

OPTIMIZATION AND CHARACTERIZATION OF GABA PRODUCTION BY *LACTIPLANTIBACILLUS PLANTARUM* PPKMB1 AND PRELIMINARY CYTOTOXICITY AGAINST HCT-116 COLORECTAL CANCER CELLS

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ABSTRACT

γ -Aminobutyric acid (GABA) is a bioactive non-protein amino acid, and Lactic Acid Bacteria (LAB) offer a food-grade route to its production. A GABA-producing isolate from raw cow milk was identified as *Lactiplantibacillus plantarum* PPKMB1 (GenBank MZ951173) by phenotypic and 16S rRNA analyses. It survived pH 3.0 (>90%, 3 h), tolerated >0.8% ox bile and 4–8% NaCl, showed 35% hydrophobicity and was γ -haemolytic. GABA production was peaked at 48 h, initial pH 7.0 and 40 °C, with xylose (464.2 μ g/mL) and tryptone (610.8 μ g/mL) as the best carbon and nitrogen sources. TLC (R_f 0.672), UV-visible and FTIR spectroscopy and GC-MS (retention time 2.963 versus 2.912 min for the standard) supported GABA identity, and GC-MS quantified 862.39 mg/L in the purified sample. An L1-derived fraction (6.25–100 μ g/mL, 24 h) reduced HCT-116 colorectal cancer cell viability from 97.4% to 31.4% (MTT assay), with a preliminary IC_{50} of approximately 76 μ g/mL; doxorubicin (5 μ M) gave 32.3%. Because the fraction was not pure GABA, the cytotoxicity cannot be attributed to GABA alone, and mechanistic and normal-cell studies are required.

KEYWORDS: γ -aminobutyric acid; *Lactiplantibacillus plantarum*; fermentation optimization; GC-MS; FTIR; HCT-116; MTT assay; colorectal cancer

1. INTRODUCTION

γ -Aminobutyric acid (GABA) is a four-carbon, non-protein amino acid formed by the α -decarboxylation of L-glutamate, a reaction catalysed by the pyridoxal-5'-phosphate-dependent enzyme glutamate decarboxylase (GAD) [1, 2]. In the mammalian central nervous system, GABA is the principal inhibitory neurotransmitter, but interest in the molecule which extends well beyond neuroscience. Dietary and microbially derived GABA has been associated with antihypertensive, anxiolytic, sleep-promoting, antidiabetic, anti-inflammatory and antioxidant effects, and GABA-enriched foods which are marketed in several countries as functional products [3, 4]. Peripheral GABA is also increasingly recognised as a gut–brain axis signal, with intestinal bacteria contributing substantially to luminal and circulating GABA [5]. Outside the nervous system, GABA receptors are expressed in epithelial tissues, including the colon, and GABAergic signalling influences the proliferation, migration and immune microenvironment of several tumours [6, 7]. This aspect is particularly relevant to colorectal cancer, which ranked third in incidence and second in mortality worldwide in 2022 [8, 9].

Chemical synthesis of GABA is poorly suited to food applications, and plant enrichment yields comparatively small amounts. Microbial fermentation, particularly with LAB, has therefore become the preferred route to natural GABA [1, 10]. LAB possess a GAD system, comprising the *gadB*-encoded decarboxylase and the GadC glutamate/GABA antiporter, which consumes an intracellular proton with each decarboxylation and thereby helps the cell withstand acid stress [2, 10]. GABA yields vary widely between species and strains and are strongly determined by culture conditions: initial pH, temperature, incubation time, the concentration of glutamate or monosodium glutamate, cofactor availability and the carbon and nitrogen sources all modulate GAD expression and activity [10–12]. Recent studies have accordingly combined screening of new isolates with stepwise or statistical optimization, reporting yields from a few hundred milligrams to several grams per litre [11, 13–16]. Dairy environments, including raw and spontaneously fermented milk, remain productive sources of GABA-producing LAB [17, 18].

Among LAB, *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*) is exceptionally versatile. It colonises dairy, plant, meat and gastrointestinal niches, has a long history of safe use in fermented foods and it is widely employed as a probiotic [19–21]. Strains of this species tolerate gastric acid and bile, adhere to intestinal epithelium, show antimicrobial and immunomodulatory activity, and several are efficient GABA producers [2, 11, 14, 22]. *L. plantarum* metabolites have also attracted attention in cancer research: cell-free supernatants and postbiotic fractions of different strains have reduced the proliferation of colorectal and gastric cancer cells, induced apoptosis or autophagy, and interfered with pro-tumorigenic signalling in vitro [23–26]. Postbiotics,

defined as preparations of inanimate microorganisms and/or their components that confer a health benefit, are attractive because they avoid the safety concerns associated with administering live bacteria [27]. Despite this progress, few studies have linked the optimization and analytical confirmation of GABA production by an individual dairy *L. plantarum* isolate to a biological read-out relevant to colorectal cancer. Most GABA optimization studies stop at yield, whereas most anticancer studies of *L. plantarum* use crude supernatants without characterizing their GABA content. Moreover, the role of GABA in colorectal cancer is itself debated: exogenous and microbially produced GABA inhibited colon cancer cell proliferation through GABA-A and GABA-B receptors in some models [28, 29], whereas tumour-derived GABA promoted β -catenin signalling and immune evasion in others [30, 31]. In earlier screening work, we isolated *L. plantarum* PPKMB1 from raw cow milk and observed that it produced more GABA than a co-isolated *Lactiplantibacillus pentosus* strain. The aims of the present study were therefore to (i) confirm the identity and key probiotic attributes of PPKMB1, (ii) optimize five fermentation factors governing its GABA production, (iii) confirm the product using complementary chromatographic and spectroscopic methods and (iv) obtain a preliminary estimate of the cytotoxicity of an L1-derived, GABA-containing fraction against HCT-116 cells. The novelty of the work lies in integrating strain characterization, process optimization, analytical confirmation and an initial anticancer screen for a single dairy isolate, while explicitly defining what the cytotoxicity data can and cannot show.

2. MATERIALS AND METHODS

2.1 Isolation and Identification of *Lactiplantibacillus plantarum* PPKMB1

Raw cow milk was collected aseptically near Eachanari, Coimbatore, India, and held at 4 °C until processing. Media and analytical-grade chemicals were from HiMedia, NICE Chemicals and Merck. Serial dilutions (1 mL) were plated on de Man, Rogosa and Sharpe (MRS) agar and incubated at 37 °C for 42 h, and colonies with typical LAB morphology were sub-cultured to purity; one isolate (L1) was retained. Presumptive identification used colony and cell morphology, Gram staining, catalase, motility and fermentation type. The 16S rRNA gene was amplified with primers 63F (5'-CAGGCCTAACACATGCAAGTC-3') and 1387R (5'-GGGCGGGGTGTACAAGGC-3') and sequenced on an ABI 3500xL Genetic Analyzer. The sequence was compared with GenBank entries by BLASTn, placed in a phylogenetic tree with related reference sequences and deposited in GenBank.

2.2 Evaluation of Probiotic Characteristics

Cells from a 24-h MRS culture were washed and resuspended in saline. Acid tolerance was tested in MRS broth at pH 2.5, 3.0 and 6.0 (control) containing 1% pepsin at 37 °C, with viable counts on MRS agar at 0, 1, 2 and 3 h [14, 18]. Bile tolerance was examined in MRS broth with 0.1–8.0% ox bile by optical density; growth at 0.3% bile was taken to indicate tolerance. NaCl tolerance (0–15%) was assessed by turbidity. Cell-surface hydrophobicity was measured by microbial adhesion to hydrocarbon: cells in PBS (pH 7.2) were read at 600 nm (A_0), vortexed with the hydrocarbon, and the aqueous phase was read again (A); hydrophobicity (%) = $[(A_0 - A)/A_0] \times 100$. Haemolysis was recorded on sheep blood agar.

2.3 Growth Kinetics and Production of GABA

The isolate was grown in MRS broth at 37 °C from a fresh 24-h inoculum. Growth was followed spectrophotometrically at 24-h intervals, and biomass was estimated by weighing the cell pellet from a fixed culture volume. The basal GABA-production medium contained glucose (25 g/L), soya peptone (25 g/L), $MnSO_4 \cdot 4H_2O$ (0.01 g/L), Tween-80 (2 mL/L) and L-glutamate (500 mM). It was inoculated and incubated in an orbital shaker (100 rpm) for 5 days, with daily sampling for biomass and GABA.

2.4 Optimization of GABA Production

GABA production was optimized in MRS broth one factor at a time: incubation period (24, 48 and 96 h at 37 °C), initial pH (5, 6, 7 and 8, adjusted with HCl or NaOH), temperature (30, 40 and 50 °C), carbon source (xylose, sucrose, maltose or cellulose) and nitrogen source (tryptone, casein or rice husk, with KH_2PO_4 included as a non-organic comparator). Cell-free supernatants were analysed by the colorimetric method (Section 2.5), and absorbance at 512 nm (A_{512}) was converted to GABA using the standard calibration.

2.5 Extraction and Analytical Characterization of GABA

Cultures (24–48 h) were centrifuged at 5000 rpm for 5 min. The cell-free supernatant was brought to 60% ammonium sulphate (12 g per 20 mL) and held at 4 °C for 12 h; the precipitate was recovered by centrifugation, dissolved in phosphate buffer and purified by column chromatography. For colorimetric estimation, 1 mL of sample was mixed with 0.6% $CuSO_4$, incubated at 40 °C for 40 min and read at 512 nm against a GABA standard (1 μ g/mL). For TLC, 2 μ L of filtered supernatant and standard were spotted on silica gel plates, developed in n-butanol:acetic acid:water (5:3:2), sprayed with 0.1% ninhydrin and dried at 60–70 °C, and R_f values were calculated; bands were eluted in 75% ethanol with 0.6% $CuSO_4$ (40 °C, 40 min) and read at 512 nm. UV–visible spectra (200–800 nm) were recorded on an LT-291 spectrophotometer (Labtronics). ATR-FTIR spectra were recorded on a Shimadzu spectrometer with a MIRacle 10 ZnSe accessory (4000–400 cm^{-1} , 4 cm^{-1} resolution, 32 scans).

GC-MS was performed on a Shimadzu GC-MS/MS triple-quadrupole system with an AOC-20i autosampler and a DB-5 column (30 m $\times$ 0.25 mm, 1 μ m film; Agilent). One microlitre was injected (split 10:1); injector, ion-source and interface temperatures were 280, 200 and 280 °C, and helium flowed at 1.10 mL/min. The oven was held at 100 °C for 4 min, raised at 10 °C/min to 320 °C and held for 11 min. Electron ionization (70 eV) with

selected ion monitoring was used, and GABA was identified by retention time and mass spectrum against a 5-ppm standard. Samples were diluted 100-fold in methanol, and concentration = (sample peak area × standard concentration × dilution factor)/standard peak area.

2.6 Preparation of the L1-Derived Test Fraction

The material tested in the cytotoxicity assay, coded 'L1', was the GABA-containing fraction recovered from *L. plantarum* PPKMB1 fermentation by the procedure in Section 2.5. It is a microbially derived fraction, not analytical-grade GABA, and may also contain co-purified metabolites such as other amino acids, peptides, organic acids or residual salts. The dried fraction was weighed and dissolved on a weight-per-volume basis in sterile vehicle [authors to specify solvent and stock concentration], filter-sterilized (0.22 µm) and stored at 4 °C protected from light. Working concentrations of 6.25, 12.5, 25, 50 and 100 µg/mL were prepared by two-fold serial dilution in complete medium immediately before use.

2.7 In-Vitro Cytotoxicity Against HCT-116 Cells

HCT-116 human colorectal carcinoma cells (NCCS, Pune, India) were maintained in high-glucose DMEM (HiMedia) with 10% fetal bovine serum and 1% antibiotic–antimycotic at 37 °C and 5% CO₂, sub-cultured every 2 days and used at passage 48. Cells were seeded at 20,000 cells per well (200 µL) in 96-well plates, allowed to attach for 24 h and exposed for 24 h to the L1-derived fraction (6.25–100 µg/mL). Controls were medium without cells (blank), untreated cells and cells treated with 5 µM doxorubicin (Sigma-Aldrich) [32].

Viability was measured by the MTT assay, based on reduction of the tetrazolium salt to formazan by metabolically active cells [33]. Spent medium was replaced with MTT (0.5 mg/mL final), the foil-wrapped plates were incubated for 3 h at 37 °C, and formazan was dissolved in 100 µL of DMSO. Absorbance was read at 570 nm (reference 630 nm) on an ELx800 reader (BioTek, USA), and viability was calculated as:

Cell viability (%) = (mean A₅₇₀ of treated cells – blank) / (mean A₅₇₀ of untreated cells – blank) × 100

Before the MTT step, cells were photographed under an inverted microscope (Biolinkz, India). The IC₅₀ was estimated by linear regression of viability against concentration ($y = mx + c$, $y = 50$) and checked with a four-parameter logistic fit.

2.8 Statistical Analysis

MTT conditions were tested in duplicate wells ($n = 2$) in one experiment and are expressed as mean ± SD. Treatments were compared with the untreated control by one-way ANOVA followed by Dunnett's test ($p < 0.05$). Viability was calculated in Microsoft Excel, and statistical and regression analyses used SciPy 1.17 (Python). Probiotic, optimization and analytical data are presented as mean values and interpreted descriptively.

3. RESULTS

3.1 Identification and Probiotic Characteristics of the Isolate

On MRS agar, isolate L1 formed circular, smooth, entire, slightly convex, creamy-white colonies of Gram-positive, non-spore-forming rods occurring singly or in short chains. It was catalase-negative, non-motile and homofermentative. The partial 16S rRNA sequence showed 99.67% similarity to *L. plantarum*, and the isolate clustered with *L. plantarum* reference sequences in the phylogenetic tree; it was designated *Lactiplantibacillus plantarum* PPKMB1 (GenBank MZ951173; Table 1).

With 1% pepsin, viable counts at pH 3.0 remained above 90% of the initial count over 3 h, similar to pH 6.0, whereas survival fell sharply at pH 2.5. PPKMB1 grew in more than 0.8% ox bile, well above the 0.3% tolerance threshold, and in 4–8% NaCl. Cell-surface hydrophobicity was 35%, and no haemolysis was observed on blood agar (γ-haemolysis), supporting its non-pathogenic character (Table 1).

Table 1. Morphological, biochemical, molecular and probiotic characteristics of *Lactiplantibacillus plantarum* PPKMB1 (isolate L1)

Characteristic	Observation
Source	Raw cow milk, Coimbatore, India
Colony morphology (MRS agar)	Circular, smooth, entire, slightly convex, creamy-white
Gram reaction / cell shape	Gram-positive rods, single or short chains
Endospore / motility	Non-spore-forming / non-motile
Catalase	Negative
Fermentation type	Homofermentative lactic acid production
16S rRNA identity (BLAST)	99.67% similarity to <i>L. plantarum</i>
GenBank accession number	MZ951173
Acid tolerance (1% pepsin, 3 h)	>90% survival at pH 3.0; marked loss of viability at pH 2.5
Bile tolerance	Growth above 0.8% ox bile (tolerant)
NaCl tolerance	4–8% NaCl
Cell-surface hydrophobicity	35%
Haemolysis (sheep blood agar)	γ-haemolysis (non-haemolytic)

3.2 Growth Kinetics and Optimization of GABA Production

Wet biomass reached 0.082 g on day 1 and declined to 0.066 g on day 2, and optical density peaked at 24 h (0.269) before decreasing at 48 and 72 h, indicating entry into the stationary phase within the first day.

GABA production depended strongly on all five factors (Table 2; Figure 1). It rose from 217.5 µg/mL at 24 h to a maximum of 474.2 µg/mL at 48 h and fell to 137.5 µg/mL at 96 h, so maximum GABA accumulation lagged peak cell density by about 24 h. An initial pH of 7.0 gave 490.8 µg/mL, about three-fold more than pH 5.0 or 6.0, and production declined at pH 8.0 (258.3 µg/mL). The optimum temperature was 40 °C (470.8 µg/mL), with lower output at 30 °C (235.8 µg/mL) and 50 °C (103.3 µg/mL).

Xylose supported the highest GABA level among carbon sources (464.2 µg/mL), followed by cellulose, maltose and sucrose. Organic nitrogen sources gave the highest values of the study: tryptone 610.8 µg/mL, casein 570.0 µg/mL and rice husk 555.0 µg/mL, compared with 242.5 µg/mL for KH₂PO₄.

Table 2. Summary of one-factor-at-a-time optimization of GABA production by *L. plantarum* PPKMB1

Factor	Levels tested	GABA range (µg/mL)	Optimum (GABA, µg/mL)
Incubation period	24, 48, 96 h	137.5–474.2	48 h (474.2)
Initial pH	5, 6, 7, 8	151.7–490.8	pH 7.0 (490.8)
Temperature	30, 40, 50 °C	103.3–470.8	40 °C (470.8)
Carbon source	Xylose, sucrose, maltose, cellulose	255.0–464.2	Xylose (464.2)
Nitrogen source	Tryptone, casein, rice husk, KH ₂ PO ₄	242.5–610.8	Tryptone (610.8)

GABA estimated colorimetrically (A_{512}) in cell-free supernatant; values are means. Individual values are shown in Figure 1.

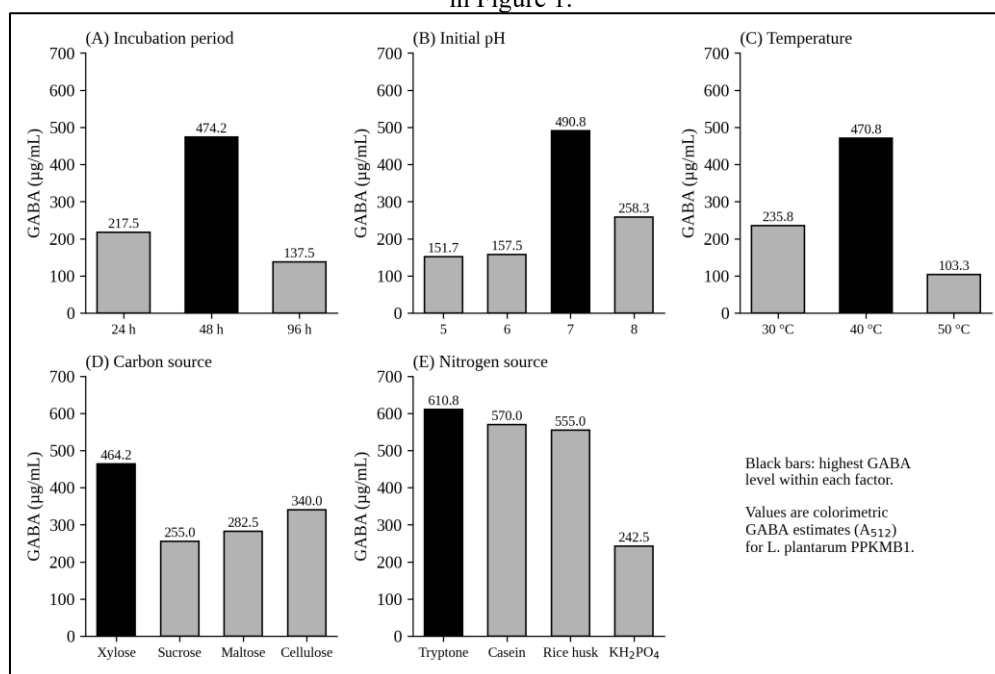


Figure 1. Effect of (A) incubation period, (B) initial pH, (C) temperature, (D) carbon source and (E) nitrogen source on GABA production by *L. plantarum* PPKMB1. Black bars indicate the highest GABA level within each factor.

3.3 Analytical Confirmation and Quantification of GABA

In the colorimetric assay, the GABA standard (1 µg/mL) gave an absorbance of 0.516 at 512 nm, and the L1 assay aliquot corresponded to 0.80 µg/mL GABA equivalents. On TLC, L1 produced a red-purple, ninhydrin-positive spot (R_f 0.672) matching the GABA standard; eluted spots gave absorbances of 0.793 (L1) and 0.837 (standard), equivalent to 0.947 µg/mL, or about 1.89 ng per 2 µL band. These values refer to small, diluted aliquots and are semi-quantitative, whereas GC-MS gives the concentration in the purified sample.

In UV-visible spectra, both the L1 sample and the standard absorbed only in the ultraviolet region (Figure 2). L1 showed a sharp maximum at 300 nm (absorbance 0.853) and the standard was annotated at 295 nm (0.771), with absorbance falling steeply above about 310 nm.

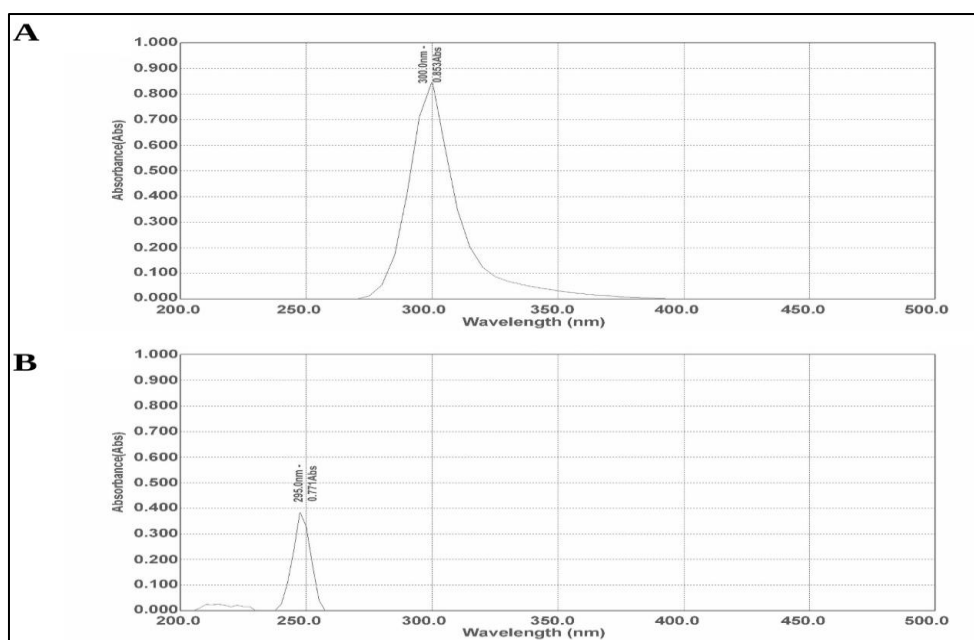


Figure 2. UV-visible absorption spectra of (A) the purified sample from *L. plantarum* PPKMB1 (L1) and (B) the GABA standard, recorded between 200 and 500 nm.

The FTIR spectrum of L1 closely resembled that of the standard (Figure 3; Table 3). Both showed a broad O–H/N–H stretching band (3278.99 and 3232.70 cm^{-1}), bands in the NH_3^+ deformation and asymmetric COO^- stretching region (1643.35/1527.62 cm^{-1} in L1; 1635.64/1550.77 cm^{-1} in the standard), C–N and C–C/C–O stretching bands near 1040–1110 cm^{-1} and identical skeletal bands at 686.66, 601.79 and 555.50 cm^{-1} . The symmetric COO^- stretch (1404.18 cm^{-1}) and CH_2 bands (1311.59 and 1265.30 cm^{-1}) of the standard were weak or absent in L1, consistent with a less concentrated or matrix-containing sample.

Table 3. Principal FTIR absorption bands of the GABA standard and the L1 sample with tentative assignments

GABA standard (cm^{-1})	L1 sample (cm^{-1})	Tentative assignment
3232.70	3278.99	O–H and N–H (NH_3^+) stretching; hydrogen-bonded water
1635.64	1643.35	NH_3^+ asymmetric deformation / COO^- stretching; H–O–H bending
1550.77	1527.62	COO^- asymmetric stretching / NH_3^+ symmetric deformation
1404.18	—	COO^- symmetric stretching
1311.59, 1265.30	—	CH_2 wagging and twisting
—	1188.15	C–N stretching / CH_2 rocking
1111.00, 1056.99	1087.85, 1041.56	C–N and C–C/C–O stretching
686.66	686.66	COO^- bending / N–H wagging
601.79, 555.50	601.79, 555.50	Skeletal deformation
501.49, 462.92, 424.34	470.63, 416.62	Skeletal and lattice vibrations

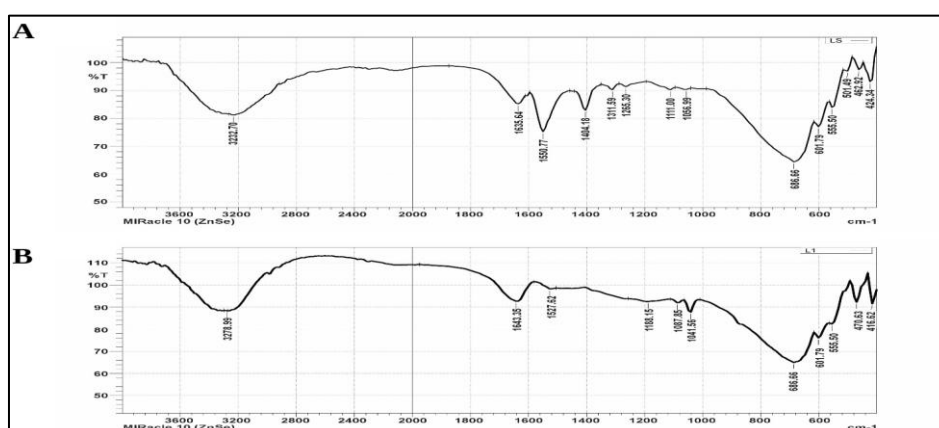


Figure 3. ATR-FTIR spectra of (A) the GABA standard and (B) the purified sample from *L. plantarum* PPKMB1 (L1), recorded between 4000 and 400 cm^{-1} .

GC-MS provided the most specific evidence (Figure 4; Table 4). The 5-ppm standard eluted at 2.912 min (peak area 15,741), and L1 gave a corresponding peak at 2.963 min (peak area 27,150). After correction for the 100-fold dilution, the GABA concentration of the purified L1 sample was 862.39 mg/L, of the same order as the colorimetric values under optimal conditions (464–611 µg/mL).

Table 4. GC-MS identification and quantification of GABA in the purified L1 sample

Sample	RT (min)	Peak area	Calculation	Dilution (MeOH)	GABA
GABA standard	2.912	15,741	—	—	5 ppm
L1 (<i>L. plantarum</i> PPKMB1)	2.963	27,150	$27,150 \times 5 \times 100 / 15,741$	100-fold	862.39 ppm

RT, retention time; MeOH, methanol; ppm, mg/L. Concentration calculated relative to the 5-ppm GABA standard and corrected for dilution.

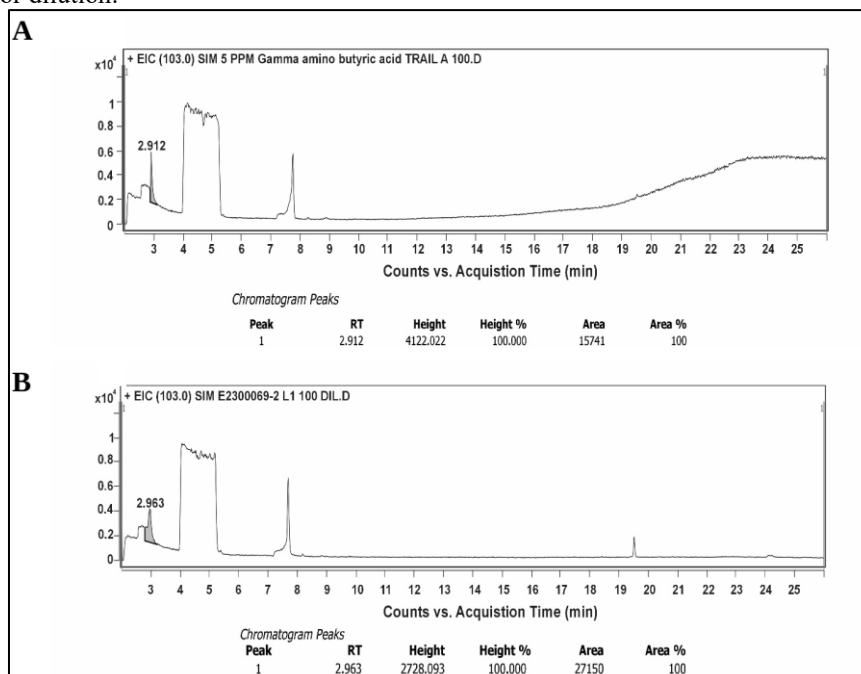


Figure 4. GC-MS chromatograms (selected ion monitoring) of (A) the 5-ppm GABA standard (retention time 2.912 min) and (B) the purified sample from *L. plantarum* PPKMB1 (L1; retention time 2.963 min).

3.4 Effect of the L1-Derived Fraction on HCT-116 Cell Viability

The L1-derived fraction reduced HCT-116 viability in a concentration-dependent manner after 24 h (Table 5; Figure 5A). Viability was unaffected at 6.25 µg/mL ($97.37 \pm 2.56\%$) and 12.5 µg/mL ($95.91 \pm 1.82\%$), slightly reduced at 25 µg/mL ($91.00 \pm 1.82\%$; $p = 0.023$) and clearly reduced at 50 µg/mL ($70.21 \pm 2.64\%$) and 100 µg/mL ($31.37 \pm 0.91\%$; both $p < 0.001$). Doxorubicin (5 µM) reduced viability to $32.30 \pm 1.40\%$ ($p < 0.001$), comparable to the highest concentration of the fraction.

Viability declined almost linearly with concentration ($y = -0.7264x + 105.32$; $R^2 = 0.990$), giving a preliminary IC_{50} of approximately 76 µg/mL (Figure 5B); a four-parameter logistic fit gave a similar value (74.0 µg/mL). Because 5 µM doxorubicin corresponds to about 2.9 µg/mL, the fraction was considerably less potent than the reference drug on a mass basis.

Table 5. Absorbance and viability of HCT-116 cells after 24 h exposure to the L1-derived fraction

Treatment	A ₅₇₀ (blank-corrected)	SD	Viability (%)	p vs. untreated
Untreated control	0.856	0.031	100.00 ± 3.63	—
Doxorubicin (5 µM)	0.277	0.012	32.30 ± 1.40	< 0.001
L1 6.25 µg/mL	0.834	0.022	97.37 ± 2.56	0.72
L1 12.5 µg/mL	0.821	0.016	95.91 ± 1.82	0.37
L1 25 µg/mL	0.779	0.016	91.00 ± 1.82	0.023
L1 50 µg/mL	0.601	0.023	70.21 ± 2.64	< 0.001
L1 100 µg/mL	0.269	0.008	31.37 ± 0.91	< 0.001

Values are mean $\pm$ SD of duplicate wells ($n = 2$); blank (medium only) mean absorbance = 0.042. p values from one-way ANOVA with Dunnett's test against the untreated control. Preliminary $IC_{50} \approx 76$ µg/mL.

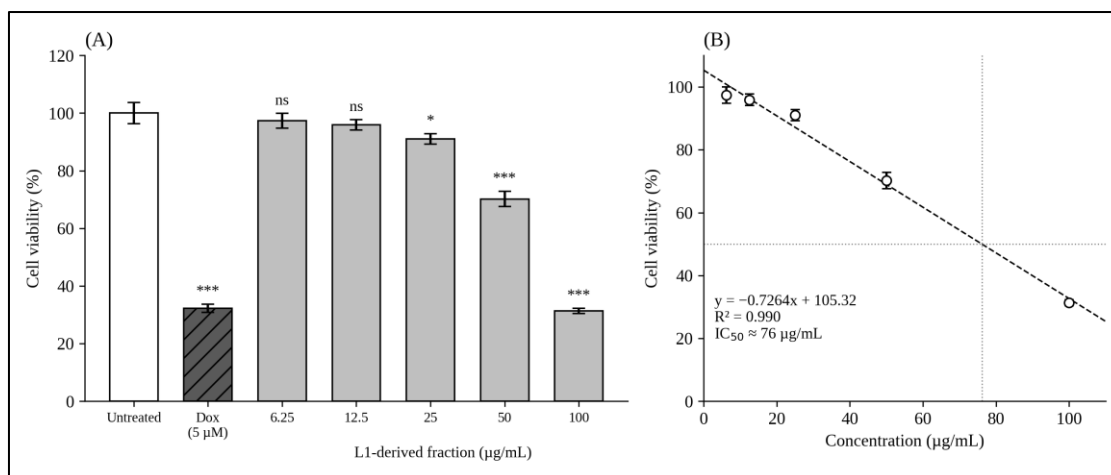


Figure 5. Cytotoxicity of the L1-derived fraction towards HCT-116 cells after 24 h. (A) Cell viability relative to the untreated control; doxorubicin (5 µM) served as the positive control. Bars show mean $\pm$ SD (n = 2); ns, not significant; *p < 0.05; *p < 0.001 versus untreated (Dunnett's test). (B) Linear regression used to estimate the preliminary IC_{50} ($\approx 76 \mu\text{g/mL}$).**

Microscopy supported these results (Figure 6). Untreated and low-concentration fields showed dense clusters of well-spread polygonal and spindle-shaped cells with intact cell–cell contacts, whereas fields representative of higher concentrations and doxorubicin showed reduced density, loss of monolayer continuity, cell shrinkage and rounded, refractile, detached cells, features associated with cytotoxic stress.

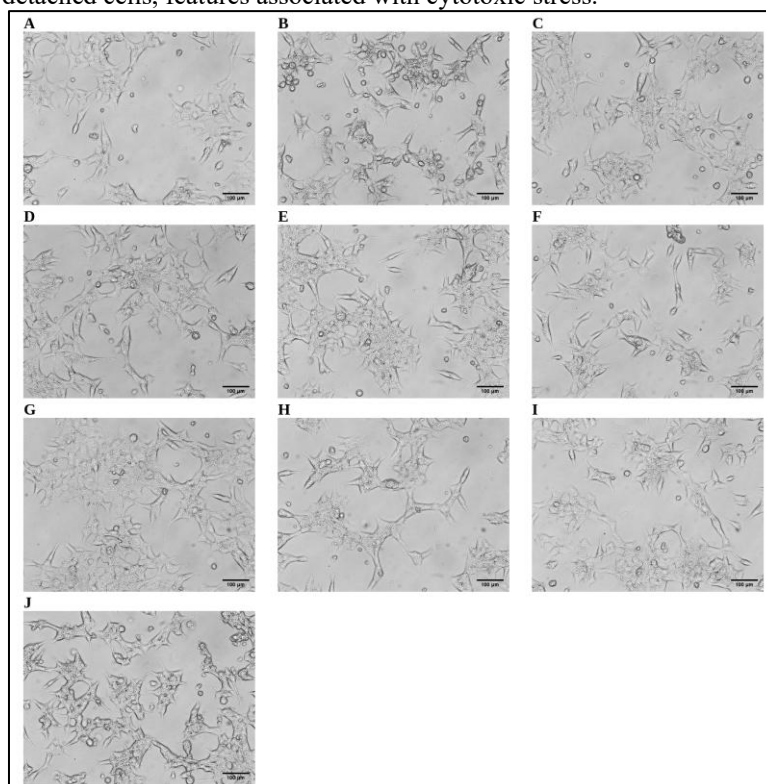


Figure 6. Representative inverted-microscope images (A–J) of HCT-116 cells recorded after 24 h in the MTT experiment, including untreated, doxorubicin-treated and L1-treated wells. Scale bar = 100 µm.

4. DISCUSSION

The first part of the study established the identity and probiotic suitability of the isolate. The phenotype and 99.67% 16S rRNA similarity place PPKMB1 within *L. plantarum*, a species with a long record of safe use in dairy and plant fermentations [19, 20]. Survival above 90% at pH 3.0 and growth in more than 0.8% bile compare well with recent raw-milk *L. plantarum* isolates, which retained 80–101% viability after pepsin–acid challenge and tolerated 0.5% bile [18], and with the GABA-producing strains FBT215 and SY1 [14, 22]. A hydrophobicity of 35% indicates moderate adhesion potential, although epithelial adhesion assays are needed to confirm it. With γ -haemolysis and salt tolerance, these traits make PPKMB1 a reasonable candidate for further safety evaluation, including antibiotic-resistance gene screening and whole-genome analysis [21].

The second part concerns fermentation conditions. The 48-h optimum, about one day after peak biomass, is consistent with the physiology of the GAD system, which is induced as the medium acidifies during late growth

and consumes protons during glutamate decarboxylation [2, 10]. The decline at 96 h probably reflects cell death and GABA catabolism [11, 34]. The optimum initial pH of 7.0 may seem to conflict with reports that *L. plantarum* GadB is most active at pH 4.0–5.0 [2, 14], but initial pH is not the pH at which decarboxylation occurs: a near-neutral start favours rapid growth, after which the culture acidifies into the range of maximal GAD activity. Strain FRT7 showed almost identical optima (pH 7.0, 40 °C, 48 h) [11], whereas other strains preferred initial values of 5.5–6.4 [12, 13]. The 40 °C optimum agrees with the thermal preference of LAB GAD enzymes [10], and the sharp decline at 50 °C indicates heat stress.

The superiority of xylose is notable because *L. plantarum* is facultatively heterofermentative and metabolises pentoses via the phosphoketolase pathway [35]; xylose may alter redox balance and acidification in ways that favour GAD activity, a hypothesis that remains to be tested. Pentose-rich agro-food residues are attractive low-cost substrates for GABA production [12, 36]. Organic nitrogen sources clearly outperformed KH_2PO_4 , which supplies no nitrogen; peptide-rich sources such as tryptone and casein supply the amino acids and growth factors that fastidious LAB require, consistent with other optimization studies [12, 13, 37]. Because one-factor-at-a-time designs cannot capture interactions, response surface methodology should now be applied [14–16].

The third part concerns the analytical evidence. Colorimetry and TLC detect primary amines and could respond to other amino acids, although the matching R_f values reduce this concern. The UV data need particular caution: GABA lacks a chromophore absorbing strongly above about 210 nm, so the maxima at 295–300 nm probably arise from derivatization products or co-purified compounds, and the UV profile is supportive rather than diagnostic. FTIR provides stronger structural evidence, as the NH_3^+ , carboxylate, C–N and skeletal bands of the sample match those of the standard. GC-MS gave the most specific confirmation; the 0.05-min retention-time difference is within normal injection variation. The yield of 862.39 mg/L exceeds that of raw-milk *Lactococcus* strains (up to about 250 mg/L) [17] but is below the 1.16–3.74 g/L reported for statistically optimized *L. plantarum* FRT7, SY1 and Lp3 [11, 13, 14]. Isotope-dilution GC-MS or validated HPLC with o-phthalaldehyde derivatization would strengthen future quantification [38].

The fourth part was a preliminary cytotoxicity assessment. The potency of the L1-derived fraction ($\text{IC}_{50} \approx 76 \mu\text{g/mL}$) lies within the range reported for LAB-derived materials: supernatant of *L. plantarum* YT013 had an IC_{50} of about 1208 $\mu\text{g/mL}$ against HCT116 cells [24], supernatant of *L. plantarum* OC01 reduced HCT116 and HT29 proliferation through ERK/mTOR inhibition and β -catenin degradation [23], and other probiotics induced caspase-dependent apoptosis in colorectal cells [25, 39, 40]. Possible explanations include GABA acting through GABA-A and GABA-B receptors, which mediated anti-proliferative effects of exogenous and bacterial GABA in colon cancer cells [28] and suppressed proliferation via the Hippo/YAP1 pathway [29], and co-purified postbiotic metabolites such as organic acids, peptides or exopolysaccharides [26, 27].

However, the cytotoxicity observed here cannot be attributed exclusively to GABA. The material tested was an L1-derived, GABA-containing fraction, not purified GABA, and no pure-GABA arm, vehicle control or receptor-antagonist experiment was included. Attribution to GABA would be justified only if purified GABA reproduced the effect at equivalent concentrations and the effect were blocked by GABA receptor antagonists. Caution is reinforced by evidence that tumour-derived GABA can activate β -catenin signalling, promote tumour growth and suppress anti-tumour immunity in colorectal and other cancers [7, 30, 31].

Further limitations must be noted. The assay used one 24-h exposure, duplicate wells and a single experiment, so p values are indicative only. MTT reflects reductase activity rather than cell number and may be affected by reducing compounds or residual salts [33]. Without a normal colon epithelial cell line, no selectivity index can be calculated, and the mode of cell death was not examined. Future work should chemically profile the fraction, compare it with purified GABA and receptor antagonists, repeat assays in at least three independent experiments at 24–72 h, include normal cells and assess apoptosis, reactive oxygen species and cell cycle.

5. CONCLUSION

A GABA-producing lactic acid bacterium isolated from raw cow milk was identified as *Lactiplantibacillus plantarum* PPKMB1 (GenBank MZ951173) and showed acid, bile and salt tolerance and γ -haemolysis. GABA production was highest after 48 h at an initial pH of 7.0 and 40 °C, with xylose and tryptone as the most favourable carbon and nitrogen sources, and TLC, UV–visible, FTIR and GC-MS analyses confirmed GABA, quantified at 862.39 mg/L. The L1-derived fraction reduced HCT-116 viability in a concentration-dependent manner (preliminary $\text{IC}_{50} \approx 76 \mu\text{g/mL}$). As this fraction was not pure GABA, the effect cannot be attributed solely to GABA, and mechanistic and normal-cell selectivity studies are required.

Acknowledgements and Conflict of Interest

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