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Screening Lactic Acid Bacteria from locally fermented products for probiotic potential

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Abstract

Lactic acid bacteria (LAB) from fermented coconut sap and sugar cane were characterized and screened for probiotic potential. A total of 5 species of LAB from these local products were identified and characterized. These are Lactobacillus brevis, Lactobacillus collinoides, Lactobacillus fermentum, Lactobacillus plantarum and Lactobacillus pentosus. Six prototypes of probiotic cocktails were formulated with varying combinations. These prototypes were assayed for acid tolerance, bile resistance and antibacterial activity. All probiotic cocktails were acid tolerant with pH ranging from 2 to 4. Growth of LAB at pH 2 was significantly slower than those at pH 3 and 4 ($p<0.001$). LAB in all the cocktails were bile resistant but growth was significantly reduced ($p<0.001$). Test for antimicrobial potential revealed that two probiotic cocktails composed of L. collinoides, L. plantarum, and L. fermentum inhibited all the test bacteria ($p=0.00$). Overall, cocktails composed of L. collinoides, L. fermentum and L. plantarum were acid tolerant, bile resistant and exhibited inhibitory potential against the test bacteria. These cocktails can be further assayed to confirm their probiotic potential.

Keywords: Probiotics, Lactic Acid Bacteria (LAB), antibacterial activity, fermented food, probiotics.

Introduction

Lactic acid bacteria (LAB) are gram-positive, catalase-negative anaerobic and non-spore forming bacteria which may range from aerotolerant to strictly anaerobic. They can either be bacillus (rod-shaped) or coccus (round)[1]. Commonly, these belong to the genus *Lactobacillus*. LAB have been found to exhibit probiotic properties, and are one of the most common components of probiotics today. These probiotics are preparations of products containing microorganisms, which target the specific microflora of the consumers, giving health benefits[2]. Probiotic LAB can be found as part of the natural flora of foods that ferment spontaneously, which include dairy products[3]. However, due to the prevalence of lactose intolerance, popularity of probiotics has shifted from common dairy sources to non-dairy sources which can be any fermented food like vegetables and fruits[4-7].

Local fermented products in the Philippines have not been widely screened for probiotic potentials. As such, the study would screen LAB that will be isolated from local fermented products like coconut sap (*tuba*) and sugar cane (*basi)* for probiotic properties. Most probiotics are lactose-based while this study explores the non-dairy sources of probiotics. Sugarcane and Coconut sap are readily consumed fermented products in the local communities.

A good probiotic should survive well in the gastrointestinal tract. This is an important criterion because as a food supplement, probiotics are ingested before it reaches its specific target cell. This survivability includes adhesion to the intestinal cells for competition with other organisms, acid resistance for the gastric juice produced by the stomach, and tolerance to bile in the small intestine[8]. The use of probiotics instead of antibiotics or to supplement antibiotics has a great potential to address the problem of the spread of antibiotic resistance.

The study aims to screen lactic acid bacteria (LAB) isolated from local fermented products for probiotic properties. Specifically, it aims to identify isolated LAB using morphological and biochemical tests, formulate probiotic cocktail prototypes, and determine probiotic potential through acid tolerance test, bile resistance test and antimicrobial activity. The study will have a significant contribution towards the standardization of formulating an effective probiotic cocktail. These formulated probiotic cocktails will have the potential to be used as cheaper alternatives to commercial probiotic products such as probiotic drinks, making probiotics more available and accessible in the Philippines. Furthermore, it will also serve as baseline information for future research on LAB consortium and probiotics.

Methodology

Collection and Preparation Phase: A day-old fermented coconut sap *(tuba)* and sugarcane juice *(basi)* were used as the local products. For each product, a 500mL composite was formulated. The composite was obtained by mixing 5 100mL containers of each product, coming from 5 different sources. The composites served as the samples in the study, along with a

500-mL composite of commercial probiotic product, which served as control.

Nutrient agar, deMan, Rogosa and Sharpe (MRS) agar and MRS broth were prepared by mixing agar powder and broth powder in boiling distilled water until components were fully dissolved. The mixtures were then autoclaved for sterilization at 121°C, 103.42kPa for 15 min. Preparation of nutrient agar was done by dispensing 20mL of agar on sterile petri dishes. For broths, 10 mL of broth was poured in 20mL sterile test tubes.

Isolation and Identification: The samples were serially diluted with distilled water (1:9 v/v) up to a concentration of 10^{-8}. Each diluted sample was streaked on nutrient agar and was incubated anaerobically at 37°C for 24h. All distinct colonies were isolated by repetitive plating on nutrient agar until pure cultures were obtained.

The morphology of the colonies from each culture was examined. Colony color, form, margin, and elevation were identified. Gram-staining was done. Gram-stained specimens were examined under the microscope using oil immersion objective (OIO). Purple-stained bacteria were identified as gram-positive, while pink-stained bacteria were identified as gram-negative. Its bacterial shape was also determined through the use of Bergey's Manual of Determinative Bacteriology[9]. Lastly, endospore staining was performed. The presence of green round spores indicated the presence of spores, thus a positive test.

Catalase test was performed. For catalase test, effervescence after inoculating a loop of bacteria in hydrogen peroxide indicated the presence of catalase, a positive test. Only the gram-positive, catalase-negative and non-endospore forming rods and cocci cultures were kept. The cultures were transferred on de Man, Rogosa and Sharpe (MRS) agar plates. Colonies which grew on MRS agar were presumptively identified as LAB and were further identified using API 50 CHL fermentation assay kits (BIOMERIEUX SA, France). These were then kept on MRS agar slants at 4°C until further use, while sub-culturing on a weekly basis were also done on MRS plates.

Formulation of Probiotic Cocktail and Characterization of Probiotic Cocktail: A method described by Mnif et al.[10] was modified in formulating a probiotic cocktail. Three probiotic cocktail prototypes were formulated per sample. A prototype consisted of a unique combination of LAB isolated from the samples, initially cultured on MRS broth. Its optical density (measured using Thermo Fisher Scientific Model Genesys 10-s) at 600nm (OD) was adjusted to 0.2, to standardize the amount of LAB to be used. The broths of the desired LAB components for the prototype were then mixed, in equal amounts in a separate test tube, creating 1 prototype of probiotic cocktail.

For acid tolerance, the pH level of MRS broth in a test tube was measured, which served as control. MRS broth of different pH levels were prepared in different test tubes, ranging from pH 1.0-4.0, at intervals of 1. This was done by adding 1N HCl in the tubes, dropwise. 1mL of probiotic cocktail was then pipetted into each test tube. The initial optical density of each test tube at 600nm was measured. The tubes were then incubated at 37°C for 24 hours. After 3 hours of incubation, the optical density was measured, followed by another measurement after 24 hours. A change in optical density between 3 and 24 hours indicated acid tolerance. This was done in 30 replicates. Optical densities were compared using T-test (for significant difference between 2 groups) and One-way Analysis of Variance (ANOVA) with Tukey post hoc test (for significant differences between 3 or more groups).

For bile salt resistance, two test tubes containing 1mL of probiotic cocktail were prepared. The initial optical density of each test tube, at 600nm, was measured. One (1) gram of oxgall-bile was added to one tube, while the other remained as is. The tubes were then incubated at 37°C for 24 hours. After 3 hours of incubation, the optical density of each tube was measured, followed by another measurement after 24 hours. A change in optical density between 3 and 24 hours indicated bile resistance. This was done in 30 replicates. Optical densities between 2 groups were compared using T-test.

For antibacterial activity, prepared antibiotic disks of erythromycin, ticarcillin and meropenem were obtained from a local supplier. Disks from probiotic cocktail were prepared by dipping 6-mm diameter chipboard to 20µL of probiotic cocktail. Bacterial strains subjected to testing were *Escherichia coli, Pseudomonas aeruginosa* and *Staphylococcus aureus*. These strains were cultured on Mueller-Hinton agar plates. Antibiotic and probiotic disks were scattered on the plates, which were then incubated for 24 hours at 37°C. The zone of inhibition of each disk was then measured. Each was done in 30 replicates. The zones of inhibition were compared using one-way analysis of variance (ANOVA) with Tukey post hoc test.

Results and discussion

Isolation and Identification of LAB: Figure-1 shows the typical characteristics of LAB isolated from fermented products. LAB are gram positive and endospore forming rods. A total of 10 distinct colonies were isolated from fermented coconut sap *(tuba)* coded as CS; six (6) distinct colonies were isolated from fermented sugarcane juice *(basi)* coded as SJ, while only 1 distinct colony was isolated from the commercial probiotic product coded as Y.

Table-1 summarizes the colonial and morphological characteristics of the isolated bacteria, while Table-2 summarizes its biochemical test results. Based on the tests performed, isolates CS 3, CS 4, CS 8, CS 10, SJ 1, SJ 3, SJ 4, and Y 1 were presumptively identified as LAB. LAB colonies are generally classified as: small colonies, ranging from beige to white in color, circular to irregular in shape, elevation may be flat, raised, or convex and usually they have entire margins[1].

Other morphological characteristics of LAB include being gram-positive and non-endospore forming rods or cocci[1]. As gram positive bacteria, they possess a thick peptidogly can cell wall which retain the crystal violet stain and have no outer membrane, as opposed to gram-negative bacteria[11]. According to Sharma and Mishra[12], gram-positive bacteria are less pathogenic compared to gram-negative bacteria, making them more suitable as potential probiotics. As non-endospore forming, LAB do not form spores when subjected to environmental stresses, making them sensitive to environmental changes, which may be one reason why they are difficult to culture.

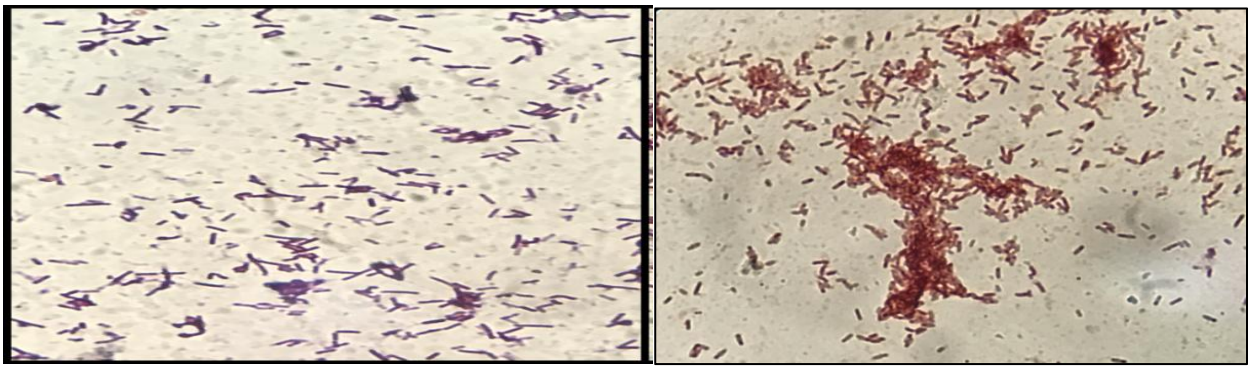

Figure-1: Typical Morphological Characteristics of (Gram-positive (L) and non-endospore forming (R) rods).

Table-1: Colonial and morphological characteristics of isolated bacteria.

Isolate Code	Colony				Bacterial		
	Color	Form	Elevation	Margin	Shape	Gram Staining	Endospore Staining
CS 1	White	Irregular	Flat	Undulate	Rod	-	-
CS 2	Off White	Circular	Raised	Undulate	Rod	-	-
CS 3	Off White	Circular	Raised	Undulate	Rod	+	-
CS 4	Off White	Circular	Raised	Entire	Rod	+	-
CS 5	Off White	Filamentous	Lobate	Lobate	Rod	-	-
CS 6	White	Circular	Irregular	Irregular	Rod	-	-
CS 7	Orange	Circular	Raised	Entire	Coccus	-	-
CS 8	Off White	Circular	Raised	Undulate	Rod	+	-
CS 9	Yellow	Circular	Entire	Entire	Coccus	-	-
CS 10	White	Circular	Raised	Entire	Rod	+	-
SJ 1	Off White	Circular	Raised	Entire	Rod	+	-
SJ 2	Orange	Circular	Raised	Entire	Coccus	-	-
SJ 3	Off White	Circular	Raised	Entire	Rod	+	-
SJ 4	Off White	Circular	Raised	Undulate	Rod	+	-
SJ 5	Orange	Filamentous	Raised	Filiform	Coccus	-	-
SJ 6	Off White	Irregular	Umbonate	Undulate	Rod	-	-
Y 1	Off White	Circular	Raised	Entire	Rod	+	-

Table-2 shows the identified species of LAB with the use of API 50 CHL kit. The % ID and remarks are based on the closeness of the isolated strains to the identified species and purity of the isolated strains respectively. A total of 5 species of lactic acid bacteria belonging to the genus *Lactobacillus* were identified from both local fermented products. Two (2) species were unique to fermented coconut sap namely: *Lactobacillus brevis* and *Lactobacillus pentosus*. Only 1 species was unique to fermented sugarcane juice which was *Lactobacillus fermentum*, while 2 species were common to both products which were: *Lactobacillus plantarum* and *Lactobacillus collinoides*. From the commercial probiotic product, only 1 species of LAB was identified, which was *Lactobacillus delbrueckii ssp. bulgaricus.*

Formulation of Probiotic Cocktails: A total of 7 probiotic cocktails consisting of LAB consortium were formulated. 3 of these were derived from LAB isolated from the fermented coconut sap sample (labeled as "PC"); the other 3 cocktails were derived from LAB isolated from the fermented sugarcane juice sample (labeled as "PS"), while 1 cocktail was formulated from LAB isolated from the commercial probiotic product (labeled as "PY"). The list of formulated cocktails is shown in Table-3.

These cocktails contain unique LAB consortium combinations. According to Wasilewska[13], bacterial components in a consortium may enhance one another, allowing more efficient growth and metabolic processing to occur. LAB cocktails were arbitrarily prepared since there is no known standard in formulating probiotics. Formulating probiotics from LAB consortia has been a trend in current research studies.

Table-2: API 50 CHL Biochemical Identification.

Isolate code	Species Identification	% ID	Remarks
CS 3	Lactobacillus brevis1	84.5%	Acceptable Identification
CS 4	*Lactobacillus pentosus*	99.9%	Excellent Identification
CS 8	*Lactobacillus collinoides*	99.9%	Good Identification
CS 10	*Lactobacillus plantarum 1*	99.9%	Acceptable Identification
SJ 1	*Lactobacillus fermentum 2*	98.2%	Good Identification
SJ 3	*Lactobacillus plantarum 2*	99.1%	Very good identification
SJ 4	*Lactobacillus collinoides*	99.7%	Very good identification
Y 1	*Lactobacillus delbrueckii ssp bulgaricus*	99.7%	Very good identification

Table-3: LAB prototypes of probiotic cocktails.

Cocktail code	LAB present
PC 1	L. brevis 1, L. pentosus, L. plantarum 1
PC 2	L. brevis 1, L. plantarum 1
PC 3	L. collinoides, L. pentosus, L. brevis 1, L. plantarum
PS 1	L. collinoides, L. plantarum 2
PS 2	L. collinoides, L. fermetum
PS 3	*L. collinoides, L. plantarum 2, L. fermentum*
PY 1	L. delbrueckii ssp. bulgaricus

Acid Tolerance of Probiotic Cocktails: For all probiotic cocktails, no significant difference in optical densities was observed at different times of measurement in pH 1. For pH 2, pH 3 and pH 4, there were significant differences between the optical densities measured after 3 and 24 hours for each pH level (Figure-2). Also, in all cocktails, the optical densities after 24 hours at pH 2 were significantly higher than those of pH 1. For pH 3, 4 and 6.5 (control), the optical densities had no significant difference, while they were significantly higher than those of pH 1 and 2. These statistical data imply that no growth of LAB can be observed in an environment with pH 1. Also, it implies that all probiotic cocktails were acid-tolerant from pH 2 to 4 as they were able to grow after 3 hours. Lastly, it showed that growth of LAB in the cocktail was slower in pH 2, while normal growth resumed at pH 3 and pH 4.

No growth of LAB was observed among the probiotic cocktails at pH 1 since low pH does not only slow down the bacteria's growth, but also cause damage, which ultimately leads to a loss of cell viability. At pH 2, 3 and 4, growth was observed since LAB are acid-tolerant up to a certain extent. Unlike in pH 3 and 4, LAB in pH 2 had slower growth because the environment was still too acidic for normal metabolism and growth to occur[14]. The acid tolerance of LAB can be accounted to its F0F1-ATPase mechanism and cell membrane structure[15,16].

A decrease in extracellular pH would activate the F0F1-ATPase, leading to the production of ATP. ATP then couples with H+ ions, which exits the cell, maintaining homeostasis within the cell despite the acidic environment[15]. Also, Membranes of LAB are impermeable to extracellular protons and lactate molecules produced by the bacteria during fermentation, preventing the cell from being affected ions contributing to acidity[16]. Acid tolerance is an essential characteristic of a probiotic. Since probiotics are meant to be ingested by organisms, gastrointestinal survivability, which includes acid tolerance, is a must[8].

Bile Resistance of Probiotic Cocktails: Results show that the optical densities after 24 hours were significantly higher than the optical densities after 3 hours, for all cocktails (Figure-3). In addition, after 24 hours, the cocktails without bile had significantly higher optical densities than those with bile. These data imply that LAB in the cocktails were bile resistant, as they continued to grow after 3 hours. It also implies that bile caused a slower growth of LAB.

Bile resistance is also an important characteristic of a probiotic. Along with acid tolerance, it indicates gastrointestinal survivability, which is necessary for probiotics as it passes through the consumers' gastrointestinal tract[8]. Some of the most common mechanisms that mediate resistance to bile are active efflux of bile acids/salts, alteration of cell membrane by producing exopolysaccharides for protection and production of bile-salt hydrolases[17-19]. Bile-salt hydrolases (BSHs) are enzymes which catalyze the deconjugation of glycine and taurine links of bile salts, leading to its inactivation[20]. The results obtained were similar to the results of the studies conducted by Njoki et al.[14], Ruiz et al.[20] and Abbas & Mahasneh[21] where in LAB was found to be bile resistant. In fact, their studies showed that it was resistant up to a bile concentration of 1%.

Antibacterial Activity of Probiotic Cocktails: Figure-4 shows the zones of inhibition (ZOI) of the different antibiotic and probiotic disks against *S. aureus, E. coli* and *P. aeruginosa,* respectively. Based on the results, the antibiotic Meropenem showed the highest inhibition against all test bacteria, while antibiotic Erythromycin showed the least inhibition. For the probiotic disks, PS 3 showed the highest ZOI against all test bacteria. PY 1 showed the least inhibition against *S. aureus*and *P. aeruginosa*, while PC 1 showed the least inhibition against *E. coli* (Table-8 for a complete list of the average zones of inhibition)

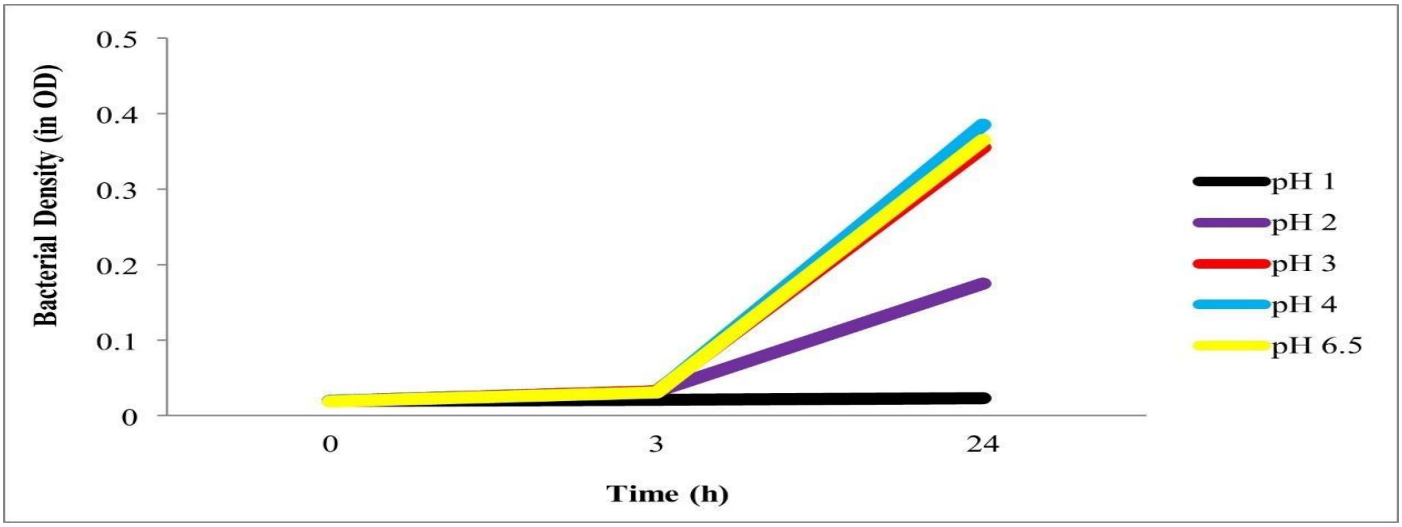

Figure-2: Bacterial growth (measured at OD_{600}) of probiotic cocktails subjected to varying pH.

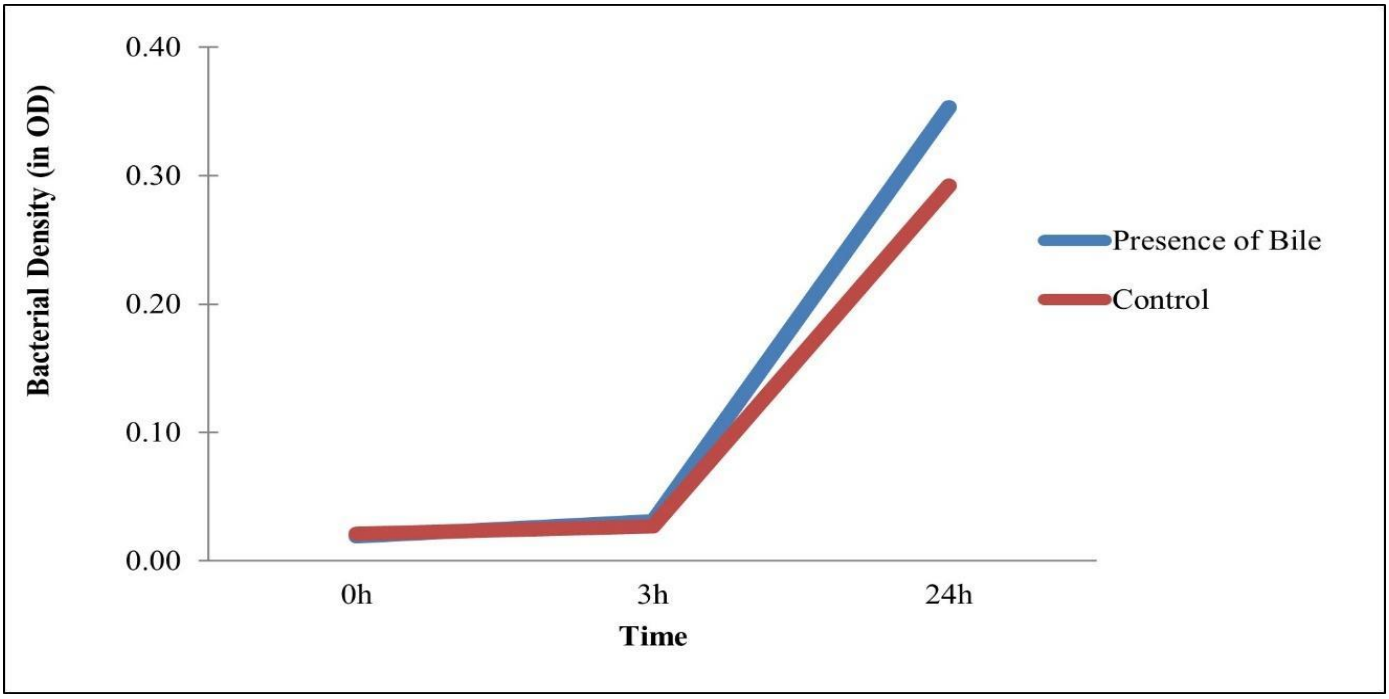

Figure-3: Bacterial density (measured at OD_{600}) of probiotic cocktails exposed to bile.

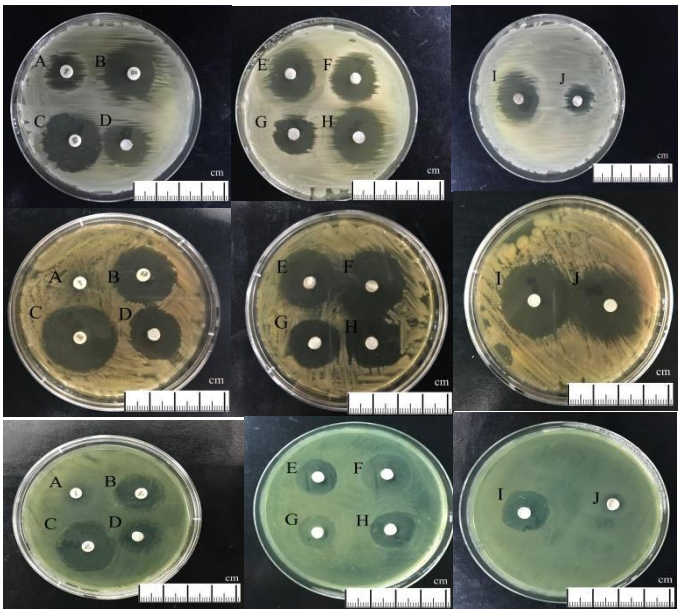

Figure-4: Zone of inhibition of antibiotic and probiotic disks against: (top) *E. coli* (middle) *S. aureus* (bottom) *P. aeruginosa.* (A) Erythromycin (B) Ticarcillin (C) Meropenem (D) PC 1 (E) PC 2 (F) PC 3 (G) PS 1 (H) PS 2 (I) PS 3 (J) PY 1.

All probiotic cocktails showed a larger zone of inhibition against all test bacteria than Erythromycin. This implies that all probiotic cocktails were more effective than Erythromycin in controlling certain pathogenic bacteria. Among the probiotic cocktails, PS 3 showed the largest zone of inhibition against all test bacteria comparable to Meropenem. All other cocktails showed similar zones of inhibition to Ticarcillin except PS 3 showing superior antibacterial activity against all test bacteria (Figure-5). The cocktails used had a good potential for antimicrobial activity against common human pathogens.

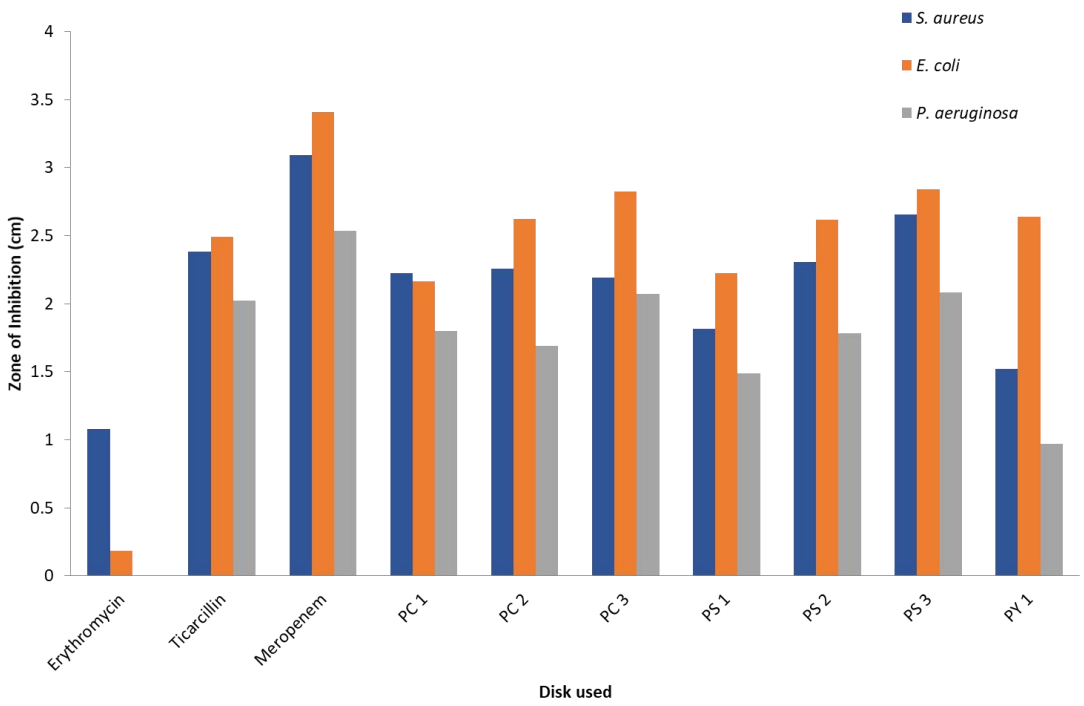

Figure-5: Antibacterial activity of probiotic discs to test bacteria.

Several factors affect the antibacterial activity exhibited by LAB. These include: bacteriocins, bacteriocin-like substances and acid production. Bacteriocins are complex peptides produced by LAB. Different LAB produce different bacteriocins and these bacteriocins have specific antimicrobial activity which may be of broad or narrow range. The difference in the zones of inhibitions of the different cocktails may be accounted to the variation in bacteriocin production[22]. Most bacteriocins have been found to commonly inhibit gram-positive bacteria, while only a few have been tested to be effective against gram-negative bacteria. *L. plantarum* and *L. fermentum* have been widely used as a source of bacteriocins as these species produce many kinds of bacteriocins, which include those that inhibit gram-negative bacteria[23]. This may explain why all cocktails showed a significant degree of inhibition as each cocktail contain either of these species, while PS 3 consistently had the highest degree of inhibition as it contained both. Other gram-positive bacteria may also produce bacteriocins. However, LAB bacteriocins have sparked more interest as they are found in food, and they possess the GRAS status, making them safe for consumption[24]. Bacteriocin-like substances (BLS) are substances which are similar to bacteriocins, but do not qualify as such. These substances have also been found to have antagonistic effects on both gram-positive and gram-negative bacteria[25]. Production of these BLS may also be a factor on the antibacterial activity of the probiotic cocktails. Lastly, acid production by the fermentative nature of LAB also contributed to the antibacterial activity of the probiotic cocktails. LAB metabolized sugars in order to produce organic acids which lower the pH of the growing medium, promoting the production of hydrogen peroxide[26,27]. The alteration of pH made the environment less suitable for the growth of other microorganisms, exhibiting antibacterial activity.

For quantifying probiotic potential, results for each assay were assigned as positive or negative. For antibacterial activity, only those which were susceptible (S) were considered as positive. Table-4 shows the summary of the results of all probiotic characterization assays performed.

Overall, PS 2 and PS 3 had the highest probiotic potential, testing positive for 4 out 5 assays conducted. On the other hand, PC 1 and PS 1 had the lowest probiotic potential. Only *P. aeruginosa* was resistant to all probiotic cocktails. Probiotic cocktails PS 2 and PS 3 had the highest antibacterial activity. These cocktails composed of *L. collinoides, L. plantarum,* and *L. fermentum* (PS 2) and *L. collinoides,* and *L. fermentum* (PS 3). The table above indicates that PS 2 and PS 3 are good probiotic candidates.

This research successfully established the following gaps in the literature: (1) identified LAB from fermented coconut sap and sugarcane, (2) the identified LAB can be grown in pure cultures under laboratory conditions, (3) microbial proportions can be standardized. Considering that there is no known standard for an effective probiotic, this study demonstrated that probiotics from non-lactose sources can be good alternatives of commercially available probiotics that are lactose-based.

Table-4: Summary of probiotic characterization assays.

Probiotic cocktail / disk used	Acid tolerance	Bile resistance	Antibacterial Activity		
			S. aureus	*E. coli*	*P. aeruginosa*
PC 1	+	+	-	-	-
PC 2	+	+	-	+	-
PC 3	+	+	-	+	-
PS 1	+	+	-	-	-
PS 2	+	+	+	+	-
PS 3	+	+	+	+	-
PY 1	+	+	-	+	-

Conclusion

Lactic acid bacteria (LAB) were isolated from local fermented products, *tuba* (fermented coconut sap) and *basi* (fermented sugarcane juice). All LAB identified were gram-positive, catalase-negative and non-endospore forming rods, belonging to the same genus, *Lactobacillus*. A total of 5 species were identified from both samples namely: *L. brevis, L. pentosus, L. collinoides, L. plantarum, and L. fermentum.* From these, 7 probiotic cocktail prototypes were formulated, which consisted of different consortia of LAB except for the commercial probiotic product, wherein a monoculture of *L. deblrueckii ssp. bulgaricus* was used. All probiotic cocktails were acid tolerant from pH 2 to 4. In addition, growth was found to be slower at pH 2, while it was normal at pH 3 and 4. Also, all probiotic cocktails were bile resistant. However, presence of bile showed slower growth of LAB in the probiotic cocktail. For antibacterial activity, PS 3 was the most effective in inhibiting all pathogenic strains. However, it was less effective compared to the antibiotic meropenem. PY 1, which was a monoculture, was the least effective against *S. aureus*and *P. aeruginosa*, while PC 1 was the least effective against *E. coli*. Overall, cocktails PS 2 and PS 3 showed the highest probiotic potential, testing positive for 4 out 5 assays, while PC 1 and PS 1 showed the lowest probiotic potential, testing positive for only 2 assays.

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