Chiang Mai J. Sci. 2018; 45(2) : 717-730
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

Selection and Validation of Carbohydrate-utilizing Bacteria as a New Probiotic Candidate to Develop Probiotic-supplemented Thai Rice Cultivar Product

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Received: 16 November 2016
Accepted: 6 March 2017

ABSTRACT

The objectives of this study were to validate new strains of probiotics and to develop probiotic-supplemented rice products. One hundred and fifty-seven bacteria were isolated from bees, and their probiotic properties were investigated. Among these, only two strains were selected based on ability to utilize carbohydrates from rice grains. The carbohydrate-utilizing bacteria were identified as *Bacillus amyloliquefaciens* C11AM2 and *Bacillus subtilis* C12AM3 on the basis of morphological, biochemical and molecular analysis. These two probiotic candidate strains could survive in simulated gastrointestinal tract conditions involving both short and long digestion periods. From the results of the antimicrobial activity, *B. amyloliquefaciens* C11AM2 could inhibit growth of *Vibrio cholerae* and *Vibrio parahaemolyticus*. Both probiotic candidates did not produce virulent factors i.e. hemolysin and lecithinase. The production of probiotic-supplemented rice product revealed that the survival of probiotic strains was maintained in both encapsulated and steamed rice products, using 3 rice cultivars. The survival rates of the 2 probiotic strains were 55-60% after 2 months of storage and the products incorporating probiotic strains could be stored at 4°C for at least 2 months. This study indicated Thai rice cultivars are potential carriers of probiotic culture and are suitable for novel functional food products.

Keywords: probiotic, functional food, Thai rice cultivar, intestinal pathogens, shelf-life

1. INTRODUCTION

Currently, functional food products are regarded as benefits to human health. Therefore, the development of functional dietary supplements has increased in the food industry. One of the high demand dietary supplements is probiotic products which are now generally popular with consumer. The market share of probiotic

products is about 10% of the global functional food market and is becoming a substantial and increasing sector of economic importance [1-2].

Research on new techniques, processing, carrier matrices and bacterial strains is currently expanding to enhance the survival of probiotic strains when exposed to the heat, bile, high acid and pressure in a digestive tract. The selection of a preferable probiotic strain is one of the most important criteria for human use. The general probiotic strains used in commercial products belong to the genera *Lactobacillus* and *Bifidobacterium* [3]. The genus *Bacillus* is not prohibited with regards to food safety as recommended by the Food and Drug Administration (FDA) of the United States and furthermore, *B. subtilis* conforms to the Qualified Presumption of Safety (QPS) proposed by the European Food Safety Authority (EFSA) [4]. One important advantage of the use of *Bacillus* spp. is spore formation that can be delivered through the digestive tract, survive in the gastric juices, produce antimicrobial substances, grow well in various carrier matrices and can maintain viability after storage at room temperature [4-8].

In addition to preferable probiotic strains, the food materials used as carrier matrices are a vital key for probiotic production. According to the Food and Agriculture Organization/World Health Organization, probiotics can confer a health benefit to the human host when delivered in adequate numbers [9]. A great deal of carriers will help maintain the viability of probiotic strains during storage and administration to the digestive tract. In general, the well-known probiotic products are dairy products such as milk, cheese and yogurt. The non-dairy probiotic products require an excellent environment for supporting the survival of probiotics that is more challenging than in dairy matrices [3]. In addition to enhance of probiotic survival, the nutritional value, cost and sensory satisfaction should be considered. Rice (*Oryza sativa*) is an excellent source of nutrition that includes carbohydrates, essential elements, fibers and some functional substances such as antioxidants. Rice is a staple food for a large proportion of the world's population, especially in Asia. Rice cultivation occurs in many regions of Thailand and the production of rice in 2015 was forecast to be 19.8 million tonnes [10]. Thai glutinous rice cultivars, derived from Indica rice, have a long kernel length and are part of daily meals in north and northeastern Thailand [11]. The Thai colored rice cultivars i.e. black, red, brown and purple rice that are composed of various pigments particularly anthocyanins, have potential antioxidant activity [12]. So, the ideal use of Thai rice cultivars as carriers for probiotics was approached to develop new multifunctional food products.

Thus the objectives of this study were to search for new probiotic candidate that had potential probiotic properties i.e. the absence of virulent factors, the ability to produce antimicrobial substances and to survive in high acid and alkaline conditions, and to develop Thai rice cultivars as potential carrier matrices for identified probiotic strains.

2. MATERIALS AND METHODS

2.1 Sample Collection and Raw Materials

Brood cells and the nest materials of Thai-native bees, *Apis cerana* were collected from Mae Rim District, Chiang Mai Province. The collections were made in 2015. Total of 17 beehives were collected. All samples were stored at -20°C until use [13].

Samples of 9 cultivars of rice grains (*Oryza sativa*) 105 jasmine brown, 105 jasmine, Riceberry, glutinous black, 105 germinated-

jasmine, red jasmine, germinated-red jasmine, glutinous Luem Pua and black jasmine rice, and oats were purchased from local markets in Thailand. The rice grains were dried in an oven (Memmert, Germany) at 60°C for 12 h until a moisture content of 4-5% (w/w) was reached. The dried rice bran was ground to a particle size of 5 mm using a high-speed blender and then filtered through a sieve tube.

2.2 Tested Microorganisms

Nine pathogenic bacteria i.e. *Aeromonas hydrophila* KPS01, *Escherichia coli* KPS01, *Proteus vulgaris* KPS01, *Pseudomonas aeruginosa* KPS01, *Salmonella typhimurium* KPS01, *Serratia marcescens* KPS01, *Staphylococcus aureus* KPS01, *Vibrio cholera* KPS01 and *Vibrio parahaemolyticus* KPS01 were tested for antimicrobial activity. All pathogens were obtained from the Faculty of Veterinary Science, Kamphaeng Saen campus, Kasetsart University.

2.3 Isolation of Bacteria from Bees

Samples of adults, brood cells, pollens and honey were sorted from nest materials. The bacteria were isolated from 1 g of each sample using the serial dilution technique. Briefly, The samples of adults, brood cells and pollens were ground, suspended in 10 mL of sterile water, and vortexed for 1 min. The samples of raw honey were aseptically squeezed, diluted in approximately 10 volumes of sterile water, and vortexed for 1 min. One milliliter of successive decimal dilution were spread on de Man Rogosa and Sharpe (MRS; Merck, Germany) agar containing bromocresol green and supplemented with 25 $mg.mL^{-1}$ of cycloheximide prior to incubating at 37°C for 48 h. To obtain pure isolate, the cultures were streaked on MRS agar and incubated at 37°C for 48 h. Colonies of different shapes were randomly picked from the agar plates. Each pure isolate was preserved on MRS agar, stored at 4°C and subcultured every 2 months. The cultures were also kept at -20°C in 20% (v/v) glycerol as a cryoprotective agent for long term preservation.

2.4 Screening of Bacteria Capable of Carbohydrate Utilization

The ability of bacterial isolates to hydrolyze carbohydrate was performed using agar plates containing 9 cultivars of rice grains, with oats as the control. To test for carbohydrate utilization, the point-inoculation was performed. Briefly, the colony of each pure culture isolated from bees was picked by needle. The culture was then pointed or touched into the surface of modified carbohydrate agar plates containing 1% peptone, 1.2% agar and 3% ground rice grains. The plates were then incubated at 37°C for 5 days, prior to observation for the appearance of a clear zone. Iodine solution was added to stain the carbohydrate displayed dark blue and to investigate obvious clear zones of hydrolysis. The diameters (millimeter, mm) of clear zones were measured. All experiments were carried out using three replications. The bacterial isolates that could hydrolyze carbohydrate were selected for further experiments.

2.5 Identification of Carbohydrate-Utilizing Bacteria

2.5.1 Conventional method

The carbohydrate-utilizing bacterial strains were primarily identified according to traditionally morphological criteria and biochemical characterization [14-15]. The carbohydrate-utilizing bacterial strains were tentatively identified according to morphological criteria including colony growth pattern, pigment production, spore formation. The biochemical characterization were tested including catalase, motility, oxidation and fermentation (O-F) and

Voges-Proskauer test; hydrolysis of gelatin, casein, pectin, urea, lipid and starch; production lecithinase; gas from glucose, sucrose, mannitol and lactose; growth in media containing 2, 5 and 7% NaCl; growth at 5, 20, 30 and 50°C; growth at media pH 6, 7, 8 and 9. For motility test, the cultures were stabbed into the semi-solid deep tube agar, incubated at 37°C for 5 days, prior to observation for the appearance of growth and motility.

2.5.2 Molecular method

The genomic DNA of bacterial isolates was extracted by the method of Martin-Platero et al. (2007) [16]. An almost complete 16 Svedberg units ribosomal ribonucleic acid (16S rRNA) gene (~1.5 kb) was amplified using the universal primer pair 20F (5′-AGTTTGATCCTGGCTC-3′) and 1540R (5′-AAGGAGGTGATCCAGCC-3′) [17]. The PCR products were purified using Nucleo Spin® Gel and a PCR Clean-up Kit (Macherey-Nagel, Germany) following the manufacturer's protocol. The purified PCR products were directly sequenced by the First base company, Malaysia using primers 20F and 1540R as sequencing primers. The identities of nucleotide sequences of the 16S rRNA gene obtained were subjected to BLAST analysis using the NCBI database (http://www.ncbi.nlm.nih.gov).

2.6 Assessment of Probiotic Properties

2.6.1. Simulated gastrointestinal condition: pH transit tolerance

The procedure was performed according to Axelsson (1993) [18] and Bao et al. (2010) [19] with some modification. The selected bacteria were examined for their ability to grow in simulated gastrointestinal conditions. One milliliter of bacterial cell suspensions (approximately 10^7 cfu.mL^{-1}) was transferred to 10 mL of MRS broth, pH 2.5 and incubated at 37°C for 2 h. The viable cells were counted using a dilution plate method. After that, the cultures were centrifuged at 3,000 ×*g* and 4°C for 15 min to obtain packed cells. The cell pellets were then transferred to 10 ml of MRS broth at pH 8 and incubated at 37°C for 3 h. The viable cells were counted using a serial-decimal dilution plate method. The experiments were done in three replications. A graph of bacterial survival was performed. The values were expressed as log of colony forming units per mL (cfu.mL^{-1}).

2.6.2 Antimicrobial activity

The inhibition of intestinal pathogenic bacteria by carbohydrate-utilizing bacterial strains was conducted using an agar well diffusion method as described by Tulumoglu et al. (2013) [20] with some modifications. Each carbohydrate-utilizing bacterial isolate was cultivated in MRS broth at 37°C for 48 h. The culture broth was centrifuged at 2,000 ×*g* and 4°C for 10 min to obtain the supernatant. Concomitantly, the tested organisms were grown in nutrient broth (NB, Merck®, Germany) at 37°C for 18 h (the optical density at 540 nm (OD_{540}) was 0.5, corresponding to approximately 10^6-10^7 cfu.mL^{-1}) prior to swabbing onto nutrient agar (NA) plates. Wells (6 mm diameter) were punched in each plate and 100 µL of cell-free supernatant was added in each well. The MRS medium was used as a negative control and 0.1 mg.mL^{-1} of streptomycin was used as a positive control. The plates were then incubated at 37°C for 48 h and each zone of inhibition was measured. The experiments were done in three replications.

2.6.3 Haemolytic activity

Each strain was point-inoculated on blood agar (7% bovine blood) and incubated

at 37°C for 48 h. Some bacteria produced extracellular enzymes that lyse red blood cells were reported as hemolytic activity according to Forbes et al., 2002 [14]. The experiments were performed in duplicate and *Staphylococcus aureus* was used as a positive control.

2.7 Media Optimization

The strains were inoculated in MRS broth and incubated at 37°C for 18 h to obtain culture inoculum with an OD_{540} of 0.5. An aliquot of 20 mL of culture was applied in 200 mL of modified media consisting of modified MRS No.1, modified MRS No.2 or bean extract media prior to incubating at 37°C and 150 rpm for 18 h in a reciprocal shaker. The MRS broth served as the control. The optical density at 540 nm was measured after 24 h of incubation. The modified MRS No.1 consisted of glucose 20 $g.L^{-1}$, yeast extract 4 $g.L^{-1}$, magnesium sulfate 0.04 $g.L^{-1}$ and manganese sulfate 0.2 $g.L^{-1}$. The modified MRS No.2 consisted of glucose 20 $g.L^{-1}$, peptone 4 $g.L^{-1}$, magnesium sulfate 0.04 $g.L^{-1}$ and manganese sulfate 0.2 $g.L^{-1}$. Bean extract media consisted of green beans 20 $g.L^{-1}$, soybeans 20 $g.L^{-1}$ and coconut sugar 5 $g.L^{-1}$.

2.8 Development of Probiotic-Supplemented Thai Rice Products

2.8.1 Preparation of probiotic bacterial culture

Two probiotic candidate strains were inoculated in 10 mL of MRS broth and incubated at 37°C for 18 h. A cell aliquot was then transferred to modified medium consisting of 1%(w/v) soybean and 1%(w/v) green bean with a concentration of 10%(v/v) inoculum, incubated at 37°C and 150 rpm for 24 h in a reciprocal shaker. The cells were harvested using centrifugation for 10 min at 3,000 ×*g* and 4°C. The pellets were washed once using 0.8% normal saline. The cell pellets with 20-30% of humidity were conducted using centrifugation with the same conditions.

2.8.2 Preparation of Thai rice cultivars grains

A sample of each rice grain was milled using blender and winnowed through a 0.5 mm-diameter sieve. The granulated Thai rice cultivars were then prepared for probiotic-supplemented Thai rice products using the two procedures; Procedure 1: each granulated rice grain sample was baked in an oven (Memmert, Germany) at 105°C for 48 h to eliminate humidity and then pasteurized at 77°C for 15 min. Procedure 2: each granulated rice grain sample was steamed using moist heat at 100°C for 20 min and pasteurized at 77°C for 15 min. The rice grains were put into plastic bags containing silica gel, tightly packed and stored at 4°C for further experiment.

2.8.3 Description of the processing method

The granulated rice was formulated at various proportion of probiotic culture using 2 processes; Process 1: each capsule consisting of 0.5 g of baked-granulated rice grains was mixed with probiotic cell pellets in the ratio of 1:9 (bacterial cells to rice grains) prior to encapsulating using capsule filling machine. Process 2: steamed-granulated rice grain was mixed with bacterial cell pellets in the ratio of 1:9. The products were put into opaque plastic bags contained silica gel, tightly packed and stored at 4°C.

2.8.4 Enumeration of bacterial culture in probiotic products

The measurement of cell viability during storage was carried out after 7, 14, 21, 28, 35, 42, 49 and 60 days of incubation using a serial dilution plate technique on MRS agar.

The results were expressed in log cfu.g^{-1} of products. The survival rate (%) was then calculated after 2 months of incubation using the following equation (Bao et al. 2010) [19].

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

N_1 represents the total viable count of bacterial strains after treatment.

N_0 represents the total viable count of bacterial strains before treatment.

2.8.5 Investigation of storage stability and microbiological safety of products

The physical changes in the color, odor and pH value of products were observed during storage. The microbiological safety of probiotic-supplemented Thai rice products during storage was determined by enumeration of the total aerobic microbial count, *Escherichia coli* and total combined yeast and mold according to the United States Pharmacopeia-National Formulary (USP-NF) recommendation [21].

2.9 Nutritional Analysis

The 500 mg (1 capsule) of probiotic-supplemented riceberry cultivar rice product was subjected to Institute of Food research and Product Development, and was nutritional analyzed using Thai Compendium of methods for food analysis, 1st ed. 2003.

2.10 Statistical Analysis

The results are reported throughout as mean ± standard deviation. A significantly different value was tested using Duncan's test and one-way ANOVA ($P \leq 0.05$) with the SPSS v.16 software (SPSS Inc., Chicago, IL, USA).

3. RESULTS AND DISCUSSION

3.1 Isolation of Bacteria and Screening of Carbohydrate-Utilizing Strains

Isolation of microorganisms from new natural habitats has led to the discovery of novel strains and natural products for many applications. There are few reports of the isolation of bacteria from *Apis cerana* (native Thai bee). In this study, 17 nests of *A. cerana* were collected from northern Thailand. It was considered that this diversified microbial community could encourage the utilization of carbohydrate of Thai rice cultivars. In the present study, the representatives of different cultural colony were chosen from each part of bee sample. A total of 157 bacterial isolates were isolated from the samples collected from bees. The pure culture isolated from adults, brood cells, pollens and honey were 50, 53, 37 and 17 isolates, respectively. Most of the isolated bacteria were rod-shaped, Gram-positive and spore-forming bacteria. Among these bacteria, only 2 isolates of bacteria namely C11AM2 and C12AM3, that were isolated from the adults of bees could exhibit ability to utilize the carbohydrates of all cultivars of rice grains (Table 1). This finding is associated with the research of Ratithammatorn [22] who demonstrated that *Lactobacillus amylovorus* could consume native Thai glutinous rice starch and corn starch from the medium plates. Bioconversion of small molecules of carbohydrate materials to lactic acid can be achieved much more effectively by coupling the enzymatic hydrolysis of substrates and microbial fermentation. Starch from various plants is an interesting raw material due to its low cost and wide availability. Likewise, rice contains high starch content and is available in all regions, particularly in Southeast Asia. Rice is the major agricultural product by

value for Thailand. There are many varieties of native Thai rice that have high nutritional and medicinal value. For instance, Riceberry can reduce the risk of hyperlipidemia, hypertension, diabetes and cancer. Glutinous Luem Pua rice has high contents of flavonoids which are antioxidant residues. In addition, black jasmine rice contains high volume of fibers, selenium and niacin that are essential for the human body [11-12]. In this study, it was found that Riceberry, glutinous Luem Pua and black jasmine rice were utilized by the 2 isolates of bacteria. This seemed to indicate that these 3 cultivars of rice could be potential carriers for probiotic-candidate bacteria. Ultimately, the isolates C11AM2 and C12AM3 were selected for further study due to their capacity for carbohydrate utilization.

Table 1. Clear zones (mm) of carbohydrate utilization of rice grains and oat on the agar plates by the strain C11AM2 and C12AM3.

Material	Bacterial strain	
	C11AM2	C12AM3
oat	8.16±0.76^{a}	2.16±0.28^{b}
105 jasmine brown	9.33±0.57^{a}	4.33±0.28^{e}
105 jasmine	12.00±1.00^{b}	0.66±0.28^{a}
Riceberry	14.00±1.00^{c}	0.66±0.28^{a}
glutinous black	16.33±2.08^{d}	3.50±0.50^{d}
105 germinated-jasmine	16.66±0.57^{d}	4.50±0.50^{e}
red jasmine	21.50±0.50^{e}	3.16±0.28^{cd}
germinated-red jasmine	22.00±1.00^{ef}	2.66±0.28^{bc}
glutinous Luem Pua	23.33±0.57^{f}	7.50±0.50^{g}
black jasmine	35.50±0.50^{g}	6.33±0.57^{f}

$^{a-g}$Values in the columns with different letters in superscript were significantly different (P<0.05).

3.2 Identification of Bacteria Capable of Carbohydrate Utilization

The strains C11AM2 and C12AM3 were motile and spore-forming, Gram-positive bacteria. They could not produce any pigments. The morphological and biochemical identification revealed that both strains were *Bacillus*. The strain identification was confirmed by 16s RNA gene determination. The 16S rRNA sequence of strain C11AM2 and C12AM3 was comprised of 1,379 and 1,367 nucleotides, respectively. The sequence comparison using BLAST of strain C11AM2 and C12AM3 proposed a close relationship with *Bacillus amyloliquefaciens* (99% similarity) and *B. subtilis* (99% similarity), respectively.

Several studies reported that many species of bacteria had been isolated from samples from brood, adult, nectar and pollen of bees. The isolation of bacteria was found mostly in tropical and warm regions and in *Apis mellifera* (honey bees), *Apis cerana japonica* (Japanese honey bees) and *Bombus* spp. (Bumblebees) [23-25]. In this study, *Bacillus* spp. were found mostly in bee habitat due to their capacity to survive in all environments such as spore formation and antibiotic production [23]. Furthermore, *B. amyloliquefaciens* C11AM2 and *B. subtilis* C12AM3 could utilize the carbohydrates of all rice grains. These two

bacterial strains were proposed as probiotic candidates that used native Thai rice cultivars as a carrier. According to several reports on the use of *Bacillus* spp. in probiotic products, it was concluded that some strains are safe for human use, for instance, *B. subtilis, B.licheniformis, B. clausii, B. pumilis, B. polymyxa* and *B. indicus*. However, very limited information is publicly available about the application of *B. amyloliquefaciens* in humans [4-8]. Interestingly, there has been much research concerned about the safety of *Bacillus* spp. in humans. According to the WHO and European Union regulations, it is suggested that the phenotypic and genotypic characterization based on taxonomic identification including 16S rRNA gene sequencing of a candidate probiotic should be described [4].

3.3 Assessment of Probiotic Properties

3.3.1 pH transit tolerance

The survival of the two probiotic candidates in the simulated stomach fluid and small intestine fluid in terms of acid tolerance and pH transit was determined. After 5 h of incubation in media, the viable cells of *B. amyloliquefaciens* C11AM2 and *B. subtilis* C12AM3 were 2.7×10^4 and 3.2×10^4 cfu.mL^{-1}, respectively (Figure1). In order to select probiotics for human use, survival in the gastrointestinal (GI) tract and health benefits of selected strains must be conferred [9]. This study simulated the conditions of the GI tract and demonstrated the growth of bacterial cultures to survive both short and long digestion periods. The results seemed to indicate that the two probiotic candidates could resist these conditions. Reports of the *in vitro* probiotic properties of *Bacillus* spp. are relatively scarce, in comparison with the abundant information on lactobacilli and other probiotic strains [26, 27]. It may be concluded from consideration of Sanders et al. (2003) [7] that some germination of spores may occur *in vivo*, but the role of these different cell states in mediating physiological interactions *in vivo* is still unresolved. It is interesting that spore-forming bacteria can be a potential probiotic when administered into the gastrointestinal tract.

There are many experiments of ability to survive in simulated GI tract, for instance, resistance to bile salt, resistance to artificial gastric juice, resistance to bile acid, tolerance to simulated gastric juice, pH shift, pH resistance at different pH and time. Each experiment usually was done separately. The experiment of our study was applied from pH transit which simulated stomach and small intestine. Previous studies revealed various experiments of simulated human GI tract condition of probiotic strains such as survival in artificial stomach and small intestine that contain bile salt but reports on pH transit are scarce. Our experiment focused on monitoring the amounts of probiotic candidate strains at 2 pH values in continuous time. The result really showed bacterial cell changes from initial pH (simulated upper GI tract condition) to final pH (simulated lower GI tract condition) with certainly time. From our result and previous studies of probiotic strains, it is an essential data for supporting an idea of ability to survive in simulated GI tract conditions. However, the ability to survive in GI tract should be tested in epithelial cell culture or in *in vivo* animal studies for more sure/accurate results. A further step may be to investigate the ability of probiotic candidates to survive *in vivo* in the GI tract.

3.3.2 Antimicrobial activity

Testing of the ability of selected strains to inhibit the growth of intestinal pathogenic bacteria showed that only *B. amyloliquefaciens* C11AM2 could inhibit growth of *Vibrio*

cholerae and *Vibrio parahaemolyticus* with inhibition zones of 10.6±0.4 and 9.1±0.1 mm, respectively. There are few reports about the growth inhibition of *V. cholerae* and *V. parahaemolyticus* by *Bacillus* spp. This research is relevant to the study of Das et al. (2013) [28] which found that *B. amyloliquefaciens* FPTB16 displayed inhibition zones against *Edwardsiella tarda*, *Aeromonas hydrophila*, *V. harveyi* and *V. parahaemolyticus*. *V. cholerae* is an important pathogen in many developing countries, where it can cause devastating disease and economic burdens. The cholera affected the expenses of health-care and public health organization, and caused a loss of economic worldwide [29]. Indeed, Vibrios are one of the most common organisms in surface waters of the world. They occur in both marine and freshwater habitats and in associations with aquatic animals. *V. cholerae* and *V. parahaemolyticus* are serious pathogens of humans. Interestingly, this study found that this probiotic candidate strain could exhibit the ability to minimize one of the most important pathogenic bacteria. Also, the production of antimicrobial substances by *Bacillus* spp. has been widely reported, but the published data on growth inhibition of *V. cholerae* are scarce. In particular, this study reported that the strains *B. amyloliquefaciens* C11AM2 that is amylolytic bacteria had high ability to inhibit growth of *Vibrio* spp. Hence, a further step may be to evaluate the antimicrobial substances produced by the two strains, which could then find potential use in food products.

3.3.3 Haemolytic activity

From the results of hemolysin production of the two probiotic candidates, it was found that both *B. amyloliquefaciens* C11AM2 and *B. subtilis* C12AM3 did not have haemolytic activity. In addition, the biochemical characteristics of the two strains revealed that all strains were negative for lecithinase. These two aspects of strong haemolytic and lecithinase activity are very necessary to indicate the presence of cytotoxic phospholipase that is relevant to the virulent factor [4]. This research is in accordance with the findings of Cao et al. (2011) [30] and Sorokulova et al. (2008) [4] who investigated *B. subtilis* VKPM B2335 and *B. amyloliquefaciens* G1and reported they had no haemolytic and lecithinase activity. In summary, the absence of haemolytic activity is a very important characteristic for probiotics to be considered as safe and,as such, *B. amyloliquefaciens* C11AM2 and *B. subtilis* C12AM3 seem to be safe for human use.

3.4 Media Optimization

The results of the experiment on the utilization of alternative nutrient sources for probiotic strains, showed that both probiotic strains could grow well on all media, particularly bean extract medium (Table 2). Even though the results of growth of probiotic strain on MRS medium were significantly the highest, bean extract medium was an interesting medium for probiotic strains as it is natural, low cost and more available. In fact, *Bacillus* strains can grow well on various sources of nutrient. The cell growth measurement results showed that the growth rates of the two probiotics were high, so the selection of other nutrient sources to obtain high yield cells was determined. In addition, natural sources which are less toxic are suitable for the food industry. The use of various kinds of bean as natural sources for the growth of *Bacillus* spp. has been widely evaluated. The well-known soya-based food products are Nutto or Thao-nao that are favourite fermented foods in Asian countries, particularly Japan and Thailand.

Table 2. Optical density at 540 nm representing the growth of probiotic strains in various medium after 18 h of incubation.

Medium	Bacterial strain	
	B. amyloliquefaciens C11AM2	*B. subtilis* C12AM3
Modified MRS No.1	1.2348±0.00^{c}	1.5200±0.00^{d}
Modified MRS No.1	0.6312±0.00^{a}	1.1636±0.00^{b}
Bean extract	1.0140±0.00^{b}	1.1322±0.00^{a}
MRS	1.2450±0.00^{c}	1.4118±0.00^{c}

$^{a-d}$Values in the columns with different letters in superscript were significantly different (P<0.05).

3.5 Development of Probiotic-supplemented Thai Rice Cultivar Products

Three cultivars of Thai rice; Riceberry, glutinous Luem Pua and black jasmine rice that were selected on the basis of the highest activity of carbohydrate utilization, were evaluated for their capacity as a potential carrier for probiotic strains. It was interesting that the survival rates of the two probiotic strains were slightly decreased in all cultivars of rice grains, and in both processes encapsulated and steamed rice grains (Figure 2-5, Table 3). Indeed, the survival rates of *B. amyloliquefaciens* C11AM2 in encapsulated grains were slightly higher than steamed grains, whereas, the survival rates of *B. subtilis* C12AM3 in steamed grains were slightly higher than in baked-encapsulated grains. As a result, the shelf-life of these products was up to 2 months in the refrigerator. This seemed to be indicative that the encapsulation and steaming were suitable for maintaining the survival of probiotic strains in rice carriers. Several previous studies reported that low humidity carriers could enhance the viability of probiotic bacteria. The fruit powders of apple, banana and strawberry were good carriers for *L. plantarum* 299v; however, the researcher suggested that the techniques used should be carefully monitored. The challenged in probiotic production is to maintain viable cell number during incubation and storage. Food materials which have different physicochemical properties may affect the survival of probiotic bacteria [1]. Consequently, suitable methods to maintain the viability of probiotic strains must be determined. Several reports revealed that microencapsulation could protect probiotics against acid stress, allowing cells to survive in the stomach and intestines [3]. As well, in our study, the two methods of processing showed promise to maintain the viability of probiotic strains. Notwithstanding, further examination, such as nutrient composition of products, adhesion of bacterial cells to rice matrices and other materials of encapsulation, should be approached.

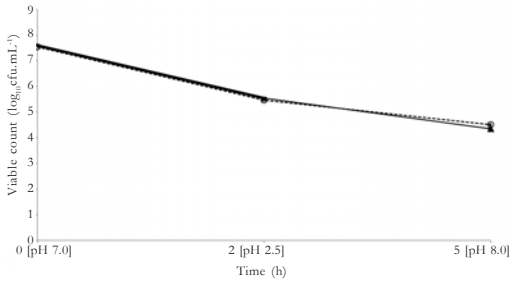

Figure 1. Viable cell count of *B. amyloliquefaciens* C11AM2 (○) and *B. subtilis* C12AM3 (▲) in pH 2.5 fluid for 2 h of incubation and then in pH 8.0 artificial fluid for 3 h of incubation (in continuous time) at 37°C. Each point represents the mean ± standard deviation of three independent experiments.

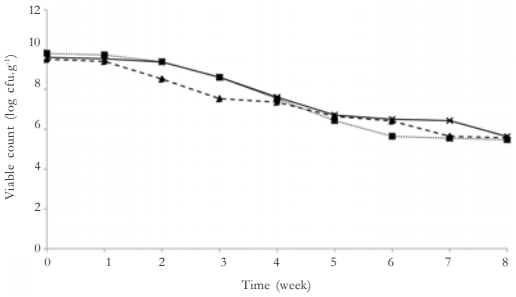

Figure 2. Viable cell count of *B. amyloliquefaciens* C11AM2 in encapsulated rice grains of cultivar Riceberry (---■---), glutinous Luem Pua (---▲---) and black jasmine rice (---×---) after 8 weeks of incubation at 4°C.

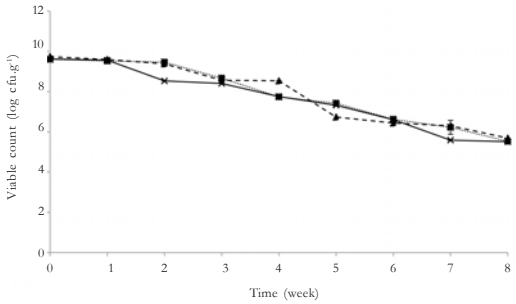

Figure 3. Viable cell count of *B. subtilis* C12AM3 in encapsulated rice grains of cultivar Riceberry (---■---), glutinous Luem Pua (---▲---) and black jasmine rice (---×---) after 8 weeks of incubation at 4°C.

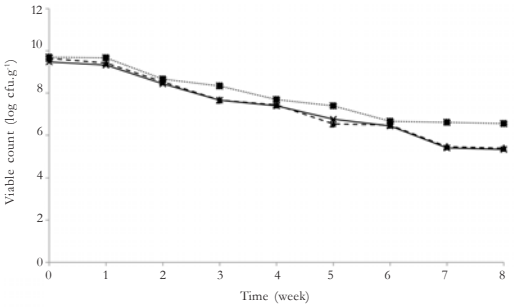

Figure 4. Viable cell count of *B. amyloliquefaciens* C11AM2 in steamed rice grains of cultivar Riceberry (---■---), glutinous Luem Pua (---▲---) and black jasmine rice (---×---) after 8 weeks of incubation at 4°C.

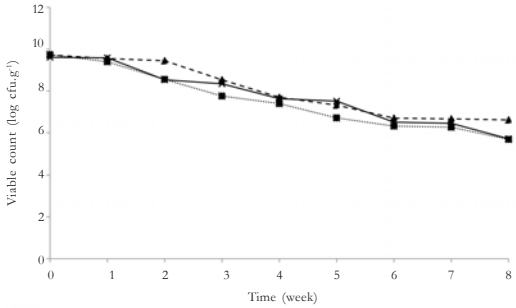

Figure 5. Viable cell count of *B. subtilis* C12AM3 in steamed rice grains of cultivar Riceberry (---■---), glutinous Luem Pua (---▲---) and black jasmine rice (---×---) after 8 weeks of incubation at 4°C.

Table 3. Survival rate (%) of *B. amyloliquefaciens* C11AM2 and *B. subtilis* C12AM3 in Riceberry, glutinous Luem Pua and black jasmine rice products after 2 months of incubation.

Process	*B. amyloliquefaciens* C11AM2			*B. subtilis* C12AM3		
	Riceberry	glutinous Luem Pua	black jasmine	Riceberry	glutinous Luem Pua	black jasmine
1. Encapsulated rice grains	55.81±0.09	58.56±0.05	58.58±0.11	57.54±0.03	58.41±0.04	57.27±0.19
2. Steamed rice grains	57.52±0.20	55.34±0.17	55.41±0.15	58.31±0.04	58.00±0.68	59.47±0.78

Observation of physical changes in color, odor and pH value of products during storage, revealed that all the probiotic-supplemented products had a similar appearance. The color of products after 2 months of storage was pale or faded from the original color and a fermented odor of products was detected, even though it was not a strong odor. Thus, the changed color and odor of all products was considered acceptable. In addition, the pH values of the products slightly decreased from approximately 6.1-6.6 to 5.4-5.8. These experiments demonstrated that the probiotic-supplemented products from both processing procedures were stabilized after storage and the processing extended the long-term shelf-life of the products. The results showed that growth of *E. coli*, yeasts and molds was not detected after 2 months of storage. The total aerobic bacterial counts were approximately 10^4-10^5 cfu.g^{-1}, however, bacteria were the probiotic candidate strains. According to some essentially morphological, biochemical and molecular identification of aerobic bacteria found on agar plates. The results showed that these bacteria were 2 strains of probiotic strains (*B. amyloliquefaciens* C11AM2 or *B. subtilis* C12AM3). Hence, our study revealed that the amounts of contaminants were considered acceptable and safe for human use.

The advantages of these products can be further developed to facilitate storage at room temperature as the products contained spore-forming probiotic strains. Moreover, spores are capable of surviving at the low pH of the gastric barrier [31]. We propose that *B. amyloliquefaciens* C11AM2 can be a new probiotic strain and both types of rice products had a well-formulated probiotic content. Even though capsulation is an extra cost, it can result in increased profit in higher-value food markets. Finally, the probiotic-supplemented products must be developed based on the ability to deliver health benefits through a body part of humans. However, these preliminary studies should be further evaluated by considering the adhesion to epithelial cells, *in vivo* toxicity test or sensory analysis.

3.5 Analysis for Food Nutrition

The nutritional profile of probiotic-supplemented riceberry was characterized and showed that each capsule (100g) contained total carbohydrate (include dietary fiber) 60.47 g, protein (factor 5.95) 21.66 g, dietary fiber 2.36 g, total sugar 1.6 g, sodium 57.9 mg, saturated fat 0.59 g, total fat 2.1 g, vitamin B1 0.02 mg, calcium 10.74 mg and iron 0.9 g. The dietary supplement had high carbohydrate and protein that serve high amounts of energy for human daily diet, while the amounts of saturated fat were low and had no cholesterol. Therefore, this product is prone to develop high-value rice products.

4. CONCLUSIONS

Among the bacteria isolated from bees, two strains *B. amyloliquefaciens* C11AM2 and *B. subtilis* C12AM3 exhibited maximum carbohydrate-utilization activity with robust probiotic properties. The probiotic candidates passed most the *in vitro* requirements recommended by FAO/WHO (2002) [9] and could survive during simulated gastrointestinal conditions of both short and long digestion periods. The result really showed the resistance of pH stress condition of bacterial cells from initial pH to final pH with certainly time. Indeed, the strains also did not exhibit haemolytic activity. Interestingly, the strain *B. amyloliquefaciens* C11AM2 had high ability to inhibit important intestinal pathogens such as *V. cholerae* and *V. parahaemolyticus*. The *Bacillus* strains were

able to survive food product processing and during storage at 4°C for 60 days, the cell reductions were minimal. Although further *in vivo* tests are required, this research indicated that the 2 strains are potential probiotic candidates ideally as supplements in the food industry. Indeed, rice and product processing can be used to maintain the stability of probiotic bacteria during storage and can be employed for formulating novel functional food products. This approach should be the starting point and will benefit the development of probiotic-supplemented rice products.

ACKNOWLEDGEMENTS

This research was supported by grants from the Faculty of Liberal Arts and Science, Kasetsart University and from the Division of Research Management and Academic Service, Kasetsart University Kamphaeng Saen Campus. We also thank the Kasetsart University Research and Development Institute (KURDI).

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