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Antioxidant Potential of Yogurts Produced from Milk of Cows Fed Fodder Supplemented with Herbal Mixture with Regard to Refrigerated Storage

Magdalena Stobiecka , Jolanta Król * and Aneta Brodziak

Department of Quality Assessment and Processing of Animal Products, Faculty of Animal Sciences and Bioeconomy, University of Life Sciences in Lublin, Akademicka 13, 20-950 Lublin, Poland; magdalena.stobiecka@student.up.edu.pl (M.S.); aneta.brodziak@up.lublin.pl (A.B.)
* Correspondence: jolanta.krol@up.lublin.pl; Tel.: +48-81-4456822

Abstract: The aim of the study was to assess the potential of milk from herbal blend-fed cows to be used for the production of yogurts exhibiting increased antioxidant potential with regard to the duration of refrigerated storage of the products. Bulk milk (control—CM and experimental—EM) intended for the production of yogurts was provided by a dairy cattle breeding farm. The milk samples were analyzed to determine their basic chemical composition (the content of dry matter, fat, and total protein including casein), hygienic status (somatic cell count (SCC) and total microbial count (TMC)), and antioxidant activity (FRAP, DPPH, and ABTS assays). Pasteurized milk was used to manufacture natural yogurts with the use of starter cultures YC-X11 (Chr. Hansen, Hørsholm, Denmark). Changes in physicochemical traits (acidity, nutritional value, and water activity) and antioxidant activity (FRAP, DPPH, and ABTS assays) occurring during 21-day refrigerated storage of the yogurts were determined. The analyses revealed that the yogurts had higher antioxidant potential than the milk, irrespective of the determination method. Additionally, the experimental yogurts produced from milk obtained from the cows fed fodder supplemented with an herbal mixture exhibited significantly higher antioxidant activity than the control yogurts. The antioxidant potential of the yogurts changed during the refrigerated storage. It should be emphasized that their antioxidant activity significantly increased during the first two weeks (until day 14) but decreased by 15–20% in the following week.

Keywords: antioxidant activity; herbs; milk; yogurts; storage

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1. Introduction

Currently, consumers are increasingly paying attention to the quality of consumed products, especially to their health value. Fermented milk products, mainly yogurts, have been gaining in popularity recently [1,2]. Due to its high nutritional value and excellent therapeutic and sensory properties, yogurt is one of the oldest and most popular fermented milk products consumed worldwide [3,4]. It should be emphasized that the fermentation process carried out using lactic acid bacteria is accompanied by a release of bioactive peptides and free amino acids with various biological activity, including antioxidant effects, from milk proteins [5–7]. Although the beneficial properties of yogurt have long been known, scientists are constantly trying to improve its functional properties and provide new yogurt-based products that will be attractive to consumers [8–10]. Research in this field is focused on the enhancement of the antioxidant activity of manufactured products with the maintenance of an appropriate flavor and aroma profile [7,11,12]. Products being a rich source of antioxidants positively affect the antioxidant status of the organism. Therefore, such products are able to reduce the risk of development of many lifestyle diseases. It also increases the overall resistance of the organism to diseases and infections, slows down the process of organism aging, and reduces the frequency of neurological diseases, e.g., Parkinson's and Alzheimer's diseases or cerebral ischemia [1,3,5,6]. Numerous studies indicate that the antioxidant status of dairy products can be modified with the use of natural plant materials (fruits, vegetables, herbs) exhibiting high content of phenolic compounds and carotenoids in the production process [13–16]. Additionally, these additives have an impact on the sensory traits of the final product and contribute to an extension of the product shelf life via inhibition of the lipid oxidation process during refrigerated storage [12,17–19]. It should be emphasized that the bacterial cultures used exert a substantial impact on the antioxidant potential value. As indicated in many studies, fermented products containing probiotic strains are characterized by substantially increased antioxidant activity [3,7,11]. The level of the antioxidant status of milk used as a raw material for the dairy industry can be modified with the use of natural additives in cow nutrition. Herbal mixtures or post-production residues provided by the food industry are used most frequently [20–23].

In a previous study [20], the authors assessed the effect of the addition of herbal blends to the feed ration for Holstein-Friesian cows on the level of antioxidant potential of milk. It was shown that the use of the herbal additive significantly increased the antioxidant potential of milk. From the nutritional point of view, this seems to be particularly important for the protection of the organism against the harmful effects of oxidative stress. To meet the expectations of modern consumers, the present research was undertaken to assess the potential of milk of herbal blend-fed cows to be used for the production of yogurts characterized by increased antioxidant potential with regard to the duration of storage of the products.

2. Materials and Methods

2.1. Research Material

Bulk milk obtained from a farm specializing in breeding Holstein-Friesian dairy cattle was the research material. Detailed information on the breeding conditions, health status, and nutrition of the animals is presented in Stobiecka et al. [20]. The addition of a standardized blend of dried herbs (oregano 25%, thyme 25%, cinnamon 15%, and purple coneflower 35%) was the experimental factor. This study is a continuation of the research conducted by Stobiecka et al. [20]. During the experiment, 10-l bulk milk samples (control and experiment) were collected three times and used for the production of yogurts.

Milk Analysis

The basic chemical composition, i.e., the content of total protein, fat and dry matter (Infrared Milk Analyzer, Bentley, Chaska, MN, USA), casein content [24], potential acidity (in Soxhlet-Henkl's degree (°SH) [25], active acidity (pH value using a CP-401 pH meter (Elmetron, Zabrze, Poland)), and total microbial count (TMC) in CFU/mL [26,27] were determined in each bulk milk sample. The somatic cell count (SCC) was measured (Somacount 150, Bentley, Chaska, MN, USA) to assess the hygienic status of the raw material.

2.2. Yogurt Production

The yogurts were produced using the water bath (thermostatic) method, according to Glibowski et al. [28]. The yogurts were stored for analysis at 4–6 °C until the next day (approximately 14 h). The yogurts were retested every 7 days for 21 days (days 0, 7, 14, and 21) [29]. The scheme of the production of the yogurts is presented in Figure 1.

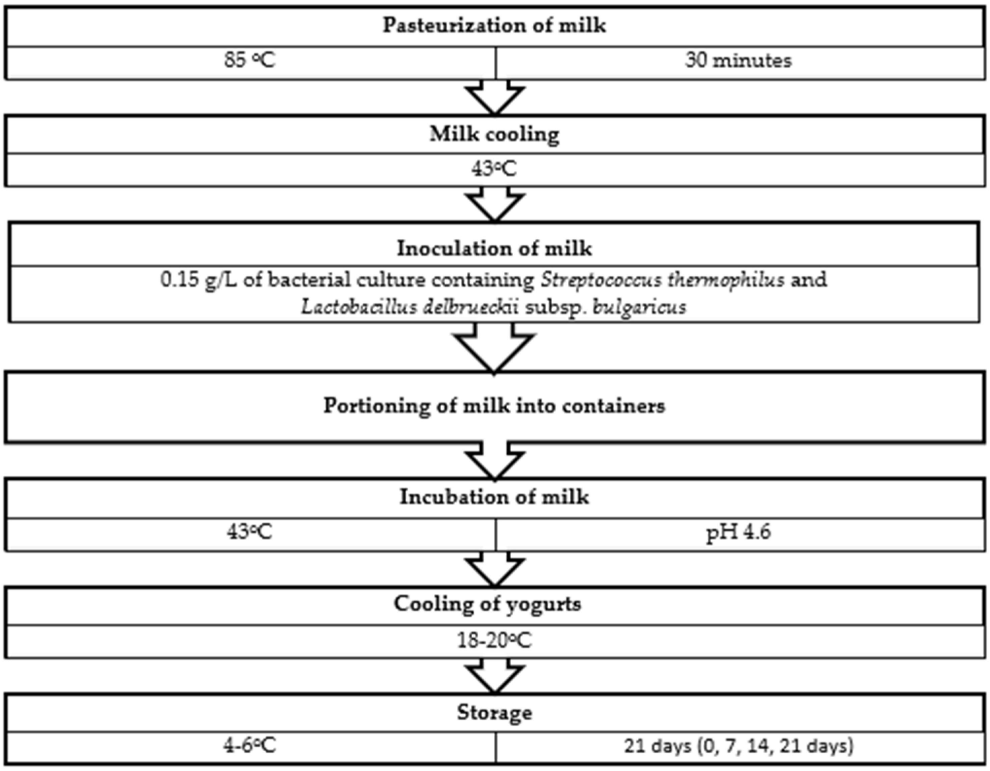


Figure 1. Scheme of production of yogurts (authors' scheme).

2.3. Analysis of Yogurt

2.3.1. Basic Chemical Composition

The yogurts were analyzed to determine the protein (Kjeldahl method) [30], fat (gravimetric method), and dry matter content (oven-drying method) [31].

2.3.2. Acidity

Active acidity was determined using a pH–meter, while potential acidity (°SH) was established with the titration method [32] and expressed as lactic acid content (%).

2.3.3. Water Acidity

The water activity (aw) of the yogurts was measured using a HygroLab C1 water activity meter (Rotronic, Bassersdorf, Switzerland) [29].

The measurements of basic chemical composition, acidity, and water acidity were made in triplicate.

2.4. Determination of Antioxidant Capacity of Milk and Yogurts

The antioxidant activity of the bulk milk and yogurts was determined with three methods, i.e., FRAP (Ferric Reducing Antioxidant Power assay) [33], DPPH (2,2-diphenyl-1 picrylhydrazyl assay) [34], and ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay [35] assays.

The results were expressed as milligrams of Trolox equivalents (TE) per 100 mL of sample.

The methodology is described in detail by Stobiecka et al. [20].

2.5. Statistical Analysis

The statistical analysis of the results was performed with one- and multi-way analysis of variance (ANOVA) in StatSoft Inc. Statistica ver. 13.1 (Dell, Round Rock, TX, USA). Significant differences between the means were determined with Tukey's test at a significance level p (alpha) = 0.05 and 0.01. The results are presented as the means ± standard deviation (SD).

3. Results and Discussion

3.1. Basic Physicochemical Parameters in Milk

The quality of raw milk is one of the main determinants of the value of dairy products. Only raw material with an appropriate hygienic status and chemical composition yields a wholesome, durable, and tasty product that fully meets consumers' expectations. Additionally, the composition and quality of raw milk determines its technological suitability and ensures appropriate quality and durability of finished dairy products [36]. Table 1 presents the physicochemical traits and hygienic status of the milk processed into the yogurts in this study. Milk intended for processing should have appropriate acidity indicating its freshness, i.e., the pH value should be within the range of 6.6–6.8, and the titratable acidity value should be in the range of 6.0–7.5 °SH [37]. Lower pH values and higher titratable acidity indicate the overacidity of milk. As shown in this study, the milk was characterized by appropriate acidity, indicating its freshness, regardless of the type of TMR doses administered to the cows. The results also indicate that the high hygienic quality of the raw milk met the requirements of Commission Regulation (EC) No. 1662/2006 [38], as evidenced by the total microbial count below 100,000/mL and the somatic cell count below 400,000/mL (Table 1). An important component of milk determining its suitability for processing is the content of total protein, including casein. Regardless of the group, the processed milk exhibited a high content of protein (control group—3.47%; experimental group—3.51%) and casein (2.80 and 2.85%, respectively).

Table 1. Acidity, basic chemical composition, and hygienic status of bulk milk used for the production of yogurts.

Parameter	Control Group	Experimental Group
Active acidity (pH)	6.72 ± 0.04	6.73 ± 0.05
Potential acidity (°SH)	6.86 ± 0.10	6.82 ± 0.08
Dry matter (%)	13.09 ± 0.20	13.19 ± 0.44
Total protein (%)	3.47 ± 0.52	3.51 ± 0.62
Casein (%)	2.80 ± 0.41	2.85 ± 0.54
Fat (%)	4.27 ± 0.89	4.30 ± 0.87
Lactose (%)	4.65 ± 0.17	4.72 ± 0.13
SCC (thous./mL)	204 ± 48	183 ± 62
TMC (thous. CFU/mL)	7.2×10^4	5.5×10^4

TMC—total microbial count; SCC—somatic cell count.

3.2. Antioxidant Activity of Milk and Yogurts

Table 2 presents the antioxidant potential of the bulk milk and yogurts determined with three methods, i.e., FRAP, DPPH, and ABTS. Regardless of the experimental group and the method used, the yogurts had higher antioxidant activity than the bulk milk. Noteworthy, compared to the CM samples, the DPPH scavenging activity of the control yogurts (CY) was twofold higher (an increase from 1.14 to 2.52 mg Trolox/100 mL) ($p \leq 0.01$). The activity assessed in the FRAP and ABTS assays increased by approximately 60–70%. Similar differences were found in the experimental group, i.e., the experimental yogurts (EY) had significantly ($p \leq 0.01$) higher antioxidant potential values than the milk (EM) in this group. Many studies [39–41] have found that lactic fermentation has a positive effect on the antioxidant activity of manufactured products. Peptides and free amino acids released during milk fermentation increase the antioxidant capacity of products and inhibit lipid peroxidation [39]. Additionally, the use of herbal mixtures in nutrition has been shown to improve the antioxidant potential of milk and yogurts. Our previous study [20] showed that the addition of a herbal mixture to the feed ration for cows contributed to a significant increase in the content of bioactive components with antioxidant properties in milk, i.e., whey proteins and lipophilic vitamins. The antioxidant capacity of milk increased as well. Similarly, other authors [12,23,42,43] reported that the use of herbal blends in cow nutrition improved the nutritional value of milk via an increase in the content of bioactive components (unsaturated fatty acids, whey proteins, vitamins) in milk and dairy products and, consequently, an increase in their antioxidant potential. Irrespective of the determination methods employed, the yogurts from the experimental group (EY) had approximately 30% higher antioxidant potential values ($p \leq 0.01$) than the control product (CY). The higher antioxidant activity of the EY samples than that of CY was probably associated with the addition of natural antioxidants, i.e., phenolic compounds present in the herbal blends. Plant extracts may contribute to an increase in the level of endogenous antioxidants and reduction of free radicals [44].

Table 2. Antioxidant activity of milk and yogurts in mg Trolox/100 mL.

Method	Milk		Yogurts	
	CM	EM	CY	EY
ABTS	3.02 ^{AX} ± 0.26	4.03 ^{BX} ± 0.23	4.98 ^{AY} ± 0.28	6.87 ^{BY} ± 0.86
DPPH	1.14 ^{aX} ± 0.15	1.25 ^{bX} ± 0.18	2.52 ^{AY} ± 0.16	3.26 ^{BY} ± 0.28
FRAP	8.97 ^{AX} ± 1.13	13.1 ^{BX} ± 2.05	17.13 ^{AY} ± 0.65	22.58 ^{BY} ± 0.98

ABTS—2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay; DPPH—2,2-diphenyl-1 picrylhydrazyl assay; FRAP—Ferric Reducing Antioxidant Power assay. CM—control milk; EM—experimental milk; CY—control yogurts; EY—experimental yogurts. ^{a,b}—significant differences at $p \leq 0.05$; ^{A,B}—significant differences at $p \leq 0.01$. ^{X,Y}—significant differences at $p \leq 0.01$.

3.3. Acidity of Yogurts during 21 Days of Storage

Acidity is an important parameter determining product quality [1]. The initial active acidity (pH) of the yogurts made from the control milk (without the addition of herbs) and the herbal milk yogurts were similar, i.e., 4.59 and 4.60, respectively (Figure 2). During storage, the pH value of the yogurts gradually decreased and reached the lowest value, i.e., 4.41 and 4.43, respectively, on storage day 21. Significant differences ($p \leq 0.05$) were noted only between the initial (day 0) and final (day 21) storage time. An exception was the experimental yogurt, as its pH value on storage day 7 slightly increased in comparison with the value recorded on day 0. The same changes were noted in the case of potential acidity (Figure 3). In the process of sugar fermentation, lactic acid bacterial strains used to manufacture yogurts produce from 0.6 to 1.0% lactic acid, which is responsible for the specific sensory traits and durability of the product. The titrimetric acidity of the experimental yogurts was within the normal range (at least 0.6% lactic acid content) [31]—Figure 3. The lowest content of lactic acid was recorded on storage day 0, i.e., 0.85% in the control yogurts and 0.83% in the experimental samples. During storage, the acidity of the CY samples gradually increased and reached the highest value on day 21 (0.95%). The potential acidity of the experimental yogurt (EY) slightly decreased by 0.04% after 7 days of storage and then increased significantly ($p \leq 0.05$) to 0.98% on day 21. The increase in the lactic acid content and the decrease in pH in stored yogurts are caused by the fermentation activity of microorganisms present in yogurt inocula. In refrigeration conditions, bacteria still decompose lactose, but the process is much slower than at the optimum temperature for thermophilic bacteria [1,29,45]. Various authors [46,47] have reported an increase in active acidity (reduction of pH) with a simultaneous increase in lactic acid content in yogurts during 28-day storage. Amadarshanie et al. [48] recorded a decrease in pH to 3.58 and an increase in lactic acid content to 1.24% during 21-day storage of yogurts. Najgebauer-Lejko et al. [49] found that the acidity of yogurts was influenced by both the storage time and the addition of tea. As expected, the initial pH value steadily decreased from 4.65 to 4.36 ($p < 0.05$) during 28-day storage. The pH values in the tea-supplemented yogurts were significantly ($p < 0.05$) lower (by 0.09–0.15 units) than the mean value determined in the control yogurts. A similar trend in pH was found after the addition of *Moringa oleifera* extracts to milk [50]. The effect of the storage time on pH was also significant ($p < 0.001$), as significant differences were observed between storage day 1 and the other storage time points. In their study, Ogunyemi et al. [4] found that the addition of spice extracts exerted no significant effect on pH changes and lactic acid content during the fermentation process. As reported by Amirdivani and Baba [51], herbal yogurts were characterized by a faster pH-reduction rate than control samples. In a study conducted by Shori [52], the pH values in rosemary-, dill-, and oregano-supplemented yogurts were significantly ($p < 0.05$) lower than in the control in all storage periods. Similar trends were reported in a study of yogurt supplemented with an aqueous solution of fennel and stored for 21 days [53].

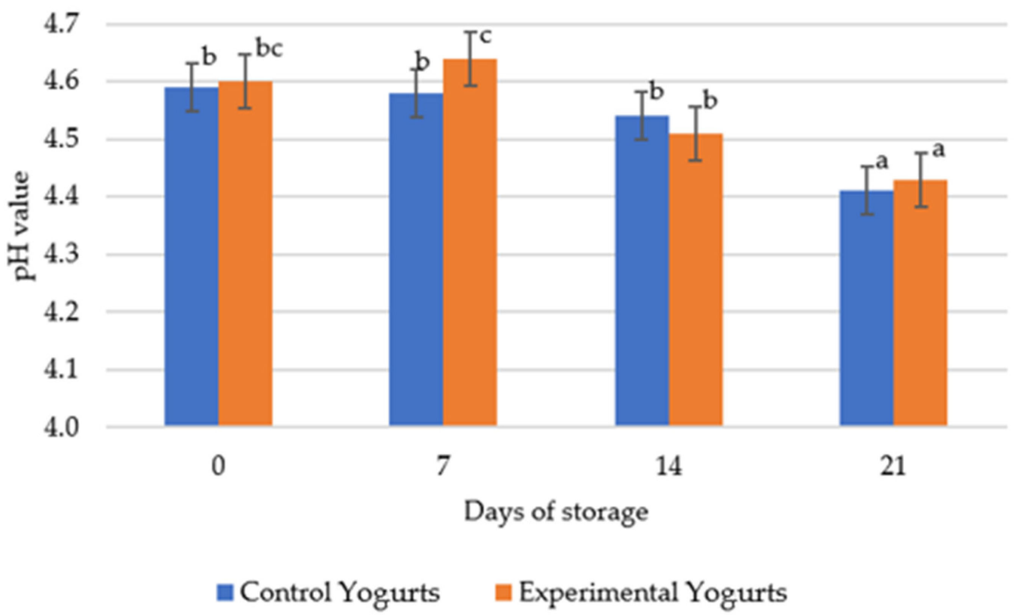


Figure 2. Changes in the pH value in the analyzed yogurts during 21 days of storage. a, b, c—significant differences between the day of storage within the yogurt type at $p \leq 0.05$.

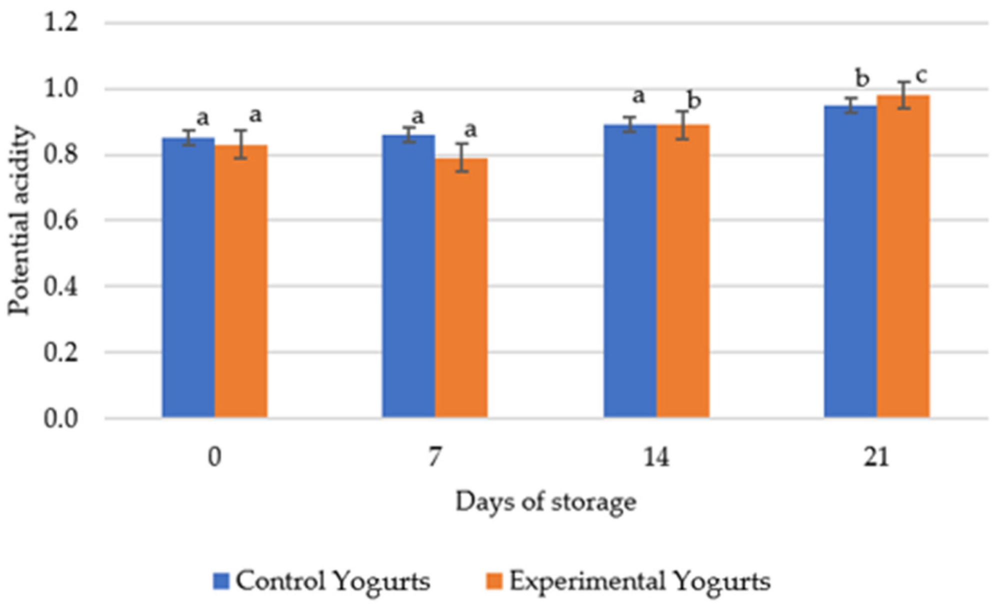


Figure 3. Changes in the lactic acid content (%) in the analyzed yogurts during 21 days of storage. a, b, c—significant differences between the days of storage within the yogurt type at $p \leq 0.05$.

3.4. Water Activity of Yogurts during 21 Days of Storage

Table 3 shows changes in water activity (aw) in the yogurts analyzed during 21 days of storage. This parameter can be used to determine the course of biochemical reactions, the stability of food sensory traits, the growth of microorganisms, and, primarily, the storability of food products [46]. Adverse reactions affecting food quality are largely related to the activity rather than the content of water in the product [54]. The higher the aw index, the faster the multiplication of microorganisms facilitated by the water used by microorganisms in their processes. The water activity in the present study ranged from 0.939 to 0.961 and was lower in the control yogurt. During storage, the a_w value slightly increased, but the differences were not significant. Similar trends were reported in other studies [29,46].

Table 3. Water activity and basic chemical composition of yogurts during 21 days of storage (mean ± SD).

Yogurt Type	Day of Storage	Water Activity	Total Protein (%)	Fat (%)	Dry Matter (%)
CY	0	0.939 ± 0.007	3.46 ^{b} ± 0.19	4.27 ± 0.17	12.33 ^{B} ± 0.19
	7	0.942 ± 0.006	3.44 ^{ab} ± 0.11	4.23 ± 0.11	12.25 ^{B} ± 0.26
	14	0.954 ± 0.011	3.38 ^{ab} ± 0.14	4.19 ± 0.14	12.06 ^{AB} ± 0.28
	21	0.960 ± 0.008	3.25 ^{a} ± 0.16	4.16 ± 0.15	11.58 ^{A} ± 0.31
EY	0	0.943 ± 0.010	3.52 ^{b} ± 0.13	4.30 ± 0.09	12.38 ^{B} ± 0.25
	7	0.949 ± 0.007	3.49 ^{ab} ± 0.16	4.28 ± 0.11	12.31 ^{B} ± 0.20
	14	0.955 ± 0.009	3.42 ^{ab} ± 0.10	4.25 ± 0.12	12.15 ^{B} ± 0.29
	21	0.961 ± 0.012	3.30 ^{a} ± 0.15	4.20 ± 0.10	11.66 ^{A} ± 0.17

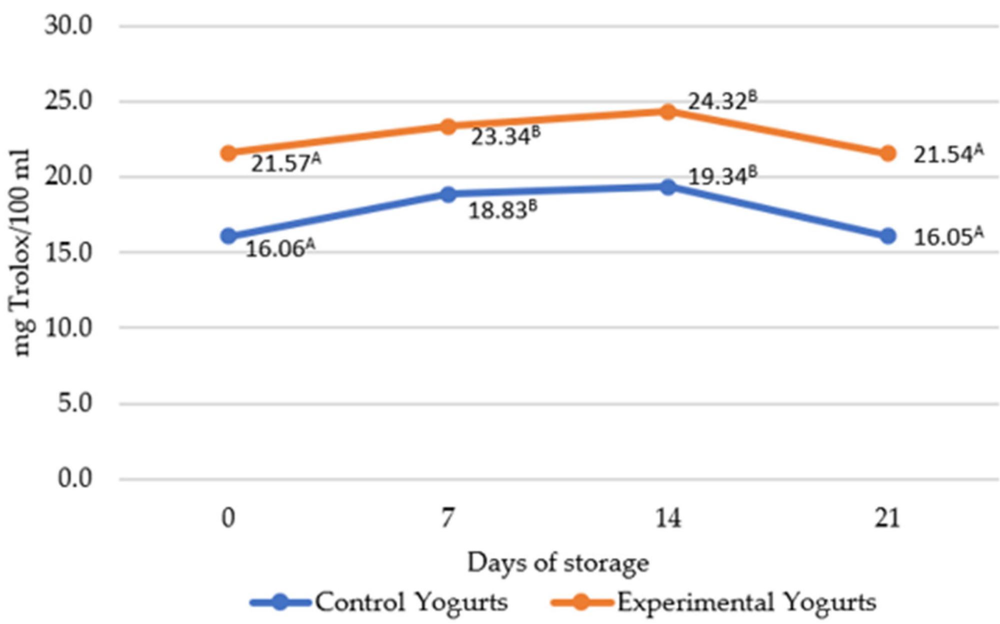
CY—control yogurts; EY—experimental yogurts; ^{a,b}—significant differences at $p \leq 0.05$; ^{A,B}—significant differences at $p \leq 0.01$.

3.5. Basic Chemical Composition of Yogurts during 21 Days of Storage

The results of the assessment of the basic nutritional value are presented in Table 3. There were no significant differences in the composition of the analyzed groups of yogurts (CY vs. EY); however, the yogurts produced from the herbal milk had a slightly higher content of dry matter, fat, and total protein, which was associated with the higher content of these components in the raw material. Regardless of the research group, the content of the analyzed components gradually decreased during storage. There were significant differences in the content of dry matter ($p \leq 0.01$) and protein ($p \leq 0.05$) in CY and EY between the initial (day 0) and the final (day 21) time points of cold storage. The content of dry matter was reduced by 0.72% in CY and by 0.75% in EY. In turn, the reduction of the protein content was estimated at 0.21% and 0.22% in CY and EY, respectively. Atwaa et al. [53] found no significant effect of the addition of aqueous extracts of fennel seeds on the composition of yogurt, i.e., protein and fat content, compared to the control sample.

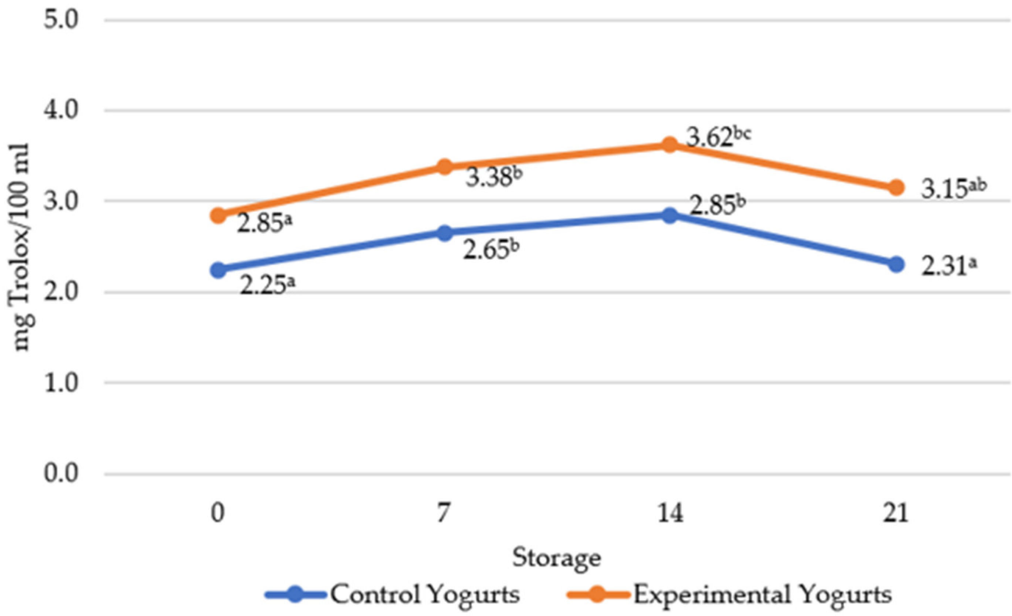
3.6. Antioxidant Activity of Yogurts during 21 Days of Storage

Figure 4a–c show the antioxidant activity of the yogurts stored for 21 days in refrigeration conditions. In general, regardless of the determination method used (FRAP, DPPH, ABTS), the antioxidant status of the EY samples was significantly higher than in the control group. A significant increase in their antioxidant activity was noted during the first two weeks of storage (until day 14). In comparison to day 0, the activity of the EY samples determined using the DPPH and ABTS assays increased by approximately 30%, i.e., from 2.85 to 3.62 mg Trolox/100 mL and from 5.97 A to 7.86 mg Trolox/100 mL, respectively. The iron ion reducing power (FRAP) also increased by 15%, i.e., from 21.57 to 24.32 mg Trolox/100 mL. Similar changes were recorded in the group of the CY samples. In the consecutive week of storage, the antioxidant potential of the yogurts declined. On day 21, the EY samples had a significantly ($p \leq 0.01$) lower (by approx. 15%) free radical scavenging capacity (ABTS and DPPH assays) and iron ion chelation capacity (FRAP) compared to day 14. The decrease in the antioxidant activity of the CY samples was higher, i.e., 20%. The literature does not provide reports on the use of milk by cows receiving herb-supplemented fodder for yogurt production. In turn, there are many literature reports [55–67] on the use of various additives as natural sources of antioxidants in the manufacture of yogurts. Enriched yogurts have been shown to have higher antioxidant activity than natural (control) products throughout the storage period. Muniandy et al. [68] reported a significant effect of the addition of green, white, and black tea on the antioxidant activity (FRAP, DPPH) of yogurts during 21-day refrigerated storage. Additionally, the addition of extracts from red ginseng (*Panax ginseng*) [69] or blackberry flowers (*Rubus ulmifolius*) [70] may increase the antioxidant capacity of yogurts. In the present study, the EY yogurts exhibited higher antioxidant activity than the CY samples during the 21-day storage period. In their study, Shori and Baba [71] used Neem (*Azadirachta indica*) leaf extracts as an additive and noted an increase in the free radical scavenging activity (DPPH) of manufactured yogurts versus traditional yogurts until storage day 14, followed by a decline in the activity. In another study conducted by Shori [52], yogurts supplemented with aqueous extracts of rosemary, dill, and oregano exhibited significantly higher ($p < 0.05$) activity (61.15 ± 1.2, 58.92 ± 1.3, and 66.97 ± 0.7 µg GAE/mL, respectively) than the control (34.79 ± 1.0 µg GAE/mL). During the 21-day storage period, the values decreased significantly ($p < 0.05$) in both herb-enriched and control groups [48]. Atwaa et al. [53] found that the antioxidant activity of yogurts supplemented with an aqueous solution of fennel seeds increased significantly ($p < 0.05$) in comparison with natural yogurt samples. In turn, a study conducted by Amirdivani and Baba [51] showed that herbal yogurts had higher ($p < 0.05$) antioxidant activity (DPPH) than natural samples throughout the storage period, but the activity gradually declined between storage days 7 and 28. Yilmaz-Ersan et al. [41] reported a significant increase in the DPPH and ABTS values during the first two weeks of storage of kefir made from cow milk and a decrease in these activities in the third week of storage. Lisak Jakopović et al. [50] observed higher ($p < 0.001$) values of antioxidant activity (FRAP) in moringa fruit-enriched yogurts. A significant increase in the FRAP value was noted during 28 days of storage, but the activity declined in the consecutive weeks. Many authors suggest that the high oxidative stability of yogurt during the first storage weeks is associated with the release of antioxidant peptides during milk fermentation [3,11,68].



(a)

Figure 4. *Cont.*



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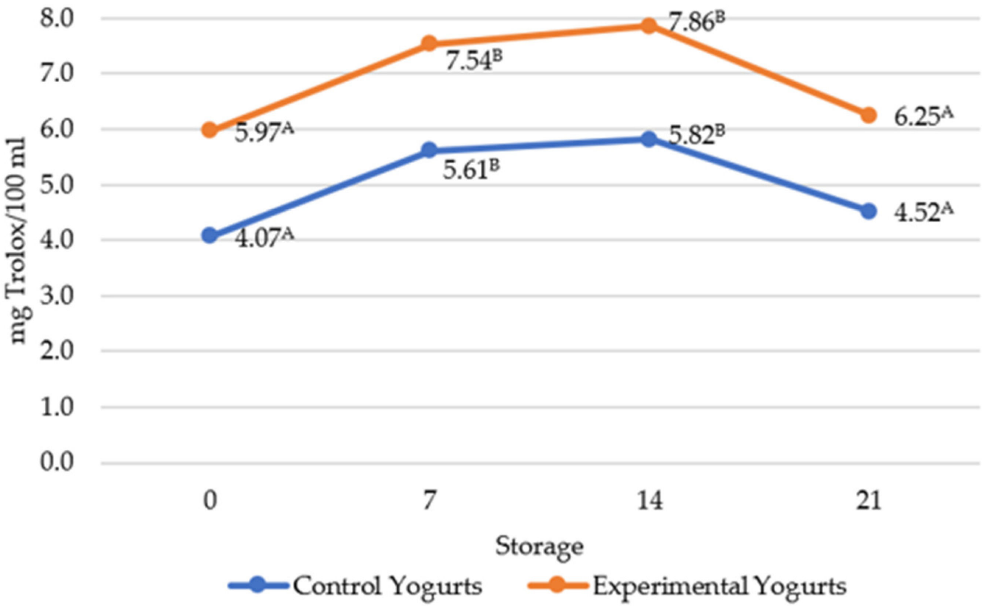

(**c**)

Figure 4. Antioxidant activity of yogurts during storage in mg of Trolox equivalent per 100 mL of sample (**a**) FRAP; (**b**) DPPH; (**c**) ABTS assay. a,b,c—significant differences at $p \leq 0.05$; A,B—significant differences at $p \leq 0.01$.

4. Conclusions

The study has confirmed that fermentation of milk contributes to an increase in the antioxidant activity of manufactured products. Regardless of the group and the research method used, the yogurts had significantly higher antioxidant activity than the milk. Irrespective of the determination method employed, the experimental yogurts were characterized by approximately 30% higher antioxidant potential than the control products, which was probably associated with the introduction of natural antioxidants, i.e., phenolic compounds contained in the herbal mixture. No significant differences were observed in the chemical composition of the yogurts. During the storage period, a significant increase in the antioxidant potential of the yogurts was noted in the first two weeks, and a decline in this activity in the third storage week. The decrease in the antioxidant activity of the experimental yogurts was lower, which indicates a higher level of oxidative stability of yogurts produced on the basis of milk obtained from the cows fed fodder supplemented with the herbal mixture.

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