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Utilisation of yoghurt bacteria and probiotic combination enhanced melatonin content and antioxidant activity of yoghurt

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Abstract

Melatonin and its derivatives positively influence human circadian rhythms. Yoghurt is often suggested as a bedtime food because it contains tryptophan which can be converted to melatonin. However, the impact of yoghurt bacteria (YC) and probiotics on producing melatonin and its derivative content in yoghurt during fermentation has not yet been reported. Therefore, the present work investigated the effect of YC and probiotics on enhancing tryptophan, serotonin, and melatonin during yoghurt fermentation. Cow milk yoghurt was prepared using different combinations of YC (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, MP-YC, control); YC and *Lactobacillus acidophilus* and *Bifidobacterium animalis* subsp. *lactis* (MP-BY); *S. thermophiles*, *L. acidophilus*, and *B. animalis* subsp. *lactis* (MP-BT); and YC with *L. acidophilus* (MP-LA). Combining YC and probiotic mixtures had no impact on pH and total acidity after fermentation for 8 h. Yoghurt containing probiotics and YC had higher melatonin content ranging from 3.54 to 6.67 ng/g dry weight than the control, whereas serotonin was not detected. Tryptophan content significantly improved in all yoghurt samples ranging between 8.52 and 10.17 µg/g. The antioxidant activity of MP-BY and MP-BT was significantly enhanced compared with the control. The findings suggested a synergistic effect between YC and probiotics, highlighting the potential of probiotic yoghurt as a functional food rich in melatonin, tryptophan, and antioxidants, which could benefit and support human circadian rhythms and overall health.

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Introduction

Yoghurt is a widely consumed fermented milk product made using *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* as the starter bacteria (Sieuwerts *et al.*, 2010). Yoghurt provides a rich source of essential nutrients and bioactive compounds, and its consumption trends are increasing. Copious research has investigated how to add value to yoghurt by the addition of probiotics, prebiotics, and various plant extracts to formulate functional yoghurt containing more beneficial substances.

Probiotic yoghurt refers to any yoghurt that contains live microorganisms that provide health benefits when consumed in sufficient amounts (Bai *et al.*, 2020). Most probiotics belong to lactic acid-producing bacteria (LAB), typically found in yoghurt, fermented milk, and other fermented foods. The bacteria *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* do not survive in the gastrointestinal tract, and do not function significantly as probiotics, as they cannot colonise the human gut (McFarland, 2015). Some probiotic bacteria grow slowly in milk due to a lack of essential probiotic activity and acid production, which impact product texture. Therefore, mixing probiotic strains with yoghurt-starting bacteria is recommended to reduce fermentation time (Damin *et al.*, 2008).

Current trends in probiotic yoghurt manufacture involve inoculating probiotic strains along with yoghurt starter bacteria during fermentation to stimulate probiotic growth. The selection of probiotic strains is based on their safety, nutritional value, health-promoting properties, and interactions between bacterial strains to enhance performance, yoghurt quality, and survival during storage. Various species of lactobacilli and bifidobacteria are generally applied to probiotic yoghurt products. Combinations of YC with

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bifidobacteria such as *B. breve*, *B. lactis*, *B. longum*, and *B. animalis*, or with lactobacilli such as *L. acidophilus*, *L. rhamnosus*, and *L. johnsonii* were reported by Bourrie *et al.* (2016). *B. animalis* subsp. *lactis* and *L. acidophilus* have recently gained significant attention for their role in promoting human health due to their diverse bioactive properties.

Antioxidant activity helps to prevent oxidative stress that often leads to chronic illnesses such as cardiovascular diseases, cancers, and neurodegenerative disorders. Recent research focusing on the antioxidant potential of these probiotic strains has highlighted their ability to scavenge free radicals, reduce lipid peroxidation, and modulate the body's antioxidant defence systems (Vougiouklaki *et al*., 2023). The antioxidant effects of *B. animalis* subsp. *lactis* have been linked to its production of antioxidant metabolites such as glutathione and exopolysaccharides, which help neutralise harmful oxidative agents (Kim *et al*., 2020). Similarly, *L. acidophilus* has been shown to enhance antioxidant enzyme activities such as superoxide dismutase and catalase, further contributing to cellular protection against oxidative damage (Feng and Wang, 2020). These findings underscore the potential health benefits of incorporating these probiotics into the human diet, particularly in mitigating oxidative stress-related conditions.

Yoghurt is recommended as a bedtime food because it is easy to consume, and contains tryptophan which transforms to melatonin, a hormone that improves sleep efficiency. Dairy products such as milk, cheese, and yoghurt are good sources of tryptophan (Punia *et al.*, 2020). Biasiolo *et al.* (1995) reported total non-protein tryptophan in plain yoghurt made from whole or skimmed milk at 3.45 and 3.25 mg/kg, respectively, or five- to six-fold higher than in whole and skim cow milk (2.76 and 2.44 mg/kg, respectively), while Tillisch *et al.* (2013) found that food intake containing bifidobacteria improved sleep quality due to enhanced serotonin secretion.

Melatonin (N-acetyl-5-methoxytryptamine), a neurohormone that acts as an endogenous antioxidant, is synthesised from tryptophan in the pineal gland at the centre of the cerebrum. Melatonin plays a key role in regulating circadian rhythms and sleep in humans while exhibiting strong antioxidant properties by quenching oxidation and enhancing the activity of antioxidant enzymes such as glutathione peroxidase, superoxide dismutase, and catalase (Aguilera *et al*., 2015). Serotonin, a monoamine neurotransmitter derived from tryptophan, is known to influence mood, and linked to mood disorders that arise from low serotonin levels (Richard *et al*., 2009). Previous studies demonstrated that starter cultures mixed with different probiotic bacterial strains improved bioactive compounds and antioxidant capacity. However, the effects of using mixed yoghurt cultures and probiotics on improving melatonin, serotonin, and tryptophan contents, as well as yoghurt antioxidant activity during fermentation, have not been assessed. Therefore, the present work investigated the effects of co-culture of YC with different probiotics as mixed cultures on melatonin and tryptophan contents, antioxidant capacity, and physical properties of yoghurt during fermentation.

Materials and methods

Chemicals and materials

Whole milk powder (4% full fat) was purchased from a local supermarket in Maha Sarakham Province, Thailand. Freeze dried-direct vat set yoghurts including (1) YC-380 (*S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*), (2) ABY-3 (*S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *B. animalis* subsp. *lactis*, and *L. acidophilus*), (3) ABT-5 (*S. thermophilus*, *B. animalis* subsp. *lactis*, and *L. acidophilus*), and (4) LA-5 (*L. acidophilus*) were obtained from Chr. Hansen Ltd. (Hoersholm, Denmark).

Starter culture preparation

Starter cultures were prepared following the method of Felix da Silva *et al.* (2017). Reconstituted milk was prepared by dissolving full-fat milk powder (1 kg) in distilled water to obtain 14% total solids, and then homogenising the mixture in a homogeniser (Blue House BH5521HB, Turkey) before pasteurisation at 63 ± 2°C for 30 min and cooling to 42 ± 1°C before inoculation by freeze dried-direct vat sets of different yoghurt cultures. Four commercial mixed cultures (treatments) used in the present work included (1) YC-380 (designated as MP-YC), (2) ABY-3 (designated as MP-BY), (3) ABT-5 (designated as MP-BT), and (4) YC-380 with LA-5 (strain ratio 1:1) (designated as MP-LA). Sterile containers were filled with pasteurised reconstituted milk (250 mL) aseptically. A lyophilised culture (0.02% w/v) of each treatment was added to the milk,

and incubated in an incubator (Binder, Germany) at 42 ± 1°C for 6 h to generate the starter cultures. Each culture contained viable cell counts of each strain at more than 8 log CFU/g, and these were further used for yoghurt production.

Yoghurt production

Plain yoghurt was produced by reconstituting whole milk powder in distilled water to obtain 14% total solids, then homogenised and pasteurised to 63 ± 2°C for 30 min (Yang *et al.*, 2012) before cooling to 42 ± 1°C and inoculating with 4% yoghurt starter culture (designated as 0 h fermentation time). All inoculated milk samples were placed in pasteurised plastic cups, and incubated at 42 ± 1°C. Yoghurt samples were randomly removed every 4 h (0, 4, and 8 h) until pH 4.5 - 4.6 was obtained. The yoghurt samples were then cooled to 4°C to retard fermentation, and subjected to further analyses.

Determination of pH and titratable acidity

The pH values of the yoghurt were measured using a pH meter (Fiveeasy FE20, Mettler Toledo). Samples were taken from the incubator hourly until a pH of 4.5 - 4.6 was achieved. The samples were cooled to room temperature (25 ± 2°C) before measuring the pH. The titratable acidity (TA) was assessed by neutralising the yoghurt's acids with 0.1 N NaOH, using phenolphthalein as an indicator to determine the acidity in grams of lactic acid per 100 grams of yoghurt (Felix da Silva *et al.*, 2017).

Determination of melatonin, serotonin, and tryptophan

Analyses of melatonin, non-protein tryptophan, and serotonin in the yoghurt were performed following Pranil *et al.* (2021) and Nontasan *et al.* (2022). The yoghurt sample (2.5 g) was dissolved in 10 mL of 80% methanol, and mixed by sonication in an ultrasonic bath (Powersonic 420, Korea) for 30 min in the dark. Then, the mixture was shaken by a shaking incubator (LSI-1005R, Korea) at 200 rpm, 4°C, for 16 h, and centrifuged (Universal 320R, Germany) at 5,000 rpm at 4°C for 5 min. The obtained extract was purified using a Sep-Pak C18 Solid Phase Extraction (SPE) cartridge (Waters, USA) following Pranil *et al.* (2021). The purified extract obtained was then passed through a 0.22 μm syringe filter into an amber glass vial before LC-MS/MS analysis.

LC-MS/MS analysis

Melatonin, serotonin, and tryptophan levels were analysed using a Shimadzu 20ADS Liquid Chromatograph paired with a Shimadzu 8030 Mass Spectrometer (Shimadzu Corporation, Japan) operating in electrospray ionisation mode (ESI). The stationary phase was an InertSustain® C18 column (2.1 × 150 mm, 3.5 μm) from GL Science Inc. (Japan), while the mobile phase consisted of 0.45% formic acid in HPLC-grade water (solvent A) and acetonitrile (solvent B). The flow gradient was set as follows: from 0 - 5 min at 80:20% (A:B), 5 - 6 min at 50:50% transitioning to 0:100%, 6 - 9 min at 0:100%, and finally from 9 - 10 min back to 80:20%, with a flow rate of 0.25 mL/min and an injection volume of 2 μL. An external standard curve was constructed to quantify the contents of melatonin, tryptophan, and serotonin, with results reported as ng/g on a dry basis (Pranil *et al.*, 2021).

Determination of antioxidant activity

The DPPH radical scavenging assay was conducted following the method described by Sangsopha *et al.* (2020). A 1.0 mL aliquot of extract was combined with a 0.05 mM DPPH solution (in a 1:2, v/v ratio), and mixed thoroughly. For the control reaction, ethanol was added to the DPPH solution. The absorbance of each mixture was measured at 517 nm. The scavenging activity was expressed as the yoghurt concentration that inhibited 50% of the radicals (IC_{50}), determined by plotting the yoghurt concentration against the corresponding percentage of inhibition. Lower IC_{50} values indicate a stronger radical scavenging activity.

The ABTS radical scavenging activity was evaluated following the method described by Sangsopha *et al.* (2020). The ABTS solution was prepared by mixing 7 mM of ABTS with 2.45 mM potassium persulfate (1:1, v/v), and then left to stand at room temperature for 24 h. Subsequently, the ABTS solution was diluted with distilled water to obtain an absorbance value of 0.7 ± 0.005 at 730 nm. Then, 170 μL of the extract and 30 μL of ABTS solution were mixed and incubated at room temperature for 10 min. The absorbance of the mixture was measured at 730 nm. The scavenging activity was reported as the yoghurt concentration that inhibited 50% of the radicals (IC_{50}).

The FRAP value was evaluated following the method described by Sangsopha *et al.* (2020). Ferrous

sulphate was used as the standard to construct a calibration curve. The results were expressed as µM $FeSO_4$ equivalent per gram of sample on a dry basis.

Determination of colour

Colour analysis was performed using a chromameter (Minolta CR-300, Japan) with results expressed as the Hunter system L*, a*, and b* coordinates (Pranil *et al.*, 2021).

Determination of texture

Yoghurt textural attributes were measured using a texture analyser (TA-XT plus, UK) (Yang *et al.*, 2012) at 4 ± 1°C. Texture profile analysis (TPA) was applied with a cylinder probe (P/20 diameter 20 mm) using a pre-test speed of 1 mm/s, post-test speed of 5 mm/s, test speed of 5 mm/s, and distance of 30 mm.

Determination of syneresis

Yoghurt syneresis was assessed following the method of Jeong *et al.* (2018) with minor adjustments. A 10 g yoghurt sample was centrifuged (Universal 320R, Germany) at 2,300 rpm for 6 min at 4°C. The clear liquid that separated from the yoghurt was then collected and weighed. Syneresis was expressed as the percentage of serum weight that separated from the yoghurt during centrifugation.

Statistical analysis

All treatments were performed in triplicate, with results reported as means ± standard deviations using SPSS Statistics version 21. The data underwent one-way analysis of variance (One-way ANOVA) within a completely randomised design, and Duncan's multiple range test was applied at $p \leq 0.05$ to identify significant differences between mean values.

Results and discussion

Change of pH and titratable acidity during yoghurt fermentation

Changes in the pH values of yoghurts fermented by YC and YC with probiotics are shown in Figure 1A.

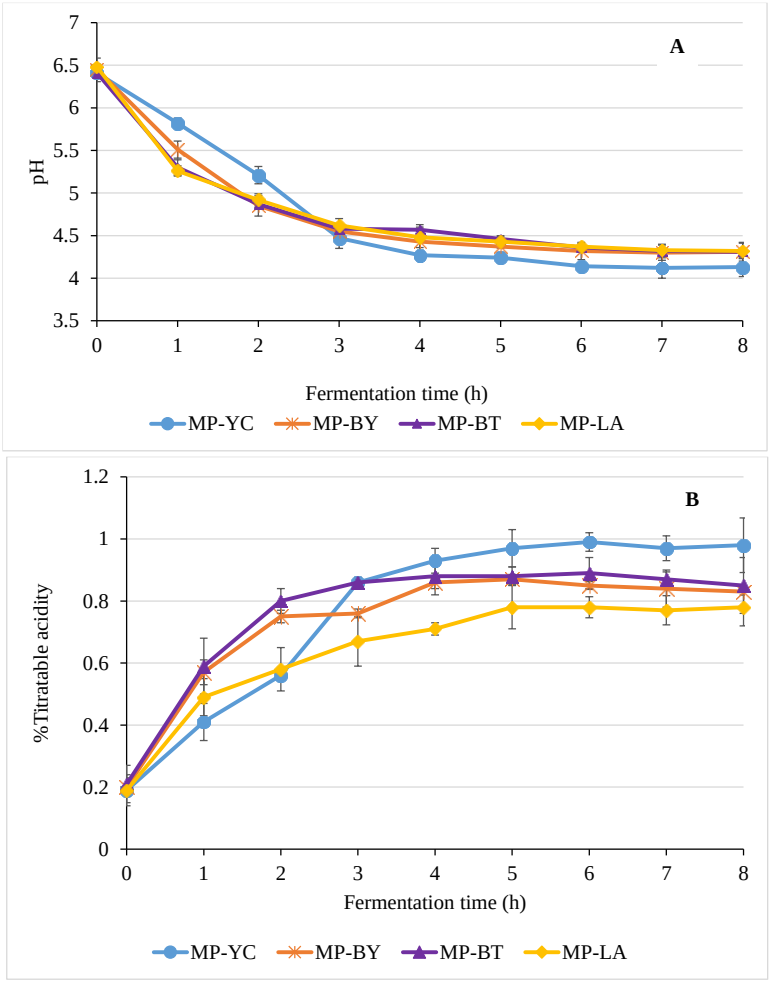

Figure 1. pH value **(A)** and titratable acidity **(B)** during yoghurt fermentation by different mixed cultures.

The pH value during yoghurt fermentation decreased with increasing fermentation time. The pH of milk at the beginning of the experiment before the starter cultures were added ranged between 6.65 and 6.67. When the starter cultures were added, pH decrease varied from 6.41 to 6.48. At the end of 8 h of fermentation, pH values of yoghurt MP-YC, MP-BY, MP-BT, and MP-LA decreased from 6.41, 6.45, 6.41, and 6.48 to 4.11, 4.31, 4.32, and 4.31, respectively. Fermentation using a four-strain mixture of YC and two probiotics (MP-BY), or three-strain mixture (MP-BT and MP-LA), or two-strain mixture (MP-YC), had no significant ($p > 0.05$) effect on the rate of pH decrease over time. The pH value and TA reached the desired value after 4 h; however, the present work aimed to evaluate the impact of co-culture YC with probiotics on melatonin, antioxidant activity, and yoghurt quality characteristics. Therefore, fermentation time was prolonged until 8 h, the period normally used for conventional yoghurt fermentation to allow the milk to curd, develop flavour, and produce some bioactive compounds.

The TA progressively increased, whereas the pH slowly decreased over fermentation time. The TA of yoghurt produced from MP-YC, MP-BY, MP-BT, and MP-LA increased significantly from 0.19, 0.20, 0.21, and 0.18% to 1.07, 0.83, 0.85, and 0.78%, respectively (Figure 1B). Yoghurt fermented by YC and probiotic cultures, MP-YC, MP-BY, and MP-BT required 3 h to reach the desired pH (approximately 4.5), whereas yoghurt obtained from MP-LA took 4 h to reach 4.48 (Figure 1A). All yoghurt samples contained total bacteria counts between 9.2 and 10 log CFU/g. The decrease in pH and increase in TA were caused by lactose fermentation and release of lactic acid by YC and probiotics. Increasing acidity and decreasing pH values were not significantly different in yoghurt obtained from different YC mixtures and probiotics, concurring with Correa and Cuenca (2022). All yoghurt samples were added with *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, the conventional yoghurt cultures, while changes in pH and TA were attributed to *L. delbrueckii* subsp. *bulgaricus*, the main acid producer (Tamime and Thomas, 2017).

Effect of yoghurt bacteria and probiotic combinations on melatonin, serotonin, and tryptophan contents

Changes in melatonin and its metabolite concentrations, including serotonin and tryptophan, in yoghurts fermented with YC mixed with probiotics are shown in Table 1. Concentrations of melatonin, serotonin, and tryptophan in whole milk powder were measured before inoculation with the starter cultures. Results revealed tryptophan as the only metabolite that could be detected (5.00 µg/g dw) in milk powder used as raw material for yoghurt preparation. Melatonin and serotonin were not detected because whole milk contained amounts below the detection limit used in the present work. At 0 h of fermentation, when different combinations of starter cultures were transferred into the pasteurised reconstituted milk, the initial melatonin and tryptophan concentrations increased. However, only melatonin in MP-BY yoghurt was observed (3.31 ng/g dw). Melatonin content increased significantly ($p \leq 0.05$) after fermentation for 4 and 8 h, with highest melatonin level observed in MP-BY (four-strain mixture) (6.67 ng/g), whereas melatonin levels in MP-LA, MP-BT, and MP-YC were 3.54, 3.56, and 2.16 ng/g, respectively. Melatonin content in yoghurt fermented by YC mixed with probiotics was higher than recorded in previous reports, in both raw milk and yoghurt. Sangsopha *et al.* (2020) reported melatonin content in raw cow milk at only 0.03 ng/mL, while melatonin content in plain yoghurt at 14.45 pg/g was reported by Karunanithi *et al.* (2014), and 126.7 pg/g in probiotic yoghurt studied by Kocadağlı *et al.* (2014).

In the present work, melatonin concentration increased by 3-fold in yoghurt fermented with YC mixed with two probiotics compared to yoghurt fermented with YC at only 2.16 ng/g. When both *B. animalis* subsp. *lactis* and *L. acidophilus* were involved in the mixed culture as the four-strain mixture (MP-BY), melatonin was detected at 0 h, and then increased until the highest value at 8 h. By contrast, when only YC (two-strain mixture, MP-YC) was used, or *S. thermophilus* was combined with *B. animalis* subsp. *lactis* and *L. acidophilus* (three-strain mixture, MP-BT) or YC was combined with *L. acidophilus* (three-strain mixture, MP-LA), melatonin content was lower than MP-BY. A synergistic relationship was found between YC and probiotics, with both *B. animalis* subsp. *lactis* and *L. acidophilus* resulting in higher melatonin production.

Serotonin content was also determined but not detected in all samples because the serotonin amount was below the limit of detection in the present work. This finding was supported by Bertazzo *et al.* (2016),

Table 1. Melatonin (ng/g dw), tryptophan (µg/g dw), and serotonin contents of yoghurt at different fermentation times compared with unfermented milk powder.

Treatment	Melatonin			Tryptophan			Serotonin		
	0	4	8	0	4	8	0	4	8
Milk powder	ND	-	-	5.00 ± 0.94^{B}	-	-	ND	-	-
MP-YC	ND	ND	2.16 ± 0.03^{C}	$5.40 \pm 0.^{12bB}$	10.74 ± 0.66^{aA}	10.17 ± 0.27^{a}	ND	ND	ND
MP-BY	2.31 ± 0.01^{b}	3.63 ± 0.05^{aA}	6.67 ± 0.21^{aA}	5.25 ± 0.01^{bB}	4.73 ± 0.54^{bD}	9.25 ± 0.11^{a}	ND	ND	ND
MP-BT	ND	ND	3.56 ± 0.12^{B}	6.15 ± 0.68^{bB}	7.97 ± 0.08^{aC}	8.52 ± 0.83^{a}	ND	ND	ND
MP-LA	ND	3.37 ± 0.01^{bB}	3.54 ± 0.10^{aB}	8.08 ± 0.53^{bA}	8.76 ± 0.46^{abB}	9.22 ± 0.66^{a}	ND	ND	ND

Uppercase superscripts in similar columns and lowercase superscripts in similar rows for melatonin and its derivative contents indicate significant difference ($p \leq 0.05$). ND: not detected, (-): not determined.

who reported that 5-hydroxytryptophan, the precursor of serotonin in the serotonin pathway, was found in low concentrations in yoghurt.

Non-protein tryptophan or free tryptophan contents in yoghurt prepared from YC and different probiotics are presented in Table 1. Yoghurt fermentation increased free tryptophan concentration compared with unfermented milk (5.00 µg/g). At 0 h, after the starter culture was added to the milk, tryptophan contents were 5.40, 5.25, 6.15, and 8.08 µg/g for MP-YC, MP-BY, MP-BT, and MP-LA, respectively. After fermenting for 4 and 8 h, the tryptophan contents significantly improved in all samples, varying from 4.73 to 10.74 µg/g. Results concurred with Yılmaz and Gökmen (2018), who reported that free tryptophan levels in commercial yoghurts ranged from 3.2 to 13.4 mg/kg dw. Changes in free amino acid concentrations were due to the different bacteria applied in yoghurt preparation because free amino acid levels are affected by different strains and interactions between the bacteria cultures involved in yoghurt fermentation (Bertazzo *et al.*, 2016). One serving of 130 g of set yoghurt contained 630 ng of melatonin and 1,300 µg of free tryptophan providing beneficial impacts to health. The probiotic yoghurt contained higher melatonin levels than other melatonin-rich foods, such as melatonin-rich milk powder (80 pg/g of powder) (Bae *et al.*, 2016). The present work was the first to report an increase in melatonin content in yoghurt through the combination of yoghurt cultures and probiotics.

Research studies have shown that probiotic strains, including *L. acidophilus* and *B. animalis* subsp. *lactis*, have a significant effect on increasing tryptophan in yoghurt, which in turn increases melatonin production. The fermentation process facilitated by yoghurt cultures, including *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* influences tryptophan metabolism, further enhancing melatonin content. The synergistic effects of probiotic strains combined with conventional yoghurt bacteria optimise these bioactive compounds, creating a functional food with enhanced health benefits (Taverniti and Guglielmetti, 2011). Combining *L. acidophilus* with *B. longum* increases melatonin concentrations in yoghurt, contributing to its sleep-regulating and antioxidant capacities (Feng *et al.*, 2018). Consuming melatonin and tryptophan-enriched yoghurt offers significant health benefits, particularly by supporting sleep regulation and reducing oxidative stress (Srinivasan *et al*., 2011). Melatonin plays a crucial role in managing the body circadian rhythm and promoting restful sleep, making it especially beneficial for individuals facing sleep disturbances or irregular sleep patterns. Tryptophan, as a precursor to melatonin and serotonin, further supports mood regulation and cognitive function, enhancing the overall impact of these nutrients on mental health. Melatonin's powerful antioxidant properties help protect cells from oxidative stress, which is linked to aging and chronic illnesses such as cardiovascular diseases and neurodegeneration. Regular intake of melatonin and tryptophan-enriched yoghurt serves as a functional food that improves sleep quality, and also promotes cellular health and mental well-being, making it a promising dietary intervention for overall human health (Ikram *et al*., 2021).

Effect of YC and probiotics on yoghurt antioxidant activity

The antioxidant activities (IC_{50}) of yoghurt fermented with YC and YC combined with one or two probiotics evaluated by DPPH and ABTS radical scavenging and FRAP are shown in Figures 2A, 2B, and 2C. The smaller the IC_{50} value, the greater the scavenging rate. The DPPH antioxidant activity of all yoghurt samples significantly improved after fermentation for 4 and 8 h ($p \leq 0.05$). The IC_{50} values of MP-YC and MP-LA yoghurt initially increased but then decreased after fermenting for 8 h. The MP-YC, MP-BY, and MP-BT yoghurt samples showed significantly stronger DPPH radical scavenging at 8 h, with IC_{50} values 44.61, 39.72, and 42.90 mg/mL, respectively, than the MP-LA yoghurt (52.11 mg/mL) ($p \leq 0.05$). The IC_{50} of ascorbic acid on DPPH scavenging with greatest antioxidant activity (8.24 mg/mL) is also shown for comparison with the yoghurt samples.

The antioxidant properties of yoghurt using the ABTS radical scavenging method revealed that yoghurt fermented with different mixed cultures and during the first 4 h of fermentation time had significantly different IC_{50} values ($p \leq 0.05$). However, after 6 to 8 h of fermentation, the antioxidant activity of all the samples improved but was not significantly different with IC_{50} values of 13.96, 14.38, and 15.12 mg/mL for MP-YC, MP-BY, and MP-BT yoghurt, respectively, whereas MP-LA had the lowest antioxidant activity at 8 h (IC_{50} = 16.03

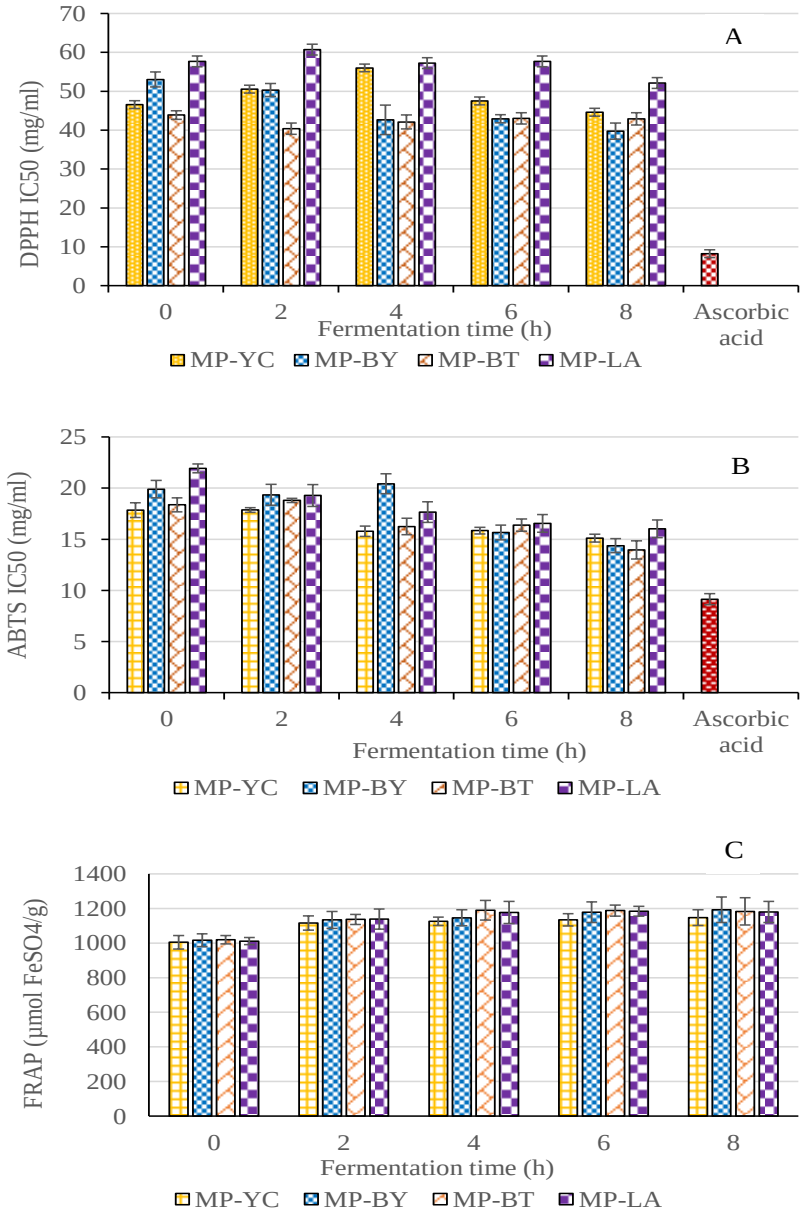

Figure 2. Yoghurt antioxidant activity assessed by DPPH **(A)**, ABTS **(B)**, and FRAP **(C)** fermented by different mixed cultures.

mg/mL). The highest IC_{50} value was observed in MP-BT (13.96 mg/mL), while the IC_{50} of ascorbic acid as the antioxidant standard was 9.14 mg/mL.

The FRAP assay measures the ability to reduce Fe^{3+}-TPTZ (ferric tripyridyltriazine) iron complexes of antioxidants. MP-BY and MP-BT yoghurts were significantly more potent for reducing ferric to ferrous ions compared to plain yoghurt MP-YC. The FRAP values of all yoghurt samples increased after fermenting for 2 h, and were then stable between 2 and 8 h of fermentation. The FRAP values of MP-YC, MP-BY, MP-BT, and MP-LA at 8 h fermentation were 1147.23, 1192.37, 1183.19, and 1179.64 µm $FeSO_4$/g dw, respectively, and not significantly different (p > 0.05). Antioxidant compounds increased during fermentation. This finding was supported by Shori *et al.* (2022), who found that total phenolic and flavonoid contents of yoghurt fermented by *L. casei* ATCC 393 increased, and these compounds expressed antioxidant properties. Probiotics can chelate positively charged minerals such as iron, zinc, copper, cobalt, and manganese. The chelating capacity of probiotic strains is due to the physiological chelators that exist in the intracellular cell-free extract of probiotics. YC and probiotics contain antioxidant enzymes including superoxide dismutase that catalyses the decomposition of superoxide free radicals into

hydrogen peroxide and water. This enzyme acts as a regulator of free radical formation (Landis and Tower, 2005).

Specific probiotic strains, such as *L. acidophilus* and *B. animalis* subsp. *lactis*, play pivotal roles in boosting antioxidant activity, by directly scavenging free radicals, and enhancing the body's endogenous antioxidant defences through increased production of antioxidant enzymes like superoxide dismutase and catalase (Akan, 2022). Research demonstrated that probiotic combinations significantly improved the yoghurt's antioxidant potential, largely due to increased levels of bioactive peptides and fermented metabolites with strong radical-scavenging activity (Famelart *et al*., 2024).

Effect of starter and probiotic type on yoghurt texture

The texture profile analysis (TPA) parameters including firmness, consistency, and cohesiveness of yoghurt samples are presented in Table 2. Firmness values of all the yoghurt samples were significantly different ($p \leq 0.05$), ranging from 3.79 to 8.06 N. Changes in texture characteristics during yoghurt fermentation were recorded after incubation at 42°C for 2 h. Firmness or hardness is an important yoghurt property that controls its quality and acceptability. Adequate firmness without syneresis is essential for good yoghurt quality. Yoghurt jelly-like texture results from the three-dimensional network structure of milk proteins induced by decreasing pH and increasing acidity during milk fermentation. The firmness values of yoghurt samples in the present work were comparable with results reported by Yilmaz-Ersan and Topcuoglu (2022). They found that yoghurt prepared from reconstituted skim milk powder had firmness of 267.70 g (or 2.62 N), higher than the average value of industrial yoghurt (1.55 N) determined by Kose and Yil (2018). Firmness of fermented milk products is impacted by many factors including the starter culture, total soluble solid, and protein content of the product. Higher hardness of yoghurt was related to longer incubation time, while lower yoghurt incubation time adversely impacted the textural quality (Sah *et al.*, 2016).

The cohesiveness values of all the yoghurt samples were not significantly different ($p > 0.05$), ranging between -1.39 and -1.59 N. Our results were higher than reported by Costa *et al.* (2015), who found that the cohesiveness value of natural yoghurt was -0.29 N (-30.29 g). This might have occurred due to their having comparable proximate milk protein content after preparation from whole milk powder. The consistency of the samples was also not significantly high in yoghurts with added probiotics compared with the control at the end of fermentation (8 h).

Effect of starter and probiotic type on yoghurt syneresis

Syneresis or whey separation is a textural defect in yoghurt affected by coagulum fracture, due to low protein and fat contents, and high mineral content. In the present work, yoghurt syneresis values were significantly different ($p \leq 0.05$) after fermentation for 8 h. Lowest syneresis values were found in MP-YC and MP-LA (21.36 and 17.41%, respectively), corresponding to the higher firmness of MP-YC and MP-LA than MP-BY and MP-BT. These results concurred with Yonezawa *et al.* (2010), who found that fermented milk prepared using multi-strain probiotics had better texture and nutrition than milk produced using single-strain probiotics.

The TPA results highlighted the differences, with probiotic yoghurts (MP-BY and MP-BT) demonstrating lower firmness and higher syneresis than in conventional yoghurt and MP-LA due to many factors. Specific probiotic strains produce less acid compared to conventional yoghurt cultures, resulting in a softer texture. Probiotics interact with milk proteins less effectively than standard yoghurt cultures, leading to less coagulation and a creamier, less firm product. These findings aligned with Cui *et al.* (2021), who reported that the firmness of yoghurt made from cow milk significantly decreased with the addition of probiotics.

Effect of starter and probiotic type on yoghurt colour

Colour values of the yoghurts are shown in Table 2. The type of bacteria and probiotic combination had no effect on the colour values. This result concurred with Mani-López *et al.* (2014), who reported colour values of yoghurt ranging from 74.06 to 77.94 for lightness (L*), -3.01 to -3.14 for redness (a*), and 11.18 to 10.42 for yellowness (b*). The whiteness in milk results from the presence of colloidal particles such as milk lipid globules and casein micelles which scatter light in the visible spectrum. The yoghurt samples had negative a* values indicating greenness, and positive b* values indicating yellowness, due to the presence of

Table 2. Texture profile analysis, syneresis, and colour values of yoghurts fermented by different mixed cultures at 8 h of fermentation.

Treatment	Firmness (N)	Consistency (N.sec)	Cohesiveness (N)	Index of viscosity (N.sec)	Syneresis (%)	Colour		
						L*	a*	b*
MP-YC	8.06 ± 0.69^{a}	8.67 ± 0.82	-1.51 ± 0.10	-2.35 ± 0.08	21.36 ± 0.67^{b}	74.06 ± 0.45	-3.01 ± 0.04	10.42 ± 0.07
MP-BY	3.79 ± 0.49^{b}	9.16 ± 0.22	-1.59 ± 0.18	-2.64 ± 0.25	28.25 ± 1.99^{a}	77.94 ± 0.74	-3.11 ± 0.03	11.01 ± 0.06
MP-BT	4.20 ± 0.32^{b}	8.18 ± 1.08	-1.39 ± 0.08	-2.41 ± 0.40	31.61 ± 2.29^{a}	74.86 ± 3.91	-3.14 ± 0.19	11.18 ± 0.32
MP-LA	7.12 ± 1.22^{a}	9.19 ± 0.27	-1.58 ± 0.27	-2.40 ± 0.17	17.41 ± 1.10^{c}	75.48 ± 0.61	-3.02 ± 0.02	10.78 ± 0.02

Lowercase superscripts in similar columns indicate significant difference ($p \leq 0.05$).

beta-carotene and other carotenoids in dairy products. The colour of yoghurt varies depending on many factors, but mainly depends on the pigments in the cow feed.

Conclusion

Yoghurt fermented using a mixture of yoghurt bacteria and probiotics had significantly higher melatonin content and antioxidant activity than conventional yoghurt. The combination of four strains (*S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *B. animalis* subsp. *lactis*, and *L. acidophilus*) yielded the highest melatonin content at the end of fermentation. The tryptophan content of all yoghurt samples also increased. The combination of probiotic cultures with yoghurt cultures increased antioxidant activity evaluated using DPPH, ABTS, and FRAP. The combination of multi-strain YC and probiotics had comparable TA and pH values, colour values (L*, a*, and b*), and TPA (consistency and cohesiveness) to conventional yoghurt, whereas the addition of probiotics altered some physicochemical and textural properties. Probiotic yoghurt enriched with melatonin and tryptophan, as well as high antioxidant activity, emphasised health benefits over texture. The present work highlighted the potential of functional yoghurt containing melatonin to improve sleep quality, boost immune function, and reduce oxidative stress. These findings position yoghurt as a functional food rich in melatonin, tryptophan, antioxidants, and probiotics, providing valuable insights for the dairy industry in developing new products through optimal strain combinations.

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