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Isolation and Biochemical Characterization of Lactic Acid Bacteria from "Seet", a Traditional Buttermilk

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Abstract

The effective group of food supplements known as paraprobiotics offer greater health advantages than conventional nutritional goods, therefore they can be considered functional foods. The traditional buttermilk "Seet," which is made from milk sourced from nearby Hisar, Haryana, communities, was used to collect paraprobiotic bacteria. Following observation of growth, bacteria were isolated on MRS agar media, and pure culture was produced by sub-culturing on the same medium. By morphological analysis, Gram staining, and additional identification by particular biochemical tests, the purity of each culture was established. All the bacterial strains were Gram-positive. Twenty four bacterial isolates were characterized morphologically, culturally, and biochemically, and 13 of them were found to be Lactobacillus spp. The results of the isolates tests for catalase, oxidase, urease, nitrate reduction, Simmon's citrate, MRVP, Oxidative and Fermentative and Caesin Hydrolysis. All the strains exhibited negative test for catalase, oxidase, urease, nitrate reduction and Simmon's citrate, but showed positive fermentative and casein hydrolysis test. Maximum samples positively responded to MR test. Few samples (S5, S10, S11, S15, S16 and S24 exhibited VP positive test. A total of 11 strains belonging to 5 genera (*Lactobacillus, Pediococcus, Lactococcus, Weissella* and *Leuconostoc)* were identified.

Keywords: Biochemical properties, Fermentation, Isolation, Lactobacillus, MRVP

Introduction

Paraparaprobiotics are microorganisms that have special positive effects on people and animals. These organisms not only maintain a healthy gut flora, but also good health (Schrezenmeir and Verse, 2001). Bifidobacterium enterococcus, Lactobacillus, and

Streptococcus species are the paraprobiotics bacteria. These bacteria actively work to protect the host's immune system from dangerous germs (Soccol et al., 2010). When given in sufficient doses, paraprobiotics must benefit the host's health. Paraprobiotics must satisfy a number of essential requirements, including bile salt and gut pH tolerance, antibiotic sensitivity, and growth suppression of other dangerous organisms (Shripada et al., 2020). The vast majority of bacteria in the human colon produce a variety of chemicals with health-promoting properties (Butera et al., 2022). In order to promote the health of the hosts, diet and functional foods are crucial modulators of the gut microbiota (Johan et al., 2011). In the current state, paraprobiotics are a prominent and widely utilized category of food supplements. Because paraprobiotics offer much more health advantages than conventional dietary items, they can be considered functional foods (Cumming et al., 2011). High-quality clinical data with a scientific foundation has shown that paraprobiotics can be effective in treating a variety of viral diseases, colon cancer, immunological modulations, and other chronic gastrointestinal inflammatory conditions. Bacteriocins are produced by paraprobiotics bacteria's antimicrobial compounds and are utilized to fight food-rotting organisms and improve food safety (Teame et al., 2020). According to Banger et al., (2022), during fermentation, the bacterium converts carbohydrates into carbon dioxide, organic acid, and alcohol. More lactic acid is produced during fermentation by lactic acid bacteria (LAB), which are described as gram-positive, non-motile, non-sporulating bacteria (Pelinescu et al., 2009). According to Hoque et al. (2010), these are typically regarded as secure and appropriate for medical uses. They are microaerophilic or anaerobic, and they are acid-tolerant organisms with rod- or coccus-shaped cells (Zhang et al., 2014). LAB starts the rapid and acceptable acidification of source materials by creating various organic acids obtained from carbohydrates.

According to De Vuyst and Leroy (2007), LAB can produce lactic acid, bacteriocin, ethanol, exopolysaccharides, specific enzymes, and flavoring chemicals. LAB has a long history of making and consuming cheese. Consumers prefer fermented foods over unfermented meals because of their organoleptic qualities. Because of the formation of acid, LAB lowers pH below 4°C and prevents the growth of pathogenic organisms. According to Ananou et al. (2007), these pathogenic microorganisms taint milk and cause illnesses. For the preservation of food, lactobacilli are employed as starter cultures for fermented vegetables, milk products, sausages, and inoculants. According to Giraffa et al. (2010), lactobacilli are also suggested for the generation of nutraceuticals. There is ample evidence from additional

research showing paraprobiotics affect innate and acute immunity in a number of ways, including by altering T-cell responses, enhancing Th1 responses, and reducing Th2 responses (Guarner et al., 2003). It has been discovered that creating biological medicinal formulae with microbial and synergistic strains in both the colon and the gut can result in a more noticeable Paraprobiotic effect. In comparison to their separate components, these improved products may be more potent and have a stronger stimulating and protective effect (Bomba et al., 2002).

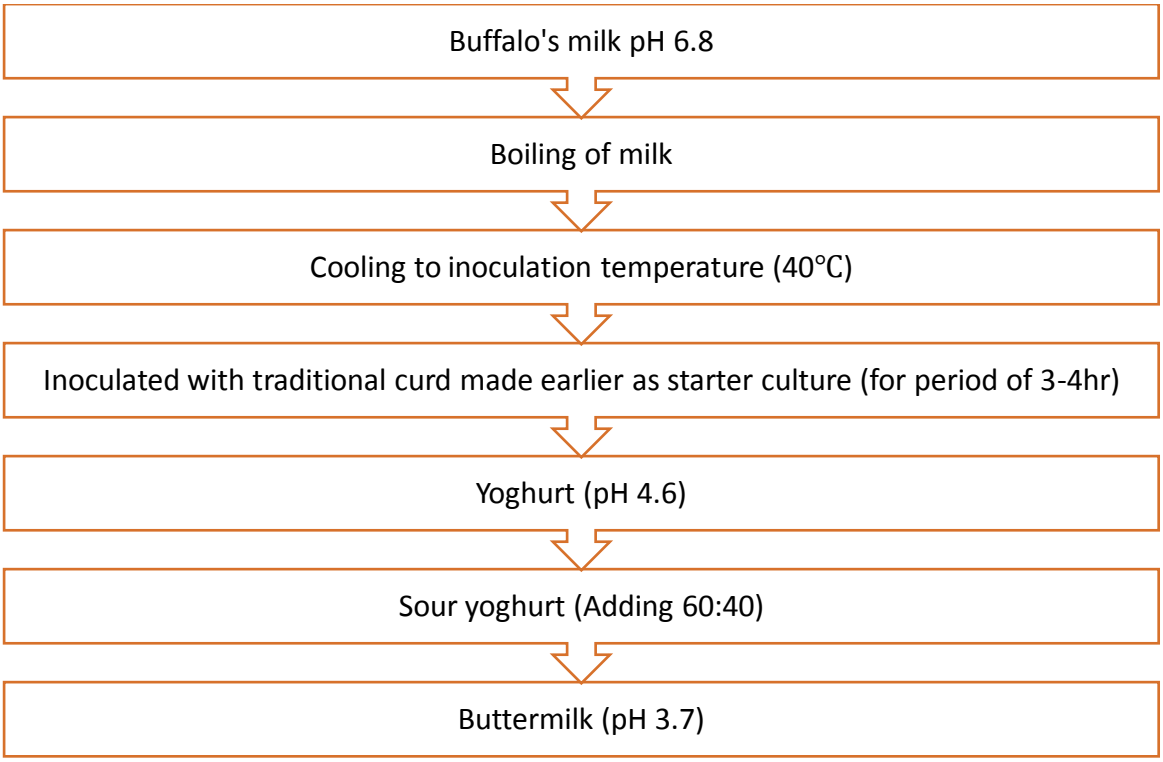
Materials and methods

Sample gathering

In the beginning, a market study was conducted to determine whether buffalo milk was commercially available in the Hisar area markets. The study used buffalo milk that was gathered from 8 separate dairy farms in Hisar, Haryana. The procedure shown in figure 1 was then followed to prepare 24 buttermilk samples.

Seet preparation

Samples of buffalo milk were gathered from Hisar's neighborhood dairy farms. All samples were collected in sterile 250 mL vials and delivered within 36 hours to the lab while being refrigerated (4oC). The obtained buffalo milk had a pH of 6.8 at first. For inoculation, milk was heated, and then cooled to 40°C (Ali et al., 2019). To create yogurt (pH 4.6), the prepared milk was then inoculated with typical curd that had been previously created as a starter culture. A manufactured mixture of buttermilk called "seet" with a pH of 3.7 was then obtained by adding water in a 60:40 ratio (Figure 1). For later usage, the "seet" was kept in the refrigerator. As a result, 24 samples of seet in total were created.



Isolation of bacteria that are paraprobiotics

The pour plate technique and serial dilution were used. The seet sample was diluted 10-fold and suspended in 90 cc of sterile water. Then, 1 ml of the dilution 10-1 sample was taken to dissolve in 9 ml of distilled water (dilution 10-2), and so on, up to the dilution 10-7. A 1 ml sample from each dilution was then obtained and put on an MRS agar medium (Kim et al., 2021). After that, it was cultured for 72 hours at 37 °C to induce growth. The distinctive LAB colonies were carefully removed from the incubated plates and streaked onto MRS agar medium for additional purification. In order to retain the grown-out cultures at 4°C in a refrigerator for further research, the distinct single colonies were picked up and injected into MRS broth in the culture vials. S1 through S24 were the code numbers given to the isolates.

Investigation of colony traits

On MRS agar medium, the morphological traits of each isolate's colony were investigated by incubation for a predetermined amount of time. Shape and stain reaction, two cultural characteristics of isolates, were noted (Kumar, 2015).

Gram Staining

A drop of sterile distilled water was added to the centre of the slide along with a loop full of inoculum from a young culture. The inoculation needle's tip was used to spread the

suspension thinly throughout the slide. By lightly heating the lower surface of the slide three to four times, the smear was dried in the air and secured. Each smear was then covered with crystal violet for 30 seconds, and each slide was then briefly rinsed with distilled water (Nikita and Hemangi, 2012). Following a 60-second Gram's iodine solution coating, each slide was cleaned with 95% ethyl alcohol, followed by distilled water, and then drained. Gram-positive bacteria are those that appear purple, while Gram-negative bacteria are those that appear pink.

Bacterial colony morphology

Visual observations of morphology were made. It covers the elevation of bacterial colonies as well as their form, color, and edges.

Biochemical analysis of isolates

Growth Test with Various Salt Concentrations

The de Mann Rogosa Sharpe broth (MRS broth) media were infected with a single colony of bacteria at concentrations of 5%, 6.5%, and 10% NaCl. The cultures were then incubated for seven days at 37°C. The creation of silt in the media was a sign of bacterial growth. (Esmaeilzadeh et al., 2012)

Test of Growth at Various Temperatures

The de Mann Rogosa Broth (MRS broth) media was infected with a single colony of bacteria and cultured for seven days at 14°C and 37°C. The creation of silt in the media was a sign of bacterial growth.

Motility test

In a test tube, one colony of bacteria was added to the sulfide indole and motility (SIM) media. At 37°C, the test tube was incubated for 48 hours. The growth of bacteria on the medium is observed during the motility test. Bacteria that spread across the media or grow on its surface produce positive results, whereas those that exclusively grow in the immediate vicinity of the insert produce negative results (Shields and Cathcart, 2011).

Catalase test

By applying two drops of 3% hydrogen peroxide at 24 hours of aged cultures on a glass slide, the catalase test was carried out (Reiner, 2010). The creation of oxygen bubbles during a catalase test indicates the presence of the catalase enzyme, which breaks down H_2O_2 into water (H_2O) and oxygen (O_2).

Test for oxidase

The test isolate's overnight cultures were spotted on sterile trypticase soya agar (TSA)-coated plates, and the plates were incubated for 24 hours at 30°C. Oxidase discs placed in Petri plates on the growth of culture after incubation appear blue within a few minutes, indicating a favourable result. A negative is indicated by no color shift (Ifeanyi et al., 2019).

Urease test

This test was carried out using Christensen's Urea Agar. A part of a well-isolated colony was streaked across the surface of a urea agar slant. For 48 to 7 days, tubes are incubated in ambient air at 35 to 37 °C with the cap left unfastened. Observe the growth of color for up to seven days. Yellow color denotes a negative urea test, while pink color denotes a positive urea test (Brink, 2010).

Test for Nitrate Reduction

Nitrate reduction is a crucial requirement for identifying and classifying various bacterial species. As a result, the isolates were cultured in trypticase nitrate broth for 24 hours at 37°C. Sulphanilic acid (0.8% in 5N acetic acid) and -naphthylamine (0.5% in 5N acetic acid) were added to the tubes after the incubation period. A positive nitrate reduction test result was indicated by the development of red or pink color (Cook, 1950).

Test for Simmon's citrate

A well-isolated colony was extracted using a sterile inoculating needle from an 18–24-hour culture. The surface of the slant was then streaked to inoculate the citrate agar tubes. To guarantee enough aeration, the test tube caps were left off. The tube was then incubated aerobically for up to 4 days at 35–37°C. The test tubes were checked every day for four days until the results were discarded as inconclusive. If present, the color change was seen. Growth with a color change from green to bright blue along the slant serves as evidence of a

positive test. No growth or color change, and the slant's color remaining green, are indicators of a negative test (Cheng et al., 2012).

Methyl red and Voges Proskauer's test

MR test: The test culture was injected into sterile glucose-phosphate broth tubes and incubated at 30°C for 48 hours. Five drops of the methyl red indicator were added to each tube after incubation, and they were then gently shaken. Yellow color output was regarded as negative for the test while red color production was seen as favourable (Test, 2016).

VP test: Test cultures were added to the pre-sterilized glucose-phosphate broth tubes and cultured there for 48 hours at 37°C. Barrit's reagent A and Barrit's reagent B were added after incubation, and the development of pink color in the broth was regarded as a positive result for the test.

Oxidative and fermentative test (O/F Test)

Tryptone and the indicator bromothymol blue are both ingredients in OF medium. The medium's addition of sugars serves as the fermentable carbohydrate. Inoculated into two tubes of each OF Medium were pure isolated colonies of the test organism (from an 18–24hour culture). After being inoculated, one tube was covered with melted paraffin or mineral oil to create an anaerobic environment (Bruce et al., 1983). The air was allowed to enter the other tube. For 14 days, tubes were incubated aerobically at 35 degrees Celsius. Every day, the test tube's color is scrutinized. In this medium, bacteria either grow by using tryptone, which creates an alkaline reaction (dark blue hue), or by using glucose, which produces an acidic reaction (changing bromothymol blue to yellow).

Test for Casein Hydrolysis

The purpose of this test is to distinguish between various organisms based on their synthesis of the exoenzyme proteinase (caseinase), which may be utilized to break down the casein protein. The test organism is injected on milk agar in a zigzag pattern and allowed to grow there for 37 hours. The growth of each of the bacterial test organisms on the milk agar plate cultures is checked for the presence or absence of a distinct region, or zone of proteolysis. Clearing that is seen around or beneath colony expansion (hydrolysis) is indicative of a positive test. Negative test results show no clearance above, below, or around the inoculum (Medina et al., 2007).

Sugar Fermentation Test

For this test, carbohydrate broth was employed. A fresh culture (18–24 hours old) of sample bacteria was injected into sterile inoculating loop culture broth. Tubes were incubated at 35±2°C for 18-24 hours. There was color shift. Positive fermentation caused the media to turn reddish-orange to yellow. No medium color shift (reddish-orange) indicated negative fermentation. (Hedberg et al., 2008)

Results and Discussion

Isolation and LAB population

Here, the specifics of the findings related to the isolation and population of LAB from seet are discussed. LAB was isolated on MRS (Mann, Rogosa, and Sharpe's agar) medium from 24 seet samples. Twenty of the twenty-four samples had LAB population positive tests results. (Table 1)

Table 1: LAB population count in Seet samples and API 50 CH test species identification

S.No.	Sample Code	Lactic Acid Bacteria Population (CFU/ml)	Species Identified
1	S1	$9.9x10^4$	*Lactobacillus acidophilus*
2	S2	$9.6x10^4$	*Pediococcus acidilactici*
3	S4	$10.7x10^4$	*Lactococcus lactis*
4	S5	$12.5x10^4$	*Lactobacillus paracasei*
5	S6	$13.0x10^4$	*Lactococcus lactis*
6	S7	$13.5x10^4$	*Lactobacillus acidophilus*
7	S8	$14x10^4$	*Lactobacillus brevis*
8	S10	$11.87x10^4$	*Lactobacillus bulgaricus*
9	S11	$15.7x10^4$	*Pediococcus acidilactici*
10	S12	$12.7x10^4$	*Lactobacillus casei*
11	S13	$12.8x10^4$	*Lactobacillus casei*
12	S15	$11.6x10^4$	*Lactobacillus bulgaricus*
13	S16	$14.3x10^4$	*Lactobacillus helveticus*
14	S17	$15.5x10^4$	*Lactobacillus delbrueckii*

15	S18	$10x10^4$	*Weissella confusa*
16	S19	$9.9x10^4$	*Weissella confusa*
17	S20	$9.8x10^4$	*Lactobacillus delbrueckii*
18	S21	$10.8x10^4$	*Lactobacillus delbrueckii*
19	S22	$9.4x10^4$	*Lactobacillus delbrueckii*
20	S24	$15.1x10^4$	*Leuconostoc citrivorum*

In S22 and S15, respectively, the LAB population in fermented food products varied from 9.4 to 15.7 x 104. For the purpose of making buttermilk, starter culture was utilized. Additionally, the incubation temperature during growth and nutrient content were typically well-suited to the needs of microorganisms. Other microorganisms found in the finished product must deal with a variety of selective and competitive pressures, such as salts, organic acids, ethanol, anaerobiosis, and low pH.

Morphological characteristics

Microorganisms on MRS agar plates were cultured to determine morphological properties. Results relating to colony features and the gram reaction are addressed below. While cell morphology was seen under a microscope using Gram stain, cell shape, and elevation, colony morphology included color, shape, and edges (Table 2).

Table 2: Morphology characteristics of the isolates

Sample code	Colony Morphology			Cell Morphology		
	Shape	Colour	Margin	Cell Shape	Gram Stain	Elevation
S1	Circular	Creamy White	Entire	Bacilli	Positive	Slightly raised
S2	Circular Occurs in Tetrad	White	Entire	Cocci	Positive	Flat
S4	Spherical	Bright red or brown	Entire	Cocci	Positive	Curved
S5	Circular	Creamy white	Entire	Bacilli	Positive	Curved

S6	Spherical	Bright red or brown	Entire	Cocci	Positive	Curved
S7	Circular	Creamy White		Bacilli	Positive	Slightly raised
S8	Circular	Green	Entire	Rod shaped	Positive	Slightly raised
S10	Circular	Blue	Entire	Rectangular	Positive	Curved
S11	Circular Occurs in Tetrad	White	Entire	Cocci	Positive	Flat
S12	Circular	Pink	Entire	Rod	Positive	Curved
S13	Circular	Pink	Entire	Rod	Positive	Curved
S15	Circular	Blue	Entire	Rectangular	Positive	Curved
S16	Oval	White yellowish color	Entire	Rod	Positive	Curved
S17	Spherical	Yellow	Entire	Slender	Positive	Curved
S18	Circular	White	Entire	Coccbacilli	Positive	Curved
S19	Circular	White	Entire	Coccbacilli	Positive	Curved
S20	Spherical	Yellow	Entire	Slender	Positive	Curved
S21	Spherical	Yellow	Entire	Slender	Positive	Curved
S22	Spherical	Yellow	Entire	Slender	Positive	Curved
S24	Oval	Creamy	Entire	Cocci	Positive	Curved

We isolated, and morphologically and culturally described 24 isolates. The isolates have various colors. The MRS agar medium required the LAB isolates 48–72 hours to initiate their growth. The 24 isolates were divided into ten rod-shaped isolates (S1, S5, S7, S8, S10, S12, S13, S15, S18, and S19), two circulars with chain-shaped isolates (S2, S11), two oval-shaped isolates (S16, S24), and six spherical isolates (S4, S6, S17, S20, S21, and S22). The majority of isolates were of various shades of white, including creamy white, white, and yellow white. However, only a few colonies also display red, blue, green, and pink colours (Table 2). The gram-positive response was present in all 24 isolates. According to Konings et al. (2000), LAB is a gram-positive bacterium that mostly ferments lactic acid into the culture medium

and is widely regarded as harmless. The current results are consistent with other research that found isolates in buttermilk to contain different species of the Lactobacillus genus, including Lactobacillus acidophilus, Lactobacillus bulgaricus, and Lactobacillus lactis. According to research by Salvetti et al. (2012), Pithva et al. (2014), and Rao et al. (2015), Lactobacillus spp. gram-positive in nature. Chain, cocci, rod, and ovoid forms were found in the morphological LAB isolates from milk by Waisse and Waisse, (2016).

Biochemical analysis of isolates

Growth Test with Various Salt Concentrations

To ascertain the capacity of bacterial growth at various salt concentrations, salt endurance tests were conducted. The ability of each genera of lactic acid bacteria to grow on plates containing various NaCl salt concentrations varies. The findings of the salt endurance tests indicated that the isolates could grow at concentrations of 6 to 10% NaCl.

Test of Growth at Various Temperatures

A temperature endurance test was conducted to find out whether bacteria could grow at various temperatures. The results of the tests revealed that the isolates could endure various temperatures. Both isolates are capable of growing at 14 and 37 degrees Celsius. The creation of silt in the media was a sign of bacterial growth. Temperatures between 10 and 45 °C are considered mesophilic for lactic acid bacteria development and is the ideal temperature for the growth of lactic acid bacteria. The temperature at which lactic acid bacteria thrive best varies depending on the genus.

Motility test

The purpose of the motility test is to identify whether the bacteria are motile or not, as well as whether they have flagella that are used for movement. Testing for motility on the isolates revealed that they are not motile. Due to the fact that the growth only occurred along the line of inoculation, isolates are classified as non-motile. According to this finding, none of the LAB isolates have flagella. Buffalo milk curd-derived lactic acid bacteria are nonmotile. Non-motile bacteria are a characteristic of lactic acid bacteria in the genus Lactobacillus**.**

Catalase test

Some bacteria need oxygen in order to produce hydrogen peroxide, an unhealthy by-product of aerobic metabolism. Although lactic acid bacteria are anaerobic bacteria, they do not need

oxygen to survive. Additionally, catalase, an enzyme that breaks down hydrogen peroxide into water and oxygen, is not produced by lactic acid bacteria. No gas bubbles were created during the current research project, validating the results of the negative catalase test for all isolated strains of bacteria and proving they are all anaerobes. The current findings were in line with Ismail et al., 2019 where catalase negative activity was exhibited by lactic acid bacteria in cow's milk Kefir. Amelia et al., 2020 further investigated lactic acid bacteria isolated from *dadiah* All were found to be catalase negative.

Test for oxidase

Using a redox dye called tetramethyl-p-phenylene-diamine, the oxidase test can identify the presence of a cytochrome oxidase system in bacteria that will catalyze the transit of electrons between electron donors. Positive outcomes are indicated by the dye's reduction to a deep purple tint. All of the bacterial cultures in the current study were unable to oxidize the colorless reagent. According to Mamata et al. (2017), the isolates lacked cytochrome C, which allows them to use free oxygen in their energy metabolism. The current findings agreed with those made by Ouoba et al. (2009), who found that LAB isolated from traditional African alkaline fermented foods were oxidase negative. Mamata et al. (2017) claim that specific Lactobacillus isolates, namely Lactobacillus delbrueckii ssp. A negative oxidase response was identified by Bulgaricus from curd and paraprobiotic drinks. Even in the experiments by) Gomaa, 2017 and Saif et al. (2016), Lactobacillus isolates showed a negative response.

Urease test

Amino acids are decarboxylated to produce urea. Ammonia and CO_2 are produced when urea is hydrolyzed. The solution becomes more alkaline due to the creation of ammonia, and the pH change is indicated by phenol red, which turns from pale orange at pH 6.8 to magenta (pink) at pH 8.1. Within 24 hours, rapid urease-positive microbes turn the entire medium pink. Negative organisms generate no color change or yellow as a result of acid production, while weakly positive organisms may require several days. All of the isolated isolates in the current study project tested negative for urease, indicating that the organisms did not fall under the Enterobacteriaceae family. The results obtained were in accordance with Jang (2014), who isolated lactic acid bacteria from kimchi and infant feces that showed urease test negative.

Test for nitrate reduction

Nitrate reductase, which is produced by nitrate-reducing organisms, converts nitrate into nitrite. As a result, nitrous acid is created when produced nitrite and acetic acid react. A colorless diazonium salt (diazotized sulfanilic acid) is created when the nitrous acid and sulfanilic acid are diazotized. When the colorless nitrite-sulfanilic acid and dimethyl-naphthylamine (-naphthol) are mixed, a red azo dye (p-Sulfobenzene-azo-naphthylamine) is created that is water soluble. Current results showed no red hue, showing that the bacteria isolated at the time did not manufacture nitrate reductase enzyme. According to Kavitha et al. (2016), lactobacillus acidophilus from dahi samples reacted negatively. The findings of the current investigation were consistent with those of Sneha et al. (2017) who revealed that curd and fruit juice did not contain any LAB.

Test for Simmon's citrate

An organism's capacity to use citrate as a source of energy is evaluated using citrate agar. Citrate serves as the only source of carbon and inorganic ammonium salts serve as the only source of nitrogen in the agar media. Citrate is an intermediate metabolite in the Krebs cycle, which indicates aerobic respiration, hence the organism's growth is a sign that it is being used. Negative results, on the other hand, suggested fermentation. Both Monica et al. (2012) and Mamata et al. (2017) found that several lactic acid bacteria isolates from non-dairy Paraprobiotic drinks displayed negative citrate utilization test results. The findings of Ankita and Jayanti (2015), who reported on the paraprobiotic characteristics of Lactobacillus Spp, were identical to those of the current study. from a few local dairy products failed a test for citrate use. Lactobacillus acidophilus from a dahi sample displayed a negative response, according to Kavitha et al.'s (2016) research. According to Ankur et al. (2017), citrate consumption was not present in lactic acid bacteria isolated from fermented foods. According to Timothy (2017), curd and milk-derived Lactobacillus bulgaricus exhibited unfavorable behaviors.

Methyl Red and Voges Proskauer's test

Methyl Red and Voges Proskauer tests, two distinct procedures, are used to distinguish between the two main categories of facultative anaerobic bacteria, or acid- and acetoin-forming bacteria. Methyl red test further confirmed that the isolated microbes in the current research work followed the lactic acid fermentation pathway. Methyl red test is based on the principle of fermentation of glucose to pyruvic acid and oxidation of pyruvic acid to other

acids such as lactic, acetic, and formic acids which results in decrease in pH. These results agreed with Kamel et al., (2012) who stated that lactic acid bacteria in fermented dairy products showed a positive reaction. In the present study, S1, S2, S4, S6, S7, S8, S11, S12, S13, S17, S20, S18, S19, S21, and S22 exhibited MR positive but VP negative, thus confirming the fact that bacteria is lowering down the pH. According to Apoorva et al. (2013), Lactobacillus. cultivum, L. brevis from beverages and dairy products (curd, buttermilk, paneer whey) showed favorable effects. In the Voges Prausker test reaction carried out by Guessas and Kihal (2004), the common characteristics of Lactobacillus species revealed negative results. According to Hossain et al. (2010), yoghurt-derived Lactobacillus isolates exhibited a negative response. The findings of the current investigation are consistent with those of Rhaiem et al. (2016) who found that LAB strains of the Voges Proskauer test did not exhibit any color changes. The methyl red test was negative for the isolates S5, S10, S11, S15, S16, and S24, but they tested positive for VP. The current findings are consistent with those made by Jagadeeswari et al. (2010) that Lactobacillus isolated from conventionally fermented meals showed adverse effects. Ankur et al. (2017) concluded that while some LAB isolates from fermented foods responded positively to the methyl red test, some did not. According to Timothy (2017), the previous study also demonstrated that Lactobacillus bulgaricus curd from milk and curd, respectively, showed adverse effects.

Oxidative/fermentative test

Organic compounds known as carbohydrates contain carbon, hydrogen, and oxygen in the proportion (CH_2O) n. Depending on their enzyme complement, various organisms utilize carbohydrates in different ways. The O/F test has been widely utilized as a tool for the biochemical differentiation of microorganisms since the pattern of fermentation is a trait of specific species, genera, or groupings of organisms. Hugh and Leifson created OF media to distinguish between fermentative bacteria, which can produce acid under both aerobic and anaerobic conditions, and oxidative bacteria, which can only manufacture acid from carbohydrates under aerobic conditions. A golden tint developing in the medium indicates a successful carbohydrate utilization test. Only the open tube develops a yellow hue during oxidative catabolism. While in both open and closed tubes, fermentative catabolism results in the production of a yellow colour. The current analysis verified that all isolated strains had yellow coloring in both test tubes (open and closed), indicating that the route was fermentative. This outcome provided additional evidence that bacteria are facultative anaerobes.

Test for Casein Hydrolysis

The primary protein (phosphoprotein) in milk is called casein and has the molecular formula C81H125N22O39P. 'Milk protein' is another name for it. A proteolytic exoenzyme caseinase with casein hydrolyzing activity can be produced by some bacteria. Casein is broken down into more manageable, soluble amino acid units by caseinase. Only some species of bacteria can hydrolyze casein because not all bacteria can produce this enzyme. The casein hydrolysis test was developed to classify bacteria and identify them phenotypically based on this feature. All of the isolated strains in the current experiment were able to exhibit modest proteolytic activity. The results corroborated Garcia-Cano et al.'s 2019 investigation, which concluded that lactic acid bacteria isolated from dairy products were potential makers of proteolytic proteins. Proteolytic activity of L. lactis and L. Brevis was further validated in 2017 by Bounouala et al.

Sugar Fermentation test

The biological Carbohydrate Fermentation Test evaluates bacteria's capacity to ferment a specific carbohydrate and identifies them by their fermentation pattern. Different bacterial groups have different dietary and biochemical needs. Another example is bacteria using carbohydrates (sugars): some can use one type and others can't. Different bacterial groups may metabolize the same sugar in different ways, producing acids, alcohol, gas, or both. This ability of bacteria to ferment a carbohydrate can help classify and identify microorganisms. (Gunkova et al., 2021)

S.No.	SAMPLE	SPECIES IDENTIFIED	SUGAR FERMENTED											
			Gluco	Sucro	Galact	Lactos	Melibi	Arabi	Cellob	Malto	Sorbit	Manni	Raffin	Xylos
1	S1, S7	*Lactobacillus acidophilus*	+	+	+	+	+	+	+	+	-	-	-	+/-
2	S2, S11	*Pediococcus acidilactici*	-	+	+	+	-	+	-	+	+/-	-	+/-	-
3	S4,	*Lactococcus*	+	-	+	+	-	-	-		-	+/-	+	+/-

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	S6	*lactis*												
4	S5	*Lactobacillus paracasei*	+	+	+	+	+	-	+	-	+/-	-	-	-
5	S8	*Lactobacillus brevis*	+	+	+	+	+	+	-	+	-	-	+	-
6	S10, S15	*Lactobacillus bulgaricus*	+	-	-	+	-	+	+/-	-	+/-	-	+	+/-
7	S12, S13	*Lactobacillus casei*	+	+	+	+	+	-	+	+	+	+	-	+/-
8	S16	*Lactobacillus helveticus*	+	-	-	-	+	-	+	-	+	+	-	+/-
9	S17, S20, S21, S22	*Lactobacillus delbrueckii*	+	+	+	+	+	-	-	+	-	-	-	-
10	S18, S19	*Weissella con fusa*	+	+	+	-	-	+	-	+/-	-	-	+/-	+
11	S24	*Leuconostoc citrivorum*	+	+	+	+	-	+	+		+/-	-	-	-

+ = Positive fermentation; - = Negative fermentation; +/- = Variable reaction

On basis of sugar fermentation test isolates were differentiated as follows. S2 and S1 showed glucose negative confirming them to be *Pediococcus acidilactici* . Isolates S4, S6,

S10,S15,S16 all exhibited sucrose negative. But among these S4 and S6 showed galactose-positive reaction thus exhibiting *Lactococcus lactis* species (Gunkova et al., 2021). Now among S10, S15 and S16, S10, S15 is lactose positive but S 16 is lactose negative thus differentiating S10 and S15 to be *Lactobacillus bulgaricus* (Rhaiem et al., 2016) and S 16 to be *Lactobacillus helveticus.* S18 and S19 gave lactose, Mellibiose negative but Xylose positive thus differentiating it from other isolates and was found to be *Weissella confusa* (Hedberg et al., 2008). In case of S1 and S7 both Arabinose and Cellobiose test were positive but in case of S5 Arabinose positive and cellobiose negative was obtained thus differentiating among these isolates. S1 and S7 was found to be *Lactobacillus acidophilus* (Gunkova et al., 2021) and S8 was identified as *Lactobacillus brevis.* On the same lines, S11 was distinguished from S1 as it was found to be Mellbiose negative and later on identified as *Leuconostoc citrivorum.* S5 differed from S12 and S13 as the former was Maltose negative and latter was Maltose positive. S5 was identified as *Lactobacillus paracasei* and S12 and S13 was found to *Lactobacillus casei, Lactobacillus delbrueckii* was found to be Arabinose as well as cellobiose negative but Maltose positive (Hedberg et al., 2008). This test confirmed a total of 11 strains belonging to 5 genera (*Lactobacillus, Pediococcus, Lactococcus, Weissella* and *Leuconostoc).*

Conclusion

Based on analysis of the results, it can be concluded that the results of isolation and characterization of different bacterial isolates from Seet got 5 genera of bacteria, that are found to be gram-positive and non-motile. Biochemical tests revealed negative catalase, oxidase, urease, citrate, and nitrogenase activity. MRVP test was positive for the maximum isolated species of *Lactobacillus*. Sugar fermentation test further confirmed that isolates S1, S5, S7, S8, S10, S12, S13, S15, S16, S17, S20, S21, S22 was bacteria from *Lactobacillus* genus and isolate S2, S11 was *Pediococcus* genus. Isolate S4 belonged to the *Lactococcus* genera, S18 and S19 belonged to the genera *Weissella* and S20 belonged to the genera *Leuconostoc.*

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