Changes in nutritional content in raw milk of the Simmental breed cows fed with different forage combinations

Farklı yem kombinasyonları ile beslenen Simmental ırkı ineklerin çiğ sütünde besin içeriğindeki değişiklikler

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ABSTRACT

Objective: In this study, the biochemical compositions of fatty acids, amino acids, fat-soluble vitamins (ADEK), cholesterol and phytosterols were examined in raw milk samples of Simmental breed cows fed with different forage combinations.

Methods: Milk samples were provided from their farm in Kovancılar district of Elazığ province in Türkiye. The cows were fed in different forage combinations the spring season. These were Group 1: cows fed with Beet pulp+Factory feed+Hay (n: 10); Group 2: cows fed with Maize silage+Barley+Hay (n: 10), and Group 3: cows free-fed in pasture (n: 10).

Milk samples were taken on the 15th day of feeding and brought to the laboratory. Biochemical analyses were performed on the milk samples brought to the laboratory in accordance with the cold chain rules. After the milk samples went through a number of biochemical processes, fatty acid, ADEK vitamins, cholesterol and amino acid analyses were performed. The analysis of cholesterol and ADEK vitamins was performed with a high performance liquid chromatography (HPLC) device, and

ÖZET

Amaç: Bu çalışmada, farklı yem kombinasyonları ile beslenen Simmental ırkı ineklerin çiğ süt örneklerinde yağ asitleri, aminoasitler, yağda çözünen vitaminler (ADEK) ve fitosterollerin biyokimyasal bileşimleri incelenmiştir.

Yöntem: Süt örnekleri Elazığ iline bağlı Kovancılar ilçesindeki çiftlikten sağlanmıştır. İnekler bahar mevsiminde farklı yem kombinasyonlarında beslenmiştir. Bunlar; Grup 1: Pancar küspesi+Fabrika yemi+Saman ile beslenen inekler (n: 10); Grup 2: Mısır silajı+Arpa+Saman ile beslenen inekler (n: 10) ve Grup 3: Merada serbest beslenen inekler (n: 10) olarak gruplandırılmıştır.

Beslenmenin 15. gününde süt örnekleri alınarak laboratuvara getirilmiştir. Soğuk zincir şartlarına uygun olarak laboratuvara getirilen süt örneklerinin biyokimyasal analizleri yapılmıştır. Süt örnekleri birtakım biyokimyasal işlemlerden geçtikten sonra, yağ asidi, ADEK vitaminleri, kolesterol, fitosterol ve aminoasit analizleri yapıldı. Kolesterol ve ADEK vitaminlerinin analizi yüksek performanslı sıvı kromatografisi (HPLC) cihazı, aminoasit ve yağ asitlerinin analizi ise gaz

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analysis of amino acids and fatty acids was performed with gas chromatography (GC) and FID detector.

Results: 18 amino acids were detected in the milk samples examined. Compared to Group 1, it was found that the amounts of arginine (p<0.001), isolosine (p<0.05), methionine (p<0.05), threonine (p<0.05), phenylalanine (p<0.001), lysine (p<0.05) and tryptophan (p<0.001) amino acids increased statistically, while the amounts of alanine (p<0.001), glycine (p<0.05), leucine (p<0.05), aspartic acid (p<0.01) and glutamic acid (p<0.05) decreased statistically in Group 3. Compared to Group 1, it was found that the amounts of arginine (p<0.001), threonine (p<0.05), phenylalanine (p<0.001) and lysine (p<0.05) amino acids increased statistically, while the amounts of alanine (p<0.001), glycine (p<0.001), proline (p<0.01), aspartic acid (p<0.05), glutamic acid (p<0.001), histidine (p<0.001) and tryptophan (p<0.05) decreased statistically in Group 2. Compared to Group 1, the amounts of lauric acid (p<0.05), myristic acid (p<0.01), pentadecanoic acid (p<0.05), palmitic acid (p<0.01), stearic acid (p<0.001), oleic acid (p<0.001) and cholesterol (p<0.05) were statistically decreased, while the amounts of linoleic acid (p<0.001), alpha-linolenic acid (p<0.001), dihomo-gamma-linolenic acid (p<0.05), vitamin K2 (p<0.001), γ-tocopherol (p<0.001), vitamin D2 (p<0.001), α-tocopherol (p<0.001), α-tocopherol acetate (p<0.05), vitamin K1 (p<0.001), stigmasterol (p<0.01), β-sitosterol (p<0.05) were statistically increased in Group 3. Compared to Group 1, the amounts of stearic acid (p<0.001), oleic acid (p<0.01), α-tocopherol acetate (p<0.001) and β-sitosterol (p<0.05) were statistically decreased, while the amounts of vitamin K2 (p<0.001), γ-tocopherol (p<0.01), vitamin D2 (p<0.01), α-tocopherol (p<0.01), vitamin K1 (p<0.01) and stigmasterol (p<0.01) were statistically increased in Group 2. It was determined that the amounts of palmitic, oleic, myristic, stearic, lauric, linoleic, palmitoleic and pentadecanoic acids were found to be above 1% in the fatty acid composition of milk among the groups, respectively. It was determined that there were significant changes in the amount of some fatty acids among the groups. In addition, it was determined

kromatografi (GC) ve FID dedektörü ile yapılmıştır.

Bulgular: İncelenen süt örneklerinde 18 aminoasit tespit edilmiştir. Grup 1 ile karşılaştırıldığında, arginin (p<0,001), isolözin (p<0,05), metionin (p<0,05), threonin (p<0,05), fenilalanin (p<0,001), lizin (p<0,05) ve triptofan (p<0,001) aminoasitlerinin miktarlarının istatistiksel olarak arttığı, alanin (p<0,001), glisin (p<0,05), lösin (p<0,05), aspartik asit (p<0,01) ve glutamik asit (p<0,05) miktarlarının istatistiksel olarak Grup 3'te azaldığı tespit edilmiştir. Grup 1 ile karşılaştırıldığında, arginin (p<0,001), threonin (p<0,05), fenilalanin (p<0,001) ve lizin (p<0,05) aminoasitlerinin miktarlarının istatistiksel olarak arttığı, alanin (p<0,001), glisin (p<0,001), prolin (p<0,01), aspartik asit (p<0,05), glutamik asit (p<0,001), histidin (p<0,001) ve triptofan (p<0,05) miktarlarının istatistiksel olarak Grup 2'de azaldığı belilenmiştir. Grup 1 ile karşılaştırıldığında laurik asit (p<0,05), miristik asit (p<0,01), pentadekanoik asit (p<0,05), palmitik asit (p<0,01), stearik asit (p<0,001), oleik asit (p<0,001) ve kolesterol (p<0,05) miktarlarının istatistiksel olarak azaldığı, linoleik asit (p<0,001), alfa-linolenik asit (p<0,001) ve dihomo-gamma-linolenik asit (p<0,05), vitamin K2 (p<0,001), γ-tokoferol (p<0,001), vitamin D2 (p<0,001), α-tokoferol (p<0,001), α-tokoferol asetat (p<0,05), vitamin K1 (p<0,001), stigmasterol (p<0,01), β-sitosterol (p<0,05) düzeylerinin Grup 3'te istatistiksel olarak arttığı belirlendi. Grup 1 ile karşılaştırıldığında stearik asit (p<0,001), oleik asit (p<0,01), α-tokoferol asetat (p<0,001) ve β-sitosterol (p<0,05) miktarları istatistiksel olarak azalırken, vitamin K2 (p<0,001), γ-tokoferol (p<0,01), vitamin D2 (p<0,01), α-tokoferol (p<0,01), vitamin K1 (p<0,01) ve stigmasterol (p<0,01) miktarlarının istatistiksel olarak Grup 2'de arttığı görülmüştür. Gruplar arasında sütün yağ asidi bileşiminde sırasıyla,- palmitik asit, oleik asit, miristik asit, stearik asit, laurik asit, linoleik asit, palmitoleik asit ve pentadekanoik asit miktarlarının %1'in üzerinde bulunduğu saptanmıştır. Gruplar arasında bazı yağ asidi miktarlarında önemli değişimlerin olduğu görülmüştür. Ayrıca that there were significant changes in the amounts of cholesterol, phytosterol and some ADEK vitamins among the groups.

Conclusion: According to these data, it is seen that the use of different feed combinations in animal nutrition by dairy producers is very important for enriching the biochemical content of milk. It has been determined that the nutrient content of milk changes depending on different feed combinations and feeding in the flora. In addition, it has been found that the milk content of cows fed in pasture is more significantly enriched with nutrients necessary for a healthy life.

Key Words: Amino acids, chemical composition, dairy biochemistry, lipids, raw milk, vitamins

gruplar arasında kolesterol, fitosterol ve bazı ADEK vitaminlerin miktarlarında önemli değişimlerin olduğu tespit edilmiştir.

Sonuç: Bu verilere göre, süt üreticilerinin hayvan beslenmesinde farklı yem kombinasyonlarını kullanmasının sütün biyokimyasal içeriğinin zenginleşmesi için oldukça önemli olduğu görülmektedir. Sütün besin içeriğinin farklı yem kombinasyonları ve florada beslenmeye bağlı olarak sütün biyokimyasal bileşiminin değiştiği saptanmıştır. Ayrıca merada beslenen ineklerin süt içeriğinin sağlıklı yaşam için gerekli besinlerce daha belirgin bir şekilde zenginleştiği bulunmuştur.

Anahtar Kelimeler: Aminoasitler, çiğ süt, kimyasal bileşim, lipitler, süt biyokimyası, vitaminler

INTRODUCTION

The liquid obtained from the milk glands of mammals such as cows, sheep, goats, buffaloes, donkeys, horses, and camels is called milk (1). Cow milk and dairy products have been part of the human diet, from birth to old age, for millennia. Milk and dairy products are important foodstuffs in daily life as well as being used extensively in the preparation of many foods. Milk is an excellent food that contains micro and macronutrients essential for healthy living. At the same time, human beings get an important part of their daily fat, carbohydrates, essential amino acids, vitamins and minerals from milk and dairy products (2, 3). Milk compound in dairy cows is acknowledged to be influenced by herbal forage compound, flora composition, and plant development status and rumen activity (4, 5). Milk yield and the enrichment of milk in terms of nutrients are related to the feeding of animals. Animal nutrition is very important for high meat and milk yield in livestock. As a result, cows need to be fed with different forage and forage combinations to prevent protein, vitamin and mineral deficiencies but also to increase milk yield (6). Due to the nutritional and economic value of cow's milk, fertile dairy cow breeds have been successfully selected, which has resulted in significant success in milk production over the years. With significant improvements in breeding and feeding and animal husbandry practices, milk production has now far exceeded the needs of the offspring. This excess milk is then sold in grocery stores for the purpose of human nutrition as fresh pasteurized milk. It is also used in the production of foods such as yogurt, butter, cheese, cream, ice cream, milk powder which are important in human nutrition (7). Cow's milk consists of approximately 5% lactose, 3.2% protein, 4% lipid, 3.5% vitamin and 0.7% mineral, but these figures may vary depending on animal nutrition and environmental factors and other factors. Milk is a very important food for bone and dental health. However, it has been recent data shown that milk contains nutrients necessary for healthy nutrition, as well as some active compounds important for healthy living (8).

In order to sustain all biological activity in a healthfully, the metabolism needs protein. One of the most important sources of protein in the human diet is milk, provides about 32 g protein/L. Its protein fraction can be divided into two parts, soluble protein and insoluble protein. Soluble proteins (whey proteins), describe 20% of milk protein fraction, while the insoluble, (caseins), describe 80 %. Both groups are high-quality proteins. It is classified as a source of amino acids with physiological properties suitable for humans (9). Apart from its biological function, some bioactive compounds formed as a result of enzymatic hydrolysis of milk proteins and some bioactive peptides have been shown to play an important role in the protection of human health. These bioactive compounds have been found to show antibacterial, antiviral, antifungal, antioxidant, antimicrobial, antihypertensive, antithrombotic and immunomodulatory properties (8). Research is needed on the effects of different forage combinations and regional flora on milk composition in terms of human health and nutrition. It has been reported that there are changes in the biochemical composition of raw milk in studies conducted with different feed combinations (9, 10). It is seen that the feeds used in this study are primarily used in animal nutrition in our country. According to the literature research, it seems that the effect of these feed combinations on the biochemical composition of raw milk has not been studied. It was aimed to investigate the effect of this feed combination on raw milk biochemistry. This study investigated changes in the compound of amino acids, fatty acids, lipophilic vitamins, cholesterol, and phytosterols in raw milk obtained from cows living in the same environment but feeding on different feed combinations.

MATERIAL and METHOD

1. Feeding of Animals

In this study, the cow's milk was obtained from animal farm in Kovancılar District of Elazig province. The cows were from the same farm. They were four years old. These cows have usually given birth. The animals on this farm were fed in three different ways the spring season. These;

I. Group: Cows fed with Beet pulp (4 kilograms)+Factory feed (6 kilograms)+Hay (4 kilograms) (n:10),

II. Group: Cows fed with Maize silage (4 kilograms)+Barley (6 kilograms)+Hay (4 kilograms) (n:10),

III. Group: Cows fed freely in pasture (n:10).

In this feed mixture, animals were given 14 kilograms per day (Morning 7 kilograms-Evening 7 kilograms). Hay and maize silage are prepared by the farmers in the area. Raw milk samples were taken on the 15th day of feeding and brought to the laboratory, and implemented biochemical processes. Ethical permission is not required in these studies. The cows were milked by hand. The milk of each cow was taken separately. The cows were milked at five o'clock. Milking was done after dinner. The necessary hygiene conditions were paid attention to during milking. The analyses of milk samples were performed in the laboratories of the Biology Department of Fırat University. Raw milk samples were taken into 50 ml tubes directly after milking. The raw milk samples were brought to the laboratory in accordance with the cold chain rule and used in biochemical processes.

2. Extraction of lipids

The extraction of lipids from the raw milk samples was realized according to (11). For this purpose, milk samples were centrifuged (at +4°C at 9050 g for 5 minutes-Hettich Universal 320) after homogenizing 3:2 (v/v) in the isopropanol-hexane mixture (Sigma Aldrich). Consequently, this supernatant liquid was exercised for the determination of fatty acids,

Table 1. Factory feed composition

Analytical components	
Crude protein	%19
Raw oil	%3.5
Raw cellulose	%9
Ash	%9
Vitamins and trace elements	
Vitamin A	10000000 IU
Vitamin D	2000000 IU
Vitamin E	40000 mg
Manganese	50000 mg
Iron	50000 mg
Zinc	80000 mg
Copper	12000 mg
Iodine	500 mg
Selenium	300 mg
Biotin	1000 mg

lipophilic vitamins, cholesterol and sterol.

3. Measurement of lipophilic vitamins, cholesterol and phytosterols

Methyl alcohol (Sigma Aldrich) KOH (Sigma Aldrich) (5 %) solution was added on milk examples taken for lipophilic vitamins, cholesterol and sterol analysis and was immediately mixed. Milk samples were kept for 15 minutes at 85 °C temperatures. After the samples were cooled to room temperature, 5.0 mL of pure water was added and mixed. Lipophilic phase in the hexane were extracted. Hexane (Sigma Aldrich) mixture was removed by nitrogen gas. It was solved in 1.0 mL (60/40%, v/v) acetonitrile methanol (Sigma Aldrich) solution and was analyzed. The analysis was performed on the Shimadzu brand High-Performance Liquid Chromatography (Shimadzu-HPLC) device. For analysis, a solution of acetonitrile methanol (60/40%, v/v) was used as the carrier phase. Mobile phase flux rate was identified as 1.0 mL minute DAD-UV detector was utilized for analysis of lipophilic vitamins. Nucleodur LC 18 (15 x 4.6 cm, 5µm; MN USA) column was utilized as HPLC column. Detection wavelength 326 nm was used for lipophilic vitamin retinol, 202 nm was utilized for vitamin E, vitamins D and K cholesterol and phytosterols (12, 13).

4. Preparation of fatty acid methyl esters from milk sample

For chromatographic analysis, fatty acids must be converted into methyl ester derivatives. For this purpose, the acid-catalyzed esterification method was utilized. According to the method, the hexane isopropanol phase was transferred to the test tubes. 5.0 mL 2% methanolic sulfuric acid (Riedel-De-Haën) was added and thoroughly mixed (14, 15). This mixture was kept for about 15 hours at 55 °C temperatures for methylation. At the end of the time, the test tubes were cooled to room temperature, and then 5.0 mL of 5% sodium chloride (Sigma Aldrich) was added and thoroughly mixed. The formed fatty acid methyl esters were extracted with 5.0 mL hexane. Then the hexane phase was removed and 5.0 mL of 2% $KHCO_3$ (Sigma Aldrich) was added. The test tubes waited five hours for phase separation. Solvents in the mixture containing methyl esters were removed with the help of nitrogen gas (45°C). Then the methyl esters were dissolved in chloroform (Sigma Aldrich) and analyses were performed on the gas chromatography device (Shimadzu 2010 Plus).

5. Gas chromatographic analysis of fatty acid methyl esters

Conditions of analysis, the column temperatures, detector, and injection valve were 110–215, 245, and 275°C, in turn. The analysis time was set at 28 minutes. Helium gas was utilized as the bearer gas. Before the analysis of the fatty acid methyl ester of milk samples, analyses of the standard fatty acid methyl ester mixture were performed. At this stage, the retention times of each fatty acid were determined (16).

6. Amino acid analysis

Derivatization of amino acids with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA (Sigma Aldrich)) reasons the synchronous sialylation of the amino and carboxyl groups in a step by step with modifications method explained by Buch et al. (17). Each sample protein was hydrolyzed by the 6 M HCl (Riedel-De-Haën) (0.5% phenol) at the 110°C for 24 hours and then examples were added 1.0 mL of the studying solution (1.5 µg) and then vaporized to exact dryness. Then, 10 µL of dimethylformamide and 60 µL of MTBSTFA were added and the flask was closed with a cap. Eventually, the examples were warmed at 70°C for 20 minute to obtain the chemical derivatization of amino acid and the derivatives were analyzed by Gas Chromatography-Mass Spectrometer (Shimadzu-GC–MS).

7. Analysis of amino acids in gas chromatography

The analysis of amino acid derivatives, Shimadzu gas chromatograph modified for glass-capillary work and FID detector were utilized. Amino acid derivatives were distinguished on a 20 m Supelco Slb 5 ms capillary column (Supelco, Sigma, 0.25 mm ID 0.25 µm film thickness) working with helium bearer gas (45 cm sec) under the following temperature program: from 120 to 150°C at 120°C minute (five minute hold), to 240°C at 7°C minute and eventually to 285°C at 20°C min (18 minute hold). The temperature of the injector and detector was kept fixed at 240, 300°C, in turn. The identification of amino acid derivatives was based on compare of their FID chromatogram and detention times with those of real acknowledgement (18).

8. Statistical analysis

Data was analyzed using SPSS package (version 15.0; SPSS Inc., Chicago, IL, USA). The results were shown as mean±standard error. Statistical evaluation was used in analysis of variance (ANOVA) and post-hoc test (LSD). A $P<0.05$ was accepted significant.

RESULTS and DISCUSSION

1. Amino acids distribution in milk of Simmental cows fed with different nutrients (%)

Milk and dairy products are recognized as food products of high nutritional and dietary value. They are important sources of food that ensure normal human growth and development as well as vital functions of the body (19-21). Milk and its products is a rich source of nutrients and considered by many as a valuable element of diet for all age groups especially for infants, children and elderly. Cow's milk contains many bioactive components that boost the physiological processes in the body. The compound of milk alters significantly across species because milk composition is related to the nutrient needs of newborns from each species (22). The biochemical content of milk can vary depending on different factors such as nutrition, genetic characteristics, cow's health and physiological status (22, 23). Milk and dairy products are considered to be a good source of protein in the nutrition. Cow's milk, which contains high quality protein, has an average of 3-3.5% protein. Cow's milk protein is a heterogeneous mixture of non-protein compounds containing enzymes and small amounts of nitrogen, based on casein and whey proteins (24). The major amino acids found in whey protein are leucine, isoleucine, valine and lysine, while the major amino acids found in casein are histidine, methionine, and phenylalanine (9). In our study, eighteen amino acids were detected in milk samples. Cow's milk has been found to be an important food in terms of protein (Table 2). According to raw milk amino acids analysis of Simmental breed cows fed with different rations, the amounts of aspartic acid, cysteine, lysine, phenylalanine and leucine amino acids were higher than other amino acids. In general, it was found that these amino acid values were higher than other amino acid values (Table 2). In studies, it has been determined that these essential amino acids are more present than other essential amino acids (25). It has been reported that the phenylalanine essential amino acid is found more often than other essential amino acids, especially in Group 2 and Group 3. It is important for the quality of milk that the proteins in milk contain high levels of essential amino acids (Table 2).

Compared to Group 1, glycine ($p<0.001$), proline ($p<0.01$), aspartic acid ($p<0.05$), glutamic acid ($p<0.001$), histidine ($p<0.001$) and tryptophan ($p<0.05$) amino acid amounts were statistically decreased, while arginine ($p<0.001$), alanine ($p<0.001$), threonine ($p<0.05$), phenylalanine ($p<0.001$) and lysine ($p<0.05$) amino acid amounts were statistically

Table 2. Amino acids distribution in milk of Simmental breed cows fed with different forage combinations (%)

Amino acids	Group 1	Group 2	Group 3
Arginine	0.49±0.02	1.17±0.18[d]	1.46±0.11[d]
Alanine	7.89±0.78	2.91±0.26[d]	1.91±0.15[d]
Glycine	6.70±0.56	1.87±0.41[d]	3.71±0.89[b]
Valine[1]	6.51±0.67	6.93±0.88	7.56±1.33
Leucine[1]	9.02±0.89	9.51±1.12	8.65±1.67[b]
Isoleucine[1]	4.77±0.34	4.45±0.67	6.45±0.97[b]
Proline	3.97±0.32	1.66±0.08[c]	3.40±0.79
Methionine[1]	1.45±0.05	1.54±0.07	2.01±0.75[b]
Serine	6.40±0.47	7.25±1.11	5.34±1.09
Threonine[1]	3.89±0.36	5.29±0.86[b]	6.71±1.34[b]
Phenylalanine[1]	5.86±0.77	12.32±1.78[d]	13.56±2.06[d]
Aspartic acid	13.57±1.16	10.65±1.34[b]	9.34±1.19[c]
Cysteine	9.61±1.11	8.01±2.21	8.73±0.87
Glutamic acid	4.24±0.78	1.08±0.03[d]	2.76±0.56[b]
Lysine[1]	8.57±0.97	10.09±1.25[b]	10.89±1.36[b]
Histidine[1]	1.96±0.09	0.52±0.04[d]	2.09±0.67
Tyrosine	4.22±0.29	5.19±0.69	4.56±1.08
Tryptophan[1]	0.91±0.03	0.54±0.01[b]	1.11±0.56[d]

[1]: Essential amino acids

One-way analysis of variance (ANOVA) LSD post hoc test, ($p<0.05$)

[a]: The difference between the groups is not statistically significant ($p>0.05$)
[b]: The difference between the groups is statistically significant ($p<0.05$)
[c]: The difference between the groups is statistically more significant ($p<0.01$)
[d]: The difference between the groups is statistically most significant ($p<0.001$)

increased in Group 2. It was determined that the changes in the amino acid values of valine, leucine, isoleucine, methionine, serine, cysteine and tyrosine were statistically insignificant (Table 2). Compared to Group 1, it was determined that the amounts of arginine ($p<0.001$), isoleucine ($p<0.05$), methionine ($p<0.05$), threonine ($p<0.05$), phenylalanine ($p<0.001$), lysine ($p<0.05$) and tryptophan ($p<0.001$) amino acids statistically increased, while the amounts of alanine ($p<0.001$), glycine ($p<0.05$), leucine ($p<0.05$), aspartic acid ($p<0.01$) and glutamic acid ($p<0.05$) amino acids statistically decreased in Group 3. It was determined that the changes in valine, proline, serine, cysteine, histidine and tyrosine amino acid values were statistically insignificant (Table 2).

When amino acid levels were compared between the groups, it was found that arginine and lysine amino acids were high in the second and third groups ($p<0.05$, $p<0.001$). When amino acid levels were compared between the groups, amino acid levels such as isoleucine, methionine, threonine, phenylalanine and tryptophan were determined to be high in the third group ($p<0.05$, $p<0.001$). When amino acid levels were compared between the groups, it was found that alanine, aspartic acid and glutamic acid levels decreased in the second and third groups (respectively $p<0.001$, $p<0.05$, $p<0.01$, $p<0.001$, $p<0.05$). It was determined that there was a decrease or increase in amino acid levels other than the above-mentioned amino acids at different rates between the groups. But these changes are not statistically significant (Table 2).

In this study, we observed that there were significant changes in the milk obtained from cows grazing freely in the pasture and that the milk content was enriched in terms of nutrients. This is also true in terms of protein content. This may be linked to the amount of pasture cows receive more nitrogen through nutrition and the conversion of excess nitrogen into milk protein. Studies have announced that changes in amino acid composition in milk are related to the amount of nitrogen taken by nutrition (22).

2. Fatty acid composition in milk of Simmental cows fed with different nutrients (%)

Fatty acid composition in milk depends on factors related to animal race, lactation stage, ruminal fermentation and nutrition. In fact, the fatty acid composition of milk depends on nutrition and ruminal microbial activity. Milk is generally made up of saturated fatty acids (70%) and unsaturated fatty acids (30%) (9). In the fatty acid compound of the milk content, palmitic acid (C16:0), oleic acid (C18:1), myristic acid (C14:0), stearic acid (C18:0), lauric acid (C12:0), linoleic acid (C18:2), palmitoleic acid (C16:1) and pentadecanoic acids (C15:0) were determined to be more. According to milk analysis, the dominant saturated fatty acids are palmitic (C16:0), stearic and (C18:0) myristic acids (C14:0), while the dominant unsaturated fatty acids are oleic (C18:1), linoleic (C18:2) and alpha-linolenic acids (18:3 n-3) (Table 3).

In general, according to our findings, it was found that saturated fatty acid level decreased ($p<0.05$, $p<0.01$, $p<0.001$), the unsaturated fatty acid level increased ($p<0.01$, $p<0.001$) in Group 3 compared to Group 1 and Group 2 (Table 3). There were significant changes in milk fatty acid composition in Group 3 compared to Group 1 and Group 2 in terms of human health. In Group 3, the reduction in the amount of saturated fat acid is very important for human health. The C18:0 (Stearic acid) fatty acid and C18:1 (Oleic acid) fatty acid levels were reduced in Group 2 and Group 3 compared to Group 1 ($p<0.01$, $p<0.001$). The level of saturated fatty acids (C14:0-Myristic acid, C18:0-Stearic acid) in Group 1 was increased compared to Group 3. The level of saturated fatty acids (C18:0-Stearic acid) in Group 1 was increased compared to Group 2 ($p<0.001$). Compared to group 2, the level of oleic (C18:1-Oleic acid unsaturated fatty acid) acid increased significantly in Group 1 ($p<0.01$). In Group 3, it was found that the amount of some saturated fat acid decreased compared to other groups. Also in Group 3, the levels of linoleic and alpha-linolenic fatty acids, which are essential fatty acids, were increased compared to other groups

Table 3. Fatty acids distribution in milk of Simmental breed cows fed with different forage combinations (%)

Fatty acids	Group 1	Group 2	Group 3
[1]Caprylic acid (8:0)	0.14±0.01	0.90±0.05[d]	0.70±0.03[d]
[1]Capric acid (10:0)	0.36±0.06	3.68±0.78[d]	2.68±1.04[c]
[1]Lauric acid (12:0)	**5.35±1.08**	**4.87±0.88**	**3.87±1.06[b]**
[1]Myristic acid (14:0)	**14.04±1.89**	**14.30±1.09**	**12.30±2.07[c]**
[2]Miristoleik asit (14:1)	0.35±0.05	0.26±0.01	0.36±0.01
[1]Pentadecanoic acid (15:0)	1.49±0.45	1.42±0.33	1.02±0.04[b]
[2]15:1	0.29±0.02	0.43±0.02[b]	0.23±0.06
[1]Palmitic acid (16:0)	**38.05±2.67**	**39.89±2.76**	**35.20±2.81[c]**
[2]Palmitoleic acid (16:1)	1.79±0.22	2.16±0.16[b]	2.75±0.06[b]
[1]Heptadecanoic acid (17:0)	0.79±0.08	0.64±0.04	0.74±0.07
[2]17:1	0.63±0.07	0.32±0.01	0.42±0.03[b]
[1]Stearic acid (18:0)	**11.18±1.67**	**7.74±1.03[d]**	**6.74±1.04[d]**
[2]Oleic acid (18:1)	**22.19±2.23**	**19.88±2.67[c]**	**17.88±2.71[d]**
[2]Linoleic acid (18:2)*	**2.34±0.26**	**2.29±0.67**	**8.29±0.88[d]**
[2]Gamma-linolenic acid (18:3 n-6)	0.32±0.04	0.15±0.01	1.15±0.04[d]
[2]Alpha-linolenic acid (18:3 n-3)*	**0.28±0.03**	**0.32±0.02**	**3.32±1.08[d]**
[1]Arachidic acid (20:0)	0.10±0.01	0.14±0.01	0.74±0.05[c]
[2]Gadoleic acid (20:1)	0.11±0.02	0.21±0.02	0.81±0.06[c]
[2]Eicosadienoic acid (20:2)	0.13±0.01	0.17±0.01	0.37±0.04[b]
[2]Dihomo-gamma-linolenic acid (20:3)	0.18±0.02	0.23±0.02	0.43±0.05[b]

[1]: Saturated fatty acids
[2]: Unsaturated fatty acids
*: Essential fatty acid

One-way analysis of variance (ANOVA) LSD post hoc test, ($p<0.05$)

[a]: The difference between the groups is not statistically significant ($p>0.05$)
[b]: The difference between the groups is statistically significant ($p<0.05$)
[c]: The difference between the groups is statistically more significant ($p<0.01$)
[d]: The difference between the groups is statistically most significant ($p<0.001$)

(Table 3). It is possible that due to free nutrition in pasture, the level of saturated fat acid decreases and the level of unsaturated fat acid increases in milk. It is reported that there is a correlation between saturated fatty acid consumption and diabetes with cardiovascular diseases (22, 26). It has been stated that there may be a decrease in the level of saturated fatty acids in milk due to free nutrition in pastures (27, 28). It has been stated that the milk fat acid composition of cows may vary depending on diet. It has been reported that oleic acid, polyunsaturated fatty acids and saturated medium-chain fatty acids are particularly affected in milk depending on the diet (26). It has been reported that linoleic (C18:2), alpha-linolenic and (C18:3 n-3) oleic acid (C18:1) levels may increase in raw milk due to free feeding in the pasture (29). This fatty acid-rich diet is very important for human health (8, 29, 30). Many studies have recognized the importance of diet in regulating and improving health-promoting and diseaseinhibiting groups of fatty acid in cow milk. Milk fatty acid profile varies depending on external and internal factor (26, 29). Furthermore, seasonal differences in pasture, flora of the region and characteristics of forage will result in differences in milk fatty acids composition (31). According to nutrition, fatty acids such as C8:0 (Caprylic acid), C10:0 (Capric acid) and C16:1 (Palmitoleic acid) were found to be higher in Group 2 and Group 3 in milk. In Group 3 such as, C18:2 (Linoleic acid), C18:3 (Gamma-linolenic acid) (n-6), C18:3 (Alpha-linolenic acid) (n–3), C20:0 (Arachidic acid), C20:1(Gadoleic acid), C20:2 (Eicosadienoic acid), C20:3 (Dihomo-gamma-linolenic acid) fatty acids high, C12:0 (Lauric acid), C14:0 (Myristic acid), C16:0 (Palmitic acid), C18:0 (Stearic acid) and C18:1 (Oleic acid) fatty acids were low (Table 3).

3. Changes in the composition of cholesterol, lipophilic vitamins and phytosterols in the milk of Simmental cows fed with different nutrients (μg/10 mL)

There was no difference in the amount of retinol in milk between the groups. However, the levels of K2 vitamin, α-tocopherol, D2 vitamin, γ-tocopherol, K1 vitamin and stigmasterol were observed to rise at different rates in Group 2 and Group 3 ($p<0.05$, $p<0.001$). In Group 3, there was a partial decrease in cholesterol level in milk samples ($p<0.05$). In Group 1 and Group 3, partial increase in ß-sitosterol level was determined in milk samples ($p<0.05$) (Table 4).

In addition to being involved in the structure of cell membranes, cholesterol is a molecule necessary for the synthesis of bile acids and steroid hormones. High plasma cholesterol levels are considered to be an important risk factor for cardiovascular diseases. Mortality rates from cardiovascular disease are high all over the world. Cardiovascular diseases and other metabolic diseases have been reported to be related to high fat intake (32). The most important sterol found in milk is cholesterol (3 mg/g fat, equivalent to 100 mg/L cow's milk), but phytosterols are also present (33). Cholesterol levels were significantly reduced in Group 2 and Group 3 ($P<0.05$) compared to Group 1 (Table 4). It has been stated that there may be changes in the amount of cholesterol in the milk of farm animals according to dietary fat (34, 35). It is emphasized that low cholesterol diet is important against cardiovascular diseases and metabolic diseases (36). Our study shows that the amount of cholesterol in the milk of naturally fed animals has decreased (Table 4). Therefore, low cholesterol content in the milk of naturally fed animals is very important for healthy living. Hypercholesterolemia is evaluated as an important cardiovascular risk factor. In terms of cardiovascular health, healthy diet and healthy lifestyle are very important. The diet rich in phytosterols (Stigmasterol, ß-Sitositerol) is recommended for the treatment of hypercholesteroemia. Phytosterols are a widely used approach in lowering plasma cholesterol levels (37). The amount of stigmasterol was significantly increased in Group 2 and Group 3 compared to Group 1 ($p<0.01$, $p<0.01$). The amount of ß-sitosterol was significantly reduced in Group 2 compared to Group 1 ($p<0.05$). The amount of ß-sitosterol was significantly increased in Group 3 compared to Group 1 ($p<0.05$) (Table 4). In Group 3, phytosterol (Stigmasterol, ß-Sitosterol) level significantly increased in milk. In general, group 1 and Group 2 are important sources of phytosterol (Stigmasterol, ß-Sitosterol) (Table 4). Sterol rich diet is a useful dietary strategy to reduce the risk of cardiovascular and metabolic disease. Epidemiological studies have shown that cardiovascular diseases can be prevented based on positive changes in diet and lifestyle. The main plant-derived sterol in farm animal's milk is ß-sitosterol (33). It was determined that there were changes in sterol content in milk due to the feeding of cows. Researchers have stated that changes in milk composition may occur when animal nutrition is interfered with (33, 34). Milk and dairy products are an important component of a healthy diet due to their capacity to provide vitamins, minerals, macronutrients and micronutrients prominent for,

Table 4. Distribution of lipophilic vitamins, cholesterol and phytosterols in milk of Simmental breed cows fed with different forage combinations (μg/10 mL)

	Group 1	Group 2	Group 3
Vitamin A (Retinol)	0.03±0.001	0.03±0.001	0.04±0.001
Vitamin K_2	0.09±0.01	0.34±0.09[d]	0.65±0.07[d]
γ-tocopherol	**1.12±0.07**	**3.12±0.97[c]**	**5.34±0.78[d]**
Vitamin D_2	0.31±0.03	0.79±0.04[c]	1.34±0.05[d]
Vitamin D_3	0.15±0.01	0.46±0.03	0.57±0.03
α-tocopherol	**0.64±0.07**	**1.47±0.06[c]**	**2.67±0.67[d]**
α-Tocopherol acetate	**73.53±2.76**	**33.87±2.78[d]**	**87.67±3.09[b]**
Vitamin K_1	0.68±0.08	2.02±0.89[c]	3.04±0.88[d]
Cholesterol	74.34±2.87	66.64±2.09	56.78±1.88[b]
Stigmasterol	8.16±0.98	18.21±1.12[c]	34.78±2.06[c]
β-Sitosterol	661.23±3.45	531.23±5.56[b]	711.78±7.18[b]

One-way analysis of variance (ANOVA) LSD post hoc test, ($p<0.05$)

[a]: The difference between the groups is not statistically significant ($p>0.05$)
[b]: The difference between the groups is statistically significant ($p<0.05$)
[c]: The difference between the groups is statistically more significant ($p<0.01$)
d: The difference between the groups is statistically most significant ($p<0.001$)

up growth, flourish and tissue care (38). Vitamin A is particularly substantial in up growth, flourish, immunity, and eye health. Its content in milk depends fundamentally on fat quantity, but also on factors like animal nutrition and season. In general, fatty milks are richer in vitamin A (9). In our study, there were no significant changes in vitamin A level in the milk of cows fed with different foods (Table 4). Retinol comes from the liver to the mammary glands, where it is esterified and then given to milk (33). The amount of vitamin D in cow's milk is very low, but some countries supplement vitamin D in milk and dairy products from different sources. In previous studies, vitamin D levels (5 and 35 IU/L) in cow's milk were stated to be low. Recently, people's interest in vitamin D has increased due to its protective property against some important diseases. Studies have reported that vitamin D protects against cancer, diabetes and heart disease (39). In addition, vitamin D has important effects on muscle and bone health due to calcium absorption (9). The amount of fat-soluble vitamins in cow's milk varies depending on the breed, nutrition, physiological conditions and health of the animal. It has been reported that the amount of vitamin E (alpha-tocopherol-0.2 and 1.0 mg/L) and vitamin A in milk may vary depending on nutrition, forage content and forage quantity (40). The amount of vitamin D and vitamin K in milk varies depending on the time the animal and food stay in the sun and the function of the rumen (33). Vitamin K2 ($p<0.001$, $p<0.001$) and vitamin D2 ($p<0.01$, $p<0.001$) were significantly increased in Group 2 and Group 3 compared to Group 1 (Table 4). Because the animals live in the same environmental conditions, the difference in milk content may have been due to the forage content. Studies have shown that the biochemical content of milk can vary depending on nutrition (33). The amount of δ-tocopherol and α-tocopherol was significantly increased in Group 2 and Group 3 compared to Group 1 ($p<0.01$, $p<0.001$). The amount of α-tocopherol acetate was significantly increased in Group 1 and Group 3 compared to Group 2 ($p<0.001$, $p<0.05$)

(Table 4). Alpha-tocopherol is the fundamental manner of vitamin E available in cow's milk, referring 84–92% of the total, whereas gamma-tocopherol and alpha-tocotrienol conduce approximately 5% each one (33). According to feeding with different forage combinations, the amount of tocopherol is higher in the milk of cows grazing freely in the pasture.

In general, the amount of fat-soluble vitamins depends on the fat content of milk. When the total amount of fat or any amount of fatty acid changed in milk and dairy products, the level of fat-soluble vitamins can also change. In order to increase the vitamin content of these products, it is necessary to increase the content of synthetic or natural vitamins or their precursors in the forage. It has been reported that there may be significant changes in the micronutrient and macronutrient composition of milk due to forage and forage content (33).

In conclusion; the effect of regional flora and different forage combinations on milk composition (amino acids, fatty acid, sterol, lipophilic vitamins) was evaluated for the first time in this study. It was determined that flora caused significant changes in milk composition. In this study, changes in amino acid, fatty acid, cholesterol, lipophilic vitamin and sterol content were examined in milk samples of cows living in the same conditions but fed different forage combinations. Milk samples were taken in the spring. As a result, milk content changes depending on the feeding of animals, especially milk content of cows fed freely in pasture is found to be enriched in terms of nutrients necessary for healthy living.

ETHICS COMMITTEE APPROVAL

* This study does not require Ethics Committee Approval.

CONFLICT OF INTEREST

The author declares no conflict of interest.

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