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Evaluation of safety aspects and oral probiotic potential of *Weissella cibaria* strain isolated from Idli batter

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Abstract
Weissella cibaria strains, beyond their role in traditional fermentations, are being studied for their probiotic potential, particularly in preventing periodontal diseases. These strains also enhance food safety, nutrition, and sensory quality. Among them, *W. cibaria* MTCC 5948 has demonstrated notable functional and safety attributes. *In vitro* tests on *W. cibaria* MTCC 5948 assessed 10 safety parameters, confirming its sensitivity to tetracycline and erythromycin but resistance to several other antibiotics. The strain was negative for hemolytic reaction, phenylalanine degradation, indole production, gelatin liquefaction, nitroreductase activity and beta-glucronidase activity indicating a low risk of harmful effects. The enzyme profiling highlighted activities such as lipase, arylamidases, phosphatases, and galactosidases. Under oral conditions, the strain demonstrated promising survival rates of 67.95% and 95.16% after lysozyme and hydrogen peroxide exposure.

Keywords: *Weissella cibaria* MTCC 5948, lactic acid bacteria, fermented food, traditional

Introduction

Lactic Acid Bacteria (LAB) are Gram-positive, non-spore-forming bacteria, commonly appearing as cocci or rods, that primarily produce lactic acid through carbohydrate fermentation. These bacteria are crucial in spontaneous fermentation processes, with key genera including *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus*, and *Weissella* (Jin *et al*., 2019) [1]. Lactic acid bacteria with potential probiotic benefits primarily belong to the genera *Lactobacillus* and *Bifidobacterium*. Recently, there has been growing interest in studying the probiotic effects of *Weissella* species. The genus *Weissella*, which is part of the lactic acid bacteria group, is commonly found in various foods and is also a natural component of the human microbiome. (Kim *et al*., 2023) [2]. The bacterial composition of *Idli*, a traditional naturally fermented Indian food, was examined using high-throughput Illumina amplicon sequencing across different taxonomic levels. Genera such as *Weissella*, *Lactococcus*, and *Enterococcus* demonstrated increased abundance (Kavitake *et al*., 2022) [3]. Certain species of *Weissella* are regarded as potential probiotics because of their capacity to endure the challenging conditions of the gastrointestinal tract in both humans and animals. Some of these species have shown promise in the treatment and prevention of periodontal diseases, skin conditions, and atopic dermatitis. Additionally, others are employed as starter cultures in food fermentation due to their ability to produce exopolysaccharides, or as protective cultures thanks to their production of weissellicin, a type of bacteriocin (Russo *et al*., 2023) [4]. They are often classified as opportunist pathogens, which seems to be a limit for using as probiotic in food and feed (Nath *et al*., 2020) [5]. Moreover, many species of *Weissella* show inherent resistance to vancomycin. Therefore, the *Weissella* genus is important from both medical and industrial viewpoints. As the efficacy of probiotics depends on the specific strain, it is essential to verify the identity, characterization, and non-pathogenicity of the genotype or phenotype to ensure safety for human use. (Saarela *et al*., 2019) [6]. Several *Weissella* species isolated from food and animal sources have demonstrated health-promoting properties, including cholesterol-lowering, antioxidant activity, immunomodulatory effects, oral health benefits, and anticancer properties (Unnikrishnan and Sreeja, 2021) [7].

At the same time their safety aspects are controversial. Even microorganisms, such as lactic acid bacteria (LAB), which belong to a group of bacteria generally recognized as safe (GRAS) (Joint FAO/WHO 2002), must be assessed for their potential virulence. Key virulence factors linked to pathogenicity include antibiotic resistance, the production of extracellular proteins like haemolysin and gelatinase, as well as surface proteins and substances involved in aggregation (Silva *et al*., 2019) [8]. The oral cavity is a sophisticated ecosystem that has evolved a diverse microbial population. Probiotics can form biofilm and protect oral tissues from oral illnesses by forming biofilm. Caries, periodontal disorders, and halitosis can all be treated or prevented using probiotics (Anilkumar *et al*., 2012) [9]. This *in vitro* study was aimed to assess the safety aspects as well as the oral probiotic potential of *Weissella cibaria* strain isolated from indigenous fermented food idli batter.

Materials and Methods

The strain

The strain *Weissella cibaria* MTCC 5948 was sourced from the Culture Collection of the Dairy Microbiology Department, SMC College of Dairy Science, Kamdhenu University, Anand. It was grown in MRS medium by incubating at 37 °C for 24 hours and was stored at 5 °C. Sub-culturing was performed every 10 days throughout the study. The positive control organisms used included *E. coli* O157H7 ATCC 43888, *Listeria ivanovii* ATCC 19119, *Proteus vulgaris* ATCC 6896, *Pseudomonas aeruginosa* ATCC 27853, and *Lactobacillus rhamnosus* ATCC 53103. All cultures were obtained from Himedia (Mumbai, India) as KWIK STIK™ lyophilized cultures. The lyophilized pellets of these organisms were activated according to the culture producer's instructions and maintained in their respective selective growth media under the prescribed growth conditions.

Antibiotic resistance

The antibiotic resistance of *Weissella* strain was tested using the Kirby-Bauer disk diffusion method given by Nath *et al*. (2020) [5]. Standard antibiotic discs, including Gentamicin, Vancomycin, Tetracycline, Kanamycin, Ampicillin, Norfloxacin, Rifampicin, Methicillin, Streptomycin, and Erythromycin, were used. MRS agar plates were prepared, and the active culture was spread on the surface. After drying for 10 minutes, antibiotic discs were placed on the plates, which were incubated at 37 °C for 24 hours. The inhibition zone diameters were then measured using a zone scale.

Hemolytic activity

The haemolytic activity of *Weissella* strain was evaluated using a modified method by Wang *et al*. (2020) [10]. Sheep blood agar plates containing 7% sheep blood were inoculated with active cultures and incubated at 37 °C for 48 hours. *Listeria ivanovii* ATCC19119 and *Lactobacillus helveticus MTCC 5463* were used as positive and negative controls, respectively. Haemolysis types—β (clear zones), α (green zones), and γ (no zones)—were assessed post-incubation.

Mucin degradation

The mucin degradation ability of *Weissella* strain was evaluated using the method described by Kang *et al*. (2019) [11]. A 1 mL aliquot of active culture was inoculated into 10 mL of the following media: (i) MRS basal medium supplemented with 0.3% partially purified mucin (type III, Sigma-Aldrich, Bangalore, India), (ii) MRS broth with 1% glucose, (iii) MRS broth without glucose, and (iv) MRS broth containing 1% glucose and 0.3% mucin. *Lactobacillus helveticus* MTCC 54163 served as the negative control. The tubes were incubated at 37 °C for 24 hours. Bacterial growth was measured by recording the absorbance at 600 nm at intervals of 0, 8, and 24 hours.

D-lactic acid production

The production of D-lactic acid by *Weissella* strain was determined using the method described by Kang *et al*. (2019) [11]. One milliliter of active cultures was inoculated into 10 milliliters of skim milk and incubated at 37 °C for 24 hours. After incubation, the cell-free supernatant was collected by centrifugation at 10,000 rpm for 10 minutes at 4 °C. Unfermented milk (pH adjusted to 4.6) was used as another control. D-lactic acid in the supernatant was quantified using the D-lactate colorimetric assay kit (SIGMA-ALDRICH, Bangalore, India).

Urease activity

The method described by Kang *et al*. (2019) [11] was followed, the urea agar was prepared by supplementing nutrient agar with 2% urea and 0.0012% phenol red. A loopful of active culture from each *Weissella* strain was streaked onto the urea agar plates. *Proteus vulgaris* ATCC6896 was used as the positive control. The plates were incubated at 37 °C for 24 hours. After incubation, a color change in the medium to pink or red was considered a positive reaction

Enzymatic profiling of *Wesisella* strain

The β-glucuronidase activity and enzyme profiling of *Weissella cibaria* was assessed using the API-ZYM kit (Biomeruix, Germany), which allows for rapid evaluation of 19 enzymatic reactions with minimal sample quantities. The bacterial suspension was prepared by centrifuging and resuspending the cells, then adjusting the density to McFarland standard 3. The suspension was added to the API-ZYM strips, which were incubated at 37 °C for 4.5 hours. After incubation, reagents were added, and the color reactions were assessed. A scale from 0 to 5 was used to interpret the results, where 0 was negative, and values 2-5 were considered positive reactions.

Nitroreductase activity

Nitroreductase activity was assessed by converting 4-nitrobenzoic acid to 4-aminobenzoic acid, following the method of Kang *et al*. (2019) [11] with modifications. A 50 ml culture was centrifuged at 5000 rpm for 10 minutes, and the pellets were washed with phosphate-buffered saline (pH 7.2). The pellets were then sonicated for 7 minutes at 55% amplitude, 15 °C, with 15 pulses. The suspension was centrifuged again at 5000 rpm for 10 minutes. *E. coli* O157:H7 ATCC 43888 served as the positive control. The supernatant was collected and transferred to a clean test tube. To this, 4-nitrobenzoic acid (30 mg/L, Sigma-Aldrich), 1 ml of trichloroacetic acid (0.21%), and 1 ml of supernatant were added and incubated at 37 °C for 1 hour. The production of 4-aminobenzoic acid was detected via diazotization in the presence of 0.35 ml of 0.007% sodium nitrite (Himedia, Mumbai, India), followed by the addition of 1 ml of 0.35% NEDD (N-(1-naphthyl ethylenediamine dihydrochloride), Sigma-Aldrich) at 4 °C. A pink to purple color indicated a positive reaction

Gelatin liquefaction

The gelatin liquefaction activity of *Weissella* strain was assessed using the method described by Kang *et al*. (2019) [11], gelatin media. Each tube was inoculated with 1 mL of the test organism and incubated at 37 °C for seven days. Pseudomonas aeruginosa ATCC 27853 was used as the positive control. The gelatin tubes were removed daily from the incubator and placed at 4 °C to observe for liquefaction. Liquefaction at 4 °C was considered a positive reaction.

Phenylalanine degradation

The phenylalanine degradation activity was tested following a modified method by Kang *et al*. (2019) [11]. Phenylalanine agar slants were prepared, and a loop full of active culture was streaked onto the slant, then incubated at 37 °C for 24 hours. *Proteus vulgaris* ATCC6896 served as a positive control. After incubation, ferric chloride was added to the slant, and a green color within 1 to 5 minutes indicated a positive reaction.

Oral probiotic potential

The oral probiotic potential of *Weissella* strain was evaluated interms of (i) lysozyme resistance on bacterial growth and (ii) hydrogen peroxide resistance on bacterial growth

Lysozyme resistance on bacterial growth

The lysozyme resistance of *Weissella* strains was evaluated using the method described by Jang *et al*. (2016), with modifications. An inoculum of 10 ml (with a cell concentration adjusted to 10^9 cells/ml) from a 24-hour culture was centrifuged at 5000 rpm for 10 minutes at 4 °C. The resulting pellets were washed once with PBS buffer (pH 7.2) and resuspended in 10 ml of PBS buffer (pH 7.2) containing 0.1 mg lysozyme/ml (Himedia, Mumbai, India). The tubes were incubated at 37 °C for 90 minutes. Microbial growth was assessed by plating on MRS agar at 0, 30, and 90 minutes, followed by incubation at 37 °C for 24-48 hours. Survival (%) was calculated at both 30 and 90 minutes.

$$\text{Survival Ratio (\%)} = \frac{\text{Cell numbers (Log cfu/ml) in Lysozyme containing PBS}}{\text{Cell numbers(Log cfu/ml) in control PBS}} \times 100$$

Hydrogen peroxide resistance on bacterial growth

The potential of hydrogen peroxide to inhibit growth in *Weissella* strain was assessed by monitoring the survival ratio on MRS agar. The method described by Jang *et al.* (2016) was followed with some modifications. Specifically, 0.2 ml of inoculum (OD at 600 nm = 5 × 10^8 cells/ml) from a 24-hour culture was used. This inoculum was added to 10 ml of MRS broth in tubes containing 1 mM hydrogen peroxide (LOBA Cheme, Mumbai, India). The tubes were incubated at 37 °C for 1, 3, and 6 hours. After incubation, bacterial survival (%) was determined by plating on MRS agar.

$$\text{Survival Ratio (\%)} = \frac{\text{Cell numbers (Log cfu/ml) in } H_2O_2 \text{ containing MRS broth}}{\text{Cell numbers(Log cfu/ml) in control MRS broth}} \times 100$$

Results and Discussions

The antibiotic resistance of *Weissella cibaria* MTCC 5948 was evaluated against ten antibiotics: Gentamicin, Vancomycin, Tetracycline, Kanamycin, Ampicillin, Norfloxacin, Rifampicin, Methicillin, Streptomycin, and Erythromycin. The results, shown in the Table2 and Figure 1. The strain was sensitive to tetracycline and erythromycin, resistant to gentamicin, vancomycin, kanamycin, norfloxacin, methicillin, streptomycin and intermediate resistant to rifampicin. Le *et al*. (2018) [13] reported that *W. cibaria* strain FB069 was resistant to Kanamycin (with a minimum inhibitory concentration (MIC) of ≥128 µg/mL), Vancomycin (MIC ≥64-128 µg/mL), and Tetracycline (MIC ≥32 µg/mL). Kang *et al*. (2019) [11] found that *W. cibaria* strain CMU exhibited strong resistance to Kanamycin and Vancomycin. Additionally, Liu *et al*. (2024) [14] found that all isolates of *W. cibaria* showed resistance to Vancomycin but were susceptible to Ampicillin, Erythromycin, Tetracycline, Chloramphenicol, and Clindamycin, which aligns with the results obtained for our strain.

W. cibaria MTCC 5948 did not show hemolysis on blood agar plates, whereas the positive control, *Listeria ivanovii* ATCC 19119, exhibited beta-hemolysis (Figure 2). A similar finding was reported by Huy *et al*. (2020) [15] for *W. cibaria* HN05, which was isolated from the gastrointestinal tract of Pacific white shrimp.

The mucin degradation activity of the *Weissella* strain was assessed by growing it in four different conditions: (i) MRS basal medium with 0.3% partially purified mucin, (ii) MRS broth with 1% glucose, (iii) MRS broth without glucose, and (iv) MRS broth with 1% glucose and 0.3% mucin. The growth curve of the *Weissella* strain in the various media is shown in Figure 3. The results indicated that both *W. cibaria* MTCC 5948 and the control probiotic *L. helveticus* MTCC 5463 (V3) failed to grow in the mucin-containing medium, suggesting that these strains are not capable of degrading mucin *in vitro*. Reported similar findings, noting that *W. cibaria* isolates from aquatic sources were unable to degrade mucin *in vitro*.

The strain did not exhibit gelatinase activity, while the positive control, *Pseudomonas aeruginosa* ATCC 27853, caused liquefaction of the gelatin medium (Figure 4). This observation aligns with a similar report by Ojkenle *et al.* (2017) [17], where *W. cibaria* WD2 tested negative for gelatin liquefaction.

The *Weissella cibaria* MTCC 5948 strain tested negative for urease activity, while the control organism, *Proteus vulgaris* ATCC 6896, exhibited a positive result (Figure 5). The *Weissella* strain also tested negative for indole production (Figure 6). The positive control organism, *E. coli* O157:H7 ATCC 43888, produced a red-colored ring upon the addition of Kovac's reagent, indicating a positive reaction (Figure 6). The *Weissella* strain displayed a negative phenylalanine degradation reaction, while the control *Proteus vulgaris* ATCC 6896 was positive (Figure 7). Our *Weissella* strain also tested negative for nitroreductase activity, in contrast to control *E. coli* O157:H7 ATCC 43888, which showed a positive reaction (Figure 8).

Kang *et al*. (2019) [11] examined the safety aspects of *Weissella cibaria* CMU and CMS1, and found that these strains did not harbor virulence genes. The strains produced trace amounts of D-lactate, suggesting that they are capable of producing D-lactic acid. According to the biochemical characteristics of *Weissella* species, *W. cibaria* produces both D- and L-lactic acid. Reported that administering probiotic bacteria that produce D-lactic acid is safe and does not lead to long-term increases in blood D-lactic acid levels.

The API Zym profile of *Weissella cibaria* MTCC 5948 is presented in Table 3 and Figure 9. The strain tested positive for the enzymes lipase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, naphthol-AS-BI phosphohydrolase, alpha-galactosidase, beta-galactosidase, and beta-glucosidase activities, while it tested negative for beta-glucuronidase activity.

Analyzed the enzyme profiles of *W. cibaria* isolates from aquatic sources using the API ZYM test. The isolates demonstrated activities including β-glucuronidase, acid phosphatase, Naphthol-AS-BI phosphohydrolase, and β-galactosidase. Similarly, Kang *et al*. (2019) [11] reported that the CMU and CMS1 strains exhibited acid phosphatase and Naphthol-AS-BI phosphohydrolase activities, similar to the findings for our *W. cibaria* strain.

Hydrogen peroxide in the oral cavity is produced by oral bacteria, leukocytes, and salivary glands. It is utilized in the peroxidation reaction of salivary peroxidase to generate antibacterial products and water. Resistance to hydrogen peroxide (H_2O_2) is a key factor in selecting oral probiotic bacteria. *Weissella cibaria* MTCC 5948 demonstrated a survival rate of >50% after being exposed to hydrogen peroxide for 6 hours which is considered a very good survival rate. The survival rate of the organism gradually decreased from 1 hour to 6 hours of incubation (Table 4).

Weissella cibaria MTCC 5948 demonstrated survival of 95.16% after a 90-minute lysozyme treatment (Table 5). This suggests that the strain exhibits resistance to lysozyme and could be considered for use as an oral probiotic. The results of both lysozyme and hydrogen peroxide treatments indicated the potential of *Weissella cibaria* MTCC 5948 as an oral probiotic. Similar findings were reported by Jang *et al*. (2016), who observed that *W. cibaria* CMU maintained over 90% viability after 90 minutes of lysozyme exposure, with a viability of 92.83% after hours of exposure.

Table 1: Details *Weissella Cibaria* strain used in the study

Bacterial Name	Lab code	Source of isolation	NCBI Accession number
Weissella cibaria MTCC 5948	92	Idli batter	JN792466

Table 2: Antibiotic resistance (Inhibition Zone in mm) of *Weissella cibaria* MTCC 5948

Antibiotic (Concentration)	Zone of Inhibition(mm)
Gentamicin (10 µg)	12.33±1.52 [R]
Vancomycin (30 µg)	14.66±0.57 [R]
Tetracycline (30 µg)	21.66±1.15 [S]
Kanamycin (30 µg)	0.00±0.00 [R]
Ampicillin (5 µg)	12.00±1.01 [R]
Norfloxacin (10 µg)	0.00±0.00 [R]
Rifampicin (5µg)	15.33±1.52 [I]
Methicillin (5 µg)	0.00±0.00 [R]
Streptomycin (10 µg)	14.00±1.01 [R]
Erythromycin (15 µg)	23.00±1.01 [S]
R= Resistant (0 to 15 mm), I= Intermediate (15.01 to 20.00), S= Sensitive (more than 20mm), as per the guide lines of Clinical and Laboratory Standards Institute (2011) 26th Edition.	

Table 3: APIZYM profiling of *Weissella cibaria* MTCC 5948

No	Enzyme Assayed For	API ZYM reaction	Result
1	Control	0	-
2	Alkaline phosphatase	0	Negative
3	Esterase (C4)	0	Negative
4	Esterase Lipase (C8)	0	Negative
6	Lipase (C14)	2	Positive
7	Leucine arylamidase	2	Positive
8	Valine arylamidase	3	Positive
9	Cystine arylamidase	2	Positive
10	Trypsin	0	Negative
11	α-chymotrypsin	0	Negative
12	Acid phosphatase	2	Positive
13	Naphthol-AS-BI-phosphohydrolase	2	Positive
14	α-galactosidase	2	Positive
15	β-galactosidase	2	Positive
16	β-glucoronidase	0	Negative
17	α-glucosidase	2	Positive
18	β-glucosidase	3	Positive
19	N-acetyl-β-glucosaminidase	0	Negative
20	α-mannosidase	0	Negative
21	α-fucosidase	0	Negative
0 corresponds to a negative reaction; 1, 2, 3 or 4 are intermediate reactions depending on the level of intensity (2, 3, 4 or 5 being considered as positive reactions).			

Table 4: Survival ratio (%) of *Weissella cibaria* MTCC 5948 After Hydrogen peroxide treatment at different time intervals

Survival Ratio (%)		
1 Hour	3 Hour	6 Hour
97.02±0.54	87.411±0.94	67.95±1.7

Table 5: Survival ratio (%) of *Weissella cibaria* MTCC 5948 After lysozyme treatment at different time intervals

Survival Ratio (%)	
30 Minutes	90 Minutes
96.32±0.24	95.16±0.08

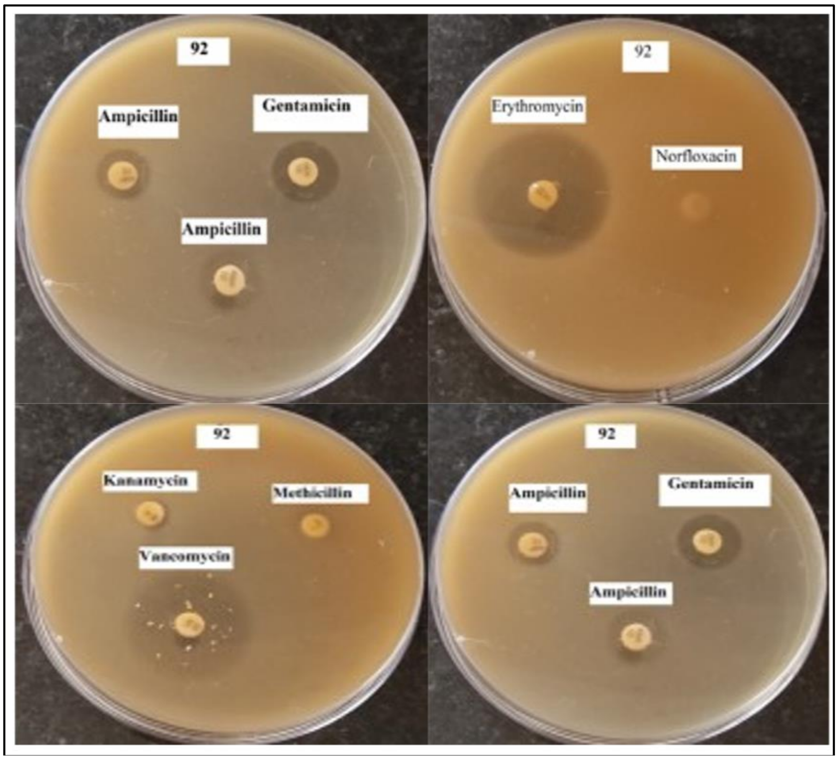

Fig 1: Antibiotic resistance of *Weissella cibaria* MTCC 5948

Fig 2: Haemolytic activity of *Weissella cibaria* MTCC 5948

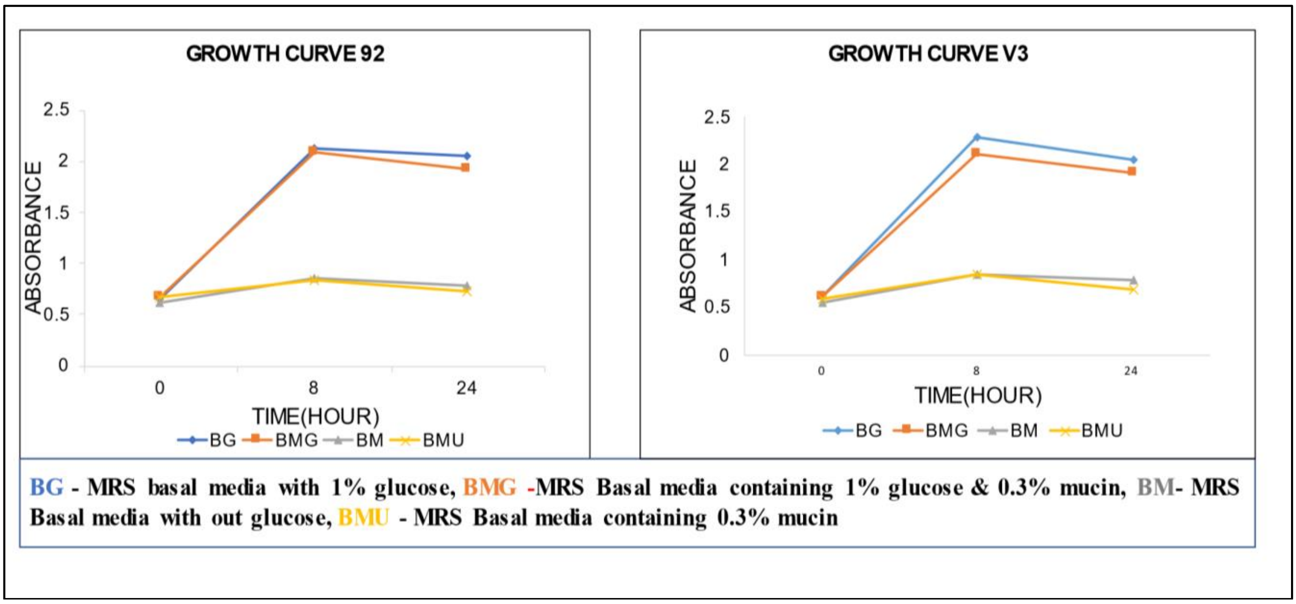

Fig 3: Mucin degradation activity of *Weissella cibaria* MTCC 5948

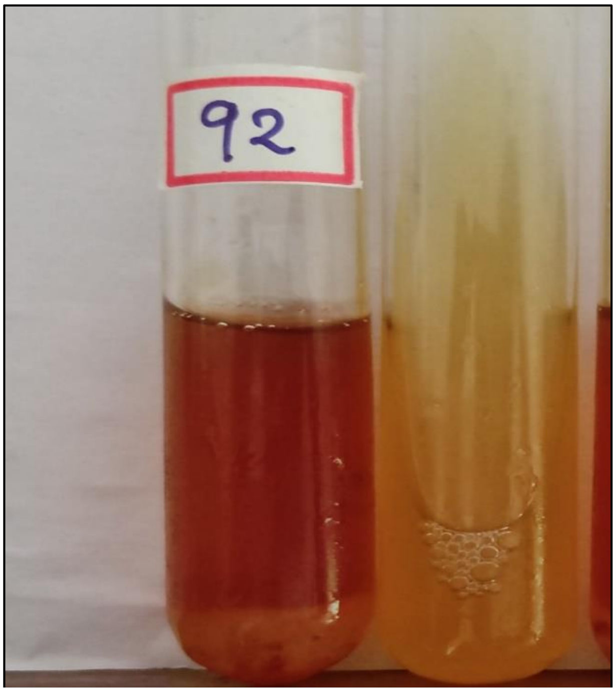

Fig 4: Gelatine liquefaction activity of *Weissella cibaria* MTCC 5948

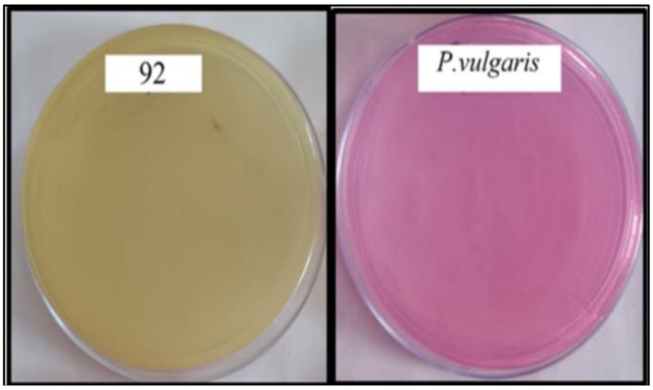

Fig 5: Urease activity of *Weissella cibaria* MTCC 5948

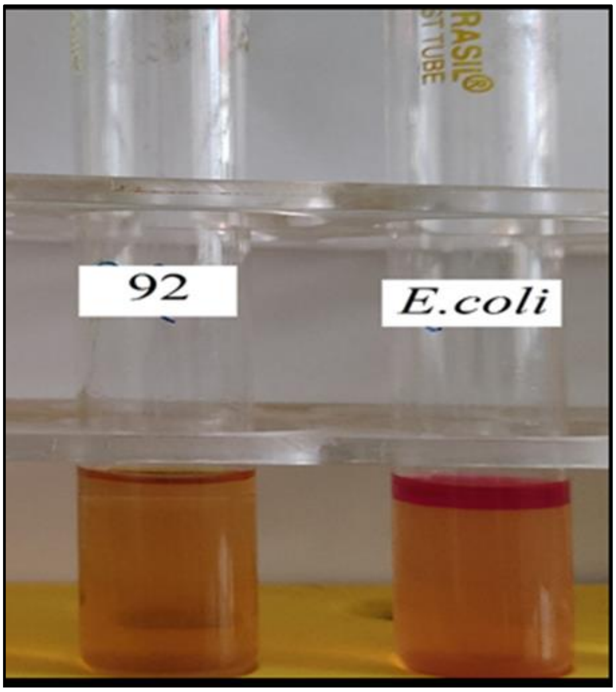

Fig 6: Indole production activity of *Weissella cibaria* MTCC 5948

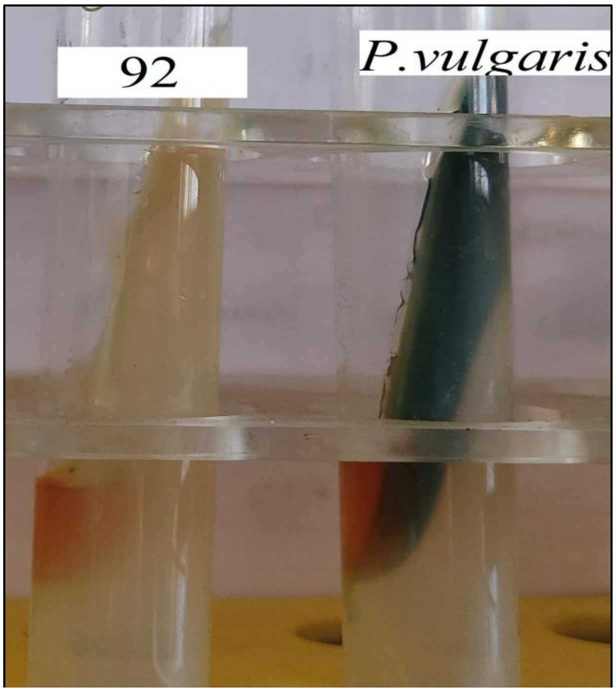

Fig 7: Phenylalanine degradation activity of *Weissella cibaria* MTCC 5948

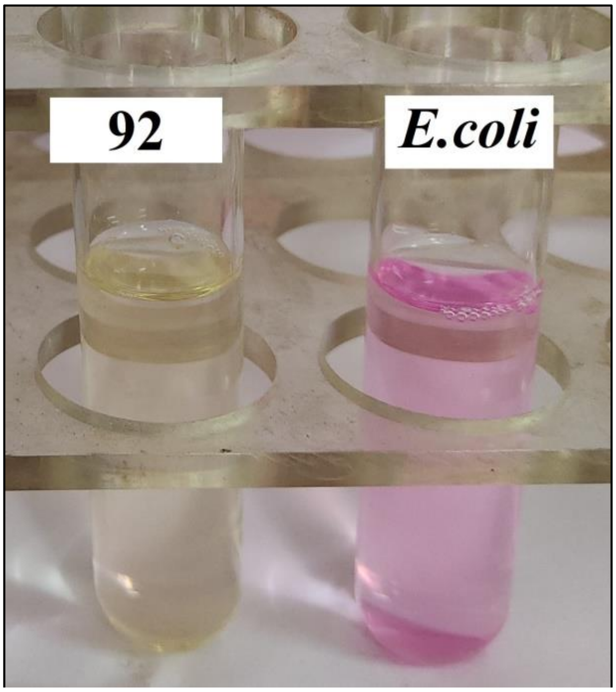

Fig 8: Nitroreductase activity of *Weissella cibaria* MTCC 5948

Fig 9: API ZYM reactions of *Weissella cibaria* MTCC 5948

Conclusion

The *in vitro* safety evaluation of *Weissella cibaria* MTCC 5948 included 10 key assessments, revealing sensitivity to tetracycline and erythromycin, resistance to several antibiotics, and absence of harmful activities like hemolysis and mucin degradation. *W. cibaria* MTCC 5948 showed sensitivity to tetracycline and erythromycin but resistance to several antibiotics. It tested negative for harmful activities, was non-hemolytic, and produced D-lactic acid with uncertain implications. Enzymatic profiling revealed diverse activities, supporting its functionality. *W. cibaria* MTCC 5948 demonstrated high survival ability under oral conditions, making it suitable for oral probiotic formulations. While promising, further *in vivo* studies, gene transferability assessments, and virulence gene investigations are needed to confirm its safety and efficacy.

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