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In-vitro evaluation of probiotic properties of some *Bacillus* strains isolated from catfish in Makurdi, Nigeria

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ABSTRACT: This study was designed to evaluate potential probiotic bacteria isolated from indigenous freshwater catfish (*Clarias anguillaris*) for productive performance and health status of catfish in the year 2022. The probable probiotic bacteria (*Bacillus species*) were isolated and identified from the skin and intestine of catfish (*C. anguillaris*) using standard procedures. The identified *Bacillus* species were screened for probiotic properties such as antagonistic (inhibitory) properties, bacteriocin production ability, pH and bile tolerance, amylolytic; protease and lipolytic activities and virulence tests were performed using standard procedures. Successful bacteria were molecularly characterized using polymerase chain reaction (PCR) and sequenced for definitive identification. Species with DNA sequences that matched closely with typed probiotics from National Center for Bioinformatics (NCBI) data base were picked as prospective probiotic bacteria. Ninety (90) and eighty-six (86) bacterial species from skin and intestine respectively were recorded. Five (5) and eleven (11) *Bacillus* species from skin and intestine respectively were subjected for screening for probiotic capability. All the *Bacillus* species isolated, characterized in this study showed positive response to the probiotic properties as possible probiotic bacteria. All the *Bacillus* species inhibited more than 1 indicator organism with minimum of 11 mm diameter zone of inhibition (DZI) during antagonistic test and all possess ability of bacteriocin production except Bsp4 and Bsp7.

Keyword: *Bacillus, Clarias anguillaris,* Isolation, Makurdi, Probiotic.

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INTRODUCTION

Aquaculture is commonly referred to as fish farming (Souza, 2018 because fish farming is the principal form of aquaculture (Wang *et al*., 2021) which implied some sort of intervention in the rearing process to enhance production processes such as regular stocking, feeding, and protection from diseases and predators. The rapid growth of aquaculture is closely linked to its significant contribution to global food production, raw materials for industries and pharmaceutical use and aquatic organisms for stocking and/or for ornamental trade increased dramatically in the past recent decades (FAO, 2010), which made the need for aquaculture to increase globally. The demand of fisheries in developed and developing countries has continued to grow due both to population growth and increased per-capita consumption. Fish and fish products is the cheapest source of protein and an important cash crop improving the livelihood of both rich and peasant in many regions of the world (FAO, 2016) that has contributed for 1% of all global trade in value terms (Bhatnaga and Davi, 2013). However, the aquatic organisms are like any other terrestrial animals, they can experience problems related to disease conditions either due to deterioration of the environment or from ill handling that results in some serious economic losses. Conventional approach adopted to prevent and control disease occurrence in aquaculture is the use of antimicrobial drugs (Alderman and Hastings, 2004), but Rodgers and Furones (2009) had observed that limited success had been achieved. Disease treatments are often ineffective, costly and result in development of drug resistant pathogens, product, residues in tissues and environmental contamination (Senok *et al*., 2005). The indiscriminate use of antibiotics even without diagnosis resulting to giving incorrect dose, have led to an increase frequency of drug resistant microorganisms and multiple antibiotics resistance (Muteeb *et al.*, 2023). There have been serious growing concerns on the public health threat, which not only in human medicine, but also in aquaculture (Jahangiri and Esteban, 2018). The consequences of antibiotic use have become a global issue, leading to restrictions or bans on their use in food producing animals. One of most significant technology that evolved in response to disease control problem is the use of beneficial bacteria (probiotics) which have been considered a valid alternative to prophylactic use of antibiotics for control of bacterial pathogens in aquaculture and promoting the growth of animals. There is therefore growing advocacy to shift from the use of the troublesome antimicrobial agents to use a safer technology that is free from public health hazard. The application of beneficial (probiotic) bacteria which control pathogens through variety of mechanisms of action is globally viewed to be the best alternative to antibiotic treatment. The use of probiotics in human (Rocha-Ramírez *et al*., 2017) and aquaculture have been documented which is focused on the probiotic ability to improve the immune response and nutritional parameters of the host through efficient feed conversion ratio (Rini *et al*. 2014). Other significances are stimulation of appetite, improvement of nutrition by production of vitamins, detoxification, and breakdown of indigestible compound (Jahangiri and Esteban, 2018) which are not obtainable with antibiotics. The United State Food and Drug Administration (FDA) designated probiotics as 'generally recognized as safe' (GRAS) according to Arora *et al*. (2019). The term probiotic is derived from two Greek words, 'Pro and Bios' which means 'for life' is defined as live microbial which when administered adequately or appropriate confers health benefits to the host (FAO, 2001; FAO/WHO, 2002). Due to aquaculture peculiarities' probiotics could be defined as live microbial supplement that is administered via artificial or natural feed or directly into rearing water which provide benefits to the animal by improving the water quality, enhancing nutrient utilization, and the general performance of the host. The aim of the study was to isolate, identify *Bacillus* species from the skin and intestinal tract of indigenous catfish (*Clarias anguillaris*) and to screen for potentials of probiotics *In-vitro* for catfish production

MATERIALS AND METHODS

Sampling Methods

Apparently healthy fish samples of *Clarias anguillaris* of both sexes with mean weight and total length of 96±28 grams and 24±22 centimeters respectively were obtained randomly from FUAM fish hatchery complex (N = 115) and 6 homestead fish ponds (N = 135) in Makurdi metropolitan between July to September 2022. The fishes were caught randomly from either concrete or earthen ponds using dragging net measuring 6x2m^2 with mesh size of 2cm^2 by two trained fishermen between 8.00 to 10.00 hours in the morning. The fish species were identified with the help of pictorial fish diagrams compiled by Olaosebikan and Raji (2004). Immediately after collection on each sampling day, the fish samples were trans-

ported live in a 50 liters Jerrycan containing water from the same source of fish to the laboratory of the Federal University of Agriculture, Markudi where the bacterial sample cultivation was carried out.

Sample collection for cultivation of bacteria isolates.

The fish was euthanized using MS222 (Tricaine methane-sulfonate) (Lin *et al*., 2014) and placed on a disinfected table. Aseptically, samples for bacterial culture were obtained from the skin and gastrointestinal tract of the *C. anguillaris* using sterilized surgical kit and hand gloves as described by Abareethan and Amsath (2015). Sterile swab sticks were used to swab the skin of the fish for sample collection. After collection of sample from the skin, the abdominal region of the fish was disinfected with 70% ethanol. The gut region was incised to expose the gastrointestinal tract using sterile scalpel blade. The intestine was incised longitudinally and materials from the intestinal lumen were obtained using sterile wire loop. Each sample obtained from the skin and intestine was inoculated onto brain heart infusion agar (BHIA, LAB048, UK) plate and incubated for 18-24hrs at 37^{o}C (Cowan and Steel, 1993). Pure cultures were obtained by using sterilized wire loop to pick discrete colonies with varying morphologies and sub-cultured on separate fresh culture plates using the same agar. These isolates were grouped into Gram positive coci or rods and Gram negative using Gram stain (LAB, Nigeria) and stock cultured on Nutrient agar (TM MEDIA, India) slant and stored at 4^{O}C until further use.

Phenotypic identification of *Bacillus* species as bacteria of interest

All Gram-positive rods were identified by following a Gram-positive identification flow chart provided by Bergey's Manual of Determinative Bacteriology using standard procedures. Tests including carbohydrate fermentation tests (glucose, lactose, maltose, manitol, fructose, arabinose, sucrose, rhamnose, xylose, sorbitol), enzyme production tests (catalase, oxidase, urease, citrate), motility test, nitrate reduction test, Voges Proskauer, methyl red test, amylase test, NaCl growth test, growth at 55^{o}C and starch hydrolysis test were conducted.

Molecular characterization of *Bacillus* species, using polymerase chain reaction (PCR).

Genomic DNA (gDNA) was extracted from each isolate using the bacterial DNA Extraction Kit (QuickExtract™ Epicentre, USA) and modified protocol of Baranzoni (2014). Briefly, 0.5 mL of the bacterial culture grown in brain heart infusion broth (BHIB, LAB048, UK) for 24hrs at 37^{o}C was centrifuged at 1,700 x g (5,000 rpm) for 10 minutes in a micro-centrifuge to pellet the cells. The supernatant was decanted and the cell pellet was washed once with 0.5 ml of sterile distilled water, and thereafter re-centrifuged at 1,700 x g (5,000 rpm) for 10 minutes. The supernatant was decanted. Hundred microliters (µL) of QuickExtract bacterial DNA Extraction Solution (Epicentre /USA) containing Rnase A was added to the cell pellet. Then 1 µL of Ready-Lyse Lysozyme solution was added to each tube and mixed gently by inversion to be sure that both the bacteria cells and the Ready-Lyse Lysozyme are dispersed in solution. Then the suspension was incubated at room temperature for 15 minutes and thereafter was centrifuged at 12,000 rpm for 20 minutes. Add 30µL 10% sodium dodecyl sulfate (SDS, Oxoid USA) and 3µL proteinase K, and gently invert and incubate at 50°C for 60 minutes. Centrifuge at 12,000 rpm for 15 minutes and carefully decant the supernatant and thoroughly dry pellet in a 50°C incubator. The pellet was re-suspended in 50 µL Tris-EDTA (TE) buffer and allow pellet to sit overnight at 4°C as the DNA. The extracted and purified DNA was stored on FTA™ Micro Card.

Amplification of the 16S rDNA gene was carried out by PCR using universal primers 27F and 1492R targeting the V1 to V9 variable regions by PCR followed by sequencing (sense and antisense) of the 1465bp amplified products using primers 27F, 1492R, 518F and 800R as previously described by Sontakke *et al.* (2009). A consensus sequence covering the entire amplified region was then assembled using the Bio-Edit Software (STABvida, Portugal**)**. Earlier a 2µL of PCR product and 5µl of DNA ladder were loaded onto a 1.5% Agarose gel, stained with ethidium bromide and electrophoresed at 120V for 20 minutes. Gel image was photographed using the Bio-Rad documentation unit (Molecular imager®, Crescent Lab, USA). Identification of each isolate was carried out by querying each consensus sequence to sequences in the GenBank using the basic local alignment search tool – BLAST. The most similar bacterial species was found in the GenBank by using BLAST search (http://www.ncbi.nlm.nih.gov/).

Antagonistic capability tests of *Bacillus* species

All isolates of *Bacillus* species identified were subjected to screening process for probiotics using well diffusion agar assay (WDAA) according to modified

method of Hemaiswarya *et al*. (2013). Standardized suspension of the potential pathogenic bacteria was obtained by suspending colonies of potential pathogen (seed organism) in normal saline. Sterile swab stick was immersed in the standard suspensions and the surface of nutrient agar plate was inoculated by streaking the swab stick several times over the entire agar surface to obtain uniform inoculum and thereafter, wells of 3mm in diameter were punctured into the agar using cork-borer. 0.2 mL of potential probiotic (*Bacillus* species) culture with approximately 1 x 10^8 cfu/mL obtained from BHIB (LAB048-UK) was added into the wells and incubated for 18-24hrs at 37°C. Positive results for potential probiotics were signified by clear zone of inhibition around the wells. The screening was performed in factorial combinations in such a way that every single *Bacillus* species was tested against all the potential fish pathogens (seed organism) selected.

In another experiment, the test isolates, grown in MRS broth, were centrifuged for 15 minutes at 16xg at 4°C. The supernatants were harvested in separate Eppendorf tubes and the sediments were discarded. A drop (0.1ml) of 1x10^8 McFarland standards of potential pathogenic bacteria was seeded on the agar plates and sterile swap stick was used to spread the inoculum on the entire surface of the agar plates. After the seeding, well of 3mm in diameter were bored into the seeded agar plates using Cork Borer. The supernatants of the putative bacteriocin were added into the wells and incubated and clear zones of inhibition around wells indicated positive for bacteriocin production.

The Bile and pH tolerance test

The effects of bile on the growth of probiotic strains were examined using modified methods by Vine (2004). Test strains were grown in MRS (TM MEDIA, TM147-India) broth at 37 °C for 24 hours. Different bile concentrations [(0%, 0.1%, 0.2% and 0.3% (w/v)] were prepared by dissolving oxygal bile salt in MRS (TM MEDIA, TM147-India) broth. The various concentrations of the bile solutions were inoculated with 100µl of test isolate were incubated and thereafter, the growth rate was assessed by measuring the optical density (OD) using spectrophotometer at 600nm. All the experiments were performed in triplicate. To know the ability of a probiotic to survive the transit of the gastrointestinal tract of the host, different pH levels such as 1.3, 3.0, 5.0 and 7 (control) were prepared by using 1% HCl and 1M NaOH to Man Roggsa sharp (MRS TM MEDIA, TM147-India) broth to obtain a desired pH. These were dispensed into universal bottles with control bottles containing only the MRS broth. All the broth media were autoclaved at 121°C for 15 minutes and after cooling, were inoculated with 0.2ml of overnight broth culture of *Bacillus* species and incubated at 37°C for 24hrs. The optical density (OD), as growth rate of bacteria, was measured by spectrophotometer (UV 2100 UNICO USA) at 600nm. The tolerance of each testing isolate to the different pH was determined as the percentage reduction in absorbance in relation to the growth medium with pH 7 (control).

Digestive enzyme production test

Amylase production test was done using a modified method of Rini *et al*. (2014) and Sneha *et al*. (2014) to know the ability of *Bacillus* species to hydrolyze carbohydrate. Pure colonies of the test isolates were inoculated onto Nutrient agar to which 2% starch was added and incubated. The incubated plate was flooded with 1% potassium iodide and allowed for 15-30-minute incubation at room temperature. The ability to hydrolyze carbohydrate was signified by the formation of yellow zone around the colonies.

The ability of probiotic bacteria to hydrolyze protein and fats was tested using the method of Sneha *et al.* (2014) and Widanarni and Dedi (2015) with some modifications for the assessment of lipolytic activity of the isolates. Nutrient agar was prepared and enriched with 2% olive oil. The *Bacillus* species to be tested were grown on the olive oil enriched agar and was incubated. After incubation, the plate was flooded with saturated copper sulfate ($CuSO_4$) solution and allow at room temperature for 15 minutes. The fats hydrolysis was indicated by appearance of green coloration on the colonies.

This experiment was aimed at assessing *Bacillus* species with proteolytic activity.

A modified method described by Bhaskar *et al.* (2007) was employed to assess *Bacillus* species having proteolytic potential. To Nutrient agar (TM MEDIA, TM341-India) enriched with 2% skimmed milk (LP 0031, Oxoid), about 3-5 discrete colonies of the test isolate were packed and smeared on the center of the surface of the agar plate followed by incubation. Those isolates that hydrolyze protein showed zones of clearance around the colonies were regarded as protease producers.

All data collected were analyzed by Analysis of

Variance (ANOVA) using the statistical package for the Social Sciences (SPSS) version 21 of 2012 and the variant means were separated by Duncan's Multiple Range Test. Significance was accepted at the probability level of 95% ($P \leq 0.05$) confidence interval.

RESULTS

A total of 176 isolates of bacteria was obtained from 530 samples from the skin and gastrointestinal tract of *Clarias anguillaris* and were subjected to phenotypic identification (Table 1). Ninety-eight (98) isolates were Gram-negative and 78 were Gram-positive. Of the Gram-positive rods, forty-one (41) were bacilli and while thirty-seven (37) were found to be Gram positive cocci. Gram negative bacteria recorded the highest prevalence (55.7%), Gram positive cocci (21%), Gram positive rods (14.2%) and *Bacillus* recorded the least (9.1%) as presented on table 1.

Molecular identification of *Bacillus* strains isolated from the skin and intestinal tract of catfish (*C. anguillaris*) using 16S rDNA sequencing.

The result of molecular identification is presented on table 2 below. The homology blast of the sequences generated for the 13 isolates yielded a 100% identity with 12 *Bacillus* species and 1 *Clostridium sporogenes*. The 12 *Bacillus* species strains were distributed into five species; *Bacillus subtilis* (n=5),

Table 1. Cultural microscopic groupings of bacteria isolated and Biochemical characteristics of *Bacillus* strains on the skin and gastrointestinal tract of *C. anguillaris*

Isolates	Number	Prevalence (%)
Bacillus species	16	9.1
Other Gram-positive rods	25	14.2
Gram-positive cocci	37	21.0
Gram-negative rods	98	55.7
Total	176	100

Isolates code	Gram stain	Amylase	Glucose	V-P	Cell size ⩾1µm	Swollen cell with spores	Citrate test	6.5%Nacl growth	Mannitol fermentation	Growth at 55°C	Nitrate reduction test	Catalase	M/Red Test	Arabinose	Motility	Starch hydrolysis	*Bacillus* species
Bsp1	+	-		-	+	-	-	+	-	-	-	+	+	+	+	-	*B. subtilis*
Bsp2	+	+	+	+	NA	+	+	+	-	-	+	+	+	-	+	-	*B. subtilis*
Bsp3	+	+	+	-	+	-	-	+	-	-	-	+	-	-	+	+	*B. cereus*
Bsp4	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	*B. insolitus*
Bsp5	+	+	+	+	+	-	-	+	-	-	-	+	-	+	-	-	*B. polymyxia*
Bsp6	+	+	+		+	+	+	+	+	-		+	-	-		-	*B. macquariensis*
Bsp7	+	+	+	+	NA	-	-	+	-	-	-	+	-	+	-	-	*B. polymyxia*
Bsp8	+	+	+	+	NA	+	+	+	-	-	+	+	+	-	+	-	*B. macquariensis*
Bsp9	+	-	-	-	+	+	+	+	-	-	-	+	-	+	-	-	*B. sphericus*
Bsp10	+	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	*B. insolitus*
Bsp11	+	+	+	+	NA	-	-	+	-	-	-	+	-	+	-	+	*B. lechinoformis*
Bsp12	+	+	+	+	NA	-	+	+	-	-	-	+	-	+	-	-	*B. amyloliquifaciens*
Bsp13	+	+	+	-	+	-	+	+	+	-	+	+	-	+	-	+	*B. velezensis*

Keys: + = positive, - = negative, NA = not applicable

B. cereus (n=2), *B. velezensis* (n=3), *B. amyloliquefaciens* (n=1) *and B. safensis* (n=1) and *Clostridium sporogenes* (n=1)

The combination of highest identity, total score and query over values were used to attribute the suggested species. The sequencing of isolate Bsp6 with primer 518F and 800R failed and the most represented species was *Clostridium sporogenes* (MK341728.1) with identity similarity of 100%. Although, the preliminary identification and the confirmatory identification did not agree 100% on the *Bacillus* strains finally identified, 12 were *Bacillus* species and 1 *Clostridium* species.

Antagonistic capability testing

The result of the antagonistic capability testing is presented in table 3 below.

The result showed that Bsp1 inhibited 6 out of the 7 seed bacteria and was effective against 2 strains of *Pseudomonas fluorescens-25*. Bsp4 inhibited 5 of the seeded bacteria, Bsp6 and Bsp12 inhibited 4, while the rest of the selected *Bacillus* species, except Bsp3 and Bsp9 that inhibited only 2 of the testing bacteria, inhibited 3. All the 13 *Bacillus* species selected inhibited more than 1 indicator organisms with good zone of inhibition against the seeded bacteria in this experiment.

Bacteriocin Production test

The inhibitory activity revealed the presence of bacteriocin in these Bacillus species. There were varying degrees of inhibitions according to the effectiveness of organism in the well and or sensitivity of the seeded organism. Table 4 showed the result of the *Bacillus* species producing bacteriocin against different indicator organisms. The results revealed that some *Bacillus* species produced bacteriocin against some strains and could not produce against other strains of the same species of indicator organism. Bsp10 produce bacteriocin against 85.7% of the indicator organisms. Others are Bsp3 and Bsp5 (71.5%), Bsp9, Bsp11, Bsp12 (30.6%), Bsp13 (28.6%), Bsp1, Bsp2, Bsp6, Bsp8 (14.3%), while Bsp4 and Bsp7 could not produce bacteriocin against any of the selected indicator organism. In this study, Bsp3, Bsp5 and Bsp10 are regarded as good bacteriocin producers followed by Bsp9, Bsp11, and Bsp12.

The tolerance of the *Bacillus* species in different pH levels

The tolerance of *Bacillus* species to different pH levels showed that all the 13 *Bacillus* species survived in all the pH including the highly acidic level (pH 1.3) as shown in Figure 1. The results significant differences among Bsp3, Bsp4, Bsp5, Bsp6, Bsp7, Bsp10, Bsp12 and Bsp13 compared to the

Table 2. *Bacillus* species distribution and Percentage similarity of identified strains against reference strains in the GenBank

Sample ID	Suggested spp	Accession number	Identity percentage (%)
Bsp1	Bacillus subtilis	MK085082.1	100
Bsp2	Bacillus subtilis	CP026608.1	100
Bsp3	Bacillus cereus	MN122695.1	100
Bsp4	Bacillus subtilis	MN099359.1	100
Bsp5	Bacillus subtilis	MK085082.1	100
Bsp6	Clostridium sporogenes	MK341728.1	100
Bsp7	Bacillus cereus	MN122695.1	100
Bsp8	Bacillus subtilis	MN099359.1	100
Bsp9	Bacillus velezensis	CP041145.1	100
Bsp10	Bacillus amyloliquefaciens	MN099360.1	100
Bsp11	Bacillus velezensis	CP041145.1	100
Bsp12	Bacillus velezensis	CP041145.1	100
Bsp13	Bacillus safensis	MF980894.1	100

Table 3. Antagonistic capability test of Bacillus species against known fish pathogens

Bacillus species	Fish pathogens								
	Aeromonas hydrophila	Klebsiella . pneumoniae	Pseudomonas fluoroscens-25	Stenotrophila maltophilia	Vibrio alginolyticus	Flavobacteria. ordaratus	Flavobacteria meningosepticum	No isolates inhibited	Mean zone of inhibition (mm)
Bsp1	+	+	++	+	+	-	+	6	13
Bsp2	+	-	-	-	-	-	+	3	15
Bsp3	-	-	-	-	+	-	+	2	25
Bsp4	+	+	+	+	-	-	+	5	17
Bsp5	+	+	-	+	-	-	-	3	14
Bsp6	+	+	+	-	-	+	-	4	16
Bsp7	-	+	-	+	+	-	-	3	13
Bsp8	-	-	++	+++	-	+	-	3	12
Bsp9	-	-	-	-	-	+	+	2	11
Bsp10	+	-	-	+	-	-	-	3	16
Bsp11		+++	+++	-	-	-	-	3	13
Bsp12	-	+	++	-	-	+	-	4	15
Bsp13	+	-	+	-	-	-	-	3	16

Key: + represents positive antagonistic 1, ++ positive against 2 strains, +++ positive against 3 strains and – negative antagonistic capability against indicator organism

Table 4. Bacteriocin production by Bacillus species against varied indicator organisms

Selected *Bacillus* species	Indicator organisms							
	Aeromonas hydrophila	Klebsiella pneumoniae	Pseudomonas fluoroscens-25	Stenotrophila maltophilia	Vibrio alginolyticus	Flavobacteria ordaratus	Flavobacteria meningosepticum	Produced bacteriocin against (n)
Bsp1	±	-	-	-	-	-	-	1
Bsp2	-	±	-	-	-	-	-	1
Bsp3	±	±	±	+	-	-	±	5
Bsp4	-	-	-	-	-	-	-	-
Bsp5	-	±	±	+	-	+	±	5
Bsp6	-	-	±	-	-	-	-	1
Bsp7	-	-	-	-	-	-	-	-
Bsp8	-	±	-	-	-	-	-	1
Bsp9	-	±	±	-	±	-	-	3
Bsp10	+	+	+	+	+	-	±	6
Bsp11	±	±	±	-	-	-	-	3
Bsp12	±	-	-	-	±	-	±	3
Bsp13	-	±	-	-	+	-	-	2

Key: - = Negative result, + = positive results, ± = positive but, negative against other strains

control (pH 6.5). The pH tolerances of Bsp3, Bsp4, Bsp5, Bsp6, Bsp7, Bsp10, Bsp11, and Bsp12 were significantly lower ($P \leq 0.05$) compared to control. The lowest tolerance in pH (pH 1.3) was recorded in Bsp6 and Bsp7. The tolerance of all isolates decreased with increase in the acidic level and revealed that Bsp1, Bsp2, Bsp8, Bsp9, Bsp12 and Bsp13 can tolerate low acidic environment (pH 1.3) as well as pH 6.5. The log CFU^{-1} demonstrates the trends of growth (multiplication) in different pH levels. The multiplications were uniform, close to the control (pH 6.5) in all levels except for Bsp4, Bsp5 Bsp6, Bsp10 and Bsp11. The uniformity of the growth was more pronounced in Bsp1, Bsp2, Bsp3, Bsp8, Bsp9, Bsp12 and Bsp13.

The bile tolerance test

The growth performances of the *Bacillus* species in bile solutions were found to be good in almost all level of concentration. The result shows that the colony forming units (CFU) ranges from $1.9x10^7$ to $1.9x10^9$ in 0% bile solutions, 5.0×10^7 to $1.4x10^9$ in 0.1% bile, $5.7x10^7$ t0 $1.3x10^9$ for 0.2% bile, while 4.9×10^7 to $1.4x10^9$ for 0.3% were observed in Table 5. All these *Bacillus* species performed similar to those in the control (0%) groups. The tolerance of the isolates to simulated bile solution according to the colony forming units in descending order were as follows: Bsp10 ($1.4x10^9$), Bsp11 ($8.2x10^8$), Bsp1 ($5.6x10^8$), Bsp13 ($4.0x10^8$), Bsp12 and Bsp2 ($2.8x10^8$) each. The multiplication of Bsp3 was more or less uniform in all the levels of the bile solutions. Bsp4, Bsp6, and Bsp11 recorded highest growth in 0.2%bile of $6.4x10^{7,}$ $9.4x10^7$ and $9.4x10^8$ respectively, while the growth at 0% recorded the least ($1.9x10^7$) for Bsp4 and $2.3x10^8$ at 0.3%. Bsp10 break the record of multiplication reaching $1x10^9 CFUmL^{-1}$

The multiplications of all the isolates in control (0%) were high above 8 Log $CFUmL^{-1}$ except Bsp3, Bsp6 and Bsp7 and Bsp10 that recorded the highest $LogCFUmL^{-1}$ above 9 all through the bile concentrations (Figure 2). The trends of multiplication of all isolates were really good, which multiplied above 7log $CFU^{-1}mL$.

The survival of the *Bacillus* species in different bile concentrations is described in table 6. The survival of Bsp2 in 0.2% and 0.3% were higher than those in control which dropped even lower in 0.1%. The survivability of Bsp3 was uniform which rose in 0.2% generally, however, Bsp1 survived better. The trend of survival of Bsp6 increases steadily from 0% and reach a pick observed in 0.3%. The general survivals of Bsp1and Bsp10 are the top most followed by Bsp11. The survivals of the isolates in high concentration of bile (0.3%) were observed higher than control in Bsp2, Bsp6, Bsp7, and Bsp9. While Bsp2, Bsp4, Bsp9, Bsp11, showed better survival

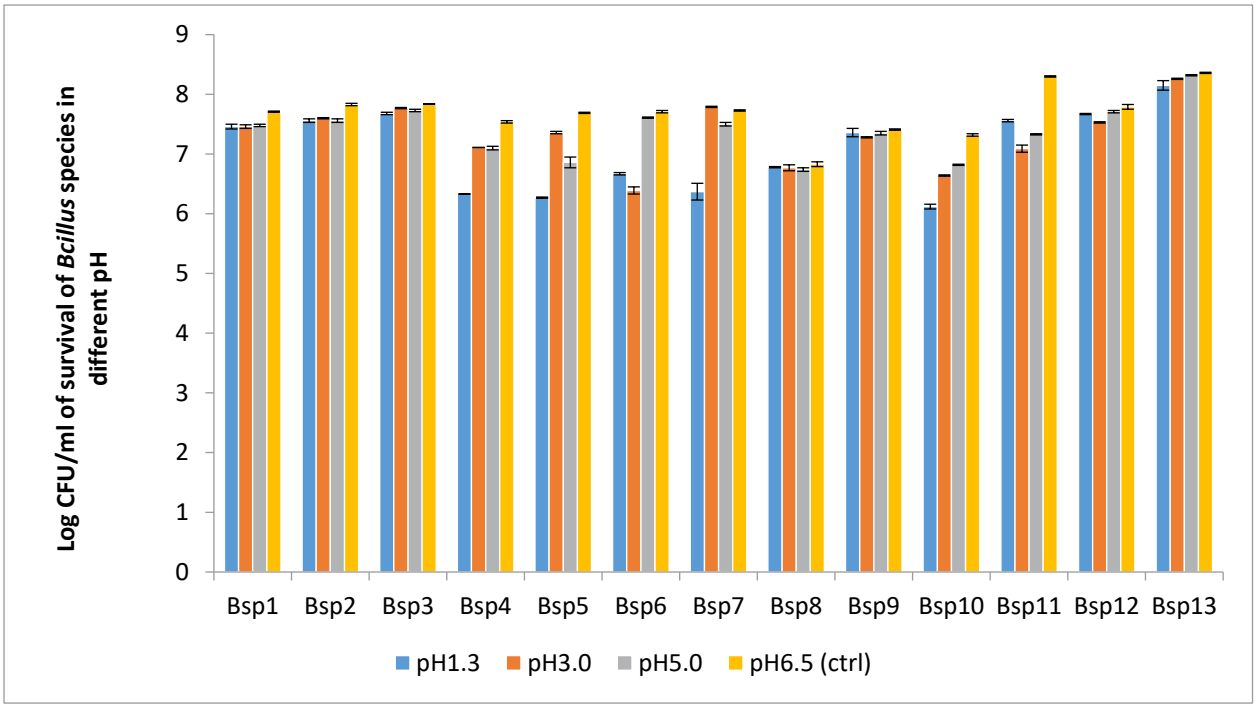

Figure 1. Log colony forming units (CFU^{-1}) of *Bacillus* species in different pH levels.

Table 5. Growth performance of *Bacillus* species at various bile concentrations

	Colony forming unit ($CFUmL^{-1}$)			
Isolate	**0%**	**0.1%**	**0.2%**	**0.3%**
Bsp1	$7.4 x10^8$	$5.3 x10^8$	$5.8 x10^8$	$5.6 x10^8$
Bsp2	$1.7 x10^8$	$6.1x10^7$	$3.0 x10^8$	$2.8 x10^8$
Bsp3	$5.4 x10^7$	$5.0 x10^7$	$5.7 x10^7$	$4.9 x10^7$
Bsp4	$1.9 x10^7$	$5.9 x10^7$	$6.4 x10^7$	$5.1 x10^7$
Bsp5	$3.2 x10^8$	$1.1 x10^8$	$1.2 x10^8$	$2.0 x10^8$
Bsp6	$2.9 x10^7$	$5.0 x10^7$	$9.4 x10^7$	$2.3 x10^8$
Bsp7	$6.3 x10^7$	$8.4 x10^7$	$8.4 x10^7$	$1.8 x10^8$
Bsp8	$9.4 x10^7$	$6.2 x10^7$	$8.6 x10^7$	$7.0 x10^7$
Bsp9	$1.8 x10^8$	$7.2 x10^7$	$2.3 x10^8$	$2.6 x10^8$
Bsp10	$1.9 x10^9$	$1.4 x10^9$	$1.3 x10^9$	$1.4 x10^9$
Bsp11	$6.6 x10^8$	$7.9 x10^8$	$9.4 x10^8$	$8.2 x10^8$
Bsp12	$4.3 x10^8$	$5.2 x10^8$	$1.0 x10^8$	$2.8 x10^8$
Bsp13	$6.2 x10^8$	$1.8 x10^8$	$3.6 x10^8$	$4.0 x10^8$

in 0.2%bile solution compared to the control. There was highly significant difference ($P \leq 05$) in the survival of Bsp2, Bsp4, Bsp6 Bsp8, Bsp9, Bsp12 and Bsp13 across the simulated bile solution. So Bsp2, Bsp4, Bsp6, Bsp7, Bsp9 and Bsp11 survived better in concentrated bile (0.3%) more than the control (0.0%) as shown in Table 6.

So, the growth performances of Bsp2 (0.833±0.04), Bsp4 (0.697±0.05), Bsp6 (1.303±0.12), Bsp7 (1.187±0.56) and Bsp9 (0.817±0.03) are the isolate that survived better than those in the control groups are considered to be good. All isolate with significant ($P \leq 0.05$) difference compared to control (0.0%) are

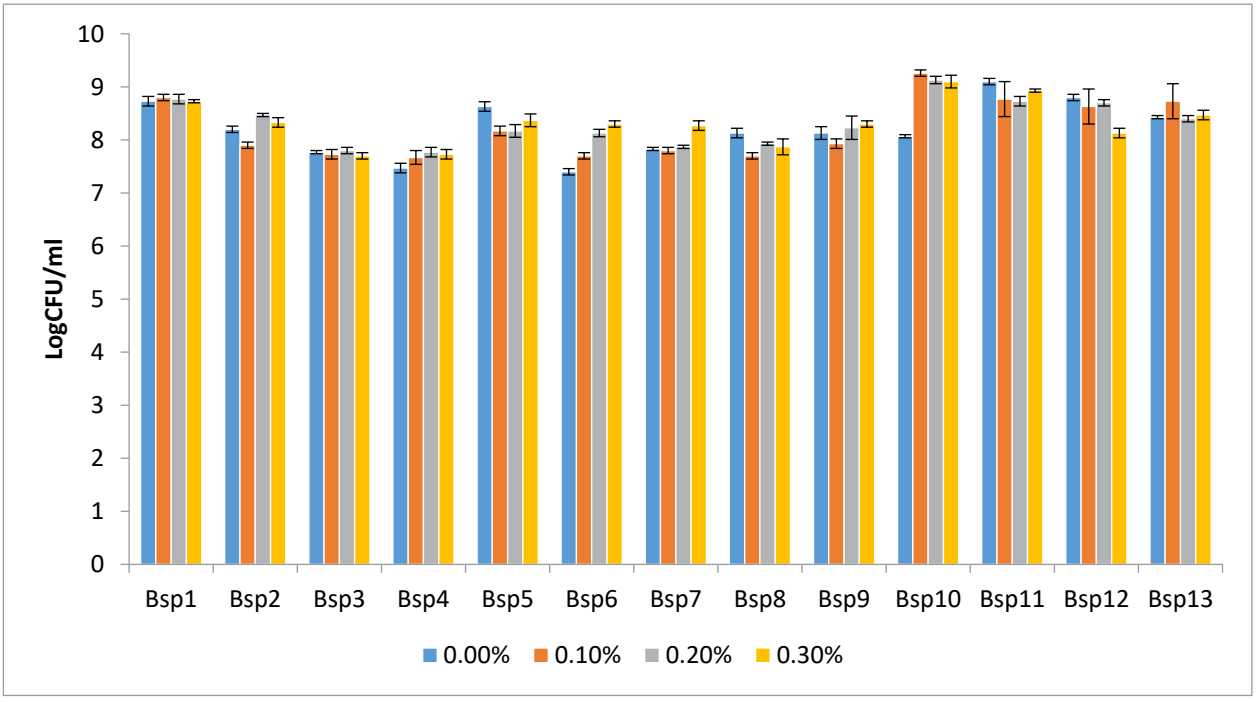

Figure 2. Log CFU/ml of *Bacillus* species at different concentration of simulated bile solution.

Table 6. The survival of *Bacillus* species in different bile concentrations

	Optic Density ± SD			
Isolates	**0.0% bile**	**0.1% bile**	**0.2% bile**	**0.3% bile**
Bsp1	1.933±1.00^{a}	0.960±0.05^{a}	1.045±0.11^{a}	1.020±0.14^{a}
Bsp2	0.610±0.11^{b}	0.330±0.03^{c}	0.853±0.06^{a}	0.833±0.04^{a}
Bsp3	0.304±0.02^{a}	0.297±0.02^{a}	0.310±0.02^{a}	0.293±0.02^{a}
Bsp4	0.377±0.07^{b}	0.770±0.07^{a}	0.820±0.11^{a}	0.697±0.05^{a}
Bsp5	0.390±0.08^{a}	0.247±0.04^{b}	0.257±0.04^{b}	0.313±0.01^{ab}
Bsp6	0.490±0.08^{b}	0.440±0.15^{b}	0.647±0.09^{b}	1.303±0.12^{a}
Bsp7	0.503±0.27^{b}	0.650±0.19^{ab}	0.947±0.06^{ab}	1.187±0.56^{a}
Bsp8	0.770±0.11^{a}	0.573±0.03^{b}	0.650±0.04^{ab}	0.623±0.04^{b}
Bsp9	0.633±0.08^{a}	0.367±0.02^{c}	0.743±0.03^{a}	0.817±0.03^{a}
Bsp10	0.760±0.05^{a}	0.627±0.15^{a}	0.680±0.06^{a}	0.610±0.08^{a}
Bsp11	0.457±0.06^{a}	0.550±0.12^{a}	0.570±0.10^{a}	0.587±0.13^{a}
Bsp12	0.667±0.01^{a}	0.767±0.09^{a}	0.310±0.01^{c}	0.507±0.10^{b}
Bsp13	0.757±0.04^{a}	0.360±0.10^{c}	0.517±0.05^{b}	0.567±0.05^{b}

Different superscripts ^{abc} in the same row indicate significant difference (P≤ 0.05)

Table 7. Digestive tract enzyme (Amylase, Lipase and Protease) production of the isolated *Bacillus* species

Isolate	**Amylase**	**Lipase**	**Protease test and zone of clearance (mm)**	
Bsp1	+	+	-	-
Bsp2	+	-	-	-
Bsp3	-	+	+	10
Bsp4	-	+	+	25
Bsp5	-	+	-	-
Bsp6	-	+	-	-
Bsp7	+	+	-	-
Bsp8	-	+	+	20
Bsp9	-	+	-	-
Bsp10	+	+	-	-
Bsp11	-	-	+	18
Bsp12	-	-	+	23
Bsp13	-	+	+	38

Bacillus species that survived better in all the levels of concentration of bile.

The result of digestive enzyme production tests

All the *Bacillus* species were assessed for their ability to produce amylase, lipase and/or protease. The results are presented on table 7 below.

The physiological tests in this study revealed the production of detectable amylase, protease and/ or lipase. The results showed that Bsp1, Bsp2, Bsp8 and Bsp10 produced amylase on 20% starch enriched media. For Lipolytic test the result showed that all the isolates except Bsp2, Bsp11 and Bsp13 were positive in the hydrolysis of lipids on 2% olive oil enriched agar. For protease production, Bsp3, Bsp4, Bsp8, Bsp11, Bsp12 and Bsp13 produced protease with Bsp13 recording the highest diameter zone of

clearance of 38mm. Four (30.7%), ten (76.9%), and six (46.2%) out of 13 isolates were positive for amylase, lipase and protease production respectively. Seven (53.9%) isolates produced at least 2 of the 3 enzymes and 5 (38.5%) strains produced only 1 of the 3 enzymes, while none produced all the 3 enzymes as seen in table 7 above.

DISCUSSION

The central aim was to isolate, screen and select indigenous *Bacillus* species as potential probiotic bacteria that would be used for disease control to attain maximum yield in fish production in aquaculture. Characterization of the *Bacillus* species and evaluation of their effects on the productive performance and health status of the *C. anguillaris* was the expected end of the study. The choice of *Bacillus* species as probiotic bacteria of interest from the general selection in this study was in consideration of the beneficial results obtained using these bacterial and their contribution to the probiotic world. They have been shown to possess good attributes and have a wider range of action in human, animal and aquatic environment (Anee *et al*., 2021; Tarnecki *et al*., 2019) which offers higher acid and bile tolerance, and better stability during heat processing and thrive at low temperature storage (Somashekaraiah *et al*., 2019).

The twelve *Bacillus* species characterized were *Bacillus subtilis* (5 strains), *B. cereus* (2), *B. velezensis* (3), *B. amyloliquefaciens* (1) and *B. safensis* (1). *Clostridium sporogenes* (1) was also characterized and screened successfully alongside with *Bacillus* species, probably because, there is record of its being potential probiotic bacteria (Guo *et al*., 2020). The isolation, characterization and use of these *Bacillus* species on fish elsewhere as potential probiotics have been documented by other workers including *B. velezensis* (Wang *et al*., 2020), *B. subtilis* (Hussain *et al*., 2013; Zuenko *et al*., 2017), *B. amyloliquifaciens* (Afrin and Bhuiyan, 2023) and *C.sporogenes* (Guo *et al.,* 2020).

The tolerance to pH and bile recorded in this study was one of the most important factors affecting the survival of bacteria in gastric juice. This potential served as one essential characteristics of probiotic candidate since they have to pass through the host's stomach to reach the site of action (the intestine).

All the twelve *Bacillus* species and the *Clostridium sporogenes* subjected to antagonistic test and bacteriocin production test, inhibited more than 1 indicator organism with good diameter zone of inhibition (DZI). This is an indication of having good attributes for potential probiotic bacteria that produce inhibitory substances or ability to inhibit the growth of another as mode of action (Krishna *et al*., 2015) subject to fulfilling other criteria. The reports of *Bacillus* species to produce metabolites and inhibitory substances have been documented in a study (Kuebutornye *et al*., 2020). The inhibitory effects of *Bacillus* species which were successfully recorded in this study agrees with the report of (Krishna *et al*., 2015) who isolated *Bacillus* species which successfully inhibited *Aeromonas hydrophila* due to the inhibitory substances. By producing antimicrobial compounds, the probiotic organism gain edge over other microorganisms to survive in the adverse condition of gastrointestinal tract of animal. Probiotic bacteria produce substances including bacteriocin, hydrogen peroxide, siderophore, lysozymes, and protease with bactericidal or bacteriostatic effects on the other microbial populations.

The survival of these *Bacillus* species in acidic environment and bile solution was significantly good in all pH levels and various bile concentrations. The significant difference in the growth of this *Bacillus* species in bile and low pH corroborated with the work of Sahadeva *et al.* (2011) and Hassan *et al.* (2015) who investigated the antibacterial, acid and bile tolerance of commercial probiotic bacteria. They reported that acid and bile tolerance as an important criterion for a good probiotic that tolerate high acid and bile solution which is present in the stomach of animals.

The growth performances of the entire *Bacillus* species generally were uniform ranging from 80-100% in this study. The significant difference recorded in the survival of *B. cereus, B. amyloliquefaciens* and *B. velezensis* across the simulated bile solution indicated a good attribute for survival in the duodenum of catfish. The survival rate was similar to the trend portrayed in acid tolerance test according to the report of Sahadeva *et al.* (2011). The successful production of digestive enzymes (amylase, lipase and protease) by these groups of *Bacillus* species in this study was reported by Zokaeifar *et al.* (2012). The amylase, protease and lipase activities showed significant difference ($P \leq 0.05$) among all the *Bacillus* species screened. The findings of the study are supported by Ziaei-Nejad *et al.* (2006) who observed higher digestive enzyme activities in shrimp when treated with *Bacillus* species compared to the

control. A good digestive enzyme activity recorded in this study shows a better digestive potential and subsequently correlate to the increase in productive performance of animal to be treated with these *Bacillus* species. Probiotics are expected to have direct growth promoting effect on the host by nutrient uptake or providing nutrient which could result in improve digestibility and weight gain (Ezema, 2013). This was evident in this study possibly due to the digestive enzyme productions by these *Bacillus* species. This result is also in agreement with the work of Sayed *et al.* (2011), who reiterated the importance of the ability of probiotic bacteria to produce digestive enzymes. The ability of probiotic bacteria to produce hydrolytic enzymes enhances the digestion of nutrients in the gastrointestinal tract of fish and increase degradation of ingested food.

CONCLUSION

All the 12 *Bacillus* species and a *Clostridium sporogenes* isolated, screened and characterized from indigenous catfish are found to be potential probiotics and so will have beneficial effects in the production of fish as shown from the result of this study. *Bacillus cereus, B. subtilis, B. amyloliquefaciens* and *B. velezensis* showed significant difference in survival rate in simulated bile indicated a good attribute for survival in the duodenum of catfish. The similar survival rate portrayed in acid tolerance test and the successful production of digestive enzymes (amylase, lipase and protease) by these potential probiotics with better digestive enzyme production potential and subsequently correlate to the increase in productive performance of animal to be treated with these *Bacillus* species, made them to be selected as good potential probiotic that will have direct growth promoting effect on the host, which was evident due to the digestive enzyme productions.

Compliance with Ethical Standards

All experiments in this study associated with fish complied with animals welfare ethical approval (FVM-UNN-IACUC-2019-0925) obtained from Faculty of Veterinary Medicine Institutional Animal Care and Use Committee, University of Nigeria, Nsukka

Conflict of Interest

The authors declare that they have no conflicts of interest.

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