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DOES THE HUMAN PROBIOTIC BACTERIUM *ESCHERICHIA COLI* NISSLE (1917) AFFECT GROWTH PERFORMANCE AND IMMUNE RESPONSE OF NILE TILAPIA *OREOCHROMIS NILOTICUS*?

by

RAZAN SALMAN ZEIN EDDINE

A thesis
submitted in partial fulfillment of the requirements
for the degree of Master of Science
to the Department of Biology
of the Faculty of Arts and Sciences
at the American University of Beirut

Beirut, Lebanon
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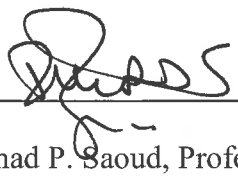

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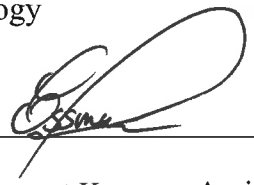

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AN ABSTRACT OF THE THESIS OF

Razan Salman Zein Eddine for Master of Science
Major: Biology

Title: Does the human probiotic bacterium *Escherichia coli* Nissle (1917) affect growth performance and immune response of Nile tilapia *Oreochromis niloticus*?

Probiotics are used in aquaculture as health and growth promoters as well as prophylaxis to reduce the use of antibiotics. *Escherichia coli* Nissle 1917 (EcN) is a human probiotic known to outcompete intestinal pathogens and strengthen the tight junctions of the intestinal barrier in mammals. We were not able to find literature that discusses the use of human probiotics as piscine probiotics. The aim of the present work was to determine the best way of incorporating EcN into fish feed as well as to assess the effects of EcN on survival, growth and immune response of Nile tilapia *Oreochromis niloticus*.

EcN was extracted from Mutaflor® capsules, grown in BHI broth and sprayed on the diets of the fish at various concentrations and offered to them for 8 weeks. At the end of the 8 weeks survival, growth, condition index, HSI, VSI and hematological parameters were evaluated. A group of EcN fed fish were also used to test the persistence of EcN in tilapia after reverting to the control diets. Another group of EcN fed fish was challenged with *Aeromonas hydrophila* and *Edwardsiella tarda* either by feeding or through injection. Results showed that EcN does not have a significant effect on survival and growth of Nile tilapia ($p > 0.05$). However, EcN has a significant effect on total white blood cell counts, hemoglobin, thrombocytes, lymphocytes, respiratory burst and HSI ($p < 0.05$).

Dietary EcN did not affect survival and growth and was not able to permanently colonize the guts of tilapia. Hematological parameters suggest an effect on the immune system of the fish offered EcN and but there was no difference in survival between probiotic offered fish and controls with regards to pathogen challenged individuals. Therefore, we can conclude that EcN was not able to act as a probiotic in tilapia and the immune response observed was probably targeting the EcN itself. The decrease in respiratory burst is probably caused by the iron chelating systems of EcN, whilst the decrease in liver weight is related to intestinal inflammation and stress caused by the EcN in the guts of the fish. EcN was not effective in tilapia but might work as a probiotic in fish that have a natural flora of intestinal *E. coli*.

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ABBREVIATIONS

%	Percent
BHI	Brain Heart Infusion
CFU	Colony Forming Unit
EcN	*Escherichia coli* Nissle
FBW	Final Body Weight
Hb	Hemoglobin
Hct	Hematocrit
HSI	Hepatosomatic Index
K	Fulton's Condition Index
LB	Luria Bertani
LPS	Lipopolysaccharide
OD	Optical Density
RBC	Red Blood Cell
S	Survival
SD	Standard Deviation
SE	Standard Error
TAN	Total Ammonia Nitrogen
TTP	Total Plasma Protein
VSI	Viscerosomatic Index
WBC	White Blood Cell

CHAPTER I
INTRODUCTION

Aquaculture is one of the fastest-growing food production industries in the world, providing more than half of all fish for human consumption (FAO, 2016). Increasing demand for food fish is causing an increase in intensive aquaculture. High stocking densities cause degradation of water quality and stress the aquacultured species, thus affecting growth and making animals more susceptible to bacterial infections (Pulkkinen *et al.*, 2010; Sundberg *et al.*, 2016), leading to serious economic losses. To treat bacterial diseases, fish farmers often use antibiotics (Cabello, 2006), resulting in the proliferation of drug-resistant bacteria that affect both fish and public health (Santos & Ramos, 2018). Accordingly, scientists and health workers are focusing on prophylaxis measures, including the use of probiotics (Irianto & Austin, 2002), rather than treat with antibiotics.

A. Probiotics in aquaculture

Probiotics are live microorganisms offered to a host as a feed supplement to improve the host's health status (Fuller, 1989). Probiotics can either be naturally present in the intestinal microbiota of the host (autochthonous probiotics) or be probiotics to other species (allochthonous probiotics). For bacteria to be denominated as probiotics, they must not be pathogenic to the host species or the consumer of said host species. Moreover, probiotics must not exhibit a plasmid-mediated resistance and should be able to tolerate low pH and bile salts (Merrifield *et al.*, 2010). In other words, when administered in feeds, probiotics must be able to survive the passage through the digestive system and to proliferate in the colon (FAO/WHO, 2001).

Probiotics used in aquaculture include both Gram-positive and Gram-negative bacteria, as well as various microalgae, yeasts and bacteriophages. The first probiotic reported to have been used in aquaculture was *Bacillus toyoi*, a growth promoter for

yellow tail, *Seriola quinqueradiata* (Kozasa, 1986). Subsequently, probiotics were used to improve water quality either by decreasing the amount of ammonia and nitrite in the water (Porubcan, 1991b) or by reducing biochemical oxygen demand (Porubcan, 1991a). Starting in the late 1990s, most probiotics in aquaculture have become feed additives used to stimulate growth, enhance appetite, and stimulate immune response (Irianto & Austin, 2002).

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). To decrease viable microbial counts, some probiotics act by competitive exclusion (Balcazar et al., 2007; Lazado, Caipang, Brinchmann, & Kiron, 2011), blocking pathogenic bacteria from colonizing the gut surface. Other probiotics produce antibacterial compounds that can inhibit pathogens (Balcazar et al., 2007; Zapata & Lara-Flores, 2013; Zhou, Wang, Yao, & Li, 2010). Probiotics can also consume nutrients and chemicals essential for pathogen growth and survival thus making them unavailable (Gram, Melchiorsen, Spanggaard, Huber, & Nielsen, 1999). Furthermore, some probiotics modify bacterial enzymatic activity to alter the microbial metabolism of pathogens (Goldin & 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, Kobayashi, Katagiri, Maita, & Endo, 2006).

B. Nile tilapia

Tilapias, are fresh water fishes that belong to the Cichlidae family (El-Sayed, 2006). They are the second most aquacultured species in the world after carps (FAO, 2014). Tilapias are able to adapt to a wide range of environmental conditions, are easy to handle, and are relatively resistant to aquaculture stress when compared to other finfish species (Welker & Lim, 2011). Nevertheless, tilapias are prone to bacterial pathogens

such as *Aeromonas hydrophila* and *Edwardsiella tarda* (Standen *et al.*, 2015). Previous studies reported that *A. hydrophila* and *E. tarda* isolated from several fish species, including Nile tilapia *Oreochromis niloticus* and red hybrid tilapia, were multi-resistant to a variety of antibiotics (Belém-Costa & Cyrino, 2006; Lee & Wendy, 2017; Vivekanandhan, Savithamani, Hatha, & Lakshmanaperumalsamy, 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, 2015). Various probiotics were found to have positive effects on growth, feed conversion and immune response of tilapia (El-Haroun, Goda, & Kabir Chowdhury, 2006; Ferguson et al., 2010; Jatoba et al., 2011; Standen et al., 2015; Utami & Suprayudi, 2015). However, the probiotics used were autochthonous to tilapias and thus susceptible to be resisted by evolving bacterial pathogens.

C. Aeromonas hydrophila

Aeromonas hydrophila is a rod-shaped, gram negative non-spore forming facultative anaerobe found in fresh water. *A. hydrophila* is ubiquitous in limnetic environments and is naturally present in the gastrointestinal tract of fish. *A. hydrophila* is a secondary invader, often preceded by stress caused by poor water quality or by other infections or even bad handling (Scullion, 2008). In fish, *A. hydrophila* causes "Hemmorhagic Septicemia", also known as "Motile Aeromonas Septicemia" (MAS) (Bullock, Conroy, & Sniesko, 1971). When infected, fish display various symptoms such as bloated appearance, pale gills, swimming abnormalities, exophthalmia, and sudden death (Ibrahem, Mostafa, Arab, & Rezk, 2008; Swann & White, 1991). There are numerous reports of *A. hydrophila* infection in Nile tilapia (Fernandes et al., 2019; Ibrahem et al., 2008; Noor El Deen, Dorgham, Hassan, & Hakim, 2014; Yardimci & Aydin, 2011), many of which led to serious economic losses caused by morbidity and mortality.

D. Edwardsiella tarda

Edwardsiella tarda is a motile gram-negative facultative anaerobe, typically found in fresh and brackish water environments. In fish, *E. tarda* causes edwardsiellosis also known as Edwardsiella septicemia. Fish infected with *E. tarda* are characterized by a swelling in the abdominal region, abnormal swimming, and exophthalmia (Mohanty & Sahoo, 2007). *Edwardsiella tarda* is known to infect Nile tilapia, affecting blood parameters by decreasing hematocrit values and erythrocyte counts and increasing leukocyte counts (Karasu Benli & Yavuzcan Yildiz, 2004). *E. tarda* is a zoonotic bacterium, and is recognized as a major pathogen in aquaculture (Haenen, 2017), leading to serious losses in the industry.

E. Escherichia coli Nissle 1917

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 can modulate immune responses, suppress the development of allergic reactions (Bickert *et al.*, 2009), and treat ulcerative colitis (Schultz *et al.*, 2004) as well as improve the general health of the consumer. *E. coli* Nissle is known to outcompete intestinal pathogens (Hancock, Dahl, & 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 should be safe to offer to aquacultured fish destined for human consumption. No literature was found that discusses the presence of EcN in the gut microbiota of tilapia. Accordingly, we investigated whether EcN can play the role of an allochthonous probiotic in the gut of aquacultured Nile tilapia. We assessed *Escherichia*

coli Nissle's effects on survival, growth, intestinal colonization, hematological factors, and immune response of Nile tilapia.

CHAPTER 2
MATERIALS AND METHODS

A. Fish acquisition and maintenance

Nile tilapia *Oreochromis niloticus* used in the present work were spawned at the aquaculture research laboratory of the American University of Beirut (AUB), Lebanon. Broodstock were maintained in outdoor 1 m^3 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. Larvae were collected, placed in a separate tank and sex-reversed using α- methyl testosterone in the feed. Larvae were then maintained in an outdoors recirculation system until used in the research.

B. Preliminary study

A pilot experiment was performed first to determine whether our tilapia had EcN in their guts and then to assess whether commercial EcN human probiotic would get established in tilapia guts and whether it might affect growth. First, we checked for the presence of EcN in the guts of Nile tilapia after starving them for 24 hours. Several fish were randomly taken, euthanized by overdose of tricaine methane sulphonate (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). The plates were incubated for 24 hours at 37ºC.

Escherichia coli Nissle was purchased from a local apothecary as commercial capsules, Mutaflor®. Powder was extracted from the capsules (2.5 - 25×10^9 CFU/capsule) and a small portion was transferred with a sterile pipette tip to liquid

Luria-Bertani (LB) medium. The liquid EcN culture was then added to cryovials containing 80% glycerol. The cryovials were stored at -80°C. To determine an EcN growth curve, EcN cultures were taken from the frozen stock and grown on RAPID'*E. coli* 2 (BioRad) agar plates. The plates were incubated for 24 hours and violet colonies of EcN were collected and re-cultured (heavy streaking) then stored in glycerol stocks for later use. Concurrently, single colonies were isolated and allowed to grow in LB broth. The optical density (at 600 nm OD_{600}) of the culture was measured using a spectrophotometer (time points every two hours). The OD_{600} of the culture was used to determine a growth curve (optical density vs. time) (Fig.1). The culture was plated every other hour on Rapid 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 (Fig. 2).

A test was performed to determine the best way to incorporate EcN in the diets of Nile tilapia, and to test the effect of EcN on the growth of the fish. In order to find the best way of incorporating EcN in fish feed, two 1 Kg batches of feed mix were prepared and an EcN liquid culture (10^9 CFU/ml) added to each batch. In the first batch, EcN was mixed with the ingredients before extrusion to get a final concentration of 10^8 CFU/Kg. In the second batch, EcN was sprayed on the diet after manufacturing to get a final concentration of 10^8 CFU/Kg. Diets were spread on RAPID'*E. coli* 2 plates on a weekly basis to check for the presence of EcN. Results showed that when EcN was sprayed on the diet after manufacture, the probiotic remained viable for at least four weeks, whilst when EcN was mixed in the diet before extrusion, the probiotic did not survive. Juvenile tilapias were stocked into twelve indoors 52L glass tanks each with its own submerged biological filter. Each four tanks were randomly assigned one of the treated diets (EcN sprayed on the feed or mixed with the ingredients before

manufacture), or the control diet without EcN. Fish were offered the diets at 5% body weight daily divided into two equal feedings, six days a week for six weeks.

C. Main Experiment

Based on the results of the preliminary study, an experiment was preformed where EcN was sprayed on diets at 10^6, 10^7, and 10^8 CFU/Kg of feed and offered to tilapia. Sex-reversed Nile tilapia juveniles (6.8 ± 1.13g; mean ± SD) were stocked into an indoors research system consisting of four batteries of three tanks (180-L fiberglass tanks), each connected to a biological filter. Fresh batches of diets were prepared every other week to ensure the viability of EcN. Each three tanks were randomly assigned one of the treatments, and the control group was offered a diet sprayed with LB medium only. Photoperiod was set at (14:10) (light:dark). Total ammonia nitrogen and nitrite nitrogen levels were tested weekly using a HACH Aquaculture Test Kit, Model FF-3.

Water parameters (temperature, oxygen concentration, pH, salinity) were monitored daily and maintained at the levels conducive to good growth of tilapia. Temperature was set at 26 – 29°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.

Figure 1. Standard growth curve (Time points vs. OD_{600}) of *Escherichia coli* Nissle 1917.

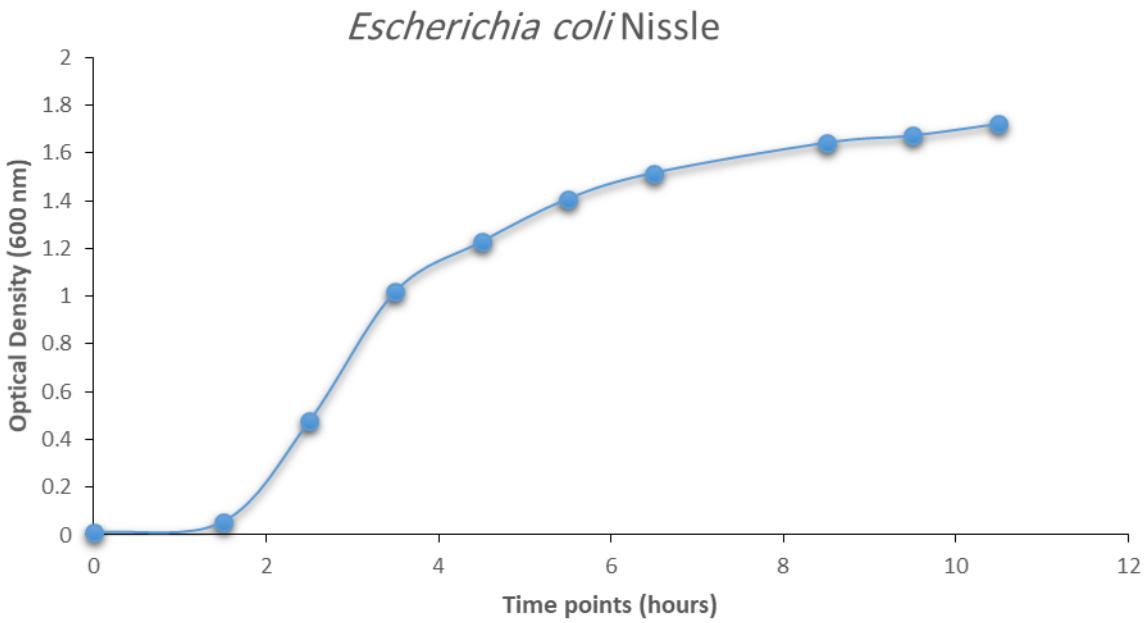


Figure 2. Standard curve (OD_{600} vs. Log CFU/ml) of *Escherichia coli* Nissle 1917 (EcN).

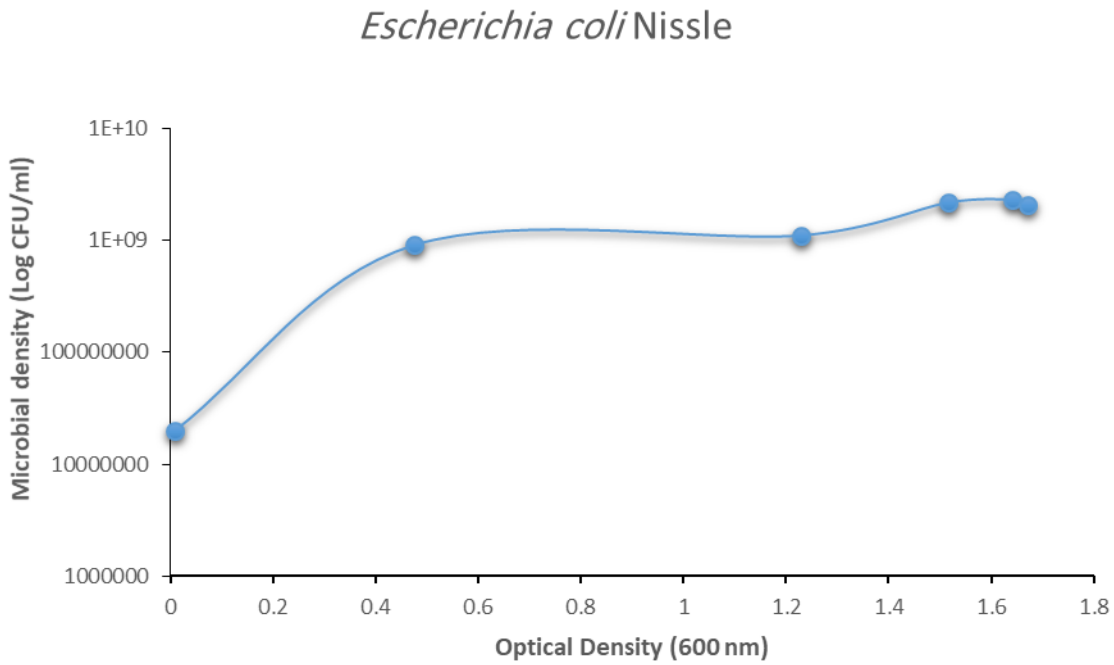
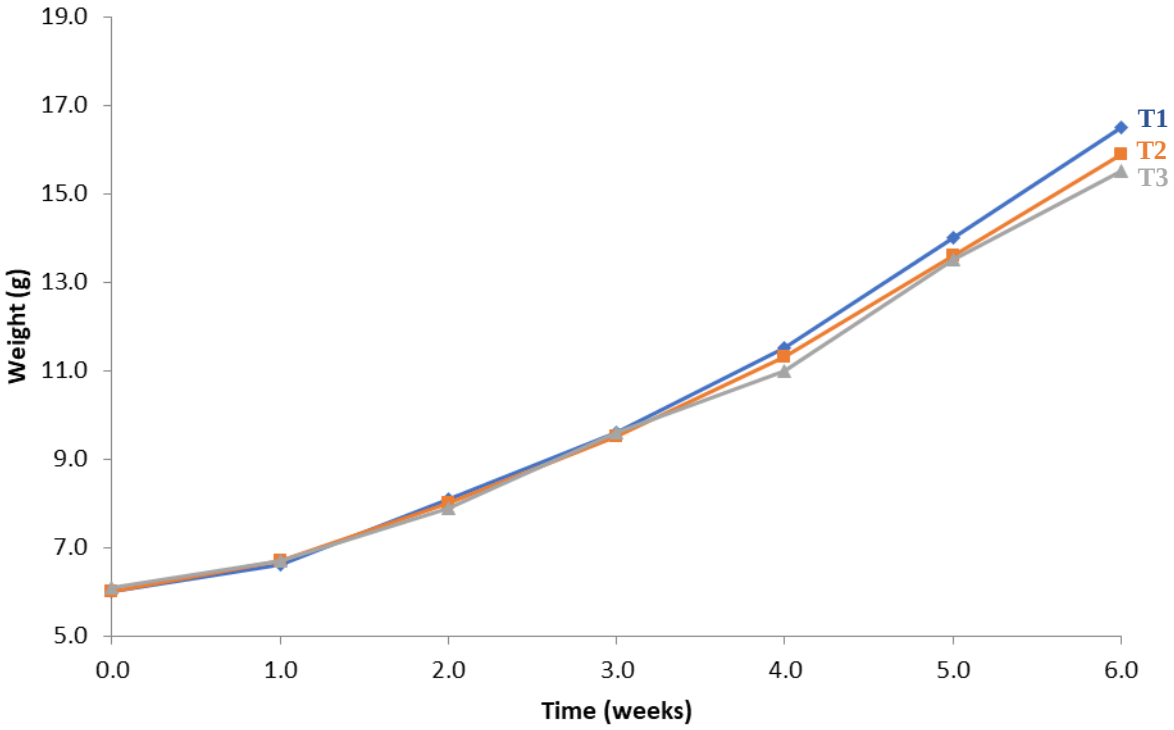


Figure 3. Growth (g) of juvenile *Oreochromis niloticus* offered a control diet (T1), a diet sprayed with *Escherichia coli* Nissle (EcN) (T2), a diet with EcN incorporated within the mix (T3) over six weeks.



D. Fish growth rates and feeds utilization

At the beginning of the feeding experiment, Nile tilapia fingerlings were fasted for 24 hours, size sorted by hand then the weights and lengths of 40 fish recorded. Fish were offered experimental diets at 5% of their 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: $K = (W/L^3) \times 100{,}000$, where W = fish weight (g) and L = total length (mm).

Nine to twelve fish per treatment were removed, weighed and dissected. For each fish, liver and viscera were extracted, weighed and used to calculate hepatosomatic (HSI) and viscersomatic (VSI) indices:

HSI (%) = 100 x (liver weight [g] /whole fish weight [g])

VSI (%) = 100 x (viscera weight [g]/whole fish weight [g]).

E. EcN colonization of the gut

Throughout the feeding experiment culture-dependent analysis was performed to test for EcN colonization of the guts of Nile tilapia. At the end of every week, two fish were randomly removed from each treatment, euthanized by overdose of tricaine methane sulphonate (MS222; Pharmaq, Fordingbridge, UK) followed by destruction of

Table 1. Averages of water quality parameters (Temperature, salinity, total ammonia nitrogen (TAN), nitrite, pH) measured on a weekly basis during the growth experiment.

Parameter	**Mean ± SD**
Temperature (ºC)	26.28 ± 0.48
Salinity (ppt)	2.8 ± 0.77
TAN (ppm)	0.27 ± 0.12
Nitrite-N (ppm)	0.1 ± 0.08
pH	7.67 ± 0.29

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.

F. Persistence of EcN after reverting to non-supplemented diets

After termination of the feeding period, we checked for persistence of EcN in the guts in the absence of EcN-supplemented feed. At the end of the eight week-period, we stopped offering the fish the EcN-rich diets and randomly took three fish from each tank and assessed EcN numbers in the gut. The remaining fish were 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.

G. Hematological parameters

After termination of the growth experiment, tilapias were fasted for 24 hours. Three fish per tank were randomly removed, anesthetized, and blood samples collected from their caudal arch using a heparinized 1ml syringe and a 25 gauge needle. The blood was used to determine total red and white blood cells counts, white blood cell differential counts, hemoglobin, hematocrit, total plasma proteins and respiratory burst.

To get the total red and white blood cells counts, 20µL of blood were added to 4ml of Natt-Herrick's stain in heparinized tubes (Nutt & Herrick, 1952). The samples were mixed vigorously, held at room temperature for five minutes, and then loaded into both chambers of a modified Neubauer hemocytometer. Blood cells were counted, using 40x objective lens. To get white blood cells differential counts, a drop of blood was smeared on a microscope slide and fixed with ethanol. The slides were stained with Wright-Giemsa stain. On each slide, 800 WBC were counted (Klontz, 1994) . Hemoglobin (g/dL) was determined using the cyanmethemoglobin procedure by adding

0.02ml of whole blood to 5ml of Drabkin's solution and measuring the absorbance using a spectrophotometer at 540nm (Larsen & Snieszko, 1961). Hematocrit (%) was determined using the (Klontz, 1994) indirect method. Blood samples were filled in capillary tubes, spun in a centrifuge at 10,000 g for 5 minutes, and then placed in a hematocrit reader to determine hematocrit value. Plasma was separated from the red blood cells in the capillaries used for hematocrit determination, and total plasma protein (g/dL) was determined by using a veterinary refractometer (Alexander & Ingram, 1980). Respiratory burst was determined using the (Secombes, 1990) procedure. Heparinized blood samples were placed into 'U' well microtiter plates (50 µL/well) and incubated for two hours at 22ºC. Supernatant was removed and the wells were washed three times with phosphate buffered saline (PBS). 50 µL of 0.2% Nitroblue Tetrazolium (NBT) and phorbol myristate acetate (PMA) were added to each well, and the samples were incubated for one hour at 22ºC. The cells were fixed with 100% methanol for 2-3 minutes. The wells were then washed 3 times with 70% (v/v) methanol and allowed to air dry. 60µL of 2M Potassium Hydroxide (KOH) and 70µL of Dimethyl sulphoxide (DMSO) were added to each well, air bubbles were removed and absorbance was measured at 540 nm.

H. Culture of pathogenic bacteria

Edwardsiella tarda (ATCC15947) was acquired from American Type Culture Collection (ATCC) in the form of freeze-dried pellets. One freeze-dried pellet was aseptically transferred into 1 ml of nutrient broth and the mixture was homogenized. The culture was then transferred to a tube containing 5 ml of nutrient broth, homogenized and incubated at 37°C. The incubated liquid culture was used the next day to prepare glycerol stocks. *Aeromonas hydrophila* (ATCC43874) was acquired from glycerol stocks at the immunology laboratory at AUB. To increase *Aeromonas hydrophila* virulence, 0.1 ml of *Aeromonas hydrophila* liquid culture was injected into

five fish. None of the injected fish died, so we dissected a fish and extracted its liver. The liver was homogenized in Brain Heart Infusion (BHI) broth, then plated on BHI plates. *Aeromonas hydrophila* colonies were then transferred to BHI broth and incubated at 30°C. The broth was subsequently injected in fish resulting in their death. The BHI plates were used to prepare new *A. hydrophila* stocks.

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 using a spectrophotometer. For *Aeromonas hydrophila*, OD was recorded every two hours at 600 nm (OD_{600}) (Fig. 4) and 100 µL of the culture streaked on BHI plates and incubated at 30°C. CFU counts were determined using viable counts-spread plate method, and used to establish an OD vs. CFU curve (Fig. 5). For *Edwardsiella tarda* OD was recorded every hour at 540 nm (OD_{540}) (Fig. 6) and 100 µL of the culture was streaked 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 (Fig. 7).

I. 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 indoors research system, consisting of

Figure 4. Standard growth curve (Time points vs. OD_{600}) of *Aeromonas hydrophila.*

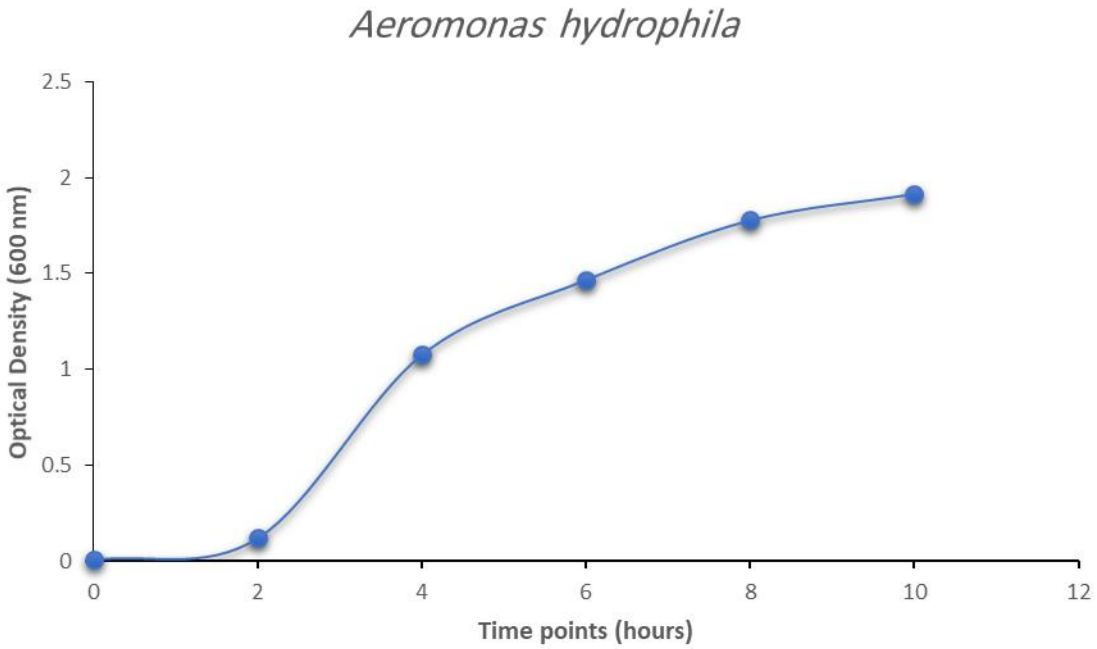


Figure 5. Standard curve (OD_{600} vs. Log CFU/ml) of *Aeromonas hydrophila.*

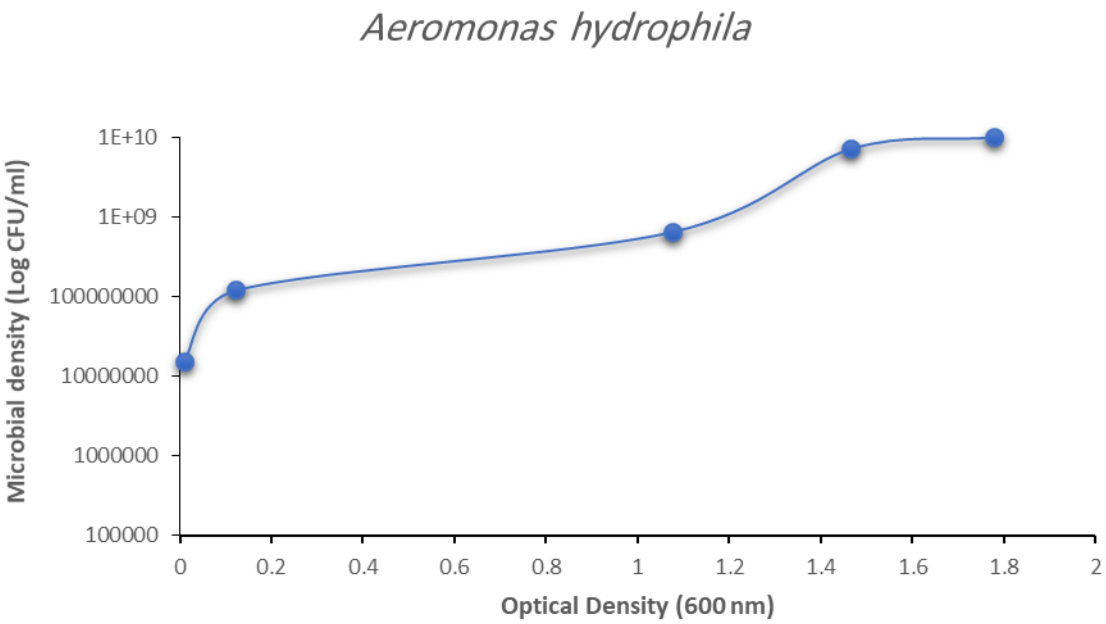


Figure 6. Standard growth curve (Time points vs. OD_{540}) of *Edwardsiella tarda.*

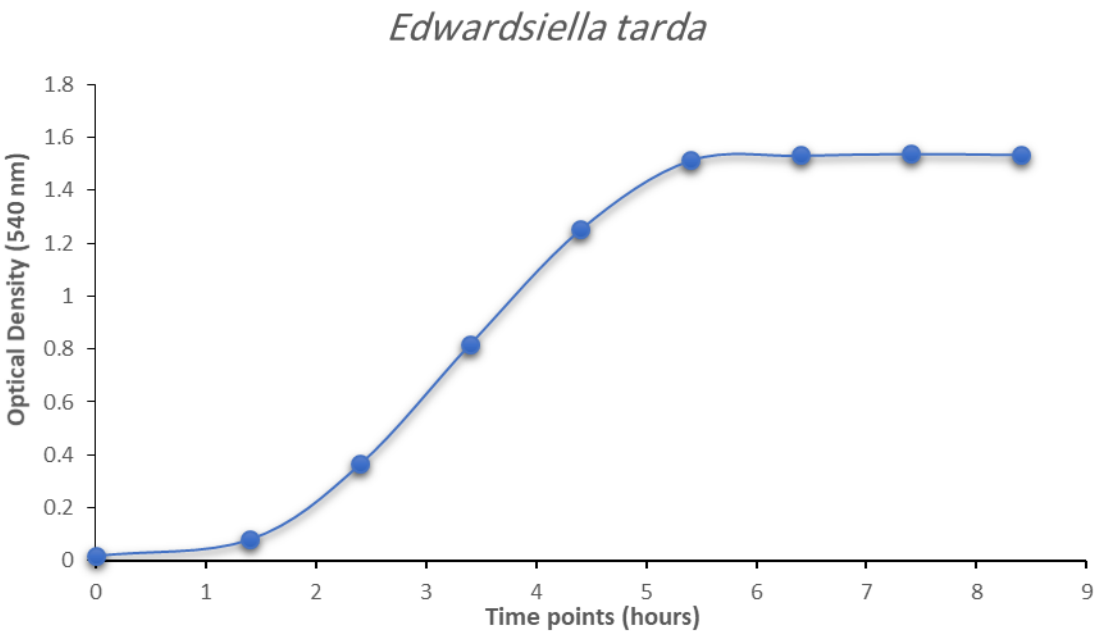
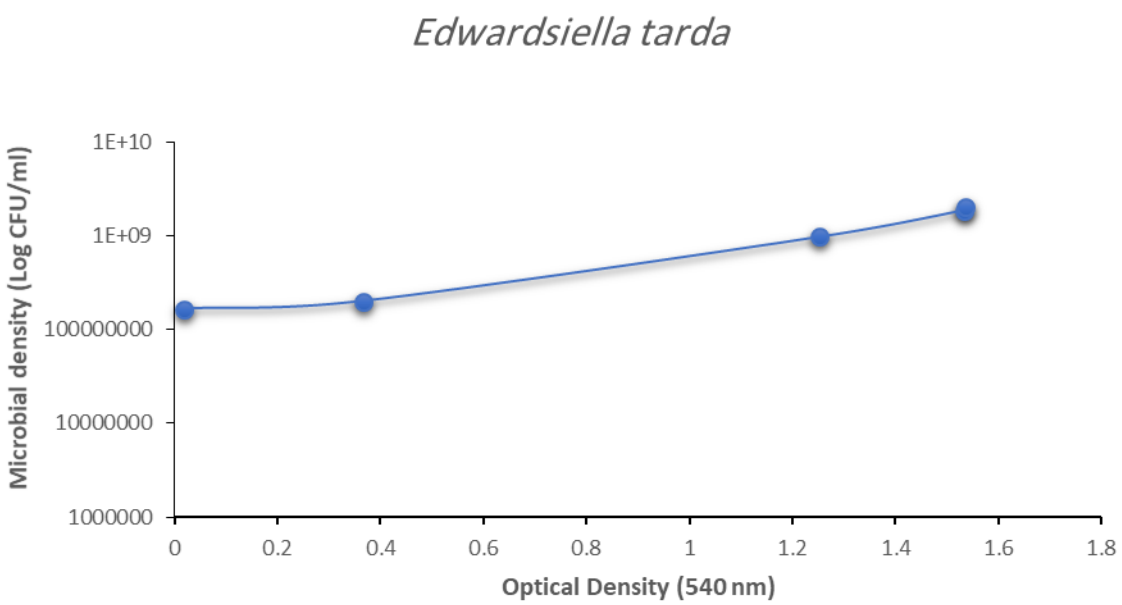


Figure 7. Standard curve (OD_{540} vs. Log CFU/ml) of *Edwardsiella tarda.*



sixteen 180-L fiberglass tanks connected to a biological filter and settling tank and offered EcN-rich diet (at 10^7 CFU/ Kg) 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 several concentrations of the pathogenic bacteria *Aeromonas hydrophila* and *Edwardsiella tarda* per group. To prepare the bacterial solutions, *Aeromonas hydrophila* (ATCC43874) and *Edwardsiella tarda* (ATCC15947) were 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 5 hours at 30°C (*A. hydrophila*) and 4 hours at 37°C (*E. tarda*), to reach an optical density (OD) of 1.3 and 1.23, respectively, corresponding to 10^8 CFU/ml for both bacteria. For each bacterium two dilutions (10^6 and 10^7 CFU/ml) were prepared. The experimental design also included eight control groups of 10 fish per group. Treated fish were those offered the probiotic diet then injected with the pathogen. The treatments and controls were: 1- Probiotic + *E. tarda* (10^6); 2- Probiotic + *E. tarda* (10^7); 3- Probiotic + *E. tarda* (10^8); 4- Probiotic + *A. hydrophila* (10^6); 5- Probiotic + *A. hydrophila* (10^7); 6- Probiotic + *A. hydrophila* (10^8); 7- Control 1:Physiological saline (2 tanks); 8- Control 2: Brain Heart Infusion broth (2 tanks); 9- Control 3: *E. tarda* (10^6); 10- Control 4: *E. tarda* (10^7); 11- Control 5: *E. tarda* (10^8); 12- Control 6: *A. hydrophila* (10^6); 13- Control 7: *A. hydrophila* (10^7); 14- Control 8: *A. hydrophila* (10^8). After the injection, the fish were placed in 52-L glass tanks and survival monitored for 96 hours. Dead fish were removed and recorded when discovered.

The second challenge consisted of offering the fish 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 (Fig. 5 and 7).

Fish feed was sprayed with either *A. hydrophila* or *E. tarda* to get a final concentration of 10^7 CFU/Kg.

The experimental setup consisted of four indoors recirculation systems, each consisting of three tanks (180-L fiberglass tanks) connected to a biological filter. Fifteen sex-reversed Nile tilapia juveniles (28 ± 0.2 g; mean ± 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/Kg 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.

J. 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 ± 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 $p < 0.05$.

CHAPTER 3
RESULTS

A. EcN colonization of the gut

Results of the preliminary study suggest that *Escherichia coli* is not naturally present in the guts of Nile tilapia. Furthermore, there were no significant differences in survival and growth among the control group and the EcN groups in the preliminary tests (Fig. 3). Some EcN colonization of the gut was detected in EcN fed fish at the end of the six week-trial.

In the main experiment EcN cell 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 cell counts dropped drastically after the second week, and remained relatively 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 (Table 2, Fig. 11).

B. Persistence of EcN after reverting to non-supplemented diet

After reverting to the non-supplemented diets for a week *Escherichia coli* Nissle cell 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 (Table 3).

C. Fish survival and growth rates

In all treatments, *O. niloticus* juveniles survived and grew in weight and length (Table 4). The various EcN concentrations did not have a significant effect on growth of

Table 2. Weekly viable *Escherichia coli* Nissle (EcN) counts (CFU/g) in the guts of Nile tilapia offered control feed, 10^6 EcN feed, 10^7 EcN feed or 10^8 EcN feed for 8 weeks.

		Control	**10^6 EcN**	**10^7 EcN**	**10^8 EcN**
Week	**Fish**	**CFU/g**	**CFU/g**	**CFU/g**	**CFU/g**
1	**1**	0	25	17	33
1	**2**	0	25	7	37
2	**1**	0	0	2	5
2	**2**	0	0	0	0
3	**1**	0	0	0	3
3	**2**	0	0	2	3
4	**1**	0	0	0	0
4	**2**	0	0	0	0
5	**1**	0	2	7	23
5	**2**	0	0	7	17
6	**1**	0	3	40	3
6	**2**	0	2	10	17
7	**1**	0	0	8	12
7	**2**	0	2	20	42
8	**1**	0	0	73	3
8	**2**	0	0	77	10
8	**3**	0	7	3	0
8	**4**	0	0	53	3
8	**5**	0	10	10	0
8	**6**	-	3	3	0
8	**7**	-	0	3	0
8	**8**	-	10	0	0
8	**9**	-	7	3	0

Table 3. Viable *Escherichia coli* Nissle (EcN) counts (CFU/g) in the guts of Nile tilapia in treatments (10^6 EcN, 10^7 EcN and 10^8 EcN) at the end of the feeding experiment (Initial colonization) and after 1 week of reverting to the non-supplemented diet (Final colonization).

Treatment	**Initial colonization[a] (CFU/g) ± SE**	**Final colonization[b] (CFU/g) ± SE**
10^6 EcN	4 ± 1.5	1 ± 1
10^7 EcN	25 ± 10.9	5 ± 2.3
10^8 EcN	2 ± 1.1	1 ± 1

a: N= 9 fish
b: N= 3 fish

Figure 8. Viable *Escherichia coli* Nissle (EcN) cell counts (CFU/g) in the guts of Nile tilapia offered 10^6 EcN diet for 8 weeks.

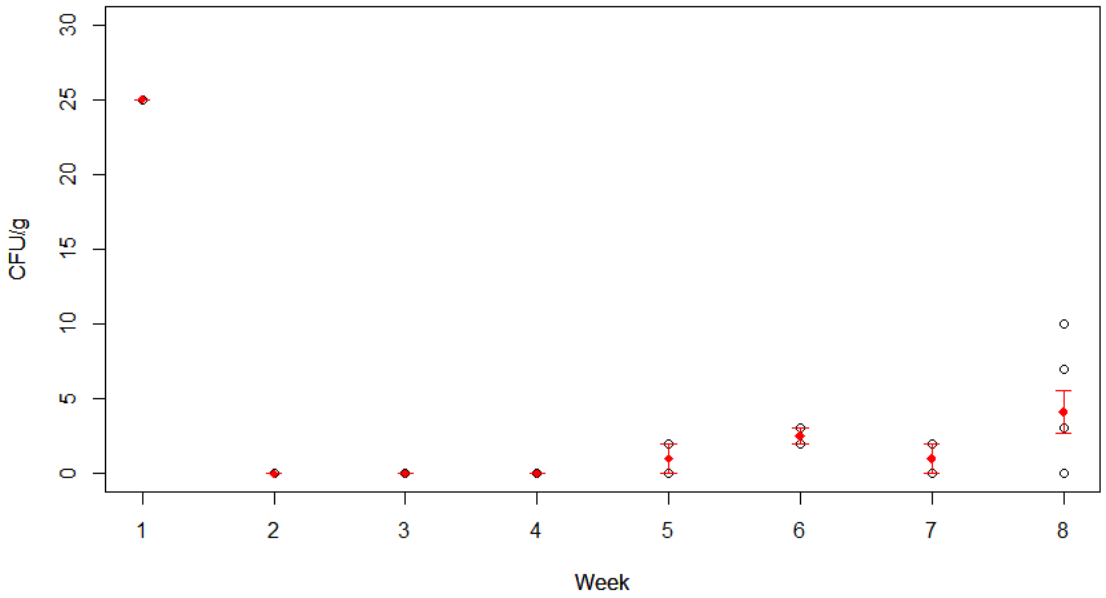


Figure 9. Viable *Escherichia coli* Nissle (EcN) cell counts (CFU/g) in the guts of Nile tilapia offered 10^7 EcN diet for 8 weeks.

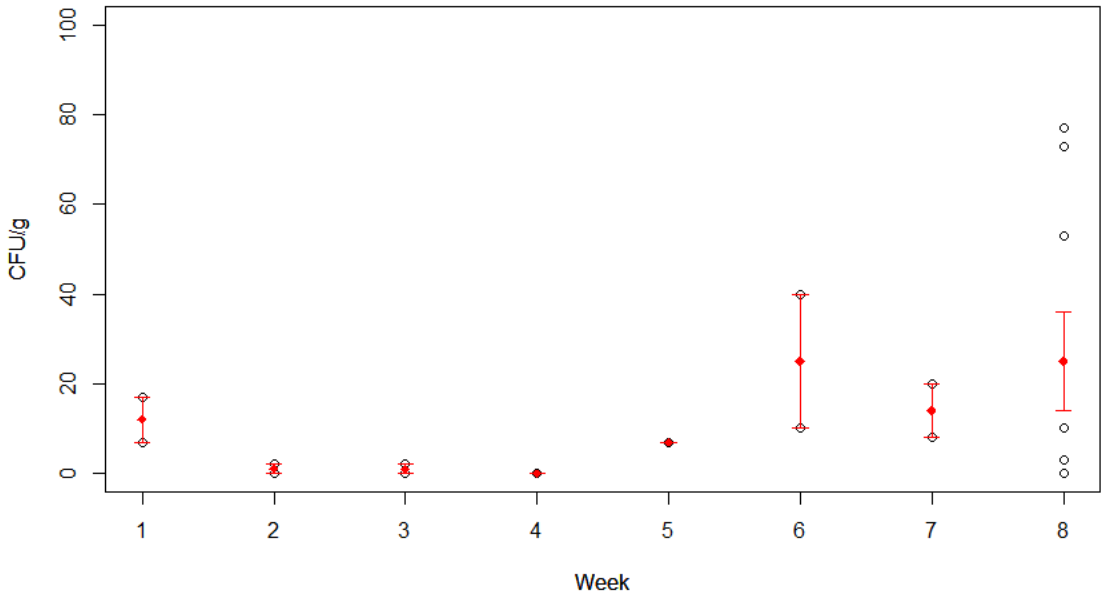


Figure 10. Viable *Escherichia coli* Nissle (EcN) cell counts (CFU/g) in the guts of Nile tilapia offered 10^8 EcN diet for 8 weeks.

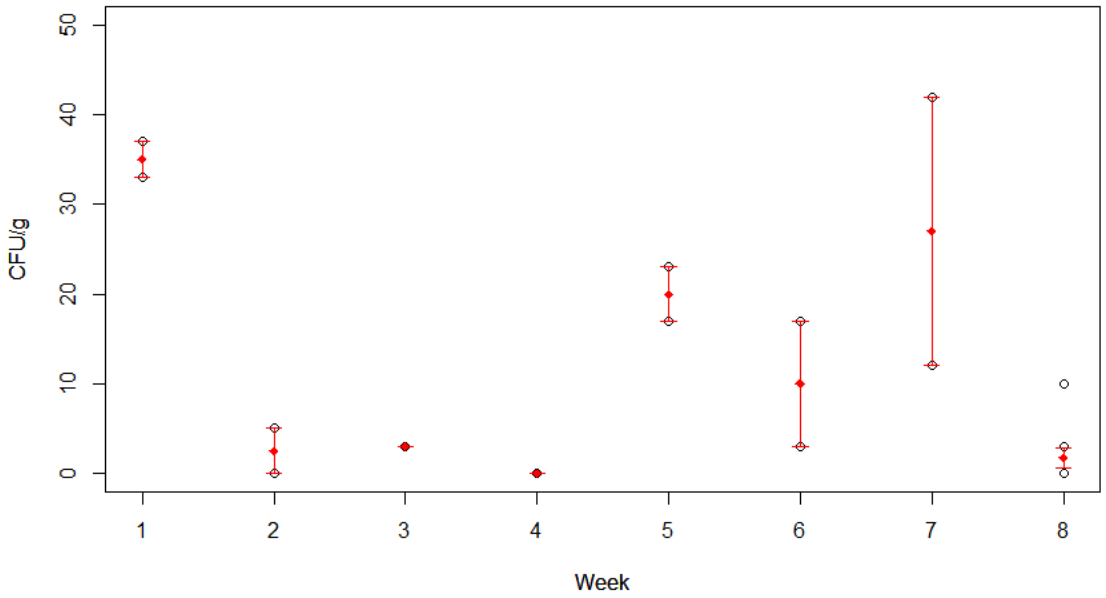
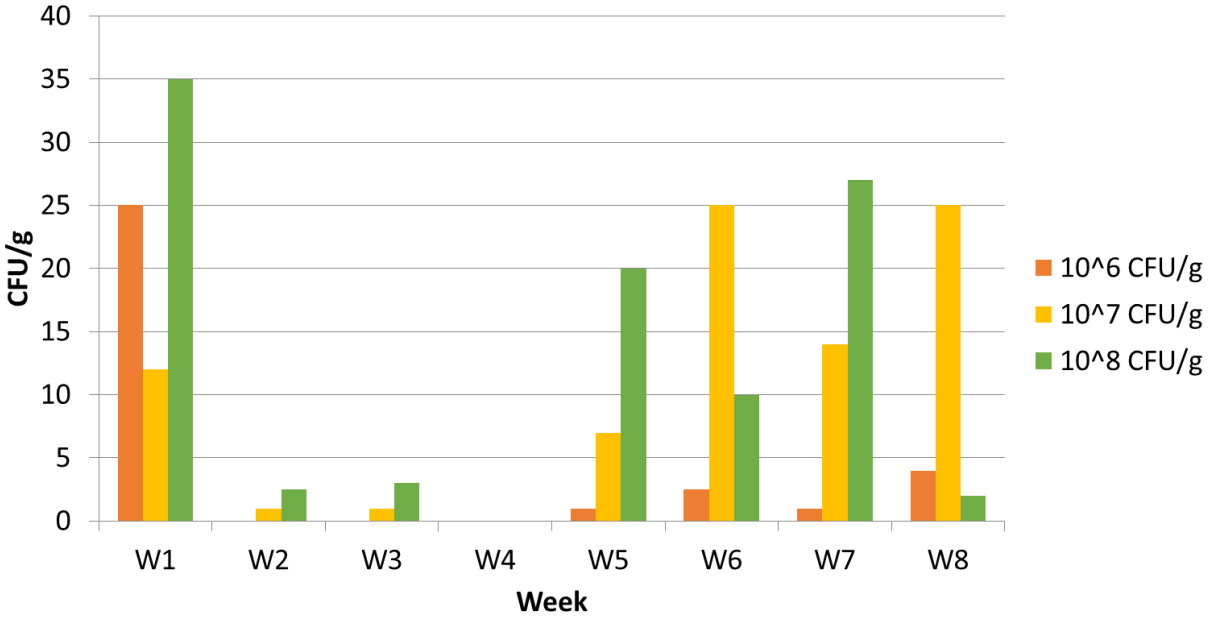


Figure 11. Weekly viable *Escherichia coli* Nissle (EcN) counts (CFU/g) in the guts of Nile tilapia offered control feed, 10^6 EcN feed, 10^7 EcN feed or 10^8 EcN feed for 8 weeks.



Nile tilapia (p value>0.05). 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 10^7 CFU/Kg. However, Fulton's condition index (K) was significantly larger in 10^7 EcN fish than in control fish ($p < 0.05$).

Hepatosomatic index did not vary among fish offered control diet (1.05 ± 0.17 %) and fish offered 10^6 and 10^7 EcN (1.01 ± 0.1 % and 0.89 ± 0.26 % respectively). However, the hepatosomatic index of the fish offered 10^8 EcN (0.75 ± 0.14 %) was significantly less than the HSI of control fish (p value<0.05). There were no significant differences among viscerosomatic indices of fish offered the control diet (7.39 ± 0.86 %), 10^6 EcN (7.50 ± 0.47 %) and 10^8 EcN (7.02 ± 0.44 %). However, viscerosomatic index of fish offered 10^7 EcN (8.16 ± 0.63 %) was significantly greater than the viscerosomatic indices of fish in all other treatments ($p < 0.05$).

D. Hematological parameters

There were no significant differences in hematocrit and total plasma protein among treatments (p value > 0.05). Hematocrit values ranged from 23.33 ± 3.43% in fish offered the control diet to 25.67 ± 6.71% in fish offered diets with 10^6 EcN. Total plasma protein values ranged between 4.26 ± 0.41g/ dL (for 10^7 EcN) and 4.91 ± 0.69 g/dL (for the control).

Hemoglobin concentrations were significantly different among treatments ($p < 0.05$) with the control fish having the least hemoglobin in their blood (10.54 ± 1.49 g/dL). On the other hand, respiratory burst in the control (0.38 ± 0.12) was significantly greater than respiratory burst in all treatments. Respiratory burst in the 10^8 EcN treatment (0.16 ± 0.06) was less than in all other treatments (Table 5).

Figure 12. Growth in average individual body weight (g) of juvenile *Oreochromis niloticus* offered a control diet (T1), diets sprayed with *Escherichia coli* Nissle (EcN) at 10^6 CFU/Kg (T2), at 10^7 CFU/Kg (T3), at 10^8 CFU/Kg (T4) over eight weeks.

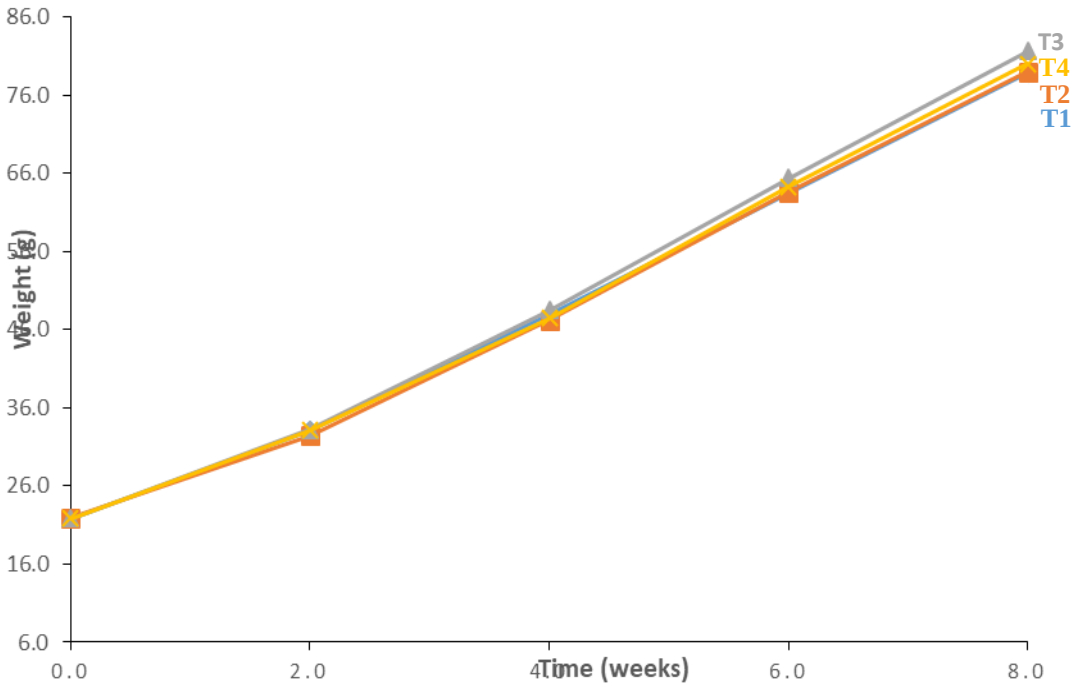


Table 4. Survival (S; %), final body weight (FBW; g), final length (FL; cm), Fulton's condition index at harvest (K), hepatosomatic (HSI; %) and viscerosomatic (VSI; %) indices of Nile tilapia, *Oreochromis niloticus*.

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

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

*PSE: Pooled standard error

Table 5. Hemoglobin (Hb; g/dL), hematocrit (HCT; %), total plasma protein (TPP; g/dL), and respiratory burst of Nile tilapia, *Oreochromis niloticus*. Values in the same column with different superscripts (a, b) are significantly different from each other ($p < 0.05$)

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

*PSE: Pooled standard error

Total red blood cell counts did not vary significantly among treatments ($p > 0.05$), (Table 6). Total white blood cell counts varied significantly among treatments with the greatest value ($1.05 \pm 0.49 \times 10^5$ cells/µL) observed in fish offered the 10^7 EcN feed. There were no significant differences in neutrophils and monocytes among treatments ($p > 0.05$). However, thrombocyte and lymphocyte percentages varied significantly among treatments ($p < 0.05$), 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 ($29.7 \pm 9.8\%$), (Table 6).

E. Challenging Nile tilapia, *Oreochromis niloticus*, with bacterial pathogens

EcN did not improve survival of tilapia injected with *E. tarda* or *A. hydrophila*. Survival of fish injected with 10^6 and 10^7 CFU/ml *A. hydrophila* was nearly 100% in both EcN and control fish (Table 8). 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 whilst 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 weren't apparent in the EcN fed group. No mortalities were recorded in fish injected with physiological saline or with BHI broth.

When tilapia were offered *A. hydrophila* and *E. tarda* in the feed, no mortalities were recorded neither in the control groups nor in EcN groups (Table 9). 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.

Table 6. Total red blood cells (TRBCs; $\times 10^6/\mu L$), total white blood cells (TWBCs; $\times 10^5/\mu L$), and differential white blood cells count (in %) for Nile tilapia, *Oreochromis niloticus*. Values in the same column with different superscripts (a, b) are significantly different from each other ($p < 0.05$)

Treatment	TRBCs	TWBCs	Neutrophils	Thrombocytes	Lymphocytes	Monocytes
Control (No EcN)	1.66^{a}	0.56^{c}	1.33^{a}	82.2^{a}	14.57^{b}	1.56^{a}
10^6 EcN	1.98^{a}	0.80^{b}	1.00^{a}	$78.4^{a,b}$	18.78^{b}	2.22^{a}
10^7 EcN	1.64^{a}	1.05^{a}	1.42^{a}	69.8^{b}	29.70^{a}	1.67^{a}
10^8 EcN	1.77^{a}	1.02^{a}	1.42^{a}	$78.7^{a,b}$	17.64^{b}	2.20^{a}
PSE*	0.09	0.06	0.22	2.71	2.30	0.32

Table 7. Fish mortality rates after 96 hours of injecting them with one of the controls or with *Edwardsiella tarda*.

Treatment	**Number of dead fish/10**	**% Mortality**	**% Survival**
Control 1– Physiological saline 1	0	0	100
Control 1– Physiological saline 2	0	0	100
Control 2– BHI 1	0	0	100
Control 2– BHI 2	0	0	100
Control 3– 10^6 *E. tarda*	0	0	100
Control 4– 10^7 *E. tarda*	0	0	100
Control 5– 10^8 *E. tarda*	3	30	70
Treatment 1– 10^6 *E. tarda*	0	0	100
Treatment 2– 10^7 *E. tarda*	0	0	100
Treatment 3– 10^8 *E. tarda*	4	40	60

Table 8. Fish mortality rates after 96 hours of injecting them with one of the controls or with *Aeromonas hydrophila*.

Treatment	**Number of dead fish/10**	**% Mortality**	**% Survival**
Control 1– Physiological saline 1	0	0	100
Control 1– Physiological saline 2	0	0	100
Control 2– BHI 1	0	0	100
Control 2– BHI 2	0	0	100
Control 3– 10^6 *A. hydrophila*	0	0	100
Control 4– 10^7 *A. hydrophila*	0	0	100
Control 5– 10^8 *A. hydrophila*	8	80	20
Treatment 1– 10^6 *A. hydrophila*	1	10	90
Treatment 2– 10^7 *A. hydrophila*	0	0	100
Treatment 3– 10^8 *A. hydrophila*	8	80	20

Table 9. Fish mortality rates after 4 days of offering them one of the control feeds or one of the treatments.

Treatment	% Mortality	% Survival
Control 1– 10^6 *E. tarda*	0	100
Control 2– 10^7 *E. tarda*	0	100
Control 3– 10^8 *E. tarda*	0	100
Control 4– 10^6 *A. hydrophila*	0	100
Control 5– 10^7 *A. hydrophila*	0	100
Control 6– 10^8 *A. hydrophila*	0	100
Treatment 1– 10^6 *E. tarda*	0	100
Treatment 2– 10^7 *E. tarda*	0	100
Treatment 3– 10^8 *E. tarda*	0	100
Treatment 4– 10^6 *A. hydrophila*	0	100
Treatment 5– 10^7 *A. hydrophila*	0	100
Treatment 6– 10^8 *A. hydrophila*	0	100

CHAPTER 4

DISCUSSION

Probiotics are used to improve the health of aquacultured animals, and improve their disease resistance. Moreover, interest in the use of probiotics as a sustainable solution to decrease use of antibiotics in aquaculture is increasing. Most probiotics used in aquaculture are autochthonous to the organisms being reared (Irianto & Austin, 2002). These probiotics are often isolated from the species itself and therefore are sure to be safe to the host (Hai, 2015). 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, the continuous interaction between pathogens and autochthonous probiotics could drive the pathogens to evolve and become less susceptible to the probiotic's effects. Alternatively, 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 allochtonous fish is not guaranteed.

A. Colonization and growth

Our preliminary study showed that *Escherichia coli* was not present in the gastrointestinal tract of our *Oreochromis niloticus* although present in the ambient water. 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 the probiotic 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. *Escherichia coli* Nissle was not able to permanently colonize the guts of Nile tilapia. Instead, we observed a transient colonization that is mostly apparent in the 10^7 CFU/ Kg treatment. However, our results showed that *E. coli* Nissle colonization of tilapia guts was dependent on the continuous supply of the probiotic because EcN was detected in guts only when the probiotic-supplemented feed was offered to the fish. After reverting to the control diets EcN practically disappeared in the fish guts.

Previous studies on mammals showed that a permanent colonization by EcN is only achieved in gnotobiotic models (Vlasova *et al.*, 2016), neonates (Lodinová-Žádníková & 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 outcompete the intruder and prevent successful EcN colonization (Sonnenborn & Schulze, 2009).

The present study also showed that EcN – supplemented feed did not significantly affect the growth performance of Nile tilapia. EcN was unable to permanently colonize the guts of Nile tilapia, and thus the probiotic was unable to affect digestive enzymes or nutrients uptake in the fish.

B. Hepatosomatic index

Hepatosomatic index is used to assess the health of fish (Goede, 1990). HSI is affected by ambient environmental conditions and nutritional conditions of fish. Our results showed a decrease in HSI as the concentration of EcN in the diets increases. A smaller liver is usually associated with chronic stress in the fish (Cech Jr, Wilson, & Crosby, 1998), that can be caused by the introduction of a foreign microorganism,

pathogenic or not, into the body of the fish (Francis-Floyd, 1992). Accordingly, we can assume that EcN in the diets of Nile tilapia was sufficiently recognized as a foreign organism as to alter the physiological status of the fish leading to a decrease in liver growth.

C. Hematological parameters

Hematological parameters include total red and white blood cell counts, hemoglobin, hematocrit and total plasma proteins, and are a reflection of the health and immune status of fish (Svobodova, Pravda, & Palackova, 1991). In the present study, EcN induced a significant increase in the total white blood cells, reaching a maximum of 1.05×10^5 cells/μL in fish offered the 10^7 CFU/Kg diet. White blood cell counts tend to increase in the presence of an infection. This possibility of an infection that did not cause mortality is 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 & Schulze, 2009). Although 10^7 CFU/Kg feed induced an increase in WBCs, we observed a decrease in WBCs when dietary EcN was increased to 10^8 CFU/Kg EcN. According to Sakai (1999), an overdose of an immunostimulant might suppress the immune responses in the host, which could result in a decrease in WBCs similar to what we observe in the present 10^8 CFU/Kg EcN treatment. Moreover, whilst EcN didn't affect counts of neutrophils and monocytes, the 10^7 CFU/Kg 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. 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.

D. Respiratory burst

Respiratory burst is a series of reactions where phagocytic cells release reactive oxygen species such as hydrogen peroxide (H_2O_2) and superoxide (O_2^-). Our results showed that the respiratory burst was greater in the control than in the EcN treatments. One explanation for the decreasing respiratory burst would be that EcN chelated the iron needed for the Fenton reaction to occur. In vivo, respiratory burst occurs via the Fenton reaction (Equations 1 and 2) which is catalyzed by Fe ions (Trenam, Blake, & Morris, 1992).

$$O_2^- + Fe^{+++} \longleftrightarrow (Fe^{+++} - O_2^- / Fe^{++} - O_2^-) \longleftrightarrow O_2 + Fe^{++} \quad \text{Equation 1}$$

$$Fe^{++} + H_2O_2 \longrightarrow Fe^{++} + \cdot OH + OH^- \quad \text{Equation 2}$$

EcN takes up ferric ions by producing various siderophores such as aerobactin, hemin- and citrate-dependent iron acquisition system, etc. (Sonnenborn & 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.

E. Challenging Nile tilapia, *Oreochromis niloticus*, with bacterial pathogens

Bacterial challenges were performed by incorporating *A. hydrophila* or *E. tarda* into the feed or injecting the pathogens directly into the fish. When the pathogens were administered to the fish in the feed, results showed that EcN did not affect survival of Nile tilapia. Usually, EcN can protect the host from infections by promoting the synthesis of tight junction proteins (Zyrek et al., 2007), thus restoring the integrity of tight junctions and preventing bacterial entry (Lu, Yang, & Hu, 2014). However, in our study we can assume that the tight junctions did not play a role in preventing *A. hydrophila* or *E. tarda* infection because both the control groups and EcN groups had no mortalities, which suggests that *A. hydrophila* and *E. tarda* at the concentrations offered in the feed appear to be harmless when ingested.

When the pathogens were administered to the fish by injection, EcN did not improve the survival of Nile tilapia. Previous studies reported that probiotics can have immunomodulating properties that would stimulate the immune system of the fish and thus protect it from pathogens (Aly *et al.*, 2008; Pirarat *et al.*, 2006). However, in the present study, because EcN couldn't permanently colonize the guts of tilpia, we assume that it was not able to improve the immunity of the fish to allow it to fight *A. hydrophila* and *E. tarda.* Alternatively, the slightly higher mortality in the EcN fed groups could be caused by the stress induced by the exposure to a foreign microorganism, EcN, into the gut of the fish.

F. Conclusion

The present study indicates that *Escherichia coli* Nissle was unable to act as a probiotic in *Oreochromis niloticus*. EcN was unable to significantly affect the growth of Nile tilapia nor prevent death upon infection by a pathogen. The EcN- induced increase in TWBC could be explained as an immune reaction against EcN itself unrelated to its

assumed probiotic properties. Therefore, 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 a promising solution to the use of antibiotics. Allochthonous probiotics comprise a wide range of candidates to select from, and the problem is in ensuring that these probiotics are able to colonize the GI tract of the host.

An interesting observation in the present work is that although the culture water contained a huge amount of *E. coli*, the guts of the tilapia did not and resisted colonization by EcN. We were not able to find literature reporting presence or absence of *E. coli* in tilapia although there are reports of the bacteria in the guts of various other fishes.

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