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Isolation and Identification of Potential Probiotics Bacteria from the Gut of *Oreochromis niloticus* and *Clarias gariepinus* in Uganda

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Authors' contributions

This work was carried out in collaboration between all authors. Authors CDK, SM and AS designed the study, performed the statistical analysis, wrote the protocol, supervised the work, wrote the first draft of the manuscript and managed literature searches. Authors MBM, AT and NLM did the laboratory experiments, managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Bacterial infections remain a hindrance to aquaculture expansion globally. Increased fish mortality and poor performance resulting from ill health has forced farmers to resort to the use of antibiotics globally. However, prolonged use of these drugs in aquaculture is becoming restrained as pathogens develop resistance to drugs and unpredicted long term effect on public health. Alternative approaches to control disease are proposed of which probiotics have come forward.

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Therefore, the purpose of this study was to identify potential probiotic bacteria in the guts of fish from different sampling sites around Kampala. Fish were obtained from selected cages, ponds, tanks and hatcheries around Kampala, including different parts of Lake Victoria. The fish were gutted and the guts aseptically swabbed with subsequent culture on both general purpose and selective media. The identification of various isolates was based on gram staining and biochemical tests. Probiotic screening was done using the agar spot method. Results revealed complete growth across all samples. The total microbial load was highest in the samples from the lake (1204.8±12.7 × 10^5cfu/g). Out of the three probiotic genera isolated, only *Lactobacillus spp* (LB) and *Lactococcus spp* (LC) showed antibacterial activity against selected pathogenic bacteria. The activity of LB was significantly ($p< 0.0001$) higher against *Streptococcus spp* (17.0±0.2 mm) as compared to *Proteus* at 9±0.02 mm and *Pseudomonas* (7.5±0.2 mm). *Lactobacillus spp* did not show any antimicrobial activity against *Staphylococcus aureus*. For *Lactococcus spp*, probiotic activity was only detected against *Proteus spp* (5.5±0.2 mm). Although our study shows that *Lactobacillus spp* and *Lactococcus spp* possess probiotic activity against a number of pathogenic bacteria, characterization of these isolates is paramount before further manipulation.

Keywords: Probiotics; gut; Oreochromis niloticus; Clarias gariepinus; Aquaculture; Uganda.

1. INTRODUCTION

Fish remains a vital resource in Uganda where it significantly contributes to employment, food security and foreign exchange [1,2]. Evidence from the Uganda Bureau of Statistics revealed that in 2005 the sector employed over 1.3 million people in that year alone and the country earned over $143 million in foreign exchange monies. Indeed, according to Esteban et al. [3], in 2009 the global production of fish had increased by 7.5%. Since 2000, aquaculture has slowly come forward as one of the fastest growing food production sectors in the world [4]. In Uganda, this boom in aquaculture has seen the emergency of the Nile tilapia (*Oreochromis niloticus*) and catfish (*Clarias gariepinus*) as main fish species on the market [5].

The aquaculture sector in Uganda however has challenges impeding its progress [6,7]. Foremost, is the problem of fish disease that significantly constrains aquaculture expansion [8]. This constraint in disease has forced farmers to use antibiotics as therapeutic and disease control agents [9]. However, use of antibiotics might alter gut microbial communities inducing resistant bacteria populations, with significant public health outcomes [10]. Indeed, with pathogens becoming resistant and with accumulation of drug residues in fish tissues, use of antibiotics is becoming limited to cure bacterial infection and prevent fish mortality in aquaculture is becoming limited as pathogens develop resistance to the drugs and accumulation of antibiotic residues in fish tissues [11].

The demand for environmental friendly aquaculture has seen research into alternative disease prevention methods such as use of non-pathogenic bacteria called probiotics [12,13]. A number of probiotic bacteria belonging to the lactic acid bacteria (*Lactobacillus*), *Vibrio*, *Bacillus* and *Pseudomonas* genera's have been proposed [13]. Indeed, in aquaculture a number of commercially formulated probiotics are being tried but success rates differ. It is possible that for a given probiotic to be effective, they need to be isolated from the same environment where they will work. Therefore, the aim of the current study was to isolate and identify potential probiotic bacteria in the gut of Nile Tilapia (*Oreochromis niloticus*) and Catfish (*Clarias gariepinus)* from different production systems around Kampala. Results from this study will guide future studies in probiotic formulations and subsequent testing in aquaculture.

2. MATERIALS AND METHODS

2.1 Study Design

A total of 45 Nile tilapia (*Oreochromis niloticus*) and 45 Catfish (*Clarias gariepinus*) were randomly collected from different locations. The sites included Mulungu landing site on Lake Victoria, hatcheries in Kawempe, ponds and tanks at Kajjansi Research Institute and Kitinda cages in Lake Victoria, around Kampala district in Uganda. Freshly captured fish were immediately put in sterile polythene bags, stored and transported in iceboxes to the Microbiology Laboratory at the College of Veterinary Medicine,

Animal Resources and Biosecurity at Makerere University. In the laboratory, immediately on arrival, fish were gutted and the guts aseptically swabbed with subsequent culture on sterilized media plates and incubated at 37℃ for 24 hours. The isolates were then sub cultured to obtain pure cultures and that were further identified using Gram staining and biochemical tests [14].

2.2 Bacteria Isolation and Identification

The bacteriological media of Nutrient agar, MacConkey agar, Potato Dextrose Agar (PDA), de Man, Rogosa and Sharpe agar (MRS), Mannitol Salt Agar (MSA), Thiosulfate-citrate-bile salts-sucrose agar (TCBs) and Blood agar were prepared according to manufacturer's instructions (Sigma-Aldrich, USA). The media were sterilized at 121℃ for 15 minutes in an autoclave and were poured into sterilized disposable plastic petri dishes. The petri dishes were then stored in the incubator after the media had set. The fish were slit along the ventral line to expose the gut. The gut was then cut and a sterile cotton swab was rubbed over the contents and lining. The swabs were then swirled into sterilized peptone water. The peptone water was serially diluted into five dilutions of 9ml. A quantity of 0.1ml of 10^{-3} and 10^{-5} dilution was inoculated onto Nutrient Agar, MacConkey agar, TCBS, PDA, MSA agar plates in duplicates and spread using a sterile glass rod, then incubated aerobically for 24 hours at 37℃ and anaerobically for MRS agar plates at the same temperature for 48 hours, this was strictly done for the first batch of fish samples.

The colony count was calculated by dividing the bottom of the Petri dish into four and the sum of bacterial count was multiplied by the dilution factor. Each distinct colony was further sub cultured on freshly prepared Nutrient agar and for evaluation of purity and colonial morphology. Smears from the isolates were subjected to Gram stain to determine their Gram reaction and biochemical test to genus level previous described by Cheesbrough [15].

2.3 Probiotic Activity Screening

The probiotic strains were screened for antimicrobial activity against selected pathogens using an agar spot method as described previously [16]. Briefly, cultures that were kept overnight for *Lactobacillus spp, Bacillus spp* and *Lactococcus spp* were spotted onto the surface of MRS agar (1.2% w/v agar, 0.2% w/v glucose) plates with subsequent anaerobically incubation for 24 hours at 37℃. The indicator species (*Staphylococcus aureus, Streptococcus spp, Proteus spp and Pseudomonas spp*) were inoculated into 7 ml of soft agar medium (nutrient broth containing 0.7% w/v) to a final concentration of approximately 10^5cfu/g. The soft media were later poured on the plates and incubated for 24 at 37℃. Zones of clearance were later measured in millimetres.

2.4 Data Analyses

All statistical analyses were done using Graphpad 6.0 statistical software. Total microbial load across sampling sites and fish species were done using a Two-way ANOVA. Significant differences in antibacterial activity across the different pathogenic bacteria were analysed using a One-way ANOVA set at significance level of ($P< 0.05$), followed by Multiple comparisons between groups (sampling sites and pathogenic bacteria strains) using Tukey`s multiple comparison test, differences were taken as significant at $P< 0.05$.

3. RESULTS

3.1 Total Microbial Load

In order to determine the total number of viable micro-organisms from the gut, we measured the total microbial load, results are shown in Fig. 1. The results revealed that sampling sites significantly ($p<0.0001$) influenced the total microbial load. On comparison between sampling sites, gut total microbial load was significantly higher ($p< 0.05$) in lakes ($1204.8\pm12.7 \times 10^5$ cfu/g) and cages ($1096.5\pm13.9 \times 10^5$ cfu/g) as compared to ponds ($424.5\pm14.1 \times 10^5$ cfu/g), tanks ($134.5\pm11.3 \times 10^5$ cfu/g) and hatcheries ($100.6\pm2.1 \times 10^5$ cfu/g). On comparison between tilapia and catfish, gut total microbial load in tilapia from ponds was significantly higher ($p< 0.05$) as compared to catfish from the same source. From other sampling localities, gut microbial load did not significantly differ ($p>0.05$) between the two-fish species.

3.2 Prevalent Bacteria Isolated from the Gut

From the gut of *Oreochromis niloticus*, the most prevalent bacteria isolated were: *Escherichia coli* (100%), *Proteus* spp (56%), *Enterobacteria* spp (52%) and *Staphylococcus* spp (42%, Table 1).

The least isolated bacteria were: *Bacillus* spp (24%), *Klebsiella* spp (20%), *Streptococcus* spp (18%) and *Pseudomonas* spp (17%). In *Clarias gariepinus*, the most commonly isolated bacteria were: *Escherichia coli* (100%), *Proteus* spp (56%), *Pseudomonas* spp (48%), *Enterobacteria* spp (48%) and the least isolated being *Staphylococcus* spp (38%), *Bacillus* spp (28%), *Klebsiella* spp (16%) and *Streptococcus* spp (14%, Table 2).

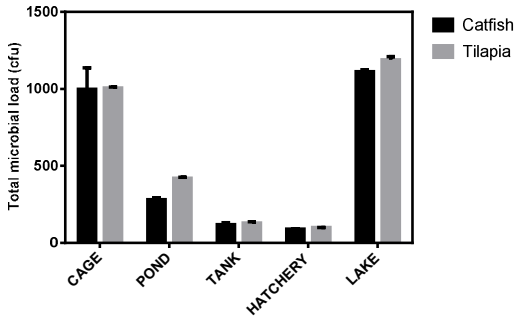

Fig. 1. Total microbial load in both tilapia and catfish from selected sampling systems

3.3 Antimicrobial Activity of Lactic Acid Bacteria on Pathogenic Bacteria

The antimicrobial activity of both *lactobacillus* and *Lactococcus* was significantly ($p < 0.0001$) influenced by the pathogenic bacteria used. The activity of *Lactobacillus* was highest on *Streptococcus spp* (17±0.2 mm) as compared to *Proteus spp* (9±0.2 mm) and *Pseudomonas spp* (7.5±0.2 mm). No activity was detected against *Staphylococcus aureus* (Fig. 2A). For *Lactococcus spp*, activity was only detected for *Proteus spp* (5.5±0.2 mm, Fig. 2B).

4. DISCUSSION

The adverse effects of bacterial diseases are a major concern to the aquaculture industry especially those dealing in economically viable species like the Nile Tilapia and the Catfish [8]. Due to increased fish mortality and poor performance [17], farmers have turned to the use of chemotherapeutic agents like antibiotics to salvage their investments. The prolonged use of antibiotics has significant public health consequences on the environment as well as spawning antibiotic resistant pathogens [18]. As an alternative, probiotic organisms have been suggested [19].

In this study, the sampling site significantly influenced the total microbial load in both species with lake and cage samples recording the highest load. Gut microbial load was significantly higher in fish from lakes and cages. The results obtained in this study for microbial load especially in lake samples have been similarly

Table 1. Prevalent isolates by site of sampling in tilapia

Pathogens	Cage	Pond	Hatchery	Tank	Lake	Total
Escherichia coli	10(100%)	10(100%)	10(100%)	10(100%)	10(100%)	50(100%)
Proteus spp	8(80%)	7(70%)	1(10%)	4(40%)	8(80%)	28(56%)
Lactococcus spp	6(60%)	3(30%)	4(40%)	5(50%)	9(90%)	27(54%)
Enterobacteria spp	5(50%)	6(60%)	3(30%)	5(50%)	7(70%)	26(52%)
Lactobacillus spp	5(50%)	4(40%)	4(40%)	4(40%)	7(70%)	24(48%)
Pseudomonas spp	7(70%)	6(60%)	0	3(30%)	7(70%)	23(46%)
Staphylococcus spp	3(30%)	5(50%)	2(20%)	5(50%)	6(60%)	21(42%)
Bacillus spp	0	4(40%)	1(10%)	2(20%)	5(50%)	12(24%)
Klebsiella spp	2 (20%)	1(10%)	1(10%)	2(20%)	4(40%)	10(20%)
Streptococcus spp	2 (20%)	1(10%)	0	3(30%)	3(30%)	9(18%)

Table 2. Prevalent isolates by site of sampling in catfish

Pathogens	Cage	Pond	Hatchery	Tank	Lake	Total
Escherichia coli	10(100%)	10(100%)	10(100%)	10(100%)	10(100%)	50(100%)
Lactococcus spp	7(80%)	7(70%)	6(60%)	5(50%)	9(100%)	34(68%)
Lactobacillus spp	5(50%)	4(30%)	6(40%)	4(40%)	6(60%)	25(50%)
Pseudomonas spp	7(70%)	6(60%)	1(10%)	3(30%)	7(70%)	24(48%)
Enterobacteria spp	5(50%)	5(50%)	3(30%)	5(50%)	6(60%)	24(48%)
Staphylococcus spp	3(30%)	5(50%)	2(20%)	3(30%)	6(60%)	19(38%)
Bacillus spp	1(10%)	4(40%)	1(10%)	3(30%)	5(50%)	14(28%)
Klebsiella spp	2(20%)	1(10%)	0	2(20%)	3(30%)	8(16%)
Streptococcus spp	2(20%)	1(10%)	0	1(10%)	3(30%)	7(14%)

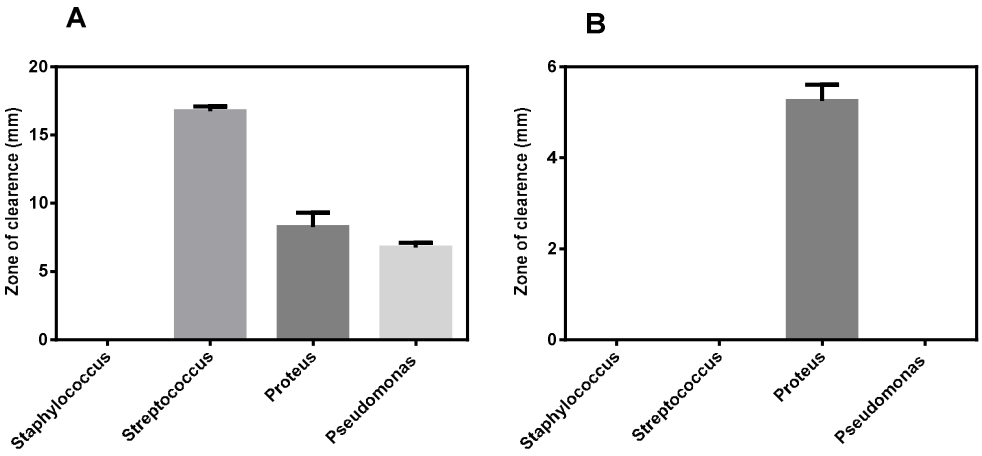

Fig. 2. Probiotic activity of *Lactobacillus* (A) and *Lactococcus* (B) on pathogenic bacteria

reported elsewhere [20,21], pointing to habitant contamination. It is further postulated that tropical areas provide ambient temperatures suitable for bacterial replication [22]. The differences between the bacterial counts in individual fish in this study were supported by Spanggaard et al*.* [23].

According to our study, there were 10 genera of bacteria isolated from the gut. *Escherichia coli* were present in all fish samples as previously shown in other studies [20,24]. Our work is in agreement with work by Emikpe et al. [20] and Gedrich et al. [24] who found other faecal coliforms like *Enterobacter* to make up more than half of total isolates. These coliforms in the gut would point to normal gut flora.

Our results also conform to those obtained by Shinkafi and Ukwaja [22] who also isolated the genus *Bacillus*. Other genera we isolated in our study like *Proteus, Pseudomonas* and *Staphylococcus* were also previously confirmed from the gut of catfish in the fresh water habitats in Ondo State in Southwest Nigeria [25]. *Streptococcus* and *Klebsiella* have been associated with fish ill health [26]. S*treptococcal spp* has been similarly isolated in the intestinal tract of numerous fresh water fish [24]. The bacteria isolated in the fish some opportunistic and others outright pathogenic did not cause mortalities, it is probable that fish had developed protective measures.

According to this study, three probiotic genera were isolated from the gut of both the Nile tilapia and the catfish. These included; *Lactobacillus, Lactococcus* and *Bacillus.* Our study is in agreement with previous studies confirming the presence of lactic acid bacteria in the gut [27-30]. However, unlike *lactobacillus*, reports on the presence of *Lactococcus* in freshwater fishes are scarce.

In this study, *Lactobacillus* had the highest antimicrobial activity against all the selected pathogens except against *Staphylococcus* as previously reported by Ringo et al. [31] and Kato et al. [30]. Its activity was highest against *Streptococcus* with a zone of inhibition of 17±0.2 mm, followed by *Proteus* (9±0.2 mm) and least for *Pseudomonas* (7±0.2 mm). Our findings are in agreement with previous studies showing *Lactobacillus spp* as having a broad inhibitory spectrum against indicator organisms [32,33]. However, Ashraf et al. [34] revealed that all lactobacilli tested (except *L. delbruceki*) inhibited the growth of *S. aureus* and thus were different from the results of the current study. *Lactococcus spp* also showed antimicrobial activity but only against *Proteus* (5±0.2 mm) as previously reported by Kato et al [29] in bacteria isolated from surfaces of tilapia and catfish. These results were also in accordance to Zhou et al*.* [35] who reported the presence of *Lactococcus* in tilapia to inhibit the fish pathogen, *Aeromonas hydrophila.* The mechanism of antibacterial activity in probiotic lactic acid bacteria appears to be multifactorial [36] and might be due to bacteriocins or production of organic acids.

Surprisingly, in our study, *Bacillus spp* did not show any antimicrobial activity against the selected pathogenic bacteria. These results are not in accordance with Sugita et al. [37] who

reported that *Bacillus subtilis* from fish gut produced antibacterial substances. Our results also varied from those of Krishnan et al. [38] who found probiotic activity in *B. subtilis* and *B. licheniformis.* The possible explanation for this is that the *Bacillus* species isolated in the current study might be different and further characterisation might be important to elucidate this.

5. CONCLUSION

In conclusion, fish harbor a wide spectrum of bacteria within the gut some of which might be normal flora and others apparently pathogenic. *Lactobacillus spp* and *Lactococcus spp* demonstrated antibacterial activity against selected potentially pathogenic bacteria, all pointing to the pathogenic nature of these isolates from the gut of both the Nile tilapia and Catfish. However, before these genera could be exploited further, characterization to identify the exact species is paramount.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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