Isolation and in vitro evaluation of Potential Probiotics from Traditional Fermented Foods of Goa

Dissertation submitted to Goa University for partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

By

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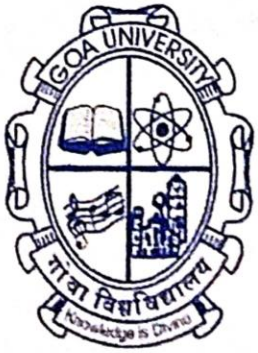
Under the guidance of

Dr. Samantha Fernandes D'Mello



SCHOOL OF BIOLOGICAL SCIENCES AND BIOTECHNOLOGY

GOA UNIVERSITY

DECLARATION

I hereby declare that the data presented in this Dissertation entitled, "**Isolation and in vitro evaluation of Potential Probiotics from Traditional Fermented Foods of Goa"** is based on the results of investigations carried out by me in the Discipline of Biotechnology at the School of Biological Sciences and Biotechnology, Goa University under the Supervision of Dr. Samantha Fernandes D'Mello and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations / experimental or other findings given in the dissertation.

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CERTIFICATE

This is to certify that the dissertation report "**Isolation and in vitro evaluation of Potential Probiotics from Traditional Fermented Foods of Goa**" is a bonafide work carried out by Ms. Siddhi Ramesh Tembkar under my supervision/mentorship in partial fulfilment of the requirements for the award of the degree of Master of Science in Biotechnology at the School of Biological Sciences and Biotechnology

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CONTENTS

List of abbreviations

%	**Percentage**
°C	**Degrees Centigrade**
CFU/mL	**Colony forming units per milliliters**
cm	**Centimeters**
FAO	**Food and Agricultural Organization**
g	**Grams**
g/L	**Gram per liter**
GIT	**Gastrointestinal tract**
h	**Hour(s)**
L	**Litre**
m	**Metre**
mg/L	**Milligram per litre**
mg/mL	**Milligram per milliliter**
min	**Minute(s)**
mL	**Milliliter**
mm	**Millimeter**
mM	**Millimolar**
nm	**Nanometer**
OD	**Optical density**
PBS	**Phosphate buffered saline**
pH	**Hydrogen ion concentration**
WHO	**world health organization**
TCBS Agar	**Thiosulfate Citrate Bile Salt Sucrose**

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INTRODUCTION

CHAPTER 1: INTRODUCTION

Fermented foods are a rich source of beneficial microorganisms which play an important role in converting raw food components into edible products through enzymatic actions. It can be defined as 'foods and beverages produced through enzymatic actions of beneficial microorganisms under controlled growth parameter' (Dimidi et al., 2019)'.

Fermented foods can be classified based on the raw materials used during their preparation. Briefly it is classified into lentil based, plant sap and crude solar salt. Goan fermented foods and beverage include sur, nero, gauthi mith, tor, sukhe bangde, madda feni, kaju feni, salted fish (Furtado et al., 2020).

Some examples of fermented foods include tempeh, kombucha, sauerkraut, sourdough bread, miso, etc. which have proven to boost immunity, improve gastrointestinal health. Kefir which is one of the most studied fermented foods showed beneficial effect in lactose malabsorption. (Dimidi et al., 2019).

Fermented foods contain live microorganisms called probiotics which when administered in a required amount have proven to boost human health by improving digestion, enhancing immunity and combating gut pathogens, (Leeuwendaa et al., 2022). Their mode of action includes competing for receptors, exclusion of pathogens, production of bacteriocins, production of different enzymes which affects the growth of Enterobacter (Gill et al., 2019). It has been observed that intake of probiotics also reduces risk of high cholesterol, diabetes, hypertension, etc. The bioactive products formed during fermentation have high concentration of vitamins such as Riboflavin, folate, vitamin K which helps in controlling blood pressure levels. (Sanlier et.al.2019).

Most of the fermented foods have Lactic acid bacteria as probiotics, mainly *Lactobacillus Bacillus, Bifidobacterium, Pediococcus,* etc. as probiotic microorganisms (Soemarie et al 2020). Probiotics reported from Goan fermented food *viz Khazrache Boll or Bollo* includes several yeast species like *S. cerevisiae* (Pereira et al 2020).

Lentil and cereal-based fermented foods are good alternatives against dairy based fermented foods for the lactose intolerant population. Lentils and cereals are rich in carbohydrates, fiber, proteins, vitamins, minerals, and their high protein content makes them a better alternative for probiotic use (Verni et al., 2020). Yogurt alternatives produced by using lentils and cereals showed good antioxidant capacity and high levels of antifungal phenolic compounds (Boeck et al., 2013).

The present study focusses on isolating and evaluating potential probiotic organisms from cereal and lentil-based traditional fermented foods of Goa.

LITERATURE REVIEW

CHAPTER 2: LITERATURE REVIEW

Probiotics are live microorganisms which when administered in a required amount have proven to boost human health by improving digestion, enhancing immunity and combating gut pathogens (Leeuwendaa et al 2022). In 2001, United Kingdom's Food and Agriculture Organization along with World Health Organization, defined probiotics as 'live microorganisms when taken in appropriate amount gives health benefit to the host' (FAO/WHO ,2002). Probiotics have applications inwide domains which includes aquaculture, livestock, poultry, to treat disease or different disorders like nervous system disorders. These probiotic supplements benefit their host by hindering the growth of pathogenic bacteria, secreting enzymes which helps in digestion, colonization to the gut epithelial cells which hinders attachment of pathogens to the gut lining and interacting with gut microbiota to improve health, producing antibacterial compounds like bacteriocin (Raheem et al 2021, Karimi et al 2018).

Probiotics should be safe to the host including humans and animals, should have resistance to antibiotics, capacity to survive in adverse intestine conditions like high pH and bile salt concentrations. (Diaz et al 2019).

2.1 Probiotic benefits to the host;

2.1.1 Improving Epithelial barrier

Epithelial barrier is a major defense barrier which protects the host from the environment and with the direct contact to pathogens. These include epithelial junction adhesion complex, production of antimicrobial agents and secretion of immunoglobulin A (Diaz et al 2019).

Most pathogens at primary stage of infection require to attach to the mucosa lining of the gut. Probiotic microorganisms attach to the active site of the mucosa lining hence there is no place for the attachment of pathogens. Therefore, attachment to the mucosa ling is an important criterion while choosing probiotic strain. This can be determined by the hydrophobicity test called bacterial adhesion to hydrocarbons.

2.1.2. Production of antimicrobial agents

Probiotic microorganisms produce metabolites which includes hydrogen peroxide, lysozymes, proteases amylases, lipases etc. Lactic acid bacteria produce bacteriocins which are proven to have inhibitory activity against pathogens (Fernandes et al 2019**).** Antagonist activity also includes autoaggregation and coaggregation of probiotic strain, reduction of pathogens to intestine (Choi et al 2018).

2.1.3. Extracellular enzymes production

Many enzymes are produced by probiotics bacteria. Amylase production is important as it carries out hydrolysis of starch to form small oligosaccharides (Padmavathi et al 2018). Proteases cleaves proteins like casein into smaller peptides which enables them to move across cytoplasmic membrane. Lipase breaks lipids into a simpler form which helps in absorption by the human body (Manasian et al 2020).

2.2 In vitro tests for the selection of probiotic

2.2.1. Bile salts and pH tolerance;

Small intestine has a high concentration of bile salt. Therefore, it is important that the probiotic bacteria should tolerate the bile salt concentration which enables them to survive in the gut. Different lactobacilli strains are studied for bile salt survival. Bile tolerant bacteria are a good model for understanding the molecular mechanisms involved in bacterial tolerance to bile salts. (Ruiz et al 2013).

Stomach contains acid which can drop up to pH 2, which makes it important criteria for probiotic organisms to survive in the stomach. The pH in the body ranges from 2 to 7.8.it can be determined by the method in which isolates are incubated at different pH concentrations followed by measuring optical density (Yadav et al 2016).

2.2.2 Enzyme production

Many enzymes are produced by probiotic bacteria. Amylase production is important as it carries out hydrolysis of starch to form small oligosaccharides (Padmavathi et al 2018). Most of the studies are done on lactic acid bacteria for amylase production, some of these includes, *Lactobacillus amylovorus*, *Lactobacillus plantarum*, *Lactobacillus manihotivorans*, *Lactobacillus fermentum* (Padmavathi et al 2018).

Proteases cleaves proteins like casein into smaller peptides which enables them to move across cytoplasmic membrane. *Lactobacillus sanfranciscensis* is an important strain which is

widely used in the baking industry for production of bread especially from wheat (Kieliszek et al 2021).

Lipase breaks lipids into simpler forms which helps in absorption by the human body. Lactobacillus *rhamnosus GG* and *Bifidobacterium longum NCC 2705* are examples of protease producing bacteria (Manasian et al 2020).

2.2.3 Cell surface hydrophobicity

Cell surface hydrophobicity is the property of the cell by which they determine to live in either aqueous solution or hydrophobic surrounding. Those bacteria that are hydrophobic in nature will choose to live in hydrophobic environment and others which are water loving, will grow in an aqueous environment. Hydrophobic activity of the cell depends on the surface proteins and the receptors exposed. These proteins might not be always exposed. The physiological conditions, nutritional conditions and growth parameters determine the availability of the surface proteins. It can be measured by bacterial adhesion to hydrocarbons (toluene, xylene, n-hexane.) Those having more cell surface hydrophobicity will remain attached to the hydrocarbons whereas those that are hydrophilic will move to the aqueous environment (Danchik & Casadevall 2021).

2.2.4 Antimicrobial activity

Probiotic bacteria have a property of binding to the intestine cell and hence they compete with the pathogenic bacteria for the same. Most probiotic can produce compounds like bacteriocin,

a secondary metabolite produced by certain bacteria which acts against potential pathogens and hinders their growth (Karimi et al 2018). *Enterococcus faecalis* UGRA10, *Enterococcus faecium* AL41, *Enterococcus faecium* CLE34, *Lactococcus lactis* DPC3147 are some examples of probiotics producing bacteriocin (Hernández et al 2021). Another mechanism of antimicrobial property involves production of exopolysaccharides. Evidences includes different *Lactobacillus* bacteria *like L. planturam, L. garvieae* who are proven to inhibit both gram negative and gram-positive bacteria (Abdalla et al 2021).

2.2.5. Gastric acid tolerance

Gastric juice is produced in the stomach which mainly contains enzymes like pancreatin and pepsin that helps in digestion of food. Other than this pH in the stomach drops to around 2.5-3 during digestion. Hence it is important that the probiotic microorganisms should survive in gastric juice. Gastric acid tolerance is a test to measure the survival rate of bacteria in stimulated gastric acid juice. *Lactobacillus brevis* is an example of probiotic bacteria produced from pickle (Tokatl et al 2015).

2.2.6. Cellular aggregation

Most of the bacteria attach themselves by receptors on their cell surface. This allows attachment of the same bacteria to each other and the property is known as autoaggregation or cellular autoaggregation. This helps in forming conization in the intestine and their attachment to the epithelium (Nath et al 2021).

2.2.7. Antioxidant Activity by DPPH Free Radical Scavenging Activity

Oxidative stress can show adverse effects on cells such as DNA hydroxylation, lipid paraoxidation and can even lead to apoptosis. Hence it is important to reduce the effect of oxidative stress. Probiotics have proven to reduce oxidative stress by showing antioxidant activity (Wang et al 2017).

2.2.8 Phenol tolerance

Gut Bacteria have the ability to deaminate aromatic amino acids which leads to the production of phenol (Yadav et al 2016) that may have a role in cancer (Bone et al 1976). A potential probiotic should exhibit the tolerance to phenol to survive, which can be tested by inoculating it in 0.4% of phenol and measuring its growth. (Yadav et al 2016).

2.2.9 Safety assessment tests

2.2.9.1 Hemolytic activity

Hemolysis test is a safety assessment test which checks if the hemolytic activity of the bacteria. Bacteria induce erythrocyte lysis or lysing of red blood cells which are responsible to carry oxygen blood through production of hemolysins, which bacteria require for their growth and hence harmful if ingested. Therefore, it is important that a good probiotic must be devoid of hemolytic activity (Shaw et al 2017).

2.2.9.2 Gelatinase production

Collagen is a primary building material of bone, skin, ligaments in animals. When collagen degrades gelatin forms. Some bacteria produce gelatinase, an enzyme that degrades gelatin which results in disruption of bone density and other health issues. It is important that probiotic organisms should no produce gelatinase enzyme (Liu et al 2015)

TABLE 1: Some examples of probiotics

Microorganisms reported as probiotics	Source	Mode of action	References
Lactobacillus. planturam	dough	Production of extracellular enzyme amylase	Padmavathi et al., 2018
Lactobacillus brevis	pickle	Survival in gastric acid and pepsin tolerance	Tokatl et al., 2015
Lactobacillus reuteri	Human breast milk	pH tolerance	Łubiech et al., 2020
Weissella confusa	Sour rice	Pancreatin tolerance	Nath et al., 2021
Lactobacillus plantarum	Fish and pork	Cholestrol lowering	Sirilun et al., 2010
Saccharomyces cerevisiae	-	Antibacterial effects	Igbafe et al., 2020
Bifidobacterium longum	Baby faeces isolate	Antioxidant activity	Stevens et al., 2017

2.3 Fermented foods

Fermented foods are a rich source of beneficial microorganisms which play an important role in converting raw food components into edible products through enzymatic actions. Fermented foods contain probiotics.

Traditionally fermentation practices are carried out in Goa. Some examples of Goan fermented food and beverages include sur, nero, feni, suke bangde, chepni tor which have great pH

tolerance, antioxidant effects, etc.

Saccharomyces Cerevisiae DABRP5 obtained from Khazrache boll showed good gastric juice tolerance, extracellular enzyme production and pancreatin tolerance. Khazrache boll is a Goan traditional fermented food which is made by grinding wheat, fresh coconut, coconut jaggery with toddy. (Pereira et al 2021)

Fugiche pole is a Goan fermented food which is prepared by soaking rice and urid dal overnight and grinding with water to make fine paste. This mixture is kept for fermentation and then cooked. Sanna is prepared by soaking rice overnight and grinding with fresh coconut and toddy. Fermentation of this batter is carried out for 7-8 h and then steamed.

Nachni ambil is prepared by adding buttermilk to ragi flour. This mixture is fermented overnight and then boiled.

AIM

To isolate and screen for potential probiotic microorganisms from lentil and cereal-based traditional fermented foods of Goa.

OBJECTIVES

1. Isolation of microorganisms from traditional cereal and lentil based fermented foods of Goa.
2. In-vitro screening of the isolated microorganisms for probiotic potential.
3. Partial characterization and safety assessment of the potential isolates.

MATERIALS AND METHODS

CHAPTER 3:MATERIALS AND METHODS

3.1 ISOLATION OF POTENTIAL PROBIOTIC MICROORGANISMS

3.1.1 Isolation of bacteria from Fugiche pole

40 grams of rice was mixed with 10 grams of urid dal, soaked overnight and ground to get a fine paste. Following overnight fermentation, the sample was serially diluted and plated on Nutrient agar plates to obtain colonies at zero hour, three hours and six hours (Schoenborn et al., 2004). The plates were incubated at 37℃ for 24 h. The number of isolates obtained was noted and the colony morphology of the unique colonies was recorded.

3.1.2 Isolation of bacteria from Nachni ambil

A total of 20 grams of ragi flour was fermented with 40 mL buttermilk for 6 hours. Sample was serially diluted and plated on Nutrient agar plates at zero hour, three hours and six hours of fermentation. The plates were incubated at 37℃ for 24 h. The number of isolates obtained was noted and the colony morphology of the unique colonies was recorded.

3.1.3 Isolation of bacteria from Sanna

50 grams of rice was soaked overnight and then ground with 70 grams of fresh coconut and 200 mL of toddy to obtain a paste. Fermentation was carried out for 6 hours and the sample was serially diluted and plated 24 h. The number of isolates obtained was noted and the colony

morphology of the unique colonies was recorded on Nutrient agar plates at zero-hour, three hour and six hours. The plates were incubated at 37℃ for 24 h.

3.1.4 Isolation of bacteria from Khazrache boll

40 grams of wheat was ground with coconut jaggery, fresh grated coconut and toddy to obtain a fine paste. Sample was serially diluted and plated on Nutrient agar plates at zero hour, six hours and three hours to get colonies. The plates were incubated at 37℃ for 24 h. The number of isolates obtained was noted and the colony morphology of the unique colonies was recorded.

3.2 Screening for potential probiotic organisms:

The morphologically unique isolates that were obtained from the traditional fermented foods were further screened for their probiotic potential in vitro.

3.2.1 Enzyme production (Fernandes et al., 2019)

A total of 96 isolates were evaluated for their ability to produce enzymes that could promote food digestion in humans.

3.2.1.1 Amylase production

The isolated cultures were spot inoculated on minimal agar plates (appendix) supplemented with 1% starch and incubated at 37 °C for 24 hours. Lugol's iodine was used to identify starch degradation. The diameter of zone of clearance was measured in mm.

The isolated culture was spot inoculated on minimal agar plates (appendix) supplemented with 1% casein agar. Plates were incubated at 37 °C for 24 hours and then flooded with Coomassie brilliant blue. The diameters of zones of clearance were measured in mm.

3.2.1.2 Protease production:

The isolated cultures were spot inoculated on 1% casein agar plates and incubated at 37°C for 24 hours to get a zone of clearance. The diameters of the zones were measured in mm.

3.2.1.3 Lipase production:

The isolated cultures were spot inoculated on 1% tributyrin agar plates and incubated at 37°C for 24 hours to get a zone of clearance. The diameters of the zones were measured in mm.

15 cultures exhibiting highest zones of clearance and more than one enzyme production, were further used to screen for antioxidant activity, antimicrobial activity and ability to survive in the harsh conditions of the gastrointestinal tract.

3.2.2 Antioxidant Activity by DPPH free radical scavenging activity (Pereira et al., 2020)

To test for anti-oxidant potential of the isolates, 1 mL of overnight grown cultures was centrifuged at 5000 rpm for 15 minutes. Pellets were washed with PBS twice and resuspended in 0.8 mL of DPPH (1,1-diphenyl-2-picrylhydrazyl) reagent and vortexed. These tubes were incubated at 37 °C for 30 minutes. The initial absorbance at time zero and final at 30 minutes was recorded at 517 nm. The scavenging activity was calculated using the formula;

$$scavanging\ activity = \left(\frac{ODcontrol - ODsample}{ODcontrol}\right) \times 100$$

3.2.3 Preliminary Antimicrobial testing by cross-streak method (Karimi et al 2018)

To assess the antimicrobial potential of the selected isolates, the cultures were cross-streaked against two environmental pathogens. The environmental pathogens were isolated from sewage water by plating on EMB agar, TCBS agar and MacConkey agar.

The selected potential probionts were streaked on Muller Hiltons Agar plates by cross-streak method and incubated at 37 °C overnight. The isolated environmental pathogens (*E. coli and Vibrio* sp.) were cross-streaked perpendicularly to check for antimicrobial activity. Plates were incubated at 37 °C for 24 hours. Bacterial inhibition along the line of streak indicated antimicrobial activity (measured in mm).

3.3. Assessing the ability of the isolates to survive in the gastrointestinal tract

3.3.1 pH and bile salt tolerance

The test for pH and bile salts is an essential parameter to assess if the potential probiotic can survive the acidic pH of the stomach and the strong bile salts of the gastrointestinal tract. The isolates were individually inoculated in Nutrient broth with pH 2, 5 and 7.8 and incubated overnight at 37 °C. The optical density was measured at 600 nm (Fernandes et al., 2019)

Similarly, the overnight grown fresh cultures were inoculated in Nutrient broth supplemented with 0%, 3% and 6% of bile salt mixture (Himedia RM009) and incubated overnight at 37°C. Bacterial growth was determined by measuring the absorbance at 600 nm. (Choi et al., 2018).

3.3.2 Hydrophobicity testing

Hydrophobicity test was performed to evaluate the adhesive property of the isolates in the gut. The isolates were grown overnight in Nutrient broth and centrifuged to obtain a pellet. The pellet was resuspended and washed twice with 1 X PBS.

1 ml of the suspension was taken and equal number of hydrocarbons were added to it (toluene and n-hexadecane). This suspension was vortexed and left undisturbed for 1 hour. After 1-hour, the aqueous solution was removed and the absorbance was measured at 600 nm (Pereira et al., 2021).

Percent hydrophobicity was calculated as follows:

$$\%\, Hydrophobicity = (1 - \frac{ODfinal}{ODinitial}) \times 100$$

3.3.3. Gastric juice tolerance

To assess the tolerance of the cultures to gastric enzyme pepsin, the isolates were exposed to gastric juice tolerance test. It was determined by inoculating the selected isolates in stimulated gastric juice which was supplemented with pepsin. Stimulated gastric juice was prepared by adding 7 mM KCl, 45 mM $NaHCO_3$ and 125 Mm NaCl. The pH of the juice was adjusted to 3, followed by addition of 3 g/L pepsin. The potential isolates that were grown overnight in Nutrient broth were centrifuged, resuspended in PBS and added to the gastric juice-pepsin medium. The tubes were incubated for 24 hours at 37 °C and the absorbance was recorded at 600 nm (Nath et al 2019).

3.3.4 Cellular autoaggregation

To check the bacterial binding ability, the isolates were grown overnight in Nutrient broth and were centrifuged at 5000 rpm for 10 minutes. The pellet obtained was washed twice with PBS (pH 7.2). The initial absorbance was noted at 600nm. The cultures were incubated at 37 °C for 2 hours and the absorbance was noted at 600 nm (Nath et al 2019).

$$\%\, autoaggregation = (\frac{ODfinal - ODinitial}{ODinitial}) \times 100$$

3.3.5 Phenol tolerance

To check phenol tolerance, the isolates were grown overnight in Nutrient broth and were centrifuged at 5000 rpm for 10 minutes. The pellet obtained was washed twice with PBS (pH 7.2) 004% phenol was incubated for 24 hours at 37 °. Final absorbance was noted at 600 nm.

3.4 Safety assessment

3.4.1 Hemolysis test

To test if the isolates produce hemolysin toxin that could lyse blood cells, hemolysis test was performed on human blood agar. The selected isolates were spot inoculated on human blood agar plates and incubated at 37°C for 24 hours. A clear hemolysis zone round the bacterial colony indicated β-hemolysis, no change in agar around the colony was recorded as ɣ-hemolysis. (Fernandes et al., 2019).

3.4.2 Gelatinase production

Gelatin is a degraded form of collagen. Presence of gelatinase gene is an indicator of virulence To check the ability of the isolates to produce gelatinase that could degrade gelatin and harm the bones, gelatinase production test was performed. Gelatin agar butts were prepared and cultures were stab inoculated in it and incubated at 37 °C for 48 hours. To check gelatinase production, tubes were refrigerated for 1 hour. Those who showed liquification are gelatinase positive (Pereira et al 2021).

3.4.3 Antibiotic susceptibility test

Antibiotic susceptibility test was performed by disc diffusion method to evaluate the presence of antibiotic resistance strains. Ten different antibiotic discs *viz.* chloramphenicol(30µg), Kanamycin(30µg), Streptomycin(10µg), Metronidazole(10µg), Ciprofloxacin (5 µg), Penicillin (10 µg), Erythromycin (15 µg), Tetracycline (30 µg), Lincomycin (10µg), Polymyxin (10 µg) were placed on lawn of selected isolates grown on Muller Hiltons agar and incubated at 37 °C for 48 hours (Fernandes et al., 2019). The strains were categorized as susceptible, intermediate and resistant as recommended by the Clinical and Laboratory Standards Institute (CLSI),2013.

3.4.4 Biocompatibility test

To assess the ability of the isolates to survive together as a mixed consortium, the potential isolates were streaked on Nutrient agar plates in the center and the other cultures were cross streaked perpendicularly against it. The plates were incubated at 37 °C for 48 hours (Fernandes et al., 2019).

3.5 Morphological and biochemical characterization of the isolates

3.5.1 Cell morphology

The isolates were Gram stained using standard protocol as described by Tripathi et al 2022.Briefly, a smear was prepared and heat fixed. Crystal violet was added and kept for 1 minute followed by adding iodine for 1 minute and decolorizing with Gram's decolorizer followed by applying counter stain, safranin for 1 minute (Tripathi et al 2022).

SEM Analysis: A thick smear of the samples was prepared on 1×1 cm slide and was incubated overnight with 2.5% glutaraldehyde followed by ethanol wash of 50%, 60%, 70%, 80%, 90% for 10 minutes each and lastly with 100 % ethanol for 30 minutes. Samples were sputter coated with gold and magnified images obtained (Evo 18, Carl Ziess, Germany) with magnification from 2 KX TO 40 KX.

3.5.2 Biochemical test kit (Himedia)

Biochemical test was carried out as per manufacturer's instructions. KB009, HiCarbo™ biochemical test kit was used for 5 selected isolates.100µl of the previously overnight grown culture was added in each well and test kit was incubated at 37 °C for 24 hours

RESULTS AND

DISCUSSION

CHAPTER 5: RESULTS AND DISCUSSION

5.1. Isolation of microorganisms from Goan fermented foods.

Total of 141 colonies were isolated out of which, 36 colonies were isolated from Fugiche pole (SP), 75 from Nachni ambil (SN), 20 from sanna (SS) and 10 from Khazrache boll (SB). All the isolates showed different colony characteristics.

5.1.1 Isolation of microorganisms from Fugiche pole

36 colonies were isolated from Fugiche pole. Colony characteristics for all the colonies was recorded. Some of the representative colony characteristics are noted below:

TABLE 2: Colony characteristics of the representative colonies from Fugiche pole (SP)

	SP1	**SP2**	**SP3**
color	White	White	White
margin	Umbonate	Entire	Entire
texture	Smooth	Smooth	Slimy
elevation	Raised	Pulvinate	Flat
consistency	Slimy	Slimy	Smooth
Size	0.4 cm	0.2 cm	0.5 cm
Opacity	Opaque	Opaque	Translucent
form	Irregular	circular	Irregular

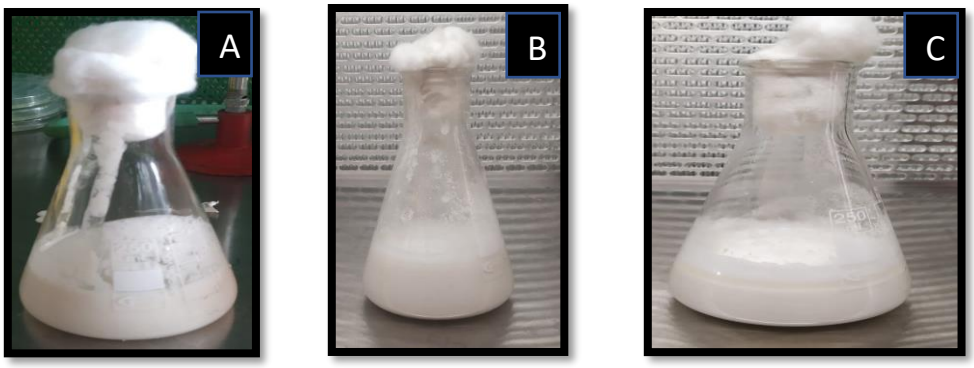

Fig. 1: Flask with fermentation of Fugiche pole batter at (A) Zero h (B) Three h (C) Six h

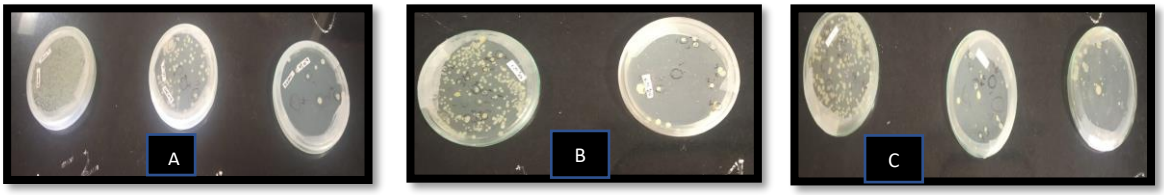

Fig. 2: Plates spread plated with sample at (A) Zero hour (B)Three (C) Six hours

5.1.2 Isolation of microorganisms from Nachni ambil

Total 75 colonies were isolated from Nachni ambil. Colony characteristics of all the colonies were performed. Some of the colony characteristics are listed below:

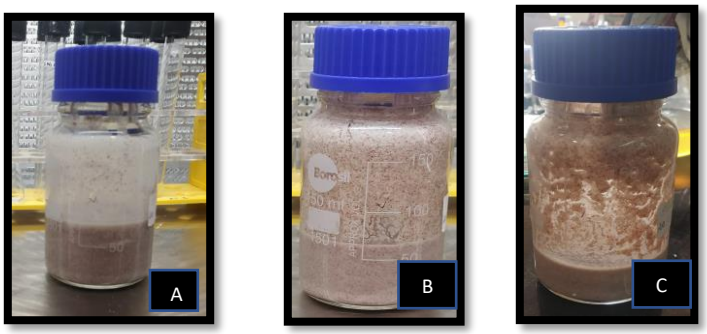

Fig. 3: Reagent bottles showing fermentation of Nachni ambil batter at (A)Zero hour (B)Three hours (C)Six hours

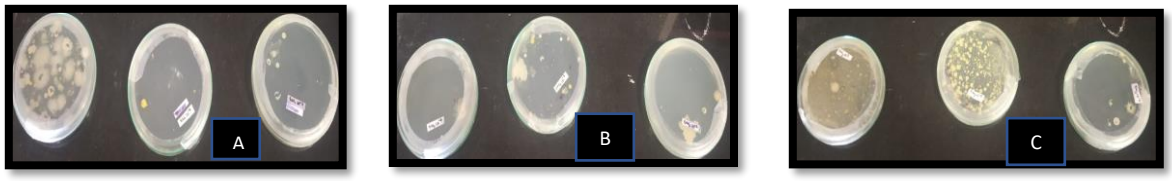

Fig. 4: Plates spread plated with sample at (A) Zero hour (B)Three hours (C)Six hours

TABLE 3: Colony characteristics of representative colonies from Nachni ambil

	SN1	**SN2**	**SN3**
color	White	crème	White
margin	Entire	Entire	Entire
texture	rough	wrinkled	Slimy
elevation	flat	Pulvinate	convex
consistency	Slimy	grainy	grainy
Size	2.8cm	0.2cm	0.8cm
Opacity	Opaque	Opaque	opaque
form	Irregular	circular	circular

5.1.3 Isolation of microorganisms from Sanna

A total of 20 colonies were obtained from Sanna batter. Colony characteristics of all the colonies were noted. Some of the colony characteristics of representative isolates are listed in Table 4.

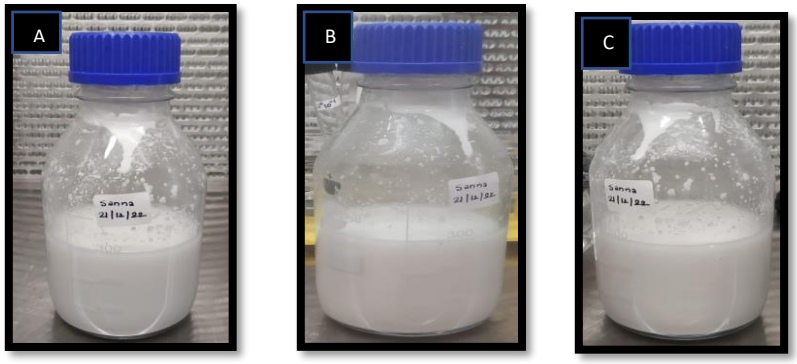

Fig. 5: Reagent bottle showing fermentation of Sanna batter at (A)Zero hour (B)Three hours (C) Six hours.

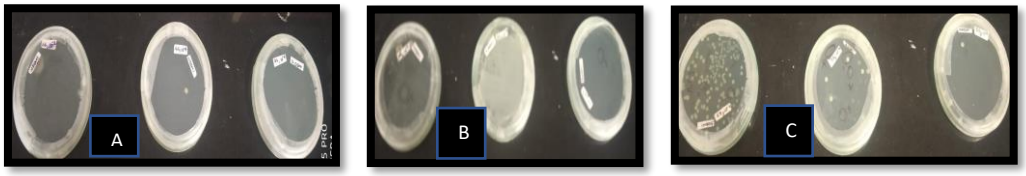

Fig. 6: Plates spread plated with sample at (A) Zero hour (B)Three hours (C)Six hours

TABLE 4: Colony characteristics of representative colonies from Sanna batter.

	SS1	**SS2**	**SS3**
color	Crème	White	White
margin	Curved	Entire	Erased
texture	Mucoid	Smooth	Slimy
elevation	flat	Pulvinate	convex
consistency	Slimy	grainy	rough
Size	0.7 cm	1.3 cm	0.2 cm
Opacity	Opaque	Opaque	opaque

5.1.4 Isolation of microorganisms from Khazrache boll

Total 10 colonies were obtained from Khazrache boll. Colony characteristics of all the colonies was studied. Some of the colony characteristics of representative colonies are listed below.

Fig. 7: Reagent bottles showing fermentation of Khazrache boll batter at (A)Zero hour (B)Three hours (C) Six hours

TABLE 5: Colony characteristics of representative colonies.

	SB1	**SB2**	**SB3**
color	Crème	White	White
margin	entire	Erased	Erased
texture	Smooth	Smooth	Slimy
elevation	flat	Pulvinate	convex
consistency	Slimy	Shiny	rough
Size	0.3cm	-	0.2cm
Opacity	Opaque	Opaque	opaque
form	circular	punctiform	circular

5.2 Screening of potential probiotic organism

5.2.1 Extracellular enzyme production

Spot inoculation of cultures showed the ability of the isolates to produce extracellular enzymes *viz.* amylase, protease and lipase. A total of 30 isolates showed extracellular enzyme production, out of which, 17 showed amylase production, 10 showed lipase production and 10 isolates showed protease production. Among these, SN16 showed highest amylase production with 21.6 ± 3.21 mm zone of clearance. SS6 showed highest protease production with 20.6±1.52 mm zone of clearance. Maximum lipase production was observed in SN49 with 18.0 ± 1.0 mm zone of clearance.

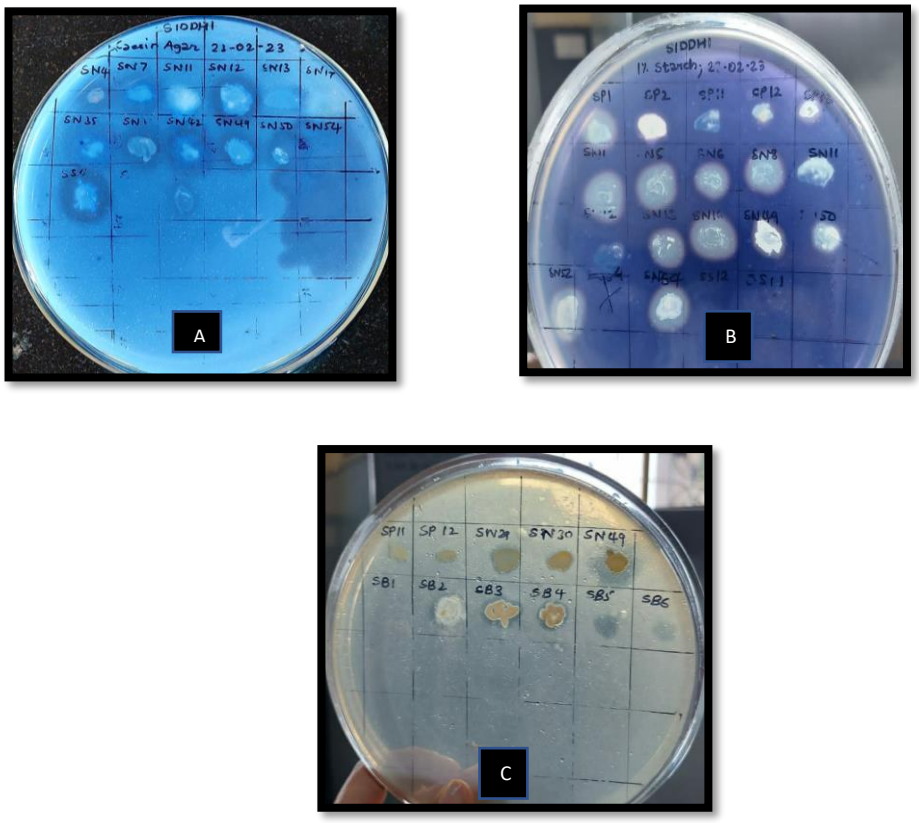

Fig. 8: Plates showing extracellular enzyme production (A)Amylase production, (B) Protease production, (C)Lipase production

Table 6: Extracellular enzyme production of selected isolates determined by measuring the zone of clearance in (mm)

Sr. no	cultures	Amylase	Protease	Lipase
1	SP1	18.6±0.57	N. D	N. D
2	SP2	11.6±1.5	N. D	N. D
3	SP11	11.0±1.5	N. D	8.3±1.15
4	SN12	7.3±2.8	N. D	6.0±1.0
5	SP13	11.3±0.5	N. D	N. D
6	SN1	21.6±1.5	N. D	N. D
7	SN5	19.6±0.5	N. D	N. D
8	SN6	17.3±0.5	N. D	N. D
9	SN8	19.6±1.15	N. D	N. D
10	SN11	15.0±2.64	13.0±1.0	N. D
11	SN12	13.0±1.0	17.3±0.57	N. D
12	SN13	15.3±1.15	13.0±1.0	N. D
13	SN16	21.6±3.21	N. D	N. D
14	SN49	14.0±2.0	15.6±1.52	18.0±1.0
15	SN50	13.3±1.15	N. D	N. D
16	SN51	20.0±3.6	N. D	N. D
17	SN54	19.6±1.15	N. D	N. D
18	SN4	N. D	12.6±2.50	N. D
19	SN7	N. D	17.6±0.57	N. D
20	SN35	N. D	14.0±1.15	N. D
21	SN36	N. D	12.6±1.15	N. D
22	SN42	N. D	19.6±2.08	N. D
23	SS6	N. D	20.6±1.52	N. D
24	SN29	N. D	N. D	12.6±0.57
25	SN30	N. D	N. D	8.0±1.75
26	SB2	N. D	N. D	14.6±1.15
27	SB3	N. D	N. D	14.6±2.08
28	SB4	N. D	N. D	13.0±0.57
29	SB5	N. D	N. D	10.6±0.57
30	SB6	N. D	N. D	7.6±0.57

Where “N.D” represents “Not detected”

5.2.2Antioxidant activity by DPPH free radical scavenging activity

Antioxidant test is the test which shows the ability of the bacteria to survive against free radical oxygen species and reduce their harmful effects on the host. This was determined by DPPH free radical scavenging activity. Out of the 15 isolates that were screened, SN42 showed maximum scavenging activity of 96.24%; while the least activity was observed by SN49 with 9.39 ± 0.001%. Table 7 reports the antioxidant activity of the selected 15 isolates.

TABLE 7: Antioxidant activity (%) of the selected isolates.

Colony	**Scavenging activity (%)**
SP1	-
SN1	90.9 ± 0.003
SN5	95 ± 0.001
SN8	-
SN12	73 ± 0.003
SN16	26 ± 0.004
SN49	9.39 ± 0.001
SN51	32.33 ± 0.001
SN54	27.81 ± 0.001
SN7	-
SN42	96.24 ± 0.002
SS6	31.95 ± 0.002
SB2	47.3 ± 0.02
SB3	15.7 ± 0.001
SB4	-

5.2.3 Preliminary Antimicrobial testing by cross-streak method

Environmental pathogens were isolated from sewage water samples. Yellow colonies of *Vibrio* sp. was observed when plated on TCBS agar, while *E. coli* showed green metallic sheen on EMB agar.

When culture was cross streaked on the plate with the environmental pathogens,they did not show antimicrobial activity against the selected pathogens. Antimicrobial activity of the cultures is the property by which they inhibit the growth of the potential intestinal pathogens. Hence protecting the host. None of the tested cultures showed antimicrobial effect against the selected environmental pathogens.

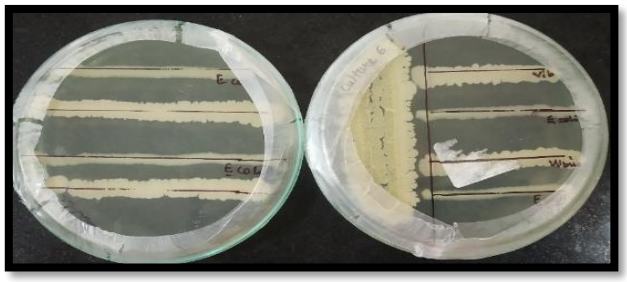

Figure 10: Plate showing negative results for antimicrobial activity against environmental pathogens *E. coli* and *Vibro* sp. by cross-streak method.

Figure 11: Picture showing isolated environmental pathogens (A) *Vibrio* sp. (B)*E. coli*

5.3 Assessing the ability of the isolates to survive in the gastrointestinal tract

5.3.1 pH tolerance and bile salt tolerance

The gastrointestinal tract contains a wide range of pH as well as high concentration of bile juices. Therefore, it is an important criterion for the selection of potential probiotic strain. The probiotic bacteria should be able to survive in the high concentration of bile juices and wide range of pH. 15 isolates were selected who gave more than one extracellular enzyme productions. Out of 15 screened cultures, all survived in the bile salt concentration ranging from 0% to 6%. and wide range of pH from pH from 2 to 7.8.

TABLE 8: Table showing pH tolerance, bile salt tolerance **(Absorbance at 600 nm)**

cultures	Bile salt tolerance (600 nm)			pH tolerance (600 nm)		
	0%	3%	6%	2	5	7.8
SP1	++	+	-	-	+++	+++
SN1	+	+	+	-	+	+
SN5	++	++	+	-	+++	+++
SN8	+++	++	+	-	-	-
SN12	+	+	+	-	-	-
SN16	+	+	-	-	++	++
SN49	+	+	++	-	+	-
SN51	+++	+++	+	-	-	-
SN54	++	+	+	-	++	+
SN7	+	+++	+	-	-	+
SN42	+	+	+	+	+++	+++
SS6	+++	+	+	-	+	-
SB3	+	+	+	-	-	-
SB3	+	+	+	-	-	-
SB4	+	+	+	+	-	-

O.D. <0.05: indicated as "- "; O.D. between 0.051 to 0.10: "+ ", O.D between 0.11 to 0.20 "++", absorbance > 0.21= "+++"

5.3.2 Hydrophobicity testing

Cell surface hydrophobicity is an ability of bacteria to depend on their surface receptor proteins. This property helps them to stay attached to the epithelial layer of the intestine and restrict the adhesion of the pathogens. Percent hydrophobicity was calculated by the formula mentioned in methodology. 15 isolates were screened for cell surface hydrophobicity, out of which SN1 showed highest % hydrophobicity with 89% followed by SN42 with 42%, whereas SB2 showed least % hydrophobicity of 43%.

TABLE 9: Cell surface hydrophobicity of the selected isolates.

Sr. No	Isolate	% hydrophobicity (%)
1	SP1	61.1$^{\mathbf{M}}$
2	SN1	89$^{\mathbf{H}}$
3	SN5	75$^{\mathbf{H}}$
4	SN8	77$^{\mathbf{H}}$
5	SN12	46$^{\mathbf{M}}$
6	SN16	54$^{\mathbf{M}}$
7	SN49	66$^{\mathbf{M}}$
8	SN51	51$^{\mathbf{M}}$
9	SN54	64$^{\mathbf{M}}$
10	SN7	62$^{\mathbf{M}}$
11	SN42	80$^{\mathbf{H}}$
12	SS6	87$^{\mathbf{H}}$
13	SB2	43$^{\mathbf{M}}$
14	SB3	59$^{\mathbf{M}}$
15	SB4	50$^{\mathbf{M}}$

Where, % hydrophobicity between 66.66 - 100 % indicates 'high hydrophobicity – superscripted as **H**'; between 33.37 %to 66.66% indicates medium hydrophobicity – superscripted as **M**, 0% to 33.36% indicates low hydrophobicity – Superscrip **L.**

5.3.3 Gastric juice tolerance

One of the primary criteria for selection of potential probiotic microorganism is to survive the extreme conditions in the stomach. As stomach contains pepsin, an enzyme which helps in digestion; and pH ranging between 2 to 3, it is important that the probiotic should survive in this harsh environment.

15 isolates were selected to perform gastric juice tolerance test, out of which SN 8 showed highest growth in stimulated gastric juice.

TABLE 10: Ability of selected isolates to survive the simulated gastric juice

Sr.no	sample	Gastric juice tolerance (OD at 600 nm)
1	SP1	+
2	SN1	+
3	SN5	++
4	SN8	+++
5	SN12	+
6	SN16	+
7	SN49	+
8	SN51	++
9	SN54	++
10	SN7	++
11	SN42	++
12	SS6	++
13	SB2	++
14	SB3	++
15	SB4	+

"+" indicates growth showing OD between **0.01 to 0.1**; **"++"** indicates growth showing absorbance between **0.11 to 0.20, "+++"** indicates growth showing absorbance between **0.21 – 0.4,** after 24h.

5.3.4 Cellular autoaggregation

Cellular aggregation allows microorganisms to colonize in the intestine and attach with each other to form a layer that adheres to the gut epithelial. Hence, not allowing pathogens to adhere to the gut epithelial.

15 isolates were selected for the test. It was seen that the % auto-aggregation increased with SN49 giving highest 1962.8% autoaggregation (TABLE 11).

TABLE 11: Cellular autoaggregation of the selected isolates.

Sr. No.	Isolate	% auto-aggregation
1	SP1	90.4
2	SN1	53.6
3	SN5	267.6
4	SN8	230
5	SN12	131
6	SN16	275.43
7	SN49	1962.8
8	SN51	405.7
9	SN54	325.7
10	SN7	355
11	SN42	485.7
12	SS6	378
13	SB2	146
14	SB3	296
15	SB4	241

5.3.5 phenol tolerance

Phenol tolerance enables to check the probiotic survival in the gut, as some microorganisms deaminate food particles to produce phenol which can produce a toxic effect to the host.

15 isolates were selected to perform phenol tolerance. SN5 exhibited highest phenol tolerance

TABLE 12: phenol tolerance of selected isolates (Absorbance 600 nm)

Sr. No	Isolates	Phenol tolerance at 600nm
1	SP1	+
2	SN1	++
3	SN5	+++
4	SN8	+
5	SN12	+
6	SN16	+
7	SN49	+
8	SN51	+
9	SN54	++
10	SN7	+
11	SN42	+
12	SS6	+
13	SB2	+
14	SB3	+
15	SB4	+

At 600 nm, **"-"** indicates **no growth** or growth with **OD <0.05**; **"+'** indicates growth with absorbance between **0.051 to 0.10**, **'++"** indicates growth with absorbance between **0.11 to 0.20**, '+++**"** indicates absorbance **> 0.21.**

5.4 Safety assessment

5.4.1 Hemolysis activity

Some microorganisms produce hemolysin toxin which can lyse the cell and can give toxic effects to the host. Hemolysis activity is the preliminary test performed to select the potential probiotic which are non-toxic to the host.

15 isolates were selected to perform hemolysis test. 4 isolates showed complete hemolysis (β- hemolysis) which was determined by the zone of clearance.11 isolates did not exhibit hemolysis (α- hemolysis). None of the isolates showed partial hemolysis (γ- hemolysis).

TABLE 13: Hemolytic activity of the selected isolates

Sr. No	Isolates	Type of hemolysis
1	SP1	No (ɣ) hemolysis
2	SN1	No (ɣ)hemolysis
3	SN5	Beta hemolysis
4	SN8	No (ɣ) hemolysis
5	SN12	No (ɣ) hemolysis
6	SN16	No (ɣ) hemolysis
7	SN49	No (ɣ) hemolysis
8	SN51	No (ɣ) hemolysis
9	SN54	Beta hemolysis
10	SN7	Beta hemolysis
11	SN42	Beta hemolysis
12	SS6	No (ɣ) hemolysis
13	SB2	No (ɣ) hemolysis
14	SB3	No (ɣ) hemolysis
15	SB4	No (ɣ) hemolysis

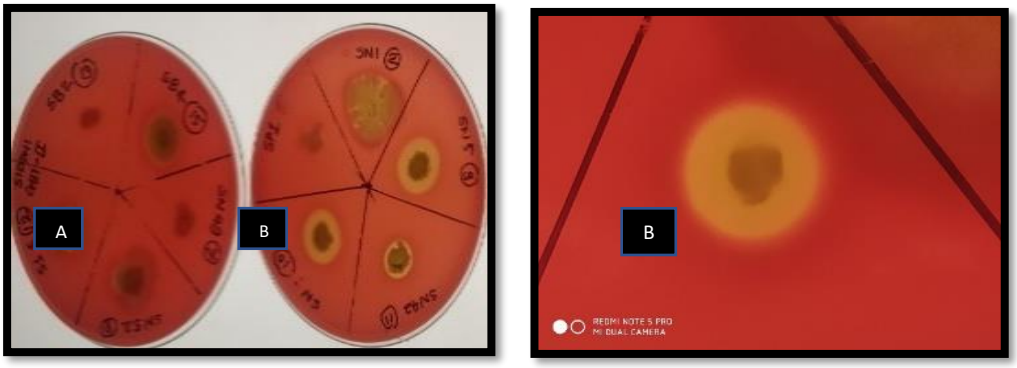

Fig 12: Plates showing (A) No or ϒ hemolytic activity (no zone of clearance) and (B) plate showing beta hemolysis (to the right) indicated by clear zone of clearance.

Five best isolates which did not give hemolytic activity and exhibited probiotic potential, were chosen for further screening.

5.4.2. Gelatinase production

5 best isolates were screened for gelatinase production. Some microorganisms have the ability to produce virulence through gelatinase production Hence these isolates are not fit for consumption.

None of the culture showed positive test for gelatinase production.

Fig. 13: Image depicting negative test for gelatinase production

5.4.3 Antibiotic susceptibility test

Antibiotic susceptibility test was performed by disc diffusion method to evaluate the presence of antibiotic resistance strains. Ten different antibiotic discs *viz.* chloramphenicol (30µg), Kanamycin (30µg), Streptomycin (10µg), Metronidazole (10µg), Ciprofloxacin (5 µg), Penicillin (10 µg), Erythromycin (15 µg), Tetracycline (30 µg), Lincomycin (10µg), Polymyxin (10 µg) were used.

TABLE 14: Antibiotic susceptibility of the selected isolates.

ANTIBIOTICS	SN1	SN16	SN49	SN51	SS6	SENSITIVITY CRITERION (mm)
Chloramphenicol	31^{s}	29.1^{S}	21.5^{S}	27.8^{S}	27.8^{S}	S (≥18), I (13 - 17), R (≤12)
Kanamycin	17.3^{I}	17.6^{S}	19.5^{S}	18^{S}	16.5^{I}	S (≥18), I (14 - 17), R (≤13)
Streptomycin	16.6^{S}	18.8^{S}	22.5^{S}	18^{S}	21.6^{S}	S (≥15), I (12 - 14), R (≤11)
Polymyxin	ND	ND	ND	ND	ND	No interpretative criteria
Metronidazole	ND	ND	ND	ND	0.88^{R}	S (≥21), I (16 - 22), R (≤16)
Ciprofloxacin	20.5^{I}	ND	17.1^{I}	ND	16.33^{I}	S (≥21), I (16 - 20), R (≤15)
Penicillin	26.5^{S}	ND	16.8^{I}	ND	12.1^{R}	S (≥22), I (12 - 21), R (≤12)
Erythromycin	26^{S}	ND	20.3^{I}	ND	19^{S}	S (≥23), I (14 - 22), R (≤13)
Tetracycline	25.3^{s}	ND	27.6^{S}	ND	22.3^{S}	S (≥15), I (12 - 14), R (≤11)
Lincomycin	ND	ND	ND	ND	15.1^{R}	S (≥22), R (≤15)

"R" indicates resistance, "I" indicates intermediate and 'S" indicates sensitivity, ND indicates Not detected (No zone of clearance = sensitive) according to Clinical and Laboratory Standards Institute (CLSI),2013

Fig. 14: Antibiotic susceptibility test of SS6 by disc diffusion method.

5.4.4 Biocompatibility

Biocompatibility of bacteria was determined by cross streak method.5 isolates were chosen for this test. It was seen that the bacteria did not inhibit the growth of each other when cross streaked.

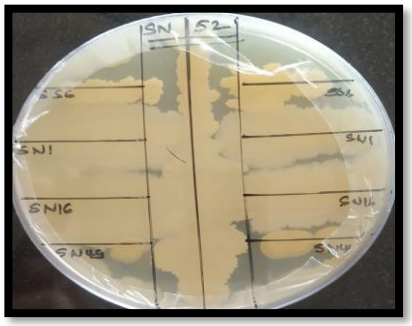

Fig. 15: Plate showing biocompatibility of the selected isolates

5.5 Morphological and Biochemical characteristics of the isolates

5.5.1 Cell morphology and colony characteristics

Colony morphology of the 5 isolates were studied and noted below.

TABLE 15: Colony characteristics of 5 isolates

Colony	**SN1**	**SN16**	**SN51**	**SN49**	**SS6**
Color	White	Creme	Crème	Crème	White
Margin	Entire	Erase	Curved	Entire	Undulate
Elevation	Flat	Pulvinate	raised	Flat	raised
Texture	Rough	Wrinkled	Rough	Smooth	rough
Consistency	Grainy	Smooth	Grainy	Slimy	slimy
Size	28mm	4mm	4mm	2mm	16mm
Opacity	Opaque	Opaque	opaque	Opaque	opaque
Form	Irregular	circular	irregular	circular	irregular

5.5.2. Gram staining and SEM analysis

Gram staining of the 5 isolates was performed to study the cell wall characteristics. The characters have been represented in Table 8.

TABLE 16: Gram characteristics of the 5 potential probiotic isolates

Isolate	**Gram character**
SN1	Gram positive rods
SN16	Gram positive rods
SN49	Gram positive cocci
SN51	Gram positive cocci
SS6	Gram positive rods

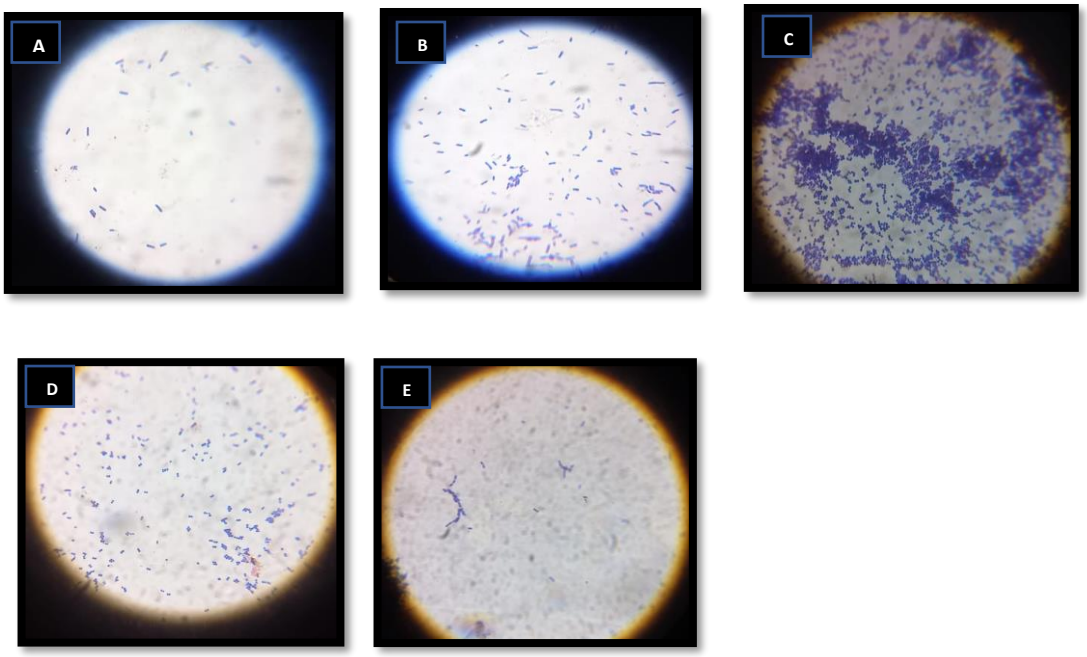

Fig.16: Study of Gram characteristics (A) SN1 (B) SN16 (C) SN49 (D) SN51 (E) SS6

SEM Analysis

Fig. 17 represents the morphology of the best 3 cultures (isolated from Nachni ambil and Sanna), showing maximum positive results for probiotic potential.

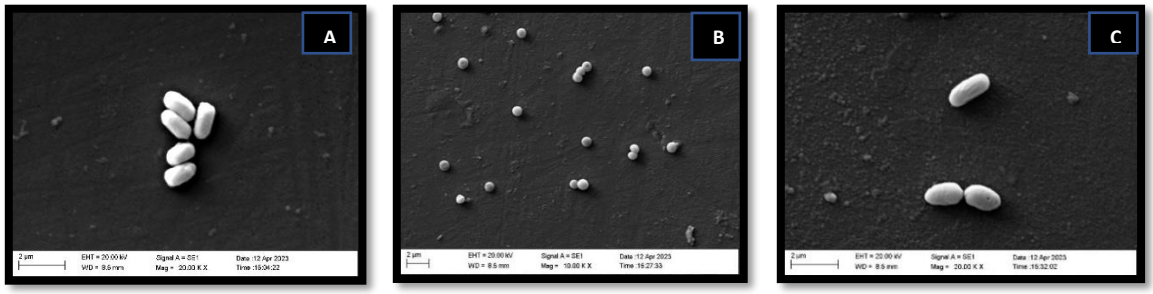

Fig. 17: Morphology of the best 3 isolates studied by Scanning Electron Microscope (A) SN1, (B)SN49, (C)SS6

5.5.2 Biochemical tests

Biochemical test KB009 (Himedia) was used to test carbohydrate utilization by the isolates. Lactose, Xylose, Maltose, Fructose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, LArabinose, Mannose, Insulin, Sodium gluconate, Glycerol, Salicin, Dulcitol, Inositol, Mannitol, Adonitol, rabitol, Erythritol, Alpha-Methyl-D-Glucoside, Rhamnose, Cellobiose, Melazitol, alpha-methyl-D mannoside Xylitol, ONPG, Esculin hydrolysis, D-Arabinose, Citrate utilization, Malonate utilization, Sorbose are the sugars available for detection in the kit. Isolates showed the results as shown in Table 17.

TABLE 17: Carbohydrate utilization of selected isolates

Sr. No	SUGARS	SN1	SN16	SN51	SN49
1	**Lactose**	+	±	±	+
2	**Xylose**	+	+	+	+
3	**Maltose**	+	+	+	+
4	**Fructose**	+	±	±	+
5	**Dextrose**	+	+	+	+
6	**Galactose**	+	+	+	+
7	**Raffinose**	+	+	+	+
8	**Trehalose**	+	+	+	+
9	**Melibiose**	+	+	+	+
10	**Sucrose**	+	±	±	+
11	**l-arabinose**	+	+	+	±
12	**Mannose**	+	±	±	+
13	**Insulin**	±	±	±	-
14	**Sodium gluconate**	-	-	+	-
15	**Glycerol**	±	±	±	+
16	**Salicin**	-	+	+	±
17	**Dulcitol**	-	-	-	-
18	**Inositol**	±	±	±	-
19	**Sorbitol**	-	-	-	-
20	**Mannitol**	-	-	-	-
21	**Adonitol**	-	-	-	-

22	**Arabitol**	-	-	-	-
23	**Erythritol**	-	-	-	-
24	**Alpha-methyl glucoside**	-	-	-	±
25	**Rhamnose**	±	±	±	±
26	**Cellobiose**	-	+	±	±
27	**Melazitose**	-	-	±	-
28	**Alpha- methyl d mannoside**	-	-	-	-
29	**Xylitol**	±	-	-	-
30	**ONPG**	+	-	-	-
31	**Esculin hydrolysis**	-	±	+	+
32	**D-arabinose**	-	+	±	-
33	**Citrate utilization**	+	+	+	+
34	**Malonate utilization**	+	+	+	+
35	**sorbose**	-	-	-	-
36	**control**	**control**	**control**	**control**	**control**

"+" indicates carbohydrate utilization by selected isolates, "-" indicates no carbohydrate utilization by the selected isolates and "±" indicates partial carbohydrate utilization by selected isolates.

Fig. 18: Utilization of carbohydrates by representative culture SN16

DISCUSSION

Goa is located on the western coast of India. Traditionally, fermentation is carried out in almost every Goan house. Based on the preparation procedure, Goan fermented foods can be divided into three types *viz* lentil and cereal based, coconut sap based and fermented beverages mainly from cashew fruit; feni (Furtado et al 2020). Probiotics can be obtained from fermented food. Earlier, probiotics were reported from kharza boll; a Goan fermented food. This study focuses on Goan traditional lentil and cereal based fermented foods *viz* Fugiche pole, Sanna, Khazrache boll and Nachni ambil.

Screening of potential probiotic organisms

Extracellular enzyme production

Probiotics produce different extracellular enzymes which can help in boosting digestion and enhancing the nutrient absorption by the host. Amylase hydrolyzes starch to produce small oligonucleotides which are easy to absorb by human body (Padmavathi et al 2018). SN16 showed highest amylase production with 21.6 ± 3.21 mm zone of clearance which indicates that it can hydrolyze starch to form oligosaccharides. Similar study was done on *S. cerevisiae* obtained from bolo batter and was concluded that this yeast strain produced amylase (Pereira et al., 2021). Proteases cleaves proteins like casein into smaller peptides which enables them to move across cytoplasmic membrane (Fernandes et al., 2019). SS6 showed highest protease production with 20.6 ± 1.52 mm zone of clearance, enabling it to cleave proteins efficiently. Lipase breaks lipids into simpler form which helps in absorption by human body (Manasian et al., 2020). Maximum lipase production was observed in SN49 with 18.0 ± 1.0 mm zone of

clearance which indicates that isolates can produce lipase enzyme which aids in absorption of food. *S. cerevisiae* obtained from bollo batter showed Amylase, Lipase and Protease production (Pereira et al., 2021)

Antioxidant activity

Oxidation causes stress in the cell which disrupt its function leading to DNA hydrolysis and severe conditions like apoptosis of the cell. Probiotics have antioxidative property which can help the cell to restore the balance between its oxidative stress (Wang et al 2017). Therefore, it is important that potential probiotics should have antioxidant property. *Lactobacillus casei* KCTC 3260, showed high antioxidant activity (Wang et al 2017). Similar results were obtained as SN1 showed antioxidant activity of 90 % followed by SS6 and SN49 which indicates that the final selected isolates can be used as a potential probiotic.

Antimicrobial testing

Some probiotic organisms have the ability to produce secondary metabolites like bacteriocin, hydrogen peroxide which is lethal for pathogenic bacteria. *Lactobacillus plantarum* showed antimicrobial property against *E. coli* (Karimi et al., 2018). None of the isolates showed antimicrobial property against *E. coli* and *Vibrio*. But they might be able to show antimicrobial effects with other pathogens. In this study only *E.coli* and *Vibrio* was chosen to see antimicrobial effect. It is recommended to check the antimicrobial activity of the strains against clinical pathogens in future, which was beyond the scope of this study.

Survival in the Gastrointestinal tract

pH and bile salt tolerance

One of the basic criteria to screen the potential probiotic organism is their survival in gastrointestinal tract. Gastrointestinal tract contains high concentration of bile salts and a wide range of pH. Therefore, it is important that the probiotic bacteria should tolerate the bile salt concentration which enables them to survive in the gut. Different Lactobacilli strains are studied for bile salt survival (Ruiz et al., 2013). The screened isolates showed substantial bile salt tolerance, making them ideal probiotic candidates. Similar study was done by Pereira on *S. cerevisiae* (Pereira et al 2021)

Adhesion is the important property of probiotic bacteria which helps them to attach to the epithelial cells and enables to survive for longer period of time, ensuring that it does not wash off easily with liquid in the gut (Shangpliang et al ., 2017). This was measured by bacterial adhesion to hydrocarbons (Pereira et al., 2021). SN1 exhibited highest hydrophobicity followed by SS6 with 89.9 % and 87 % hydrophobicity.

Gastric juice contains pepsin; an enzyme which aids in digestion. It is important that the probiotic should tolerate the concentration of pepsin as well as pH of the stomach (if it drops to 2). Therefore, it is important that the potential probiotic should be able to survive in the stomach. It was seen that *Weissella confusa* survived stimulated gastric juice (Nath et al 2021). Similar results were obtained in our study, where SN8 exhibited highest gastric juice tolerance.

Cellular autoaggregation helps bacteria to colonize in the intestine and attach to the intestinal epithelium. Good probiotic should exhibit this property. (Xu et al 2009). It also helps to protect the host from pathogens (Pereira et al 2021). Bacterial cultures have the ability to bind with each other and attach to the gut of the host (Soemarie et al 2021). SN49 showed highest

autoaggregation followed by SN49 which indicates that they can attach to each other to form a layer which will compete for binding of pathogens to the gut.

Gut Bactria have the ability to deaminate aromatic amino acids which leads to the production of phenol (Yadav et al., 2016) that may have a role in cancer (Bone et al., 1976). A potential probiotic should exhibit the tolerance to phenol to survive (Yadav et al., 2016). From the selected isolate, SN51 showed highest phenol tolerance activity, which suggests that it may have some role in cancer and can even stay active in adverse condition of the human gut making it a potential probiotic.

Safety assessment

Hemolysis activity

Some organisms produce hemolysin which can cause toxic effects in the host. Hence not advisable for humans. *Bacillus subtilis* did not show α hemolysis whereas *Bacillus licheniformis*, showed β hemolysis (Fernandes et al., 2019). 15 isolates were selected to perform hemolysis test. 4 isolates showed complete hemolysis (β- hemolysis) which was determined by the zone of clearance. 11 isolates did not exhibit hemolysis (α- hemolysis) whereas four isolates exhibited hemolysis, which states that they produce hemolysin.

Gelatinase production

Some microorganisms produce gelatinase which contributes to their virulence, which can adversely affect the host. Yeast isolated from bollo batter did not exhibit gelatinase production (Pereira et al 2021). In the present study, no gelatinase was produced by the potential isolates.

SUMMARY

Four cereal and lentil based Goan traditional fermented foods were used in this study. Total 141 colonies were obtained. Colony characteristics of all the colonies was studied.

96 unique colonies were chosen for screening of potential probiotic characteristics. Enzyme production of all 96 colonies was carried out to check if they can produce extracellular enzymes *viz* amylase, protease and lipase. 15 best colonies giving enzyme activity with more than one enzyme production was chosen for further screening. Antioxidant test was carried out to check the ability of the isolates to produce antioxidant activity. Antimicrobial testing was carried out for 15 isolates to check their survival against pathogens (*E. coli* and *Vibrio*). None of the isolates showed antimicrobial activity which suggest that they might exhibit antimicrobial activity against other pathogens.

Selected isolates were tested for survival in the gastrointestinal tract: pH tolerance, bile salt tolerance, hydrophobicity test, gastric juice tolerance, cellular autoaggregation which were the preliminary tests for selecting potential probiotic organisms.

Safety assessment was carried out which includes hemolysis test, gelatinase production, biocompatibility test and antibiotic susceptibility test. Four cultures showed β hemolysis. Five cultures which did not show hemolysis was selected for further screening. None of the isolates showed gelatinase production.

Biochemical test was performed by using KB009 test kit (Himedia). Cell morphology indicated the organisms were Gram positive rods and Gram positive cocci.

CONCLUSION

- The five selected organisms (SN1, SN16, SN49, SN51, SS6) isolated from Nachni ambil and Sanna showed all the characteristics of a potential probiotic organism.
- The study reveals that the traditional lentil and cereal-based fermented foods of Goa are ideal sources of probiotics of commercial importance.

FUTURE PROSPECTS

In this study the probiotic organisms are isolated from Goan fermented foods *viz*. Fugiche pole, Sanna, Nachni ambil and Khazrache boll. Probiotics have a wide range of application in the food industry, as they are proven to improve digestion, boost immunity, etc. The current study focuses on the different in vitro tests required for the selection of probiotics.

The future prospects of this current study involves:

- Identification of the selected isolates by DNA isolation and genomic sequencing.
- Assessing the anti- inflammatory and anti- diabetic effect of selected isolates.
- To prepare a consortia of the isolated probiotic.
- In vivo study of the probiotic, followed by additional safety evaluation tests in mice models.

APPENDIX

- **CHEMICALS AND REAGENTS USED**

Nutrient agar	Hi Media Pvt
Nutrient broth	Hi Media Pvt
Bile salt mixture	Hi Media Pvt
Pepsin	Hi Media Pvt
n-hexadecane	Hi Media Pvt
Toluene	Hi Media Pvt
PBS	Hi Media Pvt
antibiotics	Hi Media Pvt
Blood agar base	Hi Media Pvt
Gelatin agar	Hi Media Pvt
Sodium chloride	Sigma Adrich Pvt
KCl	Hi Media Pvt
$NaHCO_3$	Hi Media Pvt
Phenol	Hi Media Pvt
Mueller Hinton agar	Hi Media Pvt
DPPH (1,1-diphenyl-2-picrylhydrazyl)	Hi Media Pvt
Gram's crystal violet	Hi Media Pvt
Gram's iodine	Hi Media Pvt
Lugol's iodine	Hi Media Pvt
Coommessie brilliant blue	Hi Media Pvt
Safranine	Hi Media Pvt
Agar powder	Hi Media Pvt
Ethanol	Hi Media Pvt
Biochemical kit	Hi Media Pvt
Starch powder	Hi Media Pvt
Casein agar	Hi Media Pvt
Tributyrin agar base	Hi Media Pvt
Tributyrin	Hi Media Pvt
Sudan black II	

- **MINIMAL MEDIA USED**

Na_2HPO_4	6g
KH_2PO_4	3g
NaCl	0.5g
NH_4Cl	1g
Agar powder	20g
Distilled water	Make volume to 1000 ml

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