https://doi.org/10.48047/AFJBS.6.Si4.2024.3474-3488

African Journal of Biological Sciences

Isolation, Screening And Characterization Of Dairy Isolated Probiotics

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Article History
Volume 6, Issue Si4, 2024
Received : 05 June 2024
Accepted : 25 June 2024
Published: 23 July 2024
doi:
10.48047/AFJBS.6.Si4.2024.3474-3488

ABSTRACT

In the industrialized world, functional foods have become a part of an everyday diet and are demonstrated to offer potential health benefits beyond the widely accepted nutritional effects. Probiotics are live microorganisms that confer health benefits when administered to the host in adequate amounts. Probiotics have been emerging as a safe and viable alternative to antibiotics in human health, besides improved immunomodulation and nutrient digestibility; in feeds, probiotics have shown drastic reductions of gastrointestinal tract invading pathogens. Dairy products can be a valuable source of value-added foods, especially when combined with a healthy diet. These probiotic dairy foods enhance the host's oral cavity and gut flora, stimulate the growth and catabolism of health-promoting bacteria, and improve the microbial balance in the gut microbiota.

In order to verify the probiotic isolates' qualities, a series of tests including phenol, acid, bile salt, and NaCl tolerance have been performed on them. Additionally, antibiotic susceptibility and antibacterial assays were used to confirm the isolates' potency, and hemolytic activity was used to determine the isolates' safety. The best probiotic isolate was chosen based on the above experiments and was subjected to molecular identification.

Keywords: Probiotic, functional foods, Immunomodulation, Gastrointestinal tract, Dairy.

INTRODUCTION

Probiotics are referred to the live microorganisms that in their appropriate numbers can bring in health benefits and avert some of the ill effects of metabolic and microbial processes in host system.The term "probiotics" originates from the combination of latin word "pro" and Greek word "biotikos" meaning "for life" it emphasizes their role in promoting a healthy microbial environment in the body (Reid *et al.,* 2003). Probiotics improve microbial balance of the digestive system, particularly in the gastrointestinal tract these are beneficial to the body through a number of processes, including the reduction of intestinal pH, preventing the colonization of pathogenic microorganisms its invasion, and altering the host immune response. Probiotics have gained more attention from researchers and consumers in recent years. A growing amount of clinical research

on the potential benefits of probiotic bacteria in preventing and treating oral illnesses is gaining attention (Williams, 2010 and Keller *et al.,*2011). Although this highlights the potential importance of this developing discipline, there is still more work to be done in order to standardize the term probiotic and determine which strains of microorganisms truly meet the requirements to be considered as probiotics (Hill *et al.,* 2014 and Merenstein *et al.,* 2023). Dairy products and cultured probiotic cultures have been present since human civilization, with references found in the Bible and Hindu literatures. These products, including leben, dahi, kefir, and koumiss, were used medicinally before germ discovery (Kumar *et al.,* 2015 and Shortt, 1999). In 1950s and 1960s, due to the use of antibiotics and their adverse effects on humans and agricultural animals, there was a surge in interest of viable microbial supplements, leading to the development of these products which are still widely consumed until today (Fuller, 1999). The digestive system, from the oral cavity to the anal region has an uneven distribution of bacterial microbiota, which significantly impact various functioning like immune system development, nutrient utilization through metabolic activities and their uptake. The digestive system's uneven distribution of bacteria contributes to the complexity of human health (Cani and Delzenne, 2009 and Ley *et al.,* 2006).

Therapeutic interventions like radiation, immunosuppressive medication, and antibiotics can alter the flora's balance in the gut and resilience is relevant to the oral microbiome, which is affected by external factors like host behavior, habits, lifestyle, etc. Poor oral hygiene can lead to a mature biofilm on oral surfaces, causing gingivitis, dental caries, tartar, oral ulcers, cancer, halitosis, etc. Disease-associated microbial communities are resilient and difficult to return to health-associated microbiota without proper treatment. To reestablish microbial equilibrium and prevent illnesses, dairy and non-dairy probiotics can be introduced through oral supplementation. Regular consumption of probiotic-containing food is recommended (Soccol *et al.,* 2010 and Gupta and Garg, 2009). Milk and its products are essential for a healthy diet due to their bioactive compounds and essential nutrients. Consuming fermented dairy functional foods regularly provides indirect health benefits and supports oral health by promoting a diverse range of microbes that trigger immune responses and create biofilms to prevent pathogen growth (Chugh *et al.,* 2020). The oral cavity is crucial for the entry of microorganisms associated with eating habits and natural entrants, as it plays a significant role in food processing and digestion. Understanding the microbial diversity and functioning in the oral cavity is essential, consuming probiotic bacteria from dairy products or natural sources enhances value addition, restores gut/digestive flora, and preserves epithelial barrier function, promoting gut health(Kaur*et al.,* 2022). Dairy products are widely available with probiotic *Lactobacillus* and *Bifidobacteria* as starters. While most probiotic microbes discovered in the past have been linked to food, recent research in microbiota has opened up new avenues for discovering novel probiotics from non-food sources, including species that are not yet considered to be "safe" (Zoumpopoulou *et al.,* 2017). Dairy foods provide an optimal environment and substrate for probiotic bacteria, buffering them during their passage through the intestinal tract, aiding in lactose digestion, removing toxicity, and contributing to the synthesis of essential nutrients (Urrutia-Baca *et al.,* 2024). Probiotics have various biological functions beyond the gastrointestinal system, including antibacterial, antimutagenic, antigenotoxic, lactose tolerance improvement, cancer prevention, and immunomodulation (Oelschlaeger, 2010 and Sanders, 2000). However, in order for bacteria to be categorized as probiotic microorganisms, certain criteria need to be fulfilled which includes assessment of their probiotic potentials. The microorganisms must be administered in adequate amounts and be viable in the digestive system. They also need to be genetically identified, named, and categorized in accordance with the most recent nomenclature. Probiotics should be assessed for their pathogenicity and ability to tolerate bile concentration and digestive acids (Collado *et al.,* 2008).

The aim of this study is to isolate some unique wild probiotics from the dairy source particularly from the milking animals which have been grazed naturally in the wild environment instead of feeding them with the commercially produced, prepackaged fodder. The samples were collected from the remote village areas from the districts of Dharwad and Vijayapura. Collected samples were cultured to isolate wide range of bacterial pure cultures which were then screened for probiotic potential properties and finally identifying it with the molecular identifications for further studies.

1. MATERIALS AND METHODS

1.1. Sample collection :

Samples were collected from milking animals that had grazed naturally (wild) and without being fed any packaged commercial fodder in rural areas. Milk samples sourced from buffalo, goat, and sheep were aseptically collected in the midst of the milking process in a pre-sterilized 5ml culture vial, while the curd samples collected were traditionally homemade (Zhang *et al.,* 2022). These samples were brought to the laboratory in a thermos flask filled with ice to keep them cold for better viability of cultures and were immediately inoculated on specific media for growth and isolation of microorganisms for further experiments.

1.2. Isolation of pure cultures:

Microbial isolates were obtained through crowd plate technique by spreading 100µl of the collected sample on different agar media (MRS, LURIA, NA and BHI) to get a crowd plate isolation of microbial cultures. Distinct colonies were selectively picked and streaked on fresh media plates to obtain pure cultures and then maintained in broth with glycerol at 4℃ as stock cultures for further use.

1.3. Microscopic observation:

The purity and morphological identification of the isolates were validated using Gram's staining technique, in which single colony was stained according to standard protocol and examined under oil immersion (100x). The colony morphology and microscopic characteristics were photographed and recorded.

1.4. NaCl tolerance:

NaCl plays an important role in human physiology by being electroneutral in regulation of the intestine physiology. The NaCl concentrations in the stomach and intestine can vary from 1% to 10%. Higher concentration of NaCl adopted probiotic isolates can very well adopt and function as probiotics in the human gut. To determine the tolerance of the isolates against NaCl concentrations (2%, 4%, and 8%) fresh cultures were inoculated with three concentrations of NaCl in luria media and incubated at 37°C for 24 to 48 hrs. Only media was used as negative control results were determined by observing the turbidity in the inoculated tubes (Rahman*et al.,* 2015 and Chakraborty and Bhowal, 2015) the incubated tubes were used to check the viability of the isolates by inoculating them on the agar media.

1.5. Bile salt tolerance:

Tolerance to bile is one of the most important properties of probiotic microbes, it determines the ability of these microorganisms to survive in the small intestine, and consequently the ability to adopt help the microbe to be functional as a probiotic in the small intestine. Luria broth amended with three different concentrations of bile salt (0.05%, 0.1% and 0.5% equating with variable

concentrations in the small intestine) was prepared and was inoculated with the isolates to be tested. The tubes containing the inoculated broth were incubated at 37℃ for 4hrs. Afterwards, the cultures were withdrawn from the incubator and were streaked on to fresh luria agar media plates and kept for incubation at 37℃ for 24 hrs. to check their viability after subjecting the isolates to the bile salt concentrations (Yadav *et al.,* 2016).

1.6. Acid tolerance:

To understand the gastric juice adaptability of the probiotics, the need for culturing the isolates in varying concentration of acids can determine the organism's strong ability to adopt and function as probiotics. The isolates were cultured in luria broth at 37℃ for 24 hours. After incubation, the broth containing the culture was centrifuged at 3700×g for 10 min at 4 ℃ to get a cell pellet. The supernatant was discarded, leaving behind the pellet, which was redissolved in the fresh broth with pH 2 concentration. This bacterial suspension was left at room temperature for 4 hours, then streaked onto luria solid medium and incubated at 37℃ for 24 hours. The plates were observed for viable colonies of the test bacterial isolates. (Zhao *et al.,* 2022 and Song *et al.,* 2015).

1.7. Phenol tolerance:

This phenol tolerance is important for isolates to survive the gastrointestinal conditions, where the gut bacteria have the ability to deaminate aromatic amino acids that are derived from dietary proteins and may lead to formation of phenols (Yadav *et al.,* 2016; Divisekera *et al.,* 2019; Singhal *et al.,* 2019). To determine the phenol tolerance ability of the isolates, Luria broth with 0.1%, 0.2%, 0.3% and 0.4% of phenol concentration were prepared. Fresh cultures were inoculated in each different concentration respectively and incubated at 37 ℃ for 24-48 hrs., uninoculated media was used as negative control. Results were determined by observing turbidity after 24 h and 48 h and no growth was found in negative control (Mannan *et al*., 2017).

1.8. Antibiotic susceptibility test:

The safety of probiotics is coupled to their intended use as probiotics and not as an opportunistic pathogen.The antibiotic susceptibility was tested against four antibiotics (Penicillin, Clindamycin, Gentamycin and Erythromycin). The test organisms were spread onto Mueller Hinton agar plates and the antibiotic discs were placed on the agar plate and incubated at 37℃ for 24 hrs. The zone was observed around the discs to which the test organism was susceptible (Beatrice *et al.,* 2020).

1.9. Antibacterial activity:

The isolates were tested against Gram-positive and Gram-negative pathogens namely, *Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 9144, *Pseudomonas aeruginosa* ATCC 8650 and *Escherichia coli* ATCC 4157 respectively with well diffusion method. Mueller Hinton agar plates were prepared, the pathogenic bacterial isolates were spread onto the plate, and after drying, wells were made in the agar media and 100µl of the test probiotic isolates were added to the respective wells and left to dissociate in the agar for an hour and then the plates were then incubated for 24 hrs. at 37℃ and observed (Cizeikiene and Jagelaviciute, 2021 and Zeng *et al.,* 2020).

1.10. Hemolytic activity:

When the safety of probiotic is concerned, lack of hemolytic activity is important during the selection of probiotic strains, because such strains are non-virulence and lack of hemolysin ensures that virulence will not appear among the bacterial strains (FAO/WHO 2006). The hemolytic activity of isolates was assessed using Columbia agar containing 5% sheep blood. The probiotic

isolate was inoculated and incubated at 37°C for 48 hours. The isolates were classified based on red blood cell lysis around the colonies, with green zones (α-hemolysis), clear zones (β-hemolysis), and no zones (γ-hemolysis) on Columbia blood agar plates. Strains with γ-hemolysis were considered safe (Yasmin*et al.,* 2020 and Khushboo *et al.,* 2023).

1.11. Molecular identification:

The selected potential probiotic isolate DGM 7 that possessed most of the probiotics functional characteristics was identified on the basis of 16S rRNA gene sequencing. DNA was isolated from the culture using QIAGEN DNeasy UltraClean Microbial Kit (Cat. No. / ID: 12224-50). Its quality was evaluated on 1.0% agarose gel, a single band of high-molecular weight DNA has been observed. Fragment of 16S rRNA gene was amplified by 16SrRNA-F and 16SrRNA-R primers. A single discrete PCR amplicon band of 1500 bp was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants QIAGEN QIAquick PCR Purification Kit (Cat. No. / ID: 28104). Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 16S-F and 16S-R primers using BDT v3.1 Cycle sequencing kit on Applied Biosystems™ MiniAmp™ Plus Thermal cycler and analyzed on ABI 3730xl Genetic Analyzer. The obtained sequence was compared and molecularly identified with the available nucleotide databasefrom the NCBI GenBank using the BLAST search(https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2. RESULTS

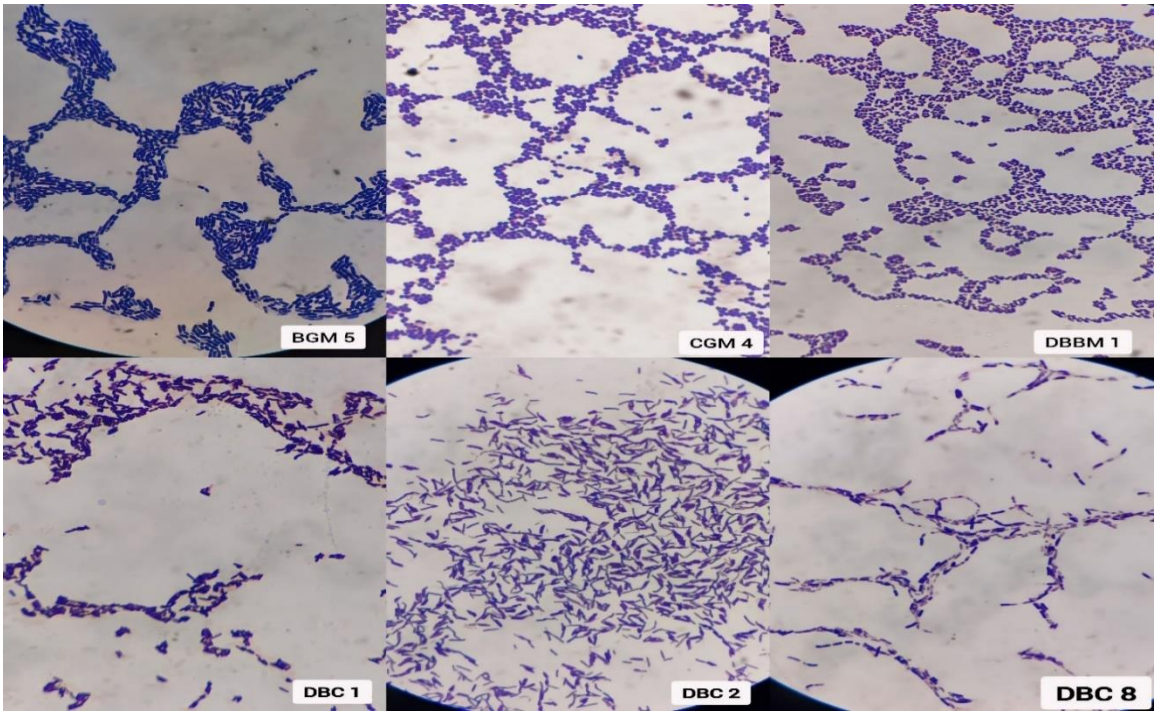
2.1. Isolation:

The isolations were done by crowd plate technique followed by pure culturing of the isolates for the further experiments. About fifty organisms were isolated and maintained as pure cultures on which further tests were performed for their identification and analyzing the efficiency of them to be utilized as a probiotic source.

Fig. 1: Pure cultures obtained from dairy source.

2.2. Microscopic observation:

All the isolates were observed under the microscope to determine their Gram's nature by Gram staining method. About half of the isolates were found to be Gram-positive and other half of the isolates were Gram-negative. The isolates were observed under 100x magnification to record the morphology of the isolates. Only Gram-positive isolates were selected for the further study.



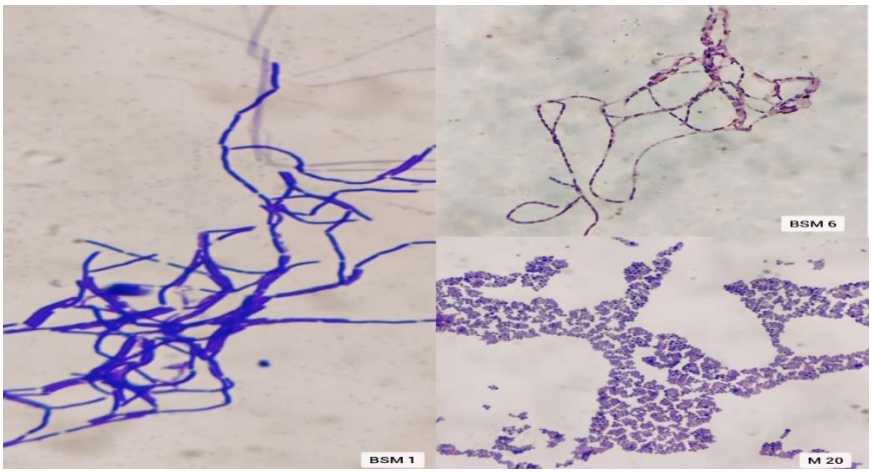


Fig 2: Microscopic images of some isolates.

2.3. NaCl tolerance:

All the 26 isolates were tested against three different concentrations of NaCl (2%, 4%, and 8%). Nearly all the isolates were found to be tolerant to 2% and 4% NaCl concentration and there were about 14 isolates which were tolerant to 8% NaCl concentration and 12 of them were found to be intolerant. The salt tolerant isolates were selected for further studies.

2.4. Bile salt tolerance:

The bacteria that are intended for use as probiotics must be resistant to bile salts and other digestive system inhibitors (Pyar and Peh, 2014). For this, Luria broth containing 0.05%, 0.1% and 0.5% Bile salt concentrations were prepared to determine the bile salt tolerance ability of the isolates. About 25 isolates were able to tolerate the 0.5% bile concentration and only 1 isolate was intolerant to the bile salt. The tolerant isolates were subjected to further assays.

2.5. Acid tolerance (pH):

Isolates ought to remain viable and active throughout the GIT's transit, product storage, and manufacturing processes (Ayyash *et al.,* 2021). In order to assess this ability to withstand acidity, luria broth with pH 2 was prepared inoculated with the isolates, and then allowed to incubate for four hours. Viability of the isolate was assessed by streaking on newly prepared media plates. About 15 isolates showed tolerance to the low pH and were found to be viable, whereas the remaining 11 isolates were determined to be nonviable.

2.6. Phenol tolerance:

The chosen cultures in the current investigation were tested for phenol tolerance at various phenol concentrations, including 0.1%, 0.2%, 0.3%, and 0.4%. the isolates were inoculated, incubated and observed for the turbidity in the form of growth which shows the tolerance of the isolates to the different phenolic concentrations. About 19 isolates showed tolerance to 0.3% phenol concentration and others could not resist the phenolic concentrations and hence there was no growth in the test-tube which shows the intolerance of the isolates.

Table 1 :Table showing the results of NaCl, bile, acid and phenol tolerance of the test isolates.

S.No.	Isolate	NaCl (8%)	Bile (0.5%)	Phenol (0.3%)	Acid (pH 2)
1	M 2	+	+	+	-
2	M 3	+	+	+	+

3	M 5	+	+	+	+
4	M 7	+	+	+	–
5	M 17	–	+	+	–
6	M 19	–	+	+	–
7	M 20	+	+	+	–
8	DGM 5	–	+	–	–
9	DGM 6	+	+	+	–
10	DGM 7	+	+	+	+
11	BSM 6	–	+	–	–
12	BSM 5a	+	+	+	+
13	BSM 5b	–	+	–	+
14	DBC 1	+	+	–	+
15	DBC 2	+	+	–	+
16	DBC 3	+	+	+	–
17	DBC 5	+	+	+	+
18	DBC 6	+	+	+	–
19	DBC 7	–	+	+	–
20	DBC 8	–	+	+	+
21	DBM 1	–	–	+	+
22	DBM 3	–	+	+	+
23	DBM 4	–	+	–	+
24	DBBM 2	–	+	–	+
25	DBBM 4	–	+	+	+
26	BCS	+	+	+	+

2.7. Antibiotic susceptibility test:

Out of 26 isolates only 10 isolates were subjected to antibiotic susceptibility testing using the agar disc diffusion method of which the results are presented in the table 2. It was found that all the isolates were resistant to penicillin except DBC3 and DGM5. About 7 isolates except BCS, DBC3 and DGM6 were found to be resistant to clindamycin. Isolates M3, DBM3, DBC2, DBC5, and BSM6 were resistant to erythromycin. All the isolates were found to be susceptible to gentamycin. Only DGM5 and DBC3 was found to be susceptible to penicillin. DGM7 was found to be resistant to penicillin and clindamycin and was found to be susceptible to erythromycin and gentamycin.

Table 2:Data of antibiotic susceptibility showing strains.

S.no.	Isolates	Zone of inhibition in mm			
		Penicillin	Clindamycin	Gentamycin	Erythromycin
1	BCS	–	13mm	15mm	15mm
2	M 3	–	–	10mm	–
3	DBM 3	–	–	10mm	–
4	DBC 2	–	–	10mm	–
5	DBC 3	14 mm	10 mm	12mm	14 mm
6	DBC 5	–	–	10mm	–
7	BSM 6	–	–	13mm	–
8	DGM 5	10mm	–	13 mm	13 mm
9	DGM 6	–	12mm	12mm	10mm
10	DGM 7	–	–	14mm	10 mm

2.8. Antibacterial activity:

After evaluating all of the 26 isolates' positive test findings, from the previously carried out tests were subjected for the antibacterial activity assay.The antibacterial activity was tested against Gram-positive and Gram-negative pathogens namely, *Bacillus cereus, Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Escherichia coli* respectively with agar well diffusion method. Only 12 isolates (M2, DGM5, DGM6, DBBM2, DBBM4, DBM1, BCS, M3, DBM3, DBC2, BSM6, DGM7) were screened and selected based on their antibacterial activity for the further assays.

Isolates DBM1, BCS, M3, DBM3, DBC2, BSM6 and DGM7 showed zone of inhibition against all the pathogenic bacteria. M2 showed zone of inhibition against *P. aeruginosa* only, DGM5 and DGM6 showed zone of inhibition towards *P. aeruginosa* and *E. coli*. DBBM2 had no zone of inhibition against *S. aureus*, DBBM4 showed zone of inhibition against *P. aeruginosa* and *B. cereus.* The zone of inhibition diameters in mm is shown in the table 3.

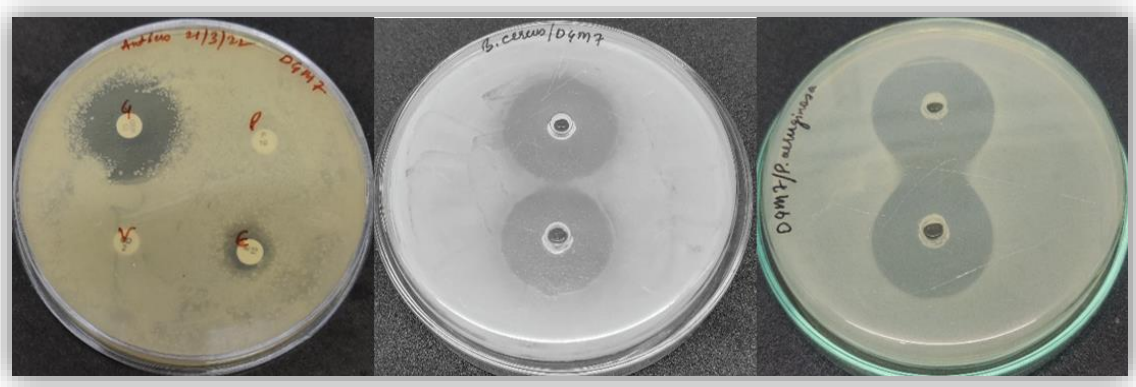


Fig 3: Antibioticsusceptibility and antibacterial activity of DGM7 isolate.

Table 3: Zone of inhibition (diameter in mm) of the selected isolates against the pathogenic isolates (antibacterial activity).

S.no.	Isolate	Zone of inhibition in mm ((n≥3) ± s.d.)			
		P. aeruginosa	*E. coli*	*B. cereus*	*S. aureus*
1	M 2	10.33 ± 0.57	–	–	–
2	DGM 5	11.66 ± 0.57	12.0 ± 1.0	–	–
3	DGM 6	10.66 ± 0.57	10.33 ± 0.57	–	–
4	DBBM 2	11.33 ± 0.57	11.66 ± 1.15	10.33 ± 0.57	–
5	DBBM 4	11.66 ± 0.57	–	12.33 ± 0.57	–
6	DBM 1	11.0 ± 1.0	12.33 ± 0.57	13.66 ± 0.57	10.33 ± 0.57
7	BCS	15.33 ± 0.57	14.33 ± 0.57	15.0 ± 1.0	14.0 ± 1.0
8	M 3	10.33 ± 0.57	11.0 ± 1.0	10.0 ± 0.0	10.66 ± 0.57
9	DBM 3	13.66 ± 0.57	12.66 ± 0.57	12.0 ± 1.0	12.33 ± 1.15
10	DBC 2	10.66 ± 1.15	10.66 ± 0.57	10.66 ± 0.57	11.33 ± 1.52
11	BSM 6	15.66 ± 0.57	14.66 ± 0.57	15.66 ± 0.57	14.33 ± 0.57
12	DGM 7	24.0 ± 1.0	23.33 ± 0.57	20.0 ± 1.0	21.33 ± 1.52

2.9. Hemolytic activity:

Out of all the isolates, one isolate (DGM7) was selected as a probiotic based on the above conducted assays, for which this isolate has shown positive results. The selected isolate was

subjected to hemolytic activity to assess its safety. The isolate inoculated in the plate showed no hemolytic activity as no zone formation was observed around the colony, which indicates the γ-hemolytic activity of the isolate. The isolates that show γ-hemolysis are generally considered safe to be used as a probiotic.

Fig 4:Hemolytic activity of DGM7 isolate.

2.10. Molecular identification:

Among all the isolates, DGM 7 possessed all the probiotic potential tests like tolerance to NaCl (8%), bile salt (0.5%), acid (pH-2), phenol (0.3%) conducted on it. Also, showed resistant to penicillin and clindamycin, and it possessed antibacterial activity against tested pathogenic bacteria. It was also found that DGM7 had γ-hemolysis activity which indicates its safety to be used as a probiotic. The isolate was further confirmed through 16S rRNA gene sequence analysis. On the basis of molecular identification, purified PCR products were sequenced and compared with the information in the NCBI database. BLAST search disclosed that isolated bacterium as *Bacillus velezensis*. which exhibited a similarity index (100.0 %) to other *B. velezensis* strains. Therefore, the isolate was a strain of *B. velezensis* and designated as *B. velezensis*DGM7. The 16S rRNA sequence was deposited to NCBI Genebank data base under the accession number PP832844.

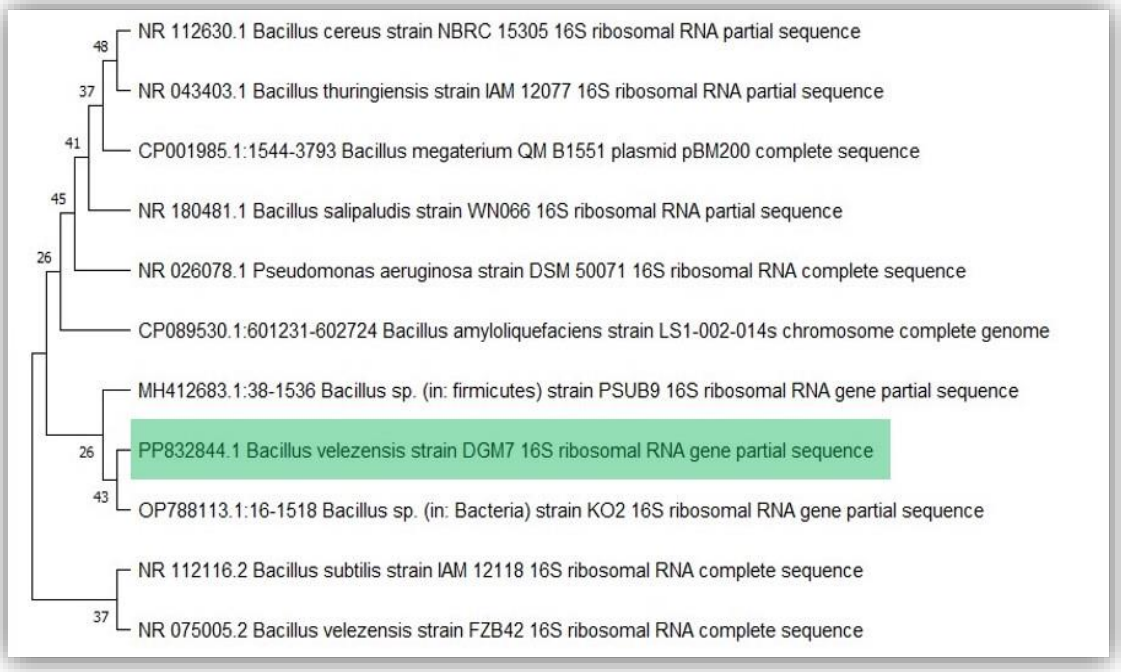


Fig 5: Phylogenetic tree of DGM7 isolate.

3. DISCUSSION:

The selection procedures for probiotics that were employed in this study are based on in vitro criteria that have been previously documented by the FAO/WHO committee (Joint FAO/WHO Working Report, 2002). The process involves examining the organism's behavior in environments similar to the GIT to select promising isolates that can endure these conditions and be further studied for potential probiotic culture applications (Zago *et al.,* 2011). In order to select the best probiotic isolate with the greatest probiotic potential, a preliminary screening of the isolates based on their probiotic potential was conducted. In our present study, NaCl tolerance, bile salt tolerance, acid and phenol tolerance, antibiotic susceptibility, antibacterial activity, and hemolytic activity were conducted to screen and select the best isolate to be used as a probiotic, which was further confirmed by molecular identification.

In our present study NaCl tolerance was tested using 8% NaCl concentration, finding 14 isolates including *B. velezensis* DGM7tolerant and others intolerant. This aligns with previous studies by Liu et al. (2009) and Prabhurajeshwar and Chandrakanth (2017), which showed *Lactobacillus spp*. could withstand 1-6% NaCl and showed great growth at 1-5% NaCl. Bile salt resistance is crucial for selecting a probiotic strain for effective performance in the human gastrointestinal tract. Bacteria must be resistant to acid and bile salt, as they travel through the acidic environment and alkaline pH of the intestine. A bile salt concentration range of 0.15-0.5% is suggested for probiotics Papadimitriou et al., (2015). In this study, 26 isolates showed growth at different concentrations of bile, with 25 able to grow at 0.5% bile. This finding is consistent with previous studies by Raisagar and Shukla (2022) and Andriani et al., (2017), where probiotic cultures like *B. subtilis* and *B. licheniformis* showed the greatest growth at 0.3% and 0.5% bile salt concentrations. Also, Pundir et al., (2013), reported bile salt tolerance in LAB isolates where the range was found to be at 0.5% to 2.0% bile concentrations. Probiotics' ability to withstand acidic environments is essential for their survival and efficacy, particularly in the harsh gastrointestinal tract. Studies have found that probiotic strains must be able to tolerate low pH values, usually between pH 1 and pH 3, in order to survive the stomach's acidic environment and successfully colonize the gut (Raisagar and Shukla, 2022). *Lactobacillus* species can thrive effectively at an acidic pH of 2-5 and can withstand a wide pH range of 2-8 (Prabhurajeshwar and Chandrakanth, 2017). Hyronimus et al., (2000) stated that spore forming lactic acid producing bacteria (SFLAB) such as *Bacillus* species survive in acid conditions (pH 2.5-3.0) Wang et al. (2010) study found that 11 *Bacillus* strains were acid-resistant to pH 2.5 and had survival rates of over 90%. These strains were further tested in simulated gastric juice at pH 2.0, revealing their high survival rates.

Phenols, byproducts of colonic protein metabolism and degradation, can have harmful consequences on the gut mucosa and suppress probiotic bacteria. Phenol tolerance is a necessary characteristic for probiotics (Jena *et al.,* 2013 and Raisagar and Shukla., 2022). In our study, 19 isolates including *B. velezensis* DGM7 showed tolerance to 0.3% phenol concentration, while others could not resist it. High survival rates were observed in LAB isolates like *Lactobacillus brevis, L. plantarum*, and *Pediococcus* at 0.4% phenol concentration, decreasing with increased concentration. They could tolerate a maximum of 0.6% phenol (Divya *et al.,* 2012). Previous studies by Panda et al. (2017)have shown that *Enterococcus* and *Bacillus* isolates with phenol resistance have a high survival rate up to 0.2% phenol, which progressively drops to less than 30% at 0.5% phenol. Also, Dabiré et al. (2022) reported that *B. dakarensisB54* showed the lowest viability (52.90%), while *B. cabrialesii F26* showed the highest survivability (88%) at a 0.4% phenol concentration.

Probiotic strains like *B. subtilis* have antibacterial properties, preventing pathogenic bacteria infection or invasion. The *Bacillus* genus produces various antimicrobial substances, including peptide and lipopeptide antibiotics, which are effective against Gram-positive and Gram-negative

bacteria (Ritter *et al.,* 2018). According to Panda et al. (2017), SB10 and JC13 showed antibacterial activity against *Staphylococcus aureus, Klebsiella*, and *E. coli*, while *Pseudomonas* did not show any antibacterial activity. IFM22 showed moderate antibacterial activity against *S. aureus, Klebsiella*, and *E. coli*, with zones of 1.5 mm, 1 mm, and 0.5 mm, respectively.

Hemolytic activity analysis is regarded as a safety factor when adopting probiotic strains (Ritter *et al.,* 2018). Non-hemolytic strains are generally safe for hosts, while those with hemolytic activity are pathogens. Hemolytic microorganisms disrupt red blood cells, release hemoglobin, and produce hemolysins, which can have cytolytic effects on host cells and decrease hemoglobin content as a Fe source (Golnari *et al.,* 2024). Our study reveals that *B. velezensis* DGM7 exhibits γ-haemolysis also, Panda et al. (2017) stated, Isolates *Enterococcus* and *Bacillus* were found to be non-haemolytic (γ- haemolysis) on 5 % sheep blood agar.

Overall, the assessment of the isolates' probiotic properties in this study demonstrated their capacity to endure challenging gastrointestinal-like environments and manifest probiotic potential. According to our findings, the isolate identified as *Bacillus valenzensis* DGM7 may be a promising probiotic whose ingestion may enable its growth in the gastrointestinal system, where it could enhance consumers' health.

4. CONCLUSION:

Isolated *Bacillus valenzensis* DGM7 from dairy source (goat milk) has probiotic properties that make them potential candidates for probiotic applications in maintaining oral health. *Bacillus valenzensis* DGM7 strain showed tolerance to acid, phenol, bile and salt concentrations used in the above studies. The strain also showed antibacterial activity against Gram-positive and Gram-negative pathogens tested against it and showed resistance to antibiotics like penicillin and clindamycin. The organism was confirmed to be safe for consumption as probiotics as it showed γ-hemolysis which is generally considered safe for a probiotic to be used.*Bacillus valenzensis* DGM7 might become a perfect probiotic system due to its probiotic potential properties. This strain being dairy isolate thus enhances their ease of application in the human health. Further, in vivo evaluation and safety profile studies are required to determine its real-life applications.

ACKNOWLEDGEMENTS:

This research was financially supported by University Research Scholarship (URS), Karnatak University Dharwad, DST-FIST grant to Department and Department of Science and Technology, KSTePS (Karnataka Science and Technology Promotion Society).

REFERENCES:

1. Andriani, Y., Safitri, R., Rochima, E., & Fakhrudin, S. D. (2017). Characterization of Bacillus subtilis and B. licheniformis potentials as probiotic bacteria in Vanamei shrimp feed (Litopenaeus vannamei Boone, 1931). *Nusantara Bioscience*, *9*(2), 188-193.
2. Ayyash, M. M., Abdalla, A. K., AlKalbani, N. S., Baig, M. A., Turner, M. S., Liu, S. Q., & Shah, N. P. (2021). Invited review: Characterization of new probiotics from dairy and nondairy products—Insights into acid tolerance, bile metabolism and tolerance, and adhesion capability. *Journal of Dairy Science*, *104*(8), 8363-8379.
3. Beatrice, A. A., Joseph, M. M., Rahma, U. M. Y., & Tochi, B. (2020). Probiotic potential of lactic acid bacteria isolated from traditionally fermented legume products of Western Kenya. *African Journal of Food Science*, *14*(10), 373-377.
4. Cani, P. D., & Delzenne, N. M. (2009). The role of the gut microbiota in energy metabolism and metabolic disease. *Current Pharmaceutical Design*, *15*(13), 1546-1558.

5. Chakraborty, A., & Bhowal, J. (2015). Isolation, identification and analysis of probiotic properties of Lactobacillus spp. from selected regional dairy product. *International Journal Of Current Microbiology and Applied Sciences*, *4*(6), 621-628.
6. Chugh, P., Dutt, R., Sharma, A., Bhagat, N., & Dhar, M. S. (2020). A critical appraisal of the effects of probiotics on oral health. *Journal of Functional Foods, 70*, 103985.
7. Cizeikiene, D., & Jagelaviciute, J. (2021). Investigation of antibacterial activity and probiotic properties of strains belonging to Lactobacillus and Bifidobacterium genera for their potential application in functional food and feed products. *Probiotics and Antimicrobial Proteins*, 1-17.
8. Collado, M. C., Meriluoto, J., & Salminen, S. (2008). Adhesion and aggregation properties of probiotic and pathogen strains. *European Food Research and Technology*, *226*, 1065-1073.
9. Dabiré, Y., Somda, N. S., Somda, M. K., Compaoré, C. B., Mogmenga, I., Ezeogu, L. I., ... & Dicko, M. H. (2022). Assessment of probiotic and technological properties of Bacillus spp. isolated from Burkinabe Soumbala. *BMC Microbiology*, *22*(1), 228.
10. Divisekera, D. M. W. D., Samarasekera, J. K. R. R., Hettiarachchi, C., Gooneratne, J., Choudhary, M. I., & Gopalakrishnan, S. (2019). Isolation and identification of lactic acid bacteria with probiotic potential from fermented flour of selected banana varieties grown in Sri Lanka.*Journal Of The National Science Foundation Of Sri Lanka*, 47 (1): 3 – 16.
11. Divya, J. B., Varsha, K. K., & Nampoothiri, K. M. (2012). Newly isolated lactic acid bacteria with probiotic features for potential application in food industry. *Applied Biochemistry and Biotechnology*, *167*, 1314-1324.
12. Fuller, R. (1999). Probiotics for farm animals. *Probiotics: A Critical Review.*, 15-22.
13. Gill, P. K. (2023). Lactic Acid Bacteria as Probiotics: Current Status and Future Prospects. *Asian Journal Of Microbiology And Biotechnology*, 120-139.
14. Golnari, M., Bahrami, N., Milanian, Z., Rabbani Khorasgani, M., Asadollahi, M. A., Shafiei, R., & Fatemi, S. S. A. (2024). Isolation and characterization of novel Bacillus strains with superior probiotic potential: comparative analysis and safety evaluation. *Scientific Reports*, *14*(1), 1457.
15. Gupta, V., & Garg, R. (2009). Probiotics. *Indian Journal Of Medical Microbiology*, *27*(3), 202-209.
16. Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., ... & Sanders, M. E. (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews Gastroenterology & Hepatology*, *11*(8), 506-514.
17. Hyronimus, B., Le Marrec, C., Sassi, A. H., & Deschamps, A. (2000). Acid and bile tolerance of spore-forming lactic acid bacteria. *International Journal Of Food Microbiology*, *61*(2-3), 193-197.
18. Jena, P. K., Trivedi, D., Thakore, K., Chaudhary, H., Giri, S. S., & Seshadri, S. (2013). Isolation and characterization of probiotic properties of lactobacilli isolated from rat fecal microbiota. *Microbiology And Immunology*, *57*(6), 407-416.
19. Joint FAO/WHO Expert Committee on Food Additives. Meeting. (2006). *Compendium of food additive specifications: joint FAO/WHO expert committee on food additives: 67th meeting 2006* (Vol. 3). Food & Agriculture Organization.
20. Kaur, H., Kaur, G., & Ali, S. A. (2022). Dairy-based probiotic-fermented functional foods: An update on their health-promoting properties. *Fermentation*, *8*(9), 425.
21. Keller, M. K., Hasslöf, P., Stecksén-Blicks, C., & Twetman, S. (2011). Co-aggregation and growth inhibition of probiotic lactobacilli and clinical isolates of mutans streptococci: an in vitro study. *Acta Odontologica Scandinavica*, *69*(5), 263-268.

22. Khushboo, Karnwal, A., & Malik, T. (2023). Characterization and selection of probiotic lactic acid bacteria from different dietary sources for development of functional foods. *Frontiers In Microbiology*, *14*, 1170725.
23. Ley, R. E., Peterson, D. A., & Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, *124*(4), 837-848.
24. Liu, C. H., Chiu, C. S., Ho, P. L., & Wang, S. W. (2009). Improvement in the growth performance of white shrimp, Litopenaeus vannamei, by a protease-producing probiotic, Bacillus subtilis E20, from natto. *Journal Of Applied Microbiology*, *107*(3), 1031-1041.
25. Mannan, S. J., Rezwan, R., Rahman, M. S., & Begum, K. (2017). Isolation and biochemical characterization of Lactobacillus species from yogurt and cheese samples in Dhaka metropolitan area. *Bangladesh Pharmaceutical Journal*, *20*(1), 27-33.
26. Merenstein, D., Pot, B., Leyer, G., Ouwehand, A. C., Preidis, G. A., Elkins, C. A., ... & Sanders, M. E. (2023). Emerging issues in probiotic safety: 2023 perspectives. *Gut Microbes*, *15*(1), 2185034.
27. Oelschlaeger, T. A. (2010). Mechanisms of probiotic actions-a review. *International Journal Of Medical Microbiology*, *300*(1), 57-62.
28. Panda, S. H., Goli, J. K., Das, S., & Mohanty, N. (2017). Production, optimization and probiotic characterization of potential lactic acid bacteria producing siderophores. *AIMS Microbiology*, *3*(1), 88.
29. Papadimitriou, K., Zoumpopoulou, G., Foligné, B., Alexandraki, V., Kazou, M., Pot, B., & Tsakalidou, E. (2015). Discovering probiotic microorganisms: in vitro, in vivo, genetic and omics approaches. *Frontiers In Microbiology*, *6*, 129543.
30. Prabhurajeshwar, C., & Chandrakanth, R. K. (2017). Probiotic potential of Lactobacilli with antagonistic activity against pathogenic strains: An in vitro validation for the production of inhibitory substances. *Biomedical Journal*, *40*(5), 270-283.
31. Pundir, R. K., Kashyap, S. R. N., & Kaur, A. (2013). Probiotic potential of lactic acid bacteria isolated from food samples: an in vitro study. *Journal Of Applied Pharmaceutical Science*, *3*(3), 085-093.
32. Pyar, H., & Peh, K. K. (2014). Enteric coating of granules containing the probiotic Lactobacillus acidophilus. *Acta Pharmaceutica*, *64*(2), 247-256.
33. Rahman, S. M. K. (2015). Probiotic properties analysis of isolated lactic acid bacteria from buffalo milk. *Arch Clin Microbiol*, *7*(1), 5-10.
34. Raisagar, A., & Shukla, S. (2022). Evaluation of Probiotic Potential of Selected Lab Cultures. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences*, *24*(2), 269-274.
35. Reid, G., Jass, J., Sebulsky, M. T., & McCormick, J. K. (2003). Potential uses of probiotics in clinical practice. *Clinical Microbiology Reviews*, *16*(4), 658-672.
36. Ritter, A. C., Paula, A., Correa, F., Veras, F. F., & Brandelli, A. (2018). Characterization of Bacillus subtilis available as probiotics. *Journal of Microbiology Research*, *8*(2), 23-32.
37. Sanders, M. E. (2000). Considerations for use of probiotic bacteria to modulate human health. *The Journal Of Nutrition*, *130*(2), 384S-390S.
38. Shortt, C. (1999). The probiotic century: historical and current perspectives. *Trends In Food Science & Technology*, *10*(12), 411-417.
39. Singhal, N., Singh, N. S., Mohanty, S., Singh, P., & Virdi, J. S. (2019). Evaluation of probiotic characteristics of lactic acid bacteria isolated from two commercial preparations available in Indian market. *Indian Journal Of Microbiology*, *59*, 112-115.

40. Soccol, C. R., de Souza Vandenberghe, L. P., Spier, M. R., Medeiros, A. P., Yamaguishi, C. T., De Dea Lindner, J., ... & Thomaz-Soccol, V. (2010). The potential of probiotics: a review. *Food Technology and Biotechnology*, *48*(4), 413-434.
41. Song, M., Yun, B., Moon, J. H., Park, D. J., Lim, K., & Oh, S. (2015). Characterization of selected Lactobacillus strains for use as probiotics. *Korean Journal For Food Science Of Animal Resources*, *35*(4), 551.
42. Urrutia-Baca, V. H., Hernández-Hernández, S. N., Martínez, L. M., Dávila-Vega, J. P., & Chuck-Hernández, C. (2024). The Role of Probiotics in Dairy Foods and Strategies to Evaluate Their Functional Modifications. *Food Reviews International*, *40*(1), 434-456.
43. Vijaya Kumar B., Vijayendra, S. V. N., & Reddy, O. V. S. (2015). Trends in dairy and non-dairy probiotic products-a review. *Journal Of Food Science And Technology*, *52*, 6112-6124.
44. Wang, Y., Zhang, H., Zhang, L., Liu, W., Zhang, Y., Zhang, X., & Sun, T. (2010). In vitro assessment of probiotic properties of Bacillus isolated from naturally fermented congee from Inner Mongolia of China. *World Journal of Microbiology and Biotechnology*, *26*, 1369-1377.
45. Williams, N. T. (2010). Probiotics. *American Journal Of Health-System Pharmacy*, *67*(6), 449-458.
46. World Health Organization. (2005). Fruit and vegetables for health: report of the Joint FAO.
47. Yadav, R., Puniya, A. K., & Shukla, P. (2016). Probiotic properties of Lactobacillus plantarum RYPR1 from an indigenous fermented beverage Raabadi. *Frontiers In Microbiology*, *7*, 1683.
48. Yasmin, I., Saeed, M., Khan, W. A., Khaliq, A., Chughtai, M. F. J., Iqbal, R., ... & Tanweer, S. (2020). In vitro probiotic potential and safety evaluation (hemolytic, cytotoxic activity) of Bifidobacterium strains isolated from raw camel milk. *Microorganisms*, *8*(3), 354.
49. Zago, M., Fornasari, M. E., Carminati, D., Burns, P., Suàrez, V., Vinderola, G., ... & Giraffa, G. (2011). Characterization and probiotic potential of Lactobacillus plantarum strains isolated from cheeses. *Food Microbiology*, *28*(5), 1033-1040.
50. Zeng, Y., Li, Y., Wu, Q. P., Zhang, J. M., Xie, X. Q., Ding, Y., ... & Wang, J. (2020). Evaluation of the antibacterial activity and probiotic potential of Lactobacillus plantarum isolated from Chinese homemade pickles. *Canadian Journal Of Infectious Diseases And Medical Microbiology*, *2020*, 1-11.
51. Zhang, W., Lai, S., Zhou, Z., Yang, J., Liu, H., Zhong, Z., ... & Peng, G. (2022). Screening and evaluation of lactic acid bacteria with probiotic potential from local Holstein raw milk. *Frontiers In Microbiology*, *13*, 918774.
52. Zhao, X., Hu, R., He, Y., Li, S., Yang, J., Zhang, J., ... & Xue, T. (2022). Screening of isolated potential probiotic lactic acid bacteria from Sichuan pickle for cholesterol lowering property and triglycerides lowering activity. *Food Science and Technology*, *42*.
53. Zoumpopoulou, G., Pot, B., Tsakalidou, E., & Papadimitriou, K. (2017). Dairy probiotics: Beyond the role of promoting gut and immune health. *International Dairy Journal*, *67*, 46-60.