Chapter 4

The Quality Characteristics of a New Sustainable Functional Kefir Fortified with *Spirulina plantensis* Microalgae During Storage

Özge Duygu Okur[1]

Özgün Burcu İşbecer[2]

Abstract

The purpose of this study was to reveal the quality characteristics of sustainable functional kefir enriched with *Spirulina platensis* (*S. platensis*) microalgae, known for its health effects, during storage (4°C) for 21 days. For this purpose, four kefir samples were produced by using *S. platensis* microalgae in different amounts (A: 0% (control), B: 0.25%, C: 0.50%, D: 1%). The physicochemical, microbiological, sensory, phenolic contents and antioxidant properties of the kefir samples were investigated during day 1, day 7, day 14 and day 21 of storage.

One of the main results was that the addition of *S. platensis* powder was seen to increase *L. bulgaricus* and *S. thermophilus* counts in the kefir samples during storage ($P < 0.05$). Among the samples, the D sample had the highest total phenolic and antioxidant activity content (respectively 1033.75 mg GAE L^{-1} and 15.78 mM TE) ($P < 0.05$).

1. INTRODUCTION

Kefir, which is formed as a result of the activity of a number of specific microorganisms, is a functional fermented milk product originating from the Caucasus. Lactic acid and acetic acid bacteria and several yeast species

1 Corresponding author, Asst. Prof. Dr., Department of Food Engineering, Faculty of Engineering, Zonguldak Bulent Ecevit University, Zonguldak, TURKEY, oduyguokur@beun.edu.tr, 0000-0002-5483-2983

2 M.S.c., Department of Import and Export, Antalya Seed Certification Test Directorate, Republic of Turkey Ministry of Agriculture and Forestry, Antalya, TURKEY, ozgunburcu.isbecer@tarimorman.gov.tr

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coexist in kefir in a symbiotic association, and this microflora is responsible for acidic-alcoholic fermentation (Garrote et al. 2001; Irigoyen et al. 2005).

Kefir consumption has gained popularity in contemporary times due to its potential anticarcinogenic, antitumoral, anti-inflammatory, antimicrobial, probiotic, and prebiotic properties. Additionally, it is associated with benefits such as enhanced lactose tolerance, reduced cholesterol levels, and positive effects on both the immune system and hypocholesterolemic responses (John and Deeseenthum 2015; Sharifi et al. 2017; Yilmaz et al. 2022). Diversifying kefir's nutraceutical advantages can be achieved through the implementation of applicable strategies, such as enriching kefir with specific ingredients capable of bestowing distinctive and valuable properties upon the beverage (John and Deeseenthum 2015; Sharifi et al. 2017; Arslan 2015; Aiello et al. 2020).

Microalgae have been proposed as new model organisms in dietary supplements in animal and human nutrition (Spolaore et al. 2006; Enzing et al. 2014), as well as in wastewater bioremediation (Craggs et al. 1996) and biotechnological applications (Demirbas and Demirbas 2011). Capable of surviving under very harsh conditions compared to other algae, *Spirulina* is a cyanobacteria or blue-green microalgae with a spiral cellular structure (Ghaeni et al. 2014). With its excellent nutritional value and high protein content, *Spirulina* is the best known strain (Spolaore et al. 2006). The two most commonly used Spirulina species in nutritional supplements are *S. platensis* and *Spirulina maxima* (*S. maxima*). *Spirulina* is a rich source of nutrients, namely high quality proteins, gamma linolenic acid, sulfolipids, glycolipids, polysaccharides, carotenoid, omega-3 and omega-6 polyunsaturated fatty acids, vitamin E, vitamin A, various B vitamins, and minerals such as magnesium, potassium, calcium, zinc and selenium (Belay 2002; Tang and Suter 2011). Therefore, as a potential therapeutic agent, it is effective in the treatment of diseases caused by oxidative stress (Makhlouf and Makhlouf 2012). *Spirulina* has particular therapeutic effects such as reduction of hyperlipidemia and obesity, protection against some cancers, increasing intestinal lactobacilli, reducing nephrotoxicity, reducing blood cholesterol, enhancing the immune system and radiation protection (Jimenez et al. 2003; Patel and Goyal 2013; Finamore et al. 2017; Lafarga et al. 2020). In addition, *Spirulina* contains many functional bioactive components with antioxidant and anti-inflammatory activities such as phycobiliprotein C-phycocyanin (Riss et al. 2007) and phenolic phytochemicals (Machu et al. 2015). In the food industry, due to these functional properties, *S. platensis* can be used in the production of functional foods (Fadaei et al. 2013). Moreover, a trend has begun to add microalgae (cyanobacterial biomass) in fermented

milk products to increase the functional product properties by promoting the viability of probiotics and also to improve their nutritional properties (Varga et al. 2002; Beheshtipour et al. 2013). *Spirulina* is frequently used for human consumption in various forms: tablets, capsules, or food additives such as dairy products (Varga et al. 2002; Guldas and Irkın 2010; Malik et al. 2013; Agustini et al. 2016; Barkallah et al. 2017; Darwish 2017; Szmejda et al. 2018; Çelekli et al. 2019; Narayana and Kale 2019; Çelekli et al. 2020; Bosnea et al. 2021), gelatin desserts (Gouveia et al. 2008), and bakery products (De Marco et al. 2014; Ashoush and Samar 2019; Grahl et al. 2020; Da Silva et al. 2021).

This study aimed to determine the potential effect of adding microalgae on the quality properties (physicochemical, microbiological, sensory, phenolic content and antioxidant properties) during storage in a new type of sustainable functional kefir produced by adding *S. platensis* powder. When the enrichment studies on kefir as a functional product were examined, no study was found on the use of microalgae such as *S. platensis*, especially in milk kefir. Hence, it is anticipated that this research will add value to the current body of published literature.

2. MATERIALS AND METHODS

2.1. Materials

The entirety of the UHT cow's milk utilized in the research was acquired from Pınar Sut Co. located in Izmir, Turkey. The commercial freeze-dried kefir starter culture was sourced from Danisco-Biolacta in Poland. *S. platensis* powder was obtained from Cyanotech Corp. Hawaii, USA, while 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and gallic acid were obtained from Acros (Morris Plains, NJ, USA). Folin Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). Potassium persulphate ($K_2S_2O_8$), sodium carbonate (Na_2CO_3) and ethanol (C_2H_5OH) were purchased from Sigma Aldrich (St. Louis, MO). All chemicals and reagents were of analytical grade.

2.2. Kefir production

Kefir production was carried out according to García et al. (2006) with some modifications. The freeze-dried kefir culture underwent activation in skimmed milk prior to inoculation. The inoculated skimmed milk designated for use as the culture was incubated at 25°C until reaching a pH of 4.6, followed by overnight storage at 4°C. The whole UHT milk underwent

heat treatment at 85°C for 10 minutes, after which it was enriched with *S. platensis* powder at concentrations of 0.25%, 0.50%, and 1% (w/v). Subsequently, the milk samples were cooled to 25°C and inoculated with a 3% kefir culture, then packaged into 200 mL glass containers. Incubation continued at 25°C until a pH value of 4.6 was achieved. After fermentation, the samples were cooled and stored at 4°C for 21 days for the analyses. Four different kefir samples were produced, namely A: control kefir, B: kefir supplemented with 0.25% *S. platensis* powder, C: kefir supplemented with 0.50% *S. platensis* powder and D: kefir supplemented with 1% *S. platensis* powder (Figure 1)

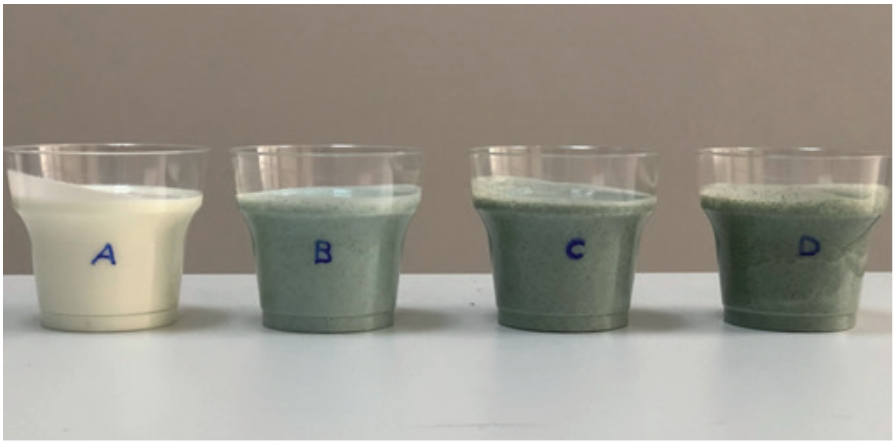

Figure 1. Kefir Samples, A: Control kefir, B: Kefir supplemented with 0.25% S. platensis powder, C: Kefir supplemented with 0.50% S. platensis powder, D: Kefir supplemented with 1% S. platensis powder

2.3. Analytical methods

The titratable acid content was assessed following the method outlined by Case et al. (1985), and the total solids content was determined in accordance with AOAC (1990) guidelines. Titratable acidity was characterized through titration with 0.1 N NaOH until reaching a pH of 8.1, with results expressed as a percentage of lactic acid. The pH value of the content was measured using a digital device (Schott Instruments, Lab 860, Germany).

2.4. Microbiological analysis

An aliquot of 1 mL of each kefir sample was mixed with 9 mL of sterile saline solution (0.85 g 100 mL^{-1}) in a tube. The suspension was homogenized for 1 minute and serial dilutions were prepared using the same

saline solution. The total bacterial count (TBC), *Lactobacillus* counts and *Streptococcus* counts were determined by the pour plate technique. TBC was determined using plate count agar and incubation was for 48 hours at 35°C according to Houghtby et al. (1992). M17 agar (Biolife) was used to count *Streptococcus* in the kefir samples and incubated aerobically at 37°C for 72 hours according to Torriani et al. (1996). MRS agar (Biolife) was used to count *Lactobacillus* in the kefir samples according to Tharmaraji and Shah (2003) and plates were incubated under anaerobic condition at 37°C for 72 hours.

2.5. Determination of the Total Phenolic Content

In the assessment of phenolic content in kefir, gallic acid served as the standard, and the Folin Ciocalteu method (Singleton and Rossi 1965) was employed. The absorbance value was measured at a wavelength of 760 nm using a spectrophotometer (Shimadzu Scientific Instruments, Inc., Tokyo, Japan). The concentration of total phenolic content in kefir was expressed in gallic acid equivalent (GAE), determined through a standard gallic acid curve. The results are reported as mg GAE L^{-1}.

2.6. Determination of the Total Antioxidant Activity

2,2′-azinobis (3-ethylbenzothiazoline)-6-sulfonic acid (ABTS-TEAC) Assay

The 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt ($ABTS^+$) radical cation was generated by combining 7 mM of ABTS stock solution with 2.45 mM of potassium persulfate. The resulting $ABTS^+$ radical cation was then diluted with PBS to achieve a pH of 7.4 and an absorbance of 0.70 (±0.02) at 734 nm, equilibrated at 30°C. Spectrophotometric measurement of $ABTS^+$ inhibition against Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchromane-2-carboxylic acid) was conducted, with absorbance recorded at a wavelength of 734 nm using a spectrophotometer (Shimadzu Scientific Instruments, Inc., Tokyo, Japan). TEAC values for the samples were calculated from the Trolox standard curve and expressed as Trolox equivalents (in μmol mL^{-1} of sample) (Re et al. 1999).

2.7. Sensory evaluation

Sensory evaluation of the kefir samples was conducted by 10 trained panelists (4 to 5 males, 4 to 5 females) using sensory assessment scorecards. The selected panelists voluntarily gave their consent to do the sensory

evaluation of the proposed product. Kefir samples were evaluated in terms of appearance, texture, homogeneous structure, color, taste, mouthfeel, odor and general acceptability on storage days 1, 7, 14 and 21. The evaluation was carried out based on eight criteria on a 10-point scale (Lawless and Heymann 1999).

2.8. Statistical analysis

Three measurements of each parameter were carried out at separate times. The experimental data was analyzed for variance (one way ANOVA) with SPSS 16 version software (SPSS 2017). The data presented are given as mean values with standard errors. For each principal effect, a multiple comparison of treatment means was performed using Tukey's pair-wise comparison at an α-level of 5%.

3. RESULTS AND DISCUSSION

3.1. Physicochemical composition

The physicochemical composition of kefir samples are given in Table 1. It was determined that the addition of *S. platensis* to the kefir samples increased the titratable acidity and dry matter contents ($P < 0.05$). Also, the addition of *S. platensis* caused a decline in pH values of the kefir samples. This decline was probably due to the stimulatory effect produced by *S. platensis* on the growth of *L. bulgaricus*, which was also supported by the higher viable cell counts of *L. bulgaricus* in the kefir samples with *S. platensis* during storage (Beheshtipour et al. 2013). Similar results were found by Varga et al. (2002); Gyenis et al. (2005); Molnár et al. (2005); Molnár et al. (2009); Guldas and Irkin (2010); Narayana and Kale (2019) and Çelekli et al. (2020).

Table 1. Physicochemical properties of the kefir samples

Sample	Storage periods (day)	Titratable acidity (LA%)	pH	Dry matter (%)
A	1	0.83 ± 0.00^{Aa}	4.56 ± 0.00^{Aa}	12.83 ± 0.03^{Aa}
	7	0.87 ± 0.00^{Aa}	4.49 ± 0.00^{Ab}	12.83 ± 0.03^{Aa}
	14	0.91 ± 0.00^{Aa}	4.48 ± 0.00^{Ab}	12.83 ± 0.03^{Aa}
	21	0.87 ± 0.00^{Aa}	4.46 ± 0.00^{Ac}	12.83 ± 0.03^{Aa}
B	1	0.86 ± 0.01^{Aa}	4.49 ± 0.00^{Ba}	12.90 ± 0.00^{Aa}
	7	0.90 ± 0.02^{Aa}	4.47 ± 0.00^{Bb}	12.90 ± 0.00^{Aa}
	14	0.95 ± 0.01^{Ab}	4.45 ± 0.00^{Bc}	12.90 ± 0.00^{Aa}
	21	0.92 ± 0.00^{Aa}	4.47 ± 0.00^{Ab}	12.90 ± 0.00^{Aa}
C	1	0.90 ± 0.02^{Aa}	4.48 ± 0.00^{Ba}	13.05 ± 0.02^{Ba}
	7	0.97 ± 0.04^{Ba}	4.45 ± 0.00^{Cb}	13.05 ± 0.02^{Ba}
	14	1.04 ± 0.04^{Bb}	4.44 ± 0.00^{Bb}	13.05 ± 0.02^{Ba}
	21	0.97 ± 0.01^{Ba}	4.43 ± 0.00^{Bc}	13.05 ± 0.02^{Ba}
D	1	0.96 ± 0.02^{Ba}	4.44 ± 0.00^{Ca}	13.40 ± 0.07^{Ca}
	7	0.99 ± 0.01^{Ba}	4.42 ± 0.00^{Db}	13.40 ± 0.07^{Ca}
	14	1.08 ± 0.00^{Bb}	4.43 ± 0.00^{Ca}	13.40 ± 0.07^{Ca}
	21	1.05 ± 0.01^{Bb}	4.43 ± 0.00^{Ba}	13.40 ± 0.07^{Ca}

A: Control kefir, B: Kefir supplemented with 0.25% S. platensis powder, C: Kefir supplemented with 0.50% S. platensis powder, D: Kefir supplemented with 1% S. platensis powder.

$^{a-c}$***Different lowercase superscripts depict the statistical difference within a row between time ($P < 0.05$).***

^{ABC}***Different uppercase superscripts depict the statistical difference between the mean values of kefir samples ($P < 0.05$).***

3.2. Microbiological analysis

Figure 2 presents the total microbial counts for all the kefir treatments during the storage period at 4°C. There were significant differences in the total aerobic mesophilic bacteria (TAMB) counts between the control and kefir with *S. platensis*. The TAMB count was influenced not only by adding *S. platensis* but also by the storage time ($P < 0.05$). The results presented here are similar to those found by Suzery et al. (2018), and Patel et al. (2019).

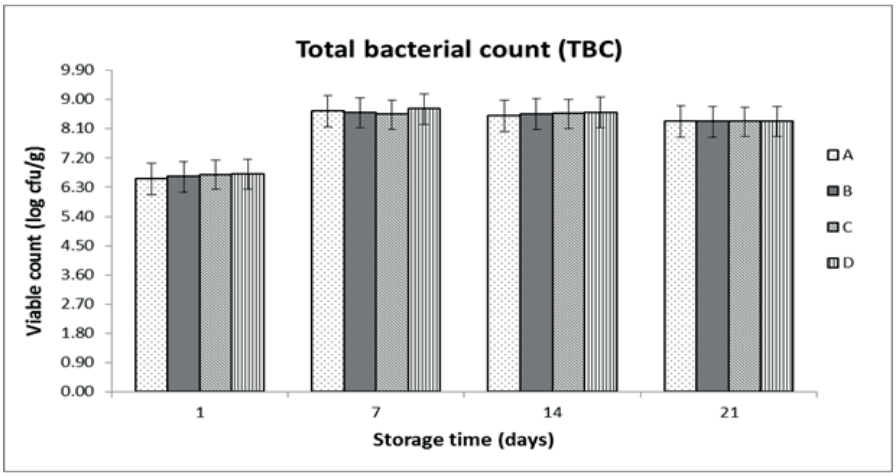

Figure 2. The effect of adding S. platensis on the total microbial counts (log cfu g^{-1}) in kefir during storage: A: Control kefir, B: Kefir supplemented with 0.25% S. platensis powder, C: Kefir supplemented with 0.50% S. platensis powder, D: Kefir supplemented with 1% S. platensis powder

The storage period significantly affected the *L. bulgaricus*, *S. thermophilus* and total bacteria counts ($P < 0.05$). Data in Figure 3 and 4 present the effects of *S. platensis* enrichment on the viability of *L. acidophilus* and *S. thermophilus* in kefir samples during storage.

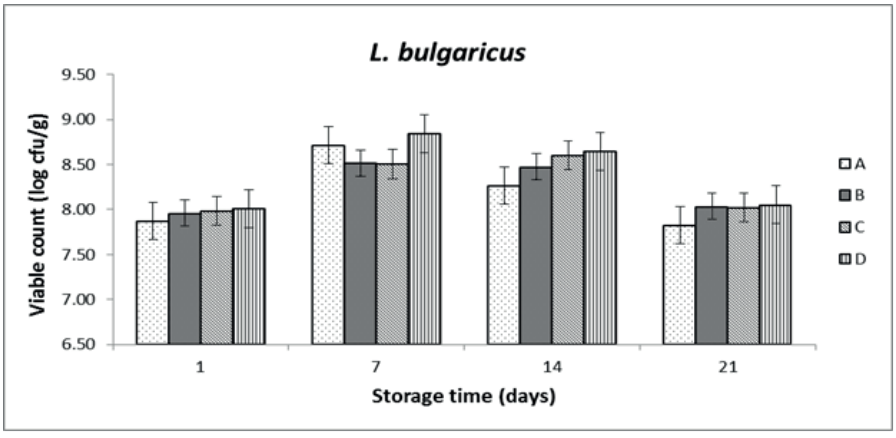

Figure 3. The effect of adding S. platensis on the viability (log cfu g^{-1}) of L. acidophilus in kefir during storage: A: Control kefir, B: Kefir supplemented with 0.25% S. platensis powder, C: Kefir supplemented with 0.50% S. platensis powder, D: Kefir supplemented with 1% S. platensis powder.

As illustrated in Figures 3 and 4, the introduction of *S. platensis* powder into the kefir samples, in comparison to the control samples, resulted in elevated counts of *L. bulgaricus* and *S. thermophilus* throughout the storage period. Microalgae have been reported to stimulate the growth of lactic acid bacteria, and increase their viability and acid production by providing essential compounds such as adenine, hypoxanthine, free amino acids, vitamins, minerals etc (Beheshtipour et al. 2013; Omar et al. 2019). Similar results were reported by Fadaei et al. (2013); Mocanu et al. (2013); Agustini et al. (2017); Narayana and Kale (2019); Patel et al. (2019); Atallah et al. (2020) and Çelekli et al. (2020).

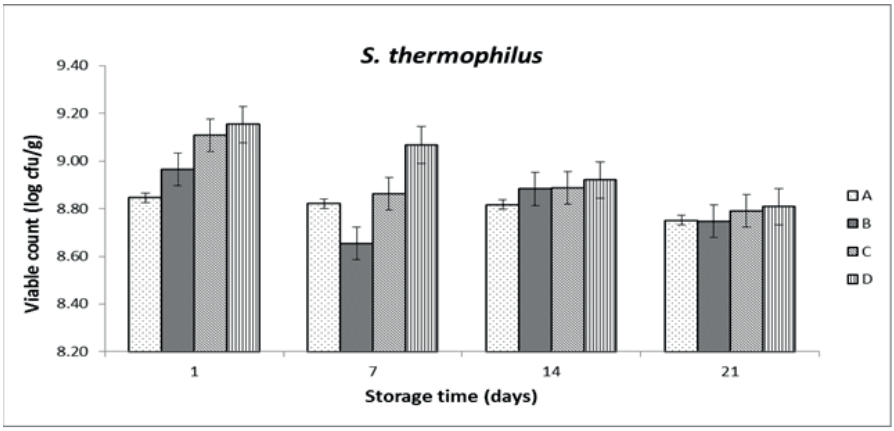

Figure 4. The effect of adding S. platensis on the viability (log cfu g^{-1}) of Streptococcus thermophilus in kefir during storage: A: Control kefir, B: Kefir supplemented with 0.25% S. platensis powder, C: Kefir supplemented with 0.50% S. platensis powder, D: Kefir supplemented with 1% S. platensis powder

Also, because of the alkaline character of *S. platensis* and having considerable buffering capacity, *Spirulina* significantly stimulated the acid production and increased growth rates of lactic acid bacteria during the fermentation process and even during the 1st week of storage. However, viability percentages declined slowly thereafter similar to other study results (Beheshtipour et al. 2013; Guldas and Irkın 2010; Atallah et al. 2020).

3.3. Evaluation of total antioxidant activity and phenolic contents

Figures 5 and 6 depict the alterations in the total antioxidant activity and total phenolic content of kefir samples, respectively. The lowest total antioxidant activity value (7.65 mM TE) was detected in the control kefir samples (A) on the 7th day of storage, and the highest total antioxidant

activity value (15.78 mM TE) was detected in the sample D on the 21st day of storage (Figure 5).

Recent investigations have demonstrated the considerable antioxidant and phenolic content found in microalgae (Goiris et al., 2012; Machu et al., 2015; Barkia et al., 2019). Phenolic compounds, known for their antioxidant capacity, have the ability to interact with free radicals without compromising their stability (Gershwin and Belay, 2007). Phenolic acids, tocopherols and ß-carotene, which are known to exhibit antioxidant properties, are included in the composition of *Spirulina*. *S. platensis* are bioactive-rich microalgae of great potential as a source of natural antioxidant (Ghaeni et al. 2014; Jacob-Lopes et al. 2019).

The incorporation of microalgae was found to exert a significant impact on the antioxidant capacity of all samples in comparison to the control kefir samples ($P < 0.05$). Especially, the addition of 1.0% *S. platensis* was found to approximately double the antioxidant capacity from 7.68 to 15.03 mM TE on day 1 of storage. Research indicates that the antioxidant activity and phenolic content in dairy products, including yogurt, cheese, ice cream, and fermented milk, show an elevation in the presence of microalgae (O'Sullivan et al., 2014; Barkallah et al., 2017; Darwish, 2017; Szmejda et al., 2018; Atallah et al., 2020; Aydemir and Öner, 2020).

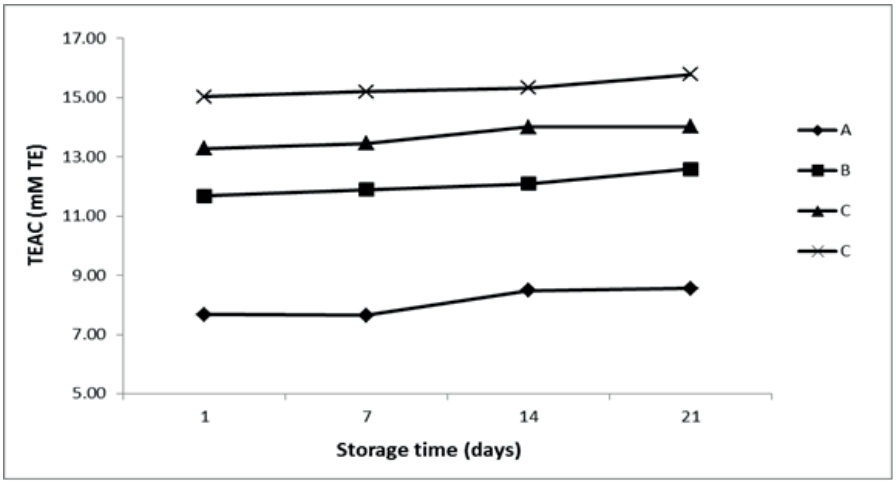

Figure 5. The total antioxidant activity of kefir samples during storage at 4°C for 21 days: A: Control kefir, B: Kefir supplemented with 0.25% S. platensis powder, C: Kefir supplemented with 0.50% S. platensis powder, D: Kefir supplemented with 1% S. platensis powder

S. platensis, one of the most important microalgae, has been used as a food in Asian countries for a long time due to its high nutritional value and is gaining importance in European countries. This microalgae contains many high-value bioactive ingredients such as proteins, phenolics, antioxidants, carbohydrates, lipids, vitamins, pigments and other phytonutrients (Li et al. 2007; Hajimahmoodi et al. 2010; Priyadarshani and Rath 2012; Vo et al. 2015).

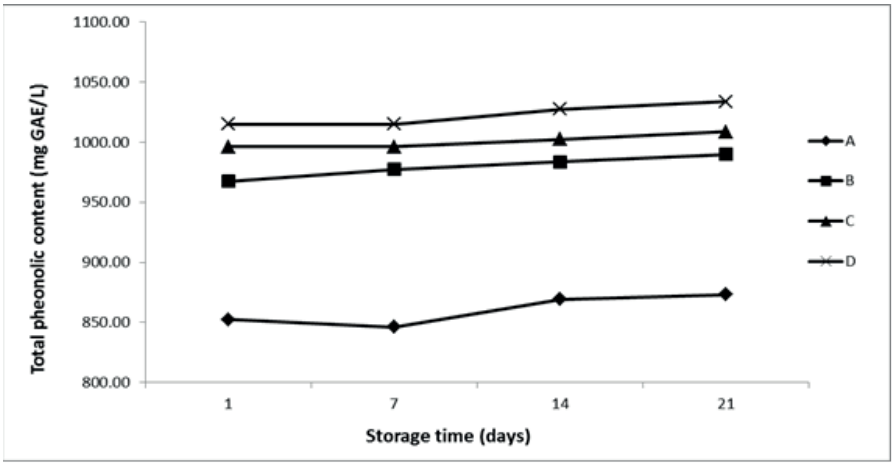

Figure 6. The total phenolic content of kefir samples during storage at 4°C for 21 days: A: Control kefir, B: Kefir supplemented with 0.25% S. platensis powder, C: Kefir supplemented with 0.50% S. platensis powder, D: Kefir supplemented with 1% S. platensis powder

The lowest total phenolic content (846.25 mg GAE L^{-1}) was found in control kefir samples (A) on day 7 of storage, while the highest total phenolic content (1033.75 mg GAE L^{-1}) was detected in sample D on day 21 of storage (Figure 6). The addition of *S. platensis* significantly affected the total phenolic content of all samples when compared to the control kefir samples ($P < 0.05$).

Particularly, the inclusion of 1.0% *S. platensis* was observed to enhance the total phenolic content from 852.50 to 1015 mg GAE L^{-1} on the first day of storage. These findings align with the results reported by O'Sullivan et al. (2014), Barkallah et al. (2017), Darwish (2017), Szmejda et al. (2018), Atallah et al. (2020), and Aydemir and Öner (2020).

3.4. Sensory analysis

The sensory evaluation results for the kefir samples are depicted in Figures 7 and 8. Overall, among the kefir samples, the control kefir (A) and the kefir enriched with 0.25% *S. platensis* powder (B) consistently earned the highest ratings across all sensory attributes. Concurrently, the kefir supplemented with 1% *S. platensis* powder consistently received the lowest scores for sensory properties ($P < 0.05$). Both the control kefir (A) and the kefir enriched with 0.25% *S. platensis* powder (B) garnered favorable ratings from the panelists for positive sensory attributes, as well as for having a uniform texture and mouthfeel. Generally, sensory scores declined in all kefir samples over the course of storage. Treatments with higher microalgae concentrations (0.50 and 1%) had lower sensory acceptability for all organoleptic properties compared to the control ($P < 0.05$).

Spirulina, like most microalgae, are known to have an undesirable odor. The addition of *S. platensis* will produce an odor that tends to be fishy or unpleasant. The fishy odor of *S. platensis* comes from its minerals contents (Beheshtipour et al. 2013). Also, higher amounts of added *S. platensis* gave a darker green color to the kefir due to the blue–green pigment (phycosianin) in it (Machu et al. 2015). These properties were noticed by the panelists as an inappropriate sensory attribute (appearance), especially in samples C and D. Alternatively, it is suggested that 1% *S. platensis* powder can be used in fruit kefir or fruit flavored kefir samples without adversely affecting the organoleptic properties. In this way, negative sensory properties can be suppressed by the addition of fruit flavoring agents.

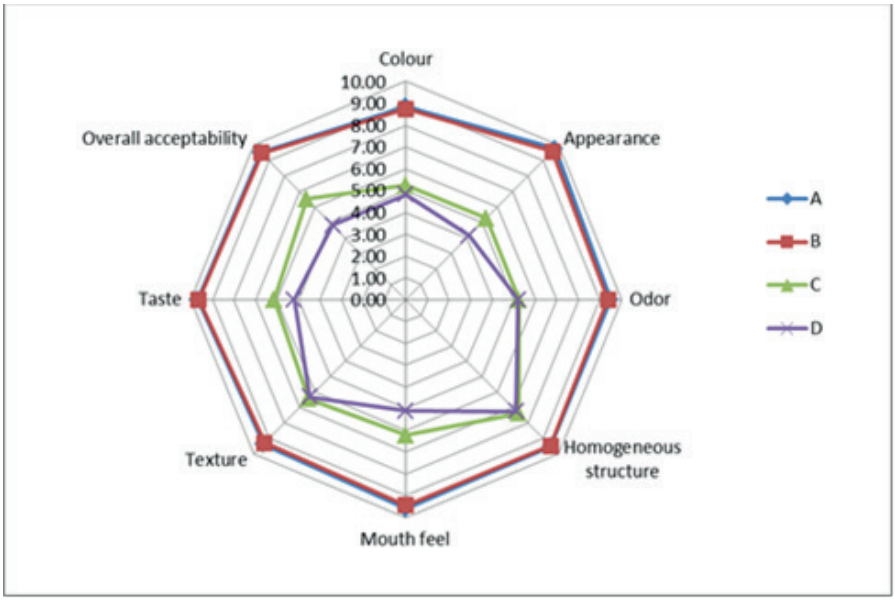

Figure 7. Sensory properties of kefir samples at day 1 of storage: A: Control kefir, B: Kefir supplemented with 0.25% S. platensis powder, C: Kefir supplemented with 0.50% S. platensis powder, D: Kefir supplemented with 1% S. platensis powder

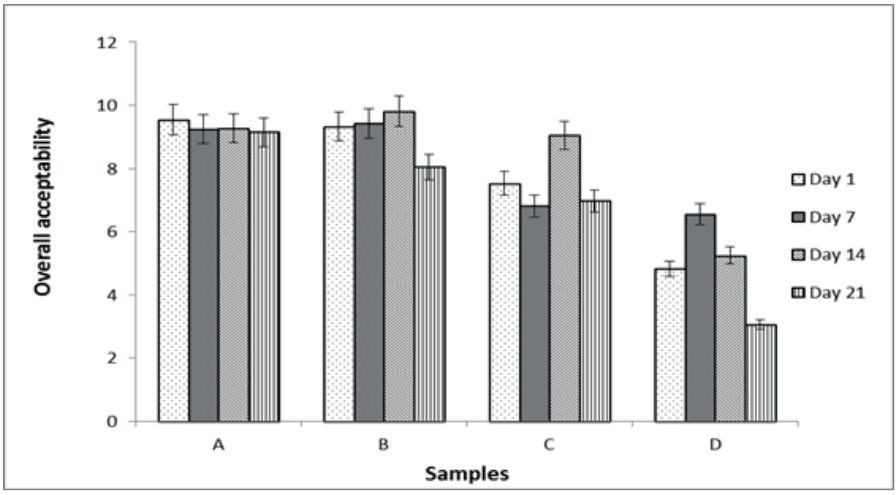

Figure 8. Overall acceptability of kefir samples during storage: A: Control kefir, B: Kefir supplemented with 0.25% S. platensis powder, C: Kefir supplemented with 0.50% S. platensis powder, D: Kefir supplemented with 1% S. platensis powder

The close alignment of values between the control kefir (A) and the kefir supplemented with 0.25% *S. platensis* powder (B) across various sensory

evaluation criteria indicates that the quantity of *S. platensis* powder employed should adhere to a specific ratio. These findings are also supported by Guldas and Irkın (2010); Malik et al. (2013); Agustini et al. (2016); Barkallah et al. (2017); Behir et al. (2019); Çelekli et al. (2019); Narayana and Kale (2019); Atallah et al. (2020) and Bosnea et al. (2021).

4. CONCLUSION

In this study, *S. platensis* powder was successfully used in kefir samples and according to the results obtained, it was determined that the addition of *S. platensis* powder significantly increased the antioxidant activity and phenolic content of kefirs. The addition of *S. platensis* resulted in higher dry matter, titratable acidity, and lower pH in kefir samples during storage. It was also shown that the addition of *S. platensis* powder to kefir samples increased *L. bulgaricus* and *S. thermophiles* counts compared to control samples during storage. Also, according to the observed results, the addition of *Spirulina* had a beneficial effect on the survival of the bacterial starter culture. On day 1 of storage, the control kefir (A) and kefir supplemented with 0.25% *S. platensis* powder (B) received the highest scores of 9.54 and 9.50, respectively, in terms of overall acceptability. *S. platensis* can be successfully used in kefirs to further enhance its health benefits as a functional additive. In conclusion, it can be inferred that *S. platensis* has the potential to contribute to the production of a novel, sustainable functional kefir with favorable sensory attributes and antioxidant properties, contingent upon the concentration of microalgae utilized.

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