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RESEARCH ARTICLE OPEN ACCESS

Gamma-aminobutyric Acid (GABA) Producing Probiotic *Lactiplantibacillus Pentosus* Isolated from Fermented Spider Plant (Pak Sian Dong) in Thailand

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Abstract

Psychobiotics are defined as the organisms that can provide the mental health benefit. The possible mechanism of psychobiotics is manipulation of neurotransmitter production and neurotransmitter production by the microbes. The lactobacillus group has been reported for the potential of neurotransmitter production, especially γ-aminobutyric acid (GABA) which is an important inhibitory neurotransmitter. Therefore, GABA can be used for relaxation and applied in various psychiatric disorders. The aim of this study was determination of lactic acid bacterial isolates from Pak Sian Dong in Thailand for GABA producing ability. The results found that there were 3 isolates, SF66, SF80 and SF82, which revealed the ability to produce glutamic acid decarboxylase (GAD) enzyme. The GABA were detected by thin layer chromatography (TLC) and high-performance liquid chromatography (HPLC) in the bacterial culture containing 3% monosodium glutamate. The survival in gastrointestinal synthetic condition found that only SF66 isolate showed the authentic percentage of survival then this isolate was selected. From the identification, the isolate was identified as *Lactiplantibacillus pentosus* and was designated as *L. pentosus* SF66 which exhibited with the potential for further investigation and development to be psychobiotics.

Keywords: Psychobiotics, Probiotics, Gamma-Aminobutyric Acid, Pak Sian Dong

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INTRODUCTION

Spider plant or bastard mustard (*Cleome gynandra* L.) is a kind of plant which is known as Pak Sian in Thailand and these plants are world-wide distributed.[1] Spider plant can be fermented to preserve this plant and produce the type of fermented food.[2] During fermentation process, several microorganisms can grow and generate their products which lead to the signature taste of the fermented spider plant (Pak Sian Dong). For the community of microorganism in fermented spider plant, there are a group of lactic acid bacteria (LAB), such as *Pediococcus pentosaceus, Lactiplantibacillus plantarum* and *Levilactobacillus brevis*, and these bacteria were characterized for probiotic activities.[3]

Probiotics are commonly defined as the live organisms that can provide beneficial effects to the hosts.[4] Various aspects of probiotics can be used such as prevention of infection, promotion of the health and treatment of the diseases. Interestingly, the approach for using the probiotics as psychobiotics, which is the probiotic that capable of giving profit to nervous system, is in the trend. From consuming of glutamic acid and using activity of glutamic acid decarboxylase (GAD) enzyme, several LAB have ability to produce γ-aminobutyric acid (GABA) which is an important neurotransmitter for the nerves as described above. For instance, *L. plantarum* which is isolated from Thai fermented food exhibited GABA production.[5] Moreover, the isolation of *Lactobacillus buchneri* from Kimchi had been determined for GABA production and studied the effect on neuronal cells.[6]

GABA is one of various neurotransmitters in cerebral cortex that is synthesized by GABAergic neuron and plays an important role in inhibitory neurotransmission. Abnormal GABA metabolism may induce abnormal cortical excitability.[7] Biosynthesis of GABA requires the decarboxylation reaction of glutamate catalyzed by GAD.[8] Not only central nervous system but also gastrointestinal tract require GABAergic neurotransmission via interneuron of the enteric nervous system. GABAergic signaling in the gut is involved in the activation of GABA receptor that modulate gastrointestinal motility and mucosal function.[9]

The bidirectional communication between the brain and the enteric nervous system, called gut-brain axis, is the network that regulates gastrointestinal homeostasis as well as higher cognition by integrating and linking brain function associated with emotion and cognition with gastrointestinal function by mechanisms such as neuroendocrine and immune response. Moreover, gut-brain axis can be influenced by the commensal gut microbiota.[10] In accordance with impermeability of the blood-brain barrier to GABA; nevertheless, there is some evidence that small amount of GABA can pass through the blood-brain barrier.[11]

Previous study showed that mice chronically administered with *L. rhamnosus* (JB-1) had alterations in GABA receptor mRNA in the brain.[12] Moreover, reduced stress hormone, corticosterone, and behavior related to anxiety and depression were also found. On the contrary, these results were not found in vagotomized mice.[13] The results suggest that vagus nerve may play a key role in microbiome gut-brain axis.

Due to the discovery of GABA producing LAB which had been previously isolated from diverse fermented food and beneficial effect of probiotic on gut-brain axis and, therefore, the goals of this study are screening for GABA producing LAB which isolated from Pak Sian Dong and characterizing the isolates for using as probiotic bacteria.

MATERIALS AND METHODS

Bacterial isolation and culture condition

Pak Sian Dong were collected from local area in Pathum Thani province and used as the source for LAB isolation. The LAB were isolated by spreading the sample on MRS agar containing $CaCO_3$ and nystatin. After incubation at 37°C for 48 h, the colonies with clear zone were picked from the plate and then characterized for lactobacilli properties which included gram positive, rod shape and catalase negative. The selected isolates were grown in MRS broth and stored at -80°C in glycerol until used.

Glutamic acid decarboxylase (GAD) activity and thin layer chromatography (TLC)

GAD activity was measured with color changing of indicator in reagent. The reagent

for GAD activity was prepared. To determine GAD activity, the pellet of overnight culture was collected by centrifugation at 10,000 rpm for 2 min. The pellet was washed twice with normal saline solution to remove culture medium. A 500 µl of The GAD activity reagent (a liter of water which consisted of 1 g of L-glutamic acid, 300 µl of TritonX-100, 90 g of NaCl and 0.05 g of bromocresol green) was added to the pellet and mixed well then incubated at 37°C for 4 h. The color changing of the reagent was observed and blue color was indicated as strong GAD activity which remarked as positive result.[14]

For TLC, the isolate was inoculated in MRS containing 3% (w/v) of monosodium glutamate and the supernatant was collected from 48 h of the culture. The culture supernatants were spotted on TLC plate (Loba Chemie, India) and GABA was used as standard positive control. The solvent which containing butanol: acetic acid: distilled water (5:3:2) was prepared and used as mobile phase. After TLC running, the plate was sprayed with ninhydrin solution and placed at 70°C for 15 min.[15] Finally, the bands were observed and compared with GABA standard control then picked the positive isolates for further study.

GABA quantification by HPLC

Following the method which described by Kanklai et al.[15] and Li et al.[16] with slightly modification. In brief, the LAB isolates were inoculated and culture in MRS containing 3% (w/v) of monosodium glutamate and the supernatant were collected after incubation for 48 h. The supernatant cultures were dried with lyophilization and resuspended with 1000 µl of the mixture which consisted of ethanol: water: triethylamine (2: 2:1). The derivatization was done by adding 80 µl of the solution which consisted of ethanol: water: triethylamine: phenyl isothiocyanate (7: 1: 1: 1) and incubated in the dark environment at room temperature for 20 min. After derivatization, the GABA content was analyzed by HPLC (Shimadzu, Japan) with ODS-3 column (4.6 x 250 mm, 5 µm). The GABA standards were prepared for 0.1, 0.25 0.5 0.75 and 1.0 mg/ml.

Acid and bile tolerance

The acid tolerance of the isolate was determined with synthetic gastric fluid incubation. Overnight culture of the isolate was washed twice with normal saline solution and prepared bacterial suspension by adjusting with McFarland 0.5. After preparation of bacterial suspension, a 100 µl of suspension was added to 900 µl of synthetic gastric fluid and then incubated at 37°C for 3 h. For control tube, the suspension was added to normal saline solution instead of synthetic gastric fluid. The survival of the isolate was calculated by plate count technique on MRS agar compared with control.[17]

To examine resistant to bile, MRS with 0.3% (w/v) of bile was prepared and inoculated with bacterial isolate. After incubation at 37°C for 24 h, viable plate count was performed by using MRS agar and then calculated for percentage of survival.

***In vitro* hydrophobicity**

Adhesion to hydrocarbon was determine to assess ability of cell adherence. The overnight culture was washed twice with normal saline solution and the concentration of cell suspension was prepared the optical density (OD) to 0.5 at 600 nm (A0). The suspension was pipetted 1200 µl and mixed with 200 µl of xylene for 1 min. After mixing, the tube containing bacterial suspension and xylene was statically placed at room temperature for 1 min. Subsequently, the aqueous phase was collected and measured the OD (A1) and calculate for percent of hydrophobicity by following formula (A0-A1 x 100) /A0.[17]

Blood hemolysis and gelatinase determination

Sheep blood agar (SBA) were used to determine blood hemolysis. The isolates were streaked on the SBA and incubated at 37°C for 48 h. The hemolysis activity positive was presented as clear zone surrounding colony of LAB isolates while negative result was showed as the growth without clear zone.

For gelatinase activity, MRS containing 12% gelatin was prepared in test tube and inoculated with LAB isolates by stabbing. After incubation at 37°C for 24 h, inoculated tubes were placed at 4°C to assess the hydrolyzing by gelatinase. The positive was presented as liquefaction of medium containing gelatin while negative was presented as solidified medium.

Bacterial isolate identification

The isolate, which had ability to produce GABA and showed high percentage of survival in synthetic gastric fluid and bile containing condition, was identified by API 50 CHL kit (bioMérieux, France) for phenotypic identification and determined the 16 s rRNA sequence for molecular identification. To differentiate between *L. plantarum* and *L. pentosus*, the primers and PCR condition for *recA* gene were used with slightly modification.[18]

Antibiotic susceptibility profile

Ten antibiotics, which included ampicillin, chloramphenicol, ciprofloxacin, erythromycin, kanamycin, nalidixic acid, novobiocin, penicillin, tetracycline and vancomycin, were selected to examine the susceptibility profile of the isolate. The procedure was done following Yasiri et al.[19]

RESULTS

Screening for GABA producing lactic acid bacteria

The 96 isolates were isolated and revealed lactic acid bacterial characteristics from Pak Sian Dong. The LAB isolates were screened for GABA producing ability by determination of GAD activity. There were 3 isolates, SF66, SF80 and SF82 (Figure 1), which changed indicator color from yellow to blue in GAD reagent representing an ability to produce GAD enzyme. All 3 isolates were cultured and collected the supernatants to run with TLC. The GABA expected bands, which could be assumed from comparison with the GABA standard, showed on the TLC plate after sprayed with ninhydrin and developed by heating (Figure 2). This result brought these 3 isolates for probiotic determination.

Table. Probiotic characteristic determination for the selected isolates and safety examination

Isolates	Probiotic characteristics			Safety examinations	
	Gastric fluid survival rate (%)	Bile salt survival rate (%)	Hydrophobicity (%)	Blood hemolysis test	Gelatinase test
SF66	36.71	91.88	85.05	No hemolysis	Negative
SF80	0	75.06	85.09	No hemolysis	Negative
SF82	0	83.31	89.46	No hemolysis	Negative

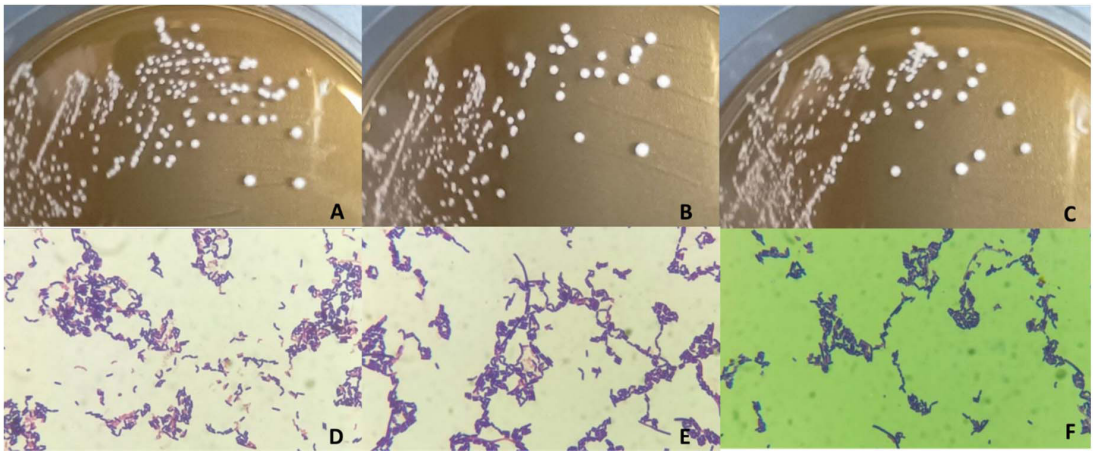

Figure 1. The colony of LAB isolates on MRS agar were showed on the upper row. The isolates SF66, SF80 and SF82 were represented with A, B and C, respectively. The Gram's staining results of LAB isolates were showed on the lower row. The isolates SF66, SF80 and SF82 were represented with D, E and F, respectively.

Probiotic characteristics and antibiotic susceptibility

To assess colonizing characteristics, survival in simulated gastrointestinal tract conditions and hydrophobicity were determined. The isolate SF66 had ability to survive in the condition which contained acid and bile salt while SF80 and SF82 did not survive in these conditions (Table). This result indicated that SF66 isolate has potential to use as orally administered probiotics. According to ability of survival rate in gastrointestinal tract condition, only SF66 isolate was selected to investigate in further experiments.

For *in vitro* hydrophobicity, the percentage of hydrophobicity was 63.29 for the SF66 isolate. According to criteria from Mota et al, the percentage more than 50 was defined as hydrophobic characteristic.[17] Hydrophobicity is a force that is related with ability to attach the epithelial cells and microorganisms can use this cellular mechanism to adhere the surface of host cells.[20] This suggested that SF66 isolate was able to colonize in the intestine and provide beneficial effect to their host.

According to safety of LAB isolate, blood hemolysis and gelatinase test were determined. The results showed that there was no beta hemolysis from the isolate which inoculated on sheep blood agar and liquified gelatin was also not observed. Therefore, safety of probiotics, the strains should not express virulence activity which including blood hemolysis and gelatinase activity. Thus, the isolate in this study could be assigned as a safe probiotic.

The antibiotic susceptibility profile of the SF66 isolate was determined and the results found that the SF66 isolate was susceptible to chloramphenicol, erythromycin, novobiocin, penicillin and tetracycline.

GABA quantification and identification of GABA producing LAB

The GABA content from LAB SF66 was quantified by HPLC. After determination, the GABA content of LAB SF66 supernatant was 0.9 mg/ml.

The isolate was identified by using API 50 CHL commercial identification kit combined with and the result showed that the SF66 was shown possibility to be *Lactiplantibacillus pentosus*. The 16s rRNA sequencing was needed for confirmation. However, *L. plantarum* and *L. pentosus* showed highly identity from 16s rRNA

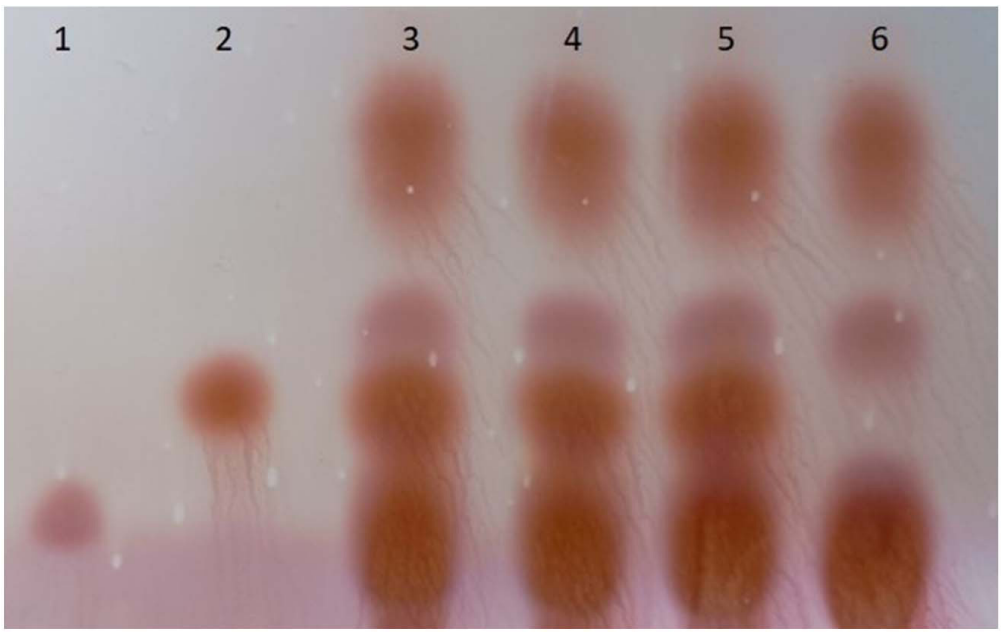

Figure 2. The supernatant of MRS with 3% MSG cultures was determined for GABA production by TLC. Lane 1; MSG, Lane 2; GABA standard, Lane 3; isolate SF66, Lane 4; isolate SF80, Lane 5; isolates SF82, Lane 6; *L. plantarum* SF21 which is non-GABA producing strain.

sequencing then the amplification of *recA* gene by multiplex PCR was performed and the result found that the product of PCR was approximately 218 bp, which could be interpreted as the specific size for *L. pentosus*. From the above results, this novel isolate was *L. pentosus* firmly and could be designated as *L. pentosus* SF66.

DISCUSSION

The colonizing properties are the essential characteristics of commensal bacteria. It is crucial for novel LAB isolation for using probiotics as an oral administration.[21] This study found that the isolate SF66 could resist harsh condition of gastrointestinal tract, which were gastric fluid and bile salt containing environment, and had high percentage of hydrophobicity that related with ability to attach the surface of epithelial cells. Besides probiotic and colonizing properties, the safety of LAB was commonly concerned for screening the novel strain to use as probiotics. From the results, the isolate SF66 revealed the susceptible to 5 from 10 antibiotics and presented with no hemolysis and gelatinase activity. According the above results, this noticed that novel LAB SF66 which isolated from Pak Sian Dong has ability to produce GABA with safe and good colonizing characteristics.

The probiotic characteristics for the LAB isolates from Pak Sian Dong were presented in numerous reports. As for example, the study of Yasiri and Seubsasana[22] revealed that bile salt hydrolase and uricase activity of *L. brevis* can be applied for hypercholesterolemia and hyperuricemia. In addition to antimicrobial activity, LAB which is/are isolated from Pak Sian Dong showed ability to inhibit gram-positive and gram-negative bacteria. Moreover, *L. pentosus* which is isolated from Pak Sian Dong was reported for GABA producing activity and could be developed as a novel probiotic drink.[23]

From GABA quantification by HPLC, the LAB SF66 could produce 0.9 mg/ml of GABA which was determined from 48 h supernatant of the MRS containing 3% MSG culture. The previous study reported the concentration of GABA which was produced by LAB with various amount.[24,15,3] The concentration of GABA related with several variable parameters such as starter inoculum, MSG concentration and glucose supplementation.[25] According the amount of GABA from this study, this suggested that the optimization for high yield production was needed to be investigated in the future.

From bacterial identification, the sequencing of 16s rRNA showed the high identity of the isolate with *L. plantarum* and *L. pentosus*. Unambiguous identification of these species has been reported and solved by using *recA* gene amplification.[18,26] Regarding to the result of identification kit and *recA* gene amplification, the novel isolate was finally characterized and identified as *L. pentosus*. This result suggested that the identification of LAB isolates probably need more than one methods to characterize the species for achieving the rigid identification results.

The isolation of *L. pentosus* from Pak Sian Dong has been reported previously.[3] In general, *L. pentosus* is a gram positive, rod shape which can be detected in various environments including plants, animals and fermented foods. Several studies reveald that *L. pentosus* can be used as probiotics in various aspects such as antimicrobial activity,[27] cholesterol reduction[28] and immunomodulation.[29] Using as probiotics with antimicrobial activity, *L. pentosus* SLC13 which is/are isolated from mustard pickles exhibit broad spectrum inhibitory activity for gram-positive and gram-negative bacteria.[30] For ability to reduce cholesterol, feeding with *L. pentosus* KF923750 in animal model found significant lowering of cholesterol and triglyceride in blood plasma.[28] In addition, *L. pentosus* S-PT84 which is/are isolated from pickles accomplished in modulation of cytokines production.[31]

The capability of GABA production could be observed in various several L. pentossus strains which are isolated from various sources, e.g., fermented mulberry fruits,[24,15] natural black Conservolea olives[32] and Thai pickle weed.[23] The advantages of GABA producing bacteria were shown in various aspects. In accordance with ability to convert MSG to GABA, reduction of MSG absorption was determined in mice which was administered with *L. brevis* G-101 and could be assumed to diminishing of MSG side effects.[33] For immunomodulation activity, the GABA producing *L. brevis* showed ability to regulate cytokine production in mesenteric lymph node cells and

also cause autophagy in some populations of these cells.[34] Moreover, improvement of glucose homeostasis and lipid metabolism were observed in the mice which provided with GABA producing *L. brevis* strains.[35] From several reports above, the lactobacilli with ability to produce GABA have the potential for using in health improvement and promotion aspects.

In conclusion, the GABA producing LAB strains could be isolated from Pak Sian Dong, and this indicates that Pak Sian Dong is a healthy diet with a possibility to be a source of beneficial LAB. The potential isolate in this work was identified and designated as *L. pentosus* SF66. According to ability to survive in simultaneous gastrointestinal tract condition and present with high hydrophobicity, this isolate can be applied to use as a probiotic for oral administration as well. The GABA producing isolate from this study can potentially be used in the functional food aspect. However, more studies are further needed to investigate for GABA highest yield condition and an approval in animal models.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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