Bacterial microflora in the gastrointestinal tract of Nile tilapia, *Oreochromis niloticus,* cultured in a semi-intensive system

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ABSTRACT. This experiment measured total bacterial numbers in the gastrointestinal regions of semi-intensively cultured tilapia, *Oreochromis niloticus* L. (Cichlidae). Mean bacterial numbers were higher in both anterior and posterior gut than in stomach. The percentage of isolated species and the percentage of isolates from any particular species varied significantly among gastrointestinal tract regions. *Aeromonas hydrophila, Aeromonas veronii, Burkholderia cepacia, Chromobacterium violaceum, Citrobacter freundii, Escherichia coli, Flavimonas oryzihabitans* and *Plesiomonas shigelloides* were the most frequently isolated Gram-negative bacilli. From these samples, only *Plesiomonas shigelloides* was present in both gastrointestinal regions, with higher number in the posterior gut (76%), when compared to anterior gut (4.8%) and stomach (0.6%). *Aeromonas hydrophila* (0.6%), *Escherichia coli* (7.4%), and *Flavimonas oryzihabitans* were isolated only in the stomach, and *Citrobacter freundii* and *Burkholderia cepacia* were found only in the posterior gut. *Chromobacterium violaceum* was the dominant bacteria isolated from stomach and anterior gut, with 90% and 55%, respectively. Unidentified organisms comprised 0 - 39.3% of the gastrointestinal microflora.

Key words: tilapia, bacterial flora, gastrointestinal tract, *Oreochromis niloticus*

RESUMO. Flora bacteriana de tilápia do Nilo, *Oreochromis niloticus,* cultivada em sistema semi-intensivo. A flora bacteriana de diferentes partes do trato gastrintestinal de tilápia *Oreochomis niloticus* L. (Cichlidae) foi determinada. O número médio de bactérias foi maior no intestino anterior e posterior quando comparado ao estômago. A porcentagem total de espécies bacterianas isoladas e a porcentagem de espécies isoladas em uma espécie particular variaram significativamente entre as regiões do trato gastrintestinal. *Aeromonas hydrophila, Aeromonas veronii, Burkholderia cepacia, Chromobacterium violaceum, Citrobacter freundii, Escherichia coli, Flavimonas oryzihabitans* e *Plesiomonas shigelloides* foram os bacilos Gram-negativos encontrados com maior freqüência. Destas espécies, somente *Plesiomonas shigelloides* esteve presente em cada região do trato gastrintestinal, apresentando maior número no intestino posterior (76%), quando comparado com o intestino anterior (4.8%) e o estômago (0.6%). *Aeromonas hydrophila* (0.6%), *Escherichia coli* (7.4%), e *Flavimonas oryzihabitans* foram isoladas somente do estômago, e *Citrobacter freundii* e *Burkholderia cepacia* foram encontradas somente no intestino posterior. *Chromobacterium violaceum* foi a espécie dominante isolada do estômago e do intestino anterior com 90% e 55%, respectivamente. Organismos não identificados compreendem 0 – 39.3% da microbiota gastrintestinal.

Palavras-chave: tilápia, microbiota bacteriana, trato gastrintestinal, *Oreochromis niloticus.*

Introduction

The Nile tilapia, *Oreochromis niloticus* L. (Cichlidae), is the world's major cultured species of tilapia, with 603,034 tons in 1996 (FAO, 1998). In general, cultured species show a generation adaptation to: confinement, water physical characteristics, artificial diet and husbandry manipulations (Pagés *et al.*, 1995). Recently, there has been a greater understanding of the need to study bacteria in the context of their environment and their host's physiology and this led to the conclusion that bacterial diseases of fish are almost invariably stress-related (Inglis *et al.*, 1993).

The establishment of a balanced gut microflora is important in animal health and digestive function and it can possibly occur in fish, where a resident microflora has been suggested (Munro *et al.*, 1994). The microbiology of fish gastrointestinal tract has been subjected to many researches. Characteristics of the microenvironments at various locations throughout the alimentary tract influence both the taxonomic composition and the numerical abundance of bacteria present (Horsley, 1977). The gastrointestinal tract of fish can influence nutrition, growth, and disease susceptibility. The microflora may be essential in fish that feed on recalcitrant materials or on material lacking vitamins, which the microflora can synthesise (Trust and Sparrow, 1974).

The aim of the present study was to determine the bacterial flora in the gastrointestinal tract of semi-intensively cultured tilapia (*Oreochromis niloticus)*. The level of semi-intensive management is common for small-scale tilapia operations with limited capital, or where high-quality food is unavailable.

Material and methods

Fish. Nile tilapia, *O. niloticus*, cultured semi-intensively came from the Estação Experimental de Piscicultura, Universidade Estadual de Maringá. The fish used were averaged 400-1,000g in weight and were between 6 and 9 months aged. The climate at Maringá City region in northwest of Paraná State, Brazil belongs to type CW´h (Köppen classification) and is called tropical with two seasons (fall, last spring and early morning) and two dry seasons (winter and early spring). The annual rainfall is about 1582.2mm. The annual mean temperature is approximately 22ºC (Deffune *et al.*, 1994).

Sample preparation. Three randomly chosen fish were sacrified and the gastrointestinal tract was removed from each sample with sterile dissecting instruments. The gastrointestinal tract was divided into three sections: stomach, anterior gut and posterior gut. The stomach was longitudinally opened and all the three sections were gently agitated for 2-3 minutes in sterile saline, to remove the contents. The sections were transferred to tubes containing a 9.0mL 0.85% NaCl solution and 15 to 20 glass beads, agitated with a mixer for 2 minutes. The homogenates were serially diluted to 10^{-6} in 9-ml volumes of sterile saline solution 0.85%.

Media and enumeration procedures. Samples of 0.6mL of homogenized sections and dilutions of these in saline solution 0.85% were spread on agar plates. A similar treatment was applied to the four successive decimal dilution tubes of water pond and fed ration. Media for bacterial analyses were obtained from Difco Laboratories, Detroit, Mich., or from BBL Microbiology Systems, Cockeysville, Md. The solid media employed and the bacteria enumerated were as follows: Plate counts agar, total cultivable; EMB and MacConkey, members of the family of *Enterobacteriaceae* and other Gram-negative bacteria. Numbers reported are an average of five replicate plates.

Isolation and identification of species. The isolation was made by dilution from the positive 10^{-6} tubes. Dilution (0.1mL) was plated on the appropriate enriched medium solidified with 1.5% purified agar (Difco). The plates were incubated at 28ºC for 24-48h. The microorganisms were identified according to standard methodologies using biochemical tests (Holt *et al.*, 1994), as well as standard techniques (MacFaddin, 1980; Murray *et al.*, 1995) used in our laboratory, and BBL Crystal Identification Systems (Becton Dickinson).

Results and discussion

The bacteriological qualities of the tilapia culturing pond water and the fed ration were studied (Figure 1). The approximate number of total cultivable, Gram-negative and Gram-positive bacteria was enumerated on four separate samples, took during a 8-month period. Indicative of stability, the number of organisms obtained from both pond water and fed ration varied less than 2 orders of magnitude between samplings separated by 2 months. The numbers are based on colony counts on solid media.

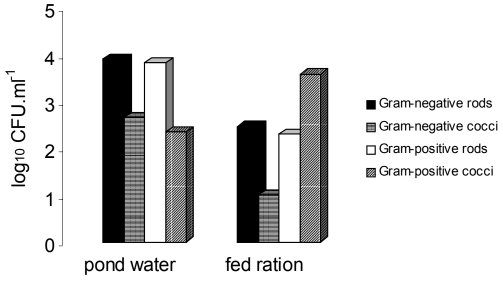

Figure 1. Quantification of different groups of microorganisms from pond water, which is culturing tilapia, and fed ration.

The number of cultivable cells in the stomach, anterior gut and posterior gut varied from 1.5 x 10^4

to 1.6 x 10^5 with the mean value of all samples being 1.5 x 10^4 viable cells per organ. These communities are mainly composed of Gram-negative rods (*Aeromonadaceae Burkholderiaceae, Neisseriaceae, Enterobacteriaceae, Pseudomonadaceae,*). The physiological types of bacterial flora in the three gastrointestinal locations varied minimally (data not shown).

The predominant microorganisms isolated from the three gastrointestinal regions are presented in Table 1. The total percentage of species isolated, as well as the percentage of isolates from any particular species, varied significantly among gastrointestinal regions. *Aeromonas hydrophila, Aeromonas veronii, Burkholderia cepacia, Chromobacterium violaceum, Citrobacter freundii, Escherichia coli, and Flavimonas oryzihabitans* and *Plesiomonas shigelloides* were the most frequently isolated glucose fermenting and nonfermenting Gram-negative bacilli. In spite of that, only *Plesiomonas shigelloides* was present in each gastrointestinal region, with higher number in the posterior gut (76%), compared to anterior gut (4.8%) and stomach (0.6%). *Aeromonas hydrophila* (0.6%), *Escherichia coli* (7.4%), and *Flavimonas oryzihabitans* were isolated only from stomach, and *Citrobacter freundii* and *Burkholderia cepacia* were found only in the posterior gut. *Chromobacterium violaceum* was the dominant species isolated from stomach and anterior gut, with 90% and 55%, respectively. Unidentified organisms comprised 0 - 39.3% of the gastrointestinal microflora.

Table 1. Predominant bacteria isolated from gastrointestinal tract of the tilapia *Oreochromis niloticus*.

Microorganism	Stomach	Anterior gut	Posterior gut
Aeromonas hydrophila	0.6	0	0
Aeromonas veronii	0	0.2	0.3
Burkholderia cepacia	0	0	3.7
Chromobacterium violaceum	90.0	55.5	0
Citrobacter freundii	0	0	13.0
Escherichia coli	7.4	0	0
Flavimonas oryzihabitans	1.1	0	0
Plesiomonas shigelloides	0.6	4.8	76.0
Unidentified sp	0	39.3	6.6

Values are mean percentages from four independent experiments

Differences in the taxonomic composition of gastrointestinal microflora among the three regions were expected, because the gastric fluids of tilapia are quite acidic. This normal indigenous flora can act competitively, excluding pathogens, inhibiting colonization and, consequently, preventing infection. The presence of these groups is regulated by local, ecological and physiological conditions in each of the areas, which differ from each other. Some of these situations are non-specific, including amount and type of different available nutrients, pH, and/or redox potential. Some are specific, such as the nature of the adhesion factors for each of the bacterial groups in the epithelial cells or mucus gel (MacMillan and Santucci, 1990).

Aeromonas spp are ubiquitous bacteria, frequently isolated from food, drinking water and aquatic environments (Handfield *et al.*, 1996). These species have long been known to cause different infections in animals such as fish, reptiles, and amphibians. These pathogens have also been associated with several human infections, such as gastroenteritis, peritonitis, endocarditis, and urinary tract and wound infections (Krovacek *et al.*, 1994).

Aeromonas hydrophila is an important bacterial fish pathogen, responsible for considerable losses in the aquaculture industry. *A. hydrophila* can cause disease in a wide range of animal species, including humans. However, mesophilic, motile aeromonads are most noted for causing haemorrhagic septicaemia in fish (Kawula *et al.*, 1996). They are known to occur in the intestinal tract of different fish species (Cahill, 1990).

Aeromonas veronii is an opportunistic pathogen of spotted sand bass commonly found in aquaculture facilities (Leonard *et al.*, 2000) which may affect fish health, development and survival, especially during summer, when the temperatures are most appropriate for *Aeromonas* growth.

Bacterial diseases associated with *Aeromonas* and other genera of chitinolytic bacteria (e.g. *Vibrio*, and *Pseudomonas)* have also been reported in Brazilian prawn hatcheries (Lombardi and Labao, 1991), where they led to "black spot" bacterial necrosis and gill obstruction.

The *Burkholderia (Pseudomonas) cepacia* complex currently comprises five genomic species (Vandamme *et al.,* 2000). The significance of *Burkholderia (Pseudomonas) cepacia* as a component of the gastrointestinal microflora is unknown.

Chromobacterium violaceum is a Gram-negative rod isolated from soil and water in tropical and subtropical regions. Both pigmented and nonpigmented strains exist, although the nonpigmented strains are rare. Chemoorganotrophs, having mainly a fermentative metabolism. Although infections caused by *C. violaceum* are rare among mammals, Lee *et al.* (1999) reported two cases of human infection caused by both pigmented and nonpigmented strains of *C. violaceum*.

Perhaps the best known and most easily recognized of the Gram-negative, facultatively anaerobic bacilli are those that currently make up the family Enterobacteriaceae. The family Enterobacteriaceae, or enteric, as they are commonly called, includes a group of bacteria that inhabit the intestinal tract of humans and other animals. Some species are permanent residents, others are found in only a fraction of the population, and others are present only as disease conditions agents. Most enteric are active fermenters of glucose and other carbohydrates. Enteric microorganisms have evolved several mechanisms for handling acid stress. *E. coli* can survive in extreme acid conditions of pH 2.5 or less, for a number of hours *in vitro* (Arnold and Kaspar, 1995; Benjamin and Datta, 1995; Lin *et al.,* 1995).

The three prevalent species facultative anaerobic Gram-negative bacteria that belong to the family of *Enterobacteriaceae* were identified as *Escherichia coli, Citrobacter freundii* and *Plesiomonas shigelloides.* The bacterium *Citrobacter freundii* has been isolated from naturally infected aquarium fish species (*Poecilia* sp., *Xiphophorus clemenciare*, *Carassius auratus*, and *Astronotus ocelattus*). The infection cause grave clinical symptoms, such as skin ulcerative lesions, distended abdomen, pale liver, darkened spleen, watery kidney, bloody exsudate in gastrointestinal system. In therapeutic, experimental injection with oxytetracycline controlled the infection (Aydin *et al.*, 1997).

De Paola *et al.* (1995) studied the effect of oxytetracycline-medicated food on antibiotic resistance of Gram-negative bacteria from fish intestines and water in catfish pond. They have reported that *P. shigelloides, A. hydrophila*, and *C. freundii* were frequently isolated during spring and fall; *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter* spp were isolated primarily in spring. These authors verified that these species were similarly prevalent in pond water and catfish intestines, except that *P. shigelloides* and *A. hydrophila* were most abundant in catfish intestines and in pond water, respectively.

Plesiomonas shigelloides was present in each gastrointestinal region, with higher number in the posterior gut, when compared to anterior gut and stomach. It is worldwide distributed, primarily in aquatic inhabitants found in seawater and fresh water and in association with aquatic animals. Several species are pathogenic for humans, fish, eels, and frogs, as well as for other vertebrates and invertebrates.

Bacteria belonging to the genus *Plesiomonas* are grouped as facultatively anaerobic, having both respiratory and fermentative type of metabolism. They occur in fish and other aquatic animals and in a variety of mammals, associated with diarrhoea and occasional opportunistic infection in humans. *P. shigelloides* is a common pathogen in tropical regions, whereas it is rarely isolated in temperate climates. *P. shigelloides* has been related in tetrahibrids of *Oreochromis* sp (Pink tilapia), at Venezuela central region. In this study, a high incidence of *P. shigelloides* was determined (73%), being higher in the intestinal tract (60%), followed by skin (36.7%) and gills (26.67%), without any correlation among then (Mendonza and Hernández, 1999).

In the present work, *Flavimonas oryzihabitans* were only isolated from stomach. The type and only species of the genus *Flavimonas*, the bacterium, *F. oryzihabitans,* is placed in the group of Gram-negative aerobic/microaerophilic rods, having a strictly respiratory type of metabolism; it can use oxygen as a terminal electron acceptor. Found in the general environment, it is apparently saprophyte or commensal of humans and other warm-blooded animals, in which they may occasionally prove being pathogenic (Wan *et al.,* 1998). Little is known about the presence of *F. oryzihabitans* as a component of gastrointestinal microflora.

In the present study, the most common species found in the gastrointestinal tract of Nile tilapia belong to eight Gram-negative bacterial species: *Aeromonas hydrophila, Aeromonas veronii, Burkholderia cepacia, Chromobacterium violaceum, Citrobacter freundii, Escherichia coli, Flavimonas oryzihabitans,* and *Plesiomonas shigelloides.* The presence of various organisms which are potentially pathogenic to humans, in fish alimentary tract, is suggestive; its significance in initiating human diseases, unknown. However, the presence of potential human pathogens suggests that fish either improperly handled, undercooked or consumed raw may cause various diseases to susceptible individuals.

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