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

Running title: Melatonin Content in Soy Milk Yogurt

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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 yogurt contains melatonin and antioxidants and has beneficial health properties. Many previous authors have investigated soy milk yogurt produced by probiotic bacteria. This is the first study to explore the effect of using yogurt bacteria co-cultured with probiotics to improve melatonin content and antioxidant activity. This research investigated the melatonin, serotonin, and tryptophan contents, antioxidant activity, physical characteristics, and sensory properties of soy milk yogurt bacteria co-cultured with different probiotics.

Experimental approach. Soy milk was fermented using four combinations of yogurt bacteria and probiotics including *Lactobacillus bulgaricus* and *Streptococcus thermophilus* (control, SB-YC),

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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). The yogurt samples were determined for melatonin, serotonin, and tryptophan content using LC-MS/MS, antioxidant activity, and quality characteristics including syneresis, texture profile analysis, color, and sensory evaluation.

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

Novelty and scientific contribution. Co-culturing yogurt bacteria and probiotics (*Bifidobacterium lactis, Lactobacillus acidophilus*; SB-BY) or *L. acidophilus* (SB-LA) improved melatonin production, antioxidant activity, and overall yogurt quality, providing valuable insights for developing functional foods to promote sleep and health.

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

INTRODUCTION

The growing consumer knowledge of the health benefits of functional foods is the main driver of the high demand for plant-based food products. There has also been an acceleration in the release of plant-based food products from startups and leading manufacturers in the market. The soy milk-based dairy industry continues to grow, reaching a market value of US$27.3 billion by 2022, four times the market size of plant-based meat. Plant-based dairy products have gained widespread acceptance, while Asian consumers have traditionally consumed soy milk for generations. However, developing protein functionality in products requires further investigation (*1,2*). The potential of soybean (*Glycine max* (L.) Merrill) to reduce the risk of chronic illnesses such as cancer, cardiovascular disease (CVD), menopausal symptoms, and hyperlipidemia has been subjected to extensive research (*3*). Previous research indicated that soybean seeds contain relatively high levels of melatonin (8.35 ng/g DM) and tryptophan compared to other food ingredients (*4*).

Melatonin (*N*-acetyl-5-methoxytryptamine) is a neurotransmitter produced by the pineal gland located in the center of the cerebrum which is involved in regulating sleep cycles. 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) share structural similarities with 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 rhythms (*6,7*). In mammals, melatonin is secreted by the pineal gland at night and then spreads to the cerebrospinal fluid and bloodstream. Melatonin levels decline rapidly during the day (*8*). Melatonin also exhibits powerful antioxidant properties by destroying the reaction and increasing the activity of antioxidant enzymes (*9*). In plants, melatonin helps to reduce stress from environmental conditions. However, studies on melatonin levels in fermented foods and 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 yogurt can be produced as a functional food enriched with melatonin. Cow milk yogurt 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 yogurt are rich sources of tryptophan (*11*). Tillisch *et al.* (*12*) reported that consuming foods containing bifidobacteria enhanced sleep quality by increasing serotonin secretion. Traditionally, yogurt is made by fermenting milk with *S. thermophilus* and *L. bulgaricus*. The development of probiotic yogurt has gained traction due to its added health benefits. Probiotic yogurt is defined as yogurt containing live microorganisms that confer health advantages when consumed in adequate amounts (*13*). The production process often involves co-culturing probiotic strains with traditional yogurt starter bacteria to encourage probiotic growth during fermentation. Probiotic strains are selected based on safety, nutritional benefits, health-promoting properties, and their interactions with other bacteria to enhance performance, yogurt quality, and stability during storage. Common probiotic strains used in yogurt 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 through their ability to scavenge free radicals, reduce lipid peroxidation, and enhance the body's antioxidant defenses (*15*). *B. animalis subsp. lactis* produces antioxidant metabolites such as glutathione and exopolysaccharides, which neutralize oxidative agents (*16*), while *L. acidophilus* boosts the activity of antioxidant enzymes like superoxide dismutase and catalase, protecting cells from oxidative stress (*17*).

Previous research has shown that combining starter cultures with various probiotic strains enhances bioactive compound levels and antioxidant properties in yogurt. However, the impacts of mixed yogurt bacteria and probiotics on melatonin, serotonin, and tryptophan levels, as well as the antioxidant properties of yogurt during fermentation, remain unexplored. This study investigated the effect of co-culturing yogurt bacteria with different probiotic strains on melatonin and tryptophan levels, antioxidant capacity, and specific physical properties of yogurt during fermentation. Developing a functional soy milk yogurt enriched with melatonin and tryptophan presents an interesting opportunity.

MATERIALS AND METHODS

Raw materials and chemicals

Soybean flour was purchased from Bangyai Supply Ltd. (Nonthaburi Province, Thailand). Freeze dried-direct vat set yogurt culture and probiotics including (1) YC-380 (*Streptococcus thermophilus*, *Lactobacillus delbruekii* ssp. *bulgaricus)*, (2) ABY-3 (*S. thermophilus*, *L. delbruekii* ssp. *bulgaricus, Bifidobacterium animalis* ssp. *lactis* and *Lactobacillus acidophilus*) (3) ABT-5 (*S. thermophilus*, *B. lactis*, and *L. acidophilus*) and (4) LA-5 (*L. acidophilus*) was obtained from Chr. Hansen Ltd. (Hoersholm, Denmark). The other analytical and HPLC grade chemicals were standards of melatonin, serotonin, and tryptophan (Sigma-Aldrich Chemical Co., St. Louis, MO, USA) while acetone nitrile, methanol, and ethanol were obtained from LabScan (Dublin, Ireland) and 98 % formic acid was sourced 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 (models 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 yogurt.

Preparation of yogurt

The soy milk for yogurt 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, before adding sugar (2 % *m*/*M*) and filtering, pasteurizing at (72±2) °C for 15 min, and immediately cooling to (42±1) °C before adding the starter culture. For yogurt fermentation, soy milk at (42±1) °C was inoculated with 4 % *m*/*V* of each yogurt starter culture. All inoculated soy milk samples were transferred into sterile plastic cups and incubated at 43 °C using an incubator (model 30-750; Memmert, Schwabach, Germany). The resulting soy milk yogurt samples were labeled 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, respectively. To monitor the change of soy milk during fermentation and to obtain the most suitable fermenting time, yogurt samples were randomly taken from the incubator at 0, 2, 4, 6, and 8 h for physical property analysis (pH, titratable acidity, texture profile, syneresis, and color). Portions of the yogurt 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. The freeze-dried samples were then analyzed for melatonin and its derivatives, antioxidant activity, and Fourier transform infrared (FT-IR) spectroscopy.

Physical analysis

The pH value of soy milk yogurt was measured using a pH meter (FEP20-FiveEasy; Mettler Toledo, Greifensee, Switzerland). Titratable acidity (% TA) (g of lactic acid/100 g yogurt) with 0.1 M NaOH was used to neutralize the acids contained in the yogurt using phenolphthalein as an indicator (*18*). Color analysis was performed using a chroma meter (CR-400; Konica Minolta, Tokyo, Japan) and reported as Hunter System *L**, *a** and *b** coordinates (*19*). Soy milk yogurt syneresis was analyzed following Jeong *et al.* (*20*) with minor changes. A 10 g aliquot of yogurt was centrifuged (Universal 320R; Andreas Hettich, Tuttlingen, Germany) at 478×*g* for 6 min at 4 °C. The clear liquid that separated from the yogurt was weighed. Syneresis was displayed as the percentage of serum separated by a specific centrifugation. The texture characteristics of the yogurt were analyzed by a texture analyzer (TA-XT plus; Stable Micro Systems, Godalming, Surrey, UK) (*21*). Texture profile analysis (TPA) was performed by compressing twice instead of the consumer chewing, with a cylinder probe (P/20 diameter 20 mm(with pre-test 1 mm/s, post-test 5 mm/s, test-speed 5 mm/s, and distance 30 mm.

FT-IR spectroscopy measurement

Variations in the functional components of the soy milk yogurt were determined by FT-IR spectroscopy and recorded using a Frontier and Spotlight System (Spotlight 200i; PerkinElmer, Waltham, MA, USA). FT-IR spectra were obtained for wave numbers from 4000 to 400 cm^{-1}, with a 4 cm^{-1} spectral resolution summarizing 8 scans (*22*).

Determination of melatonin, serotonin and tryptophan

The investigation of free melatonin, tryptophan, and serotonin in soy milk yogurt was modified from the methods of Nontasan *et al.* (*23*). A 2.5 g aliquot of freeze-dried soy milk yogurt 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, Kyonggi-Do, Korea) at 4 °C for 16 h, followed by centrifugation at 2236×*g* at 4 °C for 5 min. The extract was cleansed using a Sep-Pak C18 Solid Phase Extraction (SPE) cartridge (Waters, USA) (*24*).

An LC-MS/MS analysis of melatonin, serotonin, and tryptophan was performed using a Liquid Chromatograph coupled with a Mass Spectrometer (Shimadzu 20ADS-8030; Shimadzu Corporation, Kyoto, Japan) operated in electrospray ionization mode (ESI). The stationary phase was a C18 column (XBridge™ 3.5 μm 2.1×150 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 for calculating the quantitation of melatonin (ng/g), tryptophan, and serotonin contents (μg/g) of dry mass yogurt (DM).

Determination of antioxidant activity

A precisely measured 2.5 g of freeze-dried soy milk yogurt was liquefied in 20 mL of 80 % ethanol. This mixture was shaken for 16 h at 27 °C in a shaking incubator. The yogurts were centrifuged at 1430×*g* for 15 min and filtered through a Whatman No.1 filter. The resulting soy milk yogurt extract was stored in an amber glass bottle at 2–6 °C before being used to assess antioxidant activity (*23*).

The DPPH radical scavenging activity of soy milk yogurt extract was performed (*25*). A 100 µL aliquot of DPPH (0.2 mM) in ethanol was added to 100 µL of soy milk yogurt 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 recorded at 517 nm. The scavenging activity was reported as IC_{50}, calculated using the dose inhibition curve by plotting yogurt concentration against the corresponding inhibition percentage. A greater effect in radical scavenging activity is indicated by a lower IC_{50} value.

An ABTS solution (7 mM ABTS combined with 2.45 mM potassium persulfate) was used to determine ABTS radical scavenging activity (*25*). A volume of 170 µL aliquot of soy milk yogurt 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 determined at 734 nm using a microplate reader (SPECTROstar Nano 24V-DC 60w S/N 601-1387; BMG Labtech, Ortenberg, Germany).

The ferric reducing antioxidant power (FRAP) was evaluated (*26*). Ferrous sulfate was used as the standard and a calibration curve was plotted. Then, 20 µL of soy milk yogurt extract was mixed with 900 µL of the FRAP reagent. This buffer was prepared by mixing acetate buffer (pH 3.6), 20 mM $FeCl_3$ and 10 mM 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 (593 nm) was read (SPECTROstar Nano 24V-DC 60w S/N 601-1387; BMG Labtech, Ortenberg, Germany). Results were expressed as µg of $FeSO_4$ equivalent/g sample (dry basis).

Sensory evaluation

The sensory evaluation of yogurt was performed by 50 untrained male and female panelists at least 20 years old, and approved by Mahasarakham University (approval number: 065–436/2565). Sensory preferences were rated as appearance, color, odor, flavor, 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. Different samples within 2 days after yogurt production were served to each panelist in a white plastic cup at a temperature not exceeding 10 °C and tested from left to right. The liking 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 for all soy milk yogurt samples were performed in triplicate. The mean values and standard deviation of the results were reported. 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 21 (*28*).

RESULTS AND DISCUSSION

pH and titratable acidity of soy milk yogurt

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

The titratable acidity values of the soy milk yogurts are shown in Fig. 1b. The TA increased with fermentation time from 0.20 to 0.99 % at 0 and 8 h. SB-BY (0.21–0.99 %) and SB-LA (0.20–0.93 %) had higher TA than yogurt fermented by SB-YC (0.23–0.75 %) and SB-BT (0.24–0.74 %). Acidification is crucial in food preservation through fermentation, as the metabolic activity of bacteria accumulates acid, lowering the pH and inhibiting the growth of spoilage bacteria (*29*). The pH values in this study concurred with Grasso *et al.* (*30*) who reported that the pH values in commercial soy milk yogurts ranged from 3.99 to 4.56. This pH range could be attributed to soy milk compositions, yogurt bacteria, lactic acid, and the ingredients used in yogurt production. Specific pH ranges influence the action of certain gelling agents in soy milk yogurt (*31*). The TA values of the yogurts were 0.45–0.55 and 0.74–0.99 % at 0 h and 8 h fermentation, respectively and comparable to results reported by Grasso *et al.* (*30*) for commercial soy milk yogurts. The increase in TA resulted from the accumulation of lactic acid and other organic acids produced through fermentation of the starter culture (*32*).

Concentration of melatonin, serotonin and tryptophan

Melatonin, serotonin, and tryptophan contents in soy milk yogurts are shown in Table 1. Yogurt fermented with different types of bacteria and probiotic cultures gave diverse melatonin content. The melatonin content in soybean flour was 17.67 ng/g. Melatonin was detected in SB-BY yogurt at fermentation times of 4 and 8 h at levels of 12.83 and 21.20 ng/g, respectively while melatonin in SB-YC and SB-LA was detected after fermentation for 8 h (23.51 and 11.86 ng/g, respectively). The melatonin content found in SB-BY and SB-YC was not significantly different. These results indicated that soy milk yogurt fermented with yogurt bacteria and yogurt bacteria co-cultured with two types of probiotics gave higher melatonin production than probiotic cultures alone.

The tryptophan content in soybean flour was 159.07 ng/g. The tryptophan content in SB-YC and SB-BY yogurt increased at 4 h (175.33 and 303.10 ng/g, respectively) and then decreased at 8 h of the fermentation process ($p \leq 0.05$), showing the opposite effect to SB-BT. The SB-LA tryptophan content increased with fermentation at 4 and 8 h (292.75 and 396.18 ng/g, respectively). Juhnevica-Radenkova *et al.* (*33*) reported that the availability of tryptophan is an important factor in determining the concentration of indolic compound products *i.e*. melatonin. Yogurt bacteria and probiotics use tryptophan to produce serotonin and then melatonin isomers during malolactic fermentation.

In this study, serotonin was undetectable in both raw materials (soybean flour) and soy milk yogurt while Nontasan *et al.* (*23*) reported that serotonin content in germinated soybean, germination under normal and salt stress conditions ranged between 37 and 57 ng/g. The melatonin and tryptophan levels in soy milk yogurt fermented by YC mixed with *L. acidophilus* and *B. lactis* were higher than in soy milk yogurt fermented by SB-BT. Kocadağlı *et al.* (*34*) explained that food samples such as bread and beer fermented with yeast contained relatively high levels of melatonin compared to other food samples. Low concentrations of melatonin were found in probiotic yogurt (0.9 ng/g) while yeast-fermented foods contain more melatonin than bacterial-fermented foods. Yogurt fermented with *L. delbrueckii* ssp. *bulgaricus* OLL1073R-1 has also been evaluated for its sleep-promoting effects. Self-reported sleep quality improved in the yogurt group compared to the control group. The yogurt group also reported improved perceptions of general health and vitality (*35*). This study also revealed that storage at 4 °C for 28 days influenced the levels of melatonin, serotonin, and tryptophan in soy milk yogurt (data not shown). By day 21, melatonin was significantly degraded, while tryptophan and serotonin levels remained relatively stable throughout the storage period. These findings highlighted that probiotic fermentation effectively preserves tryptophan and serotonin in soy milk yogurt but presents challenges in maintaining melatonin stability during prolonged storage. Enhancing storage conditions by utilizing improved packaging or adding natural antioxidants may help to reduce melatonin degradation. Melatonin doses ranging from 0.3 mg to 1 mg effectively regulate the sleep-wake cycle, particularly in cases of mild insomnia or jet lag. The physiological roles and health impacts of melatonin and serotonin are intricate and interrelated. Normal endogenous melatonin levels are typically adequate for physiological functions, while factors like diet and enzymatic activity significantly influence serotonin availability and its conversion into melatonin. These elements collectively impact sleep regulation and other biological processes (*36*).

Antioxidant activity of soy milk yogurt

The antioxidant activity results evaluated by DPPH radical scavenging and ABTS were expressed as IC_{50} values, the lower 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 and significantly higher than the control yogurt, SB-YC (Fig. 2a). The SB-BT and SB-LA samples consistently showed the lowest IC_{50} values (highest antioxidant activity) at 4-8 h of fermentation. Differences among formulations were statistically significant ($p \leq 0.05$), particularly at later fermentation stages. Soy milk yogurts showed higher antioxidant capacity measured by the ABTS assay, while SB-LA exhibited the highest ABTS IC_{50} (0.51 mg/mL) compared to the other yogurt formulations ($p \leq 0.05$). The SB-LA formulation exhibited the lowest IC_{50} values at 6 and 8 h, showing superior antioxidant capacity compared to the other samples (Fig. 2b). The antioxidant properties evaluated by FRAP (Fig. 2c) revealed that all soy milk yogurts showed antioxidant potential, especially yogurt fermented with the probiotic, *L. acidophillus*. The FRAP values of SB-YC, SB-BY, SB-BT, and SB-LA were 2331.04, 1928.01, 2468.92, and 2577.86 μg$FeSO_4$ equivalent/g DM, respectively. Yogurt containing probiotics had higher FRAP values compared to the control. The FRAP values in the sample initially decreased. At the end of the fermentation, the highest FRAP value was observed in SB-LA yogurt ($p \leq 0.05$).

The results of the three antioxidant activity assays (DPPH, ABTS, and FRAP) showed that adding probiotic cultures in combination with yogurt cultures increased the antioxidant activity with the greatest activity found in SB-LA yogurt. During fermentation, the antioxidant activity of soy milk yogurt improved significantly at 4-6 h due to optimal enzymatic activity that broke down complex compounds into bioactive components like phenolics and flavonoids. By 8 h, the activity decreased or stabilized but remained higher than unfermented samples (0 h). This enhancement was influenced by the natural richness of soy milk in isoflavones and phenolics and the varying enzymatic potentials of different microbial strains (*37*). Our results concurred with previous studies. The combined use of probiotics with yogurt starters increased antioxidant activity during storage compared to day 0 (*38*). Changes in DPPH, FIC (ferrous ion chelating), and FRAP radical scavenging activities were recorded in fermented soybean and almond milk and combinations using 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 showed a higher percentage of scavenging activity than the corresponding control, and samples prepared in the same treatment without using the starting culture. Fermented almond milk by *L. casei*, *L. acidophilus*, and *L. plantarum* had higher scavenging activity than *L. rhamnosus* on the 1st day (*39*). The antioxidant activity in *Lycium barbarum* yogurt also increased during the first 2 weeks of storage and was significantly higher than in plain yogurt (*29*).

Color, syneresis, and texture characteristics of soy milk yogurt

The color characteristics of soy milk yogurt are presented in Table 2. The soy milk yogurts were yellowish and showed significantly different ($p \leq 0.05$) color values when fermented by other cultures. The highest *L** value (75.15) was observed in SB-BY, while the highest *a** (0.23) and *b** (15.41) values were found in SB-LA. Color values are essential for qualitative assessments of food quality. The visual assessment of food color closely corresponds with consumer or taster evaluations, helping to establish standard criteria for comparing instrumental measurements (*40*). The brightness (*L**) of yogurt is related to the particle size of the fat and protein globules. This affects light reflection and scattering ability. The size of these particles is greatly influenced by the choice of unit operation and the processing parameters used. Soy milk yogurt showed the *b** in yellow and the results agreed with Grasso *et al.* (*29*). The *a** (red-green axis) values were negative for all yogurts. The soy milk containing 100 % extract had the lowest brightness value (*L**=53.40).

The syneresis properties of soy milk yogurts (Table 2) were affected by the yogurt bacteria in combination with different probiotics. The highest syneresis (40.92 %) was found in SB-BY. Syneresis, the separation of whey protein on the surface of yogurt, is a significant and noticeable defect that can negatively impact consumer product acceptance (*19*). The syneresis of soy milk yogurts ranged between 30.08 and 40.92 %. Cui *et al.* (*41*) reported that syneresis in soy milk yogurt fermented by yogurt starters alone (YFL-901, consisting of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus*) was significantly higher compared to yogurts made using a combination of yogurt starters and probiotics like *L. rhamnosus* (LGG), *B. animalis* subsp. *lactis* BB-12, and *L. acidophilus* La-5. Most soy milk yogurt products showed a small increase in syneresis. These findings suggested that the addition of probiotics enhanced the water-holding capacity of the yogurt, significantly reducing whey formation. Probiotics like BB, LA, and LGG are known to produce exopolysaccharides which improve yogurt texture by interacting with free water in a gel-like structure, thus helping to reduce syneresis.

The texture profile analysis results are shown in Table 2. YC and YC co-cultured with probiotics showed a significantly different texture from the control (SB-YC). The soy milk yogurt had higher firmness, consistency, cohesiveness, and index of viscosity than the yogurt. Soy milk yogurts have lower protein contents and differences in the protein coagulation properties reduce consistency compared with milk yogurt with no adding thickener, especially at low pH, affecting the visual appearance (*42*).

FT-IR profiles of soy milk yogurt

FT-IR analysis was conducted to assess the changes in the chemical profile compositions of soy milk yogurt during fermentation. FT-IR spectral data in the range 4000–400 cm^{-1} collected from soy milk yogurts and soybean flour (control) are shown in Fig. 3. All soy milk yogurt samples had FT-IR spectrum at 3300–2800, 1700–1600, 1550–1249, and 550–400 cm^{-1}. Soybean flour as a raw material shows more than 30 peaks while the number of peaks decreased during yogurt fermentation and showed similar patterns of FT-IR spectra because the yogurt bacteria and probiotic cultures applied in this study fermented soy milk similarly. The deconvolution of amine bands was constituted by at least four spectra at 3200–2800, 1650, 1538, and 1240 cm^{-1} corresponding to amine bands of the N–H (amino group) stretching vibrations (*43*). 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) and 1550 cm^{-1} (amide II band, 40% C–N stretching and 60%N–H bending vibration) (*44*). The primary locations of fatty acid peaks were 2960, 2929, and 1740 cm^{-1}. The asymmetric and symmetric CH_2 stretching modes were associated with peaks located at 2928 and 2860 cm^{-1}, respectively (*45*). According to Greulich *et al.* (*46*), 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 located 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 (*47*).

Sensory assessment of soy milk yogurt

Sensory evaluation of the soy milk yogurt samples was evaluated by 50 volunteers aged 20-30 years, with results shown in Fig. 4. The SB-YC, SB-BT, and SB-BY samples had higher values in appearance, color, texture, flavor, and acceptability than SB-LA ($p \leq 0.05$). The appearance and color characteristics of SB-YC, SB-BY, and SB-BT were not significantly different ($p > 0.05$) from commercial yogurt. For overall acceptability, SB-YC, SB-BY, SB-BT, and SB-LA were not significantly different ($p > 0.05$). The panelists rated the preference of SB-LA the lowest compared with the other groups of probiotics, possibly due to the dark color and low consistency with the results of brightness (L^*) and consistency values (Table 2). These findings, especially the yogurt flavor, were supported by Tian *et al.* (*48*) who reported on the impact of co-fermenting four probiotics, *L. acidophilus*, *L. plantarum*, *L.*

rhamnosus, and *L. casei* with conventional yeast on yogurt flavor. 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 yogurt samples containing various probiotics during refrigerated storage, highlighting distinct flavor profiles linked to specific probiotic strains.

CONCLUSIONS

The study demonstrated that different bacterial combinations significantly affect the melatonin and tryptophan contents and the quality characteristics of soy milk yogurt. Fermentation with SB-BY and SB-YC yielded the highest melatonin levels, while SB-LA produced the most tryptophan. SB-LA yogurt had the strongest antioxidant activity with notable DPPH, ABTS, and FRAP values, indicating potent free radical scavenging ability. The inclusion of mixed yogurt bacteria and probiotics improved the color parameters (*L**, *a**, *b**), syneresis, and texture profile analysis of soy milk yogurt. Sensory evaluations indicated the highest scores for yogurt fermented with *S. thermophilus* co-cultured with *L. acidophilus* and *B. lactis*. The co-culture of probiotics and yogurt bacteria successfully enriched soy milk yogurt with melatonin, tryptophan, and antioxidants, supporting the creation of a functional food product with potential sleep-promoting and health benefits. Our findings provide a foundation for developing functional yogurts and offer a compelling strategy for future food innovations. However, further research is required to investigate the impact of storage conditions on shelf-life and changes in melatonin, tryptophan levels, antioxidant activity, and quality attributes of soy milk yogurt.

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CONFLICT OF INTEREST

The authors report no conflicts of interest.

AUTHORS' CONTRIBUTIONS

Treechada Utaida was responsible for the study's conception, data collection, data analysis, and interpretation, as well as performing the analyses and drafting the text. Anuchita Moongngarm contributed to the study's conception, sought research funding, provided critical revision, drafted the article, revised the manuscript, and gave final approval for the published version. Pariyaporn Itsaranuwat was involved in the study's conception and methodology.

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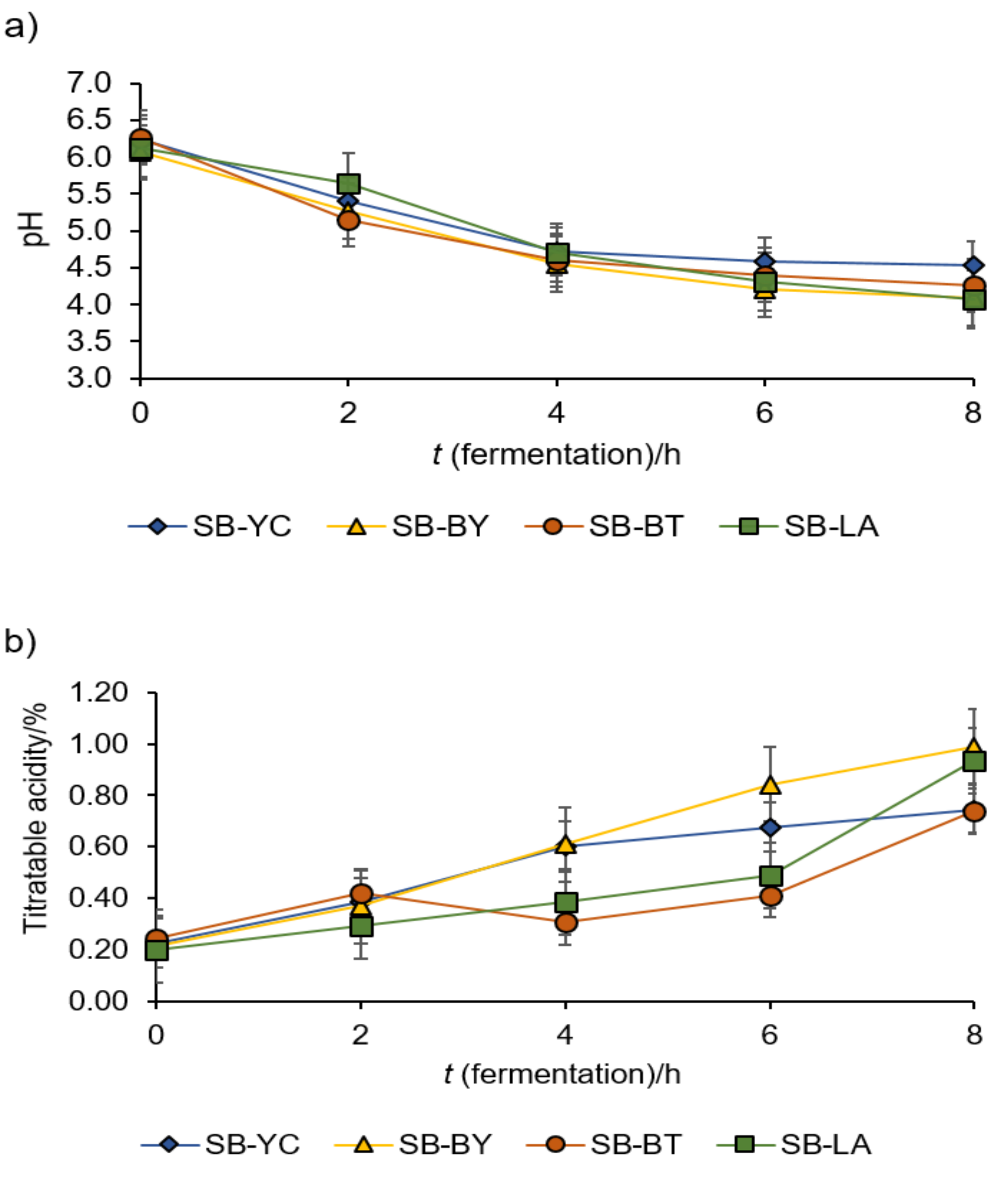
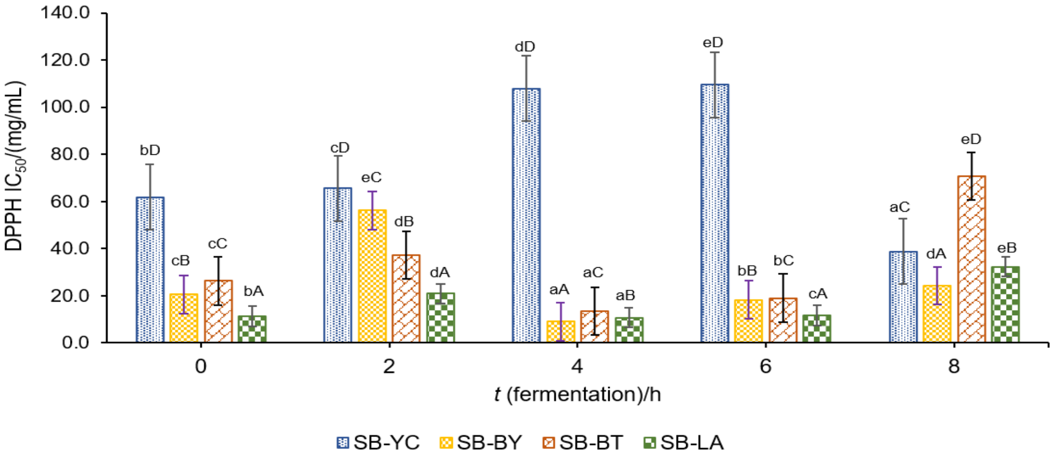
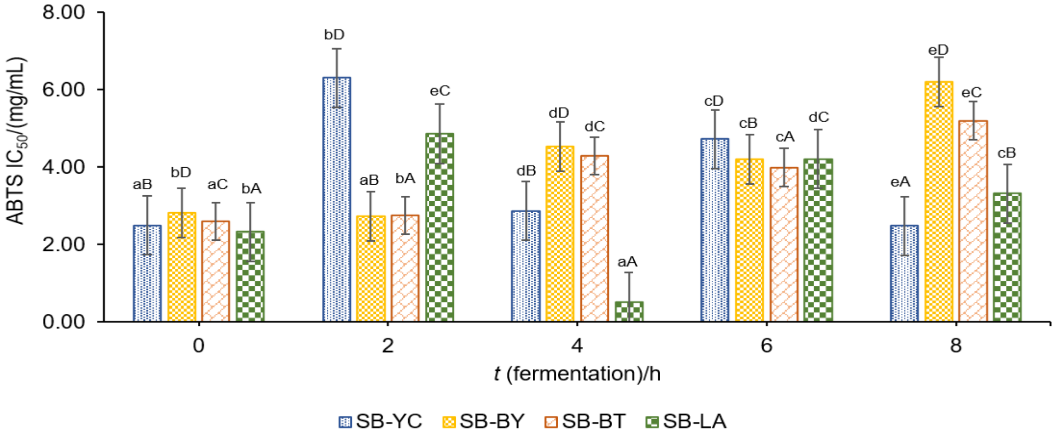


Fig. 1. Change in: a) pH value and b) titratable acidity during fermentation of soy milk yogurts. SB-YC (control) refers to soy milk yogurt fermented by YC-380 (*S. thermophilus*, *L. delbruekii* ssp. *bulgaricus*), SB-BY refers to soy milk yogurt fermented by ABY-3 (*S. thermophilus*, *L. bulgaricus,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

a)


DPPH IC50/(mg/mL)
t (fermentation)/h
SB-YC SB-BY SB-BT SB-LA

b)


ABTS IC50/(mg/mL)
t (fermentation)/h
SB-YC SB-BY SB-BT SB-LA

c)

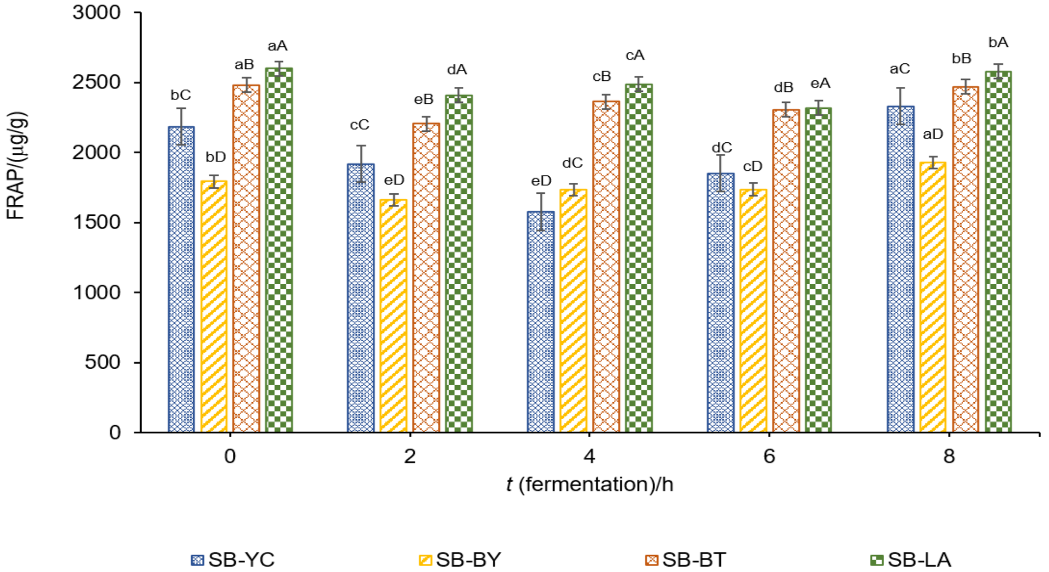

FRAP/(µg/g)
t (fermentation)/h
SB-YC SB-BY SB-BT SB-LA

Fig. 2. Antioxidant activity of soy milk yogurts measured by: a) DPPH IC_{50}, b) ABTS IC_{50} and c) FRAP measurement. SB-YC (control) refers to soy milk yogurt fermented by YC-380 (*S. thermophilus*, *L. delbruekii* ssp. *bulgaricus*), SB-BY refers to soy milk yogurt fermented by ABY-3 (*S. thermophilus*, *L. bulgaricus, B. animalis* ssp. *lactis* and *L. acidophilus*), SB-BT refers to soy milk yogurt fermented by ABT-5 (*S. thermophilus, B. lactis* and *L. acidophilus*), and SB-LA refers to soy milk yogurt fermented by LA-5 (*L. acidophilus*) mixed with YC-380 (strain ratio 1:1). Capital letters comparing yogurt bacteria and probiotic types and lowercase letters comparing fermentation time with different superscripts are significantly different ($p \leq 0.05$)

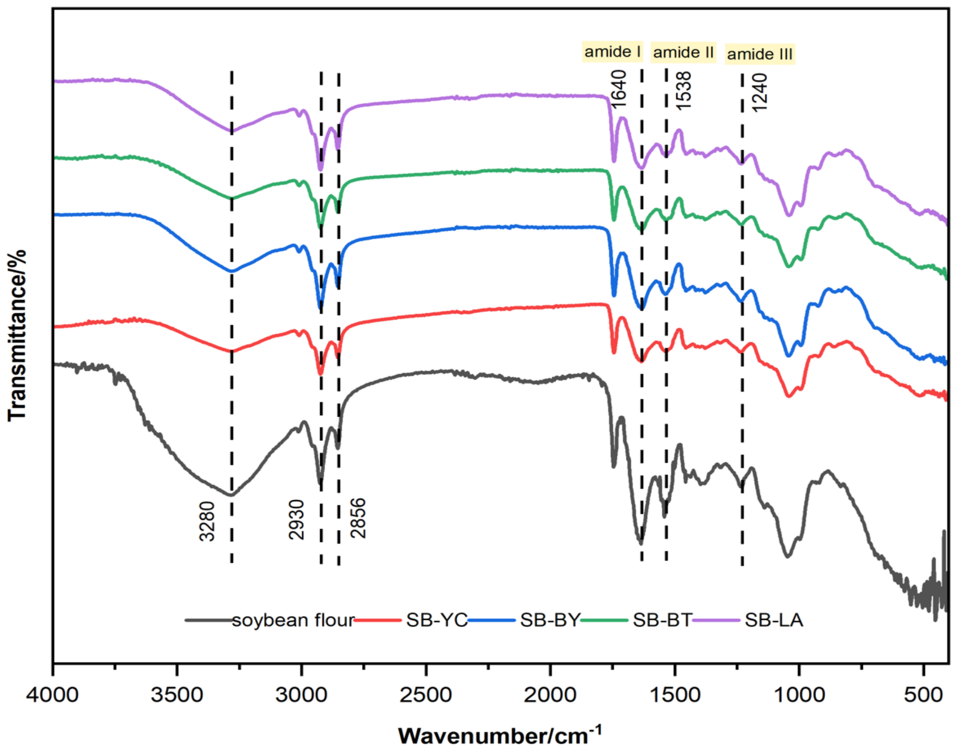


Fig. 3. FT-IR spectra of soy milk yogurts at 8 h of fermentation time. Soybean flour as a raw material, SB-YC (control) refers to soy milk yogurt fermented by YC-380 (*S. thermophilus*, *L. delbruekii* ssp. *bulgaricus*), SB-BY refers to soy milk yogurt fermented by ABY-3 (*S. thermophilus*, *L. bulgaricus, B. animalis* ssp. *lactis* and *L. acidophilus*), SB-BT refers to soy milk yogurt fermented by ABT-5 (*S. thermophilus, B. lactis* and *L. acidophilus*), and SB-LA refers to soy milk yogurt fermented by LA-5 (*L. acidophilus*) mixed with YC-380 (strain ratio 1:1)

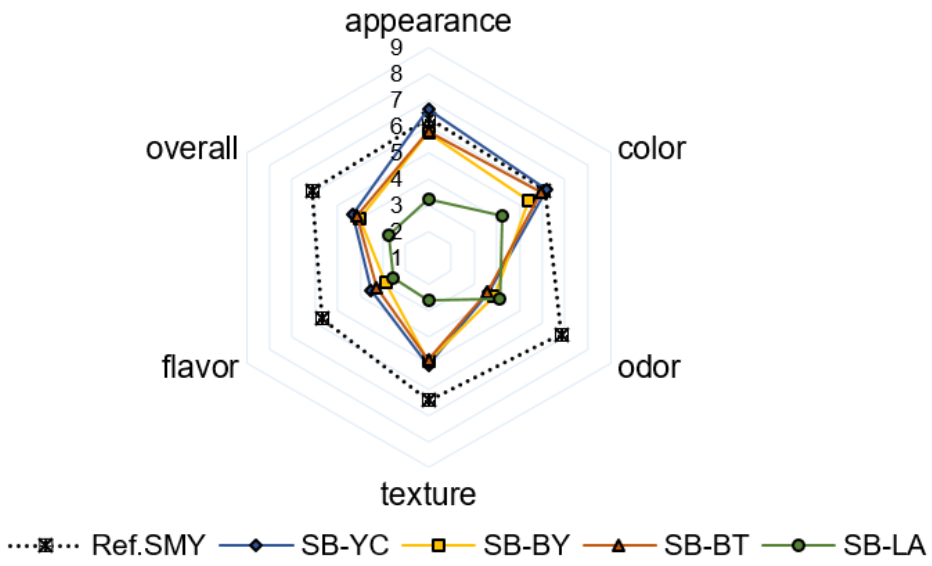


Fig. 4. Sensory analysis of soy milk yogurts. Ref. SMY refers to reference soy milk yogurt (commercial soy milk yogurt), SB-YC (control) refers to soy milk yogurt fermented by YC-380 (*S. thermophilus*, *L. delbruekii* ssp. *bulgaricus*), SB-BY refers to soy milk yogurt fermented by ABY-3 (*S. thermophilus*,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

Table 1 Melatonin and tryptophan contents of soy milk yogurts under different fermentation times compared with soybean flour.

Treatment	Melatonin *w*/(ng/g DM)			Tryptophan *w*/(ng/g DM)		
	0	4	8	0	4	8
SB-YC	$(1.26\pm0.19)^{b}$	ND	$(23.51\pm0.02)^{aA}$	$(128.69\pm0.55)^{A}$	$(175.33\pm0.75)^{B}$	$(57.52\pm0.13)^{C}$
SB-BY	ND	$(12.83\pm1.72)^{b}$	$(21.20\pm0.86)^{aA}$	$(27.66\pm0.98)^{bB}$	$(303.10\pm0.59)^{aA}$	$(76.73\pm0.94)^{bC}$
SB-BT	ND	ND	ND	$(103.70\pm0.99)^{aA}$	$(32.76\pm0.81)^{bC}$	$(101.05\pm0.57)^{aBC}$
SB-LA	ND	ND	$(11.86\pm0.10)^{B}$	$(41.49\pm0.14)^{cB}$	$(292.75\pm0.19)^{bA}$	$(396.18\pm0.11)^{aA}$

Capital letters within the columns and lowercase letters within a row for each sample of melatonin and tryptophan contents, with different superscripts are significantly different ($p\leq0.05$) and ND represents an amount that was not detected. SB-YC (control) refers to soy milk yogurt fermented by YC-380 (*S. thermophilus*, *L. delbruekii* ssp. *bulgaricus*), SB-BY refers to soy milk yogurt fermented by ABY-3 (*S. thermophilus*, *L. bulgaricus, B. animalis* ssp. *lactis* and *L. acidophilus*), SB-BT refers to soy milk yogurt fermented by ABT-5 (*S. thermophilus, B. lactis* and *L. acidophilus*), and SB-LA refers to soy milk yogurt fermented by LA-5 (*L. acidophilus*) mixed with YC-380 (strain ratio 1:1)

Table 2. Mean values and standard deviations of the physical properties of the soy milk yogurts at 8 h of fermentation

Treatment	Firmness/N	Consistency/(N•s)	Cohesiveness/N	Viscosity index/(N•s)	Syneresis/%	Color		
						*L**	*a**	*b**
SB-YC	(24.23±3.37)[b]	(65.14±8.63)[b]	(2.49±2.52)[a]	(0.89±0.40)[a]	(36.30±0.47)[c]	(74.27±0.52)[b]	(-0.43±012)[a]	(13.83±0.85)[b]
SB-BY	(40.51±2.55)[a]	(109.65±1.01)[a]	(6.48±1.18)[b]	(2.22±0.19)[b]	(40.92±1.28)[d]	(75.15±0.42)[a]	(-0.01±0.03)[b]	(12.95±0.01)[c]
SB-BT	(17.96±1.91)[c]	(37.33±3.82)[c]	(4.07±0.15)[ab]	(2.65±0.15)[b]	(30.08±0.77)[a]	(74.07±0.66)[b]	(0.08±002)[c]	(12.85±0.39)[c]
SB-LA	(26.1±0.50)[b]	(4.22±0.14)[d]	(0.58±0.13)[a]	(0.45±0.08)[a]	(34.24±0.51)[b]	(71.45±0.38)[c]	(0.23±0.13)[d]	(15.41±0.46)[a]

Different lowercase superscripts within columns for each sample of values are significantly different ($p \leq 0.05$). SB-YC (control) refers to soy milk yogurt fermented by YC-380 (*S. thermophilus*, *L. delbruekii* ssp. *bulgaricus*), SB-BY refers to soy milk yogurt fermented by ABY-3 (*S. thermophilus*, *L. bulgaricus, B. animalis* ssp. *lactis* and *L. acidophilus*), SB-BT refers to soy milk yogurt fermented by ABT-5 (*S. thermophilus, B. lactis* and *L. acidophilus*), and SB-LA refers to soy milk yogurt fermented by LA-5 (*L. acidophilus*) mixed with YC-380 (strain ratio 1:1)