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Gamma-Aminobutyric Acid (GABA) Producing *Lactobacillus plantarum* TSUB-17 and Probiotic Properties for Using as Probiotics Additive in Swine Feed

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Abstract

Due to the use of probiotics in animal husbandry has recently increased, the selection of high-quality probiotic strains is crucial. Since the uses of probiotics for animal feed supplements have to be applied at high doses of bacteria to be effective, the cultures' safety is essential. This study successfully isolated the novel *Lactobacillus* sp. from swine fecal samples and identified it as *L. plantarum* TSUB-17. The *L. plantarum* TSUB-17 exhibited excellent probiotic properties, including 100 % resistance to 3 % acids and 1 % bile salts, and effectively inhibits pathogenic bacteria such as *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Salmonella Typhimurium* (isolated ESBL strain). *L. plantarum* TSUB-17 was also found to adhere to the intestinal wall and form a biofilm. Furthermore, the bacteria have revealed some unique properties of GABA production and detected the glutamate decarboxylase B (*gad*B) gene similar to *L. brevis* both in nucleotide sequence and secondary structure of the glutamate decarboxylase enzyme that was deduced from the nucleotide. The isolated bacteria also have antioxidant activity, and most importantly, the isolated strain was safe; it is non-hemolysis and susceptible to the tested antimicrobials. Therefore, *L. plantarum* TSUB-17 is suitable for further development as a probiotic additive in animal feed.

Keywords: *Lactobacillus*, GABA production, Probiotics properties, Swine feces, Antioxidant

Introduction

Probiotics are beneficial microorganisms that have been used by both humans and livestock. In livestock, probiotics stimulate growth and improve the quality of metabolism and gut microbiota [1]. Simultaneously, some strains of probiotics can inhibit pathogenic bacteria in the gastrointestinal tract [2]. Therefore, using probiotics is supposed to reduce the use of antimicrobials in the animal husbandry process. In addition, probiotics can produce various secondary metabolites that benefit animal health, such as gamma-aminobutyric acid (GABA), which is currently an interesting substance. Gamma-aminobutyric acid or GABA, is an amino acid not classified as a protein. It is synthesized from glutamic acid by the decarboxylation pathway. GABA acts as a neurotransmitter in the central nervous system of humans and animals, promoting relaxation, reducing stress, affecting muscle building and reducing obesity through enhanced insulin action [3,4]. In livestock, many exciting studies indicate that GABA reduces stress in weaning pigs [5]. It also significantly affected pigs' average daily growth rate [5]. In laying hens, supplementation with GABA has been found to positively affect egg quality in terms of improved laying hen quality [6]. It was also found that GABA increases the immune responses in chickens during stress conditions [7]. Therefore, GABA is a valuable metabolite that can be applied to humans and livestock.

Sources of GABA can be found in both plants and animals. In plants, GABA is found in many dried tea leaves, whole grains, tomatoes and legumes, but the most common is germinated brown rice [8]. This is because GABA is a substance involved in the degradation process in grains. In addition, humans and animals can synthesize GABA via the tri-carboxylic acid (TCA) cycle using pyruvate as a precursor obtained from the glycolysis to convert into alpha-ketoglutarate and glutamate and finally to GABA [9,10]. As mentioned above, many probiotic bacteria are able to synthesize GABA [11,12]. Interestingly, a

comparison study of GABA production between germinated brown rice and probiotic *Lactobacillus* demonstrated that the bacteria produce higher GABA [13,14]. Therefore, *Lactobacillus* is another crucial source of GABA production.

The majority of GABA-producing *Lactobacillus* species are *L. brevis*, and certain are found in *L. plantarum* [15-17]. Since the mechanism for the production of GABA in *Lactobacillus* depends on the enzyme glutamate decarboxylase [10]. This enzyme is encoded by the glutamate decarboxylase (*gad*) gene, which is widely distributed in *L. brevis* and *L. plantarum.* Although GABA-producing *Lactobacillus* can be isolated from various sources such as fermented foods and dairy products [18-20], they can be used to develop probiotics products. However, the development of effective probiotics for livestock should be based on bacteria isolated from the same genus of animals. Therefore, this study aimed to characterize GABA production and determine the probiotic properties of *L. plantarum* TSUB-17, which was isolated from a swine feces sample.

Materials and methods

Bacterial isolation and Matrix Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) identification

Lactobacillus plantarum TSUB-17 was isolated from pig feces samples. The feces were prepared as a suspension using approximately 1 g of feces in 9 mL sterile normal saline, and then the sample was diluted sequentially 10-fold. Then 0.1 mL at the 10^{-3} - 10^{-5} dilution was spread on de Man, Rogosa, Sharpe (MRS) agar adjusted pH to 3.0 with 1 M HCL and supplemented with 25 µg/mL amphotericin B as antifungal. The medium was placed in an anaerobic jar (Schuett-Biotech; Germany), and the air inside was removed to keep the minimum oxygen exposure with a vacuum pump [21], then incubated at 37 °C. After 72 h, colonies were randomly selected. To presumptively test of the *Lactobacillus* isolates, catalase test and Gram staining by the *Lactobacillus* group present negative catalase results and Gram-positive with bacilli-shape. The obtained isolated bacteria were stored in a −80°C freezer for further study.

For the step of identification, in addition to 16S rRNA gene sequencing, reciprocal confirmation was performed by the Matrix-Assisted Laser Desorption Ionization Time-Of-Flight (MALDI-TOF) assay. The assay was based on a fresh colony (18 h) analyzed by MALDI Biotyper (Bruker Daltonik GmbH: Germany; Office of Scientific Instruments and Testing, Prince of Songkla University).

GABA production analysis

The *L. plantarum* TSUB-17 was grown in a 200 mL Erlenmeyer flask containing 50 mL MRS medium supplemented with 2 g/L (w/v) monosodium glutamate (MSG) as a precursor and was then incubated at 37 °C for 5 days. The cell free supernatant (CFS) was collected by centrifuging the cells at 12,000 rpm for 10 min. The CFS was then filtered with a 0.22 µm syringe filter, and collected in 15 mL tubes and stored at −80 °C for further analysis. To measure the GABA concentration, the γ-aminobutyric acid Enzyme Linked Immuno Sorbent Assay (ELISA) kit (ELISA Kit for GABA of all species; Abbexa: UK) was performed as described by the manufacturer. Finally, 50 µL of the stop solution was immediately added and measured using a microplate reader (EZread 2000 microplate reader: Biochrom; UK) at a wavelength of 450 nm to determine the GABA concentration by comparing it with the values of the standard GABA solution (Gamma-aminobutyric acid; Himedia: India) [22].

Glutamate decarboxylase (*gad*) B gene analysis and bacterial identification by 16S rRNA gene sequencing

DNA extraction was performed using a PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific: USA). The 16S rRNA gene amplification was carried out with these following primers, 27-F (5'-agagtttgatcctggctcag-3') and 1492-R (5'-ggttaccttgttacgactt-3') [23] and *gad*B gene amplification was performed with the primers PLANre-F (5'-cgcggatccatggcaatgttatacggtaaa-3') and PLANre-R (5'-ccggaattctcagtgtgtgaatccgtatttctt-3') [24]. The PCR reaction composition of both genes e.g. 16S rRNA and *gad*B amplification was 1×PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM dNTPs, 0.5 pmol primer each and 1.25 U Taq polymerase (Platinum Direct PCR Universal Master Mix; (ThermoFisher). The cycling conditions were as follows: Initial activation at 95 °C for 10 min; 30 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 90 s. The final cycle was 72 °C for 5 min. Approximately 1,500 bp of those 2 PCR products were purified with a PCR purification kit (SpinClean™PCR Purification Kit; South Korea. The DNA sample was then sent for nucleotide sequencing analysis at Biobasic, Canada. The obtained nucleotide result was further compared with the GenBank database.

Comparative study of glutamate decarboxylase

The structural analysis of the glutamate decarboxylase enzyme was conducted through deductive analysis using the Bioedit program [25]. The deduced amino acid sequence was used to predict the protein's secondary structure using the Swiss-model program [26].

The radical scavenging activities assay

Cell free supernatant (CFS) of *L. plantarum* TSUB-17 was prepared by culturing the bacteria in MRS broth for 24 h, the supernatant was then collected by centrifugation at 12,000 rpm for 5 min. The assay was performed in a 96-well plate by adding 100 µL of the CFS, then adding 100 µL of 0.6 mM DPPH solution (0.6 % w/v DPPH in methanol), shaking to homogeneity [27]. Ascorbic acid at concentrations of 10, 20, 40, 60, 100 and 200 µg/mL were used as standard antioxidants. The sample was incubated at room temperature in the dark for 30 min, then the absorbance was measured using a microplate reader (EZread 2000: Biochrom; UK) at a wavelength of 517 nm. The color of the solution would change from purple to yellow if antioxidants were available. The percentages of antioxidant activity was calculated by the following formula:

$$\% \text{ radical scavenging activity} = [(A_{DPPH} - A_{sample})/A_{DPPH}]$$

where, A_{DPPH} - control reaction absorbance; A_{sample} - testing absorbance [27].

Antibacterial activity

Anti-pathogenic bacteria were carried out using an agar well diffusion test. Briefly, the *L. plantarum* TSUB-17 was cultured in MRS broth medium, incubated at 37 °C for 24 h, centrifuged at 12,000 rpm for 5 min, and CFS was collected. The tested bacteria were *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (DMST 20637), *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumoniae* (ATCC 700603) and *Salmonella* Typhimurium (isolated ESBL strain) [28]. Approximately 1 - 2 colonies of fresh (18 h) tested bacteria were transferred to nutrient broth (NB) and incubated for 45 min or until turbidity of the bacteria culture reaches McFarland No. 0.5. The prepared bacterial suspension was then spread onto the Mueller-Hinton agar. After that, a 6 mm in diameter was then corked in each well and 100 µL of *L. plantarum* cell free supernatant was added. The plates were incubated overnight at 37 °C for 24 h and the clear zone diameter of each isolate against indicator pathogens was measured.

Acid and bile salt tolerance

Acid and bile salt tolerance assay was performed as described previously [29]. Approximately 10^8 CFU/mL of *L. plantarum* TSUB-17 was prepared. To assess the survival rate in acidic conditions, 0.5 mL of bacterial suspension was transferred into 4.5 mL MRS broth and adjusted to pH 3 with 1 M HCl. For the bile salt tolerance, 0.5 mL of bacterial suspension was transferred into 4.5 mL of MRS broth containing 1 % (w/v) of bile salt (Hi-media, Mumbai, India). Both assays was incubated at 37 °C for 0 and 3 h. The survival rate of bacteria at 0 and 3 h were then determined by single plate-serial dilution spotting (SP-SDS) [30]. The survival rate was later calculated as the following equation;

$$\text{Survival rate} = (N_1/N_0) \times 100$$

where, N_0 is the initial bacterial load, and N_1 is the number of surviving bacteria.

Adhesion to Caco-2 cell

Adhesion to $Caco_{-2}$ cells followed the method described previously [31]. Briefly, approximately 10^5 of Caco-2 cells (Purchased from the American Type Culture Collection, ATCC, Rockville, MD, USA. Lot: 70032505,) were cultured on 96-well plates with Dulbecco's modified eagle's minimal essential medium (DMEM) supplementing 10 % (v/v) fetal bovine serum and incubated at 37 °C in a CO_2 incubator for 7 days. The bacterial suspension was prepared as described in acid and bile tolerance. Before testing, the confluent cells were washed twice with PBS (pH 7.4), and then added 100 µL of 10^8 CFU/mL bacterial suspension in 1× serum free DMEM into each well, incubated at 37 °C in a 5 % CO_2 atmosphere for 2 h. At the specified time, the medium was aspirated, and the unbound bacterial cells were removed by carefully washing twice with PBS solution. Then, 0.1 % triton X-100 was added and incubated at room temperature for 10 min to detach the adherence bacteria from Caco-2 cells. The detached bacterial cells were serially diluted and spread on MRS agar and incubated for 48 h at 37 °C in anaerobic conditions. The counting of

the number of bacterial colonies that grow on agar plates and calculating the percentage of adhesion cells as follows the equation;

% adhesion capacity = (number of bacteria attached to cells/total number of cells) × 100 [32].

Each experiment was performed in triplicates.

Biofilm formation

Biofilm formation was carried out according to the method described previously [33]. The 10^8 CFU/mL of *L. plantarum* TSUB-17 suspension was prepared as mentioned above. The bacterial suspension was diluted 1:100 into MRS broth supplemented with 5 % glucose. Then 100 µL of the dilution was added to a 96-well plate and anaerobically incubated at 37 °C for 72 h. After that, each bacterial cell was removed, and each well was gently washed twice with 200 µL sterile water and let the air dry for 24 h before staining with a 0.1 % (v/v) crystal violet solution (Hi-media, Mumbai, India) for 30 min. After discarding the excess crystal violet, add 200 µL of absolute ethanol to solubilize the adhering crystal violet for 20 min. The solubilized crystal violet measured the optical density at 600 nm. In this assay, biofilm formation *P. aeruginasa* was used as a positive control and *E. coli* DH5α was used as a negative control. Each isolate was performed in triplicates.

Antimicrobial resistance and hemolytic activity

Antibiotic resistance was performed by disk diffusion method according to the Clinical & Laboratory Standards Institute (CLSI) [34]. A total of 9 antibiotics (Hi-media, Mumbai, India) were tested, including ampicillin (25 µg), cephalothin (30 µg), chloramphenicol (30 µg), ciprofloxacin (10 µg) erythromycin (15 µg), gentamicin (120 µg), norfloxacin (10 µg), streptomycin (10 µg) and vancomycin (30 µg). The bacterial was standardized by adjusting the turbidity equal to McFarland standard No. 0.5 in normal saline solution and swabbed in 3 directions onto Muller Hinton agar (MHA) plates. After that, antibiotic discs were applied and incubated under anaerobic conditions at 37 °C for 18 - 24 h. Then, the inhibition zone diameters, including the diameter of the disc were measured.

For the hemolysis assay, an overnight culture of bacteria was streaked on 5 % sheep blood agar plate and incubated at 37 °C for 24 h. After incubation, the hemolytic activity was recorded by observation of a clear zone of hydrolysis around the colonies and was then identified as beta hemolysis, alpha hemolysis or gamma hemolysis.

Results and discussion

***Lactobacillus* isolation and identification**

The colony, was able to grow on low pH of MRS agar and showed gram-positive bacilli-shaped, catalase-negative, and no hemolysis on sheep blood agar, was selected. The 16S rRNA analysis was used to identify the isolated bacterial colony. Nucleotide blast with only the type strain from GenBank showed 99.18 % identity to the *Lentiplantibacillus plantarum* NR_104573 (*Lactobacillus plantarum*), and the reciprocal confirmation by MALDI-TOF revealed a similar genus and species result. The isolate was then named *L. plantarum* TSUB-17. We are quite confident that the identification of bacteria obtained in this study was highly accurate. Because it is based on reliable 2-principles method that produces consistent test results. Recently, a comparative study on the identification of 364 clinically anaerobic bacteria was recently reported. All isolates were identified by 2 principal methods, 16S rRNA sequencing, and MALDI-TOF analysis. The results indicated that 87.3 % were correctly identified at the species level by MALDI-TOF analysis, which suggests that the MALDI-TOF is a reliable technique for identifying bacteria [35].

GABA production and *gad*B gene identification

GABA production was assayed by an ELISA kit. The results showed that *L. plantarum* TSUB-17 produced 1,364 ng/mL. Production of GABA was consistent with the detection of the *gad*B gene; in addition, the nucleotide sequence analysis of *the gad*B gene was identical to that of the *gad*B gene found in *L. brevis*. The amino acid sequence deduced from the nucleotide sequence was conducted and analyzed using the SWISS-MODEL program [26] and also confirmed that it had the identical amino acid sequences as *L. brevis* CGMCC 1306 glutamate decarboxylase to 81.73 % (**Figures 1A** and **1B**). A comparative study of the secondary structure of glutamate decarboxylase from *L. plantarum* TSUB-17 and *L. brevis* CGMCC 1306 showed that the protein structure was typical and was consistent with the glutamate decarboxylase structure of *L. brevis* CGMCC 1306 used as a comparison. The enzyme structure is a homotrimer with a

peptide chain of approximately 469 amino acids. Pyridoxal-5'-phosphate, a coenzyme, is bound in 3 regions of the structure (**Figures 1C** and **1D**).

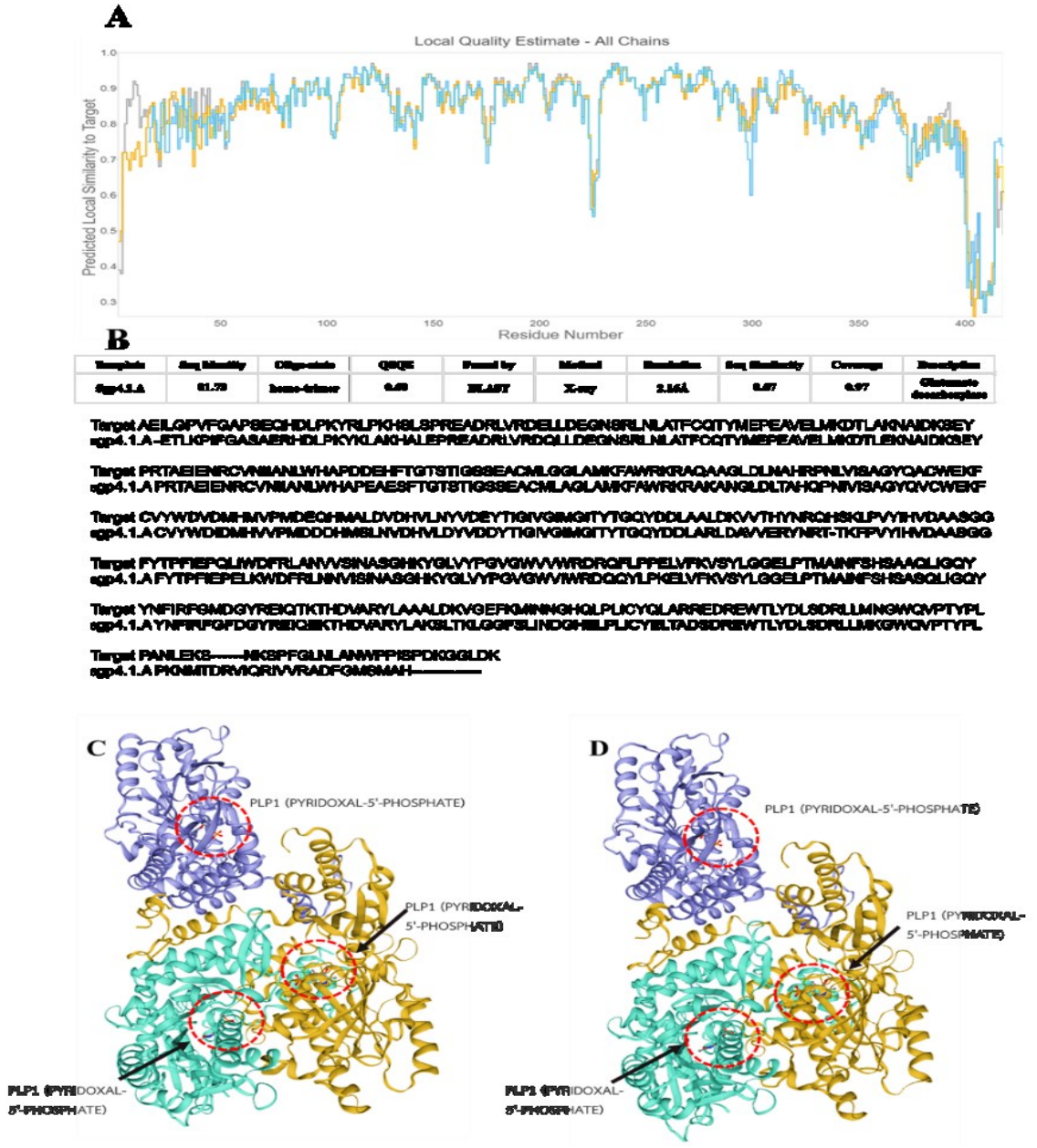

Figure 1 A) - B) Predicted local similarity of amino acid sequence deduced from nucleotide sequencing of *gad*B gene from *L. plantarum* TSUB-17 showed local similarity with glutamate decarboxylase of *L. brevis* CGMCC 1306 and C) - D) comparison of simulation secondary protein structure of deduce amino acid between *L. plantarum* TSUB-17 and *L. brevis* CGMCC 1306. The position in the circle indicated by the arrow is the location of cofactor, pyridoxal-5'-phosphate. Protein's secondary structure prediction using the Swiss-model program [26].

Probiotic properties

An essential characteristic of probiotics, when used as an additive in animal feed, is their safety, since they must be supplemented at high doses for effective animal growth. Therefore, non-hemolysis or γ-hemolysis is the principal criterion for the selection of *Lactobacillus* in this study, followed by antimicrobial susceptibility properties. The result demonstrated that *L. plantarum* TSIB-17 was a γ-hemolysis strain. An interesting result revealed that among the 7 from 9 antimicrobial agents, they were highly susceptible to

erythromycin, gentamicin and ampicillin, which are still used to treat several infectious diseases in humans and animals. However, it was found to resist to the 2 drugs tested such as vancomycin and norfloxacin (**Table 1**).

Table 1 Antimicrobial susceptibility profiles of *L. plantarum* TSUB-17.

Strains	Antibiotics susceptibility by the disc diffusion agar method								
	Cep (30 µg)	**Amp (25 µg)**	**Van (30 µg)**	**Nor (10 µg)**	**Cip (10 µg)**	**Chl (30 µg)**	**Gen (120 µg)**	**Ery (15 µg)**	**Str (10 µg)**
TSUB-17	S (18)	S (33)	R (6)	R (6)	S (20)	S (30)	S (33)	S (36)	S (20)

Antibiotics: Cep: Cephalothin; Amp: Ampicillin; Van: Vancomycin; Nor: Norfloxacin; Cip: Ciprofloxacin; Chl: Chloramphenicol; Gen: Gentamicin; Ery: Erythromycin, Str: Streptomycin. Susceptibility (diameter of clear zone): S: Sensitive; R: Resistance (Antibiotic susceptibility interpretation was performed according to the CLSI) [34].

Inhibition of pathogenic bacteria was considered as one of the potential probiotic properties, the *L. plantarum* TSUB-17 was able to inhibit all tested pathogenic bacteria. The CFS of the bacteria was particularly good at inhibiting gram-negative bacteria i.e. *E. coli*, followed by *K. pneumoniae*, *S.* Typhimurium and *P. aeruginosa* with an inhibition zone of 30, 23, 19 and 18 mm, respectively. The supernatant showed the least effect against *S. aureus*, with an inhibition zone of only 16 mm (**Table 2**).

Other properties tests, including acid tolerance, bile salt tolerance, biofilm formation, adhesion to Caco-2 cell and radical scavenging activity revealed that the *L. plantarum* TSUB-17 had various potential probiotics properties such as the bacteria could tolerate acid at pH 3.0 at 3 h with a survival rate of 100 %, while the survival rate in 1 % bile salt at 3 h was 100 %. Biofilm formation was as high as that of *P. aeruginosa* (positive control) with an OD value of approximately 2.5, while *E. coli* DH5α non-biofilm forming strain yielded an OD of only 0.5. The adhesion ability with Caco-2 cells was approximately 58 %. In addition, the cell free supernatant showed a radical scavenging activity of 60.2 % when compared to ascorbic acid.

Table 2 Antimicrobial activity and probiotic properties of *L. plantarum* TSUB-17.

Strains	Antimicrobial activity (Diameter of inhibition clear zone in mm.)									
	***E. coli* ATCC 25922**	***S. aureus* DMST 20637**	***P. aeruginosa* ATCC 27853**	***K. pneumoniae* ATCC 700603**	***S.* Typhimurium ESBL strain**	**Acid tolerance (%)**	**Bile salt tolerance (%)**	**Biofilm formation (OD)**	**Adhesion to Caco2 cell (%)**	**Radical scavenging activities (%)**
TSUB-17	30	16	18	23	19	100	100	2.5	58	60.2

This probiotic strain of *L. plantarum* TSUB-17 isolated from swine manure was performed by subtractive screening focusing on the safety of the probiotic as the initial feature, the isolated strain must not lyse red blood cells to make sure that it cannot cause disease in animals. One requirement for using probiotics in animal feed is that the probiotic dose is as high as 10^9 CFU/kg of feed [36]. Hemolysis ability is the major virulence factor of *Lactobacillus.* Hence, probiotic *Lactobacillus* should not exhibit hemolysis activity [37].

The following safety concern is antimicrobial resistance, where the mechanism of antimicrobial resistance may be on the transposon chromosome or on a plasmid that can be transferred to other bacteria. The *L. plantarum* TSUB-17 is considered very safe and has an interesting advantage as it is still susceptible

to many drugs. Importantly, it is susceptible to macrolides, aminoglycosides and beta-lactam, the primary drugs used to treat many infectious diseases. There is a study report that lactic acid bacteria used as a feed additive in animal feed showed approximately 37 % resistance to the aminoglycosides group [38]. In addition, report *Lactobacillus* isolated from chicken showed 42 % macrolides resistance [39]. However, we found that the *L. plantarum* TSUB-17 is resistant to vancomycin and norfloxacin, of which resistance to both drugs of Lactobacillus species is typically classified as intrinsic resistance [40,41]. A report by Goldstein *et al*. [42] indicated that vancomycin resistance is the best-characterized intrinsic resistance in lactobacilli. The minimal inhibitory concentration (MIC) value of vancomycin to lactobacilli averages more than 64 µg/mL. *Lactobacillus plantarum* TSUB-17 isolated in this study was also resistant to norfloxacin. A previous study reported that lactobacilli isolate from commercial probiotic products showed a high resistance rate of approximately 54.83 % [43]. Consistent with studies of *Lactobacillus* isolated from probiotics in dietary supplements showing 100 % resistance to norfloxacin [44]. It has been reported that the resistance mechanism to norfloxacin is naturally or intrinsically resistant. Intrinsic resistance is when a bacterial species naturally resists to a particular antimicrobial without mutating or receiving another mobile genetic element. This means that antimicrobials are unable to treat those bacteria.

This newly isolated *L. plantarum* TSUB-17 could produce GABA However, the production of GABA is low compared to previous studies showing that *L. brevis* produces up to 500 µg/mL of GABA [45]. These may be due to this study, a low concentration, of MSG, the precursor of GABA production, was supplemented in MRS broth by only 0.002 % whereas previous reports indicated that high levels of production of GABA in *L. brevis*, about 5 % of the MSG, were added [45]. The optimal conditions for its GABA production will be further investigated to obtain a high yield of GABA production. A possible trend is that *L. plantarum* TSUB-17 produces high levels of GABA as detected in the *gad*B gene and the amino acid sequence of the glutamate decarboxylase with 81.73 % homology to *L. brevis.* The simulated secondary structure of glutamate decarboxylase revealed that the structure obtained from *L. plantarum* TSUB-17 matched that of *L. brevis* CGMCC 1306 as homotrimers and had a binding site for pyridoxal-5'-phosphate, a cofactor in the structure of the same 3 positions.

The most efficient GABA producers lactobacilli, were *L. brevis* [16,46], and *L. plantarum* [44]. This according to the previous report indicated the major source of GABA producing probiotic bacteria was lactic acid bacteria especially *L. brevis* and *L. plantarum*. Moreover, it was found that the ability to produce GABA in *L. brevis* and *L. plantarum* was correlated with presentation of *gad*B gene [47].

For initial screening of MRS with an acidic pH condition was expected to isolate acid-tolerance *Lactobacillus*. As expected, the study showed that *L. plantarum* TSUB-17 was able to survive in pH 3. This property suggests that it has a high chance of survival when administered as an additive in animal feed. As for other probiotic properties, overall, the *L. plantarum* TSUB-17 showed good probiotic properties. High tolerance to bile salts is probably imply that the bacteria produce enzymes bile salt hydrolase, which help to reduce cholesterol content in food. It has been reported that the *Lactobacillus* could produce this enzyme that has a high tolerance to bile salts [48]. The production of bile salt hydrolase should be further investigated in terms of quality and quantity. We are quite confident that the isolates used as probiotics for animals can be well colonized in the animal's gastrointestinal tract, as evidenced by their biofilm formation and adhesion to Caco-2 cells. Interestingly, the isolates were found to have excellent inhibitory properties against pathogenic bacteria, especially gram-negative bacteria. This is likely due to the high levels of acid production by bacteria. The results corresponded with the previous report, it was found that high acid producing *Lactobacillus* strains were able to inhibit gram-negative pathogenic bacteria efficiently [49,50]. Its high acid-forming properties need further study as it could be applied in animal feed preservation.

Conclusions

This study was able to successfully isolate newly *L. plantarum* TSUB-17 that possesses good probiotic properties such as a resistance to high levels of acid and bile salts, and can produce GABA and consisted of *gad*B genes with a similar to *gab*B of *L. brevis*. Moreover, this isolate exhibited an inhibition of pathogenic bacteria effectively *in vitro* and had a relatively high radical scavenging activity. Therefore, this *L. plantarum* TSUB-17 is promising to further develop as a probiotic supplement in animal feed for enhance animal growth performance and stimulate immune system.

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