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Evaluation of antibacterial activity and susceptibility antibiotics of native lactic acid bacteria from tilapia (*Oreochromis niloticus*) in Ivory Coast

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Abstract

Lactic Acid Bacteria probiotics play a crucial role in improving aquaculture productivity however, their use required beforehand a rigorous selection. This study appeared as a preliminary research in LAB potential probiotics selection. The aim of study was to select LAB strains potentially probiotic isolated from gut intestine of tilapia (*Oreochromis niloticus*) based on antibacterial activity and susceptibility antibiotics tests. The antibacterial activity was carried by agar well diffusion method against five pathogen indicators (*E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, *P. aeruginosa* ATCC 27853, *P. mirabilis* JCM1669, and *S. aureus* ATCC 25913). The susceptibility antibiotic test was performed by disc diffusion method by 12 antibiotics use. Seventy-two (72) from 154 LAB isolates completed the antibacterial activity test. With 79.08% inhibition, *S. aureus* was the pathogen most inhibited, whereas E. coli had the least inhibition, at 61.44%. In terms of antibiotic susceptibility testing, out of the 72 LAB isolates from earlier studies, only 25 LAB isolates demonstrated resistance to ciprofloxacin, gentamycin, and oxacillin and sensitivity to nine out of the twelve antibiotics employed. The 25 LAB isolates seemed suitable candidates for more probiotic tests.

Keywords: Lactic Acid Bacteria; Probiotic; Antibacterial activity; Susceptibility antibiotic; Aquaculture; Tilapia fish

1. Introduction

One major contributor to the global food supply is aquaculture. Asia presently supplies 90% of the aquaculture produced globally, making it one of the industries expanding at the fastest rate. Fish is more widely available and less expensive in tropical countries when compared to other sources of animal proteins [1]. The increasing demand for fish around the world offers opportunities as well as challenges [2]. In recognition of their multifunctional and technological qualities, probiotics have attracted a lot of attention in aquaculture systems. These properties include boosting growth performance, enhancing immunity, resisting infections, and improving water quality [3-4]. According to Latif et al. [5] and Plaza-Diaz et al. [6], probiotics have been shown to have various mechanisms of action, such as competitive exclusion of pathogens, competition for nutrients, adhesion site competition, improved digestion, contributions to macro-and micronutrients, immunomodulation, and neurotransmitter production. Lactic acid bacteria and yeast, which are recognized growth promoters that preserve the microbial balance in the gut, are among the most researched probiotic microorganisms and their uses in aquaculture [7-9]. Since the turn of the 20th century, lactic acid bacteria (LAB), which are GRAS (generally recognized as safe) species, have been used as starters in a variety of food industries, including aquaculture [9;10]. Lactic acid bacteria (LAB) represent the most common important group of microorganisms used as probiotics for animals [11]. In the fish production sector, lactic acid bacteria (LABs) are utilized to improve feed conversion efficiency, digestion, protein efficiency ratio, survival, and resistance to infections. They also

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prevent antinutritional elements in feeds and avoid intestinal problems. They also strengthen fish's immune systems. By increasing microbial efficiency, LABs can be employed in aquaculture to enhance bacterial growth and monitoring [12]. In order to identify the microbial strains with probiotic potential, various functioning, safety, and storage criteria have been developed [13-16]. This means that the most promising strains can be selected for further investigation. Antibacterial activity remains among most important highlighted parameters for the selection of probiotics [17]. The LAB can provide antitoxin effects, compete with pathogens for vital nutrients, and prevent pathogens from adhering to the intestinal wall [11]. The production of many metabolites, including organic acids, hydrogen peroxide (H_2O_2), and bacteriocins, is linked to the antibacterial action of LAB [18]. Thus, this study aimed to assess the antibacterial activity of lactic acid bacteria strains isolated from gut intestine of Tilapia fish (*Oreochromis niloticus*).

2. Material and methods

2.1. Enumeration of lactic acid bacteria

The international AFNOR standard NF ISO 15214 [19] was used to isolate lactic acid bacteria. Lactic acid bacteria were isolated by spreading 0.1 mL of decimal dilutions on the surface of MRS agar, which had previously been poured and solidified in Petri dishes. The inoculum was then spread using a sterile spreader. The plates were incubated anaerobically at 30 °C for 24 hours in a jar containing a lighted candle. It should be noted that anaerobic conditions are created as soon as the candle is extinguished. All plates containing between 15 and 150 well-isolated colonies were counted.

2.2. Testing for catalase

For this test, a drop of hydrogen peroxide was placed on a slide and a small amount of the same colony from the fresh state is added using a sterile Pasteur pipette; the result is then observed with the naked eye. If bubbles appear, the microorganism produces catalase, otherwise it is said to be catalase negative.

2.3. Gram staining

Gram staining consisted of applying several stains to a given smear. The smear prepared as above was dried next to the Bunsen burner and then fixed over a flame with alcohol at 70 °C. The smear is then covered with gentian purple for 1 minute. The slide was rinsed with tap water and then covered with lugol for 1 minute. A few drops of alcohol are poured onto the slide in a tilted position for discolouration and the slide is then rinsed with tap water and covered with fuschin for 30 seconds. The slide was rinsed again with tap water, dried and then observed under a light microscope at objective X100. Before observation, a drop of immersion oil was placed on the slide. This test enabled the colour, shape and size of the cells to be observed.

2.4. Bacterial strains and culture conditions

Hundred and fifty four LAB strains were identified in the gut of tilapia (*Oreochromis niloticus*) which were collected from the Oceanologic Research Center's aquaculture farm in the Ivory Coast. As indicator strains, five pathogenic bacteria were employed: *S. aureus* ATCC 25913 (American Type Culture Collection (ATCC), Manassas, VA, USA); *P. mirabilis* JCM1669 (University Nangui Abrogoua of Ivory Coast); *P. aeruginosa* ATCC 27853; *E. coli* ATCC 25922; and *K. pneumoniae* ATCC 43816. LAB strains were typically cultivated for 24–48 hours at 37 °C in microaerophilic conditions (5% CO_2) in MRS (De Man, Rogosa, and Sharpe) broth or agar (Oxoid Limited, Hampshire, United Kingdom). The reference pathogenic bacteria were cultured for 18–24 hours at 37 °C in aerobic conditions using tryptic soy broth (TSB) or agar (TSA) (Scharlab S.L., Barcelona, Spain). Before being employed in assays, all strains were subcultured twice and kept at -20 °C in an appropriate culture medium containing 30% (v/v) glycerol (Scharlab S.L., Barcelona, Spain).

2.5. Antibacterial activity of LAB isolates

With some modifications, the agar well diffusion method described by Balouiri et al. [20] was used to record the antagonistic activities of the LAB isolates against five pathogen indicators (*E. coli* ATCC 25922, *K. pneumoniae* ATCC 43816, *P. aeruginosa* ATCC 27853, *P. mirabilis* JCM1669, and *S. aureus* ATCC 25913). The LAB isolates were centrifuged at 10,000 x g for five minutes at 4 °C after being cultivated in MRS broth for 48 hours at 37 °C. Filtration with sterile 0.22 µm Millipore filters (VWR International, Rosny-sous-Bois, France) produced cell-free supernatants (CFS). A sterile Petri dish measuring 90 mm was filled with 1 mL of the overnight pathogen culture (adjusted OD600 nm to 0.2 ± 0.05, or around 10^7–10^8 CFU/mL). Next, about 20 mL of TSA that had been cooled to 45 °C was added, and the mixture was gently homogenised until solidification occurred. Six mm diameter wells were aseptically punctured with a sterile tip, then filled with 100 µL of CFS tested and incubated for twenty-four hours at 37 °C. Positive inhibition was defined as a clear zone surrounding each well that measured 1 mm or more, indicating the antibacterial activity of the CFS.

2.6. Susceptibility to antibiotics

The CLSI [21] disc diffusion method was used to assess the antibiotic susceptibilities of the LAB strains. Twelve (12) antibiotics from eight different classes were used: beta-lactams (Penicillin: PEN 6µg; Amoxicillin: AML 10µg; Oxacillin: OX 5µg); Cephalosporins (Cephalothin: CN 30µg); Aminoglycosides (Gentamicin: GM 10µg; Kanamycin: KAN 1 mg; Streptomycin: STR 500µg); Quinolones (Ciprofloxacin: CIP 5µg); Cyclines (Tetracycline: TE 30µg); Rifampicin (Rifampicin: RAM 30µg); Carbapenems (Imipenem: IPM 10µg); and Phenicols (Chloramphenicol: C 30µg). Oxoid Limited (Hampshire, UK) supplied all of the antibiotics. MRS agar plates were inoculated with 100 µL of fresh LAB cultures (adjusted to the OD600 nm at 0.2± 0.05) and then allowed to dry. On the agar MRS plates, antibiotic discs were added, and the mixture was incubated for 48 hours at 37 °C. As per the Clinical and Laboratory Standards Institute CLSI [21], the zone of inhibition's diameter was measured and categorized as sensitive (S), intermediate resistant (IR), or resistant (R).

3. Results

3.1. Characteristics of Lactic Acid Bacteria isolates

3.1.1. Catalase test

All isolates were tested for their ability to produce catalase or not. The absence of bubbles in the drop of hydrogen peroxide after the addition of a colony of isolates showed that the isolates did not produce catalase, which remains one of the characteristics of lactic acid bacteria.

3.1.2. Gram coloration

Gram staining of the isolates showed a violet stain showed a violet coloration indicating Gram+. Figure 1 is an illustration of an isolate which occurs in clusters and is Gram+ Bacilles.

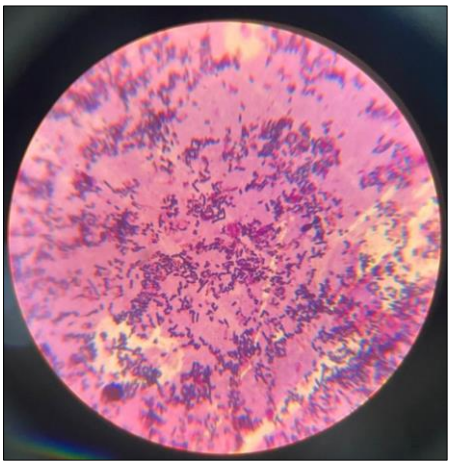

Figure 1 Gram coloration of LAB isolate Gram+ and Bacille

3.2. Antibacterial activity of lactic acid bacterial isolates

The antibacterial activity of lactic acid bacterial isolates was assessed using the well method against the pathogens *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Proteus mirabilis* and *Klebsiella pneumoniae*. In addition, the inhibitory power of lactic acid bacteria isolates was reflected by the appearance of a clear halo around the well, while the absence of a clear halo indicated an inability to inhibit (Figure 2). In this antibacterial activity test, only the lactic acid bacterial isolates that were able to inhibit all 5 pathogens were retained for the rest of the study. The smallest inhibition diameter of 7 mm was obtained against *S. aureus*, while the largest diameter recorded was 23 mm was against *K. pneumoniae*. The distribution of inhibitory lactic acid bacteria isolates presented in Figure 3 showed that out of the 154 isolates tested, 72 isolates were able to inhibit all pathogens, i.e. 5 pathogens, i.e. 49.98±1.92 %. Isolates capable of inhibiting 4, 3, 2 and 1 pathogens represented 20.13±1.6%, 13.19±0.9%, 9.72±0.5% and 6.94±0.3% respectively. Generally, LAB isolates showed high inhibition rates, whatever the pathogen. Inhibition rates ranged from 61.44±0.83% and 79.08±1.93% (Figure 4). The most inhibited pathogen was *S. aureus* (79.08±1.93%) and the least inhibited was *E. coli* (61.44±0.83%).

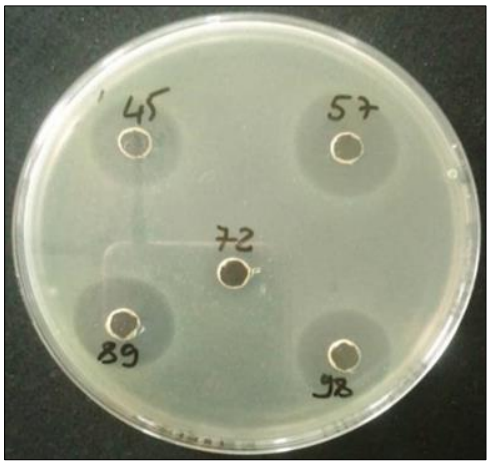

Figure 2 Clear zone indicating pathogen inhibition by LAB isolate

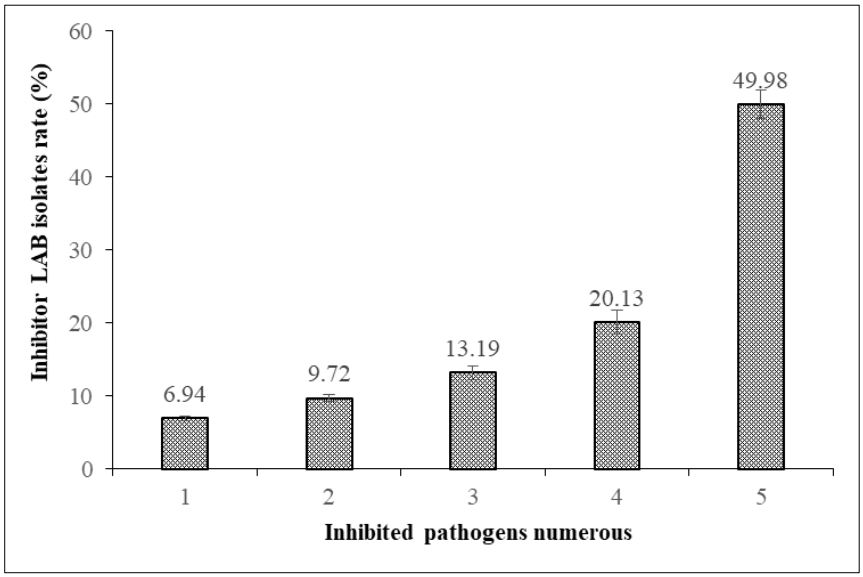

Figure 3 Inhibitor LAB isolates rates

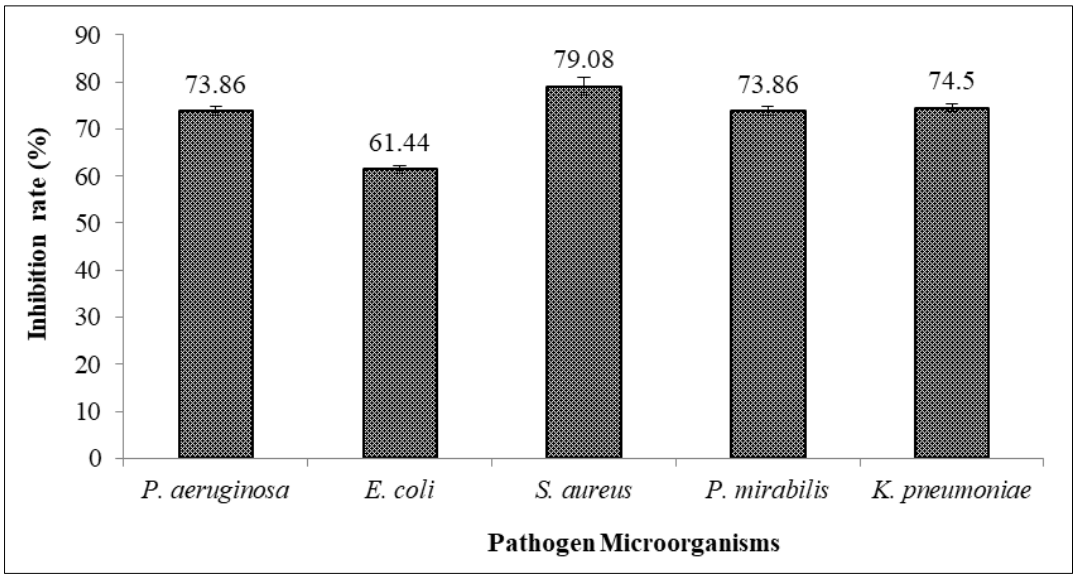

Figure 4 Rate of inhibited pathogen microorganisms

3.3. Antibiotic susceptibility testing of LAB isolates

Antibiotic susceptibility testing was carried out using the disc diffusion method (Figure 5). For this test, 8 families of antibiotics out of a total of 12 antibiotics were used: Beta-lactams (Penicillin: 6 µg; Amoxicillin: 10 µg; Oxacillin: 5 µg), Cephalosporins (Cephalotin: 30 µg), Aminosides (Gentamicin: 10 µg; Kanamycin: 1 mg; Streptomycin: 500 µg), Quinolones (Ciprofloxacin:5 µg), Cyclines (Tetracycline: 30 µg), Rifamycin (Rifampicin: 30 µg), Carbapenems (Imipenem: 10 µg), Phenicoles (Chloramphenicol: 30 µg). Only isolates (72 isolates) that passed the inhibition test against the 5 pathogens were subjected to the antibiotic sensitivity test. In this test, only isolates showing sensitivity to the maximum number of antibiotics were selected. Of the 72 isolates tested, 25 showed sensitivity to 9 of the 12 antibiotics. For this selection test, 25 isolates were selected, whose inhibition diameters are shown in Table 3. All the isolates selected showed multi-drug resistance to Oxacillin (100%), Gentamycin (100%) and Ciprofloxacin (100%).

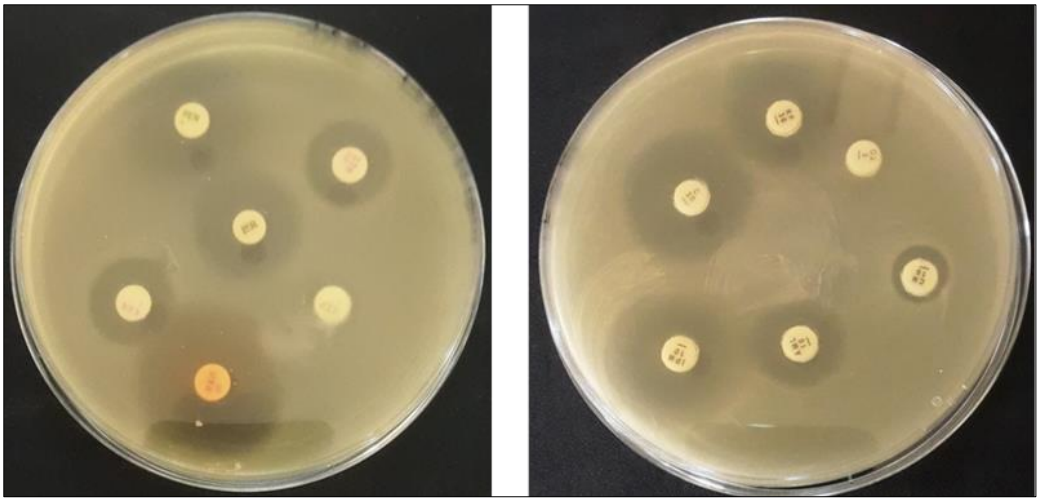

Figure 5 Antibiogram of LAB isolate

Clear zone indicating the sensitivity to antibiotic.

Table 5 Antibiotic susceptibility pattern of LAB isolates

Isolates	AML	OX	C	PEN	CN	TE	KAN	CIP	STR	RAM	IPM	KF
LAB 187	18S	10R	30S	32S	12R	20S	20S	0R	22S	32S	30S	25S
LAB 45	25S	0R	28S	27S	11R	26S	21S	14R	30S	35S	26S	22S
LAB 81	26S	0R	30S	25S	8R	21S	23S	0R	21S	39S	37S	30S
LAB 194	25S	0R	30S	27S	12R	20S	22S	0R	22S	35S	28S	24S
LAB 156	27S	0R	30S	28S	10R	22S	23S	0R	23S	40S	32S	25S
LAB 84	27S	0R	31S	28S	10R	20S	23S	0R	30S	40S	36S	23S
LAB 82	28S	0R	30S	30S	7R	24S	25S	10R	20S	37S	37S	30S
LAB 83	24S	0R	30S	28S	8R	20S	28S	0R	30S	40S	37S	28S
LAB 96	30S	0R	29S	30S	14R	25S	20S	0R	25S	32S	25S	27S
LAB 195	23S	0R	29S	27S	12R	21S	24S	0R	20S	36S	38S	30S
LAB 137	20S	0R	24S	22S	10R	22S	20S	15IR	28S	23S	32S	18IR
LAB 145	27S	0R	25S	25S	10R	21S	20S	0R	29S	30S	33S	22S
LAB 134	25S	0R	30S	25S	11R	20S	24S	0R	28S	36S	36S	23S
LAB 189	25S	0R	30S	20S	10R	20S	23S	0R	21S	35S	35S	25S
LAB 197	27S	0R	27S	24S	12R	25S	21S	12R	17IR	34S	26S	24S
LAB 98	23S	0R	30S	25S	10R	19IR	23S	0R	19IR	38S	40S	30S
LAB 100	23S	0R	26S	20S	10R	23S	19IR	13R	17IR	24S	30S	20S
LAB 196	25S	0R	30S	23S	12R	27S	23S	0R	21S	32S	25S	22S
LAB 143	27S	0R	30S	30S	12R	22S	23S	0R	25S	40S	35S	30S
LAB 166	23S	0R	25S	25S	0R	20S	20S	12R	23S	30S	25S	23S
LAB 197	27S	0R	27S	24S	12R	25S	21S	12R	17IR	34S	26S	24S
LAB 92	23S	0R	27S	25S	11R	18IR	22S	13R	28S	36S	30S	23S
LAB 153	23S	0R	30S	23S	9R	24S	23S	0R	28S	37S	40S	29S
LAB 209	22S	0R	32S	25S	10R	23S	24S	0R	20S	40S	38S	30S
LAB 166	23S	0R	25S	25S	0R	20S	20S	12R	23S	30S	25S	23S

Resistant (R) ≤14 mm; intermediate resistant (IR) 15–19 mm; susceptible (S) > 19 mm

4. Discussion

For several decades, probiotics have been researched for the treatment of fish diseases, primarily for commercial purposes [22]. Beneficial microorganisms are taking the place of growth promoters and antimicrobial drugs. Some candidate probiotics produce antibacterial compounds that inhibit the growth of harmful microorganisms that are harmful to the intestinal microbiome, which causes them to adhere to stomach surfaces and prevent pathogenic growth. Fish pathogenic bacteria compete with one another for nutrients and attachment sites in the stomach in order to prevent pathogenic growth. Several probiotics used in aquaculture have demonstrated direct antibacterial activity when tested for direct antibacterial activity against known pathogens [23-24]. Microorganisms' selection for aquaculture required some criteria. Antibacterial activity and antibiotic sensitivity were among most important criteria. Various bacterial pathogens, which can indicate different sources of contamination, include *Escherichia* sp., *Klebsiella* sp., *Staphylococus* sp., *Proteus* sp., and *Pseudomonas* sp. These pathogens were recovered from fish [25-27]. All LAB isolates showed strong growth inhibition of all reference pathogens: *P. aeruginosa*, *E.coli*, *S. aureus*, *P. mirabilis*, and *K. pneumonia.* These results were similar with those found by Pilet et al. [28]. These latters reported that after screening lactic acid bacteria isolated

from fish and fish products, out of 338 isolates tested for antibacterial activity, only 22 showed antibacterial activity. In this research, from 154 isolates tested, 72 isolates exhibited an antibacterial activity. Even if the metabolites responsible of antibacterial activity in this research were not studied, acoording Girma and Aemiro. [29], the antibacterial activity of LAB could be due to bacteriocins, diacetyl, organic acids, carbon dioxide, and hydrogen peroxide. Antibiotics have long been used in the aquaculture sector to stop infections from harming the crop. Furthermore, this resulted in the development of resistance mechanisms in bacteria and an imbalance in the gut microbiota of aquatic species, both of which had an impact on their health [30-31]. Due to antibiotic-resistant probiotic bacteria have the potential to either directly or indirectly transfer antibiotic-resistant genes to pathogenic bacteria, antibiotic susceptibility is a critical prerequisite from a safety perspective. This method necessitates proof that the LAB strain is not resistant to antibiotics used in veterinary and human medicine. Isolates that completed the antibiotic susceptibility test after the antibacterial activity test were susceptible to the highest number of antibiotics. All LAB isolates were susceptible or intermediate resistant to 9 antibiotics and resistant to 3 antibiotics for the 12 antibiotics used. In contrast to the findings of Coppola et al. [32], who found that lactic acid bacteria were resistant to the common antibiotics, including ampicillin, chloramphenicol, and penicillin. Our study's LAB isolates shown resistance to ciprofloxacin, gentamycin, and oxacillin. Given that penicillin, ampicillin, and chloramphenicol are among of the antibiotics most often used in aquaculture, our findings are nevertheless significant. Moreover, antibiotic sensitivity is a changeable and strain-specific characteristic. Thus, from 72 LAB isolates tested, 25 LAB isolates were showed the sensitivity to 9 on 12 antibiotics used. When selecting probiotic lactic acid bacteria, resistance to antibiotics remains a shortcoming. According to Bhattacherjee et al. [33] and Rhodes et al. [34], the increasing and abusive use of antibiotics has given rise to resistant bacteria through the transfer of resistance plasmids between bacteria. The acquisition of resistance to a given antibiotic may be accompanied by resistance to one or more other antibiotics without the bacterium having been in contact with them. This phenomenon is linked to the frequent presence of several resistance genes on the same plasmid (extra-chromosomal DNA that can be transmitted between bacteria). Antibio-resistance in ichthyopathogenic bacteria is a very worrying phenomenon not only for animal health but also for public health. The possibility of transferring the antibiotic resistance gene from a bacterium in the aquatic environment to a human pathogen cannot be ruled out. The use of broad-spectrum antibiotics is therefore inadvisable, or even prohibited, and reducing the use of antibiotics is an absolute necessity to improve the image of aquaculture production and limit the development and transfer of resistance to human pathogens.

5. Conclusion

In this study, 154 LAB strains isolated from intestine of Tilapia *(Oreochromis niloticus*) were tested as potentially probiotic through antibacterial activity and antibiotic susceptibility. From 154 LAB isolates, 72 LAB isolates were completed the antibacterial activity test. *S. aureus* was pathogen which most inhibited with 79.08% while *E. coli* was least inhibited with 61.44%. Regarding susceptibility antibiotics test, from previous 72 LAB isolates latters, only 25 LAB isolates exhibited a sensitivity to 9 on 12 antibiotics used and showed resistance to ciprofloxacin, gentamycin, and oxacillin. On basis of antibacterial activity and antibiotic susceptibility tests, 25 LAB isolate seemed potential probiotics for aquaculture but either analyses will be required before definitive selection.

Compliance with ethical standards

Disclosure of conflict of interest

All authors have no conflict of interest to disclose.

Statement of ethical approval

The work research complies with the current animal welfare laws in Ivory Coast. The experimental animals' Tilapia (*Oreochromis niloticus*) is not an endangered fish; the provisions of the Govt. of Ivorian's Wildlife Protection Act of 1965 are not applicable for experiments on this Tilapia. All experimental protocols were approved by the University Nangui Abrogoua ethics committee. All methods are reported following ARRIVE guidelines.

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