



## Original Research Article

# Assessment of Novel Strains of Intestinal Lactic Acid Bacteria (LAB) Isolated from the Infant Faeces as Potential Use of Probiotics

Sangeeta Huidrom<sup>1\*</sup>, Manab Deka<sup>2</sup>, RK Singh<sup>1</sup> and Narotam Sharma<sup>1</sup>

<sup>1</sup>Central Molecular Research Laboratory, Department of Biochemistry, Shri Guru Ram Rai Institute of Medical and Health Sciences, Dehradun – 248001, India

<sup>2</sup>Department of Biological Sciences, Gauhati University, Guwahati - 781014, India

\*Corresponding author

## A B S T R A C T

### Keywords

Infant faeces, *in vivo* colonization, probiotics, 16S rDNA, *Weissella paramesenteroides*

Probiotic properties of lactic acid bacteria (LAB) isolated from the faecal samples of infant were examined for their ability to grow at low pH, tolerance to different concentrations of bile salts, antimicrobial properties and the best four from the *in vitro* test were selected for the *in vivo* colonisation studies. In the *in vivo* test, the isolates were fed to mice at the concentration of  $10^{10}$  cfu/ml and faecal samples of mice were collected at 0, 3, 7 days. *In vivo* results showed that *Leuconostoc paramesenteroid* ST9 was able to colonize the intestine of the mouse. Among the tested strains, *Leuconostoc paramesenteroid* ST9 was found to be a novel probiotic as it fulfil all the basic requirements of probiotics. The strain was confirmed by using 16S rDNA and identified as *Weissella paramesenteroides* strain FMA204. This new probiotic strain would act as a promising probiotic supplement for the human consumption.

## Introduction

In 400 B.C., Hippocrates have been quoted as saying “death sits in the bowels” and “bad digestion is the root of all evil” (Hawrelak and Myers, 2004). This quotation showed that the role played by the intestine for a healthy life has been recognized long back. The good bacteria present in the gut are linked with the wellbeing of an individual. Probiotic is a relatively new term meaning ‘for life’ and is associated with those microorganisms which are beneficial for maintaining good health in human and animals. Probiotics have been recently received special attention due to its application as alternative medicine.

Although known since a long time, only in the last two decades probiotics have started to receive major attention from researchers and several studies have been carried out on the effects of probiotics microorganisms, using different formulae and with numerous purposes of preventing or treating diseases (Mercenier *et al.*, 2002; Sartor, 2005). There is an increasing interest in the development of adjunct or alternative therapies based on bacterial replacement using probiotics isolated from the natural intestinal flora (Forestier *et al.*, 2001; Collado *et al.*, 2007).

Probiotics can be defined as “living

microorganism which when administered in adequate amount gives health benefits to the host by improving its intestinal microbial balance". Several microorganisms under the name "probiotics" have been proposed and used in a wide range of clinical trials, ranging from diarrhoeal disease to cancer prevention (Fuller, 1994; Kaur *et al.*, 2001). The therapeutic benefits of probiotics reported include treatments of conditions including gastrointestinal disorders, hypercholesterolemia, lactose intolerance, suppression of procarcinogenic enzyme, immunomodulation and treatment of food-related allergies (Begley, 2005; Ouwehand *et al.*, 2003; Rodriguez *et al.*, 2010).

LAB are commonly used as a probiotics as they are considered as "generally recognized as safe" (GRAS) organisms and its history of beneficial health effect. However the probiotic potential of different bacterial strains even within the same species differs. Different strains of the same species are always unique, benefits may only pertain to particular strains and cannot be extrapolated to other strains. Therefore it is necessary to characterise the strain for its probiotic attributes.

A number of requirements have been identified for strains to be an effective probiotic microorganism. The strain should be of human origin for human application, the microbes must survive through the gastrointestinal tract that is they should survive both the stomach and bile acid and should have antimicrobial activities against common pathogen. It should adhere to the mucosal surface and they should show some health benefit to the host. Accurate identification of probiotics is an essential task. Conventional method of identification is difficult and not reliable for the species confirmation. Molecular technique based on 16S rDNA has been extensively used for bacterial phylogeny (Woese *et al.*, 1990) for

reasons including its universal distribution among bacteria (Weisburg *et al.*, 1991). The aim of the present research work is to evaluate the probiotic attributes of lactic acid bacteria from the faecal sample of infant to search for a novel probiotic of human origin.

## **Materials and Methods**

### **Bacterial strains**

A total of 70 LAB was isolated from the healthy infant faeces below 2 years old in MRS medium (Himedia M641, India). All the isolates were identified by phenotypic methods. The isolates were preserved in the MRS broth supplemented with 15% (v/v) glycerol and kept at -20°C. The strains were activated prior to assay by subculturing three times in MRS broth (Himedia M369, India). Pathogenic *E. coli* was obtained from the Department of Biotechnology, Gauhati University, India. *E. coli* was inoculated in nutrient broth.

### **Tolerance to Inhibitory Substances**

Tolerance to varying concentration of pH and bile were evaluated. Overnight culture of the isolates were incubated in MRS broth adjusted to pH (2.5 and 5) and bile [0.05%, 0.5% and 1% (w/v)] and incubated for 24 hour and growth of the cultures were monitored spectrophotometrically at 600 nm.

### **Antimicrobial profile**

Antimicrobial activities were assayed by using blank disc methods against the indicator strain, *E. coli*. The blank discs dipped in the producer strain were kept on the agar plates of indicator strains. Then the plates were kept at 4°C for diffusion and incubated at 37°C for 24 hour and zone of inhibition were measured in millimeter.

### **Antibiotic Resistance test**

Eight antibiotic discs tetracycline (30µg), norfloxacin (10µg), ampicillin (10µg), kanamycin (30µg), penicillin G (10 units), nalidixic acid (30µg), gentamicin (120µg), vancomycin (30µg) were used to determine the antibiotic resistance of the isolates by disk diffusion method.

### ***In vivo* colonisation test**

*Animals:* Three months old Swiss albino mice were used after quarantine periods of one month. The pathogen-free animals were housed individually in cages. A total of 6 mice were used. Mice were housed individually per cage and a cycle of 12 hour of light and 12 hour of dark were maintained under controlled room temperature. All the animals were fed with conventional diet *ad libitum* and water was available for 24 hours. The cages were changed twice a week.

*Bacterial isolates for in vivo assay:* The isolates which had desirable *in vitro* properties were selected for further *in vivo* test. The bacterial isolates *E. faecium* SC10, *L. acidophilus* SH10, *L. acidophilus* SN2 and *Leuconostoc paramesenteriod* ST9 were used for feeding the mice.

### **Antibiotic marking of the bacterial isolates**

The isolates were grown in MRS broth at 37°C overnight. The cultures were added to MRS agar with 100µg/ml rifampicin (Hi Media Laboratories Pvt. Ltd., Mumbai) and incubated for 1-2 days at 37 °C (Frece *et al.*, 2005). The resistant colonies were picked up and inoculated into the MRS broth and kept at 37 °C for 24 hours. This selectable trait would enable the strain to be readily enumerated from faecal sample on MRS agar supplemented with rifampicin. Cells

were harvested by centrifugation at 10,000g for 5 minutes, supernatant were discarded and pellets were washed with 0.85% NaCl and resuspended in 0.85% NaCl at concentration of 10<sup>10</sup> cfu per ml

*Intragastric administration of bacterial isolates to mice:* Healthy mice received the bacterial isolates at a daily dose of 10<sup>10</sup> cfu/ml with blunt-ended needle (1ml) with rubber tubing for 7 days. Control mice had received 0.5 ml of sterile 0.85% NaCl orally with blunt-ended needle and were maintained under identical conditions as the test groups. Faecal samples were collected from each mouse in a sterile jar for different days during the feeding periods and day one after the feeding periods and analysis took place during 24 hours of sample collection. The experiments were repeated three times for each strain.

### **Identification of probiotic strains by 16S rDNA sequencing molecular identification**

Genomic DNA was isolated from the selected isolates by using DNA Extraction Solution, Cat No. 612104680501730. The ~1.5 kb 16S rDNA fragment was amplified by using the consensus primers and *Taq* DNA polymerase. The reaction mixture of the PCR consists of 20ng of Genomic DNA, 1.0µl of dNTP mix (2.5mM each), 100ng of forward and reverse primer, 1×Taq Buffer A (10X) and 3 U *Taq* DNA polymerase enzyme. Then glass distilled water was added to make 50µl. PCR reactions were performed with the following program: 35 cycles which consist of predenaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 2 min which is followed by a final extension of 10 min at 72°C. Then amplified PCR products were loaded on 1.0% agarose gel along with StepUp TM 500bp DNA ladder. The

amplified PCR product was purified and sequenced using the forward and reverse primer.

### Sequence analysis

Sequence data which was obtained was aligned by using the National Center for Biotechnology Information (NCBI) GenBank and RDP database and sequences were analyzed for finding the closest homologous microbes by using nucleotides homology and phylogenetic tree.

### Statistical analysis

Results were expressed as mean  $\pm$ SD of triplicates for each sample. The data was analyzed using one way analysis of variance (ANOVA) of the SPSS v.21 (Statistical Package for Social Science Inc. Chicago, IL, USA). The *p* value less than 0.05 were regarded as significant difference between means using Tukey's Honestly Significance Difference for multiple pair-wise comparisons.

## Results and Discussion

### Tolerance to low pH and bile

*In vitro* studies only partially mimic the gut ecosystem but act as a useful screening tool for the selection of LAB for further *in vivo* testing. As reported in some previous cases, none of the LAB strains grew at pH 2.5 and only very few were reasonably acid tolerant (Jacobsen *et al.*, 1999). As compared to earlier reports, our findings were interesting as the isolates were able to grow at pH 2.5 after 24 hour of incubation. All the isolates were able to grow at pH5 and the isolates SW8, ST7, SH10, SW5, SH7, ST10, SC4, SW9, SA1, SH9, SC10, SH1, ST9, SN8, SN7, SE, ST4 and S16 were able to grow at pH 2.5.

Bile tolerance is considered one of the essential properties required for lactic acid bacteria to survive in the small intestine (Ibrahim and Benzkorovainy, 1993). According to Pancheniak and Soccol (2005), isolates that showed 0.3% tolerance to bile could be used as probiotic for swine. All the isolates grew at 0.05% bile. The isolates ST8, SW8, SH10, SC10, ST9, SW5, SA1, SW9, S16, SN8, SH9, SH, ST6, SN2, SC4 and SH2 were the most resistant isolates at 0.5% and 1% bile.

### Antimicrobial Activity

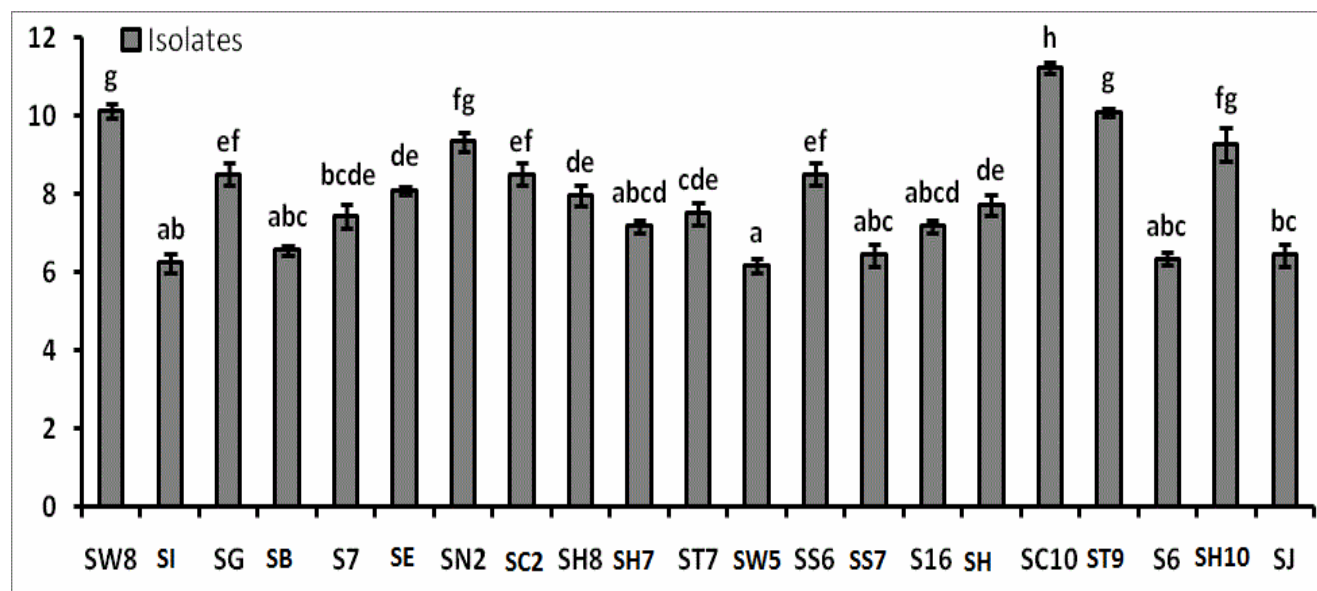
In order to have an impact on the colonic flora it is important for probiotic strains to show antagonism against pathogenic bacteria via antimicrobial substance production (Saarela *et al.*, 2000). The isolates SN2, SW8, SC10, ST9 and SH10 showed the highest zone of inhibition against pathogenic *E. coli* (Fig.1).

### *In vivo* colonization

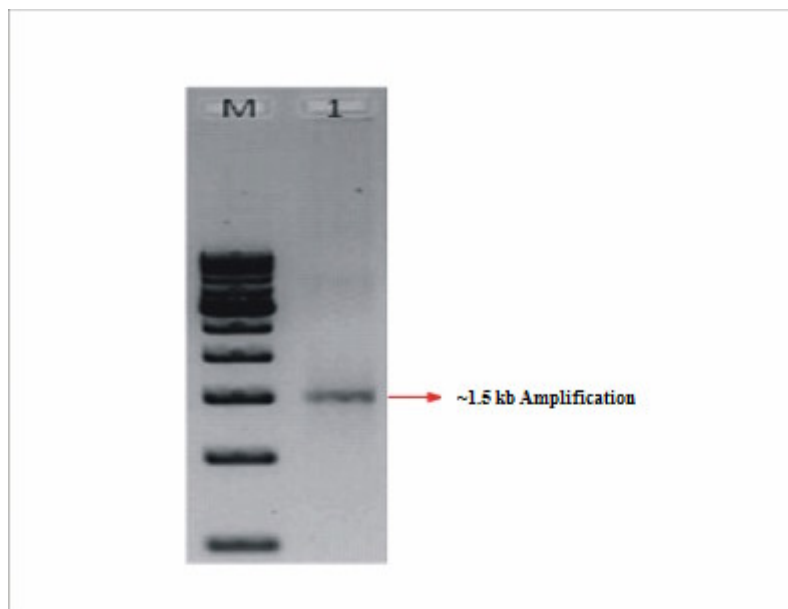
The adhesion of the bacteria to intestinal epithelium represents the first step in the colonization process (Tuomola *et al.*, 2001), and adhesion characteristics are important when selecting probiotics. Lactic acid bacteria can preferentially occupy a space or form a biofilm on the surface of intestinal lining that would otherwise be colonized by a pathogen. Thus LAB induces a competitive environment. Irrespective of the route of administration, the *L. plantarum* strains were detected for longer periods than was *L. salivarius* UCC118 in the feces of mice (Pavan *et al.*, 2003). In our *in vivo* colonisation study *Leuconostoc paramesenteroid* ST9 was able to transiently persist in the GIT of the mouse since they were detected in the faecal sample of mice during the feeding periods and after the

feeding was stop as tabulated (Table 1).

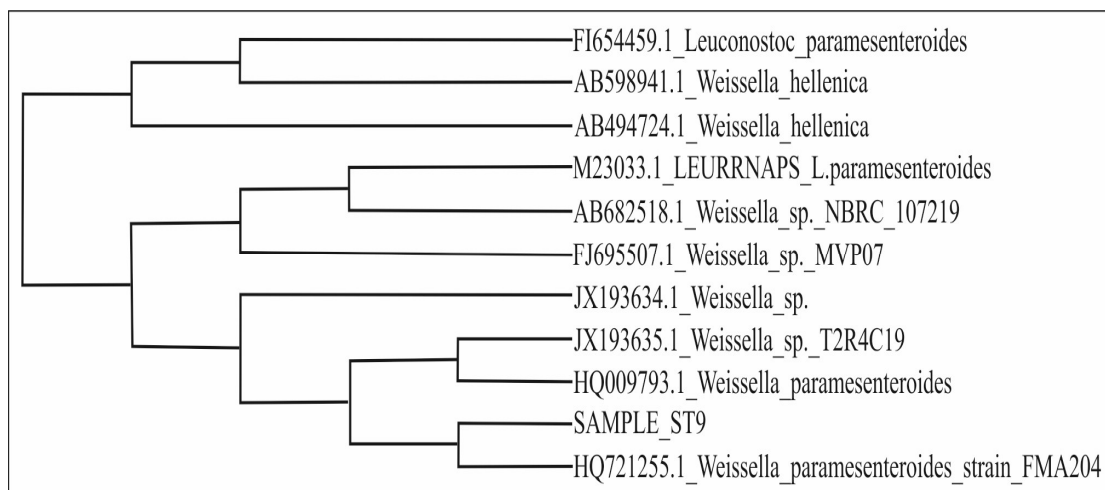
**Figure.1** Zone of inhibition of the isolates against *E. coli*. Mean values (columns) with different letters indicate statistically significant difference at  $P < 0.05$ , as determined by one-way ANOVA incorporating Tukey's Honestly Significance Difference for multiple pair-wise comparisons. Bars (column) represent the average of 3 replicate measurements of three independent experiment.



**Figure.2** The PCR product of 16S rDNA gene on 1% agarose gel. Lane 1: PCR amplification of isolates ST9, Lane M: StepUp™ 500bp DNA ladder (Cat. # 612651970501730).



**Figure.3** Phylogenetic tree of the sample ST9 based on the 16S rDNA analysis constructed using neighbour joining method



**Table.1** Bacterial count in faeces of mice at four sampling time












Days	Log c.f.u/g of faeces				
	Control	ST9	SH10	SC10	SC2
0	N.D.	N.D.	N.D.	N.D.	N.D.
3	N.D.	6.61±0.12	N.D.	N.D.	N.D.
7	N.D.	6.31±0.14	5.77±0.83	N.D.	N.D.
8	N.D.	6.32±0.80	N.D.	N.D.	N.D.

**Table.2** Aligned Sequence Data of the sample ST9 (1403 bp)

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CTTTAATTGATCTGACGAGCTTGCTCTGATTTGATTTTATCTGACAAAAGAGTGGCGAACGGGTGAGTAACACGTGGGTAAC
CTACCTCTTAGCAGGGGATAACATTTGGAAACAAGTGCTAATACCGTATAATACCAACAACCGCATGGTTGTTGGTTGAAA
GATGGTTCTGCTATCACTAAGAGATGGACCCGCGGTGCATTAGCTAGTTGGTAAGGTAACGGCTTACCAAGGCAATGATGC
ATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCATACTCCTACGGGAGGCAGCAGTAGGGGAATC
TTCCACAATGGGCGCAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGTGTTCGGCTCGTAAAACACTGTTATAAGAG
AAGAACGGCACTCAGAGTAACGTTCAGTGTGTGACGGTATCTTACCAGAAAAGGAACGGCTAAAATACGTGCCAGCAGCCGC
GGTAATACGTATGTTCCAAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAA
GCCCTCAGCTCAACTGAGGAATGGCTTTGGAACTGGATGACTTGAGTGCAGTAGAGGAAAGTGGAACTCCATGTGTAGCG
GTGAAATGCGTAGATATATGGAAGAACCAGTGGCGAAGGCGGCTTCTGGACTGTAAGTACGTTGAGGCTCGAAAGTG
TGGGTAGCAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGATGTTTCGAGGGTTTCCGCCCTTG
AGTGTGCGCAGCTAACGCATTAAGCACTCCGCCCTGGGGAGTACGACCCGAAGGTTGAAACTCAAAGGAATTGACGGGGACCC
GCACAAGCGGTGGAGCATGTGGTTAATTGGAAGCAACGCCGAAGAACCTTACCAGGCTTACATCCCTTGCTAATCCTAG
AAATAGGACGTTCCCTTCGGGACAAGGTGACAGGTGGTGCATGGTTGTCGTGAGTGTGCTGAGTGTGCTGAGTGTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTATTATTAGTTGCCAGCATTGATGGGCACTCTAGTGAAGTGTGAGGCTGCGGTTGAGGTTAAG
GGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACCTGGGCTACACACGTGCTACAATGGCATATACAACGAGTC
GCCAACCCGCGAGGGTGGGCTAATCTCTTAAAGTATGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGG
AATCGCTAGTAATCGCGGATCAGCAGCCGCGGTGAATACGTTCCCGGCTTGTACACACCGCCCGTCCACACCATGAGAG
TTTGTAAACACCCAAAGCCGGTGGGCT
    
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**Table.3** Alignment view using combination of NCBI GenBank and RDP database (Sample ST9)

Alignment View	ID	Alignment results	Sequence Description
	<a href="#">SAMPLE ST9</a>	1	Studied sample
	<a href="#">HQ721255.1</a>	0.99	<i>Weissella paramesenteroides</i> strain FMA204
	<a href="#">HQ009793.1</a>	0.99	<i>Weissella paramesenteroides</i>
	<a href="#">JX193634.1</a>	0.99	<i>Weissella</i> sp.
	<a href="#">JX193635.1</a>	0.99	<i>Weissella</i> sp. T2R4C19
	<a href="#">FJ695507.1</a>	0.99	<i>Weissella</i> sp. MVP07
	<a href="#">AB682518.1</a>	0.99	<i>Weissella</i> sp. NBRC 107219
	<a href="#">M23033.1</a>	0.98	<i>LEURRNAPS L.paramesenteroides</i>
	<a href="#">AB598941.1</a>	0.98	<i>Weissella hellenica</i>
	<a href="#">AB494724.1</a>	0.98	<i>Weissella hellenica</i>
	<a href="#">FJ654459.1</a>	0.98	<i>Leuconostoc paramesenteroides</i>

### Genotypic identification

Accurate identification of bacterial isolates is an essential task for the bacterial strain to be used as probiotics. Conventional methods of identification such as colony morphology, fermentation patterns, serotyping or some combination of these has limitation as it is not efficient to classify the bacterial isolates taxonomically as it is difficult and time-consuming, and when phenotypic methods are used to identify bacteria, interpretation of test results can involve a substantial amount of subjective judgement. Phylogenetic analysis based on comparisons of 16S ribosomal DNA (rDNA) sequence data is now routinely used in the determination of taxonomic relationships between microorganisms and in the design of taxon-specific probes for use in microbial identification and molecular ecology studies (Amman *et al.*, 1993; Ludwig and Schleifer, 1994; Olsen *et al.*, 1994; Ward *et al.*, 1995). Molecular identification was done to

confirm the identity of the ST9 strain by 16S-rDNA gene analysis. The ~1.5 kb 16S rDNA fragment of the isolates (Fig.2) was amplified by using consensus primers and *Taq* DNA polymerase. The generated PCR product was sequenced using the forward and reverse primer and the sequence data was aligned, the sequence data of the sample ST9 is shown in Table 2. After sequence alignment, the 16S rDNA sequences of the sample ST9 was compared to 16S rDNA sequence of other bacteria available in the Gen Bank of National Center for Biotechnology Information (NCBI) by BLAST software and RDP database as shown in Table 3.

Phylogenetic tree was constructed by using the neighbour joining method in order to find the closest homologous microbes as shown in figure 3. Based on nucleotides homology and phylogenetic analysis the isolates ST9 showed sequence homology with *Weissella paramesenteroides* strain

FMA204 (Gen Bank Accession Number: HQ721255.1). Nearest homolog of ST9 sample was found to be *Weissella paramesenteroides* (Gene Bank Accession Number: HQ009793.1) as shown in Table 3. The other close homologs which were 0.99 alignment were *Weissella sp.* (GeneBank Accession Number: JX193634.1), *Weissella sp.* T2R4C19 (GeneBank Accession Number: JX193635.1), *Weissella sp.* MVP07. (Gene Bank Accession Number: FJ695507.1), *Weissella sp.* NBRC 107219 (Gene Bank Accession Number: AB682518).

In conclusion, the characterization of the new probiotic strain *Weissella paramesenteroides* ST9 in the present research work would open a new horizon in the field of biotherapy. The result showed that this strain is an ideal probiotic strain as it is able to resist the stress factor of low PH, bile, possess antimicrobial activity and colonized the gastrointestinal tract of the mouse as the isolates were detected in the faeces of the mouse. This novel probiotics would act as a good probiotics supplement for the human consumption. However large numbers of controlled clinical trials and technological properties such as strain stability, viability in products are needed for the commercialization of these probiotics.

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