Description of the cultivable bacterioma of the digestive tract and gills of *Oreochromis niloticus* and isolation of bacteria with probiotic potential

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Abstract

The use of antibiotics in aquaculture has led to multiple problems, the most worrying of which worldwide is the emergence of resistance from pathogenic bacteria. In this context, the use of probiotics in aquaculture is presented as an alternative to antibiotics. This work aims to isolate bacteria with probiotic potential from the gills and the digestive tract of Tilapia niloticus usable in aquaculture. Its implementation first calls for the enumeration and description of the total bacterioma and the two organs' lactic bacterioma. Then, to evaluate the antagonistic activities of the bacteria described vis-à-vis the pathogenic strains Escherichia coli and Staphylococcus aureus by Fleming's method.

The work carried out on wild (Bandama river) and farmed (fish farm) Tilapia has made it possible to isolate 206 bacterial strains (non-demanding lactic flora and total flora) from the gills and digestive tract of this fish. The microbial load is higher in the gills than in the digestive tract in all of the fish sampled. Antimicrobial activity tests revealed 35 strains with probiotic potential, including five strains with antagonistic activity against E. coli and S. aureus simultaneously, 8 strains with antagonistic activity against E. coli against 27 strains with anti-S. Aureus activity. These strains with probiotic potential come mainly from the digestive tract. Furthermore, these strains with probiotic potential are higher in wild fish than in farmed fish. The 35 strains of interest isolated after further studies could be used as a probiotic in aquaculture.

Keywords: Oreochromis niloticus, probiotics, bacteriome, aquaculture

INTRODUCTION

The World Health Organization defines probiotics as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"; to be labeled a piece of probiotic, scientific evidence for the health benefit would have to be documented[1-3]. Their applications, which originally extended to humans and land farm animals, widened in early 1980 to aquatic animals such as fish thanks to Yasuda and Taga [4]. Fish is a protein commodity widely consumed worldwide. The FAO estimates that 1 billion people depend on fish as the main source of animal protein.

Fish is a protein product widely consumed worldwide. The FAO estimates that 1 billion people depend on fish as the main source of animal protein. It provides essential proteins to people in developing countries at affordable prices [5]. Global fish production is estimated at 148.5 million tonnes per year, including 88.6 million tonnes of capture fish and 59.9 million tonnes of aquaculture products per year [5]. World opinion has become aware that fishery resources are limited. Despite increasingly sophisticated fishing methods, world catches have leveled off and are likely to decrease. Aquaculture could, therefore, significantly contribute to reducing this deficit [6]. According to FAO projections, by the 2030s, aquaculture will become the main source of fish supply. To maintain the current per capita consumption level, global aquaculture production will have to reach 80 million tonnes by 2050. [7]

But this sector encounters multiple problems which it would be advantageous to solve, mainly those related to the very often excessive use of antibiotics in this sector. These molecules are used in breeding either to treat infected animals or to prevent bacteriosis. Therefore, this last reason leads breeders to systematic use in the absence of microbial infection since, according to them, antibiotics have properties of "growth stimulator" in these animals [8-11]. Thus in aquaculture, the massive use of antibiotics can have negative impacts not only in public health but also in the environment [12]. It is thus noted that due to their low bioavailability, more than 60% of the antibiotics administered to fish orally are excreted unchanged in the fecal matter. Therefore, these antibiotics are found in the aquatic environment, thus contaminating water, sediments, and living animal or plant organisms. No matter how small the number of antibiotics released into the environment will still be sufficient to generate or select resistant forms of bacteria[13, 14]. Studies have found that sediments near fish farms that have used large amounts of antibiotics repeatedly have a higher frequency of bacterial resistance to antibiotics. Some farms also show a number of multi-resistant bacteria [15, 16].

To deal with this problem, we thought of alternative ways to the use of antibiotics. In this perspective, we are interested in the notion of the holobiont, which considers the host and the associated microbiota as the true evolutionary unit of adaptation. The microbiota makes it possible to amplify genetic plasticity and increase the speed of adaptation to variations and aggressions of the environment. The microbiota of aquatic animals could thus be a still little explored source of microorganisms producing antimicrobial substances. Therefore, this present project consists of identifying microorganisms antagonistic towards pathogenic bacteria from marine animals (mollusks, crustaceans, and fish) with a view to exploitation as a probiotic in aquaculture. Hence the interest of this work which aims to identify from the intestinal (digestive tract) and gill (gills) microbiome of Tilapia all microorganisms with probiotic potential.

The general objective is to isolate bacteria with probiotic potential from the gills and the digestive tract of Tilapia niloticus for aquaculture. Its implementation calls first on the description of the bacterioma of the digestive tract and the gills of Tilapia. Then among the bacteria described, we will isolate those who will have a probiotic potential against two (2) pathogens, *E. coli*, and *S. aureus*.

II. MATERIALS AND METHODS

A. Sampling and packaging

The fish (*O. niloticus*) were sampled in the Ferké 2 aquaculture farm (NE) and the Bandama river (NS). They were transported to the laboratory in different containers. Their different weights and sizes were immediately measured using a scale and a ruler, respectively. Aseptically, an incision was made on the peritoneal cavity with a scalpel to extract the entire digestive tract. The intestine and its contents were weighed and then ground using a sterile mortar and then packaged in Eppendorf tubes. Likewise, the gills were isolated, weighed, crushed, and then packaged in Eppendorf tubes.

B. Seeding and bacterial enumeration

One (1) gram of homogenate (gut, gill separately) is homogenized in a test tube containing 9 ml of sterile distilled water. From the homogenate, a series of dilutions were carried out from 10^{-1} to 10^{-5} . The various dilutions were seeded on nutrient agar (GN) and agar of Man, Rogosa, and Sharpe (MRS) and then incubated at a temperature of 37° C for a minimum period of 24 hours. After growth and counting of the bacterial colonies observed, the enumeration of bacteria was obtained in CFU/ml (colony-forming unit per milliliter) according to the following formula[17]:

Equation 1: Enumeration in CFU/ml

$$N = \frac{\sum C}{d(n1 + 0, 1n2)V}$$

With

 ΣC : a sum of colonies of the counted dishes

V: volume of the inoculum (0.1 ml) d: dilution retained

n1: number of boxes corresponding to the first dilution selected

n2: number of dishes corresponding to the second dilution selected

N: number of colonies in (CFU/ml)

The final count was reduced to the number of colonies forming units per gram (CFU/g) of the digestive tract or gills according to the following formula:

Equation 2: Enumeration in CFU/g of organ $N' = \frac{N}{c}$

N': number of colonies forming units per gram of organ C: concentration (g/ml) of the comminuted organ (digestive tract or gills) in the mother solution.

C. Highlighting of the antimicrobial activity of isolated strains

The method used to demonstrate the antimicrobial activity of isolated microorganisms is the spot method, also called the Fleming method (Moroh 2013, Samadoulougou-Kafando et al. 2019).

Antimicrobial activity was assessed using reference strains ATCC (American Type Culture Collection). The strains concerned are *Escherichia coli* ATTC 25922 and *Staphylococcus aureus* ATTC 25923.

First of all, the pathogenic bacteria and the various bacterial strains isolated from the digestive tract and the gills of the fish were cultured for 24 hours at 37 $^{\circ}$ C. Then a 3-hour and 4-hour precultures of E coli and S aureus respectively were carried out. One (1) ml of the pathogenic strains to be tested was spread on the MH agar (Mueller-Hinton) and then dried at room temperature for 15 to 20 minutes under a laminar flow hood. Then by means of the buttoned tip of the Pasteur pipette, the strains isolated from the fish are deposited on the agar previously inoculated with E Coli or S aureus.

The Petri dishes are then incubated at 37° C for a period of 24 to 48 hours. At the end of this time, the inhibition halo's presence marks an antagonistic activity of the fish bacteria against the pathogenic strain concerned.

III. RESULTS AND DISCUSSION

A. Results

a) Enumeration and description of the cultivable bacteriome

The enumeration of the cultivable bacteriome by the culture media (MRS and GN) of each organ (digestive tract and gills) of the different individuals revealed great variability in the bacterial load.

There is a higher load of the cultivable nondemanding total bacteriome than the lactic bacteriome. These results presented in Figure 10 and Figure 11 are observed both on the gills and the digestive tract of each fish (bred and wild). Furthermore, the count also revealed that the gills of almost all of the fish individuals studied (bred and wild) have a microbial density greater than that of the digestive tract. These results concern the nondemanding cultivable total bacteriome and the cultivable lactic bacteriome.

In terms of the microscopic description of the bacterioma, Gram staining allowed us to have information on the type and form of bacteria, their size, their mode of association, as well as the degree of purity of the strains.

The Camembert diagram presented in Figure 14 and Figure 15 show, from Gram staining, a heterogeneous distribution of the different types of bacteria in the organs (digestive tract and gills) of individuals (wild and farmed).

In wild fish (NS), the proportions in the gills are dominated by Gram-negative cocci (51.92%) followed by Gram-positive cocci (36.54%). Gramnegative and Gram-positive bacilli each represent 5.77% of the cultivable bacteria. The digestive tract of these fish contains 51.22% Gram-negative cocci); 31.71% Gram-positive cocci; 9.75% Gram-negative bacilli and 7.32% Gram-positive bacillus.

The proportions in the gills of farmed fish include 60.87% Gram-negative cocci, 34.78% Gram-positive cocci and 4.35% Gram-negative bacillus. As for the digestive tract of farmed fish, there is 80.49% of Gram-negative cocci and 19.51% of Gram-positive cocci.



Figure 1: Distribution of the bacterial load in the digestive tract and gills of wild fish (Digestive tract; Gills)



Gills)



Figure 3: Distribution of types of bacteria in the wild fish studied



Figure 4: Distribution of types of bacteria in the farmed fish studied



Figure 5: Distribution of types of bacteria in all the fish studied

B. Probiotic potential of isolated strains

All the microbial strains to the exact number of 206 (lactic bacteria and aerobic mesophiles) have been tested to find out whether they have antimicrobial activity against *Escherichia coli* ATTC 25922 and *Staphylococcus aureus* ATTC 25923 by the spot method.

A total of 35 strains were found to be potential probiotic candidates, including 5 strains with activity against *E. coli* and *S. aureus*. Eight (8) strains have activity against E. coli and 27 strains with anti S. aureus activity. The results are reported in Table 3.

The proportions of bacteria with probiotic potential on all bacteria isolated from Tilapia in the various organs are estimated at 57.14% on the digestive tract and 42.86% on the gills.In addition, the proportion of lactic bacteria with probiotic potential is estimated at 25.71% (including 33.33% from the digestive tract and 66.66% from the gills) while that of the total non-demanding flora is 74.28 %. (Of which 46.15% from the gills and 53.85% from the digestive tract).

Concerning the forms (cocci and bacilli) the percentages are as follows: 91.43% for the cocci forms divided into 65.7% cocci with Gram-negative (CG-) and 25.71% for cocci with Gram-positive (CG +). The total proportion of bacilli comes back to 8.57% including 5.71% for Gram-negative bacilli (BG-) and 2.86% for Gram-positive bacilli (BG +).

The percentage of Gram-negative bacteria is estimated at 75.43% and that of Gram-positive bacteria is 28.57%.

Tableau 1: Proportion of bacteria with probiotic potential in wild fish individuals

fish	number of bacteria	number of bacteria with probiotic potential	Rate
NS01	11	4	36.36%
NS02	14	0	0.00%
NS03	13	2	15.38%
NS04	7	3	42.86%
NS05	12	3	25.00%
NS06	10	2	10.00%
NS07	12	1	8.33%
NS08	13	5	30.77%
NS09	9	5	44.44%

Tableau 2: Proportion of bacteria with probiotic
potential in individuals from farmed fish

fish	number of bacteria	number of bacteria with probiotic potential	Rate
NE01	9	1	11.11%
NE02	12	0	0.00%
NE03	11	1	9.09
NE04	10	0	0.00%
NE05	12	0	0.00%
NE06	11	0	0.00%
NE07	11	1	9.09%
NE08	12	4	33.33%
NE09	17	6	35.29%



Figure 6: Proportion of bacteria with probiotic potential in farmed and wild fish

IV. DISCUSSION

The study was carried out on 18 individuals of the same species (*O. niloticus*), including nine (9) sampled on a fish farm in the Ferké 2 area. The nine (9) other individuals come from the Bandama river located in the same area. The objective of this work was to be able to isolate gills and the digestive tract of Tilapia, microorganisms with antimicrobial activity against pathogenic bacteria. These isolated microorganisms are bacteria that are non-pathogenic to fish, living commensally in its organs, and serving as a protective barrier against pathogens.

The enumeration of the bacterioma made it possible to highlight the presence of lactic acid bacteria and nondemanding total flora in the digestive tract and the gills of fish (wild and farmed). These results are in agreement with those of Jeni, Bouhaouala-Zahar [18]and Chemlal-kherraz [19] who observed the presence of lactic bacterioma respectively in the digestive tract and the gills. The presence of nondemanding total flora in the gills and the digestive tract of fish (wild and farmed) is also confirmed by the authorsKapetanovic, Kurtovic [20] and Austin [21].

The non-demanding lactic and total microbial load is generally higher in the gills than in the digestive tract of different individuals (NE and NS). This is explained by the fact that commensal bacteria in fish come mainly from the environment of the fish and its food. To reach the digestive tract, these bacteria pass through the gills, which act as a filter. The microorganisms in the fish ecosystem pass through microbranchial filters before reaching the other organs[21]. The microbial density in the digestive tract varies from 10 3 to 109 CFU/g according toRingo, Bendiksen [22] which is consistent with our results.

Macroscopic observation of the bacterial population of the individuals studied revealed great morphological variability (shape, color and appearance) within the different colonies. The vast majority of bacteria isolated from the digestive tract and gills of *O. niloticus* are cocci. Bacilli are in small quantities or do not exist especially in farmed fish.

This dominance is explained by the environment of the fish, by the dominant flora of its ecosystem, its diet and its physiological state. In addition, the time taken from sampling to laboratory can also favor a high proportion of Gram-negative bacteria which are indicators of fish spoilage.

According to Zhang, Sun [23] the bacterioma of a fish depends on several factors, namely its environment, its diet, its age and its physiological state. The proportion of bacterioma with higher probiotic potential in wild fish from the Bandama river is explained by the ecosystem and the diet of these fish. Indeed in natural environment *O. niloticus* feeds on plankton on the surface of the waters. As a result, its organs are therefore colonized by microorganisms living in the environment, while in the farming system, the diet of this fish is mostly composed of agricultural waste and commercial food. Also, the fish farm's irrigation

system is supplied by rainwater and probably discharges from nearby industrial agricultural farms. However, these waters probably contain pesticides from these industrial agricultural farms. All these reasons could considerably influence the quality of the microorganisms living with these fish, which is why the proportion of microorganism with probiotic potential in farmed fish is lower than that of natural fish.

V. CONCLUSION

The present study was carried out with the aim of isolating from the microbiome of the digestive tract and gills of *O. niloticus* bacteria with probiotic potential for use in aquaculture. Given the importance of aquaculture in the global protein diet and the problem of the use of antibiotics in this sector, world opinion is now turning to their alternative: probiotics. Indeed, the use of antibiotics in order to improve the zootechnical and health performance of fish has led to the appearance of health crises for humans and their environment, of which the most worrying on a global scale remains pathogenic bacteria multi resistant.

Preliminary studies have isolated 206 bacterial strains (lactic bacterioma and non-demanding total flora) from the gills and digestive tract of Tilapia. It turns out that the organs of this fish are the home of bacteria that must be studied with interest.

Then the antibacterial activity tests carried out on all of the isolated strains revealed 35 strains with probiotic potential including 5 strains with antagonistic activity against *E. coli* and *S. aureus* at the same time, 8 strains exhibiting antagonistic activity against *E. coli* screws and 27 strains with anti *S. aureus* activity.

In view of the results, the future prospects for this work are enormous. It will be a question of deepening research in order to identify the strains with probiotic potential isolated, to study their mode of antibacterial action, to test their probiotic character by the tests of selection criterion answering to the name "probiotic". Subsequently, the challenges will be the formulation of food from strains that have responded positively to the criteria for probiotic selection. Finally, in-vitro tests on individuals will follow in order to study the viability of these strains. After all these tests, the product can be offered to aquaculturists.

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