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## Effect of the Human Probiotic Bacterium *Escherichia coli* Nissle (1917) on performance and immune response of Nile tilapia *Oreochromis niloticus*

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### ABSTRACT

If a human probiotic is beneficial for fish, its incorporation into diets would be much easier to accept. The present work assessed the effect of human probiotic *Escherichia coli* Nissle 1917 (EcN) on growth, feed conversion and immune response of Nile tilapia *Oreochromis niloticus*. Diets with various EcN concentrations were offered to fish for 8 weeks. The fish were also challenged with pathogenic bacteria to assess whether EcN improved immunity. Dietary EcN did not affect survival and growth. Hematology suggests an effect on the immune system but EcN did not improve survival of challenged tilapia. Accordingly, the immune response was probably targeting the EcN itself. A decrease in respiratory burst was probably caused by the iron chelating systems of EcN, while decrease in liver weight was related to intestinal inflammation and stress caused by the EcN. Tilapia appear not to harbor intestinal *Escherichia coli* even when surrounding water is replete.

### KEYWORDS

*Escherichia coli* Nissle; probiotic; immunity; Nile tilapia

Modern aquaculture is evolving from extensive and semi-intensive stocking numbers to more intensive farms. This intensification compromises water quality, and stresses the aquacultured species, affecting their growth and making them more susceptible to bacterial infections (Pulkkinen et al. 2010; Sundberg et al. 2016). To treat bacterial diseases, fish farmers often use antibiotics (Cabello 2006), a problematic procedure because of the proliferation of drug-resistant bacteria that affect both fish and public health (Santos and Ramos 2018). Accordingly, many researchers are now focusing their work on prophylaxis measures, including the use of probiotics (Irianto and Austin 2002).

Tilapias, the second most aquacultured species in the world after carps (FAO 2014), are prone to bacterial pathogens such as *Aeromonas hydrophila* and *Edwardsiella tarda* (Dong 2018). *Aeromonas hydrophila* causes septicemia, ulceration and exophthalmia (Ibrahim et al. 2008). *Edwardsiella tarda* affects internal organs such as liver, kidney, spleen and muscle (Mohanty and Sahoo 2007). Previous studies reported that

*A. hydrophila* and *E. tarda* isolated from several fish species including tilapias were multi-resistant to a variety of antibiotics (Belém-Costa and Cyrino 2006; Lee and Wendy 2017; Vivekanandhan et al. 2002). Therefore, hoping to find alternatives to the use of antibiotics, various researchers attempted to identify probiotics that would improve tilapia growth and health (van Hai 2015a). Various probiotics were found to have positive effects on growth, feed conversion and immune response of tilapia (El-Haroun, Goda, and Kabir Chowdhury 2006; Ferguson et al. 2010; Jatoba et al. 2011; Standen et al. 2015; Utami and Suprayudi 2015). However, the probiotics used were autochthonous to tilapias and thus susceptible to be resisted by evolving bacterial pathogens.

Probiotics have various modes of action. They can reduce viable counts of pathogenic organisms in the gut, or have an effect on microbial metabolism, or stimulate host immunity (Fuller 1989). Some probiotics act by competitive exclusion (Balcazar et al. 2007; Lazado et al. 2010), thus blocking pathogenic bacteria from colonizing the gut surface. Other probiotics produce antibacterial compounds that can inhibit pathogens (Balcazar et al. 2007; Zapata and Lara-Flores 2013; Zhou et al. 2010). Probiotics can also consume nutrients and chemicals essential for pathogen growth and survival thus making them unavailable (Gram et al. 1999). Furthermore, some probiotics modify the bacterial enzymatic activity to alter the microbial metabolism of pathogens (Goldin and Gorbach 1984). Lastly, probiotics can act by stimulating the immune system of the host, increasing the activity of various cell components such as immune cells, antibodies, inflammatory cytokines, etc. (Pirarat et al. 2006).

*Escherichia coli* Nissle 1917 (EcN), brand-name Mutaflor®, is a strain of motile *E. coli* (Wassenaar 2016) that is completely nonpathogenic to humans (Zyrek et al. 2007) and marketed as a human probiotic. In humans, EcN modulates immune responses, suppresses the development of allergic reactions (Bickert et al. 2009), and treats ulcerative colitis (Losurdo et al. 2015) as well as improves the general health of the consumer. *E. coli* Nissle is also known to outcompete intestinal pathogens (Hancock, Dahl, and Klemm 2010) and strengthen the tight junctions of the intestinal barrier (Zyrek et al. 2007) thus reducing infection via the gut. Because EcN exhibits beneficial probiotic activity in humans and other terrestrial animals, we were interested in investigating possible effects that *E. coli* Nissle could have on Nile tilapia. Moreover, because EcN is consumed by humans as a probiotic, it is safe to offer to aquacultured fish destined for human consumption. The present work investigated whether EcN can play the role of an allochthonous probiotic in the gut of aquacultured tilapia. We assessed *Escherichia coli* Nissle's effects on survival, growth, intestinal colonization, hematological factors, and immune response of Nile tilapia.

## Materials and methods

The present work was performed at the aquaculture research laboratory of the American University of Beirut (AUB), Lebanon. Nile tilapia *Oreochromis niloticus* broodstock were maintained in outdoor 1 m<sup>3</sup> circular tanks connected to a biological filter in a recirculation aquaculture system and offered a 40% crude protein, 6% lipid commercial feed (Rangen Inc., Buhl, Idaho, USA) twice daily to apparent satiation. Produced larvae were collected, placed in a separate tank and sex-reversed using  $\alpha$ -methyl testosterone in the feed. Larvae were then maintained in an outdoors recirculation system until used. A pilot experiment found that tilapia did not have EcN or any other type of *E. coli* in their guts and that after three weeks of offering EcN to tilapia juveniles, the bacteria established in the digestive system of the fish.

*Escherichia coli* Nissle was extracted from Mutaflor<sup>®</sup> capsules (2.5–25 × 10<sup>9</sup> CFU/capsule) and used to prepare 80% glycerol stocks. A growth curve (OD vs. CFU) was determined using the viable counts-spread plate method. A broth culture of EcN was then grown in Luria-Bertani (LB) medium to a density of 10<sup>9</sup> CFU/ml and was sprayed post extrusion on diets at 10<sup>6</sup>, 10<sup>7</sup>, and 10<sup>8</sup> CFU/g of feed offered to the fish. Fresh batches of diets were prepared every other week to ensure the viability of EcN. Fifteen sex-reversed Nile tilapia juveniles (6.8 ± 1.13 g; mean ± SD) were stocked per tank in an indoors research system consisting of sixteen 180 L fiberglass tanks connected to a biological filter. Each three tanks were randomly assigned one of the treatments, and the control group was offered a diet sprayed with LB medium only. For each treatment, a fourth tank (dissection tank) was stocked with 20 fish and offered one of the experimental feeds. Two fish were removed from this fourth tank every week, intestines removed aseptically and checked for EcN colonization.

Photoperiod was set at (14:10) (light:dark). Temperature was maintained around 28°C. Salinity and dissolved oxygen were measured daily using a YSI Model 85 oxygen meter (Yellow Springs Inc., OH, USA) and were 2.8 ppt and 6.5 mg/l, respectively. pH was measured daily with a commercial hand-held pH meter and ranged between 7.7 and 8.2. Total ammonia nitrogen and nitrite nitrogen were assessed weekly using a HACH Aquaculture Test Kit, Model FF-3 and remained very low.

## Fish growth and feed utilization

Nile tilapia fingerlings were fasted for 24 hours, size sorted by hand then the weights and lengths of 40 fish recorded. Fish were stocked and offered experimental diets at 5% body weight daily, divided into four equal feedings, six days a week for eight weeks. Fish in each tank were group weighed every other week

and ration adjusted accordingly. At the end of the eight week-period, fish were group weighed and final weights and lengths of individual fish recorded. Fulton-type condition index (K) was calculated:

$$K = (W/L^3) \times 100000, \text{ where } W = \text{fish weight(g)} \text{ and } L = \text{total length(mm)}.$$

Three fish per tank were removed, weighed and dissected. For each fish, liver and viscera were extracted, weighed and used to calculate hepatosomatic (HSI) and viscerosomatic (VSI) indices:  $\text{HSI (\%)} = 100 \times (\text{liver weight [g]} / \text{whole fish weight [g]})$

$$\text{VSI(\%)} = 100 \times (\text{viscera weight[g]} / \text{whole fish weight[g]}).$$

### ***EcN colonization of the gut***

Culture-dependent analysis was performed to test for EcN colonization of the guts of the fish. At the end of every week, two fish were randomly removed from the dissection tank in each treatment, anesthetized using tricaine methane sulfonate (MS222; Pharmaq, Fordingbridge, UK) followed by destruction of the brain. The guts were then aseptically removed, ground and spread onto agar plates (RAPID'E. coli 2 – BioRad) to assess for EcN colonization in the guts. At the end of the eight-week feeding period, three fish from every tank were dissected and EcN colonization of the gut assessed.

### ***Persistence of EcN after reverting to non-supplemented diets***

After termination of the eight-week feeding period and data collection, remaining fish were returned to their tanks and offered the control diet for 7 days. At the end of the week, three fish were again randomly removed from each tank, their guts homogenized with peptone, and the liquid mixture cultured on RAPID'E. coli 2 – BioRad plates to test for the presence of EcN.

### ***Hematological parameters***

At the end of the eight-week feeding period, the fish were fasted for 24 hours. Three fish per tank were randomly removed, anesthetized, and blood samples collected from their caudal arch using a heparinized 1 ml syringe and a 25-gauge needle. The blood was used to determine total red and white blood cell counts, differential white blood cell counts, hemoglobin, hematocrit, total plasma proteins and respiratory burst. To determine total red and white blood cell counts, 20  $\mu\text{l}$  of blood were added to 4 ml of Natt-Herrick's stain in heparinized tubes. Blood samples were then loaded into a Neubauer hemocytometer and blood cells counted (Natt and Herrick 1952). To get white

blood cells differential counts a drop of blood was smeared on a microscope slide. The slides were stained with Wright–Giemsa stain and 800 WBCs counted and differentiated (Klontz 1994). Hemoglobin (g/dL) was determined by Cyanmethemoglobin procedure (Larsen and Snieszko 1961). Hematocrit (%) was determined using the indirect method for hematocrit measurement (Klontz 1994). Total plasma protein (g/dL) was determined by using a veterinary refractometer (Alexander and Ingram 1980). Finally, respiratory burst was determined as described by (Secombes 1990).

### ***Culture of pathogenic bacteria***

*Edwardsiella tarda* (ATCC15947) and *Aeromonas hydrophila* (ATCC43874) were acquired from American Type Culture Collection (ATCC) in the form of freeze-dried pellets and glycerol stocks were prepared. Cultures of the bacterial strains were streaked on Brain Heart Infusion (BHI) plates and incubated for 18–24 hours. Single colonies were isolated and allowed to grow in a BHI broth to determine a growth curve (Time vs. OD). The optical density of the cultures was measured at 600 nm (OD<sub>600</sub>) for *A. hydrophila* and at 540 nm (OD<sub>540</sub>) for *E. tarda*. 100 µL of each culture was streaked every other hour on BHI plates that were incubated at 37°C. CFU counts were determined using viable counts-spread plate method and used to establish an OD vs. CFU curve.

### ***Challenging Nile tilapia, *Oreochromis niloticus*, with bacterial pathogens***

To test whether *Escherichia coli* Nissle (1917) (EcN) is a fish probiotic that helps in disease prevention, tilapias were stocked into an indoor research system consisting of sixteen 180 L fiberglass tanks connected to a biological filter and settling tank and offered EcN-rich diet (at 10<sup>7</sup> CFU/g) for six weeks. At the end of the six weeks, two challenges were performed. The first challenge consisted of injecting six groups of ten fish (18.6 ± 5.53 g; 0.1 ml per fish) with one of three concentrations (10<sup>6</sup>; 10<sup>7</sup>; 10<sup>8</sup>) of the pathogenic bacteria *Aeromonas hydrophila* and *Edwardsiella tarda*. Additionally, eight control groups of 10 fish were used. Treated fish were those offered the probiotic diet then injected with the pathogen. The treatments and controls were: 1- Probiotic + *E. tarda* (10<sup>6</sup>); 2- Probiotic + *E. tarda* (10<sup>7</sup>); 3- Probiotic + *E. tarda* (10<sup>8</sup>); 4- Probiotic + *A. hydrophila* (10<sup>6</sup>); 5- Probiotic + *A. hydrophila* (10<sup>7</sup>); 6- Probiotic + *A. hydrophila* (10<sup>8</sup>); 7- Control 1: Physiological saline (2 tanks); 8- Control 2: Brain Heart Infusion broth (2 tanks); 9- Control 3: *E. tarda* (10<sup>6</sup>); 10- Control 4: *E. tarda* (10<sup>7</sup>); 11- Control 5: *E. tarda* (10<sup>8</sup>); 12- Control 6: *A. hydrophila* (10<sup>6</sup>); 13- Control 7: *A. hydrophila* (10<sup>7</sup>); 14- Control 8: *A. hydrophila* (10<sup>8</sup>). After injection, fish were placed in 52 L aerated glass

tanks and survival monitored for 96 hours. Dead fish were removed and recorded when discovered.

The second challenge consisted of offering the fish pathogenic bacteria in the diet. Each bacterium was streaked on Brain Heart Infusion (BHI) plates and incubated for 18–24 hours. Single colonies were isolated and allowed to grow in BHI broth for 4 hours at 30°C (*A. hydrophila*) and 4 hours 30 minutes at 37°C (*E. tarda*), to reach an optical density (OD) of 1.07 and 1.2, respectively, equivalent to  $10^8$  CFU/ml. Fish feed was sprayed with either *A. hydrophila* or *E. tarda* to get a final concentration of  $10^7$  CFU/g.

The experimental setup consisted of four indoors recirculation systems, each consisting of three 180 L fiberglass tanks connected to a biological filter. Fifteen sex-reversed Nile tilapia juveniles ( $28 \pm 0.2$  g; mean  $\pm$  SD) were stocked into each of the 12 tanks. Fish in two of the systems were offered a diet with EcN at  $10^7$  CFU/g and fish in the other two systems were offered diets without EcN (control) for six weeks. Thereafter, treatments were assigned as: 1- Control fish offered *A. hydrophila* diet; 2- EcN fish offered *A. hydrophila* diet; 3- Control fish offered *E. tarda* diet; 4- EcN fish offered *E. tarda* diet. Fish were offered the feed twice a day to apparent satiation, for four days and survival monitored and recorded.

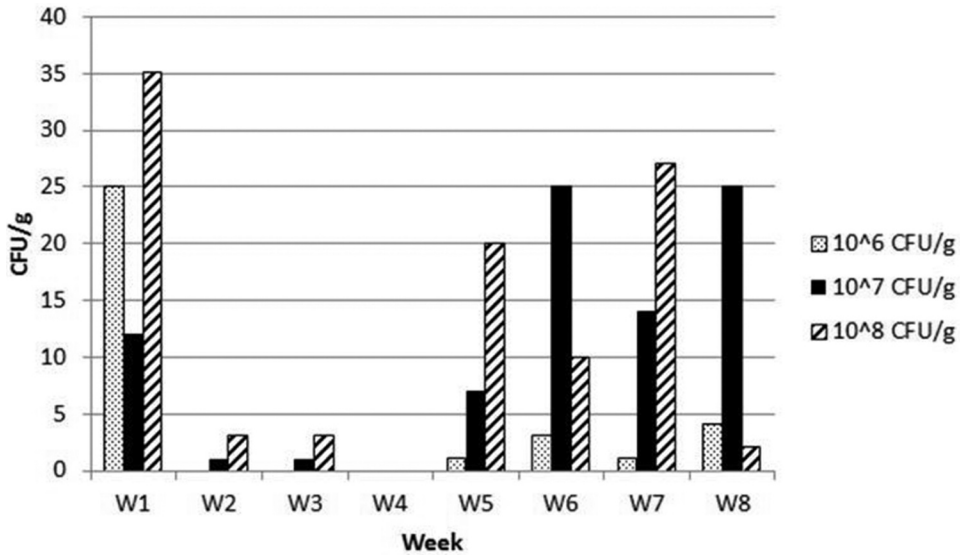
### **Statistical analysis**

Statistical analysis was performed using SAS (V.9.2, SAS Institute Inc., Cary, North Carolina, USA). All data were reported as mean values  $\pm$  standard deviation of the mean and compared using one-way ANOVA. Significant differences among means were analyzed using Student Newman–Keuls (SNK) mean separation test. Differences among treatment means were considered significant at  $p < .05$ .

## **Results**

### ***EcN colonization of the gut***

*E. coli* Nissle counts in the guts of the fish at the end of the first week were relatively high (25 CFU/g in  $10^6$  EcN; 12 CFU/g in  $10^7$  EcN; 35 CFU/g in  $10^8$  EcN). However, EcN counts decreased drastically after the second week, and remained low, varying between 0 and 5 CFU/g, until the fifth week. After the fifth week, EcN counts increased again reaching a maximum of 77 CFU/g at the end of week 8 in the  $10^7$  EcN treatment (Figure 1). After reverting to the non-EcN supplemented diets for a week, EcN counts in the gut decreased from 4, 25, and 2 CFU/g to 1, 5, and 1 CFU/g in  $10^6$ ,  $10^7$ , and  $10^8$  EcN treatments, respectively.



**Figure 1.** Weekly viable *Escherichia coli* Nissle (EcN) counts (CFU/g) in the guts of Nile tilapia offered control feed, 10<sup>6</sup> EcN feed, 10<sup>7</sup> EcN feed or 10<sup>8</sup> EcN feed for 8 weeks.

**Fish survival and growth rates**

Survival was 100% in all treatments and the fish grew in weight and length (Table 1). However, the various EcN concentrations did not have a significant effect on growth of the fish. The final weights ranged between 80.8 ± 15.25 g (mean ± SD) for the control to 86.3 ± 16.51 g for the fish offered EcN at 10<sup>7</sup> CFU/g. Fulton’s condition index (K) was significantly larger in 10<sup>7</sup> EcN fish than in control fish (*p* < .05).

Hepatosomatic index did not vary among fish offered the control diet (1.05 ± 0.17%) and fish offered 10<sup>6</sup> and 10<sup>7</sup> EcN (1.01 ± 0.1% and 0.89 ± 0.26%, respectively) (Table 1). However, the hepatosomatic index of fish offered 10<sup>8</sup> EcN (0.75 ± 0.14%) was significantly less than the HSI of control fish. There were no significant differences among viscerosomatic indices of fish offered the control diet (7.39 ± 0.86%), 10<sup>6</sup> EcN (7.50 ± 0.47%) and 10<sup>8</sup> EcN (7.02 ± 0.44%). However, viscerosomatic index of fish offered 10<sup>7</sup> EcN

**Table 1.** Survival (S; %), final body weight (FBW; g), final length (FL; cm), Fulton’s condition index at harvest (K), hepatosomatic (HSI; %) and viscerosomatic (VSI; %) indices of Nile tilapia, *Oreochromis niloticus*.

Treatment	S	FBW	FL	K	HSI	VSI
Control (No EcN)	100 <sup>a</sup>	80.8 <sup>a</sup>	16.71 <sup>a</sup>	1.71 <sup>b</sup>	1.05 <sup>a</sup>	7.39 <sup>b</sup>
10 <sup>6</sup> EcN	100 <sup>a</sup>	83.5 <sup>a</sup>	16.72 <sup>a</sup>	1.77 <sup>a,b</sup>	1.01 <sup>a</sup>	7.50 <sup>b</sup>
10 <sup>7</sup> EcN	100 <sup>a</sup>	86.3 <sup>a</sup>	16.86 <sup>a</sup>	1.80 <sup>a</sup>	0.89 <sup>a,b</sup>	8.16 <sup>a</sup>
10 <sup>8</sup> EcN	100 <sup>a</sup>	85.6 <sup>a</sup>	16.93 <sup>a</sup>	1.75 <sup>a,b</sup>	0.75 <sup>b</sup>	7.02 <sup>b</sup>
PSE*	-	2.31	0.14	0.02	0.056	0.200

Values in the same column with different superscripts are significantly different from each other (*p* < 0.05).

\*PSE: Pooled standard error.

( $8.16 \pm 0.63\%$ ) was significantly greater than the viscerosomatic indices of fish in all other treatments.

### Hematological parameters

There were no significant differences in hematocrit and total plasma protein among treatments (Table 2). Total red blood cell counts did not vary significantly among treatments ( $p > .05$ ) (Table 3). Total white blood cell counts varied significantly among treatments with the greatest value ( $1.05 \pm 0.49 \times 10^5$  cells/ $\mu\text{L}$ ) observed in fish offered the  $10^7$  EcN feed. There were no significant differences in neutrophils and monocytes among treatments. However, thrombocyte and lymphocyte percentages varied significantly among treatments ( $p < .05$ ), with fish offered the  $10^7$  EcN feed exhibiting the lowest proportion of thrombocytes ( $69.8 \pm 11.5\%$ ) and the greatest proportion of lymphocytes ( $29.7 \pm 9.8\%$ ). Additionally, hemoglobin concentrations were significantly different among treatments with the control fish having the least hemoglobin in their blood ( $10.54 \pm 1.49$  g/dL). Respiratory burst in the control ( $0.38 \pm 0.12$ ) was significantly greater than respiratory burst in all treatments. Respiratory burst in the  $10^8$  EcN treatment ( $0.16 \pm 0.06$ ) was less than in all other treatments.

### Challenging Nile tilapia, *Oreochromis niloticus*, with bacterial pathogens

EcN did not improve the survival of tilapia injected with *E. tarda* or *A. hydrophila*. Survival of fish injected with  $10^6$  and  $10^7$  CFU/ml

**Table 2.** Hemoglobin (Hb; g/dL), hematocrit (Hct; %), total plasma protein (TPP; g/dL), and respiratory burst of Nile tilapia, *Oreochromis niloticus*.

Treatment	Hb	Hct	TPP	RB
Control (No EcN)	10.54 <sup>b</sup>	23.33 <sup>a</sup>	4.91 <sup>a</sup>	0.38 <sup>a</sup>
$10^6$ EcN	13.08 <sup>a</sup>	25.67 <sup>a</sup>	4.58 <sup>a</sup>	0.22 <sup>b</sup>
$10^7$ EcN	11.56 <sup>a,b</sup>	24.25 <sup>a</sup>	4.26 <sup>a</sup>	0.19 <sup>b</sup>
$10^8$ EcN	11.03 <sup>a,b</sup>	23.50 <sup>a</sup>	4.33 <sup>a</sup>	0.16 <sup>b</sup>
PSE*	0.59	1.14	0.20	0.02

Values in the same column with different superscripts are significantly different from each other ( $p < 0.05$ ).

\*PSE: Pooled standard error.

**Table 3.** Total red blood cells (TRBC;  $\times 10^6/\mu\text{L}$ ), total white blood cells (TWBC;  $\times 10^5/\mu\text{L}$ ), and differential white blood cell count (in %) for Nile tilapia, *Oreochromis niloticus*.

Treatment	TRBC	TWBC	Neutrophils	Thrombocytes	Lymphocytes	Monocytes
Control	1.66 <sup>a</sup>	0.56 <sup>c</sup>	1.33 <sup>a</sup>	82.2 <sup>a</sup>	14.57 <sup>b</sup>	1.56 <sup>a</sup>
$10^6$ EcN	1.98 <sup>a</sup>	0.80 <sup>b</sup>	1.00 <sup>a</sup>	78.4 <sup>a,b</sup>	18.78 <sup>b</sup>	2.22 <sup>a</sup>
$10^7$ EcN	1.64 <sup>a</sup>	1.05 <sup>a</sup>	1.42 <sup>a</sup>	69.8 <sup>b</sup>	29.70 <sup>a</sup>	1.67 <sup>a</sup>
$10^8$ EcN	1.77 <sup>a</sup>	1.02 <sup>a</sup>	1.42 <sup>a</sup>	78.7 <sup>a,b</sup>	17.64 <sup>b</sup>	2.20 <sup>a</sup>
PSE*	0.09	0.06	0.22	2.71	2.30	0.32

Values in the same column with different superscripts are significantly different from each other ( $p < 0.05$ ).

\*PSE: Pooled standard error.

*A. hydrophila* was nearly 100% in both EcN and control fish. Fish injected with  $10^8$  *A. hydrophila* exhibited (80%) mortality in both control and EcN groups. Similarly, no mortalities were recorded in both the control groups and EcN groups injected with  $10^6$  *E. tarda* and  $10^7$  *E. tarda*. However, EcN fish injected with  $10^8$  *E. tarda* exhibited 40% mortality while control fish injected with *E. tarda* had 30% mortality, not significantly different from the treatment group. Fish that were in *E. tarda* control group, mainly those injected with  $10^8$  CFU/ml, had a bloated abdomen, characteristic of literature descriptions of *E. tarda* infection. Interestingly, the symptoms of disease were not apparent in the EcN fed group. No mortalities were recorded in fish injected with physiological saline or with BHI broth.

When the fish were offered *A. hydrophila* and *E. tarda* in the feed, no mortalities were recorded in the control groups and in EcN groups. However, the control group fish were refusing the feed sometimes and exhibiting an agitated behavior while EcN fish were feeding normally and appeared to be calmer.

## Discussion

Usually, probiotics used in aquaculture are autochthonous to the organisms being reared (Irianto and Austin 2002). These probiotics are often isolated from the species itself and therefore are sure to be safe to the host (van Hai 2015b). The use of autochthonous probiotics usually guarantees successful colonization but narrows the range of probiotic organisms that can be used to those indigenous to the host. Moreover, historical interactions between pathogen and autochthonous probiotic allow pathogens to evolve and become less susceptible to the probiotic's effects. Allochthonous bacteria that are not normally present in the GI tract of the host offer potential resistance to a wider range of pathogens but colonization of the gut of an allochthonous fish is not guaranteed. In the present study, EcN did not significantly affect the growth performance of Nile tilapia nor permanently colonize the guts of the fish. Thus, the probiotic was unable to affect digestive enzymes or nutrient uptake in the fish. Consequently, any discussion of its probiotic effect on fish growth in comparison to that of other well-known probiotics is moot.

## Colonization and growth

A very interesting finding of the present work was that although culture water of the fish was full of *E. coli*, the gastrointestinal tract of our *Oreochromis niloticus* contained none. During the first week of offering EcN-rich diets to tilapia, EcN got established in the gut of the fish. Presumably, the immune system of the fish was not yet primed to resist the initial colonization by the probiotic, resulting in high EcN counts in the guts (between 12 and 35 CFU/g). By the end of the first week, the immune system of tilapia seemed to have

identified EcN as a foreign organism, causing EcN counts to drop between the second and fifth week. After the fifth week, EcN colonization started to increase again suggesting a reduction in the potency of the immune system. However, even after colonization, EcN counts decreased when EcN in the feed was discontinued. A transient colonization was observed, mainly in the  $10^7$  CFU/g treatment but the bacteria decreased in the gut as soon as their supply in the diet was discontinued. Previous studies on mammals showed that a permanent colonization by EcN is only achieved in gnotobiotic models (Vlasova et al. 2016), neonates (Lodinová-Žádníková and Sonnenborn 1997) or previously antibiotic-treated individuals. In these cases, the internal microbiota is either unable to interfere with EcN's introduction or is already altered by the antibiotic treatment and thus cannot resist the colonization. In healthy individuals, however, the indigenous microbial species appear able to out-compete the intruder and prevent successful EcN colonization (Sonnenborn and Schulze 2009).

### ***Hepatosomatic index***

Results showed a decrease in HSI as the concentration of EcN in the diets increased. A smaller liver is usually associated with chronic stress in the fish (Heath 2017). The introduction of a foreign microorganism, pathogenic or not, into the body of the fish could cause such a stress (Francis-Floyd 1992). We postulate that EcN in the diets of Nile tilapia was sufficiently recognized as a foreign organism and thus altered the physiological status of the fish resulting in decreased liver growth.

### ***Hematological parameters***

EcN induced a significant increase in total white blood cell counts which tends to suggest the presence of an infection. However, no mortality was observed probably because EcN has a modified lipopolysaccharide (LPS) that grants the probiotic immunomodulating properties that stimulate the immune system without being toxic to the host (Sonnenborn and Schulze 2009). Although  $10^7$  CFU/g feed induced an increase in WBCs, we observed a decrease in WBCs when dietary EcN was increased to  $10^8$  CFU/g EcN. Sakai (1999) suggested that an overdose of immunostimulant might suppress the immune responses in the host, which could result in a decrease in WBCs, similar to what we observed. Moreover, while EcN did not affect counts of neutrophils and monocytes, the  $10^7$  CFU/g treatment caused a significant decrease in thrombocytes accompanied by a significant increase in lymphocytes. The increase in lymphocytes would suggest that the immune system recognized EcN as a foreign organism and was fighting its intrusion into the body of the fish but the decrease in thrombocytes suggests that the treated fish probably do not

have intestinal lesions since thrombocytes are primarily involved in hemostasis (Merrifield et al. 2011). An explanation of what might be happening is that intestinal lesions caused by dietary soybean meal (Merrifield et al. 2011) can lead to deformation of enterocytes and exposure of the intestinal tight junctions. When EcN is added to the diet, cross-talk between EcN and enterocytes promote the synthesis of tight junction proteins that restore the integrity of the gut (Zyrek et al. 2007). We thus suggest that in the control fish, soybean meal present in the diets caused lesions in the intestines leading to a proliferation of thrombocytes. However, in the treated fish, EcN strengthened the junctions between the enterocytes, which mitigated intestinal lesion problems and thus thrombocyte counts decreased. Accordingly, we postulate that if EcN is offered to fish that have *E. coli* in their guts, we might observe a positive effect on the fish.

### ***Respiratory burst***

Respiratory burst was greater in the control than in the EcN treatments. Previous studies suggest that the use of probiotics such as *Enterococcus faecium* (Wang et al. 2008), *Bacillus subtilis* and *Lactobacillus acidophilus* (Aly et al. 2008), or a mixture of *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Aspergillus oryzae* (Iwashita et al. 2015) enhances the innate immunity of Nile tilapia by increasing the respiratory burst activity in the fish. One explanation for the decreasing respiratory burst in the present work would be that EcN chelated the iron needed for the Fenton reaction to occur. In vivo, respiratory burst occurs via the Fenton reaction which is catalyzed by Fe ions (Trenam, Blake, and Morris 1992). EcN takes up ferric ions by producing various siderophores such as aerobactin, hemin- and citrate-dependent iron acquisition system, etc. (Sonnenborn and Schulze 2009). EcN also possesses a ferrous iron uptake system (EfeU) known as YcdN (Große et al. 2006). Accordingly, present results suggest that EcN chelated the iron from the feed, thus lowering iron levels in the blood and decreasing the respiratory burst observed in tilapias offered the probiotic treatments.

### ***Challenge with bacterial pathogens***

Survival of tilapia following challenge with *A. hydrophila* and *E. tarda* was not improved by dietary EcN supplementation. Usually, EcN protects hosts from infection by promoting the synthesis of tight junction proteins (Zyrek et al. 2007), thus restoring the integrity of intestinal tight junctions and preventing bacterial entry into the body (Lu, Yang, and Hu 2014). Dietary probiotics can also have immunomodulating properties that stimulate the immune system of fish and thus protect them from pathogens (Aly et al. 2008; Pirarat et al. 2006). However, in the present study, because EcN could not permanently colonize

the guts of tilapia, we assume that it was not able to promote tight junction formation nor improve fish immunity. The slightly higher mortality in the EcN fed groups could have resulted from stress induced by exposure of the gut to foreign microorganism, EcN.

In conclusion, EcN was not recognized as a probiotic in tilapia but rather as a foreign organism that triggered an immune response. Regardless, the potential use of allochthonous probiotics on aquatic species remains promising, especially as a means to decrease antibiotic use. Allochthonous probiotics comprise a wide range of candidates to select from, and the problem remains in ensuring that said probiotics are capable of colonizing the GI tract of the host. Moreover, EcN could still be a very good choice of probiotic for fish that have a natural *E. coli* gut flora.

### Disclosure statement

No potential conflict of interest was reported by the authors.

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