JMBFS
Journal of Microbiology, Biotechnology and Food Sciences
International peer-reviewed scientific online journal
Published by Faculty of Biotechnology and Food Sciences

CHARACTERISATION OF *Lactiplantibacillus plantarum* B13 AND ITS SYNBIOTIC INTERACTION WITH SELECTED PREBIOTICS FOR THE PRODUCTION OF γ-AMINOBUTYRIC ACID (GABA)-ENRICHED FERMENTED MILK

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https://doi.org/10.55251/jmbfs.11041

ARTICLE INFO

Received 16. 2. 2024
Revised 23. 6. 2025
Accepted 8. 10. 2025
Published 1. 12. 2025

Regular article

ABSTRACT

The study was conducted to produce γ-aminobutyric acid (GABA)-enriched fermented milk by formulating synbiotics through the amalgamating of the potential probiotic strain, *Lactiplantibacillus plantarum* B13, isolated from 'Budu' (fermented fish sauce), with a variety of prebiotics (inulin, galactooligosaccharides, sorbitol, maltodextrin, corn starch, apple, and pear) at varying concentrations (1, 2 and 3 % (w/v)). The fermentation process was carried out in skimmed milk, maintained at 37 °C, and agitated at 150 rpm for 48 hours. The findings indicate that prebiotic supplementation, particularly at a concentration of 3 % (w/v), significantly enhanced GABA production, lactic acid levels, and the proliferation of *L. plantarum* B13. Among the tested prebiotics, inulin emerged as the most effective in synergising with *L. plantarum* B13. This combination yielded the highest production of GABA (8.326 ± 0.0488 g/L), lactic acid (41.45 ± 0.167 g/L), and the highest viable cell counts (8.747 ± 0.0058 log10 CFU/mL) in fermented milk. Additionally, the antioxidant activity, assessed through DPPH radical scavenging, revealed that the inclusion of apple and pear as natural prebiotics sources at 3 % (w/v) resulted in the highest values (42.37 ± 2.352 % and 37.38 ± 0.8094 %, respectively). Thus, a synbiotic formulation comprising *L. plantarum* B13 and inulin at 3 % (w/v) is identified as the most favourable, demonstrating promising potential in the development of functional foods and beverages.

Keywords: probiotic, prebiotic, synbiotic, γ-aminobutyric acid, *Lactiplantibacillus plantarum*, fermented milk

INTRODUCTION

Lactic acid bacteria (LAB) have been extensively utilised in the food industry, specifically in the production of fermented foods (**Pannerchelvan *et al.*, 2023a**). In recent years, γ-aminobutyric acid (GABA) producing LAB have garnered considerable interest due to multiple health benefits that have high application potentials in the food and health-oriented industry as well as their generally recognised as safe (GRAS) status. GABA, a non-protein amino acid with four carbon atoms, is synthesised from L-glutamate by the enzyme glutamic acid decarboxylase (GAD) and the cofactor pyridoxal-5-phosphate (PLP) (**Sarasa *et al.*, 2019**). High GABA synthesis by LAB is associated with both adequate glutamic acid levels in the dietary matrix and its GAD activity (**Pannerchelvan *et al.*, 2023b**). A considerable proportion of GABA-producing LAB species have been extracted from fermented foods and subsequently employed in the production of naturally nutritious foods fortified with GABA (**Pannerchelvan *et al.*, 2023a**).
GABA-enriched foods and beverages provide a natural and cost-effective way to obtain the beneficial effects of GABA. Numerous studies have been conducted to support the claims of the health benefits of daily GABA consumption through natural and GABA-enriched foods and beverages. GABA-enriched fermented milk produced by *Lactobacillus brevis* DL1-11 have been demonstrated to improve sleep and reduce anxiety-related behaviour in mice following oral administration of 33.33 mg/kg body weight of the product (**Yu *et al.*, 2020**). A study by **Kanehira *et al.* (2011)**, reported that a 50 mg dose of GABA dissolved in a beverage significantly helped to reduce the psychological and physical fatigue in 30 healthy human subjects, nine whom had been diagnosed with chronic fatigue. Another study found that candidates experienced a decline in alpha wave over time while performing an arithmetic duty (**Yoto *et al.*, 2012**). However, those that orally received a 100 mg dose of GABA showed a smaller decline compared to those that did not receive GABA. Meanwhile, daily oral consumption of defatted rice germ containing 26.4 mg of GABA has been shown to effectively treat neurological disorders (**Okada *et al.*, 2000**). The study demonstrated that consuming GABA-enriched rice germ three times a day helped to alleviate common mental symptoms such as sleeplessness, depression and somnipathy during menopausal and the presenile period.
Skimmed milk can serve as a medium for cultivating probiotics to produce GABA-enriched fermented milk (**Galli *et al.*, 2022**). It contains lactose, vitamins (A and D), and minerals (particularly Ca, K and Na) which are essential for promoting the growth of *L. plantarum* (**Solval *et al.*, 2020**). Fermented milk produced using two different starter combinations (*L. lactis* and *L. rhamnonus* or *L. paracasei*) was found to have a maximum GABA content of 185.81 ± 24.0 and 319.72 ± 27.15 mg/L, respectively (**Galli *et al.*, 2022**). Similarly, **Ramos and Poveda (2022)** successfully produced fermented milks using *L. paracasei* Lb41 and *L. plantarum* Lb56, which exhibited GABA concentrations of approximately 200 mg/L. Additionally, these fermented milk products demonstrated higher viscosity than the control and possessed favourable and distinctive sensory attributes. Meanwhile, **Lee *et al.* (2022)** developed a fermented dairy product with higher levels of beneficial substances using a stepwise co-fermentation process involving *B. subtilis* HA and *L. plantarum* EJ2014. This co-fermented milk was successfully enriched with beneficial components such as γ-PGA, GABA, peptides, and probiotics. Furthermore,it exhibited a unique fragrance reminiscent of seasoned cheese.
The combination of prebiotics and probiotics results in the formation of synbiotics, which serve as a source of nourishment for microorganisms while providing the host with beneficial bacteria (**Singla and Chakkaravarthi, 2017**). A synbiotic product enhances the survival and implantation of live microbial dietary supplements in the gastrointestinal system by selectively promoting the growth and/or activating the metabolism of specific health-promoting bacteria, thereby benefiting the host. To create a synbiotic, it is necessary for the probiotic to have a greater effect when combined with a prebiotic than when used alone, and vice versa (**Kearney and Gibbons, 2017**). Nevertheless, incorporating synbiotics into food remains a significant challenge, as several factors must be considered: (1) the type of bioactive compound (probiotic, prebiotic, synbiotic), (2) the food matrix (solid or liquid), including its complexity or simplicity, (3) the food processing method, as it is crucial to incorporate the bioactive component at the appropriate stage to preserve its chemical function and properties, and (4) sensory acceptability (as many studies focus solely on ensuring that the bioactive compound is present in the minimum quantities required to provide a benefit to the consumer without negatively affecting the sensory characteristics of the product) (**González-Herrera *et al.*, 2020**). Despite these challenges, combining specific probiotics with ideal prebiotic substrates, known as synbiotics, can be a highly effective therapeutic approach, offering the combined benefits of both probiotics and prebiotics (**Yadav *et al.*, 2022**).

GABA, a principal neurotransmitter, holds significant potential for health-promoting benefits. However, its systematic incorporation into dairy products through a synbiotic approach remains a nascent area of research. This limitation stems from the complexity inherent in synbiotic development, which necessitates the strategic pairing of a specific prebiotic with a compatible probiotic strain. Therefore, this study investigates the use of selected prebiotics to establish a synbiotic effect with *Lactiplantibacillus plantarum* B13, aiming to enhance GABA synthesis in fermented milk products.

MATERIALS AND METHODS

Maintenance of *L. plantarum* B13 strain

The *L. plantarum* B13 strain used in this study was originally isolated from *budu*, a traditional fermented fish sauce commonly consumed in Malaysia. The *L. plantarum* B13 strain (GenBank accession number of ON482178.1) was deposited (UPMC 1492) at the Microbial Culture Collection Unit (UNiCC), Universiti Putra Malaysia. The broth culture was prepared by transferring 100 µL of the stock culture into 10 mL de Man Rogosa-Sharpe (MRS) broth medium and incubating for 48 hours at 37 °C in an incubator (SI-50D, Protech, Malaysia). The culture was then transferred onto an MRS agar plate and incubated for another 48 hours at 37 °C. A single colony was picked and transferred into fresh MRS broth, followed by 24 hours of incubation at 37 °C. Glycerol stock culture was prepared by mixing 40 % (v/v) glycerol with the cell culture in a 1:1 ratio. The glycerol stock culture was stored at -80 °C for further use.

In vitro characterisation of potential probiotic *L. plantarum* B13

Acidic pH, bile salts and phenol tolerance: As described by **Mohd Yusof *et al.* (2020)**, the inoculum was initially prepared by transferring 1 mL of stock culture into 9 mL of MRS broth, followed by incubation at 37 °C for 24 hours.
For the acidic pH tolerance test, 1 mL of the overnight culture was transferred into 9 mL of MRS broth, with the pH adjusted to 2.5 and 3.5 using hydrochloric acid. The samples were then incubated at 37 °C for 4 hours.
For the bile salt tolerance test, the overnight culture was transferred into MRS broth containing 0.1 %, 0.3 %, and 0.5 % (w/v) oxygall **(Lu *et al.*, 2024)**, followed by incubation at 37 °C for 4 hours. Serial dilution was performed, and the diluted samples were inoculated onto MRS agar using spread plate method to determine the number of viable cells at 0 hour and 4 hours. The survival rate of the cells was calculated using the following equation:

$$\text{survival rate (\%)} = \frac{\text{Number of viable cells survived } (\frac{\text{CFU}}{\text{mL}})}{\text{Number of initial viable cells inoculated } (\frac{\text{CFU}}{\text{mL}})} \text{ x } 100\%$$

For the phenol tolerance test, 1 mL of the overnight culture was inoculated into 9 mL MRS broths containing 0.2 % and 0.5 % (w/v) phenol. MRS broth without phenol was used as a control. The cultures were incubated at 37 °C for 24 hours, and the OD_{600} readings were recorded by a UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany). Subsequently, the three different cultures were inoculated onto MRS agar using spread plate method and incubated for 24 hours. The number of viable cells was then counted.
Cell surface hydrophobicity and cellular autoaggregation: The experiments were conducted using the microbial adhesion to solvents (MATS) method, as reported by **Rosenberg *et al.* (1980)**, with minor modifications. The culture was initially prepared and incubated at 37 °C for 24 hours. The overnight culture was then centrifuged (Microfuge® 16, Beckman Coulter, USA) at 2097 xg for 10 minutes, followed by washing twice with phosphate-buffered saline (pH 7.4). The pellets were collected and resuspended in phosphate-buffered saline to achieve an absorbance reading of 0.8 at 600 nm (A_0). The solvent and culture suspension were mixed with a1:1 ratio and vortexed for 2 minutes. The mixture was then allowed to phase-separate at 37 °C for 30 minutes. After that, the aqueous phase was collected, and its absorbance was measured at 600 nm (A_1). Three different solvents were used: toluene (an apolar solvent), chloroform (monopolar and acidic solvent) and ethyl acetate (monopolar and basic solvent). The percentage of hydrophobicity was calculated using the following equation:

$$\text{Hydrophobicity (\%)} = \frac{[\text{Initial optical density } (A_0) - \text{ Final optical density } (A_1)]}{\text{Initial optical density } (A_0)} \text{ x } 100\%$$

The method described by **Giri et al. (2018)** was followed to access cellular autoaggregation. Briefly, the overnight grown culture was centrifuged at 2097 xg for 10 minutes, followed by washing twice with phosphate-buffered saline. Then, the pellets were resuspended in phosphate-buffered saline to achieve an absorbance reading of approximately 1.0 at 600 nm (A_0). The culture suspension was vortexed for 30 seconds and subsequently incubated at 37 °C. After that, OD_{600} readings were recorded at certain time points (1, 4 and 24 hours) using a UV-visible spectrophotometer. The percentage of cellular autoaggregation was calculated using the following equation:

$$\text{Autoaggregation (\%)} = \frac{[\text{Initial optical density } (A_0) - \text{ Final optical density } (A_1)]}{\text{Initial optical density } (A_0)} \text{ x } 100\%$$

Exopolysaccharide (EPS) production: According to **Nambiar *et al.* (2018)**, the MRS agar was supplemented with 5 % (w/v) glucose before inoculating *L. plantarum* B13 onto the medium. The plates were then incubated at 37 °C for 24 to 48 hours. By using the loop touch method, the sticky or viscous characteristics of the strain were visually assessed to ascertain the EPS-production.
Antibacterial activity: The antibacterial activity of the strain was assessed using the agar well diffusion method according to **Mohd Yusof et al. (2020)** with some modifications. The antibacterial activity of *L. plantarum* B13 was tested against Gram-positive (*Staphylococcus aureus* and *Bacillus subtilis*) and Gram-negative pathogens (*Escherichia coli* and *Salmonella typhimurium*). All pathogens were obtained from the Department of Microbiology, Faculty of Biotechnology and Biomolecular Science, Universiti Putra Malaysia. For the test cultures, glycerol stocks of each microorganism were prepared by growing them on sterile nutrient agar for 24 hours at 37 ˚C. A loopful of a single colony from each strain was then inoculated into 10 mL of nutrient broth and incubated for 24 hours at 37 ˚C. For the assay, a lawn culture of each strain was prepared by spreading the broth culture evenly over the surface of Mueller Hinton Agar (MHA). Four wells, approximately 8 mm diameter, were made on the surface of the agar plate using a sterile pipette tip. Then, 100 µL of the supernatant from the samples (centrifuged at 9000 × g for 10 minutes and filtered) was pipetted into the wells. A volume of 20 µL of 10 µg ampicillin (a reference dose that complies with the standards set by the **Clinical and Laboratory Standards Institute (CLSI, 2023)**), was used as the positive control, while distilled water served as the negative control. A positive result was indicated by the appearance of a clear zone, which was measured in millimetres.

Preparation of prebiotics

Inulin (Sigma-Aldrich™, USA), galactooligosaccharides (EvaChem®, Malaysia), sorbitol (SB), maltodextrin (EvaChem®, Malaysia), corn starch (Cap Bintang®, Selangor, Malaysia), apple (*Malus pumila*), and pear (*Pyrus communis*) were selected as prebiotic sources in this study. Each prebiotic was prepared in three different concentrations: 1 %, 2 %, and 3 % (w/v). Due to the inherent differences in the physical state of the fruits, both apple and pear were cut into smaller cubes, weighed, and finely ground using a handheld blender before being added to the fermentation medium. These steps were essential to increase the total surface area available for interaction with *L. plantarum* B13.

Profiling of *L. plantarum* B13 batch cultivation in skimmed milk

The overnight culture of *L. plantarum* B13 (10 % v/v) was inoculated into skimmed milk (Sunlac, New Zealand), supplemented with 1 % (w/v) monosodium glutamate (MSG) (Sigma-Aldrich™, USA), for a total fermentation volume of 50 mL in a 250 mL shake flask. Incubation was carried out in an incubator shaker (IS-971R, Lab Companion, USA) at 150 rpm and 37 °C for 54 hours. Sampling was done for the measurement of cell growth, GABA, lactic acid concentrations, as well as pH changes.

Production of GABA-enriched fermented milk supplemented with prebiotics

The culture of *L. plantarum* B13 (10 % v/v) was inoculated into skimmed milk, supplemented with 1 % (w/v) MSG, and individually tested with various prebiotics, including inulin (INU), galactooligosaccharides (GOS), sorbitol (SB), maltodextrin (MD), corn starch (CS), apple (A) and pear (P), at concentrations of 1 %, 2 %, and 3 % (w/v), each in a separate shake flask. The selection of these three prebiotic concentrations was based on the method described by **Zahid *et al.* (2021),** with some modifications. All samples were prepared in triplicates to ensure data reliability and reproducibility. The total volume fermentation volume for each batch was set at 50 mL. Fermentation was halted at 48 hours, and the samples were centrifuged (Microfuge® 16, Beckman Coulter, USA) at 15000 × g for 10 minutes. The supernatant was collected for the measurement of cell growth, GABA and lactic acid concentrations, and antioxidant activity.

Measurement of cell viability by colony forming unit (CFU)

Serial dilution was initially performed on the samples before spread plate culture on MRS agar. A 100 µL volume of each diluted sample was pipetted onto the agar and evenly spread using a sterile glass rod. The plates were then incubated at 37 °C for 48 hours in an incubator (SI-50D, Protech, Malaysia). CFU analysis was conducted and recorded after the 48-hour incubation period. The number of cells per mL of the original cell culture (CFU/mL) was measured as follows:

$$\text{Number of cells per mL (CFUs/mL)} = \frac{\textbf{\textit{No.of colonies}} \times \textbf{\textit{Dilution factor}}}{\textbf{0.1 \textit{mL}}}$$

GABA analysis

GABA production by the bacterial strains during fermentation was analysed using thin layer chromatography (TLC) and colorimetric estimation methods.

The TLC technique, as described by **Rayavarapu *et al.* (2021)** and **Villegas *et al.* (2016)** with some modifications, was employed for the qualitative analyse GABA synthesis. A 2 µL volume of the supernatant was extracted and applied onto TLC 60F254 aluminium sheets (Merck, Darmstadt, Germany). These sheets were subsequently immersed in a solution containing n-butanol, acetic acid, and water in a 5:3:2 ratio, which served as the mobile phase. The presence of GABA was indicated by the appearance of a red-coloured spot on the TLC plate. This spot was compared to a standard GABA reference after applying a 1.0 % ninhydrin reagent solution and incubating at 70 °C for 30 minutes in a dryer (FDD-720, Protech, Malaysia).
The colorimetric estimation method described by **Dikshit and Tallapragada (2015)** was used to further analyse GABA. Following TLC, the detected GABA spots were collected by scraping them into a powdered form. The resulting powder was then transferred into a glass tube containing 3 mL of borate buffer (pH 7) and 0.5 mL of ninhydrin reagent (Macklin®, Shanghai, China; 0.8 % concentration dissolved in acetone). The sample was then vigorously mixed using a vortex mixer before placed in a water bath at 70 °C for 20 minutes. Absorbance was measured at 570 nm using a UV-visible spectrophotometer (UviLine 9400, SI Analytics, Germany). A standard curve was generated using a standard GABA compound at various concentrations (0 to 10 µg/µL) to determine the GABA concentration in the samples. The blank was prepared by mixing equal volumes of borate buffer and ninhydrin reagent, without the addition of any samples.

Lactic acid analysis

The supernatant sample was used to determine the lactic acid concentration by mixing 25 µL of each centrifuged sample with 1000 µL of a 0.2 % iron (III) chloride ($FeCl_3$) solution and vortexing to ensure thorough mixing (**Borshchevskaya *et al.*, 2016**). The absorbance was measured at 390 nm using a UV-Vis spectrophotometer within 15 minutes, ensuring the solution's colour remained stable. The reference solution for the spectrophotometer was prepared using 2 mL of the fermentation medium without any cells. A lactic acid standard curve was generated with concentrations ranging from 0.02 to 0.2 mg/µL.

pH changes

For pH measurement, the remaining fermentation liquid, after sampling for GABA and lactic acid analysis, was used to determine the pH value. The pH probe of the pH meter (Eutech pH 700, Thermo Fisher Scientific, United States) was immersed in the fermentation liquid, and the recorded pH value was noted.

Antioxidants activity by DPPH radical scavenging activity

Antioxidant activity (free radical scavenging) was determined according to the method described by **Son and Lewis (2002)**. A 500 µL volume of freshly prepared 0.06 mg/mL 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution was aliquoted into a methanol-containing supernatant sample (25 µL + 475 µL methanol) and thoroughly mixed before being incubated in the dark for 30 minutes at room temperature. Methanol was used as a blank, while an equal volume of methanol and DPPH (1:1) served as the control. After incubation, absorbance was measured at 517 nm using a UV-Vis spectrophotometer. DPPH free radical scavenging activity (RSA) was calculated as a percentage using the following formula:

$$RSA\ (DPPH)\ (\%) = \frac{Control\ Absorbance\ (A_{control}) - Sample\ Absorbance\ (A_{sample})}{Control\ Absorbance\ (A_{control})} \times 100$$

Statistical analysis

All experimental data were analysed using GraphPad Prism 9.0 (San Diego, CA, USA). Statistical differences between the means were evaluated using one-way analysis of variance (ANOVA) with a significance level of $p < 0.05$. Multiple comparisons of means were conducted using Tukey's multiple range test. All experiments were performed in triplicate. The mean values and standard errors of each sample were presented as means ± standard error of the mean (means ± SEM).

RESULTS AND DISCUSSION

In vitro characterization of potential probiotic *L. plantarum* B13

Tolerance to acidic pH, bile salts and phenol: It is essential for probiotics to withstand both acidic and alkaline conditions to survive in the human's gastrointestinal tract (**Prete *et al.*, 2020**). In the duodenum, bile secreted from the gallbladder breaks down the lipid membrane of cells, causing leakage of cell contents and ultimately leading to cell death. Therefore, to reach the small intestine and maintain their viability for a prolonged period while exerting beneficial effects, probiotics LAB must demonstrate tolerance to bile and acidic pH. As shown in **Table 1**, *L. plantarum* B13 remained viable at pH 2.5 and 3.5 after 4 hours of incubation, with a higher survival rate at pH 3.5 compared to pH 2.5 that equivalent to 91.62 % and 65.62 %, respectively.
L. plantarum B13 also demonstrated ability to survive in the presence of bile salts. Cell viability slightly decreased after 4 hours of incubation in bile salt concentrations of 0.1 % (w/v), 0.3 % (w/v), and 0.5 % (w/v). The strain remained viable with survival rates of 87.93 % and 60.44 % when grown in medium containing 0.1 % (w/v) and 0.3 % (w/v) bile salts, respectively. However, when the bile salt concentration was adjusted to 0.5 % (w/v), the survival rate of *L. plantarum* B13 declined to 50.00 %. This indicates that higher bile salt concentrations reduce cell viability (log CFU/mL). According to **Nath *et al.* (2020)**, high bile salt concentrations damage the cell membrane by breaking down lipids and fatty acids, leading to increased cell permeability and ultimately causing cell death. However, considering that the average bile salt concentration in the human gastrointestinal tract ranges from 0.03% to 0.3% (w/v), a survival rate exceeding 50% can be considered indicative of good bile tolerance in the intestinal environment (**Ou *et al.*, 2022**). The findings of this study align with previous research by **Kouadio et al., (2024)**, which reported that *L. plantarum* FS46, isolated from fermented Dockounou paste, exhibited a survival rate of 68.62 % after 4 hours of exposure to 0.3 % bile salts. Similarly, *L. plantarum* TA4, isolated from the Malay traditional fermented food (*Tapai pulut*), was reported by **Mohd Yusof *et al.* (2020)** to survive at pH 2.5 and 3.5, with survival rates of 79 % and 90.5 %, respectively. At a bile salt concentration of 0.3 % (w/v), *L. plantarum* TA4 maintained a survival rate of 83.2 %. Additionally, a potential probiotic *L. plantarum* L7, isolated from a traditional rice-based fermented beverage "bhaati jaanr" was found to remain viable at pH 2.0 and 3.0, as well as in a 0.3 % bile salt concentration (**Giri *et al.*, 2018**). Variations in survival rates among different *L. plantarum* strains in bile salts may be attributed to differences in membrane resilience, stress response efficiency, and environmental adaptation, suggesting that their original habitats influence their tolerance levels (**Prete et al., 2020**). Therefore, based on the results of this study, *L. plantarum* B13 is likely capable of surviving and remaining viable in acidic conditions and the presence of bile salts in the gastrointestinal tract, highlighting a key characteristic by probiotic strains.
Additionally, *L. plantarum* B13 also demonstrated the ability to survive in a medium containing phenol. After 24 hours of incubation, the control (without phenol) exhibited the highest cell viability (8.947 log CFU/mL), followed by the 0.2 % (w/v) phenol with a cell viability of 8.644 log CFU/mL, and the 0.5 % (w/v) phenol with a cell viability of 7.893 log CFU/mL. The performance was superior to that of *L. plantarum* TA4, which was previously demonstrated to maintain only approximately 5 log CFU/mL at 0.5 % (w/v) phenol (**Mohd Yusof *et al.*, 2020**). Phenol is a toxic metabolite that suppresses bacterial growth in the gut (**Sharma *et al.*, 2022**). Therefore, it is essential for probiotics to tolerate limited amounts of phenol to survive in the gastrointestinal tract, as phenol is produced when gut microorganisms deaminate various aromatic amino acids derived from dietary and endogenous proteins. A phenol concentration of 0.4 % or higher may have a strong inhibitory effect on probiotic cell viability (**Nandha and Shukla, 2023**).

Cell surface hydrophobicity, cellular autoaggregation and exopolysaccharide production: Autoaggregation ability and cell surface hydrophobicity are two independent traits, commonly used as indirect methods to assess the bacterial adhesion capacity. The autoaggregation ability of probiotic strains is essential for bacterial adherence and plays a crucial role in biofilm formation. Meanwhile, bacterial adhesion capacity is closely associated with cell surface properties (**Canzi *et al.*, 2005**). Exopolysaccharides (EPS) are fundamental components of the extracellular biofilm matrix and contribute significantly to biofilm developement and bacterial persistence (**Nguyen *et al.*, 2020**).
Toluene, chloroform, and ethyl acetate represent a non-polar solvent, a mono-polar electron-accepting solvent, and a mono-polar electron-donating solvent, respectively (**Klopper *et al.*, 2018**). As shown in **Table 2**, *L. plantarum* B13 exhibited the strongest adherence to chloroform (72.48 %), followed by toluene (25.31 %) and ethyl acetate (15.77 %). When comparing the polar solvents (chloroform and ethyl acetate), the results indicate that *L. plantarum* B13 displayed a greater affinity to the acidic solvent (electron accepting) chloroform than for the basic solvent (electron-donating), ethyl acetate. This suggests that electrostatic interactions between the bacterial cell surface and the solvent played a key role. Electrostatic interactions occur between oppositely charges ions, such as cations and anions. Furthermore, the high affinity of *L. plantarum* B13 for chloroform and its low affinity for ethyl acetate suggest that this strain may have the potential to colonise intestinal mucus, which carries a net negative charge (**Klopper *et al.*, 2018**). According to **Polak-Berecka *et al.* (2014)**, *L. rhamnosus* PEN and *L. rhamnosus* E/N also showed a greater tendency to adhere to chloroform, with adherence rates of 81.62 % and 76.49 %, respectively, compared to ethyl acetate and hexadecane, confirming their hydrophilic nature. Similarly, the results indicate that *L. plantarum* B13 exhibited low adherence to the non-polar solvent (toluene), suggesting that this strain has low hydrophobicity. According to **Colloca *et al.* (2000)**, cell surface hydrophobicity is influenced by the composition of surface polymers in hydrophobic interaction.
It was observed that a longer incubation time led to increased autoaggregation activity in *L. plantarum* B13. The highest autoaggregation activity (73.03 %) was recorded after 24 hours of incubation, compared to 1 hour (19.98 %) and 4 hours (33.80 %). Similarly, **Mohd Yusof *et al.* (2020)**, reported that *L. plantarum* TA4 achieved its highest autoaggregation activity (99.33 %) after 24 hours of incubation. In another study assessing the aggregation ability of various *Lactobacillus* isolates (from the gut of native (desi) chickens), it was discovered that three out of twenty *L. reuteri* strains exhibited significantly high

autoaggregation potential, ranging from 57 to 98 % after 5 hours of incubation (**Aziz *et al.*, 2019**). Generally, autoaggregation percentages are classified as follows: values above 70 % are considered high, between 20 % to 70 % as medium, and below 20 % as low (**Rahman *et al*., 2008**).

Table 1 Tolerance of *L. plantarum* B13 under different pH, bile salt and phenol concentrations

Cell tolerance under different pH for the incubation time of 0 and 4 hours			
pH	**log CFU/mL**		**Survival rate (%)**
	0 hour	**4 hours**	
2.5	8.241 ± 0.0299[a]	8.050 ± 0.1223[a]	65.62
3.5	8.294 ± 0.0375[a]	8.255 ± 0.0563[a]	91.62
Cell tolerance under different bile salt concentrations for the incubation time of 0 and 4 hours			
Oxgall concentration (%)	**log CFU/mL**		**Survival rate (%)**
	0 hour	**4 hours**	
0.1	6.461 ± 0.0424[a]	6.406 ± 0.0120[a]	87.93
0.3	5.259 ± 0.0507[b]	5.034 ± 0.1129[b]	60.44
0.5	4.040 ± 0.0560[c]	3.690 ± 0.3012[c]	50.00
Cell tolerance under different phenolic concentrations			
Phenol concentration (%)	**log CFU/mL**		
Control (no phenol)	8.947 ± 0.0058[a]		
0.2	8.644 ± 0.0081[b]		
0.5	7.893 ± 0.0037[a]		

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

The results indicated that *L. plantarum* B13 did not produce EPS. According to **Wang *et al*. (2010)**, EPS production in microorganisms is influenced by the composition of the growth medium and cultivation conditions (ie., temperature and pH). For instant, in the optimization of medium formulation for *L. plantarum* MTCC 9510, lactose, yeast extract, and ammonium sulphate were found to have a significant impact on EPS production, which peaked after 72 hours of incubation (**Ismail and Nampoothiri, 2010**). Changes in EPS production at different temperatures (20, 30 and 37°C) were observed for several *L. paracasei* strains, with an increase in EPS recorded at the lowest temperature (**Bengoa *et al*., 2018**). Furthermore, *Lactobacillus* species have been reported to produce high amounts of EPS at 35 °C and pH range of 6.5 to 7.0 (**Ismail and Nampoothiri, 2010**). In this study, culture medium was initially adjusted to pH 5.5, which did not appear to favour EPS production by *L. plantarum* B13.

Table 2 Cell surface hydrophobicity activity with different organic solvents, cellular autoaggregation assessment with different incubation time and EPS production ability of the *L. plantarum* B13 strain

Cell surface hydrophobicity (%)			**Cellular autoaggregation (%)**			**EPS Production**
Chloroform	**Toluene**	**Ethyl acetate**	**1 hour**	**4 hours**	**24 hours**	
72.48 ± 0.1245[a]	25.31 ± 0.2138[b]	15.77 ± 0.4304[c]	19.98 ± 0.1004[c]	33.80 ± 0.2090[b]	73.03 ± 0.0580[a]	-

The results are expressed as mean (n = 3) ± SD. Different letters in the rows of each group are significantly different based on Tukey's test at $p < 0.05$.

Antibacterial activity: The antimicrobial effect of the cell-free supernatant of *L. plantarum* B13 was evaluated against both Gram-positive and Gram-negative pathogenic bacteria. Generally, the diameter of the inhibition zone classifies bacteria as resistant (≤9 mm), moderately sensitive (10-11 mm), or sensitive (≥12 mm) to the sample (**Sarker *et al*., 2014**). As shown in Table 3, the cell-free supernatant of *L. plantarum* B13 was considered moderately sensitive, exhibiting the highest antimicrobial activity against *S. aureus* with an inhibition zone of 11.50 mm. In contrast, the other three bacteria (*E. coli*, *S. typhimurium* and *B. subtilis*) showed similar inhibition zone diameters of 10 mm. The antibacterial activity of *L. plantarum* B13 against *E. Coli*, *S. typhimurium* and *S. aureus* was notably lower compared to *L. plantarum* T4, which demonstrated inhibition zones of 19.33mm, 16.67 mm and 19.00 mm, respectively, as previously reported by **Mohd Yusof *et al*. (2020)**. The variation in final pH recorded for both strains (pH 3.84 for *L. plantarum* B13 and pH 3.41 for *L. plantarum* T4) may have influenced their inhibitory activity. This suggests that the two strains likely produced different organic acids acting as inhibitory substances, leading to different antimicrobial effects (***Vizoso Pinto et al. 2006***). Additionally, some studies have utilised viable probiotic cells instead of cell-free supernatant for antimicrobial testing. For example, **Adeniyi *et al.* (2015)**, reported that viable cells of *Enterococcus hirae* CO6A exhibited a stronger antimicrobial effect against *E. coli* CB6 than its cell-free supernatant, with inhibition zones of 26 mm and 10 mm, respectively. Similarly, **Benítez-Serrano *et al.* (2018)**, demonstrated the antimicrobial activity of the cell-free supernatant of *L. plantarum* and *L. brevis* against *S. typhimurium* and *E. coli*. Meanwhile, *L. plantarum* strain GCC19M1 exhibited antimicrobial activity against a broad spectrum of pathogens, including *Bacillus cereus, Acinetobacter johnsonii, Pseudomonas aeruginosa, Stenotrophomonas maltophilia, Cedecea davisae* and *Achromobacter spanius* (**Nath *et al*., 2020**). In general, antimicrobial activity is often associated with bacteriocins produced by LAB strains and their stability under acidic conditions (**Alizadeh Behbahani *et al*., 2020**). Beside bacteriocins, probiotics can synthesise various other antimicrobial compounds, including organic acids (ie., lactic acid, acetic acid, propionic acid, and succinic acids), hydrogen peroxide, ethanol, diacetyl, acetoine, acetaldehyde, carbon dioxide, reuterin and reutericyclin, as part of their metabolism pathways (**Adeniyi *et al*., 2015**). The organic acids produced by probiotics contribute to acidity, creating unfacourable environment for certain pathogenic bacteria to proliferate. Additionally, these organic acids can permeate the cell membranes of pathogenic bacteria, leading to structural changes in the phospholipid content within the cells (**Arena *et al*., 2016**).

Table 3 Antibacterial activity of cell-free supernatant of *L. plantarum* B13 against different bacteria strains

Sample	**Tested bacteria strain**	**Zone of inhibition (mm)**
***L. plantarum* B13**	*E. coli*	10.00 ± 0.1414[d]
	S. typhimurium	10.00[d]
	S. aureus	11.50 ± 0.0707[c]
	B. subtilis	10.00[d]
Ampicillin (Positive control)	*E. coli*	11.0 ± 0.0707[b]
	S. typhimurium	14.0 ± 0.1061[a]
	S. aureus	11.0 ± 0.1414[c]
	B. subtilis	18.0 ± 0.1414[b]
Distilled water (Negative control)	*E. coli*	0.00[e]
	S. typhimurium	0.00[e]
	S. aureus	0.00[e]
	B. subtilis	0.00[e]

The results are expressed as mean (n = 3) ± SD. Different letters in the column of each group are significantly different based on Tukey's test at $p < 0.05$.

Growth, GABA, lactic acid and pH changes profiles of *L. plantarum* B13 in fermented milk

Fig. 1(A) illustrates the growth curve of *L. plantarum* B13 cultured in a skimmed milk medium, incubated at 37 °C with an agitation speed of 150 rpm over 54 hours. The initial concentration of *L. plantarum* B13 was recorded to be 6.637 log CFU/mL, reflecting the cell density in the inoculum culture. During the first 12 hours, a slight increase in cell viablity was observed, reaching 6.724 log CFU/mL, before further rising to a maximum of 6.821 log CFU/mL.

During the initial 12-hour phase of fermentation, *L. plantarum* B13 exhibited a slow rate of GABA biosynthesis (Fig. 1 (B)). The GABA production phase became evident from the 24th hour, reaching a concentration of 1.681 g/L, followed by a gradual increase to its peak at the 48th hour, where a maximum concentration of 1.862 g/L was recorded. After reaching this peak, there was a noticeable decrease in GABA levels as fermentation progressed, culminating in a diminished yield by the 54th hour. This trend suggests a potential shift in metabolic activity or the depletion of essential substrates required for sustained GABA synthesis. According to **Atanda *et al.* (2007)**, GABA synthesis initiates during the bacterial growth phase and intensifies during the stationary phase due to heightened GAD activity, which is an endogenous enzyme produced in response to acidic conditions.

The synthesis of lactic acid was also tracked, revealing that rapid production commenced within the first 3 hours of fermentation and increase progressively over time (Fig. 1 (B)). Similar to GABA, lactic acid production peaked at the 48th hour, registering a concentration of 26.54 g/L. Compared to GABA, lactic acid concentrations were markedly higher, which aligns with the typical metabolic profile of LAB, where lactic acid is the predominant metabolite produced (**Othman *et al.*, 2017; Pannerchelvan *et al.*, 2024**).

Similarly, the pH of the fermentation medium exhibited a consistent downward trajectory, starting at 6.58 at the 0th hour and descending to 5.93 by the 54th hour (Fig. 1 (A)). This inverse relationship between lactic acid concentration and pH is anticipated, as the accumulation of lactic acid contributes to increased acidity in the fermentation medium.

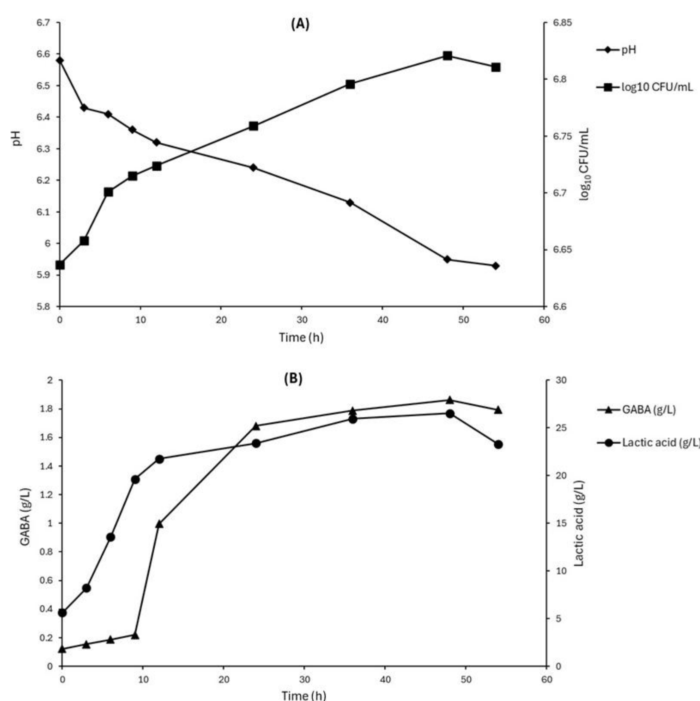

Figure 1 The profiles of (A) cell growth based on $\log_{10}$ CFU/mL (■) and pH changes (♦) and (B) production of GABA (▲) and lactic acid (●) of skimmed milk fermentation by *L. plantarum* B13 at 37 °C and 150 rpm for 54 hours.

Production of GABA-enriched fermented milk with supplementation of prebiotics by *L. plantarum* B13

Based on Fig. 2 (A), the highest CFU/mL was observed when prebiotic inulin (INU) was employed in the fermented milk medium. Notably, high values were recorded from 1 % (w/v) onwards, reaching 8.477 log CFU/mL at 1 % (w/v), 8.644 log CFU/mL at 2 % (w/v) and peaking at 8.747 log CFU/mL at 3 % (w/v). In constrast, the lowest values were recorded when corn starch (CS) was used, with log CFU/mL values starting at 6.879 at 1 % (w/v), increasing to 7.123 ± 0.0077 at 2 % (w/v), and reaching a peak at 7.338 at 3 % (w/v). The data also revealed a progressive increase in bacterial growth with increasing concentrations of galactooligosaccharides (GOS), sorbitol (SB), apple (A), pear (P), and maltodextrin (MD). Specifically, at 3% (w/v), the highest values recorded were 8.644 log CFU/mLfor galactooligosaccharides (GOS), 8.468 for sorbitol (SB), 8.277 apple (A), 7.893 for pear (P), and 7.527 for maltodextrin (MD). Compared to the highest cell count (6.637 log CFU/mL) obtained in fermented milk without the addition of prebiotic (CTRL), these findings highlight the role of each supplemented prebiotic in escalating the growth of *L. plantarum* B13. Overall, a noticeable pattern emerges in which higher prebiotic concentrations correspond to increased bacterial growth, indicating a dose-dependent effect. Among the tested prebiotics, the well-known sources, inulin (INU) and galactooligosaccharides (GOS) (**Vinayak *et al.*, 2021**), exhibited the greatest capacity for bacterial proliferation, suggesting a synbiotic effects that may be particularly effective in promoting the growth of *L. plantarum* B13. **Savedboworn *et al.* (2017)** demonstrated that 2 % inulin was the optimal concentration for promoting the growth of probiotic *L. plantarum* TISTR 2075 when fermented in *Plai Ngahm Prachin Buri* rice extract. This concentration resulted in a viable cell count of exceeding 8.94 log CFU/mL after 24 hours of fermentation at 37 °C. Similarly, **Rahimiyan-Heravan *et al.* (2020)** reported that the combination of *L. plantarum* 1085 (ATCC 8014) and inulin not only increased cell counts but also improved fertility parameters and reproductive capabilities.

As observed for cell viability, the highest GABA production was occurred at a concentration of 3 % (w/v) for all tested prebiotics (Fig. 2(B)). At this concentration, inulin (INU) yielded the highest GABA level at 8.326 ± 0.0488 g/L, followed closely by galacto-oligosaccharide (GOS) with a concentration of 7.217 ± 0.2687 g/L. In contrast, the lowest GABA production was recorded when maltodextrin (MD) and corn starch (CS) were used as prebiotics, achieving 3.662 ± 0.0488 g/L and 2.686 ± 0.1490 g/L, respectively. When compared to the highest GABA concentration (1.862 g/L) obtained from fermented milk without prebiotic (CTRL), the addition of prebiotics positively influenced GABA production by *L. plantarum* B13 that corresponding to the lowest GABA of 1.864 g/L (for corn starch (CS)) at 1 % (w/v)). Furthermore, the data delineates a positive correlation between increasing prebiotic concentration and GABA synthesis. This observed rise in GABA levels concomitant with higher prebiotic concentrations suggests a substrate-dependent enhancement of microbial GABA biosynthesis, offering potential strategies for optimising the fermentative production of this bioactive compound. The findings also indicate that *L. plantarum* B13 is capable of utilising the supplemented prebiotics, which also served as a carbon source (**You *et al.*, 2022**), alongside with trace amounts of lactose present in the skimmed milk (**Solval *et al.*, 2020**).

Fig. 2 (C) illustrates the variations in lactic acid concentrations synthesised by *L. plantarum* B13 when different prebiotics were utilised as substrates. The data show a clear positive correlation between prebiotic concentration and lactic acid production. Specifically, inulin (INU) exhibited the most substantial lactic acid generation, commencing at 38.68 ± 0.025 g/L at 1 % (w/v), escalating to 39.55 ± 0.063 g/L at 2 % (w/v), and peaking at 41.45 ± 0.167 g/L at 3 % (w/v). These results align with the findings of **Savedboworn *et al.* (2017)**, who reported that after 24 hours of fermentation, the presence of 2% and 3% inulin had resulted in drastically elevated lactic acid production, reaching maximum concentrations of 0.13 % and 0.16 %, respectively. Conversely, a discernible decrement in lactic acid production by *L. plantarum* B13 was observed when progressing from left to right across the different prebiotic types. The order of efficacy in lactic acid production is as follows: Inulin (INU) > Galactooligosaccharides (GOS) > Sorbitol (SB) > Apple (A) > Pear (P) > Maltodextrin (MD) > Corn Starch (CS). Notably, corn starch (CS) was the least effective prebiotic, with its highest concentration (3 % w/v) yielding 28.27 ± 0.021 g/L. This study suggests that the carbon source for lactic acid production was derived not only from trace amounts of lactose in the skimmed milk but also mainly from the supplemented prebiotics. This is evident in the lactic acid concentration recorded in all fermented milks, with the lowest value (27.19 g/L for corn starch (CS) at 1 % w/v) still exceeding the maximum lactic acid concentration (26.54 g/L) produced in fermented milk without the addition of prebiotic (CTRL).

As depicted in Fig. 2 (D), apple (A) demonstrated a notably high efficacy in DPPH radical scavenging activity, registering a substantial 42.37 % ± 2.352 at a concentration of 3 % (w/v). This was closely followed by pear (P), which exhibited a comparable scavenging activity of 37.38 % ± 0.8094 at the same concentration. These findings underscore the significant antioxidant potential of these prebiotics, particularly in comparison to others in the study. For instance, corn starch (CS) manifested a markedly lower activity, recording only 12.15 % ± 1.764 at 3 % (w/v). In general, **Vougiouklaki *et al.* (2023)** assert that LAB, including *L. plantarum* serve as an exceptional source of biological antioxidants. This finding underscores the premise that the incorporation of prebiotics can substantially augment antioxidant efficacy, suggesting a synergistic interaction in promoting antioxidant activities. Furthermore, the high scavenging activity exhibited by apple (A) aligns with the findings of **Boyer and Liu (2014)**, who reported that apples are exceptionally rich in a diverse range of phytochemicals, including quercetin, catechin, phloridzin, and chlorogenic acid, all of which possess potent antioxidant properties. With regard to inulin (INU), extensive research indicates that this prebiotic exhibits relatively low DPPH radical scavenging activity, alongside modest ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging activity and limited ferric reducing antioxidant power (**Shang *et al.*, 2018**; **Zhou *et al.*, 2019**). This characterisation is substantiated by its relatively low DPPH radical scavenging activity, quantitatively measured at 20.64 % ± 0.2700 at 3 % (w/v). Similarly, galactooligosaccharides (GOS) (16.67 % ± 0.2698), sorbitol (SB) (16.43 % ± 0.7138) and maltodextrin (MD) (14.25 % ± 0.8424) demonstrated closely aligned efficacies at their maximal supplementation concentration of 3 % (w/v). The presence of antioxidant activity (7.15 % ± 3.522) in GABA-enriched fermented milk without the addition of prebiotic (CTRL) may be attributed to the *L. plantarum* B13 strain itself. LAB are known to possess inherent antioxidant properties and have demonstrated effective antioxidant activity (**Nakagawa and Miyazaki, 2017**). In particular, *L. plantarum* ST63HK exhibited a DPPH radical scavenging activity of 35.60 %, highlighting the natural antioxidant characteristics of LAB as a valuable alternative to synthetic antioxidants (**Rwubuzizi *et al.*, 2023**).

Additionally, **Cukkemane *et al.* (2020)** elucidated that the antioxidant potential of synbiotics stems from their ability to activate and translocate nuclear factors. These factors, in turn, stimulate the activation of enzyme systems responsible for protecting against oxidative damage, generate essential antioxidant molecules, and mitigate the harmful effects of singlet oxygen and free radicals.

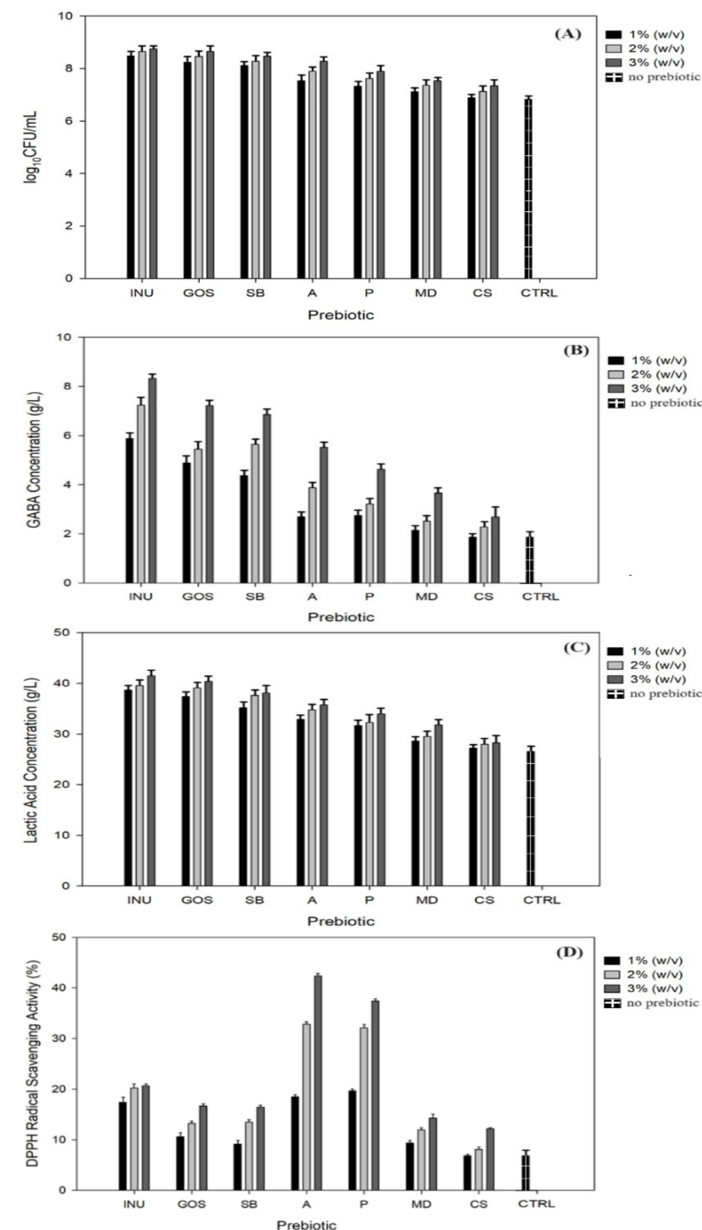

Figure 2 Colony forming unit (CFU/mL) **(A)**; production of GABA (g/L) **(B)**; production of Lactic acid (g/L) **(C)**; and antioxidant activity (%) **(D)** of *L. plantarum* B13 analysed from the fermentation of probiotic *L. plantarum* B13 supplemented with different types and concentrations (1, 2 and 3 % (w/v)) of prebiotics in skimmed milk as the medium. The results are expressed as mean (n = 3) ± SD. Abbreviations: INU (Inulin), GOS (Galacto-oligosaccharides), SB (Sorbitol), A (Apple), P (Pear), MD (Maltodextrin), CS (Corn Starch), and fermented milk without the addition of prebiotic as control (CTRL).

CONCLUSION

The potential probiotic attributes of *L. plantarum* B13 were demonstrated through several *in-vitro* assessments including a high survival rate in acidic and bile salt conditions, a high number of viable cells count in the presence of phenol, autoaggregation activity of 73.03 %, a hydrophilic nature due to high adherence towards chloroform (72.48 %) and exhibited antimicrobial effects. This study also highlighted a synbiotic approach, whereby the combination of *L. plantarum* B13 and prebiotics effectively enhanced GABA production in fermented milk. Inulin at 3 % (w/v) was identified as the most potent prebiotic for boosting both GABA synthesis and viable cell counts. All tested prebiotic mixtures resulted in substantial lactic acid production, with inulin, galacto-oligosaccharides, and sorbitol proving to be the most effective. In contrast, maltodextrin and corn starch performed poorly, indicating strain-specific interactions and their unsuitability for establishing a synbiotic relationships with *L. plantarum* B13. Prebiotics derived from natural sources, such as apples and pears, exhibited remarkable antioxidant properties. This finding holds significant promise for the development of functional foods and underscores the intricate interplay between prebiotics and probiotics in food science and nutrition.

Conflict of interest: The authors declare that there is no conflict of interest.

Acknowledgments: This research was funded by the Universiti Putra Malaysia under Geran Putra GP-IPS (UPM-IPS/2023/9742200).

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