

Article

Synergistic Evaluation of *Lactobacilli* on Probiotic Viability and Antioxidant Properties in Almond and Cow Milk

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Abstract

Lactobacilli are important probiotic groups recognized for their numerous health-promoting properties. This study investigated how four probiotic strains, *Lactocaseibacillus rhamnosus* (Lr), *Lactobacillus acidophilus* (La), *Lactiplantibacillus plantarum* (Lp), or *Lactocaseibacillus casei* (Lc), affected post-acidification, viable cell counts (VCCs), total phenolic and flavonoid contents (TPCs and TFCs, respectively), and antioxidant activity of fermented almond milk (FAM) and its combination with cow's milk (CM) at different concentrations (75:25, 50:50, and 25:75) during 1, 7, 14, and 21 days of storage. All FAM and its mixture with CM showed significantly greater ($p < 0.05$) post-acidification than their respective controls throughout storage. Viable cell counts in all samples ranged from 5.9 to 6.8 log cfu/mL, which were higher than those of the controls (3–4 log cfu/mL; $p < 0.05$). Total phenolic contents in FAM/CM (75:25 and 50:50 and 25:75)-Lc increased more than twofold (95.82 ± 0.003 and 105.71 ± 0.008 and 101.02 ± 0.071 $\mu\text{g GAE/mL}$; $p < 0.05$) compared to the controls (19–40 $\mu\text{g GAE/mL}$) by the end of the third week. *Lbs. rhamnosus* enhanced ($p < 0.05$) TFCs in FAM/CM (25:75) after the first day of storage. All lactobacilli strains improved the antioxidant activity in all treated samples during storage. In conclusion, the combination of fermented almond milk with cow's milk may serve as an excellent carrier for *Lbs. rhamnosus*, *Lab. acidophilus*, *Lpb. plantarum*, and *Lbs. casei*, which exhibit antioxidant activity.



Academic Editor: Alessandra Pino

Received: 26 June 2025

Revised: 17 October 2025

Accepted: 21 October 2025

Published: 29 October 2025

Citation: Al Zahrani, A.J.; Shori, A.B.; Altammar, K.A. Synergistic Evaluation of *Lactobacilli* on Probiotic Viability and Antioxidant Properties in Almond and Cow Milk. *Fermentation* **2025**, *11*, 616. <https://doi.org/10.3390/fermentation11110616>

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Keywords: probiotics; cow milk; almond milk; lactobacilli; viability; antioxidant activity

1. Introduction

Milk is widely recognized as the most nutritious food. It supplies several nutrients that are essential for the developing stages of our bodies [1]. Cow's milk contains 87% water, along with various types of nutrients, such as proteins (3.4%; e.g., caseins and whey proteins), lactose (4.6%), fat (4.2%), amino acids, minerals (0.8%), vitamins (0.1%; e.g., retinol, vitamin B2, and vitamin B12), calcium, phosphorus, iron and other minerals that balance our body functions [2]. It comprises various bioactive components that improve physiological processes in the human body [3]. The structure and composition of cow's milk change based on several factors such as breeding, feeding techniques, cow care, lactation stage, and seasons [4,5]. Furthermore, the dairy milk market in Saudi Arabia registered a significant annual growth rate of 4.11% from the Interval 2013 to 2018 [6]. According to analysts' forecasts, the Saudi dairy sector is expected to continue expanding during 2020–2024 in line with global trends. More broadly, dairy product consumption per capita

in developing countries is increasing at a rate of 0.8–1.7% per year [7], reflecting a growing demand for dairy-based foods worldwide.

Plant-based dairy products have a long history in both Eastern and Western cultures and are usually available in the market [8]. While certain plant milk products contain minimal protein and calcium, plant alternatives to milk are used to substitute for cow's milk in dietary supplements due to their low allergic and intolerance difficulties, lactose-free, fat-free, and healthy properties [9]. As a result, it has been noted that plant-based milk is increasing in interest among individuals [10], which has changed the direction of the nutritional areas to satisfy consumer wants and provide health-promoting and anti-aging approaches [11]. Plant-based milk substitutes are essential to vegetarian food production since they are used as an integral ingredient in a wide range of dietary food products like plant-based cheese and yogurt-like products. According to Mintel, plant-based milk consumption has increased by 19% from 2018 to early 2019. For instance, almond milk sales have increased by 10% [12].

Almond milk (AM) is a traditional product that can be used as a substitute for cow's milk in individuals who are lactose-intolerant, allergic to milk, or prefer to avoid dairy products [13]. AM was first identified at the beginning of the 1990s as free of lactose, lipids, or saturated fat. AM is ideal for people with a lactose sensitivity and has a low-calorie content of 30–60 kcal per 100 mL. Moreover, almond products are considered healthy foods and are widely used due to their therapeutic characteristics, especially by Persians, Chinese, and Indians who habitually consume them for medical purposes in their traditions [14,15]. The beneficial health impacts of almond milk consumption are related to its composition of macro- and micronutrients [16]. It includes several minerals, phytochemicals, and fatty acids that significantly lower cardiovascular risk [17]. It also serves as a powerful antioxidant, anti-inflammatory, antihyperlipidemic, anticancer, and strengthens the body's defenses [18,19].

Almond milk contains beneficial components, such as campesterol, beta-sitosterol, stigmasterol folate, vitamin E, vitamin B3, and arabinose sugar [20]. Several secondary metabolites, such as polyphenols, have been detected in almond blanch liquids, almond hulls, and almond covers [21]. The most abundant polyphenols were hydrolysable tannins, flavonoids, and proanthocyanidins. Moreover, phenolic acids, lignans, isoflavones, and stilbenes were also detected in reasonable concentrations in almonds [22]. Almonds and their co-products contain a variety of flavonoids, with the most abundant compounds being flavan-3-ols, dihydrokaempferol, and (–)-epicatechin. However, dihydroquercetin, galocatechin gallate, epicatechin gallate, and epicatechin glycoside were also detected in small amounts [23,24]. Flavonols are the most abundant flavonoid types in almond milk, including kaempferol, quercetin, isorhamnetin, rutinoides, galactosides, and morin [25,26]. Furthermore, almonds are considered a good source of phenolic compounds, which enhance the color, astringency, and shelf life of almonds, as well as many other biological functions [27]. Previous research has indicated that catechin is the primary phenolic found in almond milk [28,29]. The effects of pasteurization, roasting, and storage on the polyphenol content of almond skins have been widely studied. In the study by Bolling et al. [21], roasting reduced total phenols without affecting flavonoids or phenolic acids.

In the previous 20 years, almond consumption has increased significantly [30], demonstrating that these nuts have transformed from a handy snack to a vital component of the diet for preserving human health, both in the public population and suppliers [22]. Previous research has linked almond intake to a variety of health advantages, including the modification of blood lipid levels [31,32], glucose levels [33,34], the regulation of body weight [15,35], and prevention of several illnesses, such as cardiovascular diseases [16,36], diabetes [34,37], and obesity [35,38]. Nowadays, the market for probiotic dairy products is increasing as people become more aware of their potential health benefits. Dairy products are believed to be

beneficial to health [39] and are considered an essential dietary category [40]. Furthermore, several studies have demonstrated the health benefits of consuming these products [41–44].

Antioxidants are defined as compounds that prevent, minimize, delay, or eliminate oxidative damage to a target molecule, and include any sort of molecule found in vivo that directly scavenges reactive oxygen species (ROS) or indirectly acts to upregulate antioxidant defenses or suppress ROS formation [45]. Antioxidant compounds can prevent free radical formation and suppress oxidative chain reactions [46,47]. Consequently, continuous replenishment of antioxidant resources, either endogenously or through supplementation, is needed to counteract excess free radicals. Natural antioxidants occur primarily in plant phenolics that can be present in any part of the plant. Furthermore, plants generate secondary metabolites in many forms, such as flavonoids and phenolic acids. Plants generate various kinds of supplementary metabolites, such as lignans, tannins, flavonoids, and phenolic acids [48–51]. Artificial antioxidants have been utilized to protect foods from oxidative damage [52]. However, several artificial antioxidants have been reported to possess potential health risks, including carcinogenic effects [52]. As a result, natural antioxidants are favored over artificial ones and are widely accepted by consumers due to their perceived safety [53]. In fact, due to the limitations on the use of artificial antioxidants, the market for health-promoting natural antioxidants in foods is growing [54].

Lactic acid bacteria (LAB) are a useful category of bacteria that may preserve food through lactic acid fermentation. They are often found in milk and dairy products, but they may also be isolated from plant or animal sources [55]. The selection of *Lbs. rhamnosus*, *Lab. acidophilus*, *L. plantarum*, and *Lbs. casei* in this study was based on their well-documented probiotic potential [56], strong fermentative capacity [57], and beneficial effects on the physicochemical and bioactive properties of fermented dairy and plant-based products [58]. Furthermore, it induces a fast decrease in pH and minimizes nutrient losses. Some of these lactobacilli can generate compounds such as hydrogen peroxide, carbon dioxide, diacetyl, or bacteriocins during metabolism, resulting in an environment unfavorable for some microbial pathogens' growth [59] and thereby improving product stability and quality.

The selected strains possess distinct functional attributes relevant to the objectives of this study. Among lactobacilli species, *Lpb. plantarum* demonstrates remarkable adaptability, stress tolerance, and antibacterial activity against various pathogens [60,61], along with the ability to survive gastrointestinal transit [62]. *Lbs. rhamnosus* has been extensively investigated in dairy products in many countries [63]. It is reported to boost short-chain fatty acid (SCFA) production in the gastrointestinal tract [64]. SCFAs can regulate pH and exert anti-inflammatory and antimicrobial properties [65]. *Lbs. casei* exhibits strong adherence to the intestinal mucosa and colonizes the gut. In addition, it is used as a synbiotic supplement to enhance the health properties [66–68]. Moreover, they contribute to flavor creation in foods because of their capacity to produce flavor compounds in fermented products [66]. Meanwhile, *Lab. acidophilus* is widely used in fermented foods due to its capacity to produce lactic acid and antimicrobial compounds, as well as its broad inhibitory spectrum against pathogens [69]. Consequently, the present study focused on the influence of *Lbs. rhamnosus*, *Lab. acidophilus*, *Lpb. plantarum*, or *Lbs. casei* on post-acidification, viable cell counts, total phenolic and flavonoid contents, as well as the antioxidant activity of fermented almond milk (FAM), and its combination with cow's milk (CM) at different concentrations (75:25, 50:50, and 25:75) during 1, 7, 14, and 21 days of storage.

2. Materials and Methods

2.1. Chemicals and Reagents

All chemicals and solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Man–Rogosa–Sharpe (MRS) agar was purchased from HiMedia (Mumbai, India).

2.2. Almond Milk Preparation

A slightly modified version of the wet method-cooked slurry technique outlined by Yu et al. [70] was used to prepare almond (*Prunus dulcis* (Mill.) DA Webb) milk. Almond seeds were purchased from a local store. One hundred grams of cleaned almond seeds were soaked in distilled water (1:9 *w/w*) for sixteen hours at room temperature (22 ± 2 °C) and then ground three times for 10 min using a grinding machine (Philips 600 W, Koninklijke Philips N.V, Amsterdam, The Netherlands). To prevent foaming, the slurry was boiled in an electric oven (Esco Isotherm, Laboratory Ovens, Zhengzhou, China) at 100 °C for 10 min with constant mixing. The heated mixture was filtered through a 100-mesh sieve to separate the almond milk from its solid residue, then refrigerated at 4 °C and used in subsequent experiments within twenty-four hours.

2.3. Preparation of Starter Cultures

A selection of pure species including *Lbs. rhamnosus* ATCC 7469 [71,72], *Lpb. plantarum* ATCC 14917 [73], *Lbs. casei* ATCC 393 [74], and *Lab. acidophilus* ATCC 4356 [75] were obtained by Ain Shams University (National Committee of Microbiology). These probiotics were kept at -80 °C. The lactobacilli species were prepared following Al Zahrani et al. [76], with minor modifications. In brief, sterile 10 mL aliquots of MRS broth (HiMedia, India) were inoculated with the obtained lactobacilli strains (100 µL of each). The samples were then incubated for 48 h at 37 °C. 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.

2.4. Preparation of Fermented Almond Milk

Fermented almond milk (FAM) and its combination with cow's milk (CM) were prepared, namely FAM/CM (75:25), FAM/CM (50:50), and FAM/CM (25:75), using four strains of lactobacilli: *Lbs. rhamnosus* (Lr), *Lab. acidophilus* (La), *Lpb. plantarum* (Lp), or *Lbs. casei* (Lc). One liter of cow's milk and its combination with almond milk were heated at 40 °C. The starter culture (2% *v/v*; containing 10^5 cfu/mL of each strain) was applied to each sample separately and incubated at 40 °C for 9 h [77]. All samples were distributed into sterile disposable plastic bottles and kept in a refrigerator at 4 °C throughout the storage period, and analyses were conducted on days 1, 7, 14, and 21. Control (C) samples were prepared in the same manner but without the starter culture.

2.5. Measurement of pH and Titratable Acidity (TA)

The pH measurements were conducted on fermented milk samples following the instructions provided by Shori [78]. The titratable acidity of the fermented milk samples was determined using the following method: 1 g of the fermented milk sample was weighed and homogenized in a 50 mL test tube containing 9 mL of distilled water. After adding three drops of 1% phenolphthalein indicator, the mixture was titrated with 0.1 mol/L sodium hydroxide until a pinkish end-point lasting 30 s was reached. The titratable acidity was calculated by converting the volume of sodium hydroxide using the equation below.

$$\text{TA}(\%) = \frac{10 \times V_{\text{NaOH}} \times 0.009 \text{ g} \times 0.1}{W} \times 100\%$$

10 = dilution factor, V_{NaOH} = volume of NaOH used to neutralize the lactic acid, 0.009 = conversion factor (1 mL NaOH (0.01 N) neutralizes 0.009 g of lactic acid), 0.1 = Normality of NaOH, W = weight of sample.

2.6. Viable Cell Count (VCC)

Lactobacilli colony counts in the four fermented milk samples were estimated using Man–Rogosa–Sharpe agar (MRS), as previously described by Yong et al. [79]. One milliliter of each processed fermented milk specimen was combined with 9 mL of 0.15% sterile buffered peptone water. Serial dilutions were then prepared up to 10^{-5} using peptone water, and the pour plate technique was applied. All plates were incubated for 48 h at 37 ± 1 °C and the viable cell count was calculated as colony-forming units (CFUs) using the following formula:

$$\text{CFU/mL} = \text{the total number of colonies formed} \times \text{dilution factor of sample/1 mL of sample}$$

(CFU): colony-forming unit.

2.7. Aqueous Extract Preparation

Ten milliliters of each fermented milk specimen was homogenized in dH₂O (2.5 mL) and incubated for 10 min in a water bath (Benchmark Scientific, Sayreville, NJ, USA) at 45 °C [80]. Following calibration of the mixtures to a pH of 4.0 using 0.1 mol/L HCL, they were re-incubated for 10 min. The mixtures were then centrifuged for 10 min at $5000 \times g$, and the supernatant was neutralized to a pH of 7.0 using 0.1 mol/L sodium hydroxide. After a second centrifugation for 10 min, the supernatant was used to determine the TPCs, TFCs, DPPH, FIC, and FRAP.

2.8. Determination of Total Phenolic Content (TPC)

TPC was determined using the Folin–Ciocalteu colorimetric assay, as previously described by Maleki et al. [81]. Briefly, 0.5 mL of Folin–Ciocalteu reagent was mixed with 5 mL of dH₂O and 1 mL of 95% ethanol and then combined with 1 mL of each sample extract, or standard solutions of gallic acid (10–100 µg/mL), and kept at room temperature (22 ± 2 °C) for 5 min. One milliliter of anhydrous Na₂CO₃ solution (5% *w/v*) was added to the samples in the dark at 25 °C. After 1 h, absorbance was measured at 750 nm using a spectrophotometer (Genesys 10 UV, Thermo Scientific, Waltham, MA, USA). TPC was calculated using the gallic acid standard curve and expressed as µg GAE/mL.

2.9. Determination of Total Flavonoid Content (TFC)

The aluminum chloride colorimetric technique was used to estimate the total flavonoid content of the sample. According to Al-Ghafari et al. [82], 75 µL of a 5% sodium nitrite solution and 1.25 mL of dH₂O were mixed with each aliquot (250 µL) of the extract. After 5 min of incubation, 150 µL of 10% AlCl₃ solution and 0.5 mL of 1 M NaOH solution were added. The solution was then immediately diluted with 275 µL of dH₂O. Absorbance was measured at 510 nm using a spectrophotometer (Genesys 10 UV). TFC was expressed as catechin equivalent, and a standard curve was generated using a series of catechin dilutions ranging from 0 to 500 g/mL.

2.10. Antioxidant Activity Assay

2.10.1. Determination of Radical Scavenging Activity

The free radical scavenging activity of all fermented milk samples was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method. DPPH reagent (3 mL; 60 mM) dissolved in 95% ethanol was added to 250 µL of fermented milk aqueous extract [78]. The mixture was incubated in the dark at room temperature (22 ± 2 °C) for 1 h. Absorbance (Abs) at 517 nm was measured against a control (250 µL of ethanol instead of the extract) using a spectrophotometer (Genesys 10 UV). The radical scavenging activity was calculated as follows:

$$\text{Radical scavenging activity (\%)} = (\text{Abs control at 517 nm} - \text{Abs sample at 517 nm} / \text{Abs control at 517 nm}) \times 100$$

2.10.2. Determination of Ferrous Ion Chelating (FIC) Ability Assay

The FIC assay of all fermented milk samples was performed as described by Shori [83]. Solutions of 2 mM iron (II) sulfate hydrate ($\text{FeSO}_4 \cdot x\text{H}_2\text{O}$) and 5 mM ferrozine were diluted 20-fold prior to use. Then, 1 mL of diluted $\text{FeSO}_4 \cdot x\text{H}_2\text{O}$ solution was mixed with 1 mL of fermented milk aqueous extract and 1 mL of diluted ferrozine solution. The mixture was incubated for 10 min at 25 °C. Absorbance (Abs) at 562 nm was measured against control (1 mL of dH_2O instead of the extract) using a spectrophotometer (Genesys 10 UV). The FIC ability of samples was calculated as follows:

$$\text{FIC ability (\%)} = (\text{Abs control at 562 nm} - \text{Abs sample at 562 nm} / \text{Abs control at 562 nm}) \times 100$$

2.10.3. Determination of Ferric Reducing Antioxidant Potential (FRAP) Assay

The reducing power of fermented milk extracts was measured by assessing the reduction of $\text{Fe}^{3+}(\text{CN})_6$ to $\text{Fe}^{2+}(\text{CN})_6$, as described by Shori [83]. FRAP reagent (3.6 mL) containing 300 mM acetate buffer, 8 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) reagent, and 20 mM FeCl_3 solutions at a ratio of 10:1:1 was mixed with 400 μL of fermented milk aqueous extract or iron (II) sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) standard solutions (0.3–1.0 $\mu\text{g/mL}$). The mixture was incubated in a water bath (37 °C) for 10 min before absorbance was measured at 593 using a spectrophotometer (Genesys 10 UV). The results were calculated against an $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ standard curve and expressed as mM Fe^{2+} equivalent/mL (mM Fe^{2+} E/mL).

2.11. Statistical Analysis

Each experiment was performed in triplicate ($n = 3$). Data are presented as mean $\pm$ SEM. The results were analyzed using one-way ANOVA (SPSS 20.0; Chicago, IL, USA) and the general linear model. Differences between means were assessed using linear regression analysis, Tukey's test, Duncan's new multiple range test, and the descriptive test. A p -value of <0.05 was considered statistically significant.

3. Results

3.1. Determination of pH and Treatable Acidity (TA) of Fermented Almond Milk with Cow's Milk Using Lactobacilli

Table 1 presents the variations in pH and TA of fermented CM and its mix with AM inoculated with four strains of lactobacilli (Lr, La, Lp, or Lc), compared with their respective controls over a 21-day storage period at 4 °C. *Lbs. rhamnosus* in FCM (100) and its combinations with AM significantly ($p < 0.05$) reduced pH compared to their respective controls throughout the 21 days, except for FCM (100) on day 7 (Table 1). No significant changes in pH were observed in La-, Lp-, and Lc-inoculated FCM (100) and its combinations with AM over the experiment period.

After 21 days of storage, all treated samples of FCM (100) and its combination with AM showed significantly higher TA values ($p < 0.05$) than their respective controls (Table 1). Among other treatments, Lr and Lp were the major contributors to the significantly ($p < 0.05$) highest TA value of FCM (100) on the first day of storage (0.63% LAE), which increased to 0.83 ± 0.057 % LAE for FCM (100)-Lr on day 7. FCM (100)-Lc showed a significant ($p < 0.05$) increase in TA (0.99 ± 0.001 % LAE) compared to La (0.81 ± 0.100 % LAE) on day 21. Among the mixed samples, FAM/CM (75:25) exhibited the significantly highest TA ($p < 0.05$) due to the presence of Lp and Lc (0.63 ± 0.1 % LAE) on day 1. No significant differences in TA were observed among all probiotic strains in FAM/CM combinations on day 21 of storage.

Table 1. The difference in pH values and titratable acidity of the selected four lactobacilli in fermented cow's milk, and its combination with almond milk (25%, 50%, 75%, and 100%) contrasted to their respective controls within 21 days and keeping them at 4 °C.

Sample	pH				Titratable Acidity (TA) (% Equivalent of Lactic Acid)			
	Preservation Duration (Days)				Preservation Duration (Days)			
	1	7	14	21	1	7	14	21
FCM (100)-C	5.47 ± 0.03 ^c	4.84 ± 0.04 ^{b c}	4.77 ± 0.02 ^d	4.58 ± 0.01 ^c	0.45 ± 0.10 ^a	0.54 ± 0.00 ^a	0.60 ± 0.05 ^a	0.69 ± 0.05 ^a
FCM (100)-Lr	4.50 ± 0.01 ^a	4.41 ± 0.01 ^a	4.24 ± 0.10 ^a	4.06 ± 0.15 ^a	0.63 ± 0.10 ^c	0.83 ± 0.05 ^d	0.87 ± 0.05 ^d	0.90 ± 0.10 ^c
FCM (100)-La	4.97 ± 0.02 ^b	4.79 ± 0.05 ^b	4.62 ± 0.02 ^c	4.51 ± 0.07 ^c	0.54 ± 0.10 ^b	0.65 ± 0.05 ^b	0.74 ± 0.05 ^b	0.81 ± 0.10 ^b
FCM (100)-Lp	4.49 ± 0.05 ^a	4.46 ± 0.01 ^a	4.41 ± 0.01 ^b	4.33 ± 0.04 ^b	0.63 ± 0.10 ^c	0.72 ± 0.10 ^c	0.81 ± 0.10 ^c	0.92 ± 0.05 ^c
FCM (100)-Lc	4.84 ± 0.02 ^b	4.72 ± 0.02 ^b	4.60 ± 0.01 ^c	4.52 ± 0.02 ^c	0.54 ± 0.10 ^b	0.63 ± 0.10 ^b	0.72 ± 0.10 ^b	0.99 ± 0.00 ^d
FAM/CM (75:25)-C	5.24 ± 0.12 ^c	4.93 ± 0.04 ^d	4.63 ± 0.03 ^d	4.52 ± 0.03 ^d	0.27 ± 0.10 ^a	0.29 ± 0.05 ^a	0.42 ± 0.05 ^a	0.45 ± 0.10 ^a
FAM/CM (75:25)-Lr	4.12 ± 0.01 ^a	3.96 ± 0.06 ^a	3.91 ± 0.02 ^a	3.85 ± 0.07 ^a	0.56 ± 0.05 ^b	0.63 ± 0.10 ^b	0.74 ± 0.05 ^c	0.83 ± 0.05 ^b
FAM/CM (75:25)-La	4.41 ± 0.03 ^b	4.24 ± 0.03 ^c	4.15 ± 0.03 ^b	4.10 ± 0.04 ^c	0.56 ± 0.05 ^b	0.63 ± 0.10 ^b	0.81 ± 0.10 ^d	0.90 ± 0.10 ^c
FAM/CM (75:25)-Lp	4.15 ± 0.03 ^a	4.15 ± 0.01 ^b	4.10 ± 0.02 ^b	4.02 ± 0.04 ^b	0.63 ± 0.10 ^c	0.65 ± 0.05 ^c	0.72 ± 0.10 ^b	0.90 ± 0.10 ^c
FAM/CM (75:25)-Lc	4.38 ± 0.02 ^b	4.33 ± 0.02 ^c	4.27 ± 0.03 ^c	4.16 ± 0.06 ^c	0.63 ± 0.10 ^c	0.65 ± 0.05 ^c	0.72 ± 0.10 ^b	0.81 ± 0.10 ^b
FAM/CM (50:50)-C	4.76 ± 0.03 ^d	4.65 ± 0.04 ^d	4.54 ± 0.02 ^d	4.47 ± 0.07 ^d	0.45 ± 0.10 ^a	0.54 ± 0.10 ^a	0.63 ± 0.10 ^a	0.65 ± 0.05 ^a
FAM/CM (50:50)-Lr	4.02 ± 0.02 ^a	4.00 ± 0.03 ^a	3.98 ± 0.01 ^a	3.87 ± 0.06 ^a	0.78 ± 0.05 ^d	0.81 ± 0.10 ^c	0.87 ± 0.05 ^c	0.92 ± 0.15 ^b
FAM/CM (50:50)-La	4.38 ± 0.07 ^b	4.27 ± 0.06 ^c	4.12 ± 0.04 ^b	4.08 ± 0.08 ^b	0.63 ± 0.10 ^b	0.72 ± 0.10 ^b	0.90 ± 0.10 ^d	0.99 ± 0.10 ^c
FAM/CM (50:50)-Lp	4.30 ± 0.02 ^b	4.18 ± 0.02 ^b	4.14 ± 0.02 ^b	4.10 ± 0.03 ^b	0.72 ± 0.10 ^c	0.81 ± 0.10 ^c	0.90 ± 0.10 ^d	0.99 ± 0.10 ^c
FAM/CM (50:50)-Lc	4.57 ± 0.06 ^c	4.39 ± 0.01 ^c	4.3 ± 0.010 ^c	4.22 ± 0.04 ^c	0.65 ± 0.05 ^b	0.72 ± 0.10 ^b	0.81 ± 0.10 ^b	0.99 ± 0.10 ^c
FAM/CM (25:75)-C	4.93 ± 0.02 ^d	4.87 ± 0.06 ^d	4.51 ± 0.03 ^d	4.49 ± 0.03 ^c	0.51 ± 0.05 ^a	0.56 ± 0.05 ^a	0.63 ± 0.10 ^a	0.72 ± 0.10 ^a
FAM/CM (25:75)-Lr	4.05 ± 0.01 ^a	4.01 ± 0.01 ^a	3.96 ± 0.12 ^a	3.94 ± 0.01 ^a	0.63 ± 0.10 ^b	0.78 ± 0.05 ^b	0.83 ± 0.05 ^b	1.01 ± 0.05 ^c
FAM/CM (25:75)-La	4.48 ± 0.08 ^b	4.40 ± 0.03 ^b	4.29 ± 0.01 ^b	4.21 ± 0.02 ^b	0.81 ± 0.10 ^e	0.87 ± 0.05 ^e	0.92 ± 0.15 ^d	1.01 ± 0.05 ^c
FAM/CM (25:75)-Lp	4.58 ± 0.05 ^c	4.51 ± 0.02 ^c	4.35 ± 0.05 ^b	4.20 ± 0.08 ^b	0.69 ± 0.05 ^c	0.81 ± 0.10 ^c	0.90 ± 0.10 ^c	0.99 ± 0.10 ^b
FAM/CM (25:75)-Lc	4.82 ± 0.13 ^d	4.52 ± 0.04 ^c	4.44 ± 0.03 ^c	4.25 ± 0.06 ^b	0.74 ± 0.05 ^d	0.83 ± 0.05 ^d	0.99 ± 0.10 ^c	1.08 ± 0.10 ^d

Results are shown as mean ± SEM. *Lactocaseibacillus rhamnosus*: Lr; *Lactobacillus acidophilus*: La; *Lactiplantibacillus plantarum*: Lp; *Lactocaseibacillus casei*: Lc; fermented cow's milk = FCM, almond milk = AM; and control = C. ^{a-e} means with different superscript letters in a column are significantly different ($p < 0.05$).

3.2. Viable Cell Counts of Lactobacilli of Fermented Almond Milk with Cow's Milk

Table 2 shows the variation in viable cell counts (VCCs) of fermented CM and its mixture with AM inoculated with four strains of lactobacilli (Lr, La, Lp, or Lc), compared with their respective controls over 21 days. A significant increase in viability was observed in all lactobacilli-treated samples compared to their respective controls (Table 2). Furthermore, regardless of concentration, all starter cultures exhibited similar ($p > 0.05$) viable cell counts. However, FCM (100)-La displayed significantly lower ($p < 0.05$) VCCs than CM (100)-Lr on days 14 and 21. All treatments maintained viability between 5.9 and 6.8 log cfu/mL during storage at 4 °C.

Table 2. The difference in viable cell counts (VCCs; log cfu/mL) of the four types of lactobacilli of fermented cow's milk, and its mixture with 100%, 75%, 50%, and 25% almond milk compared to the corresponding controls over 21 days and keeping them at 4 °C.

Sample	Viability Count (log cfu/mL)			
	Preservation Duration (Days)			
	1	7	14	21
FCM (100)-C	4.870 ± 0.029 ^a	4.760 ± 0.126 ^a	4.694 ± 0.081 ^a	4.652 ± 0.110 ^a
FCM (100)-Lr	6.690 ± 0.014 ^c	6.746 ± 0.036 ^c	6.800 ± 0.019 ^d	6.662 ± 0.017 ^e
FCM (100)-La	6.172 ± 0.130 ^b	6.856 ± 0.049 ^c	6.019 ± 0.020 ^b	5.903 ± 0.036 ^b
FCM (100)-Lp	6.621 ± 0.128 ^c	6.783 ± 0.038 ^c	6.738 ± 0.119 ^d	6.543 ± 0.167 ^d

Table 2. Cont.

Sample	Viability Count (log cfu/mL)			
	Preservation Duration (Days)			
	1	7	14	21
FCM (100)-Lc	6.154 ± 0.027 ^b	6.260 ± 0.015 ^b	6.344 ± 0.010 ^c	6.312 ± 0.019 ^c
FAM/CM (75:25)-C	3.853 ± 0.042 ^a	3.797 ± 0.088 ^a	3.622 ± 0.136 ^a	3.562 ± 0.083 ^a
FAM/CM (75:25)-Lr	6.672 ± 0.015 ^d	6.763 ± 0.013 ^d	6.555 ± 0.014 ^d	6.515 ± 0.037 ^d
FAM/CM (75:25)-La	6.277 ± 0.016 ^c	6.257 ± 0.025 ^b	6.247 ± 0.047 ^b	6.215 ± 0.002 ^b
FAM/CM (75:25)-Lp	6.280 ± 0.112 ^c	6.331 ± 0.016 ^c	6.375 ± 0.038 ^c	6.403 ± 0.114 ^c
FAM/CM (75:25)-Lc	6.179 ± 0.027 ^b	6.734 ± 0.057 ^d	6.768 ± 0.067 ^e	6.601 ± 0.022 ^d
FAM/CM (50:50)-C	3.888 ± 0.111 ^a	4.167 ± 0.109 ^a	4.326 ± 0.124 ^a	4.389 ± 0.108 ^a
FAM/CM (50:50)-Lr	6.689 ± 0.010 ^d	6.753 ± 0.025 ^d	6.706 ± 0.048 ^b	6.516 ± 0.089 ^b
FAM/CM (50:50)-La	6.119 ± 0.148 ^b	6.192 ± 0.085 ^b	6.670 ± 0.102 ^b	6.597 ± 0.024 ^b
FAM/CM (50:50)-Lp	6.496 ± 0.047 ^c	6.626 ± 0.018 ^c	6.707 ± 0.087 ^b	6.606 ± 0.071 ^b
FAM/CM (50:50)-Lc	6.169 ± 0.041 ^b	6.692 ± 0.050 ^c	6.857 ± 0.074 ^c	6.624 ± 0.024 ^b
FAM/CM (25:75)-C	3.836 ± 0.071 ^a	4.010 ± 0.051 ^a	4.212 ± 0.148 ^a	4.246 ± 0.030 ^a
FAM/CM (25:75)-Lr	6.676 ± 0.020 ^d	6.791 ± 0.017 ^d	6.424 ± 0.010 ^b	6.225 ± 0.008 ^b
FAM/CM (25:75)-La	6.183 ± 0.032 ^b	6.187 ± 0.122 ^b	6.442 ± 0.028 ^b	6.723 ± 0.046 ^c
FAM/CM (25:75)-Lp	6.430 ± 0.032 ^c	6.649 ± 0.058 ^c	6.697 ± 0.063 ^c	6.731 ± 0.017 ^c
FAM/CM (25:75)-Lc	6.136 ± 0.031 ^b	6.735 ± 0.024 ^d	6.792 ± 0.064 ^c	6.695 ± 0.058 ^c

Results are shown as mean ± SEM. *Lactocaseibacillus rhamnosus*: Lr; *Lactobacillus acidophilus*: La; *Lactiplantibacillus plantarum*: Lp; *Lactocaseibacillus casei*: Lc; fermented cow's milk = FCM; almond milk = AM; and control = C. ^{a–e} means with different superscript letters in a column are significantly different ($p < 0.05$).

3.3. Determination of Total Phenolic and Flavonoid Contents of Fermented Almond Milk with Cow's Milk Using *Lactobacilli*

Table 3 shows the variations in TPCs and TFCs of fermented CM, and its mixture with AM inoculated with four lactobacilli strains (Lr, La, Lp, or Lc), compared with their respective controls over 21 days of storage. All FAM/CM combination samples showed a significant ($p < 0.05$) increase in TPCs compared to their respective controls throughout the 21 days of storage. Among other treatments, the lowest TPC ($p < 0.05$) was observed in La for 1-day-old FAM/CM (50:50). In FAM/CM combinations, the maximum TPC was recorded for FAM/CM (75:25)-Lc (88.27 ± 0.049 µg GAE/mL; $p < 0.05$) compared to other treatments (68–75 µg GAE/mL) on day 1. In addition, FAM/CM (75:25)-La exhibited the highest TPC (101.16 ± 0.009 µg GAE/mL; $p < 0.05$) on day 7, followed by a significant ($p < 0.05$) decrease to 55.49 ± 0.147 µg GAE/mL by day 21. Moreover, the highest TPC in FAM/CM (25:75) was observed on day 1 due to the presence of Lr, while the highest TPC in FAM/CM (50:50) was detected on days 7 and 14, attributed to the presence of Lp and Lc, respectively (Table 3). At the end of storage, TPC was significantly reduced ($p < 0.05$) in FAM/CM (25:75)-Lr (55.6 ± 0.016 µg GAE/mL) compared to La, Lp, and Lc (>95 µg GAE/mL) at the end of storage.

The highest TFC ($p < 0.05$) for La was observed in FAM/CM (75:25) on days 14 and 21. TFCs decreased significantly in FAM/CM (50:50)-Lc (20.17 ± 0.005 µg/g) compared to other treatments (>22 µg/g) at the end of storage. Moreover, *Lbs. rhamnosus* inoculated in FAM/CM (50:50) showed significantly higher TFCs ($p < 0.05$) compared to other samples (42.02 ± 0.002 and 35.06 ± 0.001 µg/g) on days 7 and 14, respectively. FAM/CM (25:75)-Lr exhibited the maximum TFC ($p < 0.05$) compared to other cultures during the first two weeks of storage (>43 µg/g), followed by a substantial decrease to 23.85 ± 0.003 µg/g on day 21.

Table 3. The differences in total phenolic content and total flavonoid content of the selected four lactobacilli of fermented cow's milk, and its combination with almond milk (25%, 50%, 75%, and 100%) contrasted to their respective controls within 21 days and keeping them at 4 °C.

Sample	TPCs (μg GAE/mL)				TFCs (μg/g)			
	Preservation Duration (Days)				Preservation Duration (Days)			
	1	7	14	21	1	7	14	21
FAM/CM (75:25)-C	58.71 ± 0.003 ^a	26.84 ± 0.065 ^a	25.74 ± 0.00 ^a	40.43 ± 0.108 ^a	19.77 ± 0.00 ^a	20.02 ± 0.003 ^a	18.7 ± 0.049 ^a	18.02 ± 0.004 ^a
FAM/CM (75:25)-Lr	72.67 ± 0.034 ^c	74.75 ± 0.003 ^d	93.03 ± 0.105 ^e	98.27 ± 0.057 ^e	21.43 ± 0.002 ^b	25.27 ± 0.002 ^b	26.75 ± 0.001 ^c	23.35 ± 0.008 ^c
FAM/CM (75:25)-La	75.56 ± 0.089 ^d	101.2 ± 0.009 ^e	89.15 ± 0.043 ^d	55.49 ± 0.147 ^b	20.62 ± 0.001 ^b	20.79 ± 0.008 ^a	33.43 ± 0.005 ^d	43.55 ± 0.020 ^e
FAM/CM (75:25)-Lp	68.27 ± 0.052 ^b	52.52 ± 0.034 ^c	62.45 ± 0.013 ^b	71.09 ± 0.005 ^c	24.62 ± 0.019 ^c	20.67 ± 0.004 ^a	18.75 ± 0.006 ^a	22.24 ± 0.002 ^b
FAM/CM (75:25)-Lc	88.27 ± 0.049 ^e	39.48 ± 0.007 ^b	81.68 ± 0.097 ^c	95.82 ± 0.003 ^d	20.53 ± 0.011 ^b	20.22 ± 0.008 ^a	19.75 ± 0.004 ^b	29.83 ± 0.014 ^d
FAM/CM (50:50)-C	18.45 ± 0.112 ^a	13.54 ± 0.021 ^a	25.71 ± 0.00 ^a	32.78 ± 0.013 ^a	22.31 ± 0.01 ^a	18.22 ± 0.003 ^a	18.17 ± 0.005 ^a	17.06 ± 0.003 ^a
FAM/CM (50:50)-Lr	53.18 ± 0.020 ^d	27.35 ± 0.005 ^b	57.5 ± 0.077 ^b	84.86 ± 0.003 ^b	35.17 ± 0.003 ^c	42.02 ± 0.002 ^e	35.06 ± 0.001 ^e	31.09 ± 0.002 ^c
FAM/CM (50:50)-La	28.42 ± 0.013 ^b	29.70 ± 0.013 ^c	69.62 ± 0.126 ^c	95.71 ± 0.102 ^d	30.73 ± 0.003 ^b	22.79 ± 0.006 ^d	26.76 ± 0.014 ^c	32.61 ± 0.013 ^d
FAM/CM (50:50)-Lp	50.47 ± 0.115 ^d	69.95 ± 0.095 ^e	82.63 ± 0.158 ^d	89.84 ± 0.100 ^c	35.96 ± 0.009 ^c	20.16 ± 0.040 ^c	28.61 ± 0.007 ^d	32.28 ± 0.025 ^d
FAM/CM (50:50)-Lc	46.51 ± 0.165 ^c	33.14 ± 0.059 ^d	93.69 ± 0.109 ^e	105.71 ± 0.008 ^e	22.86 ± 0.008 ^a	19.03 ± 0.001 ^b	19.79 ± 0.003 ^b	20.17 ± 0.005 ^b
FAM/CM (25:75)-C	23.29 ± 0.010 ^a	22.08 ± 0.001 ^a	14.13 ± 0.00 ^a	19.40 ± 0.064 ^a	18.86 ± 0.00 ^a	18.93 ± 0.00 ^a	19.94 ± 0.00 ^a	18.39 ± 0.006 ^a
FAM/CM (25:75)-Lr	76.04 ± 0.003 ^e	82.04 ± 0.029 ^d	84.17 ± 0.009 ^c	55.6 ± 0.016 ^b	60.59 ± 0.001 ^e	45.37 ± 0.006 ^c	43.21 ± 0.003 ^d	23.85 ± 0.003 ^d
FAM/CM (25:75)-La	61.38 ± 0.166 ^d	52.30 ± 0.114 ^c	96.07 ± 0.006 ^d	99.88 ± 0.022 ^d	47.82 ± 0.010 ^d	21.72 ± 0.002 ^b	22.06 ± 0.056 ^b	22.14 ± 0.007 ^c
FAM/CM (25:75)-Lp	35.52 ± 0.047 ^b	84.72 ± 0.079 ^e	99.81 ± 0.087 ^e	95.78 ± 0.017 ^c	43.24 ± 0.094 ^c	19.17 ± 0.003 ^a	23.44 ± 0.002 ^c	23.49 ± 0.006 ^d
FAM/CM (25:75)-Lc	46.04 ± 0.049 ^c	39.08 ± 0.014 ^b	63.87 ± 0.075 ^b	101.02 ± 0.071 ^d	20.9 ± 0.003 ^b	22.58 ± 0.004 ^b	22.19 ± 0.010 ^b	19.97 ± 0.009 ^b

Results are shown as mean ± SEM. *Lactocaseibacillus rhamnosus*: Lr; *Lactobacillus acidophilus*: La; *Lactiplantibacillus plantarum*: Lp; *Lactocaseibacillus casei*: Lc; fermented cow's milk = FCM; almond milk = AM; control = C. ^{a-e} means with different superscript letters in a column are significantly different ($p < 0.05$).

3.4. Antioxidants Properties (DPPH, FRAP, and FIC) of Fermented Almond Milk with Cow's Milk Using Lactobacilli

All treated samples of FCM (100) and its combination with AM exhibited a significant ($p < 0.05$) increase in DPPH radical scavenging activity compared to their respective controls throughout 21 d of storage, except for 7-, 14-, and 21-day-old FCM (100)-Lr (Figure 1A–D). Among other treatments, the highest DPPH activity ($p < 0.05$) was observed for FCM (100)-La and Lp (97.45 ± 0.008 and $94.01 \pm 0.006\%$, respectively) on the first day of storage, and for FCM (100)-Lc ($90.91 \pm 0.013\%$) on day 14. FCM (100) containing *Lbs. rhamnosus* exhibited the lowest DPPH activity ($p < 0.05$) among all samples throughout the 21 days of storage (Figure 1A). In FAM/CM combinations, DPPH scavenging activity did not show significant differences among all cultures during the first two weeks of storage, except for fresh FAM/CM (75:25, 50:50, and 25:75)-Lr and 7-day-old FAM/CM (25:75)-Lr, which displayed the significantly ($p < 0.05$) lowest activity compared to other treatments. However, FAM/CM (50:50 and 25:75)-Lr exhibited a significant ($p < 0.05$) increase in DPPH scavenging activity, reaching the maximum values of 93 ± 0.030 and $97.63 \pm 0.002\%$, respectively, compared to other samples ($<85\%$) on day 21.

All treated samples of FCM (100) and its blends with AM exhibited a significant ($p < 0.05$) enhancement in FRAP activity compared to their respective controls during 21 days of storage, except for 1-day-old FCM(100)-La, 7-day-old FCM (100)-La and Lc, and 14 and 21-day-old FCM (100)-La, Lp, and Lc (Figure 3A). Notably, FCM (100)-Lr and Lp showed significantly ($p < 0.05$) higher FRAP activity compared to other treatments within the first two weeks of storage. On day 1, FAM/CM (75:25)-Lr (0.766 ± 0.005 mM Fe^{2+} E/mL) and FAM/CM (25:75)-La (0.922 ± 0.063 mM Fe^{2+} E/mL) exhibited the highest ($p < 0.05$) FRAP values among all combinations. Furthermore, FAM/CM (25:75)-Lp demonstrated superior ($p < 0.05$) FRAP activity on day 7 (Figure 3D). By day 14, FAM/CM (25:75)-Lr showed significantly higher ($p < 0.05$) FRAP activity (0.78 ± 0.013 and 0.65 ± 0.008 mM Fe^{2+} E/mL, respectively) compared to other formulations. At the end of the storage period (day 21), FCM (100)-Lr maintained the highest FRAP activity (>0.55 mM Fe^{2+} E/mL). Overall, Lr and La markedly ($p < 0.05$) enhanced the FRAP value of FAM/CM (75:25 and 50:50 and 25:75) relative to Lp and Lc on day 21.

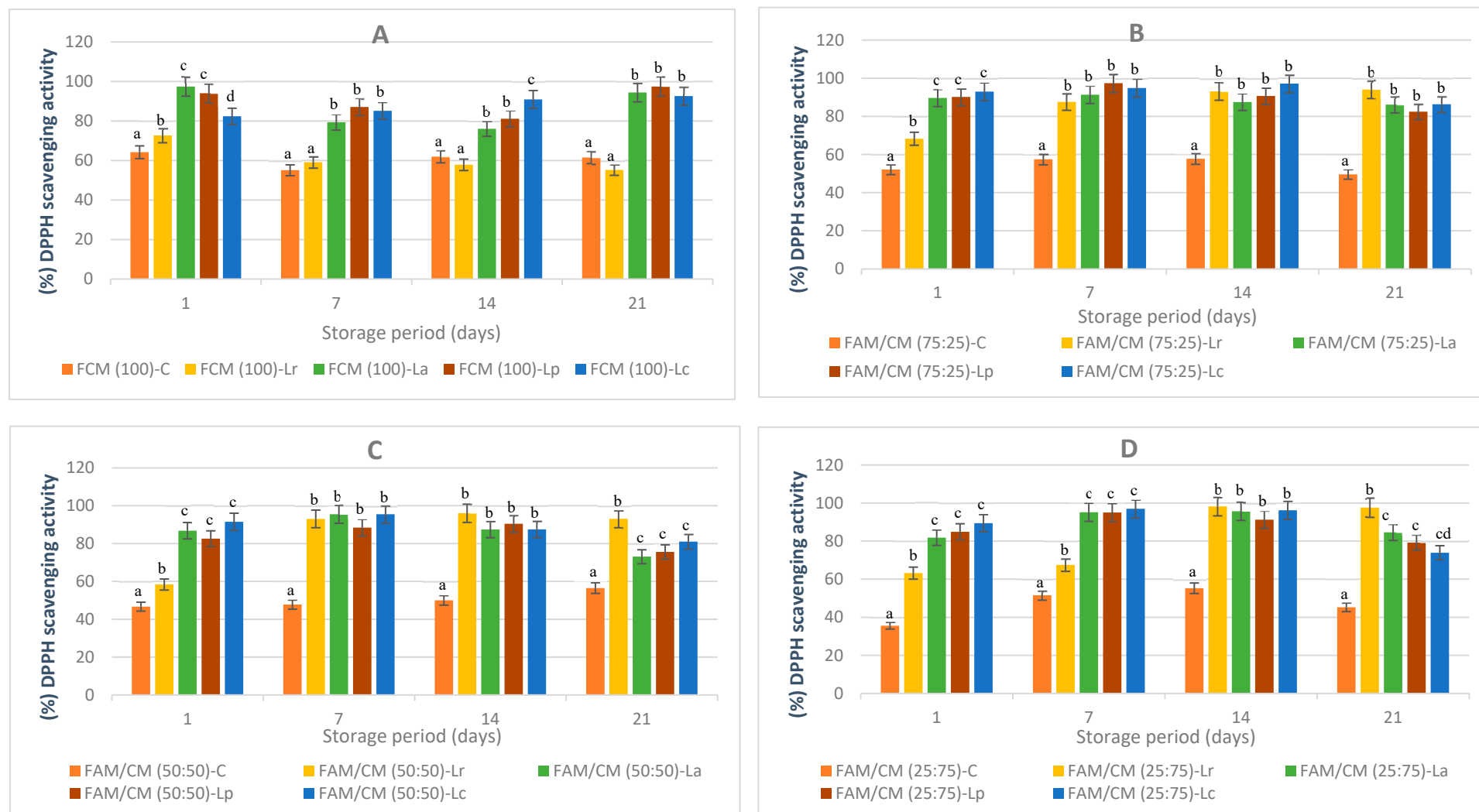


Figure 1. Variation in DPPH scavenging activity (%) of four lactobacilli of FCM and its combination with AM using different concentrations ((A) = 100% FCM, (B) = 75:25% FAM/CM, (C) = 50:50% FAM/CM, (D) = 25:75% FAM/CM) compared to their respective controls (C) within 21 days of storage at 4 °C. a–d means with different superscript letters in a column are significantly different ($p < 0.05$).

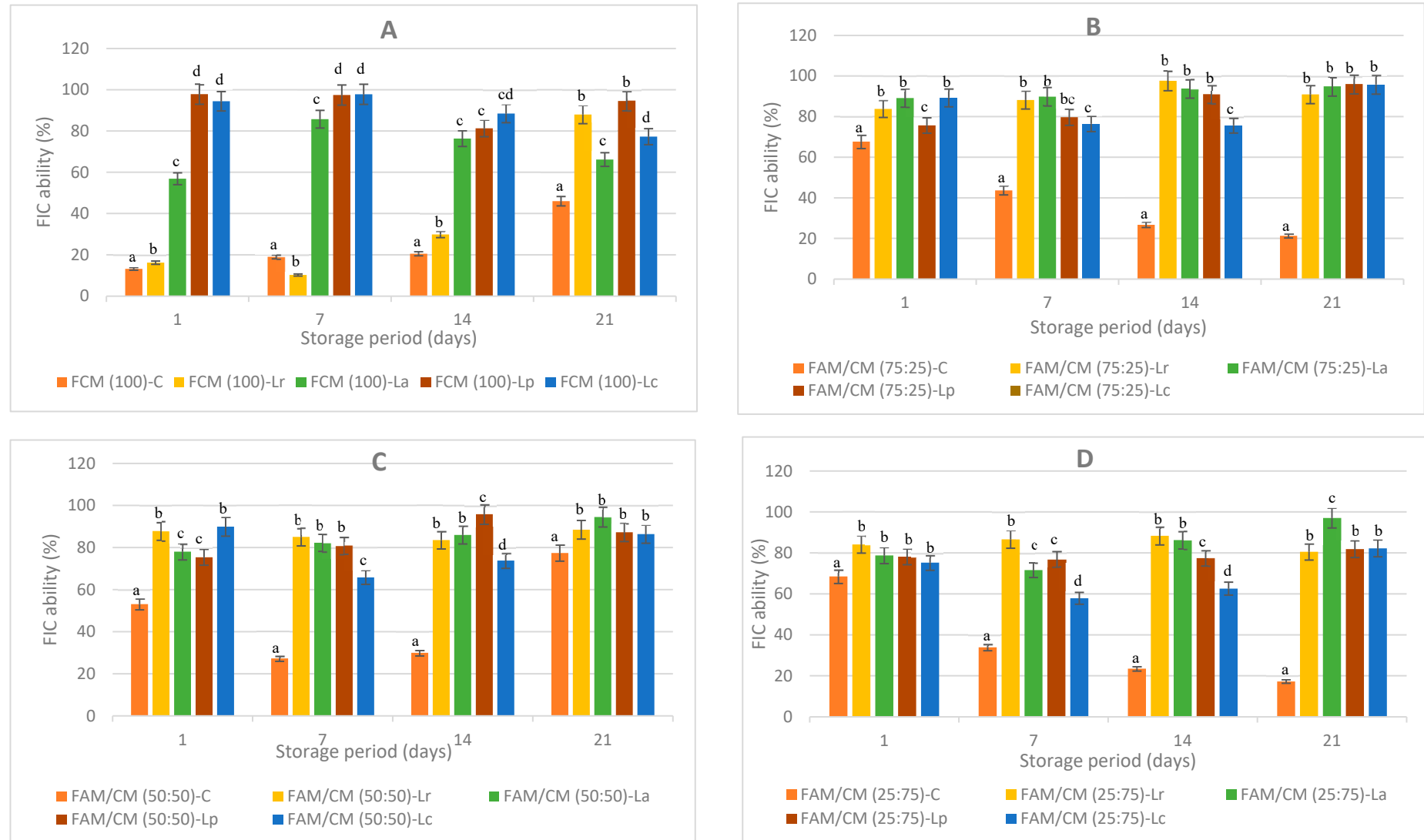


Figure 2. Variation in (FIC; %) of four lactobacilli of FCM and its combination with AM using different concentrations ((A) = 100% FCM, (B) = 75:25% FAM/CM, (C) = 50:50% FAM/CM, (D) = 25:75% FAM/CM) compared to their respective controls (C) within 21 days of storage at 4 °C. ^{a–d} means with different superscript letters in a column are significantly different ($p < 0.05$).

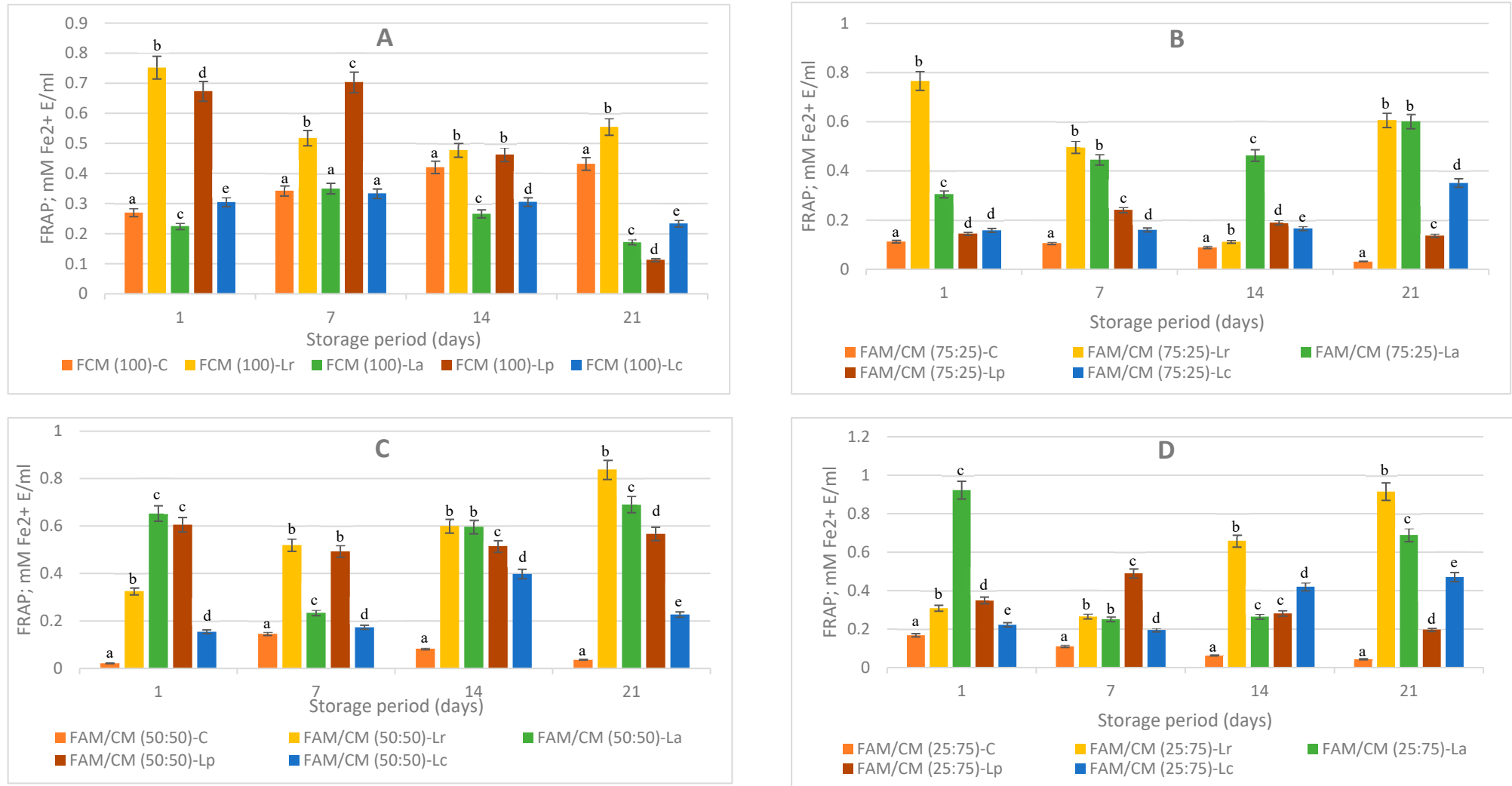


Figure 3. Variation in (FRAP; mM Fe²⁺ E/mL) of four lactobacilli of FCM and its combination with AM using different concentrations ((A) = 100% FCM, (B) = 75:25% FAM/CM, (C) = 50:50% FAM/CM, (D) = 25:75% FAM/CM) compared to their respective controls (C) within 21 days of storage at 4 °C. ^{a-e} means with different superscript letters in a column are significantly different ($p < 0.05$).

4. Discussion

Post-acidification leads to the accumulation of acid during refrigerated storage [84]. Titratable acidity is a crucial indicator for evaluating the structural quality of fermented milk products [85]. Several factors influence the titratable acidity of milk, including the starter culture, fermentable sugars, proteolysis, cold storage, concentration and type of milk, temperature, and fermentation time [86,87]. Lactic acid bacteria mainly produce lactic and acetic acids through the metabolism of carbohydrates [88]. All of the fermented samples in this study exhibited higher TA values than their respective controls. Furthermore, the acidity in FCM (100) was enhanced by Lr and Lp, whereas the acidity of FAM/CM (75:25) was greatly enhanced by Lp and Lc. Additionally, the acidity in FAM/CM (50:50) was increased by the starting culture Lr and Lp. Therefore, the inclusion of lactobacilli probiotics into cow's and almond milk, as well as their mixture, enhanced the accumulation of acidity, resulting in a decrease in pH and an increase in TA during storage. Previous studies have reported that the inclusion of lactobacilli probiotic strains in fermented cow and almond milk improves lactic acid production during fermentation [89–92]. Additionally, the properties of almond products enhance the growth of probiotics, particularly lactobacilli bacteria, thus enhancing the production of acid during the fermentation process and storage [91,93,94]. Numerous studies have demonstrated that almonds serve as an excellent substrate for probiotic bacteria, especially lactobacilli species [95–97]. This effect is attributed to the phytochemicals and nutrients in almond milk, which promote probiotic growth and consequently enhance post-acidification. Likewise, *Lbs. rhamnosus* and *Lpb. plantarum* significantly increased the acidity of cashew milk-based yogurt [98], while *Lpb. plantarum* also greatly enhanced the acidity of coconut milk [99].

During fermentation, the viability of lactobacilli probiotic cultures—namely, *Lr.*, *Lp.*, *La.*, and *Lc.* in cow's milk and its combinations with almond milk—was enhanced compared to their respective controls. The viability of probiotic cultures at various concentrations of fermented cow's milk and its mixtures with almond milk during storage did not differ significantly ($p > 0.05$). The findings of this study are consistent with previous reports that demonstrated high viable cell counts in fermented almond milk and its combinations with dairy milk containing lactic acid bacteria during storage [94,96,100,101]. Wansutha et al. [95] reported that the VCCs of several lactobacilli probiotic strains in AM ranged from 8.97 to 9.12 log cfu/mL after 24 h at 37 °C. In the present study, all lactobacilli strains remained viable in all samples for up to 21 days, maintaining counts above 6 log cfu/mL. Similarly, He & Hekmat [102] observed that *L. rhamnosus* LGR-1 in almond beverages reached 10^7 CFUs/mL after 28 days of storage. Fermented almond-based drinks containing probiotics have been shown to maintain stable probiotic viability over extended storage periods [103]. Soy and almond milks contain nutrients such as dietary fiber, protein, oligosaccharides, and polyphenols, which may serve as substrates supporting microbial growth during fermentation [104–106]. According to Mandalari et al. [107], almond extracts harbor a variety of bacteria, including *Bifidobacteria*, *Micrococcus*, *Lactobacillus*, and *Bacillus*. Moreover, almond peels contain several bioactive prebiotic components, such as xylooligosaccharides, polysaccharides, hemicellulose, and dietary fiber, that may promote LAB development throughout fermentation and storage [108].

Plant phenolics and flavonoids play crucial roles in enhancing human health and protecting the body against environmental stressors [109]. Recent studies have demonstrated that polyphenolic compounds are among the most beneficial and health-promoting phytochemicals in plants and that a diet lacking these compounds may increase the risk of developing certain illnesses [110,111]. Polyphenols are considered essential dietary components due to their strong biological activities and significant therapeutic value [112]. In recent years, there has been a growing consumer preference for natural food ingredients,

and researchers have increasingly explored the potential health benefits of plant-derived extracts, which are often used in foods and nutraceutical supplements [113]. From a food science perspective, polyphenols are critical for determining the organoleptic, antioxidant, and overall qualities of the food [114]. The current study shows that the addition of lactobacilli bacteria to almond milk and its combination with cow's milk during fermentation significantly increased the total phenolic and flavonoid contents compared to the control samples during the 21-day storage period. All fermented samples exhibited significantly higher TPC values than their controls, which can be attributed to the phenolic compounds naturally present in almond seeds [17]. Almond kernels contain phenolic substances such as α -tocopherols, kaempferol, naringenin, catechin, protocatechuic acid, vanillic acid, and benzoic acid derivative [115]. A remarkable increase in TPCs was observed in FAM/CM (75:25)-Lc on day 1 of storage and in FAM/CM (50:50)-Lc during the last two weeks. This finding suggests that the increase in phenolic content during fermentation was caused by the addition of lactobacilli to the almond–cow's milk mixture. Similar results were reported in previous studies [116,117]. Moreover, on day 7 of storage, FAM/CM (50:50)-Lp showed a significant improvement in TPC. It has been reported that *Lpb. plantarum* produces certain enzymes, such as feruloyl esterases, that facilitate the release of antioxidant phenols, thereby increasing the phenolic content [118,119]. In the current study, FAM/CM (75:25)-La substantially increased TPC to ~101.2 mg/100 mL on day 7 of storage. Topcuoglu & Yilmaz-Ersan [96] demonstrated that probiotic yogurt prepared with almond milk exhibited a TPC ranging between 22 and 50 mg/100 g. According to their study, the TPC continued to increase for 21 days in fermented almond milk inoculated with *S. thermophilus*, *L. delbrueckii subsp. bulgaricus*, *L. acidophilus*, and *B. animalis subsp. Lactis*. This increase was attributed to the presence of beneficial components in almond milk, including β -sitosterol, folate, vitamin E, niacin (B3), and arabinose [96]. Additionally, the ability of *Lpb. plantarum* to elevate the levels of aglycones in fermented almond milk may be associated with the observed rise in TPC during storage [77]. The production of β -glucosidase by *Lpb. plantarum* can convert the isoflavone glycosides in plant milk into bioactive aglycones [120–122]. Furthermore, differences in β -glucosidase activity were observed among the various starter cultures during milk fermentation [122,123].

FAM/CM (75:25)-La enhanced TFCs compared to Lr, Lp, and Lc after 1 week of storage. Lactobacilli can improve the bioavailability of flavonoids in fermented almond milk combined with cow's milk [124,125]. The activity of β -glycosidase from almond emulsion was higher than that of isoflavone glycosides towards isoflavone glycosides p-nitrophenol- β -d-glucopyranoside [126]. According to Ahn-Jarvis et al. [127], the isoflavone aglycone concentration was significantly increased by adding raw almonds, resulting in ~30% of the total isoflavones being in the form of aglycones. Furthermore, FAM/CM (25:75) with *Lbs. rhamnosus* showed the highest TFC compared to other lactobacilli throughout two weeks of storage. Flavonoid concentration in almond milk may be increased by adding *Lbs. rhamnosus* during fermentation, which may provide health benefits by delaying the degradation of phytochemicals [128]. Increased total phenolic and flavonoid contents are associated with enhanced free radical scavenging capacity, which ultimately improves the antioxidant properties of fermented milk [129]. Lc in FAM/CM (75:25 and 50:50) negatively affected the TFCs over 14 days of storage. Similarly, Lc in FAM/CM (25:75) reduced TFCs ($p < 0.05$) after the same period. According to Taşkın and Bağdatlıoğlu [130], the starter culture may degrade polymeric phenolics during refrigerated storage, leading to this decrease. Probiotic microorganisms possess specific enzymes, such as phenolic acid decarboxylases, which can reduce polyphenol levels in the final product [78,131].

Plants that contain polyphenols, as antioxidants, may protect cellular components from oxidative stress and age-related diseases [132,133]. Phenolic compounds can donate

electrons or hydrogen, which may terminate chain reactions or chelate ions, thereby inhibiting the Fenton reaction [134]. While secondary antioxidants employ Fenton mechanisms to prevent free radical formation, primary antioxidants directly scavenge free radicals straight [135]. DPPH and FRAP assays evaluate the sample's primary antioxidant properties, whereas the FIC assay assesses the sample's secondary antioxidant capabilities [136]. A single antioxidant assay cannot fully represent the antioxidant potential of food samples [137]. To evaluate free radical scavenging ability, DPPH accepts an electron or hydroxyl radical, whereas FRAP measures the antioxidants' capacity to transfer an electron from ferric to ferrous iron [138,139]. Polyphenols are widely recognized as potent natural antioxidants due to the abundance of hydroxyl groups in their structures, which facilitate free radical scavenging [45,140]. However, at high concentrations and in the presence of copper and iron at an elevated pH, phenolic compounds may act as pro-oxidants. Flavonoids efficiently inhibit lipid peroxidation due to their lower redox potential compared to peroxyl radicals [141]. A diet rich in flavonoids is associated with a reduced risk of certain cancers and lower mortality from stroke, as flavonoids help prevent blood clots and arterial blockages [142]. Because of its high polyphenol content, almond milk possesses excellent antioxidant properties [96]. In the present study, DPPH, FIC, and FRAP values of the final products varied significantly depending on the type of probiotic starter cultures (*Lactobacilli* spp.) and milk concentration used during fermentation. Except for FRAP for FCM (100)-La and Lc, all fermented samples exhibited higher DPPH, FIC, and FRAP than their corresponding controls after at least one week of storage. Furthermore, the addition of starting cultures (La, Lp, or Lc) in FCM (100) enhanced DPPH scavenging activity and FIC capability by more than 50% after 2 weeks of storage. Stobiecka et al. [143] reported similar findings regarding the antioxidant activity of milk from different cow breeds. Similarly, almond extracts demonstrated strong metal ion chelation efficacy due to antioxidant polyphenols [25]. The inclusion of *Lbs. rhamnosus* in fermented CM (100) and FAM/CM (50:50 and 25:75) increased FRAP activity more than other lactobacilli species at the end stage of storage. Shori et al. [98] indicated that *Lab. rhamnosus* is the principal hydrogen donor in cashew milk yogurt. Fermentation reduces polyphenol concentration, which may facilitate protein and carbohydrate digestion as well as mineral absorption, thereby enhancing the antioxidant effect of fermented foods. Consequently, it can be inferred that fermented cow's milk and its combination with almond milk produced using various lactobacilli strains may serve as an excellent source of antioxidants [25].

5. Conclusions

Our current research investigated the use of cow's milk and its combinations with almond milk as primary substrate for fermentation by four types of probiotic lactobacilli strains, namely *Lbs. rhamnosus* (Lr), *Lab. acidophilus* (La), *Lpb. plantarum* (Lp), and *Lbs. casei* (Lc) over 1, 7, 14, and 21 days of storage at 4 °C. Significant heterogeneity was observed depending on the starter cultures and milk concentration employed. In all fermented samples, the lactobacilli enhanced antioxidant activity, VCCs, TPCs, TFCs, and post-acidification throughout storage. During the storage period, all probiotic lactobacilli spp. remained viable ($>6 \log \text{ cfu/mL}$). Moreover, La, Lp, and Lc in fermented cow's milk samples exhibited an increase in FIC ability and DPPH scavenging activity by more than 50% during 2 weeks of storage. The presence of Lr and La in all fermented combination samples FAM/CM (75:25, 50:50, and 25:75) enhanced FRAP activity at the end of storage. All four probiotic lactobacilli strains influenced the functional and nutritional properties of fermented cow's milk and its almond milk combination, contributing to their antioxidant potential. However, this study has certain limitations. The primary focus was on assessing antioxidant activity, VCCs, TPCs, TFCs, and post-acidification during storage, whereas possible changes in the

overall nutritional composition (e.g., proteins, fats, carbohydrates, vitamins, and minerals) were not examined. Since fermentation can influence these nutritional parameters, future studies should include detailed nutritional analyses to provide a more comprehensive understanding of how fermentation affects the nutritional value of the final product. Additionally, this study was limited to four lactobacilli strains, whereas other probiotic species, such as *Bifidobacterium* spp., may exhibit distinct or synergistic effects that could further enhance the functional properties of fermented products. Moreover, almond milk was the only plant-based substrate utilized; incorporating other plant-based substrates (e.g., soy, oat, or coconut milk) in future studies could provide broader insights into fermentation behavior, microbial interactions, and nutritional outcomes.

Author Contributions: A.J.A.Z.: methodology, resources, formal analysis, investigation, validation, writing—original draft. A.B.S.: conceptualization, formal analysis, project administration, supervision, writing—original draft, writing—review and editing. K.A.A.: formal analysis, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

Funding: This project was funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant No. (IPP: 77-247-2025).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Acknowledgments: The authors, therefore, gratefully acknowledge the technical and financial support of the DSR.

Conflicts of Interest: The authors declare no conflicts of interest.

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