Screening and Characterization of *Weissella cibaria* Isolated from Food Source for Probiotic Properties

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ABSTRACT
This study was aimed to determine the probiotic potential of lactic acid bacteria (LAB) isolated from various food sources such as cow milk, goat milk and idli batter. The strain *Weissella cibaria* (KTSMBNL 28) isolated from goat milk showed more antibactericidal activity than other strains from cow milk and idli batter. This strain was able to tolerate pH 3.0 which maintained in gastric fluid and resist upto1% of bile salt retained in intestinal fluid. Moreover, KTSMBNL 28 recorded highest level of hydrophobicity (35-70%) and its non haemolytic property ensured the safe mode of application. Among the 15 antibiotics tested, KTSMBNL 28 showed resistance towards penicillin, vancomycin and nalidixic acid. Therefore, *Weissella cibaria* KTSMBNL 28 which showed good probiotic properties and could be attribute beneficial effects to mankind.

GENERAL TERM
Probiotics, lactic acid bacteria, hydrophobicity,

KEYWORDS
Weissella cibaria, antibactericial activity, acid and bile tolerance, antibiotics resistance.

1. INTRODUCTION
Probiotics are live microorganism, which bring about health benefits when administered in adequate amounts and hence, the consumption of probiotic food is very popular worldwide. The probiotic strains must possess the ability to overcome the low pH and high concentration of bile salt to enter into the site of action in a viable state [1]. Among the probiotics, lactic acid bacteria (LAB) have long played vital roles in fermented food technology which include species of the genera *Lactococcus, Enterococcus, Pediococcus, Leuconostoc, Lactobacillus, Streptococcus, Vagococcus, Tetragenococcus* and *Weissella* [2]. *Weissella cibaria* was first described by Bjorkroth and al [3], later found in various sources and some of them play important roles in food fermentation [4, 5]. Bacteriocins have been identified in many strains of LAB as microbial inhibitors that are ribosomal synthesized antimicrobial peptides usually active against closely related species [6]. These peptides adhere to the cell membrane and create pores through which cellular material diffuse. Some bacteriocins interfere with cell wall synthesis or the activity of DNase and RNase [7]. The present study was designed to screen the bacteriocin producing *Weissella cibaria* strains from various food sources such as cow milk, goat milk and idli batter for their probiotic properties which may exert beneficial effects to mankind.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of LAB from Food Source
The samples such as goat milk, cow milk and idli batter were collected and stored in ice. All samples were serially diluted with sterile peptone water. Appropriate dilutions were made with same and 0.1ml aliquot was spread plated onto Lactobacilli MRS (deMan, Rogosa and Sharpe) agar plate containing 1% $CaCO_3$. Colonies forming a clear zone on the MRS agar plate were selected. Identification was done by physiological and biochemical tests. Species level identification was done by PCR (Biorad T100, USA) with 8F (AGAGTTTGATCCTGGCTCAG) and 1492R (TACGGCTACCTTGTTACGACTT) primers. The amplified fragments were cleaned using EZ-10 Spin column PCR (BioBasic), sequenced and submitted in NCBI.

2.2 Production of Crude Bacteriocin
The isolated strain was grown in MRS broth and maintained aerobically at 37 °C for 24 h. After incubation, the cells were removed from the growth medium by centrifugation (10,000 rpm for 15 min, 4 °C). The cell free supernatant was adjusted to pH 6.0 using 1N NaOH and it was used as crude bacteriocin [8]. The crude bacteriocin was concentrated to one tenth of the original volume by lyophilizer (Christ, Germany) and the solution was designated as bacteriocin like inhibitory substance (BLIS). For synergistic activity BLIS was mixed with 1ml of 1 % of EDTA and filter sterilized by 0.22µm membrane filter paper (Millipore, India) to study the anti-microbial activity.

2.3 Detection of Antagonistic Activity using Well Diffusion Method
The antagonistic property of isolated LAB species was determined by well diffusion method [9]. For swapping, 18 h old LAB cultures were used in Muller Hinton agar plate. Multiple wells with diameter of 5mm were made in each of

the plates. A 50µl of concentrated BLIS with and without EDTA were used and the plates were incubated at 37 °C for 24 h [10]. The inhibition zone was measured in millimeter using antibiotic zone scale (HiMedia, India).

2.4 Resistant to pH and Bile Salt

To explore the survival of LAB strain under acidic condition, the strain was harvested by centrifugation (5000 rpm, 10 min at 4 °C). Pellets were washed once in PBS (pH 7.2) and resuspended in PBS at pH 3, 4 and 7 and incubated at 37 °C for 3 h. Viable microorganisms were enumerated at the 0, 1, 2 and 3 h with spread plate techniques and growth was monitored at OD 600nm in UV – Visible spectrophotometer (UV-1700, Shimadzu). To investigate bile tolerance of LAB, the strain was harvested as above and inoculated in MRS medium containing 0.3, 0.5 and 1% bile (Oxoid). During the incubation at 37 °C for 4 h, viable colonies were enumerated for every hour with spread plate technique and growth was measured at 600nm.

2.5 Hydrophobicity Test

The strain grown in MRS broth at 37 °C for 18 h was used to study the bacterial adhesion to hydrocarbons (BATH). Cells were harvested, washed twice with PBS and resuspended in the same solution and the optical density (OD 600nm) was determined. Equal volume of cell suspension and n-hexadecane were added and vortexed for 2 min. Aqueous and organic phases were allowed to separate for 30 min at room temperature. The aqueous phase was collected and optical density (OD 600nm) was determined before (reading 1) and after 30 min (reading 2). The experiments were conducted in triplicates. The percentage hydrophobicity was calculated as follows:

$$\% \text{ Hydrophobicity} = [(OD_{600 \text{ reading } 1} - OD_{600 \text{ reading } 2} / OD_{600 \text{ reading1}})] \times 100$$

2.5 Haemolysis Test

The isolates were streaked on MRS agar supplemented with 5% blood to check for hemolysis [11].

2.5 Antibiotic Sensitivity Test

The antibiotics discs used for susceptibility assay were vancomycin, ciprofloxacin, penicillin, chlorampenicol, erythromycin, tetracycline, tobramycin, cefotaxime, nalidixic acid, streptomycin, gentamycin, cefuroxime, kanamycin, oxacillin, and ampicillin. LAB swabbed agar plates were incubated at 37 °C for 24 h with antibiotic discs and the zone of inhibition were measured.

3. RESULT AND DISCUSSION

3.1 Identification of LAB

Bacterial growth was observed on MRS agar plate, after 24 h of incubation. Totally 16 colonies were isolated based on colony morphology and pure cultures were stored in agar tube. Out of 16 colonies, 4 colonies (Cow milk-1, Goat milk-1, Idli batter-2 colonies) were selected on the basis of lactic acid formation and the acids produced by the isolated microbes were neutralized by the calcium carbonate in MRS agar and the zone was observed (Figure 1). From the 16S rRNA sequence analysis, the identified strains were named as *Weissella cibaria* KTSMNBL 27 (cow milk), *Weissella cibaria* KTSMNBL 28 (goat milk), *Weissella cibaria* KTSMNBL 29 (Idli batter) and *Weissella cibaria* KTSMNBL 30 (Idli batter) with accession number KC987948 to KC987951.

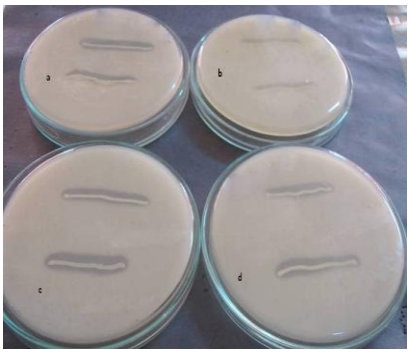

Fig 1: Clear zone formation in isolated different colonies

3.2 Antagonistic Activity

The antagonistic activity of 4 isolated strains were tested against the enteropathogens (Table 1). The strain *Weissella cibaria* KTSMBNL 28 which showed strong inhibitory activity against *Klebsilla pneumoniae*, Vibrio cholerae, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Shigella* sp. and *Salmonella typhimurium* was selected for further studies. The isolated strain *Wiesella cibaria* was able to inhibit the growth of human enteropathogens and these results also indicate the presence of the BLIS by zone of inhibition against the test organism. Bacteriocins have been reported to be inhibitory against several other bacteria [8]. Possession of BLIS by *Weissella cibaria* is an indication that the bacteria can be used as probiotics and biopreservative.

Table 1. Antagonistic activity of *Wiessella Cibaria* against various human enteropathogens

Test microorganisms	Zone of inhibition (mm)			
	KTSMBNL 27	KTSMBNL 28	KTSMBNL 29	KTSMBNL 30
Staphylococcus aureus	17	20	13	13
Klebsiella pneumoniae	±16	21	21	18
Salmonella typhimurium	-	11	-	-
Vibrio cholera	±9	13	-	±10
Pseudomonas aeruginosa	-	12	13	±12
Shigella sp.	11	21	±18	-

3.3 Tolerance under Acidic and Bile Conditions

Bacteria that tolerate the low pH in stomach acidic condition is one of the major concern in probiotics. Although in the stomach, pH can be as low as 1.0 in most *in vitro* assays pH 3.0 has been chosen due to the fact that significant decrease in the viability of strains occurs at pH 2.0 and below [12]. The strain KTSMBNL 28 had more than 89% of viability after 4 h in acid condition. While increasing the acidic condition the growth of *Weissella cibaria* was gradually decreased.

Although the bile concentration of the human gastro intestinal tract varies, the mean bile concentration of intestine is believed to be 0.3% w/v and the staying time is suggested to be 4 h [12]. Strains were detected in 0.3%, 0.5% and 1 .0 during 4 h. The colony forming units values were observed in MRS plate. According to the results the strain was resistant to 0.3%, 0.5% and 1 .0 and shows >80% viability. While increasing the concentration of bile, the growth rate of *Weissella cibaria* was gradually decreased. Several strains of *Lactobacillus* are hydrolyze bile salts by using bile salt hydrolase enzymes [13] which makes the isolates to survive at bile salt condition**.**

3.5 Hydrophobicity Test

The *Weissella cibaria* strains in our present study showed hydrophobicity from 35 to 70% with hexadecane. The determination of microbial adhesion to hexadecane is a way to estimate the ability of a strain to adhere to epithelial cells and it is a valid qualitative phenomenological approach [14]. Adherence of bacterial cells is usually related to cell surface characteristics. Cell surface hydrophobicity is a non-specific interaction between host and microbial cells. The initial interaction may be often reversible, weak and precedes subsequent adhesion processes mediated by more specific mechanisms involving cell surface proteins and lipoteichoic acids [15, 16].

3.6 Haemolysis Test

The strain *Weisella cibaria* KTSMBNL 28 produced no hemolysis, due to the inability to produce hemolytic zones on the medium [11].

3.7 Antibiotic Sensitivity Test

In the present study KTSMBNL 28 showed resistance towards penicillin, vancomycin and nalidixic acid, intermediate to kanamycin, gentamycin and tobramycin and sensitive to all other antibiotics (Table 2). Resistance may be inherent to a bacterial genus or species, but it may be acquired through mutations, exchange of genetic material and incorporation of new genes [18]. Potential probiotic LAB may act as reservoir of antibiotic resistance genes and transfer the gene horizontally to the other bacteria present in the human GIT is possible. Special purpose probiotics for use in combination with antibiotics have been developed through the introduction of multiple resistances to the bacteria [17].

TABLE 2. Antibiotic sensitivity test of *Wiessella cibaria* KTSMBNL 28

Antibiotics (µg)	Diameter of zone of inhibition (mm)			LAB strain (mm)
	Resistant	Intermediate	Sensitive	
Ampicilin (10)	13	14-16	17	36
Erythromycin (15)	13	14-22	23	25
Streptomycin (10)	11	12-14	15	15
Kanamycin (30)	13	14-17	18	14
Cefuroxime (30)	14	15-17	18	31
Gentamycin (10)	12	13-14	15	14
Vancomycin (30)	-	-	15	-
Oxacilin (5)	10	11-12	13	15
Nalidixicacid (30)	13	14-18	19	-
Penicillin (10)	28	-	29	28
Cefotaxime (30)	14	15-22	23	42
Tobramycin (10)	12	13-14	15	14
Tetracycline (30)	14	15-18	19	37
Ciprofloxacin (5)	15	16-20	21	21
Chloramphenicol (30)	12	13-17	18	37

4. CONCLUSION

The results obtained from the research indicated that among 16 strains studied, 4 strains produced lactic acid in basic screening test; only one strain *Weissella cibaria* KTSMBNL 28 showed good antagonistic activity against various human pathogens and also good resistance to gastro intestinal conditions. However, further study is required to carry out the potential application of probiotics in various fields.

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