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Multidrug Resistant Strains Inhibition by *Bacillus* Species from the Gut of *Oreochomis niloticus* and *Pomacea canaliculata*

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ABSTRACT

Antibiotic resistance is widespread in clinical settings, indicating a serious problem with infectious disease treatment. Novel strategies such as using natural products derived from microbes are being explored, generating increased research interest to address this issue. Here, the antimicrobial property of gut-associated *Bacillus* species against multidrug-resistant (MDR) strains; methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli* producing extended-spectrum beta-lactamase (EsβL *E. coli*), and *Pseudomonas aeruginosa* producing metallo beta-lactamase (MβL *P.aeruginosa*) was evaluated using a cross-streak method and agar diffusion assay. The *Bacillus* isolates inhibited MRSA and ESβL *E. coli* with an average zone of inhibition of 9.57 ± 33.40 mm and 5.07 ± 32.69 mm, respectively, in the cross-streak method. The cell-free supernatant (CFS) of ten *Bacillus* species demonstrated anti-MRSA activity but was ineffective against ESβL *E. coli* and MβL *P. aeruginosa*. The relative enzyme activities of ten *Bacillus* isolates were determined *in vitro*, and amylase, caseinase, cellulase, lipase, and gelatinase production were confirmed. Isolates were identified as *Bacillus siamensis*, *Bacillus velezensis*, and *Bacillus subtilis* through biochemical tests and 16s rRNA sequence analysis. Minimum inhibitory concentrations (MICs) of the CFSs against MRSA range is between 12.5 and 25%. *Bacillus* species isolated from fish and snail guts exhibited antibacterial activity against MRSA. Therefore, it is imperative to confirm the presence of anti-MRSA active compounds in *Bacillus* CFS and characterize them further to determine their suitability for antimicrobial drug development.

Keywords: Anti-MRSA, *Bacillus* species, cross-streak method, multidrug resistance, *Oreochomis niloticus*, *Pomacea canaliculata*

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INTRODUCTION

Antibiotic resistance has emerged as a major global public health concern and a likely future crisis. Antibiotic-resistant microorganisms are widespread and have been associated with a wide variety of hospital- and community-acquired infections. While prevalence varies by country, it is more prevalent in immunocompromised individuals and those suffering from chronic diseases (Ley et al., 2006; Pitout & Laupland, 2008; Sivasubramanian et al., 2012).

Staphylococcus aureus, *Pseudomonas aeruginosa*, and *Escherichia coli* have been linked to a variety of infections associated with a high morbidity and mortality rate worldwide (Davies & Davies, 2010). Due to their increased virulence and novel mechanisms for deactivating antibiotic effects, these bacteria are dubbed "superbugs." As a result, the terms methicillin-resistant *Staphylococcus aureus* (MRSA), extended-spectrum beta-lactamase-producing *Escherichia coli* (EsβL *E. coli*), and metallo beta-lactamase-producing *Pseudomonas aeruginosa* (MβL *P.aeruginosa*) were coined to refer to bacteria developing resistance to different antibiotic classes (Löffler et al., 2010).

The conventional approach of treating infections caused by multidrug-resistant strains (MDR) with a single antibiotic in high doses may no longer be effective, as repeated use may result in a cycle of drug resistance (Hu et al., 2015). Similarly, prolonged use of synthetic antibiotics has been reported to cause irreversible damage to the kidney, liver, and other internal organs, as well as ototoxicity and pernicious anemia (Mingeot-Leclercq & Tulkens, 1999; Reuter, 2001; Hooper & Macpherson, 2010; Kabir, 2009; Duary et al., 2011).

As antimicrobial resistance increases, however, antimicrobial drug development is slowing (Doron & Davidson, 2011). Hence, there is a need to search for novel antibiotics and alternative treatments continuously. The discovery of new antibacterial compounds from natural sources with high target specificity has remained a big challenge to health professionals and researchers. Bioactive marine and terrestrial natural products derived from plants (Ureta et al., 2019), animals (Mariottini & Grice, 2016; Lirio et al., 2018), algae (Besednova et al., 2020), and bacteria (Ramachandran et al., 2014; Fuego et al., 2021), are receiving increased attention as drug discovery candidates (Dias et al., 2012).

Utilizing natural products as antibiotic sources has been viewed to reduce, if not eliminate, adverse drug effects. For example, microbial sources of natural products metabolites produced by *Bacillus subtilis,* the model organism for Gram-positive bacteria, are promising candidates for antibiotic production. It is well established that *B. subtilis* can produce a diverse array of structurally unrelated antimicrobial compounds (Hao et al., 2014; Culligan et., 2013). With *Bacillus* species' ubiquity in nature and reported potential as a source of natural products, it is unquestionably worthwhile to decipher additional biological activities, particularly those related to MDR control. While numerous attempts have been made to elicit antimicrobial activity from diverse *Bacillus* species against common human pathogens (Yilmaz et al., 2006; Caulier et al., 2019; Horng et al., 2019), only a few have

been undertaken to uncover the potential of gut-associated *Bacillus sp*ecies from fish and snails against multidrug-resistant clinical strains. Thus, this study examined the antibacterial properties of *Bacillus* species isolated from the guts of snails (*Pomacea canaliculata*) and fish (*Oreochomis niloticus*) against three (3) resistant bacterial pathogens: MRSA, ESβL *E. coli*, and MβL *P. aeruginosa*.

MATERIALS AND METHODS

Specimen Collection

The fish (*O. niloticus*) and snail (*P. canaliculata*) samples were collected in the provinces of Laguna (14°23′N 121°29′E), Batangas (14°06′N 121°01′E), and Pampanga (15°04′N 120°43′E), which are recognized to be the Philippines' three leading aquaculture and agribusiness provinces (PSA, 2016). Samples were collected, placed in a sterile polypropylene bag containing habitat water, and sent to the Polytechnic University of the Philippines, Research Institute for Science and Technology, Microbiology and Parasitology Laboratory for dissection and isolation of *Bacillus* species. All fish samples were examined for the key morphological characteristics of *O. niloticus* described by Trewavas (1983), including 15–18 dorsal spines with 11–13 dorsal soft rays and three anal spines with nine–11 anal soft rays. The most distinctive feature is the presence of regular vertical stripes running the entire length of the caudal fin (Figure 1a). The taxonomy and nomenclature of the fish are described in Table 1. On the other hand, snail samples were examined for morphological characteristics of *P. canaliculata* as described by Rawlings et al. (2007). These are globose shells with five to six whorls and a thick, indented suture separating them. The opening or aperture of the shell is large and oval to round. Snails range in size from 45 to 75 mm in length and 40 to 60 mm in width. The snail's body is yellow to brown to black (Figure 1b). The taxonomy and nomenclature of the snail are described in Table 2.

Bacillus Species Isolated from the Guts of *O. niloticus* and *P. canaliculata*

Bacteria were isolated from fish guts using the Sarkar and Ghosh (2014) method. For 24 h, fish samples were fasted to detect indigenous intestinal bacteria and eliminate most non-indigenous bacteria associated with food. Then, cold immersion was used to euthanize the fish (Wilson et al., 2009). Before dissection, the fish samples were washed with sterile distilled water to remove any undesired materials and then scrubbed with sterile gauze and 70% ethanol. Next, the fish's gastrointestinal tract (GI) was dissected under sterile conditions. The gut was located and dissected, and one gram of fish intestine was homogenized by adding one milliliter of sterile water and grinding with a sterile mortar and pestle. The homogenates were then transferred to a 20-mL tube containing 9 mL sterile water, vortexed, and serially diluted to a final concentration of 10^{-6}. Subsequently, 0.1 mL of the homogenates were plated on Tryptic Soy Agar (TSA) plates (Himedia, India) from

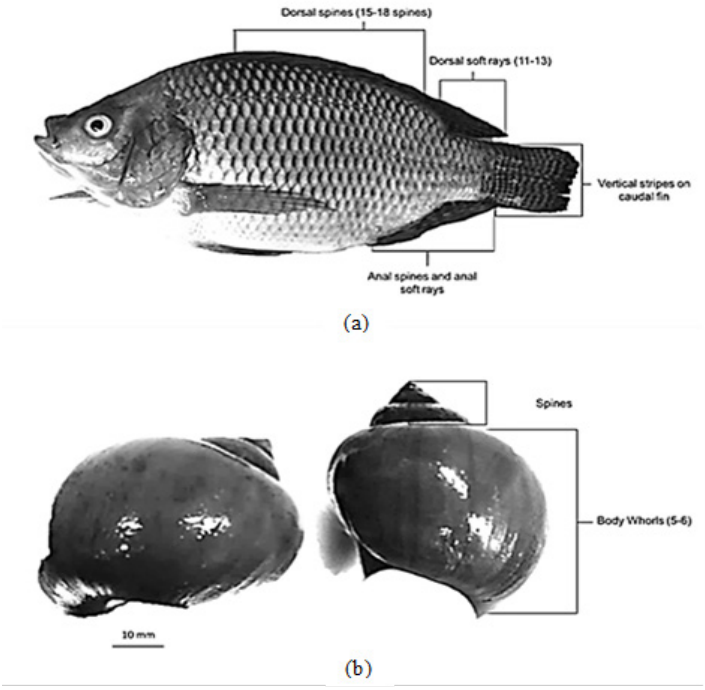

Figure 1. External morphology of (a) *Oreochomis niloticus* (tilapia), (b) *Pomacea canaliculata* (golden apple snail)

Table 1

Taxonomy and Nomenclature of Oreochromis niloticus *(Linnaeus, 1758)*

Taxonomic Rank	**Taxon**
Kingdom	Animalia
Subkingdom	Bilateria
Infrakingdom	Deuterostomia
Phylum	Chordata
Subphylum	Vertebrata
Infraphylum	Gnathostomata
Superclass	Actinopterygii
Class	Teleostei
Superorder	Acanthopterygii
Order	Perciformes
Suborder	Labroidei
Family	Cichlidae
Genus	*Oreochromis* Günther, 1889
Species	*Oreochromis niloticus* (Linnaeus, 1758) – Nile tilapia

Source. Integrated Taxonomic Information System (ITIS) (USGS, 2013)

Table 2

Taxonomy and Nomenclature of Pomacea canaliculata *(Lamarck, 1822) (Linnaeus, 1758)*

Taxonomic Rank	Taxon
Kingdom	Animalia
Subkingdom	Bilateria
Infrakingdom	Protostomia
Superphylum	Lophozoa
Phylum	Mollusca
Class	Gastropoda
Subclass	Prosobranchia
Order	Architaenioglossa
Family	Ampullariidae
Genus	*Pomacea* Perry
Species	*Pomacea canaliculata* (Lamarck, 1822) – Apple snail, Golden apple snail

Source. Integrated Taxonomic Information System (ITIS) (USGS, 2013)

each dilution and incubated at 37°C for 24 h (Koleva et al., 2014). Grown mixed cultures were examined for colonies resembling *Bacillus* species and carefully separated using the quadrant streaking method.

The method described by Godoy et al. (2013) was used to isolate bacteria from snail gut. Adult snails with 35–40 mm shell lengths were fasted for 24 h prior to dissection to clear their guts of any partially digested food and isolate indigenous bacteria. Next, cold immersion was used to euthanize the snails. This time, second-step euthanasia was performed via immersion in 70% alcohol and 10% neutral-buffered formalin. Snail shells were separated and discarded to obtain the flesh and locate the gut. Snail flesh was dissected aseptically in sterile Petri plates. A gram of snail intestine was homogenized and ground using a sterile mortar and pestle after adding 1 mL of sterile water. Serial dilutions of the homogenates up to a 10^{-6} dilution were performed, and 0.1 mL of each dilution was inoculated onto TSA plates and incubated at 37°C for 24 h. The microbial colonies from the mixed culture plates were carefully observed and then separated using the quadrant streaking method to obtain a pure culture. Individual isolates were maintained in 20% glycerol (LOBA Chemie, India.) and stored at -20°C.

Screening for Presumptive *Bacillus* Isolates

Bacillus species were identified preliminarily using phenotypic and biochemical characterization as described elsewhere (Whitman, 2009; Slepecky & Hemphill, 2006).

The Gram staining reaction, the presence of endospore, motility, colony characteristics, aerobic growth, and catalase reaction were all used as criteria for presumptive identification of *Bacillus* species (Table 3).

Table 3
Criteria for presumptive identification of Bacillus *species*

Phenotypic Characteristics	Expected Observation
Gram stain	Gram-positive, large rod-shaped cells
Endospore	Positive, either central, terminal, subterminal
Catalase	Positive
Motility	Positive
Colony	Large colonies, confluent growth, dry or moist, undulate, crusty colonies.

Gram staining was performed on freshly grown cultures following the standard staining procedure (Bartholomew & Mittwer, 1952). Isolates that appeared Gram-positive and are rod shape were further examined for the presence of endospores using the standard endospore staining technique (Reynolds et al., 2009), in which vegetative cells appear red, and endospores appear round-green when viewed under oil-immersion objectives.

Multidrug-Resistant Test Strains

Three antibiotic-resistant clinical isolates (MRSA, ESβL *E. coli*, and MβL *P.aeruginosa*) were used as test strains and obtained from the culture collections of the DLSU Microbial BioBanks**,** Manila, Philippines. The clinical strains' origins and antibiotic susceptibility profiles are described in Table 4 (Valle et al., 2016).

Table 4
Multidrug-resistant bacterial strains and their corresponding resistance phenotypes

MDR Bacterial Strains	Resistant Phenotypes
Methicillin-resistant *Staphylococcus aureus* A1	Trimethoprim-sulfamethoxazole, Cefoxitin, Oxacillin, Penicillin
Escherichia coli ESβL (+)	Ampicillin, Cefepime, Cefotaxime, Ceftadizime, Ceftriaxone
Pseudomonas aeruginosa MβL (+)	Amikacin, Cefepime, Ceftadizime, Imipenem, Meropenem

Preliminary Screening of Gut *Bacillus* Isolates for Antibacterial Activity

The cross-streak method, adapted from Williston et al. (1947), was used to screen antibiotic-producing *Bacillus* spp. against test pathogens in the preliminary screening stage. Each *Bacillus* species stock culture was streaked in a straight line across the center of Mueller Hinton Agar (MHA) plates (HiMedia, India). The plates were incubated at 37°C for 24 h to enable the bacteria to produce the antibiotic substance, which was then diffused into the agar medium (Waksman, 1962). Following incubation, each MDR strain (MRSA, ESβL *E. coli*, and MβL *P.aeruginosa*) was inoculated using a single streak (25 mm) perpendicular to the *Bacillus* growth. It was then incubated at 37°C for an additional 23 h. With a Vernier caliper, the zone of inhibition (ZOI) formed at the point of contact between the test bacteria and *Bacillus* spp. was measured in millimeters. The presence of ZOIs throughout the perpendicularly streaked *Bacillus* isolates suggests that they may possess antibacterial activity against the test bacteria (Oskay, 2009). *Bacillus* spp. that exhibited ZOI to at least one (1) of the three multidrug-resistant (MDR) bacterial strains were chosen for cultivation and the production of cell-free supernatant (CFS).

Gut *Bacillus* Cell-Free Supernatant (CFS) Antibacterial Activity Against Multidrug-Resistant Pathogens

Bacillus spp. isolates exhibiting antibacterial activity against at least one of the three test bacterial pathogens used in the cross-streak test were chosen and cultured to produce cell-free supernatant (CFS), using the method by Aminnezhad et al. (2015). *Bacillus* isolates were added to sterile Tryptic Soy Broth (TSB) (HiMedia, India) and incubated at 37°C for 24 h. The broth cultures were standardized to match the 0.5 McFarland standard to achieve a bacterial density of approximately 1.5 x 10^8 CFU/mL. The standardized broth cultures were inoculated into a 250-mL Erlenmeyer flask containing 100 mL sterile TSB and incubated at 37°C for 48 h on a platform shaker at 120 rpm to allow for aeration, prevent pellicle formation to ensure homogeneous cultures (Kivanç et al., 2014). After 48 h, the broth cultures were centrifuged for 20 min at 9,000 rpm. Then, the supernatant was transferred to a sterile vessel and filtered using a polyethersulfone (PES) membrane filter unit with a 0.22 µm pore size (Whatman Puradisc® 25 AS, United Kingdom). The resulting filtrates constituted the cell-free supernatant (CFS), was reserved, and stored at -20°C until use.

The antibacterial activity of the CFSs was determined using an agar well diffusion assay against drug-resistant test bacteria (Bell & Grundy 1968). A sterile swab was used to swab the drug-resistant test organism onto Mueller-Hinton Agar (MHA) (HiMedia, India) plates. The swabbed plates were punched through the agar with a sterile borer to create wells with 8-mm diameter. Subsequently, 100 µL of crude *Bacillus* CFSs was added to the wells and incubated at 37°C for 24 h. As an antibiotic control, cefoperazone (TM Media, India) was used, while wells containing sterile distilled water served as the negative

controls. Antibacterial activity was determined by measuring the growth-free inhibition zones in millimeters using a Vernier caliper. *Bacillus* isolates with the highest average ZOI were identified and further characterized biochemically and for enzyme activity. The test was done in three replicates, and data were expressed in the mean of the three replicates ± standard deviation for the *Bacillus* CFS and the antibiotic control.

Biochemical Characterization of Antibiotic-Producing *Bacillus* Isolates

Numerous studies have established strong correlations between enzymatic activity and antimicrobial peptide production in antibiotic-producing bacteria (Salazar & Asenjo, 2007; Godoy et al., 2013; Ahmad et al., 2013; Hassan et al., 2013; Xu et al., 2015), indicating that enzyme-producing bacteria can produce antimicrobials. Therefore, biochemical analyses and enzyme production assays; amylase, caseinase, cellulase, gelatinase, and lipase tests were performed. The reference manual describes the procedures for performing standard biochemical and enzyme tests (Bernard, 2015).

Molecular Identification of Antibiotic-Producing *Bacillus* Species

A commercial microbial DNA extraction kit (QIAGEN, UltraClean® Microbial DNA Isolation Kit) was used to isolate genomic DNA from bacterial broth cultures. DNA extraction procedures were carried out according to the instructions included in the package manual. The extracted DNA of *Bacillus* species was analyzed and run in 1% agarose using agarose gel electrophoresis (AGE). After 35 minutes, the power supply was set to 90 volts, and the gels were viewed under a UV transilluminator (Lee et al., 2012). The presence of bands in each well indicates that DNA extraction was successful. The 16S rRNA gene of *Bacillus* spp. isolates were amplified using PCR (Biometra T cycler Gradient). Each PCR reaction mixture contained 50 ng genomic DNA, 10 uM forward primer (27F), 10 uM reverse primer (1492R) (Lane, 1991), 10x Taq buffer, 25 mM, 5U/L Taq polymerase, and 10 mM DNTPs. The thermal cycling conditions (Table 5) were followed as described elsewhere (Hengstmann et al., 1999). AGE was used to determine the presence of bands on the gel associated with the generated PCR products (Lee et al., 2012). The amplicons were sequenced at Macrogen, Inc. in Seoul, Korea.

Table 5

Polymerase Chain Reaction (PCR) conditions for 16s rRNA gene amplification

Process	Temperature	Duration/Cycle
Denaturation	94°C	1 minute, 30 cycles
Annealing	52°C	1 minute, 30 cycles
Extension	72°C	1 minute, 30 cycles

The 27F and 1492R sequences (27F 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R 5'-GGTTACCTTGTTACGACTT-3') were edited, aligned, and analyzed as described previously (Miranda et al., 2007). The Macrogen, Inc.-provided partial 16s sequences of *Bacillus* spp. were assembled and edited using bioinformatics software, Seaview version 3.2 and MEGA 6. Consensus sequences were submitted to BLAST (Basic Local Alignment Search Tool) to identify closely related type material/sequences based on the query coverage results. Sequences with at least 95% query coverage were downloaded from GenBank (Altschul et al., 1997). Seaview version 3.2 was used to align the sequences to *Bacillus* spp. 16S rRNA gene sequences. A maximum-likelihood tree was constructed using the MEGA 6 software (Tamura et al., 2013), and a phylogenetic tree of the samples' closely related species was constructed using the neighbor-joining method (Saitou & Nei, 1987). For the neighbor-joining tree construction, the bootstrap value was set to 10,000.

Minimum Inhibitory Concentration (MIC) of *Bacillus* spp. CFS

The minimum inhibitory concentration (MIC) of the top ten *Bacillus* spp. CFS inhibitory for the test pathogens was determined using the clinical and laboratory standards institute's microdilution plate method (CLSI, 2009) and the method by Klančnik et al. (2010). The method included serial dilutions of *Bacillus* spp. CFS, mixing with test bacteria, incubation, and addition of a viable-cell color indicator (Rezasurin) to determine the lowest concentration inhibiting the growth of test pathogens. Columns 1 to 10 contained the representative *Bacillus* CSFs from fish and snail serially diluted from a high to a low concentration (rows A to H). The sterile and positive-growth controls were placed in columns 11 and 12.

The microplates were incubated at 37°C for 24 h, with each well containing a final reaction volume of 110. Following incubation, 10 µL of 1% Resazurin sodium solution (Himedia, India) was added to each well as a color indicator and incubated for an additional 1 h. Wells that retain a purple or violet color indicate negative bacterial growth, whereas wells that appear red or pink indicate positive bacterial growth (Palomino et al., 2002). The MICs were determined as the lowest concentration of CFS capable of inhibiting visible growth of microorganisms as determined by the viable-cell color indicator.

RESULTS AND DISCUSSION

Bacillus Species Isolated from the Guts of *O. niloticus* and *P. canaliculata*

A total of 227 bacterial isolates were isolated from the guts of 30 fish and 30 snails. Sixty (26%) of the 227 isolates were presumptive *Bacillus* species based on their phenotypic and biochemical characteristics (Table 6).

Table 6
Bacterial isolates from the gut of O. niloticus *and* P. canaliculata *from different sampling sites*

Sampling Sites	*O. niloticus* (tilapia) n=30	*P. canaliculata* (golden apple snail) n=30
Batangas (14°06′N 121°01′E)	30	24
Laguna (14°23′N 121°29′E)	25	67
Pampanga (15°04′N 120°43′E)	40	41
Total isolates per species	95	132
Total bacterial isolates	227	

Bacillus isolates exhibited general characteristics consistent with those described in Bergey's Manual of Determinative Bacteriology, Vol. III, The Firmicutes (Whitman, 2009); rod shape, endospore formation, positive catalase reaction, and confluent growth under aerobic conditions (Figure 2). The bacterial isolates were found to have raised, moist, dry, or crusty colonies with undulating margins as observed on the plates. While *Bacillus* spp. were successfully isolated from the gut of Nile tilapia (Leelavatch et al., 2011; Van Horn et al., 2011), only a few studies established their presence in golden apple snails.

One of the characteristics that distinguish members of the genus *Bacillus* from other bacteria genera is the formation of endospores. Here, the bacterial isolates were found to produce endospores. The observed refractile endospores were found throughout the samples in a variety of locations. Thirty-four isolates were found to have subterminal endospores, while 26 were found to have central endospores. No isolate possessed terminal endospores. Isolates produced free-endospores visible as green round-refractile structures when immersed in oil. These proteinaceous multilayered structures surrounded by glycoprotein-containing exosporium are the dormant cell forms of a variety of bacilli that develop in response to limiting conditions such as nutrient deprivation, demonstrating the bacteria's ubiquity in a variety of environments, including the animal gut. These dormant spores are resistant to a variety of adverse environmental conditions, including desiccation, radiation, and extremely high temperatures (Driks, 2002; Chada et al., 2003; McKenney et al., 2012). The findings provide compelling support for the presumptive identification of gut *Bacillus* species.

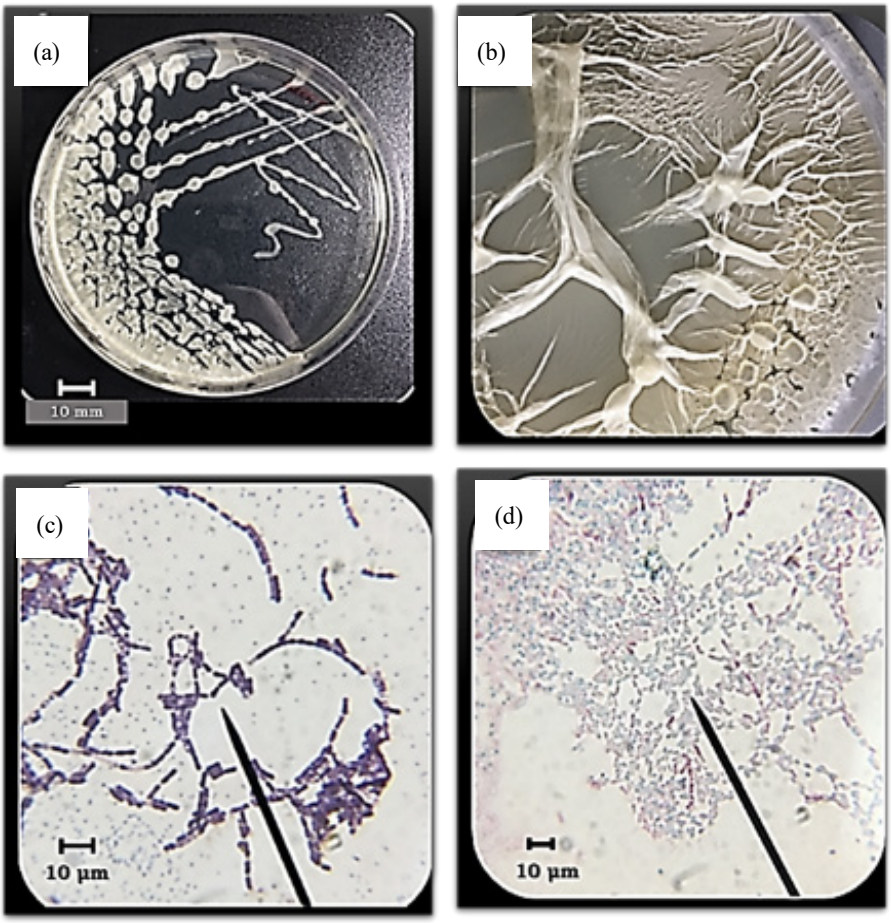

Figure 2. Bacillus species isolated from the guts of tilapia and snails used in the experiment. (a). *Bacillus* sp. colony (PTSC8) demonstrating large, frosty colonies; (b) magnified photograph of *Bacillus* (PTSC8) on culture plate demonstrating biofilm formation (wrinkled-colonies); (c) Gram staining result demonstrating purple, rod-shaped organisms; characteristics of Gram-positive bacilli; and (d) spore staining reveals red vegetative cells and spores appear as green dots when viewed under oil-immersion objectives.

Biofilm development was found in the isolates (Figure 3). The extracellular matrices of *Bacillus* spp. biofilms are crucial for bacterial cell survival and dispersal into the environment, especially in gut habitats where chemicals such as gastric acid, bile, and mucus impact bacterial survival (Gorbach, 1996). The observation of *Bacillus* colonies in the study is consistent with existing literature, as colony biofilms are formed when *Bacillus* spp. are grown on solid agar, promoting the expression of genes involved in extracellular matrix formation. Within a few days of incubation, this condition results in the growth of complex wrinkled colonies (Branda et al., 2001).

Based on the observed characteristics of the isolates attributed to the members belonging to the genus *Bacillus*, this investigation established the presence of *Bacillus* species in the guts of both fish and snail sampled. *Bacillus* spp. colonization of the intestines of fish and snails can be linked to the animals' diet, interaction with soil, and rearing settings that are typically inhabited by endospore-forming bacteria (Woiwode et al., 1993; Van Horn et al., 2011; Giatsis et al., 2014; Standen et al., 2015; Ghosh et al., 2017). When microorganisms, particularly endospore-forming bacteria, are ingested by fish and snails, they quickly adapt to the GI tract environment and develop a symbiotic relationship with the host (Saha et al., 2006).

The tilapia samples for this investigation were obtained from rearing farms bred and produced for human consumption. Fish in aquaculture farms in the Philippines are fed with commercial and non-commercial diets containing probiotics (Woiwode et al., 1993). These probiotics aid in the growth and stimulation of the host's immune system. A study examined the effect of probiotic-supplemented diets on the microbial communities in the gut of tilapia fish and discovered that fish fed with probiotics had an increased abundance of lactic acid bacteria and *Bacillus* species (Standen et al., 2015).

Pomacea canaliculata is a very invasive snail species that originated in Central and Southern America. Their rapid population growth is attributed to their excellent adaptability, large food intake, high reproductive capacity, and the lack of an active predator to feed on them (Lach et al., 2000; Cowie, 2002; Rawlings et al., 2007; Oscoz et al., 2010; Yang et al., 2017). Snails are generally considered herbivores, eating largely on vascular plants and plant material high in protein and calcium (Raut & Barker, 2002; Lodge et al., 1998; Carlsson & Lacoursière, 2005). Their ability to do so is dependent on the existence of gut symbionts capable of secreting lytic exoenzymes that degrade complicated substances to their simplest forms (Godoy et al., 2013). Numerous studies have established strong correlations between enzymatic activity and antimicrobial peptide production in antibiotic-producing bacteria (Salazar & Asenjo, 2007; Godoy et al., 2013; Ahmad et al., 2013; Hassan et al., 2013; Xu et al., 2015), indicating that enzyme-producing bacteria can produce antimicrobials. This study is based on the premise that the gut microbiota contains a vast supply of microorganisms capable of producing various compounds with diverse activities. Though snails are considered highly invasive in agriculture, they were used in this study as a source of potentially antibiotic-producing gut bacteria due to their capacity to digest complex materials.

Preliminary Screening of Gut *Bacillus* Isolates for Antibacterial Activity

In this study, sixty (60) *Bacillus* isolates were screened for antibacterial activity. Thirty exhibited varying degrees of antimicrobial activity against MDR strains. *Bacillus* species with the greatest average inhibition zone were chosen for CFS production and tested against pathogens using an agar well diffusion assay (Figure 4 and Table 7).

Thirty (50%) of the 60 presumptively identified *Bacillus* species exhibited antimicrobial activity when tested using the cross-streak method. Twenty-eight (93%) of the 30 exhibited activity against MRSA, while 16 (53%) exhibited activity against ESβL *E. coli*. It is worth noting that no *Bacillus* isolates were antimicrobial against MβL *P. aeruginosa*. Additionally, 16 of 30 (53%) exhibited activity against MRSA and ESβL *E. coli*.

The *Bacillus* isolates demonstrated a mean zone of inhibition against MRSA (9.57 ± 3.40 mm) and EsβL *E. coli* (5.07 ± 2.69 mm). Interestingly, most *Bacillus* isolates exhibited inhibitory effects against MRSA, with PSSC8 exhibiting the highest inhibitory effect (16.97 ± 0.81 mm) and BTSC23 exhibiting the highest inhibitory effect (10.73 ± 0.25 mm) against ESβL- *E. coli*. The current study corroborates previous findings by Powthong and Suntornthiticharoen (2017) and Kivanç et al. (2014) regarding the use of *Bacillus* isolates to inhibit *S. aureus*. These inhibitory activities could be attributed to the isolates' ability to secrete broad-spectrum antimicrobial peptides (AMPs) capable of killing closely related bacteria by damaging the cell membrane and inhibiting the biosynthesis of cell wall components (Wimley & Hristova, 2011; Malanovic & Lohner, 2016).

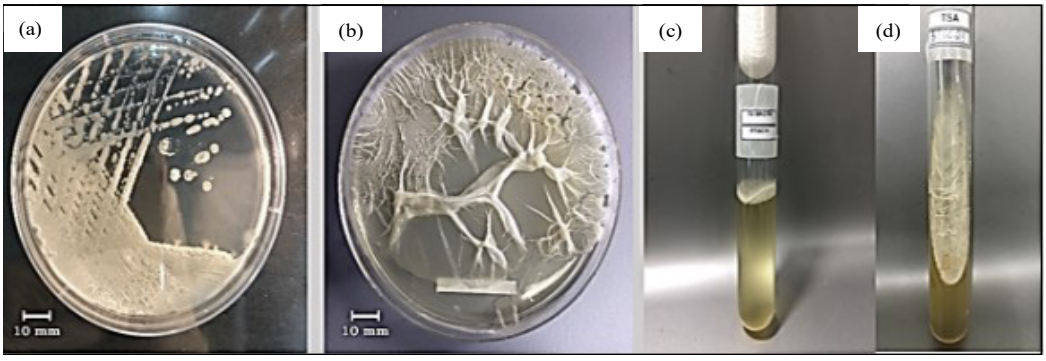

Figure 3. Bacillus species isolates grown aerobically. *Bacillus* isolates grown aerobically in a variety of media. (a) Quadrant-streak growth on TSA plate (PTSC8); (b) *Bacillus* strain BSSC22 biofilm formation; (c) *Bacillus* strain PTSC11 growth on liquid medium with pellicle formation; and (d) Bacillus strain BTSC23 growth on TSA slant.

Table 7

Cross-streak method results of gut Bacillus *spp. against MDR strains*

Bacillus species strain code	MRSA (mm)	ESβL *E. coli* (mm)	MβL *P. aeruginosa* (mm)
LSSC28	2.06 ± 0	NI	NI
LSSC29	13.92 ± 0.22	0.82 ± 0.62	NI

Table 7 *(Continue)*

Bacillus species strain code	MRSA (mm)	ESβL *E. coli* (mm)	MβL *P. aeruginosa* (mm)
LSSC30	12.56 ± 4.33	NI	NI
LSSC31	11.86 ± 2.68	3.39 ± 1.49	NI
LSSC32	7.24 ± 6.52	NI	NI
LTSC8	6.62 ± 0.46	NI	NI
LTSC10	6.89 ± 0.7	NI	NI
LTSC12	8.06 ± 1.73	NI	NI
LTSC19	4.47 ± 0.33	NI	NI
LTSC25	8.79 ± 2.45	6.49 ± 1.61	NI
BSSC17	8.84 ± 3.13	NI	NI
BSSC22	11.65 ± 0.76	NI	NI
BSSC19	11.15 ± 2.5	NI	NI
BSSC23	13.53 ± 2.1	NI	NI
BSSC21	9.06 ± 0.01	NI	NI
BTSC23	8.27 ± 0.9	10.73 ± 0.25	NI
BTSC15	8.96 ± 0.09	2.01 ± 0.79	NI
BTSC28	NI	2.75 ± 0.09	NI
BTSC29	4.68 ± 0.69	3.22 ± 0.55	NI
BTSC30	NI	6.23 ± 2.08	NI
PSSC5	8.86 ± 3.39	7.33 ± 1.51	NI
PSSC8	16.97 ± 0.81	6.16 ± 3.42	NI
PSSC9	9.87 ± 3.17	2.88 ± 0.59	NI
PSSC10	9.9 ± 5.65	2.95 ± 1.72	NI
PSSC11	13.01 ± 0.16	3.59 ± 2.66	NI
PTSC6	14.09 ± 1.08	9.03 ± 2.91	NI
PTSC8	7.88 ± 0.61	6.66 ± 3.85	NI
PTSC9	7.98 ± 0.59	7.36 ± 1.14	NI
PTSC10	6.51 ± 4.66	NI	NI
PTSC11	14.21 ± 0.64	4.55 ± 0.69	NI
Mean ZOI	9.57 ± 3.40	5.07 ± 2.69	-

The ZOI means are expressed in mm ± SD. NI= No inhibition against test organism.

Members of the genus *Bacillus* act produce numerous AMPs as inhibitors of the growth of closely related bacteria (Stein, 2005). However, no inhibition of MβL *P. aeruginosa* by *Bacillus* isolates was observed. It could be because MβL *P. aeruginosa* has developed mechanisms for hydrolyzing a variety of antimicrobial peptides. Transferable plasmids (integron-associated gene cassettes) provide an explanation for the organism's inherent resistance to antibiotics (Poirel & Nordmann, 2002). *P. aeruginosa's* resistance to antimicrobials may also be explained by its ability to modify the lipopolysaccharide's cell wall properties via the addition of enzymes to the phosphate groups within Lipid A and the core oligosaccharide components (Olaitan et al., 2014), thereby altering antimicrobials' specificity for the target cell wall.

Gut *Bacillus* Cell-Free Supernatant (CFS) Antibacterial Activity Against Multidrug-Resistant Pathogens

Remarkably, *Bacillus* spp. CFSs demonstrated a range of activities against the indicator pathogens tested in the study, with the most susceptibilities against MRSA. Ten (10) out of the 30 *Bacillus* isolates CFS have an antagonistic effect on MRSA (Table 8). The isolate of PTSC8 exhibited the highest antimicrobial activity (18.34 ± 0.35 mm) against MRSA. However, none of the isolates CFSs exhibited inhibitory activity against EsβL *E. coli* or MβL *P. aeruginosa*.

Table 8

Agar -well diffusion assay results using Bacillus *spp. cell-free supernatant (CFS)*

CODE	MRSA (mm)	ESβL *E. coli* (mm)	MβL *P. aeruginosa* (mm)
PTSC8	18.34 ± 0.35	NI	NI
LTSC10	17.61 ± 0.69	NI	NI
PTSC9	17.35 ± 0.57	NI	NI
PSSC11	17.17 ± 0.32	NI	NI
LTSC25	17.11 ± 1.12	NI	NI
BSSC22	17.09 ± 1.53	NI	NI
PSSC8	16.97 ± 1.56	NI	NI
PTSC11	16.38 ± 1.22	NI	NI
BSSC23	14.92 ± 1.02	NI	NI
BTSC23	12.67 ± 0.18	NI	NI
Mean	16.56 ± 1.63	-	-

Key: NI= No inhibitions. All data are expressed in millimeters (mm) ± standard deviation. Cefoperazone breakpoints for *S.aureus*, *E.coli* and *P.aeruginosa* = ≥21mm susceptible (S), 16–20 mm intermediate (I) , and ≤15 mm resistant (R) (CLSI, 2012).

The strains' resistance to *Bacillus* CFS could be explained by the pathogens' deactivation mechanisms, including hydrolysis of antibiotics or modification of the antibiotics' target site in the resistant bacterial cell (Bonnet, 2003; Spratt, 1994). *Escherichia coli* strains are a rapidly evolving group of bacteria that have acquired the ability to hydrolyze a wide variety of antibiotics. The changes in the beta-lactam antibiotics' substrate on the surface of the target bacterial cell confer resistance (Philippon et al., 1989). *P. aeruginosa* has been reported to be resistant to a variety of drug classes. Unfortunately, the mechanism underlying its rapid evolution and complex resistance is unknown (Bergen et al., 2010; Johansen et al., 2008). *P. aeruginosa's* resistance may be mutational or non-mutational (Nicas & Hancock, 1983). Among the non-mutational mechanisms, *P. aeruginosa* was shown to tolerate AMPs by restructuring its outer membrane via enzymes to the phosphate groups of the LPS Lipid A region, a process mediated by the genes arnBCADTEF-PA3559 (PA3552-PA3559) (Jochumsen et al., 2016). Epand et al. (2016) discussed the Gram-negative bacterial cell wall's function as a barrier to antibiotics and AMPs. Gram-negative bacterial pathogens generally have distinct cell wall structures than Gram-positive bacteria, contributing to their increased virulence and resistance to AMPs. The outer membranes have a reduced permeability, preventing antibiotic molecules from entering. While some use secondary adaptive resistance mechanisms such as efflux and enzymatic antibiotic modification, others do not.

The current study established that not all *Bacillus* species are capable of producing antimicrobials, implying that this ability is strain-specific. While MRSA strains developed resistance to several antimicrobials by altering their cell-surface components, this study observed inhibitory effects of *Bacillus* spp. isolates, correlating with previous research on anti-MRSA of *Bacillus* strains (Jeyanthi & Velusamy, 2016; Chalasani et al., 2015; Aslim et al., 2002; Aunpad & Na-Bangchang, 2007). MRSA's sensitivity to CFS may be attributed to *Bacillus* isolates releasing antimicrobial peptides (AMPs) that act on the cell wall and membrane of Gram-positive bacteria (Somsap et al., 2016; Salazar & Asenjo, 2007). Although the presence of these AMPs was not confirmed in this study, several studies on *Bacillus* antimicrobials (Jeyanthi & Velusamy, 2016; Chalasani et al., 2015; Aslim et al., 2002; Aunpad & Na-Bangchang, 2007) indicate that the ZOIs on the plates were primarily due to the action of AMPs, given that all standard protocols for CFS production including the diffusion assay's use of antibiotics and sterile controls, were followed.

The narrow-spectrum antibacterial activity of *Bacillus* species supports Ramachandran et al. (2014), in which an antimicrobial substance isolated from wild-type *Bacillus* species was found to be antagonistic only against Gram-positive *Staphylococcus aureus, Streptococcus pyogenes*, and *Enterococcus faecalis*. Similarly, *B. laterosporus* demonstrated antibacterial activity against test pathogens via the production of bacteriocins, an AMP whose mode of action involves the destruction of the pathogen's cell membrane integrity,

interfering with cellular molecules, and ultimately leading to bacterial cell lysis and death (Somsap et al., 2016).

A similar study conducted by Lertcanawanichakul and Sawangnop (2011) discovered that when the cross-streak method was used as the initial investigation for isolating antibiotic-producing *Bacillus* species, inhibitory effects were observed in both MRSA and ESβL test trains because all metabolites and AMPs were present and being produced during the assay period. However, when *Bacillus* species CFS was used in the agar well diffusion method, the growth conditions and/or preparation of the CFS (centrifugation and filtration) may have influenced antibiotic production, resulting in a decrease in the inhibitory activity of the metabolites.

Biochemical Characterization of Antibiotic-Producing *Bacillus* Isolates

The ten *Bacillus* species that showed inhibitory activities against test pathogens are positive for catalase, Voges-Proskauer, and motility but negative for indole, methyl red, urease gas formation, and sulfide production. Citrate utilization tests revealed that LTSC10, LTSC25, PSSC8, PTSC8, BSSC22, and BSSC23 were all positive, whereas PSSC11, PTSC11, PTSC9, and BTSC23 were all negative. The hydrolytic enzyme activities of standardized *Bacillus* spp. strains were determined. All the ten antibiotic-producing *Bacillus* spp. strains produced enzymes, as indicated by the zones of clearance (Figure 5) on corresponding enzyme plates. Numerous authors evaluated *Bacillus* species' enzyme production to determine their potential for antimicrobial production (Ramachandran et al., 2014; Lirio et al., 2020), as a probiotic agent in fish (Latorre et al., 2016), as an agent for organic material degradation (Oumer & Abate, 2017), and as agents for wastewater treatment (Sonune & Garode, 2018). The diversity of applications demonstrates why the genus *Bacillus* is regarded as the primary source of microbial enzymes (Monisha et al., 2009). Numerous studies have established strong correlations between enzymatic activity and antimicrobial peptide production in bacteria that produce antibiotics (Salazar & Asenjo, 2007; Godoy et al., 2013; Ahmad et al., 2013; Hassan et al., 2013; Xu et al., 2015), indicating that bacteria that produce enzymes can also produce antimicrobials. Bacteria produce enzymes involved in the degradation of complex materials due to genes encoding for these expressions (Cantarel et al., 2009). However, as demonstrated in this work, not all isolated *Bacillus* species with positive enzyme activity possess antimicrobial characteristics. It is worthwhile to investigate further how this phenomenon occurs, particularly with *Bacillus*, which was previously discovered to make enzymes, and more specifically into the factors that contribute to the expression of genes essential for antimicrobial synthesis.

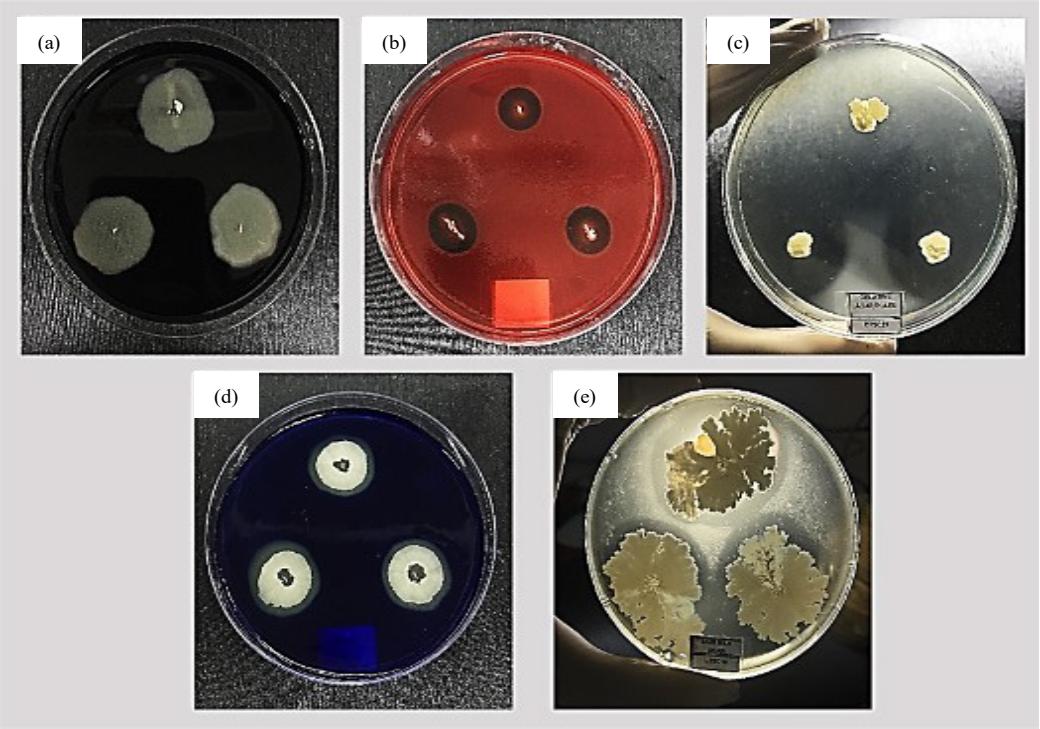

Figure 5. Enzyme activity assay results of the top-performing *Bacillus* strains. The *Bacillus* species exhibiting antimicrobial activity were evaluated for their enzyme production. (a) Amylase test on starch agar (PSSC8); (b) cellulase test on CMC agar (LTSC10); (c) gelatinase test on a gelatin agar plate (BTSC23), (d) lipase test on Tween20 agar (PSSC8), and (e) caseinase test on skim milk agar (LTSC10).

Molecular Identification of Antibiotic-Producing *Bacillus* Species

The ten *Bacillus* species with the highest antibacterial and enzyme production performance were further identified molecularly via 16s rRNA gene sequencing. The results of the blasted *Bacillus* spp. sequences are summarized in Table 9, and the constructed neighbor-joining tree is shown in Figure 6.

Table 9

BLASTn results of the gut-associated Bacillus *spp. 16s rRNA gene sequences*

	NCBI BLAST Hits				
Bacillus strain Code	Source	Query Coverage	E-value	Identity %	Scientific name and NCBI Accession (Gene ID)
LTSC10	Laguna Tilapia gut	98%	0	99.80%	*Bacillus velezensis* NR_075005.2

Table 9 *(Continue)*

Bacillus strain Code	NCBI BLAST Hits				
	Source	Query Coverage	E-value	Identity %	Scientific name and NCBI Accession (Gene ID)
LTSC25	Laguna Tilapia gut	98%	0	99.80%	*Bacillus velezensis* NR_075005.2
PSSC11	Pampanga Snail gut	98%	0	99.46%	*Bacillus siamensis* NR_117274.1
PSSC8	Pampanga Snail gut	98%	0	99.46%	*Bacillus siamensis* NR_117274.1
PTSC11	Pampanga Tilapia gut	98%	0	99.46%	*Bacillus siamensis* NR_117274.1
PTSC8	Pampanga Tilapia gut	98%	0	99.46%	*Bacillus siamensis* NR_117274.1
PTSC9	Pampanga Tilapia gut	98%	0	99.46%	*Bacillus siamensis* NR_117274.1
BSSC22	Batangas Snail gut	98%	0	99.93%	*Bacillus subtilis* NR_112686.1
BSSC23	Batangas Snail gut	99%	0	99.93%	*Bacillus subtilis* NR_027552.1
BTSC23	Batangas Tilapia gut	98%	0	99.46%	*Bacillus siamensis* NR_117274.1

Neighbor-joining tree of gut *Bacillus* species 16s rRNA gene sequences. The tree was constructed using neighbor-joining analysis of the 16s rRNA sequences of gut-associated *Bacillus* species compared to the known type sequences in the NCBI BLAST database. The bootstrap parameter was set to 10,000. *Pseudomonas aeruginosa* KP866815.2 was used as an outgroup.

According to the generated results, two *Bacillus* spp. samples from Laguna tilapia were identified as *Bacillus velezensis* (NR 075005.2). In contrast, samples from Pampanga tilapia, Pampanga snail, and Batangas tilapia were identified as *Bacillus siamensis* (NR 117274.1). Samples from Batangas snail as *Bacillus subtilis* (NR 112686.1 and NR_027552.1) The high percentage of identity (99%) and E-value of zero (0) indicate that the sequences of the samples are highly similar to those of known type material in the GenBank database. This same group of bacteria has also been found in the guts of fish, snails, and various water environments (Sumpavapol et al., 2009; Oyeleke et al., 2012; Rani et al., 2014; de Jesus et al., 2016; Zhou et al., 2018; Yi et al., 2018). Interestingly, the identified species, *B. siamensis*, *B. velezensis*, and *B. subtilis*, have been investigated extensively for their

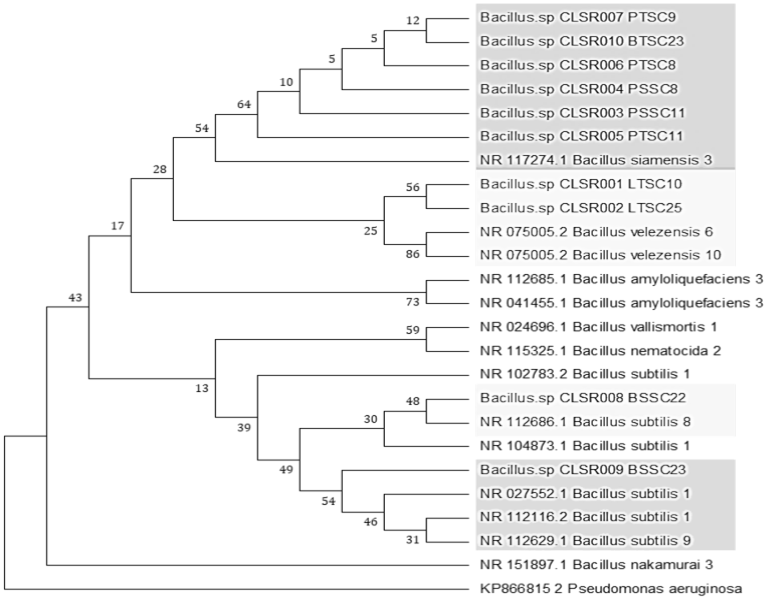

Figure 6. Neighbor-joining tree of gut *Bacillus* species 16s rRNA gene sequences. The tree was constructed using neighbor-joining analysis of the 16s rRNA sequences of gut-associated *Bacillus* species compared to the known type sequences in the NCBI BLAST database. The bootstrap parameter was set to 10,000. *Pseudomonas aeruginosa* KP866815.2 was used as an outgroup.

probiotic potential in the aquaculture industry, specifically for promoting defense and immunity against fish pathogens (Kong et al., 2017). Additionally, *B. siamensis* produced metabolized products and synthesized the functional ingredient levan, a critical prebiotic for intestinal microbiota growth (Thakham et al., 2020). Furthermore, the identified species produced antimicrobial peptides that inhibited pathogen growth and increased the integrity of the fish's intestinal lumen's tight junctions, resulting in increased protective functions against invading pathogens (Zhou et al., 2018; Meidong et al., 2017).

The 16s rRNA gene sequence has been the most frequently used genetic marker to study bacterial phylogeny and taxonomy. It is found in almost all bacteria, is highly conserved, and is large enough (1,500 bp) for downstream bioinformatics. In addition, the bioinformatics of the 16s rRNA gene sequence has the attractive potential of identifying isolates at the genus and species level, which is a limitation of the current biochemical profiling of bacterial isolates (Janda & Abbott, 2007).

Among the complex microbial communities found in the animal gut, those belonging to the phylum Firmicutes predominate (Lagier et al., 2012; Hiergeist et al., 2015; Eckburg et al., 2005). Additionally, studies have revealed that approximately half of the bacteria found in the animal gut are Gram-positive, aerobic, and anaerobic bacteria belonging to the genera *Bacillus*, *Clostridium*, and others in the Firmicutes (Verschuere et al., 2000;

Bäumler & Sperandio, 2016), which may be explained by these bacteria' ability to form endospores, one of the most robust cellular structures known (Stubbendieck et al., 2016). The present findings on the molecular identification of *Bacillus* species from the guts of fish and snails provide compelling evidence for the association of gut microbial communities of endospore-forming bacteria in the animal gut.

Minimum Inhibitory Concentration (MIC) of *Bacillus* spp. CFS

The microdilution plate method was used to determine the minimum inhibitory concentration of *Bacillus* species CFSs isolated from fish and snails. At a minimum concentration of 12.5% v/v CFS, eight (8) of the *Bacillus* CFS inhibited the growth of the test pathogen (MRSA), whereas PTSC11 and BTSC23 had MIC at a 25% v/v CFS concentration. No inhibitory effects were observed against ESβL *E. coli* or MβL- *P. aeruginosa*, which was consistent with the previous agar-diffusion test results. The results of the MIC assay are summarized in Table 10.

Table 10

Minimum Inhibitory Concentration (MIC) results of top antibiotic-producing Bacillus *spp*

	MDR Strains		
Bacillus strain Code	MRSA	ESβL *E. coli*	MβL- *P. aeruginosa*
LTSC10	12.50	NI	NI
LTSC25	12.50	NI	NI
PSSC11	12.50	NI	NI
PSSC8	12.50	NI	NI
PTSC11	25.00	NI	NI
PTSC8	12.50	NI	NI
PTSC9	12.50	NI	NI
BSSC22	12.50	NI	NI
BSSC23	12.50	NI	NI
BTSC23	25.00	NI	NI

Key: NI= no inhibition. Values are in % concentration of *Bacillus* CFS.

It may be necessary to determine the MIC of a potential antibacterial agent that will inhibit the growth of the target pathogen. Antimicrobial breakpoints are monitored in clinical settings using this method to confirm organism resistance and susceptibility and determine the most effective and appropriate dose of antibiotics, thereby preventing the development of antibiotic resistance in individuals receiving antibiotic treatment (Alikhani et al., 2015; Srivastava, 2015). The result of the MIC test on crude CFS from *Bacillus*

strains at third dilution is consistent with previous reports on the MIC activity of bacterial CFS (Kumar et al., 2013; Chalasani et al., 2015).

MRSA strains that frequently cause nosocomial infections develop resistance to antimicrobial agents by substituting D-alanine for teichoic acid on their cell surface, increasing MIC (Peschel & Collins, 2001; Peschel, 2002). *Bacillus* species isolated from the guts of *O. niloticus* and *P. canaliculata* indicated antimicrobial activity against MRSA in this study. As established in this work, however, not all isolated *Bacillus* species with positive enzyme activity have antimicrobial properties. It is worthwhile to conduct additional research into how this phenomenon occurs, particularly with *Bacillu*s, which was previously shown to synthesize enzymes, and more precisely into the factors that lead to the expression of antimicrobial synthesis-related genes.

CONCLUSION

Natural products derived from microorganisms are constantly being investigated as potential alternatives for combating pathogens and preventing the spread and development of antibiotic resistance. *Bacillus* species from the guts of *Oreochomis niloticus* (Nile tilapia) and *Pomacea canaliculata* (golden apple snail) were found to inhibit MRSA growth. *Bacillus* CFS, on the other hand, was ineffective against Gram-negative, multidrug-resistant indicator strains. Hydrolytic enzyme assays were performed and confirmed that gut *Bacillus* isolates certainly produce enzymes as shown in their biochemical and enzymatic activity profiles to substantiate the notion that antibiotic-producing bacteria can produce hydrolytic enzymes. *Bacillus velezensis* (KY694464.1), *Bacillus siamensis* (KY643639.1), and *Bacillus subtilis* (CP002905.1 and CP020102.1) were identified as the gut-associated bacteria as confirmed by 16s rRNA gene sequence analysis. The minimum inhibitory concentration exhibited by the *Bacillus* spp. CFS against MRSA ranges from 12.5% to 25% concentration with no inhibitory effects observed against ESβL *E. coli* and MβL *P. aeruginosa*.

Bacillus CFS constituent analysis must be performed to identify the active antibacterial molecules responsible for the antibacterial activity against MRSA. It may include protein analysis of novel compounds produced endogenously by *Bacillus* species. *Bacillus* species found in the guts of *O. niloticus* and *P. canaliculata* may also be investigated in the future for their probiotic potential. It is necessary to optimize the *Bacillus* culture media and conditions to maximize the production of antimicrobial peptides (AMPs) and other related compounds. It is possible to conduct additional research on the *Bacillus* strains' inhibitory effects on other multidrug-resistant pathogen strains. Synergism experiments between *Bacillus* CFS and commonly used antibiotics may be conducted to ascertain possible interactions between the molecules present in the CFS and the antibiotics in use, as well as to further elucidate synergistic mechanisms in the control of multidrug-resistant pathogens.

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