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Co-Cultivation of Yoghurt Bacteria with Probiotics Increased Melatonin Content and Enhanced the Antioxidant Activity of Soy Milk Yoghurt

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SUMMARY

Research background. Functional foods that improve sleep quality are attracting increasing attention. Melatonin is a key component that regulates circadian rhythms in humans. Soy milk yoghurt contains melatonin and antioxidants and has beneficial health properties. Many authors have investigated soy milk yoghurt produced by probiotic bacteria. This is the first study to explore the effect of using yoghurt bacteria co-cultivated with probiotics to improve melatonin content and antioxidant activity. This study investigates the melatonin, serotonin and tryptophan contents, antioxidant activity, physical and sensory properties of soy milk yoghurt bacteria co-cultured with different probiotics.

Experimental approach. Soy milk was fermented with four combinations of yoghurt bacteria and probiotics, namely *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (control, SB-YC), *Bifidobacterium lactis*, *Lactobacillus acidophilus*, *L. bulgaricus* and *S. thermophilus* (SB-BY), *B. lactis*, *L. acidophilus* and *S. thermophilus* (SB-BT) and *L. acidophilus*, *L. bulgaricus* and *S. thermophilus* (SB-LA). Melatonin, serotonin and tryptophan content in yoghurt samples were determined using liquid chromatograph coupled with a mass spectrometer (LC-MS/MS). Apart from that, antioxidant activity and quality characteristics including syneresis, texture profile, colour and sensory properties were evaluated.

Results and conclusions. The highest melatonin mass fractions were found in soy milk yogurt fermented with SB-BY (21.20 ng/g) and SB-YC (23.51 ng/g), while the highest tryptophan mass fraction was found in SB-LA (397.18 ng/g). Fermentation with different bacterial culture combinations resulted in various antioxidant activities. The yoghurt fermented with SB-LA had the strongest antioxidant activity, as indicated by DPPH IC_{50} (10.69 mg/mL), ABTS IC_{50} (0.51 mg/mL), and FRAP expressed as $FeSO_4$ (2577.86 μg/g) assays. The addition of a mixture of yoghurt bacteria and probiotics improved the colour values (*L**, *a** and *b**), syneresis and texture profiles of the soy milk yoghurt. Sensory evaluation showed that yoghurt fermented with *S. thermophilus, L. acidophilus* and *B. lactis* received a favourable overall liking score. The successful co-culture of probiotics (*B. lactis* and *L. acidophilus*) with yoghurt bacteria produced soy milk yoghurt enriched with melatonin, tryptophan and antioxidants while maintaining acceptable quality characteristics.

Novelty and scientific contribution. The co-cultivation of yoghurt bacteria and probiotics (*Bifidobacterium lactis, Lactobacillus acidophilus*; SB-BY) or *L. acidophilus* (SB-LA) improved melatonin production, antioxidant activity and the overall yoghurt quality and provided valuable insights for the development of functional foods that can promote sleep and health.

Keywords: bifidobacteria; *Lactobacillus acidophilus*; plant-based milk; prebiotic; serotonin

INTRODUCTION

Growing consumer awareness of the health benefits of functional foods is the main reason for the high demand for plant-based food products. The launch of plant-based

food products by startups and leading manufacturers has also accelerated. The soy milk market was estimated at USD 16.01 billion in 2024 and is projected to reach USD 23.59 billion by 2033, representing a significant growth trend for the soy-based dairy industry (*1*). Plant-based dairy products have become widely accepted, while Asian consumers have traditionally consumed soy milk for generations. However, the development of protein functionality in products requires further investigation (*2*). The potential of soybean (*Glycine max* (L.) Merrill) to reduce the risk of chronic diseases such as cancer, cardiovascular disease (CVD), menopausal symptoms and hyperlipidaemia has been the subject of extensive research (*3*). Previous studies have shown that soybean seeds contain relatively high mass fractions of melatonin (8.35 ng/g) and tryptophan compared to other food components (*4*).

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neurotransmitter produced by the pineal gland located in the centre of the cerebrum and is involved in regulating sleep cycle. Melatonin is synthesized from tryptophan *via* serotonin. During the day, the pineal gland produces serotonin, which stimulates awakening and at night it produces melatonin, which induces sleepiness (*5*). Indole compounds like tryptophan, serotonin and indole-3-acetic acid (IAA) have structural similarities to melatonin, a biological indoleamine. It is now recognized that melatonin functions as a biological regulator of mood, sleep cycles, immune system stimulation, seasonal reproductive physiology, retinal physiology, and heart rhythm (*6*,*7*). In mammals, melatonin is secreted by the pineal gland at night and then enters the cerebrospinal fluid and bloodstream. Melatonin levels drop rapidly during the day (*8*). It also has powerful antioxidant properties by destroying the chemical bond and increasing the activity of antioxidant enzymes (*9*). In plants, melatonin helps to reduce environmental stress. However, studies on the melatonin content in fermented foods and the microbial synthesis of melatonin are limited. The mechanisms of melatonin synthesis in microorganisms are similar to those in plants, with tryptophan serving as a precursor for synthesis through the important intermediate, serotonin (*10*).

Plant-based food products are now attracting significant attention as sustainable future options. Among these, soybeans stand out due to their high nutritional value. Soy milk yoghurt can be produced as a functional food enriched with melatonin. Cow's milk yoghurt is often recommended as a bedtime snack for its ease of consumption and tryptophan content which the body converts into melatonin—a hormone that promotes better sleep. Dairy products like milk, cheese and yoghurt are rich sources of tryptophan (*11*). Tillisch *et al.* (*12*) reported that consuming foods containing bifidobacteria improved sleep quality by increasing serotonin secretion. Traditionally, yoghurt is made by fermenting milk with *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. The development of probiotic yoghurt has gained attraction due to its added health benefits. Probiotic yoghurt is defined as yogurt containing live microorganisms that provide health benefits when consumed in adequate amounts (*13*). The production process often involves co-culturing probiotic strains with traditional yoghurt starter bacteria to promote probiotic growth during fermentation. Probiotic strains are selected for their safety, nutritional benefits, health-promoting properties and their interactions with other bacteria to improve performance, yoghurt quality and stability during storage. Common probiotic strains used in yoghurt include various species of *Lactobacillus* and *Bifidobacterium*. Strains such as *B. breve*, *B. lactis*, *B. longum*, *B. animalis*, *L. acidophilus*, *L. rhamnosus* and *L. johnsonii* are frequently reported (*14*).

Recent studies have highlighted the antioxidant potential of these probiotics, as they are able to scavenge free radicals, reduce lipid peroxidation and enhance the body's antioxidant defenses (*15*). *B. animalis* ssp. *lactis* produces antioxidant metabolites such as glutathione and exopolysaccharides, which neutralize oxidative agents (*16*), while *L. acidophilus* increases the activity of antioxidant enzymes like superoxide dismutase and catalase and protects cells from oxidative stress (*17*).

Previous studies have shown that the combination of starter cultures with different probiotic strains increases the content of bioactive compounds and antioxidant properties of yoghurt. However, the effects of mixed yoghurt bacteria and probiotics on melatonin, serotonin and tryptophan content, as well as the antioxidant properties of yogurt during fermentation, remain unexplored. This study investigates the effect of co-culturing yoghurt bacteria with different probiotic strains on melatonin and tryptophan mass fractions, antioxidant capacity and specific physical properties of yoghurt during fermentation. The development of a functional soy milk yoghurt enriched with melatonin and tryptophan is an interesting new product.

MATERIALS AND METHODS

Raw materials and chemicals

Soybean flour was purchased from Bangyai Supply Ltd. (Nonthaburi Province, Thailand). Freeze-dried direct vat set yoghurt culture and the following probiotics: (*i*) YC-380 (*Streptococcus thermophilus*, *Lactobacillus delbruekii* ssp. *bulgaricus)*, (*ii*) ABY-3 (*S. thermophilus*, *L. delbruekii* ssp. *bulgaricus, Bifidobacterium animalis* ssp. *lactis* and *Lactobacillus acidophilus*), (*iii*) ABT-5 (*S. thermophilus*, *B. lactis* and *L. acidophilus*) and (*iv*) LA-5 (*L. acidophilus*) were obtained from Chr. Hansen Ltd. (Hørsholm, Denmark). The other analytical and HPLC grade chemicals were standards of melatonin, serotonin and tryptophan (Sigma-Aldrich Chemical Co., Merck, St. Louis, MO, USA), while acetonitrile, methanol and ethanol were obtained from LabScan (Dublin, Ireland) and 98 % formic acid was purchased from Loba Chemie Pvt. Ltd. (Mumbai, India).

Preparation of starter cultures

The starter cultures were prepared following Felix da Silva *et al.* (*18*) with slight modifications. A lyophilized culture of each bacterium (0.2 % *m*/*V*) was added to the soy milk and incubated in an incubator (model 30-750; Memmert, Schwabach, Germany) at 43 °C for 6 h. Each starter culture contained a viable bacterial cell count ranging from 8.2 to 8.5 log CFU/mL. The starter cultures were used for soy milk fermentation to prepare the yoghurt.

Preparation of yoghurt

The soy milk for yoghurt fermentation was prepared by reconstituting soybean flour with distilled water to obtain 12 % total solid. The soy milk was homogenized using a hand blender (BH5521HB 950w-50/60 Hz; Tarman Dis Ticaret A.S., Istanbul, Turkey) and preheated at 50 °C for 5 min, followed by the addition of sugar (2 % *m*/*V*) and filtering, pasteurization at (72±2) °C for 15 min and then cooling to (42±1) °C before adding the starter culture. For yoghurt fermentation, soy milk at (42±1) °C was inoculated with 4 % (*m*/*V*) of each starter culture. All inoculated soy milk samples were transferred into sterile plastic cups and incubated at 43 °C in an incubator (model 30-750; Memmert, Schwabach, Germany). The obtained soy milk yoghurt samples were labelled SB-YC (control), SB-BY, SB-BT and SB-LA, corresponding to fermentation by YC-380, ABY-3, ABT-5 and a 1:1 ratio of YC-380:LA-5 strains (Chr. Hansen Ltd.), respectively. To monitor the change of soy milk during fermentation and to obtain the most suitable fermentation time, yoghurt samples were randomly taken from the incubator at 0, 2, 4, 6 and 8 h to analyze physical properties (pH, titratable acidity, texture profile, syneresis and colour). Portions of the yoghurt from each treatment (co-culture) and incubation time were freeze-dried (Beta 1-8 LSCbasic; Martin Christ Gefriertrocknungsanlagen, Osterode, Germany) to achieve a water activity (a_w) of 0.3–0.5. Melatonin and its derivatives, antioxidant activity and Fourier transform infrared (FTIR) spectroscopy measurements of the freeze-dried samples were then analyzed.

Physical analysis

The pH value of soy milk yoghurt was measured using a pH meter (FEP20-FiveEasy; Mettler Toledo, Greifensee, Switzerland). Titratable acidity (TA) of yoghurt was determined by titrating the lactic acid with 0.1 M NaOH using phenolphthalein as an indicator (*18*). Colour was analyzed using a chroma meter (CR-400; Konica Minolta, Tokyo, Japan) and reported as Hunter System *L**, *a** and *b** coordinates (*19*). Soy milk yoghurt syneresis was analyzed following Jeong *et al.* (*20*) with minor changes. A mass of 10 g of yoghurt was centrifuged (Universal 320R; Andreas Hettich, Tuttlingen, Germany) at 478×*g* and 4 °C for 6 min. The clear liquid that separated from the yoghurt was weighed. Syneresis was expressed as the percentage of serum separated by centrifugation (Universal 320R; Andreas Hettich). The texture properties of the yoghurt were analyzed by a texture analyzer (TA-XT plus; Stable Micro Systems, Godalming, Surrey, UK) (*21*). The samples were pressed twice with a cylindrical probe (P/20 diameter 20 mm) to mimic chewing with a velocity of 1 mm/s before the test, 5 mm/s after the test, a test speed of 5 mm/s and distance of 30 mm.

FTIR spectroscopy measurement

Variations in the functional components of the soy milk yoghurt were determined by FTIR spectroscopy and recorded using a Frontier and Spotlight System (Spotlight 200i; PerkinElmer, Waltham, MA, USA). The sample powder was kept at room temperature ((27±2) °C), and portions of 2 mg were compressed into pellets (1–2 mm thick). FTIR spectra were collected in the 4000–400 cm^{-1} range with a spectral resolution of 4 cm^{-1}, averaging 8 scans (*22*).

Determination of melatonin, serotonin and tryptophan

The investigation of free melatonin, tryptophan and serotonin in soy milk yoghurt was modified from the methods of Nontasan *et al.* (*23*). A mass of 2.5 g of an aliquot of freeze-dried soy milk yoghurt was dissolved in 20 mL of 80 % ethanol and sonicated in an ultrasonic bath (LUC-420; Daihan Labtech, Kyonggi-Do, Korea) for 30 min. The blend was then shaken in a shaking incubator (LSI-1005R; Daihan Labtech) at 4 °C for 16 h, followed by centrifugation (Universal 320R; Andreas Hettich) at 2236×*g* and 4 °C for 5 min. The extract was cleansed using a Sep-Pak C18 solid phase extraction (SPE) cartridge (Waters, MA, USA) (*24*).

Melatonin, serotonin and tryptophan were analyzed using a liquid chromatograph coupled with a mass spectrometer – LC-MS/MS (20ADS-8030; Shimadzu Corporation, Kyoto, Japan) operated in electrospray ionization mode (ESI). The stationary phase was a C18 column (XBridge™ 3.5 µm, 2.1 mm×150 mm; Waters, Dublin, Ireland). The mobile phases were 0.45 % formic acid in HPLC grade water (solvent A) and acetonitrile (solvent B). The injection volume was 2 µL. Calibration curves of the endogenous standards were obtained and used to calculate the mass fraction of melatonin (ng/g), tryptophan and serotonin (µg/g) on dry mass basis of yoghurt.

Determination of antioxidant activity

A precisely measured 2.5 g of freeze-dried soy milk yoghurt were liquefied in 20 mL of 80 % ethanol. This mixture was shaken for 16 h at 27 °C in a shaking incubator. The yoghurt samples were centrifuged (Universal 320R; Andreas Hettich) at 1430×*g* for 15 min and filtered through a Whatman No. 1 filter paper. The obtained soy milk yoghurt extract was stored in an amber glass bottle at 2–6 °C before the evaluation of antioxidant activity (*23*).

The DPPH radical scavenging activity of soy milk yoghurt extract was measured (*25*). A 100-µL aliquot of DPPH (0.2 mM) in ethanol was added to 100 µL of soy milk yoghurt extract in a 96-well plate and mixed thoroughly for 1 min using a

microplate reader shaker (SPECTROstar Nano 24V-DC 60w S/N 601-1387; BMG Labtech, Ortenberg, Germany). The mixtures were kept at room temperature for 30 min in the dark. The absorbance was measured at 517 nm. The scavenging activity was reported as IC_{50}, calculated using the concentration inhibition curve by plotting the yoghurt concentration against the corresponding inhibition percentage. A higher radical scavenging activity is indicated by a lower IC_{50} value.

An ABTS solution (7 mM ABTS combined with 2.45 mM dipotassium peroxydisulfate) was used to determine ABTS radical scavenging activity (*25*). A volume of 170 µL aliquot of soy milk yoghurt extract was mixed with 30 µL of the ABTS solution in a 96-well plate, shaken and kept at room temperature for 10 min. The absorbance of the mixture was measured at 734 nm using a microplate reader (SPECTROstar Nano 24V-DC 60w S/N 601-1387; BMG Labtech).

The Fe(III) reducing antioxidant power (FRAP) was evaluated (*26*). Fe(II) sulfate was used as the standard and a calibration curve was plotted. Then, 20 µL of soy milk yoghurt extract were mixed with 900 µL of the FRAP reagent. FRAP was prepared by mixing acetate buffer (pH=3.6), 20 mM $FeCl_3$ and 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl at a ratio of 10:1:1. The mixture (200 µL) was placed in a 96-well plate and kept in the dark at 37 °C for 10 min before the absorbance at 593 nm was measured (SPECTROstar Nano 24V-DC 60w S/N 601-1387; BMG Labtech). Results were expressed as µg of $FeSO_4$ equivalents per g sample (dry basis).

Sensory evaluation

The sensory evaluation of the yoghurt samples was carried out by 50 untrained panellists, comprising 15 men and 35 women aged 20–60. Participants were recruited through a call for voluntary participation and the study protocol was approved by Mahasarakham University (approval number: 065–436/2565). Sensory preferences were rated as appearance, colour, odour, flavour, texture (flexibility) and acceptability using a 9-point hedonic scale with 9=like extremely and 1=dislike extremely. The sensory evaluation was conducted by serving five randomly coded samples with three numbers in a random order. Each panellist was served different samples within 2 days of yoghurt production in a white plastic cup at a temperature not exceeding 10 °C and tested from left to right. The preference of each sample was then rated with a sample code. The panelists rinsed their mouths with water between each sample (*27*).

Statistical analysis

The treatments of all soy milk yoghurt samples were performed in triplicate. The mean values and standard deviation of the results were reported. The results were analyzed by ANOVA for a completely randomized design, with Duncan's multiple comparison test at $p \leq 0.05$ applied to determine significant differences using IBM SPSS Statistics v. 21 (*28*).

RESULTS AND DISCUSSION

pH and titratable acidity of soy milk yoghurt

The changes in the pH values of soy milk yoghurt showed similar patterns in all samples fermented with different yoghurt cultures (**Fig. 1a**). The pH gradually decreased during fermentation from 6.26 to 4.5–4.6 (desired pH) in 6 h for SB--YC, while the yoghurt fermented with YC and probiotics (SB--BY, SB-BT and SB-LA) reached the desired pH in 4 to 5 h.

The titratable acidity (TA) values of the soy milk yoghurt samples are shown in **Fig. 1b**. The TA increased with fermentation time from 0.20 to 0.99 % from 0 to 8 h. SB-BY (0.21–0.99 %) and SB-LA (0.20–0.93 %) had higher TA than the yoghurt fermented by SB-YC (0.23–0.75 %) and SB-BT (0.24–0.74 %). Acidification is crucial for the preservation of food by fermentation, as the metabolic activity of bacteria accumulates acid, lowers the pH and inhibits the growth of spoilage bacteria (*29*). The pH values in this study were consistent with those of Grasso *et al.* (*30*), who reported that the pH values in commercial soy milk yoghurt ranged from 3.99 to 4.56. This pH range could be attributed to the composition of soy milk, the yoghurt bacteria, lactic acid and the ingredients used in yoghurt production. Specific pH ranges influence the effect of

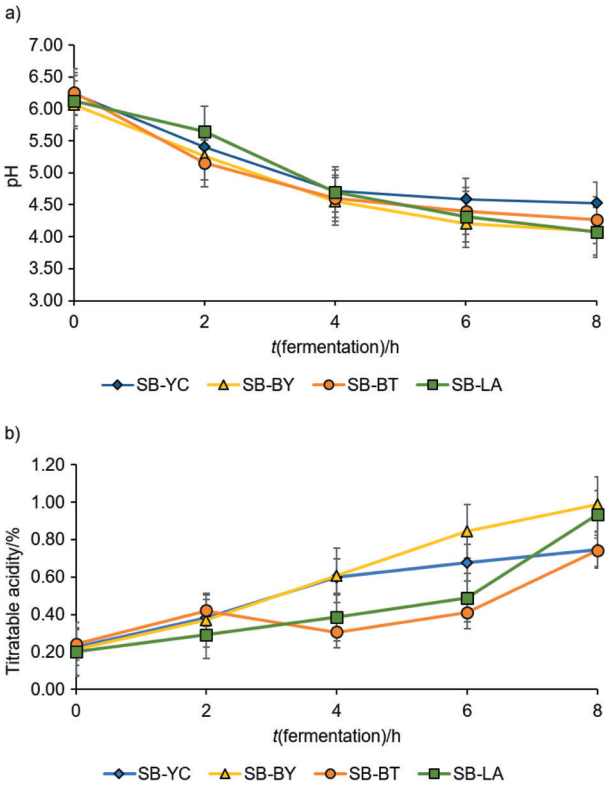

Fig. 1. Change in: a) pH value and b) titratable acidity during fermentation of soy milk yoghurt samples. SB-YC (control) is the soy milk yoghurt fermented by YC-380 (*Streptococcus thermophilus* and *Lactobacillus delbruekii* ssp. *bulgaricus*), SB-BY is the soy milk yoghurt fermented by ABY-3 (*S. thermophilus*, *L. bulgaricus, B. animalis* ssp. *lactis* and *L. acidophilus*), SB-BT is the soy milk yoghurt fermented by ABT-5 (*S. thermophilus, B. lactis* and *L. acidophilus*) and SB-LA is the soy milk yoghurt fermented by LA-5 (*L. acidophilus*) mixed with YC-380 (strain ratio 1:1)

certain gelling agents in soy milk yoghurt (*31*). The TA values of the yoghurt samples were 0.45–0.55 and 0.74–0.99 % at 0 and after 8 h of fermentation, respectively, and were comparable to the results reported by Grasso *et al.* (*30*) for commercial soy milk yogurt. The increase in TA was due to the accumulation of lactic acid and other organic acids produced by the fermentation of the starter culture (*32*).

Mass fraction of melatonin and tryptophan

Melatonin and tryptophan mass fractions in soy milk yoghurt, as determined by LC-MS/MS, are shown in **Table 1**. Yoghurt fermented with different types of bacteria and probiotic cultures gave different melatonin mass fraction. The melatonin mass fraction in soybean flour was 17.7 ng/g. After fermentation for 4 and 8 h, melatonin mass fraction in SB-BY yoghurt was 12.8 and 21.2 ng/g, respectively, while in SB-YC and SB-LA, it was 23.51 and 11.9 ng/g, respectively, after 8 h of fermentation. The melatonin mass fraction found in SB-BY and SB-YC was not significantly different. These results indicate that soy milk yoghurt fermented with yoghurt bacteria and yoghurt bacteria co-cultured with two types of probiotics produced more melatonin than probiotic cultures alone.

The tryptophan mass fraction in soybean flour was 159.1 ng/g. The tryptophan mass fraction in SB-YC and SB-BY yoghurt increased after 4 h (175.3 and 303.1 ng/g, respectively) and then decreased after 8 h of fermentation ($p \leq 0.05$), showing the opposite effect to SB-BT. The tryptophan mass fraction in SB-LA increased with fermentation for 4 and 8 h (292.8 and 396.2 ng/g, respectively). Juhnevica-Radenkova *et al.* (*11*) reported that the availability of tryptophan is an important factor in determining the content of indolic compound products, *i.e.* melatonin. Yoghurt bacteria and probiotics use tryptophan to produce serotonin and then melatonin isomers during malolactic fermentation.

In this study, serotonin was undetectable in soybean flour and soy milk yoghurt, while Nontasan *et al.* (*23*) reported that serotonin mass fraction in soybean germinated under normal and salt stress conditions ranged between 37 and 57 ng/g. The melatonin and tryptophan mass fractions in soy milk yoghurt fermented by YC mixed with *L. acidophilus* and *B. lactis* were higher than in the soy milk yoghurt fermented by SB-BT. Kocadağlı *et al.* (*33*) explained that food samples such as bread and beer fermented with yeast contained relatively high amounts of melatonin compared to other food samples. Low mass fractions of melatonin were found in probiotic yoghurt (0.9 ng/g), while yeast-fermented foods contain more melatonin than bacterium-fermented foods. Yoghurt fermented with *L. delbrueckii* ssp. *bulgaricus* OLL1073R-1 has also been evaluated for its sleep-promoting effects. Self-reported sleep quality improved in the yoghurt group compared to the control group. The yoghurt group also reported improved perceptions of general health and vitality (*34*). This study also showed that storage at 4 °C for 28 days influenced the mass fractions of melatonin, serotonin and tryptophan in soy milk yoghurt (data not shown). By day 21, melatonin was significantly degraded, while tryptophan and serotonin mass fractions remained relatively stable throughout the storage. These results show that fermentation with probiotic cultures effectively preserves tryptophan and serotonin in soy milk yoghurt, but that maintaining melatonin stability during prolonged storage is a challenge. Improving storage conditions through improved packaging or the addition of natural antioxidants could help reduce melatonin degradation. Melatonin mass from 0.3 to 1 mg effectively regulate the sleep-wake cycle, especially in cases of mild insomnia or jet lag. The physiological functions and health effects of melatonin and serotonin are complex and interrelated. Normal endogenous melatonin amounts are usually sufficient for physiological functions, while factors like diet and enzymatic activity significantly influence serotonin availability and its conversion to melatonin. Together, these elements affect sleep regulation and other biological processes (*35*).

Antioxidant activity of soy milk yoghurt

The results for antioxidant activity evaluated by DPPH radical scavenging and ABTS were expressed as IC_{50} values; the lower the value, the higher the antioxidant activity. The DPPH IC_{50} values of SB-BY, SB-BT and SB-LA were 9.03, 13.59 and 10.69 mg/mL, respectively, and significantly higher than the control yoghurt, SB-YC (**Fig. 2a**). The SB-BT and SB-LA

Table 1. Melatonin and tryptophan mass fractions expressed on dry mass basis of soy milk yoghurt samples under different fermentation times compared to soybean flour as control

Treatment	*t*(fermentation)/h					
	0	4	8	0	4	8
	w(melatonin)/(ng/g)			*w*(tryptophan)/(ng/g)		
SB-YC	$(1.3\pm0.2)^{b}$	N.D.	$(23.51\pm0.02)^{aA}$	$(128.7\pm0.6)^{A}$	$(175.3\pm0.8)^{B}$	$(57.5\pm0.1)^{C}$
SB-BY	N.D.	$(12.8\pm1.7)^{b}$	$(21.2\pm0.9)^{aA}$	$(27.7\pm1.0)^{bB}$	$(303.1\pm0.6)^{aA}$	$(76.7\pm0.9)^{bC}$
SB-BT	N.D.	N.D.	N.D.	$(103.7\pm1.0)^{aA}$	$(32.8\pm0.8)^{bC}$	$(101.0\pm0.6)^{aBC}$
SB-LA	N.D.	N.D.	$(11.9\pm0.1)^{B}$	$(41.5\pm0.1)^{cB}$	$(292.8\pm0.2)^{bA}$	$(396.2\pm0.1)^{aA}$

Soybean flour: *w*(melatonin)=17.7 and *w*(tryptophan)=159.1 ng/g. Mean values with different capital letters in superscript within a column and with different lowercase letters in superscript within a row are significantly different ($p \leq 0.05$), N.D.=not detected. SB-YC (control) is the soy milk yoghurt fermented by YC-380 (*Streptococcus thermophilus* and *Lactobacillus delbruekii* ssp. *bulgaricus*), SB-BY is the soy milk yoghurt fermented by ABY-3 (*S. thermophilus*, *L. bulgaricus*, *B. animalis* ssp. *lactis* and *L. acidophilus*), SB-BT is the soy milk yoghurt fermented by ABT-5 (*S. thermophilus*, *B. lactis* and *L. acidophilus*) and SB-LA is the soy milk yoghurt fermented by LA-5 (*L. acidophilus*) mixed with YC-380 (strain ratio 1:1)

samples showed consistently the lowest IC_{50} values (highest antioxidant activity) after 4 to 8 h of fermentation. Differences among formulations were statistically significant ($p \leq 0.05$), particularly at later fermentation stages. Soy milk yoghurts showed higher antioxidant capacity measured by the ABTS assay, while SB-LA had the highest ABTS IC_{50} (0.51 mg/mL) compared to the other yoghurt formulations ($p \leq 0.05$). The SB-LA formulation had the lowest IC_{50} values after 6 to 8 h of fermentation and better antioxidant capacity than the other samples (**Fig. 2b**). The antioxidant properties evaluated by FRAP (**Fig. 2c**) showed that all soy milk yoghurt samples had

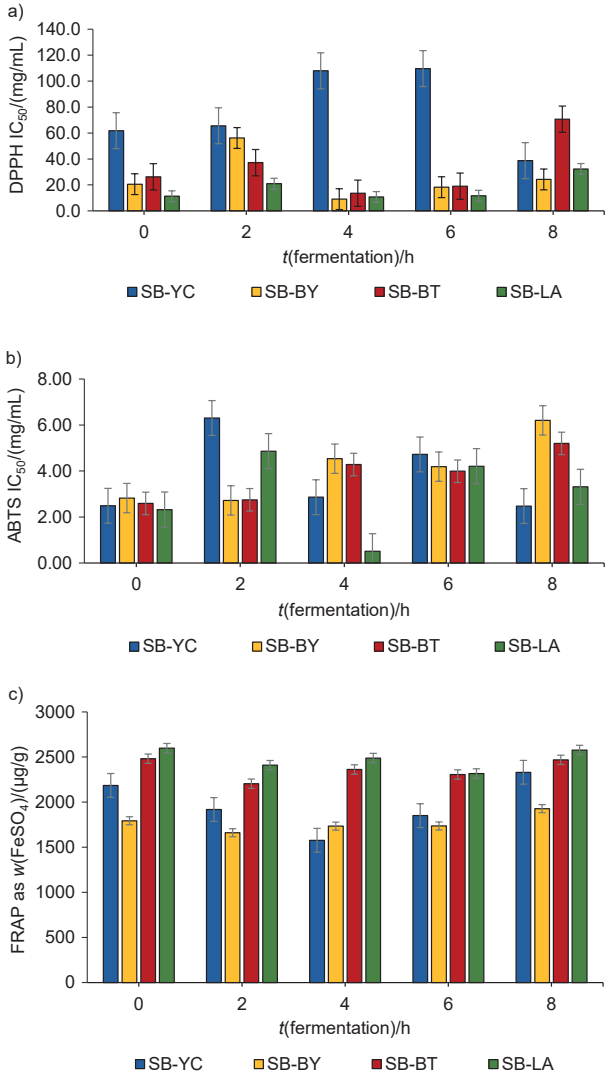

Fig. 2. Antioxidant activity of soy milk yoghurt samples measured by: a) DPPH IC_{50}, b) ABTS IC_{50} and c) FRAP. SB-YC (control) is the soy milk yoghurt fermented by YC-380 (*Streptococcus thermophilus* and *Lactobacillus delbruekii* ssp. *bulgaricus*), SB-BY is the soy milk yoghurt fermented by ABY-3 (*S. thermophilus*, *L. bulgaricus, B. animalis* ssp. *lactis* and *L. acidophilus*), SB-BT is the soy milk yoghurt fermented by ABT-5 (*S. thermophilus, B. lactis* and *L. acidophilus*) and SB-LA is the soy milk yoghurt fermented by LA-5 (*L. acidophilus*) mixed with YC-380 (strain ratio 1:1). Different capital letters indicate significant differences between yoghurt bacteria and probiotic cultures and different lowercase letters indicate significant differences between fermentation times ($p \leq 0.05$)

antioxidant potential, especially the yoghurt fermented with the probiotic *L. acidophillus*. The FRAP values, expressed as $FeSO_4$ equivalents on dry mass basis, of SB-YC, SB-BY, SB-BT and SB-LA were 2331.04, 1928.01, 2468.92 and 2577.86 μg/g, respectively. Yoghurt containing probiotics had higher FRAP values than the control. The FRAP values of all samples initially decreased. At the end of fermentation, the highest FRAP value was observed in SB-LA yoghurt ($p \leq 0.05$).

The results of the three antioxidant activity assays (DPPH, ABTS and FRAP) showed that the addition of probiotic cultures in combination with yoghurt cultures increased the antioxidant activity with the highest activity found in SB-LA yoghurt. During fermentation, the antioxidant activity of soy milk yoghurt improved significantly after 4 and 6 h due to optimal enzymatic activity that broke down complex compounds into bioactive components like phenols and flavonoids. After 8 h of fermentation, the activity decreased or stabilized, but remained higher than in the unfermented samples (0 h). This improvement was influenced by the natural richness of soy milk in isoflavones and phenols and the different enzymatic potential of the different microbial strains (*36*). Our results were consistent with previous studies. The combined use of probiotics with yoghurt starters increased the antioxidant activity during storage compared to day 0 (*37*). Changes in DPPH, Fe(II) ion chelating and FRAP radical scavenging activities were recorded in fermented soybean and almond milk with different starter cultures (*L. rhamnosus* ATCC 7469, *L. plantarum* ATCC 14917, *L. casei* ATCC 393 and *L. acidophilus* ATCC 4356) during 21 days of storage. All samples had a higher percentage of scavenging activity than the corresponding control and the samples prepared in the same treatment without using the starting culture. Almond milk fermented by *L. casei*, *L. acidophilus* and *L. plantarum* had higher scavenging activity than *L. rhamnosus* on the 1st day (*38*). The antioxidant activity in *Lycium barbarum* yoghurt also increased during the first 2 weeks of storage and was significantly higher than in plain yoghurt (*29*).

Colour, syneresis and texture characteristics of soy milk yoghurt

The colour characteristics of soy milk yoghurt are shown in **Table 2**. The soy milk yoghurt samples were yellowish and had significantly different ($p \leq 0.05$) colour values when fermented by other cultures. The highest L^* value (75.2) was observed in SB-BY, while the highest a^* (0.2) and b^* (15.4) values were found in SB-LA. Colour values are essential for qualitative assessment of food quality. The visual assessment of food colour closely corresponds to consumer or taster evaluations and helps to establish standard criteria for the comparison of instrumental measurements (*39*). The lightness (L^*) of yoghurt is related to the particle size of the fat and protein globules. This has an effect on light reflection and scattering ability. The size of these particles is greatly influenced by the choice of processing unit and the processing parameters used. Soy milk yoghurt showed the b^* in yellow and the results were in

Table 2. Physical properties of the soy milk yoghurt samples after 8 h of fermentation

Treatment	Firmness/ N	Consistency/ (N·s)	Cohesiveness/ N	Viscosity index/(N·s)	Syneresis/ %	Colour		
						*L**	*a**	*b**
SB-YC	(24.2±3.4)^{b}	(65.1±8.6)^{b}	(2.5±2.5)^{a}	(0.9±0.4)^{a}	(36.3±0.5)^{c}	(74.3±0.5)^{b}	(-0.4±01)^{a}	(13.8±0.8)^{b}
SB-BY	(40.5±2.6)^{a}	(109.6±1.0)^{a}	(6.5±1.3)^{b}	(2.2±0.2)^{b}	(40.9±1.3)^{d}	(75.2±0.4)^{a}	(-0.01±0.03)^{b}	(12.95±0.01)^{c}
SB-BT	(18.0±1.9)^{c}	(37.3±3.8)^{c}	(4.1±0.2)^{ab}	(2.6±0.2)^{b}	(30.1±0.8)^{a}	(74.1±0.7)^{b}	(0.08±002)^{c}	(12.8±0.4)^{c}
SB-LA	(26.1±0.5)^{b}	(4.2±0.1)^{d}	(0.6±0.1)^{a}	(0.45±0.08)^{a}	(34.2±0.5)^{b}	(71.4±0.4)^{c}	(0.2±0.1)^{d}	(15.4±0.5)^{a}

Mean values with different letters in superscript within a column are significantly different ($p \leq 0.05$). SB-YC (control) is the soy milk yoghurt fermented by YC-380 (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*), SB-BY is the soy milk yoghurt fermented by ABY-3 (*S. thermophilus*, *L. bulgaricus, B. animalis* ssp. *lactis* and *L. acidophilus*), SB-BT is the soy milk yoghurt fermented by ABT-5 (*S. thermophilus, B. lactis* and *L. acidophilus*) and SB-LA is the soy milk yoghurt fermented by LA-5 (*L. acidophilus*) mixed with YC-380 (strain ratio 1:1)

agreement with those of Grasso *et al.* (*30*). The *a** values (red--green axis) were negative for all yoghurts. The soy milk with 100 % extract had the lowest lightness value (*L**=53.4).

Syneresis, the separation of whey protein on the surface of yoghurt, is a significant and noticeable defect that can negatively impact consumer acceptance of the product (*19*). The syneresis properties of soy milk yoghurt samples (**Table 2**) were affected by the yoghurt bacteria in combination with different probiotics. The highest syneresis (40.9 %) was found in SB-BY. The syneresis of soy milk yoghurts ranged between 30.1 and 40.9 %. Cui *et al.* (*40*) reported that syneresis in soy milk yoghurt fermented by yoghurt starters alone (YFL-901, consisting of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) was significantly higher than in yoghurts made using a combination of yoghurt starters and probiotics like *L. rhamnosus* (LGG), *B. animalis* ssp. *lactis* BB-12 and *L. acidophilus* LA-5. Most soy milk yoghurt products had a small increase in syneresis. These results suggested that the addition of probiotics improved the water-holding capacity of the yoghurt and significantly reduced the formation of whey. Probiotics like BB, LA and LGG are known to produce exopolysaccharides, which improve yoghurt texture by interacting with free water in a gel-like structure and thus help reduce syneresis.

The results of texture profile analysis are shown in **Table 2**. YC and YC co-cultured with probiotics had a significantly different texture from the control (SB-YC). The soy milk yoghurt had higher firmness, consistency, cohesiveness and index of viscosity than the control yoghurt. Soy milk yoghurts have lower protein contents and differences in the protein coagulation properties reduce consistency compared to milk yoghurt without added thickener, especially at low pH, which affects the visual appearance (*41*).

FTIR profiles of soy milk yoghurt

FTIR analysis was carried out to evaluate the changes in the chemical composition of soy milk yoghurt during fermentation. The FTIR spectral data in the 4000–400 cm^{-1} range collected from soy milk yoghurt samples and soybean flour (control) are shown in **Fig. 3**. All soy milk yoghurt samples had FTIR spectra at 3300–2800, 1700–1600, 1550–1249 and 550–400 cm^{-1}. Soybean flour as a raw material showed more than 30 peaks, while the number of peaks decreased during yoghurt fermentation and showed similar patterns of FTIR

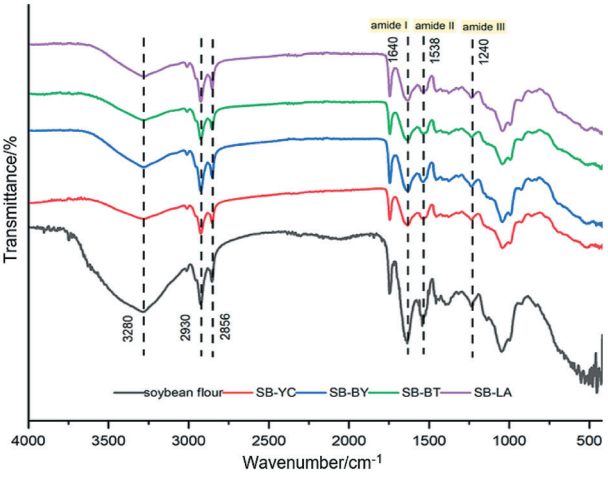

Fig. 3. FTIR spectra of soy milk yoghurt samples after 8 h of fermentation. Soybean flour as a raw material, SB-YC (control) is the soy milk yoghurt fermented by YC-380 (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*), SB-BY is the soy milk yoghurt fermented by ABY-3 (*S. thermophilus*, *L. bulgaricus, B. animalis* ssp. *lactis* and *L. acidophilus*), SB-BT is the soy milk yoghurt fermented by ABT-5 (*S. thermophilus, B. lactis* and *L. acidophilus*) and SB-LA is the soy milk yoghurt fermented by LA-5 (*L. acidophilus*) mixed with YC-380 (strain ratio 1:1)

spectra because the yoghurt bacteria and probiotic cultures used in this study fermented soy milk similarly. The deconvolution of amine bands showed that they consisted of at least four peaks at 3200–2800, 1650, 1538 and 1240 cm^{-1} corresponding to amine bands of the N–H (amino group) stretching vibrations (*42*). The peak at 1640 cm^{-1} was assigned to water (O–H) and amide I band (80 % C=O stretch, 10 % C–N stretch and 10 % N–H bending vibrations) and 1550 cm^{-1} (amide II band, 40 % C–N stretching and 60 % N–H bending vibrations) (*43*). The primary fatty acid peaks were 2960, 2929 and 1740 cm^{-1}. The asymmetric and symmetric CH_2 stretching modes were associated with peaks at 2928 and 2860 cm^{-1}, respectively (*44*). According to Greulich *et al.* (*45*), protein is typically detected by the absorbance of amide I (1600–1700 cm^{-1}), caused by amide stretching of the C=O bond, amide II (1510–1570 cm^{-1}), caused by N–H bond bending vibrations, and amide III (1350–1200 cm^{-1}), caused by the combination of N–H and C–N stretching in the plane. A study of amide I, amide II and amide III found peaks at 1640, 1538 and 1240 cm^{-1}, respectively (**Fig. 3**). The high sensitivity of the amide I band has been used to explain secondary protein structures,

but further deconvolution methods are required to separate the peaks (*46*).

Sensory evaluation of soy milk yoghurt

Sensory evaluation of the soy milk yoghurt samples was carried out by 50 volunteers and the results are shown in **Fig. 4**. The SB-YC, SB-BT and SB-BY samples had higher values for appearance, colour, texture, flavour and acceptability than SB-LA ($p \leq 0.05$). The appearance and colour characteristics of SB-YC, SB-BY and SB-BT were not significantly different ($p > 0.05$) from those of commercial yoghurt. Regarding overall acceptability, SB-YC, SB-BY, SB-BT and SB-LA were not significantly different ($p > 0.05$). The panellists rated the preference of SB-LA lowest compared to the other groups of probiotics, possibly due to its dark colour and low consistency with the results of the lightness (L^*) and consistency values (**Table 2**). These results, especially yoghurt flavour, support those of Tian *et al.* (*47*), who reported the effects of co-fermentation of four probiotics, *L. acidophilus*, *L. plantarum*, *L. rhamnosus* and *L. casei* with conventional yeast on yoghurt flavour. Ketones and aldehydes were the most abundant volatile compounds, with *L. casei* and *L. acidophilus* contributing significantly to the production of secondary volatile metabolites. Electronic nose analysis effectively differentiated yoghurt samples containing various probiotics during refrigerated storage, highlighting different flavour profiles associated with specific probiotic strains.

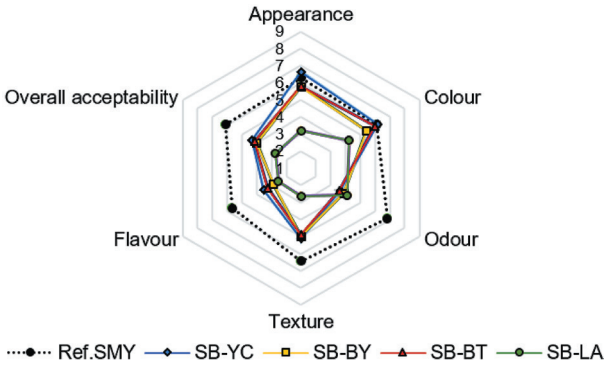

Fig. 4. Sensory analysis of soy milk yoghurts. Ref.SMY is the reference soy milk yoghurt (commercial soy milk yoghurt), SB-YC (control) is the soy milk yoghurt fermented by YC-380 (*S. thermophilus* and *L. delbruekii* ssp. *bulgaricus*), SB-BY is the soy milk yoghurt fermented by ABY-3 (*S. thermophilus*, *L. bulgaricus, B. animalis* ssp. *lactis* and *L. acidophilus*), SB-BT is the soy milk yoghurt fermented by ABT-5 (*S. thermophilus, B. lactis* and *L. acidophilus*) and SB-LA is the soy milk yoghurt fermented by LA-5 (*L. acidophilus*) mixed with YC-380 (strain ratio 1:1)

CONCLUSIONS

The study showed that different bacterial combinations have a significant effect on the melatonin and tryptophan content and the quality characteristics of soy milk yoghurt. Fermentation with SB-BY and SB-YC yielded the highest melatonin mass fraction, while SB-LA produced the most tryptophan. SB-LA yoghurt had the strongest antioxidant activity with notable DPPH, ABTS and FRAP values, indicating a strong free radical scavenging ability. The addition of mixed yoghurt bacteria and probiotic cultures improved the colour parameters (L^*, a^* and b^*), syneresis and texture profile analysis of soy milk yoghurt. The sensory evaluations resulted in the highest scores for yoghurt fermented with *Streptococcus thermophilus* in co-culture with *Lactobacillus acidophilus* and *Bifidobacterium lactis*. Co-culture of probiotics and yoghurt bacteria successfully enriched soy milk yoghurt with melatonin, tryptophan and antioxidants, supporting the creation of a functional food product with potential sleep-promoting and health benefits. Our results provide a basis for the development of functional yoghurt and offer a compelling strategy for future food innovation. However, further research is needed to investigate the effects of storage conditions on shelf-life and changes in melatonin and tryptophan content, antioxidant activity and quality characteristics of soy milk yoghurt.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

AUTHORS' CONTRIBUTIONS

T. Utaida was responsible for the conception of the study, data collection, data analysis and interpretation, as well as for conducting the analyses and drafting the text. A. Moongngarm contributed to the conceptualization of the study, sought research funding, provided critical revision, drafted the article, revised the manuscript and gave final approval for the published version. P. Itsaranuwat was involved in the design and methodology of the study.

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