藍藻沈澱物作為功能性食品的抗氧化能力和益生元特性

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摘要

藍藻之所以作為膳食補充品是因富含蛋白質、碳水化合物、脂質、維生素和色素等營養物質，因此，在食品工業中被廣泛地應用。 雖然藍藻有不同的研究在探討蛋白質萃取的方式，將其活性成分所具有的抗氧化和益生元特性應用在乳製品上，但現有文獻中還沒有關於藍藻沈澱物的理化特性研究。本主題為首次研究萃取蛋白質後的藍藻沈澱物具有的抗氧化活性、發酵試驗和益生元的生長特性。其研究結果顯示藍藻沈澱物具有抗氧化、清除自由基、螯合亞鐵離子能力和還原三價鐵能力的作用。 此外，加入藍藻沈澱物的優格顯著影響其乳酸乳球菌和雙叉雙歧桿菌等益生菌的生長，而縮短發酵時間。 總結來說，藍藻沈澱物具有的天然成分在作為優格和相關產品的營養和功能特性上具有其開發潛力。

關鍵詞：藍藻沈澱物、抗氧化、益生菌、優格

Antioxidant Capacity and Prebiotic properties of *Spirulina Platensis* Precipitate as a Functional Food

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Abstract

Spirulina is a well-known source of valuable food supplements due to its richness in nutrients such as proteins, carbohydrates, lipids, vitamins, and pigments. *Spirulina platensis* is widely applied technologically and functionally in the food industry. Although there are different studies on the antioxidant and prebiotic properties of *Spirulina platensis* and on applying different methods of protein extraction with bioactive compounds to functional dairy food, researches on the physical and chemical characteristics of *Spirulina platensis* precipitate (SPP) have not been found in the literature. This study was the first attempt to use SPP after protein extraction to assess antioxidant activities, fermentation tests, and the growth of probiotic bacteria. The in vitro study showed that SPP showed antioxidant, radical scavenging, and ferric iron chelating activities, and reduced ferric ions. In addition, SPP significantly affected the growth of *Lactococcus lactis* and *Bifidobacterium bifidum* of probiotic bacteria in SPP-enriched yogurt to decrease the fermentation time. Overall, SPP has great potential as a natural ingredient with nutritional and functional properties of yogurt and related products.

Keywords: *Spirulina platensis* precipitate (SPP), Antioxidant activities, Probiotic bacteria, Yoghurt

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I. Introduction

Microalgae have been considered a source of food and functional food products to supply under-exploited crops in the human diet. Cultivation of microalgae has a superior yield than that terrestrial crops without competing with land and resources. Microalgal-derived proteins have higher essential amino acid profiles than meat, poultry, and dairy products [1]. In recent years, many studies have shown the impact of these microalgae supplementation on animal or human health, such as the hypolipidemic effect , protective effect against diabetes and obesity [2], antihypertension [3], and an inhibitory effect of leukemia and anemia induced from cadmium toxication [4].

Blue-green algae (cyanobacteria), a simple prokaryote, are one of the primitive life forms on Earth. A few species of edible blue-green algae, including *Nostoc*, *Spirulina*, and *Aphanizomenon*, have been used as food for over a thousand years[5]. *Spirulina platensis* are unicellular and filamentous blue-green algae which was classified into the genera of *Spirulina*, belonging to the oxygenic photosynthetic bacteria. The *Spirulina* is the best source of single cell protein (SCP) characterized by the composition of the biomass and digestion easily [6]. The blue-green alga *Spirulina platensis* has shown that high nutritional content attributes by more than 70% protein, amino acid, essential fatty acids, minerals, and vitamins to meet the dietary requirements of the growing population [7]. Microalgae develop a natural protective system, such as producing antioxidants and pigments (chlorophylls, carotenes, and phycobiliproteins) due to exposure to free-radical and high oxidative stresses in extreme environmental conditions [1]. Phycocyanins, belonging to phycobiliproteins produced from *Spirulina platensis*, have demonstrated the properties of hepatoprotective, anti-inflammatory, and antioxidant for the benefit of human beings [8].

One of the most commonly used strategies is serial extraction steps, in combination with a disruption step to degrade the cell wall to facilitate protein extraction [9]. The culture conditions such as PH, ammonium sulfate concentration, resuspension volume/initial volume ratio, and strain affect vary of microalgae cultivation. Rempel et al.[10] and Salla et al. [11] reported that the biomass of *Arthrospira platensis* possesses valuable composites and a higher biochemical propensity to carbohydrate and protein accumulation. A study conducted that hydrolysate of the residual fractions from *Arthrospira platensis* protein extraction existed nutrient to promote yeast growth and ethanol production sufficiently [12]. Therefore, it is crucial to allocate their fractions to different processes of microalgae in full for application to different products.

Currently, probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* are reported to be beneficial for the gastrointestinal tract and utilized for probiotic fermented milk products [13]. Meanwhile, the contained probiotics food as healthy food is considered to be useful bacteria with the prebiotics [14]. Many studies have shown that adding *Spirulina* promotes the growth of probiotic or lactic acid bacteria [15]. *Spirulina platensis* significantly enhances the growth of intestinal *Lactobacillus* [16]. So, another important issue is the effect of the residual fractions from *Spirulina platensis* protein extraction on the probiotics bacteria throughout fermentation. The aim of this study was to investigate the antioxidative activity of *Spirulina platensis* precipitate from protein extraction on the growth of *Lactococcus lactis* and probiotics bacteria in yogurt.

II. Materials and methods

1. Preparation of *Spirulina platensis* precipitate (SPP)

SPP obtained from the protein extraction process of *Spirulina platensis*. The crude extract protein was dissolved in 10 times the volume of pure water and repeated freeze-thaw cycles at -25°C. Protein extraction was

conducted by centrifuging at 17,000 x g to obtain phycocyanins, and then the precipitate was reserved and collected. Next, the precipitate froze at −18 °C and lyophilized to powder, defined as SPP.

2. Total antioxidant activity

Total antioxidant activity was conducted by the 3-ethylbenzothiazoline-6-sulfonic acid diammonium salt radical cation (ABTS) test described by Re et al. [17] with a slight modification. The ability of the SPP to scavenge ABTS.+ radical cation was compared to that of the butylated hydroxyanisole (BHA) as the standard antioxidant. The total time needed to carry out each assay was approximately 6 min, including ABTS radical generation by peroxidase, adding antioxidants, and acquiring the final absorbance value. The absorbance decrease was determined from the difference between the A730 values before and 5 min after sample addition. Antioxidant activity was calculated as moles of ABTS radical dot+ quenched by 1 mol of Trolox.

3. DPPH radical scavenging activity

The DPPH radical scavenging activity was conducted by the method of by Brand-Williams et al. [18]. The capacity of SPP to scavenge the lipid-soluble 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, which results in the bleaching of the purple color, absorbance was read at 517 nm. The initial concentration of DPPH radicals was 100 μM for all antioxidant–radical reactions. The antioxidant–radical reactions were carried out for 5 min in the dark at ambient temperature.

4. Fe^{2+} chelation assay

The ferrous ion chelating activity was studied by using the method described by Puntel et al. [19]. The reaction mixture (2.15 ml) was shaken vigorously and left at room temperature for 5 min containing 500 μl CAF (0–20 mg ml−1), 50 μl FeCl2 (2 mM), and 1.6 ml deionized water. 100 μl of ferrozine (5 mM in methanol) was added, mixed, and left for another 5 min to complex the residual Fe^{2+} and measured the absorbance of the Fe^{2+}–ferrozine complex at 562 nm against a blank. EDTA was used as a positive control.

5. Fe^{3+} reducing activity assay

Fe^{3+} reducing ability of SPP, the $Fe^{3+}(CN-)6 - Fe^{2+}(CN-)6$ reduction method was used by Gülçin et al.[20]. The reaction mixtures contained 0.8 mL acetate buffer (50 mM, pH 4.5), 0.4 mL Ferrozine solution (1%, w/v), 0.75 mL sample, and 0.05 mL $FeCl_3$ (20 mM, freshly prepared). The reactions were conducted for 15 min. The absorbance value was recorded at 700 nm.

6. Fermentation test

The fermentation tests with the inoculation of SPP in 5L tank, which the volume of inoculum is 10% (V/V) with a *Lactococcus lactis* counts of $2.13*10^7$ CFU/mL at 37°C (pH 5.5) for 48 hours with anaerobic fermentation was carried out.

7. Experimental design of SPP- enriched yoghurt in probiotic bacteria

The fresh whole milk 200 g was mixed with different probiotic bacteria (1 or 2g) with the homogenizer using an automatic yogurt maker at 42 °C for different durations while observing the solid yogurt. Freeze-dried probiotic bacteria powder obtained from Grape King Bio Ltd. was used in this study. Different probiotic bacteria and treatments of spirulina-enriched yogurt were designed as detailed below.

Number	Probiotic bacteria
BL-905	Bifidobacterium bifidum
BL-907	Bifidobacterium infantis
MZ-196	Lactobacillus salivarius
ML-059	Lactococcus lactis

Treatments	Details
Control	Plain yoghurt without the addition of SPP
SPP1	Yoghurt + 0.2% SPP powder
SPP2	Yoghurt + 0.4% SPP powder
SPP3	Yoghurt + 0.6% SPP powder

8. Color evaluation

The evaluation of the color samples was carried out using a colorimeter (NE 4000, NIPPON DENSHOKU, Japan). The CIE L*a*b* color space values were recorded were recorded after three-day of the sampling time.

9. Instrumental texture analysis

Yogurts were analyzed for texture parameters. Texture Analyzer, Brookfield CT-3 (U.S.A), measured texture parameters like hardness, adhesiveness, cohesiveness, springiness, chewiness, and gumminess. Textural properties were analyzed (TPA texture analysis) by performing two sequential compression tests with a cylindric-shaped probe set target of 2.5 mm and separated by a rest phase of 5 s. All the measurements were carried out in triplicate. Texture parameters were calculated using the software provided by TexturePro CT software (version 1.3).

10. Statistical analysis

Results are presented as mean values ± SD. Each antioxidant activity assay was done three times to determine the measurements reproducibility. The data in the experiments were analyzed statistically by one-way ANOVA and Duncan's s multiple range test using SPSS (version 21.0).

III. Results and Discussion

1. Antioxidant activity or Antioxidant capacity of SPP

(1) ABTS (2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) scavenging activity

This study demonstrates the effect of the SPP on ABTS (cationic and hydrophilic radicals) based on the BHA as standard (Fig. 1). The figure generally obtained the free radical scavenging exhibited a high antiradical activity by the SPP. The degree of hydrophobic and hydrophilic types, antioxidant amino acids, and positive charges are considered to be the factors that influence hydrolysates on free radicals scavenging [21]. In this study, the antioxidant activity of SPP on radical scavenging was found.

(2) DPPH (1,1-diphenyl-2-picrylhydrazyl), ferrozine (3-(2-pyridyl)-5-6-diphenyl-1,2,4-triazine-4′,4″ disulfonic acid sodium salt) free radical scavenging activity

As shown in Fig. 2, the scavenging activities of SPP on DPPH radicals (anionic and lipophilic radicals) exhibited a high antiradical activity. It was reported that phycocyanin extracted from cyanobacteria had a strong antioxidant property, which could be explained mainly by Selenium-containing phycocyanin (hexamer, (αβ)6), commonly found aggregate of phycobiliproteins [22]. Moreover, unlike other plants, S. platensis is a source of

large-scale commercial production of organic Se [23] and organic Se is mainly distributed in water-soluble protein molecules [24]. The result, from this study, the antioxidant activity of SPP supported by DPPH radical-scavenging activity.

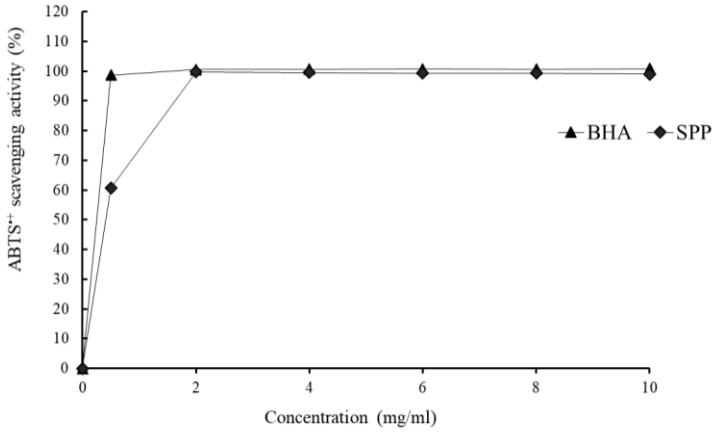

Fig.1 *Antiradical activity of SPP by ABTS assays*

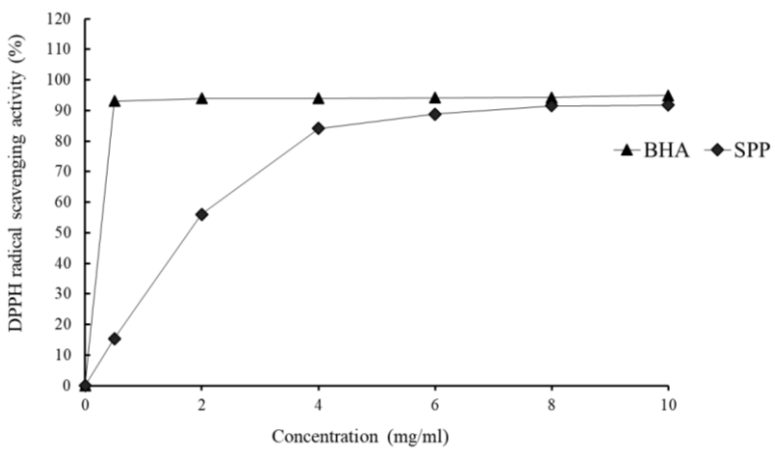

Fig.2 *Antiradical activity of SPP by DPPH assays*

(3) Fe^{2+}chelation activity

One of the strategies for reducing oxidative chain reactions and factors threatening is the removal of these metal ions, which can eliminate or decrease lipid oxidation of food for human health [25]. Akbarbaglu et al. conducted a study to find the type and concentration of *Spirulina platensis* peptides on the chelating activity of Fe and Cu peroxidation ions. Various factors affected the chelation activity, such as the release of amino acids, alkaline types, and more access to carboxylic and amino groups through different Enzymatic hydrolysis [26]. The results of this study suggested a significant effect of SPP from protein extraction on the chelating activity of Fe peroxidation ions (Fig. 3).

(4) Fe^{3+} reducing activity

The reducing properties are associated with the presence of compounds, which exert their action by breaking the free radical chain by donating a hydrogen atom [27]. Many studies have reported the phenolic compounds to have redox properties due to acting as reducing agents, hydrogen donating ability, and singlet oxygen quenchers, indicating the antioxidant capacity of phenolic compounds [28]. The reducing power of antioxidants causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous (Fe^{2+}). The reducing powers of SPP are shown in Fig.4. Thus, the SPP might have amounts of reductones, which may act as electron donors, react with free radicals, and terminate the radical chain reaction.

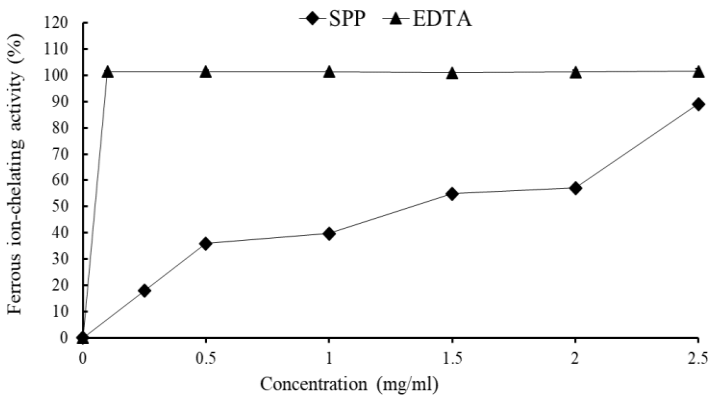

Fig. 3 *Chelating activity of Fe^{2+} ions by SPP*

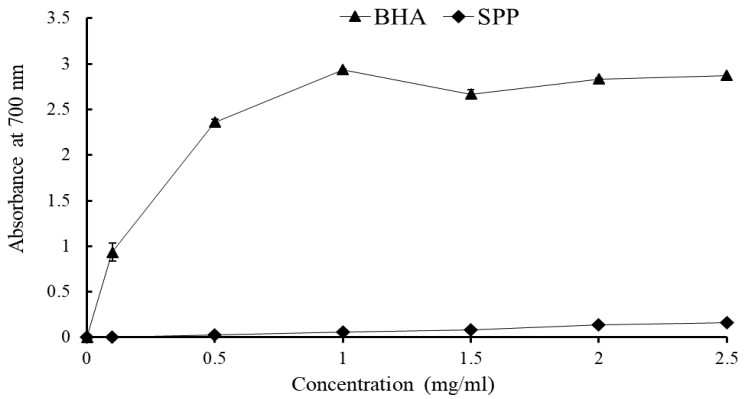

Fig. 4 *Fe^{3+}-reducing activity (FeRA) by SPP*

2. Nutritional analysis of SPP

Bensehaila et al. (2015) reported that *Spirulina platensis* contain a percentage of protein, lipids, and total sugars around 60.3%, 7.3%, and 17.6%, respectively. Lipids represent 6 to 8% of the dry weight from *Spirulina platensis*, giving the advantage of less lipid oxidation and rancidity phenomena easily. Meanwhile, lipids had high levels of polyunsaturated fatty acids, including linolenic acid (ω-6), which is a precursor of arachidonic acid [29]. Lipids are separated into a saponifiable fraction (83%) and a non-saponifiable fraction (17%), which of them are pigments, paraffin, sterols, and terpene alcohol [30]. The fatty acid profile of S. platensis depends upon the strain, mainly 18 carbon atoms of polyunsaturated fatty acids and as a good source of ϒ- linolenic acid (GLA). In addition, *Spirulina* also had lipid of monogalactosyl- and sulfoquinovosyl-diacylglycerol as well as phosphatidylglycerol [31]. The result of the nutritional analysis performed on SPP is by SGS in Taiwan as follows. The lipid of SPP was 8.50±0.01%, particularly palmitic acid (4.32±0.01%) and stearic acid (0.15±0.01%), measured as a percentage of its dry weight. Palmitic acid (16:0, PA) is the most common saturated fatty acid found in the human body, and its tissue content seems to be controlled around a well-defined concentration because the intake is counterbalanced by PA endogenous biosynthesis via de novo lipogenesis, not from changes in its intake influence its tissue concentration [32]. On the other hand, stearic acid is one of the most naturally abundant saturated fatty acids found to lower LDL cholesterol compared with other saturated fatty acids, but not when compared to unsaturated fatty acids [33].

3. Suitability of SPP for *Lactococcus lactis* Growth

As shown by bacterial counts of growth (Table 1), SPP in anaerobic fermentation demonstrated to be an

appropriate substrate for *Lactococcus lactis* growth. The recent study by Çelekli et al. (2019) focused on *S. platensis* at different percentages of concentrations on the growth of S. *thermophilus*, L. delbrueckii spp. bulgaricus, *L. acidophilus*, and *B. lacti* have significantly increased after fermentation [34]. *Spirulina platensis* has been reported to provide a perfect environment for the Bifidobacterium's survival through the entire functional milk storage time [35]. *Spirulina platensis* also improve the growth of lactic acid bacteria in synthetic media and dairy products [36].In addition, adding the lactulose had a beneficial effect on the growth of *Lactobacillus acidophilus* was better than *Bifidobacterium animalis subsp. lactis* in fermentation [37]. The results are in accordance with the abovementioned studies, which reported that SPP was highly favorable for the viability of *Bifidobacterium bifidum* within 48 hr during fermentation.

Table 1 *Fermentation test of SPP*

Group	Initial count (CFU/ mL)	48 hr
Control (MRS)	$2.13 *10^7$ CFU/ mL	$2.70 *10^8$ CFU/ mL
1% SPP in MRS	$2.13 *10^7$ CFU/ mL	$8.9 *10^8$ CFU/ mL

4. Fermentation time influence by SPP-enriched yogurt

The fermentation time of yogurt for *Bifidobacterium bifidum* (BL-905), *Bifidobacterium infantis* (BL-907), *Lactococcus lactis* (ML-59), *Lactobacillus salivarius* (MZ-196) was 24, 24, 24, and 18 hours, respectively. There are probiotics commonly used in fermented milk products, whose probiotic organisms have the benefit of protecting the intestine from the damaging effects of harmful bacteria. *Bifidobacterium* is a member of the dominant microbiota such as *Bifidobacterium bifidum* and *Bifidobacterium infantis* (i.e.,$>10^8$–10^9 colony forming unit (CFU)/g using culture methods), both in human feces and in the content of the caecal lumen by culture or quantitative PCR [38]. Another research on two lactic acid bacteria (LAB) strains, Lactococcus lactis BGBU1-4 and Lactobacillus salivarius BGHO1 showed the potential of LAB to ameliorate Listeria infections but suggested different immunological effects [39]. This study showed that *Bifidobacterium bifidum* with SPP in yogurt was observed at 0.6 percent, resulting in a significant decrease in the fermentation time of yogurt. The dose of SPP based on a previous study evaluated the sensory and textural properties of yogurt with different levels of spirulina (0.2%, 0.4%, 0.6%, and 0.8%) added [40].The previous study published that cultures of *Bifidobacterium animalis* have a higher concentration of oligosaccharides than the other bifidobacteria; however, *Bifidobacterium breve* showed higher β-galactosidase activities and had lower lactose concentrations after the fermentation process than the other yogurt [41]. Many authors reported the capacity of bifidobacteria to synthesize galacto-oligosaccharides[42] and survive in numbers higher than 10^6 cells/g of product to produce high lactase and transglycosylation activities for desirable organoleptic qualities [43]. In vitro study, exopolysaccharides significantly increased the growth of Lactobacillus sp. and Bifidobacterium sp. and increased the production of short-chain fatty acids for 48 h fermentation. Thus, exopolysaccharides may enhance various other functional properties of yogurt, such as textural, rheological, antioxidant, and prebiotic potential [44]. Moreover, a study of the potential of *Spirulina* for a stimulatory effect on the microflora of probiotic yogurt showed the viability of *Bifidobacterium bifidum*, *S. salivarius ssp*. *thermophilus* and *L. delbrueckii ssp*. *Bulgaricus* enhanced by the addition of cyanobacterial biomass, indicating that bioactive substances in *Spirulina* provide an excellent view of functional dairy foods [45]. Similar results as the reduction of fermentation in mixing of SPP at 6 % of yogurt was observed, speculated that the *Bifidobacterium bifidum* of yogurt would contribute to fermentation time, even the number of viable probiotic bacteria.

5. Color measurement of SPP-enriched yogurt

Color parameters (L*, a*, and b*) of yogurt samples were measured due to influence consumer preferences

as well as the shelf life of products. Table 2 shows the different scores towards color for control and other groups of SPP-enriched yogurts. The L* attributes show that control yogurts have higher lightness values than those of 0.2%, 0.4%, and 0.6% SPP-enriched yogurts ($p < 0.05$). The samples of SPP-enriched yogurt prepared with different percentages of SPP powder were characterized by the lowest a* and b* values ($p < 0.005$), which indicates that the yellow color decreased toward a greenish color. This could be explained mainly by partly existing SPP in chlorophylls, allophycocyanin, or c-phycocyanin. The addition of SPP into the yogurt changed the color of this milk product from green to blue based on the added SPP concentration. This result corresponded with the data reported by Barkallah (2017), the strong stability of Spirulina color. [46].

6. Texture analysis of SPP- enriched yoghurt

The mean (± S.D.) values of hardness, adhesiveness, cohesiveness, springiness, gumminess, and chewiness index of texture analysis of control and treatment yogurt (SPP 0.2%, SPP 0.4%, and 0.6% SPP) are presented in Table 3. However, no difference was observed with regard to hardness, adhesiveness, cohesiveness, springiness, gumminess, and chewiness index between the control and other SPP groups, which might be due to the low amount of SPP-enriched yogurt used in texture analysis. A study of the texture of yogurt in the 1% of *Spirulina platensis*, reported that have the highest total solid and protein content and decreased viscosity values during storage time [34]. The parameter of viscosity may be associated with the different value physicochemical characteristics of exopolysaccharides produced by the culture of the samples [35]. The effect of the increase over time on the viscosity of the yogurt was the formation of hydrogen bonds between the protein strands found in yogurt [47]. Additionally, Spirulina can enhance yogurt's viscosity through water absorption of extracellular carbohydrates produced, indicating it could reduce the content of stabilizers needed for yogurt manufacturing [48]. Furthermore, the addition of SPP could be sufficient to accelerate the end of fermentation and conserve the final milk product's textural properties and sensory acceptability.

Table 2 *Color analysis with chromameter (NE4000 L*a*b*)*

The percentage of SPP	L*	a*	b*
0	89.67 ± 0.08 [a]	1.29 ± 0.06 [a]	12.21 ± 0.18 [a]
0.2	84.75 ± 1.30 [b]	-1.54 ± 0.24 [b]	8.02 ± 0.68 [b]
0.4	79.20 ± 2.80 [c]	-2.88 ± 0.34 [c]	6.24 ± 0.47 [c]
0.6	79.21 ± 0.60 [c]	-3.147 ± 0.18 [c]	6.18 ± 0.34 [c]

Data are presented as the mean ± SD (n =3). Statistical analysis was performed using one-way ANOVA with Duncan's multiple-comparison test. Different letters indicate significant differences among groups at the level of $p < 0.05$.

Table 3 *Texture analysis of SPP-enriched yoghurt*

The percentage of SPP	Hardness(g)	Adhesiveness	Cohesiveness(ml)	Springiness(mm)	Gumminess(g)	Chewiness(ml)
0	29.00±1.50[a]	0.19±0.27 [a]	0.45±1.39[a]	0.82±0.28 [a]	11.9±40.5[a]	0.17±0.29 [a]
0.2	30.17±5.05[a]	0.24±0.04 [a]	0.17±0.02[a]	1.17±0.24 [a]	5.033±1.35[a]	0.06±0.005 [a]
0.4	33.5±13.54[a]	0.17±0.05 [a]	0.26±0.09[a]	1.14±0.1 [a]	9.6±6.58[a]	0.11±0.08 [a]
0.6	23.50±1.73[a]	0.29±0.14 [a]	-0.31±0.4[a]	0.87±0.11 [a]	-7.66±9.90[a]	0.07±0.09 [a]

Data are presented as the mean ± SD (n =3). Statistical analysis was performed using one-way ANOVA with Duncan's multiple-comparison test. Different letters indicate significant differences among groups at the level of $p < 0.05$.

IV. Conclusion

Our data have demonstrated the antioxidant activity found in the Spirulina platensis precipitate (SPP) after protein extraction. SPP still had great potential for enhancing the growth of probiotic bacteria and efficient use as an innovative and attractive additive in various food applications, including dairy and non-dairy products. In this study, the addition of SPP at different concentrations of yogurt impacted the growth of *Bifidobacterium bifidum* and the color of L*, a*, and b* values of SPP-enriched through fermentation time. SPP demonstrated that the part of the precipitate presented sufficient nutrients and nutritional values of ingredients for probiotic bacteria to perform the fermentation.

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