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Effects of *Spirulina platensis* Addition on Growth of *Lactobacillus plantarum* Dad 13 and *Streptococcus thermophilus* Dad 11 in Fermented Milk and Physicochemical Characteristics of the Product

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Abstract

Background and Objective: *Spirulina platensis* includes excellent bioactive compounds, which provide health-promoting effects. However, use of *Spirulina platensis* in foods includes limitations due to its unpleasant flavor and taste for some people. Fermented milk products include typical taste and flavor of fresh acid and can be combined with *Spirulina platensis*. Probiotics of *Lactobacillus plantarum* Dad 13 and *Streptococcus thermophilus* Dad 11 isolated from traditional buffalo milks can be used as starter culture strains for the fermented milks. This study investigated lactic acid bacterial growth, acid production and physicochemical characteristics of the fermented milks with *Spirulina platensis*.

Material and Methods: Milk fermentation was carried out using addition of various concentrations (0.15, 0.3, 0.45 and 0.6%) of *Spirulina platensis* powder and then microbial cell growth, acid production and antioxidant activity were investigated. Fermented milks with selected concentrations of *Spirulina platensis* were assessed within 24 h of fermentation at 37 °C using single and mixed cultures to study various aspects of cell growth, acid production, viscosity, water holding capacity and color.

Results and Conclusion: Increases of *Spirulina platensis* concentrations in fermented milks increased the microbial cell growth, acid production and antioxidant activity. During milk fermentation by adding 0.3% of *Spirulina platensis*, cells propagated and total lactic acid bacteria and probiotic cell counts reached to respectively 8.73-9.19 and 8.92 log CFU ml^{-1} after 24 h. The titratable acidity reached to 1.08% and pH decreased to 4.41. Viscosity increased significantly after 12 h of fermentation, compared to the controls. Fermented milks with *Spirulina platensis* addition by *Lactobacillus plantarum* Dad 13 and *Streptococcus thermophilus* Dad 11 cultures can be developed as alternative functional fermented milk products.

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1. Introduction

Spirulina (*S.*) *platensis* is a cyanobacteria species, which includes compounds of excellent nutritional values. It has been investigated for high protein contents (~74%) and essential amino acids (EAA); of which, leucine, valine and isoleucine are present at highest values [1]. Furthermore, that microorganism includes bioactive compounds such as pigments (phycocyanin, chlorophyll and carotenoid) [2], vitamins A, B, E and K, Fe, Ca, Mn, Zn and unsaturated fatty acids (UFA) [3]. Compounds of *S. platensis* have been shown to provide health-promoting effects such as antioxidant [4], anti-cancer, anti-inflammatory, anti-viral and immune system booster effects [5]. Introduction of *S. platensis* in food products includes public resistance due to the unpleasant flavor and taste of *S. platensis* for some people, caused by the oxidation of UFAs and minerals that produces metallic off-flavors [6]. Fermented milk products include typical taste and flavor of fresh acids, resulted from carbonyl compounds [7]. Thus, it may be combined with *S. platensis*. Furthermore, fermented milk is the most popular functional food with high acceptance and is regularly consumed by people on long-term diets [3]. Several studies have assessed benefits of *S. platensis* in dairy products. Beheshtipour et al. [6] reported that the addition of 1% of *Arthrospira* (*A.*) *platensis* powder into yogurts increased cell growth of the probiotic mixture of *Lactobacillus* (*L.*) *acidophilus* LA-5 and *Bifidobacterium lactis* BB-12 (reaching up to 8.42 log CFU ml^{-1}). Pan-utai et al. [8] showed that yogurt fermentation by 0.5% of *A. platensis* IFRPD 1182 and mixed cultures of *L. bulgaricus* YC-X11 and *Streptococcus* (*S.*) *thermophilus* YC-X11 produced a higher acidity than that the control yogurt did. Moreover, addition of *S. platensis* into the fermented milk can improve its physical characteristics such as increasing viscosity and water holding capacity (WHC) [9-11] and chemical characteristics such as contents of minerals and antioxidants [12]. However, ability of lactic acid bacteria (LAB) to utilize *S. platensis* biomass varies within various microbial strains and fermentation conditions.

Usually, LAB are widely used in Indonesian traditional fermented foods. Several studies have isolated indigenous LAB from Indonesian fermented foods [13-16]. In fact, *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 are indigenous LAB isolated from dadih, an Indonesian fermented buffalo milk. The authors' recent studies reported that *L. plantarum* Dad 13 potentially served as a probiotic strain because it could survive in volunteers' digestive tract, increase populations of *Lactobacilli* and *L. plantarum* in faeces of healthy people and prevent growth of pathogenic *Escherichia coli* [14,17]. Safety aspects of this probiotic strain have been studied [15]. It can be inoculated as a single starter or mixed culture to produce probiotic fermented milks [18,19]. Mixed cultures of *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 have been used as starter cultures in fermented milks, including sensory acceptance similar to that of commercial yogurts [20]. These cultures show their antioxidant activity and ability to ferment black soymilks [13]. Effects of *S. platensis* addition into the fermented milks with *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 are still unknown. Therefore, the objective of the current study was to investigate physicochemical characteristics and viabilities of *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 in fermented milks with *S. platensis*.

2. Materials and Methods

2.1 Materials

S. platensis powder (*Spirulina* TN57, Indonesia) and fresh milk (PT Sukanda Djaya, Indonesia) were purchased from the local supermarkets. The *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 were provided by the Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. De Mann Rogosa Sharpe (MRS) (Merck, Germany), bacteriological agar (Oxoid, USA) and calcium carbonate ($CaCO_3$) (Merck, Germany) were used for MRS media. Phenolphthalein (PP), 2,2'-diphenyl-1-1-picrylh-ydrazyl (DPPH) and molybdate reagent were purchased from Sigma-Aldrich, USA. Sucrose (PT, Sugar Group Company, Indonesia) and skim milk (PT, Mirota KSM, Indonesia) were purchased from the local supermarkets.

2.2 Bacterial Strain and Growth Condition

Culture stock was stored at -20 °C as 1:1 mixture with 20% of sterile aqueous sucrose solution and 10% of skim milk. Culture was inoculated in MRS broth and incubated at 37 °C for 24 h. Then, culture was inoculated using MRS agar deep tube at similar conditions and rejuvenated every two weeks as working cultures. For starter culture preparation, cultures from working cultures were inoculated in MRS broth media and incubated at 37 °C for 24 h. Then, cultures were reinoculated in skim media and incubated at 37 °C for 24 h. The LAB cell number in starter culture was calculated before inoculation into milk and expressed as colony-forming unit (CFU) ml^{-1}.

2.3 Milk Fermentation

The first stage included milk fermentation with the addition of various concentrations (0.15, 0.3, 0.45 and 0.6%) of *S. platensis* powder. Fermented milk was prepared based on the protocol by Barkallah et al. [12] with some modifications. Fresh milk, skimmed milk (2%), sucrose (8%) and *S. platensis* powder were mixed, pasteurized at 80 ºC for 30 min, cooled down and inoculated with the starter cultures (1% v v^{-1}). Fermentation proccess was carried out at 37 °C for 24 h using a single culture (either *L. plantarum* Dad 13 or *S. thermophilus* Dad 11) and mixtures of the two cultures. Cell count, titratable acidity (TA), pH and antioxidant activity of the milk were assessed before and after the fermentation (24 h). The selected concentration of *S. platensis* powder was further studied for its fermentation patterns (second stage). The cell counts, TA, pH, viscosity and color were assessed every 6 h during the fermentation. The WHC, minerals and phycocyanin contents were analyzed after 24 h of fermentation. For the milk fermentation using mixed cultures, numbers of LAB and *L. plantarum* were enumerated using MRS and LPSM agar media, respectively.

2.4 Enumeration of the Bacterial Population [19,21]

Number of LAB was calculated using serial dilution and pour plate methods and MRS agar containing 0.5% $CaCO_3$. Colonies with clear zones were counted after incubation at 37 °C for 48 h and expressed in colony-forming units (log CFU ml^{-1}). Enumeration of probiotic *L. plantarum* in mixed cultures was carried out using spread plate method on *L. plantarum* selective media (LPSM) agar. The LPSM agar was prepared based on a method by Bujalance et al. [21]. Colonies of *L. plantarum* were counted and expressed as colony-forming units (log CFU ml^{-1}) after incubation at 37 °C for 48 h.

2.5 Assessment of Acid Production [19]

Acid production was assessed using titratable acidity (TA) and pH measurements. The TA was measured via sample titration using 0.1 N NaOH with 1% of phenolphthalein as indicator. Moreover, pH measurement was carried out using pH meter (Eutech pH, USA). Calibration with standard buffer (pH 4 and 7) was carried out before the analysis.

2.6 2,2'-diphenyl-1-picrylhydrazyl Radical Scavenging Assay [22]

Sample preparation began with addition of 1 ml of sample into 5 ml of 70% methanol using shaker water bath (Sibata WS-240, Japan) at 120 rpm for 1 h at room temperature. Then, sample was macerated at 4 °C for 24 h in dark. This was centrifuged (Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany) at 3000 g for 15 min. The crude extract was collected and stored at -20 °C until the antioxidant activity analysis was carried out. The antioxidant activity was assessed based on the extract ability to scavenge 2,2'-diphenyl-1-picrylhydrazyl (DPPH). Sample extract (1 ml) was added to 3 ml of 0.1 mM DPPH solution and incubated for 30 min in dark. Methanol was used as control. The absorbance was measured using spectrophotometer (Shimadzu UV-1601, Japan) at 517 nm. The radical scavenging activity was calculated using the following equation:

$$RSA\ (\%) = \left(1 - \frac{samples\ absorbance}{control\ absorbance}\right) \times 100\% \quad \text{Eq. (1)}$$

2.7 Viscosity and Water Holding Capacity Assessments [23]

Viscosity of the samples were assessed using Brookfield Viscometer (Model DV2T, Brookfield Engineering Laboratories, USA) after stirring the product for 30 s at 4 °C. Samples were assessed using spindle no. 4 at 60 rpm. For the assessment of WHC, 5 g of the samples (Y) were transferred into a 15-ml tube and centrifuged at 4500 g for 10 min at 4 °C (Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany). The pellet was weighed (W) and WHC of the samples was calculated using the following equation:

$$WHC\ (\%) = \left(1 - \frac{W}{Y}\right) x\ 100 \quad \text{Eq. (2)}$$

2.8 Color Analysis of the Fermented Milks [8]

Color of the samples were assessed using colorimeter (Konica Minolta, Chroma Meter, CR400, Japan). The instrument was standardized using standard white plates. Color analysis was expressed from three values of lightness (L*) as 0−100 (black to white), a* as green (-) to red (+) and b* as blue (-) to yellow (+).

2.9 Mineral Content Analysis of the Fermented Milks [24,25]

Calcium (Ca), phosphorus (P) and iron (Fe) in the fermented milk products were analyzed. A sample of 5 ml was incinerated using muffle furnace at 550 °C for 6 h. Then, 50 ml of HNO_3 (1:3) were added to the ash, which was crushed until dissolved. Solution was filtered and the supernatant was collected. The Ca content was assessed using titrimetric method. Moreover, P and Fe contents were assessed using spectrophotometric methods.

2.10 Phycocyanin Content Analysis of the Fermented Milks [8]

Sample (1 ml) was centrifuged (Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany) at 5000 rpm for 10 min. Then, the supernatant was mixed with 1 ml of distilled water and sonicated (Eyela Sonicator Cleaner, Singapore) for 30 min. Supernatant solution was centrifuged (Centrifuge 5810 R, Eppendorf AG, Hamburg, Germany) at 10000 rpm for 5 min and the supernatant was collected and assessed using spectrophotometer (Shimadzu UV-1601, Japan) at 615 and 652 nm. Phycocyanin content was calculated using the following equation:

$$Phycocyanin = \frac{(\lambda\ 615\ nm - 0.474.\lambda\ 652\ nm)}{5.34} \quad \text{Eq. (3)}$$

2.11 Statistical Analysis

Results were provided as means ±SD (standard deviation). Analysis was carried out using one-way ANOVA and Duncan's multiple comparison test if significant differences were detected ($p < 0.05$). Furthermore, statistical analysis was carried out using IBM SPSS Statistics Software v.26 (IBM, USA) ($n = 4$).

3. Results and Discussion

3.1 Growth and Acid Production of LAB in Fermented Milk with Various Addition of Spirulina platensis

The initial LAB count was approximately 6.8 log CFU ml^{-1} and reached to 8.49 log CFU ml^{-1} after 24 h of milk fermentation at 37 °C for single and mixed cultures (Table 1). The higher the addition of *S. platensis*, the greater the number of LAB in the final products. Addition of *S. platensis* significantly increased ($p<0.05$) the cell growth.

Table 1. Cell count, titratable acidity, pH and antioxidant activity of the fermented milks with various quantities of *Spirulina platensis* biomasses, added before the fermentation (37 °C, 24 h)

Starter Cultures	Spirulina	Cell Counts (log CFU ml $^{-1}$)	Titratable Acidity (%)	pH-value	Radical Scavenging Activity (%) Initial	Radical Scavenging Activity (%) Final
Lactobacillus plantarum Dad 13 Total LAB	0%	8.33±0.13^{a}	0.88±0.02^{a}	4.96±0.01^{a}	16.02±0.99^{a}	34.05±1.81^{d}
	0.15%	8.65±0.10^{b}	1.02±0.03^{b}	4.85±0.04^{b}	17.97±1.14^{ab}	36.76±1.50^{e}
	0.3%	8.77±0.11^{bc}	1.04±0.06^{b}	4.77±0.04^{c}	19.43±0.50^{bc}	41.75±1.66^{f}
	0.45%	8.81±0.09^{c}	1.19±0.02^{c}	4.55±0.02^{d}	20.86±0.89^{c}	44.03±1.76^{g}
	0.6%	9.10±0.06^{d}	1.28±0.03^{d}	4.53±0.01^{d}	20.90±0.97^{c}	44.69±2.08^{g}
Streptococcus thermophilus Dad 11 Total LAB	0%	8.50±0.12^{a}	0.78±0.08^{a}	4.78±0.07^{a}	16.02±0.99^{a}	30.57±0.76^{d}
	0.15%	8.79±0.08^{b}	0.94±0.03^{b}	4.52±0.07^{b}	17.97±1.14^{ab}	33.32±1.25^{ef}
	0.3%	8.86±0.07^{bc}	1.05±0.03^{c}	4.33±0.02^{c}	19.43±0.50^{bc}	35.22±2.14^{f}
	0.45%	9.00±0.07^{cd}	1.23±0.02^{d}	3.89±0.04^{d}	20.86±0.89^{c}	40.58±2.44^{g}
	0.6%	9.08±0.12^{d}	1.27±0.01^{d}	3.81±0.01^{e}	20.90±0.97^{c}	42.12±0.63^{g}
Mixed Cultures Total LAB (*Lactobacillus plantarum*)	0%	8.63±0.05^{a} (8.21±0.11^{a})	0.85±0.02^{a}	4.61±0.06^{a}	16.02±0.99^{a}	31.09±2.77^{d}
	0.15%	8.79±0.04^{b} (8.38±0.21^{b})	1.10±0.06^{b}	4.33±0.02^{b}	17.97±1.14^{ab}	35.01±2.18^{e}
	0.30%	8.92±0.07^{c} (8.66±0.07^{c})	1.26±0.02^{c}	4.27±0.03^{c}	19.43±0.50^{bc}	37.10±1.92^{e}
	0.45%	9.07±0.09^{d} (8.76±0.10^{c})	1.33±0.03^{d}	3.75±0.03^{d}	20.86±0.89^{c}	42.23±1.79^{f}
	0.60%	9.16±0.04^{e} (8.80±0.05^{d})	1.34±0.03^{d}	3.66±0.04^{e}	20.90±0.97^{c}	42.63±1.25^{f}

* Results are provided as mean ± SD. Results for each strain with different superscripts were significantly different (p<0.05) from Duncan's multiple range test. Cell counts for single culture were measured as total LAB using MRS medium, while the mixed cultures were measured as total LAB using MRS medium and *Lactobacillus plantarum* using LPSM medium. The values in parentheses showed the cells count *of Lactobacillus plantarum* in fermented milk with *Spirulina platensis* addition.

Cell count of probiotic *L. plantarum* Dad 13 was slightly lower than the total count of LAB. Beheshtipour et al. [6] reported that cell growth of the probiotic mixtures of *L. acidophilus* LA-5 and *B. lactis* BB-12 in yogurts with *A. platensis* addition (up to 1%) was significantly greater than that in the control, which reached to 8.42 log CFU ml^{-1} after fermentation at 40 °C. Results reported by Yamaguchi et al. [26] showed mild increases in bacterial population of the mixed cultures of *L. acidophilus* LA-5, *B. lactis* BB-12 and *S. thermophilus* in freeze-dried yogurts by addition of *S. platensis* (3.4 × 10^{7} CFU g^{-1}), compared to control yogurt (2.2 × 10^{7} CFU g^{-1}). This study showed a higher population of LAB in fermented milk with addition of *S. platensis.* Fermentation of probiotic *L. plantarum* Dad 13 was optimum at 37 °C and hence called mesophilic LAB [19]. Celekli et al. [27] reported that growth of *S. thermophilus* in ayran added with various concentrations of *S. platensis* was not significantly different from that without addition of *S. platensis*, which was 8.78–8.9 log CFU ml^{-1} after fermentation at 42 °C. It can be assumed that the ability of LAB to utilize *S. platensis* biomass for their growth varies within various strains and fermentation conditions.

Natueally, LAB utilize lactose and other sugars for their growth and metabolic activities, producing acids. The initial TA was nearly 0.2%. After the milk fermentation without *S. platensis* addition, TA increased to 0.77–0.88%. Higher additions of *S. platensis* (0.15, 0.3, 0.45 and 0.6%) resulted in greater acid productions. The TA reached to 1.2 and 1.33% for single and mixed cultures, respectively (Table 1). Production of acid decreased pH. Results showed that the higher the *S. platensis* addition was, the lower pH of the fermented milk was. Moreover, addition of more than 0.3% *S. platensis* resulted in pH lower than 4.0. Pan-utai et al. [8] reported that yogurt fermentation by mixed cultures of *L. bulgaricus* YC-X11 and *S. thermophilus* YC-X11 with 0.5% *A. platensis* IFRPD 1182 addition at 40 °C for 4 h produced 0.78% TA and pH 4.21. Furthermore, TA and pH of control samples were 0.55% and 4.39, respectively. Bchir et al. [10] reported similar results as TA and pH of yogurts produced using mixed cultures *L. bulgaricus* and *S. thermophilus* PAL YOG 3-30 D and added with 0.5% of *S. platensis* were 0.8% and 4.7, respectively. Higher TA values were reported by Agustini et al. [11] as TA and pH of yogurts with 1% of *S. platensis* addition using mixed cultures of *L. bulgaricus*, *S. thermophilus* and *L. acidophilus* were 1.56% and 4.71, respectively after 6 h of fermentation at 43 °C. Positive effects of *S. platensis* on cell growth and metabolic activities might be due to the nutritional compounds of *S. platensis.* The *S. platensis* biomass includes amino acids (AA) [1], B-group vitamins and various minerals such as Mg, Fe and Mn [3], which include potentials as growth factors for *L. plantarum* Dad 13 and *S. thermophilus* Dad 11. Moreover, *S. platensis* biomass contains exopolysaccharides, hypoxa-nthine and adenine. These elements can stimulate growth and activity of LAB [3].

3.2 Antioxidant Activity of the Fermented Milks with Various Concentrations of Spirulina platensis

Results showed that higher *S. platensis* addition led to a higher antioxidant activity of the milk (Table 1). Milk fermentation by single or mixed cultures increased antioxidant activity of nearly two folds after 24 h of fermentation at 37 °C. However, increases in antioxidant activity during the fermentation was similar within various concentrations of *S. platensis* and starter cultures. It is noteworthy that antioxidant activity of *S. platensis* did not change during fermentation. These results were in line with phycocyanin contents of this study. The phycocyanin contents were relatively constant during milk fermentation in single and mixed cultures. Liu et al. [4] showed that antioxidant activity of the fermented milks containing 0.6% of *S. platensis* powder using mixed cultures of *L. acidophilus*, *B. bifidum*, *L. casei*, *B. infantis*, *B. longum* and *L. lactis* was ±33% after 48 h of fermentation. The current results showed higher antioxidant activities at shorter times of fermentation (24 h). Higher antioxidant activities were also reported by Barkallah et al. [12], who stated that yogurts with *S. platensis* addition of 0.25% using mixed cultures of *L. bulgaricus* YC-X11 and *S. thermophilus* YC-X11 included an antioxidant activity of 52.41% after 4 h of fermentation at 43 °C.

During the fermentation, antioxidant activity of the fermented milks without *S. platensis* addition significantly increased from 16.02 to 30.57–34.05%. It could occure because metabolic activity of the LAB produced bioactive compounds that acted as antioxidants. Fermentation of black soymilks using *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 increased their antioxidant activities due to the free aglycone isoflavone, produced from isoflavone glycoside hydrolysis by β-glucosidase [13]. Taha et al. [28] reported that fermentation of buffalo milk yogurts by mixed cultures of yogurt starters, *L. acidophilus* 20552 ATCC and *L. helveticus* CH 5 produced peptides with high antioxidant and antibacterial activities. It occured because these starter cultures produced enzymes that hydrolyzed milk proteins into bioactive peptides. Further studies are needed to investigate proteolytic activity and formation of the bioactive peptides in fermented milks using *L. plantarum* Dad 13 and *S. thermophilus* Dad 11. The present results showed that fermented milks containing various quantities of *S. platensis* biomass increased cell growth, acid production and antioxidant activity of samples after 24 h of fermentation. However, fermented milks containing more than 0.3% *S. platensis* biomass included a final pH of less than 4.0, which was too acidic and could affect physical and sensory characteristics of the product. Rani et al. [29] demonstrated that pH less than 4.0 could lead to body and texture defects such as gel shrinkage and syneresis. Therefore, addition of 0.3% *S. platensis* into milks prior to fermentation was selected for further studies.

3.3 Profile of Milk Fermentation with Addition of 0.3% of S. platensis: Cell Growth and Acid Production

Cell growth patterns of a single culture of *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 in fermented milks with 0.3% *S. platensis* biomass were similar to those without addition of *S. Platensis*; however, higher cell populations were achieved (Figure 1).

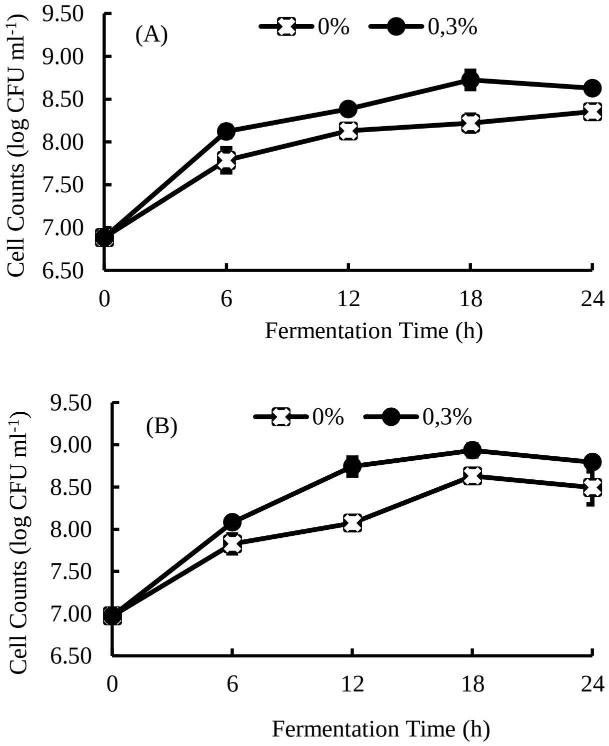

Figure 1. Cell growth patterns during milk fermentation with 0.3% of *Spirulina platensis* biomass using single cultures of (A) *Lactobacillus plantarum* Dad 13 and (B) *Streptococcus thermophilu*s Dad 11 (37 °C, 24 h)

Moreover, mildly different growth patterns were seen in milk fermentation with 0.3% *S. platensis* cultivated by the mixed culture (Figure 2). The LAB in milk fermentation with 0.3% *S. platensis* faster propagated and reached to the stationary phase, compared to the control sample which propagated rapidly until Hour 12 of the fermentation, while the control sample propagated until hour 18 of fermentation. Similar acid productions were achieved in fermented milks inoculated with single and mixed cultures. Acid production showed similar profiles of fermentation without and with *S. platensis* addition; however, higher TA and lower pH were achieved with addition of *Spirulina* biomass (Table 2). These results revealed that addition of *S. platensis* supported cell growth and metabolic activities. This was seen possibly because the cyanobacteria provided AAs and minerals that were essentials for these cultures.

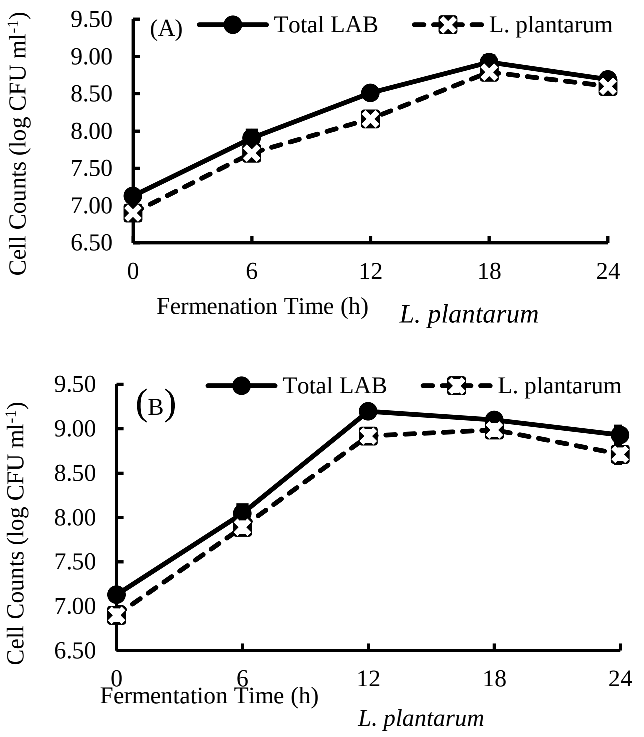

Figure 2. Cell growth patterns during milk fermentation (A) without *Spirulina platensis* and (B) with 0.3% *Spirulina platensis* addition using mixed cultures of *Lactobacillus plantarum* Dad 13 and *Streptococcus thermophilus* Dad 11 (37 °C, 24 h)

Biomass of *S. platensis* includes 17 AAs that can support growth of LAB [1]. Culture of *L. plantarum* needs arginine, glutamic acid, leucine, valine and cysteine or methionine, while S. *thermophilus* needs histidine and cysteine or methionine [30]. Furthermore, *S. platensis* includes several minerals such as Mg, Fe and Mn [3] needed by LAB for the enzymatic reactions [31].

3.4 Viscosity during Milk Fermentation with 0.3% Spirulina platensis Addition

Viscosity and WHC are important physical parameters in assessing quality of fermented milk products. Viscosity profiles during milk fermentation showed a similar pattern using either single or mixed cultures (Figure 3). Viscosity increased significantly ($p < 0.05$) after 12 h of fermentation. After this time, viscosity of the fermented milks with *S. platensis* addition was almost two folds greater than those without *S. platensis* addition. Viscosity of the fermented milks with single and mixed cultures increased from 2.04-2.44 Pa.s (without *S. platensis*) to 3.73-4.05 Pa.s (with 0.3% *S. platensis*). Moreover, WHC of the fermented milks with 0.3% *S. platensis* (64-67%) was mildly higher after 24 h of fermentation, compared to the controls (60-62%) (Figure 4).

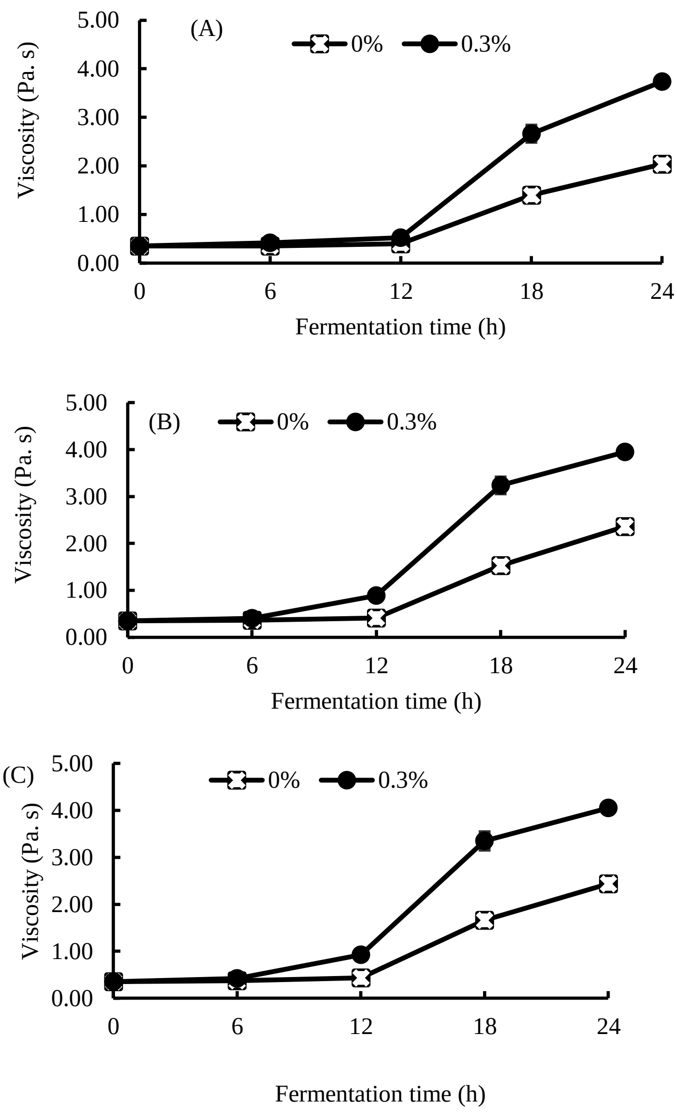

Figure 3. Viscosity patterns during milk fermentation with 0.3% *Spirulina platensis* addition using single cultures of (A) *Lactobacillus plantarum* Dad 13 and (B) *Streptococcus thermophilus* Dad 11 and (C) mixed cultures of *Lactobacillus plantarum* Dad 13 and *Streptococcus thermophilus* Dad 11 (37 °C, 24 h)

Bchir et al. [10] reported that addition of 0.3% of dried *S. platensis* into yogurts with *L. bulgaricus* and *S. thermophilus* PAL YOG 3-30 D significantly increased the viscosity to 0.85 Pa.s, compared to the control (0.70 Pa.s) after 4 h of fermentation at 42 °C. Atallah et al. [9] stated that addition of 1% of *S. platensis* into yogurts with a mixture culture of *L. bulgaricus* and *S. thermophilus* respectively increased viscosity and WHC to 16.3 Pa.s and 56% after fermentation at 42 °C. Results of the current study demonstrated higher WHC values than those results of Pan-utai et al. did [8], who reported that yogurts with mixed cultures of *L. bulgaricus* YC-X11 and *S. thermophilus* YC-X11 as well as 0.3% dried *S. platensis* addition included WHC values of nearly 55% after 4 h of fermentation at 43 °C.

Table 2. Acid production during milk fermentation with 0.3% *Spirulina platensis* addition at 37 °C

Strains	Fermentation Time (h)	Titratable Acidity (%) 0%	Titratable Acidity (%) 0.3%	pH 0%	pH 0.3%
Lactobacillus plantarum Dad 13	0	0.23±0.01[a]	0.23±0.01[a]	6.71±0.08[a]	6.71±0.08[a]
	6	0.39±0.07[b]	0.45±0.07[b]	5.83±0.01[b]	5.77±0.16[b]
	12	0.53±0.02[c]	0.57±0.04[c]	5.49±0.09[c]	5.42±0.06[c]
	18	0.78±0.06[d]	0.90±0.04[e]	5.28±0.04[d]	5.05±0.01[e]
	24	0.84±0.01[de]	0.99±0.06[f]	4.98±0.02[f]	4.6±0.01[g]
Streptococcus thermophilus Dad11	0	0.23±0.01[a]	0.23±0.01[a]	6.71±0.08[a]	6.71±0.08[a]
	6	0.41±0.04[b]	0.44±0.03[b]	5.93±0.06[b]	5.79±0.17[c]
	12	0.53±0.01[c]	0.68±0.05[d]	5.58±0.05[d]	5.30±0.20[e]
	18	0.83±0.06[e]	0.97±0.02[g]	5.30±0.10[e]	4.90±0.14[g]
	24	0.91±0.04[f]	1.06±0.03[h]	4.81±0.01[g]	4.45±0.08[i]
Mixed Cultures	0	0.23±0.01[a]	0.23±0.01[a]	6.71±0.08[a]	6.71±0.08[a]
	6	0.35±0.02[b]	0.50±0.03[c]	5.94±0.02[b]	5.88±0.04[b]
	12	0.60±0.05[d]	0.71±0.05[e]	5.56±0.04[c]	5.33±0.01[d]
	18	0.89±0.02[f]	0.97±0.03[g]	5.13±0.07[f]	4.72±0.06[f]
	24	1.00±0.03[g]	1.08±0.02[h]	4.64±0.01[gh]	4.41±0.05[g]

* Mixed cultures are mixture of *Lactobacillus plantarum* Dad 13 and *Streptococcus thermophilus* Dad 11. Results are provided as mean ± SD. Results in each strain with different superscripts were significantly different ($p<0.05$) from Duncan's Multiple Range Test

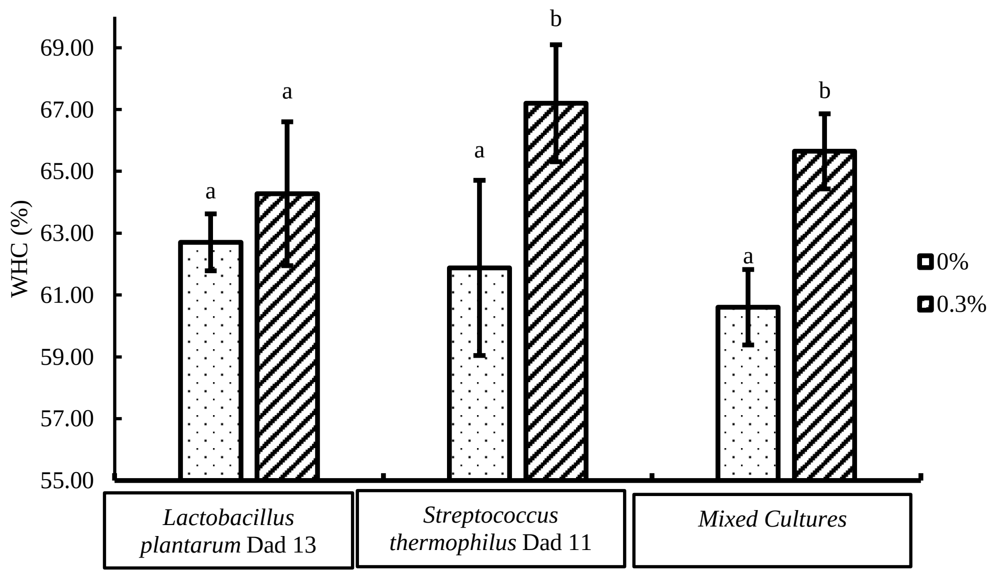

Figure 4. Water holding capacity of the fermented milks with 0.3% *Spirulina platensis* addition after 24 h of fermentation at 37 °C. Values are expressed as mean ±SD. Values of the strains with different superscripts are significantly different ($p<0.05$)

The present results were similar to those from yogurts with addition of gelling agent, stabilizer and emulsifier. Sahan et al. [32] reported that yogurts with the addition of hydrocolloid ß-glucan concentrates up to 1% included viscosity of nearly 4 Pa.s after fermentation at 43 °C. By adding *S. platensis* biomass, fermented milk products potentially do not need addition of gelling agents, stabilizers and emulsifiers to further process. Addition of 0.3% *S. platensis* increased viscosity and WHC of the fermented milks. This might be due to the exopolysaccharides produced by *S. platensis.* Sed et al. [33] reported that the addition of 0.5% of exopolysaccharides from *S. platensis* to cream products could increase their stabilizing and emulsifying characteristics, resulting in better texture characteristics. Polysaccharides of *S. platensis* included rhamnose as the major component and reached to 53%, which contributed greatly to viscosity, stabilizing and emulsifying characteristics of the polysaccharides [34].

3.5 Color Changes during Milk Fermentation with 0.3% Spirulina platensis Addition

Color changes were seen during milk fermentation, when *S. Platensis* was added. Table 3 shows that L*, a* and b* values significantly ($p<0.05$) increased until hour 18 of fermentation and then was relatively constant until the end of fermentation in single and mixed cultures. Color of the fermented milks changed from blue-green to green after 24 h of fermentation. Martelli et al. [35] reported that L*, a* and b* values increased in skimmed milk media with mixed cultures of *S. thermophilus*, *L. lactis* and *L. helveticus* with 0.25% *S. platensis* powder addition after 24 h of fermentation at 37 °C, meaning that color changed to green.

Table 3. Color Changes during milk fermentation with 0.3% *Spirulina platensis* addition for 24 h at 37 °C

Strains	Fermentation Time (h)	Color L*	a*	b*
Lactobacillus plantarum Dad 13	0	66.61±1.19[a]	-7.63±0.10[a]	2.94±0.28[a]
	6	66.02±0.80[a]	-7.39±0.09[b]	2.91±0.16[a]
	12	67.27±0.53[a]	-7.35±0.17[b]	4.03±0.12[b]
	18	74.97±1.01[b]	-6.83±0.15[c]	6.03±0.05[c]
	24	74.64±0.88[b]	-6.69±0.04[c]	7.11±0.68[d]
Streptococcus thermophilus Dad 11	0	66.61±1.19[a]	-7.63±0.10[a]	2.94±0.28[a]
	6	66.10±0.65[a]	-7.63±0.02[a]	3.18±0.50[a]
	12	66.92±0.69[a]	-7.02±0.07[b]	4.63±0.15[b]
	18	74.25±0.97[b]	-6.89±0.07[c]	6.92±0.10[c]
	24	74.68±0.67[b]	-6.67±0.08[d]	7.35±0.13[d]
Mixed Cultures	0	66.61±1.19[a]	-7.63±0.10[a]	2.94±0.28[a]
	6	66.30±0.58[a]	-7.77±0.07[a]	3.27±0.37[a]
	12	67.03±0.74[a]	-7.32±0.24[b]	6.21±0.47[b]
	18	74.74±1.05[b]	-6.75±0.13[c]	6.93±0.31[c]
	24	74.40±0.55[b]	-6.61±0.05[c]	7.39±0.06[c]

* Mixed cultures are mixture of *Lactobacillus plantarum* Dad 13 and *Streptococcus thermophilus* Dad 11. Results are provided as mean ± SD. Results in each strain with different superscripts are significantly different (p<0.05) by Duncan's multiple range test.

Barkallah et al. [12] reported that yogurts containing 0.25% of *S. platensis* biomass included low a* and b* values, showing a light green color.

Color changes of the fermented milks containing 0.3% of *S. platensis* might be due to chlorophyll degradation caused by decreases in pH during fermentation. During fermentation, pH of single and mixed cultures decreased from 6.71 to 4.41-4.6. At acidic conditions, hydrogen ions could transform chlorophylls to pheophytins by substituting magnesium ions in the porphyrin ring and releasing a phytol group to pheophorbide. Pheophytin and pheophorbide are products of chlorophyll degradation, producing an olive-green color [36].

3.6 Mineral Contents of the Fermented Milks with 0.3% S. platensis Addition

Selected minerals such as calcium, phosphorus and iron were assessed in fermented milks with *S. platensis* addition. Mineral contents of the fermented milks with 0.3% *S. platensis* addition increased significantly (p < 0.05) after 24 of h fermentation in single and mixed cultures, compared to the controls (Table 4). Calcium, phosphorus and iron respectively increased as 13, 14 and 112% for the fermented milks with 0.3% S. *platensis* addition and as 10, 7.5 and 57% for the controls after 24 h of fermentation. Barkallah et al. [12] stated that yogurts containing 0.25% of *S. platensis* and a mixed culture of *L. bulgaricus* and *S. thermophilus* included higher mineral contents than yogurts without *S. Platensis*. This was 107.7 mg 100 g^{-1} for calcium and 9.01 mg 100 g^{-1} for iron. Drago and Valencia [37] reported that calcium and iron contents respectively increased by nearly 7.37 and 36.08% in yogurts produced by a mixed culture of *L. bulgaricus* and *S. thermophilus* after 6 h of fermentation at 42 °C. Obadina et al. [38] showed that mineral contents of calcium and iron respectively increased nearly 13.55 and 21, 43% after 24 h of fermentation in soymilks fermented by a mixture of *L. acidophilus*, *S. cremoris*, *Micrococcus aureus* and *S. lactis*. Biomass of *S. platensis* is known to include high mineral contents such as calcium, phosphorus, potassium, and iron as well as trace minerals such as magnesium, zinc and selenium [3]. Therefore, fermented milks containing 0.3% of *S. platensis* biomass included higher mineral contents than that the controls did. The current results showed increases in mineral contents during the fermentation process.

Table 4. Mineral contents of the fermented milks with 0.3% *Spirulina platensis* addition (37 °C, 24 h)

Strains	Spirulina (%)	Calcium (mg 100g^{-1}) Initial	Final	Phosphorus (mg 100g^{-1}) Initial	Final	Iron (mg 100g^{-1}) Initial	Final
Lactobacillus plantarum Dad 13	0%	112.60±3.26[a]	123.51±1.77[b]	97.44±0.17[a]	105.30±1.25[c]	0.55±0.01[a]	0.87±0.01[c]
	0.3%	119.58±0.69[b]	135.33±1.60[c]	102.11±0.24[b]	116.51±0.81[d]	0.64±0.03[b]	1.37±0.03[d]
Streptococcus thermophilus Dad 11	0%	112.60±3.26[a]	124.35±2.72[b]	97.44±0.17[a]	104.74±0.65[c]	0.55±0.01[a]	0.86±0.01[c]
	0.3%	119.58±0.69[b]	135.91±2.82[c]	102.11±0.24[b]	116.03±0.21[d]	0.64±0.03[b]	1.33±0.02[d]
Mixed Cultures	0%	112.60±3.26[a]	124.27±1.15[b]	97.44±0.17[a]	104.82±0.29[c]	0.55±0.01[a]	0.87±0.01[c]
	0.3%	119.58±0.69[b]	136.09±1.33[c]	102.11±0.24[b]	116.63±0.77[d]	0.64±0.03[b]	1.39±0.02[e]

* Mixed cultures are mixture of *Lactobacillus plantarum* Dad 13 and *Streptococcus thermophilus* Dad 11. Results are provided mean ± SD. Results in each strain with different superscripts are significantly different (p<0.05) from Duncan's multiple range test

This could be due to the intracellular quantities of minerals, which were accumulated by *L. plantarum* Dad 13 and *S. thermophilus* Dad 11 during fermentation. Boyaval et al. [39] reported that the intracellular concentrations of Ca^{2+} increased at the logarithmic phase of *S. lactis.* In addition, Obadina et al. [38] demonstrated that increases in mineral contents of the fermented soymilks were affected by minerals released from chelated complex compounds through the microbial activities.

3.7 Phycocyanin Contents of the Fermented Milks with 0.3% *S. platensis* Addition

Phycocyanin is a natural proteinaceous pigment of *S. platensis* with bioactive and antioxidant activities. Phycocyanin contents were relatively constant after milk fermentation [0.220-0.248 mg g^{-1} (db)] in single and mixed cultures (Figure 5). This finding was similar to the finding by Pan-utai et al. [8], who showed that yogurts with *S. platensis* addition at a concentration of 0.1–0.3% and a mixed culture of *L. bulgaricus* YC-X11 and *S. thermophilus* YC-X11 included a phycocyanin content of approximately 0.2 mg g^{-1} (db). Barkallah et al. [12] reported that the addition of 0.25% *S. platensis* biomass to yogurts using a mixed culture of *L. bulgaricus* YC-X11 and *S. thermophilus* YC-X11 included a phycocyanin content of 0.297 mg g^{-1} (db). Wu et al. [40] demonstrated that phycocyanin included a low stability at temperatures ≥ 45 °C and pH 3-4. In this study, fermented milks were incubated at 37 °C with pH 4.46-4.6 after 24 h of fermentation. Therefore, phycocyanin contents of the fermented milks with *S. platensis* were relatively stable during fermentation.

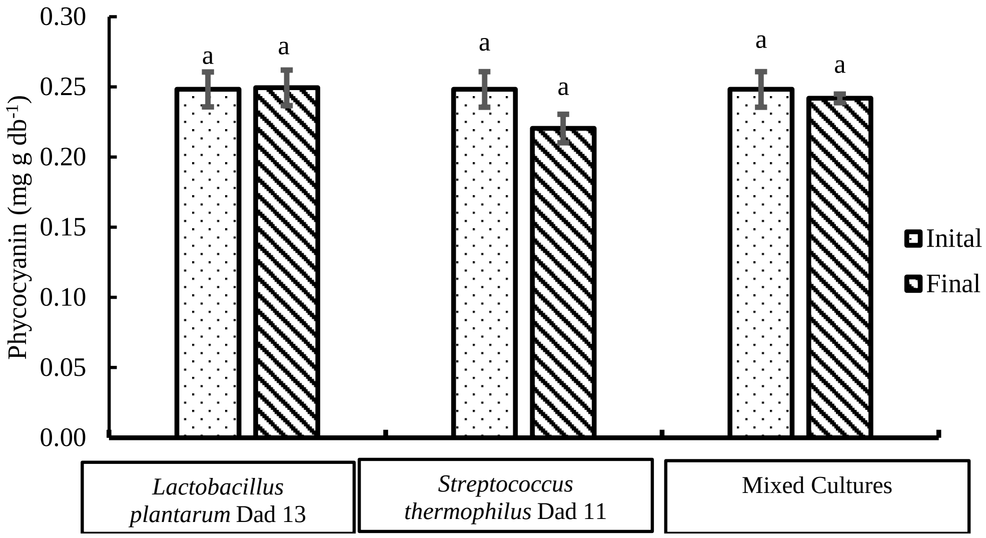

Figure 5. Phycocyanin contents of the fermented milks with 0.3% *Spirulina platensis* addition (37 °C, 24 h). Values are shown as mean ±SD. Values of the strains with similar superscripts are not significantly different ($p < 0.05$)

4. Conclusion

Increased quantities of *S. platensis* powder increased cell growth (up to 9 log CFU ml^{-1}) and acid production (titratable acid and pH of 1.34% and 3.66, respectively) in milks fermented by *L. plantarum* Dad 13 and *S. thermophilus* Dad 11. Addition of 0.3% of *S. platensis* powder significantly improved the viscosity to 4.05 Pa.s during milk fermentation. These probiotic fermented milks with 0.3% of *S. platensis* included 8.9 log CFU ml^{-1} *L. plantarum* with 35-40% antioxidant activity. This study can be addressed to develop functional fermented milks. Further studies are necessary to investigate the cell viability and physicochemical charact-ristics during storage.

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6. Conflict of Interest

The authors report no conflicts of interest.

7. Authors Contributions

Conceptualization, T.U. and E.S.R.; methodology, T.U. and E.S.R.; formal analysis, J.C.N.L.; writing and original draft preparation, J.C.N.L. and T.U.; writing, review and editing, J.C.N.L., T.U., D.A.S., R.W. and A.U.; supervision, T.U. and E.S.R.

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اثرات افزودن *اسپیرولینا پلاتنسیس* بر رشد *لاکتوباسیلوس پلانتاروم* Dad 13 /*استرپتوکوکوس ترموفیلوس* Dad 13 در شیر تخمیر شده و خصوصیات فیزیکوشیمیایی محصول

جاشوا کریسمس ناتانائل لوویدارتو[1]، اندانگ اس راهایو[۱،۲و۳]، دیان آنگراینی سوروتو[۱و۲]، راچما ویکانداری[۱و۲]، آردیکا اولفا، تیاس اوتامی[۱،۲و۳*]

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***نویسنده مسئول**

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چکیده

سابقه و هدف: *اسپیرولینا پلاتنسیس* حاوی ترکیبات زیست‌فعال عالی می‌باشد، که بر ارتقای سلامتی موثرند. با این حال، استفاده از *اسپیرولینا پلاتنسیس* در غذاها به‌دلیل طعم و مزه نامطبوع آن برای برخی افراد، محدودیت‌هایی دارد. فرآورده‌های تخمیری شیر عطر و طعم معمولی اسید تازه را دارند و می‌توانند با *اسپیرولینا پلاتنسیس* ترکیب شوند. زیست‌یارهای[1] *لاکتوباسیلوس پلانتاروم* Dad 13 و *استرپتوکوکوس ترموفیلوس* Dad 11 جدا شده از شیرهای سنتی بوفالو را می‌توان به عنوان سویه‌های آغازگر برای شیرهای تخمیر شده استفاده کرد. این مطالعه رشد باکتری لاکتیک اسید، تولید اسید و ویژگی‌های فیزیکوشیمیایی شیرهای تخمیر شده با *اسپیرولینا پلاتنسیس* را بررسی کرد.

مواد و روش ها: تخمیر شیر با افزودن غلظت‌های گوناگون (۰/۱۵، ۰/۳، ۰/۴۵ و ۰/۶ درصد) پودر *اسپیرولینا پلاتنسیس* انجام شد و سپس رشد سلول‌های میکروبی، تولید اسید و فعالیت آنتی‌اکسیدانی مورد بررسی قرار گرفت. شیرهای تخمیر شده با غلظت‌های انتخابی *اسپیرولینا پلاتنسیس* در طی ۲۴ ساعت تخمیر در دمای ۳۷ درجه سلسیوس با استفاده از کشت‌های منفرد و مخلوط برای مطالعه جنبه‌های مختلف رشد سلولی، تولید اسید، گرانروی، ظرفیت نگهداری آب و رنگ مورد ارزیابی قرار گرفتند.

یافته‌ها و نتیجه‌گیری: با افزایش غلظت *اسپیرولینا پلاتنسیس* در شیرهای تخمیرشده رشد سلول‌های میکروبی، تولید اسید و فعالیت آنتی‌اکسیدانی افزایش یافت . در طی تخمیر شیر با افزودن ۰/۳درصد از *اسپیرولینا پلاتنسیس*، سلول‌ها تکثیر یافتند و پس از ۲۴ ساعت، تعداد کل باکتری‌های لاکتیک اسید و سلول‌های زیست‌یار به‌ترتیب به ۹/۱۹-۸/۷۳ و ۸/۹۲ $\log CFU\ ml^{-1}$ رسید. اسیدیته قابل تیتراسیون به ۱/۰۸ درصد رسید و pH به ۴/۴۱ کاهش یافت. پس از ۱۲ ساعت تخمیر، گرانروی به‌طور قابل توجهی در مقایسه با گروه شاهد افزایش یافت. شیرهای تخمیر شده با افزودن *اسپیرولینا پلاتنسیس*، که به آنها *لاکتوباسیلوس پلانتاروم* Dad 13 و *استرپتوکوکوس ترموفیلوس* Dad 11 اضافه شده باشد، را می‌توان به‌عنوان جایگزین فرآورده های تخمیری فراسودمند[2] شیر توسعه داد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

[1] Probiotics
[2] Functional