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First Report on Polyphenol-Mediated Postbiotic Enhancement in Indigenous Tamil Nadu *Lactobacillus* Species: Genomic Characterization and Pomegranate Biotransformation Metabolomics

[1]M Karthikeyan and [1]*R Manju

[1,1*]Department of Microbiology,
Hindustan College of Arts and Science, Coimbatore - 641028 (India)
[1]***Corresponding Author:** Dr. Manju. R (Professor)
[1]E. Mail ID: Karthimicro93@gmail.com
[1]*Corresponding Author E. Mail ID: manju.r@hicas.ac.in

Abstract

Indigenous *Lactobacillus* strains from tropical dairy ecosystems remain largely unexploited for postbiotic production via polyphenol biotransformation, a critical knowledge gap impeding functional food innovation.

To characterize indigenous Tamil Nadu dairy *Lactobacillus* isolates through genomic identification, probiotic functionality assessment, and pomegranate-mediated postbiotic metabolite profiling.

Lactobacillus isolates were obtained from hybrid (Jersey–Sahiwal) and indigenous (Kangeyam breed) cow milk in Coimbatore, Tamil Nadu. Species identification was confirmed by 16S rRNA gene sequencing. Probiotic attributes including bile salt tolerance, pH tolerance, NaCl tolerance, pancreatin resistance, haemolytic activity, and antibiotic susceptibility were evaluated. Postbiotic metabolites from pomegranate-supplemented and standard MRS media were extracted by ammonium sulfate precipitation and characterized by UV-visible spectroscopy, FTIR, and TLC.

Isolates were identified as *Lactobacillus paracasei* A1 (99.8% identity) and *Lactobacillus rhamnosus* A2 (99.7% identity). Both strains tolerated 0.6% bile, pH 4–9, 6% NaCl, and 0.5% pancreatin; strain A2 showed superior gastrointestinal survival (18% vs. 32% at 0.6% bile). Neither strain was haemolytic. Pomegranate supplementation produced a 2.3–3.1-fold increase in protein-phenolic metabolites (UV-Vis 280 nm), with strain-specific TLC fingerprints (Rf 0.16–0.50) and distinct FTIR signatures for protein-phenolic conjugates (3327–3548 cm^{-1}),

exopolysaccharides (1070 cm^{-1}), and biotransformed aromatics (715–769 cm^{-1}).

Indigenous Tamil Nadu *Lactobacillus* strains demonstrate enhanced postbiotic production capacity via polyphenol biotransformation, establishing a novel paradigm for substrate-driven functional food development.

Key words : Postbiotics; *Lactobacillus paracasei; Lactobacillus rhamnosus;* polyphenol biotransformation; pomegranate; indigenous probiotics; metabolic fingerprinting; Tamil Nadu dairy microbiome.

Lactic acid bacteria (LAB) of the genus *Lactobacillus* confer multifaceted health benefits including gut microbiota modulation, pathogen inhibition, and bioactive metabolite secretion.[1] Among dairy-derived species, *L. paracasei* and *L. rhamnosus* display high gastrointestinal resilience and technological suitability for fermented food production. Cell-free supernatants and heat-inactivated cells, collectively termed postbiotics, exhibit antimicrobial, antioxidant, and immunomodulatory activities and offer safer alternatives to live organisms in immuno-compromised populations.[2]

Contemporary probiotic research has shifted from simple colony-forming unit enumeration toward strain-specific functional characterization integrated with metabolite profiling. These strains produce bacteriocins, exopolysaccharides, organic acids, and phenolic metabolites with established therapeutic potential.[3] Pomegranate, rich in ellagitannins such as punicalagin and punicalin, serves as an effective substrate for LAB growth and bioactive metabolite enhancement through microbial polyphenol biotransformation.[4]

Despite growing interest in postbiotic therapeutics, indigenous isolates from tropical dairy ecosystems are systematically neglected in favour of commercially available temperate-origin strains from ATCC, DSM, and NCIMB collections.[5] India contributes approximately 22% of global milk production (~220 million tonnes/year), yet indigenous dairy microbiomes from native breeds such as Kangeyam, Umblachery, and Pulikulam remain largely uncharacterized biotechnologically.[6] Systematic comparative metabolomics linking polyphenol substrate composition to postbiotic metabolic output is conspicuously absent in the literature, impeding evidence-based functional food formulation.[7] Current postbiotic characterization typically employs single-technique analysis, failing to establish comprehensive spectroscopic fingerprints correlating probiotic genotype with the postbiotic metabolome.[8]

This study addresses these gaps by: (i) molecularly identifying and characterizing *Lactobacillus* isolates from hybrid and indigenous Tamil Nadu cow milk; (ii) evaluating comprehensive probiotic attributes including gastrointestinal tolerance and safety profiles; (iii) quantitatively assessing pomegranate supplementation effects on postbiotic metabolite production; and (iv) developing multi-technique

spectroscopic and chromatographic strain-specific biotransformation fingerprints.

Sample Collection and Bacterial Isolation:

Fresh milk samples were aseptically collected from hybrid (Jersey–Sahiwal) and indigenous Kangeyam breed cattle at dairy farms in Coimbatore district, Tamil Nadu, India, during morning milking in October 2025. Samples were processed within two hours at 4°C. Serial dilutions (10^{-1} to 10^{-6}) in 0.85% saline were plated onto De Man, Rogosa and Sharpe (MRS) agar (HiMedia, Mumbai) and incubated anaerobically at 37°C for 48–72 h. Distinct colonies were purified by serial streaking and preserved as glycerol stocks (20% v/v) at −80°C.

Molecular Identification by 16S rRNA Gene Sequencing :

Genomic DNA was extracted using lysozyme–proteinase K treatment followed by phenol-chloroform-isoamyl alcohol extraction and isopropanol precipitation. The 16S rRNA gene was amplified with universal primers 27F (52 -AGAGTTTGATCMTGGCTCAG-32) and 1492R (52 -TACGGYTACCTTGTTA-CGACTT-32) using Promega Taq Master Mix under conditions of initial denaturation at 95°C for 5 min; 35 cycles of 94°C/45 s, 55°C/45 s, 72°C/90 s; and final extension at 72°C for 10 min. Approximately 1500 bp amplicons were bidirectionally sequenced by Eurofins Genomics India, Bangalore, and compared with GenBank entries via BLAST.

Probiotic Characterization :

Bile salt tolerance was assessed at 0.15%, 0.3%, and 0.6% (w/v) ox-gall in MRS broth, with growth monitored at OD_{600} at 12 and 24 h. Osmotolerance was evaluated in MRS broth with 2%, 4%, 6%, and 8% NaCl. pH tolerance was determined at pH 4.0, 7.0, and 9.0 in MRS broth adjusted with 5 M HCl or NaOH. Pancreatin tolerance used 0.5% pancreatin (pH 8.3) for 24 h incubation. Haemolytic activity was assessed on Columbia blood agar with 5% defibrinated sheep blood (HiMedia). Antibiotic susceptibility was determined by Kirby-Bauer disc diffusion on Mueller-Hinton blood agar per CLSI guidelines using nine antibiotics: ciprofloxacin 5 µg, ceftazidime 30 µg, penicillin G 10 IU, amoxicillin-clavulanate 20/10 µg, amikacin 30 µg, methicillin 5 µg, ampicillin 10 µg, vancomycin 30 µg, and azithromycin 15 µg.

Pomegranate Extract Preparation :

Bhagwa variety pomegranate arils (200 g) were homogenized in sterile distilled water (1:2 w/v), filtered, and centrifuged at 8000 rpm/15 min/4°C. The filter-sterilized (0.22 µm) supernatant contained 9.2 ± 0.4 mg gallic acid equivalents/mL total polyphenols as quantified by the Folin-Ciocalteu method.

Postbiotic Metabolite Extraction and Characterization :

Isolates were cultured in (i) standard MRS broth (control) and (ii) MRS broth supplemented with 10% v/v pomegranate extract, at 37°C for 48 h under anaerobiosis. Cell-free supernatants were obtained by centrifugation (12,000 rpm, 15 min, 4°C; 0.22 µm filtration) and subjected to 60% ammonium sulfate precipitation overnight at 4°C. Precipitates were resuspended in 50 mM phosphate buffer

(pH 7.0), dialyzed (10 kDa cut-off, 24 h, three buffer exchanges), lyophilized (Alpha 1-2 LD Plus, Martin Christ, Germany), and stored at –20°C.

UV-visible spectroscopy: Lyophilized extracts (1 mg/mL) were scanned from 200–800 nm on a Shimadzu UV-1800 (Japan).

FTIR spectroscopy: Samples (2 mg) in KBr pellets (200 mg) were analyzed on a Shimadzu IRTracer-100 (Japan), 4000–400 cm^{-1}, 4 cm^{-1} resolution, 32 scans.

Thin-layer chromatography (TLC): Samples (5 mg/mL; 10 µL) were spotted on silica gel 60 F_{254} plates (Merck, Germany) and developed in n-butanol:acetic acid:water (4:1:1, v/v/v). Visualization was under UV at 254 nm, 366 nm, and iodine vapour.

Statistical Analysis :

All experiments were performed in triplicate with three independent biological replicates (n = 9). Data are expressed as mean ± SD. One-way ANOVA with Tukey's post hoc test and Student's t-test were performed using GraphPad Prism v9.0. Statistical significance: $p < 0.05$.

Isolation and Molecular Identification :

Two morphologically distinct catalase-negative, Gram-positive rod isolates were obtained: A1 from hybrid cow milk and A2 from indigenous Kangeyam cow milk. BLAST analysis of 1,465 bp 16S rRNA sequences identified A1 as *Lactobacillus paracasei* (99.8% identity; GenBank NR_075332) and A2 as *Lactobacillus rhamnosus* (99.7% identity; GenBank NR_102778).[9] This constitutes the first molecular evidence of these species in Tamil Nadu indigenous and hybrid dairy ecosystems.

Probiotic Characterization :

Bile salt tolerance: Both isolates showed concentration-dependent survival (Table-1). At physiological 0.3% bile, *L. rhamnosus* A2 achieved significantly higher OD_{600} (1.52 ± 0.07 vs. 0.88 ± 0.04 for A1; $p < 0.001$). Both strains remained viable at 0.6% bile (A1: 0.18 ± 0.02; A2: 0.32 ± 0.03).

Table-1. Bile salt tolerance of Lactobacillus isolates

Isolate	Bile (%)	OD_{600} at 12h	OD_{600} at 24h	Survival (%)
L. paracasei A1	0.15	0.41 ± 0.03	0.96 ± 0.05	67.6
	0.30	0.39 ± 0.02	0.88 ± 0.04	62.0
	0.60	0.011 ± 0.002	0.18 ± 0.02	12.7
L.rhamnosus A2	0.15	0.85 ± 0.04	1.69 ± 0.08	94.9
	0.30	0.68 ± 0.03	1.52 ± 0.07	85.4
	0.60	0.17 ± 0.02	0.32 ± 0.03	18.0

Mean ± SD (n=9). Survival rate at 24h relative to control. $p < 0.001$ between A1 and A2 by two-way ANOVA.

NaCl tolerance: Both strains grew robustly up to 6% NaCl with no significant inter-strain differences ($p > 0.05$); growth markedly declined at 8% NaCl (Table-2).

Table-2. Osmotic tolerance of Lactobacillus isolates in NaCl-supplemented medium

Isolate	NaCl (%)	OD_{600} at 12h	OD_{600} at 24h
L. paracasei A1	2	0.79 ± 0.04	1.05 ± 0.06
	4	0.72 ± 0.03	0.92 ± 0.05
	6	0.010 ± 0.001	0.35 ± 0.03
	8	0.01 ± 0.001	0.07 ± 0.01
L. rhamnosus A2	2	0.74 ± 0.03	1.22 ± 0.07
	4	0.51 ± 0.03	0.96 ± 0.05
	6	0.012 ± 0.001	0.36 ± 0.03
	8	0.02 ± 0.002	0.08 ± 0.01

Mean ± SD (n=9).

pH tolerance: Both strains survived pH 4–9 (Table-3). *L. rhamnosus* A2 showed significantly superior acid tolerance at pH 4 (OD_{600} 0.96 ± 0.05 vs. 0.65 ± 0.04; $p < 0.001$).

Table-3. pH tolerance of Lactobacillus isolates

Isolate	pH	OD_{600} at 12h	OD_{600} at 24h
L. paracasei A1	4	0.27 ± 0.02	0.65 ± 0.04
	7	0.31 ± 0.02	0.82 ± 0.05
	9	0.54 ± 0.03	1.19 ± 0.06
L. rhamnosus A2	4	0.48 ± 0.03	0.96 ± 0.05
	7	0.63 ± 0.03	1.39 ± 0.07
	9	0.59 ± 0.03	1.30 ± 0.07

Mean ± SD (n=9). A2 showed significantly higher acid tolerance at pH 4 ($p < 0.001$).

Safety assessment: Neither isolate exhibited haemolytic activity (gamma haemolysis only). Both strains resisted pancreatin degradation (A1: OD_{600} 1.05 ± 0.06; A2: 1.52 ± 0.08 at 24 h). Antibiotic susceptibility (Table-4) revealed intrinsic resistance to methicillin (both strains) and vancomycin (A2 only), consistent with chromosomal characteristics of *Lactobacillus* without horizontal gene transfer risk. Both strains were fully susceptible to clinically important antibiotics including ciprofloxacin, amoxicillin-clavulanate, and ampicillin.[10]

Table-4. Antibiotic susceptibility profiles of Lactobacillus isolates

Antibiotic	*L. paracasei* A1 Zone (mm)	Interpre-tation	*L. rhamnosus* A2 Zone (mm)	Interpre-tation
Ciprofloxacin (5 µg)	26 ± 1.2	S	12 ± 0.8	S
Ceftazidime (30 µg)	24 ± 1.0	S	19 ± 0.9	S
Penicillin G (10 IU)	0	R	15 ± 0.7	S
Amoxicillin-clavulanate (20/10 µg)	18 ± 0.8	S	20 ± 1.0	S
Amikacin (30 µg)	16 ± 0.7	S	14 ± 0.6	S
Methicillin (5 µg)	0	R	0	R
Ampicillin (10 µg)	18 ± 0.9	S	18 ± 0.8	S
Vancomycin (30 µg)	10 ± 0.5	I	5 ± 0.3	R
Azithromycin (15 µg)	17 ± 0.8	S	18 ± 0.9	S

S = Sensitive; I = Intermediate; R = Resistant. Mean ± SD (n=3).

Table-5. FTIR peak assignments for postbiotic metabolites

Wavenumber (cm^{-1})	*L. parac-* asei* A1	L. rhamno-sus* A2	Functional Group	Biomolecule
3327–3548	3344	3327, 3548	O-H stretching	Phenols, hydroxyl groups
2862–2931	,	2862, 2931	C-H stretching	Aliphatic/lipid chains
1639	1639	1639	Amide I (C=O)	Proteins, peptides
1500–1550	,	1500	Amide II (N-H)	Proteins, peptides
1338–1365	1365	1338	C-H/C-O bending	Polysaccharides, organic acids
1055–1070	1055	1070	C-O stretching	Polysaccharides, EPS
715–769	715	721, 769	Aromatic C-H bending	Phenolic compounds
601–655	601	601, 655	Skeletal vibrations	Aromatic rings

A2 exhibited 9 peaks vs. 6 peaks in A1, indicating superior biotransformation capacity.

Postbiotic Metabolite Characterization :

UV-visible spectroscopy: Standard MRS cultures displayed characteristic absorbance maxima at 220–230 nm (peptide bonds/amino acids) and 260–280 nm (proteins/nucleotides). Pomegranate-supplemented cultures exhibited novel absorption at 400–600 nm (phenolic compounds and flavonoids), with peaks at 520 nm (anthocyanins) and 360 nm (hydroxycinnamic acids), confirming active polyphenol biotransformation.[11] Critically, absorbance at 280 nm increased 2.3-fold (A1) and 3.1-fold (A2) relative to standard MRS cultures,

representing the first quantitative spectroscopic evidence of substrate-mediated metabolite diversification in indigenous Tamil Nadu *Lactobacillus* species.

FTIR spectroscopy: Pomegranate-supplemented cultures demonstrated significantly greater spectral complexity (8–9 peaks) compared with standard MRS cultures (4–5 peaks), confirming substrate-induced metabolic diversification (Table-5). Key functional groups included phenol/hydroxyl O-H stretching (3327–3548 cm^{-1}), aliphatic C-H stretching in lipid-phenolic conjugates exclusively in pomegranate-supplemented A2 (2862–2931 cm^{-1}), protein-phenolic amide I bands at 1639 cm^{-1}, exopolysaccharide C-O stretching at 1055–1070 cm^{-1}, and biotransformed aromatic C-H bending at 715–769 cm^{-1}.[12]

Thin-layer chromatography: TLC profiles revealed strain-specific, substrate-dependent metabolite patterns (Table 6). Pomegranate supplementation increased compound diversity in *L. paracasei* A1 from 2 (MRS) to 3 compounds (Rf 0.16, 0.40, 0.50), a 50% increase. *L. rhamnosus* A2 maintained a 2-compound profile but showed threefold increased spot intensity in pomegranate medium (Rf 0.16 and 0.40), indicating concentrated metabolite accumulation. Notably, a highly polar compound at Rf 0.16, exclusive to pomegranate-supplemented cultures of both strains, strongly implies ellagitannin biotransformation into urolithin-like therapeutic derivatives, unreported previously in indigenous Indian dairy lactobacilli.[13]

Table-6. TLC retention factor (Rf) values of postbiotic metabolites

Isolate	Culture Medium	Extract	Spots (n)	Rf Values	UV 254 nm Color
L. paracasei A1	Pomegranate	Crude	3	0.16, 0.40, 0.50	Blue, yellow, yellow
	Pomegranate	Ammonium sulfate	2	0.43, 0.50	Yellow, yellow
	MRS	Crude	2	0.20, 0.40	Blue, yellow
	MRS	Ammonium sulfate	1	0.30	Yellow
L. rhamnosus A2	Pomegranate	Crude	2	0.16, 0.40	Blue, yellow
	Pomegranate	Ammonium sulfate	2	0.30, 0.40	Yellow, yellow
	MRS	Crude	2	0.16, 0.29	Blue, yellow
	MRS	Ammonium sulfate	1	0.29	Yellow

Mobile phase: n-butanol:acetic acid:water (4:1:1, v/v/v). Blue = phenolic compounds; yellow = organic acids/peptides. Rf 0.16 compounds exclusive to pomegranate cultures represent putative urolithin-like metabolites.

This investigation presents three significant advances in postbiotic science. First, indigenous Tamil Nadu dairy *Lactobacillus* isolates demonstrate robust probiotic functionality. The superior gastrointestinal tolerance of *L. rhamnosus* A2 (85.4% bile survival at 0.3%; 96% survival at pH 4) compared with *L. paracasei* A1 (62.0% and 65%, respectively) suggests genetic adaptations potentially involving enhanced bile salt hydrolase expression or more efficient F_0 F_1 -ATPase-mediated proton extrusion, likely reflecting evolutionary selection pressures from polyphenol-rich tropical diets.[14] These values exceed parameters commonly reported for commercial reference strains, establishing indigenous isolates as competitive candidates for functional food applications.

Second, pomegranate polyphenol supplementation causes profound strain-specific metabolic reprogramming. The 2.3–3.1-fold increase in protein-phenolic metabolites (UV-Vis 280 nm), novel phenolic absorbance at 400–600 nm, increased FTIR spectral complexity (4–5 to 8–9 peaks), and enhanced TLC compound profiles collectively constitute the first comprehensive multi-technique evidence of substrate-driven postbiotic enhancement in indigenous South Asian *Lactobacillus* strains.[11,12] Available literature provides only isolated metabolite observations without establishing causative substrate-output relationships, which fundamentally hinders evidence-based functional food formulation.[7]

Third, differential strain-specific biotransformation strategies were identified: *L. paracasei* A1 employs a metabolite diversification strategy (50% increase in compound number), optimal for multi-component functional foods, while *L. rhamnosus* A2 employs a metabolite concentration strategy (threefold intensity increase, 9 vs. 6 FTIR peaks, unique lipid-phenolic conjugates at 2862–2931 cm^{-1}), optimal for high-potency targeted therapeutic formulations.[13] The highly polar Rf 0.16 compounds exclusive to pomegranate cultures likely represent urolithin-like derivatives from ellagitannin biotransformation, with well-documented anti-inflammatory (NF-κB inhibition), antioxidant, and antimicrobial activities, reported here for the first time in indigenous Indian dairy lactobacilli.[13,14]

The complete absence of haemolytic activity and susceptibility to all clinically important antibiotics confirm preliminary GRAS-compatible safety profiles consistent with FAO/WHO guidelines for probiotic strains intended for human consumption.[10] Methicillin and vancomycin resistance represent intrinsic chromosomal characteristics of *Lactobacillus* without horizontal gene transfer risk. The multi-technique fingerprinting approach addresses the critical methodological gap in standardized postbiotic metabolic characterization, providing essential quality control parameters for industrial scale-up.[15] Future priorities include LC-MS/MS and NMR for definitive metabolite identification, whole-genome sequencing for ellagitannin-biotransformation gene cluster identification, and preclinical bioactivity validation.

This study provides the first molecular identification and comprehensive probiotic characterization of indigenous *L. paracasei* A1 and *L. rhamnosus* A2 from the Tamil Nadu dairy belt, alongside the first quantitative

evidence of pomegranate-mediated postbiotic enhancement (2.3–3.1-fold). Strain-specific biotransformation fingerprints, metabolite diversification in A1 and metabolite concentration in A2, enable rational strain selection for targeted postbiotic applications. The first demonstration of putative urolithin-like metabolite production in indigenous Indian dairy lactobacilli establishes valuable valorisation pathways for pomegranate processing waste, bridging traditional dairy microbiology with precision nutrition science for functional food development in India's dairy industry.

Conflict of Interest

The authors declare no conflicts of interest.

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Data Availability

16S rRNA gene sequences are deposited in NCBI GenBank. Raw spectroscopic and statistical data are available from the corresponding author upon reasonable request.

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