Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.120722

Use of pigmented rice as carrier and stingless bee honey as prebiotic to formulate novel synbiotic products mixed with three strains of probiotic bacteria

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Abstract

The aim of this study was to produce a synbiotic product containing Thai-pigmented rice as the carrier and the honey of the stingless bee as the prebiotic agent. From antibacterial activity results, all of the tested probiotic bacteria (*Limosilactobacillus reuteri* KUKPS6103, *Lacticaseibacillus rhamnosus* KUKPS6007 and *Lacticaseibacillus paracasei* KUKPS6201) had a high ability to inhibit six strains of intestinal pathogens. All three probiotic bacterial strains had high antioxidant activity according to the ABTS and DPPH scavenging assays. The three tested probiotic bacteria produced substantial reductions in the cholesterol removal percentage of 33.9-78.99%. In the prebiotic studies, stingless bee honey had high enhancing activity and the main carbohydrate components were sucrose, glucose and fructose. A synbiotic product was produced containing Thai-pigmented rice grains (cultivars Riceberry, Tubtim Chumphae and Sangyod) and stingless bee honey. During 8 weeks of storage, the viability of the probiotics in terms of mixed-strains was 7.96 ± 0.06 log CFU g^{-1} (90.25% survival rate). Microbiological safety testing stated that the amounts of contaminants were acceptable. This is the first report of an application of Thai-pigmented rice, stingless bee honey and mixed-culture probiotics as a novel functional synbiotic product.

Keywords: probiotic; prebiotic; synbiotic; pigmented-rice; stingless bee honey.

Practical Application: The characteristics of probiotic bacteria, and the enhancing activity of stingless bee honey to use as prebiotic was observed. Being used the pigmented rice cultivars as carrier and stingless bee honey as prebiotic, a synbiotic product was developed to get the heath-promoting of human wellness. Thus, mixture of prebiotics and probiotics could be an excellent method for the formulation of probiotics supplements and other food products. And, the stingless bee honey and pigmented rice much affects the nutraceutical benefits of human wellness.

1 Introduction

Probiotics is defined as "live microorganisms that, when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014). Probiotics are well-known as foods that are containing an adequate number of beneficial microorganisms in a given matrix. Probiotics are supposed to be beneficial or to prevent the production of biogenic amines and act as bactericidal components. Thus, the adhesion of probiotics would be promoted the strengthen of the beneficial effects of probiotics (Buran et al., 2022; Jiang et al., 2021; Moghadam et al., 2022). Probiotics are harmless microorganisms that, when delivered to humans in sufficient concentrations and for long enough periods of time, have certain beneficial effects on the host. In addition, probiotic bacteria boost the system by increasing intestinal cell adhesion and mucin synthesis, as well as modulating the activity of gut-associated lymphatic tissue. Lactic acid bacteria (LAB) are regarded as a major group of probiotic bacteria (Jeong et al., 2016). To be effective, probiotic bacteria must be able to survive at body temperature and be resistant to stomach acid and bile salt (Tomasik & Tomasik, 2020). In order to promote the survival and development of probiotic bacteria, oligosaccharides are added to probiotics foods (Jaimez-Ordaz et al., 2019).

To date, major groups of probiotic bacteria belong to the genera *Lactobacillus* and *Bifidobacterium*, while the genera *Lactococcus* and *Saccharomyces* are also considered probiotic bacteria. To date, there is one strain of yeast (*Saccharomyces boulardii*) used as a probiotic (Mancuskova et al., 2018). According to the evaluation criteria of probiotics in food reported by a joint FAO/WHO working party (Kumar et al., 2015), resistance to stomach acidity and resistance to bile salts are two of the most commonly used *in vitro* tests, as supported by both survival and growth investigations.

Prebiotics, non-digestible food ingredients that positively affects human health by promoting the activity and development of probiotic bacteria in the large intestine, have been the source of much research on the development of new fermented dairy food products in the dairy industry (Kavas et al., 2022). Prebiotics have beneficial physiological effects on the gastrointestinal microbiota and are a kind of dietary fibers (Hossain et al., 2021). Nondigestible oligosaccharides and polysaccharides usually exhibit prebiotic properties, such as fructooligosaccharides, galactooligosaccharides, inulin, resistant starch and lactulose derived from various plants, fruits and vegetables (Wichienchot

Received 29 Oct., 2022
Accepted 13 Dec., 2022
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& Ishak, 2017). Flours made of cereals, other vegetables edible parts, and fruits are potential sources of prebiotic fibers for improving functional quality of processed foods and the nutritional value (Pérez-Chabela & Hernández-Alcántara, 2018). Several observations have attempted to promote the growth of probiotic bacteria by adding fiber-rich fractions from herbal plants (Ahmed & Rashid, 2019), cereals (Leonard et al., 2021) and banana, passion fruit, or apple processing by-products (Santo et al., 2012).

Advances in microbial technology have established that synbiotics, which are a mix of probiotic and prebiotic products, help boost survival and hence the implantation of lively microbial dietary supplements in the gut (Tufarelli & Laudadio, 2016). Scientific evidence has shown that the synbiotic interaction between prebiotics and probiotics has a substantial impact on health. Due to the significant benefits for gut health, illness prevention, and therapy, commercial interest in functional foods containing synbiotics has steadily developed. Moreover, the term "postbiotics" can be defined as non-viable bacterial products or metabolic byproducts from probiotic bacteria that have biological activity in the host. Postbiotics are non-toxic, non-pathogenic and resistance to hydrolysis by mammalian enzymes, as these are non-viable bacterial products or metabolic byproducts from probiotics, such as bacteriocins, organic acids, ethanol, diacetyl, acetaldehydes, hydrogen peroxide, and heat-killed probiotics (Kerry et al., 2018).

Rice (*Oryza sativa* L.) is consumed as a staple food by over one-half of the world's population (Mbanjo et al., 2020). Despite the fact that pigmented or colored rice has a lower yearly consumption than white rice, it has grown in popularity among Thai customers as a result of its health benefits. When compared to ordinary varieties, red and black paddy varieties have approximately 38% more protein, 18% more crude fiber, and are higher in lysine, vitamin B1, and other minerals (Umadevi et al., 2012). Due to its healthful functional food elements, the consumption of colored rice (black and red) is rapidly increasing (Chen et al., 2012). Pigmented rice varieties provide useful qualities due to the antioxidant chemicals they contain, which can inhibit the production of reactive cell-damaging free radicals, in addition to high protein, fiber, and vitamin contents. Anthocyanins are primary metabolites found in the bran layer of rice kernels, and they have been revealed as health-promoting functional food constituents with anticancer, antioxidant, hypoglycemic, and anti-inflammatory activities (Dias et al., 2017).

Honey is one of the natural food products, which it is mainly consisted of sugars and other elements, such as amino acids, organic acids, enzymes, vitamins, minerals and flavoring substances. As a biological effect, it is rich in flavonoids and phenolic acids, which act as natural antioxidant (Alqarni et al., 2014). Stingless bee honey (honey collected from stingless bee) and honeybee honey (honey collected from honeybee) are natural product, which produced worldwide. Both two types of honey contained many biological and nutritional compounds. Stingless bee honey is well-known for its sweetness and fluid texture, it has higher nutritional value than honeybee honey. In terms of color, flavor, and viscosity, stingless bee honey differs from honey made by honeybee (Biluca et al., 2019; Almeida-Muradian et al., 2014). The antioxidant, anti-inflammatory, anti-obesity, anticancer, and antibacterial effects of stingless bee honey (SBH) can all be considered as nutraceutical benefits (Al-Hatamleh et al., 2020). The current research was established to develop a synbiotic product using probiotic bacteria (*L. reuteri* KUKPS6103, *L. rhamnosus* KUKPS6007 and *L. paracasei* KUKPS6201), Thai-pigmented rice cultivars as carriers and honey from stingless bees as a prebiotic for potential application in maintaining human health.

2 Materials and methods

2.1 Raw materials

Thai-pigmented rice cultivars (Riceberry, Tubtim Chumphae and Sangyod) and the stingless bee honey (Bankohlaenang, Songkla) used as a prebiotic in this experiment were purchased from a local market in Thailand. The rice cultivars all had Good Agricultural Practices (GAP) and Good Manufacturing Practices (GMP) certification, and were kept tightly in a refrigerator (at 4 °C) for future use.

2.2 Microorganism cultural conditions

Three probiotic bacteria (*L. reuteri* KUKPS6103, *L. rhamnosus* KUKPS6007 and *L. paracasei* KUKPS6201) that exhibited non-hemolytic activity, auto-aggregated and co-aggregated activity, tolerance to acid and bile salt, and had anaerobic growth potential, and intestinal tract disease-causing bacteria (*Aeromonas hydrophila* KPS-01, *Bacillus cereus* KPS-01, *Escherichia coli* KPS-01, *Proteus vulgaris* KPS-01, *Staphylococcus aureus* KPS-01 and *Salmonella typhimurium* KPS-01) were obtained from the culture collection of the Division of Microbiology, Faculty of Liberal Arts and Science, Kasetsart University, Kamphaeng Saen Campus, Thailand. These strains were inoculated on de Man Rogosa and Sharpe (MRS, Merck, Germany) agar or nutrient agar (NA, Merck, Germany) and incubated at 37 °C for 24-48 h. For longer use, the strains were maintained with glycerol (1 glycerol: 1 MRS v/v) and kept at -20 °C for further experiments.

2.3 Effect of growth of probiotic bacteria using dual culture method

To determine whether *L. reuteri* KUKPS6103 had any effect on the growth of *L. rhamnosus* KUKPS6007 and *L. paracasei* KUKPS6201, overnight cultures of the three bacteria were cross-streaked on MRS agar optimized for dual growth, and the plates were incubated at 37 °C for 24-48 h before the cultures were examined for any evidence of growth inhibition (Liaqat et al., 2021).

2.4 Ability to inhibit pathogenic bacteria

The antibacterial activity of probiotic bacteria was investigated using an agar well diffusion method according to Promsai et al. (2018) with some modifications. The six strains of intestinal pathogens were used as tested microorganisms. Cell-free culture supernatant (CFCS) solutions of the probiotic bacteria were obtained. Then, 6 mm wells were excised in brain heart infusion (BHI, Himedia, Mumbai, India) agar medium using a sterile cork borer. Next, indicator strain swabs were performed

using sterile cotton on the surface of the BHI medium, and each well was loaded with 100 µL of CFCS solution. The agar plates were incubated at 37 °C for 48 h, and each zone of inhibition was measured in millimeters. The experiments were replicated three times. Antibacterial activities were determined based on the assessment of a clear zone (mm) forming around the well, where bacterial growth was reduced to a well diameter (6 mm). MRS broth medium was employed as a negative control, while 0.1 mg mL^{-1} streptomycin was used as a positive control.

2.5 Investigation of antioxidant activities: $ABTS^{*+}$ radical scavenging capacity assay

The ABTS radical ($ABTS^{*+}$) is generated by oxidation of ABTS (2,2 azino-bis (3- ethylbenzothiazoline-6-sulfonic acid)) with potassium persulfate as previously described (Re et al., 1999) with some modifications. All tests were performed in triplicate. The percent inhibition as one-half the maximal inhibitory concentration (IC_{50}) of the absorbance at OD_{734} was plotted as a function of the concentration of Trolox and was used to calculate the Trolox equivalent antioxidant activity. The antioxidant activity was calculated based on the following Equations 1-2:

$$Inhibition\ (\%) = \left(\left(\begin{matrix} A_{734\ control} - \\ A_{734\ probiotic} \end{matrix} \right) / A_{734 control} \right) \times 100 \quad (1)$$

$$Antioxidant\ activity = IC_{50} standard\ Trolox / IC_{50} sample \quad (2)$$

Where, IC_{50} = one-half the maximal inhibitory concentration.

2.6 Investigation of antioxidant activities: DPPH radical scavenging capacity assay

The antioxidant activities were determined using 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma–Aldrich, Saint Louis, MO, USA) as a free radical. RSA of various concentrations (supernatant, 5-fold supernatant and 10-fold supernatant µg mL^{-1}) of each of the three probiotic bacteria and mixed culture (*L. reuteri* KUKPS6103, *L. rhamnosus* KUKPS6007, and *L. paracasei* KUKPS6201) was performed according to Mancuskova et al. (2018). Gallic acid (Merck) was used as a standard. Absorbance was measured at 517 nm using a U-5100UV/VIS spectrophotometer (Hitachi, Japan). The experiment was carried out in triplicate. Free RSA was calculated by the following Equations 3-4:

$$\%\ DPPH\ radical-scavenging = \left(\left(\begin{matrix} A_{517\ control} - \\ A_{517\ probiotic} \end{matrix} \right) / A_{517\ control} \right) \times 100 \quad (3)$$

$$Antioxidant\ activity = IC_{50} of\ gallic\ acid / IC_{50} of\ sample \quad (4)$$

Where, IC_{50} = one-half the maximal inhibitory concentration.

2.7 Determination of cholesterol assimilation

Thirty milligrams of cholesterol (polyoxyethanylcholesterol sebacate) (Sigma-Aldrich, Saint Louis, MO, USA) was dissolved into 10 mL of Milli-Q water and filter-sterilized using a 0.45 µm filter (Millipore-Bedford, MA, USA) to obtain a stock solution of cholesterol. MRS broth containing 3 g L^{-1} of bile salt oxgall (Sigma-Aldrich, Saint Louis, MO, USA) was sterilized, and 100 µL mL^{-1} of cholesterol stock solution was inoculated with 1 x 10^8 CFU mL^{-1} of activated probiotic bacterial cultures (*L. reuteri* KUKPS6103, *L. rhamnosus* KUKPS6007, *L. paracasei* KUKPS6201, mixed probiotic strains and *Bacillus cereus*) and incubated at 37 °C for 6, 12 and 24 h, respectively. Then, the solutions were centrifuged 4000 × *g* for 20 min at 4 °C. The cholesterol content in the supernatant was examined using the modified colorimetric method described in Miremadi et al. (2014). The ability of a bacterial strain to assimilate cholesterol was calculated as the percentage of cholesterol removal at each incubation using (Equation 5):

$$\%\ Cholesterol\ removal = \left[\left(\begin{matrix} 100 - residual\ cholesterol\ at \\ each\ incubation\ interval \end{matrix} \right) / 100 \right] \times 100 \quad (5)$$

2.8 Growth of probiotic bacteria in various concentration of honey

The probiotic bacteria were tested to measure the growth rate in various concentrations of honey. Deionized water containing 100, 200, 300, 400 and 500 g L^{-1} honey were sterilized by autoclaving at 110 °C for 10 min to kill all the unwanted bacteria before inoculation using the probiotic bacteria. The deionized water containing 100, 200, 300, 400 and 500 g L^{-1} honey without probiotic inoculum were used as control. The viability of the probiotic cells was determined after 24 hours of incubation.

2.9 Prebiotic properties of honey

Enhance effect of honey on probiotic bacteria

Each probiotic bacterial strain (*L. reuteri* KUKPS6103, *L. rhamnosus* KUKPS6007, *L. paracasei* KUKPS6201) and a mix of the probiotic strains were cultivated in 10 mL of MRS broth with 20 and 50 g L^{-1} honey or 20 and 50 g L^{-1} inulin (commercial oligosaccharide as a control) at 37 °C for 4 h. The growth of probiotic strains was observed by measuring the turbidity of the culture broth at 600 nm. The enhancement activities were calculated using the Equation 6 (Pangsri et al., 2015):

$$Enhancement\ activity\ (\%) = \left[(SB - CB) / CB \right] \times 100 \quad (6)$$

Where, SB is the optical density of the cell in the medium with honey or inulin and CB is the optical density of the cell in the medium without honey or inulin.

The secondary screening of prebiotic enhancement was modified from Titapoka et al. (2008). Precultures of probiotic bacteria were adjusted to an absorbance value of 0.5 at 600 nm and used as inoculum. A sample of 10 mL L^{-1} inoculum of each probiotic bacterium was transferred to 10 mL of MRS broth containing 20 and 50 g L^{-1} honey or 20 and 50 g L^{-1} inulin (commercial oligosaccharide as a control) and incubated at 37 °C for 4 h. Then, the bacterial cultures were serially diluted, spread

on MRS agar and incubated at 37 °C for 4 h. The enhanced activity on bacterial growth was examined by calculating the difference (SF-CF), where SF is the cell number with honey or inulin and CF is the cell number without honey or inulin (both measured in log CFU mL^{-1}).

Identification of carbohydrate fractions

To determine the oligosaccharide profile of honey, the honey samples were analyzed using high-performance liquid chromatography (HPLC). The samples were passed through a 0.45 µm nylon syringe filter. A sample (40 µL) was injected into a Shimadzu HPLC system consisting of a LC-20AD pump, a RID-10A detector, and two serially connected columns (both Shodex OHpak SB-802.5 HQ, 8 × 300 mm, Showa Denko K.K., Japan) with a specific guard column connected to a computer running the data analysis software program (CLASS-VP). Isocratic elution with deionized water was carried out at 60 °C and a flow rate of 0.8 mL min^{-1}.

Investigation of Short-Chain Fatty Acid (SCFAs)

Sample preparation: All probiotic bacteria were inoculated in MRS supplemented with 20 g L^{-1} stingless bee honey and incubated at 37 °C for 48 h. The bacterial culture was then centrifuged at 3000 × *g* for 10 min to obtain supernatant. After that, the supernatant was subjected to characterization using high-performance liquid chromatography (HPLC) and other studies that require ethical approval, must list the authority that provided approval and the corresponding ethical approval code.

Chromatographic conditions: The qualitative and quantitative analyses of short-chain fatty acids (SCFAs, acetic acid, butyric acid, and propionic acid) were performed by high-performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan) as described by de Sá et al. (2011) with some modification. The mobile phase consisted of 0.005 mol mL^{-1} H_2SO_4 in deionized water. This solution was filtered through a 0.45 µm Millipore membrane. The flow rate was 0.4 mL min^{-1}, and the injection volume was 20 µL. Fatty acids were analyzed using a refractive index detector (RI, Model RIS-10A). The analytical column used was an Amine HPX-87H (300 mm × 7.8 mm × 9 mm) (BIO-RAD, California, USA). The variation in temperature was 60 °C. SCFA identification was performed by comparing the retention times of standard SCFAs, and the volumes of SCFA (µg mL^{-1}) were measured under the standard curves of each SCFA.

2.10 Development of synbiotic products

Three strains of probiotic bacteria (*L. reuteri* KUKPS6103, *L. rhamnosus* KUKPS6007 and *L. paracasei* KUKPS6201) were inoculated in MRS broth and incubated at 37 °C for 24 h. Cell pellets were obtained by centrifuging the probiotic cells followed by washing with sterilized water. The probiotic cell pellet was used for the production of the synbiotic product.

Thai-pigmented rice grains (cultivars Riceberry, Tubtim Chumphae and Sangyod) were milled with a blender to produce rice powder. The rice powder was baked at 105 °C for 48 h and used as a carrier for the synbiotic production process.

The cultures of probiotic cell pellets consisted of the three types of Thai-pigmented rice cultivars supplemented with diluted stingless bee honey. The mixtures were prepared in three treatments.

Product 1: each capsule consisted of 0.5 g of baked rice powder mixed with probiotic cells at a ratio of 1:9 (bacterial cells to rice powder) prior to encapsulation using a capsule filling machine. The product was stored in an opaque, tightly sealed plastic bag at 4 °C. The viability of the probiotic cells was determined within 8 weeks of incubation.

Product 2: stingless bee honey was mixed with probiotic cells at a ratio 9:1 (honey-to-cells) and kept in a microtube. The product was stored in an opaque, tightly sealed plastic bag at 4 °C. The viability of the probiotic cells was determined within 8 weeks of incubation.

Product 3: the synbiotic product in a capsule contained 0.5 g of probiotic cell pellet, pigmented rice powder and diluted honey in the ratio (1:6:3) by encapsulation. The synbiotic product was stored in an opaque, tightly sealed plastic bag at 4 °C. The viability of the probiotic cells was determined within 8 weeks of incubation.

2.11 Microbiological safety analysis

The enumeration of probiotics, *E. coli*, yeast and molds, and coliform bacteria were analyzed using serial dilutions of the product sample in 8.5 g L^{-1} sterile NaCl (United States Pharmacopeial Convention, 2020). The developed synbiotic product was analyzed on Day 0 and then after every week of storage for 8 weeks. Suitable dilutions were spread on their respective media (MRS agar for probiotic count; eosin methylene blue (EMB) agar for *E. coli*; yeast extract-malt extract (YMA) agar for yeast and molds; BHI agar for coliform bacteria). The culture plates were incubated for 48 h at 37 °C prior to counting the microbial colonies and reported as log CFU g^{-1}. The survival percentage levels of the probiotic strains in all treatments were calculated using the formula from Savedboworn & Wanchaitanawong (2015) (Equation 7):

$$Survival\ rate\ (\%) = LogN_1 / LogN_0 \times 100 \quad (7)$$

Where, N_1 represents the number of viable cells after the treatment (CFU g^{-1}) and N_0 represents the number of viable cells before the treatment (CFU g^{-1}).

2.12 Nutritional analysis

A total of 0.5 g (500 mg) (one capsule) of synbiotic enhanced with pigmented rice cultivars and stingless bee honey was sent to the Central Laboratory Co. Ltd. (Thailand) for nutritional profiling analysis using the AOAC method (Association of Official Analytical Chemists, 2019), for food analysis.

2.13 Statistical analysis

The values were calculated as the mean ± standard deviation of individual experiments in triplicate, and viable probiotic bacteria

counts were reported in log CFU g^{-1}. The data presented are the average of the three determinations. Significantly different values were analyzed using Duncan's test and one-way ANOVA with SPSS v.20 software (SPSS Inc., Chicago, IL, USA). The chosen level of significance for all statistical tests was 5% ($p < 0.05$).

3 Results and discussion

3.1 Growth of probiotic bacteria on MRS using the dual culture method

The dual culture of three strains of probiotic bacteria produced no indication of growth inhibition among the probiotic bacteria (Figure 1). All of the probiotic bacteria had the capacity to grow well on MRS medium at the optimum incubation conditions of 37 °C for 24-48 h. After incubation, it was clear that all of the probiotic bacteria grew well on MRS medium. These probiotic bacteria were used in the production of the synbiotic products.

Several strains of lactic acid bacteria (LAB) are a major group of probiotic bacteria that are commonly found in the gastrointestinal tract of humans and animals (Jensen et al., 2012). The term 'prebiotic' has been defined as "a selectively fermented component that allows for specific changes in the composition and/or activity of the gastrointestinal microbiome, leading to improved host wellness and health" (Slavin, 2013, p. 1418). All of the probiotic strains showed the capacity to be used in the production of synbiotic products based on testing the dual growth on MRS media. The growth and metabolism of microorganisms, specifically probiotic bacterial species inhabiting the massive intestine depend upon the substrates available to them (Zhang et al., 2015).

3.2 Antibacterial activity

The results clearly indicated that all the probiotic bacteria alone and in mixed culture could produce inhibiting substances to minimize the growth of all of the pathogens (Table 1). *L. rhamnosus* KUKPS6007, *L. paracasei* KUKPS6201 and mixed-culture exhibited the superior antibacterial activity with the inhibition zone ranged 14.00, 14.33 and 13.67 mm, followed by *L. reuteri* KUKPS6103 with insignificance difference against *E. coli* KPS-01. The antibacterial activity of all probiotic bacteria and mixed-culture against *E. coli* KPS-01 was almost to that obtained against *B. cereus* KPS-01, and followed the same pattern. It is worth mentioning that the inhibitory activity of the tested probiotic supernatants was slightly less against *A. hydrophila* KPS-01 as compared to that obtained against *B. cereus* KPS-01, indicating that *A. hydrophila* KPS-01 could be less sensitive. The least activity for all probiotic was recorded (inhibition zone ranged 9.33-11 mm) against *P. vulgaris* KPS-01, while all probiotic (inhibition zone ranged 11.33-12.67 mm) was moderately active against *S. typhimurium* KPS-01 and *S. aureus*

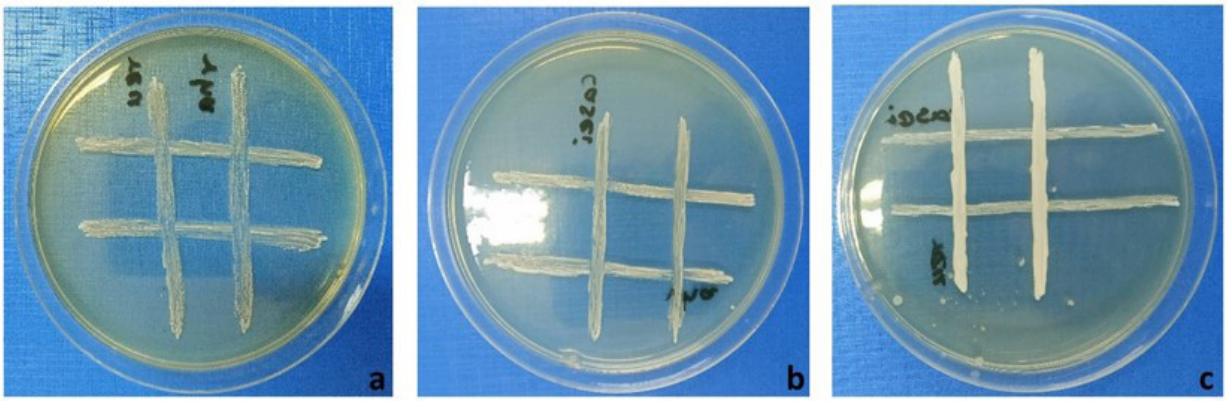

Figure 1. Growth of probiotic bacteria on dual culture plate. (a) *L. rhamnosus* KUKPS6007 and *L. reuteri* KUKPS6103, (b) *L. rhamnosus* KUKPS6007 and *L. paracasei* KUKPS6201 and (c) *L. reuteri* KUKPS6103 and *L. paracasei* KUKPS6201.

Table 1. Antagonistic activity of probiotic bacteria against foodborne pathogens.

Pathogens	Inhibition zone (mm)				
	L. reuteri KUKPS6103	*L. rhamnosus* KUKPS6007	*L. paracasei* KUKPS6201	Mixed culture	Streptomycin
A. hydrophila	12.33 ± 2.52^{a*}	11.67 ± 3.06^{a}	12.00 ± 1.00^{a}	11.67 ± 0.56^{a}	23.33 ± 1.53^{b}
B. cereus	11.33 ± 0.58^{a}	12.33 ± 0.58^{a}	12.00 ± 1.00^{a}	12.00 ± 0.00^{a}	22.67 ± 1.53^{b}
E. coli	11.67 ± 1.53^{a}	14.00 ± 1.00^{a}	14.33 ± 1.16^{a}	13.67 ± 1.16^{a}	20.67 ± 2.52^{b}
P. vulgaris	9.33 ± 1.15^{a}	11.00 ± 1.73^{ab}	10.00 ± 1.00^{a}	10.00 ± 1.00^{a}	16.33 ± 6.66^{b}
S. typhimurium	11.33 ± 0.58^{a}	12.67 ± 0.58^{a}	11.33 ± 0.58^{a}	11.67 ± 0.58^{a}	15.67 ± 2.52^{b}
S. aureus	11.33 ± 0.58^{a}	11.33 ± 0.58^{a}	11.00 ± 1.00^{a}	12.00 ± 1.00^{a}	25.33 ± 1.15^{b}

*Values represent mean ± standard deviation of three independent experiments. A different English lower case superscript letter in column represents a significantly different value in statistics using Duncan's test with a confidential level of 95% between each column.

KPS-01. The agar well diffusion method used in this test showed a useful way for selecting probiotic isolate that possessing the ability to inhibit with harmful bacteria. Rodríguez et al. (2019) revealed that LAB display a wide range of antimicrobial activities. However, some strains of LAB are known to produce bioactive molecules such as ethanol, fatty acids, hydrogen peroxide, diacetyl, reuterin and reutericyclin. Many probiotic strains produce bacteriocins and bacteriocin-like molecules that display antibacterial activity. All lactobacilli tested inhibited the growth of *E. coli* and *S. aureus* reported by Aljebourya & Mahmouda (2020). Djadouni & Kihal (2012) revealed that *Lactobacillus* spp., displayed a broad inhibitory spectrum against the indicator organisms tested. Probiotic bacteria are living microbial cells that have several beneficial health effects on humans, with most probiotic bacteria being lactobacilli that produce mainly lactic acid, antibacterial bioactive compounds, and exopolysaccharides that have antagonistic potential against the activity of intestinal foodborne pathogens.

3.3 Determination of antioxidant activity using the ABTS^{*+} assay

The antioxidant activity of probiotic bacteria was determined using the ABTS^{*+} radical scavenging assay. *L. reuteri* KUKPS6103 had the highest antioxidant activity of radical ABTS^{*+}, while *L. rhamnosus* KUKPS6007 had the lowest antioxidant activity, as presented in Table 2. There were no significant differences in antioxidant activity among the probiotic products. Lactobacilli strains are regarded as probiotic bacteria due to their ability to improve the health of hosts (Tang et al., 2016). Accumulated evidence has suggested that some Lactobacilli strains exert antioxidant activity benefiting host health after they have colonized and multiplied in the human gastrointestinal tract (Kanno et al., 2012; Ren et al., 2014). Lactobacilli strains must remain alive while subjected to digestive juice and secretions, to transfer antioxidant activity to the host (Bao et al., 2012). The ABTS^{*+}-reducing activity assay, which investigated the capacity of antioxidant substances to scavenge the ABTS produced by reacting a strong oxidizing agent ($KMnO_4$ or $K_2S_2O_8$) with the ABTS salt, was measured in this test. The longwave absorption spectrum is used to quantify the decrease in salt, was measured in this test. The longwave absorption spectrum is used to quantify the decrease in the blue-green ABTS^{*+} radical-colored solution by a hydrogen-donating antioxidant (Apak et al., 2013).

The antioxidant capacities of probiotic bacteria have been revealed in several experimental studies. The antioxidant activity has been tested using a variety of methodologies, and the results have been presented in different ways, making comparisons challenging. Probiotic bacteria may express antioxidative activity in various ways, and thus, it is usually very difficult to differentiate just one mechanism or compound responsible for the antioxidative activity. In the present work, 3 probiotic bacteria were screened for their antioxidant activity, and the strains displayed radical scavenging activity. The probiotic bacteria did not differ much in their relative amounts of antioxidant activity using ABTS^{*+} radical scavenging activity, although most antioxidant activity was produced by *L. reuteri* KUKPS6103.

3.4 Determination of antioxidant activities using the DPPH assay

The antioxidant activities of probiotic bacteria are presented in Table 3. A primary antioxidant directly scavenges free radicals, whereas a secondary antioxidant prevents the generation of free radicals via the Fenton reaction (Oh et al., 2013). The DPPH assay is a free radical scavenging activity method that is stable at room temperature and is commonly used for the determination of the antioxidant capacity of hydrophilic molecules. *L. rhamnosus* KUKPS6007 presented a significant higher antioxidant activity compared with *L. reuteri* KUKPS6103 for the DPPH method. In contrast, for the hydrophilic and lipophilic antioxidant activity test by the ABTS method (Krunić & Rakin, 2022), *L. reuteri* KUKPS6103 showed higher antioxidant activity than *L. rhamnosus* KUKPS6007 because *L. reuteri* KUKPS6103 could produce more lipophilic antioxidant molecules than *L. rhamnosus* KUKPS6007. The cell-free supernatant of *Lactobacillus* spp. is a well-known good source of antioxidant substances with distinct mechanisms in radical antioxidant responses considering the variances in scavenging capacities. *L. rhamnosus* KUKPS6007 was demonstrated to express a high capacity for DPPH radical scavenging (Xing et al., 2015).

3.5 Cholesterol removal by probiotic bacteria

Hypercholesterolemia (high blood cholesterol levels) is thought to be a major risk factor for coronary heart disorder. Thus, it is critical to reduce serum cholesterol levels to avoid such disorders. The reduction in serum cholesterol in the probiotic strains was tested *in vitro* in the presence of oxgall after 24 h at 37 °C. All three probiotic bacteria and the mixed culture had different capacities to reduce cholesterol from the medium in the range of 2.66-20.22% for 6 h, 7.33-34.06% for

Table 2. Antioxidant activity of probiotic bacteria using ABTS^{*+} scavenging assay.

Probiotic bacteria	Antioxidant activity (mg Trolox mL^{-1} extract)
L. reuteri KUKPS6103	1.564 ± 0.06^{a*}
L. rhamnosus KUKPS6007	1.514 ± 0.40^{a}
L. paracasei KUKPS6201	1.553 ± 0.02^{a}
Mixed culture	1.516 ± 0.48^{a}

*Values represent mean ± standard deviation of three independent experiments. Different lowercase superscripts represent a significant difference using Duncan's Test with a confidence level of 95%.

Table 3. Antioxidant activity of probiotic bacteria using DPPH scavenging assay.

Probiotic bacteria	Antioxidant activity (mg Gallic acid mL^{-1} extract)
L. reuteri KUKPS6103	0.033 ± 0.009^{a*}
L. rhamnosus KUKPS6007	0.096 ± 0.003^{b}
L. paracasei KUKPS6201	0.084 ± 0.005^{b}
Mixed culture	0.085 ± 0.005^{b}

*Values represent mean ± standard deviation of three independent experiments. Different lowercase superscripts represent a significant difference using Duncan's test with a confidence level of 95%.

Table 4. Growth of probiotic bacteria in various concentrations of honey.

Probiotic bacteria	Honey concentration (g L^{-1})				
	100	200	300	400	500
L. reuteri KUKPS6103	7.52 ± 0.04^{b*}	7.83 ± 0.07^{c}	7.83 ± 0.04^{c}	6.98 ± 0.17^{a}	7.14 ± 0.09^{a}
L. rhamnosus KUKPS6007	8.13 ± 0.19^{a}	7.73 ± 0.40^{a}	7.94 ± 0.12^{a}	8.19 ± 0.20^{a}	7.93 ± 0.07^{a}
L. paracasei KUKPS6201	7.75 ± 0.04^{b}	7.40 ± 0.17^{a,b}	7.79 ± 0.10^{b}	7.55 ± 0.13$^{a, b}$	7.16 ± 0.27^{a}

*Values represent mean ± standard deviation of three independent experiments. Different lowercase superscripts represent a significant difference using Duncan's test with a confidence level of 95%.

12 h and 33.9-78.99% for 24 h (Figure 2). The strain *L. rhamnosus* KUKPS6007 had a superior ability (78.99%) to reduce cholesterol from the medium and was better than the other probiotic bacteria. The lowest value of cholesterol assimilation was for the *L. reuteri* KUKPS6103 isolate. For several strains of LAB, the capacity to reduce cholesterol levels *in vitro* in model culture condition (Miremadi et al., 2014; Nagpal et al., 2012). Future investigation is necessary to assess the mechanisms involved in cholesterol removal by probiotic bacteria in the present study.

In the current study, all the investigated bacteria had some capacity to remove cholesterol from MRS broth with oxgall. Nonetheless, the degree of cholesterol reduction varied by microbial strain, with cholesterol removal involving microbial growth and the most rapid cholesterol removal occurring during the lag phase and the maximum cholesterol removal occurring after 24 h (Wang et al., 2012).

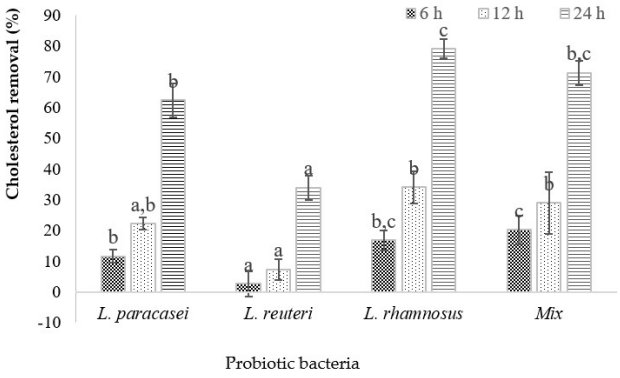

Figure 2. Cholesterol removal by probiotic bacteria and mixed culture. Different lowercase represents a significant difference using Duncan's test with a confidence level of 95%.

3.6 Growth of probiotic bacteria in various concentration of honey

Honey is a bee-derived natural food and provides many nutrients, as it is rich in minerals, carbohydrates, organic acids, phenolic acids, flavonoids, vitamins, enzymes and other proteins. Honey has been shown to be useful in the treatment of a variety of ailments, including gastrointestinal symptoms, wounds, and burns, as well as stomach protection against acute and chronic gastric lesions (Karlıdağ et al., 2021). Based on such information, the present study focused on selecting a new honey product for the development of a synbiotic product.

Although all the probiotic bacteria grew well in the various ratios of diluted honey, 500 g L^{-1} diluted honey was used in this research because of its flavor and taste. The honey was used for prebiotic activity in this experiment because of the high level of beneficial prebiotic activity associated with its honey oligo- and polysaccharides in relation to human intestinal biota. Any antagonistic action of the bacteria on human pathogens in fresh honey would make honey an attractive source of components for new prebiotic, probiotic and synbiotic supplements for humans. Thus, in this experiment, 500 g L^{-1} diluted honey was used to make the synbiotic product because of the flavor and palatability of the product. All of probiotic bacteria produced turbidity, which was an indication of the growth rate in the diluted honey (Table 4).

3.7 Prebiotic activity

The enhanced activity indicated that stingless bee honey and commercial inulin could support the growth of all probiotic bacteria (Table 5). This result was consistent with (Pangsri et al., 2015), who reported that the enhancing activity of defatted copra meal hydrolysate was high and suggested that this novel prebiotic candidate had the ability to promote probiotic bacteria. Titapoka et al. (2008) revealed that the copra meal hydrolysate had an enhancing activity of 2.15 log CFU mL^{-1} on the growth of *L. reuteri* KUB-AC5. The present study also indicated that the enhancing activity of stingless bee honey was similar to that of inulin. Notably, commercial inulin is a very expensive prebiotic source. The honey was capable of inhibiting the assembly of pathogen biofilms, suppressing adhesion activity, and reacting to full-grown biofilm. However, it didn't influence *E. coli* mature biofilm inhibition or metabolism. The LAB probiotics which comprise *L. plantarum*, *L. casei*, *L. rhamnosus, L. gasseri*, and *L. acidophilus* were shown to grow faster in honey with prebiotic potential (Fratianni et al., 2021). Therefore, stingless bee honey could be economically suitable for use as a prebiotic on an industrial scale.

3.8 Carbohydrate profile

HPLC was used to identify the carbohydrate profile of the honey sample based on its retention times for sucrose, glucose and fructose (Figure 3). Xylose was only slightly detected. Honey is mostly comprised of the monosaccharides: glucose and fructose, which make up approximately 55 to 75% of the total sugar content. There is also a complicated variety of minor carbohydrates (10-25%), mostly disaccharides and trisaccharides (Pita-Calvo et al., 2017). Although numerous attempts have been undertaken to examine the composition of honey, some of the minor carbohydrate components' identities are uncertain

Table 5. Enhancing activities of prebiotic substances.

Probiotic bacteria	Honey (g L^{-1})		Inulin (g L^{-1})	
	20	50	20	50
Enhancing activity (%)				
L. reuteri KUKPS6103	3.82	3.44	11.54	4.30
L. rhamnosus KUKPS6007	2.27	0.96	3.46	1.91
L. paracasei KUKPS6201	0.14	4.11	1.58	0.54
Mixed culture	7.39	6.28	9.20	5.19
Enhancing activity (log CFU mL^{-1})				
L. reuteri KUKPS6103	0.21	0.02	0.20	0.01
L. rhamnosus KUKPS6007	0.15	0.64	0.17	0.04
L. paracasei KUKPS6201	0.21	0.33	0.19	0.54
Mixed culture	0.11	0.04	0.01	0.08

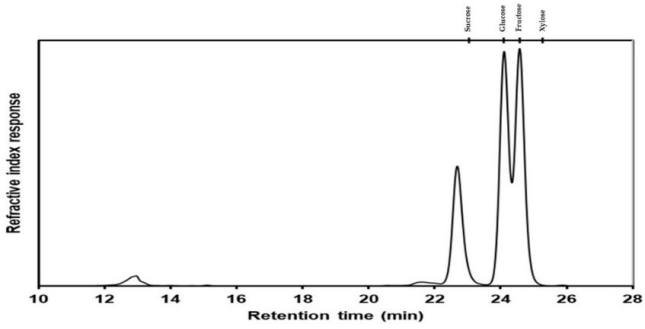

Figure 3. Analytical HPLC chromatogram of stingless bee honey, showing fraction divisions.

Table 6. Short-chain fatty acid production of stingless bee honey.

Treatment	Concentration (μg mL^{-1})		
	Acetic acid	Propionic acid	Butyric acid
Supernatant extracted from MRS broth	21.16 ± 0.13*	33.63 ± 0.10	4.89 ± 0.04
Supernatant extracted from MRS broth supplemented with honey	25.53 ± 0.11	39.73 ± 0.13	6.33 ± 0.11

*Values represent mean ± standard deviation of three independent experiments. Different lowercase superscripts represent a significant difference using Duncan's Test with a confidence level of 95%.

(Zhang et al., 2016). Certain honey components have antioxidant properties that are regarded to be good for people's health (Dezmirean et al., 2012), and honey's antimicrobial properties have been discovered in a number of investigations (He et al., 2012). More recently, honey increased the establishment of commercial *Bifidobacterium* strains in pure culture in a similar way to other commercial prebiotic oligosaccharides (fructooligosaccharides, galactooligosaccharides, and inulin) (Swears & Manley-Harris, 2021). In addition, honey's high levels of glucose and fructose, which are metabolized in the gastrointestinal tract, can promote bacterial growth in *in vitro* conditions. Ultimately, such molecules must be extracted to assess the prebiotic effects of honey oligosaccharides. Due to the oligosaccharides and low molecular weight polysaccharides attached by the β-glycosidic linkages, the prebiotic properties of honey are reported in the earlier research (Mohan et al., 2017). The possible prebiotic characteristics of honey have been investigated in various regions of the world (Mustar & Ibrahim, 2022).

Prebiotics have been reported to display various health benefits, including relieving constipation, reducing the danger of cardiovascular diseases, boosting immunity, helping to reduce cholesterol, promoting the production of bacteriocin and reinforcing gut health (Slavin, 2013). It has been suggested that *Bifidobacterium* populations in the GIT can be increased by eating foods such as natural honey that are high in prebiotics (Ajibola et al., 2012). Prebiotics are substances that help healthy and beneficial bacteria grow rapidly and perform better biologically. Honey consumption is beneficial to human digestion due to the oligosaccharides included in honey (Ajibola et al., 2012; Davani-Davari et al., 2019).

Long regarded as a high-value functional food, stingless bee honey's medicinal efficacy has remained unknown due to a lack of attribution to specific bioactive components. Aside from identifying the potential therapeutic components of stingless bee honey, the rapidly expanding consumer demand for products produced from stingless bee honey has emphasized the need for food standards to allow the authentication and provenance of such products to be established (Sousa et al., 2016).

3.9 Evaluation of short-chain fatty acids

The production of SCFAs (acetic, propionic and butyric acid) while fermenting stingless bee honey by probiotic bacteria was calculated and compared with MRS media. The large production of propionic acid was recorded in MRS supplemented with stingless bee honey (39.73 ± 0.13 μg mL^{-1}) and MRS (33.63 ± 0.10 μg mL^{-1}) (Table 6). Similarly, the production of acetic acid was the highest in sample 2 (MRS supplemented with honey) (25.53 ± 0.11 μg mL^{-1}), followed by MRS media (21.16 ± 0.13 μg mL^{-1}). The lowest amount of SCFA production in all of the samples was butyric acid. However, MRS supplemented with honey had the highest amount (6.33 ± 0.11 μg mL^{-1}) of butyric acid compared to MRS media (4.89 ± 0.04 μg mL^{-1}). Short-chain fatty acids are manufactured by the gut microbiota as end products of dietary fiber that are not degraded by human gastrointestinal enzymes

Table 7. Survival rates (%) and viable cell counts (log CFU g^{-1}) of mixed culture of probiotic bacteria in synbiotic prototype products within 8 weeks of storage (4 °C).

Time (week)	Product 1: Rice grains		Product 2: Honey		Product 3: Rice grains & honey	
	Log cell number	Survival rate	Log cell number	Survival rate	Log cell number	Survival rate
0	9.08 ± 0.06*	100.00	9.47 ± 0.26	100.00	8.82 ± 0.14	100.00
1	8.56 ± 0.10	94.27	9.79 ± 0.18	103.38	8.74 ± 0.08	99.09
2	7.91 ± 0.11	87.11	9.13 ± 0.12	96.41	8.59 ± 0.09	97.39
3	7.83 ± 0.04	86.23	8.69 ± 0.01	91.76	8.72 ± 0.18	98.87
4	7.19 ± 0.06	79.19	8.98 ± 0.08	94.83	8.29 ± 0.05	93.99
5	7.82 ± 0.01	86.12	6.50 ± 0.03	68.64	8.47 ± 0.02	96.03
6	7.27 ± 0.05	80.07	5.10 ± 0.06	53.85	8.28 ± 0.03	93.88
7	7.48 ± 0.01	82.38	3.89 ± 0.07	41.08	8.13 ± 0.03	92.18
8	7.63 ± 0.15	84.03	0.0 ± 0.0	0.00	7.96 ± 0.06	90.25

*Values represent mean ± standard deviation of three independent experiments. Different lowercase superscripts represent a significant difference using Duncan's test with a confidence level of 95%.

with the anaerobic condition of the colon (Fernando et al., 2018). Strong evidence suggests that SCFAs production is beneficial for human health. Gut microbiota can also utilize protein as a source substrate for SCFAs production during amino acid metabolism to produce isobutyrate and isovalerate (Yao et al., 2016). All probiotic strain used in current study exhibited the production of short-chain fatty acids.

3.10 Synbiotic production

To keep the high viability of the probiotics during the storage is a basic fact or requirement for probiotic products. At the time of consumption, the viability of a probiotic product is critically about 10^6-10^7 CFU mL^{-1} (Plessas et al., 2012). The present study showed that the viable cells of probiotic bacteria remained higher than 1 x 10^8 CFU g^{-1} during 60 days of storage of the synbiotic product (Table 7). However, at the same time, the cell count in Product 2 decreased sharply after storage for 4 weeks. In this experiment, probiotic bacteria were studied to assess the appropriateness of utilizing honey and a rice cultivar as a synbiotic product. As a carbon-energy source, all of the bacteria studied digested glucose and fructose, with glucose taking priority. All the probiotic bacteria survived well in the first 2 weeks of storage at 4 °C. In contrast, the viability of the product from Product 3 (the synbiotic product) remained at 1 x 10^8 CFU g^{-1} within storage for 8 weeks at 4 °C. Thus, the product for Product 3 was the best among the three probiotic products based on its good characteristics and its ability to maintain a stable viability count. The probiotic counts in the synbiotic samples were above the therapeutic minimum level (1 x 10^6 – 1 x 10^7 CFU g^{-1}) at the end of the storage period. The probiotic cells of all synbiotic product samples increased from Day 0 to Day 4 of storage, with a slight decrease thereafter. The synbiotic product samples were not contaminated with *E. coli*, yeast or mold after a shelf life of 60 days. No coliform bacteria were found in the synbiotic product sample during the entire storage period (contamination must be lower than 1 x 10^1 – 1 x 10^2 CFU g^{-1}), and thus, it was acceptable. The mixture of probiotics and prebiotics is well-known as synbiotics to enhance the probiotic cells viability and help their growth in the human gastrointestinal tract (Peredo et al., 2016). Therefore, the synbiotic products from this experiment could be safely included in functional foods for human consumption.

Table 8. Nutritional profile of synbiotic product consisting of pigmented rice and stingless bee honey.

Test items	Unit per 100 g of product
Calories	364.26 kcal
Total fat	2.46 g
Saturated fat	0.72 g
Trans fat	Not detected
Cholesterol	Not detected
Sodium	13.71 mg
Carbohydrate	78.21 g
Dietary fiber	4.11 g
Sugar	12.04 g
Includes added sugars	1.00 g
Proteins	7.32 g
Calcium	21.50 mg
Iron	1.79 mg
Potassium	263.78 mg

3.11 Analysis of food nutrition

Functional food products contain probiotics, prebiotics, vitamin, and minerals. These are discovered in such products as fermented milk and dairy products, sports drinks, baby foods, and chewing gum (Gil-Chávez et al., 2013). Prebiotics, non-digestible food ingredients, affect the host microorganisms by selectively energizing the growth, activity, or a limited number of microorganisms in the gastrointestinal tract, thus improving the host microorganism's health (Slavin, 2013). The nutritional profile of the synbiotic enhanced with the pigmented rice cultivars and the stingless bee honey was analyzed, and it was revealed that 100 g of products contained the following: calories (kcal) 364.26 g, saturated fat 0.72 g, total sugar 12.04 g, sodium 13.71 mg, carbohydrates (including fiber) 78.21 g, dietary fiber 4.11 g, protein 7.32 g, calcium 21.50 mg, total fat 2.46 g, iron 1.79 mg and potassium 263.78 mg (Table 8). The synbiotic supplement had calories, carbohydrates, dietary fiber and protein that give a high amount of energy for human daily consumption, while the amounts of saturated fat, potassium, iron and sodium were low, and it had no cholesterol. Thus, the

synbiotic product is promising for development as a high-value therapeutic product based on pigmented rice cultivars and honey. As many new reports and conference announcements target to functional foods and nutraceuticals in the food industry, interest in functional food is high in the United States. In addition, US consumers are immersed in thought with health issues, such as high blood cholesterol, cancer, and desire foods that alleviate disease (Tripathi & Giri, 2014).

4 Conclusion

Three probiotics exhibited cholesterol removal activity, and this property influenced the protection against cardiovascular disease. Moreover, they could produce antioxidant substances to reduce oxidative stress in the gut. Microbial safety was investigated in the present research work and indicated that the synbiotic product could be used as a therapeutic or functional food instead of an antibiotic. The current study stated that the synbiotic product may have a positive effect on the viability of probiotic strains in addition to the nutritional and functional value of tested products. Furthermore, in all products evaluated, refrigerated storage enhanced both the survival rates of the probiotic bacteria during storage and their survival throughout gastrointestinal transit, extending the shelf-life of the synbiotic product. This product is prone to develop high-value health products, however, the production process in industrial scale should be investigated. The studies of the quality of nutritional values, genetic stability, physico-chemical properties and long-term shelf-life should be further performed.

Acknowledgements

This research is supported by the National Research Council of Thailand (NRCT): NRCT5- RGJ63002 (Sub-Code NRCT-RGJ63002-046) under the Royal Golden Jubilee-RGJ-PhD Scholarship Program. The Kasetsart University Research and Development Institute (KURDI), Bangkok, Thailand, provided English editing assistance.

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