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Isolation and Molecular Identification of Lactic Acid Bacteria (Lab) from Nile Tilapia *(Oreochromis niloticus)* as Potential Pathogen Antagonist

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Abstract

Five lactic acid bacterial isolates from the intestinal tract of Nile Tilapia *(Oreochromis niloticus)* were tested for their antagonistic activity against common human pathogens. Only three were identified using 16S rRNA gene sequencing as *Pediococcus pentosaceus* (isolates TI-8 and TI-11) and *Enterococcus avium* (TI-17) showing 99% homology. Among the isolates, TI-8 and T1-11 (*P. pentosaceus*) viable cell cultures were found to have the highest inhibitory activity against *Escherichia coli* ATCC 25922, surpassing that of the positive control, Tetracyline. Though showing significant inhibition also to *Pseudomonas aeruginosa* BIOTECH 1335, *Staphylococcus aureus* BIOTECH 1582, *Salmonella enteriditis* BIOTECH 1963, *Salmonella typhimurium* BIOTECH 1826, and *Vibrio cholerae* BIOTECH 1967, T1-17 (*Enterococcus avium)* is not effective against *S. aureus* BIOTECH 1582. On the other hand, T1-8 and T1-11 (*P. pentosaceus*) cell free supernatant cultures exhibited inhibitory activity against *P. aeruginosa* BIOTECH 1335, *S. aureus* BIOTECH 1582, *S. enteriditis* BIOTECH 1963, *S. typhimurium* BIOTECH 1826, and *V. cholerae* BIOTECH 1967.

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The LAB isolate T1-17 (*E. avium)* exhibited an inhibitory activity against *S. enteriditis* BIOTECH and *V. cholerae* BIOTECH 1967. T1-11 (*P. pentosaceus*) was susceptible to Tetracycline and Amoxicillin; T1-8 (*P. pentosaceus*) was susceptible to Amoxicillin and Cephalexin; and T1-17 (*E. avium)* was susceptible to Amoxicillin.

Keywords: antibacterial; human enteric pathogen; Lactic Acid Bacteria; Nile tilapia .

1. Introduction

Probiotic technology notably changed the human perception concerning microorganisms. These probiotic products had been introduced to various food and drugs and represent a huge proportion of the biotechnology products available today. Moreover, they have been widely exploited due to their beneficial effects specifically for the improvement of human health.

Probiotics are viable microbial supplement which, when ingested in sufficient amount, promotes improvement in the indigenous micro-flora of the gastrointestinal tract [1,2]. The most common type of beneficial microorganism used as a probiotic is Lactic Acid Bacteria, which include *Lactobacillus acidophilus, Lactobacillus casei* and *Lactobacillus plantarum* [3].

Live lactic acid bacteria intake through dairy products have myriad beneficial effects on the gastrointestinal tract of human beings ranging from correction of lactose malabsorption, alleviation of viral and drug induced diarrhea, post-operative pouchitis, irritable bowel syndrome, inflammatory bowel syndrome, anti-neoplastic effects on human cell line, maintenance of normal insulin level in blood and also helpful to enhance the absorption of fatty acids through intestine. LAB produce these beneficial effects by restoration of normal intestinal flora, elimination of intestinal pathogens, reinforcement of intestinal barrier capacity to foreign antigens, stimulation of nonspecific immunity such as phagocytosis, stimulation of humoral immunity and production of anti-inflammatory products[4,5,6]. Beneficial microorganisms also have positive effect on lactose intolerance and malabsorption. Kinova and his colleagues [7] described these beneficial effects exhibited by Lactobacillus present in fermented milk products. Also, a review described that consumption of yogurt containing *Lactobacillus bulgaricus* and *Streptococcus thermophiles* alleviate lactose intolerance through their enzyme lactase when the product reaches the intestinal tract[8].

Lactic acid bacteria have been isolated and characterized from different sources such as vegetable wastes and fish intestines using species-specific PCR techniques and 16S rDNA/ rRNA gene sequencing[9]. Several types of researches have also revealed the presence of probiotic bacteria like *Bacillus spp*. and *Lactobacillus spp*. from the intestinal tract of common carp and the gut of blue swimming crab that are known to inhibit several pathogens[3,10]. Moreover, Flores and Novoa[11], investigated the effects of lactic acid bacteria isolated from the intestinal tract of Nile tilapia (*Oreochromis niloticus*) as a growth promoter. Their study revealed that lactic acid bacterial isolates namely, *Enterococcus faecium, Enterococcus durans, Leoconostoc sp., Streptococcus sp*. I and II have the potential to become native growth promoter comparable to *Lactobacillus acidophilus* which is an already commercialized probiotic.

In addition, several studies investigated the role of LAB as pathogen antagonist in fish and crustaceans which stabilize disease management in the aquaculture sector. Lim and his colleagues [12]. reported four probiotic bacterial isolates obtained from cultured red tilapia namely, *Paenibacillus barcinonensis* strain D12, *Paenibacillus sp*. strain D14, *Staphylococcus cohnii* strain B11 and *Bacillus megaterium* strain E28 which showed antibacterial activity against *Vibrio alginolyticus* ATCC 33839, *Aeromonas salmonicida*, and *Aeromonas hydrophila* ATCC 35654 under in vitro conditions.

However, there are limited researches that investigated the use of LAB isolates from fishes to directly target an extensive range of human enteric pathogens, thus, this study was undertaken to isolate and identify useful lactic acid bacteria from the intestinal microbiota of Nile tilapia (*Oreochromis niloticus*) as an inhibitor of different pathogens that attack the human gut and cause a wide range of human enteric maladies.

2. Methodology

2.1. Research Design

The experimental method of research was used in isolating and identifying lactic acid bacteria from intestinal tract of Nile Tilapia and their potentials as pathogen antagonists.

2.2. Sample Preparation and Plating

A total of ten (10) Tilapia, weighing 150 – 200 grams, regardless of sex, caught from Dukma River, a portion of Pampanga River, in Macabebe, Pampanga, were aseptically dissected to obtain their intestinal tract. Before dissection, the abdominal surface of each of the fish sample was scrubbed with rubbing alcohol. The entire intestinal tract was then cleansed with sterile 0.9% saline solution.

Consequently, twenty-five grams of the intestinal tract was mixed into two hundred twenty-five mL of sterile dilution water (0.1% peptone water) and homogenized for two minutes using a blender. Subsequently, a serial dilution was prepared to obtain countable colonies ranging from the original mixture 1:10 to 1:1000 or higher as needed. One mL of each dilution prepared was seeded and spread into MRS Agar plates in triplicate, then incubated in an inverted position at 35±0.5°C for 48 hours under micro-aerophilic condition.

2.3.Culture Selection and Initial Identification

After incubation, potential colonies of lactic acid bacteria were randomly picked by their colonial morphology. Selected colonies were grown in pure culture using the same media and subjected to biochemical analysis. Gram staining and catalase test were performed for preliminary identification of the selected isolates.

Lactic acid bacteria are gram positive and catalase negative[13] (Sonomoto and Yokota, 2011) thus, only the gram-positive and catalase negative isolates were screened for in-vitro antagonistic effect against selected human enteric pathogens.

2.4. Preparation of Pure Culture

Suspected colonies were streaked into MRS Agar plate for culture purification which was done three (3) times, to ensure that only a single strain of bacteria will be obtained in each of the chosen colonies.

2.5. Preparation of Isolate

A loopful of the pure culture bacterial isolates was inoculated into test tubes containing ten (10) mL MRS broth (HiMedia), and were incubated at 35±0.5°C for about 24 hours. After incubation, the broth cultures were transferred into sterile microtubes containing one (1) mL MRS broth and subsequently incubated for eighteen (18) hours. Afterward, each microtube was subjected to a ten (10) minute centrifugation using Sigma® 1-14 High-Speed Centrifuge to obtain a cell-free supernatant at 12,000 x G.

2.6. Inhibitory Activity of the LAB cell-free supernatants against Selected Pathogens

The antagonistic effects of the LAB isolates against selected human enteric pathogens were assessed using the Agar- well Diffusion Assay (Guevarra, 2005).

The selected strains of human enteric pathogens, namely, *E. coli* ATCC 25922, *S. typhimurium* BIOTECH 1826, *S. enteriditis* BIOTECH 1963, *V. cholera* BIOTECH 1967, *P. aeruginosa* BIOTECH 1335, and *S. aureus* BIOTECH 1582 were purchased from Microbiology Department of the University of the Philippines Manila and at the National Institute of Molecular Biology and Biotechnology, University of the Philippines Los Baños, Laguna. A loopful of each bacterium was inoculated heavily into five (5) mL sterile 0.1% peptone water and then compared to a McFarland standard to give an approximately $1.5x10^8$ CFU/mL of the test bacterium.Using a sterile cotton swab, the entire surface of Mueller-Hinton (MH) agar (HiMedia) was inoculated with the test organism previously grown in 0.1% peptone water. The inoculum was then allowed to dry on the surface of the medium for about fifteen (15) minutes. Consequently, the MH agar plate was divided into three (3) quadrants using a marker at the bottom of the plate. Wells were made using a cork borer, positioned at the center of each quadrant on the surface of MH agar plate.

Thirty (30) µL of the treatment or cell-free supernatants previously prepared was dropped in each agar well along with the positive control- Tetrcycline. Then, the plates were incubated at 35±0.5°C for 24 hours The zone of growth inhibition as indicated by clear zones or halo zones was measured in millimeters after the incubation period. The growth of the test organisms treated with the supernatants suspected to have bacteriocin-like inhibitory potentials was recorded.

2.7. Inhibitory Activity of the LAB Viable Cell Culture against Selected Pathogens

Using the same procedure for antibacterial screening of the cell-free supernatants, viable cell cultures were also assessed for competitive exclusion as inhibitory against the selected human pathogens. In each well of previously inoculated MH agar (HiMedia) plates, about 30 µL of the 24-hour old culture of LAB isolates were introduced. The plates were then incubated at 35±0.5°C for 24 hours, after which, the zones of inhibition

produced as indicated by clear zones or halo zones were measured in millimeters. The results were noted side by side with the negative control, 30 µL sterile MRS broth.

2.8. Antibiotic Sensitivity Assay

The same procedure was adopted from the in-vitro antibacterial screening. The lactic acid bacterial isolates served as the test organisms which were subjected to antibiotic sensitivity screening against selected antibiotics namely, T1 – Tetracycline, T2 – Amoxicillin, T3 – Cephalexin, T4 – Ciprofloxacin and T5 – Cefixime.
Each lactic acid bacterial isolates were subjected to the above-mentioned treatments. Interpretation of the results was based on Clinical and Laboratory Standards Institute document M100-S24 (CLSI, 2014). Only the negative control (distilled water) was used on a separate plate, no positive control was used.

*2.9. Molecular Identification of Bacterial Isolate***s**

LAB isolates that exhibited inhibitory activity against the selected pathogens were subjected to molecular identification using the 16S rRNA gene sequencing at the Philippine Genome Center, University of the Philippines-Diliman, Quezon City.

DNA material was extracted from a pure culture of the pre-identified lactic acid bacterial isolate that exhibited significant antibacterial activity using a DNA extraction Kit. It was amplified using 16S universal forward and reverse primers. The PCR product was purified and run in 1.5% agarose gel. Sequencing of around 750 to 1500 bp from the PCR product was done using Capillary Sequencing.

MEGA Blast 5 nucleotide sequence software was used for the analysis of the sequences and compared with the published sequences in GenBank from National Center for Biotechnology Information (NCBI).

2.10. Statistical Analysis

Data were statistically analyzed using the one-way analysis of variance (ANOVA) of the Completely Randomized Design and the Least Significant Difference test.

3. Results and Discussion

3.1. Culture Selection and Initial Identification of the Isolates

A total of twenty-one (21) colonies were selected and sub-cultured. However, only eighteen (18) colonies were able to grow during culture purification (Table 1).

Gram's Staining and Catalase Test revealed that only the isolates with the codes TI-1, TI-8, TI-11, TI-17 and TI-18 were initially presumed to be lactic acid bacteria being gram positive and catalase negative (Sonomoto and Yokota, 2011).

These five (5) presumptive lactic acid bacteria were assessed for their inhibitory potential as part of the

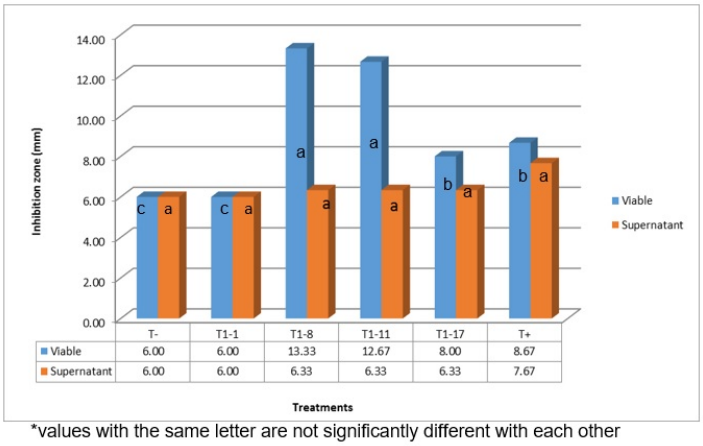
experiment in this study before validation of their identity via the molecular method. Since colonial morphology was not part of the project, random picking of colonies was done instead (Table 1).

Table 1: Gram's staining and catalase test result of the selected colonies

Code No.	Gram's Stain	Catalase Test	Code No.	Gram's Stain	Catalase Test
TI-1	(+)	(-)	**TI-10**	(-)	(+)
TI-2	(-)	(+)	**TI-11**	(+)	(-)
TI-3	(-)	(+)	**TI-12**	(+)	(+)
TI-4	(-)	(+)	**TI-13**	(-)	(-)
TI-5	(-)	(-)	**TI-14**	(-)	(+)
TI-6	(-)	(+)	**TI-15**	(-)	(+)
TI-7	(-)	(+)	**TI-16**	(-)	(-)
TI-8	(+)	(-)	**TI-17**	(+)	(-)
TI-9	(-)	(+)	**TI-18**	(+)	(-)

3.2. Inhibitory Activity of the LAB isolates against E. coli ATCC 25422

The mean zones of inhibition produced around the agar well applied with 30 µL of the broth cultures containing viable LAB cells and LAB supernatants, respectively, are shown in Figure 1a. Of the five LAB isolates, only four were tested for inhibitory activity due to the difficulty in maintaining cell culture of T1-18. Among the four isolates in the viable cell cultures, TI-8 obtained the highest inhibitory mean of 13.33 mm followed by TI-11 (12.67 mm), tetracycline (8.667 mm) and TI-17 (8.00 mm). Analysis of variance (ANOVA) revealed that these were significantly higher than the distilled water (negative control) and TI-1. In the cell- free supernatant, the zones of inhibition were relatively smaller in diameter as compared to that in viable cell culture, with 6.333 for TI-8, TI-11 and TI-17 and 7.667 for tetracycline. ANOVA revealed no significant difference among the mean zones of inhibition at 95% confidence level.



*values with the same letter are not significantly different with each other

Figure 1: Antagonistic effect of viable cell cultures and supernatants of LAB isolates against *E. coli* ATCC 25922

3.3. Inhibitory Activity of the LAB isolates against S. typhimurium BIOTECH 1826

As shown in Figure 1b, among the four viable cell culture isolates, TI-11 obtained the highest mean (11.00 mm) followed by TI-17 (10.33 mm) and TI-8 (10.00 mm). They are significantly lower than the inhibitory activity of tetracycline but significantly higher than distilled water (6.00 mm). TI-1 was comparable with the negative control, signifying the absence of inhibitory activity against the strain of *S. typhimurium*.

In the cell-free supernatants, TI-8 obtained the highest inhibition activity (9.667), followed by TI-11 (9.333) and TI-17 (9.000). They are comparable to each other, however, significantly different with the positive and negative controls. Significant differences among the means produced by the viable cell culture and cell-free supernatants were observed. The results imply that T1-8, T1-11, and T1-17 are potential pathogen antagonists.

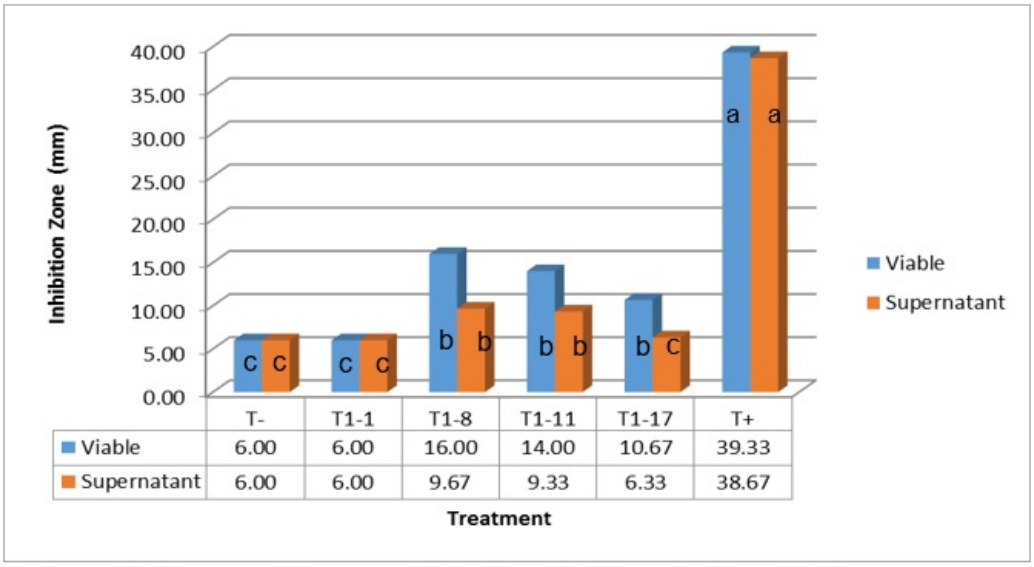


Figure 2: Antagonistic effect of viable cell cultures and supernatants of LAB isolates against

S. typhimurium BIOTECH 1826

3.4. Inhibitory Activity of the LAB isolates against S. enteriditis BIOTECH 1963

There was no significant difference among the inhibitory zones of the viable LAB culture isolates TI-11 (7.667 mm), TI-8 (7.33) and TI-11 (7.33) as presented in Figure 1c. Although they were significantly lower than tetracycline, they were significantly higher than the negative control, signifying inhibitory potential against *S. enteriditis* BIOTECH 1963.

Among the cell-free supernatant cultures, T1-8 (9.333 mm) and T1-11 (9.000 mm) were comparable with each other. They were significantly lower than the T+, but significantly higher than the negative control (6.000 mm) and T1 (6.000 mm).

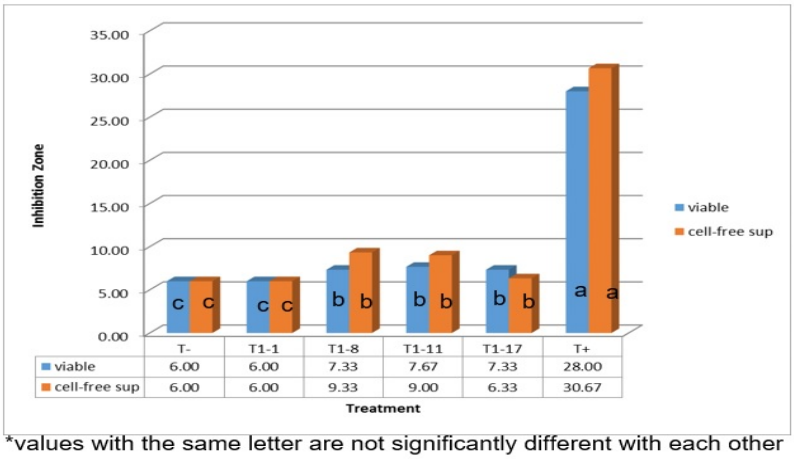


Figure 3: Antagonistic effect of viable cell cultures and supernatants of LAB isolates against*S. enteriditis* BIOTECH 1963.

3.5. Inhibitory Activity of the LAB isolates against V. cholerae BIOTECH 1967

Viable LAB cell cultures and cell free supernatant cultures of TI-8, T1-11 and T1-17 did not significantly differ as to their inhibitory activity as presented in Figure 1d. Though they were significantly lower than tetracycline, they were significantly higher than the negative control and T1-1.

The relatively great difference between tetracycline and the isolates may be because the compounds responsible for the inhibitory potential from each isolate are not yet properly isolated or purified.

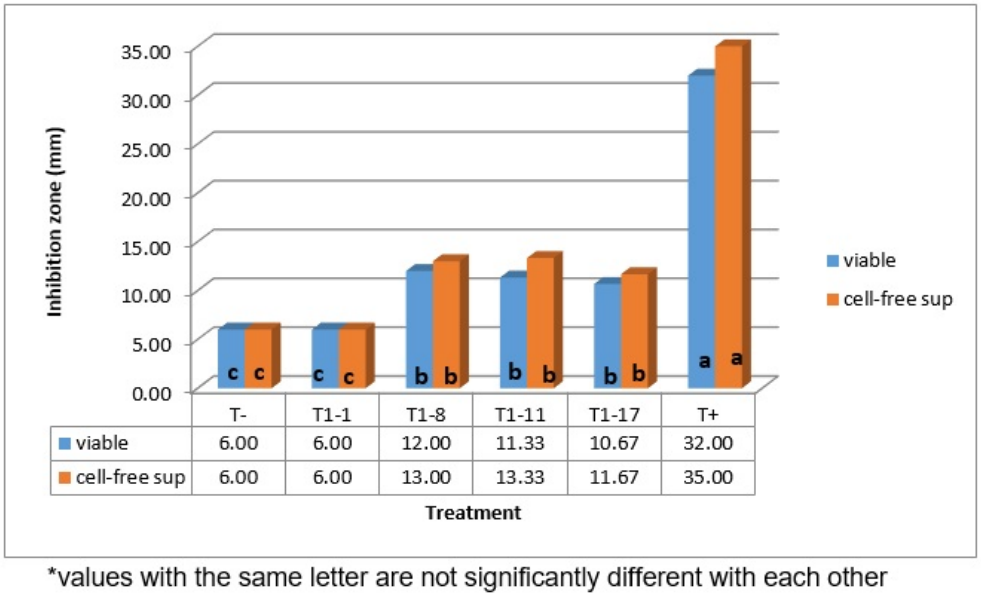


Figure 4: Antagonistic effect of viable cell cultures and supernatants of LAB isolates against *V. cholerae* BIOTECH 1967

3.6. Inhibitory Activity of the LAB isolates against P. aeruginosa BIOTECH 133

Among the four isolates, TI-11 obtained the highest mean (13.33 mm) followed by TI-17 (12.67 mm) and TI-8 (10.33 mm) as shown in Figure 1e. TI-1 did not display any sign of inhibition against the strain of *P. aeruginosa*. Signifying the absence of inhibitory activity against the strain *of P. aeruginosa*. The tetracycline produced 16.67 mm zone of inhibition which is significantly higher than the isolates. However, the inhibition difference is not relatively large which suggests that isolates TI-8, and TI-11 elicited strong inhibitory activity against *P. aeruginosa*. In the cell free supernatant, TI-8 obtained the highest inhibition with the mean of 13.33, followed by TI-11 with a mean of 12.00.

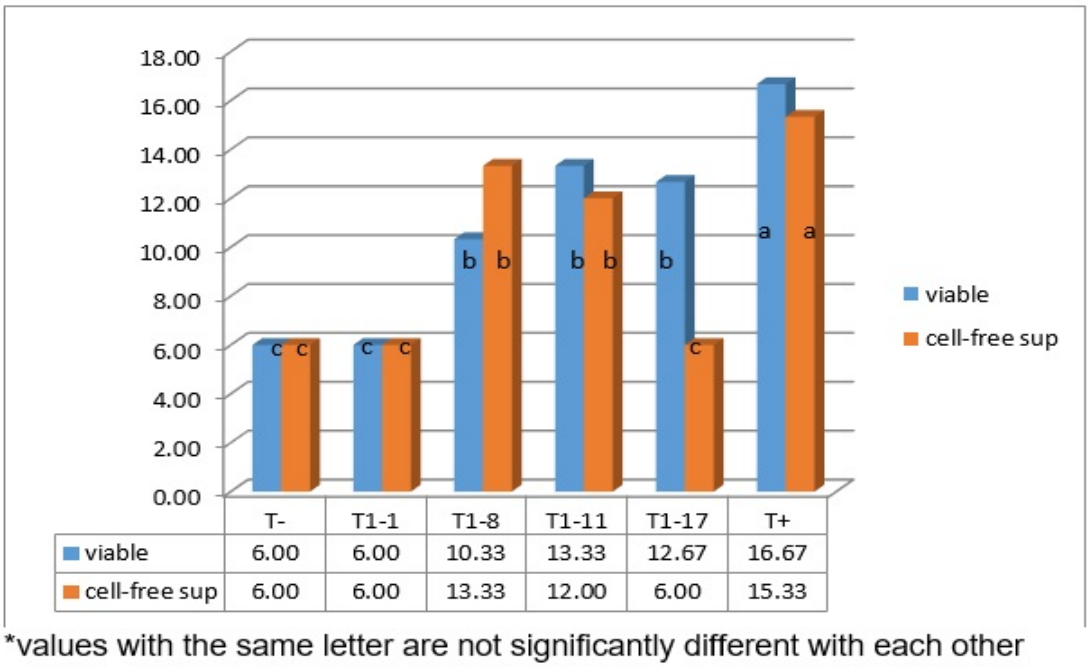


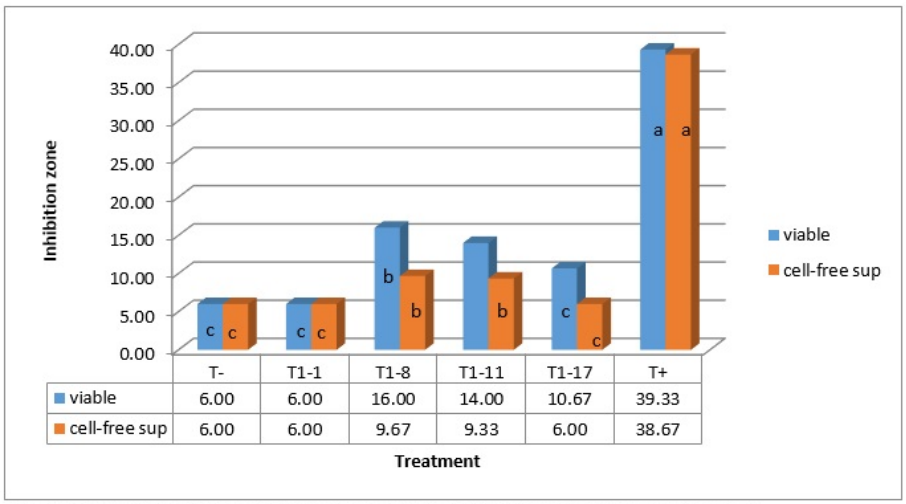
Figure 5: Antagonistic effect of viable cell cultures and supernatants of LAB isolates against *P. aeruginosa BIOTECH 133*

3.7. Inhibitory Activity of the LAB isolates against S. aureus BIOTECH 1582

As presented in Figure 1f, the viable cell cultures of TI-8 and TI-11 have comparable means of inhibition but were found to be significantly lower than the positive control but higher than TI-17. No significant difference was observed among TI-1 and negative control.

In the cell- free supernatant cultures, the same trend with the viable cell culture was observed, however, T1-17 was comparable with T- and T1, signifying no inhibitory activity against the strain of *S. aureus.*

This relatively great difference between the positive control and the isolates may be because the compounds rsponsible for the inhibitory potential from each isolate are not yet completely isolated or purified.



	T-	T1-1	T1-8	T1-11	T1-17	T+
viable	6.00	6.00	16.00	14.00	10.67	39.33
cell-free sup	6.00	6.00	9.67	9.33	6.00	38.67

*values with the same letter are not significantly different with each other

Figure 6: Antagonistic effect of viable cell cultures and supernatants of LAB isolates against S. aureus BIOTECH 1582

There were varying results observed on the inhibitory activity of lactic acid bacterial isolates against the test organisms, suggesting that the isolates have selective inhibitory potential. In fact, bacteriocin, an inhibitory protein synthesized by a bacterium *E. coli*, which is a part of the normal microbiota of the gastro-intestinal tract of human and other endotherms has a relatively narrow bactericidal spectrum which is a beneficial characteristic if the isolates will be tested as probiotic supplement [14,15].

Moreover, bacteriocins produced by Gram-positive bacteria, seldom inhibit commonly encountered enteropathogenic bacteria [16] and are usually inactive against most Gram-negative bacteria due to their outer membrane components that interfere the entry of certain hydrophobic solutes and macromolecules [17]. As normal flora of the gut, *E. coli* has many beneficial effects, such as vitamin K synthesis and inhibition of harmful bacteria from being established in the intestine via bacteriocin production and through other known mechanisms[17].

Several studies revealed that most lactic acid bacteria have inhibitory properties against several enteric pathogens, i.e, *Salmonella spp.* and *E. coli*, which may be accounted to the metabolites they produce such as enzymes, organic acids, bacteriocin and other natural antibiotics [1] (Sharma, 2011). Rauta and his colleagues [9] demonstrated that *Lactobacillus delbrueckii* has high inhibitory effect against *Proteus vulgaris, Bacillus subtilis, Pseudomonas aeruginosa, E. coli* and *Klebsiella pneumonia*. Myllyluoma and his colleagues [18] also showed that *Lactobacillus rhamnosus* inhibits growth of *H. pylori*, the causative agent of gastric ulcer. Similar undertaking by Lawalata and his colleagues [19] revealed that *Pediococcus acidilactici*, isolated from an Indonesian traditional fermented fish product, exhibits a strong activity against pathogenic bacteria. Moreover, two studies showed that lactic acid bacteria from the intestines of the Nile tilapia *(O. niloticus) such as Enterococcus faecium*, *Leuconostoc mesenteroides*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, and *Enterococcus durans* possess antibacterial activity against fish pathogens [11].

3.8. Antibiotic Susceptibility of the LAB Isolates

The susceptibility of the lactic acid bacterial isolates was tested against selected antibiotics.

As presented in Table 2, TI-1 was found to be susceptible to tetracycline and amoxicillin with means of 19.33 and 27.33 mm, respectively and resistant to the remaining test antibiotics.

The LAB isolate TI-8 was found to have an intermediate susceptibility to tetracycline with a mean of 12.22, susceptible to both amoxicillin and cephalexin with means of 36.33 and 20.33, respectively, and resistant to ciprofloxacin, cefixime and chloramphenicol with their respective mean inhibition of 8.67, 8.33 and 6.00 mm.

Moreover, TI-11 was found to have intermediate susceptibility to tetracycline (14.00 mm), susceptible to amoxicillin (35.33 mm) and cephalexin (22.00 mm), and resistant to ciprofloxacin, cefixime and chloramphenicol. Lastly, TI-17 was found to be resistant to tetracycline, cephalexin, cefixime and chloramphenicol, whereas intermediate to ciprofloxacin (16.67 mm) and susceptible to amoxicillin with a mean of 30.33. The results indicate that Tetracycline and Amoxicillin are effective against the cultures T1-1; Amoxicillin and Cephalexin are effective against the cultures T1-8 and T1-11 and Amoxicillin is effective against T1-17 culture.

Table 2: Susceptibility of LAB isolates against selected antibiotics

	Mean Inhibition Zones (mm)			
Treatments	**TI-1**	**TI-8**	**TI-11**	**TI-17**
Tetracycline	**19.33 S**	12.33 I	14.00 I	7.33 R
Amoxicillin	**27.33 S**	**36.33 S**	**35.33 S**	**30.33 S**
Cephalexin	6.00 R	**20.33 S**	**22.00 S**	6.00 R
Ciprofloxacin	6.00 R	8.67 R	10.00 R	16.67 I
Cefixime	6.00 R	8.33 R	6.00 R	6.00 R
Chloramphenicol	6.00 R	6.00 R	6.00 R	6.00 R

TI – Tilapia Intestine; Reference standard by CLSI[20]*, 2014*

	Susceptible (S)	*Intermediate (I)*	*Resistant (R)*
Tetracycline (30µg)	*≥ 15*	*12-14*	*≤ 11*
Amoxicillin (10µg)	*≥ 17*	*14-16*	*≤ 13*
Cephalexin (30µg)	*≥ 18*	*15-17*	*≤ 14*
Ciprofloxacin (5µg)	*≥ 21*	*16-20*	*≤ 15*
Cefixime (5µg)	*≥ 19*	*16-18*	*≤ 15*
Chloramphenicol (30µg)	*≥ 18*	*13-17*	*≤ 12*

3.9. Molecular Identification of the LAB Isolates

The amplicon of the target gene in the three isolates is indicated by bright bands (Figure 2). Isolate TI-1 was not included in DNA extraction due to difficulty in maintaining the culture. The isolate with code TI-1 did not yield amplicon, thus, only TI-8, TI-11, and TI-17 were subjected to sequencing.

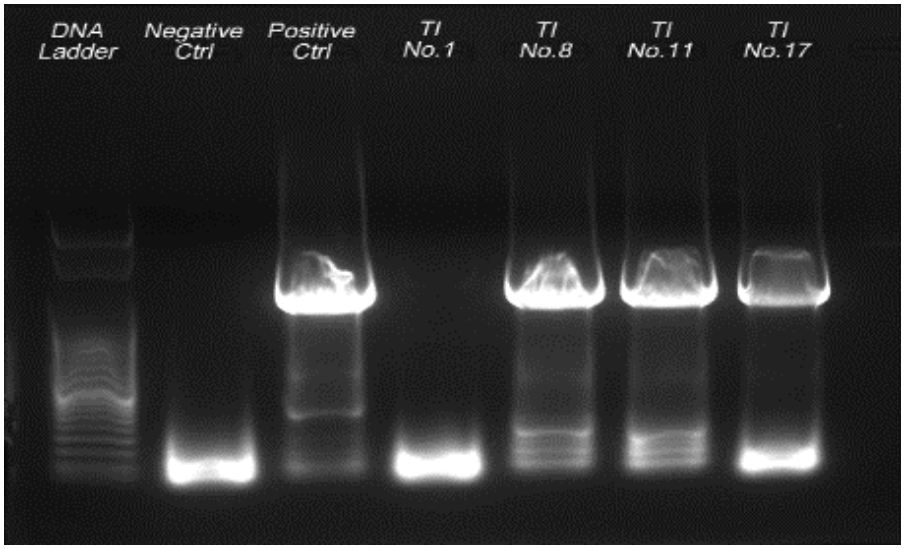


Figure 7: Amplicons of the target gene from the lactic acid bacterial isolates

Sequence analysis of the 16S rRNA gene (Figure 3a-c) revealed that TI-8 and TI-11 are both *Pediococcus pentosaceus* with an identity of 99% suggesting that they are different strains. Further, TI-17 was revealed to be *Enterococcus avium* with an identity of 99%.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pediococcus pentosaceus strain 43 16S ribosomal RNA gene, partial sequence	887	887	61%	0.0	99%	KP181624.1
Pediococcus pentosaceus strain BSS1375 16S ribosomal RNA gene, partial sequence	887	1405	100%	0.0	99%	KT351726.1
Pediococcus pentosaceus strain BSS1305 16S ribosomal RNA gene, partial sequence	887	1398	100%	0.0	99%	KT351725.1
Pediococcus pentosaceus strain BSS1109 16S ribosomal RNA gene, partial sequence	887	1405	100%	0.0	99%	KT351721.1

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pediococcus pentosaceus strain 43 16S ribosomal RNA gene, partial sequence	887	887	61%	0.0	99%	KP181624.1
Pediococcus pentosaceus strain BSS1375 16S ribosomal RNA gene, partial sequence	887	1405	100%	0.0	99%	KT351726.1
Pediococcus pentosaceus strain BSS1305 16S ribosomal RNA gene, partial sequence	887	1398	100%	0.0	99%	KT351725.1
Pediococcus pentosaceus strain BSS1109 16S ribosomal RNA gene, partial sequence	887	1405	100%	0.0	99%	KT351721.1

Figure 8: BLAST hits of the query TI-8 showing similar descriptions to *P. pentosaceus* BSS1375

Description	Max score	Total score	Query cover	E value	Ident	Accession
Pediococcus pentosaceus strain 4I1 16S ribosomal RNA gene, partial sequence	970	1409	97%	0.0	99%	KT372700.1
Pediococcus pentosaceus gene for 16S ribosomal RNA, partial sequence, strain: ZZU 64	970	1437	99%	0.0	99%	AB831184.1
Pediococcus pentosaceus gene for 16S ribosomal RNA, partial sequence, isolate: qz-201	970	1426	99%	0.0	99%	AB904769.1
Pediococcus pentosaceus strain DSPV 029SA 16S ribosomal RNA gene, partial sequence	970	1437	99%	0.0	99%	JQ322223.1

Figure 9: BLAST hits of the query TI11 showing similar descriptions to *P. pentosaceus* ZZU64

Description	Max score	Total score	Query cover	E value	Ident	Accession
Enterococcus pallens strain LSPQ-04156 16S ribosomal RNA gene, partial sequence	904	1435	99%	0.0	99%	KP793152.1
Enterococcus avium strain IN 3633 16S ribosomal RNA gene, partial sequence	904	1522	100%	0.0	99%	KC715829.1
Enterococcus sp. 2011_Ileo_MS_D3 16S ribosomal RNA gene, partial sequence	904	1512	100%	0.0	99%	JQ680075.1
Enterococcus sp. 2011_Ileo_MS_C7 16S ribosomal RNA gene, partial sequence	904	1505	98%	0.0	99%	JQ680068.1

Figure 10: BLAST hits of the query TI17 showing similar descriptions to *E. avium* IN3633

3.10. Phylogenetic Tree of the Isolates using Clustal Omega Neighbor-Joining Algorithms

Comparison of the partial 16S rRNA of the *O. niloticus* intestine associated LAB isolates *Pediococcus pentosaceus* (TI-8), *Pediococcus pentosaceus* (TI-11) and *Enterococcus avium* (TI-17) with the existing LAB sequence data from Genbank showed that all strains lay in the same clade. The three strains had a 99% identity with known *Pediococcus pentosaceus* and *Enterococcus avium*.

A phylogenetic tree of these LAB isolates was built using the Clustal Omega neighbor-joining algorithms without distance correction using different sequences from the first two BLAST query results. It can be observed that the two *Pediococcus pentosaceus* isolated from *O.*

niloticus were closely related with their common ancestor. The values indicate the dissimilarity of the isolates in which the higher the score, the lower the similarity. Moreover, the *Enterococcus avium (*TI-17) is a close relative of the former as compared to the other *Enterococcus sp.* This can be assumed that the relatedness might be due to the environment that the ancestor of the isolates inhabited, and that other *Enterococcus sp.* diverged differently because of the environment from different locale where they were isolated.

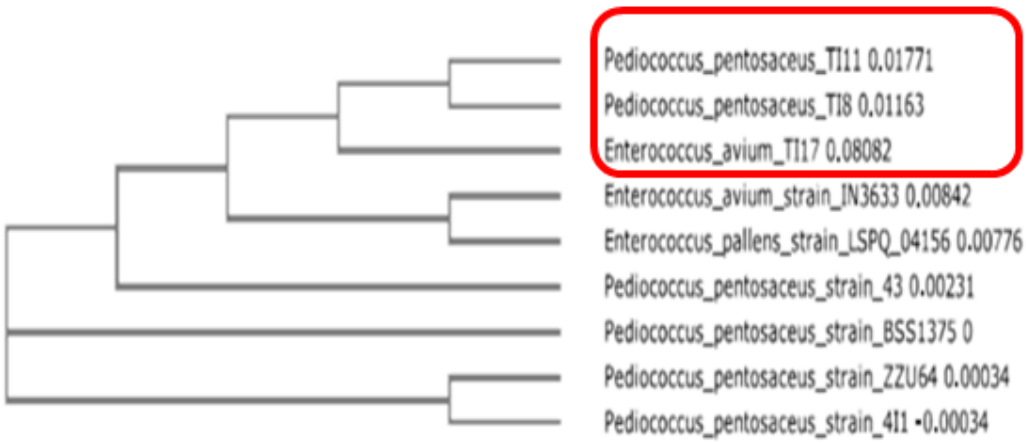


Figure 11: Phylogenetic neighbor-joining tree of the isolates based on partial 16S rRNA gene sequence in comparison with the existing NCBI database

4. Summary and Conclusion

The isolation of the three Lactic Acid Bacteria from the Nile Tilapia (Oreochromis niloticus) is a welcome development in the drug industry, mainly as possible sources of bacteriocin, to fight against human pathogens.

The two strains of *Pediococcus pentosaceus* and *Enterococcus avium* were found to be of potential source of antagonistic substances against some common human pathogens.

Among the isolates, TI-8 and T1-11 (*P. pentosaceus*) viable cell cultures were found to have the highest inhibitory activity against *E. coli ATCC 25922*, surpassing that of the positive control, Tetracyline, though showing significant inhibition also to *P. aeruginosa* BIOTECH 1335, *S. aureus* BIOTECH 1582, *S. enteriditis* BIOTECH 1963, *S. typhimurium* BIOTECH 1826, and *V. cholerae* BIOTECH 1967, T1-17 (*Enterococcus avium)* is not effective against *Staphylococcus aureus* BIOTECH 1582.

On the other hand, T1-8 and T1-11 (*P. pentosaceus*) cell free supernatant cultures exhibited the inhibitory activity against *P. aeruginosa* BIOTECH 1335, *S. aureus* BIOTECH 1582, *S. enteriditis* BIOTECH 1963, *S. typhimurium* BIOTECH 1826, and *V. cholerae* BIOTECH 1967. The LAB isolate T1-17 (*E. avium)* exhibited the inhibitory activity against *S. enteriditis* BIOTECH and *V. cholerae* BIOTECH 1967.

LAB isolates T1-8, T1-11, and T1-17 were susceptible to Amoxicillin, whereas LAB isolates T1-8 and T1-11 were susceptible to Cephalexin and possess intermediate susceptibility to Tetracycline. T1-17 had an intermediate susceptibility to Ciprofloxacin

5. Recommendation

The result is a research breakthrough that need further verification on the following:

a. Protein and proteinase treatment of the cell free supernatants should be undertaken to validate further that the inhibitory activity is caused by the protein of peptide;
b. The gene responsible for the production of the anti-biotic protein or peptide be isolated and determined; and
c. The isolates should also be tested to other pathogens aside from bacteria.

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