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In-Vitro Probiotic Characterization and Molecular Identification of Autochthonous *Bacillus* spp. from *Oreochromis niloticus*

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Abstract The escalating global aquaculture production necessitates sustainable alternatives to traditional antibiotics due to concerns regarding antimicrobial resistance and environmental contamination. This study investigated the probiotic potential and molecular identity of autochthonous bacterial isolates from the gastrointestinal tract of *Oreochromis niloticus* (Nile tilapia). Microbial populations from the upper and lower intestinal segments were enumerated and characterized using morphological, biochemical, and molecular methods. Results revealed significant spatial variation in bacterial distribution along the gastrointestinal tract, with total bacterial abundance significantly higher ($p < 0.05$) in the lower intestine than in the upper intestine. Among the bacterial groups identified, *Bacillus* spp. showed strong probiotic potential due to their high abundance, spore-forming capacity, and tolerance to gastrointestinal stress conditions. The isolates were Gram-positive, non-hemolytic, and demonstrated strong survival under acidic conditions (pH 2.5~7) and bile salt concentrations up to 1%. Antimicrobial activity assays revealed significant inhibition of pathogenic bacteria including *Escherichia coli*, *Salmonella typhi*, and *Staphylococcus aureus*, with inhibition zones reaching 34 mm. Molecular characterization using 16S rRNA gene sequencing confirmed the isolates as *Bacillus clausii*. Phylogenetic analysis showed high genetic similarity with previously reported *Bacillus clausii* strains in the NCBI database. These findings suggest that autochthonous *Bacillus clausii* isolated from *O. niloticus* possess desirable probiotic characteristics and may serve as promising candidates for functional probiotic applications in sustainable aquaculture.

Keywords Probiotics; *Bacillus clausii*; Nile tilapia; gut microbiota; antimicrobial activity; aquaculture biotechnology

1 Introduction

Global aquaculture production has expanded rapidly over the past few decades, providing a significant proportion of the world's animal protein supply (FAO, 2014). Among cultured species, *Oreochromis niloticus* (Nile tilapia) is one of the most economically important fish species globally, being the most widely cultured fish species in Africa and the second most cultured species in Nigeria (Cavalcante et al., 2020). Its importance is due to its fast growth, adaptability to diverse environmental conditions, and high consumer demand.

However, intensification of aquaculture practices has led to increased disease outbreaks caused by bacterial pathogens such as *Aeromonas*, *Vibrio*, and *Edwardsiella* species. Traditionally, antibiotics have been used to control such infections, but their widespread application has resulted in antimicrobial resistance, environmental contamination, and food safety concerns (Cabello, 2006; Defoirdt et al., 2011).

Consequently, probiotics have gained increasing attention as environmentally friendly alternatives to antibiotics. Probiotics are defined as live microorganisms that confer health benefits to the host when administered in adequate amounts (FAO/WHO, 2002). In aquaculture, probiotics have been shown to improve nutrient utilization, enhance immune responses, and suppress pathogenic bacteria (Nayak, 2010; Ringø et al., 2018).

Host-associated or autochthonous probiotics are particularly promising because they originate from the host microbiome and are therefore better adapted to colonize the gastrointestinal environment. The gut microbiota of fish plays essential roles in digestion, nutrient metabolism, immune regulation, and pathogen exclusion (Gatesoupe, 1999; Ringø and Olsen, 2014).

Among potential probiotic microorganisms, *Bacillus* species are widely studied due to their ability to produce antimicrobial compounds and extracellular enzymes, as well as their capacity to form spores that enhance survival under harsh environmental conditions (Cutting, 2011; Zorriehzahra et al., 2016).

Despite the increasing interest in probiotic development for aquaculture research in rainbow Trout (Ruholla et al., 2021), Catfish (Akanmu et al., 2016; Setufe et al., 2021), Nile Tilapia (Mohammadi et al., 2022), information on autochthonous probiotic bacteria associated with *O. niloticus* remains limited in many aquaculture systems (Cavalcante et al., 2020).

Therefore, the present study aimed to characterize the gut microbial population of *Oreochromis niloticus*, evaluate the in vitro probiotic properties of bacterial isolates, and identify promising probiotic strains using molecular techniques.

2 Materials and Methods

2.1 Sample collection and gut dissection

Healthy Nile tilapia were obtained and dissected under sterile conditions. The gastrointestinal tract was separated into upper and lower intestinal segments to investigate the spatial distribution of gut microbiota.

2.2 Bacterial isolation and enumeration

Gut contents were homogenized, serially diluted, and plated on nutrient agar plates. Colony forming units (CFU g^{-1}) were calculated after incubation. Isolation and enumeration of gut microbiota were performed following standard microbiological procedures described by Cappuccino and Welsh (2017) and Madigan et al. (2018).

2.3 Morphological and biochemical characterization

The isolates were characterized based on the following morphological and biochemical tests:

1 Gram staining was performed according to the classical method described by Beveridge (2001).

2 Endospore staining was conducted using the Schaeffer-Fulton staining technique (Schaeffer and Fulton, 1933).

3 Catalase activity was evaluated according to MacFaddin (2000).

4 Hemolytic activity was determined on blood agar following Verschuere et al. (2000).

5 These tests were used to evaluate the safety and probiotic potential of the bacterial isolates.

2.4 Acid tolerance test

Bacterial isolates were exposed to pH levels of 2.5, 3.0, 3.5, 4.0, and 7.0. Growth and survival were recorded. Acid tolerance assays were performed following methods described by Hyronimus et al. (2000) and Charteris *et al.* (1998).

2.5 Bile salt tolerance test

Bile tolerance was assessed using media containing different bile salt concentrations (0.2%, 0.4%, 0.6%, and 1.0%).Bile tolerance was evaluated according to the procedure described by Gilliland *et al.* (1984).

2.6 Antimicrobial activity assay

Antagonistic activity was evaluated against the following pathogenic bacteria:

1 *Escherichia coli*

2 *Salmonella typhi*

3 *Staphylococcus aureus*

The diameter of inhibition zones was measured in millimeters.

Antimicrobial activity was determined using the agar well diffusion method described by Tagg and McGiven (1971).

2.7 Molecular identification

Genomic DNA was extracted and amplified using universal 16S rRNA primers. PCR products were sequenced and analyzed using BLAST analysis. Genomic DNA was extracted using a modified CTAB protocol according to Saraniya and Jeevaratnam (2012).

2.8 Statistical analysis

All experiments were conducted in triplicate and data were expressed as mean ± standard deviation (SD). Statistical analyses were performed using one-way analysis of variance (ANOVA) to determine significant differences among treatments.

When significant differences were detected ($p < 0.05$), means were separated using Duncan's Multiple Range Test (DMRT). Superscript letters were used to denote statistical differences between treatments.

For microbial enumeration data, bacterial counts were $\log_{10}$ transformed prior to analysis to ensure normal distribution and homogeneity of variance.

The statistical model applied was

$Yij=\mu+Ti+\epsilon ij$

Where:

1 Yij= observed value

2 μ= overall mean

3 Ti= treatment effect

4 ϵij= experimental error

Significance was declared at $p < 0.05$.

All analyses were conducted using SPSS version 25.0 (IBM Corp., USA).

3 Results

3.1 Distribution of gut microbiota

The distribution of bacterial populations along the intestinal tract of Oreochromis niloticus is presented (Figure 1). The results indicate that the lower gut harboured consistently higher bacterial loads compared to the upper gut across all bacterial groups, including total heterotrophic bacteria, cocci, and *Bacillus* spp. This pattern may be associated with the more stable physicochemical conditions and nutrient availability in the distal intestine, which favor microbial proliferation and colonization.

Table 1 Relative abundance of bacterial groups in the gastrointestinal tract

Bacterial Group	Representative Genera	Upper Gut (CFU g^{-1})	Lower Gut (CFU g^{-1})	Probiotic Potential
Helicobacter / Fusobacterium	Opportunistic commensals	1.75×10^{1a}	6.65×10^{6b}	No
Cocci bacteria	Staphylococcus, Streptococcus	1.14×10^{6a}	3.05×10^{6b}	Variable
Bacillus spp.	Spore-forming rods	1.14×10^{6a}	3.05×10^{6b}	Yes

Different superscripts indicate significant differences ($p < 0.05$).

3.2 Morphological and biochemical characterization

3.2.1 Gram staining

Gram staining result presented shows both *Bacillus* spp. and Cocci bacteria to be gram positive while *Helicobacter spp* and *Fusobacterium spp* to be gram negative (Table 2).

3.2.2 Spore test

Table 3 shows that only *Bacillus* spp. is capable of sporulation, while the other isolates; Cocci bacteria, *Helicobacter* spp. and *Fusobacterium* spp. are non-spore forming bacteria.

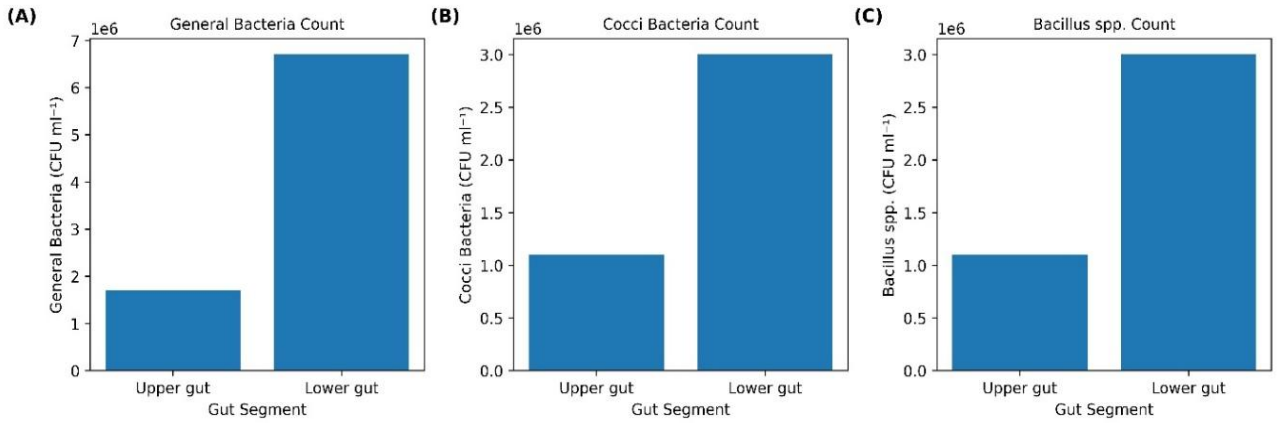

Figure 1 Comparative bacterial load in the intestinal tract of *Oreochromis niloticus*

Note: (A) Total heterotrophic bacteria, (B) cocci bacteria, and (C) *Bacillus* spp. counts in the upper and lower gut segments. Bacterial abundance is expressed as colony-forming units per millilitre (CFU mL^{-1}). The lower gut consistently exhibited higher bacterial populations than the upper gut.

Table 2 Gram staining results

Isolate	Morphology	Gram Reaction
Bacillus spp.	Rod-shaped cells	Gram positive
Cocci bacteria	Spherical cells	Gram positive
Helicobacter spp.	Spiral rods	Gram negative
Fusobacterium spp.	Spindle rods	Gram negative

Table 3 Spore staining

Isolate	Spore Formation
Bacillus spp.	Positive
Cocci bacteria	Negative
Helicobacter spp.	Negative
Fusobacterium spp.	Negative

3.2.3 Catalase and hemolysis test

The result of production of catalase and hemolytic test by the Isolates is presented in Table 4. Gas production from the isolates comes out positive for both *Cocci* and *Bacillus spp*, while both isolates were negative to hemolysis which is a good sign of potential probiotics

Table 4 Catalase and Hemolysis Test

Isolate	Catalase	Hemolysis
Bacillus spp.	Positive	Negative
Cocci bacteria	Positive	Negative

The absence of hemolytic activity suggests the isolates are non-pathogenic and safe for probiotic application.

3.3 Acid and bile salt tolerance

The results of acid tolerance and bile salt tolerance are presented in Tables 5 and 6, respectively. The results shows that selected isolates survived bile salt at all concentration, however, the tolerance of *Bacillus* spp. is greater than Cocci bacteria at all level of bile salt concentration. Also, isolates survived pH tolerance at all concentration, meanwhile, the tolerance level of *Bacillus* spp. at all pH concentration is higher in value than the value recorded for Cocci bacteria.

Table 5 Acid tolerance of isolates

Isolate	pH 2.5	pH 3.0	pH 3.5	pH 4	pH 7
Bacillus spp.	1.8±0.2	2.2±0.4	2.6±0.2	2.8±0.3	3.4±0.2
Cocci bacteria	0.4±0.1	0.7±0.2	0.9±0.1	1.1±0.2	1.8±0.3

Statistical analysis showed significantly higher acid tolerance ($p < 0.05$) for *Bacillus* isolates across all pH levels.

Table 6 Bile tolerance

Isolate	0.2%	0.4%	0.6%	1.0%
Bacillus spp.	2.8±0.3	2.2±0.1	1.9±0.2	1.6±0.1
Cocci bacteria	2.7±0.1	2.1±0.3	1.6±0.1	1.3±0.2

The superior tolerance exhibited by *Bacillus* spp. indicates their adaptation to intestinal bile conditions.

3.4 Antimicrobial activity

As presented in table 7 and figure 2, the inhibitory activity of the tested probiotic isolates against *Escherichia coli* isolates showed clear differences between *Cocci* spp. and *Bacillus* spp., as demonstrated by their respective zones of inhibition. *Bacillus* spp exhibited the highest antagonistic effect with a mean inhibition zone of 34 mm, while *Cocci* spp. produced a comparatively smaller zone of 26 mm. The significantly larger inhibition zone produced by *Bacillus* spp. indicates a stronger antimicrobial potency against the target pathogen.

Table 7 Zone of inhibition against pathogens

Pathogen	*Cocci* spp. (mm)	*Bacillus* spp. (mm)
Escherichia coli	26	34
Salmonella typhi	29	29
Staphylococcus aureus	39	39

Bacillus isolates demonstrated strong antagonistic activity, suggesting production of antimicrobial compounds.

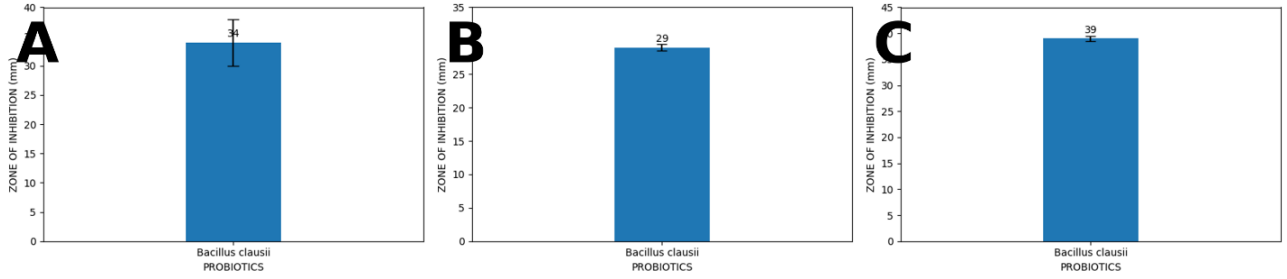

Figure 2 Diameter of inhibition zone of *Bacillus clausii* tested against A=*Escherichia coli* B=*Salmonella typhi* C=*Staphylococcus aureus* isolates.

Mean radii with different superscripts indicate significant ($\alpha_{0.05}$) difference according to DMRT.

3.5 Molecular identification

PCR amplification of the 16S rRNA gene produced single bands of approximately 1500 bp, confirming successful amplification. Sequencing analysis identified the isolate as Bacillus clausii. Phylogenetic analysis revealed a close evolutionary relationship between the identified strain and previously reported *Bacillus clausii* strains retrieved from the NCBI GenBank database (Figure 3). The identified strain showed close clustering with *Bacillus clausii* PRA25, Bacillus clausii AK31, and Bacillus clausii XJU2. Bootstrap values ranged from 86% to 98%, indicating strong support for the phylogenetic clustering.

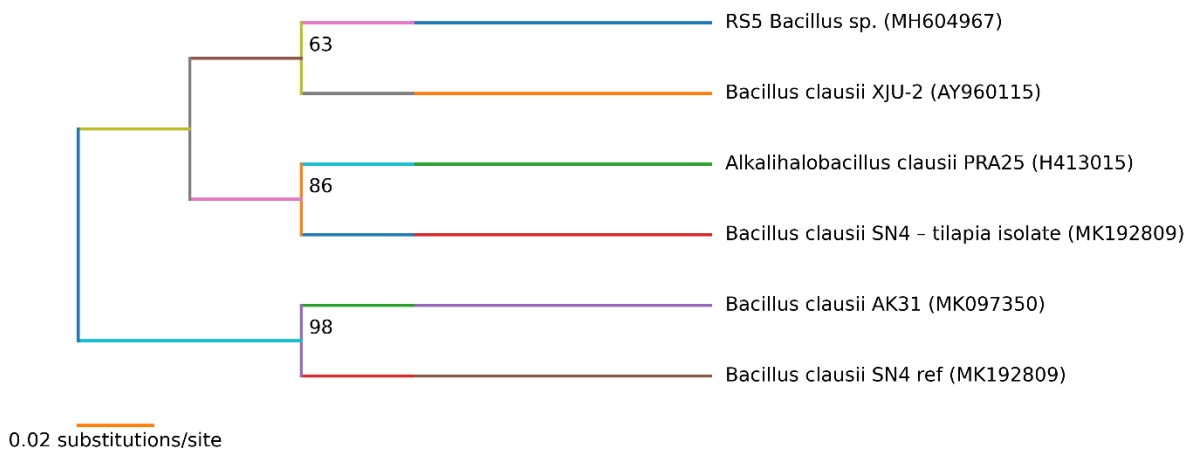

Figure 3 Phylogenetic tree based on 16S rRNA gene sequences

Note: The phylogenetic tree shows the relationship between the isolated strain *Bacillus clausii* SN4 from *Oreochromis niloticus* and closely related Bacillus species retrieved from GenBank. The tree was constructed using the Neighbor-Joining method, and bootstrap values (%) based on 1000 replicates are indicated at branch nodes. The scale bar represents 0.02 nucleotide substitutions per site.

4 Discussion

4.1 Gastrointestinal microbial distribution in *Oreochromis niloticus*

In the present study, microbial abundance was significantly higher in the lower intestine compared with the upper intestine (Table 1 and Figure 1). This pattern is consistent with the typical spatial organization of microbial communities in the gastrointestinal tract of teleost fish. TThe distal intestine generally provides more favorable conditions for microbial colonization due to lower oxygen concentrations, slower digesta transit, and greater availability of fermentable substrates. These environmental conditions promote the growth of facultative and anaerobic microorganisms that participate in fermentation processes and nutrient metabolism. Similar spatial distribution patterns have been reported in several fish species, including tilapia, salmonids, and carp, where the distal gut harbors higher microbial densities and greater microbial diversity (Nayak, 2010; Ringø et al., 2016). The higher bacterial counts observed in the lower intestine in this study therefore suggest that this region represents a major site for microbial metabolic activity and host–microbe interactions.

4.2 Probiotic potential of *Bacillus* species

Among the bacterial isolates obtained from the gastrointestinal tract of *O. niloticus*, members of the genus *Bacillus demonstrated* promising probiotic characteristics. *Bacillus* species are widely recognized as beneficial probiotic candidates in aquaculture due to several advantageous physiological traits, including their ability to form endospores, produce antimicrobial metabolites, and secrete extracellular digestive enzymes.

The spore-forming ability observed in the isolates as presented in Table 3 is particularly significant for probiotic applications. Endospores exhibit remarkable resistance to environmental stressors such as heat, desiccation, and acidic conditions. This resilience improves the stability of probiotic formulations during feed processing and storage while ensuring survival during passage through the gastrointestinal tract (Cutting, 2011; Hong et al., 2005). As a result, *Bacillus*-based probiotics are commonly incorporated into aquaculture feeds to improve digestive efficiency and disease resistance.

4.3 Safety assessment of the probiotic candidates

The safety evaluation showed that the isolates exhibited no hemolytic activity on blood agar, as shown in Table 4, indicating that they are non-hemolytic and therefore unlikely to be pathogenic. Hemolysis is commonly used as an indicator of bacterial virulence, and the absence of hemolytic activity is considered an important safety criterion for probiotic candidates (Verschuere et al., 2000). The non-hemolytic behavior observed in this study therefore

supports the suitability of these isolates for further probiotic development. Catalase activity was positive in *Bacillus* spp., indicating the ability of the isolates to produce catalase enzymes that degrade hydrogen peroxide into water and oxygen.

4.4 Acid and bile tolerance as probiotic selection criteria

Tolerance to acidic and bile environments represents a fundamental requirement for probiotic microorganisms, as they must survive the harsh conditions of the stomach and intestine before exerting beneficial effects within the host.

In the present study, the bacterial isolates demonstrated considerable survival across a wide pH range (2.5~7.0) table 5, with *Bacillus* isolates showing significantly higher tolerance compared to cocci bacteria. The ability to withstand acidic environments suggests that these isolates possess physiological adaptations that maintain cellular stability under gastric stress. Similar acid tolerance characteristics have been reported for *Bacillus clausii* strains used in commercial probiotic preparations, further supporting the robustness of this species under gastrointestinal conditions (Hong et al., 2005).

The isolates in this study exhibited tolerance to bile concentrations of up to 1% table 6, with *Bacillus* isolates showing superior tolerance compared to cocci bacteria. Such tolerance indicates that the isolates are capable of surviving within the intestinal environment where bile salts are present during digestion. Previous studies have suggested that bile tolerance in probiotic bacteria may be associated with mechanisms such as bile salt hydrolase activity and membrane modifications that protect cells from bile-induced stress (Gilliland et al., 1984). Therefore, the acid and bile tolerance exhibited by the isolates strongly supports their probiotic potential.

4.5 Antimicrobial activity against fish pathogens

The antimicrobial activity of *Bacillus* species is well documented and is often attributed to the production of bioactive compounds such as bacteriocins, lipopeptides (including surfactin, iturin, and fengycin), and organic acids (Zhou et al., 2010; Ringø et al., 2018). These antimicrobial substances inhibit pathogenic microorganisms through mechanisms such as membrane disruption, competitive exclusion, and nutrient competition. The strong inhibitory activity of *Bacillus species* against *Escherichia coli*, *Salmonella typhi* and *Staphylococcus aureus* as observed in this study is presented in table 7 and figure 2 therefore suggests that the isolates may contribute to maintaining microbial balance within the gastrointestinal tract and preventing pathogen colonization in *O. niloticus*.

4.6 Molecular identification and phylogenetic analysis

Molecular characterization using 16S rRNA gene sequencing identified the isolates as *Bacillus clausii*. This species is widely recognized for its probiotic properties and has been extensively used in both human and veterinary probiotic formulations. *Bacillus clausii* is known for its remarkable resilience to environmental stress and its ability to modulate host immune responses.

Previous studies have demonstrated that *B. clausii* can enhance innate immune responses, stabilize intestinal microbiota, and improve resistance to bacterial infections in aquaculture species (Ringø et al., 2018). The identification of this species within the gastrointestinal tract of *O. niloticus* therefore suggests that it may play a natural role in maintaining gut microbial homeostasis.

Phylogenetic analysis further confirmed the taxonomic identity of the isolate by demonstrating close genetic relationships with previously reported *Bacillus clausii* strains in the NCBI database. The high bootstrap support values (86%~98%) observed in the phylogenetic tree indicate strong confidence in the clustering of the isolate within the *B. clausii* lineage.

4.7 Significance of autochthonous probiotics in aquaculture

The identification of autochthonous probiotic microorganisms is particularly advantageous for aquaculture applications because host-associated bacteria are more likely to colonize and persist within the gastrointestinal

tract. Unlike allochthonous probiotics introduced from external sources, autochthonous strains are naturally adapted to the host's intestinal environment, which enhances their colonization efficiency and functional stability (Gatesoupe, 1999; Ringø and Olsen, 2014). The isolation of *Bacillus clausii* -like strain of *O. niloticus* therefore represents a promising step toward developing host-specific probiotic formulations that could improve fish health and aquaculture productivity.

4.8 Limitations and future research directions

Despite the promising findings obtained in this study, several limitations should be acknowledged. First, the probiotic properties of the isolates were evaluated only through in vitro assays, which may not fully reflect their performance within the complex biological environment of the fish gastrointestinal tract. Second, the study focused primarily on basic probiotic characteristics such as acid tolerance, bile tolerance, and antimicrobial activity, while other important functional properties—including enzyme production, adhesion ability, and immunomodulatory effects—were not investigated.

Future research should therefore focus on in vivo validation of the probiotic potential of the identified *Bacillus clausii* strain. Controlled feeding trials should be conducted to evaluate its effects on growth performance, feed conversion efficiency, immune responses, and disease resistance in *O. niloticus*. Additionally, advanced molecular approaches such as metagenomics and transcriptomics could provide deeper insights into the interactions between the probiotic strain, native gut microbiota, and host immune pathways.

4.9 Implications for sustainable aquaculture

Overall, the findings of this study contribute to the growing body of knowledge on fish gut microbiota and probiotic development for aquaculture. The isolation and characterization of *Bacillus clausii* from the gastrointestinal tract of *Oreochromis niloticus* highlight the potential of host-associated microorganisms as sustainable alternatives to antibiotics in aquaculture production systems. By promoting microbial balance, enhancing digestive efficiency, and suppressing pathogenic bacteria, such probiotics may play an important role in improving fish health, increasing aquaculture productivity, and supporting environmentally sustainable aquaculture practices.

Authors Contribution

O.A. Onada was responsible for conceptualization, methodology development, investigation, data curation, laboratory experiments, formal analysis, and writing the original draft. E.K. Ajani and E.F. Osho contributed to supervision, project administration, methodology validation, result interpretation, and manuscript review and editing. All authors have read and approved the final version of the manuscript.

Conflict of Interest Disclosure

The authors affirm that this research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

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