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Wpływ wybranych czynników na potencjał antyoksydacyjny mleka pozyskiwanego od krów rasy holsztyńsko-fryzyjskiej i produktów wytwarzanych na jego bazie

Effect of selected factors on the antioxidant potential of milk obtained from the Holstein-Friesian cows and products based on it

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Spis treści

Wykaz publikacji wchodzących w skład pracy doktorskiej

1. **Magdalena Stobiecka**, Jolanta Król, Aneta Brodziak. Antioxidant activity of milk and dairy products. Animals, 2022, 12 (3), 245. DOI: 10.3390/ani12030245 (MEiN = 100 pkt.; IF = 3,000).

   Indywidualny wkład w publikację: przeprowadzenie przeglądu danych literaturowych, przygotowanie i wizualizacja wersji roboczej pracy, współtworzenie odpowiedzi na recenzje.
2. **Magdalena Stobiecka**, Jolanta Król, Aneta Brodziak. Antioxidant potential of milk obtained from Holstein-Friesian cows with regard to the subsequent lactations and stage of lactation. Mljekarstvo, 2023, 73 (2), 95-104. DOI: 10.15567/mljekarstvo.2023.0203 (MEiN = 40 pkt.; IF = 1,200).

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3. **Magdalena Stobiecka**, Jolanta Król, Aneta Brodziak, Renata Klebaniuk, Edyta Kowalczuk-Vasiliev. Effects of supplementation with an herbal mixture on the antioxidant capacity of milk. Animals, 2023, 13, 2013. DOI: 10.3390/ani13122013 (MEiN = 100 pkt.; IF = 3,000).

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4. **Magdalena Stobiecka**, Jolanta Król, Aneta Brodziak. Antioxidant potential of yoghurts produced from milk of cows fed fodder supplemented with herbal mixture with regard to refrigerated storage. Applied Sciences, 2023, 13, 10469. DOI: 10.3390/ app131810469 (MEiN = 100 pkt.; IF = 2,679).

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Streszczenie

Spożywanie produktów żywnościowych bogatych w naturalne antyoksydanty poprawia status antyoksydacyjny organizmu, chroniąc go przed stresem oksydacyjnym, zmniejszając ryzyko wystąpienia szeregu chorób cywilizacyjnych. Niewątpliwie bogatym źródłem składników wykazujących właściwości antyoksydacyjne są mleko i produkty mleczne (jogurty, sery). Składniki przeciwutleniające występują głównie we frakcji białkowej i tłuszczowej mleka. Zawartość tych składników w mleku, jak również wartość jego potencjału antyoksydacyjnego można modyfikować za pomocą stosowanego żywienia zwierząt, w tym poprzez wprowadzanie różnych dodatków naturalnych. Z kolei potencjał antyoksydacyjny produktów mlecznych związany jest z jakością surowca, jak również zastosowanymi kulturami bakteryjnymi oraz dodatkami roślinnymi.

Zrealizowana dysertacja miała na celu określenie wpływu wybranych czynników na potencjał antyoksydacyjny mleka i produktów wytwarzanych na jego bazie oraz korelacji z zawartością związków o charakterze antyoksydacyjnym. Uzyskane wyniki badań wskazywały na dodatnie wysokie wartości współczynników korelacji pomiędzy poziomem potencjału antyoksydacyjnego mleka a zawartością witamin A (r=0,687) i E (r=0,664) oraz β-LG (r=0,515). Potencjał antyoksydacyjny był zatem w dużym stopniu determinowany ilością tych związków w mleku. Ponadto zanotowano ujemne korelacje (r=-0,317) pomiędzy poziomem potencjału antyoksydacyjnego a wydajnością dobową mleka, co sugeruje, że wysoka produkcyjność krów negatywnie wpływa na wartość antyoksydacyjną mleka. Istotny spadek poziomu potencjału antyoksydacyjnego mleka stwierdzono wraz z kolejną laktacją oraz w trakcie jej przebiegu. Po zastosowaniu w żywieniu krów dodatku mieszanki ziołowej istotnie zwiększyła się w mleku zawartość antyoksydantów (β-LG, laktoferyny, witamin A i E), co przełożyło się na wzrost poziomu potencjału antyoksydacyjnego mleka. Co ważne, mleko pozyskane od krów skarmianych paszą z dodatkiem ziół charakteryzowało się istotnie wyższym stopniem ochrony antyoksydacyjnej co wskazuje, że zioła są bogatym źródłem przeciwutleniaczy. Badania potwierdziły, iż fermentacja mleka prowadzi do zwiększenia aktywności przeciwutleniającej wytwarzanych produktów. Jogurty w porównaniu do mleka charakteryzowały się wyższą aktywnością antyoksydacyjną, przy czym wytwarzane na bazie mleka „ziołowego" odznaczały się wyższym statusem antyoksydacyjnym w porównaniu do kontroli przez 21dni przechowywania.

Słowa kluczowe: mleko, potencjał antyoksydacyjny, związki bioaktywne, zioła w żywieniu, jogurty

Summary

Consumption of food products rich in natural antioxidants improves the antioxidant status of the body, protecting it from oxidative stress, reducing the risk of a number of civilization diseases. Undoubtedly, a rich source of ingredients showing antioxidant properties are milk and dairy products (yoghurt, cheese). Antioxidant components are mainly found in the protein and fat fraction of milk. The content of these components in milk, as well as the value of its antioxidant potential, can be modified by animal nutrition, including the introduction of various natural additives. In turn, the antioxidant potential of dairy products is related to the quality of the raw material, as well as the bacterial cultures and plant additives used.

The aim of the doctoral dissertation was to determine the effect of selected factors on the antioxidant potential of milk and products produced on its basis and the correlation with the content of antioxidant compounds. The obtained results indicated positive high values of correlation coefficients between the level of antioxidant potential of milk and the content of vitamins A (r=0.687) and E (r=0.664) and β-LG (r=0.515). The antioxidant potential was therefore largely determined by the amount of these compounds in milk. In addition, negative correlations (r=-0.317) were noted between the level of antioxidant potential and the daily yield of milk, which suggests that high cow productivity negatively affects the antioxidant value of milk. A significant decrease in the level of antioxidant potential of milk was found with the subsequent lactation and during its course. After using the addition of herbal mixture in cows' nutrition, the content of antioxidants (β-LG, lactoferrin, vitamins A and E) increased significantly in milk, which translated into an increase in the level of antioxidant potential of milk. Importantly, milk obtained from cows fed with feed with the addition of herbs was characterized by a significantly higher degree of antioxidant protection, which indicates that herbs are a rich source of antioxidants. Studies have confirmed that fermentation of milk leads to an increase in the antioxidant activity of manufactured products. Yoghurts, compared to milk, had a higher antioxidant activity, while those made on the basis of "herbal" milk had a higher antioxidant status compared to the control for 21 days of storage.

Keywords: milk, antioxidant potential, bioactive compounds, herbs in nutrition, yoghurts

1. Wstęp

W wyniku zaburzeń metabolicznych w organizmie dochodzi do zachwiania równowagi pomiędzy reakcjami wolnorodnikowymi a przeciwutleniającymi, co prowadzi do nagromadzenia się w komórkach nadmiernej ilości wolnych rodników, które nie są obojętne dla naszego organizmu. Ich nadmiar prowadzi bowiem do licznych uszkodzeń cząsteczek białek, lipidów i kwasów nukleinowych, a w konsekwencji do rozwoju chorób nowotworowych, neurodegeneracyjnych czy układu sercowo – naczyniowego (Karasahin i in., 2021; Losada - Barreiro i in., 2017; Marcone i in., 2017; Gjorgievski i in., 2014; Miciński i in., 2013). W celu ochrony organizmu przed zmianami wywołanymi przez reaktywne formy tlenu wykształciło się wiele mechanizmów chroniących przed nadmiernym generowaniem tych cząsteczek i uczestniczących w ich modyfikacji w nieaktywne pochodne. Mechanizmy te obejmują zarówno związki pochodzenia egzogennego, jak i endogennego. Enzymatyczną barierę przeciwutleniającą stanowią wyspecjalizowane enzymy, takie jak katalaza (CAT), dysmutaza ponadtlenkowa (SOD), peroksydaza glutationowa (GPx) i reduktaza glutationowa (Kapusta i in., 2018). Drugą linię obrony stanowią przeciwutleniacze nieenzymatyczne dostarczane do organizmu wraz z żywnością, które posiadają zdolność neutralizowania reaktywnych form tlenu. Bogatym źródłem antyoksydantów jest mleko i produkty mleczne, które stanowią około 25–30% średniej dieta człowieka (Stobiecka i in., 2022; Guha i in., 2021). Składniki te występują zarówno we frakcji białkowej (kazeina, β-laktoglobulina, laktoferyna), tłuszczowej (witamina E, A, β-karoten) i wodnej (witamina C, mikroelementy: Sn, Zn, Fe, Mn) mleka (Brodziak i in., 2020; Mavangira i Sordillo, 2018; Vanitcharoen i in., 2018; Silanikove i in., 2014, Miciński i in., 2013). Spożywanie produktów stanowiących naturalne źródło przeciwutleniaczy poprawia status antyoksydacyjny organizmu oraz zmniejsza ryzyko wystąpienia szeregu chorób cywilizacyjnych. Zwiększa również ogólną odporność organizmu na zakażenia i infekcje, spowalnia proces starzenia się organizmu a także zmniejsza częstotliwość występowania chorób neurologicznych, łącznie z niedokrwieniem mózgu, chorobą Parkinsona i Alzheimera (Winiarska-Mieczan i in., 2021; Gjorgievski i in., 2014, Miciński i in., 2013). W artykule Stobiecka i in. (2022) dokonano przeglądu danych literaturowych (w oparciu o 231 pozycji) dotyczących potencjału antyoksydacyjnego mleka surowego i przetworów mlecznych (mleka, przetworów fermentowanych i sera) oraz możliwości modyfikacji jego poziomu na etapie produkcji, jak i przetwórstwa mleka. Spośród wszystkich białek w diecie człowieka, to właśnie białka

serwatkowe, a szczególnie β-laktoglobulina charakteryzuje się najwyższym potencjałem antyoksydacyjnym. Spowodowane jest to dużą zawartością aminokwasów siarkowych, szczególnie cysteiny, która jest niezbędna do syntezy glutationu (Ma i in., 2018; Yilmaz-Ersan i in., 2018). Badania Kim i in. (2019) wskazują, iż działanie przeciwutleniające oprócz β–laktoglobuliny wykazuje również α–kazeina. Białka te, dzięki właściwościom antyoksydacyjnym mogą łagodzić uszkodzenia związane ze starzeniem się organizmu wywołane stresem oksydacyjnym, poprzez hamowanie starzenia się komórek oraz wzrost różnicowania i dojrzewania mioblastów. Aktywność antyoksydacyjną wykazuje również laktoferyna, która chelatuje żelazo, co zwiększa jego biodostępność i hamuje działanie prooksydacyjne. Jednak zdolność antyoksydacyjna laktoferyny zmniejsza się proporcjonalnie do nasycenia żelazem (Cutone i in., 2020). Białka i ich frakcje stanowią cenne źródło bioaktywnych peptydów, które posiadają zdolność do wymiatania wolnych rodników, chelatowania jonów metali oraz hamowania peroksydacji lipidów (Bielecka i in., 2021; Guha i in.; 2021; El-Sayed i in., 2019). Liczne badania (El-Sayed i Awad, 2019; Marcone i in., 2017; Power i in., 2013) wskazują na interakcję między składem aminokwasowym peptydów a aktywnością antyoksydacyjną.

Spośród witamin obecnych w mleku i produktach mlecznych głównym przeciwutleniaczem są witaminy rozpuszczalne w tłuszczach, głównie witamina E, a szczególnie α-tokoferol oraz witamina A i β-karoten (Vanitcharoen i in., 2018; Kaneai i in., 2012; Celi, 2011). Ich działanie polega na zmiataniu wolnych rodników organicznych i hamowaniu peroksydacji lipidów (Mann i in., 2016). Wykazują również zdolność pochłaniania tlenu singletowego i rodników hydroksylowych skutecznie chroniąc DNA przed utlenianiem (Cichosz i in., 2017). Z uwagi na fakt, iż znajdują się w otoczkach kuleczek tłuszczowych, zapobiegają samoczynnemu utlenianiu tłuszczu mlecznego. Również witamina D_3 jest częścią nieenzymatycznego systemu antyoksydacyjnego mleka, przy czym najbardziej aktywną formą jest 1,25-dihydroksycholekalcyferol. Jej działanie przeciwutleniające polega na hamowaniu peroksydacji lipidów (Mutlu i in., 2013). Ważną rolę antyoksydacyjną w organizmie odgrywa również witamina C (kwas askorbinowy), należąca do nieenzymatycznych przeciwutleniaczy rozpuszczalnych w wodzie, jak również witaminy z grupy B, które hamują powstawanie cysteiny. Potencjał antyoksydacyjny mleka jest zatem bezpośrednio związany z zawartością składników wykazujących właściwości przeciwutleniające (Stobiecka i in., 2022).

Zawartość składników antyoksydacyjnych mleka, jak również wartość jego potencjału antyoksydacyjnego można modyfikować poprzez zastosowanie w żywieniu

zwierząt naturalnych dodatków. Najczęściej w żywieniu krów mlecznych wykorzystywane są mieszanki ziołowe. Dzięki wysokiej zawartości substancji biologicznie czynnych wywierają pozytywny wpływ na funkcjonowanie organizmu krów, co w konsekwencji przekłada się na jakość pozyskiwanego mleka (Odhaib i in., 2021; Paskudska i in., 2018). Warto zaznaczyć, że dodatki paszowe stosowane w żywieniu bydła mogą spełniać również funkcje osłonowe oraz działać jako regulatory przemiany materii (Sakowski i in., 2015; Hashemzadeh-Cigari i in., 2014). W konsekwencji przyczyniają się do wzrostu odporności zwierząt narażonych na stres (zmiany dawki pokarmowej, stres cieplny), zwiększają absorpcję niezbędnych składników odżywczych oraz podwyższają stopień ochrony antyoksydacyjnej (Sakowski i in., 2015; Rochfort i in., 2008). Aspekt ten jest szczególnie istotny w odniesieniu do wysokowydajnych zwierząt, u których obserwuje się obniżenie odporności, a w konsekwencji zwiększoną podatność na choroby (Maksymiec, 2012).

Potencjał antyoksydacyjny produktów mlecznych (jogurt, ser, kefir) związany jest z jakością surowca, a przede wszystkim obecnością i aktywnością naturalnych składników bioaktywnych w mleku. Istotny wpływ na wartość potencjału antyoksydacyjnego produktów mlecznych mają również zastosowane kultury bakteryjne oraz dodatki roślinne (Yilmaz-Ersan i in., 2018; Chen i in., 2015; Sabokbar i in., 2015; Sah i in., 2015; Gjorgievski i in., 2014; Perna i in., 2013; Shori i Baba, 2013; Kesenkas i in., 2011). Co ważne, podczas procesu fermentacji mlekowej z białek mleka uwalniane są bioaktywne peptydy i wolne aminokwasy o zróżnicowanej aktywności biologicznej, w tym działaniu przeciwutleniającym. W konsekwencji przyczyniają się do zwiększenia zdolności antyoksydacyjnej produktów i hamowania peroksydacji lipidów (Cruz-Casas i in., 2023; Guo i in., 2023). Należy podkreślić, że użyte kultury bakteryjne mają istotny wpływ na wartość potencjału antyoksydacyjnego. Jak wskazano w wielu badaniach, produkty fermentowane zawierające szczepy probiotyczne charakteryzują się znacznie zwiększoną aktywnością antyoksydacyjną (Fardet i Rock, 2018; Najgebauer-Lejko i Sady, 2015).

Zdolność antyoksydacyjna mleka i produktów mlecznych jest zatem wynikiem złożonej równowagi pomiędzy antyoksydantami a oksydantami (Fardet i Rock, 2018; Revilla i in., 2016). Bioaktywne składniki mleka i produktów mlecznych mogą skutecznie zapobiegać utlenianiu tłuszczu, a tym samym zapewniają wyższą stabilność i jakość produktu. Należy zatem dążyć do zwiększenia zawartości składników bioaktywnych w mleku, które decydują o jego potencjale antyoksydacyjnym.

2. Cel i zakres pracy

Głównym celem badawczym było określenie wpływu wybranych czynników na potencjał antyoksydacyjny mleka i produktów wytwarzanych na jego bazie oraz korelacji z zawartością związków o charakterze antyoksydacyjnym.

Zakres badań:

1. Omówienie potencjału antyoksydacyjnego mleka surowego i przetworów mlecznych oraz możliwości modyfikacji jego poziomu na etapie produkcji i przetwórstwa mleka.
2. Określenie zmian potencjału antyoksydacyjnego mleka pozyskiwanego od krów rasy holsztyńsko-fryzyjskiej w zależności od kolejnej laktacji i jej fazy.
3. Ocena wpływu dodatku standaryzowanej mieszanki ziołowej do dawki pokarmowej krów rasy holsztyńsko-fryzyjskiej na kształtowanie się poziomu potencjału antyoksydacyjnego mleka.
4. Ocena zawartości związków o charakterze antyoksydacyjnym we frakcji białkowej i tłuszczowej mleka z uwzględnieniem ww. czynników.
5. Ocena zmian potencjału antyoksydacyjnego produktów fermentowanych - jogurtów wytwarzanych na bazie ocenianego surowca podczas 21-dniowego przechowywania.
6. Określenie zależności pomiędzy zawartością antyoksydantów w mleku a jego potencjałem antyoksydacyjnym.

3. Materiały i metody

Badania zostały zrealizowane z ramach programu Ministra Nauki i Szkolnictwa Wyższego pod nazwą „Regionalna Inicjatywa Doskonałości” w latach 2019-2023, nr projektu 029/RID/2018/19, kwota finansowania 11 927 330,00 zł.

3.1. Układ doświadczalny

W ramach dysertacji: „Wpływ wybranych czynników na potencjał antyoksydacyjny mleka pozyskiwanego od krów rasy holsztyńsko-fryzyjskiej i produktów wytwarzanych na jego bazie” przeprowadzono trzy doświadczenia.

Doświadczenie I (publikacja 2: Stobiecka i in., 2023)

Celem doświadczenia pierwszego była ocena zawartości składników wykazujących aktywność antyoksydacyjną w mleku krów rasy holsztyńsko-fryzyjskiej oraz zmian całkowitego statusu antyoksydacyjnego pozyskiwanego mleka, z uwzględnieniem kolejnej laktacji i jej stadium.

Badania przeprowadzono w gospodarstwie utrzymującym krowy rasy holsztyńsko-fryzyjskiej. Produkcja mleka odbywała się w systemie intensywnym. Krowy utrzymywano w oborze wolnostanowiskowej, a ich żywienie w ciągu całego roku oparte było o pełnoporcjowy system TMR (ang. total mixed ration) – w skład dawki pokarmowej wchodziły pasze objętościowe (kiszonka z kukurydzy, sianokiszonka z traw) oraz treściwe.

Badaniami objęto 90 krów rasy holsztyńsko-fryzyjskiej, po 30 będących w każdej analizowanej laktacji (I - pierwiastki; II - wieloródki w drugiej laktacji; III - wieloródek w trzeciej laktacji). Próby mleka indywidualnie od każdej krowy pobierano w trzech terminach: 1. do 100 dni laktacji, 2. między 101 a 200 dniem laktacji 3. między 201-305 dniem laktacji. Bezpośrednio po pobraniu mleko schłodzono i przewieziono do laboratorium, gdzie oznaczano liczbę komórek somatycznych na aparacie Somacaunt150 (Bentley Instruments, USA). Do dalszych analiz pozostawiano tylko próby mleka, w których liczba komórek somatycznych nie przekraczała 400 tys./ml. Łącznie analizami objęto 262 próby mleka.

Doświadczenie II (publikacja 3: Stobiecka i in., 2023)

Celem doświadczenia drugiego była ocena wpływu zastosowanego dodatku standaryzowanej mieszanki ziół w dawce pokarmowej krów rasy holsztyńsko-fryzyjskiej na kształtowanie się poziomu potencjału antyoksydacyjnego mleka.

Badania przeprowadzono w gospodarstwie specjalizującym się w hodowli bydła mlecznego, utrzymującym krowy rasy holsztyńsko-fryzyjskiej. Do badań włączono wybrane ze stada 30 krów w 3 laktacji, będących na początku doświadczenia w I fazie laktacji (15 krów – grupa kontrolna; 15 krów – grupa doświadczalna). Układ doświadczenia przedstawia tabela 1. Żywienie krów prowadzono w systemie TMR. Podstawą dawki pokarmowej stanowiły wysłodki buraczane kiszone, kiszonka z kukurydzy oraz sianokiszonka z traw i słoma pszenna (PO) oraz mieszanka treściwa (MT), w tym śruta zbożowa gospodarska, poekstrakcyjna śruta rzepakowa, komercyjna mieszanka uzupełniająca (tabela 2). Czynnik doświadczalny stanowił dodatek standaryzowanej mieszanki suszonych ziół tj. oregano (*Origanum vulgare*) - 25%, tymianek (*Thymus vulgaris*) - 25%, kora cynamonu (*Cinnamomum zeylanicum*) - 15%, jeżówka (*Echinacea purpurea*) - 35%. Zioła podawano w formie pudru jako komponent mieszanki treściwej w ilości 3% s.m. dawki/dzień/sztukę. Grupę kontrolną stanowiły krowy, które otrzymywały jedynie PO i MT. Doświadczenie trwało 6 tygodni. Próby mleka pobierano 4-krotnie w ciągu trwania doświadczenia (termin 0, w 2, 4 i 6 tygodniu).

Tabela 1. Układ doświadczenia

Okres doświadczalny, tyg.	Grupa	
	K	D
6 tygodni	PO + MT	PO + MT + MZ (3%)
Liczba, szt.	15	15

K– krowy grupy kontrolnej żywione standardową dawką pokarmową opartą na paszach objętościowych stosowanych w gospodarstwie z dodatkiem mieszanki pasz treściwych

D– krowy grupy doświadczalnej żywione standardową dawką pokarmową opartą na paszach objętościowych, z dodatkiem mieszanki pasz treściwych i 3 % udziałem doświadczalnej, mieszanki ziołowej (MZ)

PO– pasze objętościowe

MT– mieszanka pasz treściwych

MZ– standaryzowana mieszanka ziołowa: tymianek, jeżówka, oregano, cynamon

Tabela 2. Skład komponentowy podstawowej dawki pokarmowej

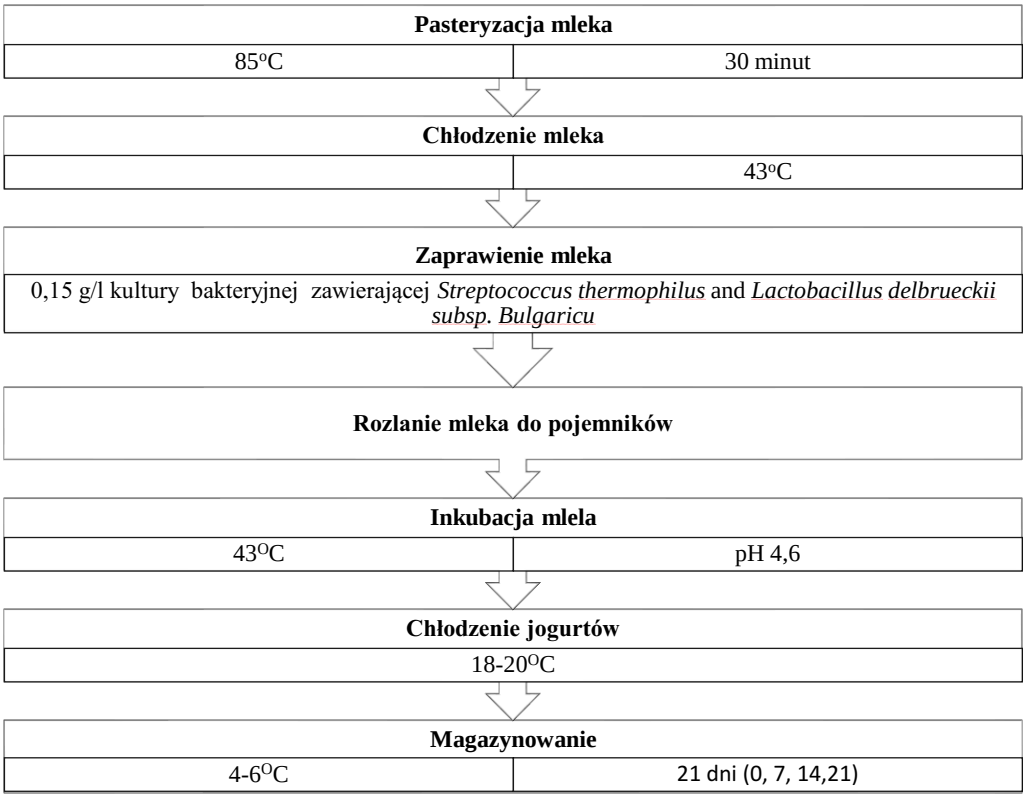
Rodzaj paszy	Ilość paszy zadawanej (kg/dzień)			
	Wydajność poniżej 25 litrów / dzień		Wydajność powyżej 25 litrów / dzień	
PO	K1	D1	K2	D2
Wysłodki buraczane kiszone	22		18	
Kiszonka z kukurydzy	15		12	
Sianokiszonka z traw	10		26,5	
Słoma pszenna	1,5		1,8	
MT				
Mieszanka treściwa*	3,0 - 6,0	3,0 - 6,0	6,5 – 9,0	6,5 – 9,0

***(K1, K2):** pszenżyto - 35%, owies - 20%, jęczmień - 18%, ziarno kukurydzy - 14%, poekstrakcyjna śruta rzepakowa - 2,5%, komercyjna mieszanka uzupełniająca – 10%, kreda pastewna i dodatki mineralno-wit. – 0,5%; **(D1, D2):** pszenżyto - 35%, owies - 20%, jęczmień - 16%, ziarno kukurydzy - 13%, poekstrakcyjna śruta rzepakowa - 2,5%, komercyjna mieszanka uzupełniająca - 10%, susz ziela lebiodki pospolitej - 3%, kreda pastewna i dodatki mineralno-wit. – 0,5%

Doświadczenie III (publikacja 4: Stobiecka i in., 2023)

Celem doświadczenia trzeciego była ocena możliwości wykorzystania mleka pozyskanego od krów żywionych paszą z dodatkiem ziół do produkcji jogurtów o podwyższonym potencjale antyoksydacyjnym z uwzględnieniem 21-dniowego czasu przechowywania.

Materiał badawczy stanowiło mleko zbiorcze, pozyskane w gospodarstwie specjalizującym się w hodowli bydła mlecznego, utrzymującym krowy rasy holsztyńsko-fryzyjskiej. Niniejsza praca jest kontynuacją badań przeprowadzonych w doświadczeniu II. W trakcie trwania doświadczenia pobierano 3-krotnie próby mleka zbiorczego (kontrola i doświadczenie) w ilości 10 l, które przeznaczano do produkcji jogurtów. W warunkach laboratoryjnych wytwarzano jogurty zgodnie ze schematem 1.



Schemat 1. Schemat produkcji jogurtów (opracowanie własne)

3.2. Metody analityczne

W próbach mleka wykonano następujące oznaczenia:

– skład chemiczny, tj. zawartość tłuszczu, białka, laktozy i suchej masy aparatem Infrared Milk Analyzer (Bentley Instruments, USA);

– zawartość kazeiny (AOAC, 2000);

– liczbę komórek somatycznych (LKS) metoda cytometrii przepływowej – aparat Somacount 150 (Bentley Instruments, USA);

– stężenie wybranych białek serwatkowych, tj. α-laktoalbuminy (α-LA), β-laktoglobuliny (β-LG), laktoferyny i krowiej albuminy serum (BSA) przy zastosowaniu wysokosprawnej chromatografii cieczowej w odwróconym układzie faz (RP-HPLC). Próbki przygotowano w oparciu o metodę opracowaną przez Romero i in. (1996) z modyfikacjami (Brodziak i in., 2012). Rozdziału białek dokonano przy wykorzystaniu chromatografu cieczowego ProStar 210 wyposażonego w detektor UV-Vis ProStar 325 (Varian, USA) i kolumnę NUCLEOSIL 300-5 C18 (Varian, USA) o długości 250 mm i średnicy 4,6 mm;

– stężenie wybranych witamin lipofilnych, tj. A, D_3 i E przy zastosowaniu metody

wysokosprawnej chromatografii cieczowej w odwróconym układzie faz (RP-HPLC) (wykorzystując chromatograf cieczowy ProStar Varian, wyposażony w detektor fluorescencyjny i kolumnę PursuitXRs 3-C18 (Varian, USA) o długości 150 mm i średnicy 4,6 mm). Próbki mleka przygotowano w oparciu o ekstrakcję tłuszczu metodą Röse-Gottlieb'a zmodyfikowaną przez Hewavitharana i in. (1996);

– potencjał antyoksydacyjny mleka oznaczono trzema metodami, tj. FRAP (Benzie i Strain, 1996), DPPH (Brand-Williams i in., 1995) oraz ABTS (Sahin i in., 2012). Próbki do analiz przygotowano ze świeżego mleka, tj. do 1 ml mleka dodano 10 ml rozpuszczalnika (roztwór 1M HCl w 95% etanol w stosunku objętościowym 15/85), wytrząsano przez 1 godzinę w temp. 40°C w wytrząsarce obrotowej 300 obr./min., a następnie próbki odwirowano w wirówce przy 8000×g (MPW-350, Med. Instruments, Polad) przez 10 min. Do oznaczania aktywności antyoksydacyjnej użyto ekstrakt.

  - ✓ metoda FRAP (Ferric Reducing Antioxidant Power) - polega na redukcji kompleksu Fe 3+ -TPTZ (2,4,6-tris(2-pirydylo)-1,3,5-triazyn) do Fe 2+ -TPTZ przy niskim pH. Redukcja objawia się pojawieniem koloru niebieskiego. Odczytów absorbancji dokonano przy długości fali 593 nm przy użyciu spektrofotometru UV-Vis (U-2900 HITACHI, Tokio, Japonia). Jako standardy do kalibracji zastosowano różne stężenia Troloxu (TE). Wyniki wyrażono w miligramach równoważników TE na 100 ml próbki;
  - ✓ metoda DPPH – polega na redukcji rodnika DPPH (2,2-diphenyl-1-picrylhydrazyl). Odczytów absorbancji dokonano przy użyciu spektrofotometru (HITACHI U-2900, Tokio, Japonia) przy 515 nm. Jako standardy do kalibracji zastosowano różne stężenia Troloxu. Wyniki wyrażono jako ilość związków przeciwutleniających zdolnych do redukcji rodnika DPPH zawartych w 100 ml próbce, równoważnych mg TE;
  - ✓ metoda ABTS – polega na określeniu stopnia zmiatania rodników ABTS+ (2,2'azyno-bis 3-etylobenzotiazolino-6-sulfonowego) wytworzonych uprzednio podczas reakcji chemicznych z nadsiarczanem potasu. Absorbancję rejestrowano przy użyciu spektrofotometru UV-Vis (Cary 100, Varian, Palo Alto, Kalifornia, USA) przy 734 nm w stosunku do ślepej próby odczynnika. Krzywą standardową przygotowano przy użyciu różnych stężeń Troloksu, a wyniki wyrażono w miligramach równoważników TE na 100 ml próbki;

– całkowity potencjał antyoksydacyjny (TAS) - oznaczono przy wykorzystaniu testów Randox (Tecan Austria GmbH, Grödig, Austria) oraz spektrofotometru UV-Vis (Cary 100,

Varian, Palo Alto, Kalifornia, USA);

– poziom cholesterolu oznaczono kolorymetryczną metodą enzymatyczną z udziałem esterazy cholesterolowej i oksydazy cholesterolowej przy użyciu zestawu Liquick Cor-CHOL (Cormay, Łomianki, Polska);

– stopień ochrony antyoksydacyjnej (DAP) obliczono na podstawie stosunku molowego między przeciwutleniaczami i utleniaczami według Pizzoferato i in. (2007).

W próbach pasz wykonano następujące oznaczenia:

– zawartość suchej masy, białka ogólnego, włókna surowego i jego frakcji, tłuszczu surowego oraz popiołu surowego (AOAC, 2011);

– wartość odżywczą pasz i dawek oszacowano na podstawie wyników analizy podstawowej pasz przy wykorzystaniu programu komputerowego Winwar 1.6, podając wartość pokarmową każdej z nich w jednostkach INRA (Strzetelski i in., 2014);

– zawartość olejków eterycznych - metodą chromatografii gazowej z wykorzystaniem układu GC-MS (Shimadzu GCMS-TQ8040).

W próbach jogurtów wykonano następujące oznaczenia:

– zawartość białka metodą Kjeldahla (PN-EN ISO 8968-1:2014);

– zawartość tłuszczu (metoda wagowa) oraz suchej masy (suszenie w temperaturze 102°C) wg PN-A-86061:2006;

– kwasowość czynną przy pomocy pH-metru (Elmetron CP-401, Polska);

– kwasowość potencjalną metodą miareczkową (IDF/ISO Standard, 1991), jako zawartość kwasu mlekowego (%);

– aktywność wody (AW) za pomocą miernika aktywności wody HygroLab C1 (Rotronic, Bassersdorf, Szwajcaria). Pomiary prowadzono w trybie AWQ i stabilizacji przez 15 min po osiągnięciu przez jogurty temperatury pokojowej;

– potencjał antyoksydacyjny określono trzema metodami, tj. DPPH, FRAP i ABTS, analogicznie jak w próbach mleka.

Wszystkie pomiary wykonano w trzech powtórzeniach.

3.3. Analiza statystyczna

Wyniki poddano analizie statystycznej za pomocą jedno- i/lub wieloczynnikowej analizy wariancji (ANOVA) w programie StatSoft Inc. Statistica ver. 13 (Dell, Round Rock, Teksas, USA). Istotne różnice pomiędzy średnimi oznaczono za pomocą testu Tukeya na poziomie istotności p (alfa) = 0,05 i 0,01. Wyniki przedstawiono jako średnie ± odchylenie standardowe (SD). Obliczono również współczynniki korelacji prostej Pearsona dla wydajności i analizowanych parametrów mleka.

4. Omówienie głównych wyników prac eksperymentalnych

4.1. Ocena zawartości zmian potencjału antyoksydacyjnego mleka pozyskiwanego od krów rasy holsztyńsko-fryzyjskiej z uwzględnieniem kolejnej laktacji i jej stadium

Produkcja mleka na świecie, w tym również w Polsce opiera się przede wszystkim na krowach rasy holsztyńsko-fryzyjskiej. Krowy tej rasy charakteryzują się wysoką wydajnością mleczną, ale są najbardziej narażone na występowanie stresu metabolicznego. Badania wskazują, iż podczas różnych procesów fizjologicznych, w tym w trakcie trwania laktacji wytwarzane są reaktywne formy tlenu. Organizm zwierzęcia wykorzystuje przeciwutleniacze, które redukują wolne rodniki (Chang i in., 2007). W przypadku nadmiaru wolnych rodników dochodzi do uszkodzeń makrocząsteczek, tj. białek, lipidów i DNA, a w konsekwencji do zaburzeń o podłożu metabolicznym, spadku wydajności czy pogorszenia jakości mleka, w tym obniżenia zawartości substancji o charakterze antyoksydacyjnym (Karasahin i in., 2021).

Celem badań była ocena zawartości składników wykazujących aktywność antyoksydacyjną w mleku krów rasy holsztyńsko-fryzyjskiej oraz zmian całkowitego statusu antyoksydacyjnego pozyskiwanego mleka, z uwzględnieniem kolejnej laktacji i jej stadium (doświadczenie I – publikacja 2). Uzyskane wyniki badań własnych przedstawione w publikacji 2 wskazują, iż całkowity potencjał antyoksydacyjny mleka zmieniał się w kolejnych laktacjach, jak również w trakcie jej przebiegu. Najwyższą pojemnością antyoksydacyjną wyróżniało się mleko pozyskiwane od pierwiastek (0,96 mmmol/l), co najprawdopodobniej należy łączyć z wyższą zawartością składników wykazujących właściwości antyoksydacyjne w ich mleku, tj. witamin A i E oraz β-LG. Nieznacznie niższym potencjałem charakteryzowało się mleko od krów będących w II laktacji (0,92 mmmol/l). W trzeciej laktacji zanotowano natomiast istotne ($p \leq 0,01$) obniżenie wartości potencjału antyoksydacyjnego mleka (o około 20%), jak również składników o właściwościach przeciwutleniaczy. Na zależności te wskazują również uzyskane istotne ($p \leq 0,01$) dodatnie współczynniki korelacji pomiędzy zawartością witamin A ($r=0,687$) i E ($r=0,664$) oraz β-LG ($r=0,515$) w mleku a wartością statusu antyoksydacyjnego mleka, co wskazuje, iż zawartość tych związków decyduje w dużym stopniu o potencjale przeciwutleniającym mleka. Z drugiej jednak strony wykazano, że status antyoksydacyjny

mleka był istotnie ($p \leq 0,05$) ujemnie skorelowany z wydajnością mleczną krów ($r = -0,317$). Wraz z kolejną laktacją zwiększała się istotnie wydajność mleczna krów, a obniżała pojemność antyoksydacyjna mleka. Podobne zależności stwierdziły Kapusta i in. (2018). Prowadząc badania na krowach wieloródkach w szczycie laktacji uzyskały istotny ($p \leq 0,01$) współczynnik korelacji pomiędzy wydajnością mleczną a TAS na poziomie $r = -0,390$. Zdaniem innych autorów (Sakowski i in., 2012; Kuczyńska i in., 2021) wyższa wydajność mleczna krów jest czynnikiem zwiększającym występowanie stresu oksydacyjnego, wpływającego na stan zdrowia zwierząt, a także skład chemiczny i jakość mleka. W badaniu Puppel i in. (2012) stwierdzono, że pojemność antyoksydacyjna mleka może być związana zarówno z suplementacją diety, jak i wiekiem krów. Modyfikacja diety krów olejem rybim i siemieniem lnianym istotnie wpłynęła na wzrost całkowitego statusu antyoksydacyjnego mleka, przy czym wyższy poziom w obu grupach żywieniowych odnotowano w mleku krów pierwiastek. Mann i in. (2016) zwrócili uwagę na wpływ fazy laktacji na zmiany statusu antyoksydacyjnego mleka. Określając status mleka pozyskiwanego od różnych ras krów (Sahiwal, Karan Fries, Holstein Frisian) wyższe wartości otrzymali dla siary oraz mleka pozyskiwanego we wczesnej fazie laktacji. Wraz z jej przebiegiem wartości potencjału antyoksydacyjnego obniżały się istotnie. Autorzy sugerują, iż wyższy poziom antyoksydantów w siarze może mieć kluczowe znaczenie dla ochrony zdrowia noworodka przed stresem oksydacyjnym. W badaniach własnych również wraz z przebiegiem laktacji stwierdzono obniżenie całkowitego statusu antyoksydacyjnego pozyskiwanego mleka, przy czym istotne różnice ($p \leq 0,05$) zanotowano pomiędzy 1 i 3 fazą laktacji. Istotne zmiany w wartości potencjału przeciwutleniającego mleka podczas przebiegu laktacji wykazali także Annie i in. (2019). Najwyższy potencjał antyoksydacyjny zanotowali dla mleka pozyskiwanego we wczesnej fazie laktacji. Badania Kapusty i in. (2018) również potwierdzają wyraźną zależność między fazą laktacji, a kształtowaniem się poziomu antyoksydantów w mleku. Najwyższy potencjał antyoksydacyjny (TAS) autorzy wykazali w pierwszych dniach laktacji, natomiast w kolejnych jego poziom ulegał stopniowemu obniżeniu. Badania innych autorów (Puppel i in., 2017; Kapusta i in., 2018) wskazują również, iż wyższym potencjałem antyoksydacyjnym charakteryzowało się mleko bodące bogatszym źródłem głównie witamin A i E.

4.2. Zastosowanie dodatku mieszanki ziół w dawce pokarmowej krów rasy holsztyńsko-fryzyjskiej i ocena jego wpływu na kształtowanie się poziomu potencjału antyoksydacyjnego mleka

Zawartość składników antyoksydacyjnych mleka, jak również wartość jego potencjału antyoksydacyjnego można modyfikować za pomocą stosowanego żywienia zwierząt (Odhaib i in., 2021; Paskudska i in., 2018). Już wprowadzenie żywienia pastwiskowego istotnie zwiększa udział składników antyoksydacyjnych w mleku, zwiększając tym samym jego potencjał antyoksydacyjny (Torre-Santos i in., 2020; Brodziak i in., 2018; Puppel i in., 2017; Cichosz i in., 2017; Sunarić i in., 2012). Poprawa jest trudniejsze do osiągnięcia w przypadku stosowania w żywieniu krów pasz konserwowanych, a zwłaszcza kiszonki (Alves i in., 2011; Dunne i in., 2009; Nielsen i in., 2004). Nielsen i in. (2004) wykazali, że wyższy udział kiszonki z kukurydzy w dawkach pokarmowych dla krów jest jednym z głównych czynników niższej zawartości witamin i przeciwutleniaczy w mleku. Alves i in. (2011) wskazują, iż kiszonka z kukurydzy, która jest najczęściej stosowana w żywieniu krów jest uboga w karotenoidy.

W żywieniu zwierząt to zioła są naturalnym źródłem przeciwutleniaczy (Embuscad, 2015). Zalicza się do ich przede wszystkim karotenoidy (ksantofile i karoteny) i polifenole (flawonoidy, antocyjany, kwasy fenolowe, stylbeny i lignany) oraz alkaloidy, terpeny, saponiny czy olejki eteryczne (Bąkowski i Kiczorowska, 2021; Bichra i in., 2020; Reddy i in., 2020). Już na etapie przechowywania pasz zabezpieczają je przed psuciem hamując proces oksydacji (Janani, 2013). Najczęściej w żywieniu zwierząt, z uwagi na właściwości antyoksydacyjne wykorzystywane są: oregano (*Origanum vulgare*), cynamon (*Cinnamonum cassia*), rozmaryn (*Salvia rosmarinus*), tymianek (*Thymus vulgaris*), majeranek (*Origanum majorana*), kurkuma (*Curcuma longa*), kminek (*Carum carvi*), imbir (*Zingiber officinale*), pokrzywa (*Urtica dioica*), czy jeżówka purpurowa (*Echinacea purpurea*) (Odhaib i in., 2021; El-Sayed i Youssef, 2019; Ulewicz-Magulska i Wesołowki, 2019; Sotek i in., 2018). Ich obecność nawet w niewielkich stężeniach w mieszankach paszowych wpływa na wskaźniki antyoksydacyjne krów mlecznych w okresie laktacji. U zwierząt otrzymujących dodatek ziół zaobserwowano wzrost aktywacji enzymów antyoksydacyjnych zarówno we krwi i mleku, które odgrywają ważną rolę w ochronie komórek przed uszkodzenie oksydacyjnym (Kolling i in., 2022; Vizzotto i in., 2021; Panchasara i in., 2019). Badania wskazują, że zastosowane zioła w diecie przeżuwaczy mają korzystny wpływa na poprawę efektywności wykorzystania pasz, modyfikację mikroflory żwacza a w konsekwencji na

zdrowotność i produkcyjność zwierząt (Panchasara i in., 2019; Prayitno i in., 2016; Wawrzynczak i in., 2000; Cardozo i in., 2006, Grabowicz i in., 2004; Greathead, 2003; Kraszewski i in., 2002). Lepsze efekty produkcyjne uzyskuje się przy stosowaniu mieszanin ziołowych niż pojedynczych ziół, głównie z powodu synergistycznego działania poszczególnych związków aktywnych (Hassan i in., 2021; Liu, 2005). Co istotne bioaktywne związki roślin wykazują wyraźną odporność na degradację mikrobiologiczną w żwaczu, nie tracąc swojej funkcjonalności (Oh i in., 2017; Gessner i in., 2015). Podęto zatem badania mające na celu ocenę wpływu dodatku standaryzowanej mieszanki ziołowej (oregano, tymianek pospolity, jeżówka purpurowa i kora cynamonu) do dawki pokarmowej krów rasy holsztyńsko-fryzyjskiej na zdolność antyoksydacyjną mleka (doświadczenie II - publikacja 3). Wykazano, że surowiec produkowany przez krowy z grupy doświadczalnej stanowił bogatsze źródło antyoksydantów w porównaniu do kontroli. Zawierał istotnie więcej białek serwatkowych, tj. β –laktoglobuliny ($p \leq 0,05$), laktoferyny ($p \leq 0,01$), lizozymu ($p \leq 0,05$) oraz witamin A ($p \leq 0,05$) i E ($p \leq 0,01$). Wyższa zawartość antyoksydantów w mleku z grupy doświadczalnej przełożyła się na wzrost potencjału antyoksydacyjnego tego mleka. Niezależnie od zastosowanej metody oznaczania (FRAP, DPPH, ABTS) surowiec pozyskiwany od krów z grupy doświadczalnej charakteryzował się istotnie wyższą wartością potencjału antyoksydacyjnego w porównaniu do kontroli. Wartości DPPH i ABTS wzrosły o około 50% w stosunku do kontroli. W przypadku testu FRAP, mleko charakteryzowało się wyższą o około 20% zdolnością chelatowania Fe2+. Wyższa aktywność przeciwutleniająca mleka w stosunku do kontroli jest związana z wprowadzeniem wraz z mieszanką ziół naturalnych przeciwutleniaczy, tj. związków fenolowych. Ekstrakty roślinne mogą przyczyniać się do wzrostu endogennych przeciwutleniaczy i redukcji wolnych rodników (Oh i in., 2017). W badaniach Paraskevakis (2015) suplementacja oregano w diecie kóz istotnie ($P < 0,001$) zwiększyła wartość antyoksydacyjną mleka wrażoną jako FRAP. W kolejnych badaniach (Uegaki i in., 2001) wprowadzono suplementację dawki pokarmowej krów rasy holsztyńskiej przez 14 dni trzema ziołami, tj. trawa cytrynowa, mięta pieprzowa i bazylia. We wszystkich przypadkach zanotowano istotny wzrost aktywności przeciwutleniającej mleka w porównaniu z kontrolą. Również w badaniach Kuczyńskiej i in., (2018) po wprowadzenie ziół wykazano prawie 3-krotny wzrost potencjału antyoksydacyjnego mleka (TAS). Z kolei w badaniach Qingru i in., (2007) po wprowadzeniu chińskiej formuły ziołowej w żywieni krów, wzrosła o ponad 40% ($P<0,01$) całkowita pojemność antyoksydacyjna pozyskiwanego mleka. Zhang i Zhao (2022) wykazali, że włączenie wyciągu z ziół chińskich na poziomie 400 i 600 mg/kg do dawki pokarmowe krów

może być korzystne dla produkcji mleka z uwagi na poprawę stanu zdrowotnego zwierząt oraz zwiększenie aktywności przeciwutleniającej.

Pizzoferrato i in. (2007) w celu określenia jakości produktu zaproponował wyliczenie stopnia ochrony antyoksydacyjnej (DAP), jako stosunek molowy między związkami przeciwutleniającymi a utleniaczami. Mleko od krów otrzymujących paszę z dodatkiem mieszanek ziołowych w porównaniu do kontroli wykazywało znacznie wyższy stopień ochrony antyoksydacyjnej, co wskazuje, że zioła są bogatym źródłem przeciwutleniaczy, które chronią cholesterol przed reakcjami oksydacyjnymi. Zdaniem Puppel i in. (2017) DAP i TAS powinny być traktowane jako biomarkery zmian antyoksydacyjnych mleka. Wyższy poziom zarówno stopnia ochrony antyoksydacyjnej, jak i całkowitego potencjału antyoksydacyjnego zapewniają lepszą stabilność i jakość produktu.

4.3. Ocena możliwości wykorzystania mleka pozyskanego od krów żywionych paszą z dodatkiem ziół do produkcji jogurtów o podwyższonym potencjale antyoksydacyjnym z uwzględnieniem 21-dniowego czasu przechowywania

Obecnie coraz więcej konsumentów zwraca uwagę na jakość spożywanych produktów, szczególnie na ich walory prozdrowotne. Coraz większym zainteresowaniem cieszą się mleczne produkty fermentowane, głównie jogurty (Brodziak i in., 2020; Granato i in., 2018; Aryana i Olson, 2017). Pomimo, że dobroczynne właściwości jogurtu znane są od dawna, naukowcy ciągle starają się poprawić jego właściwości funkcjonalne i dostarczyć nowych, atrakcyjnych dla konsumentów produktów na bazie jogurtu. Podejmowane są badania mające na celu poprawę aktywności przeciwutleniającej wytwarzanych produktów przy jednoczesnym zachowaniu odpowiedniego profilu smakowo-zapachowego (El-Sayed i Youssef, 2019; Fardet i Rock, 2018). Liczne badania wskazują, iż wartość statusu antyoksydacyjnego produktów mlecznych można modyfikować poprzez zastosowanie w procesie produkcji naturalnych surowców roślinnych (owoce, warzywa, zioła), bogatych w związki fenolowe i karotenoidy (Walkenhorst i in., 2020; El-Sayed i Youssef, 2019). Dodatkowo stosowanie tych dodatków wpływa na cechy sensoryczne produktu finalnego, a także przedłuża ich okres trwałości, opóźniając proces utleniania lipidów podczas ich chłodniczego przechowywania (Walkenhorst i in., 2020; Radzikowski i in., 2020; El-Sayed i Youssef, 2019).

We wcześniejszych badaniach (doświadczenie II – publikacja 3) wykazano, że po zastosowaniu dodatku mieszanki ziół w dawce pokarmowej krów rasy holsztyńsko-fryzyjskiej istotnie zwiększył się potencjał antyoksydacyjny mleka, co z punktu widzenia żywieniowego, wydaje się mieć szczególne znaczenie dla ochrony organizmu przed szkodliwym działaniem stresu oksydacyjnego. Wychodząc naprzeciw oczekiwaniom współczesnych konsumentów podjęto badania mające na celu ocenę możliwości wykorzystania mleka pozyskiwanego od krów żywionych paszą z dodatkiem ziół do produkcji jogurtów o podwyższonym potencjale antyoksydacyjnym z uwzględnieniem 21-dniowego czasu przechowywania (doświadczenie III - publikacja 4). Niezależnie od grupy doświadczalnej oraz użytej metody badawczej jogurty charakteryzowały się wyższą aktywnością antyoksydacyjną niż mleko zbiorcze. Warto zwrócić uwagę, że w przypadku jogurtów z grupy kontrolnej (CY) w porównaniu do mleka z tej grupy (CM) ponad dwukrotnie (przy $p \leq 0.01$) zwiększyła się aktywność zmiatania rodników DPPH (z 1,14 do 2,52 mg Trolox/100 ml). Aktywność oznaczona testami FRAP i ABTS wzrosła o około 60-70%. Podobne różnice stwierdzono dla grupy doświadczalnej, jogurty EY wykazywały istotnie ($p \leq 0.01$) wyższe wartości potencjału antyoksydacyjnego, niż mleko z tej grupy (EM). W wielu badaniach (Cais-Sokolińska i Walkowiak-Tomczak, 2021; Yilmaz-Ersan i in., 2018; Sabokbar i in., 2015) stwierdzono, że fermentacja mlekowa ma pozytywny wpływ na aktywność antyoksydacyjną wytwarzanych produktów. Podczas fermentacji mleka uwalniane są bowiem peptydy i wolne aminokwasy o różnej aktywności biologicznej, które zwiększają zdolność antyoksydacyjną produktów i hamują peroksydację lipidów (Aloğlu i Öner, 2011). Niemniej jednak, wykazano że zastosowanie mieszanki ziołowej w żywieniu prowadzi do polepszenia wartości antyoksydacyjnej mleka, jak i jogurtów wytwarzanych na jego bazie. Wcześniejsze badania autorów (Stobiecka i in., 2023) wykazały, że zastosowanie dodatku mieszanki ziół w dawce pokarmowej krów spowodowało znaczny wzrost zawartości składników bioaktywnych o właściwościach przeciwutleniających w mleku, tj. białek serwatkowych (β-laktoglobuliny, laktoferyny) oraz witamin lipofilowych (A, E). Zwiększyła się również zdolność antyoksydacyjna mleka. Wyniki badań innych autorów (El-Sayed i Youssef, 2019; Walkenhorst i in., 2020; Radzikowski i in., 2020) również wskazują, iż zastosowanie mieszanek ziołowych w żywieniu krów prowadzi do polepszenia wartości odżywczej mleka, w tym zwiększenia zawartości składników bioaktywnych (nienasyconych kwasów tłuszczowych, białek serwatkowych, witamin) w mleku i produktach mlecznych, a w konsekwencji wzrostu ich potencjału antyoksydacyjnego. Niezależnie od zastosowanej metody badawczej (FRAP,

DPPH, ABTS) status antyoksydacyjny jogurtów wytworzonych na bazie mleka ziołowego (EY) był o około 30% wyższy ($p \leq 0.01$) w porównaniu do grupy kontrolnej (CY). Wyższa aktywność przeciwutleniająca jogurtów EY w stosunku do CY jest związana prawdopodobnie z wprowadzeniem wraz z mieszanką ziół naturalnych przeciwutleniaczy, tj. związków fenolowych. Ekstrakty roślinne mogą przyczyniać się do wzrostu endogennych przeciwutleniaczy i redukcji wolnych rodników (Oh i in., 2017). Podczas przechowywania jogurtów w trakcie dwóch pierwszych tygodni (do 14 dnia) odnotowano istotny wzrost ich aktywności antyoksydacyjnej. W porównaniu do terminu "0" aktywność jogurtów EY oznaczona testami DPPH i ABTS wzrosła o około 30%, tj. odpowiednio z 2.85 do 3.62 mg Trolox/100 ml i z 5.97A do 7.86 mg Trolox/100 ml. O 15% zwiększyła się również zdolność redukowania jonów żelaza (FRAP), z 21.57 do 24.32 mg Trolox/100 ml. Dla jogurtów CY zmiany kształtowały się na podobnym poziomie. W kolejnym tygodniu przechowywania jogurtów zanotowano spadek ich potencjału antyoksydacyjnego. W 21 dniu jogurty EY charakteryzowały się istotnie niższą ($p \leq 0.01$) o około 15% zdolnością zmiatania wolnych rodników (test ABTS i DPPH), jak również zdolnością chelatowania jonów żelaza (FRAP) w porównaniu do dnia 14. W przypadku jogurtów CY spadek aktywności antyoksydacyjnej był wyższy i kształtował się na poziomie 20%. W literaturze przedmiotu nie zaleziono badań dotyczących wykorzystania mleka pozyskiwanego od krów żywionych paszą z dodatkiem ziół do produkcji jogurtów. Dostępna jest natomiast liczna literatura (Shahein i in., 2022; Rashwan i in., 2022; Ribeiro i in., 2021; Anuyahong i in., 2020) dotycząca zastosowania różnych dodatków w produkcji jogurtów, jako naturalnego źródła przeciwutleniaczy. Wykazano, że jogurty z dodatkami wykazują wyższą aktywność przeciwutleniającą w porównaniu z naturalnymi (kontrola) przez cały okres przechowywania (Muniandy i in., 2016; Jung i in., 2016; Martins i in., 2014). W badaniach własnych to właśnie jogurty EY charakteryzowały się wyższą aktywnością przeciwutleniającą w porównaniu z kontrolą CY przez 21 dni przechowywania. Shori i Baba (2013) stosując jako dodatek ekstrakt z liści Neem (*Azadirachta indica*) zanotowali wzrost aktywności wychwytywania wolnych rodników (DPPH) w wytwarzanych jogurtach w porównaniu z tradycyjnymi do 14 dnia, po czym wartości spadały. W innych badaniach Shori (2020) jogurty z dodatkiem wodnego ekstraktu z rozmarynu, koperku i oregano charakteryzowały się istotnie wyższą ($p < 0,05$) aktywnością (odpowiednio: 61.15±1.2; 58.92±1.3; 66.97±0.7 μg GAE/ml) w porównaniu z kontrolą (34.79±1.0 μg GAE/ml). W trakcie 21-dniowego przechowywania wartości istotnie zmniejszały się ($p < 0,05$) zarówno dla jogurtów ziołowych, jak i kontrolnych (Shori, 2020). Atwaa i in., (2022) stwierdzili, że aktywność przeciwutleniająca jogurtów

z dodatkiem wodnego roztworu nasion kopru włoskiego istotnie wzrosła ($p < 0.05$) w porównaniu do jogurtu naturalnego. Natomiast w badaniach Amirdivani i Baba (2011) jogurty ziołowe charakteryzowały się wyższą ($p < 0.05$) aktywnością przeciwutleniającą (DPPH), niż naturalne przez cały okres przechowywania, przy czym po 7. dniu przechowywania aktywność stopniowo zmniejszała się do 28. dnia przechowywania. Yilmaz-Ersan i in., (2018) podczas przechowywania kefirów z mleka krowiego również zanotowali istotny wzrost wartości DPPH i ABTS w pierwszych dwóch tygodniach, natomiast spadek w trzecim tygodniu przechowywania. Lisak Jakopović i in., (2022) wyższe ($p < 0,001$) wartości aktywności przeciwutleniającej FRAP zanotowali po wzbogaceniu jogurtów owocami moringa. Podczas 28 dni przechowywania, w pierwszych dwóch tygodniach stwierdzono istotny wzrost wartości FRAP, natomiast w kolejnych spadek. Liczni autorzy sugerują, że wysoka stabilność oksydacyjna jogurtu podczas pierwszych tygodni przechowywania jest związana z peptydami antyoksydacyjnymi uwalnianymi podczas fermentacji mleka (Stobiecka i in., 2022; Fardet i Rock, 2018; Muniandy i in., 2016).

Wnioski

1. Wraz z kolejną laktacją istotnie zmniejszała się zawartość składników wykazujących właściwości antyoksydacyjne, tj. witamin A i E oraz albumin (β-LG i α-LA). Zanotowano jednocześnie istotny ($p \leq 0,01$) spadek poziomu potencjału antyoksydacyjnego mleka.
2. Uzyskano wysokie wartości współczynników korelacji pomiędzy poziomem potencjału antyoksydacyjnego a zawartością witaminy A ($r=0,687$) i E ($r=0,664$) oraz β-LG ($r=0,515$), co wskazuje, ze zawartość tych związków w dużym decyduje stopniu o potencjale antyoksydacyjnym mleka.
3. Wysoka produkcyjność krów negatywnie wpływa na wartość antyoksydacyjną mleka, gdyż zanotowano ujemne korelacje ($r=-0,317$) pomiędzy potencjałem antyoksydacyjnym a wydajnością dobową mleka.
4. Mleko od krów otrzymujących dodatek mieszanki ziołowej odznaczało się wyższym poziomem związków bioaktywnych, tj. wybranych białek serwatkowych (β-LG, laktoferyny) i witamin lipofilnych (A, E), co przełożyło się na wzrost poziomu potencjału antyoksydacyjnego tego mleka.
5. Mleko pozyskane od zwierząt skarmianych paszą z dodatkiem ziół charakteryzowało się istotnie wyższym stopniem ochrony antyoksydacyjnej (DAP).
6. Jogurty w porównaniu do mleka charakteryzowały się wyższym potencjałem antyoksydacyjnym, przy czym wytworzone na bazie mleka „ziołowego” odznaczały się wyższą aktywnością przeciwutleniającą w porównaniu z jogurtami wytworzonymi na bazie mleka „kontrolnego” podczas 21 dni przechowywania.
7. Zastosowanie dodatków ziołowych w żywieniu krów zwiększyło potencjał przeciwutleniający mleka i wytwarzanych na jego bazie jogurtów, co z punktu widzenia żywieniowego, wydaje się mieć szczególne znaczenie dla ochrony organizmu przed szkodliwym działaniem stresu oksydacyjnego.

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A. Brodziak

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animals

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Review

Antioxidant Activity of Milk and Dairy Products

Magdalena Stobiecka, Jolanta Król * and Aneta Brodziak

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Simple Summary: Consumption of food products that are rich in natural antioxidants improves the antioxidant status of an organism through protection against oxidative stress and damage. Milk and dairy products (yogurt and cheese) accounting for approximately 25–30% of the average human diet are undoubtedly a rich source of compounds exhibiting antioxidant properties. The aim of the study was to present a review of literature data on the antioxidant potential of raw milk and dairy products (milk, fermented products, and cheese) and the possibility to modify its level at the milk production and processing stage. The antioxidant capacity of milk and dairy products is mainly related to the presence of sulfur amino acids, whey proteins (especially β-lactoglobulin), vitamins A, E, and C, or β-carotene. The processes of fermentation or cheese maturation are associated with the release of bioactive peptides, which are responsible for the level of the antioxidant status of the product. The use of probiotic strains significantly enhances the antioxidant status. The antioxidant status of milk and dairy products can be modified with the use of natural additives in animal nutrition or at the stage of milk processing. Herbal mixtures, seeds, fruits, and waste from the fruit and vegetable industry are used most commonly. It is worth emphasizing that regular consumption of natural dairy antioxidants minimizes the risk of development of civilization diseases (e.g., cardiovascular disease, cancer, or diabetes). It also slows down the aging process in the organism.

Abstract: The aim of the study was to present a review of literature data on the antioxidant potential of raw milk and dairy products (milk, fermented products, and cheese) and the possibility to modify its level at the milk production and processing stage. Based on the available reports, it can be concluded that the consumption of products that are a rich source of bioactive components improves the antioxidant status of the organism and reduces the risk of development of many civilization diseases. Milk and dairy products are undoubtedly rich sources of antioxidant compounds. Various methods, in particular, ABTS, FRAP, and DPPH assays, are used for the measurement of the overall antioxidant activity of milk and dairy products. Research indicates differences in the total antioxidant capacity of milk between animal species, which result from the differences in the chemical compositions of their milk. The content of antioxidant components in milk and the antioxidant potential can be modified through animal nutrition (e.g., supplementation of animal diets with various natural additives (herbal mixtures, waste from fruit and vegetable processing)). The antioxidant potential of dairy products is associated with the quality of the raw material as well as the bacterial cultures and natural plant additives used. Antioxidant peptides released during milk fermentation increase the antioxidant capacity of dairy products, and the use of probiotic strains contributes its enhancement. Investigations have shown that the antioxidant activity of dairy products can be enhanced by the addition of plant raw materials or their extracts in the production process. Natural plant additives should therefore be widely used in animal nutrition or as functional additives to dairy products.

Keywords: milk; dairy products; bioactive compounds; bioactive peptides; total antioxidant capacity

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1. Introduction

Large amounts of oxygen free radicals are produced in the human organism through natural physiological processes and contact with the external environment as well as an inappropriate diet. It should be emphasized that not only exogenous but also endogenous sources of reactive molecules are very important because they lead to an increase in the number of molecules. As a result, there is a necessity of their reduction by an organism. In an aerobic organism, 5–10% of oxygen consumed with a high-fat and high-protein diet, contaminated food, and ultraviolet irradiation is converted into free radicals [1]. In conditions of normal metabolism, the generated free radicals are neutralized by the antioxidant system of the organism. Metabolic disorders lead to disturbances in the balance between free radicals and antioxidant reactions, which results in the accumulation of an excessive amount of free radicals in cells. Their excess in the organism associated with the imbalance between active oxygen species and antioxidant substances is referred to as oxidative stress. The excess of these molecules leads to substantial damage to proteins, lipids, and nucleic acids and, consequently, can be detrimental to the human organism. They lead to the development of tumors, neurodegenerative and neoplastic diseases, and disorders in the circulatory or nervous systems and accelerate degenerative processes [2–6]. As part of the defense against changes caused by reactive oxygen species, organisms have developed many mechanisms of prevention of excessive generation of these molecules and modification thereof into inactive derivatives. These mechanisms are based on both exogenous and endogenous compounds, which constitute a complex antioxidant system with nonenzymatic and enzymatic properties. The enzymatic antioxidant barrier consists of specialized enzymes (e.g., catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and glutathione reductase) [7]. The second line of defense includes nonenzymatic antioxidants supplied to the organism with food [2,8,9]. Food antioxidants are compounds that "scavenge" free radicals through various mechanisms. They directly neutralize free radicals generated during the oxidation process, reduce peroxide concentrations, repair oxidized membranes, bind iron to reduce the production of reactive oxygen species, or neutralize ROS via the metabolism of short-chain fatty acids and free cholesterol esters [9,10]. High consumption of nutritional antioxidants protects against the risk of lifestyle diseases (e.g., cardiovascular diseases, cancer, diabetes, and obesity) [3]. It also contributes to an increase in the overall resistance of the organism to infections. An appropriate level of antioxidants in the organism is also important in the prevention of brain dysfunction. Many studies [4,6] have shown a lower incidence of such neurological diseases as cerebral ischemia, Parkinson's disease, and Alzheimer's disease in groups receiving antioxidant supplementation. The antioxidant capacity of milk and dairy products is the result of a complex balance between antioxidants and oxidants [11,12]. Oxidation processes exert a negative impact on milk quality (i.e., they shorten the shelf life and deteriorate the taste (appearance of an unpleasant aftertaste) and nutritional quality of milk) [13]. However, protein oxidation occurs independently of lipid oxidation. As shown by Havemose et al. [14], an elevated concentration of antioxidants is able to prolong the delayed phase of protein oxidation, thereby limiting the formation of dityrosine. Therefore, to improve milk properties, it is necessary to increase the level of bioactive ingredients with antioxidant properties. Antioxidant compounds play an important role in supporting and strengthening the defense mechanisms in the organism, which is useful for the prevention of some lifestyle diseases. Unfortunately, it has been proved that some synthetic antioxidants pose a potential threat in vivo [15]. Therefore, antioxidant compounds originating from natural sources are extremely valuable. One of the food sources of antioxidants is milk and dairy products. In regions and countries where milk consumption is high (North America, Australia, Europe, Argentina, and Pakistan) and amounts >150 kg/capita/year [16], milk and dairy products account for 25–30% of the average human diet [17]. These components are present in the protein (β-lactoglobulin (β-LG), lactoferrin (LF)), fat (vitamins E, A, β-carotene), and water (vitamin C, microelements: Sn, Zn, Fe, Mn) fractions [9,18–21].

The aim of the study was to present a review of literature data on the antioxidant potential of raw milk and dairy products (milk, fermented products, and cheese) and the possibility to modify its level at the milk production and processing stage.

2. Selected Nonenzymatic Antioxidants

The antioxidant potential of milk is directly associated with a content of components exhibiting antioxidant properties. Many authors emphasize that milk from dairy animals contains both enzymatic and nonenzymatic antioxidants, which are crucial in the prevention of the production of reactive oxygen species and help to strengthen the organism defense mechanism against oxidative stress [7].

2.1. Milk Proteins

Proteins are essential nutrients required for proper functioning of the human organism, providing all essential amino acids. Casein and whey proteins are the main proteins in milk. Casein accounts for approximately 80% of the total protein in cow milk. It is present mainly as micelles in macromolecular complexes. Whey proteins (i.e., α-lactalbumin (α-LA), β-LG, LF, immunoglobulins, serum albumin, and glycomacropeptides) constitute approximately 20% of milk proteins [22,23]. β-LG accounting for 50–55% is the main component of whey proteins [24,25]. It should be emphasized that human milk does not contain β-LG [26,27]. The antioxidant properties of proteins are primarily associated with their amino acid composition. Amino acids can act as antioxidants, mainly by limitation of the activity of their sulfhydryl groups (cysteine and methionine) or donation of aromatic residues (tryptophan, tyrosine, and phenylalanine). Moreover, the proper position of amino acids in protein sequences plays an important role in the antioxidant activity of proteins [28]. Whey proteins, in particular β-LG, are characterized by the highest antioxidant potential of all proteins from other food products. This is related to the high content of sulfur amino acids, especially cysteine, which is essential for glutathione synthesis [29,30]. In addition to antioxidant activity, β-LG hydrolysates have antihypertensive, antibacterial, and opioid properties [31,32]. LF has antioxidant activity as well. It was first identified in 1939 as a protein with high affinity for iron [33]. LF chelates iron, which increases its bioavailability and inhibits pro-oxidant effects. It suppresses the inflammatory response, increases the cytotoxicity of natural killer cells in vitro, and inhibits the release of ROS by leukocytes in inflammation sites [8,34]. However, the antioxidant capacity of LF decreases proportionally to saturation with iron [35]. LF stimulates the growth of the bacterial microflora by promoting the growth of selected probiotic strains. As suggested by Claeys et al. [36], LF can be completely inactivated only by thermal treatment of milk at 85 °C for 30 min.

As indicated by Kim et al. [37], in addition to the whey proteins, casein exerts an antioxidant effect as well. With their antioxidant properties, α-casein and β-LG can mitigate aging-related damage induced by oxidative stress through inhibition of cell aging and enhancement of differentiation and maturation of myoblasts. Similarly, the β-casein fraction exhibits high antioxidant activity due to the presence of proline residues. As shown by the comparison of the total antioxidant capacity of skimmed milk, casein, β-LG, α-LA, and various protein-milk mixtures reported by Cekic et al. [38], milk proteins (mainly β-LG and casein) are largely responsible for the total antioxidant capacity of milk. Many authors [26,39–41] indicated the effect of milk heat treatment on the antioxidant activity of dairy products, which is discussed in Section 5.

2.2. Bioactive Peptides with Antioxidant Properties

Proteins and their fractions are valuable sources of bioactive peptides exerting a positive effect on the functioning of the human organism [16,42]. Bioactive peptides have been classified as specific protein fragments with a positive effect on the organism [43]. The release of bioactive peptides from milk proteins in the gastrointestinal tract is a result of the action of such digestive enzymes as pepsin or pancreatic enzymes (trypsin, chymotrypsin, carboxy-, and aminopeptidases) [44,45] or milk processing with the use

of starter cultures [46]. Currently, milk proteins are regarded as an important source of bioactive peptides, which are being increasingly identified in milk protein hydrolyzates and fermented dairy products [44,47,48]. Bioactive peptides are widely used due to their numerous health benefits (e.g., antioxidant activity). Their numerous antioxidant effects are the basis for the production of functional foods, nutraceuticals, and drugs of natural origin [49]. Milk and dairy products are a source of peptides with antioxidant properties, as shown in Table 1. These peptides have the ability to scavenge free radicals, chelate metal ions, and inhibit lipid peroxidation [5,8,22]. Numerous studies [1,5,22] have reported an interaction between the amino acid composition of peptides and antioxidant activity. Peptides usually consist of 5–11 amino acid residues, including hydrophobic ones (proline, histidine, tyrosine, tryptophan, or cysteine), whose free form also has antioxidant activity [50]. In experiments consisting in the treatment of hydrolysates of whey proteins (α-LA and β-LG) with enzymes (pepsin, trypsin, chymotrypsin, thermolysin, and Corolase PP), Hernandez-Ledesma et al. [51] identified 42 peptide fragments with the WYSLAMAASDI sequence exhibiting the highest antioxidant activity. Similarly, antioxidant activity is shown by β-casein and released peptides (e.g., those with the sequences VKEAMAPK, AVPYPQR, KVLPVEK, and VLPVPEK and αs1-casein (e.g., with the YFYPEL sequence)) [52]. Timón et al. [53] identified three peptides exerting a radical scavenging effect in Burgos cheese (i.e., peptides derived from αs1-casein (SDIPNPIGSENSEKTTMPLW) and β-casein (YQQPVLGPVRGPFPIIV and LLYQQPVLGPVRGPFPIIV). A number of antioxidant biopeptides have also been isolated and identified from β-LG hydrolyzed with the use of Corolase PP [54]. It has been shown that bioactive whey peptides, including the Ile-Pro-Ala tripeptide released from β-LG, can be used in the treatment of type 2 diabetes and obesity [55]. Bioactive peptides isolated from milk (VAGTWY) and gouda cheese (LPQNIPP) can lower plasma glucose levels. Sommerer et al. [56] identified 28 small peptides with antioxidant activity from goat cheese, including 26 peptides from casein. Five new antioxidant oligopeptides from goat milk casein were identified by Li et al. [57]. In turn, gupta et al. [58] identified two milk protein peptides with the sequences VKEAMAPK and HIQKEDVPSER from cheddar cheese fermented by Lactobacillus casei sp. casei 300. Peptides consisting of Met, glu, Tyr, Lys, His, Cys, Val, and Pro have potent antioxidant activity [57,59]. As reported by girgih et al. [60], the antioxidant properties of peptides can be enhanced by the presence of Trp, Tyr, and Pro. The researchers showed that the peptides WVYY (Trp-Val-Tyr-Tyr) and PSLPA (Pro-Ser-Leu-Pro-Ala) were the most active antioxidants characterized by 67% and 58% DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) scavenging capacity and 94% and 96% metal chelating activity, respectively. As suggested by Skrzypczak et al. [61], bioactive peptides (VLPVPQK (Val-Leu-Pro-Val-Pro-Gln-Lys) and QKAVPYPQRDMPI (Gln-Lys-Ala-Val-Pro-Tyr-Pro-Gln-Arg-Asp-Met -Pro-Ile)) isolated using Lactobacillus helveticus strains seem to have a high radical scavenging potential. The stability of antioxidant peptides in a simulated gastrointestinal tract was assessed as well [62,63]. Studies showed a slow increase in the rate of free hydroxyl radical scavenging in simulated gastric conditions. This may be attributed to the pepsin-induced decomposition of antioxidant peptides contained in fermented goat milk into smaller peptides with antioxidant properties. As reported by You et al. [63], greater numbers of peptide bonds were broken down in the process of digestion with pancreatin rather than pepsin. Investigations conducted by Woo et al. [64] confirmed that hydrolysis of milk proteins effectively increased their antioxidant activity ABTS test (2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic), with the highest radical scavenging activity recorded in the process of casein digestion with trypsin.

Table 1. Biologically active peptides with antioxidant properties (own work based on: [51,54,57,58,61,65–92]).

Protein Precursors	Fragment	Sequence	References
Casein proteins			
goat milk casein	-	VYPE	[57]
	-	FGGMAH	
	-	FPYCAP	
	-	YVPEPF	
	-	YPPYETY	
Cow milk casein			
CGMP	-	VLPVPQK	[61]
	-	QKAVPYPQRDMPI	
β-casein	1–6	RELEEL	[65]
	7–16	NVPGEIVESL	[66]
	60–68	YPFPGPIN	[67]
	59–63	YGFLP	
	59–68	VYPFPGPIPN	[66]
	84–86	VPP	[68]
	106–123	HKEMPFPKYPVEPFTESQ	[66]
	111–119	FPKYPVEPF	
	114–119	YPVEPF	[67]
	142–154	SWMHQPHQPLPPT	[66]
	166–182	SQSKVLPVPQKAVPYPQ	
	169–176	KVLPVPQK	[69,70]
	170–176	VLPVPQK	
	177–183	AVPYPQR	[71]
	166–175	SQSKVLPVPQ	
	170–175	VLPVPQ	
	176–182	KAVPYPQ	
	183–190	RDMPIQAF	
	178–183	VPYPQR	[72]
	191–193	LLY	[68]
	193–202	YQEPVLGPVR	[73]
	199–209	gPVRGPFPIIV	[74]
	98–105	VKEAMAPK	[58,75,76]
	207–221	QEPVLGPVRGPFPIL	[77,78]
	207–219	QEPVLGPVRGPFP	[78]
	212–219	gPVRGPFP	[51]
	209–220	PVLGPVRGPFPI	[78]
	209–221	PVLGPVRGPFPIL	
	212–220	gPVRGPFPI	[51]
k-casein	24–33	KYIPIQYVLS	[66]
	29–41	QYVLSRYPSYGLN	
	28–30	IQY	[79]
	30–32	YVL	
	51–65	INNQFLPYPYYAKPA	[66]
	66–77	AVRSPAQILQWQ	
	81–95	NTVPAKSCQAQPTTM	
	96–106	ARHPHPHLSFM	
	108–110	IPP	[68]
	115–131	DKTEIPTINTIASGEPT	[66]

Table 1. *Cont.*

Protein Precursors	Fragment	Sequence	References
αS1-casein	1–9	RPKHPIKHQ	[80]
	7–21	KHQGLPQEVLNENLL	[66]
	26–40	APFPEVFGKEKVNEL	[81,82]
	27–35	PFPEVFGKE	[66]
	39–40	EL	[81]
	80–90	HIQKEDVPSER	[58]
	90–94	RYLGY	[82,83]
	90–96	RYLGYLE	[67]
	91–96	YLGYLE	
	92–94	LGY	
	141–143	EL	[81]
	143–149	AYFYPEL	[75,76,81,84,85]
	143–148	AYFYPE	[67]
	144–149	YFYPEL	
	145–149	FYPEL	[81]
	146–149	YPEL	
	148–149	EL	
	176–192	APSFSDIPNPIGSENSE	[66]
αS2-casein	89–95	YQKFQY	[82]
	89–91	YQK	[85]
	92–95	FPQY	
	130–138	NAVPITPTL	[86]
	171–173	YQK	[85]
	174–181	FALPQYLK	[79]
	202–207	PYVRYL	[76,79]
Cow milk whey proteins			
α-LA	19–29	WYSLAMAASDI	[54]
	50–53	YGLF	[67]
	99–108	VGINYWLAHK	[87]
β-LG	15–20	VAGTWY	[88]
	19–29	WYSLAMAASDI	[54,89]
	42–46	YVEEL	
	58–61	LQKW	[90]
	95–101	LDTDYKK	
	72–79	IAEKTKIP	[87]
	84–91	IDALNEK	[71,90]
	92–100	VLVLDTDYK	
	102–105	YLLF	[67]
	145–149	MHIRL	[54,89]
Proteins from human milk (β-casein)	154–160	WSVPQPK	[91,92]

2.3. *Vitamins*

Fat-soluble vitamins, mainly vitamin E, but especially α-tocopherol, as well as vitamin A and β-carotene, are the main antioxidants [93–95]. Their activity consists in organic free radical scavenging and inhibition of lipid peroxidation [96,97]. They also have the ability to quench singlet oxygen and hydroxyl radicals, effectively protecting DNA against oxidation [98]. Since they are present in fat globule envelopes, these vitamins prevent automatic oxidation of milk fat. Similarly, vitamin D3 is part of the nonenzymatic antioxidant system of milk, and 1,25-dihydroxycholecalciferol is its most active form. Its antioxidant effect consists in the inhibition of lipid peroxidation [99,100]. Vitamin D3 is mainly responsible for the regulation of calcium–phosphate metabolism and maintenance of calcium homeostasis in the organism [101,102]. An important antioxidant role in the organism is also played by vitamin C (ascorbic acid), which represents nonenzymatic water-soluble antioxidants.

The concentration of lipophilic vitamins in milk is directly related to animal nutrition. Higher levels of antioxidants (vitamin E, β-carotene, and retinol) were recorded in the milk of grazing cows compared with those fed concentrate- or silage-rich diets [103–107]. As shown by many authors [108,109], fresh pasture sward has a higher level of these vitamins than preserved fodder; therefore, grazing-based nutrition has a positive effect on their content in milk. Milk from grazing animals is also characterized by an increase in the content of vitamin D3 due to their exposure to UV [105,110–113]. Some investigations have shown a close relationship between the contents of β-LG and fat-soluble vitamins. As reported by Dolores-Perez and Calvo [114], the concentration of β-LG is positively correlated with the content of vitamin A, as this protein actively participates in the transport of small hydrophobic molecules (i.e., α-retinol). In turn, Bulgari et al. [115] demonstrated that the AA genotype of β-LG was associated ($p \leq 0.01$) with a higher content of vitamin D3. Studies conducted by other authors [105,112] also indicate relationships between the contents of vitamin A and β-LG in milk. The amounts of vitamin C and lipophilic vitamins in milk decrease in mammary gland inflammation, as they are utilized during oxidation processes [116]. It should be emphasized that these compounds are sensitive to light and temperature, and greater vitamin loss is caused by UV radiation [21,117].

3. Methods for Assessment of the Antioxidant Activity of Milk and Dairy Products

Various methods are used for the determination of the antioxidant activity. They are based on the SET—single electron transfer: FRAP (ferric reducing antioxidant power), ABTS (2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate), and HAT—hydrogen atom transfer: ORAC (oxygen radical absorbance capacity) and TRAP (total radical-trapping antioxidant parameter) mechanisms. In the SET methods, the reaction mixture is composed of an antioxidant and an oxidant; the latter changes color in the reduction reaction (i.e., an electron transfer from the antioxidant to the oxidant). The results obtained with this method are often converted into Trolox equivalents (TEAC—Trolox equivalent antioxidant capacity). Methods based on the mechanism of the hydrogen atom transfer (HAT) reaction are recommended for the measurement of the deactivation of free radicals resulting from the donation of a hydrogen atom by the antioxidant. The antioxidant present in the sample and the model antioxidant with a known concentration, referred to as the "molecular probe", compete with each other for the reaction with the peroxide radical. Each of these methods has a specific mechanism of action (Table 2). The methods are based on the determination of the effect of antioxidants on the rate of oxidation processes taking place in the sample (ORAC and TRAP), reduction of metal ions (e.g., iron (FRAP) or copper CUPRAC (cupric reducing antioxidant capacity)), and the ability to scavenge synthetic radicals (ABTS, DPPH) or measurements of the amount of lipid oxidation products or LDL fractions [118,119]. Three methods are most often used to assess the antioxidant activity of milk and dairy products (i.e., DPPH, ABTS, and FRAP) [120]. The ABTS method is based on 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) cation radical. It is a spectrophotometric method for the determination of the ability of antioxidants to neutralize the blue cation radical generated from ABTS under the influence of sodium persulfate, which is manifested by a decrease in the solution absorbance. The method is widely used due to its simplicity, speed, and sensitivity [121,122]. DPPH is another compound used in the measurement of the reducing ability of antioxidants towards this reagent. This assay measures the loss of DPPH color (deep purple) at 515 nm after reaction with the antioxidant. The percentage of the remaining DPPH is calculated. The DPPH radical scavenging ability is expressed in other units in addition to% inhibition [122,123]. FRAP (ferric reducing antioxidant power) measures the reduction of ferric 2,4,6-tripyridyl-S-triazine (TPTZ) to a colored solution (blue) assessed at 595 nm using a spectrophotometer. FRAP measures reducing power but cannot detect compounds that act via radical quenching (H transfer), particularly thiols and proteins [123]. However, it should be noted that, although there are a number of methods for the assessment of antioxidant properties, their results are not standardized. Unfortunately, there are often discrepancies in results obtained from the same material

analyzed using different methods and even in the case of the same material analyzed with the same method in different research laboratories [120,124].

Table 2. Selected methods for the determination of antioxidant activity (own work based on: [121,122]).

Method	Principle	Observations
DPPH	In the presence of an antioxidant compound, reduction of the purple-colored stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical to yellow 2,2-diphenyl-1-picrylhydrazine	Yellow color of the substance assessed visually or analyzed spectrophotometrically
ABTS	Antioxidants lead to the reduction of the cation radical ABTS $^{+}$ – 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate, causing discoloration of the blue-green solution	Discoloration of the solution assessed visually or analyzed spectrophotometrically
FRAP	Monitoring the antioxidant donor capacity by the measurement of the reduction of the iron(III) complex with 2,4,6-tris(2-pyridyl)-1,3,5-triazine ($[Fe^{3+} - (TPTZ)_2]^{3+}$) to an intense blue complex $[Fe^{2+} - (TPTZ)_2]^{2+}$	Spectrophotometric analysis
ORAC	Antioxidants inhibit free radical-induced oxidation of a fluorescent probe, which shows a decrease in fluorescence during the reaction	Fluorimetric analysis

4. Antioxidant Potential of Raw Milk, Effect of Animal Species, Diet, and Lactation Phase

The antioxidant potential of milk is determined by animal species, diet, and lactation phase.

As shown in some investigations [17,30,125,126], the species of the animal has a significant impact on the content of antioxidant components in milk. Compared with cow milk, which is the main material in the world production, sheep, camel, and buffalo milks contain higher levels of these ingredients, mainly β-LG, LF, vitamins A and E, and polyunsaturated acids. Interspecies differentiation in the total antioxidant capacity of milk determined using various methods has been shown (Table 3). Regardless of the method of determination, raw cow milk has the lowest antioxidant potential. In the assessment of the total antioxidant potential of raw cow and sheep milks using three methods (DPPH, ABTS, FRAP), Yilmaz-Ersan et al. [30] reported a lower antioxidant capacity of raw cow milk, which can be explained by the differences in their chemical compositions. Furthermore, Khan et al. [126] indicated statistically significantly higher values of antioxidant status (expressed as DPPH%) for buffalo milk as compared with cow milk.

Table 3. Total antioxidant capacity (TAC) of raw milk (own work based on: [30,125–130]).

Raw Material	Method				References
	ABTS•+	FRAP	DPPH	ORAC	
Cow milk	21.48 [a]	1.41 [a]	3.14 [a]	-	[30]
	-	-	24.3 [c]	-	[126]
	1033.5 [b]	-	-	667.4 [b]	[127]
			18,89 [c]		[128]
Buffalo milk	7.38 [e]	38.9 [d]	31.5 [c]	-	[129,130]
	-	-	31.8 [c]	-	[126]
			20.11 [c]		[128]
goat milk	6.80 [e]	23.45–26.71	20.86–23.22	-	[125,130]
	74.36	-	19.53–23.57 [c]	-	[131]
			18.17 [c]		[128]
Sheep milk	33.18 [a]	5.82 [a]	8.70 [a]		[30]
	7.78 [e]		27.28 [c]		[128,130]
Camel milk	-	-	18.57 [c]	-	[128]

[a] Results expressed as milligrams of Trolox equivalents (TE) per 100 mL of the sample. [b] Result expressed in μM of Trolox equivalent mg/mL. [c] DPPH%. [d] Result expressed in μmol/L. [e] Result expressed in $FeSO_4$ Eq mg/100 g.

The content of antioxidant components in milk and the value of its antioxidant potential can be modified via animal nutrition (e.g., the use of various natural additives to animal diet). Feed additives used in animal nutrition serve protective functions and act as regulators of metabolism. Consequently, they contribute to an increase in the immunity of animals exposed to stress (weaning, changes in diet, or transport), lead to efficient absorption of essential nutrients, and enhance the antioxidant protection provided by milk [132]. Examples of antioxidant activity of milk after supplementation of feed with natural plant additives are shown in Table 4. grazing significantly increases the content of antioxidant components in milk, thus increasing its antioxidant potential [93,98,105,133]. The improvement is more difficult to achieve when cows are fed preserved fodder, especially silage [134–136]. A higher proportion of maize silage in feed rations for cows is one of the main factors of the lower content of vitamins and antioxidants in milk. Alves et al. [135] demonstrated that maize silage, which is most often used in cow nutrition, has low contents of carotenoids. Pumpkin silage is regarded as a valuable source of bioactive compounds, especially carotenoids and flavonoids [137]. As demonstrated by Halik et al. [138], the addition of pumpkin silage to diet for dairy cows significantly improved the nutritional value of colostrum, including the content of carotenoids. The colostrum antioxidant status was significantly higher as well. Similar results were obtained from studies of milk from cows fed carotenoid-rich diets [139]. Santos et al. [140] reported higher antioxidant activity of milk from cows receiving grape pomace silage in their diet. In turn, the supplementation of cow diet with synthetic and natural β-carotene had no effect on its content in milk and TAS value. However, the natural β-carotene exhibited higher availability than the synthetic compound [141,142]. Delgado-Pertíñez et al. [143] used dried orange pulp (DOP) (i.e., a waste from orange juice production) as an alternative component of ruminant diet in goat nutrition. It was shown that the addition of DOP to the feed ration significantly improved the health-enhancing value of milk and increased the level of vitamin E, phenolic compounds, and milk antioxidant capacity (ABTS). grape pulp (GP) (i.e., the main by-product in wine production and a rich source of bioactive compounds) was used in animal nutrition as well. The addition of gP naturally enriched the feed with polyphenols and dietary fiber. An increase in the concentration of antioxidant bioactive compounds in milk was mainly observed. In turn, Chedea et al. [144] and Ianni et al. [145] reported no effect of a diet supplemented with gP on the health condition of cows and the chemical composition of milk. Herbs and spices are used as natural feed additives in animal nutrition. With their high content of biologically active substances (flavonoids, saponins, carotenoids, plant sterols, glucosinolates, or essential oils), they exert a positive effect on the animal organism, dairy production, and milk quality [146,147]. Importantly, spices and herbs are a natural source of antioxidants [120]. Most antioxidants contained in spices and herbs react with free radicals generated in the initial stage of autoxidation. Due to their antioxidant properties, rosemary, thyme, anise, buckwheat, black pepper, cinnamon, garlic, fenugreek, savory, and mint are used in animal nutrition most frequently [147–150]. Supplementation with 2% of a herbal mixture of yarrow, chamomile, nettle, turnip rape, plantain, and lady's mantle had a positive effect on the health of the mammary gland in cows and on the nutritional value of their milk [151]. It has been shown that the fat content in milk has an impact on its antioxidant potential. Fat-rich raw milk has a higher antioxidant value than reduced-fat milk due to the lower content of lipophilic antioxidants in fat-soluble vitamins [12,152]. Chen et al. [153] reported similar findings. They determined the total antioxidant capacity of milk using the ABTS test and showed that cow milk containing 3% of fat exhibited a substantially higher antioxidant capacity than milk with lower fat content (0.5–1.5%) or skimmed milk. Additionally, the authors revealed positive correlations between the fat content and the antioxidant capacity of milk. As reported by Puppel et al. [154,155], the antioxidant capacity of milk may be related to both the dietary supplementation and the age of cows. The modification of diets for multiparous and primiparous cows with fish oil and linseed significantly influenced the antioxidant properties of their milk. In both groups, the supplementation contributed to an increase in the total antioxidant status, with higher

values recorded in the milk from the primiparous cows. Other studies conducted by these authors [133] demonstrated that supplementation of the basic diet with maize grain improved the antioxidant capacity and antioxidant protection of milk by increasing the content of vitamin E in milk. It was also shown that addition of cow milk to green and black tea (50 mL of tea mixed with 50 mL of milk) resulted in an approximately 2.1-fold increase in their antioxidant potential [156].

The various physiological processes taking place during lactation are associated with the generation of reactive oxygen species. The animal organism utilizes antioxidants to reduce free radicals [9]. Mann et al. [71] underlined the effect of the lactation phase on changes in the antioxidant status of milk. The antioxidant capacity of milk from different breeds of cows (Sahiwal, Karan Fries, Holstein Friesian) was determined as the ability to reduce iron ions (FRAP test) and the free radical scavenging activity (DPPH test). Higher values were noted in colostrum and milk in the early stage of lactation (5–15 days). With time, the values of the antioxidant potential declined significantly. The authors suggest that higher levels of antioxidants in colostrum may be critical for the protection of the health of neonatal animals against oxidative stress. Annie et al. [157] measured the total antioxidant capacity (FRAP method) of milk produced by Vechur cows (native Kerala breed) and Malabari goats in different stages of lactation. It was shown that the antioxidant potential of milk changed significantly ($p < 0.01$) along the lactation period, with the highest potential noted in the early stage of lactation (5–15 days) compared with mid (90–120 days) and late (>150 days) lactation. As shown by Kapusta et al. [158,159], there is a clear relationship between the lactation phase and the level of enzymatic and nonenzymatic antioxidants in milk in high-yielding PHF cows. The highest total antioxidant status (TAS) in milk was demonstrated on the first days of lactation ($\geq$8), but its level was found to decrease gradually on the subsequent days.

Table 4. Antioxidant activity of milk after supplementation of feed with natural plant additives (own work based on: [140,160–170]).

Animal	Additives to the Diet	Method	Antioxidant Activity	References
Cow	Control diet (without black rice and purple corn extracted residue)	DPPH	6.96%	[160]
	2% black rice and purple corn extracted residue		7.68%	
	4% black rice and purple corn extracted residue		9.76%	
	6% black rice and purple corn extracted residue		9.27%	
	grape pomace extract	Folin–Ciocalteu	16.07 gAE mg/g	[161]
	Raw mulberry cultivars (*Yuesang 11*)	DPPH	146.04 mg of TE/g of DM	[162]
		ABTS	21.85 mg of TE/g of DM	
		FRAP	52.71 mg of TE/g of DM	
	Raw mulberry cultivars (*Chengxiansang*)	DPPH	147.78 mg of TE/g of DM	
		ABTS	19.62 mg of TE/g of DM	
		FRAP	44.71 mg of TE/g of DM	
	1% grape seed and grape marc meal extract	ABTS	283 µmol/L	[163]
	grape residue silage	Reducing power	44.6 mg gAE 1^{-1}	[140]

Table 4. *Cont.*

Animal	Additives to the Diet	Method	Antioxidant Activity	References
Sheep	Control diet	ORAC	197.09 µmol eq. Trolox/g DM	[164]
	5% *Tithonia tubiformis*		201.94 µmol eq. Trolox/g DM	
	5% *Cosmos bipinnatus*		222.79 µmol eq. Trolox/g DM	
	5% *Tagetes lucida*		255.76 µmol eq. Trolox/g DM	
	Control diet	ABTS	50.97 %	[165]
	100 g/day per head of tomato pomace		51.09%	
	100 g/day per head of grape marc		48.01%	
	75 g/day per head of exhausted myrtle berries		52.32%	
	Control diet	ABTS	71.07%	[166]
	150 mg orange peel essential oil/kg concentrate		67.79%	
	300 mg orange peel essential oil/kg concentrate		70.05%	
	450 mg orange peel essential oil/kg concentrate		76.03%	
	Control diet	FRAP	3.18%	
	150 mg orange peel essential oil/kg concentrate		3.42%	
	300 mg orange peel essential oil/kg concentrate		2.54%	
	450 mg orange peel essential oil/kg concentrate		2.99%	
	Control diet	Commercial ELISA kits	14.71 U/mL	[167]
	50 mg cinnamaldehyde, eugenol, and capsicum oleoresin/kg of diet		20.32 U/mL	
	80 mg cinnamaldehyde, eugenol, and capsicum oleoresin/kg of diet		18.17 U/mL	
goat	Fed sticky corn	DPPH	19.10%	[168]
	Anthocyanin-rich purple corn		21.58%	
	Control diet	FRAP	1.13 mmol/L	[169]
	6% date palm (*Phoenix dactylifera* L.) seed		1.43 mmol/L	
	12% date palm (*Phoenix dactylifera* L.) seed		1.45 mmol/L	
	18% date palm (*Phoenix dactylifera* L.) seed		1.59 mmol/L	
Yak	0 g/kg of astragalus root extract	Commercial ELISA kits	7.23 U/mL	[170]
	20 g/kg of astragalus root extract		7.87 U/mL	
	50 g/kg of astragalus root extract		8.04 U/mL	
	80 g/kg of astragalus root extract		8.20 U/mL	

5. Antioxidant Potential of Thermally Treated Milk

A number of studies have reported changes in the antioxidant capacity of milk subjected to thermal treatment [30,128,171]. Various reactions occur between milk compounds during thermal treatment (e.g., denaturation and aggregation of whey proteins and formation of new complexes). It is noteworthy that many studies indicate that β-LG is the most thermally unstable protein and easily undergoes thermal denaturation [26,39–41]. Investigations conducted by Liu et al. [26] confirmed this thesis, as it was shown that β-LG-free milk had approximately 50% lower antioxidant activity than skimmed milk. Thermal treatment (100 °C for 2 min) resulted in the loss of antioxidant activity of β-LG due to the blocking of thiol groups. Brodziak et al. [40] showed a statistically significant ($p \leq 0.05$ and $p \leq 0.01$) effect of the type of heat treatment on the content of undenatured whey proteins. UHT (ultra-high-temperature) milk was found to contain severalfold lower levels of β-LG, α-LA, and LF than ESL (extended shelf life) and VHT (very-high-temperature) milk. Similarly, Sakkas et al. [39] reported that peroxidase-positive HTST (high-temperature short-time) milk contained >3 g/L of undenatured β-LG. At higher heating temperatures, they recorded a successive decline in the content of undenatured proteins in milk, especially β-LG (90 °C—1132 mg/L; 100 °C—404 mg/L; 130 °C—57 mg/L). Similar findings were reported by Hammershøj et al. [41]. They achieved a low β-LG denaturation degree of 2–6% in the HTST pasteurization treatment (72 °C/15 s), whereas 30% β-LG denaturation was noted at higher HTLT temperatures (85 °C/30 s).

Processes to which milk proteins with antioxidant potential are subjected affect the total antioxidant capacity of milk. Heat treatment (temperature, time) of raw milk may inhibit or reinforce the formation of antioxidative compounds in the final product. Ertan et al. [171] determined the total antioxidant capacity of pasteurized and UHT milks

using the ABTS and Folin–Ciocalteu methods. They showed higher antioxidant capacity of pasteurized milk determined with both methods than that of UHT milk. Additionally, the total antioxidant capacity of milk was found to increase with the increasing milk fat content. Unal [172] also showed the highest antioxidant activity in full-fat UHT milk, which may be associated with higher contents of fat-soluble antioxidants. Therefore, it should be clearly stated that reducing milk fat leads to a reduction in fat-soluble vitamins. Yilmaz-Ersan et al. [30] assessed the antioxidant potential of pasteurized milk (90 °C for 10 min) using three methods (DPPH, ABT, and FRAP). The thermal treatment reduced the DPPH value but increased ABTS and FRAP, compared with raw milk. This may be related to, for example, the reducing properties of the milk, which were strongly affected by heating. Furthermore, the thermal treatment was able to increase its pro-oxidative activity through both loss of natural antioxidants and generation of new oxidizing molecules in the early stages of the Maillard reaction [173]. The increase in the antioxidant activity of sterilized milk can be attributed to the Maillard reaction (i.e., a chemical reaction between carbonyl and amino groups) mainly between lactose and lysine residues in milk proteins [174]. On the other hand, there are reports pointing to no significant differences between the values of antioxidant capacity of raw, pasteurized, and sterilized milks [121,175]. In a research by Şanlidere [175], the values of the antioxidant activity (ABTS) were 4.02, 4.47, and 4.18 mM Trolox/g, respectively. It should be emphasized that these values increased substantially during simulated gastrointestinal digestion (11.13, 12.33, and 11.88 mmol TE/g, respectively). Similarly, Cloetens et al. [121] showed no significant differences between the values of the antioxidant potential of UHT and pasteurized milk. Other studies demonstrated that pasteurization had no effect on the antioxidant capacity of milk, compared with raw milk, whereas sterilization enhanced this parameter [128]. During milk heat treatment at temperatures above 100 °C, the antioxidant capacity increases due to protein unfolding and exposure of thiol groups, potentially acting as hydrogen donors [128,173].

6. Antioxidant Potential of Dairy Products

Many studies indicate that the antioxidant potential of dairy products (yogurt, cheese, kefir) is related to the quality of the raw material and primarily the presence and activity of natural bioactive compounds in milk (i.e., amino acids (including tyrosine and cysteine) and vitamins (e.g., A and E)) [12,98]. The bacterial cultures and plant additives used also have a high impact on the value of the antioxidant potential [3,30,176–181]. It has been shown that fermented milk products (yogurt and kefir) and cheese have antioxidant properties and are able to scavenge superoxide, hydroxyl, and peroxide radicals and reactive oxygen species [153,182–188]. Milk fermentation with lactic acid bacteria contributes to the supply of a huge number of bioactive peptides and free amino acids with different biological activities [131,189]. Various investigations indicate that probiotic strains exhibit important antioxidant properties [101]. As reported by Fardet and Rock [12], probiotic yogurts have higher antioxidant activity than conventional dairy products. This is associated with the release of antioxidant peptides by probiotic strains during fermentation, which increase the antioxidant capacity of products and inhibit lipid peroxidation [101,185]. Numerous studies [3,30,190] have shown the importance of the type of probiotic strains used in the production of fermented milk. Fermented products containing *Lactobacillus acidophilus* are characterized by significantly higher antioxidant activity. In a study conducted by gjorgievski et al. [3], milk with 3.2% fat content was fermented by various microbial cultures, including symbiotic *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* and monocultures of *Lactobacillus acidophilus*, *L. casei*, and *Bifidobacterium bifidus*. Compared with the raw material, all microbial cultures increased the antioxidant activity of the fermented product determined with the DPPH method. The highest value was recorded in the case of milk fermented with the probiotic *Lactobacillus acidophilus* strain (54.86%), and the lowest activity was detected in milk fermented with the symbiotic cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (45.18%). Additionally, the authors noted that the antioxidant activity of fermented milk decreased during storage. Vir-

tanen et al. [185] determined the antioxidant activity (using ABTS tests) of milk fermented with 25 strains of lactic acid bacteria (LAB). They showed strain-specific antioxidant activity. The highest radical scavenging activity was exhibited by *Leuconostoc mesenteroides* ssp. *cremoris* strains (A and B), *Lactobacillus acidophilus* (ATCC 4356), and *Lactobacillus jensenii* (ATCC 25258), which was associated with the protein proteolysis process. Products with high scavenging activity were shown to have higher amounts of peptides in the molecular weight range of 4–20 kDa, while other products were dominated by large polypeptides and compounds below 4 kDa. Moreover, the amount of hydrophobic amino acids in these fermentates was higher. The authors also used combinations of strains in milk fermentation. A significant increase in antioxidant activity was found in the case of combinations containing the *L. acidophilus* strain, with the highest activity noted in the combination of *L. cremoris* B, *L. lactis*, and *L. acidophilus* (TAA 0.86 mmol/L). The Trolox equivalent value was over fivefold lower in the case of the *L. cremoris* B and *L. lactis* combination (TAA 0.16 mmol/L). Milk fermentation is therefore a very good method for the enhancement of the antioxidant activity of products. Concurrently, it is possible to extend the shelf life of dairy products through the inhibition of lipid peroxidation. Skrzypczak et al. [190] conducted studies to identify milk protein solutions (skimmed milk powder, α-LA, caseinoglycomacropeptide) and strains (*L. helveticus* strains B734, 141, T80, and T105; reference strain *L. helveticus* DSMZ 20075) that are desirable for the manufacture of fermented products with the best antioxidant properties. The highest increase in DPPH scavenging activity (% inhibition) was noted for skimmed milk powder solutions fermented by the *L. helveticus* DSMZ 20075 reference strain (85.98%) and *L. helveticus* T80 (81.66%). In the case of α-LA, the strongest free radical scavenging activity (66.67%) was recorded in nonfermented samples [191]. gamba et al. [127] showed an increase in antioxidant activity during kefir production from cow milk. Depending on the assessment method used, it ranged from 667.4 (ORAC) to 1033.5 µmol Trolox/mL (ABTS). In kefir produced from this milk, the antioxidant activity increased to 1403.5 and 1412.2 µmol Trolox/mL, respectively. Additionally, the authors assessed "soymilk" and showed significantly higher antioxidant activity than cow milk, which was ascribed to the higher content of polyphenols and vitamin E in the soy drink. There was no change in the activity after fermentation of kefir produced from soymilk [127]. Fiorda et al. [191] reported that kefir drinks based on both cow milk and soymilk were characterized by higher antioxidant activity than raw material. In turn, Yilmaz-Ersan et al. [192] used DPPH, ABTS, and FRAP tests to assess the antioxidant activity of kefir produced from goat milk and showed its antioxidant stability at various stages of fermentation (20 h, assessment every 4 h) and during 21 days of storage. However, a decrease in the total phenolic content in the samples was noted during both fermentation and storage. In subsequent studies [30], the authors evaluated the impact of using starter cultures (kefir grains and commercial cultures) on the antioxidant capacity of kefir from cow and sheep milk. The antioxidant capacity of the kefir samples during fermentation and on storage day 21 was assessed using three tests: ABTS, DPPH, and FRAP. It was shown that the type of milk (cow or sheep) and culture used significantly differentiated the antioxidant activity of kefir. Sheep milk and kefir drinks made from this type of milk had higher antioxidant activity than cow milk. As suggested by the authors, this should be associated with differences in the compositions of both types of milk. The authors noted fluctuations in the antioxidant activity of kefir during fermentation, probably due to the inhibition of microbial enzymes present in kefir grains activated in the initial stages of fermentation [177]. During kefir maturation, the ABTS, DPPH, and FRAP values varied. The ABTS values increased significantly until storage day 14. gupta et al. [187] reported that the ABTS and DPPH values in the case of cheddar cheese produced with the use of *Lactobacillus casei* ssp. casei 300 and *Lactobacillus paracasei* ssp. *paracasei* increased during the first 4 months of maturation and then significantly decreased. Some natural bioactive components of kefir exhibit a relatively slow rate of free radical scavenging, as large peptides and proteins are slowly hydrolyzed and thus have lower antioxidant activity [181]. As reported by Najgebauer-Lejko and Sady [193], yogurt and kefir have the highest antioxidant activity in comparison with other fermented products

available on the market. The combination of milk proteins, mainly casein and β-LG, and polyphenols may increase the antioxidant potential of fermented dairy products, which can thus become new functional foods. The presence of probiotic strains, such as *Lactobacillus casei* or *acidophilus*, enhances the antioxidant activity of yogurt as well.

Cheese is regarded as the main source of bioactive peptides due to the high protein content, the variety of proteolytic enzymes, and the degree of proteolysis during cheese ripening [194–196]. The antioxidant potential of milk increases during digestion even 2.5 times, which is associated with the release of antioxidant peptides [12]. Oner and Sardag [80] assessed the peptide profile and antioxidant activity of kashar cheese aged for 3 months. After 90 days of ripening, the cheese exhibited significantly higher antioxidant activity and a greater number of peptide peaks. Changes in antioxidant activity occurring during the maturation of cheddar cheese were reported by gupta et al. [187]. Cheddar cheese was prepared with *Lactobacillus casei* ssp. casei 300 and *Lactobacillus paracasei* ssp. paracasei 22 and without adjunct cultures. The changes in the antioxidant activity were related to the rate of formation of soluble peptides (proteolysis) in all the samples of cheeses up to the fourth month of ripening. Pisanu et al. [77] detected the presence of 187 bioactive peptides in sheep milk cheeses. Seven of these peptides showed strong antioxidant activity and were products of β-CN proteolysis. garbowska et al. [194] determined changes in the content of bioactive peptides (anserine and L-carnosine) during the maturation of cheese produced with the addition of *Lactobacillus* (*L. casei* 2639, *L. acidophilus* 2499, *L. rhamnosus* 489, and *L. delbrueckii* 49). After a 5-week maturation period, cheese supplemented with *L. acidophilus* 2499 was characterized by the highest content of L-carnosine and anserine (136.11 mg/kg in total) in comparison with other cheese variants. Revilla et al. [11] analyzed the antioxidant capacity of cheese using the ABTS method. A significant ($p < 0.05$) effect of the season of raw milk collection and the duration of the cheese ripening period on the antioxidant capacity was found. The total antioxidant capacity increased until it reached its maximum after 3 months of maturation of winter milk samples and after 1–4 months in the case of summer milk samples. A direct correlation was observed between the cheese maturation time and the TAC value ($r = 0.296$, $p < 0.01$). The antioxidant activity of the cheese was also significantly correlated with the vitamin A content ($r = 0.399$). In other studies [195], which included five French farmhouse cheese varieties—Abondance, Tomme de Savoie, Cantalet, Salers, and Rocamadour—the antioxidant activity of cheese was significantly ($p < 0.05$) correlated with fat-soluble antioxidants, including the content of β-carotene and vitamin E. In addition to bacterial cultures, enrichment with raw materials containing compounds with documented antioxidant activity has an impact on the antioxidant value of dairy products. Enrichment of milk and dairy products with natural plant additives increases the antioxidant potential (Table 5). Extensive research has focused on legume seeds, including soybeans, which are added to dairy products to increase their antioxidant potential. Soybeans contain bioactive phytochemicals (e.g., isoflavones, coumestrol, phytate, saponins, lecithin, phytosterols, and vitamin E). With such a composition, soybeans are regarded as a product and raw material with antioxidant properties contributing to the reduction of the risk of heart disease or lowering the levels of cholesterol [196–198]. Soybeans are also recognized as a product with high content of protein, fiber, vitamins, and minerals [199]. With the use of the DPPH method, Shori [200] evaluated the antioxidant activity of yogurt from cow and camel milk supplemented with soybeans. The antioxidant activity of the soybean-supplemented yogurt (from both cow and camel milks) was higher than that of the control. Similar findings were reported by gamba et al. [127], who showed that soymilk had higher antioxidant capacity than cow milk. In another study, Shori et al. [201] assessed the effect of the addition of mangosteen juice to yogurt on its antioxidant potential. The authors used the DPPH method also in this study. The control yogurt stored in refrigeration conditions for 14 days exhibited antioxidant activity in the range of 17–19%, whereas the value of this parameter in the phytomix-3+mangosteen-supplemented yogurt ranged from 60% to 62% in the first week of storage and reached up to 54% in the second week. This study, therefore, proves the possibility of extending

the shelf life of yogurt supplemented with phytomix-3+mangosteen, as indicated by the high antioxidant content recorded for 14 days of storage. In addition, mangosteen fruits contain xanthone, which neutralizes free radicals and supports the immune system [202]. Shori and Baba [178] used neem (*Azadirachta indica*) leaf extract as an additive to yogurt. *Azadirachta indica* is used in traditional medicine for the treatment of diabetes and hypertension [203]. It is characterized by high antioxidant potential due to the high content of vitamin C and riboflavin. Higher antioxidant activity (DPPH) was shown by the *Azadirachta indica*-supplemented yogurt in comparison with traditional yogurt (i.e., 30.1 ± 5.1% and 23.5 ± 5.0%, respectively) [179]. During the 28-day storage, the DPPH value increased and reached 53.1 ± 5.0% and 35.9 ± 5.2%, respectively. Dried grape pomace has also been added to yogurt as an alternative source of antioxidant dietary fiber [204]. As shown by the study, dried grape pomace can be used in the production of yogurt not only to increase the content of fiber and total phenolics but also to delay lipid oxidation during refrigerated storage.

Flavoring additives, especially fruit flavors, play an important role in the production of yogurt. Studies by Olas [205] on various types of fruit, including berries, showed that their presence in the diet may constitute an antioxidant barrier against the development of cancer or DNA mutations. Fruit additives are also an excellent prebiotic due to the presence of dietary fiber, which supports the proper function of the gastrointestinal tract. Unal [172] compared the antioxidant activity of various dairy products from the local Turkish market (UHT milk, yogurt, fresh cream cheese, and kefir) using the DPPH test. Fermented milk products containing berries (strawberry, blueberry, blackberry, raspberry) had significantly higher ($p < 0.05$) antioxidant activity than other products. Lee et al. [206] produced fermented milk with the addition of *Cudrania tricuspidata* fruit, which is rich in xanthones and flavonoids. The antioxidant activity of fermented milk determined with different methods (DPPH, ABTS, FRAP) was enhanced by the addition of *Cudrania tricuspidata*. In comparison with the control, the 3% additive concentration contributed to an increase in DPPH (from 1.94 to 3.40 µM TE/mL), ABTS (from 0.31 to 0.64 µM TE/mL), and FRAP (from 0.19 to 1.84 µM TE/mL) radical scavenging activities. It should be noted, however, that consumers evaluating the sensory value of the product accepted only the 0.5% or 1% addition of *Cudrania tricuspidata*. Ni et al. [207] enriched yogurt with extracts from salal berry and black currant pomace. The drink supplemented with black currant pomace had the greatest potential to inhibit the activity of α-glucosidase (>90%), which was probably related to the release of peptides from caseins during the fermentation process. Perna et al. [208] enriched yogurt with chestnut and sulla honeys. Compared with the control, the yogurts with honey were characterized by higher antioxidant activity (ABTS and FRAP). In particular, yogurts with the addition of chestnut honey exhibited higher ABTS and FRAP values, which were closely associated with their high antioxidant activity associated with the highest contents of phenolic acid and flavonoids. Yogurts are also supplemented with herbal additives (e.g., lemon balm, lilac flowers, or seeds such as linseed or chia). In their study, Vuksan et al. [209] reported that the consumption of 25 g of chia seeds with 50 g of glucose reduced postprandial glycemia and appetite in comparison with the control group receiving 25 g of flax with 50 g of glucose or 50 g of glucose alone. As reported by Porter and Bode [210], elderberry exhibits strong antiviral properties. Moreover, WCRF (World Cancer Research Fund International) report and meta-analyses carried out on consumers of milk and fermented milk products showed that this group of products may be a preventive factor against prostate, breast, colon, and stomach cancers [211]. The addition of fruit as a natural source of antioxidants to dairy products extends their shelf life and has an enhancing effect on human health. As indicated by Singh et al. [212], citrus peel, which is regarded as a waste, is a rich source of natural phenolic compounds and carotenoids acting as antioxidants, protecting cells from free radical damage and helping to reduce the risk of many chronic diseases. Due to the presence of antioxidant compounds, citrus peel can be used as a functional additive to food (e.g., dairy products). Ramos et al. [213] assessed the effect of green mate, cloves, lemongrass, and sweet potato pulp on the antioxidant

capacity of fermented milk. The addition of a freeze-dried extract (1 g per 100 g of product) containing 87.5% cloves and 12.5% green mate to yogurt significantly ($p < 0.05$) increased the total phenol content (to 54.14 vs. 5.28 mg gAE/100 g in plain yogurt) and FRAP antioxidant capacity (up to 289.96 vs. 31.40 mg AAE/100 g in plain yogurt) in the fermented product. The addition of sweet potato pulp improved the sensory acceptance of the fermented products. Muniandy et al. [214] reported a significant effect of supplementation with green, white, and black tea on the antioxidant activity (FRAP, DPPH, and ferrous ion chelating activity) of yogurts during 21 days of cold storage. The tea-supplemented yogurts exhibited higher antioxidant activity than the plain variant (control) throughout the storage period.

Various investigations indicate that the addition of extracts from red ginseng (*Panax ginseng*) [215] or blackberry flowers (*Rubus ulmifolius*) [216] may increase the antioxidant capacity of yogurts. Fiorda et al. [191] used various functional substrates (hydrolyzed soybean extract, colostrum, and honey) to design new probiotic drinks using kefir grains as a starter culture. Fermentation was carried out at 30 °C for 24 h, and the physicochemical composition and functional aspects were determined. It was found that the honey-based kefir drink exhibited higher antioxidant activity and sensory quality than the traditional kefir drink. Its microbiological composition showed a high level of lactic acid bacteria and yeast (over 106 CFU/mL), mainly the potentially probiotic strains of *Lactobacillus statsumensis*, *Leuconostoc mesenteroides*, *Bacillus megaterium*, and *Lachancea fermentati*. Therefore, honey may be an ideal alternative substrate for the production of a functional culture-based drink, especially for vegans and lactose-intolerant consumers. The antioxidant activity of WPC (whey protein concentrate) and sea algae (spirulina) was assessed as well. A combination of WPC and spirulina increased TAC (58 µmol/g of liver tissue) and lowered the level of cholesterol (58 mg/dL) and malondialdehyde (MDA; a lipid peroxidation marker) [217]. The authors ascribed the activity of WPC to the content of Cys and associated the activity of spirulina with the content of β-carotene, tocopherol, and phycocyanins. Enrichment of cheeses to improve their antioxidant capacity was investigated as well. Da Silva et al. [218] determined the effect of the type of extract (from whole grapes, seeds, and skins) and its concentration (0.1%, 0.2%, and 0.3%) added to cheese milk on the recovery of polyphenols in cheese. As shown by the authors, commercial grape extracts can be used as functional ingredients in the production of cheese without an adverse effect on their yield. The polyphenol recovery rate from whole grape and grape seed extracts was approximately 0.63 at a 0.1% concentration and decreased with the increasing concentration of the extract in milk. Higher rates of polyphenol recovery were observed in the case of the grape seed extracts (0.87) with no concentration effect. The authors emphasize that the consumption of several grams of such cheese can provide the same amount of polyphenols as 1 L of grape juice. Similarly, Marchiani et al. [219] showed an increase in total phenol content and radical scavenging activity (DPPH) in Italian ripened cheeses (Toma and cheddar) after supplementation with dried grape pomace (GP). As highlighted by the authors, it is necessary to add at least 1.6% of gP to achieve a significant increase in the antioxidant activity of cheese. Studies conducted by other authors also showed an increase in the oxidative stability of gP-supplemented cheeses during storage, which is attributed to the antioxidant effect of phenolic compounds contained in the additive. The sensory attractiveness of the cheeses was found to increase as well. Additionally, Marinho et al. [220] compared the antioxidant activity of cheeses that were coated or uncoated with rosemary leaves. They showed that, after 60 days of ripening, the rosemary-coated cheeses were more resistant to the oxidation process than the uncoated cheeses. In subsequent studies, the possibility of using pomegranate peel (*Punica granatum*) extract as a new natural cheese preservative was assessed [221]. Kalari cheese was treated with various concentrations of pomegranate peel extract (0%, 1%, and 2%). A significant ($p < 0.05$) effect of the extract on the oxidative stability of lipids was observed, as the treated products exhibited significantly ($p < 0.05$) lower values of TBARS (mg malondialdehyde/kg) and FFA (% of oleic acid). Similarly, pine needle extract (*Cedrus deodara* Roxb.) can be used as a new preservative in the production of Kalari cheese, as it was found to improve the oxidative stability of cheese significantly [222].

Table 5. Enrichment of milk and dairy products with natural plant additives with high antioxidant potential (own work based on: [178,213,215,223–231]).

Samples	Additives	Method	Antioxidant Activity	References
Yogurt	Control (without plain *Allium sativum)* Plain *Allium sativum* (Garlic)	DPPH	26.4 ± 0.7% 37.9 ± 0.8%	[178]
Fermented milk	Control (without herbal extract/sweet potato pulp) The optimized herbal extract—containing 87.5% clove (*Syzygium aromaticum*) and 12.5% green mate (*Ilex paraguariensis*) (1 g/100 g) The optimized herbal extract (1 g/100 g) and sweet potato pulp (15 g/100 g) Sweet potato pulp (15 g/100 g)	FRAP	31.40 ± 1.40 mg AAE/100 g 289.96 ± 46.26 mg AAE/100 g 224.95 ± 3.29 mg AAE 100 g 24.51 ± 0.85 mg AAE 100 g	[213]
Milk	Control (without red ginseng extract) Milk + red ginseng extract (100 µg/mL)	DPPH	11.8 ± 0.00 µg/mL 15.1 ± 0.5 µg/mL	[223]
Yogurt	Control (without red ginseng extract) Yogurt + red ginseng extract (100 µg/mL)	DPPH	5.8 ± 0.5 µg/mL 18.7 ± 1.1 µg/mL	
Fresh cheese	Control (without yerba mate) 1% yerba mate Control (without yerba mate) 1% yerba mate Control (without yerba mate) 1% yerba mate	ABTS DPPH FRAP 	14.59 ± 0.57% 38.76 ± 2.18% 2.93 ± 0.10% 67.30 ± 1.35% 0.14 ± 0.01 mg gAE/g 0.66 ± 0.11 mg gAE/g	[224]
Yogurt	Natural yoghurt (without green tea infusion) 10% green tea infusion	FRAP	1.04 mmol Fe^{2+} EL^{-1} 8.98 mmol Fe^{2+} EL^{-1}	[225]
	Control (without *Rosa spinosissima* fruit extract) 0.2% *Rosa spinosissima* fruits extract Control (without *Rosa spinosissima* fruit extract) 0.2% *Rosa spinosissima* fruit extract Control (without *Rosa spinosissima* fruit extract) 0.2% *Rosa spinosissima* fruit extract	FRAP DPPH ABTS 	0.07 ± 0.02 mM Trolox/L 2.45 ± 0.02 mM Trolox/L 0.86 ± 0.02 mM Trolox/L 0.86 ± 0.03 mM Trolox/L 3.18 ± 0.07 mM Trolox/L 3.33 ± 0.06 mM Trolox/L	[226]
	Control (without Argel leaf extract) 0.1 g/100 mL Argel leaf extract	DPPH	32.60 ± 0.20% 47.22 ± 0.02%	[227]
	Control (without aronia juice) 3% aronia (*A. melanocarpa)* juice Control (without aronia juice) 3% aronia (*A. melanocarpa)* juice	DPPH ABTS 	59.47 ± 0.31% 77.87 ± 0.44% 45.96 ± 0.55% 70.90 ± 0.26%	[228]
	Control (without riceberry rice extract) 0.125% riceberry rice extract 0.25% riceberry rice extract 0.5% riceberry rice extract	FRAP	5.26 ± 0.52 mmol $FeSO_4$/100 g 17.42 ± 0.43 mmol $FeSO_4$/100 g 25.64 ± 0.96 mmol $FeSO_4$/100 g 41.06 ± 2.60 mmol $FeSO_4$/100 g	[229]
	Control (without purple basil in water extract) 0.4% purple basil in water extract 1% purple basil in water extract 0.4% purple basil in powder form 1% purple basil in powder form Control (without purple basil in water extract) 0.4% purple basil in water extract 1% purple basil in water extract 0.4% purple basil in powder form 1% purple basil in powder form	ABTS DPPH 	0.67 ± 0.01 mmol TE/kg 1.17 ± 0.01 mmol TE/kg 1.76 ± 0.01 mmol TE/kg 1.42 ± 0.02 mmol TE/kg 2.94 ± 0.04 mmol TE/kg 10.66 ± 0.26% 33.16 ± 0.17% 41.92 ± 0.09% 25.32 ± 0.17% 43.42 ± 0.17%	[230]
	Control (without red ginseng extract) 0.5% red ginseng extract 1% red ginseng extract 1.5% red ginseng extract 2% red ginseng extract	DPPH	62.50 ± 4.82% 94.46 ± 2.34% 94.85 ± 0.11% 94.85 ± 0.07% 94.26 ± 0.31%	[215]
	0 % safflower petal ethanol extract 1% safflower petal ethanol extract 0 % safflower petal hot water extract 1% safflower petal hot water extract	DPPH	3.24 ± 0.62% 2.79 ± 0.85% 5.81 ± 0.61% 10.66 ± 1.21%	[231]

7. Conclusions

Summing up, it should be stated that the antioxidant capacity of milk and dairy products is mainly associated with the content of antioxidant components (i.e., proteins), which are rich sources of sulfur amino acids, vitamins A, E, and C, or β-carotene. Biopeptides generated during the fermentation or maturation of cheese also exhibit antioxidant activity. The antioxidant capacity is determined with various methods, mainly ABTS, FRAP, and DPPH assays. Research indicates differences in the total antioxidant capacity of milk between animal species, which result from the differences in the chemical compositions of their milk. Sheep and buffalo milks have the greatest capacity. The content of antioxidant components in milk and the antioxidant potential can be modified through animal nutrition (e.g., supplementation of animal diets with various natural additives). The addition of herbal mixtures or by-products from the fruit and vegetable industry to animals' rations contributes to the improvement of the nutritional value of milk through an increase in the content of bioactive compounds and antioxidant potential. The antioxidant potential of dairy products is associated not only with the quality of the raw material but also with type of heat treatment, bacterial starter cultures, and natural plant additives used in the processing stage. Fermented products, especially when used as probiotic starter cultures, have the highest antioxidant status. The antioxidant activities of products also increase using plant materials that are rich in phenolic compounds and carotenoids. However, it should be emphasized that the results may be difficult to compare, as different antioxidant activity assays are used. It is difficult to compare the results obtained with different methods, considering, for example, different values and units even within the same method (ABTS and DPPH). Neither method is a reference method, so there is no single clear point of reference. Therefore, it would be worth choosing one most objective method of status assessment and applying it in laboratories all over the world.

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Praca 2

Magdalena Stobiecka, Jolanta Król, Aneta Brodziak**.** Antioxidant potential of milk obtained from Holstein-Friesian cows with regard to the subsequent lactations and stage of lactation. Mljekarstvo, 2023, 73 (2), 95-104. DOI: 10.15567/mljekarstvo.2023.0203

Lublin, 27.09.2023

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Oświadczenie o współautorstwie

Niniejszym oświadczam, że w pracy:

Magdalena Stobiecka, **Jolanta Król**, Aneta Brodziak. Antioxidant potential of milk obtained from Holstein-Friesian cows with regard to the subsequent lactations and stage of lactation. Mljekarstvo, 2023, 73 (2), 95-104. DOI: 10.15567/mljekarstvo.2023.0203.

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Antioxidant potential of milk obtained from Holstein-Friesian cows with regard to the subsequent lactations and stage of lactation

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Abstract

The aim of the research was to assess the content of components showing antioxidant activity in the milk of Holstein-Friesian cows and changes in the total antioxidant status during subsequent lactations and their stages. The material for analysis consisted of milk collected from 90 cows (30 for each analysed lactation number: I - primaparous; II - multiparous in second lactation; III - multiparous in third lactation) during three periods: 1 - up to 100 days of lactation, 2 - 101-200 days of lactation, and 3 - 201-305 days of lactation. The basic chemical composition, casein content, somatic cell counts, selected whey proteins and fat-soluble vitamins were determined in the milk. The total antioxidant status (TAS) of milk was also measured. With the subsequent lactation, the content of components with antioxidant properties, i.e. vitamins A and E, and albumins (α-lactalbumin and β-lactoglobulin), decreased significantly ($p \leq 0.01$). Simultaneously, a decrease in the level of TAS in milk was noted. The lactation phase had a minor effect on the antioxidant potential of milk. The obtained high correlation coefficients between the value of the TAS and the content of vitamins A, E and β-lactoglobulin indicate that the content of these compounds largely determines the antioxidant potential of milk. On the other hand, the obtained negative correlations between the level of antioxidant potential and the daily milk yield ($r = -0.347$, $p \leq 0.05$) suggest that the high productivity of cows negatively affects the antioxidant value of milk.

Key words: milk; vitamins; proteins; antioxidant potential; lactation

Introduction

Milk is regarded as a valuable source of bioactive compounds. It contains many biologically active substances with antioxidant properties. Antioxidants largely limit or even prevent oxidative processes in the living organism. By scavenging and neutralizing reactive oxygen species, they play an important role in the support and enhancement of organism defence mechanisms, which is particularly important in the prophylaxis of some lifestyle diseases (Lisak Jakopović et al., 2019; Stobiecka et al., 2022). Such components are present in protein (β-lactoglobulin, lactoferrin), fat (vitamin E, A, β-carotene), and water (vitamin C, microelements: Sn, Zn, Fe, Mn) fractions (Puppel et al., 2015; Król et al., 2017; Vanitcharoen et al., 2018). It should be emphasized that whey proteins, especially β-lactoglobulin, have the highest antioxidant potential of all proteins in the human diet. This is related to the high content of sulphur containing amino acids, in particular cysteine, which is essential for glutathione synthesis (Ma et al., 2018). As reported by Kim et al. (2019), in addition to β-lactoglobulin, the antioxidant effect is also exhibited by α-casein. Due to their antioxidant properties, these proteins can inhibit cell aging and increase myoblast differentiation and maturation, thereby alleviating aging-related damage caused by oxidative stress. Antioxidant activity is also exhibited by lactoferrin, as it chelates iron and thus increases its bioavailability and inhibits the pro-oxidative effect of this element. However, the antioxidant capacity of LF declines proportionally to iron saturation (Cutone et al., 2020). Proteins and their fractions are valuable sources of bioactive peptides with an ability to scavenge free radicals, chelate metal ions, and inhibit lipid peroxidation (Bielecka et al., 2021). Fat-soluble vitamins, mainly vitamin E and especially α-tocopherol as well as vitamin A and β-carotene, are the main antioxidants in this group of compounds (Celi, 2011; Kaneai et al., 2012; Vanitcharoen et al., 2018). Their activity is based on scavenging organic free radicals and inhibiting lipid peroxidation (Mann et al., 2016). They also have the ability to absorb singlet oxygen and hydroxyl radicals, thus effectively preventing DNA oxidation (Cichosz et al., 2017). Since they are present in fat globule membranes, they prevent milk fat autoxidation. Similarly, vitamin D_3 is part of the non-enzymatic antioxidant system in milk, and 1,25-dihydroxycholecalciferol is the most active form. Its antioxidant effect is based on the inhibition of lipid peroxidation (Mutlu et al., 2013). Vitamin C (ascorbic acid) plays an important antioxidant role in the organism, by being a non-enzymatic water-soluble antioxidant, and as well as B group vitamins by inhibiting homocysteine production. Hence, the antioxidant potential of milk is directly associated with the content of compounds exhibiting antioxidant properties (Stobiecka et al., 2022).

The global dairy production, including Poland, is based primarily on Holstein-Friesian cows. The cows of this breed are characterized by high milk yields but also by the highest susceptibility to metabolic stress. As shown by some previous research findings, reactive oxygen species are produced during various physiological processes, e.g. during the lactation period. The animal organism utilizes antioxidants to reduce free radicals (Chang et al., 2007). An excess of free radicals results is damage to macromolecules, i.e. proteins, lipids, and DNA, leading to metabolic disorders, lower milk yields, or deterioration of milk quality with reduction in the content of antioxidant substances (Karasahin et al., 2021).

The aim of the study was to assess the content of components exhibiting antioxidant activity in the milk of Holstein-Friesian cows and changes in the total antioxidant status during subsequent lactations and their stages.

Material and methods

The study was carried out on a Holstein-Friesian cattle farm with an intensive milk production system. The cows were kept in a free-stall barn and their nutrition was based on the total mixed ration (TMR) system throughout the year. The feed ration was composed of roughage (maize silage, grass haylage) and concentrate fodder. The study involved 90 Holstein-Friesian cows, i.e. 30 in each lactation group (I - primiparous cows; II - multiparous cows in lactation II; III - multiparous cows in lactation III). Milk samples were collected from each cow in three terms: 1. before lactation day 100, 2. between lactation days 101 and 200, and 3. between lactation days 201 and 305. Chilled samples were transported to the laboratory where the somatic cell count (SCC) was determined using a Somacount150 device (Bentley Instruments, USA). Milk samples with the somatic cell count not exceeding 400 thousand/ml were taken for further analysis. In total, 262 milk samples were analysed. The following parameters were determined in each milk sample: the basic chemical composition, i.e. the content of total protein, fat, lactose, and dry matter (using the Infrared Milk Analyzer; Bentley Instruments, USA), the content of casein (according to AOAC (2000)), and the concentration of selected whey proteins, i.e. α-lactalbumin (α-LA), β-lactoglobulin (β-LG), lactoferrin, and bovine serum albumin (BSA), using reversed phase high-performance liquid chromatography. All samples for the whey protein determinations were prepared as in Romero et al. (1996) with modifications (Brodziak et al., 2012). Protein separation was performed using a ProStar 210 liquid chromatograph equipped with a UV-Vis ProStar 325 detector (Varian, USA). In all cases, the separations were carried out in the water-acetonitrile gradient mobile phase and NUCLEOSIL 300-5 C18 columns with 250-mm length and 4.6-mm diameter (Varian, USA). The mobile phase consisted of solvent A (90 % water, 10 % acetonitrile) and solvent B (90 % acetonitrile, 10 % water) (Sigma, Germany). A single sample was analysed for 35 min at a wavelength of λ = 205 nm and a column temperature of 37 °C. The standards were analysed in identical conditions. To this end, standard solutions of purified proteins were used, i.e. α-LA (≥85 %), β-LG (90 %), BSA (≥96 %), and

lactoferrin (90 %); they all were obtained from milk proteins (Sigma, Germany). Based on the analysis of the retention times read from individual chromatograms, the qualitative identification of individual substances was performed with the use of the Star 6.2 Chromatography Workstation program (Varian, USA). The quantitative analysis was performed with the external standard method. The RP-HPLC method was used to determine the concentration of fat-soluble vitamins, i.e. A, D_3, and E. All samples were prepared with the Röse-Gottlieb fat extraction method modified by Hewavitharan et al. (1996). The separations were performed using a PursuitXRs 3-C18 column with 150-mm length and 4.6-mm diameter (Varian, USA). The phase was a mixture of acetonitrile, methanol, water, and dichloromethane (Sigma, Germany), and the flow rate was set to 1 mL/min. The standards were analysed in identical conditions. The following standard vitamin solutions were used: (±)-α-tocopherol (vitamin E) with ≥97 % purity (HPLC), cholecalciferol (vitamin D_3) - ≥98 % (HPLC), retinol (vitamin A) - ≥99 %, and ß-carotene (provitamin A) - ≥98 % (HPLC) (Sigma, Germany). The qualitative identification of individual substances was based on the analysis of the retention times read from individual chromatograms using the Star 6.2 Chromatography Workstation program (Varian, USA). The quantitative analysis was carried out using the external standard method.

The total antioxidant status (TAS) was determined using the Randox tests (Tecan Austria GmbH, Grödig, Austria). The analysis consisted in spectrophotometric measurements of the degree of colour change of the resulting reactive radical ABTS® (2,2'-azino-di-[3-ethylbenzothiazoline sulfonate) using a UV-Vis spectrophotometer at a wavelength of 600 nm. ABTS® was incubated with peroxidase (metmyoglobin) and H_2O_2 to obtain the $ABTS^{\bullet+}$ radical cation. It is characterized by a relatively stable blue-green colour, which was measured at the aforementioned wavelength. The antioxidants present in the added sample led to weakening of the blue-green color in proportion to their concentration.

$$HX - Fe^{III} + H_2O_2 \longrightarrow X - [Fe^{IV} = O] + H_2O$$
$$ABTS^{\circledR} + X - [Fe^{IV} = O] \longrightarrow ABTS^{\bullet+} + HX - Fe^{III}$$

$HX - Fe^{III}$=Metmyoglobin

$X - [Fe^{IV} = O] + H_2O$=Ferrimyoglobin

ABTS®=2,2'-Azino-di-[3-ethylbenzothiazoline sulfonate]

The results were analysed statistically using a one-way analysis of variance in StatSoft Inc. Statistica ver. 13. Significant differences between the means were determined using Tukey's test at a significance level p (alpha) = 0.05 and 0.01. Additionally, Pearson linear correlation coefficients for the milk yield and parameters were calculated.

Results and discussion

Table 1 shows the daily milk yield (on the sampling day), SCC, and the content of basic milk components relative to the subsequent lactations and their stage. A significant ($p \leq 0.01$) increase in the milk yield was recorded during the subsequent lactations. The daily amount of milk produced by cows in lactation III was by 5 kg higher in comparison to the group of cows in lactation II and by 10 kg higher than in the group of primiparous cows. An increase in the somatic

Table 1. Cow milk yield and the basic chemical composition of milk in the subsequent lactations and the stages of lactation

Indicators	Subsequent lactation		
	I	II	III
Milk yield (kg)	$24.66^{A}\pm3.11$	$29.76^{B}\pm4.10$	$35.25^{C}\pm4.79$
Total protein (%)	$3.29^{b}\pm0.19$	$3.23^{ab}\pm0.24$	$3.19^{a}\pm0.29$
Casein (%)	$2.64^{b}\pm0.17$	$2.56^{ab}\pm0.18$	$2.52^{a}\pm0.15$
Fat (%)	$4.01^{A}\pm0.35$	$4.16^{B}\pm0.28$	$4.21^{B}\pm0.30$
Lactose (%)	4.83±0.19	4.75±0.13	4.78±0.36
Dry matter (%)	12.79±0.52	12.75±0.48	12.80±0.41
Somatic cell count (SCC) thousand/mL	$122^{A}\pm112$	$143^{A}\pm118$	$230^{B}\pm127$
	Stage of lactation		
	1	2	3
Milk yield (kg)	$33.57^{B}\pm5.29$	$30.52^{AB}\pm5.26$	$28.19^{A}\pm5.81$
Total protein (%)	$3.19^{a}\pm0.19$	$3.33^{b}\pm0.22$	$3.43^{b}\pm0.18$
Casein (%)	$2.49^{a}\pm0.15$	$2.60^{b}\pm0.17$	$2.68^{b}\pm0.14$
Fat (%)	$3.99^{A}\pm0.38$	$4.07^{AB}\pm0.30$	$4.28^{B}\pm0.27$
Lactose (%)	4.81±0.23	4.80±0.21	4.78±0.14
Dry matter (%)	$12.58^{a}\pm0.58$	$12.76^{ab}\pm0.47$	$13.05^{b}\pm0.52$
Somatic cell count (SCC) thousand/mL	$154^{A}\pm127$	$180^{AB}\pm118$	$209^{B}\pm121$

a, b - significant differences at p≤0.05; A, B, C - significant differences at p≤0.01

cell count was also noted with the increasing age of the cows. Since the somatic cell count in the analysed milk did not exceed 400 thousand/mL, it can be assumed that the permeability of the mammary gland epithelium increased with the age of the cows. This was most probably associated with earlier inflammations, which damaged the epithelium and increased its permeability even after healing (Liu et al., 2009; Litwińczuk et al., 2011). Król et al. (2014) found significant interactions ($p \leq 0.01$) between the subsequent lactations and SCC. In turn, a downward tendency in the total protein content accompanied by an increase in the fat level was observed in the subsequent lactations. Similar relationships were confirmed by other authors as well (Kuczyńska et al., 2021). The amount of produced milk decreased significantly ($p \leq 0.01$) in the consecutive lactation stages. The highest yield was recorded in the first stage of lactation. However, the milk from this stage exhibited the lowest dry matter content, including the total protein and fat. During the subsequent lactation stages, the proportion of dry matter components ($p \leq 0.05$) in the milk, e.g., the content of protein ($p \leq 0.05$) and fat ($p \leq 0.01$), increased significantly. Studies conducted by other authors (Pecka et al., 2012; Król et al., 2013; Kuczyńska et al., 2021) reported a systematic decrease in the cow milk yield accompanied by an increase in dry matter content, including fat and protein, during consecutive lactation stages.

Table 2 shows the content of whey proteins in milk, which in most cases have antioxidant activity. The content of albumins, i.e. α-LA and β-LG, decreased and significant differences were observed between lactations I and III. The differences were 0.10 g/L ($p \leq 0.05$) in the case of α-LA and 0.18 g/L ($p \leq 0.01$) in the case of β-LG. Similar trends were noted along the lactation stages, with significant differences only in the α-LA content between the first and third stages ($p \leq 0.05$). The decline in the content of α-LA may have been caused by the decrease in the milk yield at the end of lactation, as this protein is one of the main components of the lactose synthase complex involved in the control of lactation and milk secretion (Liu et al., 2007). Similar findings were reported by other authors (Heck et al., 2009; Król et al., 2013). In the case of BSA, i.e. another milk albumin, there were different relationships, i.e. its amount increased significantly ($p \leq 0.05$) with the age of the cows. As suggested by Litwińczuk et al. (2011), the BSA concentration is an indicator of the permeability of the blood-milk barrier in the mammary gland. Mastitis is the main factor increasing the permeability. Since the SCC value in the present study did not exceed 400 000/mL, it can be assumed that the age of the cows had an impact on the permeability of the mammary gland epithelium as well. It was most probably associated with previous inflammation, which caused damage to the epithelium and increased its permeability even after healing. However, there was no effect of the lactation stage on the content of this protein in the milk. The subsequent lactations were accompanied by an increase in the concentration of the main antibacterial proteins, i.e. lactoferrin and lysozyme. There were significant ($p \leq 0.05$) differences (at a level of approximately 20 mg/L) in the lactoferrin content between lactation I and III. Other authors reported significant differences in the content of lactoferrin in subsequent lactations as well (Hagiwara et al., 2003; Back and Thompson, 2005; Król et al., 2013). The content of lactoferrin and lysozyme increased with the progression of lactation, with significant ($p \leq 0.05$) differences in the lactoferrin level. Its content in the third lactation stage increased by over 25 mg/L, which corresponded to approximately 1/4 of its level in the first stage. A significant increase in the lactoferrin concentration in milk along lactation stages was demonstrated by Cheng et al. (2008), who reported a high correlation coefficient between the content of lactoferrin and the stage of lactation ($r=0.557$). The authors also found significant ($p<0.01$) negative correlations between the lactoferrin concentration and daily milk yield and significant ($p<0.018$) relationships between the lactation stage and milk yield. These correlations were confirmed in the present study. An increasing concentration of lactoferrin during the lactation stages was noted in the milk of cows characterised by a lower daily milk yield.

Table 2. Content of whey proteins in the milk in the subsequent lactations and the stages of lactation

Indicators	Subsequent lactation		
	I	II	III
β-lactoglobulin (g/L)	$3.33^{B}\pm0.44$	$3.25^{AB}\pm0.41$	$3.15^{A}\pm0.36$
α-lactalbumin (g/L)	$1.12^{b}\pm0.17$	$1.09^{ab}\pm0.15$	$1.02^{a}\pm0.15$
Bovine serum albumin (g/L)	$0.41^{a}\pm0.08$	$0.42^{a}\pm0.09$	$0.48^{b}\pm0.07$
Lactoferrin (mg/L)	$99.7^{a}\pm10.6$	$105.4^{ab}\pm36.4$	$119.6^{b}\pm17.7$
Lysozyme (µg/L)	8.21±1.88	8.56±1.74	9.19±1.16
	Stage of lactation		
	1	2	3
β-lactoglobulin (g/L)	3.28±0.38	3.30±0.45	3.25±0.39
α-lactalbumin (g/L)	$1.22^{b}\pm0.08$	$1.10^{ab}\pm0.16$	$1.08^{a}\pm0.12$
Bovine serum albumin (g/L)	0.42±0.08	0.46±0.10	0.43±0.09
Lactoferrin (mg/L)	$98.8^{a}\pm32.5$	$103.6^{ab}\pm27.8$	$123.9^{b}\pm23.4$
Lysozyme (µg/L)	8.05 ±1.98	8.64±2.28	9.08 ±2.49

a, b - significant differences at $p \leq 0.05$; A, B - significant differences at $p \leq 0.01$

The results presented in Table 3 show a significant ($p \leq 0.01$) effect of the subsequent lactations on the content of lipophilic vitamins with antioxidant activity, i.e. A and E. The milk from the primiparous cows exhibited significantly higher contents of these vitamins, compared with the milk from lactation III of the multiparous cows. The milk produced by the cows in lactation I was characterized by on average 20 % and 15 % higher content of vitamins E and A, respectively, compared to the milk from the multiparous cows. With the progression of lactation, the content of the analysed vitamins (A, E, D_3) decreased, but statistically significant differences ($p \leq 0.05$) were found only in the case of vitamin A. In comparison with the first lactation stage, the content of this vitamin in the third stage decreased by almost 30 %. Kapusta et al. (2018) reported a higher level of vitamin E at the peak of the first lactation stage, i.e. between day 29 and 70. The results of a study conducted by Strusińska et al. (2010) indicated changes in the concentration of vitamins in milk analysed in different stages of lactation; however, as emphasized by the authors, the obtained differences were largely related to the nutrition provided to the cows. During the winter period (November-March), the highest concentrations of vitamin A and E in the milk were found between lactation day 121 and 180 and for between day 61 and 120 day, respectively, but the differences were not statistically significant. The pasture-derived feed in the ration resulted in a gradual increase in the vitamin E concentration with progression of lactation. During this period, the vitamin A content in milk was higher on the first 60 days after calving. In turn, in a study of organic milk conducted by Sakowski et al. (2012), the content of vitamins A and E in milk increased significantly during the subsequent stages of lactation. The authors found the highest level of these vitamins in the fourth stage of lactation (after 250 days post calving). On contrary, Navrátilová et al. (2019) did not find significant differences in the content of vitamins A and E in the milk of lactating mares. As reported by Calderón et al. (2007), the secretion of vitamins A and E to milk is variable and depends on the lactation stage only to a limited extent. The energy balance in cows has an impact on the concentration of vitamins A and E in milk (Noziére et al., 2006).

Table 3. Content of lipophilic vitamins in the milk in the subsequent lactations and the stages of lactation

Indicators	Subsequent lactation		
	I	II	III
Vitamin A (mg/L)	$0.397^{B} \pm 0.060$	$0.353^{AB} \pm 0.070$	$0.338^{A} \pm 0.010$
Vitamin E (mg/L)	$1.371^{B} \pm 0.327$	$1.206^{AB} \pm 0.315$	$1.078^{A} \pm 0.377$
Vitamin D_3 (µg/L)	0.833±0.254	0.698±0.180	0.757±0.172
	Stage of lactation		
	1	2	3
Vitamin A (mg/L)	$0.357^{b} \pm 0.006$	$0.344^{ab} \pm 0.057$	$0.309^{a} \pm 0.041$
Vitamin E (mg/L)	1.288±0.597	1.209±0.342	1.189±0.368
Vitamin D_3 (µg/L)	0.811±0.023	0.775±0.024	0.773±0.010

A, B - significant differences at $p \leq 0.01$

As demonstrated by Dolores-Perez and Calvo (1995), the vitamin A content in milk is positively correlated with the concentration of β-LG, as this protein is actively involved in the transport of vitamin A. Similarly, other authors (Kuczyńska et al., 2011; Brodziak et al., 2018) indicated relationships between the content of these compounds in milk. This was also confirmed by the results of the present study, which showed statistically significant ($p \leq 0.01$) positive correlations between the content of vitamin A and β-LG, with a high correlation coefficient – r=0.613 (Table 4).

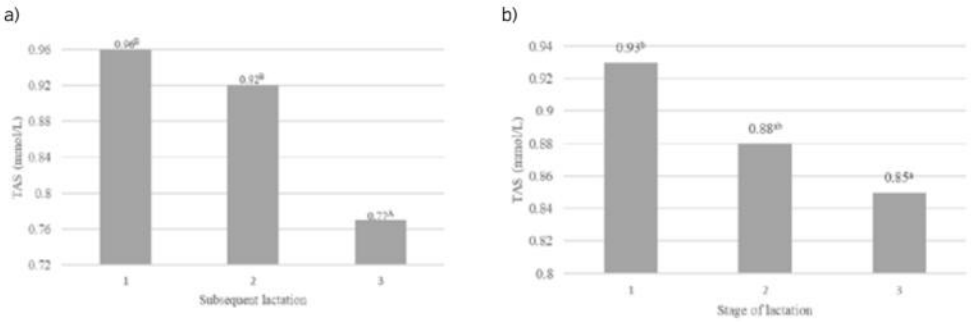
Significant ($p \leq 0.01$) positive correlation coefficients of the content of vitamin A, vitamin E, and β-LG with the total antioxidant status of milk were obtained (Table 4), which indicates that the content of these compounds largely determines the antioxidant potential of the milk. The highest values of the coefficients were recorded for the correlation between the total antioxidant status and the content of vitamin A (r=0.687) and E (r=0.664), and slightly lower values were noted for the correlation with β-LG (r=0.515). As reported by other authors (Puppel et al., 2017; Kapusta et al., 2018), milk with higher contents of vitamins A and E exhibited higher antioxidant potential.

The present results indicate that the total antioxidant status of the milk changed during the subsequent lactations (Fig. 1a) and their stages (Fig. 1b). The highest TAS was exhibited by the milk from primiparous cows

Table 4. Pearson correlation coefficients for daily yield and milk parameters

	A	E	D_3	β-LG	α-LA	Lactoferrin	BSA	Lysozyme	Yield
TAS	0.687**	0.664**	0.094	0.515**	0.120	0.194	0.092	0.117	-0.317*
Vit. A		0.201	0.018	0.603**	0.165	0.103	0.054	0.012	-0.153
Vit. E			0.119	0.098	0.052	0.092	0.006	0.000	-0.098
Vit. D_3				0.319*	0.192	0.105	0.039	0.038	0.149
β-LG					0.726**	0.029	0.044	0.112	0.059
α-LA						0.147	0.021	0.059	-0.098
Lactoferrin							0.012	0.153	0.074
BSA								0.574**	0.339*
Lysozyme									0.167

**p≤0.05; **p≤0.01*



A, B - significant differences p≤0.01; a, b - significant differences p≤0.05

Figure 1. *Value of the total antioxidant status (TAS, mmol/L) in the subsequent lactations and their stages*

(0.96 mmol/L), which was most probably associated with the higher content of components with antioxidant properties in the milk, i.e. vitamins A and E as well as ß-LG. The total antioxidant status of the milk from cows in lactation II was slightly lower (0.92 mmol/L). In turn, a significant (p≤0.01) decrease (by approx. 20 %) in the value of the antioxidant potential of milk and in the content of components with antioxidant properties was noted in the lactation III period. These relationships are also indicated by the positive correlation coefficients between TAS of milk and the content of vitamins A, E, and ß-LG (Table 4). Noteworthy, TAS of the milk was significantly (p≤0.05) negatively correlated with the milk yield (r=-0.317). The subsequent lactations were accompanied by a significant increase in the milk yield and a decrease in the antioxidant status of the milk. Similar relationships were reported by Kapusta et al. (2018), who obtained a significant (p≤0.01) correlation coefficient (r=-0.390) between the milk yield and total antioxidant status in a study of multiparous cows at the peak of lactation. As suggested by other authors (Sakowski et al., 2012; Kuczyńska et al., 2021), a higher milk yield can increase the risk of oxidative stress, which has an impact on the health of cows and the chemical composition and quality of milk. In a study conducted by Puppel et al. (2012), it was found that the antioxidant capacity of milk may be related to dietary supplementation and the age of cows. Addition of fish oil and linseed to cow rations significantly increased the total antioxidant status of milk, with a higher level in the milk from primiparous cows in both nutrition groups. Mann et al. (2016) highlighted the effect of the lactation stage on changes in the antioxidant status of milk. The authors determined the status of milk produced by different breeds of cows (Sahiwal, Karan Fries, and Holstein Frisian) and found higher values in the case of colostrum and milk collected in the early stage of lactation. In the later stages, the antioxidant potential values declined significantly. The authors suggest that elevated levels of antioxidants in colostrum may be crucial for the protection of the health of new-born calves against oxidative stress. The present study demonstrated a decrease in the total antioxidant status of the analysed milk in the consecutive stages of lactation, with significant differences (p≤0.05) between the first and third stages (Fig. 1). Significant changes in the antioxidant potential of milk during lactation were also reported by Annie et al. (2019), who showed the highest antioxidant potential of milk obtained in the early stage of lactation. The investigations conducted by Kapusta et al. (2018) also revealed a clear relationship between the lactation stage and the level of antioxidants in milk. The highest total antioxidant status was determined by the authors on the first days of lactation, but its level gradually decreased on the following days.

Conclusions

The study showed that the content of antioxidant components, i.e. vitamins A and E and albumin (ß-LG and α-LA), decreased significantly during the subsequent lactations. At the same time, a significant decrease in the value of the total antioxidant status of milk was noted. The lactation stage had a lesser effect on the antioxidant potential of milk. The values of the correlation coefficients between the total antioxidant status level and the content of vitamins A and E as well as ß-LG indicate that the presence of these compounds largely determines the antioxidant potential of milk. Additionally, there were negative correlations between the level of total antioxidant status and the daily milk yield, which suggests that high yields of milk have a negative impact on its antioxidant potential.

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Antioksidacijski potencijal mlijeka holštajn-frizijskih krava s obzirom na redoslijed i stadij laktacije

Sažetak

Cilj istraživanja bio je utvrditi koncentraciju sastojaka koji pokazuju antioksidativno djelovanje u mlijeku holštajn-frizijskih krava i promjene ukupnog antioksidacijskog statusa s obzirom na redoslijed i stadij laktacije. U svrhu istraživanja korišteni su uzorci mlijeka prikupljeni od 90 krava (po 30 za svaki analizirani laktacijski broj: I - prvotelke; II - višetelke u drugoj laktaciji; III - višetelke u trećoj laktaciji) u tri razdoblja: 1 - do 100 dana laktacije, 2 - 101-200 dana laktacije, a 3 - 201-305 dana laktacije. U mlijeku je određen osnovni kemijski sastav, broj somatskih stanica, udio kazeina, odabranih proteina sirutke i vitamina topljivi u mastima. Također je izmjeren i ukupni antioksidacijski status (TAS) mlijeka. S obzirom na redoslijed laktacije značajno se smanjivala ($p \leq 0,01$) koncentracija sastojaka s antioksidacijskim svojstvima, tj. vitamina A i E, te albumina (α-laktalbumin i β-laktoglobulin). Istovremeno je zabilježen i pad vrijednosti TAS u mlijeku. Stadij laktacije imao je manji učinak na antioksidacijski potencijal mlijeka. Visoki koeficijenti korelacije između vrijednosti TAS i koncentracije vitamina A, E i β-laktoglobulina ukazuju da udio ovih spojeva uvelike određuje antioksidacijski potencijal mlijeka. S druge strane, negativne korelacije dobivene između vrijednosti antioksidacijskog potencijala i dnevnog prinosa mlijeka ($r=-0,347$, $p \leq 0,05$) upućuju na to da visoka proizvodnja mlijeka negativno utječe na antioksidacijsku vrijednost mlijeka.

Ključne riječi: mlijeko; vitamini; bjelančevine; antioksidacijski potencijal; laktacija

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Praca 3

Magdalena Stobiecka, Jolanta Król, Aneta Brodziak, Renata Klebaniuk, Edyta Kowalczuk-Vasiliev. Effects of supplementation with an herbal mixture on the antioxidant capacity of milk. Animals, 2023, 13, 2013. DOI: 10.3390/ani13122013

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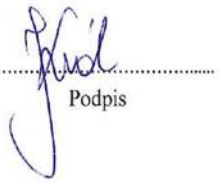

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Effects of Supplementation with an Herbal Mixture on the Antioxidant Capacity of Milk

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Simple Summary: The content of antioxidant components and the antioxidant potential of milk can be modified through animal nutrition, i.e., with the inclusion of various natural additives, e.g., herbs, seeds, or byproducts, to the feed ration. The aim of this study was to assess the effect of the addition of a standardized herbal mixture (oregano, common thyme, purple coneflower, and cinnamon bark) to the feed ration for Holstein-Friesian cows on the antioxidant capacity of milk. This study demonstrated the potential of the herbal blend to increase the content of bioactive ingredients with antioxidant properties in milk, i.e., whey proteins (β-lactoglobulin, lactoferrin) and lipophilic vitamins (A, E). The value of the antioxidant potential of milk increased as well; from a nutritional point of view, this seems to be of particular importance for the protection of the organism against the harmful effects of oxidative stress.

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Abstract: The aim of this study was to assess the effect of the addition of a standardized herbal mixture to the feed ration for Holstein-Friesian cows on the antioxidant capacity of milk. The study was carried out on a farm specialized in breeding dairy cattle. The exact study involved 30 cows in lactation III, which were in the first phase of lactation at the beginning of the experiment (15 cows—control group; 15 cows—experimental group). The nutrition supplied to the cows was based on the TMR (total mixed ration) system, with roughage and concentrate fodder used as the basis of the feed ration. The addition of a standardized blend of dried herbs, i.e., oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), purple coneflower (*Echinacea purpurea*), and cinnamon bark (*Cinnamomum zeylanicum*), was the experimental factor. Powdered herbs were administered as a component of the concentrate fodder at the dose of 3% DM ration/day/head. Milk samples were collected four times during the experiment (term 0 after the colostrum period and then after lactation weeks 2, 4, and 6). The following parameters were determined in the milk: the basic chemical composition, i.e., the content of total protein, fat, lactose, and casein; somatic cell count; content of selected whey proteins (α-lactalbumin, β-lactoglobulin, lactoferrin, BSA); and fat-soluble vitamins (A, D_3, E). Additionally, the milk antioxidant capacity (ABTS, FRAP, DPPH) was determined and the degree of antioxidant protection (DAP) was calculated. It was shown that the milk from cows receiving the herbal blend-supplemented fodder had a higher content of casein, compared to the control group. The herbal supplementation contributed to a significant increase in the content of bioactive compounds, i.e., selected whey proteins (β-lactoglobulin, lactoferrin) and lipophilic vitamins (A, E). The milk was also characterized by significantly higher antioxidant potential (regardless of the measurement method) and a higher degree of antioxidant protection (DAP).

Keywords: milk; Holstein-Friesian cows; herbal mixture; supplementation; antioxidant capacity

1. Introduction

The content of antioxidant components in milk and its antioxidant potential can be modified through animal nutrition, e.g., inclusion of various natural additives to feed rations. The dairy cow nutrition is most often supplemented with herbs. With their high content of biologically active substances, herbs exert a positive effect on the cow organism, which in turn is reflected in the quality of milk [1,2]. Of note, feed additives used in cattle nutrition can also serve protective functions and act as metabolic regulators [3,4]. Consequently, they contribute to an increased resistance of animals exposed to stress (changes in the feed ration, thermal stress), enhance the absorption of essential nutrients, and increase the degree of antioxidant protection [3,5]. This aspect is particularly important in high-yielding animals, which are characterized by reduced immunity and, consequently, increased susceptibility to diseases [6,7]. Pasture grazing significantly increases the content of antioxidant compounds in milk, thus increasing its antioxidant potential [8–12]. Improvement is more difficult to achieve when cows are fed with preserved fodder, especially silage [13–15]. As reported by Nielsen et al. [13], a higher proportion of maize silage in cow rations is one of the causes of the lower content of vitamins and antioxidants in milk. In turn, Alves et al. [14] have found that maize silage, which is most often used in cow nutrition, has a low content of carotenoids.

It should be emphasized that herbs are a natural source of antioxidants [16]. These primarily include carotenoids (xanthophylls and carotenes) and polyphenols (flavonoids, anthocyanins, phenolic acids, stilbenes, and lignans), as well as alkaloids, terpenes, saponins, and essential oils [17–19]. Already at the stage of storage, they protect fodder against spoilage through inhibition of the oxidation process [20]. Given their antioxidant properties, the following herbs are most often used in animal nutrition: oregano (*Origanum vulgare*), cinnamon (*Cinnamonum cassia*), rosemary (*Salvia rosmarinus*), thyme (*Thymus vulgaris*), turmeric (*Curcuma longa*), cumin (*Carum carvi*), ginger (*Zingiber officinale*), stinging nettle (*Urtica dioica*), and purple coneflower (*Echinacea purpurea*) [2,21–24]. Even their low concentrations in feed mixtures have an impact on the antioxidant indices in lactating dairy cows. Animals whose diets were supplemented with herbs exhibited an increase in the activation of antioxidant enzymes in both blood and milk, which play an important role in cell protection against oxidative damage [25–27]. Many studies have demonstrated that herbs used in the diet for ruminants have a beneficial effect, as they improve the feed conversion efficiency, modify the rumen microflora, and, consequently, improve animal health and performance [27–33]. Better production results are achieved upon application of herbal blends rather than individual herbs, mainly due to the synergistic effect of their active compounds [34]. Importantly, bioactive plant compounds are highly resistant to microbial degradation in the rumen and do not lose their functionality [35].

Modern consumers are becoming increasingly aware of the important role of antioxidant compounds in supporting and strengthening the defense mechanisms in the organism, which is essential for prevention of such lifestyle diseases as cardiovascular diseases, cancer, diabetes, and obesity. Extremely valuable in this respect are antioxidant compounds derived from natural sources. Similarly, milk is a source of antioxidants. They are mainly contained in the protein fraction (β-lactoglobulin (β-Lg), lactoferrin) and in the fat fraction (vitamins E and A, β-carotene) [36–39].

To meet consumer expectations, nutritional solutions for full optimization of the production potential of cows and improvement of the composition of their milk are being sought. Therefore, the present research was undertaken to assess the influence of supplementation of feed rations for Holstein-Friesian cows with a dedicated phytobiotic-rich herbal mixture on the level of antioxidant capacity of milk.

2. Materials and Methods

2.1. Experimental Material

This study was carried out on a farm specialized in breeding dairy cattle of the Holstein-Friesian breed. The experiments were approved by the 2nd Local Ethics Committee for

Animal Testing in Lublin (Resolution No 36/2011 of 7 June 2011). During this research, the herd comprised 86 dairy cows with an average milk yield of 7860 kg. The exact study involved 30 cows in lactation III selected from the herd, which were in the first phase of lactation at the beginning of the experiment (15 cows—control group; 15 cows—experimental group) (Table 1). The average body weight of the cows was 655 kg.

Table 1. Experimental design.

Experimental Design	Group	
	C	E
6 weeks	RO + CF	RO + CF + HM (3%)
number of cows	15	15

C—control group cows receiving a standard ration based on roughage used on the farm with the addition of a mixture of concentrate fodder. E—experimental cows receiving a standard ration based on roughage with the addition of a mixture of concentrate fodder and 3% of the experimental herbal mixture (HM). RO—roughage. CF—concentrate fodder mixture: triticale—35%, oats—20%, barley—16%, maize grain—14%, post-extraction rapeseed meal—2.5%, commercial supplementary mixture—10%, fodder chalk and mineral–vitamin additives—0.5%. HM—standardized herbal mixture: thyme 25%, purple coneflower 35%, oregano 25%, and cinnamon 15%.

The nutrition supplied to the cows was based on the TMR system (Table 2). The basis of the feed ration included roughage (RO): maize silage, grass haylage, beet pulp silage, wheat straw, and concentrate fodder (CF) as required. The experimental factor was the addition of a standardized blend of dried herbs: oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), purple coneflower (*Echinacea purpurea*), and cinnamon bark (*Cinnamomum Zeylanicum*). The formula of the herbal mixture selected for this study was developed during investigations carried out at the Institute of Animal Nutrition and Bromatology, University of Life Sciences in Lublin. Initially, this research covered several dozen species of herbs, e.g., purple coneflower (*Echinacea purpurea*), oregano (*Origanum vulgare*), thyme (*Thymus vulgaris*), cinnamon (*Cinnamomum*), common garlic (*Allium sativum*), licorice (*Glycyrrhiza glabra*), and caraway (*Carum carvi*). Next, the composition of the mixture was established based on the analyses of the chemical composition and content of biologically active substances in the herbs with consideration of the synergistic and antagonistic effects of compounds contained in the herbal raw materials. In addition to the basic composition, the content of essential oils in the experimental blend was determined with the method of 1-h steam distillation in the Derying apparatus. The distillation process was carried out with the addition of 400 mL of demineralized water. Samples of essential oils were diluted 100-fold with hexane (10 µL of the sample + 990 µL of the solvent) prior to further analysis. Subsequently, the diluted essential oils were subjected to chromatographic analysis using the GC–MS system (Shimadzu GCMS-TQ8040, Kioto, Japan) in the Scan mode.

Table 2. Composition of TMR administered to the experimental cows.

Item	Beet Pulp Silage	Maize Silage	Haylage	Wheat Straw	Concentrate Mixture	Herb-Supplemented Concentrate Mixture	Total
Control TMR							
kg DM/d/head	1.60	7.39	3.65	0.44	4.73	-	17.81
Share (%)	9.0	41.5	20.5	2.5	26.5	-	100
Experimental TMR							
kg DM/d/head	1.60	7.39	3.65	0.44	-	1.95	18.03
Share (%)	8.9	41.0	20.2	2.4		27.5	100

Powdered herbs were administered to the cows from the experimental group as a component of the concentrate fodder at the dose of 3% DM ration/day/head. Milk samples were collected 4 times during the experiment (after the colostrum period and then after lactation weeks 2, 4, and 6).

2.2. Analysis of the Chemical Composition and Nutritional Value of Fodder

The content of dry matter, total protein, crude fiber and its fractions, crude fat, and crude ash was determined in the feed samples in accordance with the currently applicable standards [40]. The content of nitrogen-free extractives (NFE) was calculated mathematically. Additionally, the content of some plant secondary metabolites was determined in the concentrate fodder and the herb-supplemented feed [41]. The nutritional value of the feed and rations was estimated based on the results of the basic analysis of the feed with the use of the Winwar 1.6 computer program [42], and the nutritional values were expressed in INRA units [43] (Table 3).

Table 3. Chemical composition and nutritional value of basic fodders.

Parameter	Type of Feed					
	Beet Pulp Silage	Maize Silage	Haylage	Wheat Straw	Concentrate Mixture	Herb-Supplemented Concentrate Mixture
Dry matter (%)	21.3	26.4	30.4	87.5	87.6	88.4
Content in 1 kg dry matter (g)						
Crude protein	112.1	80.9	161.0	35.0	137.1	136.8
Crude fat	8.8	27.1	33.5	15.5	23.4	23.0
Crude fiber	178.4	200.8	231.7	397.0	41.3	41.9
Ash	70.5	38.1	59.2	42.0	34.5	35.2
NFE	630.2	653.1	514.6	510.5	763.7	763.1
Content of biologically active component (%)						
Linalool	-	-	-	-	-	8.32
Cymene	-	-	-	-	-	7.02
Thymol	-	-	-	-	-	5.83
Carvone	-	-	-	-	-	2.31
Carvacrol	-	-	-	-	-	1.13
Nutritive value of 1 kg DM						
UFL	1.01	0.90	0.80	0.30	1.19	1.19
PDIN	60.0	78.0	88.0	30.0	89.9	89.9
PDIE	84.0	73.0	66.0	40.0	91.9	91.7
LFU	1.10	1.24	1.20	1.30	-	-

NFE—nitrogen-free extractives. UFL—unit for lactation. PDIE—the sum of microbial protein that could be synthesized in the rumen from available energy and the dietary protein undegraded in the rumen but truly digestible in small intestine. PDIN—the sum of microbial protein that could be synthesized in the rumen from available N and the dietary protein undegraded in the rumen but truly digestible in small intestine. LFU—fill unit for lactating cows.

2.3. Analyses of Milk

The basic chemical composition in each milk sample, i.e., the content of total protein, fat, lactose, and dry matter, was determined using the Infrared Milk Analyzer (Bentley Instruments, Chaska, MN, USA).

2.3.1. Determination of Protein

The content of casein was determined according to AOAC [44]. However, the concentration of selected whey proteins, i.e., α-lactalbumin (α-La), β-lactoglobulin (β-Lg), lactoferrin, and bovine serum albumin (BSA), was determined using reversed-phase high-performance liquid chromatography according to the methodology described by Brodziak et al. [45]. All samples for the whey protein determinations were prepared as in Romero et al. [46] with modifications of Brodziak et al. [45].

2.3.2. Determination of Vitamins

The reversed-phase high-performance liquid chromatography (RP-HPLC) was also used to determine the concentration of fat-soluble vitamins, i.e., A, D_3, and E. All samples were

prepared with the Röse-Gottlieb fat extraction method modified by Hewavitharan et al. [47]. The separations and identifications were conducted according to Brodziak et al. [11].

2.3.3. Determination of Antioxidant Capacity

The antioxidant capacity of the milk was determined with three methods, i.e., FRAP by Benzie and Strain [48], DPPH by Brand-Williams et al. [49], and ABTS by Sahin et al. [50]. The samples for the analysis were extracted from fresh milk, i.e., 10 mL of the solvent was added to 1 mL of milk (1 M HCl solution in 95% ethanol in a volume ratio of 15/85), shaken for 1 h at 40 °C in a rotary shaker at 300 rpm/min, and centrifuged in a centrifuge at 8000× *g* (MPW-350, Med. Instruments, Warsaw, Poland) for 10 min. The extract was used to determine the antioxidant activity.

FRAP Assay. The ferric reducing antioxidant power (FRAP) assay was conducted according to the method developed by Benzie and Strain [48]. The method is based on the reduction of the Fe^{3+}-TPTZ (ferric tripyridyl triazine) complex to Fe^{2+}-TPTZ at low pH. The reduction is reflected by appearance of the blue color. The absorbance readings were taken using a UV-Vis spectrophotometer (U-2900 HITACHI, Tokyo, Japan) at 593 nm. Different concentrations of Trolox were used as standards for calibration. The results were expressed as milligrams of Trolox equivalents (TE) per 100 mL of sample.

DPPH Assay. The DPPH assay was carried out as described by Brand-Williams et al. [49]. A total of 0.50 mL of the extract was transferred into a test tube, and 3.0 mL of a 6×10^{-5} M methanolic DPPH (2,2-diphenyl-1-picrylhydrazyl) solution was added. The absorbance readings were taken using a UV-Vis spectrophotometer (U-2900 HITACHI, Tokyo, Japan) at 515 nm against a reagent blank after being kept in the dark for 30 min. Different concentrations of Trolox were used as standards for calibration. The results were expressed as the amount of DPPH radical-reducing antioxidant compounds contained in a 100 mL sample equivalent to milligrams of TE after 60 min of reaction.

ABTS Assay. The total antioxidant capacity of the samples was determined using the radical cation $ABTS^{+}$ (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic)) decolorization method [50]. The determination method consists in the neutralization of the blue cation radical ABTS, which is manifested with a decrease in the absorbance of the solution. The absorbance was recorded using a UV-Vis Spectrophotometer (Cary 100, Varian, Palo Alto, CA, USA) at 734 nm against a reagent blank. A standard curve was prepared using different concentrations of Trolox, and the results are expressed as milligrams of TE equivalents per 100 mL of sample.

2.3.4. Determination of Cholesterol

The total cholesterol content was determined with the colorimetric enzymatic method with cholesterol esterase and cholesterol oxidase using the Liquick Cor-CHOL kit (Cormay, Łomianki, Poland).

2.3.5. Determination of Degree of Antioxidant Protection

Additionally, the degree of antioxidant protection (DAP) was calculated according to Pizzoferrato et al. [51]. The DAP proposed as the tracing parameter was calculated as the molar ratio between antioxidant compounds and a selected oxidation target [51]:

$$\mathrm{DAP} = \frac{\sum_{n=1}^{n} \mathrm{AC}_1\,(\mathrm{no.moles})}{\mathrm{OT}\ (\mathrm{no.moles})}$$

2.3.6. Statistical Analysis

The results were analyzed statistically using a one- and multi-way analysis of variance in StatSoft Inc. Statistica ver. 13 (Dell, Round Rock, TX, USA). Significant differences between the means were determined using Tukey's test at the 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. Milk Yield and Basic Physico-Chemical Parameters in Milk

There was no significant effect of the addition of the herbal blend on the milk yield; however, the amount of milk produced by the cows from the experimental group was higher by 2.4 kg per day (Table 4). According to many authors [4,28,52,53], higher milk production after herbal supplementation may be attributed to the galactopoietic effect of the active compounds present in the essential oils. The herbal essential oils can act as galactagogues by enhancing prolactin production and releasing somatotropins, resulting in increased glucose levels in the udder and improved milk production. The milk in the experimental group was characterized by higher levels of the basic components (dry matter, protein, fat, lactose); however, the differences were not statistically significant. For milk yield and protein content, a significant ($p \leq 0.05$) effect of the week was found. In turn, after supplementation of cow nutrition with the oregano extract (10 g/d), Kolling et al. [54] found no significant effect of this additive on the milk yield and the content of the majority of the milk components. A decrease in the milk fat content was the only effect of the supplementation. In a subsequent study conducted by Kolling et al. [25], extracts of oregano and green tea administered separately or in combination exerted a clear effect on feed intake, milk yield, and antioxidant indices in lactating dairy cows. In turn, Benchaar [55] reported that oregano oil and carvacrol administered at a dose of 50 mg/kg of dry matter in the diet did not have a beneficial effect on rumen fermentation and did not improve nutrient conversion or milk yields. The addition of 10 g of oregano extract per day in cow nutrition resulted in a significant ($p \leq 0.05$) reduction in SCC [26]. As suggested by the authors, this was associated with the antibacterial properties of oregano; its main components, carvacrol and thymol, have an impact on the properties of the cytoplasmic membrane and can inhibit bacterial adhesion to epithelial cells. In turn, no effect of the plant extracts on the milk yield and basic composition was recorded [26]. Similar conclusions were formulated by Olijhoek et al. [56] in a study on dried oregano supplementation in cow nutrition. Similar to the present study, Węglarzy et al. [57] did not find a significant effect of supplementation with fresh purple coneflower and caraway herbs on the cow milk yield. Nevertheless, the supplementation contributed to a significant increase in the content of protein and fat in the milk. In turn, as reported by Ghafaria et al. [58], the addition of cumin seeds (*Cuminum cyminum*) improved the milk yield, with a slight decrease in the fat content and cholesterol levels. In the present study, the milk from the experimental group was characterized by a lower cholesterol level, but the differences were not significant. A 25% decrease in the level of total cholesterol was found after supplementation of cow feed rations with a mixture of stinging nettle, dandelion, cumin, and chamomile herbs [59]. Cumin supplementation at a dose of 10 g/day in the diet for lactating Mehsana goats improved the milk yield, nutrient digestibility, and feed conversion rates without adverse effects on hematobiochemical parameters [60]. Nurdin et al. [61] showed that herbal supplementation (black cumin, turmeric) resulted in a decrease in the somatic cell count and significantly increased ($p \leq 0.01$) the milk yield and protein and lactose levels. The addition of lemongrass and peppermint to feed improved the health of cows and increased the production performance of both dairy and beef cattle [62,63]. Similarly, Kraszewski et al. [28] reported a positive effect of administration of herbal blends (chamomile, yarrow, agrimony, stinging nettle, ribwort plantain, St. John's wort, and lady's mantle) on cows. The authors noted an increase in the technological parameters of milk and a significant decrease (by 232,000/mL) in the somatic cell count in the experimental group. In turn, significantly ($p \leq 0.05$) higher milk yields in a group of cows receiving herbal blends (60% rosemary, 18% cinnamon bark, 18% turmeric, and 4% clove buds) compared with the control group were reported by Hashemzadeh-Cigari et al. [4]. The supplementation with the blend reduced the fat content in the milk ($p < 0.01$) but did not have an effect on the levels of other milk components, i.e., protein and lactose. After rosemary extract supplementation, Kong et al. [64] observed a significant increase in the milk yield and lactose content accompanied by a decrease in the somatic cell count. Kuczyńska et al. [65]

carried out a study on four certified organic farms and found beneficial effects of herbal blends (oregano, caraway, and rosemary) on the health of cows and improvements in the nutritional quality of milk. Supplementation of water buffalo rations with herbal blends (black pepper ginger, cinnamon, peppermint, ajwain, and garlic; 20 g/day) had no effect on the milk yield [34]. Nevertheless, the blend was shown to have potential to increase the content of milk fat and unsaturated fatty acids.

Table 4. Yield and basic chemical composition of milk.

Parameter	Control Group	Experimental Group	*p*-Value	
			Treatment	Week
Milk yield (kg)	26.8 ± 2.9	29.2 ± 5.1	ns	0.039
Dry matter (%)	13.09 ± 0.20	13.19 ± 0.44	ns	ns
Protein (%)	3.47 ± 0.52	3.51 ± 0.62	ns	0.042
Casein (%)	2.75 [a] ± 0.41	2.91 [b] ± 0.54	0.048	ns
Fat (%)	4.27 ± 0.89	4.30 ± 0.87	ns	ns
Lactose (%)	4.65 ± 0.17	4.72 ± 0.13	ns	ns
Cholesterol (mg/L)	369.34 ± 34.76	293.58 ± 55.34	ns	ns
SCC (thousand/mL)	178 ± 220	172 ± 180	ns	ns

[a, b]—significant differences at $p \leq 0.05$. ns—not statistically significant.

The milk from the experimental group contained significantly ($p \leq 0.05$) more casein, a quantitatively dominant cow milk protein with great importance for the dairy industry. The supplementation contributed to an over 5% increase in the level of this protein, i.e., from 2.75 to 2.91%. It should be emphasized that the presence of the herbal mixture in the feed ration of the experimental group of cows did not change the level of protein supplied in the fodders (PDIN and PDIE values in the concentrate fodder and herb-supplemented concentrate fodder were very close—Table 3). The amount of microbial protein produced in the rumen passing to further sections of the gastrointestinal tract (small intestine) is conditioned by the synchronization of the speed of the fermentation process of carbohydrates in the rumen, especially easily digestible ones, with the rate of decomposition of feed protein [53,66]. However, these carbohydrates increase the formation of propionic acid in the rumen, which stimulates the secretion of insulin, which may affect the uptake of amino acids by the mammary gland [67]. It can, therefore, be assumed that the provision of specific compounds contained in herbs affects the change in amino acid protein synthesis in the rumen (synthesis of microorganisms), as well as the availability of specific amino acids in the small intestine for the synthesis of milk proteins.

3.2. Bioactive Proteins in Milk

The use of phytobiotic-rich herbs has an impact on the health-promoting value of milk, which is mainly associated with the activity of phenolic compounds, i.e., flavonoids characterized by strong antioxidant and antibacterial properties [17]. The milk samples from the experimental group contained significantly higher amounts of whey proteins (by 0.09 p.p.) than the milk from the control group (Table 5). The main part of whey proteins is constituted by albumins, i.e., α-lactalbumin, β-lactoglobulin, and bovine serum albumin, the so-called blood serum albumin. Higher levels of these proteins were detected in the milk of the experimental group, with significant differences found only in the β-Lg level ($p \leq 0.05$). It should be emphasized that whey proteins, in particular β-Lg, have the highest antioxidant potential of all proteins in the human diet.

This is associated with the high content of sulfur amino acids, especially cysteine, which is indispensable for the synthesis of glutathione [68,69]. Antioxidant activity is also exhibited by lactoferrin, as it chelates iron, thereby enhancing its bioavailability and inhibiting its pro-oxidative activity. The raw material produced by the cows from the experimental group was a richer source of this protein; its level was on average 30% higher ($p \leq 0.01$) than in the control group. It also contained a 20% higher amount of lysozymes ($p \leq 0.05$). Of note, lactoferrins and lysozymes play a significant role in the protection of

the organism, as they are one of the most important components of non-specific immune mechanisms [70]. The tendencies observed in the present study are in agreement with the results reported by Kuczyńska et al. [65]. Already after the first week of application of herbal blends (oregano, rosemary, and cumin) to cow feed rations, the levels of whey proteins in milk were found to increase. In a study conducted by Klebaniuk et al. [71], herbal blends (thyme, oregano, cinnamon, and purple coneflower) administered to cows during the dry period exerted a beneficial effect on the quality of colostrum (increased content of immunoglobulins). In turn, Reklewska et al. [72] reported a higher level of lactoferrin in milk after supplementation of feed rations with the purple coneflower (*Echinacea purpurea*) herb. The content of the protein increased significantly ($p < 0.01$) during the administration of the supplement and after cessation of the supplementation (over 14 days). As suggested by the authors, the immunostimulating effect of the purple coneflower herb was associated with the presence of caffeic acid derivatives, which stimulate the activity of immune cells, have antiviral activity, and are strong antioxidants.

Table 5. Content of selected bioactive proteins in milk.

Parameter	Control Group	Experimental Group	*p*-Value	
			Treatment	Week
Whey proteins (%)	$0.64^{a} \pm 0.021$	$0.73^{b} \pm 0.064$	0.023	ns
β-lactoglobulin (g/L)	$2.99^{a} \pm 0.20$	$3.14^{b} \pm 0.23$	0.032	ns
α-lactalbumin (g/L)	0.95 ± 0.12	1.03 ± 0.11	ns	ns
Bovine serum albumin (g/L)	0.33 ± 0.06	0.36 ± 0.09	ns	ns
Lactoferrin (mg/L)	$103.77^{A} \pm 8.36$	$136.19^{B} \pm 11.19$	0.008	ns
Lysozyme (µg/L)	$5.62^{a} \pm 0.86$	$6.92^{b} \pm 0.99$	0.021	ns

$^{a, b}$—significant differences at $p \leq 0.05$; $^{A, B}$—significant differences at $p \leq 0.01$. ns—not statistically significant.

3.3. Lipophilic Vitamins in Milk

The results shown in Table 6 indicate a significant effect of the supplementation with the herbal blend on the content of lipophilic vitamins in milk. A higher level of these vitamins was found in the milk of the experimental group, with significant differences noted in the case of vitamin A ($p \leq 0.05$) and E ($p \leq 0.01$). In comparison with the control group, the milk from the experimental group contained approximately 30% and 40% higher contents of vitamins A and E, respectively. It should be emphasized that fat-soluble vitamins, primarily E and A, are the main antioxidants derived from the fat fraction protecting cells against reactive oxygen species [36,39]. Their activity consists of scavenging organic free radicals and inhibiting lipid peroxidation [73]. Similarly, Kuczyńska et al. [65] reported an increase in the content of vitamins E and A in milk fat after supplementation with a mixture of oregano, cumin, and rosemary. The concentration of vitamin E was found to increase on average by 50% after the 21-day supplementation period. These changes are explained by the authors by the content of phytobiotics and better utilization of dietary ingredients. Changes in the content of vitamins in milk found in our own research may also be a consequence of changes taking place in the rumen of ruminants, as all cows received the same mineral–vitamin additives. The observed phenomenon is a complex issue, simultaneously mobilizing for further research. Leiber et al. [74], however, showed that cows grazing on alpine pastures produced milk containing 86% higher levels of vitamin E than milk from cows fed with preserved fodder.

Table 6. Content of selected bioactive proteins in milk.

Parameter	Control Group	Experimental Group	*p*-Value	
			Treatment	Week
Vitamin A (mg/L)	$0.354^{a} \pm 0.004$	$0.503^{b} \pm 0.005$	0.018	ns
Vitamin E (mg/L)	$1.276^{A} \pm 0.38$	$2.06^{B} \pm 0.28$	0.006	ns
Vitamin D_3 (µg/L)	0.66 ± 0.16	0.75 ± 0.14	ns	ns

$^{a, b}$—significant differences at $p \leq 0.05$; $^{A, B}$—significant differences at $p \leq 0.01$. ns—not statistically significant.

3.4. Antioxidant Capacity of Milk

The modification of the cow's diet significantly increased the antioxidant potential of milk, regardless of the determination method used (FRAP, DPPH, ABTS). The raw material obtained from the experimental group of cows was characterized by significantly higher antioxidant potential than the control (Figure 1a–c). In comparison with the control, the DPPH and ABTS values increased by approximately 50%. In the FRAP assay, the milk exhibited approximately 20% higher Fe^{2+} chelation capacity. The higher antioxidant activity of these milk samples relative to the control was associated with the introduction of natural antioxidants, i.e., phenolic compounds, together with the herbal blend. Plant extracts may contribute to an increase in endogenous antioxidants and free radical scavenging [35]. In a study conducted by Paraskevakis [75], oregano supplementation of the diet for goats induced a significant ($p < 0.001$) increase in the antioxidant value of milk (expressed as FRAP). In another study by Uegaki et al. [76], the diet for Holstein cows was supplemented with three herbs, i.e., lemongrass, peppermint, and basil, for 14 days. A significant increase in the antioxidant activity of milk, compared to the control, was noted in all variants. As suggested by Yilmaz-Ersan et al. [69], the differences in the total antioxidant potential of milk can be explained by the differences in its chemical composition. Of note, the milk from the experimental group analyzed in the present study contained a higher level of antioxidants, hence the increase in its antioxidant potential. An increase in the TAS level following supplementation with herbs was reported by Kuczyńska et al. [65]. The antioxidant potential of milk from cows receiving phyto-additives increased significantly. An almost three-fold increase in the level of TAS, which indicates an increase in the degree of antioxidant protection, was detected. In a study conducted by Qingru et al. [77], the introduction of a Chinese herbal formula in cow nutrition resulted in an over 40% ($p < 0.01$) increase in the total antioxidant capacity of milk and an over 20% ($p < 0.01$) decline in the malondialdehyde (MDA) levels. As demonstrated by Zhang and Zhao [78], 400 and 600 mg/kg doses of the Chinese herb extract supplemented in cow nutrition may have a beneficial effect on milk production through improvement of animal health and enhancement of antioxidant activity. Various studies indicate that pasture grazing as part of cow nutrition has a significant impact on the content of lipophilic antioxidants in milk and, consequently, its antioxidant activity [39,79]. Santa et al. [80] reported that milk from grazing Jersey cows versus those fed TMR diets contained significantly higher levels of α-tocopherol (0.74 vs. 0.27 mg/100 g), retinol (125.62 vs. 57.51 µg/100 g), and β-carotene (0.69 vs. 0.41 µg/100 g), which was reflected in the higher antioxidant activity of the milk (3.02 vs. 2.53 µmol TE/mL).

3.5. Degree of Antioxidant Protection of Milk

For determination of product quality, Pizzoferrato et al. [51] proposed that the degree of antioxidant protection (DAP) should be calculated as a molar ratio between antioxidant compounds and oxidants. In milk, vitamins E and A are the antioxidant compounds and cholesterol is the target molecule for oxidation. Products of cholesterol oxidation exert an adverse effect on human health, as they can be absorbed in the gastrointestinal tract into the bloodstream, thus increasing the likelihood of the development of cardiovascular diseases and atherosclerosis [81,82]. It should be emphasized that the negative impact of cholesterol depends on the degree of antioxidant protection of a given product [83]. The higher the DAP value, the higher the oxidation stability of the product. The milk of cows receiving the herbal blend-supplemented fodder exhibited a significantly higher degree of antioxidant protection (DAP) (Figure 2). According to Pizzoferrato et al. [51], milk from pasture-grazed animals has a higher degree of antioxidant protection than milk produced by non-grazed animals, which indicates that green fodder is a rich source of antioxidants, protecting cholesterol against oxidative reactions. As reported by Puppel et al. [12], the highest values of the degree of antioxidant protection (DAP) and total antioxidant status (TAS) were determined in a system with pasture grazing as the basis of cow nutrition. Nevertheless, the addition of maize grain silage was shown to increase

the degree of antioxidant protection through an increase in the content of vitamin E in milk. As suggested by the authors, DAP and TAS should be considered as biomarkers of antioxidant changes in milk. Higher levels of both antioxidant protection and total antioxidant potential contribute to higher product stability and quality.

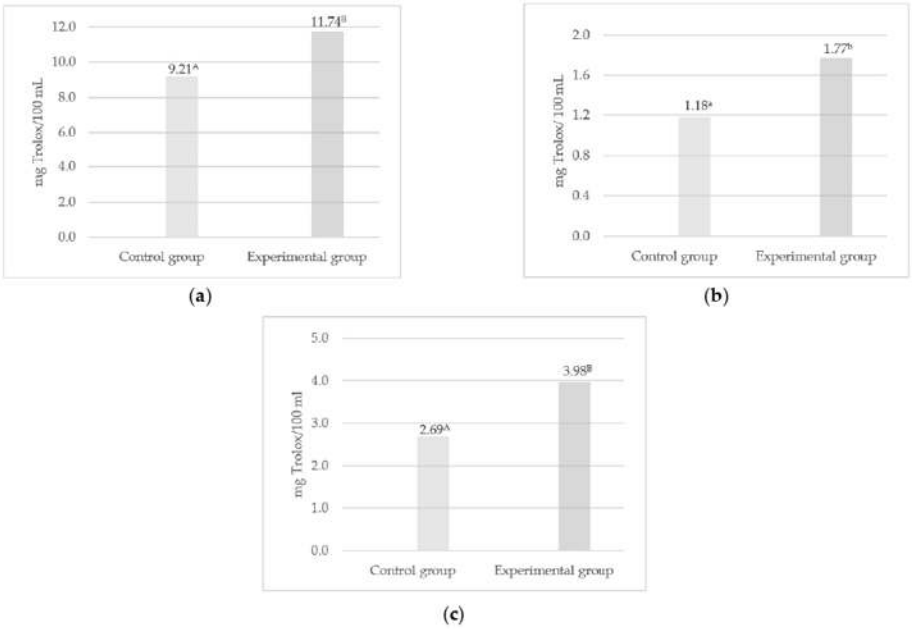

Figure 1. Antioxidant activity of milk expressed in mg of Trolox equivalent per 100 mL of sample. (**a**) FRAP—ferric reducing antioxidant power assay, (**b**) DPPH—2,2-diphenyl-1-picrylhydrazyl assay, (**c**) ABTS—2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assay. $^{a, b}$—significant differences at $p \leq 0.05$; $^{A, B}$—significant differences at $p \leq 0.01$.

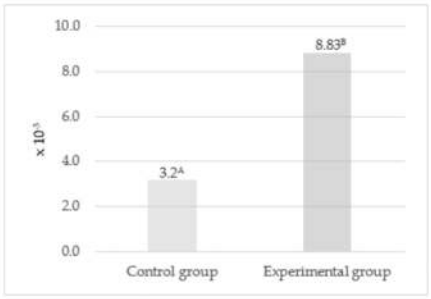

Figure 2. Degree of antioxidant protection of milk. $^{A, B}$—significant differences at $p \leq 0.01$.

4. Conclusions

The herbal mixture supplementation of cow nutrition had no significant effect on the yield and basic chemical composition of the milk. Of note, significant differences in the

content of casein, a protein of high importance for the dairy industry, were observed in favor of the experimental group. The blend was shown to increase the content of bioactive ingredients with antioxidant properties in the milk. The supplementation resulted in a significant increase in the content of selected whey proteins (β-lactoglobulin, lactoferrin) and lipophilic vitamins (A, E). The antioxidant capacity of milk increased as well; from a nutritional point of view, this seems to be especially important for the protection of the organism against harmful effects of oxidative stress. Importantly, the milk of cows receiving the herbal blend-supplemented fodder exhibited a significantly higher degree of antioxidant protection (DAP).

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Praca 4

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Lublin, 27.09.2023

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Magdalena Stobiecka, **Jolanta Król**, Aneta Brodziak. Antioxidant potential of yoghurts produced from milk of cows fed fodder supplemented with herbal mixture with regard to refrigerated storage. Applied Sciences, 2023, 13, 10469. DOI: 10.3390/ app131810469.

mój udział polegał na: sformułowaniu koncepcji badawczej, zaplanowaniu układu doświadczalnego, analizie wyników, udziale w przygotowaniu wersji finalnej manuskryptu, zatwierdzeniu wersji finalnej manuskryptu, współtworzeniu odpowiedzi na recenzje, pełnieniu roli autora korespondencyjnego.

Wyrażam zgodę na wykorzystanie niniejszej publikacji w opracowaniu pt: „Wpływ wybranych czynników na potencjał antyoksydacyjny mleka pozyskiwanego od krów rasy holsztyńsko-fryzyjskiej i produktów wytwarzanych na jego bazie" stanowiącym rozprawę doktorską Pani mgr inż. Magdaleny Stobieckiej.

.....
Podpis

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

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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.

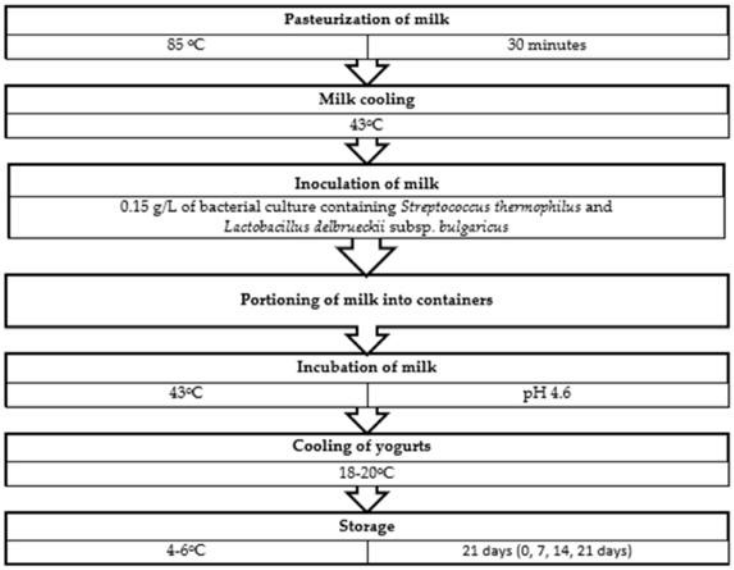


Figure 1. Scheme of production of yogurts (authors' scheme).

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 signifi-

cance 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	$3.02^{AX} \pm 0.26$	$4.03^{BX} \pm 0.23$	$4.98^{AY} \pm 0.28$	$6.87^{BY} \pm 0.86$
DPPH	$1.14^{aX} \pm 0.15$	$1.25^{bX} \pm 0.18$	$2.52^{AY} \pm 0.16$	$3.26^{BY} \pm 0.28$
FRAP	$8.97^{AX} \pm 1.13$	$13.1^{BX} \pm 2.05$	$17.13^{AY} \pm 0.65$	$22.58^{BY} \pm 0.98$

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].

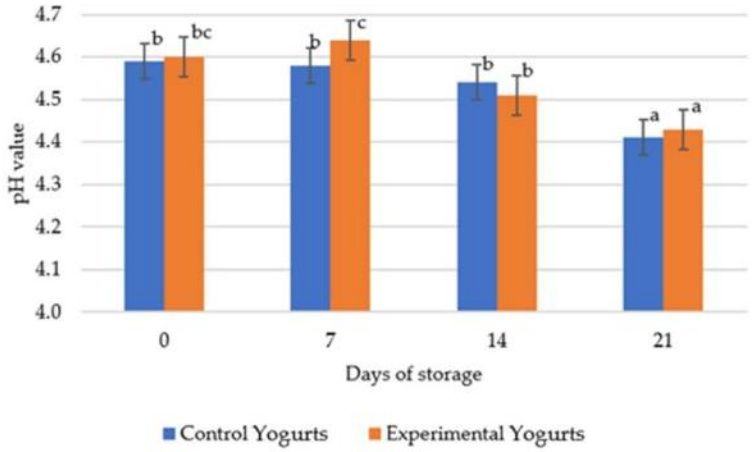


Figure 2. Changes in the pH value in the analyzed yogurts during 21 days of storage. a, b, c—significant differences between the day of storage within the yogurt type at $p \leq 0.05$.

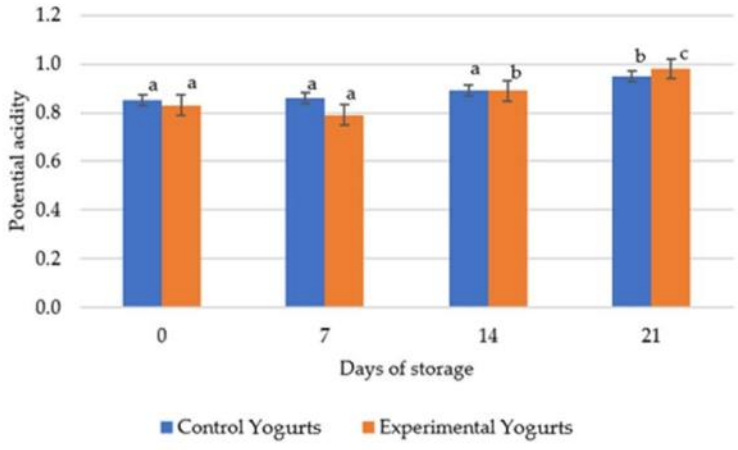


Figure 3. Changes in the lactic acid content (%) in the analyzed yogurts during 21 days of storage. a, b, c—significant differences between the days of storage within the yogurt type at $p \leq 0.05$.

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	$3.46^{b} \pm 0.19$	4.27 ± 0.17	$12.33^{B} \pm 0.19$
	7	0.942 ± 0.006	$3.44^{ab} \pm 0.11$	4.23 ± 0.11	$12.25^{B} \pm 0.26$
	14	0.954 ± 0.011	$3.38^{ab} \pm 0.14$	4.19 ± 0.14	$12.06^{AB} \pm 0.28$
	21	0.960 ± 0.008	$3.25^{a} \pm 0.16$	4.16 ± 0.15	$11.58^{A} \pm 0.31$
EY	0	0.943 ± 0.010	$3.52^{b} \pm 0.13$	4.30 ± 0.09	$12.38^{B} \pm 0.25$
	7	0.949 ± 0.007	$3.49^{ab} \pm 0.16$	4.28 ± 0.11	$12.31^{B} \pm 0.20$
	14	0.955 ± 0.009	$3.42^{ab} \pm 0.10$	4.25 ± 0.12	$12.15^{B} \pm 0.29$
	21	0.961 ± 0.012	$3.30^{a} \pm 0.15$	4.20 ± 0.10	$11.66^{A} \pm 0.17$

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

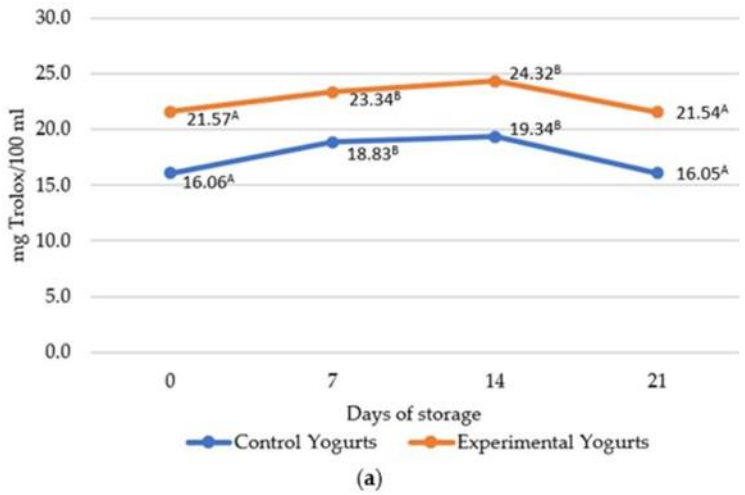
3.5. Basic Chemical Composition of Yogurts during 21 Days of Storage

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.*

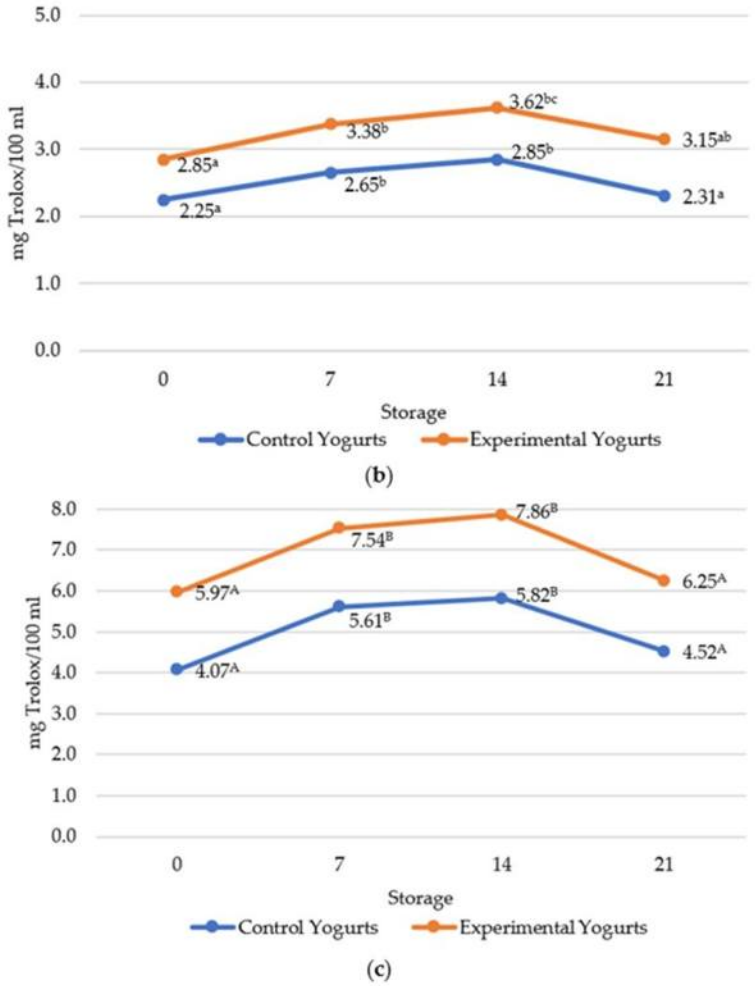


Figure 4. Antioxidant activity of yogurts during storage in mg of Trolox equivalent per 100 mL of sample (**a**) FRAP; (**b**) DPPH; (**c**) ABTS assay. a,b,c—significant differences at $p \leq 0.05$; A,B—significant differences at $p \leq 0.01$.

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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