Probiotic Potential of Indigenous *Lactobacillus* and *Yeast* Encapsulated in Alginate beads and their impact on yogurt formation

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Abstract

Lactic acid bacteria have probiotics potential which help to prevent and treat a variety of medical disorders and have significant nutritional value when used in fermented foods. This study was designed with the objective of isolation and characterization of Lactic acid-producing bacteria with potential probiotic properties from milk and commercialized yogurt samples, after encapsulated in alginate beads and evaluating potential in yogurt formation. To enhance the viability of isolated strains encapsulation with sodium alginate was done. For this study, 40 samples (20 milk, 20 yogurts) were taken from different areas of Multan, Punjab. **Method:** The 20 different isolated strains were treated for the selection of the best bacteria with the probiotic potential to survive in harsh conditions i.e. at low pH, in the presence of bile salt, gastric juice stimulant, and have the ability to produce bacteriocin. Antibacterial activity, Antioxidant test, and detection of virulence factor test were performed. Further probiotic yogurt was prepared using isolated lactobacillus and yeast strains as inoculum. Different parameters were observed, including fermentation time, pH, microbiological analysis, total solid content, water holding capacity, and synergies at different time intervals. **Results:** In this study, isolated *Lactobacillus* strains had promising probiotic characteristics they were able to tolerate acidic pH, could survive at compatible temperatures intestinal tract, were able to tolerate bile salts presence, and could have biofilm formation and bacteriocin production. Probiotic yogurt showed its microbial count increases during 4 weeks and was higher in the case of alginate beads. Similarly, total solid content increases and decreases in the case of beads. **Conclusion:** All the strains have the best probiotic properties and are used for yogurt formation. Which plays a significant role in a variety of fermentation processes. They ferment carbohydrates that exist in food.

Keywords: LAB, Encapsulation, Bacteriocin, Fermentation

1. INTRODUCTION

Probiotics are microorganisms that are added to foods as food additives and have many positive benefits on the body by restoring gastrointestinal tract microbial equilibrium. According to previous research, probiotics also have beneficial effects on intestinal inflammation, antibiotic-induced diarrhea, allergic and cancerous reactions, fat loss and host immune-mediated response, irritable bowel syndrome symptoms, and inflammation in the intestines (Karami et al., 2017). Probiotics have typically been thought of as health-promoting bacteria found in meals or as a dietary supplement. Pharma-biotics emerged as a new category of probiotics needed to minimize confusion (Lee, Song, Nam, & Lee, 2018). Probiotics after their discovery and creation which are living bacteria or *yeasts* utilized to promote the host's health and coincided with our research in food preservation, microbiologic identification techniques, and microbial biotechnology play important roles in complex interactions in microbiota that affect the host's health. Studies using a variety of animal models have demonstrated that the gut microbiota is crucial in controlling host longevity(Wu et al., 2022). The majority of probiotics are species of the genus *Bifidobacterium* and the genus *Lactobacillus.* Anaerobic, gram-positive, non-motile, and non-sporulating are the characteristics. Microbiota diversity is dynamic and individualized dependent on the influence of nutrition, exposure to ingested probiotic bacteria, environmental conditions of the intestine, and other factors linked with the organism's host that will incorporate momentary some new types in the environment (Wieërs et al., 2020). The immune system is essential in gut defense, and the gut has receptors that set off a variety of reactions. The anti-inflammatory cytokines are stimulated by some probiotic strains, such as *Lactobacillus reuteri and B. bifidum.* Antimicrobial activity against gastrointestinal infections, immune system boosting, reduction of serum cholesterol and blood pressure, anti-carcinogenic action, and improved nutritional utilization and bioavailability are the key advantages of probiotics (ŞEner, Bulut, & GÜNeŞ Bayir, 2021). The following explanation, put out by a committee of specialists made up of representatives of the World Health Organization WHO is among the simplest and most widely accepted one's probiotics are bacteria that, when given in large enough doses, have a positive effect on the patient's health. They thereby stop dysbiosis from occurring and help the patient avoid it, which results in eubiosis of the gut microbiome (Ballini et al., 2023).

It is commonly acknowledged that this collection of bacteria has a significant impact on the host in whom it resides(Abdel-Megeed, 2021). Particularly the parietal microbiota, with microbes living in food in transit and stools, known as the luminal microbiota, the microbiota organizes as a focused ecosystem and changes from one place to another. Microbiota diversity is dynamic and individualized dependent on the influence of nutrition, exposure to ingested probiotic bacteria, environmental conditions of the intestine, and other factors linked with the organism's host (Wieërs et al., 2020). Although it is widely recognized that dysbiosis in the gut refers to changes in both the quantitative and qualitative makeup of the microbiota, these changes may result in altered host-microbial interaction that can contribute to a disease state frequently accompanied by inflammation (Plaza-Diaz, Ruiz-Ojeda, Gil-Campos, & Gil, 2019). Fortunately, there has been a lot of interest in *Lactobacillus* and *Bifidobacterium* strains as potential therapeutics. Clinical trials have demonstrated the effectiveness of *L. rhamnosus*, *L. acidophilus*, and *B. longum* in the treatment of cancers of the gastrointestinal tract and colon cancer, respectively (Abdelhamid, El-Masry, & El-Dougdoug, 2019). Although the fact that yeasts are a common component of the microflora in many dairy-related products, Yeasts predominate in a variety of fermented foods made with both plant- and animal-derived materials. Yeasts can be found in a wide variety of foods, including alcoholic beverages like wines (such as fruit, palm, and rice wines), cereal-based leavened goods like sourdough and idli, dairy products like cheese and dahi, and condiments like soy sauce and papads. Yeasts are either dominant by themselves or in combination with lactic acid bacteria during natural food fermentation (Rai & Jeyaram, 2017).

Active compounds are enclosed in a protective shell during the encapsulation process, which enhances their stability, controlled release, and targeted distribution. Probiotic encapsulation is the method of protecting probiotic cells from environmental deterioration and releasing them at controlled levels under particular conditions (Utama, Oktaviani, Balia, & Rialita, 2023). The ability of yeast cells to carry both hydrophobic and hydrophilic molecules, as well as their capacity to shield encapsulated substances from heat, light, oxygen, and moisture, are just a few of its potential benefits (Tan, Huang, McClements, Sun, & Wang, 2021). Yeasts have been used as microcapsules in addition to their most well-known use in the food and fermentation industries. Yeast and LAB cells can be Used living intact, permeabilized, or even emptied of all their original cytoplasmic contents (Dadkhodazade et al., 2021).

2. MATERIALS AND METHODS

2.1 Isolation and Purification of *Lactobacillus* and *Yeast* Strains

Spread plate technique and MRS agar were used for the isolation of *Lactobacillus* and *yeast* strains. A total of 40 samples, 20 milk samples, and 20 yogurt Samples were collected from different urban and rural areas of Southern Punjab Multan in sterile bottles. Of 20 milk samples 10 were collected from households, 5 from different dairy forms, and 5 from different vending shops in Multan, Punjab, Pakistan. In 10ml of MRS broth 1ml of each sample was inoculated and placed in an incubator for 24hrs at 37 °C. After a specified period take 25µl of each sample and spread it on MRS agar plates (Innocente et al., 2016). Distinct colonies were picked and purified by repeated streaking procedure and stored at 4°C for further use.

2.2 Screening of LAB and Yeast for Probiotic Properties

Tolerance of Acidic pH, bile salt, and pepsin enzyme are three important probiotic properties. For pH tolerance strains were gown at different pH (2, 4,6). The acid tolerance potential of selected strains was calculated by using the following formula:

% age Acid Resistance= OD at different pH (2, 4, 6)/ OD at pH 7 ×100 (Kumar & Kumar, 2015)

For bile salt tolerance selected strains were inoculated in MRS broth with/without bile salt, and incubated at 37°C for 24 hrs. After incubation, optical density at 560nm was measured by using a spectrophotometer. The percentage of bile salt tolerance was calculated by using the following formula: %age of bile salt tolerance= Optical density of bacteria with bile salt/ Optical density of bacteria without bile salt*100). For pepsin tolerance, selected strains were grown in MRS broth with pepsin enzyme for 30 minutes and spread on MRS agar plates, incubated anaerobically at 37°C for 48 hrs. (Kalhoro, Nguyen, & Anal, 2019).

2.3 Morphological and biochemical Characterization of selected Strains

For morphological characterization observe colony shape, color, and texture of selected strains while biochemical identification was performed by different tests i.e. Gram staining, Endospore test, Motility test, Triple iron sugar test, Indole test, Simmon's citrate test, Vagous proskaur test, Methyl red test, H2S Production test, Oxidase test, Catalase test was performed.

2.4 Optimization study of selected Strains

Selected strains were optimized at different physical factors to find out the best growth conditions. LAB strains and yeast were grown in MRS broth at different temperatures (37 °C, 45°C), and salt concentrations (2%, 4%, 6%) in different test tubes incubated for 24 hrs. and measured their optical density at 650nm.

2.5 Antioxidant, Antibacterial and MIC Properties

Hydrogen peroxide was added to MRS media at varying concentrations (0.4 mM, 1 mM). Isolated strains were added to sterilized media, and the mixture was then cultured for 24 hours at 37°C. The same procedure was used to prepare the control, but no bacterial inoculum was used. To determine the strain resistance to hydrogen peroxide, absorbance at 600 nm was measured spectrophotometrically and compared with control (Sethi et al., 2020).

Two milliliters of DPPH (0.05 mM in ethanol) solution were combined with one milliliter of CFS. As a control, DPPH was combined with deionized water. For thirty minutes, the mixture was left at room temperature in the dark. Following incubation, the mixture was centrifuged for 10 minutes at 8,000 rpm. The absorbance of the solution at 517 nm was used to measure the strains' capacity to scavenge DPPH (Sethi et al., 2020).

Reducing power (antioxidant capacity) was performed with phosphomolydenum protocol. For the experiment, 100 working solutions (4mM ammonium molybdate, 0.6 M sulphuric acid, and 28mM sodium phosphate) and different concentrations of bacterial extracts (200,600,800µg/ml) in distilled water were prepared. After that, in separate clean test tubes, 1ml of reagent solution and 100 µl of different bacterial concentrations were added and mixed thoroughly. Ascorbic acid was kept in control. After 1 hour and 30 minutes at 90◦C tubes were incubated in a water bath. After 90 minutes of incubation, test tubes were cooled down at room temperature. Each sample absorbance was recorded at 690-700 nm. Antioxidant activity was calculated by using the following formula:

Antioxidant capacity % = (OD control – OD sample)/OD control ×100 (de Moura et al., 2021).

Biological screening for antimicrobial properties of strains was performed by agar well diffusion method. 6 selected strains(A1, A2, A3 B1, B2, B3) with the best probiotic properties were used to check their antagonistic activity against 5 different pathogens *(Klebsiella, pseudomonas aeroginosa, staph Aureus, MRSA, E.Coli* (Scillato et al., 2021).

Minimum inhibitory concentration (MIC) is the minimum amount of antimicrobial agent that inhibits visible growth of bacteria after 12h and 18 h of incubation. The indicator organism used in this was *E. coli* and strain A1. Different concentrations of supernatant (0.2, 0.4, 0.8, 1.6, 3.2, 6.4, 12.8 µg/ml) were used. Then optical density was observed at 420nm by using a spectrophotometer. The strain showed minimum inhibitory concentration against *E. coli* as compared to the non-pathogenic strain.

2.6 Detection of virulence factor for safety Assessment

The presence of virulence factor was determined by performing different tests including thermo-nuclease activity, hemolytic activity, and gelatinase activity. The thermo-nuclease activity was performed by using brain heart infusion broth specifically. Pink zone formation was observed around the edges of the tubes which is an indication of positive results otherwise considered negative with no zone formation. A hemolysis test was performed to observe the hemolytic activity of our strain. Lysis activity was shown by the zone of inhibition. All the tested strains were subjected to a gelatinase test. The ability of test strains to yield gelatinase which is a proteolytic enzyme and liquefy gelatin was done by gelatin hydrolysis. No liquefication of media shows no virulence factor present (Zarzecka, Zadernowska, & Chajęcka-Wierzchowska, 2022).

2.7 Hydrophobicity Assay

For surface hydrophobicity (H %). fresh bacterial culture was inoculated in MRS broth for 14 h at 37◦C. Through centrifugation for 5 minutes at 6000rpm cell cultures were being harvested. The pellet was taken and washed twice with PBS solution. Later it was suspended in a PBS buffer and OD was observed at 540 nm with a UV visible spectrophotometer. By taking 3ml of bacterial suspension was mixed with 1ml toluene and vortexed for 30 seconds and for temperature equilibrium, it was incubated for 10 min at 37◦C. Next, for phase separation mixture was vortexed and incubated for 1 hour at 37◦C gently, the aqueous phase was taken out OD was observed at 540 nm and H% was calculated according to the following formula (Krausova et al., 2019). Surface Hydrophobicity $H\% = (Ao - At)/Ao \times 100$. Where At and Ao represent the aqueous phase's absorbance and the original suspension absorbance.

2.8 Auto-aggregation and CO-aggregation assay

For this assay MRS broth was inoculated with fresh bacterial cultures and incubated for 14 hours at 37°C through centrifugation for 5 minutes at 6000 rpm cell cultures were being harvested. The pellet was taken in Eppendorf and washed two times with PBS buffer solution and OD was measured at 540 nm, then for 10 secs, this suspension was vortexed and kept for 15 hours at room temperature. After that, absorbance was measured at 540nm by collecting the upper layer (Krausova et al., 2019). Auto- Aggregation % = (Ao-At)/Ao ×100. Where At and Ao mean the OD values at times 5 hours and 0 hours.

The co-aggregation ability (Co-A%) of isolated strains against *E. coli* was performed. First MRS broth was inoculated with fresh bacterial cultures and incubated for 14 h at 37°C, by centrifugation at 6000rpm for 5 minutes, cell pellets were taken and then washed twice with PBS Solution. OD Was measured at 540nm. Fresh *E. coli* was also inoculated in N broth and incubated at 37◦C for 24 hrs. The tested strain and pathogenic strain were taken in equal amounts (2ml) together and vortexed for 10 sec. Each cell suspension of a single strain was labeled as a control. These tubes were placed at room temperature for 5h. OD of (Ax) strains, (Ay) strains and the third one was a mixture (Ax+y) which was measured at 540nm. Co-Aggregation percentage was given by using the following formula: Co-A% = (1- Ax+y)/Ax+Ay/2 ×100. Where x and y represented each of the two strains and (x+y) represented the mixture of strains.

2.9 Biofilm Formation Assay

The ability of biofilm production of all isolated strains was determined in test tubes. Tubes of MRS broth were prepared and inoculated with fresh selected bacterial cultures and incubated at 37°C for three days. After 72 hours, the Media was trashed out and test tubes were washed slowly about three times using distilled water without muddling biofilm present at the bottom of the test tubes (Qian et al., 2021).

2.10 Encapsulated LAB and Yeast Stability and their Impact on Yogurt

2.10.1 Microencapsulation of LAB & Yeast in Alginate Beads

For encapsulation of the selected strain, a fresh bacterial culture was prepared and formed their PBS suspension. Take 20 ml of 3% sodium alginate solution and mix it with 1 ml of bacterial suspension by vortex for 10 seconds. After that fill this suspension in a sterile syringe and extrude drop by drop in 0.1M Calcium Chloride solution. The beads were allowed to stand for 30 min to

ensure complete gelification then beads were harvested through filtration method and the rinsed beads were stored in peptone saline containing 0.05M calcium chloride (Gupta et al., 2020).

2.10.2 Physiochemical Analysis of Prepared Alginate Beads

Microbiological analysis of alginate beads was done in PBS. 1g of alginate beads were dissolved in PBS and spread on MRS agar to measure their growth in cfu/ml. For the chemical stability of beads, 30 blank beads were placed in MRS broth for 1 week after that intact beads were visually observed and recorded (Vaziri et al. 2018). The survival rate in the presence of gastric juice was determined by dissolving 1g of encapsulated probiotics and 1ml of unencapsulated probiotics in pepsin enzyme for 2 hrs, centrifuged and spread on MRS agar plates (Coghetto et al., 2016b; Coghetto et al., 2016a).

The chemical characteristics of the microcapsules were demonstrated using an infrared spectrometer (FTIR) between 4000 and 600 cm^{-1}, using 64 scans at a resolution of 4 cm^{-1}. The FTIR peaks were evaluated separately for every bacterial strain, blank beads, and beads along with bacteria (Brinques and Ayub, 2011).

2.10.3 Experimental Set-Up for Preparation of Probiotic Yogurt

A cow milk was used for the formation of yogurt. Milk was boiled at 90°C, for 10 minutes and cooled at 45°C and separated into seven equal sets (50 ml in each beaker). The experiment was designed with the following conditions. First Set=Control (Commercial yogurt cultures); Second Set = Yogurt with A1 beads; Third Set= Yogurt with A2 beads; Fourth Set = Yogurt with A3 beads; Fifth Set = Yogurt with B1 beads; Sixth Set = Yogurt with B2 beads; Seventh Set= Yogurt with B3 beads. All sets of experiments were incubated for 24 hours until a pH of 4.6-4.8 was attained. All beakers were later placed in cold storage (4◦C) for observation. Where they were stored for 4 weeks. Each week, samples were collected for the interpretation of different parameters.

2.10.4 Physical Analysis of Probiotic Yogurt (pH, Survival rate, Solid Content, WHC)

All samples of yogurt were stored for 3 weeks and physical analysis was done following the methods described by Korkmaz et al. (2021). The pH of all samples was measured on day 1, day 7, day 14 & and day 21 with a pH meter. The survival rate of probiotic bacteria used in the

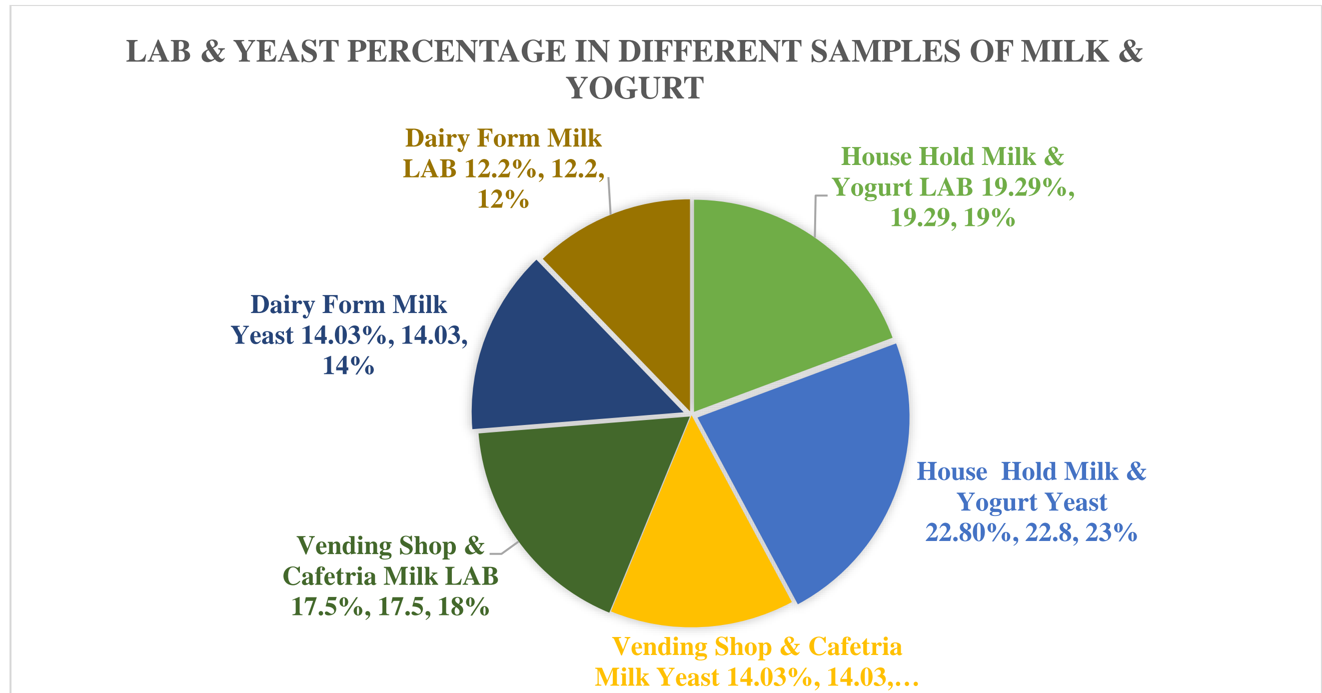
preparation of yogurt was done every week, Samples were serially diluted 10^{-1} to 10^{-7} in sterilized PBS solution and spread over the agar surface. After 24 h cfu/ml was observed. Viable numbers of cells were evaluated 1,7,14 and 21 days of storage.

The solid content was measured by placing yogurt samples overnight at 110°C. The initial weight of all yogurt samples dry weight of all samples were measured. The solid content was calculated by using the following formula: Total solid content = dry weight /wet weight ×100. For the determination of syneresis percentage, 2ml of yogurt was weighed and placed in 3 different tubes and they were placed in a centrifuge machine for 5 minutes at 2000 rpm. After centrifugation, the supernatant was separated and weighted. Syneresis was calculated by the formula: % of Syneresis = Ws/Wy ×100. Here Ws represents the weight of the supernatant after centrifugation and Wy represents the yogurt weight in the tube. The water holding capacity of yogurt samples was measured by centrifugation at 3000rpm at 10 °C for 60 min. After centrifugation supernatant was discarded and the weight of the remaining pellet was taken. Water holding capacity was expressed as the percentage relative to the initial weight of the sample (Korkmaz et al., 2021).

3. Results

3.1 Isolation and Purification of LAB and Yeast

Out of 40 dairy product samples, a total of 57 strains were isolated. Household milk & yogurt contains 19.29% LAB while 22.80% yeast, Vendor shop milk & yogurt contains 17.5% LAB, and 14.03% yeast as well, and Dairy forms milk and yogurt contains 12.2 % LAB and 14.03% Yeast strains. 28 strains were Lactobacillus and 29 strains were yeast (Fig. 1). pH of milk samples was between 6.6-6.9 and yogurt was 4.4-4.6. All the strains were purified and stored at 4°C for further analysis.



3.2 Probiotic potential of Selected strains (Acid, Bile Salt & Pepsin Tolerance)

Tolerance to acidic environments was tested for 57 strains. All strains showed viability in an acidic environment but 6 strains, 3 LAB (A1, A2, A3) and 3Yeast (B1, B2, And B3) were the best strains that showed growth in a highly acidic environment. The strains A1 (94%), A2 (91%), A3 (87%) B1 (64%) B2 (94%) B3(71%%) showed maximum survivability rate in highly acidic conditions (Table 1).

Table1: Probiotic Potential of Selected LAB and Yeast

Strains	pH Tolerance			Bile Salt Tolerance			Pepsin Tolerance
Id	pH2	pH4	pH6	With bile salt	Without bile salt	%age of survivability	
A1	1.193	1.1234	1.545	1.668	1.821	94%	1.921
A2	1.857	1.875	2.422	1.668	1.821	91%	1.989
A3	1.163	0.919	2.799	1.706	1.987	87%	0.921
B1	0.970	1.191	1.944	1.726	2.165	64%	1.286
B2	0.841	2.123	2.354	1.881	1.9321	94%	1.013
B3	1.8177	1.431	2.345	1.415	1.9910	71%	1.996

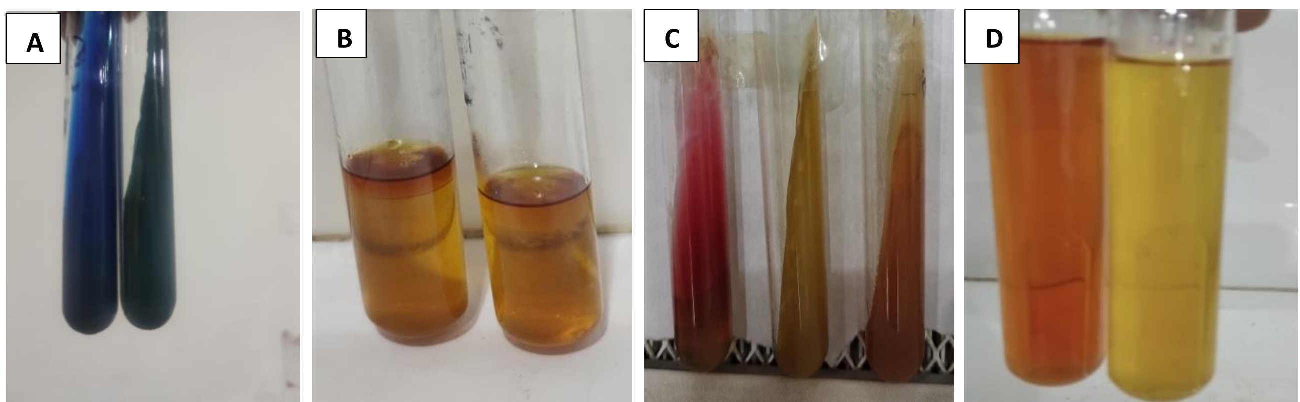
3.3 Biochemical Identification and Optimization of Selected Strains

Lactobacillus strains (A1, A2, A3) with white color and smooth margins were observed. Yeast (B1, B2, B3) with raised colonies with entire margins were observed. Different biochemical tests were performed for the identification of microbial strains. It shows that all strains were gram-positive. Catalase tests were performed to check the ability of strains to degrade hydrogen peroxide. None of the strains could catalyze H_2O_2 by producing catalase enzymes. An oxidase test was performed for the identification of bacteria none of the strains could catalyze oxidase enzyme by producing cytochrome c. A sugar fermentation test was performed and a total of six sugars were used in this test. in the case of dextrose, A2 was unable to ferment the sugar while the rest of the strains fermented this sugar and changed the color medium was observed no gas production. In the case of fructose B1 was unable to ferment the sugar while the rest of all fermented the sugar with no gas production. In the case of sucrose A3 was unable to ferment sugar. In the case of glucose B3 was unable to ferment the sugar. In the case of lactose B2, B3 was unable to ferment sugar. In the case of maltose, all strains were able to ferment. As shown in Table.

Table: Biochemical characterization of Selected LAB and Yeast

Strain	Biochemical tests										Sugar Fermentation test					
	GS	SF	CT	OT	IT	MT	H_2S	MR	SC	TSI	DE	FR	SU	GU	LA	MA
A1		-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
A2	+	-	-	-	-	-	-	-	+	+	-	+	+	+	+	+
A3	+	-	-	-	-	-	-	-	+	+	+	+	-	+	-	+
B1	+	-	-	-	-	-	-	-	+	+	+	-	+	+	+	+
B2	+	-	-	-	-	-	-	-	+	+	+	+	+	+	-	+
B3	+	-	-	-	-	-	-	-	+	+	+	+	+	-	-	+

(**GS**: gram staining, **SF**: Spore formation, **CT**: Catalase test, **OT**: oxidase test, **IT**: indole test, **MT**: Motility test, **MR**: methyl red test, **SC**: Simmons citrate test, **TSI**: triple sugar iron test, **DE**: dextrose, **FR**: fructose, **SU**: sucrose, **GU**: glucose, **LA**: lactose, **MA**: Mannitol)



A: oxidase test **B:** indole test **C, D:** Sugar fermentation test

Table 3: Growth at different temperatures and NaCl concentration

Strains	Growth at different temperatures		Growth at different % of NaCl		
	37◦C	45◦C	2%	4%	6%
Control	0.2134	0.2310	0.213	0.413	0.134
A1	1.9321	1.9842	1.867	1.837	1.921
A2	2.109	1.8910	1.523	1.231	0.981
A3	0.361	1.781	1.671	1.781	1.231
B1	1.336	1.456	1.703	1.221	1.893
B2	1.291	0.875	0.814	1.541	1.435
B3	1.081	1.642	1.462	0.974	1.240

3.4 Antibacterial Activity of selected strains:

Biological screening was performed by agar well diffusion method. For an effective probiotic strain, the strain must have the ability to inhibit the colonization of pathogens in the intestine. 6 selected strains with the best probiotic properties were selected to check their antagonistic activity against 5 different pathogens *(Klebsiella, pseudomonas aeruginosa, Staph aureus, MRSA, and E.coli).* The diameter of the zone of inhibition was varied for different strains.

Table 4: Antibacterial activity of isolates

Strains	*Pseudomonas aeruginosa*	*MRSA*	*E.coli*	*Staphylococcus aureus*	*Klebsiella*
A1	4mm	16mm	12mm	10mm	12mm
A2	12mm	4mm	8mm	4mm	14mm
A3	6mm	14mm	4mm	6mm	10mm
B1	10mm	4mm	2mm	4mm	8mm
B2	4mm	2mm	6mm	10mm	10mm
B3	6mm	6mm	4mm	6mm	6mm

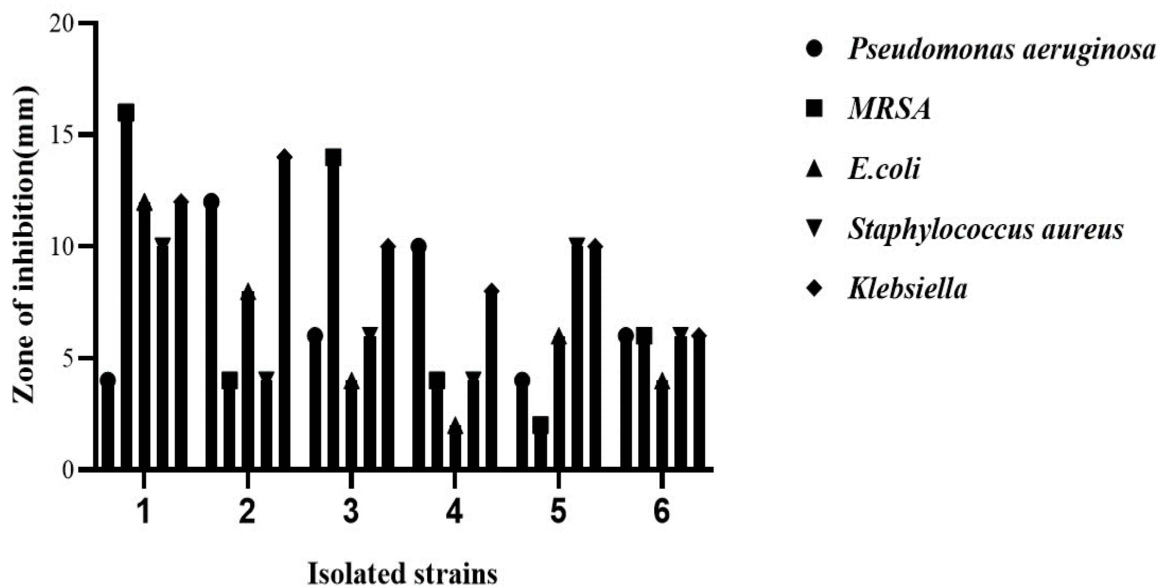
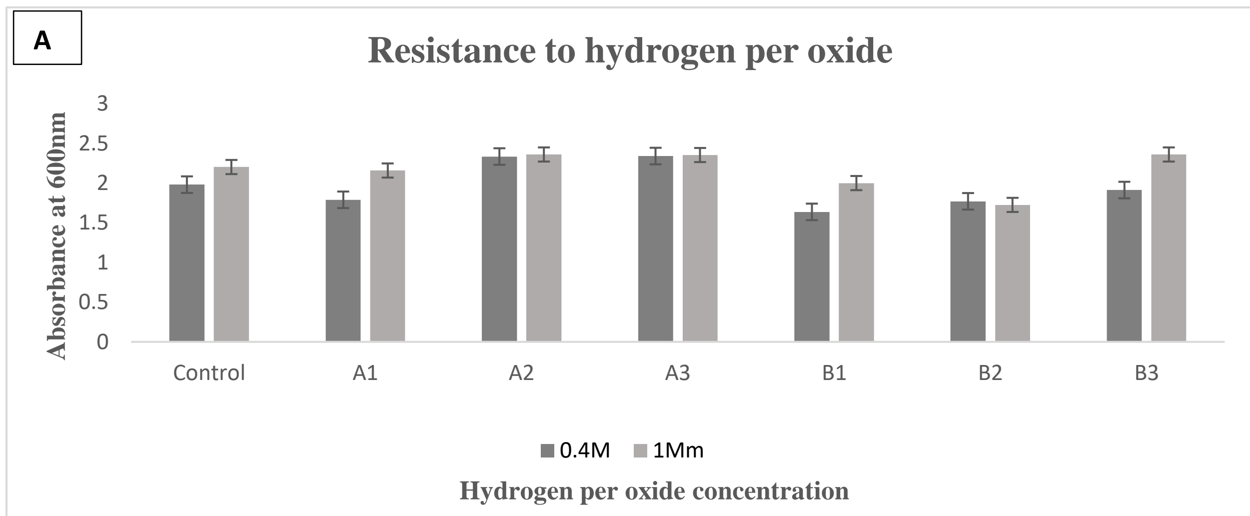


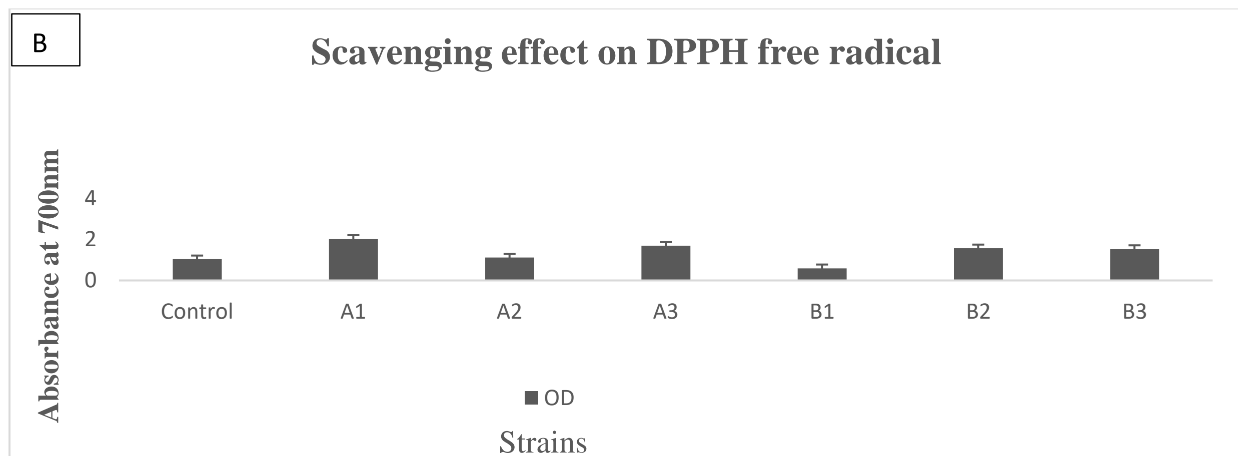
Fig 1. Antibacterial Activity of Strains

3.5 Antioxidant Activity:

Resistance to hydrogen peroxide was determined spectrophotometrically. Two different concentrations (0.4, and 1 mM) were used. Strain A2 and A3 showed maximum growth at both concentrations. Strain A1

and showed maximum growth at 0.4mM. Antioxidants demonstrate their scavenging activity by converting DPPH from its radical form to its non-radical form. Every sample's capacity to scavenge free radicals was compared to Trolox, a positive control or standard.at 517nm. Results showed that Strain A1, A3, and B2 have maximum scavenging activity. while strain B1 has the minimum scavenging activity. The reducing power of the strains was obtained by taking the OD of all strains at 700nm by the FRAP method. This test is used to evaluate the production of free radicals. All strains showed different growth patterns. Maximum growth was shown by the A3 at 40µg/ml. The least growth pattern was observed by A2 at 600µg/ml.





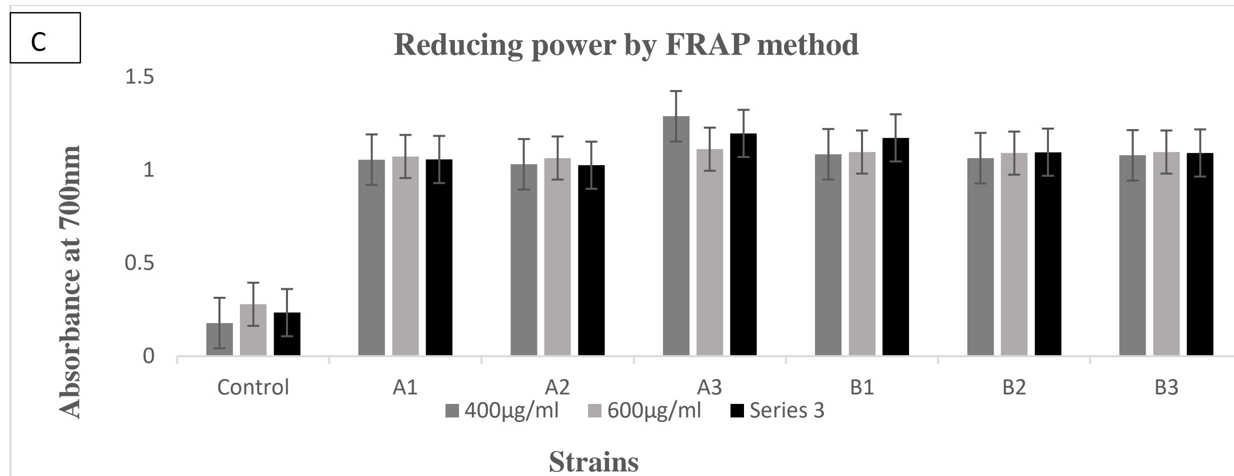


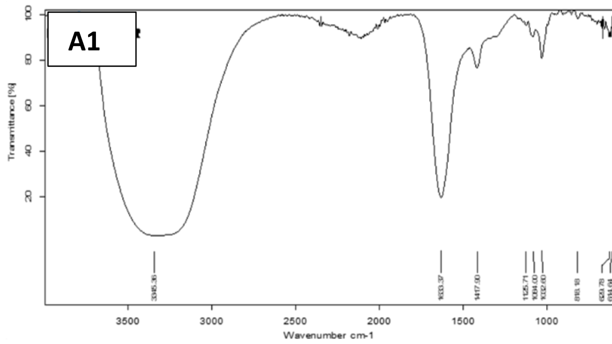
Fig2. Antioxidant assay of Strains A) Resistance at different hydrogen peroxide concentrations (0.4mM, 1mM) incubated at 37°C for 24 h. B) Scavenging effect on DPPH free radical C) Reducing power by taking OD at 700 nm after incubation at 37°C.

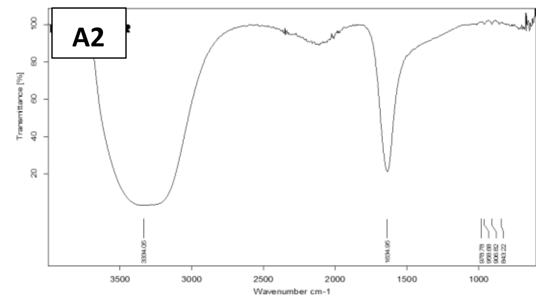
Fig 3. Encapsulated beads

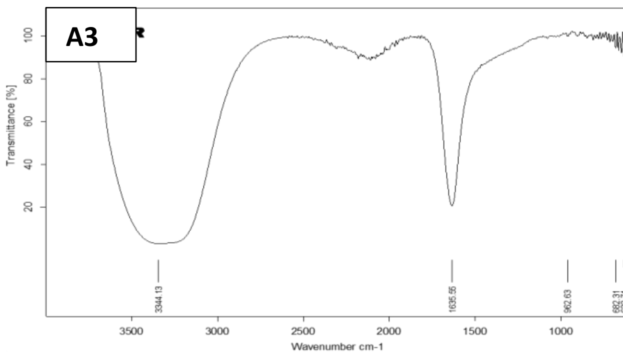
3.6 FTIR analysis of encapsulated beads

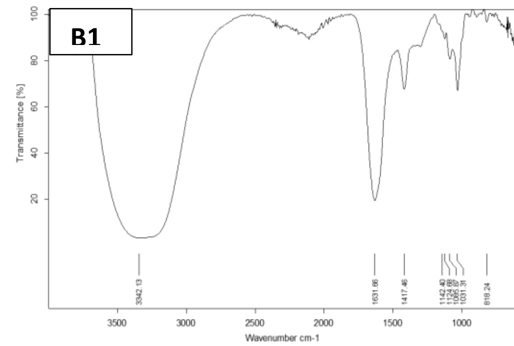
Lactobacillus and yeast were encapsulated using sodium alginate as a core material for the formation of yogurt. FTIR peaks were verified for all microbial strains. Beads with lactobacillus strain and yeast. The relative FTIR spectra of lactobacillus and yeast are in the range of 400-4000cm. the intensity of peaks was weak, medium, and strong. All strains containing peak 1 were

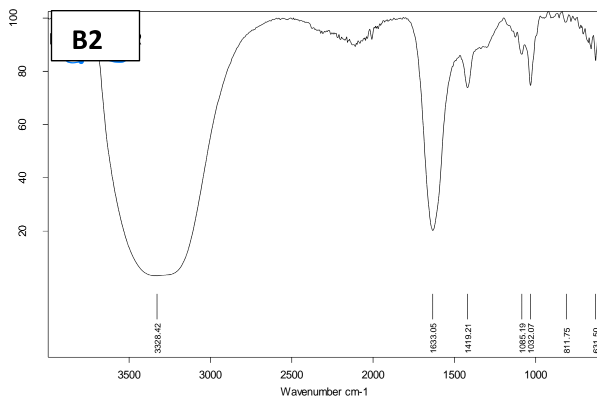
positioned between 1630-1640cm^{-1}. which indicates the C=O amide group. And Peak 2 was positioned between 3340-3350cm^{-1} which indicates the C-H Alkyne.











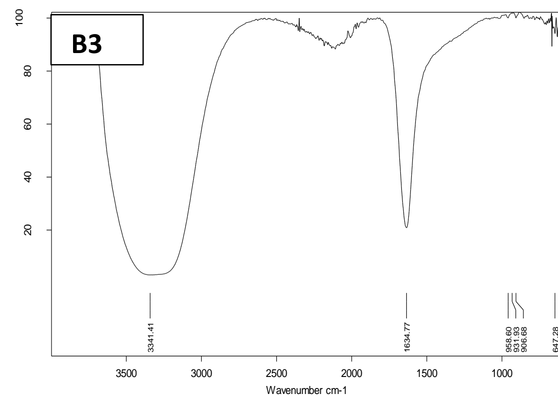


Table 7: FTIR Analysis of microencapsulation of LAB and yeast

Sample	Major IR peaks	Bond Present	Class present	Intensity
A1	1064	C-OH	Alcohol	Medium
	1125	C-O-C	Ethers	Medium
	1417	O-H	hmediumxyl	Medium
	1633	C=O	Amide	Medium
	3345	C-H	Alkyne	Strong
A2	1634	C=O	Amide	Medium
	3334	C-H	Alkyne	Strong
A3	1635	C=O	Amide	Medium
	3344	C-H	Alkyne	Strong
B1	1031	C-OH	alcohol	Medium
	1124	C-O-C	Ethers	Medium
	1417	O-H	hydroxyl	Medium
	1631	C=O	Amide	Medium
	3342	C-H	Alkyne	Strong
B2	1032	C-OH	alcohol	Medium
	1085	C-OH	alcohol	Medium
	1419	0-H	hydroxyl	Medium
	1633	C=O	Amide	Medium
	3328	C-H	Alkyne	Strong
B3	1634	C=O	Amide	Medium
	3341	C-H	Alkyne	Strong

3.7 Probiotic Yogurt Formation

By using 6 different probiotic strains in a single set yogurt was made. Yogurt was kept under observation for 4 weeks at 4 °C. During storage, different tests were observed after one week. The yogurt samples were evaluated for fermentation time, pH, microbiological analysis, total solid content syneresis, and water holding capacity. The difference of Yogurt with lactobacillus strains had less pH as compared to yogurt with yeast strains. During storage differences ($P<0.01$) were observed. Yogurt with a viable no of cells remained the same in both cases *Lactobacillus* and yeast

beads. Yogurt samples were analyzed for solid content and water-holding capacity syneresis. The solid content was less in the case of lactobacillus strains and yeast as compared to yogurt prepared by commercial starters. It is affected by the acid produced by the strains. As *Lactobacillus* produced higher acid. Syneresis of yogurt was observed during storage for 21 days. During Storage difference (p<0.01) was observed. The decrease in the values of synergetic yogurt was because of the absence of supplementary solids.

Table 8: pH and microbial analysis of Yogurt at different time intervals:

Strain	pH				Microbial analysis		
	Day 1	**Day 7**	**Day 14**	**Day 21**	**Day1**	**Day 14**	**Day 21**
A1	4.9±1	4.2±0.9	4.0±1	4.0±1	$2.\times10^{-2}$±0.5	2.0×10^{-2}±0.3	3.2×10^{-2}±0.3
A2	4.5±0.9	4.4±0.8	4.2±0.9	4.0±0.9	3.5×10^{-2}±0.3	3.5×10^{-2}±0.5	3.6×10^{-2}±0.3
A3	4.8±0.8	5.0±1	4.6±0.8	4.3±0.8	2.2×10^{-2}±0.2	4.1×10^{-2}±0.5	4.3×10^{-2}±0.3
B1	5.6±1	5.0±1	4.6±1	4.5±0.7	3.8×10^{-2}±0.5	3.6×10^{-2}±0.5	3.3×10^{-2}±0.2
B2	4.6±1	4.9±0.9	4.6±0.9	4.4±1	2.5×10^{-2}±0.5	3.7×10^{-2}±0.5	3.9×10^{-2}±0.2
B3	4.8±0.9	4.9±0.9	4.6±0.8	4.4±0.8	3.8×10^{-2}±0.5	3.6×10^{-2}±0.5	4.0×10^{-2}±0.2
control	4.5±0.8	4.0±1	3.6±0.7	3.4±0.7	4.2×10^{-2}±0.5	4.6×10^{-2}±0.5	5.2×10^{-2}±0.2

(The same values did not differ significantly (P<0.01))

Table 9: Total solid content and synergizes of yogurt at different time intervals

Strain	Total solid content				Synergetic			
	Day 1	**Day 7**	**Day 14**	**Day 21**	**Day1**	**Day7**	**Day14**	**Day21**
A1	45%	55%	57%	69%	57.3±0.5	54.2±0.5	57.2±0.5	58.0±0.5
A2	59%	68%	71%	74%	34.4±0.5	32.3±0.21	29.0±0.5	25.0±0.5
A3	38%	45%	57%	63%	45.7±0.5	46.3±0.5	48.6±0.2	49.0±0.2
B1	47%	52%	56%	67%	56.5±0.21	57.8±0.5	55±0.2	50.1±0.2
B2	43%	47%	49%	55%	50.3±0.32	55.4±0.5	56.7±0.2	57.0±0.2
B3	50%	54%	57%	62%	47.6±0.31	49.5±0.31	50.2±02	52.0±0.2
Control	39%	42%	44%	45%	45.0±0.31	47.3±0.32	51.9±0.2	53.0±o.2

A significant difference (P<0.01) was observed. The same values do not differ significantly (p<0.01)

4. DISCUSSION

Probiotics are considered as non-pathogenic living microorganisms that, taking in abundant quantities, confer a health advantage on consumers. *Lactobacillus* and B*ifidobacterium* are the widely researched probiotic microorganisms. Probiotics can create a variety of antimicrobial chemicals, including lactic and acetic acids, ethanol, bacteriocins, and other antimicrobial molecules like reuterin (Sen, 2019). Microbes such as bacteria, yeasts, and molds are common microflora in mainly lactic acid bacteria traditional and ethnic fermented foods mainly lactic acid bacteria. *yeast, Saccharomyces cerevisiae*, is an inevitable part of fermented foods, drinks, and traditional alcoholic beverages (Rabetafika, Razafindralambo, Ebenso, & Razafindralambo, 2023). Current epidemiological research suggests that dairy items can lower illness risk and improve, health, and improve life. Identifying these gaps can serve as a foundation for future study, to make better understand the possible health advantages of fermented dairy foods (Marco et al., 2021). In this study, a total of 40 samples were obtained from Multan city of Punjab Pakistan. A total of 25 strains were isolated. A total of 25 strains were isolated of which 6 with the best probiotic properties were selected (Mulaw, Sisay Tessema, Muleta, & Tesfaye, 2019). Have the ability to grow in acidic environments in the presence of bile salt and gastric juice resistance. And also able to grow under Stress conditions (high temperature and low and high salt concentration) (Wolfe et al., 2023).

A biochemical test was performed on strains having high probiotic potential. It was observed that none of the strains could catalyze catalase, oxidase, and citrate enzymes. Negative results were obtained for H_2S production. Oxidation test was also performed. All strains showed negative results. A sugar fermentation test was performed and a total of six sugars were used in this test. in the case of dextrose, A1 was unable to ferment the sugar while the rest of the strains were fermented. In the case of sucrose A2 was unable to ferment the sugar while the rest of all fermented the sugar with no gas production. In the case of fructose B1, B2, and A1 were unable to ferment sugar. In the case of maltose A2, A3, B1, and B3 were unable to ferment the sugar. An indole test was done and all strains were indole-negative (Bazireh, Shariati, Azimzadeh Jamalkandi, Ahmadi, & Boroumand, 2020). Bacteriocins are protein-containing antimicrobial chemicals generated on the ribosomes of certain bacteria. It was observed that all selected strains were able to produce bacteriocin(Gaspar et al., 2018). Probiotic bacteria also can remove the biofilm of pathogenic bacteria 90% of the strains were able to form strong biofilm. Strong biofilm was observed in *yeast*

as compared to *lactobacillus (Tarrah et al., 2019).* The hydrophobicity assay determines how repellent a molecule is to water, It plays a vital role in protein binding. The hydrophobicity assay of the 6 strains ranged from 34% to 74%. The highest cell surface hydrophobicity was exhibited (Li, Liu, Dong, Zhou, & Wang, 2015). Encapsulation is an innovative food production process that enables the preservation and control of bioactives. Extrusion is the most popular and simple technique for microencapsulation. Alginate is the most often utilized polymer for encapsulation because of its outstanding gelling capabilities. Alginate can create three-dimensional structures with calcium and magnesium ions to generate microbeads (Azam et al., 2022). Microencapsulation has been studied to improve the viability of tested microorganisms (Rajam & Subramanian, 2022).

Probiotic yogurt was prepared with selected encapsulated strains of *lactobacillus* and yeast with higher qualities of probiotics. Yogurt was observed for pH after different intervals of time from day 1 to 21day. Probiotic yogurt prepared with a commercial starter requires less fermentation time as compared to yogurt. Similarly, yogurt prepared by the yeast strains had less fermentation time as compared to yogurt prepared by *Lactobacillus.* pH of the yogurt was observed at different time intervals at 7,14,21 days during storage. pH of the yogurt was decreased due to acidification. Yogurt with lactobacillus strains had less pH as compared to yogurt with yeast strains. It can be shown that pH activity can be due to the residual activity of micro-organisms. Yogurt with a viable no of cells remained the same in both cases *Lactobacillus* and yeast beads. A significant decrease ($p<0.01$) was demonstrated same results (Hossain, Keidel, Hensel, & Diakité, 2020). Yogurt samples were analyzed for solid content and water-holding capacity syneresis. The solid content was less in the case of lactobacillus strains as compared to yogurt prepared by yeast and commercial starters. It is affected by the acid produced by the strains. As *Lactobacillus* produced higher acid also prepared probiotic yogurt

Conclusion

From this study, it was concluded that all the strains have the best probiotic properties and are used for yogurt formation. which plays a significant role in a variety of fermentation processes. They ferment carbohydrates that exist in food, with the main end product of fermentation being lactic acid. LAB and yeast strains are primarily used as starting cultures for a wide range of fermented dairy, meat, fish, fruit, vegetable, and cereal products.

Conflict of interest

The authors declare no conflict of interest.

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Author's Contribution

SI.: conceptualization, designing, investigation, and writing the original draft. **AI.:** Validation and writing. **SS.:** formal analysis, data curation, and writing. **AI:** data curation, critical review, and editing. **SB.:** validation, review, and editing. All authors have read and agreed to the published version of the manuscript.

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