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Feasibility of Genome-Wide Screening for Biosafety Assessment of Probiotics: A Case Study of *Lactobacillus helveticus* MTCC 5463

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Abstract Recent years have witnessed an explosion in genome sequencing of probiotic strains for accurate identification and characterization. Regulatory bodies are emphasizing on the need for performing phase I safety studies for probiotics. The main hypothesis of this study was to explore the feasibility of using genome databases for safety screening of strains. In this study, we attempted to develop a framework for the safety assessment of a potential probiotic strain, *Lactobacillus helveticus* MTCC 5463 based on genome mining for genes associated with antibiotic resistance, production of harmful metabolites, and virulence. The sequencing of MTCC 5463 was performed using GS-FLX Titanium reagents. Genes coding for antibiotic resistance and virulence were identified using Antibiotic Resistance Genes Database and Virulence Factors Database. Results indicated that MTCC 5463 carried antibiotic resistance genes associated with beta-lactam and fluoroquinolone. There is no threat of transfer of these genes to host gut commensals because the genes are not plasmid encoded. The presence of genes for adhesion, biofilm, surface proteins, and stress-related proteins provides robustness to the strain. The presence of hemolysin gene in the genome revealed a theoretical risk of virulence. The results of in silico analysis complemented the in vitro studies and human clinical trials, confirming the safety of the probiotic strain. We propose that the safety assessment of probiotic strains administered live at high doses using a genome-wide screening could be an effective and time-saving tool for identifying prognostic biomarkers of biosafety.

Keywords 16S rRNA · Antibiotic resistance · *Lactobacillus* spp. · Probiotics

Introduction

The awareness of the beneficial effects of probiotics in promoting gut and general health has grown over the decade, leading to a surge in the consumption of probiotic foods worldwide. Probiotic bacteria, especially strains of *Lactobacillus* and *Bifidobacterium,* isolated from fermented foods are "Generally Regarded As Safe" (GRAS), according to the American Food and Drug Administration, due to their long history of safe use in fermented foods. *Lactobacillus helveticus* strains are given the Qualified Presumption of Safety (QPS) status as they are readily identified to the species level and have rarely been indicated in opportunistic infections [1]. With new strains being identified from diverse niches like gastrointestinal tract, vagina, and honey bee stomach, the efficacy of novel strains calls for a careful case-by-case evaluation to determine whether they share the safety status like the traditional food-grade organisms [2]. Illnesses caused by lactobacilli are reported in isolated cases in elderly and immune-compromised patients, rather than as collective foodborne diseases [3–9]. Further, the risk of infection with probiotic lactobacilli is similar to that of commensal strains, presenting negligible risk to consumers [7]. With the recent developments in tools and techniques to track

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specific strains, the reports of infections and other adverse incidents traced to probiotics have surfaced. Infections caused by lactobacilli and bifidobacteria make up 0.05–0.4 % of cases of endocarditis and bacteremia, respectively. About 1.7 % of infections reported were linked directly with intensive dairy probiotic consumption by patients and not healthy individuals [10]. A number of well-controlled human studies have tracked adverse incidents and have provided data about the safety assessment of probiotic cultures [11]. Classical risk assessment approaches, like microbiological risk assessment, are not warranted for lactobacilli because they are ubiquitous in nature [12]. The safety of probiotics is dependent on the strain, intended use, host health status, dose and duration of consumption, and both the manner and frequency of administration. The degree of risk that is regarded as acceptable can vary between countries, depending on the safety standards and carriers, like food, feed, and supplements. Emerging safety risks of probiotic strains include acquisition of antibiotic resistance and virulence determinants, genetic stability, deleterious metabolic activities, potential for pathogenicity, and immunological effects [11]. To address these risks, it would be better to do a premarket risk assessment of the strain for the above-mentioned theoretical safety concerns.

Whole-genome sequencing of bacteria has recently emerged as a cost-effective and convenient approach for testing safety-related genes [13]. *L. helveticus* strains, used largely in the cheese industry as a starter culture with high proteolytic activity, have been recently characterized at the genomic level [14–18]. Safety assessment can be greatly improvised using mechanistic omics-based data [19, 20]. *L. helveticus* MTCC 5463 (from now on referred to as strain MTCC 5463) is a potential probiotic strain and the first in India to be fully sequenced. It displayed antimicrobial activity against *Bacillus cereus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Salmonella enterica* serovar Typhi, and *Escherichia coli* [21], immunomodulation in a chicken model [22] and hypocholesterolemic effects in human volunteers [23]. The strain MTCC 5463 is a vaginal isolate, initially identified as *L. acidophilus* LBKV3 by preliminary biochemical tests. The partial 16S rRNA sequencing (accession number GQ253959) (1053 bp) of the vaginal isolate showed similarities of above 97 % with that of other *L. helveticus* strains, and it was confirmed by API 50 CHL results that the strain was *L. helveticus*. The genomic library contained 119,569 reads, and assembly generated a 1,911,350-bp single chromosome. In total, 2046 coding sequence (CDS) regions and 71 RNA genes were reported. MTCC 5463 showed resistance to phenol (0.4 %), bile salt (4 %), and low pH (pH 3) with a significant antimicrobial activity against *B. cereus*, *S. aureus*, *P. aeruginosa*, *S. enterica* serovar Typhi, and *E. coli* [21]. Studies on the strain's safety, dose response, and effect on host intestinal well-being [24] have also been conducted. The strain also produced extracellular polysaccharide and was able to adhere to cells of the human carcinoma cell line HT 29 [25]. The above-mentioned adaptive and probiotic characteristics of the strain were confirmed through trait and gene matching confirming that the strain possesses the genetic arsenal required to adapt to the gut milieu [26]. The predictions of functional genes further validate the experimental evidence of adaptation and probiosis. On comparing the stress-responsive genes of MTCC 5463 and a cheese starter strain, *L. helveticus* DPC 4571, gene sets specific to the gut and dairy niche could be identified [27]. Upon completion of genome sequencing of the strain in question [17], we attempted a bioinformatic analysis to provide additional insight into the genetic basis of its safety. The criteria for safety were delineated similarly to the QPS approach (defining the taxonomic unit, collecting the body of knowledge, safety concerns like the presence of antibiotic resistance, likelihood of gene transfer, tolerance to heavy metals, virulence determinants, and production of harmful metabolites). Our approach enables rapid screening of the gene set that determines the safety of a probiotic strain and its potential to become an opportunistic pathogen. We illustrate this strategy for MTCC 5463, but it can be readily adapted for biosafety assessment of other potential probiotic strains.

Materials and Methods

Bacterial Strains, Growth Conditions, and Pyrosequencing

The culture maintenance and pyrosequencing methodology is explained in our previous publication [17, 26]. Each DNA sample was subjected to pyrosequencing (454 Life Sciences technology) based on a high-throughput sequencer (GS-FLX, Roche) according to the manufacturer's instructions. The genome was sequenced to a depth of 10X.

Identification of Antibiotic Resistance and Virulence-Related Genes

Putative virulence factors and putative antibiotic resistance genes were identified by Basic Local Alignment Search Tool (BLAST) with the Virulence Factors Database (VFDB) (http://www.mgc.ac.cn/VFs/main.htm) and Antibiotic Resistance Genes Database (ARDB) (http://ardb.cbcb.umd.edu/), respectively. Safety genes were exhaustively searched based on published literature [20, 28–30].

Results

Mobilomes

Mobile genetic elements (mobilome), such as bacteriophages, plasmids, transposons, and ISs, are important for the colonization of ecological niches, symbiosis, host–cell interaction, and pathogenicity [31].

Plasmids

The strain MTCC 5463 showed a lack of plasmids. Scanning the genome for toxin–antitoxin (TA) proteins for plasmid maintenance showed the presence of an antitoxin of TA system that was present as a nonfunctional hypothetical protein.

Prophages and Integrases

The strain lacked the presence of a complete prophage but revealed the presence of structural and regulatory genes associated with prophages such as putative prophage repressor, DNA-packaging protein NU1, and a phage-associated protein. The strain MTCC5 463 further showed the presence of group II intron-encoded maturase, integrase recombinase, and putative integrases with integrase catalytic region encoding genes (Table 1).

Transposases and Insertion Sequence (ISs) Elements

Annotations showed the presence of a large number of putative transposase genes (154 copies). Six copies of IS 1201, one of IS lhe15, and two coding sequences for the IS 4 family were observed in the strain MTCC 5463. Further analysis revealed that no core gene was clustered with the ISs restricting its transferability.

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) and Restriction/Modification (R/M) Systems

CRISPR provides acquired immunity against foreign genetic elements. The probiotic bacteria did not exhibit the presence of CRISPRs and associated proteins. A range of R/M systems like the type I, type II, and type III restriction/modification system has been observed in the genome.

Resistome: Antibiotic and Heavy-Metal Resistance

A growing biosafety concern is of the transfer of antibiotic resistance genes from commensals to pathogens in the gut (reservoir hypothesis). The genome in question was mined for the presence of resistance genes against common antibiotics.

Beta-lactam

The most common and important mechanism through which bacteria becomes resistant to β-lactams is by the expression of β-lactamases. The strain MTCC 5463 harbored three homologs of beta-lactamases of various classes. The presence of penicillin-binding proteins (PBPs) could also have been contributing to beta-lactam

Table 1 In silico analysis of safety genes (mobilome) in the genome of *L. helveticus* MTCC 5463

Category	Gene name	Gene ID AAULH_…
Prophage	Putative prophage repressor	00520
	Prophage DNA-packaging protein NU1	12306
	Phage-associated protein	01093
	Group II intron-encoded maturase	06181
	Integrase recombinase	06106, 06131
	Integrase	11866, 12081, 12406, 12411, 12796
	Integrase catalytic region encoding genes	06921 00643
	Uncategorized mobile genetic elements	05641, 08036, 08543, 09663, 10882, 03391
Insertion sequences	IS 1201	05791, 00559, 07096, 07111, 10887, AULH_03683
	IS lhe15	05601
	IS*4* family	00287, 04689
	Type I restriction/modification system, S subunit	08548
R/M systems	Type I site-specific deoxyribonuclease	08553
	Type I restriction enzyme EcoKI subunit R	08568
	Type II restriction enzyme, methylase subunit	11871, 13216
	Type III restriction/modification system	12356, 12361, 00172, 14601, 12906 13491, 00167, 14601

resistance. Although five homologs of PBPs were present in the genome, BLAST analysis showed no mutation in the sequence implying that the altered PBPs were not the resistance-conferring agents.

Fluoroquinolone

The molecular targets of fluoroquinolone in the genome, like DNA gyrase, and topoisomerase IV, showed no mutation, and aminoglycoside N3′-acetyltransferase gene was present as a hypothetical protein. Hence, it is unclear whether they encode actual proteins. The ABC transporter, ATPase, permease components, and efflux pumps could be the probable determinants of resistance against quinolones (Table 2).

Other Antibiotic Resistance Genes

Tetracycline resistance determinants, like the NADP requiring oxidoreductase and xanthine–guanine phosphoribosyltransferase, were absent in the genome. Ribosomal protection proteins having homology to elongation factors were identified.

The additional presence of ABC transporter, ATPase, permease components, and efflux pumps mentioned above may contribute toward tetracycline resistance. Other antibiotic resistance genes mined in the strain MTCC 5463 genome included macrolide-lincosamide-streptogramin B (MLSB) resistance-conferring rRNA methylases, aminoglycoside acetyltransferases, and members of the GCN5 superfamily of proteins that include the histone acetyltransferases.

Bacteriocins and Immunity Genes

Genes expressing bacteriocin helveticin, bacteriocin production-related histidine kinase, bacteriocin response regulator, and a bacteriocin ABC transporter along with homologs of immunity proteins, like PlnI, were present in the genome.

Tolerance to Heavy Metals

The strain MTCC5 463 genome carried genes involved in copper homeostasis, like the copper-transporting ATPase and the copper chaperone. It has also developed systems for the removal of excess cobalt from cells by efflux system genes to avoid toxicity. Cadmium efflux mechanisms are also exhibited by the probiotic strain, as evidenced by the presence of cadmium efflux ATPase and cadmium-translocating P-type ATPase. In addition, genes to acquire iron and nickel transport system permease proteins are present in the genome (Table 2).

Adverse Metabolic Genes

The potential of the strain MTCC5 463 to produce the biogenic amines agmatine and putrescine from arginine and its amino acid derivative, ornithine, was investigated. It was found that sequences coding for the enzymes arginine deiminase and ornithine transcarbamylase existed as pseudogenes, rendering the pathway nonfunctional (Table 2). The strain encoded ornithine decarboxylase, which catalyzed the conversion of ornithine to putrescine, while both the spermidine/putrescine ABC transporter permease component and the spermidine/putrescine import related ATP-binding protein, PotA-accommodated ornithine uptake into the cell. Putrescine itself did possess a direct harmful biologic activity. Instead, it enhanced the toxic effects of histamine and tyramine, which were not produced by the strain. The strain MTCC 5463 exhibited the orthologs for beta-glucuronidase, Lac L, and Lac M. The presence of conjugated bile salt hydrolase gene in the genome in question is a fitness factor facilitating resistance against intestinal conditions rather than a risk factor.

Virulence- and Stress-Related Genes

Factors required to survive the physiological stresses and host interaction may be termed as virulence mechanisms in the case of undesirable bacteria. The same factors are considered crucial for a symbiotic interaction in the case of probiotics and termed as fitness factors or "host interaction factors" [32]. Offensive virulence factors are an invasion, mucin degradation, cytotoxicity, hemolysis, and biofilm production. We used a set of virulence factors from the VFDB to search for the presence of such classical and defensive virulence factors in MTCC 5463 genome. Data mining the probiotic strain's genome revealed adhesion factors, including a putative sortase gene, GroEL, aggregation promoting protein, two copies of fibronectin-binding protein, S-layer protein, and a mucus-binding protein (Table 3).

Exopolysaccharide (EPS) is the probiotic effector molecules and biofilm-forming ability help in colonizing the gut. The strain MTCC 5463 also carried genes like glycosyltransferases that are important for the biosynthesis of exopolysaccharide (EPS) including bactoprenol glucosyl transferase and putative hexosyl transferase YtcC. Probiotic MTCC 5463 strain displayed the biosynthesis of glycosyltransferase protein for capsular polysaccharide synthesis and four exopolysaccharide biosynthesis proteins. The epsE gene-encoding phosphoribosyltransferase was present as a single copy. The genome also encoded ten potential uncharacterized glycosyltransferases and one galactosyltransferase. General stress adaptation genes exhibited by the strain included the universal stress protein, UspA, chaperones GroES and GroEL, Clp protease, heat-

Table 2 In silico analysis of safety genes (resistome, heavy metal, and adverse metabolic genes) in the genome of *L. helveticus* MTCC 5463

Category	Gene name	Gene ID AAULH_…
Resistome		
Beta-lactam	Class B metallo-beta-lactamase superfamily protein	05014
	Class A beta-lactamase	10045
	Ribonuclease Z	05881
	Class C beta-lactamase putative esterase	05881
	Penicillin-binding proteins	05204, 00407, 06271, 06776, 11461
Fluoroquinolones	Aminoglycoside N3′-acetyltransferase	03446
	Major facilitator superfamily efflux pump	13121
Others	Small multidrug efflux protein	00277
	Multidrug resistance protein	00978
	Multidrug ABC transporter	03236, 09950
	Bacteriocin ABC transporter	06336
	ABC-type multidrug transport system	03286, 03291, 06511, 06341, 06536, 06531 00075, 06546 08778, 08773 09718, 09518, 10117, 10582, 10587, 11546: 13036, 13141 13416, 13821
	Streptothricine-acetyl-transferase	06431
	Ribosomal protection proteins elongation factor Tu	05109
	Phospho-beta-glycosidase	00460
	Elongation factor Ts	07866
	Elongation factor P	08266, 10152
	rRNA methylase	02043, 09158
	Aminoglycoside acetyltransferases	13106, 03193, 06801, 07006
	D-ala, D-ala ligase	10747
	CheY-like receiver domain and a winged-helix DNA-binding domain	04400, 08643, 09183, 10577
	LytR/AlgR family two-component response regulator	14541
	D-lactate dehydrogenase	00367
	L-lactate dehydrogenase	05541
	Signal transduction histidine kinase	04405 00589, 12656, 12661
	Undecaprenyl pyrophosphate synthase	07851
	Dihydropteroate synthase	06851
	Dihydrofolate reductase	05431
Heavy metals		
Copper	Copper-transporting ATPase	11711
	Copper chaperone	13076
Cobalt	ABC-type cobalt transport system, permease component CbiQ	05671, 00628, 00633, 11092, 01878, 03296
	cbiO	01873
	Cobalt transport ATP-binding protein	11097
	ABC-type cobalt transport system, ATPase	11152, 11157
Cadmium	Cadmium efflux ATPase	11521
	Cadmium-translocating P-type ATPase	13126
Iron	Siderophore 2,3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin synthetase	14001
Nickel	Nickel transport system permease protein	01205, 01200
Adverse metabolic genes		
Biogenic amines	Carbamate kinase	07591, 07596
	Ornithine decarboxylase	10242
	Spermidine/putrescine ABC transporter permease components	04155, 04160

Table 3 In silico analysis of safety genes (virulence- and stress-related genes) in the genome of *L. helveticus* MTCC 5463

Category	Gene name	Gene ID AAULH_…
Virulence-related genes		
	Hemolysin III	06041
	Hemolysin a	08201
	Hemolysin-type calcium-binding repeat protein VCBS	14191
	Putative sortase gene	07706
	GroEL	02328
	Putative aggregation promoting protein	10742, 09428, 02328
	Fibronectin-binding protein	07186, 01148
	S-layer protein	01325, 12271
	Mucus-binding protein	05511 05516
	Bactoprenol glucosyl transferase	04749, 00465, 00470, 00480, 00773, 11811, 11816
	Putative hexosyltransferase YtcC	13501
	TagF/TagB/EpsJ/RodC	03006
	Membrane protein involved in the export of O-antigen and teichoic acid	11486, 11921
	6-Diaminopimelate-D-alanyl-D-alanine ligase	01552
	D-alanyl-alanine synthetase A	00828
	D-alanyl-D-alanine Carboxypeptidase	09853
	UDP-N-acetyl-D-mannosamine transferase	02956
	Polysaccharide transporters specific to O-antigen and teichoic acid	09930 11431, 14176
	Teichoic acid glycosylation protein	03611
	D-alanine esterification of lipoteichoic acid and wall teichoic acid	11466
	Lipopolysaccharide biosynthesis glycosyltransferase	00470
	Capsular polysaccharide synthesis PROTEIN	03011
	Exopolysaccharide biosynthesis protein	10282, 10287, 10292, 10297
	Phosphoribosyltransferase	10277
	Glycosyltransferases	02538, 02543, 00465, 00480, 04749, 13501, AULH_13506, 13536 11816, 11811
	Galactosyltransferase	02961
Stress-related genes		
	Universal stress protein UspA	00893
	Co-chaperonin GroES,	02323
	Chaperonin GroEL,	02328
	ATP-dependent Clp protease	01657
	Low molecular weight heat-shock protein	01235
	Heat-shock protein HtpX	00653
	Heat-shock protein GrpE,	07746
	dnaK,	07741
	Signal recognition particle-docking protein FtsY	07996
	Chaperone protein DnaJ	07736
	HtrA-like serine protease	00609
	F_0F_1 ATP synthase subunit C	04505
	F_0F_1 ATP synthase subunit B	04510
	F_0F_1 ATP synthase subunit delta	04515
	F_0F_1 ATP synthase subunit alpha	04520

shock proteins, HtpX, GrpE, DnaK, FtsY and DnaJ, HtrA-like serine protease, and F0F1 ATP synthase subunits (Table 3).

Discussion

The use of lactobacilli in food and food production is generally accepted as safe by the scientific community. Even so, the authors attempted to conduct an exhaustive safety risk analysis for two main reasons: (1) to assess the suitability of the strain MTCC 5463 for the use with immunocompromised human hosts of all ages and (2) to provide a platform whereby all future potential probiotic isolates could undergo an exhaustive safety genes assessment. The safety of a strain cannot be assessed until it is taxonomically characterized. This characterization facilitates the comparison with known variants and helps track the strain in case of outbreaks. Using a polyphasic approach, the strain under study was identified as *L. helveticus* MTCC 5463. The genomic sequence of the strain MTCC 5463 (1.91 Mb) was smaller than those of the reference dairy strain *L. helveticus* DPC 4571 (2.08 Mb) [15], but larger than the smallest reported genome *L. iners* AB-1 (1.3 MB) [33]. This is suggestive of probable alterations in the MTCC 5463 genome to adapt to the nutritionally rich milk medium and for probiotic functionality [34]. An example of this adaptability is the presence of conjugated bile salt hydrolase in the gut strain, MTCC 5463, and a nonfunctional homolog in the dairy strain *L. helveticus* DPC 4571 [27]. The RAST server predicted the closest six neighbors of the MTCC5 463 strain to be of lactobacilli, and the first three significant hits (*E* value of 1e-20 or less) to the NCBI database were of the genus *Lactobacillus.* Thus, it can be concluded that the strain has not acquired a large number of genes from other organisms. This indicates the excellent genomic stability and minimum influence of horizontal gene transfer [8]. Being devoid of plasmids, strain MTCC 5463 loses its capacity to transfer antibiotic resistance genes to gut commensals. The strain maintains an unpaired antitoxin that is rendered nonfunctional. The nonfunctionality could be due to lack of plasmids and lower competition in a milk matrix against pathogens compared to the vaginal ecosystem. The strain MTCC 5463 harbors 154 copies of IS elements with no evidence of chromosomal rearrangements, similar to the 213 IS element-loaded DPC 4571 genome. Another reason for the maintenance of the high number of IS elements could be because the protective milk/food matrix with a defined strain inoculum used for propagation promotes low levels of intra-species competition which favors clones with increased numbers of IS elements to survive [35]. Genomic stability is a required trait to ensure that probiotic attributes are not affected by long-term preservation and production. The strain MTCC 5463 has exhibited a stable technological performance for 25 years, and the whole-genome order is conserved. This provides conclusive evidence that the large number of IS elements did not cause genetic instability. Further analysis revealed that no core genes were clustered with ISs, thus restricting transfer of antibiotic resistance. In silico analysis of the genome of the strain MTCC 5463 did not reveal any complete prophages but showed the presence of isolated prophage remnants. This suggested an inactivation or elimination of integrated prophages and evolution toward a stable genome. R/M systems present in the strain further act as barriers against horizontal gene transfer. Strain MTCC 5463 lacks CRISPRs that exclude foreign DNA. The absence of CRISPRs although does not result in an increase in foreign genes. Antibiotic resistance markers for penicillin are evidently the beta-lactamases, as no alterations in penicillin-binding (PBP) sites were observed. The molecular basis for the resistance to quinolones could not be established due to the absence of mutations in the *gyrA* or *parC* genes. The chromosomal nature of the resistance determinants does not pose any risk. Lactobacilli are known to possess intrinsic resistances to tetracycline, quinolones, vancomycin, and erythromycin that support the safety of the strain MTCC 5463 [36, 37]. The stable antibiotic resistance profile of the strain MTCC 5463 is beneficial during administration along with antibiotic therapy. The antibiotic resistance observed in strain MTCC 5463 was not acquired but intrinsic resistance. According to the QPS criteria, these results provide safety assurance for the ongoing use of the strain MTCC 5463 as a probiotic. The theoretical resistance markers need to be further subjected to expression studies and transferability tests, like bacterial mating experiments. As the resistance genes are not located on the plasmid, standard protocols for showing genetic transfer are not available in the literature [38]. Further research in this area is warranted. Lactic acid bacteria can add further functionality as heavy-metal-detoxifying agents by binding and effluxing heavy metals from food and water [39]. A growing concern lies in the co-occurrence of genes conferring metal resistance and antibiotic resistance genes, which could lead to the selection of antibiotic-resistant organisms in the human gut. This concern is mitigated in strain MTCC 5463 due to the absence of mobilome-associated resistant determinants. The suggested roles of heavy-metal-transporting ATPases and copper homeostasis elements in the acid tolerance mechanisms [40] further support the role of these related genes as a survival factor for the strain in gut rather than making the strain unsafe for human consumption.

The possibility of biogenic amine-induced discomfort during probiotic intervention depends on the number of

strains in the product and the sensitivity levels of the host. No cases of biogenic toxicity have been reported during the three-controlled human clinical studies undertaken with strain MTCC 5463. The presence of ornithine decarboxylase and its expression could be a defense mechanism used by bacteria to withstand acidic environments [41] rather than for the production of biogenic amines. Similarly, the presence of D-lactate dehydrogenase in MTCC 5463 has not caused any complaint of D-lactic acidosis in human subjects. Beta-glucuronidase (BG) activity is considered a cancer risk biomarker [42], as well as an anti-tumor factor [43]. Therefore, the presence of BG in the strain MTCC 5463 genome can be concluded to be beneficial, as no detrimental activities have been reported in its long history of use. Functional metagenomic approaches have identified BG as a part of the functional core of the human microbiome of the gut ecosystem [44].

Among the vaginal microflora, bacteriocin producers achieve a competitive edge over uropathogens. This explains the presence and maintenance of bacteriocin genes, along with immunity proteins in the strain MTCC 5463. The strain MTCC 5463 did not produce bacteriocins in in vitro studies, but the presence of helveticin homologs suggests that expression of such genes may occur only under stress. The presence of pore-forming hemolysin genes may have been conserved due to the bacteria's need for sustenance, defense, and survival through menstruation in the vaginal ecosystem. Virulence factors are universally differentiated into defensive factors (that protect the bacteria from the host immune system) and offensive factors (those that harm the host). In the case of probiotics, this classification is blurred. The vast number of genes identified through VRDB correlates with virulence factors of some pathogens but relates to fitness factors in the case of probiotics. Adaptation factors like adhesins and stress proteins are important to the characteristics of probiotics and should be considered as fitness factors. Fibronectin-binding protein, mucus-binding and sortase-dependent proteins involved in bacterial–host interactions are common to lactobacilli that colonize the gastrointestinal tract [41]. Thus, we can conclude that they are not acquired virulence genes, but genes that confer niche adaptation. The molecular chaperones present in the genome include DnaK, GroEL, and GroES, and they are pivotal for long-term acid stress resistance. Clp ATPase is particularly important for the fast response of lactobacilli in adverse conditions [45]. The use of databases like ARDB and VRDB is meaningful only in case of high-coverage assemblies and high sequencing depths. The safety of the strain MTCC 5463 is further supported by human clinical evidence to claim that the strain is not associated with any intestinal disorders or discomfort.

Conclusion

Traditional methods of analyzing the safety of a strain are replaced by cost-effective next-generation sequencing based on annotations. These methods reveal the maximum potential risk. The authors second the need put up by Zhang et al. [20] for a safety-associated gene database specifically for probiotics. This database could act as a preliminary defense against unsafe and uncharacterized strains entering the market and hence the food chain. Because probiotic strains are free living in natural niches and rarely the primary cause of an infection, it is often difficult to identify specific traits that contribute to pathogenesis. Certain virulence and antimicrobial characteristics are bound to be present in the genome, as lactobacilli are known to prevent microbial spoilage. The authors further emphasize the need for using multiple databases before drawing any conclusion. The detection of the genes, or homologs thereof, does not show whether they are intact and functional. The sequences may have premature stop codons, insertions, or deletions, making them nonfunctional. Expression studies of genes could further strengthen the safety assessments. The transferability of traits needs to be further validated using conjugation and filter mating experiments. The strain under study shows no known transferable determinants for antibiotic resistance, and the resistance traits are intrinsic in nature. The strain exhibits a specific pattern of fitness and adaptive factors. The study findings provide safety assurance for the ongoing use of the strain MTCC 5463 as a probiotic.

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Compliance with Ethical Standards

Conflict of interest Senan, Prajapati, and Joshi have no affiliations with or involvement in any organization or entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this manuscript.

Ethical Statement The research has been conducted with integrity, and intellectual honesty, and human or animal subjects have not been a part of this study.

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