Probiotic characterization of *Limosilactobacillus reuteri* strain isolated from cow's milk

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LAB strains , antibiotic resistance, antimicrobial activity, probiotics, *lactobacillus reuteri*

ABSTRACT

Lactic acid bacteria (LAB) are commonly found in milk and dairy products, and their probiotic characteristics make them valuable for use in food products. Understanding the antibiotic susceptibility of LAB is crucial to ensure the safe use of LAB-based products. This study investigated the probiotic characteristics including tolerance to pH, bile salts , antibiotic susceptibility and antibacterial activity of a strain isolated from raw cow's milk. Pure colonies were identified by 16S rRNA gene sequencing as *Limosilactobacillus reuteri*. The antibacterial activities of an overnight culture of *Lactobacillus reuteri* against four test microorganisms: *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were studied using the agar well diffusion method. Antibiotic susceptibility profile of the isolated strain were tested using disc diffusion method for ciprofloxacin(8mcg), ceftazidime(10mcg), ampicillin(10mcg), penicillin(10mcg), amoxiclav(10mcg), vancomycin (30mcg), amikacin(10mcg), methicillin(5mcg), chloramphenicol(30mcg) and cefozolin(30mcg). The results indicated that *L. reuteri* isolated from cow's milk was tolerant to acidic conditions and bile salts and demonstrated significant antimicrobial activity against the test microorganisms, suggesting its potential use as a natural antibacterial agent. Additionally, the strain exhibited resistance to the antibiotics tested, highlighting its safety profile. These findings suggested that the isolated LAB strain has beneficial properties and would serve as a starter culture for safe use in commercial food products.

Introduction

Lactic acid bacteria (LAB) are a group of Gram positive, non-sporulating, anaerobic or facultative aerobic cocci or rods which produce lactic acid as one of the main fermentation products of the metabolism of carbohydrate (Hayek and Ibrahim , 2013) . *Lactobacillus* bacteria are used to process a variety of dairy products, meat, vegetables, and grains. They are also the dominant population in raw milk, where they play a key role in food fermentation and preservation. *Lactobacillus* produces antimicrobial substances such as organic acids, hydrogen peroxide, antifungal peptides, and bacteriocins which help to protect food from spoilage and harmful bacteria (Quiley *et al* 2013).

Probiotics are live microorganisms that, when consumed in adequate amounts can provide health benefits to the host (Aaraya *et al* 2002 and Hill *et al* 2014). The genera *Lactobacillus* and *Bifidobacterium* are the most common sources of probiotic species and strains. *Lactobacillus* is one type of probiotics that is considered to be safe (GRAS) and effective (Ingilin *et al* 2015). For a probiotic strain to be considered effective, it must demonstrate the ability to survive in the gastrointestinal tract, possess high resistance to gastric acids, lack any transferable antibiotic resistance genes and clearly provide benefits to the host. (Montalban-Arques *et al* 2015). There are several shreds of evidence that many strains of probiotic microorganisms can hinder the growth and activities of some of enteropathogenic bacteria (Fijan *et al* 2018). The benefits of probiotic intervention in the gut microbiota in maintaining and restoring health are becoming more well recognized, drawing increased scientific interest (Rijkers *et al* 2010)

Lactobacillus probiotics find their primary application in harnessing their positive impact on the natural microbial balance within the human intestinal tract (Hassanzadazar *et al* 2012). These beneficial effects of Lactobacilli on the host's well-being are primarily achieved through three key mechanisms: antagonistic actions against harmful pathogens, favorable adjustments to the composition of the host's microbiota, and the modulation of immune responses (Saarela *et al* 2005).

An essential attribute of probiotics is safeguarding the host's intestinal tract against pathogens. Beyond this pivotal function, probiotics have diverse applications in various domains, including pharmaceuticals and pharmacology (Granato *et al* 2010 and Ranadheera *et al* 2019). LABs have gained significant interest for their role not only as antagonists against pathogenic microorganisms but also as producers of antimicrobial compounds. Probiotic strains that are resistant to antibiotics could provide advantages to individuals whose natural

gut microbiota have experienced imbalances or substantial depletion due to the usage of different antibiotics (Salminen *et al* 1998).

The predominance of *Lactobacillus* sp. in gut microflora is a key factor to protect the body against disease-causing microorganisms (Brochers *et al* 2009). *Lactobacillus* reuteri is the most potent probiotic strain used to GIT disorder (Larroya Garcia *et al* 2019) which plays an essential role in alleviating osteoporosis and improving bone density (YU *et al* 2021). Considering the importance of probiotic bacteria in milk , the present work aimed to isolate *Lactobacillus* sp from cow milk for the study and assess the probiotic characterisation of the isolate.

Materials and methods

Collection of sample

The untreated cow milk was procured from Coimbatore district. The collection carried out using sterile sample bottles, ensuring hygiene, and transporting them to the laboratory for subsequent microbiological analysis.

Isolation of lactic acid bacteria

For isolation of lactic acid bacteria, 10ml of milk sample was homogenized with sterilized saline. Sequential dilutions (ranging from 10^{-1} to 10^{-6}) were prepared for sample, using 1ml of the homogenate. From these dilutions, 0.1ml were spread-plated onto MRS (HI-media) Agar medium. The plates were then subjected to a 48-hour incubation at 32°C. Subsequently, colonies demonstrating typical LAB characteristics were selected at random and subjected to purification through streaking on fresh MRS agar plates, which was repeated three times to obtain the pure culture. These colonies were further evaluated using both macroscopic and microscopic examinations to confirm their traits as lactic acid bacteria. Colonies displaying the general attributes of lactic acid bacteria were singled out for subsequent identification steps (Ben david *et al* 2014).

Identification of Bacteria

Identification of isolated bacteria was done using PCR method and 16S rDNA sequencing. The genomic DNA of the LAB strains was extracted using DNA Extraction Kit followed by 16S rDNA gene specific fragment was amplified by universal primers of 5′-

AGAGTTTGATCCTGGCTCAG-3′F and reverse primer 5′ AAGGAGGTGATCCAAGCCGCA-3′ R. PCR conditions consisted of 28 cycles (1 min at 94°C, 1 min at 55°C, 2 min at 72°C using thermal cycler (ABI Applied Bio system Thermal cycler, USA). PCR products were separated from agarose gel (1.5% w/v) and purified by using PCR purification kit (Genei, Bangalore, India). The 16S rRNA gene sequences were executed with Basic Local Alignment Search Tool (BLAST) through the National Centre for Biotechnological Information (NCBI), USA, and server.

Characteristics of selected isolates

The isolate was characterized for gram staining and catalase reaction. The selected isolate was tested for its ability to grow at different pH and tolerance to different concentrations of Bile salts and its safety aspects such as antimicrobial activity and antibiotic susceptibility.

Tolerance to pH

Tolerance of isolated LAB to acidic pH was determined by growing all the isolates in acidic MRS broth. Active cultures were incubated for 24 hours in MRS broth. The cells were harvested in MRS broth and was poured in test tubes and pH was adjusted to 2.0, 5.0, 7.0 with 1ml HCl and 0.5ml NaOH (Awan and Rahman, 2005). The growth was monitored for 24 h and observed the growth by presence of turbidity at 620nm using a T70 UV: VIS spectrometer.

Tolerance to bile salts

Isolate was grown in MRS broth at 37°C overnight. 0.3%(w/v) of bile salt (oxoid) was prepared and added to the 24h old active culture of selected isolates and incubated at 37°C. Control was run alongside. The bacterial growth was monitored spectrophotometrically at 600nm at 0, 3, 6, 12 and 24h (Chaudhary and Saharan,2019)

Antibiotic susceptibility test

Limosilactobacillus reuteri DIRAD was tested for their resistant to antibiotics using disc diffusion method as described by Bauer *et al* (1966). 70µl of the 24-48 hrs old culture was added and spread evenly on the plate. After swabbing antibiotic discs of Ampicillin (AMP-8), Methicillin (MET-5), Penicillin (P-10), Moxifloxacin (MO-5), Amoxyclav (AMC-10), Gatifloxacin (GAT-5), ceftazidime (CAZ-10), Chloramphenicol (C-30), Ciprofloxacin (CIP-5), Amoxycilliun sulbactam (AMS30/15) were placed on the agar plates. The diameters of inhibition zones were measured and compared with the zones suggested by NCCLS. The

zone diameter of inhibition (ZDI) were measured. Isolates were categorized as sensitive (ZDI; ≥21 mm), intermediate (ZDI; 16-20 mm), or resistant (ZDI; ≤15 mm)

Antibacterial activity test

The antibacterial activity of the isolates against *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Bacillus cereus* were determined using the agar well diffusion method. *L.reuteri* was inoculated to MRS broth and incubated for 24h at 37°C under aerobic conditions. Fresh cultures of the four targeted pathogens (100µl, 107 CFU/ml) were coated on an agar plate and dried. Well of 5mm diameter made on plates were filled with 100µl of cell-free supernatant (CFS) obtained from centrifugation of LAB cultures at 4500r/min for 10min(cell fee extract was mixed with equal amount (1:1-V/V) of ethyl acetate and incubated for 24hrs followed by evaporated), positive control (antibiotic disc- Moxifloxacin 10mcg) and negative control (ethyl acetate) were placed. The diameters of inhibition zones were measured and recorded after incubating at 37°C for 24h

Results and Discussion

The viable bacteria in the cow's milk were enumerated as 1.84 x10-6 cfu/ml(colony forming unit/millilitre) and 2.47 x10-6 cfu/ml. The inhabitant Lactobacillus species that utilize the MRS agar medium were successfully isolated. Colony characteristics of Lactobacilli isolates were studied by picking-up a single well isolated colony aseptically and transferred to selective medium to observe the growth pattern of isolates on MRS medium.Morphological characteristics of isolated Lactobacillus was found as whitish, small to larger size colonies as shown in figure-1. Biochemical tests confirmed it as gram positive and catalase negative. Colonies were plated on MRS media and pure colonies were obtained by streak plate method. In line with this findings, Misganae and Teketey (2016) found that Lactobacillus, making up 26.51%, was the dominant genus isolated from raw cow milk.

Figure-1 Colonies of the isolate

The 16sRNA gene sequence based phylogenetic tree revealed that the strain in the present study belonged to subclade of *Lactobacillus reuteri* with more similarity to other strains within the genus (The Genebank accession number is ON329807). Thus, the isolate was named *L. reuteri* DIRAD1. The phylogenetic tree was constructed by Mega5 software in figure 2.

Figure 2 The phylogenetic tree construction of *Limosilactobacillus reuteri* strain (Mega5 software)

Tolerance to pH

Based on the experimental results, it was found that *Lactobacillus reuteri* strain isolated from cow's milk exhibited good growth characteristics at pH 5.0 and 7.0 indicating its ability to thrive in environments with varying acidity levels. However, the strain showed reduced growth at pH 2 indicating that the strain's ability to survive and grow is compromised under extreme acidic conditions (figure 3). Similarly, Jacobsen et al (1999) found that while the majority of *Lactobacillus* strains in their study had difficulty in surviving at pH 2.5, some

demonstrated a certain level of tolerance. Similarly, Choo *et al* (2019) reported that L. reuteri strains isolated from the anogenital tract were able to tolerate and survive at pH 2.5 and 3.5 for a specific period. The ability to tolerate acidic conditions is a crucial characteristic for probiotic bacteria, as they must survive passage through the highly acidic environment of the stomach (pH 1.5-3.5) to reach the intestine and exert their beneficial effects.

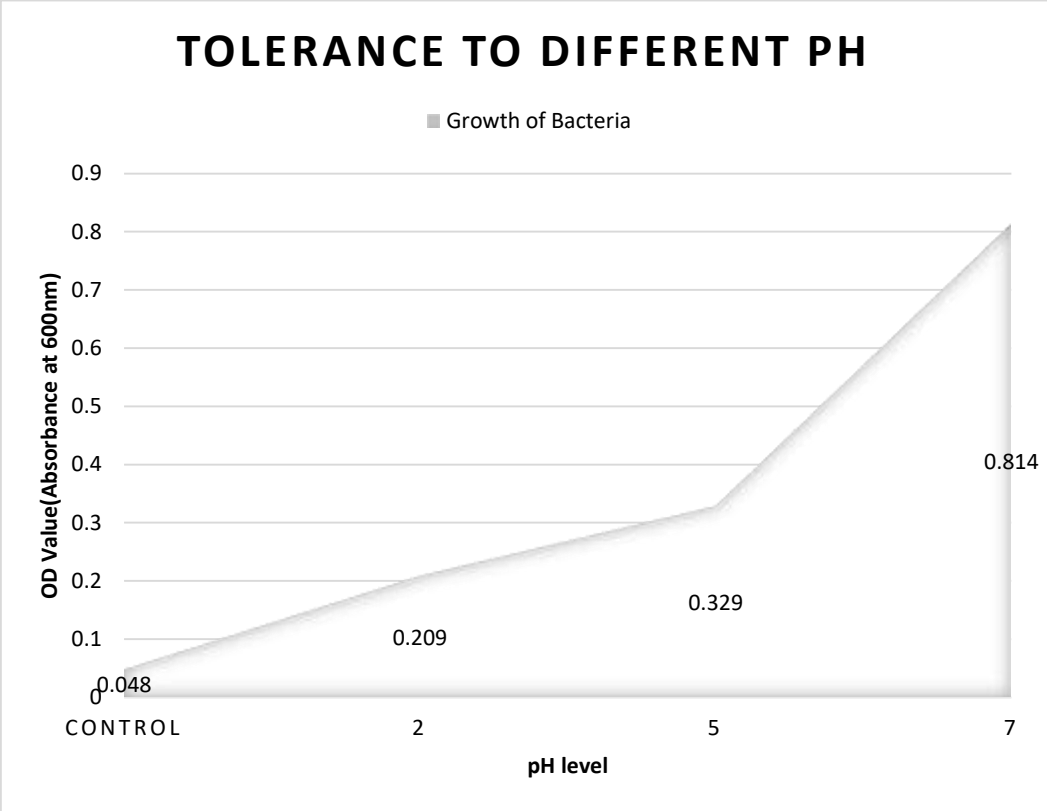

Figure 3 *Limosilactobacillus reuteri*- Tolerance to pH

Tolerance to Bile salts

L. reuteri isolates demonstrated good tolerance to bile salts, maintaining viability and growth at a concentration of 0.3% ox bile. The absorbance readings at 0, 3, 6, 24, and 48 hours showed that the isolates were able to withstand the presence of bile salts and continue growing throughout the duration of the study . These results showed that isolate possess an inherent ability to survive in the bile-rich environment of the small intestine, which is a key requirement for effective probiotic delivery to the gut. Zhang *et al* 2012 showed that *L. reuteri* ATCC 55730, a known probiotic strain, exhibited significant tolerance to bile salts, contributing to its ability to survive in the intestinal environment and exert beneficial effects. For instance, *Lactobacillus* plantarum CY2 and *Lactobacillus* paracasei CY3 isolated from yak milk were

showed growth superior to the control, after treatment with 0.3% bile salts for 4h, and decreased growth when the concentration increases (Zheng *et al* 2021). The bile salt concentrations in the gastrointestinal tract range from 0.5% to 2.5% in the first hour of digestion which decreases further in subsequent hours (Hati *et al* 2014).

Antibiotic susceptibility profile

In this study, *L reuteri* found to be resistant against ciprofloxacin,ceftazidime, amikacin, methicillin and vancomycin and sensitive and intermediate sensitive to ampicillin, amoxyclav, chloramphenicol, penicilin and cefazolin attributed to the intrinstic traits . Danielsen and Wind(2003) reported that many *Lactobacillus* species has been found to be tolerant to aminoglycoside antibiotics such as gentamicin, kanamycin, streptomycin. Similarly, Majekova *et al* (2014) noted the strain *L.reuteri* was susceptible to ampicillin, erythromycin and penicillin but resistance to vanomycin, ciproflaxin and ofloxacin. The functional qualities of probiotic strains should be assessed before in vivo administration. The antibiotic susceptibility of a probiotic is a critical factor in determining whether it can be coadministered with antibiotic (Slizewska *et al* 2021).

Antibacterial activity

Antimicrobial activity is a very significant criterion for selection of starter and probiotic culture as they form natural antagonists of potentially harmful bacteria. *Limosilactobacillus reuteri* isolated from milk was screened for their antagonistic activity against Gram-positive and Gram-negative food-borne and human pathogens. *L. reuteri* displayed varied level of inhibitory activity against tested pathogens such as *E coli*, *S.aureus*, *P.aeruginosa* and *B.cereus* as shown in the figure 4. Similarly, Jamalifer *et al* (2011) revealed that the antibacterial activity of the supernatants of these autochthonus *Lactobacilli* and also commercially *Lactobacillus acidophilus* and *Lactobacillus reuteri* were tested against 10 highly resistant strains of *P. aeruginosa.* Sekander *et al* (2023) revealed that *L. reuteri PSC102* could inhibit all the tested enterotoxigenic *E. coli* pathogenic strains in the study.

Figure 4 – Antibacterial activity of *Limosilactobacillus reuteri* against food borne pathogens

Conclusion,

In this study, *Limosilactobacillus reuteri* isolated from cow's milk was capable of tolerating acid condition and bile salts. The results confirmed that the isolated has remarkable antimicrobial activities against food-borne *E.coli, S.aureus, B. cereus* and *P. aeruginosa.* Furthermore, the strain was found to be sensitive to therapeutically important antibiotics could also contribute to the probiotic potential for maintain the microflora in gut. The results highlighted that *L.reuteri* is an efficient probiotic candidate which has potential application in food industry.

Author contribution

Divya SB: conceptualized the study; contributed to data curation, methodology, and laboratory work; and prepared, wrote, revised, and edited the original draft of the manuscript. Radhaisri S: supervised; visualized; reviewed, and edited the manuscript; and contributed to data analysis and reference search. All authors approved the submission of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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