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Isolation and Characterization of Some Food Grade Lactic Acid Bacteria for their Application as Probiotics

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ABSTRACT

Probiotics, which are living, non-pathogenic microorganisms, can enhance a person's health, immunity, and mental function when taken in large quantities together with food. In the present study, seven microbial strains (L1, L2, C1, C2, C3, X, and Y) were isolated from locally collected fresh palm sap and characterized morphologically and biochemically. Among them, two strains were yeast (L1 and C3), two were Bacillus (C2, Y) and three were Cocci (L2, C1, X). All the bacterial isolates were gram-positive and catalase-negative. They showed a broad antimicrobial spectrum against both gram-positive (*Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis MB1, Leuconostoc messenteroides Ly)* and gram-negative bacteria (*Salmonella abony, Escherichia coli).* There was little or no change in the growth after three hours of incubation at pH 2, 2.5 and 3. So all the strains were tolerant of gastric acidity. Bacterial isolates were checked for their survivability in the presence of bile salt. Strain L2 showed maximum tolerance to 0.3% bile salt. So, this strain can be further checked *in vivo* for its usefulness as a probiotic.

Keywords*: Lactic Acid Bacteria; Probiotics; Palm Sap; Antimicrobial Activity*

Introduction

In recent years, consumers have paid more attention to human health and used more natural products. As a result, there is a greater need for natural products in a variety of industries, including those that deal with the food and dairy business, the health sector, the agricultural industry, poultry and fisheries, pesticides and fertilisers. This prompted extensive study to find novel, naturally derived antimicrobial chemicals that might be utilised successfully without endangering human health, the environment, or their foods, for example. Medicinal plants, as well as marine and terrestrial creatures, including fungi and bacteria, are known sources of natural substances with valuable antimicrobial activity. Since ancient times, Lactic acid bacteria (LAB) have been present in different types of fermented foods such as curd, yogurt, cheeses, sauerkraut, sausage *etc.* They also have GRAS (Generally Recognized As Safe) status by the United States Food and Drug Administration (FDA) for human consumption (Rodríguez, 1996). During carbohydrate fermentation, LAB, which is a group of related bacteria, produces mainly lactic acid. They are gram-positive bacteria with DNA that has a GC content of under 50%. Since they lack cytochromes and porphyrins and are therefore catalase and oxidase negative, the majority of LAB are non-

spore forming rods or cocci. They are also aerotolerant anaerobes. Due to their ability to create a variety of antimicrobial substances, including lactic acid, hydrogen peroxide, di-acetyl, and bacteriocins, this group of bacteria can be employed to control microbial growth. Bacteriocins are cationic, hydrophobic, secreted anti-microbial peptides (AMP) of length 20–60 amino acids that are produced by the ribosome, which differs from antibiotics. Both gram-negative and gram-positive pathogenic bacteria that cause food to rot, infections, allergies, and cancer can be inhibited by them. They can be applied alone or in combination with other natural products or chemical drugs.

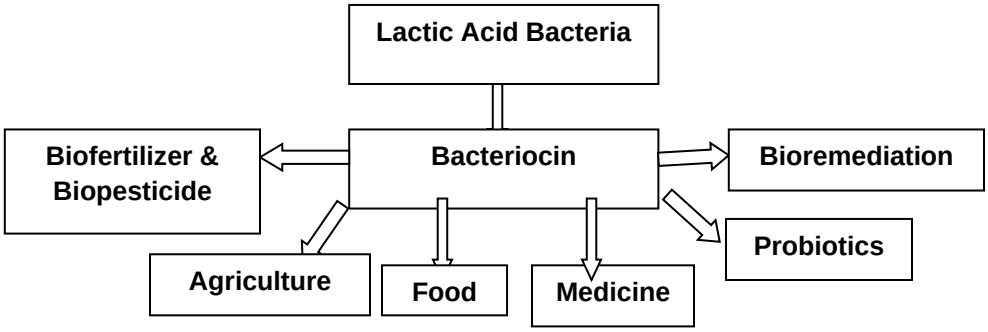

Figure 1: Application of Lactic Acid Bacteria

Probiotic microorganisms assert health benefits for the host when ingested in a suitable amount (Reuben *et al*., 2020). They are live microbial dietary supplements that benefit the host by balancing the microbial population in the intestines (Fuller, 1999). The first observations were made by Elie Metchnikoff, a Nobel Prize laureate, in 1907, who proposed that due to the intestinal microorganisms' reliance on food, it is simpler to change the flora in human systems and replace toxic microbes with beneficial ones. The most accepted probiotic LAB varieties contain various Lactobacillus species. (*Lb. acidophilus*, *Lb. johnsonii*, *Lb. casei*, *Lb. rhamnosus*, *Lb.gasseri*, and *Lb. reuteri*),genus Bifidobacteria (*Bf. bifidum*, *Bf. animalis* subsp. *lactis*, *Bf. longum* subsp. *longum*, and *Bf. longum* subsp. *infantis*) and *Lactococcus* spp. (*L. raffinolactis*, *L. lactis* subsp. *lactis*, and *L. lactis* subsp. *cremoris)* (Ouwehand & Salminen, 2003, Chassard, Grattepanche & Lacroix, 2011, Jung *et al*., 2017). LAB can be used to treat a variety of disorders brought on by pathogenic bacteria that are drug-resistant (Marco *et al*., 2017). The approval process for probiotics requires certain essential characteristics, such as resistance to bile and low pH, antibiotic susceptibility, and antimicrobial activity (Unban *et al.*, 2021). There are numerous sources from which LAB can be isolated, including sugar cane plants, milk products, animal intestines, freshwater fish, fermented foods, and chicken farms (Mulaw *et al.*, 2019).

Palm wine is a very popular drink in different parts of South and Eastern India, Africa and Philippines. It is produced from the sap of different types of palm trees. Though the types and quantity of microorganisms influence the product quality, mainly yeast and some lactic acid bacteria like *Leuconostoc mesenteriodes, Lactobacillus plantarum* etc. have been

found to ferment the sap of palm trees (Sornsenee *et al*., 2021). According to Naknean, Meenune and Roudaut (2010), the product quality is also influenced by the techniques used to tap palm trees and collect their sap, as well as by the air and the surrounding environment. In the recent studies by Fossi *et al*. (2022), it was observed that the probiotic LAB isolated from locally harvested palm wine in Cameroon exhibited cholesterol (LDL)-lowering ability both *in vivo* and *in vitro*. In another study by Ramadhanti *et al*. (2021), lactic acid bacteria with probiotic properties were isolated from palm sugar from West Sumatra, Indonesia. Immunomodulatory activity of the probiotic strains isolated from palm sap in the presence of some prebiotics was also established in a mouse model (Harahap, Munir & Hutahaean, 2023).

This study aimed to determine the types and number of lactic acid bacteria present in a sample of palm wine taken from a local area of Haldia, Purba Medinipur, as well as evaluate certain characteristics that would make them effective probiotic components.

Methodology

Isolation of Lactic Acid Bacteria

Freshly tapped palm sap was collected aseptically in sterile containers from the nearby village of Haldia and immediately brought to the laboratory in an ice box (4°C) to stop fermentation during transportation. Isolation of microbial strains was performed on the same day of collection. With slight changes, the technique of Bromberg *et al*. (2004), was adopted to carry out LAB isolation. To obtain a 1:10 dilution, 1 ml of each sample was diluted in 9 ml of 1% buffered peptone water. Using sterile distilled water, this mixture was further diluted, and 100µl of the diluted samples were applied to Lactobacillus MRS agar plates, a selective medium for lactic acid bacteria. The plates were kept at 30°C for 48–72 hours until growth became visible.

Characterization of Isolates

a) Morphological characterization:

Single colonies of the LAB isolates were picked up and performed Gram staining. The slides were observed under light microscope at 40X and 100X magnifications.

b) Biochemical characterization:

Some biochemical experiments were carried out using each isolate's 18-hour overnight culture in order to properly identify the bacterial isolates, such as Catalase reaction, carbohydrate utilization test, MR-VP test (Methyl red-Voges-Praskauer), indole production, NaCl tolerance study etc. by following standard protocols.

Detection of Antibacterial Activity of the LAB Isolates

Antibacterial activity of the LAB isolates was tested following the agar-cup assay against seven indicator strains. In this method, a bore was made using a borer with a diameter of 5mm on the MRS plates. Cultures of indicator strains were spread over the plates and 50 µl

of cell free supernatant of each isolate was tested by taking both the filtered and boiled cell-free culture supernatant (pH 7) of overnight grown isolates. Then the plates were incubated for 24hrs at 30°C for the formation of a zone of inhibition. The result was further confirmed by Spot on the Lawn method. A direct comparison was made between the diameters of zones of inhibition (mm) produced by different strains after 24hrs incubation.

Micro-organisms used:

The inhibitory spectra of the isolates were evaluated against total seven (7) number of Gram positive and Gram negative bacteria including *Staphylococcus aureus, Bacillus subtilis, Streptococcus faecalis, Salmonella abony, Leuconostoc messenteroides Ly, Enterococcus faecalis MB1*and*Escherichia coli*. Bacterial strains were procured from MTCC, Chandigarh and maintained on Trypticase Soy Broth (TSB, Himedia) media.

Acid Tolerance Study

For application as a good probiotic agent, the bacterial isolates should survive in stomach acid (pH 1.5) and bile acids (pH 2.5). So the survivability of the isolates was checked at pH 2, pH 2.5 and pH 3 following the method of Liong and Shah (2005). Isolates were grown in MRS broth for 24 hours at 37°C and centrifuged at 5000 rpm for 10 minutes at 4°C. As inoculums, 1% of this solution was added to MRS broth that had been acidified with concentrated HCl to pH 2, 2.5, and 3, and then incubated for three hours at 37°C. MRS broth that had not been acidified was used as the control. OD values (600nm) were taken at 0 and 3 hours after incubation at 37°C. Strains that showed little or no reduction in OD values were considered acid-tolerant.

Bile Salt Tolerance Study

Following the methodology outlined by Walker and Gilliland (1993), this experiment was carried out. MRS agar supplemented with 0.3% bile salt (Himedia Laboratories Pvt. Ltd., India), was prepared and each isolate was spread over and incubated at 37°C. After this incubation period, growth was compared with that of control plates of MRS agar without bile salt. The percentage of LAB colonies formed on MRS agar relative to the starting bacterial concentration was used to calculate the survival rate:

survival rate (%) = (log $CFUN_1$/ log $CFUN_0$) × 100,

where N_1 is the viable count of isolates after incubation and N_0 is the initial viable count.

Results and Discussion

Isolation of Lactic Acid Bacteria

A total of 89 bacterial colonies were obtained from the isolated plates, of which some were cream in color and some were whitish. Among them, 7 colonies (L1, L2, C1, C2, C3, X, Y) were selected and purified by the streak plate method on MRS medium, and the isolates were maintained in MRS slants at 4°C. Small, white colonies were selected as lactic acid bacteria (Figure 1).

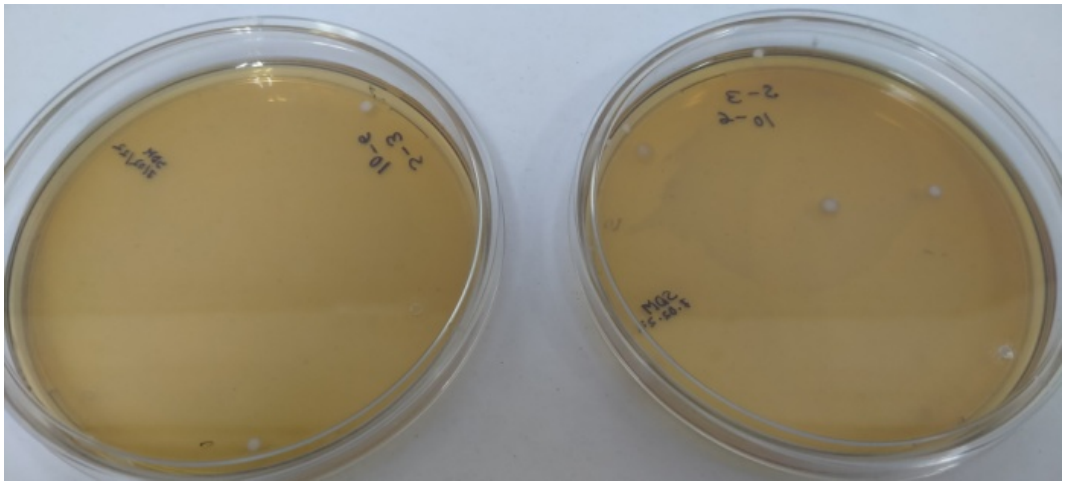

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Figure 2: Lactobacillus MRS Plates with LAB Isolates

Characterization of Isolates

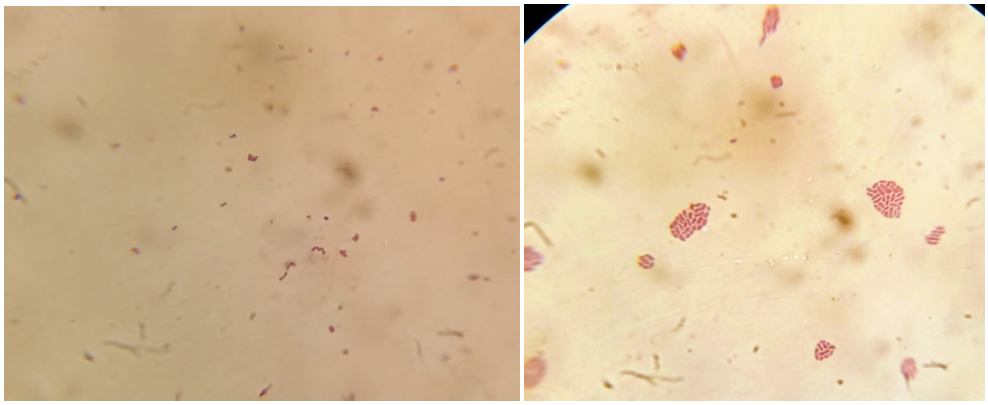
Except for L1 and C3, all the isolates were gram-positive and Catalase negative. Among the isolates L2, X, C1 appeared as cocci and C2, Y as Bacillus and two strains, L1 and C3, were yeast (Figure 2). All the isolates were negative in the Indole test. Except for C1 and Y, all the isolates were Methyl Red positive (Figure 3). All the isolates were VP and Citrate negative (Table 1). All the isolates were able to grow in the presence of 10% NaCl. The carbohydrate fermentation profiles of the isolated strains were shown in Table 2 as evidenced by the color change after 48 h of incubation (Figure 4). The fermentation pattern was compared to the standard lactic acid bacterial strains' fermentation chart. According to this test, it can be predicted that, L2, X, C1 and C3 are *Lactococci* species and C2 and Y are *Lactobacillus* species.

Table 1: Biochemical Properties of Seven Isolates

Tests	L1	L2	C1	C2	C3	X	Y
Morphology	c (y)	c	c	r	c (y)	c	r
Gram staining	+/-	+	+	+	+/-	+	+
Methyl Red	+	+	-	+	+	+	-
Voges Proskauer	-	-	-	-	-	-	-
Catalase test	+	-	-	-	+	-	-
Indole test	-	-	-	-	-	-	-
Gas from glucose	-	-	-	-	-	-	-
NaCl tolerance	+	+	+	+	+	+	+

c: round shaped, cocci; r: rod shaped, Bacillus, c(y): yeast; + : positive reaction, -: negative reaction

Source: Collected by authors



a b

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Figure 3: Observation under Light Microscope (100X) After Gram Staining of L2 (a: chain of cocci) and Y (b: rod shaped)

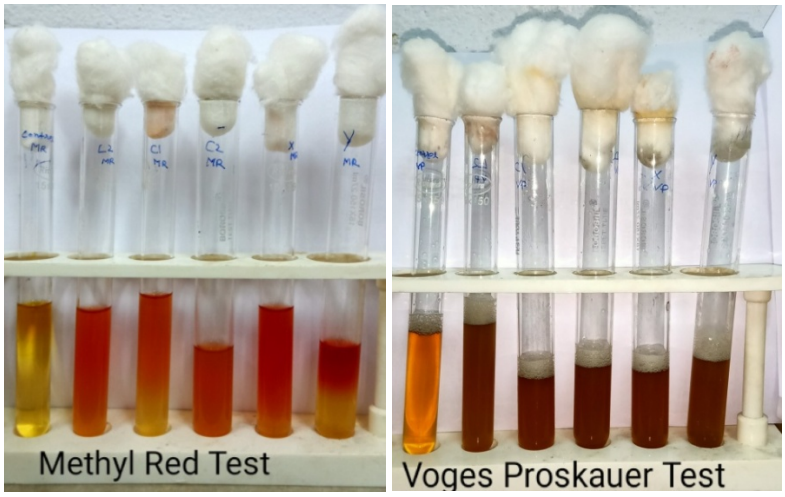

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Figure 4: IMViC Test of Isolates

Table 2: Carbohydrate Utilization Profile of Five LAB Isolates

Carbohydrates	**L2**	**C1**	**C2**	**X**	**Y**
Lactose	++	++	++	+/-	+/-
Sucrose	++	++	++	++	++
Maltose	++	++	++	++	++
Fructose	+/-	+/-	+/-	-	+/-
Dextrose	++	++	++	++	++

++: Good utilization, +/- : weak utilization, - : No utilization

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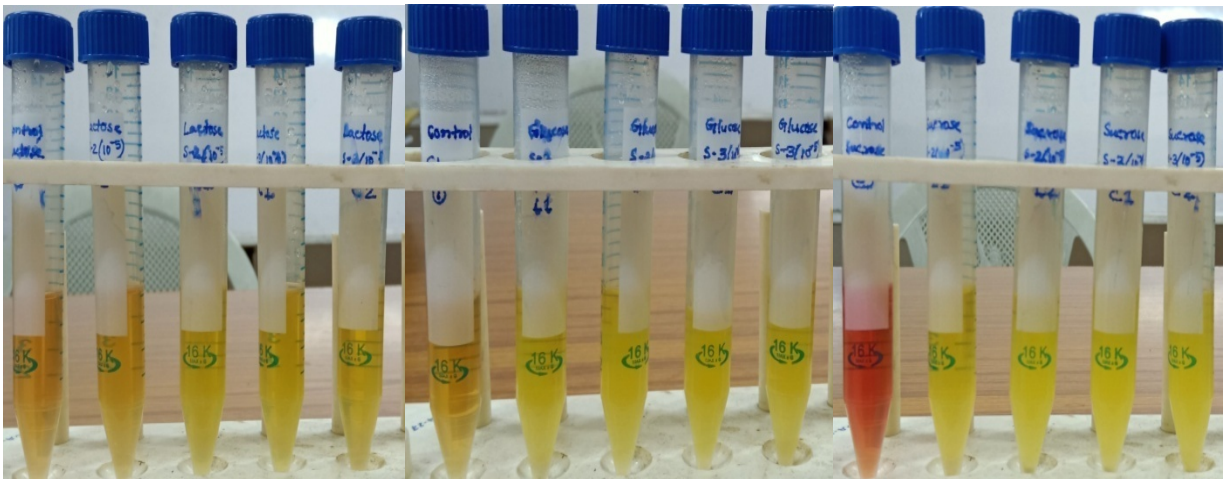

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Figure 5: Carbohydrate Utilization Test of the LAB Isolates

Antibacterial Activity of the LAB Isolates: All the five LAB strains showed broad inhibitory spectra. No isolate showed inhibitory activity against all the tested bacteria (Table 3). LAB C1and C2 has shown maximum antibacterial activity against most of the test organisms. Figure 5 showed some of the antibacterial activity plates with zone of inhibition.

Table 3: Antibacterial Spectra of Different Isolates

Indicator strains	**L2**	**C1**	**C2**	**X**	**Y**
Staphylococcus aureus	++	++	++	++	++
Bacillus subtilis	++	++	++	++	++
Streptococcus faecalis	++	++	++	++	++
Enterococcus faecalis MB1	-	+	+	-	-
Leuconostocmessenteroides Ly	-	-	+	+	+
Salmonella abony	++	++	++	++	++
Escherichia coli	+	+	-	-	-

++: Zone diameter >2cm; +: <2cm; -: no zone of inhibition

Source: Collected by authors

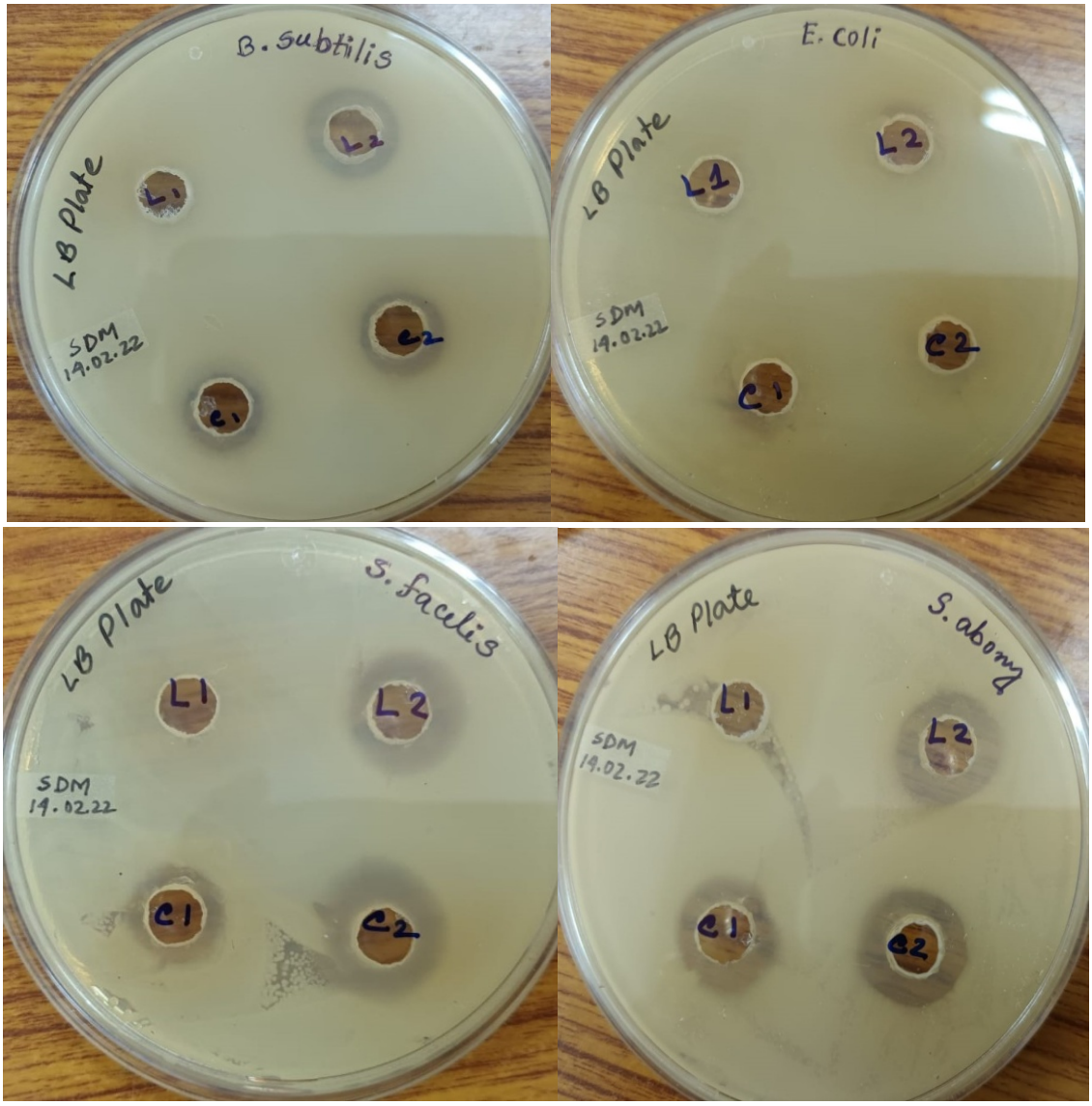

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Figure 6: Antimicrobial Activity of the Isolates Against the Pathogens Tested

Acid Tolerance Study

After 3 hrs incubation period, all the isolates showed a slight increase in OD_{600} values at pH 2 and PH 3, but at pH 2.5, there was little lessen in the OD_{600} values. After 24hr growth ceased in most of the cases, but at pH 2, all the isolates showed a little increase in values (Figure 6). So it can be concluded that all the bacterial isolates were very acid tolerant, though their growth is better at pH 2. In the stomach, where pH is around 1.5, the foods stay for 90 minutes. For this reason, the acid tolerance of the isolates was mainly observed for 3 hours.

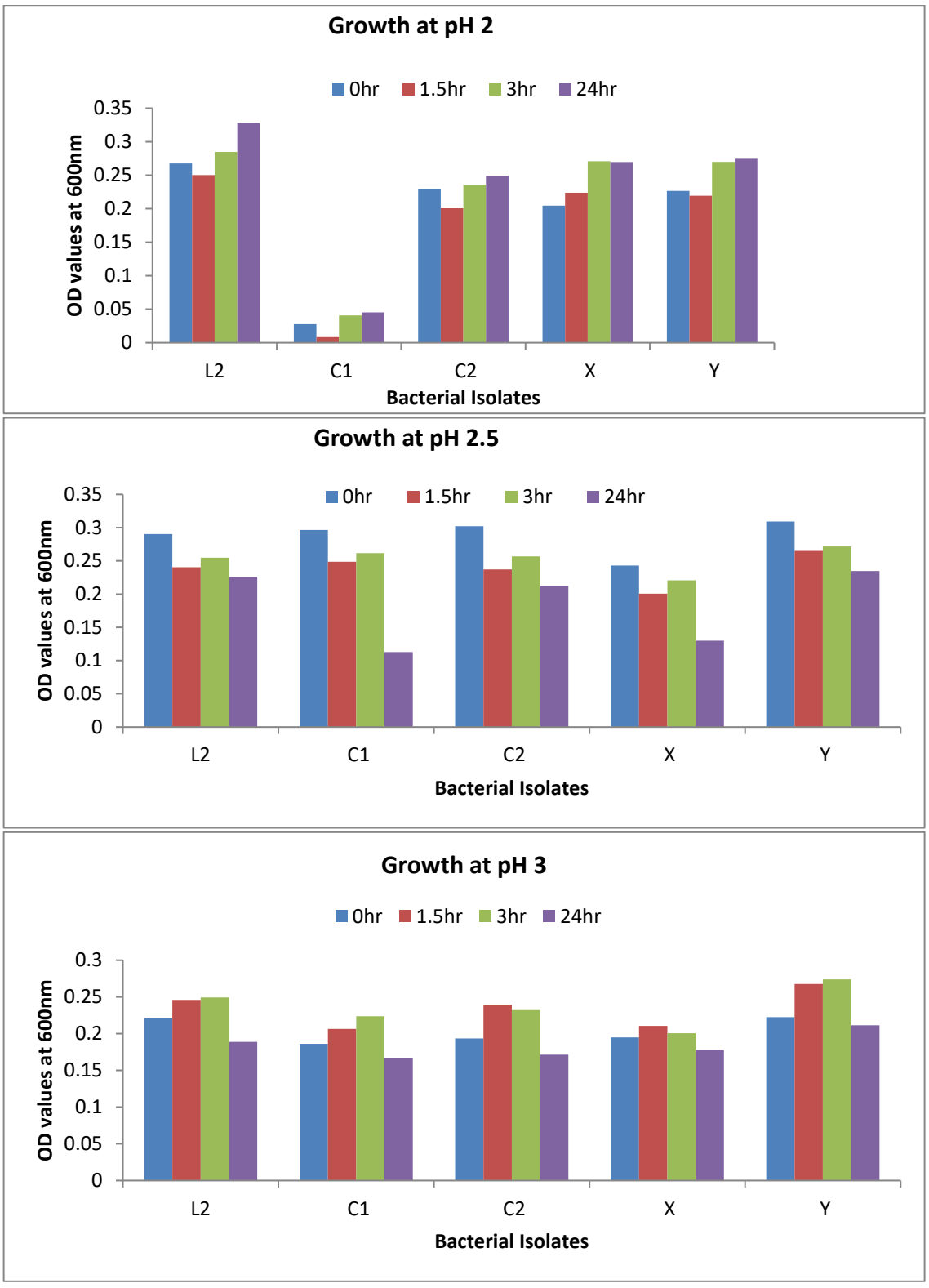

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Figure 7: Growth Pattern of Isolates at pH2, pH2.5 and pH3

Bile Salt Tolerance Study

According to Gilliland, Staley and Bush (1984), 0.3% bile tolerance is necessary for the evaluation of bile-tolerant probiotic LAB. All the isolates survived the tested bile salt concentration (0.3%). Small colonies developed after 48 hours of incubation at 37°C on bile salt agar medium. Growth was also observed in MRS broth containing bile salt. Survival rate was highest for L2, then for Y and least for X (Figure 7).

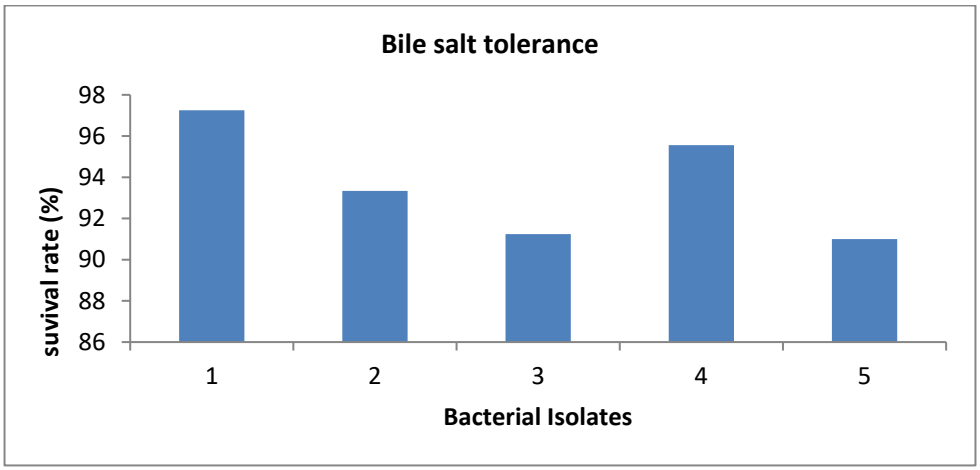

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Figure 8: Survival Rates of Isolates in Presence of Bile Salt

Conclusion

This study demonstrates that palm sap is a potential source of LAB with probiotic properties, especially strong antimicrobial activity against food borne pathogenic bacteria. Among all the isolates, two strains were yeast (L1 and C3), two were Bacillus (C2, Y), and three were cocci (L2, C1, X). All were gram-positive and catalase-negative. All the isolates showed a broad antimicrobial spectrum against both gram-positive (*Staphylococcus aureus, Bacillus subtilis, Enterococcus faecalis MB1, Leuconostoc messenteroides Ly)* and gram-negative bacteria (*Salmonella abony, Escherichia coli).* All the isolates showed little or no change in growth after three hours of incubation at pH 2, 2.5 and 3. So all the strains were tolerant of gastric acidity. All the LAB strains were very bile salt-tolerant. Strain L2 showed maximum survivability in the presence of 0.3% bile.

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