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STUDIES ON SAFETY AND SHELF STABILITY OF BACILLUS PROBIOTICS

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ABSTRACT

Safety of probiotics has always been an area of concern. The present study evaluates the safety aspects of novel probiotic *Bacillus* species as per the guidelines recommended jointly by Indian Council of Medical Research (ICMR) and Department of Biotechnology (DBT). It also includes assessment of viability during storage since the number of viable probiotic bacteria per gram of product or per serving should be mentioned on its label. Fifteen probiotic Bacillus isolates were subjected to the study of haemolytic activity, testing for enterotoxin genes by PCR technique, antibiotic sensitivity tests by Kirby Bauer method, and identification of the isolates by 16S rRNA and MALDI-TOF-MS studies. Five of these cultures were found to be non-haemolytic which is a desirable probiotic characteristic. Three of these acid and bile tolerant isolates were tested for antibiotic sensitivity. They were found to be sensitive to most antibiotics used, thus safe for consumption, in terms of antibiotic resistance transfer to normal flora and pathogens. The three Bacillus isolates tested negative for frequently encountered enterotoxin genes in *Bacillus* species-- nheA, nheB, bal, hblA, entFM and bceT. As per the WHO/FAO joint working committee recommendations, minimum viable numbers of the probiotic strains after six months storage at different temperatures was evaluated using the spread plate method. One of these cultures, identified as *Brevibacillus borstelensis* was found to be the most stable. Its GenBank accession number is MK156349. The culture has been identified to be a non-pathogenic strain. Thus, the novel prospective probiotic bacterium proved to be safe for consumption.

Keywords: MICP; Antibiotic sensitivity, Enterotoxin, MALDI-TOF-MS, Probiotic, 16S rRNA, *Brevibacillus borstelensis*

1. INTRODUCTION

Live microorganisms which are beneficial to the host when taken in adequate quantities are called probiotics [1]. Many probiotics which are marketed claim to have generally recognized as safe status [2]. Most of the probiotics used, belong to the genera *Lactobacillus* and *Bifidobacterium* and have their origins in dairy products. Efficacy and safety of probiotics has always been an area of concern for researchers. It is often asserted that probiotic species should be isolated from the gastrointestinal tract of the animal to which it is targeted to be administered. With an exception of *Saccharomyces boulardii,* which is not a normal resident of the human gastrointestinal tract but is used as a probiotic against colitis caused by *Clostridium difficile*, the use of microbial species of non-intestinal origin as a probiotic seems to be justified [3]. Consumption of large number of viable bacteria that are not normal inhabitants of intestinal tract does raise some safety issues. Among safety aspects of probiotics belonging to *Bacillus* and related genera enterotoxin production, presence of parasporal body, haemolytic activity and antibiotic resistance pattern of the culture are to be studied according to ICMR and DBT guidelines. Effects of a probiotic are strain specific. Hence it is important to identify a prospective probiotic [4].

The WHO/FAO joint working committee recommends that the label on the probiotic product should make a mention of Genus, species and strain, minimum viable numbers of each probiotic strain during its shelf-life, proper storage conditions and corporate contact details for consumer information [4].

Antibiotic susceptibility testing is considered an important part of safety assessment of probiotics. If a probiotic species has acquired resistance to antibiotics, it has a higher potential for transference when the genes are present on mobile genetic elements, such as plasmids and transposons. These mobile elements can be passed on to diverse bacteria and propagate resistance genes into variety of microbial communities [5].

Viability during Storage should be assessed, and the producer should implement procedures for monitoring the number of viable probiotic organisms in the product during storage. Number of viable probiotic bacteria per gram of product/per serving should be mentioned. The methodology to determine the number of viable cells should be given, including type of agar used and incubation parameters [6].

2. MATERIAL AND METHODS

2.1. Study of haemolytic activity

Fifteen *Bacillus* species shortlisted from our earlier study [7] having desirable probiotic characteristics like inhibitory activity against gastrointestinal pathogens and producing extracellular digestive enzymes were spot inoculated on superimposed blood agar plates. (5% citrated human blood was used to prepare the blood agar plates). Haemolytic activity was then observed as colourless, green or no zone around the growth.

2.2. Detection of Enterotoxin genes

Polymerase chain reaction has been reported as used for rapid detection of enterotoxin genes in *Bacillus cereus* [8, 9]. The five non haemolytic *Bacillus species* were screened by PCR for the presence of six enterotoxin gene targets (nheA, nheB, bal, hblA, entFM, bceT). The primers for these target enterotoxin genes, listed in table 1 were procured from Eurofilms Genomics India Pvt. Ltd.

Standard culture of *Bacillus cereus* NCIM 2106 which is an enterotoxin producing strain, positive for nhe and bal genes, was procured from National Collection of Industrial Microorganisms, NCL, Pune, India. Pure cultures of the five prospective probionts, VS-5, PL.2, WBS-1, JS-1 and VS-2 and the standard culture of *Bacillus cereus* NCIM 2106 were cultivated in Luria Bertani medium. DNA Sure tissue mini kit of Genetix brand was used to purify their genomic DNA.

Table 1: Primers for the target enterotoxin genes

Target gene	Primer	Primer sequence	Reference
nheA	NH1F	GCTCTATGAACTAGCAGGAAAC	10
	NH1R	GCTACTTACTTGATCTTCAACG	
nheB	NH2F	CGGTTCATCTGTTGCGACACG	
	NH2R	GATCCCATTGTGTACCATTGG	
bal	BalF	TGCAACTGTATTAGCACAAGC T	11
	BalR	TACCACGAAGTTTGTTCACTACT	
hblA	HblA1	GCTAATGTAGTTTCACCTGTAGCAAC	
	HblA2	AATCATGCCACTGCGTGGACATATAA	
entFM	ENTA-	ATGAAAAAAGTAATTTGCAGG	
	ENTB	TTAGTATGCTTTTGTGTAACC	
bceT	BceT1	GAATTCCTAAACTTGCACCATCTC G	
	BceT2	CTGCGTAATCGTGAATGTAGTCAAT	

2.2.1. Amplification of the targeted enterotoxin genes

PCR Master Mix (2X) from Puregene, Genetix brand was used to amplify the six enterotoxin genes, if present in the test *Bacillus species* as well as in the standard strain, *Bacillus cereus* NCIM 2106, i.e. five test cultures and one standard culture each amplified using primers for six target genes in separate reactions.

PCR Master Mix (2X) contained 0.05U/ml Taq DNA polymerase, reaction buffer, 4mM $MgCl_2$ and 0.4mM of each dNTP. For a total volume of 50µL PCR reaction mixture, 25 µL of PCR Master Mix, 0.5µM each of forward and reverse primers, 0.1 µg template DNA and nuclease free water were mixed. The samples were then gently vortexed. PCR was performed using a Palm cycler. The thermal cycling conditions for amplification included an initial denaturation at 95°C for 5 minutes, 30 cycles of denaturation step at 95°C for 1 minute, annealing at a temperature gradient from 50-60°C for 45 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 5 minutes.

2.2.2. Agarose gel electrophoresis

The PCR products were then analysed by loading 10 µL of the samples on a 1.5% agarose gel with Ethidium bromide and visualized using UV light. A 100 bp DNA ladder was also loaded on each gel.

2.3. Determination of antibiogram

Test was carried out by Kirby Bauer method as per NCCLS standard [12, 13]. Culture density of 0.12 (530 nm) was spread on Mueller Hinton agar plate. After drying the surface of the plate, the antibiotic discs were placed, and plates were incubated at 37°C for 18-20 hours. Zone of inhibition of the cultures were then measured in mm.

2.4. Shelf life and storage study

Isolates were inoculated in 50 ml of sterile sporulation broth [14], incubated on a shaker at 37°C for 1 week. Absence of vegetative cells and presence of spores was confirmed by Gram staining. Tween 80 (0.1%) was added to reduce aggregation of cells and spore pellet was harvested by centrifugation at 3000 rpm for 10 minutes. The saline suspension of the cultures was transferred to tubes to be stored at three temperatures, i.e. 0°C (Freezer), 4-10°C (Refrigerator) and 30-35°C (Room temperature). Viable count of the suspension before storage was determined and recorded as before storage viable count and thereafter at monthly intervals for six months by performing spread plate method using Nutrient agar plates.

2.5. Identification of *Bacillus* species using 16S rRNA sequencing

2.5.1. DNA extraction and quantification

DNA Extraction was carried out using HiPurA Bacterial Genomic DNA Purification Kit (Himedia, MB505). The DNA was stored at -20°C for further use.

2.5.2. PCR amplification

DNA isolated from bacteria was subjected to polymerase chain reaction (PCR) amplification using Biometra thermal cycler. The PCR reaction mix contained 2.5µl of 10X buffer, 1µl of each primer (Table 2), 2.5µl of 2.5mM of each dNTP, 2.5 Units of Taq DNA polymerase and 1µl Template DNA and 8.5µl nuclease free water. The PCR amplification cycle consisted of, a cycle of 5 min at 94°C ; 35 cycles of 1 min at 94°C , 1 min at 50°C , 2 min at 72°C ; and additionally, 1 cycle of 7 min at 72°C . The reagents used were procured from GeNei.

Table 2: Primers used for 16S rRNA region amplification

Primers	Primer Sequence (5'-3')
519F (Forward)	CAGCAGCCGCGGTAATAC
1385R (Reverse)	CGGTGTGTACAAGGCCC

2.5.3. Gel electrophoresis

Agarose Gel electrophoresis was performed (Seakem, 50004L) to analyze the size of amplified PCR product. The size obtained was approx. 850bp for 16S rRNA region.

DNA sequencing**:** The PCR product was purified using Axy Prep PCR Clean up kit (Axy gen, AP-PCR50). It was further sequenced using Applied Biosystems 3730xl DNA Analyzer USA and chromatogram was obtained.

2.5.4. Bioinformatics analysis

The DNA sequences were analyzed using online BLASTn (nucleotide Basic Local Alignment Search Tool) facility of National Center for Biotechnology Information (NCBI). The BLAST results were used to find out evolutionary relationship of bacteria.

2.6. Identification of *Bacillus species* using MALDI-TOF-MS

2.6.1. Preparation of the HCCA matrix solution

250 µl of standard solvent was added to a tube of HCCA. The HCCA was dissolved by vortexing at room temperature until the solution turned clear.

2.6.2. Loading the bacterial colony to target plate

Actively grown bacterial cultures of PL-2, VS-5 and WBS-1 were subjected to this analysis. Smear of bacteria (single colony) as a thin film directly onto a spot on a MALDI target plate. The bacterial smear was overlaid with 1 µl of HCCA solution and allowed to dry at room temperature. One µl of bacterial standard (Bruker Daltonik GmbH, Germany) was loaded on to a separate well and 1 µl of matrix was added to it. The target plate was allowed to dry at room temperature and then loaded in to the instrument AUTOFLEX speed (Bruker Daltonik GmbH, Germany).

2.6.3. Analysis on MALDI-TOF MS instrument

Mass spectra were acquired in a linear positive ion extraction mode at a laser frequency of 200 Hz within a mass range from 2,000 to 20,000 Da. The ion source 1 voltage was 19.5 kV, ion source 2 voltages were maintained at 18.2 kV, lens voltage at 7kV and the extraction delay time was 240ns. The spectra were calibrated externally using the bacterial standard calibration mixture (*Escherichia coli* extracts including the additional proteins RNase A and myoglobin, supplied by Bruker Daltonik GmbH, Germany). The MALDI biotyper software 3.0 (Bruker Daltonik GmbH, Germany) was used to identify the isolates and visualize the mass spectra.

2.6.4. Data analysis and reporting

The strains showing ≥1.7 log value with strains in database were confirmed as the member of that genus and strains showing ≥2.0 log values were confirmed as the member of that species.

2.6.5. Software

MALDI Biotyper 3.1 (Bruker Daltonik GmbH, Germany) and Flex Analysis version 3.4 (Bruker Daltonik GmbH, Germany)

3. RESULTS AND DISCUSSION

3.1. Haemolytic activity

Five of the isolates were found to be non-haemolytic and were used for further study. If the probiotic strain under evaluation belongs to a species that is a known mammalian toxin producer or of hemolytic potential, it must be tested for toxin production and hemolytic activity, respectively. [15]. Since there is a high degree of phylogenetic relatedness among members of genus Bacillus, these species must be checked for hemolysis and enterotoxin production. Also, many of the enterotoxins produced by these organisms are haemolytic [10]. Among the fifteen isolates tested for haemolysis, PL-2, AVS, ATS, WBS-1 and VS-5 were non haemolytic. Other tested isolates showed partial or complete haemolytic activity. Only the non-haemolytic strains were screened further.

Table 3: Haemolytic activity of the Bacillus isolates

Isolate code	PL-5	PL-3	PL-2	AK-1	AK-4	RE-2	Ga1-2	AVS	ATS	HS-2	HS-3	Gr2-2	WBS-1	VS-5	CTS-2
Haemolysis type	β	β	γ	β	β	β	β	γ	γ	α	α	α	γ	γ	β

Key: β= Complete haemolysis, α = Partial haemolysis, γ = No haemolysis

3.2. Detection of Enterotoxin genes:

Several species of Bacillus including isolates of *B. mycoides, B. thuringiensis, B. subtilis, B. lentus, B. circulans, B. licheniformis* and *B. laterosporus/ cereus* produced detectable toxins. The protein toxins they produced were very similar to those of *B. cereus*, hence these species may also represent a potential hazard in food products [16]. Thus, several *Bacillus species* are shown to produce enterotoxins. However, a very few researchers have reported foodborne illness associated with *Bacillus species* other than *B cereus.* [10] However, due to the phylogenetic relatedness of members of this genus, it is especially important to test for the presence of these genes in this group of organisms, in the potential probiotics.

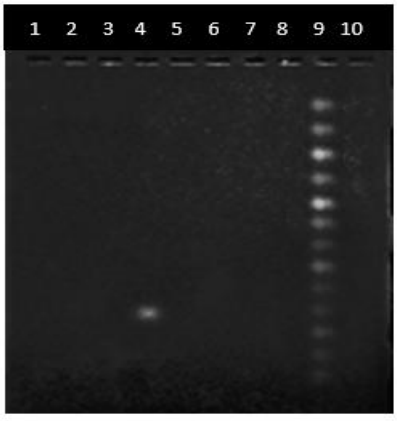

Fig. 1: Gel 1

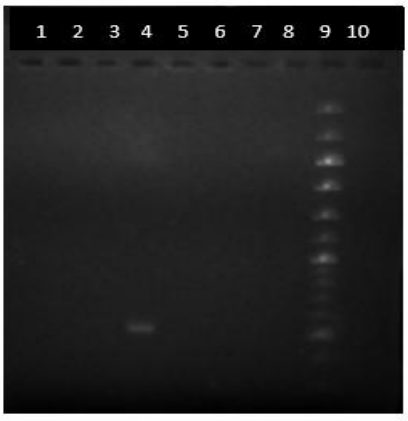

Fig. 2: Gel 2

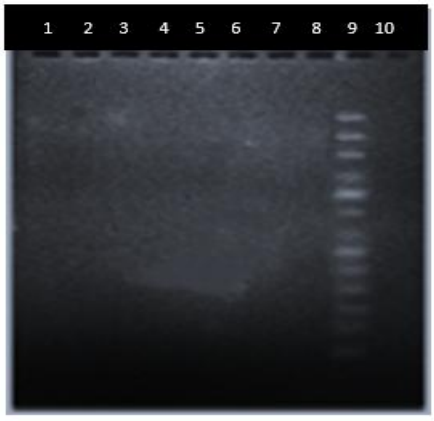

Fig. 3: Gel 3

Fig. 1-3: gel doc pictures of Agarose gel electrophoresis of PCR amplified products of the six target enterotoxin genes of the three test cultures VS-5, PL-2 and WBS-1.

Lanes 1 to 4 ---PCR product of VS-5, PL-2, WBS-1 and standard culture Bacillus cereus NCIM 2106, amplified with primer NH1 in gel1, Bal in gel 2 and EntFM gene in gel 3 respectively.

Lanes 5 to 8---- PCR product of VS-5, PL-2, WBS-1 and standard culture Bacillus cereus NCIM 2106, amplified with primer NH2 in gel 1, HblA in gel 2 and BceT gene in gel 3 respectively

Lane 9—100 bp DNA ladder (showing bands for 3000, 2000, 1500, 1200, 1000,900, 800, 700, 600, 500, 400, 300 and 200 bp)

Lane 10----- NTC (Non template control) in all three gels.

Standard culture *Bacillus cereus* NCIM 2106 shows a band in lane 4 in gel 1 and gel 2, confirming that it is positive for target genes nheA and Bal. The three isolates VS-5, PL-2 and WBS-1 were found to be negative for target nheA, nheB, Bal, HblA, EntFM and BceT enterotoxin genes. They were safe with respect to enterotoxin production.

3.3. Antibiotic sensitivity tests

Expression of antibiotic resistance and transferability of antibiotic resistance genes from ingested probiotic strains to commensal microorganisms in vivo are important characters for testing the safety of bacteria to be used as probiotics. After testing that the probiotic in question is not pathogenic, the next concern is to ensure that the strain is not a potential source of antibiotic resistance genes to other organisms of the intestinal microbial ecosystem [17].

The cultures coded as VS-5, PL-2 and WBS-1 were sensitive to most of the commonly used antibiotics except PL-2 which was resistant to Penicillin and WBS-1 which was resistant to Oxacillin and Penicillin (Table 4).

From the results of all tested antibiotics, the three isolates do not pose the danger of transferring determinants for antibiotic resistance. Interpretation as per 'Disk Diffusion Susceptibility Testing' (Kirby-Bauer Method) Spring 1997 [18].

Table 4: Antibiogram of the three Bacillus cultures

Antibiotic	Abbreviation and concentration	Zone of Inhibition in mms for VS-5	Interpretation	Zone of Inhibition in mms for PL-2	Interpretation	Zone of Inhibition in mms for WBS-1	Interpretation
Ampicillin	AMP 25	20	S	20	S	20	S
Kanamycin	K30	24	S	19	S	21	S
Oxacillin	Ox5	13	S	14	S	12	I
Chloramphenicol	C30	42	S	40	S	40	S
Ciprofloxacin	CIP5	45	S	39	S	35	S
Tetracycline	Te 30	29	S	27	S	26	S
Methicillin	M 5	37	S	39	S	36	S
Cephalothin	Ch 30	45	S	33	S	29	S
Clindamycin	Cd 2	18	I	13	I	20	I
Co-trimoxazole	Co 1.25	25	S	14	I	28	S
Erythromycin	E 15	26	S	40	S	28	S
Gentamicin	G 10	28	S	25	S	24	S
Ofloxacin	Of 5	30	S	30	S	26	R
Penicillin G	P 10	25	S	17	R	19	R
Vancomycin	Va 30	21	S	20	S	21	S
Oxacillin	Ox 1	38	S	13	S	22	S

Key: S = susceptible, R=resistant, I= intermediate

3.4. Shelf life studies

VS-5 exhibited 95.97 - 96.13% survival at the three storage temperatures up to six months**.** Storage at freezer and refrigerator temperatures gave a more consistent count of bacteria than storage at room temperature (Fig 4).

PL-2 exhibited viability of 97.81% - 98.54% at the three temperatures**.** The culture showed 98.54% survival when stored in the refrigerator at the end of 6 months of storage (Fig. 5).

WBS-1 at the end of shelf life study showed 92.61-96.98% survival. Room temperature range of 24-35 storage proved to be better with 96.98% cells viable (Fig. 6).

3.5. Identification of *Bacillus species* using 16SrRNA sequencing

The three cultures VS-5, WBS-1 and PL-2 found to be acid and bile tolerant from an earlier study [20] were identified by 16S rRNA sequencing.

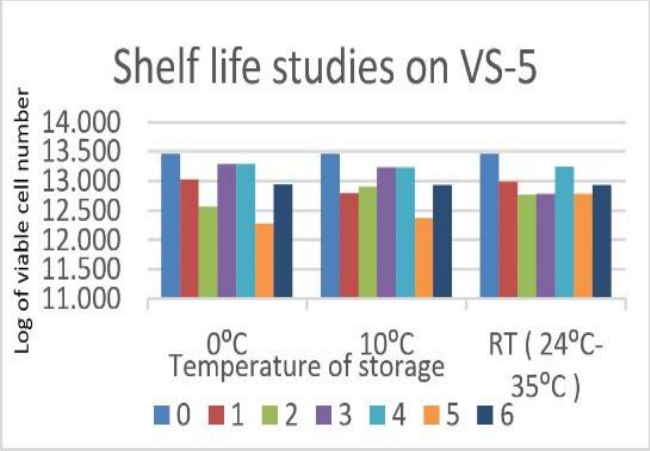

Fig. 4: Shelf life studies of VS-5

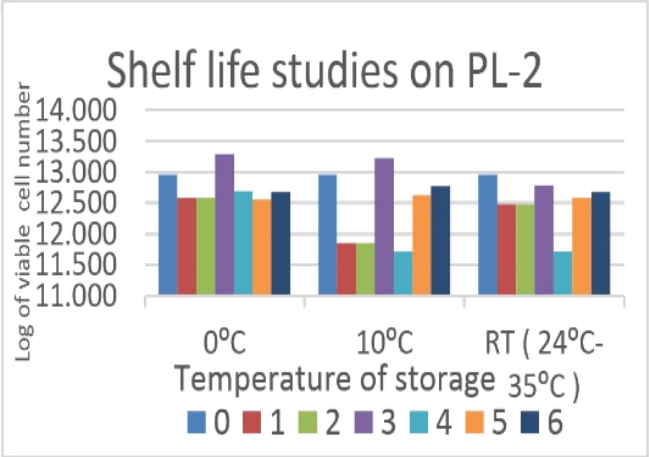

Fig. 5: Shelf life studies of PL-2

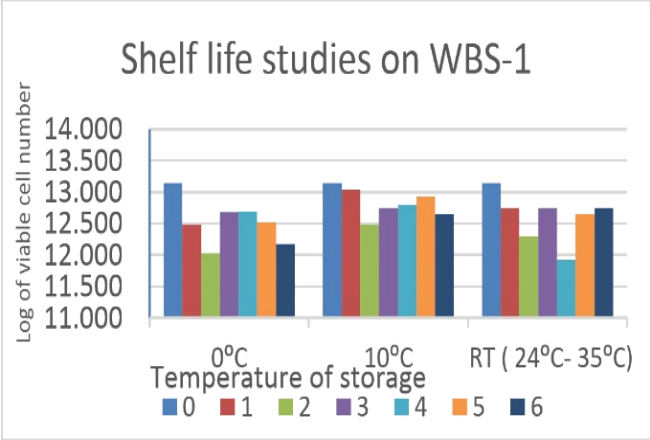

Fig. 6: Shelf life studies of WBS-1

Survival % at temperature 'T'= (Log cfu of culture after storage at T/Log cfu of culture before storage) x 100 [19].

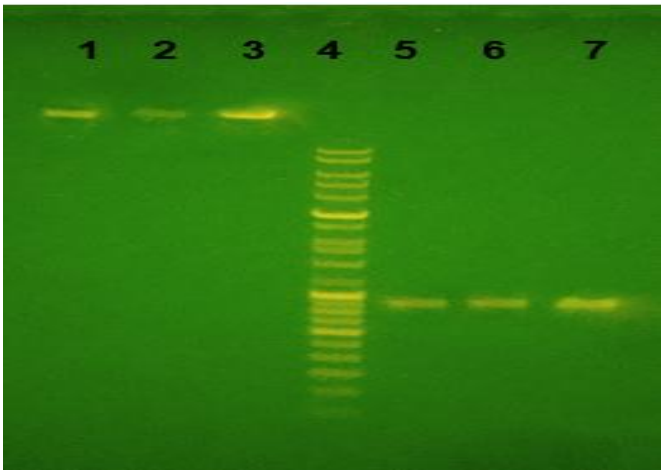

Fig. 7: Amplified partial 16S rRNA gene for bacteria

Lane 1-3: Genomic DNA of WBS-1, PL-2 and VS-5 respectively, Lane 4: High range DNA marker, Lane 5-7: Amplified PCR product of WBS-1, PL-2 and VS-5 respectively

The molecular phylogeny of sample was determined by analysing 16S rRNA gene sequences. Based on the position of sequence of the given bacterial samples in the phylogenetic tree, the closest similarities obtained are listed in table 5.

The three isolates were identified to be strains of *Brevibacillus borstelensis,* which belongs to family Paenibacillaceae, order Bacillales and Class Bacilli.

Table 5: Closest phylogenetic neighbours of samples analysed

Sample code	Closest neighbour
WBS-1	*Brevibacillus borstelensis* strain NBRC 15714 or *Brevibacillus borstelensis* strain DSM 6347 or *Brevibacillus borstelensis* strain Logan B4029
PL-2	*Brevibacillus borstelensis* strain NBRC 15714 or *Brevibacillus borstelensis* strain DSM 6347 or *Brevibacillus borstelensis* strain Logan B4029
VS-5	*Brevibacillus borstelensis* strain Logan B4029 or *Brevibacillus borstelensis* strain DSM 6347

3.6. Identification of Bacillus isolates using MALDI-TOF MS

Good quality MALDI-TOF MS spectra were generated for all strains (Fig. 8-10).

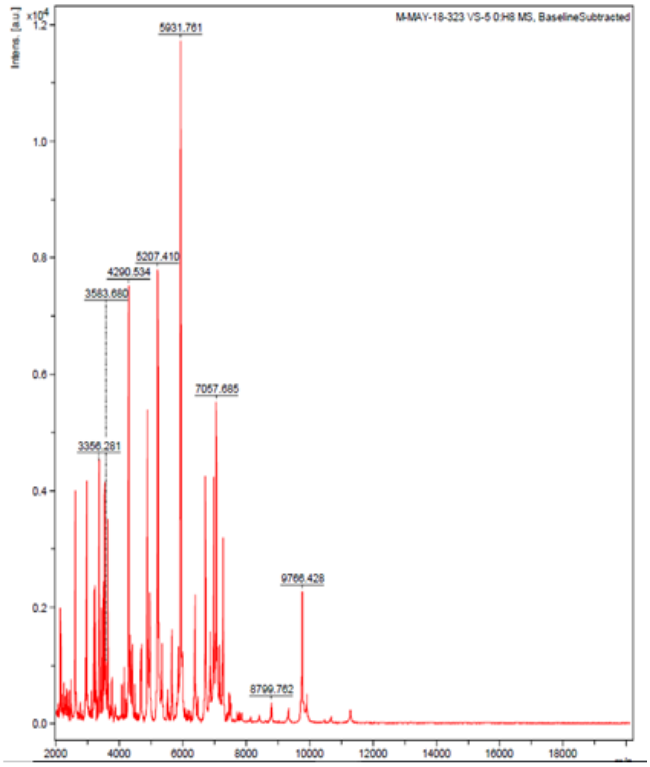

Fig. 8: MALDI-TOF MS spectra of VS-5

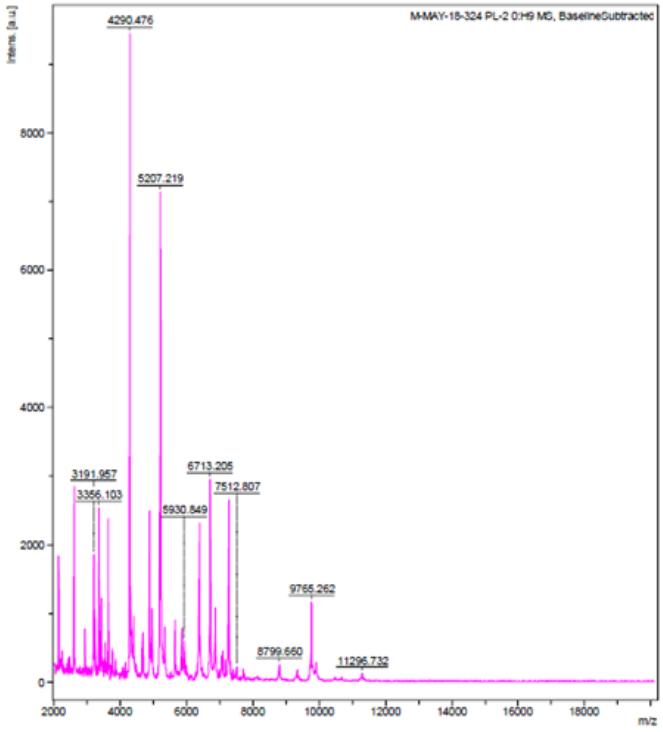

Fig. 9: MALDI-TOF MS spectra of PL-2

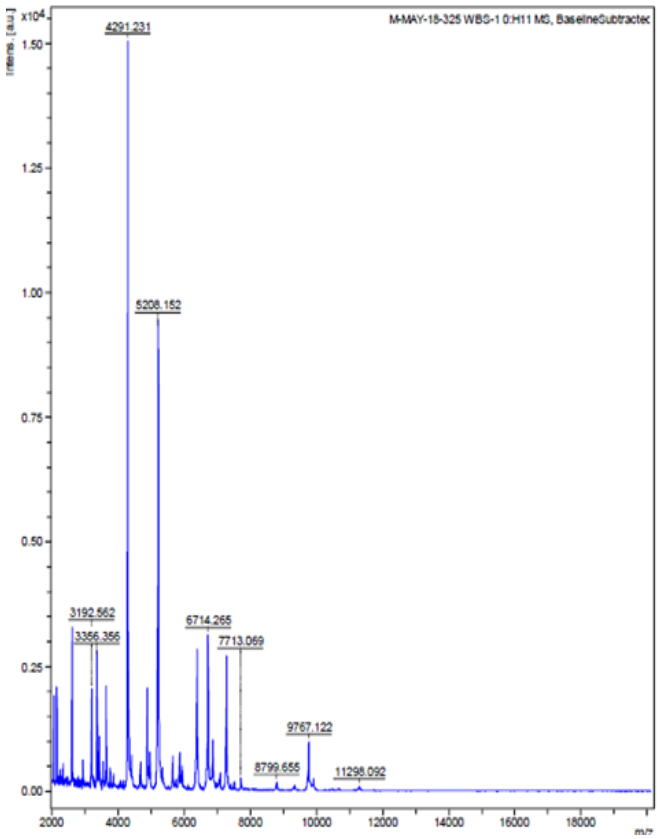

Fig. 10: MALDI-TOF MS spectra of WBS-1

The above MALDI-TOF MS spectra of bacterial strains indicate their protein profile (2-20KDa).

In PMF (peptide mass fingerprint) matching, the MS spectrum of the Bacillus isolates was compared with the MS spectra of known microbial isolates contained in the database. For species level identification of microbes, a typical mass range m/z of 2-20 kDa was used, which represents mainly ribosomal proteins along with a few housekeeping proteins [21].

Even though, culture conditions might greatly affect microbial physiology and protein expression profile [22], they do not influence microbial identification by MALDI-TOF MS. Research done in different laboratories has shown that culture conditions and culture time did not affect microbial identification by MALDI-TOF MS [23, 24].

The three isolates were submitted to Gen Bank and their accession numbers are VS-5 accession no. MK156349, WBS-1 accession no. MK970572 and PL-2 accession no. MK970573.

Table 6: Comparison with the Bruker taxonomy database using Biotyper 3.1 software the test strain

S.No.	Culture code	Organism (best match)	Score	Organism (second match)	Score
1	VS-5	*Brevibacillus borstelensis* 5_5 TUB	2.266	*Brevibacillus borstelensis* (MCC 2403)	2.207
2	PL-2	*Brevibacillus borstelensis* (MCC 2403)	2.225	*Brevibacillus borstelensis* DSM 6347T DSM	2.197
3	WBS-1	*Brevibacillus borstelensis* 5_5 TUB	2.334	*Brevibacillus borstelensis* (MCC 2403)	2.263

4. CONCLUSION

While screening for novel probiotic bacterial cultures, it is not enough to only test for desirable probiotic characteristics. It is important to follow safety guidelines before declaring a culture as a health promoter.

The three cultures which were identified as strains of *Brevibacillus borstelensis* by 16SrRNA and confirmed by MALDI TOF-MS spectra proved to be safe in terms of haemolysis, being non haemolytic. It does not possess the tested enterotoxin genes. Moreover, it is sensitive to the most commonly used antibiotics and hence does not pose danger of transmitting antibiotic resistance to the normal flora. The culture has been identified to be a non-pathogenic strain. Thus, the novel prospective probiotic bacterium proved to be safe for consumption.

5. ACKNOWLEDGEMENT

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