An *in vitro* Approach: Cholesterol Assimilation and Probiotic Characterization of *Enterococcus faecium* (JD 9) isolated from fermented foods

Allavarapu Dharma Teja and Cherukuri Pavana Jyothi*
Department of Life Sciences, GITAM School of Sciences, Vishakhapatnam, Andhra Pradesh, INDIA
*pcheruku@gitam.edu

Abstract
High plasma cholesterol levels can cause cardiovascular diseases (CVD) in humans and lactic acid bacteria (LAB) were found to be fine-tuned in maintaining cholesterol homeostasis and were considered helpful for life due to their significant impact on human and animal health when taken orally. This bacterial interaction leads to the deconjugation of bile salts by the BSH enzyme and the assimilation of cholesterol, which are particularly important. This study examined the probiotic characterisation and cholesterol assimilation by lactic acid bacteria isolated from fermented foods along with reference strains (L. acidophilus MTCC 10307 and L. rhamnosus MTCC 1408). Isolate was identified as Enterococcus faecium JD9 by 16S rRNA. All strains in this investigation can enhance the tolerant to low pH, bile salt and simulated gastric juice. It has shown catalase-negative, gram-positive, non-haemolytic activity, bile salt hydrolase activity, sugar fermentation, autoaggregation, antibiotic sensitivity and assessment of cholesterol assimilated from media.

HPLC was used to quantify the assimilated cholesterol. All strains demonstrated a remarkable capacity to assimilate cholesterol with and without bile salt, reaching a rate of cholesterol assimilation of Enterococcus faecium JD9 (78% and 96.52%), Lactobacillus rhamnosus MTCC 1408 (77% and 92.34%) and Lactobacillus acidophilus MTCC 10307 (76% and 88.45%). The results indicate that the strain isolated from fermented food has the potential to be employed as probiotics and alternative therapeutics due to their capacity to bile salt hydrolase and to reduce cholesterol levels.

Keywords: Cholesterol assimilation, Probiotics, *Lactobacillus* spp, De Man-Rogosa-Sharpe agar.

Introduction

Recent studies consistently show that high plasma cholesterol levels are closely connected to the onset of various lifestyle diseases, particularly cardiovascular diseases (CVD)[31]. Lowering plasma cholesterol is identified as a crucial factor in reducing the risk of cardiovascular disease with 1% decrease in cholesterol corresponding to a 2-3% drop in the risk of developing CVD. According to the World Health Organisation (WHO), there will be an increase in the death rate by 2030 caused by ischemic heart disease, causing a death toll of 23.5 million people[22,29]. The current hypercholesterolemia management involves dietary changes and drug treatment[19]. However, using medications like statins for extended periods may result in adverse side effects and may require a significant financial commitment to treat cardiac disorders[4].

Given these challenges, researchers actively explore alternative treatments to improve patient outcomes and alleviate symptoms to decrease the risks related to hypercholesterolemia and to enhance overall eminence of life. One promising approach is to use probiotic supplements. Probiotics are live microorganisms acknowledged for their health benefits and have been found to lower serum cholesterol levels [40]. This discovery has been under investigation since the times of Eli Metchnikoff, with Mann and Spoerry[24] notably linking probiotic intake to cholesterol reduction in a Massai population. The cholesterol-lowering effect primarily occurs in the intestine, underscoring the importance of probiotics reaching the colon alive despite the challenging conditions of high acid and bile salt concentrations in the intestine [39].

Recent research has sparked interest in utilising lactic acid bacteria (LAB) as additives to lower cholesterol levels in the human body[9]. Few *in vivo* studies have proven that lactic acid bacteria can effectively reduce cholesterol levels in rats[41], mice[15], pigs[11] and humans[23]. Few reports have supported an *in vitro* and an *in vivo* approach to lower cholesterol and low-density lipids using LAB strains such as *Lactobacillus* spp. and *Enterococcus* spp.[28] The mechanism of cholesterol reduction is either bile salt hydrolysate (BSH) activity [37] or conjugation of bile salts[8]. *E. faecium* has emerged as a probiotic with potential health benefits in the medical field and it has shown health advantages as a reducing agent for inflammation, obesity, cholesterol assimilation and anti-cancer[7,12,25].

Hence, the medical and health industries have emphasised the advantages of probiotics like *E. faecium* in the host's health through immune system activation and cholesterol assimilation[1,17,36]. Bacteria with BSH activity may contribute to easier excretion of bile salts in the faeces compared to conjugated bile salts. This process potentially reduces serum cholesterol by increasing bile salt excretion and decreasing the solubility of cholesterol, ultimately reducing cholesterol absorption in the gut. These findings

highlight a promising avenue for further exploration into LAB's cholesterol-lowering properties and their impact on bile salt metabolism and serum cholesterol regulation in a living body[16].

The current study focused on *in vitro* techniques to explore the survival capabilities of isolated and selected standard LAB in the presence of acids and bile salts tolerance, BSH. Additionally, the research seeks to investigate the cholesterol assimilation properties of these probiotic bacteria.

Material and Methods

Isolation of bacteria: Thirty-five fermented rice samples were collected from different sites in Vishakhapatnam district, Andhra Pradesh, INDIA. Isolation and microbial quantification of samples were performed under aerobic conditions. Samples were serially diluted, inoculated on MRS agar plates and incubated at 35°C until pure colonies were observed. Five LAB isolates were identified based on morphological phenotypic and biochemical (IMVIC test, catalase, haemolytic activity, sugar fermentation and growth on lactic acid differential media) and molecular characteristics[33]. Selected isolated strain *Enterococcus faecium* JD9 and two MTCC standard strains, *Lactobacillus acidophilus* MTCC 10307 and *Lactobacillus rhamnosus* MTCC 1408, were screened for tolerance to acidic conditions (low pH), bile, simulated gastric juices and haemolytic activity. These three strains were evaluated for cholesterol assimilation in the presence and absence of bile[30]. The isolated strain showed the highest cholesterol assimilation confirm the identification using 16S rRNA sequencing.

Genome analysis: The genomic RNA is purified using a QIAGEN DNeasy ultra-clean microbial kit amplified via PCR [43]. The amplification was executed using the QIAGEN QIA quick PCR purification kit (Cat. No. / ID: 28104). The universal primers used for the 16S rDNA (27F:3'AGAGTTTGATCCTGGCTCAG5' and 1492R:5' CGGTTACCTTGTTACGACTT 3') were used. The sequence was equated with the nucleotide BLAST analysis. A phylogenetic tree was constructed using MEGA 11 software version 11.0. Further, an isolated strain (*E. faecium* JD9) 16s rRNA sequence was identified and deposited in the GenBank.

Evaluations of probiotic characteristics: As per the guidelines of the Indian Council for Medicine and Research (ICMR) and the Department of Biotechnology (DBT), the probiotics were evaluated based on the haemolytic characteristics, tolerance to acid, bile and synthesised gastric juice[14,42].

Haemolytic activity: The haemolytic activity of *Lactobacillus acidophilus* 10307, *Lactobacillus rhamnosus* 1408 and *Enterococcus faecium* JD9 strains was determined by inoculation of strains on blood agar plates (5% v/v blood) and incubation at 35°C for 28 h to screen the haemolytic activity of isolated and standard strains[6].

Fermented sugar identification: Fermented sugars were identified *in vitro* using a Hi Lacto identification kit KB 020-10 KT (HIMEDIA). Each well was inoculated in this kit with 50µl of 1% overnight grown culture incubated at 35° C for 24 h. After incubation, the colour change was observed[20].

Acid and Bile Tolerance: Tolerance to low pH (acidic) and bile salts [21, 34] is a prominent characteristic of probiotics. To determine acid and bile tolerance, all three strains were inoculated and incubated in MRS media adjusted with various pH conditions (1.5, 2, 3) by 1N HCl. MRS media were supplemented with 0.5% ox bile bacteriological (Hi media) and inoculated with all three strains. The samples were collected at 0, 2 and 4 h and inoculated in MRS agar plates. Colony-forming units were observed.

Simulated gastric juice: The survivability in synthesised gastric juice was conducted using the modified simulated gastric juice, dissolving pepsin 0.3% (w/v) in 0.5% saline (v/v) at pH 3.0[35]. The three strains, *Lactobacillus acidophilus* 10307, *Lactobacillus rhamnosus* 1408 and *E. faecium* JD9 strains, were inoculated and incubated in simulated gastric juice at 35°C, 200 rpm separately. The count of viable cells was measured at every 2 h intervals and was expressed as log10 cfu/ml. Triplicates of each bacterial strain were evaluated.

Bile salt hydrolysis: The BSH activity in *Lactobacillus acidophilus* 10307, *Lactobacillus rhamnosus* 1408 and *E. faecium* JD9 strains was carried out in modified MRS plates supplemented with 0.5% bile salts. Subsequently, 50µl of bacterial strains were inoculated into 6mm diameter wells on modified MRS plates incubated at 35°C for 24 h and maintained with negative control. The precipitation observed as white zones around the wells indicates bile salt hydrolysis[37]. This method ensures the reliable evaluation of BSH activity[35] in *Lactobacillus acidophilus* 10307, *Lactobacillus rhamnosus* 1408 and *Enterococcus faecium* JD 9 strains.

Auto aggregation: Auto aggregation is one of the positive characteristics of probiotic organisms for enhancing cell-to-cell interactions within the same strains. Previously grown cultures were centrifuged, washed in phosphate buffer saline (PBS) and resuspended in PBS to adjust optical density (OD) to 0.5 at 650 nm, followed by incubation at 35°C for 2 h. The top layer was taken to measure absorbance at 650 nm. The percentage of auto-aggregation was estimated by decreasing the initial and final absorbance compared to the initial absorbance of bacterial culture. After centrifugation of 24 hrs grown cultures, re-suspend the cells washed with phosphate buffer saline and OD was adjusted (0.5 at 650 nm). Incubate at 35^{0}C for 2hrs, the top layer's 1 ml absorbance was taken at 650 nm. The percentage of auto-aggregation has been estimated by decreasing the start (A_o)

and final (A_t) absorbance compared to the initial absorbance of bacterial culture[5].

$$\%\ Auto\ aggregation = \frac{\mathrm{At} - \mathrm{A0}}{\mathrm{A0}}\ X\ 100\%$$

where A0 is the absorbance of initial time and At is the absorbance at the end time.

Antibiotic disc diffusion assay: Disc diffusion assay by "Kirby Bauer" was used to evaluate the microorganisms' susceptibility to popular antibiotics [38]. This investigation used antibiotic disks (HI Media, India) containing (AMX), (AMC), (CIP), (E), (K), (GEN) and (STP). These antibiotic discs were placed on the MRS agar plate's surface and incubated for 24 h. at 35° C. The size (mm) of the zone of inhibition was determined and these strain's antibiotic susceptibilities were interpreted according to the CLSI criteria.

Cholesterol assimilation: HPLC determined the potentiality of three strains to assimilate cholesterol. This method was determined by supplemented MRS media with the 240 µg/ml cholesterol, 0.5% bile and without bile. It was inoculated with 1% overnight grown cultures and incubated at 35° C for 24 hrs. The supernatant was separated by centrifugation (8500*g* for 12 min at 4° C) and filtered. The cell-free suspension with non-assimilated cholesterol was measured using HPLC[18].

High-performance liquid chromatography: To determine the non-assimilated cholesterol in MRS media, a chromatographic analysis was conducted on an "Agilent Technologies 1260 infinity system (USA) with a UV-DAD detector set at 205 nm". Elution was isocratic and carried at a flow rate of 0.5 ml/min using 'acetonitrile/methanol' in a 60/40 (v/v) ratio as a mobile phase. The injection volume of the sample was 25µL and the temperature 32 ºC was maintained. Zorbax C18 column of 2.1 × 100mm, filled with 3.5µm size silica atoms, acts as a stationary phase. The total analysis run time was 7 min, with cholesterol retention time noted at 4.2 min. Results were documented using the Open Lab CDS software[32].

Statistical analysis: The studies were conducted in triplicate and the mean values and ± standard deviation calculated (Microsoft Excel) were testified. The Tuckey test calculates the significance of statistical data and the p-value < 0.05 is significant

Results

Isolation and Genomic Analysis: This study aimed to isolate and characterize probiotic bacteria from fermented foods and to evaluate their capacity to assimilate cholesterol. Fermented products are preferred for their elevated sensory characteristics and potential health advantages, which arise from the presence of microorganisms and biochemical transformations during fermentation. Fermented foods serve as a reservoir for the extraction of probiotic bacteria. Rice is widely consumed as a staple food worldwide, particularly in Asia, Latin America and portions of Africa. Rice has long been used to make various traditional and ethnic fermented meals and drinks[26]. Since these bacteria undergo simultaneous evolution with harmful bacteria and fungi, they can thrive in humans' naturally microbe-rich gastrointestinal tract.

Thirty-five strains were isolated from the fermented food samples. These five isolates showed preliminary probiotic characteristics, such as Gram-positive and catalase-negative.

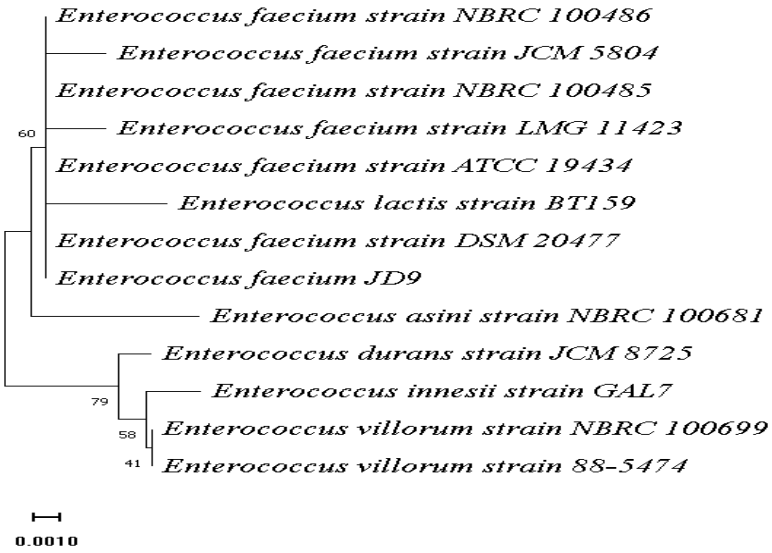

Fig. 1: Phylogenetic tree of *Enterococcus faecium* JD9 The phylogenetic tree was constructed using a 16S rRNA nucleotide sequence with a neighbourhood joining method and bootstrap support. The node values represent the bootstrap level derived from the neighbour-joining analysis of 1000 resampled data sets. The values are accession numbers

The five isolates and two standard strains, *Lactobacillus acidophilus* 10307 *and Lactobacillus rhamnoses* 1408, were screened for cholesterol assimilation. Isolated *E. faecium* JD9 has shown the capability of 78% cholesterol assimilation. Two standard strains (*Lactobacillus acidophilus* 10307 and *Lactobacillus rhamnoses* 1408) have shown 76 and 77% of cholesterol assimilation respectively. The isolated strain was confirmed as an *Enterococcus faecium* based on 16s rRNA gene sequencing and BLAST analysis. The sequence was deposited in the Gene Bank and the accession number is JD9 (Fig. 1).

Haemolytic activity: Haemolysin plays an essential role in characterizing the virulence-causing infection. Probiotics should be non-virulent and non-haemolytic. All three strains have not shown α, β, γ haemolysis in blood agar.

Fermented sugar identification: Hi Lacto identification kit provides information on the utilization of sugars by lactic acid bacteria as their potential growth factor. All the sugars were fermented by the *Enterococcus faecium* JD9, *L. acidophilus* and *L. rhamnosus* (esculin, xylose, cellobiose, arabinose, maltose, galactose, mannose, melibiose, raffinose, sucrose and trehalose) except raffinose and galactose by the *Lactobacillus* spp. (Table 1).

Bile and acidic tolerance: Tolerance to bile and acidic conditions is a significant characteristic of probiotics and plays a major role in sustainability in critical gastrointestinal conditions. All three strains have survived in the bile concentration of less than or equivalent to 0.5% i.e. 0.125 and 0.25%. The viability of all three strains was observed in the acidic medium (pH 2 and 3) up to 4h. However, the feasibility of strains was reduced to half of the strength after 4 h at pH 2 and 3. All three strains were sustained, but cell volume decreased slightly after 4 h (Table 2).

Table 1
Sugar fermentation of LAB strains by a Hi Lacto identification kit KB 020-10KT.
(Positive (+) indicates sugar fermentation and negative (-) indicates the non-fermentation of sugars)

Fermented Sugar	*Lactobacillus acidophilus* 10307	*Lactobacillus rhamnosus* 1408	*Enterococcus faecium* JD9
Xylose	+	+	+
Cellobiose	+	+	+
Arabinose	+	+	+
Maltose	+	+	+
Galactose	+	-	+
Mannose	+	+	+
Melibiose	+	+	+
Raffinose	-	+	+
Sucrose	+	+	+
Trehalose	+	+	+

Table 2
Antibiotic sensitivity of LAB strains by disc diffusion assay Amoxicillin (30 µg), Amoxiclav (30 µg), Ciprofloxacin (5 µg), Erythromycin (15 µg), Kanamycin (30 µg), Gentamycin (10 µg) and Streptomycin (30 µg). S indicates sensitivity. R indicates the Resistance number in the parentheses, indicating the concentration of the antibiotic's µg/disk

Strain	K (30)	TET (30)	STM (10)	GEN (10)	E (15)	CIP (5)	AMP (10)
Lactobacillus acidophilus 10307	R	S	R	S	R	R	S
Lactobacillus rhamnosus 1408	S	S	R	S	R	R	S
Enterococcus faecium JD9	S	S	S	R	S	R	S

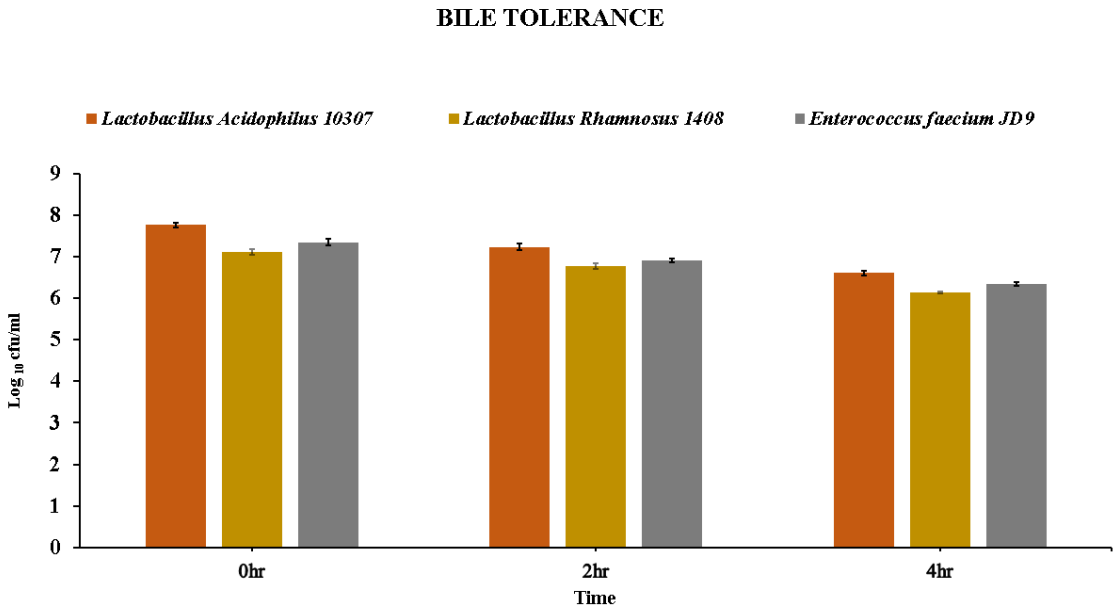

Fig. 2: 0.5% Bile Tolerance among all the strains, *E. faecium* JD 9 and *L. acidophilus* 10307 have shown the greatest survivability in the bile tolerance compared to *L. rhamnosus* 1408. Mean ± SD values of triplicate data of their independent investigation is shown here.

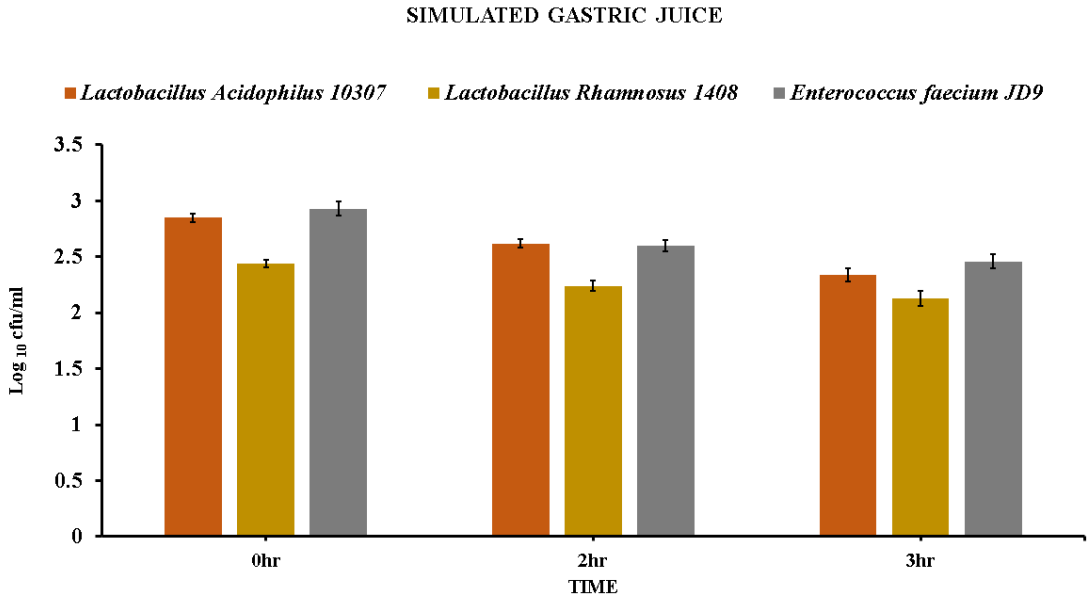

Fig. 3: Simulated gastric juice All the lactobacillus strains have shown a reduction in the cell count in simulated gastric juice conditions, compared to standard *lactobacillus* spp., isolate *E. faecium* JD9 has shown more tolerance. After 4h, no growth was observed. Mean ± SD values of triplicate data of their independent investigation are shown here.

Simulated gastric juice: Probiotic bacteria can survive in digestion, reflecting their colonization in the intestine and positively affecting the host (Fig. 3) indicating the survivability of all three strains in the simulated gastric juice (pH-3.0). The initial concentration of all three strains is Log $_{10}$ cfu/ml and after incubation, all three strains are viable in the simulated gastric juice even after 2 to 4 h.

Bile salt hydrolysis: Bile salt hydrolysis is characteristic of the probiotic property which plays a prominent role in cholesterol assimilation by converting the cholesterol into taurine and glycine and excreted through the faeces. WHO recognizes BSH as a significant factor in the selection of probiotics. The results of the BSH phenotypic evaluation confirmed that the presence of BSH in the *E. faecium* JD9

isolate was a note-worthy finding, considering the occurrence of BSH activity in bacteria isolated from fermented foods. All three strains have exhibited a white precipitation zone around the well in bile-supplemented MRS media, indicating the BSH activity.

Auto aggregation: Auto aggregation is characteristic of the accumulation of bacteria in the intestinal lining and *Enterococcus faecium* JD9 (37.92%) has shown a high percentage of auto aggregation compared to the *Lactobacillus*. spp. 10307 (22.54%) and 1408 (32.04%) (Fig. 5).

Antibiotic disc diffusion assay: Susceptibility to antibiotics is a safety assessment recommended by the WHO for probiotics, as the probiotics can potentially transfer the antibiotic resistance gene to the bacteria in the intestine. *Enterococcus faecium* JD9 exhibited the maximum susceptibility to amoxycillin amoxiclav, ciprofloxacin, gentamicin, vancomycin, streptomycin and resistance to erythromycin and kanamycin, whereas *L. acidophilus* showed resistance to streptomycin and *L. rhamnosus* showed resistance to ciprofloxacin. Both the strains exhibit resistance to kanamycin susceptible to remaining all antibiotics as shown in the table 2.

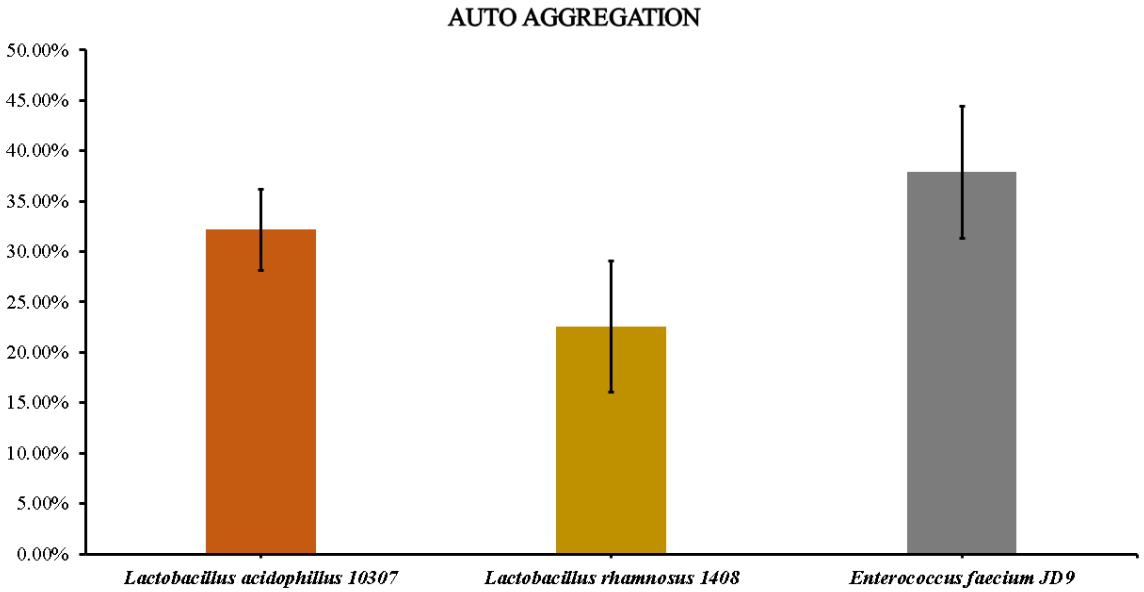

Fig. 4: Auto aggregation % Auto aggregation ability of *enterococcus faecium* JD 9, *lactobacillus rhamnosus* 1408 and *lactobacillus acidophilus* 10307.Mean ± SD values of triplicate data of their independent investigation. All the strains can potentially bind to the epithelial and mucosal surfaces.

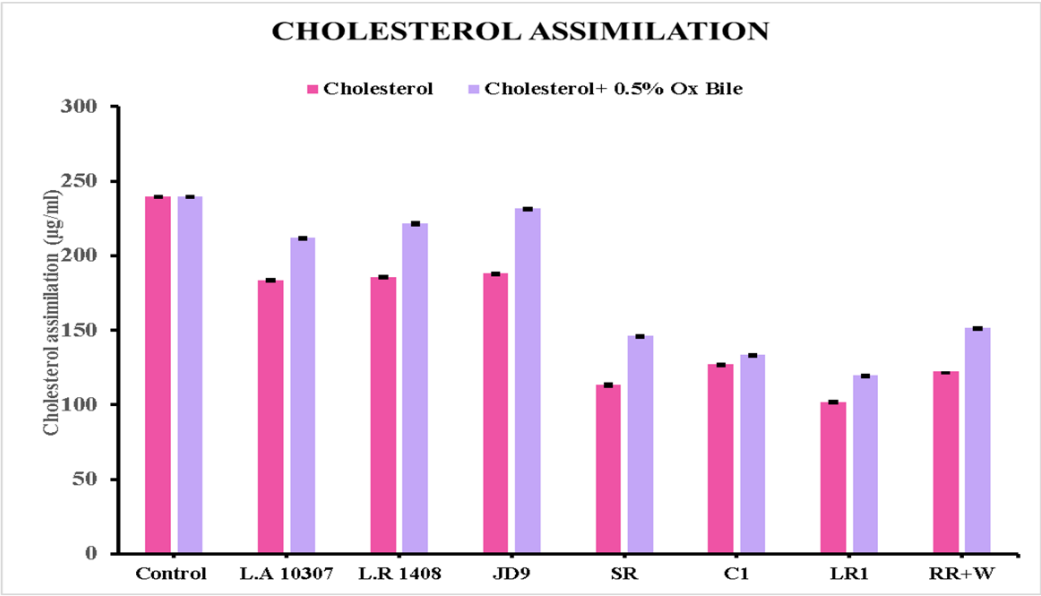

Fig. 5: Cholesterol assimilation Cholesterol assimilation by the *lactobacillus* spp. (10307 and 1408) and the isolated *Enterococcus faecium* JD9 have shown a positive effect on the cholesterol assimilation in the MRS with and without bile supplemented with the cholesterol (240µg/ml).

Cholesterol assimilation: Cholesterol assimilation from culture media by the probiotics exhibited various mechanisms that suggest the consumption of these probiotics used as a biotherapeutic agent for cholesterol as they could perform the same function in the intestine based on the *in vitro* evaluation. All the five isolates along with the standard strains have shown the assimilation of cholesterol. However, the isolate JD9 and the MTCC 10307 and MTCC 1408 have shown>70% cholesterol assimilation with and without bile salt in the media (Fig. 5).

Discussion

This study aimed to isolate and characterize probiotic bacteria from fermented foods and to evaluate their capacity to assimilate cholesterol. Fermented products are preferred for their elevated sensory characteristics and potential health advantages, which arise from the presence of microorganisms and biochemical transformations during fermentation. Fermented foods serve as a reservoir for the extraction of probiotic bacteria. Rice is widely consumed as a staple food for many people worldwide, particularly in Asia, Latin America and portions of Africa. Rice has long been used to make various traditional and ethnic fermented meals and drinks[26]. Since these bacteria undergo simultaneous evolution with harmful bacteria and fungi, they can thrive in humans naturally through microbe-rich gastrointestinal tract.

This study collected 35 household fermented food samples (curd, dosa batter and fermented rice) from Vishakhapatnam, India. Five isolates and two MTCC strains have shown the capability for cholesterol assimilation. Isolate JD9 from fermented rice has shown high cholesterol assimilation out of five isolates, identified as *E. faecium* JD9, using 16S rRNA gene sequencing. WHO recognizes BSH as a significant factor in the selection of probiotics. The results of the BSH phenotypic evaluation confirmed that the presence of BSH in the *E. faecium* JD9 isolate was a noteworthy finding, considering the occurrence of BSH activity in bacteria isolated from fermented foods.

The *E. faecium* JD9 exhibited a notable capacity to assimilate cholesterol, which was further enhanced by the presence of bile salts. Compared to other standard strains of *Lactobacillus* spp (MTCC 10307 and 1408), JD9 showed a much superior capacity to absorb cholesterol, specifically without bile 75% and with bile 96.72% in a media respectively. The cholesterol removal of the isolate (JD9) was significantly greater than the previously reported *E. faecium* VC223 55%[2]. This implies that *enterococci* might be regarded as effective probiotics if they meet the requirements for probiotic potential. For a probiotic strain to have beneficial effects on the host, it is crucial to possess certain features such as the ability to tolerate gastric juice and low pH and bile.

Isolate *E. faecium* JD9 exhibited salt tolerance and demonstrated optimal development at a temperature of 37°C. Another essential characteristic that probiotics must have to live in the human gut, is their ability to withstand the low pH levels of the stomach in an acidic environment with a pH of 3.0 for a minimum of 3-4 h. Meanwhile, the *E. faecium* JD9 isolate could tolerate for 3-4 h at pH 3.0. Furthermore, the standards and isolated strains survived in the simulated gastric juice even after 4 h. Therefore, our findings align with other research outcomes [3, 27]. Following their transit through the stomach, probiotic bacteria must confront the bile secretions found in the intestine.

The probiotic's ability to colonise the human gut is facilitated by its high tolerance to bile, ranging from 0.3 to 0.5%. Bile tolerance was crucial in assessing probiotic microorganisms' viability in the presence of bile salts. All strains exhibited high viability in the presence of 0.5% bile salt and even after being exposed for 2-4 h, these strains have demonstrated moderate auto-aggregation ability and nonhemolytic properties, suggesting their safety for future probiotic formulations. The suitability of *E. faecium* as an effective probiotic was assessed, considering the antibiotic resistance profile of the proposed probiotic. *E. faecium* JD9 and *Lactobacillus* spp (MTCC 10307&1408) resisted β lactams, fluoroquinolones, kanamycin and vancomycin in the antibiotic susceptibility tests. However, they were found to be susceptible to gentamycin.

Therefore, these strains may be suitable for human consumption. Several species of *Enterococcus* exhibit inherent resistance to β lactam antibiotics [10]. Prior research has documented a greater prevalence of aminoglycoside resistance in enterococcal strains found in dairy products[13]. However, our findings demonstrate that the isolate from fermented foods was resistant to kanamycin and remained susceptible to gentamycin. Therefore, considering the susceptibility of *E. faecium* JD9 to commonly used antibiotics against *Enterococcus* infections suggests that these isolates have the potential to be evaluated as probiotic candidates.

Conclusion

In this work, we detailed the process of isolating and characterising *E. faecium* JD9 as a probiotic isolated from a fermented food sample from Vishakhapatnam, India. Based on our findings, both the isolate and standard strains from MTCC *Lactobacillus* spp (10307 and 1408) strains coped sufficiently in the hostile environment of the GI conditions and bile salts.

Out of all the isolates tested, *E. faecium* JD9 stood out for its exceptional cholesterol-removing capabilities and sensitivity to gentamycin and these findings offer a preliminary evaluation of probiotic strains for potential use as cholesterol-lowering medicines through cholesterol absorption. These probiotic strains are considered biotherapeutics because of their capacity to assimilate cholesterol from media.

References

1. Afshin A., Sur P.J., Fay K.A., Cornaby L., Ferrara G., Salama J.S., Mullany E.C., Abate K.H., Abbafati C. and Abebe Z., Health effects of dietary risks in 195 countries, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017, *The Lancet*, **393(10184)**, 1958-1972 **(2019)**

2. Albano C., Morandi S., Silvetti T., Casiraghi M., Manini F. and Brasca M., Lactic acid bacteria with cholesterol-lowering properties for dairy applications: *In vitro* and *in situ* activity, *Journal of Dairy Science*, **101(12)**, 10807-10818 **(2018)**

3. Banwo K., Sanni A. and Tan H., Functional properties of Pediococcus species isolated from traditional fermented cereal gruel and milk in Nigeria, *Food Biotechnology*, **27(1)**, 14-38 **(2013)**

4. Bliznakov E., Lipid-lowering drugs (statins), cholesterol and coenzyme Q10. The Baycol case--a modern Pandora's box, *Biomedicine & Pharmacotherapy Biomedecine & Pharmacotherapie*, **56(1)**, 56-59 **(2002)**

5. Collado M., Grześkowiak Ł. and Salminen S., Probiotic strains and their combination inhibit in vitro adhesion of pathogens to pig intestinal mucosa, *Current Microbiology*, **55**, 260-265 **(2007)**

6. Cui X., Shi Y., Gu S., Yan X., Chen H. and Ge J., Antibacterial and antibiofilm activity of lactic acid bacteria isolated from traditional artisanal milk cheese from Northeast China against enteropathogenic bacteria, *Probiotics and Antimicrobial Proteins*, **10**, 601-610 **(2018)**

7. Divyashri G., Krishna G., Muralidhara and Prapulla S., Probiotic attributes, antioxidant, anti-inflammatory and neuromodulatory effects of Enterococcus faecium CFR 3003: *in vitro* and *in vivo* evidence, *Journal of Medical Microbiology*, **64(12)**, 1527-1540 **(2015)**

8. Duche R.T., Nwagu T.N., Singh A., Wandhare A.G., Sihag M.K., Panwar H. and Ezeogu L.I., Bile salt hydrolase and cholesterol assimilation potential of lactobacilli from Nigerian fermented foods and human sources, *Indian Journal of Dairy Science*, **75(4)**, 314-325 **(2022)**

9. Gallaher C.M., Munion J., Gallaher D.D., Hesslink Jr. R. and Wise J., Cholesterol reduction by glucomannan and chitosan is mediated by changes in cholesterol absorption and bile acid and fat excretion in rats, *The Journal of Nutrition*, **130(11)**, 2753-2759 **(2000)**

10. Grayson M., Eliopoulos G., Wennersten C., Ruoff K., De Girolami P., Ferraro M. and Moellering Jr. R., Increasing resistance to beta-lactam antibiotics among clinical isolates of Enterococcus faecium: a 22-year review at one institution, *Antimicrobial Agents and Chemotherapy*, **35(11)**, 2180-2184 **(1991)**

11. Hou G. et al, Lactobacillus delbrueckii interfere with bile acid enterohepatic circulation to regulate cholesterol metabolism of growing–finishing pigs via its bile salt hydrolase activity, *Frontiers in Nutrition*, **7**, 617676 **(2020)**

12. Huang X., Ai F., Ji C., Tu P., Gao Y., Wu Y., Yan F. and Yu T., A rapid screening method of candidate probiotics for inflammatory bowel diseases and the anti-inflammatory effect of the selected strain Bacillus smithii XY1, *Frontiers in Microbiology*, **12**, 760385 **(2021)**

13. Jaimee G. and Halami P.M., High level aminoglycoside resistance in Enterococcus, Pediococcus and Lactobacillus species from farm animals and commercial meat products, *Annals of Microbiology*, **66**, 101-110 **(2016)**

14. Joint F., WHO Working Group: Guidelines for the evaluation of probiotics in food: report of a joint FAO/WHO working group on drafting guidelines for the evaluation of probiotics in food, London, ON, Canada **(2002)**

15. Kang Y., Kang X., Yang H., Liu H., Yang X., Liu Q., Tian H., Xue Y., Ren P. and Kuang X., Lactobacillus acidophilus ameliorates obesity in mice through modulation of gut microbiota dysbiosis and intestinal permeability, *Pharmacological Research*, **175**, 106020 **(2022)**

16. Kim G.B., Yi S.H. and Lee B., Purification and characterization of three different types of bile salt hydrolases from Bifidobacterium strains, *Journal of Dairy Science*, **87(2)**, 258-266 **(2004)**

17. Kobyliak N., Conte C., Cammarota G., Haley A.P., Styriak I., Gaspar L., Fusek J., Rodrigo L. and Kruzliak P., Probiotics in prevention and treatment of obesity: a critical view, *Nutrition & Metabolism*, **13**, 1-13 **(2016)**

18. Krausova G., Hyrslova I. and Hynstova I., *In vitro* evaluation of adhesion capacity, hydrophobicity and auto-aggregation of newly isolated potential probiotic strains, *Fermentation*, **5(4)**, 100 **(2019)**

19. Kumar M., Nagpal R., Kumar R., Hemalatha R., Verma V., Kumar A., Chakraborty C., Singh B., Marotta F. and Jain S., Cholesterol-lowering probiotics as potential biotherapeutics for metabolic diseases, *Journal of Diabetes Research*, **1**, 1-14 **(2012)**

20. Kumara S.S., Bashisht A., Venkateswaran G., Hariprasad P. and Gayathri D., Characterization of novel Lactobacillus fermentum from curd samples of indigenous cows from Malnad region, Karnataka, for their aflatoxin B 1 binding and probiotic properties, *Probiotics and Antimicrobial Proteins*, **11**, 1100-1109 **(2019)**

21. Liong M. and Shah N., Acid and bile tolerance and cholesterol removal ability of lactobacilli strains, *Journal of Dairy Science*, **88(1)**, 55-66 **(2005)**

22. Lozano R., Naghavi M., Foreman K., Lim S., Shibuya K., Aboyans V., Abraham J., Adair T., Aggarwal R. and Ahn S.Y., Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010, *The Lancet*, **380(9859)**, 2095-2128 **(2012)**

23. Madjd A., Taylor M.A., Mousavi N., Delavari A., Malekzadeh R., Macdonald I.A. and Farshchi H.R., Comparison of the effect of daily consumption of probiotic compared with low-fat conventional yogurt on weight loss in healthy obese women following an energy-restricted diet: a randomized controlled trial, *The American Journal of Clinical Nutrition*, **103(2)**, 323-329 **(2016)**

24. Mann G.V. and Spoerry A., Studies of a surfactant and cholesteremia in the Maasai, *The American Journal of Clinical Nutrition*, **27(5)**, 464-469 **(1974)**

25. Mishra A.K. and Ghosh A.R., Probiotic Enterococcus faecalis AG5 mitigated high fat diet induced obesity and produced propionic acid stimulated apoptosis in 3T3-L1 pre-adipocyte, *Life Sciences*, **261**, 118292 **(2020)**

26. Mishra S., Mithul Aravind S., Charpe P., Ajlouni S., Ranadheera C.S. and Chakkaravarthi S., Traditional rice-based fermented products: Insight into their probiotic diversity and probable health benefits, *Food Bioscience*, **50**, 102082 **(2022)**

27. Nami Y., Lornezhad G., Kiani A., Abdullah N. and Haghshenas B., Alginate-Persian Gum-Prebiotics microencapsulation impacts on the survival rate of Lactococcus lactis ABRIINW-N19 in orange juice, *Lwt*, **124**, 109190 **(2020)**

28. Ooi L.G. and Liong M.T., Cholesterol-lowering effects of probiotics and prebiotics: a review of in vivo and in vitro findings, *International Journal of Molecular Sciences*, **11(6)**, 2499-2522 **(2010)**

29. Organization W.H., Cardiovascular disease, http://www.ho.int/cardiovascular_diseases/en/ (**2020**)

30. Pan D.D., Zeng X.Q. and Yan Y.T., Characterisation of Lactobacillus fermentum SM-7 isolated from koumiss, a potential probiotic bacterium with cholesterol-lowering effects, *Journal of the Science of Food and Agriculture*, **91(3)**, 512-518 **(2011)**

31. Rippe J.M. and Angelopoulos T.J., Lifestyle strategies for risk factor reduction, prevention and treatment of cardiovascular disease, Lifestyle Medicine, Third Edition, 19-36 **(2019)**

32. Ryu E.H. and Chang H.C., *In vitro* study of potentially probiotic lactic acid bacteria strains isolated from kimchi, *Annals of Microbiology*, **63**, 1387-1395 **(2013)**

33. Saavedra L., Taranto M.A.P., Sesma F. and de Valdez G.F., Homemade traditional cheeses for the isolation of probiotic Enterococcus faecium strains, *International Journal of Food Microbiology*, **88(2-3)**, 241-245 **(2003)**

34. Sahadeva R., Leong S., Chua K., Tan C., Chan H., Tong E., Wong S. and Chan H., Survival of commercial probiotic strains to pH and bile, *International Food Research Journal*, **18(4)**, 1515-1522 **(2011)**

35. Shahidi F., Mendosa A., Boylstone T. and Mohebbi M., A perception to survival of Bifidobacterium spp. in bioyoghurt, simulated gastric juice & bile solution, *World Applied Sciences Journal*, **3(1)**, 40-44 **(2008)**

36. Shehata A., Tarabees R., Basiouni S., ElSayed M., Gaballah A. and Krueger M., Effect of a potential probiotic candidate Enterococcus faecalis-1 on growth performance, intestinal microbiota and immune response of commercial broiler chickens, *Probiotics and Antimicrobial Proteins*, **12**, 451-460 **(2020)**

37. Shehata M., El Sohaimy S., El-Sahn M.A. and Youssef M., Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity, *Annals of Agricultural Sciences*, **61(1)**, 65-75 **(2016)**

38. Singhal N., Maurya A.K., Mohanty S., Kumar M. and Virdi J.S., Evaluation of bile salt hydrolases, cholesterol-lowering capabilities and probiotic potential of Enterococcus faecium isolated from rhizosphere, *Frontiers in Microbiology*, **10**, 460235 **(2019)**

39. Suganya M. and Manimegalai K., Geometric Morphometric Study of Wing Shape in Leva indica (Acrididae: Gomphocerinae) from Different Environmental Sites of Coimbatore, India, *Res. J. Chem. Environ.*, **28(1)**, 133-141 **(2024)**

40. Wang J., Zhang H., Chen X., Chen Y. and Bao Q., Selection of potential probiotic lactobacilli for cholesterol-lowering properties and their effect on cholesterol metabolism in rats fed a high-lipid diet, *Journal of Dairy Science*, **95(4)**, 1645-1654 **(2012)**

41. Xie N., Cui Y., Yin Y.N., Zhao X., Yang J.W., Wang Z.G., Fu N., Tang Y., Wang X.H. and Liu X.W., Effects of two Lactobacillus strains on lipid metabolism and intestinal microflora in rats fed a high-cholesterol diet, *BMC Complementary and Alternative Medicine*, **11**, 1-11 **(2011)**

42. Yusuf D., Nuraida L., Dewanti-Hariyadi R. and Hunaefi D., *In vitro* characterization of lactic acid bacteria from Indonesian kefir grains as probiotics with cholesterol-lowering effect, *Journal of Microbiology and Biotechnology*, **30(5)**, 726 **(2020)**

43. Zanirati D.F., Abatemarco Jr. M., de Cicco Sandes S.H., Nicoli J.R., Nunes Á.C. and Neumann E., Selection of lactic acid bacteria from Brazilian kefir grains for potential use as starter or probiotic cultures, *Anaerobe*, **32**, 70-76 **(2015)**.

(Received 04th October 2024, accepted 08th November 2024)