

Antibacterial Activity of *Weissella confusa* Isolated From Vaginal Swab of Indian Women

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Abstract— *Weissella confusa*, a lactic acid producing bacteria (LAB), is known to occur in the human samples of breast milk, vaginal fluids, saliva and faeces. The species is known to be a probiotic microorganism due to its ability to control the growth of the pathogenic microbes capable of causing food borne diseases. The isolate reported in this study showed high antimicrobial activity against *Bacillus subtilis* (MTCC 736), *Escherichia coli* (MTCC 9492), *Pseudomonas aeruginosa* (MTCC 3541), *Proteus mirabilis* (MTCC 425), *Proteus vulgaris* (MTCC 771) and *Staphylococcus aureus* (MTCC 3160). Additionally, the isolated microbe was resistant to many of the commonly used antibiotics and hence can be used to develop formulations. Thus, the isolated organism may be explored as a probiotic candidate by local administration via suppositories for the prevention and treatment of vaginal infections.

Keywords— Antibiotic susceptibility, Probiotic, Vaginal microbiota, *Weissella confusa*.

I. INTRODUCTION

Weissella belongs to lactic acid producing bacteria (LAB) group. They are placed under Firmicutes, class Bacilli, order Lactobacillales and family Leuconostocaceae. They are obligate heterofermentative organisms which produce CO₂ from carbohydrate metabolism with lactic acid and acetic acid as major end products.

This microbe is present in nutrient-rich habitats, generally associated with the human body [1]. It has been successfully isolated from human breast milk [2], human saliva [3], human feces [4], and human vagina [5, 7]. *Weissella confusa* has been so far reported from human feces [8-10].

Two *Weissella* spp., namely, *Weissella viridesens* and *Weissella Kimchii* were reported from women of South Africa [7] and Korea [5]. From the phylogenetic analysis of *Weissella kimchii* strains, it is suggested to include both *Weissella confusa* and *Weissella cibaria* [6]. In a screening test which involved 100 LAB strains, *Weissella kimchii*

PL9023 was found to produce highest quantity of hydrogen peroxide [11] and therefore it inhibited growth of pathogenic microbes, namely, *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus agalactiae*, isolated from the vaginal smears. The presence of surface glycoproteins in *W. kimchii* PL9023 promoted the adherence of the microbe on the vaginal surface. Further, *W. kimchii* PL9023 was found to produce no harmful metabolites or enzymes. Based on the results obtained, *W. kimchii* PL9023 was reported to have a great potential as probiotics for vaginal health. The presence of *Weissella confusa* as a part of the normal vaginal flora opens up possibility for using this organism as probiotic supplement in female genital tract.

The present paper makes the first report of isolation of *Weissella confusa* from the vaginal swabs of the woman of North Eastern region of India. Further, the antimicrobial activity of the isolated microbe was tested against Gram positive and Gram negative bacteria, viz., *Bacillus subtilis* (MTCC 736), *Escherichia coli* (MTCC 9492), *Pseudomonas aeruginosa* (MTCC 3541), *Proteus mirabilis* (MTCC 425), *Proteus vulgaris* (MTCC 771) and *Staphylococcus aureus* (MTCC 3160). It further presents antibiotic sensitivity test conducted against the test organisms using twelve antibiotics.

II. MATERIALS AND METHODS

A. Sample Collection

Vaginal samples were collected from the Red Cross Hospital, Karimganj, Assam after obtaining a written informed consent of the volunteers. Prior permission was obtained from the Institutional Ethical Committee, Karimganj College (Memo No. KC/IEC/2012/M-1/10, dated: 23 Jun 2012) for conducting the study. The vaginal swabs were collected by a senior gynaecologist of the Hospital. The participants were explained in local language the procedure and the purpose of the collection of the samples. The volunteers were in the age group of 18-30 years. They were all pre-menopausal, non-menstrual and either pregnant or non-pregnant. Women suffering from any vaginal disease were not included in the study. Sampling was done using a sterile swab (HiMedia laboratory Pvt. Ltd., India) from the posterior zone of the fornix of the vagina. The swabs containing the vaginal isolates were immediately transferred to the laboratory and were used for the cultivation of bacteria. The pure cultures were stored at -20 °C and -80 °C as glycerol stock for further use. The

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isolates were characterized for morphological traits and biochemical properties [12].

B. Preparation Of Culture Filtrate And Antimicrobial Assay

The culture filtrates of the microbes were prepared by incubating the selected microbial colonies in MRS broth (HiMedia Laboratory Pvt. Ltd., India) at 37 °C for 48 h. After the incubation period, the microbial cells were collected by centrifugation (7000 g, 4 °C, 10 min). Subsequently, the supernatant was collected and filter sterilized using membrane filter (pore size: 0.22 µm) [13]. Antibacterial assay of the crude bacteriocin was done by agar well diffusion method. For the purpose, Mueller-Hinton agar plates were prepared and the plates were seeded with the test organisms. The standardization of the inoculum density was done using 0.5 McFarland standards. 5 mm wells were prepared in each Mueller-Hinton agar plates. 50 µl of the crude bacteriocin was loaded in each well [14]. The plates were incubated for 24 h at 37 °C. The isolates which showed antibacterial activities were used for the further study (Table 1).

C. Characterization Of Antimicrobial Substances

The bacterial strains were grown on 25 ml of MRS broth at 37 °C for overnight. Thereafter, the cultures were centrifuged (7000 g, 4 °C, 10 min). The supernatant of each strain were divided into equal portions (5 mL each) for different assays. For the bacteriocin assay, the supernatant was treated with proteinase 1 mg/ml (HiMedia). The supernatant was adjusted to pH 6.5± 1 using 1N NaOH for the organic acid assay, whereas, the culture filtrate was treated with 0.5 mg/ml catalase (HiMedia) for the hydrogen peroxide assay. The treated supernatants were filter sterilized through 0.22 µm syringe filter (HiMedia). Antimicrobial assay was done by agar well diffusion assay as described above against *E. coli* (MTCC 9492).

D. Antibiotics susceptibility test

Antibiotics susceptibility test was done on Mueller-Hinton agar plates. The plates were seeded with *Weissella confusa* and positive control (*Lactobacillus fermentum*, a strain which is known to have sensitivity to some of the selected antibiotics). Antibiotics discs of different concentrations were put over the medium at a gap of 40 mm. The plates were incubated for 24 h at 37 °C.

E. Molecular characterization

The isolation of the microbial genomic DNA was done as per the method described by Vural and Ozgun (2011) [15] with some modifications. 5 ml of MRS broth was inoculated with the isolates and incubated for 48 h. The broth was centrifuged at 7000 g at 4 °C for 10 min. The supernatant was discarded. The cell pellets were treated with 567 µl of 1X TE buffer (Hi Media Laboratory Pvt. Ltd., India) and 2 µl of 20 mg/ml lysozyme (HiMedia Laboratory Pvt. Ltd., India). Subsequently, the treated cells were incubated for 30 min (37 °C) in a shaking water-bath (NSW-133, NSW Pvt Ltd., India). To this mixture, 3 µl of each 1% SDS and 20 mg/ml proteinase

K (Hi Media Laboratory Pvt. Ltd., India) solutions were added and further incubated for 15 min at 37 °C. Thereafter, 1 µl of 5 M NaCl was added and further incubated for 10 min at 65 °C. Thereafter, 675 µl of chloroform: isoamyl alcohol (24:1) mixture was added and centrifuged at 10,000 rpm for 5 min (Centrifuge 5418R, Eppendorf Pvt. Ltd., Germany). The supernatant (aqueous phase) was collected in a fresh tube and an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) mixture (Hi Media Laboratory Pvt. Ltd. India) was added and centrifuged at 10,000 rpm for 5 min. The supernatant (aqueous phase) was again transferred in a fresh tube, sixth volume of ice-cold isopropanol was added and centrifuged at 10000 rpm for 5 min. The supernatant was discarded and the pellet was washed with 500 µl of 70% alcohol and centrifuged at 5000 rpm for 2 min. The centrifuge tubes were dried and the pellets were dissolved in 20 µl of nuclease-free water. The concentration of DNA was measured in BioSpectrometer (Eppendorf Pvt. Ltd., Germany). If the concentration of DNA was >30 ng/µl, the samples were considered for PCR amplification. For identification of the isolate, 16S rRNA gene was amplified using universal primers 27s Forward primer (5'-AGAGTTTGTATCTGGCTCAG-3') and 1492R Reverse primer (5'-GGTTACCTTGTACGACTT-3') [16]. Each single reaction mixture (10 µl) contained 1 µl of template DNA, 1 µl of each primer (20 pM), 5 µl of Master Mix 2X (HiMedia Laboratory Pvt. Ltd., India) and 2 µl of nuclease-free water. The PCR reactions were run under the following conditions: initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 30 sec, 47 °C for 30 sec, 72 °C for 1min, and final extension at 72 °C for 3 min in S1100 thermal cycler (BioRad, USA). The PCR products were visualized in 1% agarose gel and image was taken by Gel DOC (BioRad, USA).

III. RESULTS

Weissella confusa is a Gram positive, catalase negative coccobacilli which do not form endospore. It is a facultative anaerobic bacterium which is cultivated on MRS agar in aerobic condition. The bacterium has been isolated from pregnant women along with *Klebsiella pneumoniae*. The 16S rRNA gene sequencing and subsequent analysis with bioinformatics tool (BLASTn of NCBI) showed that the bacteria exhibit 100% similarity with *Weissella confusa*. The sequence has been submitted in by BanqIt submission tool of NCBI and accession number (Table 1) was obtained.

In the present experiment, the antimicrobial activity of *Weissella confusa* was studied using pH-adjusted supernatant (Table 2). The activity was tested against pathogenic microorganisms, viz., *Bacillus subtilis* (MTCC 736), *Escherichia coli* (MTCC 9492), *Pseudomonas aeruginosa* (MTCC 3541), *Proteus mirabilis* (MTCC 425), *Proteus vulgaris* (MTCC 771) and *Staphylococcus aureus* (MTCC 3160). It has been found that the highest inhibition zone was produced against *Pseudomonas aeruginosa* (16mm) followed by *Proteus mirabilis* (15mm), *Escherichia coli* (12mm),

Bacillus subtilis (11mm) and *Staphylococcus aureus* (11mm). No antibacterial activity was observed against *Proteus vulgaris*.

The antimicrobial property is due to the production of hydrogen peroxide, bacteriocin-like particles (BLP) and acids. It has been observed that the zone of inhibition is varied when one or more than one inhibitory compound is neutralized as compared to the inhibition size of raw bacteriocin without neutralization of inhibitory substances.

Antibiotic resistance of probiotic microorganism does not pose a safety threat in itself. It is believed that some beneficial microbes, with intrinsic antibiotic resistance, are often successful when both antibiotic and probiotic are used concurrently. This helps in the immediate replenishment of the probiotic flora after the antibiotic treatment. Unfortunately, they may form a reservoir of resistance genes for the potential pathogenic microorganisms and hence may constitute a safety threat. In the present experiment, antibiotic sensitivity test was done using *Lactobacillus fermentum* (positive control). It has been found that the *Lactobacillus fermentum* is sensitive to amoxicillin (AMP), amoxycylav (AMP), chloramphenicol (C), co-trimoxazole (COT), norfloxacin (NX), streptomycin (HLS) and vancomycin (VA). However, the test organism (*Weissella confusa*) is resistant to all antibiotics except ciprofloxacin (CIP) and streptomycin (HLS).

IV. DISCUSSIONS

In the present paper, *Weissella confusa* has been reported for the first time from the vaginal swabs of the North-Eastern region of India. It has been generally agreed that *Weissella confusa* grows in different ecological habitats but it is more commonly found in sugarcane juice, milk, fermented foods and human samples [17]. It has already been stated that *Weissella kimchi* PL9023 is a potential probiotic for women because the species produces high amount of hydrogen peroxide (H₂O₂) amongst most of the LAB and it has been reported that the species inhibits the growth and the adherence of the vaginal isolates of *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus agalactiae* [11]. According to the said study, *Weissella kimchi* did not produce harmful metabolites or enzymes and hence was considered as a potential probiotic for vaginal health. Others have also hinted that *Weissella confusa* strains are excellent probiotic agents and require application studies [18]. In the said study, *Weissella confusa* 20 showed a very high adherence to the caco-2 cells even better than *Lactobacillus rhamnosus* GG. This character would be very useful for vaginal application of this species. It has been reported that there is bacteriocin-like activity (BLA) of *Weissella confusa*, which can control the growth of food-borne pathogens including *Bacillus cereus* [19]. Like previous studies, in the present study, a high degree of antibacterial activity of *Weissella confusa* was observed. Thus, the result obtained in the present experiment is in conformity with the earlier reports published by the different authors.

The isolation of *Weissella confusa* for the first time from the vaginal swabs of Indian women indicates that it is a resident microorganism in human vagina. It may also be pointed out that *Weissella confusa* was earlier isolated from human vagina [5]. This microorganism is considered as a safe probiotic agent, although, there are some clinical cases where *Weissella confusa* was found to be an opportunistic pathogen. Needless to say that the disease caused by this microorganism should be more carefully studied before recognizing it as a probiotic agent [20]. Some authors have reported the occurrence of *Weissella confusa* in association with *Klebsiella pneumonia* [21]. However, they could not explain the significance of occurrence of these two microorganisms together. In the present study too, these two microorganisms were reported from the same volunteers. It is possible that, when human vagina is invaded by the microbes which are not the normal inhabitant of the dynamic ecosystem, the body reaction facilitates the growth of microorganisms which perform the immune protective function.

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TABLE I
ANTIBACTERIAL ACTIVITY OF VAGINAL ISOLATE WHICH EXHIBITED INHIBITION ZONE AGAINST TEST ORGANISMS

Name of the organisms with GENBANK accession number	Test organisms (MTCC)					
	<i>Bacillus subtilis</i> (MTCC 736)	<i>Escherichia coli</i> (MTCC 9492)	<i>Proteus mirabilis</i> (MTCC 425)	<i>Proteus vulgaris</i> (MTCC 771)	<i>Pseudomonas aeruginosa</i> (MTCC 3541)	<i>Staphylococcus aureus</i> (MTCC 3160)
	Inhibition zone in mm (replicates of three)					
<i>Weissella confusa</i> K75 KT361205	11±1	12±1.7	15±1	-	16±2	11±1
Positive control (Antibiotics)	28	30	26	28	27	24

TABLE II
ANTIBIOTICS SUSCEPTIBILITY TEST AGAINST TEST ORGANISM *WEISSELLA CONFUSA* K75 AND *L. FERMENTUM*, A POSITIVE CONTROL

Serial No	Name of the Antibiotics	Quantity in the disc (mcg/disc)	Zone of inhibition (replicates of three)	
			Against <i>W. confusa</i> K75	Against Control (<i>L. fermentum</i>)
1.	Amoxycilin (AMP)	10	-	30±2
2.	Amoxyclav (AMP)	30	-	30±0.6
3.	Chloromphenicol (C)	30	-	10±0.6
4.	Ciprofloxacin (CIP)	5	14±0.6	-
5.	Co-Trimoxzole (COT)	25	-	12±1
6.	Methicillin (MET)	5	-	-
7.	Nalidixic acid (NA)	30	-	-

8.	Norfloxacin (NX)	10	-	10±0.6
9.	Nitrofurantoin (NIT)	300	-	-
10.	Streptomycin (HLS)	300	10±1.5	15±1.5
11.	Tetracyclin (TET)	30	-	-
12.	Vancomycin (VA)	30	-	15±0.6