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Improve the antioxidant activity and viability of *B. longum* and *B. animalis subsp lactis* in fermented soy and almond milk

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Abstract

This research focused on the impact of two strains of probiotic *Bifidobacterium spp.* i.e., *B. longum* (Bg), or *B. animalis subsp lactis* (Bc) on post-acidification, the viable cell counts (VCC), total phenolic and flavonoid contents (TPC and TFC, respectively), and antioxidant activity of fermented (F) soymilk (SM), almond milk (AM), and their combination (100%, 75%, 50%, & 25%) during 0, 7, 14, & 21 days of storage. All fermented SM, AM, and their combination showed higher ($p < 0.05$) post-acidification than their respective controls during the storage. All samples showed VCC ranging between 6.9 and 7.4 log cfu/mL compared to their respective controls (3-4 log cfu/mL; $p < 0.05$). TPC in FSM/AM (50:50)-Bg & Bc was 2 folds higher (74.3 ± 0.021 & 61.34 ± 0.037 µg GAE/mL; $p < 0.05$) than control (22.52 ± 0.168) on day 21. The presence of Bg improved ($p < 0.05$) TFC in FSM (100) after 1st day of storage. Both Bg and Bc had a positive impact on the antioxidant activity of all treated samples during storage. In conclusion, fermented soy and almond milk and their combination might serve as an effective vehicle for *B. longum*, or *B. animalis subsp lactis* with antioxidant activity.

Keywords: soymilk; almond milk; *Bifidobacterium longum*; *Bifidobacterium animalis subsp lactis*; viability; antioxidant activity.

Practical Application: Fermented soy and almond milk might act as a good vehicle for *Bifidobacterium* with antioxidants.

1 Introduction

The term "functional food" emphasizes the beneficial relationship between nutrition and health. A significant number of studies have focused on searching for alternative sources for people looking to improve their diet quality and healthy lifestyle (Pimentel et al., 2021; Shen et al., 2021; Villaño et al., 2022). Plant-based milk replacements are one of the food categories indispensable in vegan food manufacture since they are utilized as an important component in many vegan food items like plant-based yogurt, and cheese (Aydar et al., 2020). The non-dairy plant milk market is expected to exceed $38 billion by 2024, as well as be anticipated to expand by over 14% throughout 2018-2024 (Non-dairy milk market - worldwide expectation and forecast 2019-2024).

Plant-based milk products with probiotics are becoming increasingly popular due to consumers' awareness of their potential health benefits. Probiotics promote the balance and structure of microbiota, and they serve as a barrier against pathogens (Rasika et al., 2021; Xavier-Santos et al., 2022). Rai et al. (2017) indicated that the fermentation efficiency is completely dependent on the cultures utilized in the process. The bacterial genera most used as probiotics include *Bacillus, Lactobacillus, Enterococcus, Bifidobacterium,* and *Streptococcus* (Zendeboodi et al., 2020; Roobab et al., 2020; Cruz et al., 2021).

The negative impacts of oxidants include damage to biological macromolecules (e.g., DNA, proteins, and lipids) through oxidative stress, which is linked to disease development (Shehata et al., 2020). Antioxidant molecules can stop the production of free radicals and inhibit oxidative chain reactions (Guo et al., 2023; Gulcin, 2020). Natural antioxidants are mainly found in plant phenolics, which may be found in all parts of plants. Moreover, secondary metabolites are produced by plants in a variety of forms, including flavonoids, tannins, lignans, coumarins, and phenolic acids (Arribas et al., 2019; Gulcin, 2020).

Soy products have gained popularity among vegetarians due to their well-documented health advantages and high protein levels (Yu et al., 2021). Lai et al. (2013) found that fermented soymilk containing *S.thermophilus* and *B.infantis* increased total phenolic content and reduced the level of anti-nutritional components like phytates and saponins. In addition, almond contains many nutrients, phytochemicals, and fatty acids (Liu et al., 2016). Almond xylooligosaccharide (XOS) can be fermented by using several strains of *Lactobacillus* and *Bifidobacterium* (Singh et al., 2021). This is because almond seeds are a major source of prebiotics, which can increase the number of Bifidobacteria, leading to increase butyrate levels (Rocchetti et al., 2019). Moreover, almonds contain fiber and polyphenols that promote microbial fermentation in the gut, thereby impacting the composition of the gut microbiota (Barreca et al., 2020). A previous study has indicated that almonds support the growth of *Bifidobacterium* spp. and *Lactobacillus* spp. thereby inhibiting *Enterococcus* growth (Barreca et al., 2020).

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Several studies have found that *Bifidobacterium* spp. fermented soy and almond products offer a variety of therapeutic characteristics, including anti-oxidative capabilities, reduced inflammation in colitis, modulating gut bacterial growth (Wang et al., 2018; Hu et al., 2022; Singh et al., 2021), and anti-cancer activities (Lai et al., 2013; Karimi et al., 2021). Fermented soymilk with three strains of probiotics (*Bifidobacterium bifidum, Lacticaseibacillus casei, and Lacticaseibacillus plantarum*) led to decreased radical oxidative stress (ROS) production in mice on high-fat diets (Zhang et al., 2017). Fermented almond milk with different strains of probiotics may increase antioxidant activity by releasing various antioxidant components that were previously inactive (Topcuoglu & Yilmaz-Ersan, 2020). According to *in vitro* studies, phenolic and flavonoid compounds in almonds possess cytoprotective properties against oxidative stress, and DNA damage in smokers (Karimi et al., 2021). Therefore, this research focused on the impact of two strains of probiotic *Bifidobacterium spp.* i.e., *B.longum* (Bg), or *B. animalis subsp lactis* (Bc) on post-acidification, the viable cell counts (VCC), total phenolic and flavonoid contents (TPC and TFC, respectively), and antioxidant activity of fermented (F) soymilk (SM), almond milk (AM), and their combination (100%, 75%, 50%, & 25%) during 0, 7, 14, & 21 days of storage.

2 Materials and methods

2.1 Plant milk preparation

Soy and almond milk were prepared using the wet method-cooked slurry process as described by Yu et al. (2021) with minor modifications. Soy and almond seeds were purchased from a local store. Clean seeds (100 g) were soaked in distilled water (1:9 w/w) at room temperature overnight (16 h) before being grinding three times for 10 min using a grinding machine. The slurry was then boiled at 100 °C using an electric oven for 10 min with constant mixing to prohibit foam development. The heated slurry was then filtered through a 100-mesh screen to separate the milk from solid residue followed by refrigeration at 4 °C and used within 24 h.

2.2 Preparation of starter cultures

Pure strains of *Bifidobacterium longum* DSM 20219 and *Bifidobacterium animalis subsp lactis* DSM 10140 were purchased from the National Committee of Microbiology at the University of Ain Shams. All samples were stored at -80 °C. Each strain was prepared as reported previously by Shori & Baba (2015) with some modification. Briefly, sterile 10 mL aliquots of MRS broth (HiMedia, India) were supplemented with 0.05% L-cysteine hydrochloride were inoculated with 100 µL of each strain, followed by incubation at 37 °C for 48 h. The pre- inoculum cultures were prepared by transferring 1% (v/v) of activated culture to 10-mL aliquots of sterile reconstituted skim Milk (RSM) supplemented with 2% glucose and 1% yeast extract.

2.3 Preparation of fermented plant milk

Five fermented (F) soy (S)- and almond (A)- milk (M) and their combination (100%, 75%, 50%, & 25%) were prepared namely FSM (100), FAM (100), FSM/AM (75:25), FSM/AM (50:50), FSM/AM (25:75) using two Bifidobacterium sp. probiotics i.e., *Bifidobacterium longum* (Bg) and *Bifidobacterium animalis subs lactis* (Bc). One liter of each type of milk and their combination was heated at 40 °C. The starter culture (2% v/v; containing 10^5 cfu/mL of individual strains) was added to each sample individually and incubated at 40 °C for 9 hours. Control samples were prepared in the same way without using starter culture (native bacteria). Samples were aliquoted into sterile disposable plastic containers into refrigerated at 4 °C for 1, 7, 14, & 21 days.

2.4 Measurement of pH and Titratable Acidity (TA)

The pH and TA changes in all samples were examined as described by Shori (2020a) for 1, 7, 14, and 21 days.

2.5 Determination of Viable Cell Count (VCC)

The colony counts of Bifidobacterium strains in all fermented plant milk samples were determined as previously mentioned by Yong et al. (2022) using MRS-LP agar.

2.6 Preparation of aqueous extracts

Each milk sample (10 mL) was blended with 2.5 mL of distilled water and incubated in a water bath at 45 °C for 10 min (Shori, 2020a). After calibrating to pH 4.0 with HCl (0.1 mol/L), all mixtures were re-incubated in a water bath (45 °C) for 10 minutes. The mixtures were centrifuged (5000 g) for 10 min. The supernatant was then neutralized to pH 7.0 by NaOH (0.1 mol/L), followed by another 10 minutes of centrifugation, and the supernatant was utilized for additional research.

2.7 Determination of Total Phenolic Content (TPC)

The TPC was analyzed by Folin-Ciocalteu colorimetric method as previously mentioned by Shori (2013). Folin-Ciocalteu reagent (0.5 mL; 50% v/v) was added to 5 mL deionized water, One mL of ethanol (95%), and one mL of each sample extract, or standard solutions of gallic acid (10-100 µg/mL). The blend was left at room temperature for five minutes. One mL of anhydrous $Na2CO3$ solutions (5% w/v) was added to the samples in a dark place at 25 °C. After one hour, the absorbance at 750 nm was determined using a spectrophotometer. According to the gallic acid standard curve, the total phenol content was calculated and represented as µg gallic acid equivalent per milliliter (µg GAE/mL).

2.8 Determination of Total Flavonoid Content (TFC)

Total flavonoid estimation was carried out by using the aluminum chloride colorimetric method. According to Al-Ghafari et al. (2017), each aliquot (250 µL) of the extract was combined with 1.25 mL of $dH2O$ and 75 µL of a solution of sodium nitrite at a concentration of 5%. After the mixture had been incubated for five minutes, 150 µL of a 10% aluminum chloride solution and 0.5 mL of a 1 M sodium hydroxide solution were added. Immediately, the solution was diluted with 275 µL of deionized water. The absorbance was determined at 510 nm using a spectrophotometer. The total flavonoid content of extracts was expressed as equivalent, and a standard curve was created using a series of Catechin dilutions, ranging from 0 to 500 µg/mL.

2.9 Antioxidant activity assay

Determination of radical scavenging activity

The free radical scavenging activity of all fermented milk samples was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) method (Shori, 2020a).

Determination of Ferrous Ion Chelating (FIC) ability assay

FIC assay of all fermented milk samples was performed as described by Shori (2022a).

Determination of Ferric Reducing Antioxidant Potential (FRAP) assay

The reducing power of fermented milk extracts was measured by assessing the reduction of Fe3+ (CN-)6 to Fe2+ (CN-)6, as explained by Shori (2022a).

2.10 Statistical analysis

There were three separate batches for each experiment. The data is displayed as mean ± standard error mean (SEM). The significance of differences between means was assessed using one-way analysis of variance (ANOVA) at a p-value < 0.05. IBM SPSS Statistics version 20.0 was used to conduct the statistical analysis.

3 Results and discussion

3.1 Determination of pH and Titratable Acidity (TA) in fermented plant milk

Table 1 represents the changes in pH and TA of fermented SM, AM, and their combination using two strains of *Bifidobacterium* spp. (Bg and Bc) compared to the control during 21 days of storage. The pH of FSM (100)-C was slightly acidic (pH 5.87-5.68; p > 0.05) than FAM (100)-C (pH 5.95-5.69) during the storage. Combining the milk at various ratios (control) altered the pH (p > 0.05), with the lowest being in FSM/AM (5.26-4.74; 25:75) during 21 days of storage. During storage, a gradual decline (p < 0.05) in pH was noticed in FSM & FAM (100)- Bg or Bc and their combination in comparison to their respective controls (Table 1). Cold storage reduced (p < 0.05) the pH of both FSM & FAM (100))- Bg or Bc and their combination with no significant differences noted between them over 21 days of storage.

FSM & FAM (100)- Bg or Bc and their combination showed higher TA values compared to their respective controls during the storage except for FAM (100)-Bc on day 21 (Table 1). FSM (100)-Bg showed the highest acidity (0.87 ± 0.057% LAE) compared to Bc on day 14. However, FAM (100)-Bg displayed the highest TA (0.47 - 0.63% LAE; p < 0.05) than Bc (0.42 – 0.56% LAE) during 21 days of storage. There were no significant differences in TA between both Bg and Bc in FSM/AM (75:25). FSM/AM (50:50)-Bg showed the highest acidity (0.72 ± 0.10 & 0.78 ± 0.057% LAE; p < 0.05) compared to Bc (0.60 ± 0.057 & 0.65 ± 0.057% LAE) on day 7 and 14 respectively. Maximum TA was noticed for FSM/AM (25:75)-Bc as compared to Bg during 1, 7 & 14 days (Table 1).

Titratable acidity varied with a starter culture, chemical composition of milk particularly fermentable sugars, milk concentration, cold storage, temperature, and time (Costa et al., 2017). Bifidobacteria mainly produce acetic and lactic acids through carbohydrate metabolism (Shori et al., 2021). In our analysis, the TA values of all the fermented samples were greater than their corresponding controls. In addition, *B.longum* improved the acidity in FSM & FAM (100), and FSM/AM (50:50). However, *B.lacts* enhanced the acidity in FSM/AM (25:75). Thus, the addition of two Bifidobacteria strains to almond and soymilk and their combination enhanced acid accumulation during fermentation. Previous studies have demonstrated that almond products promote the viability of probiotic bacteria including Bifidobacteria leading to an increase in acid production during fermentation and storage (Shi et al., 2020; Lipan et al., 2020;

Table 1. Changes in pH values and titratable acidity (TA; % lactic acid equivalent; %LAE) of *B longum* (Bg), or *B lactis* (Bc) of fermented soymilk and almond milk and their combination (100%, 75%, 50%, & 25%) compared to control during 21 days of refrigerated storage at 4 °C.

Sample	pH				TA (% LAE)			
	Storage period (days)				Storage period (days)			
	1	7	14	21	1	7	14	21
SM (100)-C	5.87 ± 0.07	5.85 ± 0.03	5.79 ± 0.02	5.68 ± 0.01	0.27 ± 0.10	0.29 ± 0.05	0.38 ± 0.05	0.51 ± 0.05
SM (100)- Bg	4.42 ± 0.02	4.40 ± 0.01	4.29 ± 0.04	4.15 ± 0.04	0.60 ± 0.05	0.74 ± 0.05	0.87 ± 0.05	0.96 ± 0.05
SM (100)- Bc	4.50 ± 0.02	4.41 ± 0.05	4.37 ± 0.02	4.32 ± 0.03	0.65 ± 0.05	0.74 ± 0.05	0.78 ± 0.05	0.87 ± 0.05
AM (100)-C	5.95 ± 0.13	5.75 ± 0.07	5.71 ± 0.09	5.69 ± 0.09	0.29 ± 0.05	0.33 ± 0.05	0.36 ± 0.10	0.56 ± 0.05
AM (100)- Bg	4.37 ± 0.02	4.32 ± 0.03	4.27 ± 0.03	4.19 ± 0.01	0.47 ± 0.05	0.56 ± 0.05	0.60 ± 0.05	0.63 ± 0.10
AM (100)- Bc	4.29 ± 0.01	4.25 ± 0.02	4.2 ± 0.02	4.13 ± 0.03	0.42 ± 0.05	0.47 ± 0.05	0.51 ± 0.05	0.56 ± 0.05
SM/AM (75:25)-C	5.53 ± 0.06	5.26 ± 0.01	5.16 ± 0.02	5.03 ± 0.06	0.29 ± 0.05	0.36 ± 0.10	0.47 ± 0.05	0.54 ± 0.10
SM/AM (75:25)- Bg	4.42 ± 0.04	4.39 ± 0.01	4.21 ± 0.02	4.12 ± 0.05	0.56 ± 0.05	0.69 ± 0.05	0.78 ± 0.05	0.83 ± 0.05
SM/AM (75:25)- Bc	4.32 ± 0.06	4.30 ± 0.02	4.25 ± 0.03	4.16 ± 0.04	0.56 ± 0.05	0.65 ± 0.05	0.74 ± 0.05	0.81 ± 0.10
SM/AM (50:50)-C	5.64 ± 0.04	5.49 ± 0.08	5.28 ± 0.02	5.15 ± 0.02	0.27 ± 0.10	0.29 ± 0.05	0.36 ± 0.10	0.45 ± 0.10
SM/AM (50:50)- Bg	4.35 ± 0.04	4.32 ± 0.01	4.21 ± 0.11	4.01 ± 0.05	0.51 ± 0.05	0.72 ± 0.10	0.78 ± 0.05	0.83 ± 0.05
SM/AM (50:50)- Bc	4.35 ± 0.04	4.34 ± 0.01	4.28 ± 0.01	4.17 ± 0.06	0.51 ± 0.05	0.60 ± 0.05	0.65 ± 0.05	0.78 ± 0.05
SM/AM (25:75)-C	5.26 ± 0.01	5.06 ± 0.02	4.88 ± 0.03	4.74 ± 0.04	0.27 ± 0.10	0.36 ± 0.10	0.45 ± 0.10	0.54 ± 0.10
SM/AM (25:75)- Bg	4.31 ± 0.02	4.28 ± 0.02	4.19 ± 0.01	4.10 ± 0.04	0.38 ± 0.05	0.47 ± 0.05	0.56 ± 0.05	0.81 ± 0.10
SM/AM (25:75)- Bc	4.25 ± 0.02	4.18 ± 0.02	4.12 ± 0.02	4.05 ± 0.03	0.54 ± 0.00	0.63 ± 0.1	0.65 ± 0.05	0.78 ± 0.05

Shori, 2022b). Similarly, several studies found that soybean is a good carrier for probiotic bacteria, especially *Bifidobacterium* spp (Patrignani et al., 2018; Joel et al., 2021; Shori & Alzahrani, 2022). An earlier study found that fermented soymilk with probiotic Bifidobacterium strains i.e. *B. longum*-Ya3 and *B. adolescentis*-C52 enhanced the accumulation of lactic acid during the fermentation process and storage (Trufkati et al., 2021). Moreover, *B. longum* significantly improved the acidity in barley (hemp) milk (Merenkova et al., 2022).

3.2 Viable Cell Counts (VCC) of Bifidobacterium bacteria in fermented plant milk

Table 2 represents the changes in the viable cell counts in fermented SM, AM, and their combination using two strains of *Bifidobacterium* spp. (Bg and Bc) compared to the control during 21 days of storage. Both B*ifidobacterium*-treated samples had significantly higher viability than their respective controls. In addition, the two starter cultures used showed similar ($p > 0.05$) viable cell counts irrespective of the concentrations. All the treatments maintained viability ranging between 6.9 and 7.4 log throughout storage at 4 °C.

The addition of bifidobacteria i.e. *B. longum*, and *B. lacts* in soy and almond milk during fermentation enhanced their viability over their respective controls. Although there were no appreciable differences ($p > 0.05$) observed in survival rate between *B. longum*, and *B .lacts* in both fermented soy and almond milk, our results confirm a previous study that found no differences between *B. infantis* CCRC 14633 and *B. longum* B6 in fermented soymilk during storage (Wang et al., 2002). A study by Karaçalı et al. (2018) reported that the VCC of *B. longum* & *B. animalis subsp. lactis* in soymilk kefir were 7.30 & 7.25 log cfu/mL, respectively after 21 days at 4 °C. In our study, both *B. longum*, and *B. lacts* maintained the viability of 6.9 and 7.4 log cfu/mL during 21 days of storage for both soy and almond milk and their combination. Mustafa et al. (2020) found that the growth of *B. longum* BB536 has effectively increased in soymilk up to 10^6 cfu/mL after 48 h of fermentation. This is because soymilk contains enough readily available energy sources for bifidobacteria growth (Kaprelyants et al., 2020). Nutrients such as dietary fiber, protein, oligosaccharides, and polyphenols available in soy and almond milk may act as a substrate for microbial growth during milk fermentation (Liu et al., 2016; Ribeiro et al., 2023).

3.3 Determination of TPC and TFC in fermented plant milk

Table 3 represents the changes in the TPC and TFC in fermented SM, AM, and their combination using two strains of *Bifidobacterium* spp. (Bg and Bc) compared to the control during 21 days of storage. Both SM & FAM (100)- Bg & Bc samples displayed greater TPC ($p < 0.05$) than their respective controls the control during 3 weeks of storage except for FAM (100) on day 14 (Table 3). A maximum TPC was seen in FSM (100)-Bc during two weeks of storage (~ 98 µg GAE/mL) followed by a steady decline ($p < 0.05$) up to (80.12 ± 0.024 µg GAE/mL) on day 21. The presence of *B.lacts* in FAM (100) enhanced ($p < 0.05$) TPC more than *B.longum* on day 1. The TPC in FAM (100)-Bc decreased to the lowest value in the last two weeks (Table 3). The TPC of FSM & FAM(100) was the highest (120.24 ± 0.0025 & 50 ± 0.0096 µg GAE/mL; $p < 0.05$, respectively) in Bg compared to Bc on day 21.

There were no significant differences in TPC between the two starter cultures inoculated in FSM/AM (75:25 & 50:50) on day 1 of storage (Table 3). FSM/AM (75:25)-Bc demonstrated a significant increase ($p < 0.05$) in TPC compared to Bg and control during the last 2 weeks of storage. FSM/AM (50:50)-Bc showed maximum activity of TPC (69.94 ± 0.0065 µg GAE/mL; $p < 0.05$) compared to Bg on day 7. TPC in FSM/AM (50:50)-Bg & Bc was 2 folds higher (74.3 ± 0.021 & 61.34 ± 0.037 µg GAE/mL; $p < 0.05$) than control (22.52 ± 0.168) on day 21. A significant increase in TPC was observed in both FSM/AM (25:75)-Bg & Bc than control on the last 3 weeks (Table 3).

Table 2. Changes in viable cell counts (VCC; log cfu/mL) of *B longum* (Bg), or *B lactis* (Bc) of fermented soymilk and almond milk and their combination (100%, 75%, 50%, & 25%) compared to control during 21 days of refrigerated storage at 4 °C.

Sample	Viability count (log cfu/mL)			
	Storage period (days)			
	1	7	14	21
SM (100)-C	3.411 ± 0.107	3.520 ± 0.157	3.870 ± 0.051	3.887 ± 0.060
SM (100)- Bg	7.366 ± 0.052	7.358 ± 0.071	7.276 ± 0.045	7.418 ± 0.017
SM (100)- Bc	7.346 ± 0.015	7.366 ± 0.016	7.375 ± 0.105	7.328 ± 0.019
AM (100)-C	3.431 ± 0.141	3.436 ± 0.078	3.666 ± 0.125	3.539 ± 0.170
AM (100)- Bg	7.038 ± 0.097	7.333 ± 0.119	7.151 ± 0.031	7.089 ± 0.165
AM (100)- Bc	7.049 ± 0.160	7.304 ± 0.037	7.308 ± 0.078	7.257 ± 0.080
SM/AM (75:25)-C	3.593 ± 0.123	3.762 ± 0.064	4.221 ± 0.055	3.334 ± 0.141
SM/AM (75:25)- Bg	7.291 ± 0.081	7.323 ± 0.030	7.360 ± 0.074	7.216 ± 0.088
SM/AM (75:25)- Bc	7.312 ± 0.047	7.168 ± 0.031	7.260 ± 0.039	7.367 ± 0.048
SM/AM (50:50)-C	3.816 ± 0.063	3.741 ± 0.067	3.555 ± 0.107	3.509 ± 0.113
SM/AM (50:50)- Bg	7.347 ± 0.069	7.399 ± 0.088	7.278 ± 0.097	7.269 ± 0.019
SM/AM (50:50)- Bc	7.332 ± 0.044	7.20 ± 0.136	7.209 ± 0.114	7.114 ± 0.039
SM/AM (25:75)-C	3.734 ± 0.188	3.682 ± 0.076	3.597 ± 0.154	3.484 ± 0.180
SM/AM (25:75)- Bg	7.161 ± 0.062	7.224 ± 0.118	7.319 ± 0.062	7.345 ± 0.052
SM/AM (25:75)- Bc	7.085 ± 0.051	7.359 ± 0.008	7.186 ± 0.070	6.934 ± 0.161

Table 3. Changes in total phenolic content (TPC; µg GAE/mL) and total flavonoid content (TFC; µg/g) of *B longum* (Bg), or *B lactis* (Bc) of fermented soymilk and almond milk and their combination (100%, 75%, 50%, & 25%) compared to control during 21 days of refrigerated storage at 4 °C.

Sample	TPC (µg GAE/mL)				TFC (µg/g)			
	Storage period (days)				Storage period (days)			
	1	7	14	21	1	7	14	21
SM (100)-C	51.75 ± 0.003	40.43 ± 0.005	34.31 ± 0.025	30.50 ± 0.017	16.75 ± 0.002	16.70 ± 0.019	18.36 ± 0.003	19.17 ± 0.004
SM (100)- Bg	102.67 ± 0.070	91.94 ± 0.041	97.64 ± 0.023	120.24 ± 0.002	18.62 ± 0.004	17.38 ± 0.014	16.19 ± 0.002	18.42 ± 0.001
SM (100)- Bc	96.85 ± 0.026	98.85 ± 0.006	98.67 ± 0.042	80.12 ± 0.024	16.55 ± 0.003	18.36 ± 0.019	16.89 ± 0.025	16.50 ± 0.009
AM (100)-C	23.84 ± 0.001	29.00 ± 0.008	41.79 ± 0.005	22.15 ± 0.007	18.24 ± 0.012	19.75 ± 0.022	21.77 ± 0.006	22.28 ± 0.013
AM (100)- Bg	43.64 ± 0.005	37.27 ± 0.019	38.79 ± 0.019	50.00 ± 0.009	17.48 ± 0.003	19.18 ± 0.028	18.33 ± 0.004	16.25 ± 0.016
AM (100)- Bc	54.55 ± 0.009	35.82 ± 0.010	32.73 ± 0.010	38.00 ± 0.018	17.54 ± 0.001	18.25 ± 0.008	18.19 ± 0.002	17.03 ± 0.005
SM/AM (75/25)-C	55.63 ± 0.011	73.14 ± 0.008	72.41 ± 0.003	41.79 ± 0.059	23.15 ± 0.008	21.06 ± 0.004	20.05 ± 0.011	22.89 ± 0.022
SM/AM (75:25)- Bg	72.06 ± 0.009	78.30 ± 0.020	59.76 ± 0.004	45.94 ± 0.026	16.22 ± 0.003	17.23 ± 0.013	16.95 ± 0.003	16.51 ± 0.007
SM/AM (75:25)- Bc	71.52 ± 0.098	86.06 ± 0.032	87.94 ± 0.028	57.09 ± 0.008	15.93 ± 0.001	20.44 ± 0.009	18.79 ± 0.006	16.30 ± 0.003
SM/AM (50/50)-C	42.34 ± 0.030	50.32 ± 0.002	72.30 ± 0.028	22.52 ± 0.168	20.98 ± 0.017	22.22 ± 0.002	21.41 ± 0.001	18.86 ± 0.010
SM/AM (50:50)- Bg	68.06 ± 0.016	60.67 ± 0.013	69.94 ± 0.001	74.30 ± 0.021	18.33 ± 0.005	18.14 ± 0.011	16.36 ± 0.004	16.82 ± 0.005
SM/AM (50:50)- Bc	63.52 ± 0.013	69.94 ± 0.006	73.88 ± 0.010	61.34 ± 0.037	17.71 ± 0.001	20.48 ± 0.016	17.82 ± 0.031	16.24 ± 0.002
SM/AM (25/75)-C	37.68 ± 0.003	32.59 ± 0.003	30.32 ± 0.037	26.00 ± 0.026	19.01 ± 0.002	21.35 ± 0.019	23.04 ± 0.007	16.70 ± 0.019
SM/AM (25:75)- Bg	41.52 ± 0.005	61.27 ± 0.019	50.73 ± 0.003	33.21 ± 0.026	17.69 ± 0.002	18.44 ± 0.008	18.56 ± 0.007	17.51 ± 0.020
SM/AM (25:75)- Bc	35.82 ± 0.011	59.40 ± 0.013	44.97 ± 0.014	31.34 ± 0.006	18.17 ± 0.004	18.41 ± 0.032	21.89 ± 0.224	15.55 ± 0.002

In FSM (100)-Bg displayed the highest TFC (18.62 ± 0.00 µg/g; $p < 0.05$) on day 1. This followed by significant reduction to 16.19 ± 0.00 µg/g on day14. The least content of TF was observed for FSM (100)-Bc (16.5 ± 0.009 µg/g) than Bg and control on day 21. No significant difference between control and treatments on the first two weeks. However, FAM (100)-C showed higher TFC (~22 µg/g) than treatments on the last 2 weeks of storage (Table 3). All the treatments in FSM/AM (75:25) & (50:50) showed a decrease ($p < 0.05$) in TFC compared to control during 21 days of storage except for 7- and 14-day-old FSM/AM (75:25)-Bc and 7 day- old FSM/AM (50:50)-Bc that showed nearly parallel results to control (Table 3). FSM/AM (25:75)-Bg showed the lower TFC ($p < 0.05$) than Bc that showed almost similar result to control on day 14 of storage (Table 3).

Polyphenol antioxidants in plants protect against oxidative stress and age-related illnesses (Bodoira & Maestri, 2020; Hano & Tungmunnithum, 2020). The increase of TPC and TFC in both fermented soy and almond milk during storage may be related to the ability of *B. longum* and *B. lacts* to increase aglycones levels in fermented milk (Donkor & Shah, 2008). Karaçalı et al. (2018) showed that fermented soymilk kefir containing different types of *Bifidobacterium* spp. increased TPC compared to unfermented soymilk. In addition, fermented almond milk by *S. thermophilus*, *L. delbrueckii* subsp. *bulgaricus*, *L. acidophilus*, and *B. animalis subsp. Lactis* has been reported to increase TPC during 21 days of storage (Topcuoglu & Yilmaz-Ersan, 2020). Bifidobacteria can produce β-glucosidase leading to degraded isoflavone glycosides from soymilk to bioactive aglycones (Queirós et al., 2020; Peirotén et al., 2020). In addition, the levels of β-glucosidase activity were found to vary among the different starter cultures during milk fermentation (Delgado et al., 2019; Peirotén et al., 2020).

In the current study, Bc in fresh FSM & FAM (100) significantly enhanced TPC compared to other samples whereas Bg enhanced the TPC of FSM & FAM (100) on 21 days. A similar observation was also noted by Karaçalı et al. (2018) who found soymilk kefir fermented with *B. longum* or *B. animalis* subsp. *lactis* strain increased TPC. Further decreases in TPC and TFC during the last two weeks of storage suggest degradation of polyphenol by Bifidobacterium. Lactic acid bacteria possess certain enzymes like phenolic acid decarboxylases, which aid in reducing polyphenol concentrations in the products at the end of storage (Shori, 2020b).

The addition of two *Bifidobacterium* spp. (i.e. *B. longum* and *B. lacts*) into soy and almond milk during fermentation have been demonstrated to boost the phenolic and flavonoid content at varied concentrations compared to the control during storage. Higher TPC and TFC are associated with greater free radical scavenging ability, which ultimately improves the antioxidant properties of fermented milk (Sharma et al., 2021).

3.4 Antioxidant properties (DPPH, FIC, and FRAB) in fermented plant milk

Figures 1-3(A-E) represent the changes in DPPH radical scavenging activity, FIC, and FRAP in fermented SM, AM, and their combination using two strains of *Bifidobacterium* spp. (Bg and Bc) compared to the control during 21 days of storage. During storage, a higher percentage ($p < 0.05$) of scavenging activity was shown in all treatments as compared to their respective controls (Figure 1A-1E). There were no significant differences in DPPH radical scavenging activity between the two starter cultures inoculated in FSM & FAM (100) samples during the 21 days of storage (Figure 1A-1B). FAM (100)-Bg & Bc showed a significant ($p < 0.05$) decrease in scavenging activity on the day 1 (83.16% ± 0.017 & 79.69% ± 0.0107%; respectively) compared to control (26.29% ± 0.015) followed by a substantial increase during two weeks of storage (up to 90%; Figure 1B). No significant differences in DPPH scavenging activity between the two starter cultures inoculated in FSM/AM combinations during the storage. However, FSM/AM (50:50)-Bc was significantly increased to the maximum scavenging activity (96.93% ± 0.0085) than Bg on day 1.

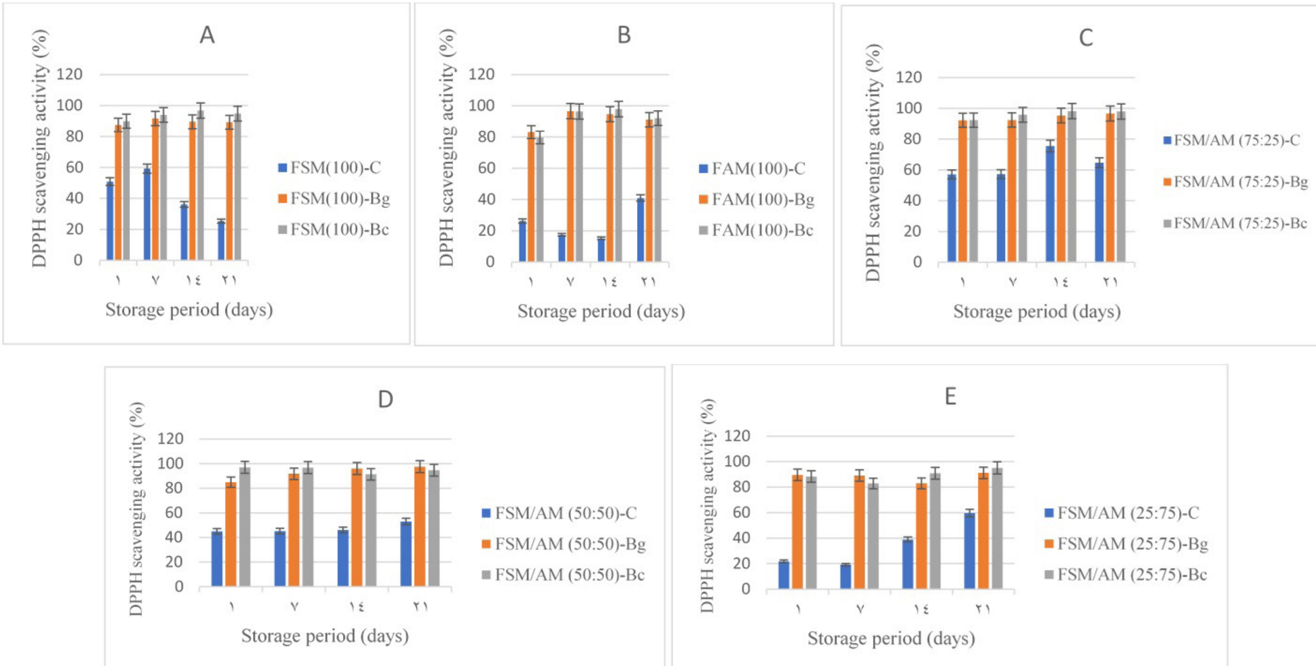

Figure 1. Changes in DPPH scavenging activity (%) of *B longum* (Bg), or *B lactis* (Bc) of fermented (F) soymilk (SM) and almond milk (AM) and their combination using different concentrations (A = 100% FSM, B = 100% FAM, C = 75:25% FSM/AM, D = 50:50% FSM/AM, E = 25:75% FSM/AM) compared to control (C) during 21 days of refrigerated storage at 4 °C. Data are presented as mean ± SEM. The level of significance was preset at $p < 0.05$ compared to control at the same storage period.

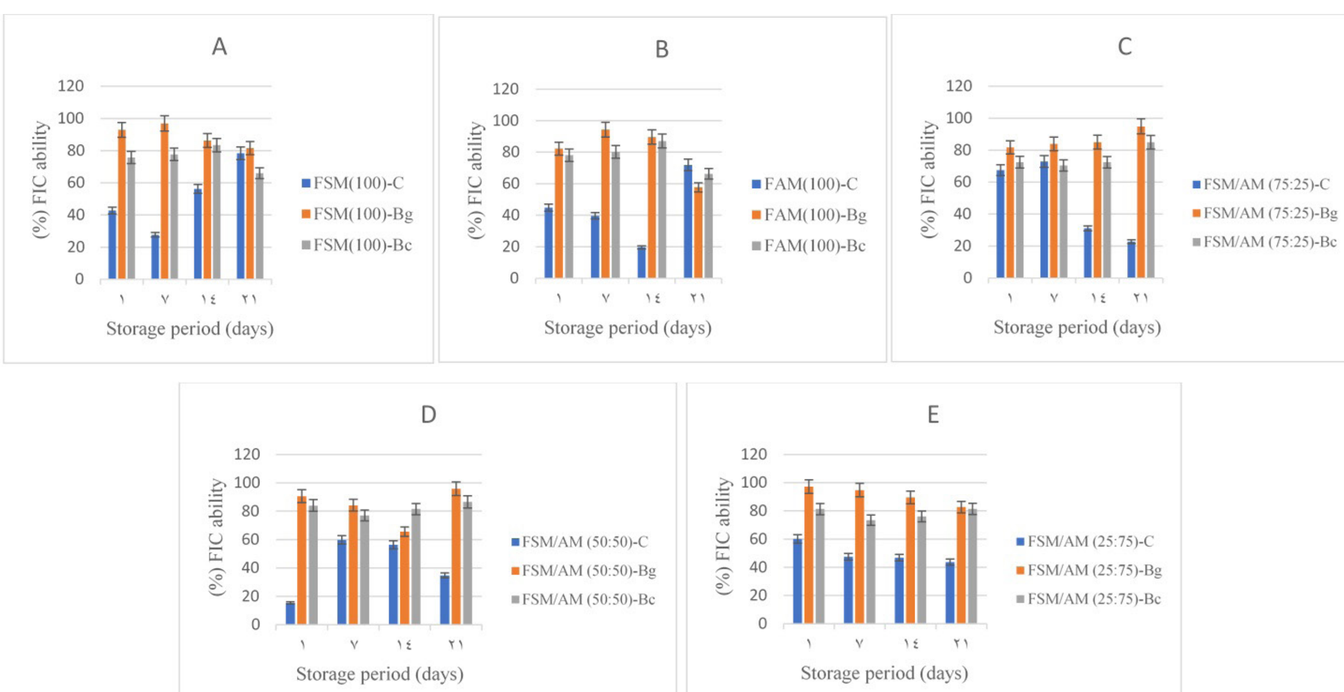

Figure 2. Changes in ferrous ion-chelating (FIC; %) of *B longum* (Bg), or *B lactis* (Bc) of fermented (F) soymilk (SM) and almond milk (AM) and their combination using different concentrations (A = 100% FSM, B= 100% FAM, C = 75:25% FSM/AM, D = 50:50% FSM/AM, E = 25:75% FSM/AM) compared to control (C) during 21 days of refrigerated storage at 4 °C. Data are presented as mean ± SEM. The level of significance was preset at $p < 0.05$ compared to control at the same storage period.

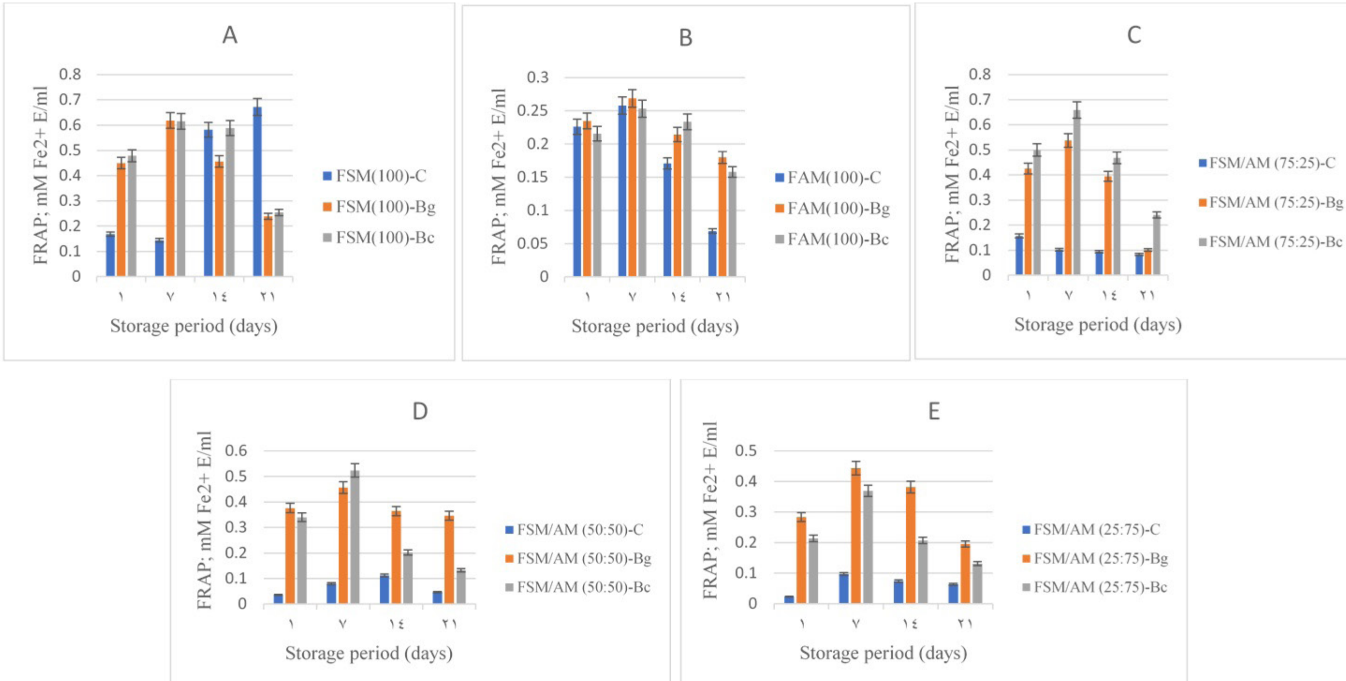

Figure 3. Changes in ferric reducing antioxidant potential (FRAP; mM Fe^{2+} E/mL) of *L B longum* (Bg), or *B lactis* (Bc) of fermented (F) soymilk (SM) and almond milk (AM) and their combination using different concentrations (A = 100% FSM, B = 100% FAM, C = 75:25% FSM/AM, D = 50:50% FSM/AM, E = 25:75% FSM/AM) compared to control (C) during 21 days of refrigerated storage at 4 °C. Data are presented as mean ± SEM. The level of significance was preset at p < 0.05 compared to control at the same storage period.

Both treated samples FSM & FAM (100) exhibited higher (p < 0.05) FIC activity than their respective controls during 14 days of storage (Figure 2A-2B). In addition, FSM (100)-Bg displayed maximum (p < 0.05) FIC activity compared to Bc during one week of storage. FAM (100)-Bg showed the highest FIC activity (94.29 ± 0.017%) than Bc (80.18 ± 0.004%) on day 7 days of storage (Figure 2B). Both Bc and Bg decreased (p < 0.05) FIC activity to 66% and 57.77% for FSM & FAM (100), respectively on day 21 of storage. FSM/AM samples demonstrated increased (p < 0.05) FIC activity than their respective controls during 21 days of storage except for FSM/AM (75:25)-Bc on day 1 and 7 (Figure 2C). FSM/AM (75:25)-Bg showed higher FIC activity than Bc over 21 days. FSM/AM (50:50)-Bc displayed higher (81.50 ± 0.015%; p < 0.05) FIC activity than Bg (65.52 ± 0.002%) on day 14 of storage. In addition, the FIC of FSM/AM (25:75)-Bg was higher than Bc during two weeks of storage.

FSM (100) with both starter cultures presented greater (p < 0.05) FRAP activity than control during one week of storage whereas, FAM (100) showed better (p < 0.05) activity during the last 2 weeks (Figure 3A-3B). Moreover, FRAP activity in FSM (100)-Bg significantly decreased (0.455 ± 0.162 mM Fe^{2+} E/mL) compared to Bc (0.588 ± 0.016 mM Fe^{2+} E/mL) on day 14. FSM/AM samples with both starter cultures exhibited higher (p < 0.05) FRAP activity than their respective controls during 21 days of storage (Figure 3C-3E). Furthermore, FRAB activity increased ~ up to 3x and 4x for FSM/AM (75:25)-Bg and Bc, respectively over two weeks. FSM/AM (75:25)-Bc achieved higher FRAP activity than Bg during 21 days of storage. Both Bg & Bc showed maximum FRAP activity in FSM/AM (75:25 & 50:50) on day 7 followed by a decline (p < 0.05) up to the 21 day (Figure 3C-3D). Maximum FRAP values was shown in FSM/AM (25:75)-Bg & Bc on day 7 whereas minimum values was seen on day 21. In addition, inoculated Bg in FSM/AM (25:75) significantly enhanced (p < 0.05) FRAP activity compared to Bc during the 21 days.

It is well known that polyphenols are among the most powerful natural antioxidants since they have several hydroxyl groups present in their structures, which help them scavenge free radicals (Gulcin, 2020; Al-Sulbi & Shori, 2022). Soy and almond milk have great antioxidant properties because of their polyphenol content (Tonolo et al., 2019; Topcuoglu & Yilmaz-Ersan, 2020). In the present study, the antioxidant activity of fermented soy and almond milk and their combination using three different methods (DPPH, FIC, and FRAP) was vary depending on the type of *Bifidobacterium* spp. (i.e. *B. longum* and *B. lactis*) and milk concentration used during the fermentation. All fermented samples showed higher DPPH and FIC than their respective controls during the storage. In addition, Bc in fresh FSM/AM (100) improved DPPH activity to about 97%. Fermented soybean with *L. plantarum* KFRI 00144, *L. delbrueckii* subsp. *latis* KFRI01181, *B. thermophilum* KFRI00748, and *B. breve* K-101 increased antioxidant activity by about 78.5% during the storage period (Pyo et al., 2005). Pham & Shah (2007) found a reduction of ~ 27% in total isoflavone glycoside content in soymilk supplemented with skim milk powder fermented by *B. animalis lactis* after 24 h. Most isoflavones in soybeans are glycosides, and they are transformed into aglycones by microbial β-glycosidase activity during fermentation (Hwang et al., 2016). Karaçalı et al. (2018) reported that *B. longum* and *B. lactis* are capable to ferment soymilk with a DPPH activity of ~16%. The peel of almonds contains bioactive prebiotic compounds such as xylooligosaccharides, polysaccharides, hemicellulose, and dietary fiber which may enhance the growth of the *Bifidobacterium* spp. during fermentation and storage (Barral-Martinez et al., 2021).

4 Conclusion

The present study investigated the use of soy and almond milk and their combination as the main substrate for fermentation by two probiotic *Bifidobacterium* strains (i.e. *B.longum* and *B. animalis subsp lactis*) during a storage period of 21 days at 4 ºC. A significant variation was seen depending on the starter cultures and the concentration of milk used. All probiotic *Bifidobacterium spp*. improved post-acidification, VCC, TPC, and antioxidant activity in fermented plant-based milk samples during storage. All probiotic *Bifidobacterium spp.* in fermented samples have maintained the viability ranging between 6.9 and 7.4 log cfu/mL during the storage period. Fermented soy and almond milk and their combination showed a potential application that might serve as an effective vehicle for *B. longum* and *B. animalis* subsp *lactis*. In addition, consumers might benefit from an innovative probiotic fermented plant-based milk with antioxidant activity.

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