' I and II, Israel. Proc Natl Acad Sci USA 102:15924–15929

Small BC, Wolters WR (2003) Hydrogen peroxide treatment during egg incubation improves channel catfish hatching success. N Am J Aquaculture 65:314–317

Sokal R, Rohlf F (1955) The principles and practice of statistics in biological research. Freeman & Company, New York, NY

Soto E, Maziel A, Riofrio A, Martinez A, Cabrejos ME (2012) *Edwardsiella ictaluri* as the causative agent of mortality in cultured Nile tilapia. J Aquat Anim Health 24:81–90

Soto E, Illanes O, Revan F, Griffin M, Riofrio A (2013) Bacterial distribution and tissue targets following experimental *Edwardsiella ictaluri* infection in Nile tilapia *Oreochromis niloticus.* Dis Aquat Org 104:105–112

Spor A, Koren O, Ley R (2011) Unravelling the effects of the environment and host genotype on the gut microbiome. Nat Rev Microbiol 9:279–290

StatSoft (2004) Electronic statistics textbook. StatSoft, Tulsa, OK. www.statsoft.com/textbook/

Stock I, Wiedemann B (2001) Natural antibiotic susceptibilities of *Edwardsiella tarda*, *E. ictaluri*, and *E. hoshinae.* Antimicrob Agents Chemother 45:2245–2255

Thavasi R, Aparnadevi K, Jayalakshmi S, Balasubramanian T (2007) Plasmid mediated antibiotic resistance in marine bacteria. J Environ Biol 28:617–621

Tinh NTN, Direckens K, Sorgeloos P, Bossier P (2008) A review of the functionality of probiotics in the larviculture food chain. Mar Biotechnol 10:1–12

USEPA (US Environmental Protection Agency) (2002) Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th edn. EPA-821-R-02-012, USEPA, Office of Water, Washington, DC

Verner-Jeffreys DW, Shields RJ, Birkbeck TH (2003) Bacterial influences on Atlantic halibut *Hippoglossus hippoglossus* yolk sac larval survival and start feed response. Dis Aquat Org 56:105–113

Verschuere L, Rombaut G, Huys G, Dhont J, Sorgeloos P, Verstraete W (1999) Microbial control of the culture of *Artemia* juveniles through preemptive colonization by selected bacterial strains. Appl Environ Microbiol 65: 2527–2533

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64:655–671

Waltman WD, Shotts EB, Hsu TC (1986a) Biochemical characteristics of *Edwardsiella ictaluri.* Appl Environ Microbiol 51:101–104

Waltman WD, Shotts EB, Hsu TC (1986b) Biochemical and enzymatic characterization of *Edwardsiella tarda* from the United States and Taiwan. Fish Pathol 21:1–8

Welker TL, Lim CE (2011) Use of probiotics in diets of tilapia. J Aquac Res Dev S1:014

Yousefian M, Amiri MS (2009) A review of the use of prebiotic in aquaculture for fish and shrimp. Afr J Biotechnol 8:7313–7318

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