The Bioscan AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES 20(2): S2: 725-730, 2025 www.thebioscan.com

Isolation and Identification of *Bifidobacterium longum subsp infantis* isolated from cow milk and its antibacterial activity

Divya SB[1*], Radhai Sri S[1], R Ragunathan[2] and Jesteena Johney[2]

[1*] Department of Nutrition and Dietetics, PSG College of Arts and Science, Coimbatore.

[1]Department of Food Processing Technology, PSG College of Arts and Science, Coimbatore.

[2]Centre for Bioscience and Nanoscience Research, Coimbatore

DOI: 10.63001/tbs.2025.v20.i02.S2.pp725-730

KEYWORDS
LAB, Bifidobacterium longum, probiotic, ABST, antimicrobial activity.

Received on:
01-02-2025

Accepted on:
18-05-2025

Published on:
09-06-2025

ABSTRACT

Bifidobacteria are common probiotics associated with health-promoting "functional foods" as well as therapeutic, prophylactic and growth supplements for humans. The study was designed to isolate and characterise *Bifidobacteria* from raw cow milk and to provide a more inclusive report for their probiotic potential. The presumptive isolate was assessed for tolerance to pH and bile salt. Subsequently, probiotic potential and safety evaluation were confirmed through antibacterial activity and antibiotic susceptibility. The most promising strain with remarkable probiotic potential was identified by 16S rRNA gene sequencing *as the Bifidobacterium longum subsp infantis* strain. The strain showed good tolerance to 0.3% bile salts after 6 hrs of exposure. The growth of *Bifidobacterium longum* was observed at 4% Nacl with an OD value of 0.684. *B.longum* showed survivability at pH ranging from 2.0, 5.0 and 7.0 through turbidity of growth confirming the acid tolerance property. In addition, the strain also exhibited antimicrobial activity (agar well diffusion method) towards intestinal pathogens, such as *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*. The antibiotic susceptibility profile of the isolated strain was tested using the disc diffusion method and found to be resistant to the antibiotics. The findings of this study showed that the B*ifidobacteria* strain isolated from raw cow milk, a promising probiotic could be used as a dietary adjunct or for the development of new functional foods.

INTRODUCTION

Probiotics are commonly consumed as part of fermented foods with specially added active live cultures, such as in yoghurt, soy yoghurt, or as dietary supplements [1]. The definition of probiotics is 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' [2,3]. Probiotics have been attributed to various benefits such as antimutagenic and anticarcinogenic properties, antiinfection properties, immune system stimulation, serum cholesterol reduction, alleviation of lactose intolerance and nutritional enhancement [4].

Several requirements have to be fulfilled by strains to be an effective Probiotic. The microbes must survive through the gastrointestinal tract that is they should survive both in the stomach and bile acid and should have antimicrobial activities against pathogens [5,6]. Strains should be safe, with minimal possibilities for antibiotic resistance transfer. Probiotic strains should be stable during the gastrointestinal passage (resistance to low pH and bile acids) and also in the product (resistance to oxygen and technological process), and they should maintain good viability and functionality during storage. It is required that probiotics survive in the gastrointestinal microbial ecosystem; adherence to the gut epithelium would enhance their survival ability [7].

The most known probiotic microorganisms are bacteria belonging to the *Bifidobacterium* genera [8]. *Bifidobacterium* is one of the most used probiotic microorganisms in the food industry due to its health-enhancing benefits [9]. One of the largest bacterial populations of the human gastrointestinal tract (GIT) is that formed by *bifidobacteria*. GIT-associated microorganisms play a pivotal role in health by maintaining a well-balanced intestinal microbiota [10]. The ability of *bifidobacteria* to exert beneficial effects on human health is a species- and strain-specific feature [9].

The *Bifidobacterium* species that inhabit the human intestinal tract are rather distinct from those that inhibit the intestines of animals [11]. They suppress harmful bacteria by controlling the pH of the large intestine through the production of lactic and acetic acids [12]. *Bifidobacterium* spp. of human origin can utilize fructose, galactose and lactose. They are the main saccharolytic bacteria found in the human colon and can utilize non-digestible oligosaccharides in the colon to produce acetic acid as well as lactic acid in the ratio of 3:2 through a unique fructose-6-phosphate phosphoketolase pathway [13].

Different species of *bifidobacteria* may exhibit various health benefits, such as regulation of intestinal microbial homeostasis, repression of carcinogenic enzymatic activities within the microbiota, production of vitamins, and the bioconversion of several dietary compounds into bioactive molecules [14].

The isolation of *bifidobacteria* as a potential probiotic culture, the antimicrobial agent is promising in a wide range of biotechnological and biomedical applications. These probiotics are potentially used as microbial culture in food systems due to their safety aspects and various functional attributes [15]. *Bifidobacteria* exhibited tremendous biotechnological and functional attributes including the ability to survive in harsh gastrointestinal conditions that seem to vary among different strains of *Bifidobacterium* due to phenotypic and genotypic

variations within the species [16]. Thus, this study aims to isolate and characterise *bifidobacteria* from cow's milk and study their probiotic proprieties *in vitro*.

2. Materials and Methods

Isolation of Bifidobacteria

The *Bifidobacteria spp* was isolated from cow's milk by serial dilution from 10^{-1} to 10^{-6}. From each dilution, 0.1 mL was spread onto *Bifidobacterium* agar and incubated at 32°C for 48 hours in anaerobic conditions. Colonies with typical LAB traits were selected, purified in MRS broth, and examined microscopically for morphology. Confirmed lactic acid bacteria colonies were then subjected to further identification steps [17]. Isolated colonies were routinely propagated in MRS broth. Further identification was done using classic microbiology tests, including Gram-staining for detecting morphology and catalase test. The probiotic characterisation, including tolerance to pH, tolerance to bile salts and tolerance to NaCl concentration of the isolate, was determined.

Tolerance to pH

Tolerance to low pH was determined by using broth assay. 40µl (2%) activated overnight culture of the isolates were incubated in 2 ml of MRS-Cys broth in triplicate which was adjusted to pH 2, 4, 5 and 7 with 1N HCl or 1N NaOH. The inoculated tubes were incubated for 48 to 72 hours at 37°C, and the tubes were observed for turbidity. The cultures which showed turbidity were monitored by determination of optical density at 600 nm in the UV-Vis spectrophotometer [18].

Tolerance to Bile salts

Bile plays an important role in the survival of bacteria in the small intestine. Food remains in the small intestine for around 4 hours till it gets absorbed. The isolated strain was screened for survival in bile concentrations. Cultures were inoculated into 10 ml MRS broth in test tubes and incubated at 37^0C overnight in anaerobic conditions. 100µl of active culture was inoculated into fresh MRS broth tubes with pH 6.5 containing 0.3% bile. The bacterial growth was measured at 600nm OD spectrophotometrically for 0, 3 hrs, 6 hrs, 24 hrs and 48 hrs [19].

Tolerance to NaCl

The tolerance of isolated culture against different NaCl concentrations was recorded by inoculating the pure culture isolates in MRS broth having 2.0, 3.0, 4.0, 6.0 and 8% (w/v) sodium chloride. After 48 h of incubation at 37°C, broths were examined for growth and results were recorded for OD value at 620 nm OD spectrophotometrically.

Identification of the isolated strain

The genomic DNA extraction from isolated and purified strains was the first step in the molecular identification of lactic acid bacteria strains. It was extracted in accordance with the DNA extraction kit. Once the DNA had been extracted, the specific genes of each bacterial species were analysed using Polymerase Chain Reaction (PCR). Simplex PCR amplification was carried out using primers targeting genes coding for 16s rRNAs. The PCR conditions were initial denaturation at 95°C for 1min, followed by 28 cycles consisting of denaturation at 95°C for 1min, hybridisation at 55°C for 30s, extension at 72°C for 30s and a final extension step for 2 min at 72°C. Then, the sequence homology was analysed by comparative studies using "The National Centre for Biotechnology Information (NCBI) and Basic Local Alignment Search Tool (BLAST)".

Antibiotic Susceptibility Test

The antibiotics susceptibility of the isolate was evaluated against antibiotics (Himedia, Mumbai) ciprofloxacin (8µg), ceftazidime (10µg), ampicillin (10µg), penicillin (10µg), amoxiclav (10µg), vancomycin (30µg), amikacin (10µg), methicillin (5µg), chloramphenicol (30µg) and cefozolin (30µg) using disk diffusion method by the Kirby-Bauer method (1966). After Incubation for 48 hr at 37ºC, the inhibition zone diameter was measured by using the Hi-media zone scale. The results were expressed as sensitive (S), intermediate (I) and resistant (R) according to the National Committee for Clinical Laboratory Standards, NCCLS [20].

Antimicrobial activity test

The antimicrobial activity of the isolated strain against the test pathogens namely *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus cereus* was determined using the agar well diffusion method The diameters of inhibition zones were measured and recorded after incubating at 37°C for 24 h under anaerobic conditions. The isolated strain *B.longum* was inoculated to MRS broth and incubated for 24 h at 37°C. Fresh cultures of the four targeted pathogens (100 µl, 10^{-7} CFU/mL) were coated on each Muller Hinton agar plate and dried. Bacterial cell extracts were prepared using ethyl acetate. Well of 5 mm diameter were made on plates and filled with 100 µl of cell-free supernatant (CFS) obtained from centrifugation of LAB cultures at 4500 rpm/min for 10 min. A sterile glass dropper was used to punch wells each on four MHA plates inoculated with test strains, in which 40 µl of supernatant from the sample was loaded into the wells. The plates were then incubated at 37 ℃ for 24 hours. The antibacterial activity was indicated by the density of bacterial growth around the discs [21].

3. Results and Discussion

In this study, the presence of *Bifidobacteria* in cow milk was investigated. Colonies were obtained from milk samples in *Bifidobacteria* agar plates. The colony counts ranged from $2.5x10^{-7}$ to $2.9 x10^{-7}$. The isolates of *Bifidobacterium* in the present study were found to be Gram-positive and branched rods that occur singly or in chains or clumps, as shown in Figure 1. The colonies of *Bifidobacterium* were found in round and off-white colonies on modified MRS agar plates. The isolate showed the catalase test negative. Subsequently, the isolate was inoculated by streak plates on *Bifidobacterium* agar and was incubated anaerobically. In this context, Yasmin *et al*. [22] reported that *Bifidobacterium* isolated from camel milk formed white to off-white, shiny, round colonies on agar plates. Similar to the present study, the *Bifidobacterium* species were characterised as Gram-positive, rod-shaped (V or Y), catalase-negative, non-motile, and non-spore-forming, consistent with observations by Milani *et al*. [23]. Further, the probiotic characterisation, such as tolerance to pH, bile salts and NaCl, was observed for the isolate.

Figure 1: Colony culture of the isolated strain

The 16sRNA gene sequence-based phylogenetic tree revealed that the strain in the present study belonged to *Bifidobacterium* with more similarity to other strains within the genus, which was identified as *Bifidobacterium longum subsp infantis* (The Genebank accession number is ON817279). The strain showed 98% similarity with *Bifidobacterium longum*. Thus, the isolate was named *Bifidobacterium longum DIRAD1*. The phylogenetic tree was constructed by Mega5 software and shown in Figure 2.

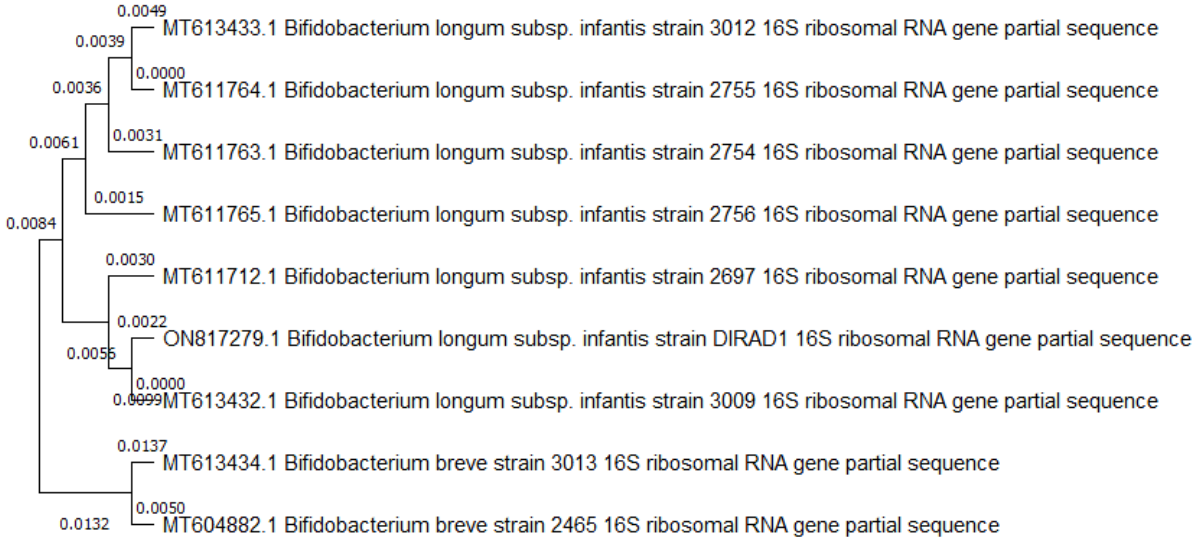

Figure 2: The phylogenetic tree construction of *Bifidobacterium longum* strain (Mega5 software)

Tolerance to pH

The mean residence time of food in the stomach is approximately 3 hours. The impact of acidic conditions on bacterial growth at pH levels of 2.0, 5.0, and 7.0 was assessed, as shown in Figure 3. In this study, the *Bifidobacterium longum* strain demonstrated the ability to survive at pH 2 after 24 hours of incubation. This indicates the strain's high tolerance to the acidic conditions typically encountered in the stomach. Maximum growth was observed by measuring the bacterial density of the isolate at pH 5 and 7, which are more neutral conditions similar to the small intestine and colon, where *Bifidobacterium* species naturally thrive. The ability of probiotics to survive, in adequate numbers, after being subjected to gastric acidity (low pH) and intestine condition (bile salts) is important to be used in the food industry [24].

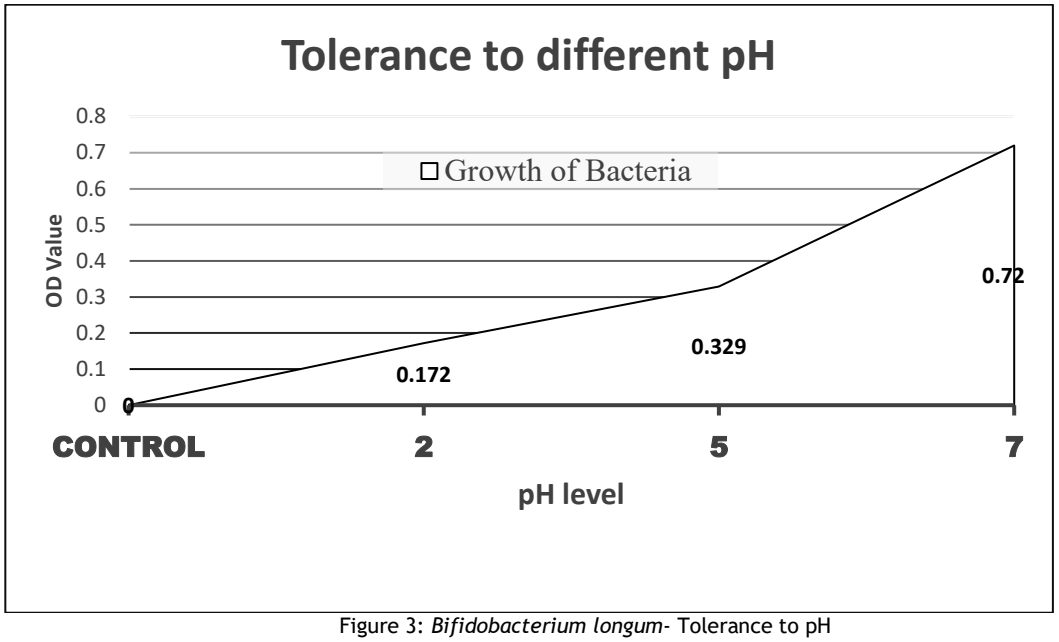

Figure 3: *Bifidobacterium longum*- Tolerance to pH

Tolerance to NaCl

In this study, the isolated probiotic demonstrated the ability to grow at NaCl concentrations ranging from 1% to 5%, with growth gradually increasing as NaCl concentration rose. Interestingly, growth increased progressively with rising NaCl concentrations within this range. This characteristic could be advantageous for the strain's survival in various environments, including food matrices with varying salt content and certain regions of the gastrointestinal tract. Dairy *et al* (2016) discuss the effects of NaCl reduction and substitution with KCl on *B. longum* This finding aligns with Rahman *et al*. [9], who reported that lactic acid bacteria could grow at NaCl concentrations from 1% to 6%. Similarly, Nannu and Chandru [25] observed good growth of *Bifidobacterium longum* at NaCl concentrations between 2% and 6%, supporting the results of the present study.

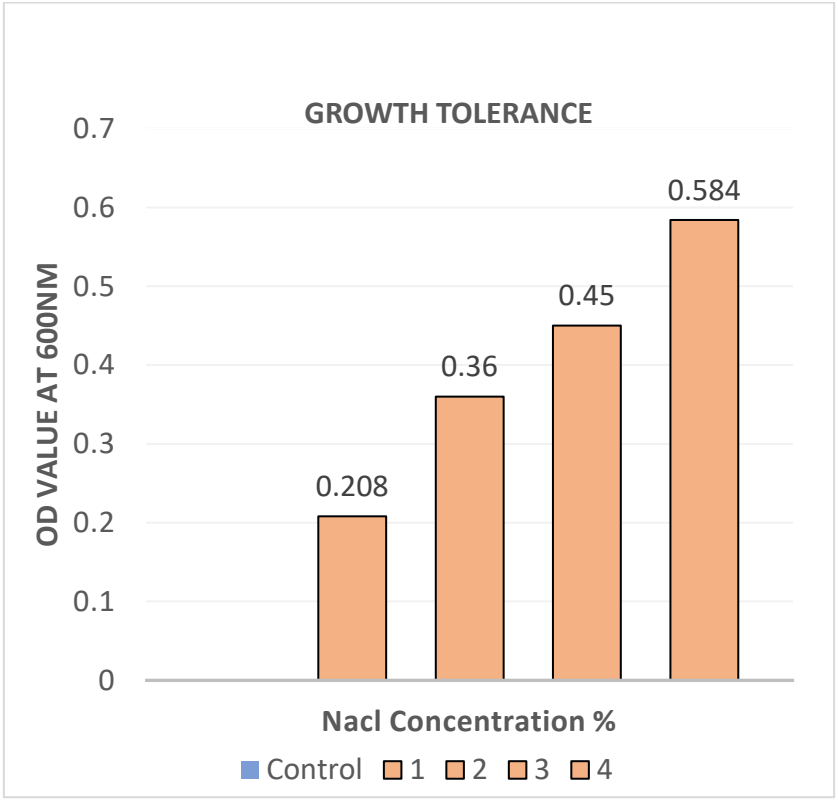

Figure 4: Shows *Bifidobacterium longum*- Tolerance to NaCl

Tolerance to Bile salts

Probiotic bacteria should be able to resist inhibitory factors in the gastrointestinal tract, such as acid and bile salts. The isolate was tested for its capacity to tolerate bile salt, which showed the ability to grow at 0.3% of bile salt concentrations. The optical density was measured by spectrophotometer after 2h, 3h, 6h, 24h and 48hrs intermission. Their ability to tolerate bile salt is shown in Figure 5. Delgado *et al*. [26] also showed that all isolates grew in the presence of 0.25% to 2% bile salt concentration, including one-third of *B.longum* isolates.

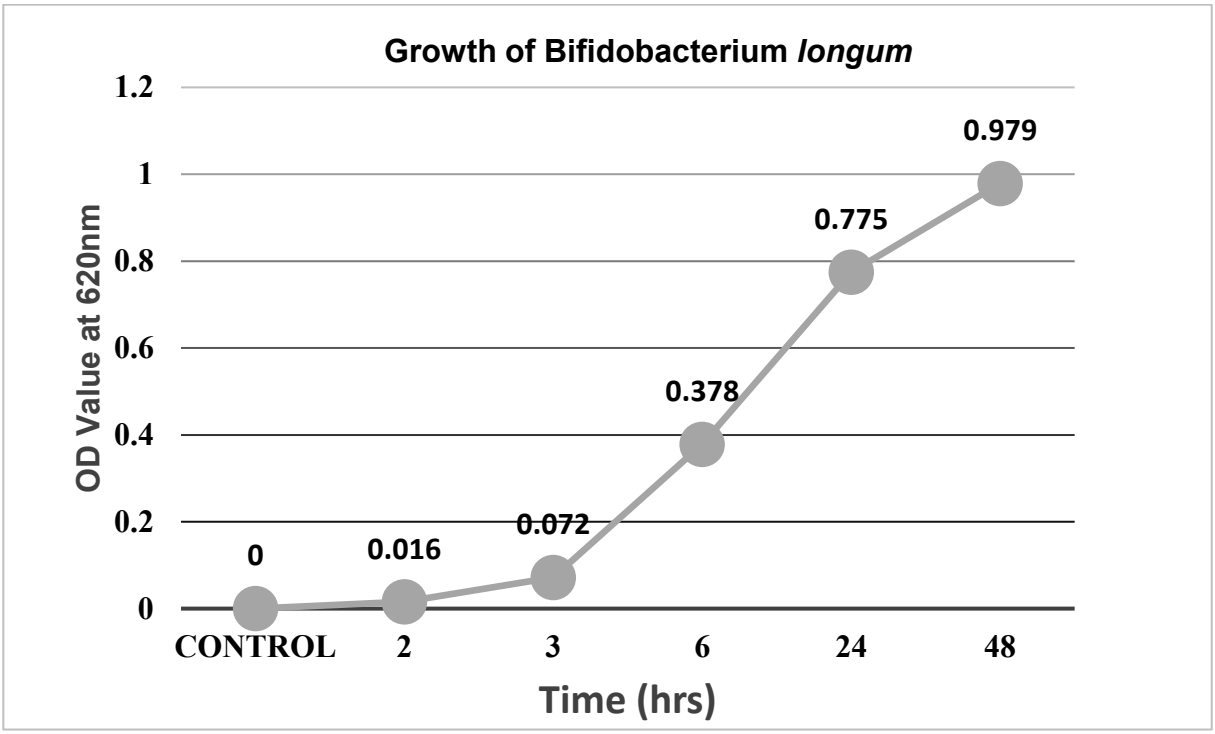

Figure 5: Tolerance against bile salts (0.3%) concentration- OD 620 nm values

Antibiotic susceptibility profile

The antibiotic susceptibility of the isolated strain was documented by using different antibiotics. The result presented in Table 1 is that the strain was resistant to Ciprofloxacin, vancomycin, cefazolin, and methicillin. Sensitive to Amoxyclav, amikacin, ceftazidime, penicillin, ampicillin and chloramphenicol. As far as safety is concerned, the isolates should have the ability to resist commonly used antibiotics. In line with the result, Mahmoudi *et al*. (2020) [AB] also showed that many strains are very susceptible to chloramphenicol, penicillin, ampicillin, erythromycin and resistant to kanamycin, vancomycin and streptomycin. *Bifidobacterium* is susceptible to antibiotics and can be consumed safely after antibiotic therapy for maintaining the balance of gut microflora.

Antibacterial activity

Antimicrobial activity is a crucial criterion for selecting starter and probiotic cultures, as these organisms serve as natural antagonists to potentially harmful bacteria. The results of the antibacterial activity, presented in Figure 6 were observed as halos of growth inhibition on agar plates, produced by the isolate against indicator pathogens. Antibacterial activity was confirmed when no growth or haziness was observed around the disc, indicating a reduced density of test strains. Conversely, the presence of growth around the disc indicated the absence of antibacterial activity.

The isolate demonstrated strong antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Bacillus cereus*, with inhibition zones exceeding 6 mm. The inhibitory properties of the isolate are likely attributed to the production of antimicrobial compounds. Among the pathogens tested, *E. coli* and *Salmonella typhimurium* were the most sensitive, whereas S. aureus was the most resistant to the isolate.

These findings are consistent with the study by Elobaid *et al*. [28], which reported that isolates 3C, 3D, and 3E exhibited antibacterial activity against *S. aureus* and *E. coli* using the agar well diffusion method. However, isolate 3D showed no inhibitory activity against *P. aeruginosa*, and none of the isolates inhibited *Klebsiella pneumoniae*. Other studies, such as Mjalawi *et al* [21], suggest that the antibacterial activity of *Bifidobacterium spp.* is linked to antimicrobial compounds released in their supernatants.

Tested Pathogens	Zone of Inhibition (mm)
Escherichai coli	10
Staphylococcus aureus	8
Bacillus cereus	13
Pseudomonas aeruginosa	10

Figure 6 – Antibacterial activity of *Bifidobacterium longum* against food-borne pathogens

CONCLUSION

This study demonstrates the promising probiotic potential of a *Bifidobacterium longum* strain isolated from cow's milk. The strain exhibited remarkable tolerance to various conditions, including survival at pH 2, growth in NaCl concentrations up to 5%, and resistance to bile salts. The observed increase in growth with rising NaCl concentrations suggests a unique adaptability to saline environments, potentially advantageous for survival in various food matrices and within the gastrointestinal tract. These findings, combined with the strain's previously demonstrated antagonistic activity against several pathogens, highlight its potential for application in food products and as a probiotic supplement.

REFERENCES

- Baita, N & Inna, Na & Muhammad Dewu, Mansur. (2023). In-vitro probiotic evaluation of Lactobacillus species. 8. 70-76.
- FAO/WHO (2001). Evaluation of health and nutritional properties of powder milk and live lactic acid bacteria: Food and Agriculture Organization of the United Nations and World Health Organization expert consultation report Rome.
- Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C. and Sanders, M. E. 2014. Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use

of the term probiotic. Nature Reviews Gastroenterology and Hepatology 11(8): 506-514.
- Mombelli B, Gismondo MR (2000). The use of probiotics in medical practice. Int J Ant Agents. 16: 531-536.
- Holzapfel, W. H. and Schillinger, U. 2002. Introduction to pre- and probiotics. Food Research International 35(2- 3): 109-116.
- Morelli L, Zonenenschain D, Del Piano M, Cognein P. Utilization of the intestinal tract as a delivery system for urogenital probiotics. J Clin Gastroenterol. 2004; 38: S107-S110.
- E. Vlkova, M. Grmanova, V. Rada, I. Homutova, S. Dubna, Selection of probiotic bifidobacteria for lambs Materials and methods, Czech J. Anim. Sci. 54 (2009) 552-565.
- Prasad J,Gill H, Smart J,Gopal PK (2000) Selection and Characterisation of Lactobacillus and *Bifidobacterium* strains for use as probiotics. Int Dairy J 8:993–1002
- Rahman SMK. Probiotic Properties Analysis of Isolated Lactic Acid Bacteria from Buffalo Milk. Arch Clin Microbiol. 2015, 7:1.
- Vaughan, E.E., Heilig, H.G.H.J., Ben-Amor, K. and de Vos, W.M. (2005) Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches. FEMS Microbiol Rev 29, 477–490
- Mitsuoka, T., 1984, Taxonomy and ecology of bifidobacteria, Bacteria and Microflora, 3(1):11-28.
- Gibson G.R, Wang X., 1994: Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J.Appl.Bacteriol.,77;412-420
- Toscano, M., De Vecchi, E., Gabrieli, A., Zuccotti, G. V., & Drago, L. (2015). Probiotic characteristics and in vitro compatibility of a combination of *Bifidobacterium* breve M-16 V, *Bifidobacterium* longum subsp. infantis M-63 and *Bifidobacterium* longum subsp. longum BB536. Annals of Microbiology, 65(2), 1079-1086. http://dx.doi.org/10.1007/s13213-014-0953-5
- Chugh, B., & Kamal-Eldin, A. (2020). Bioactive compounds produced by probiotics in food products. Current Opinion in Food Science, 32, 76-82. http://dx.doi.org/10.1016/j.cofs.2020.02.003.
- Verso, L.L.; Lessard, M.; Talbot, G.; Fernandez, B.; Fliss, I. Isolation and selection of potential probiotic bacteria from the pig gastrointestinal tract. Probiotics Antimicrob. Proteins 2018, 10, 299–312.
- Riaz, T.; Iqbal, M.W.; Saeed, M.; Yasmin, I.; Hassanin, H.A.; Mahmood, S.; Rehman, A. In vitro survival of *Bifidobacterium bifidum* microencapsulated in zein-coated alginate hydrogel microbeads. J. Microencap. 2019, 36, 192–203. [CrossRef]
- Ben-David, A., Davidson, C.E. (2014) Estimation Method for Serial Dilution Experiments. Journal of Microbiological Methods 107:214-221.
- Huidrom S and Sharma N. Isolation and Screening of Novel Isolates of Bifidobacteria from Human Milk as Potential Probiotic with Antidiarrheal Activity. Ann Appl Microbiol Biotechnol J. 2018; 2(1): 1007. https://dx.doi.org/10.36876/aamb.1007
- Rubio R, Jofré A, Martín B, Aymerich T, Garriga M. Characterization of lactic acid bacteria isolated from infant faeces as potential probiotic starter cultures for fermented sausages. Food Microbiol 2014;38:303- 11.
- NCCLS. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 4th Edn. 17. National Committee for Clinical Laboratory Standards, Wayne, PA. 1997.
- Al-Mjalawi B, Al-Awade H, Al-Hamil A, Al-Hussai A. Study Antibacterial Activity of Bifidobacterium spp. against some pathogenic bacteria isolated from patients with Cardiac Catheterization in vitro. Int J Chemtech Res. 2017;10(6):109-15.
- Iqra Yasmin, Muhammad Saeed ,Wahab Ali Khan, Adnan Khaliq, Muhammad Farhan Jahangir Chughtai, Rabia Iqbal, Saima Tehseen, Saima Naz, Atif Liaqat, Tariq Mehmood, Samreen Ahsan and Saira Tanweer (2020)., In Vitro Probiotic Potential and Safety Evaluation (Hemolytic, Cytotoxic Activity) of *Bifidobacterium* Strains Isolated from Raw Camel Milk, Microorganisms, vol : 8., 354
- Milani, C.; Lugli, G.A.; Duranti, S.; Turroni, F.; Bottacini, F.; Mangifesta, M.; Sanchez, B.; Viappiani, A.; Mancabelli, L.; Taminiau, B. Genomic encyclopedia of type strains of the genus *Bifidobacterium*. Appl. Environ.Microbiol. 2014, 80, 6290–6302.
- Chalas, R., Janczarek, M., Bachanek, T., Mazur, E., Cieszko-Buk, M., & Szymanska, J. (2016).Characteristics of oral probiotics - A review. *Current Issues in Pharmacy and Medical Sciences, 29*(1), 8-10. doi:10.1515/cipms-2016-0002
- Nannu Shafakatullah and M. Chandra (2014), Screening of Raw Buffalo's Milk from Karnataka for Potential Probiotic Strains, *Research Journal of Recent Sciences*, Vol. 3(9), 73-78, September (2014).
- S.Delgado., E. O'Sullivan, G. Fitzgerald and B. Mayo (2008), In vitro evaluation of the probiotic properties of human intestinal *Bifidobacterium* species and selection of new probiotic candidates, Journal of applied microbiology, 104, 1119-1127
- Mahmoudi, M., Khomeiri, M.., Saeidi, M., & Davoodi, H. (2020). Lactobacillus Species from Iranian Jug Cheese: Identification and Selection of Probiotic Based on Safety and Functional Properties. *Applied Food Biotechnology*, *8*(1), 47–56. https://doi.org/10.22037/afb.v8i1.29253
- Elobaid, R., Kaur, B., & Vijayalakshmi, N. (2020). Isolation and identification of Bifidobacterium from dairy products and screening its antibacterial activity. *Quest International Journal of Medical and Health Sciences*, *3*(2), 38-43. https://doi.org/10.5281/zenodo.4473214