ORIGINAL ARTICLE

Assessment of four Poly Antibiotic-resistant Bacterial Strains of *Bacillus clausii* (TIL 19T, TIL 21C, TIL 28S, TIL 30R) and their Probiotic Attributes: Implications for Safety and Therapeutic Viability

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Abstract

Objective: This study evaluated the probiotic potential and safety of four poly-antibiotic-resistant strains of *Bacillus clausii* (TIL 19T, TIL 21C, TIL 28S, and TIL 30R), a spore-forming bacterium known for supporting gastrointestinal health. **Results:** The strains demonstrated several desirable probiotic characteristics, including tolerance to gastrointestinal conditions such as bile salts, lysozyme, and intestinal transit stress, along with strong auto-aggregation and co-aggregation abilities. Acid, bile, transit, and lysozyme tolerance assays demonstrated strong gastrointestinal survival abilities in all the four strains. TIL 19T and TIL 30R displayed high survival rates in lysozyme-rich environments, and TIL 28S and TIL 30R showed strong co-aggregation with *E. coli*, indicating potential pathogen inhibition. The strains also adhered effectively to intestinal cell models, produced digestive enzymes and exopolysaccharides, reduced cholesterol levels, and exhibited antimicrobial properties. Safety assessments further supported their probiotic candidacy. The strains were heat resistant, capable of moderate biofilm formation, and did not produce harmful compounds such as biogenic amines, or DNase. Cytotoxicity testing on Vero cells revealed high cell viability, suggesting a favourable safety profile. **Conclusion:** Collectively, these findings indicate that the investigated *Bacillus clausii (Shouchella clausii)* strains possess strong probiotic and therapeutic potential, making them promising candidates for future applications in functional foods and gastrointestinal health promotion.

Key words: 16SrRNA, *Bacillus clausii*, functional food, polyantibiotic resistant strains, probiotic bacteria

INTRODUCTION

The human microbiota is a complex community of microorganisms that plays a vital role in maintaining host health through functions, such as nutrient metabolism, immune modulation, and protection against pathogens. Disruptions in this microbial balance, known as dysbiosis, have been associated with numerous diseases and health disorders. Probiotics have emerged as an

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effective strategy for restoring microbial equilibrium, with species of *Bacillus* attracting considerable attention due to their ability to withstand harsh environmental conditions and confer various health benefits, including immune regulation and pathogen inhibition.[1] In the face of the growing global challenge of antibiotic resistance, probiotics are increasingly being explored as alternative or complementary therapeutic approaches. Their capacity to enhance gut health and suppress pathogenic microorganisms highlights their potential in combating antibiotic-resistant infections while promoting overall well-being.[2] Among these probiotic candidates, *Bacillus clausii (B. clausii)* has gained particular interest because of its established safety profile, resilience, and therapeutic effectiveness in managing intestinal infections, reducing antibiotic-associated adverse effects, and exhibiting antimicrobial activity within the gastrointestinal tract.

B. clausii is a spore-forming, alkaliphilic bacterium with a long history of use in the prevention and treatment of intestinal disorders. Its ability to survive adverse gastrointestinal conditions, including exposure to gastric acidity and bile salts, contributes significantly to its probiotic efficacy.[3] The taxonomic classification of this organism has undergone several revisions over the years. Originally classified as *Bacillus subtilis*, it was reclassified as *B. clausii* based on phylogenetic analyses conducted by Nielsen *et al*. (1995).[5] With advances in molecular characterization techniques, particularly 16S *rRNA* gene sequencing and comparative protein analyses, Patel and Gupta reassigned the species to the genus *Alkalihalobacillus*, naming it *Alkalihalobacillus clausii*.[6] Further phylogenetic investigations led to propose its reclassification as *Shouchella clausii (S. clausii)*. Despite these taxonomic changes, the organism continues to be widely recognized for its probiotic properties, including tolerance to acidic and bile conditions and its ability to produce bioactive secondary metabolites that may contribute to host health.

Although substantial research has highlighted the probiotic potential of *B. clausii*, significant gaps remain in understanding the strain-specific characteristics that contribute to its therapeutic efficacy, particularly in relation to antibiotic resistance and pathogen inhibition. Limited information is available regarding the mechanisms through which different *B. clausii* strains exert their probiotic effects and their effectiveness against antibiotic-resistant pathogens. Addressing these knowledge gaps is essential for optimizing the use of this bacterium in clinical and functional food applications.

Therefore, the present study aims to investigate the probiotic properties of different *B. clausii* strains and evaluate their safety and therapeutic potential. We hypothesize that individual strains possess distinct probiotic characteristics that contribute to their ability to survive gastrointestinal conditions and inhibit antibiotic-resistant pathogens. By elucidating the mechanisms underlying their probiotic activities and assessing their safety profiles, this research seeks to support the development of innovative probiotic interventions for the prevention and management of antibiotic-resistant bacterial infections.

MATERIALS AND METHODS

Materials

Chemicals used in this study were purchased from Hi-Media (Mumbai) and Sigma-Aldrich Merck (USA). The pathogens used were procured from the National Collection of Industrial Micro-organisms, National Chemical Laboratory, Dr. Homi Bhabha Road, Pune 411 008, Maharashtra, India. Sample strains were procured from Allianz Biosciences Pvt Ltd. (ABPL), R.S. No.55/1,2, 3, Whirlpool Road, Thiruvandarkoil 605 102, Puducherry. Test strains were propagated in nutrient broth for 24 h at 37°C and maintained in glycerol stock at −20°C until further use.

Methods

ABPL bacterial isolates

Four antibiotic-resistant bacterial strains (TIL 19T, TIL 21C, TIL 28S, and TIL 30R) obtained from Allianz Bioscience Pvt. Ltd. were maintained at 4°C. Cultures were grown in nutrient broth at 37°C for 16–18 h and preserved on agar slants and glycerol stocks at −80°C.

Identification of bacterial isolates

The isolates were identified by 16S *rRNA* gene sequencing using primers 8F and 1492R. Sequence data were analyzed using BioEdit software and compared with GenBank sequences through BLAST. Phylogenetic analysis was performed using the UPGMA method in MEGA 7.0.21 software.[5]

Antibiotic susceptibility test

Antibiotic susceptibility was determined by the disc diffusion method on Mueller–Hinton agar. Overnight cultures were spread onto agar plates and tested against a panel of broad- and narrow-spectrum antibiotics. After incubation at 37°C for 24 h, inhibition zones were measured and interpreted as sensitive or resistant according to standard guidelines.

Hemolytic activity

Hemolytic activity was evaluated on 5% sheep blood agar plates incubated at 30°C for 48 h. Hemolysis patterns (α, β, or γ) were recorded according to Fadia BT *et al*.[7]

Acid and bile tolerance

Bacterial cultures (1×10^9 colony forming unit [CFU]/mL) were exposed to De Man, Rogosa, and Sharpe (MRS) broth adjusted to pH 2.0, 3.0, and 7.0 (control) for 3 h. Bile tolerance was assessed using MRS broth containing 0.3%, 0.5%, and 1% ox-bile for 4 h at 37°C. Cell viability was determined by the spread plate method.[8]

Transit tolerance assay

The strains were exposed to simulated gastric juice containing pepsin (3 g/L, pH 3) and intestinal juice containing trypsin (1 g/L, pH 8). Viable counts were determined on MRS agar after incubation at different time intervals to evaluate gastrointestinal transit tolerance.[9]

Lysozyme tolerance activity

Bacterial cultures (1×10^9 CFU/mL) were incubated in MRS broth containing 100, 200, and 300 ppm lysozyme at 37°C for 3 h. Viable cell counts were determined on MRS agar following incubation.[10]

Microbial adhesion to hydrocarbon test

Cell surface hydrophobicity was assessed using n-hexadecane and xylene. Bacterial suspensions (1×10^9 CFU/mL) were mixed with hydrocarbons, incubated for 1 h, and the absorbance of the aqueous phase was measured at 580 nm. Hydrophobicity percentage was calculated using the standard formula.

$$Hydrophobicity\ \% = (A0 - At/A0) \times 100,$$

Where A0 is the initial absorbance and at is the post-mixture absorbance of the aqueous phase.

Auto aggregationma

Auto aggregation was assessed with minor modifications to Angmo *et al*.'s (2016) protocol, using selected bacterial strains (10^9 CFU/mL). Post-vortexing, the samples were incubated at 37°C, with absorbance readings at 600 nm taken at 3 and 24 h. %Auto aggregation was calculated using the formula:

$$Auto\ aggregation\ (\%) = (1\ At/A0) \times 100$$

Where (At) and (A0) represent absorbance at time t and initial absorbance, respectively.

Co-aggregation

Equal volumes of bacterial strains and pathogens, both at 10^9 CFU/mL, will be mixed and vortexed for 10 s, then incubated at 37°C for 24 h. Samples (1 mL) will be taken at 3 h and 24 h post-incubation, and their optical density (OD) measured at 600 nm. Co-aggregation percentage is determined by the formula:

Table 1: Cytotoxicity by MTT assay

Name of Bacterial isolates	% of viability Vero cells (Concentration of bacterial cells)			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}
TIL 19T	81.6%	89.2%	95.4%	98.22%
TIL 21C	82.7%	86.2%	92.7%	94.3%
TIL 28S	81.5%	90.5%	95.7%	98.4%
TIL 30R	84.1%	89.5%	93.5%	95.8%

Table 2: Results showing the resistance (R) or sensitivity (S) of the strains toward the antibiotics

Antibiotics	Concentration	TIL 19T	TIL 21C	TIL 28S	TIL 30R
Tetracycline	10 µg/Disc	R	S	S	S
Clindamycin	2 µg/Disc	R	R	S	S
Chloramphenicol	30 µg/Disc	S	R	S	S
Methicillin	30 µg/Disc	S	R	R	R
Ciprofloxacin	5 µg/Disc	S	S	S	S
Amoxyclav	30 µg/Disc	R	R	S	S
Erythromycin	15 µg/Disc	R	R	R	R
Co-trimaxazole	25 µg/Disc	R	R	R	R
Levofloxacin	5 µg/Disc	S	S	S	S
Ampicillin	10 µg/Disc	S	R	R	R
Vancomycin	30 µg/Disc	S	S	S	S
Kanamycin	30 µg/Disc	S	S	S	S
Penicillin G	10 Units/Disc	R	R	R	R
Cloxacillin	5 µg/Disc	R	R	R	R
Ofloxacin	5 µg/Disc	S	S	S	S
Ceftrixone	30 µg/Disc	R	R	S	R
Gentamycin	10µg/Disc	S	S	S	S
Rifampicin	30 µg/Disc	S	S	S	R
Streptomycin	300 µg/Disc	S	S	R	S

Table 3: Acid and bile tolerance

Samples	CFU/mL											
	Acid concentration			Bile concentration (%)								
	pH 7 (Control)	pH 2	pH 3	Control (No Bile)	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1
TIL 19T	103×10^8	67×10^8	86×10^8	312×10^8	151×10^8	142×10^8	92×10^8	79×10^8	46×10^8	47×10^8	42×10^8	2×10^8
TIL 21C	94×10^8	61×10^8	72×10^8	269×10^8	181×10^8	113×10^8	103×10^8	81×10^8	63×10^8	41×10^8	36×10^8	39×10^8
TIL 28S	97×10^8	62×10^8	79×10^8	210×10^8	149×10^8	67×10^8	69×10^8	57×10^8	48×10^8	34×10^8	23×10^8	3×10^8
TIL 30R	157×10^8	84×10^8	91×10^8	243×10^8	162×10^8	101×10^8	97×10^8	83×10^8	87×10^8	51×10^8	42×10^8	24×10^8

CFU: Colony-forming unit

Table 4: Transit tolerance and lysozyme tolerance

Samples	CFU/mL							
	Control	2 h	4 h	8 h	Control (Without lysozyme)	Lysozyme concentration (mg)		
						100	200	300
TIL 19T	197×10^8	112×10^8	62×10^8	46×10^8	177×10^8	122×10^8	99×10^8	71×10^8
TIL 21C	153×10^8	72×10^8	23×10^8	2×10^8	140×10^8	120×10^8	102×10^8	54×10^8
TIL 28S	217×10^8	123×10^8	82×10^8	49×10^8	101×10^8	89×10^8	64×10^8	26×10^8
TIL 30R	236×10^8	72×10^8	47×10^8	36×10^8	214×10^8	187×10^8	119×10^8	74×10^8

CFU: Colony-forming unit

Table 5: Production of bacteriocin activity

Bacteriocin sample	Microorganisms used	Zone of inhibition (mm)			Average zone of inhibition (mm)	Resistance/ Sensitivity
		x	y	z		
TIL 19T	*E. coli*	-	-	-	-	Resistant
	S. aureus	26	30	26	27.3	Sensitive
	K. pneumoniae	-	-	-	-	Resistant
	B. subtilis	-	-	-	-	Resistant
	P. aeruginosa	21	23	22	22	Sensitive
TIL 21C	*E. coli*	-	-	-	-	Resistant
	S. aureus	-	-	-	-	Resistant
	K. pneumoniae	24	22	22	22.6	Sensitive
	B. subtilis	-	-	-	-	Resistant
	P. aeruginosa	-	-	-	-	Resistant
TIL 28S	*E. coli*	-	-	-	-	Resistant
	S. aureus	33	26	30	29.6	Sensitive
	K. pneumoniae	22	24	23	23	Sensitive
	B. subtilis	32	31	35	32.6	Sensitive
	P. aeruginosa	21	24	24	23	Sensitive
TIL 30R	*E. coli*	-	-	-	-	Resistant
	S. aureus	-	-	-	-	Resistant
	K. pneumoniae	-	-	-	-	Resistant
	B. subtilis	-	-	-	-	Resistant
	P. aeruginosa	-	-	-	-	Resistant

E. coli: *Escherichia coli*, *S. aureus: Staphylococcus aureus*, *K. pneumoniae*: *Klebsiella pneumoniae*, *B. subtilis*: *Bacillus subtilis*, *P. aeruginosa*: *Pseudomonas aeruginosa*

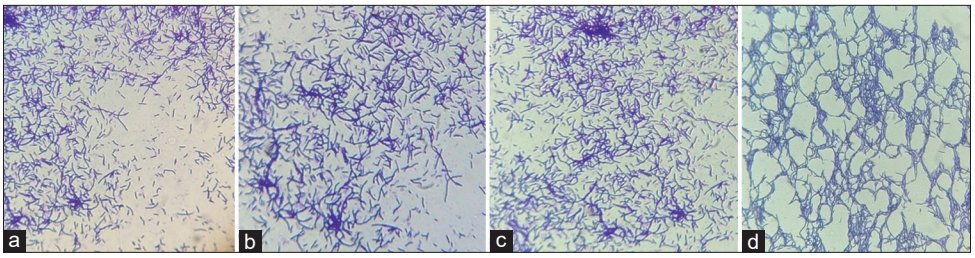

Figure 1: Depicts the Gram staining results of four bacterial samples: TIL 19T, TIL 21C, TIL 28S, and TIL 30R. Each panel within the figure corresponds to a specific sample: (a) TIL 19T, (b) TIL 21C, (c) TIL 28S, and (d) TIL 30R. It is noteworthy that all observed bacterial specimens exhibit a characteristic rod-shaped bacillus morphology

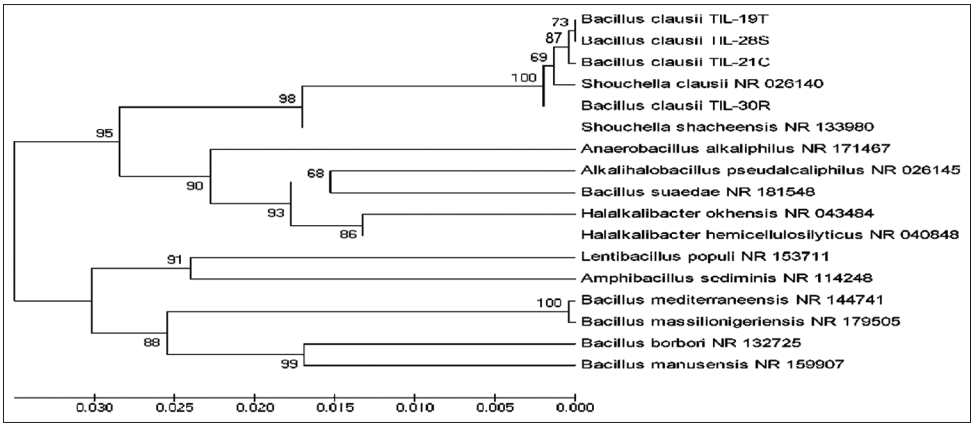

Figure 2: UPGMA tree of the identified bacterial samples TIL 19T, TIL 21C, TIL 28S, TIL 30R. UPGMA: Unweighted Pair Group Method with Arithmetic Mean

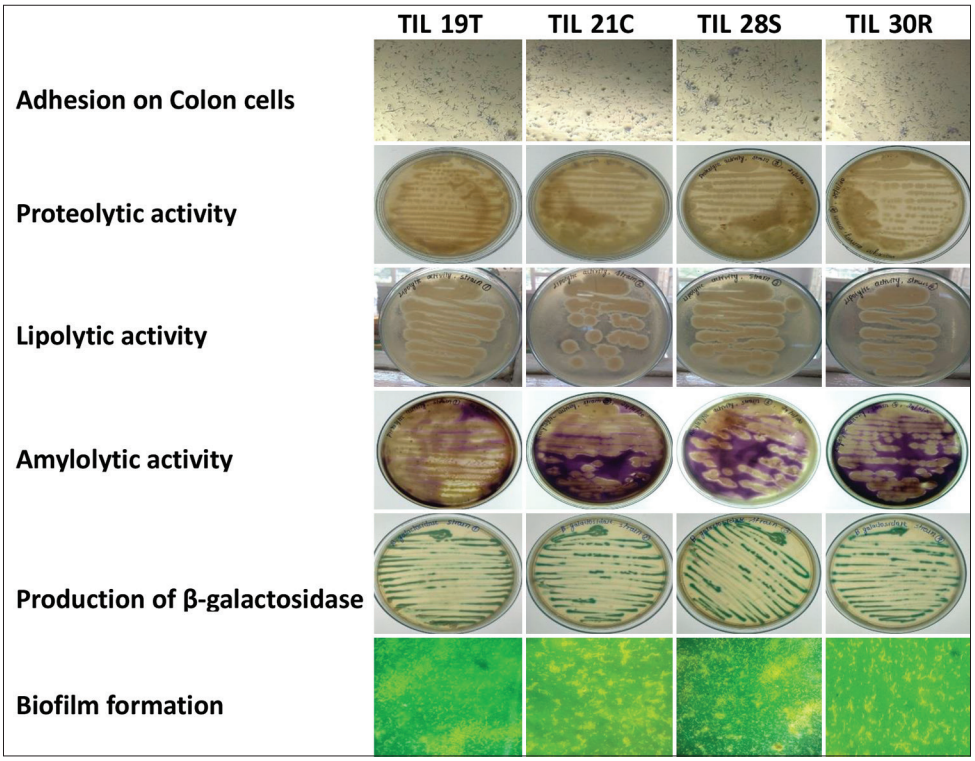

Figure 3: Depicts the comparative analysis of various *Bacillus clausii* strains (TIL 19T, TIL 21C, TIL 28S, and TIL 30R) across multiple functional parameters. These parameters include adhesion on colon cells, proteolytic activity, lipolytic activity, amylolytic activity, production of β-galactosidase, and biofilm formation

$$Coaggregation(\%) = \left(\left[\frac{ODi + ODp}{2} ODip) \right] / ODi + ODp \right) / 2 \times 100$$

Where ODi and ODp are the ODs of individual bacterial and pathogen suspensions, respectively, and ODip is the OD of the combined suspension at designated time points.

Adhesion of isolates to colon adenocarcinoma cells

Adhesion ability was evaluated using the CaCo-2 cell line following established protocols.[11-13] Cells were grown to 80% confluence, washed with phosphate-buffered saline, and incubated in serum- and antibiotic-free Dulbecco's Modified Eagle Medium. Bacterial isolates were added and incubated for 2 h at 37°C. After washing to remove non-adherent cells, bacterial adhesion was assessed microscopically and confirmed by light microscopy at 100× magnification.

Proteolytic activity

Proteolytic activity was determined by streaking isolates on skim milk agar and incubating at 30°C for 48 h. The formation of clear zones around colonies indicated casein hydrolysis and positive proteolytic activity.[15]

Lipolytic activity

Lipolytic activity was assessed on tributyrin agar plates incubated at 30°C for 4 days. Clear halo zones around colonies were considered indicative of lipid hydrolysis.[16]

Amylolytic activity

Amylase production was evaluated on starch agar plates after incubation at 37°C for 72 h. Starch hydrolysis was detected by iodine staining, with clear zones around colonies indicating positive activity.

Autolytic activity

Autolytic activity was measured by monitoring the reduction in OD630 of standardized cell suspensions incubated at 30°C for up to 24 h. The percentage of autolysis was calculated based on the decrease in OD values.

$$Autolytic\ activity(\%) = \left[ODI - \frac{ODF}{ODI} \right] \times 100$$

Classification of autolytic activity was based on the method used by Khan *et al*. (2021) and Shivangi *et al*. (2020), where 35–66% was considered good, 24–34% fair, and 0–22% poor.

Bacteriocin production

Bacterial strains were cultured in MRS broth at 37°C for 48 h. Cell-free supernatants were collected, adjusted to pH 6.5–7.0, and antimicrobial activity was evaluated using the agar well diffusion method.

Exopolysaccharide (EPS) production

EPS production was assessed in modified MRS media supplemented with different sugars. After incubation at 37°C for 3 days, EPS was extracted, precipitated with acetone, dried, and quantified by dry weight measurement.

Cholesterol removal

Cholesterol assimilation was determined by culturing isolates in cholesterol- and bile-supplemented MRS broth. Residual cholesterol was quantified spectrophotometrically using the O-phthalaldehyde method.

Heat resistance activity

Bacterial suspensions were exposed to 80°C for 5 min in skim milk. Viable counts were determined on MRS agar after incubation to evaluate heat tolerance.

β-galactosidase activity

The isolates were cultured on MRS agar containing X-gal and IPTG. Blue-green colony formation after incubation indicated β-galactosidase activity.

Biofilm formation

Biofilm formation was assessed using crystal violet staining in microtiter plates and confirmed by fluorescence microscopy using acridine orange staining.

Hydrogen peroxide, histamine, and DNase production

Hydrogen peroxide production was assessed on 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS)-Horseradish peroxidase (HRP) agar, histamine production was evaluated using histidine decarboxylase broth, and DNase activity was determined on DNase agar plates.

Cytotoxicity assay

The safety of the isolates was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay on cultured cells. Cell viability was measured spectrophotometrically following exposure to bacterial isolates.

RESULTS

The four isolates were identified as *B. clausi (S. clausii)* strains TIL 19T, TIL 21C, TIL 28S, and TIL 30R through morphological, biochemical, and 16S *rRNA* analyses (Figure 1). All strains were Gram-positive, spore-forming bacteria with typical probiotic characteristics (Figure 2). Antibiotic susceptibility testing revealed strain-specific resistance profiles, while all isolates exhibited γ-hemolysis, indicating a favorable safety profile (Table 2).

Acid, bile, transit, and lysozyme tolerance assays demonstrated strong gastrointestinal survival abilities in all the four strains (Tables 3 and 4). High cell-surface hydrophobicity, auto-aggregation, and co-aggregation abilities suggested good intestinal adhesion potential (Figure 3). Microscopic observations confirmed adhesion to epithelial cells (Table 5).

All strains exhibited protease, lipase, amylase, EPS production, β-galactosidase activity, and heat resistance. Several isolates showed antagonistic activity against pathogenic bacteria and demonstrated cholesterol removal capabilities. Moderate biofilm formation was observed, whereas hydrogen peroxide production, histamine formation, and DNase activity were absent. Cytotoxicity studies indicated high cell viability, supporting the safety of the strains.

DISCUSSION

The investigated *B. clausii (S. clausii)* strains exhibited several desirable probiotic traits, including antibiotic resistance, gastrointestinal survival, pathogen inhibition, and safety. Their ability to tolerate acidic, bile, and digestive conditions supports their potential to survive within the gastrointestinal tract and confer health benefits. Similar findings have been reported for other probiotic bacteria, where acid and bile tolerance are considered essential characteristics for successful colonization and functionality.

The high adhesion, aggregation, and biofilm-forming abilities observed in the strains may enhance intestinal colonization and competitive exclusion of pathogens. In addition, the production of beneficial enzymes, EPS, and cholesterol-lowering activity further strengthens their probiotic potential. The absence of hemolytic activity, histamine production, and DNase activity highlights their safety for potential use in functional foods and probiotic formulations. Overall, these findings support the application of *B. clausii* strains as promising probiotic candidates for promoting gut health and combating antibiotic-associated gastrointestinal disorders.

CONCLUSION

The four poly-antibiotic-resistant *B. clausii (S. clausii)* strains, TIL 19T, TIL 21C, TIL 28S, and TIL 30R, demonstrated promising probiotic properties and a favorable safety profile. Their ability to tolerate acidic, bile, and gastrointestinal conditions, along with adhesion, biofilm formation, pathogen inhibition, and cholesterol reduction, highlights their potential as effective probiotic candidates. The absence of hemolytic activity, hydrogen peroxide production, histamine synthesis, and DNase activity further supports their safety. These findings suggest that the investigated strains may help alleviate antibiotic-associated dysbiosis and contribute to gut health, warranting further clinical evaluation.

AUTHORS' CONTRIBUTIONS

R.R., A.D.W., M.N., and R.K.: Conceptualization and study design; T.V.V.: Experimental work and data analysis; S.M.: Data interpretation and manuscript drafting; R.R.: Manuscript preparation; R.R., T.V.V., A.D.W., S.M., P.T.K., E.A., B.M., M.K., and D.S.P.: Manuscript review and editing. All authors reviewed and approved the final manuscript.

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