

## Isolation and Molecular Characterization of Exo-Polysaccharide Producing *Weissella Confusa* from Buffalo Ruminal Gut

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### Abstract

Lactobacilli are the most important colonizers of the ruminant's intestinal tract and are an important source of probiotics. Among Lactic acid bacteria, Exopolysaccharide producing *Weissella confusa* has gained considerable interest due to its importance in the food industry and several health attributes. The present analysis was carried out to isolate and characterize exopolysaccharide producing *W. confusa* from buffalo ruminal gut. The initial characterization of the isolate was done morphologically and biochemically. Molecular detection of the isolate was done by PCR based amplification of 16s rRNA gene. Temperature, pH, incubation-time and media conditions were optimized for the enzyme assay. A total of 35 LAB isolates were cultured for exopolysaccharide production on MRS agar having ruthenium red dye. The pure isolates were also screened for proteolytic, amylase, lipolytic, and antibiotic susceptibility testing. Two Catalase and Oxidase negative strains were selected which shows their probiotic potential.. The two strains identified as *W.confusa* (by GenBank accession number MK128505, Mk212944), which was based on 99% nucleotide homology and phylogenetic analysis. Isolates produced amylase, protease, lipase and also displayed a high resistance range against selected antibiotics. These isolates also showed antimicrobial activity against pathogens *Listeria monocytogenes* as well as against *Staphylococcus aureus*. The isolates also produce EPS when grown on ruthenium red MRS agar. The temperature 30°C-37°C and pH 4 were found optimum for enzyme assay. This study unravelled the capability and safety of EPS producing *W. confusa* for industrial use, and other potential applications.

**Key words:** Isolation; Molecular identification; Exopolysaccharides; *Weissella confusa*

### Introduction

Buffalos, whose scientific name is "Bubalus bublis," are the biggest nutritionally and commercially valuable animals in the Bovidae family. The overall buffalo population in the world is estimated to be around 150 million. They are employed not just for flesh but also for dairy production due to their

high mass, huge physique, and ease of growth. Kundi, Nili Ravi and Azakheli buffalo are the most common buffalo species in Pakistan (1). Franzolin and Wright (2016) suggested that the rumen may contain 300–400 different species of bacteria, while contemporary techniques such as 16s r RNA gene sequence analysis and real-time polymerase chain reaction (PCR) revealed that up to 2000 species may exist. While some microorganisms are pathogens that cause infection, other microbes, known as “Probiotics”, are beneficial to the host and help it fight pathogens (2, 3). *Escherichia*, *bifidobacterium*, *Propionibacterium*, *Enterococcus*, *Weissella Spp* and *Bacillus* are examples of bacterial probiotics, however the most frequent and effective probiotics are Lactic acid bacteria (LAB) (4). Different microorganisms have the ability to produce exopolysaccharides (EPS). exopolysaccharides is made of long polysaccharides chain (5). They may be papered within the cell or in secretary form (6). More research is being done on EPS, which have a role in the flavor, texture, perception and mouth feel of fermented foods (7). *Weissella* genus has a distinctive phenotypic trait that allows it to produce dextran. As a result, certain strains of *Weissella*, such as *W. confusa* and *W. cibaria*, have attracted researchers' interest due to their great potential for producing dextran, as well as heteropolysaccharides, new non-digestible oligosaccharides, and fractions (8). These are the exopolysaccharides that are the most significant. It also receives attention because of its prebiotics potentials, which include the ability to improve metabolism and bowel function, decrease the risk of illness and diarrhoea, and promote the unique gut microbiota, which includes the *Bifidobacteria* (9). Prebiotic oligosaccharides are mostly utilized at the industrial level in the cosmetics, food, and feed sectors, as well as in the clinical setting and as dietary fibre (10, 11). Dextran and other similar oligosaccharides produced by *W. confusa* and *W. cibaria* are utilised in therapeutic settings, such as animal therapies, to enhance and activate the gut microbial flora and decrease illness risk (11, 12).

Galle and Arendt reported in 2014 that EPS may be utilized as a replacement for polysaccharides derived from plants, which are often employed in the food sector as gelling, stabilizing, thickening and texturizing agents (5). Food-related functions of EPS are important, although they are also concerned with general health issues, such as the prevention of cancer and ulcers, activation of the immune system, and reduction of cholesterol (13). EPS is mostly made by LAB, which provides a possible method for replacing hydrocolloid additives (14). The present study was piloted to isolate and characterize exopolysaccharide producing *W. confusa* from buffalo ruminal gut.

### Materials and methods

The present research was carried out at the National Agriculture Research Center (NARC) in Islamabad, Pakistan. From three distinct sites in ChataBakhtwar, Bharakhao, and Rawalpindi, rumen samples were obtained from 35 different species of younger and healthier Nili Ravi, Kundi and Azakheli buffalos. Standard microbiological procedures were followed to transfer the samples using well-labeled, clean, and sterilized Polythene bags or sterilized flasks. Before processing, the samples were transported to the NIGAB Laboratory and kept in the refrigerator to avoid external contaminants (15). The bacteria were isolated using the De Man, Rogosa, and Sharpe agar (MRS) medium. Gram staining and standard biochemical assays were used to confirm the identification of bacterial isolates. The molecular characterisation of isolated bacterial strains was conducted out using 16S rRNA gene sequencing. To do this, the isolates were first cultivated on MRS solid medium plates and then incubated at 37°C for 24 hours. A new colony was chosen with the aid of a

sterilised toothpick. Colonies were selected and immersed in a 20L TE buffer solution before being stirred. In a PCR strip tube, a 0.2(ml) suspension was produced. The PCR tube strips were put in a water bath at 95°C for 10 minutes to extract the DNA from the isolates. After that, the supernatant was obtained by centrifugation at 13000 rpm for 2-3 minutes. The DNA of the isolated bacterial templates was found in the supernatant. The pallet was dumped after centrifugation. The 16s rRNA gene was amplified using the template DNA that was acquired. The supernatant, which included 1µl of bacterial cell template DNA, was used to achieve the required Gene amplification.

Forward primer: (5'AGAGTTTGATCMTGGCTCAG-3').

Reverse Primer: (5'ACCTTGTTACGACTT3').

After that, the template DNA (1µl) and master mix (49 µl) were put in PCR tube and the reaction volume reached to 50 µl (16). For 1-2 minutes, the sample was centrifuged lightly. The PCR strips were maintained in the thermocycler for 16s rRNA Gene amplification before the reaction was begun and condition were set according to previous study (17). The amplified DNA was run on gel electrophoresis and the bands were observed through the Gel Doc System (17). PCR products from isolated isolates were sent to Macrogen (Korea) for 16S rRNA gene sequencing. With the use of the online NCBI BLAST, the acquired sequence was compared to the nucleotide database of 16S rRNA gene sequences. The top BLAST hits' phylogenetic tree was then chosen to create a phylogenetic tree. The MEGA7 bioinformatics program was used to measure evolutionary relationships and build a phylogenetic tree. The EPS production ability of isolated strains was tested similar to previous study (18). All the data was analyzed statistically by using SPSS version 23. A p value of less than 0.05 was taken as significant.

## Results

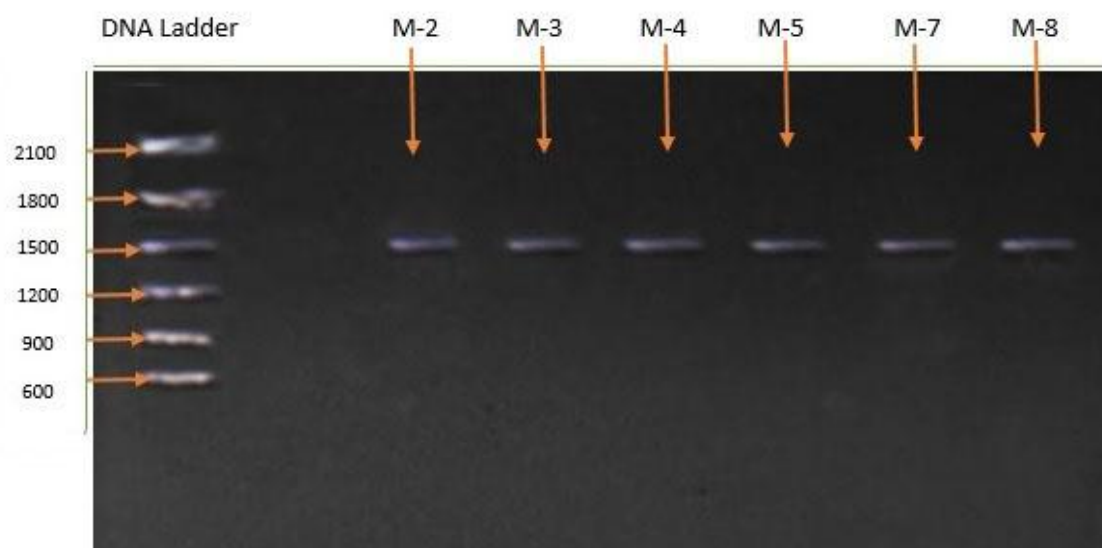
In this research, 35 buffalo rumen samples were obtained from various areas of Islamabad, namely Bharakhao, Chatabakhtwar and Rawalpindi. In the microbial biotechnology lab, the specimens were processed and analyzed. MRS (De Man, Rogosa, and Sharpe agar) agar was used to culture all of the samples. Pure growth was shown on this media by 24 isolates. For identification, the isolates were further examined by gram staining and biochemical tests (Table 1)

**Table 1: Gram staining and biochemical characteristics of isolates**

Strain ID	Gram staining	Morphology	Oxidase test	Catalase test
NMCC-M2	Gram positive	Rods	-ve	-ve
NMCC-M3	Gram positive	Rods	-ve	-ve
NMCC-M4	Gram positive	Cocci	-ve	-ve
NMCC-M5	Gram positive	Rods	-ve	-ve
NMCC-M7	Gram positive	Cocci	-ve	-ve

NMCC-M8	Gram positive	Rods	-ve	-ve
fM-13	Gram positive	Rods	-ve	-ve
fM-14	Gram positive	Rods	-ve	-ve
fM-20	Gram positive	Short rods	-ve	-ve
MF-9	Gram positive	cocci	-ve	-ve
FI-10	Gram positive	Rods	-ve	-ve

The DNA was extracted using a kit that was readily available (FavorPrep cat No-FAVNK001-2). Using the primers described in the previous section, the 16s rRNA gene was amplified. Figure 1 shows the Gel Doc system used to analyse the amplified DNA sequence. A band size of 1465-1522bp was used to confirm 16s RNA.

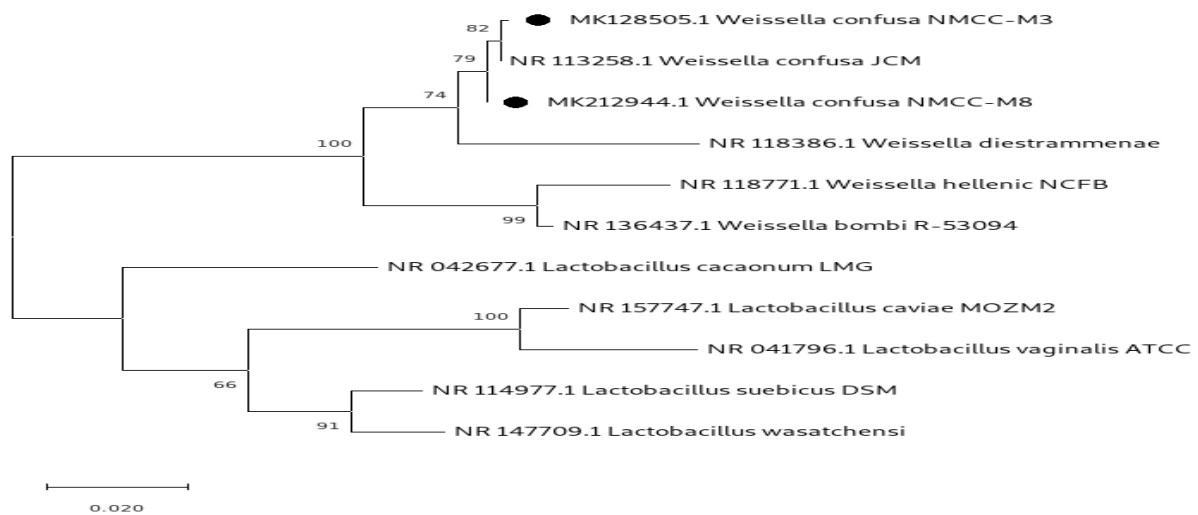


**Figure 1: Agarose gel and 16s rRNA amplified bands of isolated strains:**

The image above illustrates the amplified sequence of the 16s rRNA gene from isolated strains on an agarose gel. The 16s rRNA gene length in *W. confusa* strains with accession number (Mk128505) is 1465 base pairs (bp) in M3, whereas the 16s rRNA gene length in strains with accession number (Mk212944) is 1470 base pairs (bp). The M line depicts a DNA ladder with various sizes ranging from 600 to 2100 base pairs. The size of the isolated strains corresponded to 1500bp DNA bands. In the provided image M3 indicate lab ID (NMCC-M3) whereas M8 indicates lab ID (NMCC-M8) (NMCC-M8).

The data was analysed for phylogeny once it was sequenced and trimmed. Only NMCC-M3 and NMCC-M8 (BLAST, NCBI) showed 99 percent identity with the available sequence of *W. confusa* JCM strain accession number NR113258.1 in a sequence similarity search for all identified strains. ClustalW of MEGA7 was used to do the phylogenetic analysis, and maximum-likelihood was used as the statistical metric. The fact that both strains have a bootstrap value more than 50 indicates that

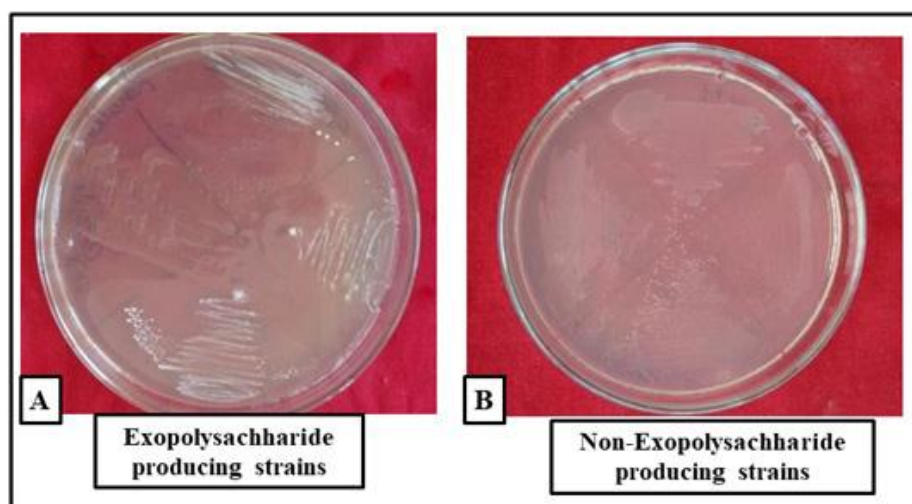
they are fully accurate and very similar to the *W. confusa* JCM strain. Figure 2 shows a phylogenetic study of *W. confusa* in relation to other LAB using the maximum-likelihood technique.



**Figure 2: Phylogenetic tree :** Based on examination of the 16S rRNA sequence, a maximum likelihood tree with 50 bootstrap values in MEGA7 shows the phylogenetic relationship of *W. confusa*. The tree was constructed through MEGA-7 by using neighbour-joining method (16).

### EPS Producing Activity

On the MRS medium with a particular indication, the suspicious LAB isolates were qualitatively tested (ruthenium red dye). Ruthenium red dye is cationic, meaning it attaches to ionic sites in the bacterial cell membrane, giving it a red colour. EPS-producing bacteria, on the other hand, do not interact with ruthenium red dye, which results in a white colony on screening medium. In the case of both isolates chosen, white colonies of isolates were found, indicating favourable findings as shown in figure 3.



**Figure 0: EPS production by the selected isolates**

## Discussion

Because of its probiotic potential and many industrial uses, LAB strains capable of generating EPS have been intensively researched. These EPS-producing LABS may be found in milk, dairy products, and the rumen gut of ruminants. The present research isolated and identified *W. confusa* that produces EPS from the ruminal gut of buffalo in order to evaluate their potential of exopolysaccharide production. Thirty five samples of buffalo faeces were collected from various locations throughout Islamabad, Pakistan. MRS medium was used to isolate lactic acid bacteria anaerobically. Colony morphology was creamy white and silky. The isolates (NMCC-M3 and NMCC-M8) were classified as round or punctiform. A previous study reported similar results (19). Gram staining was used to identify the LAB isolates morphologically. They showed purple rods under a light microscope following Gram staining, which is a hallmark of Gram-positive bacilli. Only 19 isolates were recognised as Gram-positive rods in our research. In comparison to our study same results were reported in another study done by Jose, Bunt (20). The isolates were chosen for further investigation based on biochemical analyses. Only 14 Gram-positive isolates were catalase-negative, with only 11 showing oxidase negativity among the 19 Gram-positive isolates. Barakat et al., for instance, found similar findings (21). Different settings were adjusted for the experiment in order to ensure that LAB's activity was as efficient as possible. At 30°C and 37°C, the LAB strains grew the fastest. At 45°C, though, no growth was seen. Same findings were reported by another previous study done by Lee, Park (22). LAB's probiotic capacity to survive within the gastrointestinal system is shown by their growth at 37°C. The strains were also unable to generate bacteriocins, among other things. *W. confusa* strains had previously been shown to be largely resistant to high salt concentrations, according to the authors. The strain's unique feature of acid tolerance and survival at low pH allows it to demonstrate its probiotic potential as well as its capacity to live in unfavourable circumstances (23). The strains in our research exhibited the best survivability at pH 2 for 2-3 hours. Montoro, Benomar (24) reported that *W. confusa* was shown to have similar survival behaviour at pH 2 without decreasing viability. H<sup>+</sup>-ATPase activity determines a strain's capacity to withstand high acid concentrations. Only two isolates were chosen for this study and were tested for EPS activity. On the MRS medium with ruthenium red dye as an indicator, these two isolates were then tested qualitatively for EPS production. Osmotic stress, biofilm and hazardous chemicals are all inhibited by exopolysaccharide. They are also suitable for usage in the textile, culinary, pharmaceuticals, and chemical industries. Both isolates formed white colonies on the medium, indicating that the EPS test was positive (25). The identification of LAB species is based on their physiological and biochemical properties, according to the literature. Furthermore, since 16s rRNA is the most conserved segment across various LAB strains, it is used for phylogenetic analysis. Exopolysaccharide-producing LAB was isolated from the ruminal gut of buffalo and genotypically identified using PCR-based amplification of the 16s rRNA fragment. Sequencing was used to improve the strain's characterisation. After comparing the sequence to other sequences in the Genbank database, it was determined that it was 99 percent identical to the *W. confusa* JCM strain accession number NR113258.1. Other researchers have also reported similar findings (26).

## Conclusion

Our study concludes that isolates of *W. confusa* from the ruminal gut of buffaloes showed unique exopolysaccharide production activities.

Coming scientific research is attempt on probiotics bacteria to explore novel and particular bacterial strains for the welfare of the different host such as animals and human. So, the future improvement and scientific research tendency will be.

- More features of *W. confusa* will need to be determined before such strains may be used in the industrial zone. Apart from that, the strains' safety must also be assessed.
- Further processed the isolated strains for whole genome sequencing.

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