CHARACTERIZATION OF PROBIOTIC ISOLATE, SLA FROM YOGURT

A Dissertation Submitted to St.Teresas college (Autonomous), Ernakulam in Partial Fulfilment of the Requirement for the Award For

DEGREE OF MASTER OF SCIENCE IN ZOOLOGY

SUBMITTED BY

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CERTIFICATE

This is to certify that the dissertation entitled 'Characterization of probiotic isolate, SLA from yogurt' submitted to St. Teresa's College (Autonomous), Ernakulam, in partial fulfilment of the requirement of award of degree of Master of Science in Zoology is an authentic work carried out by Ms. NEELIMA GIREESH (SM21ZOO009) in the academic year 2021 – 2023 under the guidance and supervision of Dr. Athira (External Guide) Assistant Professor, Department of biotechnology, Cochin university of science and technology and Dr. Damaris Benny Daniel (Internal Guide), Assistant Professor, Department of Zoology, St. Teresa's College, Ernakulam.

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I hereby declare that the dissertation entitled 'Characterization of probiotic isolate, SLA from yogurt' submitted to St. Teresa's College (Autonomous), Ernakulam in partial fulfilment of the requirements, for the award of the Degree of Master of Science in Zoology is a record of original research work done by me under the supervision and guidance of Dr Athira (External Guide) Assistant Professor, Department of biotechnology, Cochin university of science and technology during the period from 12th December 2022 to 12th January 2023 and Dr. Damaris Benny Daniel (Internal Guide), Assistant Professor, Department of Zoology, St. Teresa's College, Ernakulam, to the best of my knowledge and belief, this project contains no material previously published or written by another person, except where due reference is made.

NEELIMA GIREESH

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LIST OF ABBREVIATIONS

1.	%	Percentage
2.	ºC	Degree Celsius
3.	pH	Potential of hydrogen
4.	SCFAs	Short chain fatty acid
5.	IBD	Inflammatory bowel syndrome
6.	IB	Irritable bowel syndrome
7.	WHO	World health organisation
8.	FAO	Food and agriculture organization
9.	MRS	De man, Rogosa, Sharpe
10.	DNA	Deoxyribonucleic acid
11.	LAB	Lactic acid bacteria
12.	CO2	Carbon dioxide
13.	NACL	Sodium chloride
14.	EF	Enterococcus faecium
15.	BSH	Bile salt hydrolase
16.	ML	Milli litre
17.	RNA	Ribonucleic acid
18.	7MP	Lactobacillus pentose
19.	NG	Nanogram
20.	HCL	Hydrochloric acid
21.	NAOH	Sodium hydroxide
22.	OD	Optical density

23.	CFU	Colony forming unit
24.	NM	Nanometer
25.	AM	Ampicillin
26.	GM	Gentamycin
27.	E	Erythromycin
28.	C	Chloramphenicol
29.	ONPG	Ortho-Nitrophenyl-β-galactoside

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ABSTRACT

Probiotics are living cells and refer to beneficial microorganisms that may have nutritional benefits due to their various beneficial properties. Probiotic strains exhibit potent activity to improve human health. The main probiotic groups commonly used are *Lactobacillus, Bifidobacterium, Pediococcus, Lactococcus, Bacillus* and Yeast strains. Probiotics have recently received a great deal of interest in the field of microbiology, particularly for their role in normal physiology and their impact on human health during infection. The use of probiotics has shown promising results in numerous well-designed clinical studies. For example, as a therapeutic option for the treatment, prevention and management of various disorders and diseases such as gastrointestinal disorders, allergies, urogenital infections, *Helicobacter pylori* infections, inflammatory bowel syndrome and diarrhoea and colon cancer. It is currently one of the most fertile and attractive research areas due to its ability to prevent and treat communicable and non-communicable human diseases. Various clinical research results support this idea. In this work, a bacterial isolate from yogurt was characterized for its probiotic potential. Biochemical and physiological characterization of the isolate showed that the isolate is a potential probiotic.

INTRODUCTION

Microorganisms are present on the skin, in the mouth, and in the digestive tract. Humans are closely related to these microbes. The greatest concentration of microorganisms is in the digestive tract. The gastrointestinal tract has the largest surface area at about 400 m2, and the respiratory tract he is the second largest. The gastrointestinal tract is home to over 500 species of bacteria, some of which have health functions such as stimulating the immune system, protecting the host against bacterial and viral invasion, and aiding digestion. The use of antibiotics, immunosuppressive therapy, and radiation can lead to changes in composition and effects on the flora. By introducing beneficial species of bacteria into the digestive tract, it restores microbial balance and prevents disease (Gupta *et al.,* 2009).

Probiotics was first introduced in the year 1908. Nobel laureate Eli Metchnikoff suggested that the longevity of Bulgarian farmers was due to the consumption of fermented milk products. In 1965, Lilly and Stillwell first used the word probiotics to describe substances secreted by one organism that stimulate the growth of another. Probiotics are defined as "a microbial preparation or component of microbial cells that has beneficial effects on health and well-being." (Marteau *et al.,* 2002).

The term "probiotics" comes from the Greek study and means "lifetime". Probiotics are defined by FAO and WHO as “live microorganisms” that, when administered in appropriate amounts, provide a health benefit to the host. The different genera of bacteria most commonly used in probiotics are *Lactobacillus, Bifidobacterium, Escherichia, Enterococcus, Bacillus and Streptococcus*. The most commonly used strain in fungi is Saccharomyces. *Lactobacillus rhamnosus* GG is the first probiotic to receive the most clinical attention ever. Discovered in 1985. Lactobacillus strains used for fermentation in the dairy industry have failed to transplant into the intestine. *Lactobacillus rhamnosus* has been shown to have a positive effect on intestinal immunity. It increases the number of her IgA- and immunoglobulin-secreting cells in the intestinal mucosa and increases antigen uptake into Peyer's patches. Prebiotics are dietary carbohydrates that escape digestion in the upper gastrointestinal tract and alter the bacterial composition of the gut by altering the types of substrates supplied to existing microbial populations in the gut. Fructo-oligosaccharides, gluco-oligosaccharides, insulin. Both probiotics and prebiotics improve bacterial survival. (Garg *et al.,* 2009)

Properties of a probiotic

A dose of 5 billion colony forming units per day for a reasonable amount of health benefits, must have anti carcinogenic activity, produce lactic acid, reduce intestinal permeability, and be able to withstand both the acidic and alkaline conditions of the stomach and duodenum. Fermented milks, cheeses, fruit juices, wines and sausages contain lactic acid bacteria and are used as foods for human consumption. Single and mixed cultures of live microorganisms are used in probiotic preparations. (Marteau *et al.*, 2002)

Mechanism of action

Partial lactose digestive stimulation of lactose activity in the intestinal mucosa has been postulated as a mechanism for some types of diarrhoea. Lactobacilli used in the fermented milk industry have an active beta-galactosidase that reduces the concentration of lactose in dairy products. Lactic acid bacteria produce free fatty acids, hydrogen peroxide and bacteriocins to prevent the growth of foodborne pathogens in dairy products. Probiotics prevent colonization by pathogens through competitive inhibition. The gut microbiota lowers intestinal pH, releases protective intestinal metabolites, and modulates intestinal motility and mucus production and the environment of the immune system. A decrease in this gut microbiota resulted in increased antigen transport, suggesting that a normal gut microbiota maintains intestinal defense. Probiotic bacteria also regulate immunoglobulin production. Secretory IgA is a barrier against pathogens and viruses. Fermented milk yogurt increases the number of IgA.

Nutritional and therapeutic benefits of probiotics

- Microbial action in the gut increases bioavailability, quantity and digestibility of certain nutrients.
- Probiotics increase the bioavailability of iron, manganese, copper, calcium and phosphorus, and improve protein and fat digestibility in yogurt.
- Probiotics improve production of riboflavin, niacin, vitamin B6, vitamin B12, thiamine, and folic acid.

- Enzymatic hydrolysis of proteins and fats increased free amino acids and short-chain fatty acids (SCFAs).
- Probiotic bacteria prevent lactose intolerance, allergies and diarrheal diseases, reduce mutagenic and carcinogenic risk, lower serum cholesterol and stimulate the immune system.
- Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IB) can be treated with probiotics.
- Probiotics reduce the risk of cancer.
- The probiotic strains *Lactobacillus reuteri, Lactobacillus rhamnosus* and *Propionibacterium freudenreich* have been shown to improve symptoms such as constipation.

Several studies have been conducted based on the isolation of bacteria from probiotics such a yogurt. Elie Metchnikoff in 1907 was intrigued as to why certain inhabitants of the Bulgarian population lived much longer than others. He focused his study on centenarians, people who've lived past the age of 100. What Metchnikoff discovered was that villagers living in the Caucasus Mountains were drinking a fermenting yoghurt drink on a daily basis. His studies found that the drink contain probiotic called *Lactobacillus bulgaricus* which improved their health and increased their lifespan.

Fontana in 2013 isolated lactic acid bacteria from Iranian traditional yogurts. Yogurt was collected from goat, sheep, and cow milk. Morphological and biochemical characterization was done using gram staining and catalase test. Lactic acid bacteria isolated from Iranian traditional yogurt were considered as viable candidates of probiotics, based on their properties such as acid and bile tolerance.

A similar study was conducted by Ali Almasi in 2017, that lactic acid bacteria were isolated from traditional yogurt. Antibacterial test, bile salt tolerance, low pH, antibiotic susceptibility test were done. The experiment revealed various strains of lactobacillus were isolated from shiraz and cheese displayed a desirable tolerance to low pH and high bile salt.

In a study by Prabhurajeshwar *et al.*, (2015) Lactobacilli strains from commercial yogurt were isolated for checking the evolution of antimicrobial properties and their substances against pathogenic bacteria. They isolated *Lactobacillus* from four different companies of yogurt samples from the city markets of Gulbarga region. All the isolates were suspectible to clinical

antibiotics and showed good growth in the acidic condition, resistant to NACL, maximum growth was observed at pH around 6.0.

In this work, a bacterial isolate from yogurt was characterized for its probiotic properties.

AIM AND OBJECTIVE

AIM:

The aim of this study is to characterize a bacterial isolate SLA from yogurt as a potential probiotic candidate.

OBJECTIVE:

1. Biochemical characterization of probiotic isolate from yogurt.
2. Physiological characterization of the probiotic isolate
3. To check the antibiotic sensitivity and antibacterial property of the probiotic isolate.

RELAVANCE OF THE STUDY:

Characterizing a probiotic isolate has importance in health and disease for example- Microbial action in the gut increases bioavailability, quantity and digestibility of certain nutrients. Probiotics increase the bioavailability of iron, manganese, copper, calcium and phosphorus, and improve protein and fat digestibility in yogurt. Probiotics improve production of riboflavin, niacin, vitamin B6, vitamin B12, thiamine, and folic acid. Enzymatic hydrolysis of proteins and fats increased free amino acids and short-chain fatty acids (SCFAs). Probiotic bacteria prevent lactose intolerance, allergies and diarrheal diseases, reduce mutagenic and carcinogenic risk, lower serum cholesterol and stimulate the immune system. Inflammatory Bowel Disease (IBD) and Irritable Bowel Syndrome (IB) can be treated with probiotics. Probiotics reduce the risk of cancer.

Probiotics provide many health benefits by improving the balance of the intestinal microbiota and mucosal defenses against pathogen used inside.

REVIEW OF LITERATURE

The modern history of probiotics begins in the early 20th century. Pioneering research on probiotics was done by Russian scientist Ellie Mechnikov, working at the Pasteur Institute in Paris. The microbes responsible for the fermentation process were identified by Louis Pasteur when Metchnikoff was trying to understand the effects of these microbes on human health. He linked increased life expectancy in Bulgaria's rural population to regular consumption of fermented milk products such as yogurt. In 1907 he addressed the question of why certain inhabitants of Bulgaria lived much longer than others. He focused his research on centenarians over the age of 100. Metchnikoff discovered that villagers living in the Caucasus drank fermented yogurt daily. His research found that the drink contained a probiotic called *Lactobacillus bulgaricus*, which improved health and extended lifespan. People delve deeper into probiotics, and scientists discover and classify different types of probiotics. These include familiar species of *Lactobacillus acidophilus*, *Saccharomyces boulardii*, and *Bifidobacterium infantis*, all of which have different properties that have different effects on the body.The antimicrobial effects of probiotics isolated from organic yogurt against several common bacterial pathogens were studied by Hami Kaboosi et al., (2011).

The probiotic isolate was off-white, round, convex, moist with smooth edges. Gram staining of the colonies showed Gram-positive, non-spore-forming short bacilli, the results of which were found for Bifidobacterium species. It was expected. From organic yogurt. Particle-stained swabs showed both paired or long-chain Gram-positive cocci and Gram-positive non-spore-forming long bacilli, results as expected for *Streptococcus sp*. and the genus *Lactobacillus.* (Caputo et al., 2011). Antibacterial efficacy results showed that all probiotic strains isolated from various organic yogurts were able to inhibit the growth of some, but not all, selected pathogens. Probiotics were bactericidal against *S. aureus* and *P. aeruginosa* are inhibitory against *S. typhi* and inactive against *E. coli.* The results of the study showed antibacterial effects of probiotics isolated from various organic yogurts (Kaboosi *et al.*, 2011).

Probiotics provide many health benefits by improving the balance of the intestinal microbiota and mucosal defenses against pathogen used inside. Fontana isolated lactobacilli from Iranian traditional lactobacillus yogurt in 2013. The survey he conducted over 18 months and collected

96 samples. Yogurt was made from goat, sheep and milk. Collected samples were transferred to MRS broth and sub cultured on MRS agar. Morphological and biochemical characterization was performed using Gram staining and catalase test. Tolerance to acidic pH and tolerance to bile salts were used as limiting criteria for probiotic potential. 16s rDNA sequence analysis was also identified using selected lactic acid bacteria (Gil *et al.*, 2013). 47 LABs were isolated from the 96 yogurt samples isolated. Twelve of them were probiotic candidates. Six probiotic isolates belonged to *Pediococcus acidilacticii*, and his other six isolates belonged to *Lactobacillus plantarum, L. brevis, L. fermentum, L. kefiri* bile resistance. (Fontana *et al.,* 2013).

A study was conducted on the effects of probiotic bacteria isolated from yogurt produced in the city of Damietta on several pathogens. A total of 200 samples of different brands of traditional yogurt were randomly collected from local markets and supermarkets in different districts of Damiet city. Probiotics were anaerobically isolated and identified from yogurt samples in the presence of 10% CO2. (Bazalou *et al.*, 2013) A total of 17 lactobacilli were isolated in this study. Due to the antibacterial activity of isolated probiotics, microbes were used as inhibitors against several common foodborne pathogens (E. coli, Salmonella spp.). and Staphylococcus aureus) using the agar well diffusion test. Yoghurt probiotics have in vitro antibacterial activity and excellent antibacterial activity against the aforementioned indicator pathogens (Maarof *et al.,* 2013).

A study conducted by Prabhurajeshwar in 2015 isolated strains of lactobacilli from commercial yogurt to confirm the antibacterial properties and development of the substance against pathogenic bacteria. He isolated lactobacilli from his four different companies from his samples of yoghurts from the city market in the Gulbarga region. From the samples he isolated 32 lactobacillus strains, from which the top 13 Lactobacillus isolates were selected and tested for potential probiotic and antimicrobial activity against pathogenic bacteria. All isolates are sensitive to clinical antibiotics, show good growth in acidic conditions, are resistant to NACL (1-6%), bile salts (0.5-3%), and grow maximally around pH 6.0 was observed. The isolated strain also showed effective aggregation and hydrophobicity studies. Based on Prabhurajeshwar's findings, his selected *Lactobacillus* isolates were considered as potential new probiotic bacteria. Therefore, for the development of probiotic-enriched dietary supplements and human health benefits through the prevention and control of bacterial infections, it is necessary to isolate and characterize probiotic bacteria from dairy products and

their proliferation. Further extensive research may be needed to optimize the (Prabhurajeshwar *et al.*, 2015).

In 2015, a study was conducted by Haghshenas to characterize the bioactivity of *Lactobacillus* strains isolated from dairy products. Lactobacillus is isolated from sheep dairy with probiotic and anticancer activity. 100 samples were collected from yogurt and colostrum and 125 her LABs were isolated. Among these, 17 Lactobacillus strains belonging to 5 species (*L.delbruekii, L.plantarum, L.rhamnosus, L.paracasei, Lcasei*) were identified. *L. plantarum* isolated from colostrum has been questioned as a potential probiotic, showing remarkable results such as tolerance to low pH and high bile salt concentrations, susceptibility to some antibiotics, and excellent antibacterial activity. (Haghshenas *et al.*, 2015)

A study on the probiotic properties of bacteria isolated and identified from local yogurt. LAB was isolated from local yogurt and tested for its probiotic properties in in vivo mouse experiments. A total of 8 isolates were obtained from yogurt. Some of them were thermophilic Streptococcus, Lactobacillus acidophilus, Lactobacillus brevis and Bifidobacterium species. Physiological and biochemical characterization was performed and yogurt was Gram-positive, endospore-negative, catalase-negative and non-motile, which is a characterization of common probiotic bacteria. A summary experiment of sugar fermentation using 16 essential sugars confirmed the putative identification of these 4 species from yogurt samples. All species were tolerant to the synthetic stomach environment and were able to tolerate artificial bile salts. All types of yogurt showed good tolerance to low levels of phenols and sodium chloride. Isolated probiotic bacteria can also increase weight gain, lower blood cholesterol levels, and exhibit antibacterial activity in in vitro mouse experiments. The isolated and identified probiotic bacteria in this study can be further investigated for the development of probiotic products (Rahman *et al.*, 2015).

A variety of traditional dairy products provide health benefits such as enhanced nutrient absorption, toxin inactivation, and antipathogenic activity. Traditional dairy products such as yoghurt, cottage cheese, shiraz, cheese and tarquine are produced in many countries, but Iran is the most famous. A traditional Iranian dairy product was a delicious and authentic Shiraz. Shiraz is made from buttermilk or yogurt with herbs such as nigella and fennel. Probiotics affect the bioavailability of nutrients in the human body by promoting the absorption of magnesium and calcium from milk protein, digesting lactose, and producing folic acid and vitamins. A study conducted by Ali Almasi in 2017 isolated lactic acid bacteria from regular

yogurt. Antimicrobial testing, bile salt tolerance, low pH, and antibiotic susceptibility testing were performed. His LAB strain from traditional Iranian dairy. *L.plantarum* 15HN, *L.lactic* subsp. Cremoris 44L and E. mundtii50H strain was isolated. In this experiment, various lactobacillus strains were isolated from Shiraz and cheese and demonstrated desirable low pH and high bile salt tolerance, which was advantageous for antipathogenic activity and antibiotic susceptibility (Ali Almasi *et al.,* 2017).

In 2017, Mannan isolated Lactobacillus species from yogurt and cheese samples from the Dhaka region for biochemical characterization. The aim of this study was to investigate lactic acid bacteria in yogurt and cheese that could be used as potential probiotics: 15 He from cheese collected from local markets and 10 He from yogurt, totaling 25. sample was taken. Samples were collected from Dhaka city from May 2016 to he June. Collected samples were transferred to MRS broth and sub cultured on MRS agar. Twenty-five isolated bacteria were identified as *Lactobacillus* by gram staining and biochemical testing. All isolated strains were characterized for probiotic properties such as acid and salt tolerance, phenol tolerance, sugar fermentation, lactose fermentation and proteolytic activity. Acid tolerance tests were performed at pH 2, 3, 4, 5, 6, 7 and 8 in MRS broth. Results showed that all isolates survived the strongly acidic pH. All isolates survived NACL concentrations of 2% and 4%. This study showed that Lactobacillus species from yogurt and cheese samples have potential probiotic properties (Mannan *et al.,* 2017).

Hossain and many others conducted a study on probiotic bacteria isolated from yogurt samples from Rajshahi and Chittagong districts of Bangladesh, their biochemical properties, and antimicrobial activity against intestinal pathogens. In this study, 10 probiotic *Lactobacillus* spp. were isolated and analyzed according to their morphological, physiological and biochemical assays. All isolated bacteria were observed to be rod-shaped, gram-positive, catalase-negative, immobile, and coagulase-positive, indicative of typical probiotic bacteria. All isolated bacteria showed excellent low pH (3.0) and bile (0.3%) growth over 24 hours. The isolated bacteria were tested for antimicrobial activity against eight human and animal enteric pathogens and showed promising antimicrobial efficacy against selective pathogens (Hosain *et al.,* 2018).

Probiotic potential of lactic acid bacteria isolated from cheese, yogurt and poultry feces. Due to their beneficial probiotic properties, research continues on new strains of lactobacilli from yogurt, cheese, and poultry faeces as potential sources for the isolation of Lactobacilli. Based on the results, 33 isolates were selected for in vitro acid and bile tolerance testing. Of the 33

isolates, 13 isolates were tested for acid and bile tolerance and showed good tolerance (Kamai *et al.,* 2018).

Development of a probiotic milk drink using probiotic strains isolated from local yogurt. 2 types of lactic acid bacteria was isolated from his four yogurt samples that were identified based on the morphology of colonies grown on MRS medium and biochemical tests performed in the laboratory. (Islam *et al.,* 2018). Gram stain, catalase test, oxidase test, sugar fermentation, bile salt concentration 0.05% to 0.25%, pH 2.5 to pH 5.5 can be used as probiotics. NACL resistance studies showed that two isolates had slightly reduced growth during 1-4% NACL supplementation. After isolating probiotic strains from yogurt, these strains were used to develop probiotic milk beverages. These beverages were developed by mixing one controlled and the other he two with different concentrations of fruit (mango juice). Analysis of the probiotic milk drink revealed a maximum fat content of 4.50%, protein content of 3.99%, ash content of 1.90% and acid content of 0.78%. The studied storage took place at refrigerator temperature (10 °C) for 15 days and samples were taken at 3-day intervals. (Ahmadur *et al.,* 2018).

Bacterial characterization was performed by isolating Nigerian yogurt as a promising alternative to antibiotics in gastrointestinal infections. Gastrointestinal infections are endemic in Nigeria and commonly used antibiotics. Several factors contribute to its survival, including bacterial resistance to substances. This study investigated the antibacterial potency of bacteria in yogurt produced in Nigeria against enteric pathogens (Ayeni *et al.,* 2019).

Probiotic properties of *Lactobacillus helveticus* and *Lactobacillus plantarum* isolated from traditional Pakistani yogurt to assess the probiotic properties of bacteriocin-producing isolates of *Lactobacillus helveticus* and *Lactobacillus plantarum* isolated from traditional Pakistani yogurt (Shafique *et al.,* 2020). Ten bacteriocin-producing isolates were selected to test their probiotic properties. The isolates showed tolerance to acidic pH (6–6.5), bile salts (0.01–1%), and 1–7% NACL salts, showing good growth at acidic pH and resistance to 10 foodborne pathogens. demonstrated antibacterial activity (Nayab et al., 2020). These isolates have been shown to be active against *Actinobacter baumannii* and least active against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Some isolates were found to be resistant to some antibiotics such as vancomycin, gentamicin, erythromycin, streptomycin and clindamycin. This finding provides strong evidence that traditional Pakistani yogurt is a potential source of bacteriocin-producing bacteria with the added benefit of probiotic properties. , should be used

in the development of probiotic-enriched dietary supplements in Pakistan (Williamson *et al.*, 2020).

Characterization and probiotic potential of lactic acid bacteria isolated from Dadia harvested in West Sumatra was performed by Philip *et al.*, 2020. Dadia is a traditional dairy product from West Sumatra. Dadia is made by fermenting fresh buffalo milk on bamboo sticks covered with banana leaves for two days at room temperature. Dadia has a high nutritional value as it contains lactic acid bacteria with probiotic properties. intended to make a decision. LAB was discovered in Dadia using standard laboratory tests and 16s rRNA sequencing methods, confirming that LAB is Lactobacillus fermentum. Gram staining showed that the isolated bacteria were Gram-positive bacilli, catalase assay, and the fermentation type was catalase-negative and homofermentative (Rinita *et al.*, 2020).

Diwaney *et al.*, tested the cholesterol-lowering ability and vitamin B12 producing ability of bacteria isolated from yogurt. Three LAB isolates with probiotic potential, namely Enterococcus faecium (EF), Enterococcus faecium (Chole 1) and Lactobacillus pentose (7MP), were isolated from yogurt. These isolates were screened for bile salt hydrolase (BSH) activity, cholesterol-lowering properties of MRS broth, and vitamin B12 production. The study found that the 7MP isolate had the highest cholesterol-lowering potential of 48% compared to other isolates. Isolated EF and chole1 produced large amounts (1 ng/ml) of vitamin B12. These isolates were identified by 16s rRNA gene sequencing. Therefore, the use of these LAB isolates in yogurt production may provide the added value of cholesterol-lowering and vitamin B12 fortification of fermented foods (Walhe *et al.*, 2021).

MATERIALS AND METHODS

1. Microorganism

An unidentified probiotic isolate was obtained from microbiology lab of CNS. The organism was named SLA. Colony morphology was observed from streaked plates. The organism were grown in MRS (de man, Rogosa and Sharpe) agar. The temperature for the incubation of the organism is 37ºC.

2. Colony morphology

Colony morphology was studied using quadrant streaking method in MRS agar plate. The isolate was streaked in the MRS agar plate and kept for incubation at 37ºC for 24 hours.

3. Gram staining

The Gram staining is a differential staining method used to classify bacteria into gram-positive or gram-negative based on their cell wall properties. The procedure is named after Danish bacteriologist Hans Christian Gram. Gram staining leaves gram positive bacteria violet while gram negative bacteria appears as reddish pink. The organism is Gram stained using Hi-media Gram staining kit (K1001-1KT).

4. Catalase test

Overnight cultures of isolates were grown on MRS agar at +37°C for 24 h under anaerobic conditions. Catalase test was conducted by dripping two drops of hydrogen peroxide (3%) on 24 h-old cultures on a glass slide. The catalase test that shows positive reaction is characterized by the formation of oxygen bubbles that indicate the production of catalase enzyme by the test bacterium.

5. Media preparation

I. MRS media

Preparation using 16.54g MRS, 6g agar in 300ml water. The prepared media was autoclaved and was poured into petri plates.

II. Nutrient agar media

Preparation using 6g agar in 100ml water. The prepared media was autoclaved and was poured into petri plates.

6. Serial dilution experiment

Serial dilution experiment was done up to 10^15 dilution. For this 15 test tubes and 15 plates were taken. Marked up to 10^15 on the test tubes and plates. Transferred 1ml of the sample to 9ml of sterile distilled water and mixed thoroughly. The sample are then serially diluted up to 10^15. Transfed 1ml each of the dilution into sterile plate and it was spread using a spreader. Allow the plate to solidify. And the plate was incubated at 37 degree Celsius for 24 hours.

7. Quadrant streaking

Quadrant streaking in MRS media and quadrant streaking in differential media was done.

Differential media used was lactic bacteria differential broth.

8. pH and temperature tolerance

The isolate grown in MRS broth at 37 °C overnight, two set of 0.1 mL aliquots of each active cultures were inoculated to media adjusted to pH 2, 6.8 and 8 with 1 N HCL and IN NAOH kept for 24 hours and OD at 600nm was recorded and the viable numbers of bacteria were enumerated by spread plate counts of all samples using 15-fold serial dilutions.

For temperature tolerance assay the isolate was grown overnight in MRS broth at three different temperatures- 4, 37 and 25°C respectively. After incubating the culture overnight, the OD of the samples were read at 600nm and spread plating was done by serial diluting the samples. The viable count of bacteria was expressed in CFU/mL.

9. Antibiotic sensitivity test

Disc diffusion method was used to access the antibiotic susceptibility of isolate with 5 disc containing ampicillin (AM 10g), erythromycin (E 15g), chloramphenicol (C 30 3g), gentamycin (GM 10 3g) and methicillin. Agar plates were surface inoculated uniformly from broth culture of the isolate. The discs were placed on the medium with proper spacing. Inhibition-zone diameters were measures after aerobic incubation at 37ºC for 24 hours.

10. Biochemical analysis

The biochemical properties of the organism were identified using Hi-Bacillus KBO13 biochemical kit. The kit contains sterile media for Malonate, Voges Proskauer's, Citrate, ONPG, nitrate reduction, Catalase, Arginine and 5 different carbohydrate utilization tests – Sucrose, Manitol, Glucose, Arabinose and Trehalose. Organism was inoculated to each of the media present in the kit and incubated at 37ºC for 24 hours.

Table 1- Biochemical tests conducted in the study

No	Test	Principle	Original colour of the medium	Positive reaction	Negative reaction
1	Malonate	Malonate utilization	Bluish green	Dark blue	Bluish green
2	Voges Proskauer's	Detects acetoin production	Colourless/light yellow	Pinkish red	Colourless/slight copper
3	Citrate	Citrate utilization	Light green	Dark blue	Light green
4	ONPG	Detects beta galactosidase	Colourless	Yellow	Colourless
5	Nitrate reduction	Detects nitrate reduction	Colourless/light yellow	Pinkish red	Colourless
6	Catalase	Detects catalase activity	Colourless	Efferverscene coming out from the loop	No efferverscene seen
7	Arginine	Arginine utilization	Olive green to light purple	Purple/ dark purple	No change in colour or yellow
8	Sucrose	Carbohydrate utilization	Pinkish red/ red	Yellow	Red/ pink
9	Mannitol	Carbohydrate utilization	Pinkish red/ red	Yellow	Red/ pink
10	Glucose	Carbohydrate utilization	Pinkish red/ red	Yellow	Red/ pink
11	Arabinose	Carbohydrate utilization	Pinkish red/ red	Yellow	Red/ pink
12	Trehalose	Carbohydrate utilization	Pinkish red/ red	Yellow	Red/ pink

11.Antimicrobial test

Antimicrobial activity of the test strains was tested against E.coli, Staphylococcus aureus, Pseudomonas aeuroginosa and Klebsiella pneumonia by disc diffusion assay. MHA agar plates were surface inoculated uniformly with the pathogenic strains. Sterile discs were impregnated with the culture. The disc were placed on the medium with proper spacing. Inhibition-zone diameters were measured after aerobic incubation at 37ºC for 24 hours.

RESULTS

1. Quadrant streaking in MRS media

The isolate from yogurt named SLA was available in CNS laboratory, CUSAT

Colony morphology of SLA

The colonies were off white, small, round, flat, regular, opaque, and the margin was entire.

Figure 1- Quadrant streaking in MRS media

2. Quadrant streaking in differential media (Lactic Bacteria Differential Media)

Observed colonies were homo fermentative differential media. Bluish-green colonies were observed.

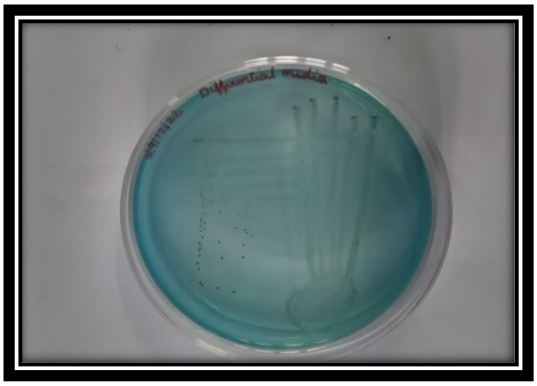


Figure 2 – Quadrant streaking in differential media

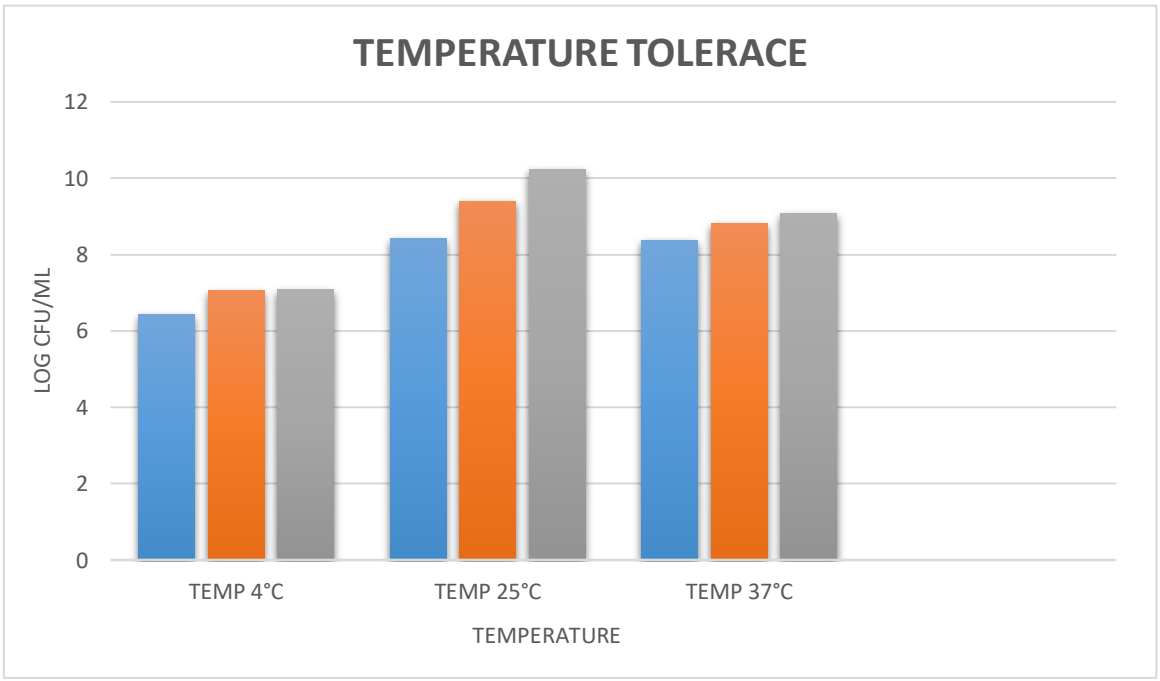
Table 2- Optical density of three different temperature and three different pH

Temperature	**OD**	**pH**	**OD**
Saline	0.000		
MRS broth	0.173		
25ºC	1.970	pH 8	1.889
25ºC	2.041	pH 8	0.435
25ºC	0.608	pH 8	0.749
37ºC	2.196	pH 6.8	2.806
37ºC	0.892	pH 6.8	1.285
37ºC	0.606	pH 6.8	1.631
4ºC	0.151	pH 2	0.140
4ºC	0.118	pH 2	0.211
4ºC	0.073	pH 2	0.222

3. Temperature tolerance

Table 3 – Colony counting of three different temperature.

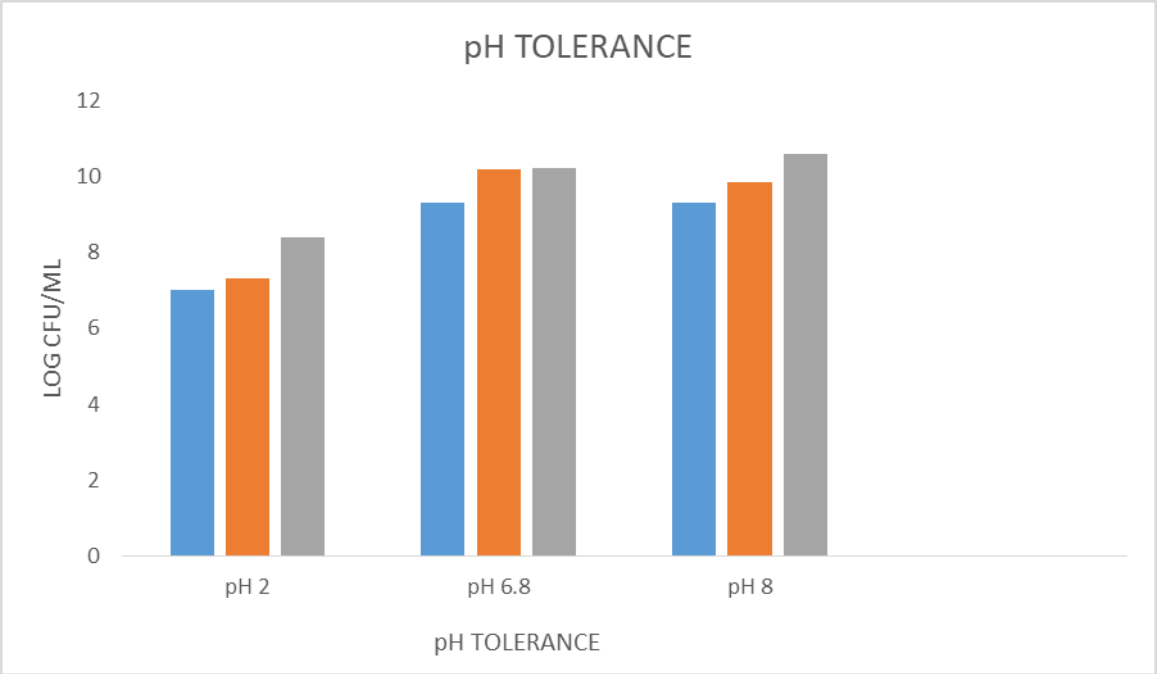
Temperature 4ºC	Colony counting	Temperature 37ºC	Colony counting	Temperature 25ºC	Colony counting
10^1	Numerous	10^1	Numerous	10^1	Numerous
10^2	Numerous	10^2	Numerous	10^2	Numerous
10^3	Numerous	10^3	Numerous	10^3	Numerous
10^4	270	10^4	Numerous	10^4	Numerous
10^5	112	10^5	Numerous	10^5	Numerous
10^6	12	10^6	241	10^6	261
10^7	3	10^7	65	10^7	250
10^8	1	10^8	12	10^8	176
10^9	NIL	10^9	5	10^9	120
10^10	NIL	10^10	6	10^10	210
10^11	NIL	10^11	7	10^11	130
10^12	NIL	10^12	8	10^12	110
10^13	NIL	10^13	NIL	10^13	90
10^14	NIL	10^14	NIL	10^14	90
10^15	NIL	10^15	NIL	10^15	NIL


TEMPERATURE TOLERACE
12
10
8
6
4
2
0
LOG CFU/ML
TEMP 4°C
TEMP 25°C
TEMP 37°C
TEMPERATURE

4. pH tolerance

Table 4 – Colony counting of three different pH

pH 6.8	Colony counting	pH 8	pH 6.8	pH 8	pH 2
10^1	Numerous	10^1	Numerous	10^1	Numerous
10^2	Numerous	10^2	Numerous	10^2	Numerous
10^3	Numerous	10^3	Numerous	10^3	Numerous
10^4	Numerous	10^4	Numerous	10^4	Numerous
10^5	Numerous	10^5	Numerous	10^5	100
10^6	Numerous	10^6	Numerous	10^6	20
10^7	200	10^7	200	10^7	25
10^8	150	10^8	70	10^8	25
10^9	17	10^9	40	10^9	16
10^10	15	10^10	28	10^10	14
10^11	10	10^11	25	10^11	NIL
10^12	NIL	10^12	25	10^12	4
10^13	NIL	10^13	35	10^13	2
10^14	NIL	10^14	6	10^14	1
10^15	NIL	10^15	NIL	10^15	2



The isolate SLA was able to thrive at three different pH and temperatures tested. The organism showed a viability of $14x10^{10}$ CFU/mL at pH 2, which confirmed the organism is acid tolerant and will be able to thrive in the acidic condition in the stomach. This is an important property of the probiotic organism. The survival at the temperatures 25 and 4°C indicates that the organism can be stored at these different temperatures which is an important characteristic useful in commercialization.

Figure 3 - Colony counting of pH tolerance

Figure 4 - Colony counting of temperature tolerance

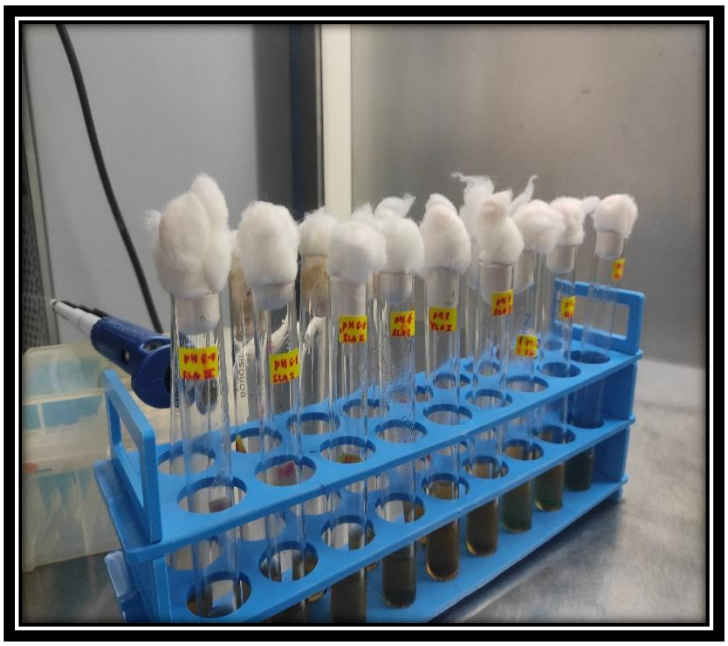


Figure 5- Inoculating SLA for checking the temperature and pH tolerance

Figure 6- Incubated the plates at 37 degree Celsius

5. Gram staining

The Bacteria was observed to be gram positive because it stained in violet colour and was cocci shaped.

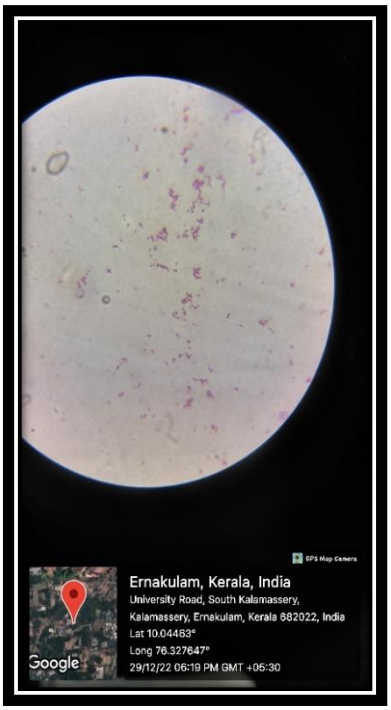


Figure 7 - Gram staining

6. Biochemical test

Samples 1-6 showed negative result, 7-8 showed positive result, 9 showed negative result and 10-12 showed positive result.

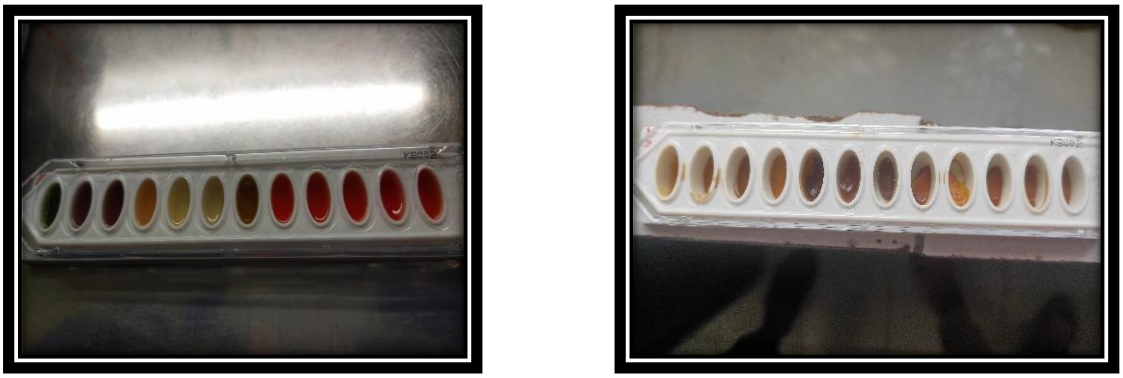


Figure 8- Showing the arrangement for biochemical test

Table 5- Showing biochemical test result.

No:	Test	Principle	Original colour of the medium	Positive reaction	Negative reaction
1	Malonate	Malonate utilization	Bluish green	----	Bluish green was observed
2	Voges proskauer's	Detects acetoin production	Colourless/light yellow	----	Colourless
3	Citrate	Citrate utilization	Light green	----	Light green was observed
4	ONPG	Detects beta galactosidase	Colourless	----	Colourless
5	Nitrate reduction	Detects nitrate reduction	Colourless/light yellow	----	Colourless
6	Catalase	Detects catalase activity	Colourless	----	No efferverscene seen
7	Arginine	Arginine utilization	Olive green to light purple	Purple/ dark purple was observed	-----
8	Sucrose	Carbohydrate utilization	Pinkish red/ red	Yellow was observed	-----
9	Mannitol	Carbohydrate utilization	Pinkish red/ red	----	Red/ pink was observed
10	Glucose	Carbohydrate utilization	Pinkish red/ red	----	Red/ pink was observed
11	Arabinose	Carbohydrate utilization	Pinkish red/ red	----	Red/ pink was observed
12	Trehalose	Carbohydrate utilization	Pinkish red/ red	----	Red/ pink was observed

7. Antimicrobial test

Zone of clearance of *Klebsiella pneumoniae* was 0.6cm, *Staphylococcus aureus* was 0.9cm, *Pseudomonas* was 0.8cm, *Escherichia coli* was 0.7cm

Figure 9- Antimicrobial test of Staphylococcus aureus

Figure 10- Antimicrobial test of Pseudomonas aeruginosa

Figure 11- Antimicrobial test of E.coli

Figure 12- Antimicrobial test of Klebsiella pneumonia

8. Antibiotic sensitivity test

Zone of clearance of Ampicillin was 1.5cm, Gentamycin was1cm, Erythromycin was 0.7cm, Chloramphenicol was1.5cm. Ampicillin and Chloramphenicol was resistant. Gentamycin and Erythromycin was intermediate.

Figure 13- Antibiotic sensitivity test of Ampicillin and Gentamycin

Figure 14- Antibiotic sensitivity test of Erythromycin and Chloramphenicol

DISCUSSION

The antimicrobial effects of probiotics isolated from organic yogurt against several common bacterial pathogens were studied by Hami Kaboosi et al., (2011). The probiotic isolate was off-white, round, convex, moist with smooth edges. Gram staining of the colonies showed gram-positive, non-spore-forming short bacilli, the results of which were found for Bifidobacterium species. It was expected. From organic yogurt. Particle-stained swabs showed both paired or long-chain gram-positive cocci and gram-positive non-spore-forming long bacilli, results as expected for *Streptococcus sp*. and the genus *Lactobacillus.* (Caputo *et al.,* 2011). Antibacterial efficacy results showed that all probiotic strains isolated from various organic yogurts were able to inhibit the growth of some, but not all, selected pathogens. Probiotics were bactericidal against *S. aureus* and *P. aeruginosa* are inhibitory against *S. typhi* and inactive against *E. coli*. The results of the study showed antibacterial effects of probiotics isolated from various organic yogurts (Kaboosi *et al.,* 2011).

In the present study also probiotic were isolates from yogurt and the present study show correlation with the previous study conducted by Kaboosi *et al.,* (2011) that the antibacterial test result showed that the probiotic strain isolated from yogurt were able to inhibit the growth against the pathogens. The pathogens selected in the present study were *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas*, *Escherichia coli*. Here also the result shows antibacterial effect of the probiotics isolated from yogurt. Gram staining conducted by the present study show Gram-positive bacteria and cocci shaped. The probiotic isolate in the present study was observed to be off white, small, round, flat, regular, opaque, and the margin was entire.

Probiotic properties of *Lactobacillus helveticus* and *Lactobacillus plantarum* isolated from traditional Pakistani yogurt. The aim of this study was to access the probiotic properties of bacteriocin-producing isolates of *Lactobacillus helveticus* and *Lactobacillus plantarum* isolated from traditional Pakistani yogurt (Shafique *et al.,* 2020). Ten bacteriocin-producing isolates were selected to test their probiotic properties. The isolates showed tolerance to acidic pH (6–6.5), bile salts (0.01–1%), and 1–7% NACL salts, showing good growth at acidic pH and resistance to 10 foodborne pathogens. demonstrated antibacterial activity (Nayab et al., 2020). These isolates have been shown to be active against *Actinobacter baumannii* and least

active against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. Some isolates were found to be resistant to some antibiotics such as vancomycin, gentamicin, erythromycin, streptomycin and clindamycin. This finding provides strong evidence that traditional Pakistani yogurt is a potential source of bacteriocin-producing bacteria with the added benefit of probiotic properties. , should be used in the development of probiotic-enriched dietary supplements in Pakistan (Williamson *et al.*, 2020).

In accordance with the previous study conducted by Shafique *et al.,* (2020) that isolated probiotic showed tolerance to three different pH. The three different pH selected are 6.8, 8 and 2. And all the 3 showed tolerance to the isolated probiotics. Temperature tolerance was also checked in the present study and all the three different taken showed tolerance to the isolated probiotic. The three different temperatures taken for the present study was 25ºC, 4ºC, and 37ºC and all the three showed tolerance to the isolated probiotic. There is no previous studies conducted for checking the temperature tolerance of the probiotic isolate. The isolate SLA was able to thrive at three different pH and temperatures tested. The organism showed a viability of $14x10^{10}$ CFU/mL at pH 2, which confirmed the organism is acid tolerant and will be able to thrive in the acidic condition in the stomach. This is an important property of the probiotic organism. The survival at the temperatures 25 and 4°C indicates that the organism can be stored at these different temperatures which is an important characteristic useful in commercialization.

Antibiotic sensitivity test result of the present study show similarity with the previous study conducted by Williamson *et al.,* 2020 that ampicillin and chloramphenicol has a zone of clearance 1.5cm and both of them are resistant. Gentamycin and erythromycin has a zone of clearance 1cm and 0.5cm respectively and both of them are intermediate.

Fontana isolated lactobacilli from Iranian traditional lactobacillus yogurt in 2013. The survey he conducted over 18 months and collected 96 samples. Yogurt was made from goat, sheep and milk. Collected samples were transferred to MRS broth and sub cultured on MRS agar. Morphological and biochemical characterization was performed using Gram staining and catalase test. 47 LABs were isolated from the 96 yogurt samples isolated. Twelve of them were probiotic candidates. Six probiotic isolates belonged to *Pediococcus acidilacticii*, and his other six isolates belonged to *Lactobacillus plantarum*, *L. brevis*, *L. fermentum*, *L. kefiri* bile resistance.

In the present study biochemical test was done. The test result is positive to arginine test, sucrose test, arabinose test, glucose test and trehalose test. And negative test result is showed to malonate teat, voges proskauer's test, citrate test, ONPG test, nitrate reduction test, catalase test and mannitol test.

Characterization and probiotic potential of lactic acid bacteria isolated from Dadia harvested in West Sumatra. Dadia is a traditional dairy product from West Sumatra. Dadia is made by fermenting fresh buffalo milk on bamboo sticks covered with banana leaves for two days at room temperature. Dadia has a high nutritional value as it contains lactic acid bacteria with probiotic properties. Gram staining showed that the isolated bacteria were Gram-positive bacilli, catalase assay, and the fermentation type was catalase-negative and homo fermentative (Rinita *et al.,* 2020).

Present study show correlation with the previous study conducted by Rinita *et al.,* (2020) and many other. In the present study, quadrant streaking in differential media was done. Lactic bacteria differential media was used and observed colonies were homo fermentative, bluish green colonies were observed.

CONCLUSION

The probiotic strains isolated from yogurt in this study are potential candidates for probiotic use. The isolate strains exhibited attractive probiotic properties such as excellent temperature and pH tolerance. All tested strains were susceptible to a range of clinically effective antibiotics.

In this study the probiotic was characterized biochemically and physiologically and the following tests were conducted antimicrobial test, antibiotic sensitivity test, Gram staining, biochemical test and checking the temperature and pH tolerance. The isolate SLA was able to thrive at three different pH and temperatures tested. The organism showed a viability of $14x10^{10}$ CFU/mL at pH 2, which confirmed the organism is acid tolerant and will be able to thrive in the acidic condition in the stomach. This is an important property of the probiotic organism. The survival at the temperatures 25 and 4°C indicates that the organism can be stored at these different temperatures which is an important characteristic useful in commercialization. Antibacterial test result showed that the probiotic strain isolated from yogurt were able to inhibit the growth against the pathogens. The pathogens selected in the present study were *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas*, *Escherichia coli*. Here also the result shows antibacterial effect of the probiotics isolated from yogurt. Gram staining conducted by the present study show Gram-positive bacteria and cocci shaped. The probiotic isolate in the present study was observed to be off white, small, round, flat, regular, opaque, and the margin was entire.

These results suggest that the isolates from the yogurt samples have potential properties essential for new probiotics. They work in real situations for human health. The use of probiotics has shown promising results in numerous well-designed clinical studies. For example, as a therapeutic option for the treatment, prevention and treatment of various disorders and diseases such as gastrointestinal disorders, allergies, urogenital infections, *Helicobacter pylori* infections, inflammatory bowel syndrome and diarrhoea and colon cancer. It is currently one of the most fertile and attractive research areas due to its potential to prevent and treat communicable and non-communicable human diseases. In summary, this study shows that probiotics isolated from yogurt have good probiotic potential.

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