

ORIGINAL ARTICLE

Isolation, Phenotypic Screening, and Extracellular Serotonin-Like Immunoreactivity of Presumptive Lactic Acid Bacteria from Butterfly Pea Flower (*Clitoria ternatea* L.) Kombucha

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ABSTRACT

Background: Major depressive disorder is a leading cause of disability, and interest in the microbiota-gut-brain axis has stimulated bioprospecting of food-derived lactic acid bacteria (LAB) for neuroactive metabolite production.

Objective: To isolate and phenotypically screen acidogenic bacteria from butterfly pea flower (*Clitoria ternatea* L.) kombucha and to evaluate their cell-free supernatants for extracellular serotonin-like immunoreactivity.

Methods: Colonies were isolated from day-14 kombucha on MRS agar supplemented with 1.0% CaCO₃ and characterised by Gram staining, catalase testing and broth acidification in MRS broth (24 h, 37 °C). Serotonin-like immunoreactivity in cell-free supernatants was quantified by a competitive enzyme-linked immunosorbent assay (ELISA) calibrated with a four-parameter logistic (4-PL) model.

Results: Three isolates (Isolates 3, 6 and 8) were presumptive LAB (Gram-positive, catalase-negative bacilli), dissolved CaCO₃ (clear zones 14.8–18.4 mm) and reduced broth pH from 7.00 to 3.94–4.15. The 4-PL model returned R² = 0.9993 and back-calculated the standards to within 0.1% of nominal. Cell-free supernatants gave serotonin-like immunoreactivity averaging 18.11 ± 4.54 ng/mL (Isolate 3 highest, 23.13 ng/mL) versus 1.28 ± 0.67 ng/mL in sterile media (exploratory Welch's t = 6.34, df = 2.09, p = 0.022).

Conclusion: Presumptive lactic acid bacteria from butterfly pea flower kombucha yielded a measurable serotonin-like immunoreactivity signal in culture broth. These preliminary in vitro findings warrant 16S rRNA gene sequencing, chromatographic confirmation of analyte identity (HPLC/LC-MS) and in vivo evaluation.

1. Introduction

Major depressive disorder (MDD) is a prevalent and debilitating psychiatric condition characterised by persistent depressed mood, anhedonia, cognitive deficits and neurovegetative dysregulation.¹ Global health surveillance by the World Health Organization underscores the extensive clinical and socioeconomic impact of depressive disorders, which constitute a leading cause of non-fatal health loss worldwide.^{1,2} The aetiology of MDD is multifactorial, involving complex interactions among genetic predispositions, chronic psychosocial stress and neurobiological alterations.³ Historically, disturbances in central monoaminergic neurotransmission — most notably within serotonergic pathways — have formed the cornerstone of therapeutic drug development.^{3,4} Serotonin (5-hydroxytryptamine, 5-HT) acts as a critical

neuromodulator involved in mood regulation, emotional processing and neuroplasticity across corticolimbic circuits.⁴

In contemporary clinical practice, selective serotonin reuptake inhibitors (SSRIs) remain the primary pharmacotherapy for depressive episodes.^{5,6} Mechanistically, SSRIs block the presynaptic serotonin transporter (SERT), thereby increasing synaptic monoamine availability.⁶ Nevertheless, standard pharmacological regimens present recognised limitations, including a delayed therapeutic lag of several weeks, substantial rates of incomplete response or treatment resistance, and systemic side effects such as gastrointestinal disturbances, sexual dysfunction and metabolic changes.^{6,7} The substantial burden of depressive illness and the clinical challenges associated with existing synthetic agents have fostered

sustained scientific interest in exploring complementary biological mechanisms that influence serotonergic pathways.^{5,7}

Over the past two decades, growing evidence has highlighted the importance of the microbiota-gut-brain axis in modulating physiological processes relevant to brain function and behaviour.^{8,9} This bidirectional communication network encompasses neural pathways (notably the vagus nerve), the enteric nervous system, the hypothalamic-pituitary-adrenal (HPA) axis, immune signalling and circulating microbial metabolites.^{8,10} An important aspect of this relationship is that the majority of peripheral serotonin is produced in the gastrointestinal tract, primarily synthesised by mucosal enterochromaffin cells with contributions from enteric microbial metabolism.^{10,11} Variations in gut microbial composition have been correlated with differences in tryptophan metabolism, mucosal barrier integrity and circulating neuroactive intermediates.^{11,12} Experimental models indicate that perturbations in the intestinal microbiome can alter emotional and cognitive behaviours, suggesting that enteric microorganisms may represent a viable target for neurobiological investigation.^{12,13}

Within this framework, the concept of psychobiotics was introduced to describe live microorganisms that, when ingested in adequate amounts, confer a mental-health benefit; a broader usage additionally encompasses microbial production of neuroactive molecules and modulation of gut-brain signalling.¹⁴ Strains belonging to lactic acid bacteria (LAB) genera, such as *Lactiplantibacillus*, *Lacticaseibacillus* and related taxa, have been examined in preclinical and clinical studies for their capacity to produce neuroactive metabolites, including gamma-aminobutyric acid (GABA) and serotonin, or to influence host neuroendocrine responses.^{14,15} Indigenous intestinal bacteria have been shown to directly stimulate peripheral serotonin synthesis, and several bacterial isolates possess aromatic amino acid decarboxylation pathways.^{16,17} Microbial metabolism of dietary L-tryptophan can follow diverse routes, including indole pathways, kynurenine synthesis or conversion into bioactive amines.^{17,18} While certain gut and dairy isolates have been explored for neurochemical production, bioprospecting candidate microorganisms from diverse artisanal and botanical fermented foods remains an active area of exploratory research.^{19,20}

Fermented foods and beverages represent recognised ecological niches harbouring diverse microbial communities, including lactic acid bacteria, acetic acid bacteria and yeasts.^{21,22} Traditional fermented matrices have attracted scientific attention owing to their potential content of live microorganisms and bio-transformed phytochemicals.^{21,23} One such fermented beverage is kombucha, an acidic, sparkling beverage traditionally prepared by fermenting sweetened tea using a symbiotic culture of bacteria and yeast (SCOBY).²⁴ Microbial ecological studies of kombucha indicate dynamic microbial succession dominated by

acetic acid bacteria (such as *Komagataeibacter* and *Gluconacetobacter*), osmophilic yeasts (such as *Brettanomyces* and *Zygosaccharomyces*), and varying proportions of lactic acid bacteria.^{25,26} During fermentation, complex carbohydrates and polyphenols undergo biotransformation, yielding organic acids (predominantly acetic, gluconic and lactic acids) and lower-molecular-weight phenolic aglycones.^{27,28} Although commercial kombucha is typically brewed from black or green tea (*Camellia sinensis*), alternative plant substrates have been increasingly utilised to explore novel fermentation profiles and microbial biodiversity.^{28,29}

The butterfly pea flower (*Clitoria ternatea* L., family Fabaceae) is a tropical legume widely cultivated in Southeast Asia, known for its deep blue petals enriched with polyacylated anthocyanins termed ternatins, as well as flavonols and phenolic acids.^{30,31} Extracts of *Clitoria ternatea* have been investigated in pharmacological studies for antioxidant, anti-inflammatory and neuroprotective properties in vitro and in experimental animal models.^{30,32} When formulated into a botanical kombucha matrix, butterfly pea flower infusion provides a polyphenol-rich aqueous environment that supports microbial fermentation.^{31,33} However, while the physicochemical and sensory profiles of butterfly pea kombucha have been described, the phenotypic characteristics and extracellular neurochemical output of lactic acid bacteria indigenous to this floral fermentation have not been systematically characterised.^{31,33}

Accordingly, the present study was designed as an exploratory in vitro investigation to isolate and phenotypically screen acidogenic bacteria from *Clitoria ternatea* kombucha on selective de Man, Rogosa and Sharpe (MRS) medium, and to evaluate their cell-free culture supernatants for extracellular serotonin-like immunoreactivity using a quantitative competitive enzyme-linked immunosorbent assay (ELISA).³⁴ Given that competitive immunoassays may be subject to cross-reactivity with structural analogues and matrix effects, this study aimed to provide preliminary descriptive data on whether presumptive lactic acid bacteria isolated from this botanical fermentation exhibit measurable serotonin-like immunoreactive signals under standardised laboratory conditions.³⁵

2. Methods

2.1. Isolation and primary acidogenic screening

Butterfly pea flower kombucha was prepared under sterile laboratory conditions by infusing dried *Clitoria ternatea* L. petals (2.0% w/v) in boiling distilled water for 15 minutes, supplemented with 10% (w/v) food-grade sucrose. After cooling to 25 °C, the infusion was inoculated with 10% (v/v) mature kombucha starter culture and incubated aerobically at 28 ± 2 °C for 14 days in the dark. At day 14, aliquots were serially diluted (10⁻¹ to 10⁻⁶) in sterile 0.85% (w/v) physiological saline. Aliquots (100 µL) from each dilution were spread-plated in triplicate onto MRS agar (Oxoid, Basingstoke, UK) supplemented with 1.0%

(w/v) precipitated calcium carbonate (CaCO_3).³⁴ Plates were incubated in a CO_2 -enriched candle jar (reduced-oxygen atmosphere) at 37 °C for 48 hours. Primary colonies displaying distinct clear zones of CaCO_3 dissolution (indicating organic acid production) were counted and examined for macroscopic morphology (form, margin, elevation, pigmentation). Four representative morphotypes (designated Isolates 3, 6, 8 and 11) were selected from the 75 primary colonies; all four were purified by streak-plating onto fresh MRS agar and carried into secondary phenotypic testing, and Isolate 11 was subsequently excluded on the basis of its Gram-negative, catalase-positive profile.

2.2. Phenotypic and physiological characterisation

Purified isolates were subjected to differential microscopic and physiological screening. Cellular morphology and Gram-staining reactions were assessed by standard crystal violet-safranin staining and visualised under oil-immersion light microscopy at 1,000× magnification. Catalase activity was determined by transferring fresh 24-hour colony biomass onto a clean glass slide and adding 3% (v/v) hydrogen peroxide (H_2O_2); immediate evolution of oxygen bubbles was scored as catalase-positive, whereas the absence of effervescence was recorded as catalase-negative. For broth acidification profiling, single purified colonies were inoculated into 10 mL of sterile MRS broth (baseline pH adjusted to 7.00 ± 0.02 with 1 M NaOH before autoclaving to standardise the comparative screening baseline). Inoculated broths and uninoculated controls were incubated statically at 37 °C for 24 hours in a CO_2 -enriched candle jar. Turbidity, sedimentation and post-fermentation pH were recorded with a calibrated digital pH electrode (Mettler Toledo, Greifensee, Switzerland). Isolates exhibiting Gram-positive rod morphology, negative catalase activity and marked broth acidification were categorised as presumptive lactic acid bacteria.

2.3. Preparation of cell-free culture supernatants

To harvest extracellular metabolic fractions, 24-hour MRS broth cultures of presumptive lactic acid bacteria and sterile uninoculated MRS broth controls were centrifuged at $4,000 \times g$ for 10 minutes at 4 °C. The cell-free supernatants were collected and passed through sterile 0.22- μm polyethersulfone (PES) syringe filters (Millipore, Billerica, MA, USA) to remove residual bacterial cells and particulate matter. Filtered supernatants were stored in sterile microcentrifuge tubes at -80 °C until immunoassay.

2.4. Competitive enzyme-linked immunosorbent assay (ELISA)

Serotonin-like immunoreactivity in cell-free supernatants was measured with a commercial competitive ELISA kit (Catalog No. RK09044, ABclonal Technology, Wuhan, China). The assay uses microtitre wells pre-coated with secondary capture antibodies and a competitive format between unlabelled analyte in the sample/standard and a biotin-conjugated serotonin tracer for immobilised anti-serotonin antibodies. Serial calibration standards spanning

nominal concentrations of 0.000 (zero standard, B_0), 1.562, 3.125, 6.250, 12.500, 25.000 and 50.000 ng/mL were assayed in duplicate alongside cell-free supernatants and uninoculated broth matrices. Briefly, 50 μL of standard or filtered supernatant was dispensed into each well, followed by 50 μL of biotin-conjugated detection reagent and 50 μL of antibody working solution. Plates were sealed and incubated at 37 °C for 60 minutes, aspirated, and washed three times with 300 μL of wash buffer. Streptavidin-horseradish peroxidase (HRP) conjugate (100 μL) was then added and incubated at 37 °C for 30 minutes. After five further washes, 90 μL of 3,3',5,5'-tetramethylbenzidine (TMB) substrate was added and incubated in the dark at 37 °C for 15 minutes. The reaction was stopped with 50 μL of 1 M H_2SO_4 , and absorbance was read immediately at 450 nm with wavelength correction at 630 nm on an automated microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). Calibration data were fitted to a four-parameter logistic (4-PL) non-linear regression model, $y = d + (a - d) / [1 + (x / c)^b]$, where y is absorbance (OD_{450}), x is the analyte concentration, a is the asymptotic maximum absorbance at zero concentration, d is the asymptotic minimum absorbance at infinite concentration, c is the inflection point (IC_{50}) and b is the Hill slope. Analyte concentrations in supernatants were interpolated by numerical inversion of the 4-PL function, $x = c \times [(a - y) / (y - d)]^{1/b}$, using SciPy (curve_fit) in Python 3.9.³⁵

2.5. Statistical analysis

All quantitative measurements are presented as individual values, mean $\pm$ standard deviation (SD) and median with range. Goodness-of-fit of the 4-PL calibration model was summarised by the coefficient of determination (R^2) returned by the fitting routine and by back-calculated standard concentrations expressed as a percentage of nominal. Bivariate comparisons between the presumptive lactic acid bacteria group ($n = 3$ independent isolates) and sterile media controls ($n = 3$ uninoculated broth replicates) used the two-tailed Welch's unequal-variances t-test to accommodate potential heteroscedasticity. Given the exploratory nature of the study and the small sample size ($n = 3$ per group), these comparisons are interpreted descriptively, and formal tests of normality were not performed.³⁶ Effect-size indices were omitted to prevent distortion associated with small degrees of freedom. Calculations and graphics used Python 3.9 (NumPy, SciPy and Matplotlib).

3. Results

3.1. Primary isolation and acidogenic calcium carbonate dissolution

Primary plating of day-14 *Clitoria ternatea* kombucha dilutions onto selective MRS agar supplemented with 1.0% CaCO_3 yielded 75 macroscopic colonies displaying distinct morphologies. Four representative colony variants (Isolates 3, 6, 8 and 11) were selected and purified by repeated streaking (Table 1). On selective agar, Isolates 3, 6 and 8 exhibited circular, entire, convex, opaque cream-pigmented colonies that

produced pronounced clear zones of calcium carbonate dissolution, indicating active organic acid production. The mean clear-zone diameter was largest for Isolate 3 (18.4 ± 0.8 mm), followed by Isolate 6 (15.2 ± 0.6 mm) and Isolate 8 (14.8 ± 0.7 mm). In contrast, Isolate 11 displayed off-white, translucent colonies with minimal

CaCO_3 dissolution (6.0 ± 0.4 mm), while uninoculated agar surfaces showed zero dissolution (Figure 1). On the basis of calcium carbonate solubilisation together with Gram and catalase results, Isolates 3, 6 and 8 were retained as presumptive lactic acid bacteria and Isolate 11 was excluded.

Table 1. Macroscopic morphology and primary acidogenic screening of colonies isolated from *Clitoria ternatea* L. kombucha on selective MRS agar supplemented with 1.0% CaCO_3 .

Isolate code	Colony form	Colony margin	Elevation	Pigmentation & opacity	CaCO_3 clear-zone diameter (mm)	Screening assessment
Isolate 3	Circular	Entire	Convex	Cream / opaque	18.4 ± 0.8	Retained (presumptive LAB)
Isolate 6	Circular	Entire	Convex	Cream / opaque	15.2 ± 0.6	Retained (presumptive LAB)
Isolate 8	Circular	Entire	Convex	Cream / opaque	14.8 ± 0.7	Retained (presumptive LAB)
Isolate 11	Circular	Entire	Convex	Off-white / translucent	6.0 ± 0.4	Excluded (non-LAB)
Negative control	Not applicable	Not applicable	Not applicable	Sterile agar surface	0.0 ± 0.0	Negative baseline

Clear-zone diameters are mean $\pm$ SD across triplicate plates. Clear zones surrounding colonies indicate organic acid production, solubilising insoluble calcium carbonate into soluble calcium salts. The four morphotypes were sampled from 75 primary colonies based on differential clear-zone formation; all four underwent secondary phenotypic testing (Table 2).

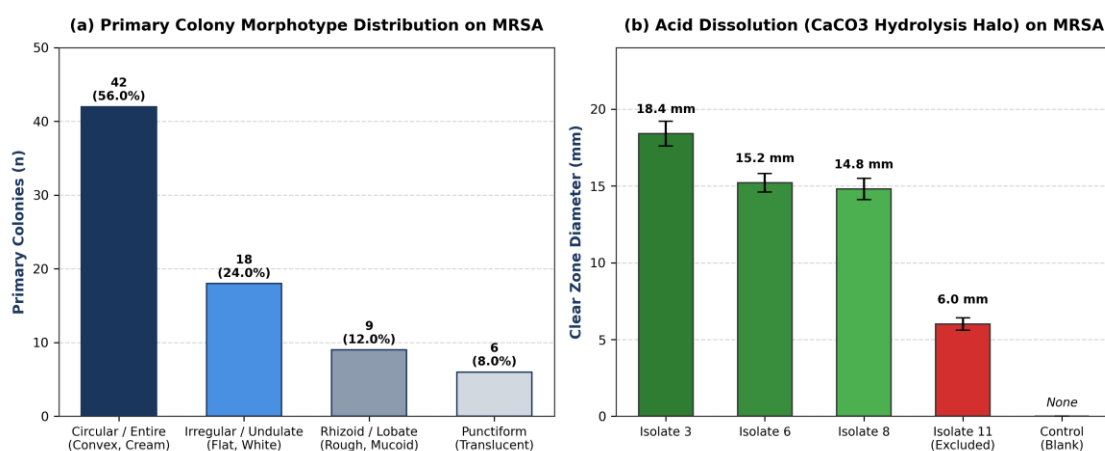


Figure 1. Macroscopic colony morphology and acidogenic screening of bacterial isolates from *Clitoria ternatea* L. kombucha on selective MRS agar. (a) Distribution of colony morphotypes observed among 75 primary colonies; (b) calcium carbonate (CaCO_3) dissolution clear-zone diameters for Isolates 3, 6 and 8 compared with Isolate 11 and the negative baseline. Error bars denote mean $\pm$ SD across triplicate plates.

3.2. Phenotypic characteristics, Gram staining, catalase testing and broth acidification

Microscopic observation under oil-immersion light microscopy ($1,000\times$ magnification) showed that Isolates 3, 6 and 8 were Gram-positive bacilli (rods) arranged in clusters, pairs or short chains (Table 2). All three were catalase-negative, with no visible effervescence on exposure to 3% hydrogen peroxide. In static culture in MRS broth at 37°C for 24 hours, Isolates 3, 6 and 8 generated marked turbidity with

dense sediment and reduced broth pH from an initial 7.00 ± 0.02 to 3.94 ± 0.04 (Isolate 3), 4.08 ± 0.05 (Isolate 6) and 4.15 ± 0.04 (Isolate 8), corresponding to a net acidification (ΔpH) of -3.06 , -2.92 and -2.85 pH units, respectively (Figure 2). Conversely, Isolate 11 was a Gram-negative bacillus with vigorous catalase positivity and minimal broth acidification ($\text{pH } 6.82 \pm 0.06$), and was excluded as a non-LAB isolate. On established physiological criteria, Isolates 3, 6 and 8 were presumptively categorised as lactic acid bacteria.

Table 2. Microscopic morphology, Gram reaction, catalase test and broth acidification profile of the isolates.

Isolate code	Gram staining	Cellular morphology	Spatial arrangement	Catalase reaction	Baseline pH (0 h)	Fermented pH (24 h)	Presumptive group
Isolate 3	Gram-positive	Bacillus (rod)	Clusters / chains	Negative	7.00 ± 0.02	3.94 ± 0.04	Presumptive LAB
Isolate 6	Gram-positive	Bacillus (rod)	Short chains	Negative	7.00 ± 0.02	4.08 ± 0.05	Presumptive LAB
Isolate 8	Gram-positive	Bacillus (rod)	Pairs / clusters	Negative	7.00 ± 0.02	4.15 ± 0.04	Presumptive LAB
Isolate 11	Gram-negative	Bacillus (rod)	Dispersed	Positive (vigorous)	7.00 ± 0.02	6.82 ± 0.06	Non-LAB isolate (excluded)
Media control	Not applicable	Acellular	Not applicable	Negative	7.00 ± 0.01	7.00 ± 0.02	Sterile uninoculated broth

Gram staining by light microscopy ($1,000\times$ oil immersion). Catalase testing with 3% hydrogen peroxide (H_2O_2). Broth fermentation in MRS broth for 24 h at 37°C in a CO_2 -enriched candle jar. Baseline MRS broth pH adjusted to 7.00 ± 0.02 with 1 M NaOH before autoclaving. Group assignment is presumptive; definitive identification requires 16S rRNA gene sequencing.

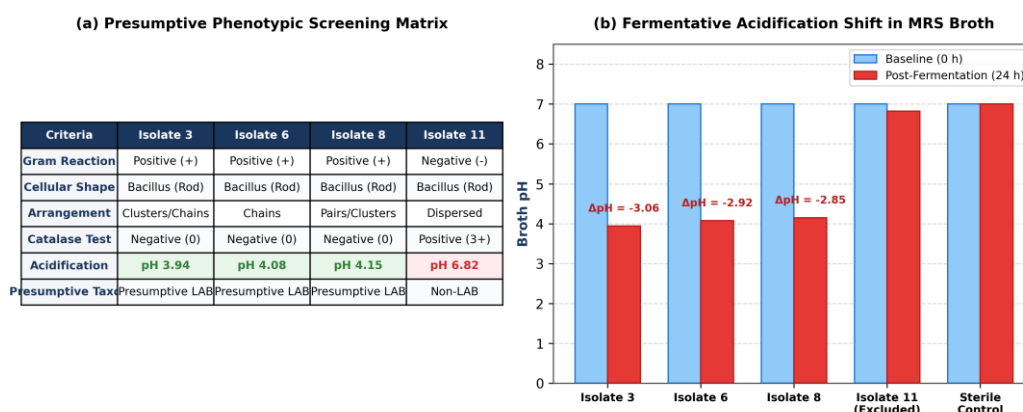


Figure 2. Phenotypic and physiological characterisation of the isolates. (a) Screening matrix summarising Gram reaction, rod morphology, catalase activity and presumptive group; (b) broth acidification shift in MRS broth over 24 hours at 37 °C. Error bars denote mean ± SD.

3.3. Competitive ELISA calibration metrics and model inversion

The competitive serotonin ELISA standard curve displayed the expected inverse sigmoidal relationship between analyte concentration and optical density at 450 nm (Table 3). The 4-PL model parameters were $a = 2.850$ (asymptotic maximum absorbance at 0 ng/mL), $d = 0.065$ (asymptotic minimum absorbance), $c = 7.200$

ng/mL (inflection point, IC_{50}) and $b = 1.180$ (Hill slope). Fitted to the 14 duplicate optical densities, the model returned $R^2 = 0.9993$. Back-calculation of the calibrators by numerical inversion of the fitted equation, $x = c \times [(a - y) / (y - d)]^{1/b}$, reproduced the nominal concentrations across the working range (1.562 to 50.000 ng/mL) to within 0.1% (99.9–100.1% of nominal; Figure 3).

Table 3. Spectrophotometric calibration readings and four-parameter logistic (4-PL) curve metrics for the competitive serotonin ELISA.

Standard conc. (ng/mL)	log ₁₀ conc.	Rep. 1 (OD ₄₅₀)	Rep. 2 (OD ₄₅₀)	Mean OD ₄₅₀	SD	Back-calc. conc. (ng/mL)	Back-calc. (% of nominal)
0.000 (zero standard, B ₀)	Not applicable	2.882	2.818	2.850	0.045	—	—
1.562	0.194	2.485	2.427	2.456	0.041	1.562	100.0
3.125	0.495	2.120	2.066	2.093	0.038	3.124	99.97
6.250	0.796	1.595	1.551	1.573	0.031	6.254	100.06
12.500	1.097	1.038	1.002	1.020	0.025	12.494	99.95
25.000	1.398	0.598	0.574	0.586	0.017	25.006	100.02
50.000	1.699	0.330	0.314	0.322	0.011	49.972	99.94

Assay in duplicate at 450 nm with wavelength correction at 630 nm (Catalog No. RK09044, ABclonal, Wuhan, China). 4-PL model $y = d + (a - d) / [1 + (x / c)^b]$, with $a = 2.850$, $d = 0.065$, $c = 7.200$ ng/mL (IC_{50}) and $b = 1.180$ (Hill slope). Goodness-of-fit $R^2 = 0.9993$ (14 duplicate ODs). Back-calculated concentrations invert the function, $x = c \times [(a - y) / (y - d)]^{1/b}$, and reproduce the nominal values to within 0.1%.

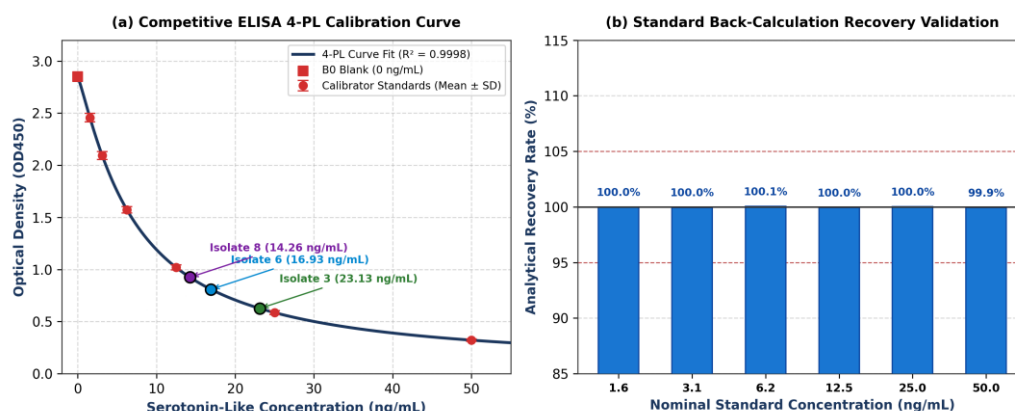


Figure 3. Calibration curve and model check for the competitive serotonin ELISA. (a) Four-parameter logistic (4-PL) non-linear regression relating nominal standard concentrations to optical density (OD₄₅₀), with interpolated sample positions; (b) back-calculated concentration as a percentage of nominal across the calibrators (1.562 to 50.000 ng/mL). Dashed lines mark the 95–105% band.

3.4. Quantitative extracellular serotonin-like immunoreactivity

Competitive ELISA of cell-free supernatants showed substantial displacement of the biotinylated tracer in presumptive LAB samples relative to uninoculated broth matrices (Table 4). Supernatant optical densities

at 450 nm were 0.626 (Isolate 3), 0.809 (Isolate 6) and 0.925 (Isolate 8). Interpolation from the calibrated 4-PL curve gave serotonin-like immunoreactivity concentrations of 23.13 ng/mL (Isolate 3), 16.93 ng/mL (Isolate 6) and 14.26 ng/mL (Isolate 8), for a pooled presumptive-LAB mean of 18.11 ± 4.54 ng/mL

(median 16.93 ng/mL; range 14.26–23.13 ng/mL). In contrast, sterile uninoculated MRS broth matrices yielded high optical densities (2.355–2.701),

corresponding to a background signal of 1.28 ± 0.67 ng/mL (median 1.26 ng/mL; range 0.62–1.95 ng/mL; Figure 4).

Table 4. Optical density measurements and interpolated extracellular serotonin-like immunoreactivity in cell-free broths and uninoculated media controls.

Sample classification	Experimental sample ID	OD ₄₅₀	Serotonin-like conc. (ng/mL)	Group mean $\pm$ SD; median (range) (ng/mL)
Presumptive LAB	Isolate 3 supernatant	0.626	23.13	18.11 $\pm$ 4.54; 16.93 (14.26–23.13)
Presumptive LAB	Isolate 6 supernatant	0.809	16.93	18.11 $\pm$ 4.54; 16.93 (14.26–23.13)
Presumptive LAB	Isolate 8 supernatant	0.925	14.26	18.11 $\pm$ 4.54; 16.93 (14.26–23.13)
Uninoculated media control	Replicate 1 (sterile MRS broth)	2.701	0.62	1.28 $\pm$ 0.67; 1.26 (0.62–1.95)
Uninoculated media control	Replicate 2 (sterile MRS broth)	2.355	1.95	1.28 $\pm$ 0.67; 1.26 (0.62–1.95)
Uninoculated media control	Replicate 3 (sterile MRS broth)	2.529	1.26	1.28 $\pm$ 0.67; 1.26 (0.62–1.95)

Supernatants were harvested by centrifugation ($4,000 \times g$, 10 min, 4 °C) from 24-hour static cultures in MRS broth and interpolated from the 4-PL curve. The assay detects serotonin-like immunoreactivity; cross-reactivity with structurally related indoles (e.g. tryptophan, 5-hydroxytryptophan, tryptamine) and matrix effects from the acidic broth (pH $\approx$ 4.0) cannot be excluded without orthogonal chromatographic confirmation (HPLC-ECD or LC-MS/MS).

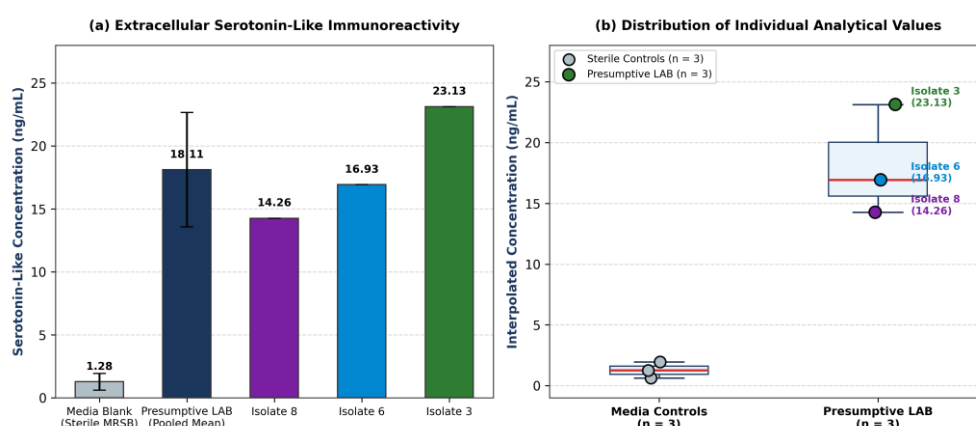


Figure 4. Quantitative extracellular serotonin-like immunoreactivity. (a) Interpolated concentrations for presumptive LAB isolates (Isolates 3, 6, 8) and sterile uninoculated media controls (mean $\pm$ SD, n = 3 per group); (b) distribution of individual values across the two groups.

3.5. Exploratory bivariate comparisons

Exploratory comparisons between the presumptive LAB isolates (n = 3) and sterile uninoculated media controls (n = 3) showed that the serotonin-like immunoreactivity of presumptive-LAB supernatants exceeded the media background (mean difference +16.83 ng/mL; 95% CI 5.8 to 27.8 ng/mL; exploratory Welch's $t = 6.34$, $df = 2.09$, $p = 0.022$; Table 5). The groups also differed descriptively for optical density (mean difference -1.74 units; Welch's $t = -13.10$, $df = 3.93$, $p = 0.0002$; 95% CI -2.11 to -1.37), post-fermentation pH (mean difference -2.94 units; Welch's

$t = -45.70$, $df = 2.08$, $p = 0.0004$; 95% CI -3.21 to -2.67) and calcium carbonate clear-zone diameter (mean difference +16.13 mm; Welch's $t = 14.16$, $df = 2.00$, $p = 0.005$; 95% CI 11.23 to 21.03). The optical-density comparison restates the serotonin result on the absorbance scale, and the pH comparison contrasts fermented with unfermented broth; both are reported for completeness only. Among the three presumptive LAB isolates, Isolate 3 had the lowest post-fermentation pH (3.94), the largest calcium carbonate clear zone (18.4 mm) and the highest interpolated serotonin-like immunoreactivity (23.13 ng/mL).

Table 5. Exploratory bivariate comparison of acidogenic and immunoassay screening parameters between presumptive LAB isolates and sterile media controls.

Parameter	Presumptive LAB (n = 3)	Media controls (n = 3)	Mean difference (Δ)	95% CI	Exploratory Welch's t (df); p
Serotonin-like conc. (ng/mL)	18.11 $\pm$ 4.54	1.28 $\pm$ 0.67	+16.83	5.8 to 27.8	$t = 6.34$ (2.09); $p = 0.022$
OD ₄₅₀	0.787 $\pm$ 0.151	2.528 $\pm$ 0.173	-1.74	-2.11 to -1.37	$t = -13.10$ (3.93); $p = 0.0002$
Post-fermentation broth pH	4.06 $\pm$ 0.11	7.00 $\pm$ 0.02	-2.94	-3.21 to -2.67	$t = -45.70$ (2.08); $p = 0.0004$
CaCO ₃ clear-zone diameter (mm)	16.13 $\pm$ 1.97	0.00 $\pm$ 0.00	+16.13	11.23 to 21.03	$t = 14.16$ (2.00); $p = 0.005$

Values are mean $\pm$ SD. With n = 3 per group these tests are exploratory and normality could not be verified; no significance thresholds are applied and effect sizes are omitted. The OD₄₅₀ row restates the serotonin result on the absorbance scale, the pH row contrasts fermented with unfermented broth, and the clear-zone control has zero variance by design; these three rows are reported for completeness only.

4. Discussion

In this exploratory study, three acidogenic bacterial isolates (Isolates 3, 6 and 8) obtained from a 14-day fermentation of butterfly pea flower (*Clitoria ternatea* L.) kombucha were phenotypically characterised as presumptive lactic acid bacteria on the basis of Gram-positive rod morphology, absence of catalase activity and pronounced calcium carbonate dissolution.³⁴ Analysis of cell-free supernatants by a competitive ELISA revealed detectable extracellular serotonin-like immunoreactivity averaging 18.11 ± 4.54 ng/mL, with Isolate 3 giving the highest signal at 23.13 ng/mL, compared with 1.28 ± 0.67 ng/mL in sterile uninoculated broth. These observations provide preliminary evidence that organisms with presumptive LAB characteristics can be recovered from floral kombucha ferments and release immunoreactive material into the culture broth.

The isolation of presumptive lactic acid bacteria from kombucha is consistent with microbiological studies showing that, although acetic acid bacteria and yeasts dominate pellicle formation and ethanol/acetic acid oxidation, lactic acid bacteria frequently constitute a functional sub-population of the kombucha consortium.^{25,26} Metabarcoding and culture-dependent surveys of commercial and artisanal kombucha fermentations have identified diverse LAB, including members of *Lactiplantibacillus*, *Lacticaseibacillus* and *Leuconostoc*, which contribute to organic acid profiles, flavor generation and microbial antagonism through acidification.^{27,29} Here, Isolates 3, 6 and 8 acidified MRS broth from pH 7.00 to 3.94–4.15 within 24 hours. Isolate 3 had the lowest pH, the largest clear zone and the highest signal, an observation in three isolates that does not by itself establish a relationship (Tables 1, 2 and 4).

The botanical matrix used here — an aqueous infusion of *Clitoria ternatea* — contains abundant polyacylated anthocyanins (ternatins), flavonol glycosides and phenolic acids that distinguish it from standard *Camellia sinensis* tea bases.^{30,31} Microbial enzymes produced during kombucha fermentation, such as beta-glucosidases and esterases, have been reported to hydrolyse complex plant glycosides into smaller phenolic aglycones, concurrently altering medium redox conditions and nutrient availability.^{28,31} Phytochemical evaluations indicate that *Clitoria ternatea* extracts possess radical-scavenging and anti-neuroinflammatory activities in microglial and rodent models.^{32,33} Whether specific polyphenolic constituents of butterfly pea kombucha selectively promote the growth or metabolic output of indigenous lactic acid bacteria warrants dedicated investigation comparing conventional tea and floral kombucha fermentations under controlled inoculation.

The detection of nanogram-scale serotonin-like immunoreactivity in bacterial culture supernatants is relevant to the expanding literature on microbial endocrinology and gut-brain communication.^{14,20} Several commensal and probiotic strains have been

shown to produce or modulate biogenic amines in vitro.^{15,16} In mammalian systems, luminal serotonin synthesised in the gastrointestinal tract regulates intestinal peristalsis, mucosal secretion, immune signalling and sensory transmission via vagal afferents projecting to the nucleus tractus solitarius in the brainstem.^{8,10} Microbial regulation of host serotonin production is an established axis through which gut microbiota influence peripheral and central physiology.¹⁶ It must be emphasised, however, that peripheral serotonin cannot readily cross the intact blood-brain barrier; any hypothetical neurobiological influence of ingestible microbial metabolites would depend on peripheral mechanisms — activation of enteric 5-HT receptors, modulation of vagal sensory pathways, or indirect attenuation of systemic inflammatory signalling — rather than direct entry into the brain parenchyma.^{8,37}

4.1. Study limitations

Several limitations must be acknowledged. First, bacterial identification relied exclusively on classical phenotypic and biochemical criteria (Gram stain, catalase, CaCO_3 clear zone and broth acidification); without 16S rRNA gene sequencing or MALDI-TOF mass spectrometry, the isolates can only be classified as presumptive lactic acid bacteria, and their genus, species and strain identities remain undetermined.³⁸ Second, measurements were derived from a single-batch fermentation with three independent isolates rather than multi-batch biological replicates, so intra-strain variability across repeated fermentations was not assessed, and no growth normalisation (OD_{600} or CFU/mL) was performed. Third, the competitive ELISA detects serotonin-like immunoreactivity; anti-serotonin antibodies may cross-react with structurally related indoles (L-tryptophan, 5-hydroxytryptophan, tryptamine), and the signal was measured at a single time point without a pH-matched or heat-killed control, so matrix effects from the acidic broth ($\text{pH} \approx 4.0$) relative to neutral standards cannot be excluded. Orthogonal chromatographic verification by HPLC with electrochemical detection (HPLC-ECD) or liquid chromatography–tandem mass spectrometry (LC-MS/MS), alongside spiked-recovery and dilution-linearity assessments in pH-adjusted media, is required to confirm analyte identity.³⁵ Fourth, because inferential comparisons were based on small samples ($n = 3$ per group), the analyses are strictly exploratory and should not be read as definitive effect estimates.³⁶ Finally, this was an in vitro microbial screening study; no neuronal cell culture, animal behavioural testing or clinical evaluation was performed, so claims regarding antidepressant efficacy or psychobiotic status cannot be made and remain speculative pending in vivo pharmacological and toxicological validation.

4.2. Context from earlier studies

Reported findings across distinct experimental systems and analytical platforms are collated in Table 6. Because concentrations reported in different matrices and assays are not directly equivalent — owing to method-dependent cross-reactivities, extraction

efficiencies and matrix interference — the table provides qualitative context only and does not support quantitative comparison with the present result.

Table 6. Context from earlier studies on neuroactive and acidogenic metabolite production across food-derived LAB and kombucha.

Study / reference	Biological matrix / microorganism	Analytical platform	Reported finding	Methodological context
Present study (2026)	Presumptive LAB (<i>Clitoria ternatea</i> kombucha)	Competitive ELISA (4-PL)	18.11 ± 4.54 ng/mL (peak 23.13 ng/mL)	Preliminary in vitro screening; serotonin-like immunoreactivity in MRS broth
Yano JM, et al. (2015) ¹⁶	Spore-forming gut bacteria / Clostridial isolates	HPLC-ECD & in vivo assays	Colonic 5-HT upregulation	Microbial regulation of peripheral serotonergic biosynthesis via enterochromaffin cells
Batista P, et al. (2023) ²⁴	Conventional kombucha (<i>Camellia sinensis</i> SCOBY)	Metabolomic profiling	Polyphenols, acetic acid, gluconic acid	Microbial succession and composition; no direct neurotransmitter quantification
Villarreal-Soto SA, et al. (2018) ²⁵	Kombucha consortium (yeast + acetic/lactic bacteria)	HPLC & enzymatic profiling	Dynamic organic acid and ethanol kinetics	Strong acidification during fermentation
Suna O, et al. (2025) ³³	<i>Clitoria ternatea</i> & <i>Morus alba</i> extracts	Rodent behavioural models	Neuroprotective & anxiolytic-like effects	Phytochemical synergy in vivo; botanical relevance of the floral matrix
Dinan TG, et al. (2013) ¹⁴	<i>Bifidobacterium</i> & <i>Lactobacillus</i> candidates	Behavioural & neuroendocrine models	Attenuated HPA stress response	Framework defining psychobiotics

Concentrations reported in different matrices and assays are not directly equivalent, owing to method-dependent cross-reactivities, extraction efficiencies and matrix interference; this table provides qualitative context only.

5. Conclusion

This exploratory study showed that acidogenic bacterial isolates with phenotypic characteristics consistent with presumptive lactic acid bacteria can be isolated from butterfly pea flower (*Clitoria ternatea* L.) kombucha, and that their cell-free supernatants gave a higher competitive-ELISA serotonin-like immunoreactivity signal (14.26 to 23.13 ng/mL) than sterile media background. Definitive species-level identification by 16S rRNA gene sequencing, orthogonal chromatographic verification of analyte identity (HPLC/LC-MS), and in vivo neurobiological evaluation are required before any functional significance within the microbiota-gut-brain axis can be inferred.

6. Declarations

6.1. Ethics approval and consent to participate

The protocol was reviewed and granted exemption from full committee review (exemption letter No. 12/IV/2026/KEP) by the Health Research Ethics Committee, Universitas Pembangunan Nasional “Veteran” Jakarta. The investigation was conducted strictly on microbial isolates and food-grade fermentation matrices, without human participants, patient records or live animal subjects; consent to participate was therefore not applicable.

6.2. Consent for publication

Not applicable; no identifiable individual data or images are presented.

6.3. Availability of data and materials

The 4-PL calibration model, microbial phenotypic records and group-level ELISA optical densities are reported in the manuscript and its tables. Duplicate-well optical densities and the raw microplate reader files, together with the analysis script, are available from the corresponding author on reasonable request.

6.4. Competing interests

The authors declare that they have no financial, commercial, professional or personal competing interests related to this manuscript.

6.5. Funding

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6.6. Author contributions

RAM: conceptualisation, methodology, investigation (kombucha preparation, LAB isolation, Gram staining, catalase assay, ELISA), formal analysis, visualisation, writing — original draft. DP: conceptualisation, methodology, supervision, validation, project administration, writing — review and editing. IR: methodology, biochemical interpretation, supervision, validation, writing — review and editing. DJ: methodology, microbiology validation, laboratory resource coordination, supervision, writing — review and editing. All authors read and approved the final manuscript.

6.7. Use of generative artificial intelligence

The authors did not use generative artificial intelligence tools in the preparation of this manuscript.

6.8. Acknowledgements

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