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Research Article

Lactobacillus plantarum in the Gut of a Marine Fish from a Caloocan Local Market

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The search for potential probiotic bacteria of value to medicine, food industry, agriculture, and aquaculture has been extended in this study where bacterial isolates from the intestines of talakitok (*Alectis* sp.), a Philippine marine fish cultured and grown using de Man-Rogosa-Sharpe agar, were used to isolate Gram-positive rods that are catalyse negative. Its identity of 93-95% with *Lactobacillus plantarum* strains was confirmed by NCBI BLAST of the 16S rRNA forward and reverse sequences.

1. Introduction

Microorganisms that thrive as natural microflora but can contribute to better growth performance and survival of their host have been called probiotic bacteria. Merrifield et al. [1] claimed that probiotic bacteria in the intestinal tract can improve micronutrient absorption, increase disease resistance, and promote the growth performance of their fish host. As a component of the animal gut natural flora, probiotic bacteria like *Lactobacillus plantarum* play an active role in the physiology of animals by activating the gut-associated lymphoid tissues (GALT) which are important components of the innate and humoral immune systems. Nayak [2] says that gastrointestinal microbiota assume active physiological roles in the fish species that can withstand changes in their environmental conditions and thus afford to maintain high rates of survival. The type of microbiota in the gut is an orchestration of the fish's choice of diet, the water temperature, and other environmental factors [3]. Enhanced nutrient absorption, disease resistance, survival rates, and greater ability to adapt to changing environmental conditions have been related to the presence of probiotic bacteria in the gut of fish [2–5].

The interest in improving aquaculture techniques recently has given much attention to the inclusion of probiotic bacteria in the diet of commercially important fish species—*Oreochromis* spp., *Decapterus* sp., *Acanthurus* sp., and *Chanos chanos*. Incidentally, most of these fish species are not in the list of threatened Pisces groups.

Adding probiotic microorganisms like *Lactobacillus plantarum* and *Lactobacillus rhamnosus* to the diet of *Oreochromis niloticus* (tilapia) is claimed to have promoted growth, digestion, disease resistance, and greater yield [6]. Gomez et al. [7] suggested that the presence of probiotic microorganisms in the gut of *Decapterus* sp. (mackerel) can promote better survival and reproductive rates. Supplementing the diet of the surgeonfish, *Acanthurus* sp., with probiotics suggested by Kohl et al. [8] may improve the digestion of cellulose in the gut of this seaweed-eating coral reef thriving fish and thus help promote ecosystem balance. Vibin et al. [9] similarly claim that the water conditions where *Chanos chanos* lives can be rid of pollutants as the probiotic bacteria in the gut of this fish can help balance excess levels of nitrogenous materials in the water.

Earlier studies by Hoseinifar et al. [6] where the diet of the common carp was enriched with probiotic bacteria exemplified by many *Lactobacillus* species claimed to have improved gut health as well as growth rates. In 2011, Roeselers et al. [10] similarly said that probiotic bacteria in the gut microbiota of zebrafish (*Danio rerio*) did benefit its survival. Ghori et al. [11] found out that the growth, feed utilization,

and hematological profile of *Labeo rohita* significantly improved by probiotic-enriched diet.

Lactobacillus plantarum, *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus delbrueckii*, and other species of probiotic bacteria that produce lactic acid have long been found to have high potentials to boost human and animal health. Currently, the search for more sources of probiotics is still in vogue as these bacteria are potential alternatives for certain antibiotics and drugs. They are also found to be competitors of pathogenic ones in the intestinal tract [12]. Probiotic benefits that go with consumption of some beneficial microorganisms in traditional foods including yogurt, cheese, and milk, associated with protection against diseases, and extended lifespan were shown by Adou et al. [13], where *Lactobacillus* from cattle milk, i.e., buffalo, camel, and goat, from feces of the same, as well from feces of fishes, was isolated; however, there was no mention of which species of fish was used.

In the effort to find bacteria that improves nutrient absorption, Asaduzzaman and Abol-Munafi [14] studied different bacterial genera including *Alcaligenes*, *Bacillus*, and *Shewanella* from the gut of a juvenile cyprinid, *Tor tambroides*. Recently, the authors were able to isolate *Enterococcus*-like bacteria from the gut of C*hanos chanos* (milkfish) which also showed antimicrobial inhibitory action against *Staphylococcus aureus* in vitro. Earlier in 2008, though Vijayavaskar and Somasundaram isolated *Bacillus* sp. from *Oreochromis mossambicus* (tilapia), *Bacillus* sp. was found to inhibit *Aeromonas hydrophila*, a common pathogen of tilapia.

There remains a great deal of task to explore the sources of potential probiotic bacteria. This study is thus aimed at

(1) isolating probiotic bacteria from the gut of a fish locally sold in the community market

(2) confirming the identity of the lactic acid bacteria using 16S rRNA sequencing

2. Methods

2.1. Sampling. A marine fish available from June to December in the Philippine waters was to be sampled in the study. Random purchase of the available marine fish was done in Bonifacio Market, Caloocan City, Metro Manila, Philippines. The criteria for selection were that the marine fish species should be absent from the list of threatened fish species, must be with intact scales, and must have bright bluish eyes and bright red gills, which are indications of freshly caught fish. Purposive convenience sampling was followed. A fish stall selling a huge fish that is locally called talakitok (*Alectis, Alectis indicus*) was selected. It happened to be the available marine fish with qualifications sought for. The fish vendor claimed that this fish was caught from the Bicol Region. The same single fish was taken as the sample of an unthreatened marine fish and hence was taken to the laboratory. The gut of the fish was excised for examination of the presence of probiotic bacteria.

2.2. Dissection of the Gut. Aseptically, the gut was excised out from the fish and then immersed in sterile distilled water. After an hour, the fish gut was placed on sterile mortar and pestle and then crushed to get a homogenous texture. The supernatant was decanted out.

2.3. Isolation of the Lactic Acid Bacteria. de Man, Rogosa, and Sharpe (MRS) agar is a culture medium specific for isolating and growing *Lactobacillus*, the genus popularly used in commercial probiotic drinks. Thirty-five grams of the MRS powder is dissolved in 500 ml distilled water, set on a water bath, and allowed to boil while continuously stirring. The culture medium is sterilized at 121°C for 15 minutes and then cooled down to 45°C before plating. Fifteen to twenty milliliters of the sterilized MRS was poured in sterilized Petri dishes.

One thousand microliters (μl) of the supernatant was inoculated into sterile plates and then poured in with fifteen milliliters (ml) of sterile de Man-Rogosa-Sharpe agar (MRSA).

2.4. Incubation. The plates were incubated at 35°C for at least three days; alternatively, the inoculated MRS plates were kept in a glass jar enriched with CO_2 and left at room temperature (25-25°C), for one week. Colony growths that developed on the MRS agar plates were subcultured in MRS agar butt slants.

2.5. Subculturing in MRS. Cream-white colonies that looked like very small specks on the plate were subcultured by incubation at the same temperature. MRS agar butt slants that grew colonies that looked mucoid to dry, but which grew deep in the butt, were Gram stained and then tested for catalase activity.

2.6. Catalase Test. A loopful of the white colony growths on MRSA butt slants were introduced on drops of hydrogen peroxide on the glass slide. Absence of bubbling indicated that the bacteria introduced were negative for catalase activity.

Rod-shaped Gram-positive rods that grew as white drier colonies both on MRSA and NA plates were nutrient agar (NA) subculturing.

Gram-positive and catalase-positive colonies from the MRS agar butt slant subcultures were considered as potential lactic acid bacteria and were subcultured in nutrient agar butt slants for 16S rDNA sequencing at the Philippine Genome Center (PGC), University of the Philippines, Diliman, Quezon City.

2.7. 16S rDNA Extraction. Identification of the pure culture of the isolates that grew on MRS agar butt slants required DNA extraction, purification, 16S rRNA amplification using PCR, and checking of the products using agarose gel electrophoresis. All of these were performed by the Philippine Genome Center at the University of the Philippines, Diliman, Quezon City.

The protocol (as indicated in Zymo Research Quick-DNATM Fungal/Bacterial Miniprep Kit) followed below involved the use of beta-mercaptoethanol (user supplied) to the genomic lysis buffer to a final dilution of 0.5% (v/v), i.e., 500 μl per 100 ml, for optical performance:

Fifty to one hundred (50–100) milligrams of wet weight bacterial cells which equates to approximately 109 bacterial cells that have been resuspended in up to 200 μl of water

or isotonic buffer (e.g., PBS) was added to a ZR Bashing-Bead™ lysis tube (0.1 mm and 0.5 mm). Add 750 μl Bashing-Bead™ buffer to tube 2.

Securing of this in a bead beater fitted with a 2 ml tube holder assembly and process at maximum speed for ≥5 minutes followed. However, the required processing time varied depending on the device and application. Centrifuging the ZR Bashing Bead™ lysis tube (0.1 and 0.5 mm) in a microcentrifuge at 10,000 × g for 1 minute thereafter was done. Up to 400 μl supernatant was transferred to a Zymo-Spin™ III-F filter in a collection tube and centrifuged at 8000 × g for 1 minute; 1200 μl of genomic lysis buffer was added to the filtrate in the collection tube; 800 μl of the mixture was transferred to a Zymo-Spin™ IICR Column 3 in a collection tube and centrifuged at 10,000 × g for 1 minute. The flow through from the collection tube was discarded, and then, the step was repeated.

200 μl DNA prewash buffer was added to the Zymo-Spin™ IICR Column in a new collection tube and centrifuged at 10,000 × g for 1 minute.

500 μl g-DNA wash buffer was added to the Zymo-Spin™ IICR Column and centrifuged at 10,000 × g for 1 minute.

The Zymo-Spin™ IICR Column was transferred to a clean 1.5 ml microcentrifuge tube and added with 100 μl (35 μl minimum) DNA elution buffer directly to the column matrix. Centrifugation at 10,000 × g for 30 seconds was done to elute the DNA.

2.8. rRNA Sanger Sequencing. The PGC adopts the Sanger or capillary sequencing that uses the chain-termination method with fluorescent ddNTPs and optics to determine the nucleotide sequence. It followed the conventional Sanger sequencing method, called "first-generation sequencing," involving PCR amplification, product qualitative detection and separation by gel electrophoresis, and purification of the amplicon through ethanol.

3. Results

Gram-positive rods that were prepared from small mucoid colonies that grew on both the MRS and NA butt slants and plates are shown in Figure 1, as they were examined under the oil immersion objective. The size of the rods is close to 0.9–1.2 μm wide and 3–8 μm long.

3.1. 16s rDNA Sequencing Results. Forward and reverse primers were used to provide the rRNA sequence of the isolates from talakitok fish. NCBI BLAST results point to 93% identity with a strain of *Lactobacillus plantarum* (accession number KX538911.1; a total query cover of cover of 95%) and 87% identity with a strain of *Lactobacillus plantarum* (accession number 658476.1), both with *E* values equal to 0.

4. Discussion of Results

The local marine fish called talakitok, of the genus *Alectis*, belongs to the Carangidae family which includes other marine fishes like the jacks, pompanos, jack mackerels, runners, and scads (Wikipedia). Massive and weighing to as much as 80 kg, talakitok can be identified as having a longitudinal faint yellowish tint along the flattened surface of its body. As a sturdy fish, it is an important part of Filipino diet. In this study, bacterial isolates from its gut were grown on de Man-Rogosa-Sharpe agar, a kind of special culture medium to grow lactic acid-producing bacteria. The bacterial isolates showed basic colony features, as being small white mucoid to dry colonies that are Gram-positive rods on microscopic examination. Characteristic of lactic acid-producing bacterial species, the isolates were likewise catalase negative observed to as such when loopfuls of colony samples did not oxidize hydrogen peroxide as evidenced by the absence of bubble formation.

The isolates were submitted to the Philippine Genome Center for identification using the forward and reverse primers of the 16S rRNA. NCBI BLAST results of the forward primers show that the isolates were 93-95% identical (95-96% cover) with strains of *Lactobacillus plantarum* (accession nos. KX538911.1 and MG551212.1), respectively, both with *E* values = 0. CLUSTAL OMEGA multiple alignment scheme inclusive of the query sequences' forward primers (unknown isolates SpA_27F and Sp5_27F) shows fully conserved regions from segments 538 to 719 with those of *Lactobacillus plantarum* strains used in the sequence analysis (Figure 2).

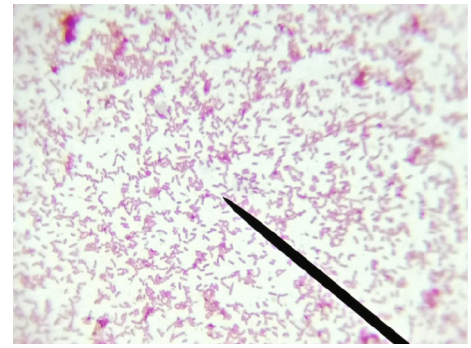

Figure 1: The Gram stain result from the small dry colonies that grew on the plates (×1000).

Figure 2: Conserved regions common to the Lactobacillus plantarum strains and the MRS isolates (SpA_27F and Sp4_27F).

Sequences producing significant alignments Download Select columns Show 100

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Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Lactobacillus plantarum strain LY21 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2259	2259	100%	0.0	100.00%	1223	KX538911.1
Lactiplantibacillus plantarum strain TMPC 4E173 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2095	2095	96%	0.0	98.57%	1204	ON138870.1
Lactiplantibacillus plantarum strain TMPC 3F725 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2093	2093	97%	0.0	98.18%	1292	OL589263.1
Lactobacillus plantarum strain 5107 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2091	2091	98%	0.0	97.79%	1482	MT463411.1
Lactiplantibacillus plantarum strain TMPC 4A121 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2091	2091	96%	0.0	98.57%	1277	OM760906.1
Lactiplantibacillus plantarum strain TMPC 46D26 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2089	2089	96%	0.0	98.41%	1255	ON506115.1
Lactiplantibacillus plantarum strain 46D26 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2089	2089	96%	0.0	98.41%	1255	OM877469.1
Lactobacillus plantarum strain 2186 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2087	2087	98%	0.0	97.79%	1471	MT604686.1

Figure 3: NCBI BLAST results of KX538911.1 which is one of the similar sequences to the query sequence SpA_27F and Sp4_27F shown in Figure 2.

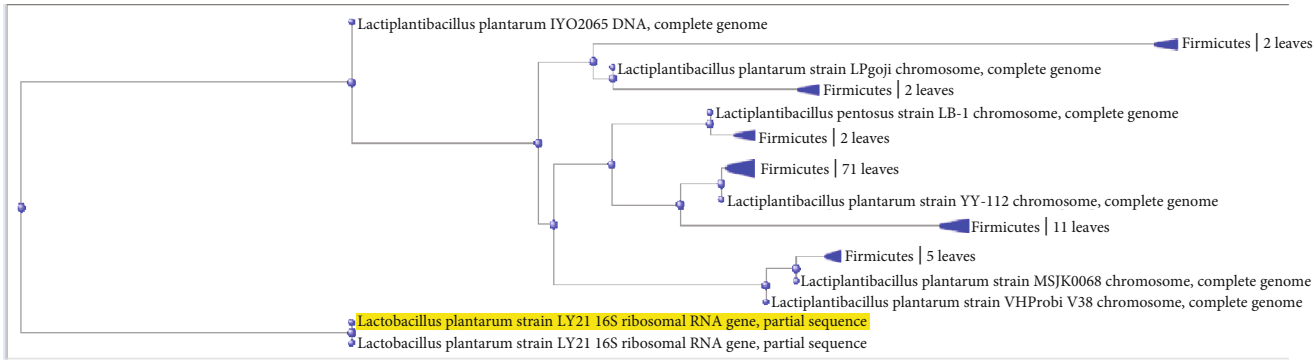
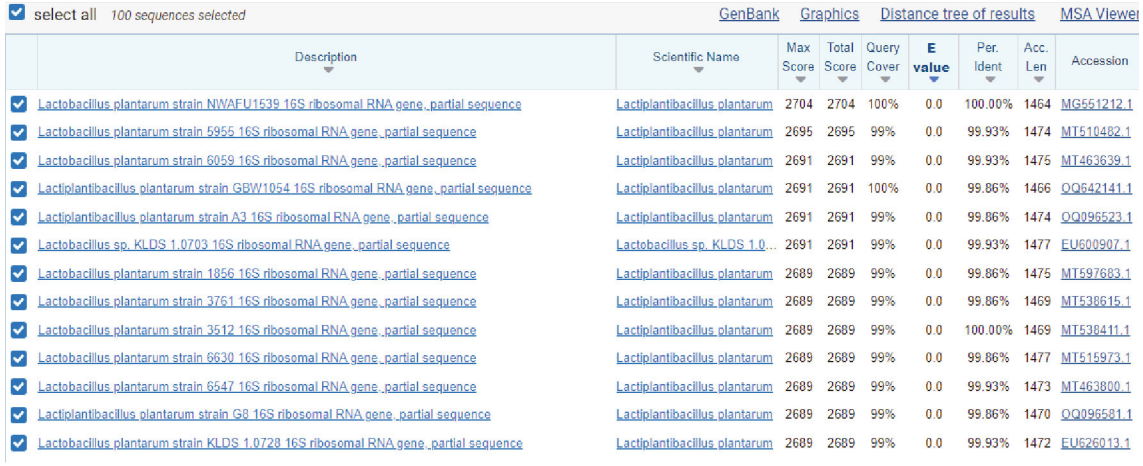


Figure 4: BLAST tree view of Lactobacillus plantarum.



select all 100 sequences selected GenBank Graphics Distance tree of results MSA Viewer

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
Lactobacillus plantarum strain NWAFU1539 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2704	2704	100%	0.0	100.00%	1464	MG551212.1
Lactobacillus plantarum strain 5955 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2695	2695	99%	0.0	99.93%	1474	MT510482.1
Lactobacillus plantarum strain 6059 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2691	2691	99%	0.0	99.93%	1475	MT463639.1
Lactiplantibacillus plantarum strain GBW1054 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2691	2691	100%	0.0	99.86%	1466	OQ642141.1
Lactiplantibacillus plantarum strain A3 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2691	2691	99%	0.0	99.86%	1474	OQ096523.1
Lactobacillus sp. KLDS 1.0703 16S ribosomal RNA gene, partial sequence	Lactobacillus sp. KLDS 1.0...	2691	2691	99%	0.0	99.93%	1477	EU600907.1
Lactobacillus plantarum strain 1856 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2689	2689	99%	0.0	99.86%	1475	MT597683.1
Lactobacillus plantarum strain 3761 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2689	2689	99%	0.0	99.86%	1469	MT538615.1
Lactobacillus plantarum strain 3512 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2689	2689	99%	0.0	100.00%	1469	MT538411.1
Lactobacillus plantarum strain 6630 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2689	2689	99%	0.0	99.86%	1477	MT515973.1
Lactobacillus plantarum strain 6547 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2689	2689	99%	0.0	99.93%	1473	MT463800.1
Lactiplantibacillus plantarum strain G8 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2689	2689	99%	0.0	99.86%	1470	OQ096581.1
Lactobacillus plantarum strain KLDS 1.0728 16S ribosomal RNA gene, partial sequence	Lactiplantibacillus plantarum	2689	2689	99%	0.0	99.93%	1472	EU626013.1

Figure 5: BLAST results for MG551212.1.

Strongly conserved regions found in the 16S rRNA sequences of the bacterial isolates from Alectis sp. gut with the strains earlier isolated from other sources are shown in Figure 2. This implies functionality of the regions of these 16S rRNA sequences that turned out to be similar to those of the gut bacterial isolates. The accession numbers to the left of the aligned sequences can be used to trace the species identity of these sequences, by simply entering each in the BLASTn box in the NCBI web software.

Figure 3 shows the BLAST results of KX538911.1 as Lactobacillus plantarum, with a query cover of 100% at 0.0 *E* value and percent identity of 100%, has a high similarity to the unknown isolate query sequences (SpA_27F and Sp5_27F) in this study.

The BLAST tree view in Figure 4 shows that Lactobacillus plantarum descends as a monophyletic group inclusive of Lactiplantibacillus plantarum strains that terminates in Firmicutes. Firmicutes are a group of Gram-positive rods that

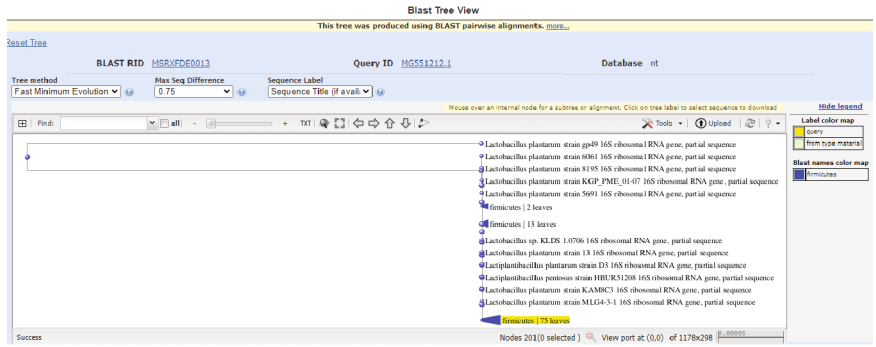


Figure 6: BLAST tree view of MG551212.1.

have very important use in the agroindustry, inclusive of Bacillus species.

Figure 5 shows that MG551212.1, another sequence that shares conserved sequences with the unknown isolates named query sequences SpA_27F and Sp5_27F (Figure 2), has 100% identity with Lactobacillus plantarum, with 100% cover at $E = 0.0$.

Figure 6 shows that the sequence MG551212.1 that shows much conserved regions with the unknown isolates (SpA_27F and Sp5_27F) mentioned in Figure 2 has the same monophyletic group, all consisting of Lactobacillus plantarum strains based on their 16S ribosomal RNA genes, the cluster with terminal nodes labeled as Firmicutes. A recent study by Liu et al. [15] found out that Proteobacteria and Firmicutes dominate the gut of a zigzag eel (Mastacembelus armatus), a nonendangered fish species, with more of the Firmicutes found in cultivated ones. The study on Tor putitora, a large fish species said to be declining in number, from the Indian Ocean, was found out to harbor an abundance of Proteobacteria [16]; however, no Firmicutes species was mentioned in the study.

A more recent finding by Andriani and Pratama [17] states that the most commonly used bacterial species for fish feed fermentation are Bacillus subtilis and lactic acid bacteria. In this study, the isolate from talakitok found to have strong identity with Lactobacillus plantarum is a potential fish feed ingredient, it being a lactic acid bacteria. Additionally, *Lactobacillus plantarum* is a nonpathogenic lactic acid-producing Gram-positive bacterium that has been long used as fermenting agent for cheese, sauerkraut, and pickles and as probiotic organism included in human diet.

Both Bacillus subtilis and Lactobacillus plantarum are Firmicutes claimed to have important applications in agroindustry.

As a heterofermentative species, it can convert sugar to alcohol in the absence of oxygen gas aside from its ability to convert oxygen to hydrogen peroxide when manganese is present. With high tolerance for hydrogen peroxide, it can convert sugar to alcohol and produce lactic acid.

As a probiotic bacterium, *Lactobacillus plantarum* is also used as treatment for gastrointestinal disorders such as irritable bowel syndrome [18]. Amit et al. [19] noted that the hematological response, the immune system, and the health of Cyprinus carpio improved with the enrichment of their diet with *Lactobacillus plantarum*. Hence, addition of this and other similar lactic acid bacteria may contribute to fish longevity in aquaculture.

5. Conclusion

The unknown bacterial isolate from the gut of Alectis sp. (talakitok), a nonendangered marine fish, was found to be identical in its 16S rRNA sequence to that of Lactobacillus plantarum. This isolate like other lactic acid bacteria was found to be Gram-positive rods that are catalase negative. Like most Firmicutes which have important applications in agroindustry, the isolate from the gut of Alectis can potentially be used as an ingredient in fish feeds to boost their growth and development.

Data Availability

I have integrated all my data in the manuscript.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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