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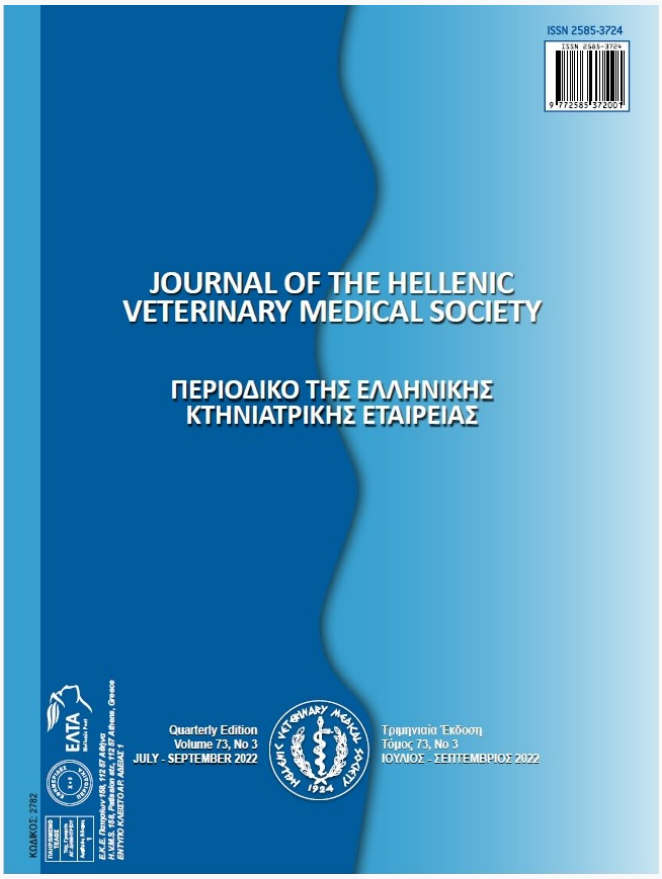

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Research article
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Metabolomics characteristics associated with milk yield and milk quality in sheep

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ABSTRACT: Standard milk tests include monitoring traits such as milk yield and quality. In addition to performing standard milk tests, it is now possible to evaluate metabolites that could act as potential biomarkers to determine milk properties. This study was conducted to identify the metabolomic parameters related to milk yield and quality in Awassi sheep. In our study, a total of 26 Awassi ewes, 13 with high milk yield and 13 with low milk yield, were examined. Liquid chromatography tandem mass spectrometry was used for metabolomic analysis. There was a statistically significant difference at different levels between the two groups in terms of aspartic acid, ornithine, anserine, and cystathionine levels ($P<0.05$ and $P<0.01$). A moderate negative correlation was determined between milk yield and aspartic acid ($r=-0.63$) and anserine ($r=-0.52$) levels ($P<0.01$). A significant negative correlation was found between lactose levels and lysine ($r=-0.50$), alpha-aminoadipic acid ($r=-0.52$), and hydroxylysine ($r=-0.51$) levels ($P<0.01$). The somatic cell count and alanine ($r=0.49$), aspartic acid ($r=0.42$), proline ($r=0.42$), alpha-aminoadipic acid ($r=0.41$), beta-alanine ($r=0.43$), and thiaproline ($r=0.43$) levels ($P<0.05$) showed positive correlation. Our results provide important insights into the metabolic events involved in sheep milk yield and milk quality which may guide further research to improve milk production and enhance the constituents of sheep milk.

Keywords: Amino acid profile; Awassi; milk; sheep; somatic cell count

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INTRODUCTION

Sheep breeds are well adapted to various rearing conditions, including areas having an arid/humid climate (Ferro et al., 2017). Milk is the major product of sheep rearing particularly in the Mediterranean coastline where the emphasis is on the production of sheep dairy products rather than sheep meat (Gerber et al., 2013). Small ruminants have higher economic importance compared to other countries due to the presence of highly specialized breeds and breeding systems in developed countries involved in dairy sheep breeding (Opio et al., 2013). Different morphological, genetic, and biochemical tools have been used to determine the yield characteristics of farm animals having an economic importance (Goldansaz et al., 2017; Yurchenko et al., 2019). Metabolomic parameters have the potential for use as low-cost biomarkers in selection studies to determine economically important yield characteristics of livestock (Sun et al., 2015; Wu et al., 2018). Samples obtained via noninvasive methods from easily accessible biological fluids, such as blood, urine, milk, and saliva, are used in metabolomic analysis (Sun et al., 2015). The determination of characteristics such as milk yield, milk quality, and feed consumption often requires labor-intensive, costly, and time-consuming techniques (Moore et al., 2009). In metabolomic studies predicting feed consumption in cattle, a prediction accuracy of 95% was obtained, which was considered as a very promising result (Karisa et al., 2014; Widmann et al., 2015). These results demonstrate that the metabolomic data has the potential to be used in animal husbandry for the identification of animals with superior characteristics. Considering the favorable results obtained in studies with cattle so far, the application of metabolomics in studies involving other animal species has been suggested by many researchers (Giallongo et al., 2016; Goldansaz et al., 2017; Lee et al., 2015; Robinson et al., 2010).

The Awassi sheep, which are considered as dairy breeds, are widely distributed across the Middle East. Studies conducted in the past years on Awassi sheep show that there is a wide variation in their yield characteristics (Ferro et al., 2017). These variations as well as breeding studies on Awassi sheep conducted in different parts of the world enabled developing high-yielding ewes (Pollott and Gootwine, 2001).

Metabolomic parameters represent biochemical end products resulting from genetic and environmental effects (Corona et al., 2012). To identify animals with superior yield characteristics among the livestock, a long-term and laborious process is required. It is important to investigate the potential of novel selection parameters in identifying new animal breeds producing a high yield of meat and milk and having rapid growth characteristics (Moore et al., 2009). Therefore, analysis of the correlations between yield characteristics obtained by performing standard performance tests and metabolites is very important (Caboni et al., 2019). In this study, it was aimed to investigate the metabolomic parameter (plasma free amino acid profile) related to milk yield and quality in Awassi sheep.

MATERIALS AND METHODS

Animals, diets, and experimental design

Necessary permissions were obtained from the Harran University Animal Experiments Local Ethics Committee for all the procedures conducted during the study (approval number: 2020-001, 01-07). A total of 26 Awassi sheep were selected from a herd of 500 sheep considering the yield records and brought to the farm animals unit operating within the Harran University Experimental Animals Research Center (Şanlıurfa /Turkey). The study animals were divided into two groups high and low-milk yield groups. These ewes were in the same period of lactation. In addition to grazing in a pasture, the animals consumed 800 g concentrated feed (18% crude protein, 10% crude cellulose, 10% ash, 3% crude oil, 0.5% phosphorus, 0.2% calcium, 2600 kcal/kg ME), wheat straw 600 g, and 1200 g corn silage. The chemical composition of the grass in the is 31% dry matter, 8% crude ash, 14% crude protein, 2% crude oil, 45% NDF (neutral detergent fiber), 32% ADF (acid detergent fiber). The grazed pasture has a low hay yield, a very short green forage period, and its botanical composition is predominantly grassy.

Collection of milk yield and milk quality data

Ewes (2-3 years old and 49.43±0.79 kg live weight) with similar lambing times were selected to investigate milk yield and quality. The milk tests were performed on day 15 following lambing, and monthly monitoring was continuously performed until 75th day of lactation. The animals used as research material were selected based on milk yield records from an estrus synchronized herd (approximately 500 heads). Among the sheep that were pregnant in the first cycle, 60 sheep that gave birth on the same day were brought to the research center. In the research center,

milk controls were started on the 15th day of lactation. Also, milk control was performed on the 45th and 75th days of lactation. According to these 3 control data, 26 sheep with the lowest and highest milk yield were determined. Metabolomics and milking properties were examined from blood and milk samples taken from these sheep on the 75th day of lactation. On testing days, the lambs and ewes were separated from each other at 18:00 pm on the previous day until after milking was performed on the evening of the next day. Milking was performed twice using a machine, at 06:00 am and 18:00 pm, and the amount of milk obtained was determined using an electronic scale (sensitivity: 1 g). In this study, traits such as milk yield and quality were determined on day 75, which corresponds to the peak of lactation. Milk content analysis was carried out by adding bronopol and natamycin to the mixture obtained from milk samples collected in the morning, noon, and evening (4:3:3) on the milk testing days. Bronopol and natamycin ensure that milk composition and somatic cell count are not affected by preventing microbial proliferation in milk samples. Combi Milk Analyzer (Bentley Combi 150, USA) was used to examine milk content (Kahraman and Yüceer Özkul, 2020).

Collection of blood samples

The blood samples required for conducting metabolomic analysis were collected from each ewe on the milk testing days before morning feed was provided to them. The blood required for amino acid analysis was collected from the jugular vein and stored in tubes containing 10 ml of ethylenediaminetetraacetic acid. The collected blood samples were centrifuged at 4500 rpm for 15 min to separate the plasma and stored at −80°C until analysis.

Metabolomic analysis

Metabolomic parameters (plasma free amino acid profile) related to milk yield were determined using a liquid chromatography tandem mass spectrometry (LC-MS/MS) device (Shimadzu, Japan) and the blood samples collected from the animals on the milk testing days when milk quality traits were examined. The derivatization method was used for analyzing the presence of free amino acids in biological fluids. Following this method, 100 µL of the sample was mixed with an internal standard mixture consisting of 20 amino acids with C13- and N15-labeled atoms prepared in 0.1 M HCl. In the second step, basic organic buffer components prepared in propanol were added to balance the pH and increase the efficiency of the derivatization reaction. In this step, protein precipitation also occurred. Then, a chloroform/isooctane mixture containing 5% alkyl chloroformate as an active ingredient was added to the mixture and kept at room temperature for 3 min. During this process, gas evolution was observed due to the production of carbon dioxide as a byproduct of the esterification reaction of alkyl chloroformate and amino acids (Tammo et al., 2021).

The derivatized amino acids were transferred to the upper phase containing organic solvents by centrifugation, and 1 µL of this phase was injected into the LC-MS/MS device. Since the molecular weight of the esterified amino acids increases and they become more volatile after the extraction and derivatization processes, the signal given by the MS device also increases. Chromatographic separation was performed using a Trimaris Amino Acid LC-MS/MS column (250 mm × 2 mm, 3 µM) containing a C18 reverse phase filler. Water, methyl alcohol, and 1 M ammonium formate in the ratio 85:14:1 were used as mobile phase A, and methyl alcohol was used as mobile phase B.

Statistical analysis and data processing

Independent samples t-test was used to evaluate milk yield and quality (fat, protein, and lactose levels; dry matter content; and somatic cell count). Results are presented as mean ± standard error of mean. Normality analysis was performed using Shapiro-Wilk test and via the visual examination of histograms. Pearson's correlation analysis was performed to determine the direction and level of the relationship between milk quality traits and metabolomic parameters. The amino acid data obtained using LC-MS/MS analysis were uploaded to the MetaboAnalyst 5.0 (https://www.metaboanalyst.ca/) server to perform multivariate statistical analysis, and none of the MetaboAnalyst normalization protocols were applied. First, principal component analysis (PCA) was performed to detect the segregation and aggregation of the groups. Then, partial least squares discriminant analysis (PLS-DA) was applied to maximize the segregation and aggregation. The variable importance in projection (VIP) scores of the amino acids contributing to the separation of the groups were calculated. Hierarchical clustering heatmaps were generated to visualize the amino acids contributing to segregation of the groups. In addition, pathway analysis was performed to identify degradations in biological pathways.

RESULTS

The biochemical values detected at baseline for the ewes included in the study are given in Table 1. These data show that the low- and high-milk yield group ewes were similar in terms of the evaluated biochemical parameters (P>0.05). There was a two-fold difference in milk yield between the study groups (Table 2) (P<0.001), and it was determined that the number of somatic cells was higher in the ewes with high milk yield (P<0.05). The difference observed between the study groups in terms of aspartic acid, ornithine, anserine, and cystathionine levels (Table 3) was statistically significant at different levels (P<0.05 and P<0.01). The correlations between milk yield, milk quality, and amino acid profiles are shown in Table 4. Moderately significant negative correlations were determined between milk yield and aspartic acid (−0.63, P<0.01) and anserine (−0.52, P<0.01) levels. Significant negative correlations were observed between lactose levels and lysine (−0.50, P<0.01), alpha-aminoadipic acid (−0.52, P<0.01), and hydroxylysine (−0.51) levels. A positive correlation was determined between somatic cell count and alanine, aspartic acid, proline, alpha-aminoadipic acid, beta-alanine, and thiaproline levels (P<0.05). PCA and PLS-DA were performed sequentially to visualize potential outliers and the sample-wise distribution of amino acids (Figure 1). First, PCA was performed to provide an overview of the differences between the low- and high-milk yield groups in terms of the 38 amino acid profiles. Two-dimensional (2D) and three-dimensional (3D) score plots of PCA are shown in Figures 1A and 1B, respectively. The analysis revealed that no clear separation and clustering occurred between the groups. PLS-DA was performed to maximize the differences between the groups. Although it provided better segregation and aggregation compared to PCA, the groups did not differ significantly. The 2D and 3D score graphs of PLS-DA are shown in Figures 1C and 1D, respectively. VIP charts generated from PLS-DA models were analyzed to determine the amino acids that discriminate the high- and low-milk yield groups. The VIP chart ranks amino acids according to their discrimination power. Citrulline, alanine, and arginine were the

Table 1. Some biochemical parameters in sheep

Parameters	Low	High	P Value
Creatine kinase (mg/dL)	356.92±35.16	316.92±25.32	0.37
Blood urea nitrogen (mg/dL)	12.30±0.85	11.53±0.48	0.44
Urea (mg/dL)	26.54±1.76	24.69±1.02	0.37
Creatinine (mg/dL)	0.44±0.06	0.47±0.05	0.65
Cholesterol (mg/dL)	64.31±3.76	61.77±3.6	0.63
Triglyceride (mg/dL)	23.08±2.27	21.31±1.43	0.52
High density lipoprotein (mg/dL)	35.85±1.47	37.54±1.65	0.45
Low density lipoprotein (mg/dL)	17.92±0.99	16.08±1.44	0.30
Aspartate transaminase (U/L)	97.62±5.35	99.08±3.53	0.82
Alanine aminotransferase (U/L)	14.62±1.18	14.77±1.22	0.93
Gamma glutamyl transferase (U/L)	59.54±7.51	59.77±6.46	0.98
Lactate dehydrogenase (U/L)	600.23±30.24	549.15±16.15	0.15
Total bilirubin (g/dL)	0.16±0.03	0.13±0.01	0.27
Total protein (g/dL)	7.08±0.17	6.97±0.08	0.57
Albumine (g/dL)	2.66±0.1	2.67±0.06	0.92

Table 2. Milk yield and some milk quality traits.

Traits	Mean ± SEM		P-Value
	Low (n=13)	High (n=13)	
Milk Yield	1100.62 ± 113.03[b]	2185.51 ± 101.05[a]	0.00***
Fat (%)	6.10 ± 0.20	5.94 ± 0.23	0.60
Protein (%)	4.96 ± 0.14	4.79 ± 0.09	0.31
Lactose (%)	5.11 ± 0.08	5.87 ± 0.04	0.06
Dry Matter (%)	17.53 ± 0.29	17.43 ± 0.30	0.83
SCC (x1000) Cell/ml	298.31 ± 87.65	116.23 ± 23.42	0.06

a,b: Values within a row with different superscripts differ significantly at P<0.001.
SCC: Somatic Cell Count.

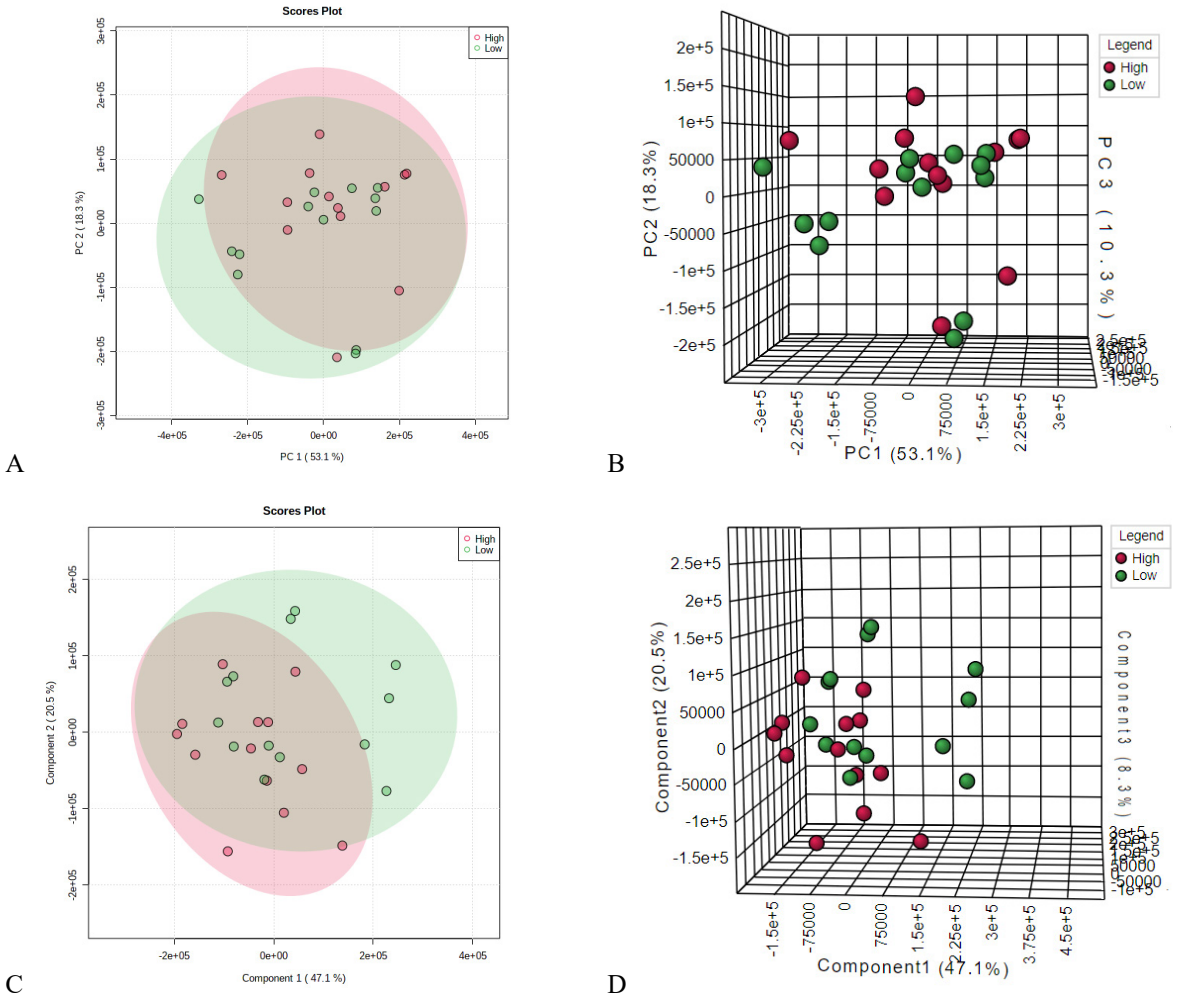

Figure 1. PCA and PLS-DA analyzes for low and high milk yield groups. PCA 2D (A) and 3D (B) score graphs. PLS-DA 2D (C) and 3D (D) score graphs

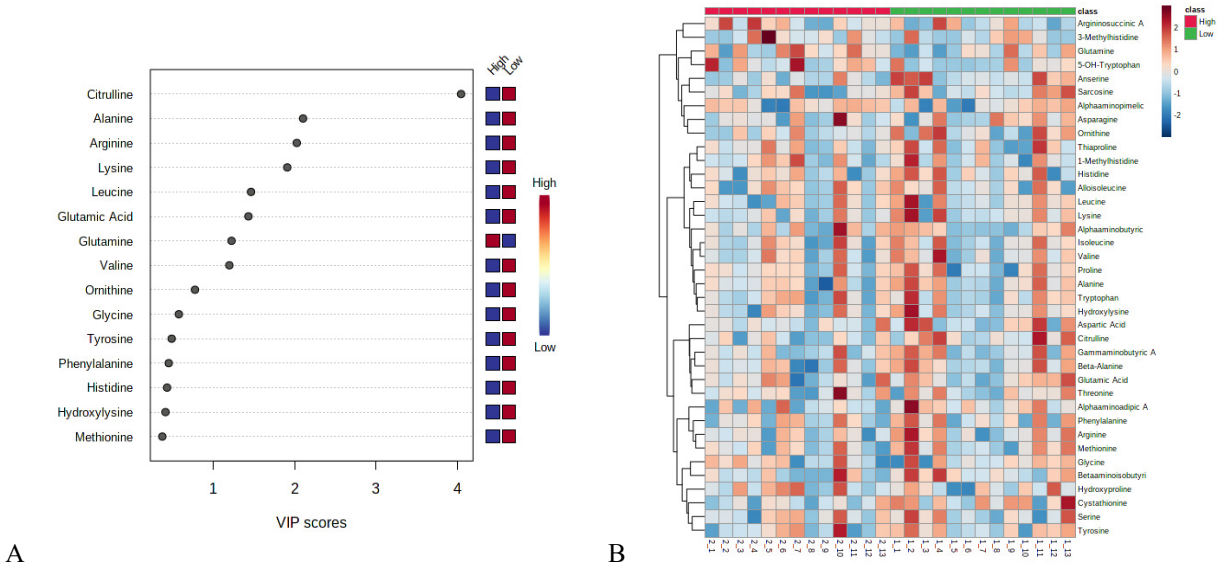

Figure 2. VIP table: essential amino acids (A) identified in decreasing order of importance. Heat map (B) showing the amino acid patterns in the groups

Table 3. Plasma free amino acid profile in sheep.

Amino Acids (µmol/L)	Mean ± SEM		P-Value
	Low (n=13)	High (n=13)	
Alanine	163.03 ± 15.63	138.35 ± 12.98	0.24
Arginine	106.47 ± 14.11	84.15 ± 9.14	0.20
Asparagine	32.56 ± 3.62	29.61 ± 4.48	0.61
Aspartic Acid	22.61 ± 2.92[a]	12.07 ± 1.83[b]	0.01**
Citrulline	152.97 ± 21.74	113.31 ± 12.25	0.13
Glutamine	57.68 ± 9.25	53.64 ± 6.94	0.73
Glutamic Acid	200.75 ± 21.10	196.78 ± 20.09	0.89
Glycine	283.85 ± 21.92	276.93 ± 23.36	0.83
Histidine	31.25 ± 3.32	27.76 ± 2.22	0.39
Leucine	91.12 ± 11.41	80.59 ± 9.83	0.49
Isoleucine	44.20 ± 4.42	40.26 ± 4.74	0.55
Alloisoleucine	0.38 ± 0.04	0.35 ± 0.04	0.53
Lysine	96.10 ± 13.32	71.50 ± 9.71	0.15
Methionine	25.18 ± 3.02	21.68 ± 2.45	0.38
Ornithine	50.60 ± 7.47[a]	30.14 ± 4.43[b]	0.03*
Phenylalanine	40.55 ± 4.49	37.20 ± 4.28	0.60
Proline	59.76 ± 6.33	51.69 ± 5.37	0.34
Serine	59.79 ± 7.55	56.09 ± 5.76	0.70
Threonine	85.31 ± 11.52	90.05 ± 11.83	0.78
Tryptophan	30.32 ± 3.61	28.31 ± 3.70	0.70
Tyrosine	51.70 ± 5.32	50.33 ± 7.05	0.88
Valine	132.77 ± 15.08	115.53 ± 13.31	0.40
Alphaaminoadipic Acid	6.32 ± 0.82	5.90 ± 0.58	0.68
Alphaaminopimelic Acid	0.69 ± 0.06	0.76 ± 0.03	0.23
Anserine	5.44 ± 0.79[a]	2.83 ± 0.48[b]	0.01**
Argininosuccinic Acid	0.04 ± 0.01	0.05 ± 0.01	0.38
Alphaaminobutyric Acid	2.35 ± 0.42	2.44 ± 0.51	0.90
Betaaminoisobutyric Acid	16.89 ± 2.11	12.36 ± 2.41	0.17
Gammaminobutyric Acid	4.43 ± 0.68	3.85 ± 0.52	0.51
Beta-Alanine	12.21 ± 1.40	11.14 ± 1.17	0.56
Sarcosine	2.09 ± 0.25	1.93 ± 0.28	0.68
Cystathionine	0.41 ± 0.06[a]	0.23 ± 0.04[b]	0.02*
Thiaproline	4.63 ± 0.65	3.61 ± 0.49	0.22
1-Methylhistidine	0.80 ± 0.09	0.73 ± 0.10	0.59
3-Methylhistidine	0.04 ± 0.01	0.04 ± 0.01	0.66
Hydroxylysine	29.87 ± 3.73	25.76 ± 3.10	0.41
Hydroxyproline	0.39 ± 0.04	0.38 ± 0.05	0.93
5-OH-Tryptophan	8.32 ± 1.02	7.17 ± 1.16	0.46

a,b: Values within a row with different superscripts differ significantly.

amino acids with the highest scores (Figure 2A). High VIP scores indicate high contribution in discriminating the two groups. A heatmap was also generated to visualize amino acid changes (Figure 2B). No significant amino acid aggregation was observed between the groups. The investigation of biological pathways may contribute to elucidating the mechanisms associated with lactation and milk quality characteristics. No biological pathways with $P<0.05$ and impact value <0 were obtained on performing pathway analysis. However, the biological pathways with an impact value of >0.1, which are considered to effectively reflect the metabolic change in pathway analysis, are shown in Table 5. In addition, the pathways with an impact value of >0.4 are shown in Figure 3. These metabolic pathways included phenylalanine, tyrosine, arginine, and tryptophan biosynthesis pathways and alanine, aspartate, glutamate, D-glutamine, D-glutamate, arginine, and proline metabolism pathways.

Table 4. Correlations Between Milk Yield and Milk Quality Traits and Plasma Free Amino Acid Levels (n=26).

Traits	Milk Yield	Fat	Protein	Lactose	Dry Matter	SCC
Alanine	-0.25	0.01	0.04	-0.50**	-0.04	0.49*
Arginine	-0.26	0.05	0.02	-0.50*	-0.08	0.34
Asparagine	-0.16	-0.01	0.25	0.10	0.15	0.16
Aspartic Acid	-0.63**	0.07	0.04	-0.371	-0.02	0.42*
Citrulline	-0.26	-0.08	0.10	-0.05	-0.01	0.29
Glutamine	-0.15	-0.17	-0.26	-0.33	-0.32	0.11
Glutamic Acid	0.03	0.01	0.08	-0.15	-0.01	0.28
Glycine	-0.10	0.05	-0.08	-0.34	-0.09	0.22
Histidine	-0.33	0.05	-0.13	-0.50**	-0.14	0.39*
Leucine	-0.24	0.03	-0.14	-0.49*	-0.15	0.31
Isoleucine	-0.18	-0.04	0.02	-0.33	-0.08	0.33
Alloisoleucine	-0.17	-0.06	0.06	-0.26	-0.06	0.33
Lysine	-0.36	0.00	-0.08	-0.50**	-0.15	0.33
Methionine	-0.22	-0.01	-0.08	-0.42*	-0.14	0.30
Ornithine	-0.49*	-0.07	-0.10	-0.48*	-0.21	0.30
Phenylalanine	-0.16	0.02	-0.09	-0.35	-0.10	0.23
Proline	-0.27	0.07	-0.06	-0.45*	-0.08	0.42*
Serine	-0.11	0.01	-0.13	-0.25	-0.10	0.20
Threonine	-0.02	-0.17	-0.25	-0.14	-0.26	0.02
Tryptophan	-0.13	0.10	-0.15	-0.28	-0.06	0.22
Tyrosine	-0.04	0.05	-0.07	-0.21	-0.04	0.20
Valine	-0.27	-0.10	-0.04	-0.36	-0.15	0.27
Alphaaminoadipic Acid	-0.14	0.29	-0.07	-0.52**	0.04	0.41*
Alphaaminopimelic Acid	0.17	-0.24	-0.26	0.15	-0.24	0.13
Anserine	-0.52**	-0.17	-0.10	-0.44*	-0.27	0.36
Argininosuccinic Acid	0.16	0.02	0.11	-0.02	0.070	-0.05
Alphaaminobutyric Acid	-0.00	-0.17	-0.05	-0.11	-0.16	0.10
Betaaminoisobutyric Acid	-0.35	-0.04	-0.14	-0.22	-0.12	0.14
Gammaminobutyric Acid	-0.20	-0.09	-0.15	-0.31	-0.18	0.31
Beta-Alanine	-0.12	0.09	0.04	-0.37	-0.00	0.43*
Sarcosine	-0.22	-0.16	-0.15	-0.26	-0.24	-0.08
Cystathionine	-0.50*	-0.05	-0.02	-0.39*	-0.16	0.06
Thiaproline	-0.40*	0.09	-0.07	-0.49*	-0.08	0.43*
1-Methylhistidine	-0.28	0.16	-0.09	-0.39*	-0.01	0.24
3-Methylhistidine	0.07	-0.24	-0.16	-0.06	-0.25	0.18
Hydroxylysine	-0.27	0.01	-0.15	-0.51**	-0.17	0.33
Hydroxyproline	-0.01	0.31	0.14	-0.03	0.26	-0.12
5-OH-Tryptophan	-0.26	0.01	0.13	-0.02	0.07	-0.05

**: Correlation is significant at the 0.01 level (2-tailed).
*: Correlation is significant at the 0.05 level (2-tailed).
SCC: Somatic Cell Count.

DISCUSSION

Sheep milk has greater utility in the production of high-fat dairy products and cheese varieties due to its high fat, protein, and dry matter contents as well as its distinctive flavor (Moatsou and Sakkas, 2019). In the literature, there are few studies investigating milk yield and metabolomic parameters in sheep. In recent years, metabolomics has been used in many fields, and it has been shown that it can help to provide detailed information regarding sheep lactation mechanism (Shi et al., 2021). In this study, specific quality traits of milk such as dry matter content; fat, protein, and lactose levels; and somatic cell count as well as lactation data, a milk yield-related parameter, were determined. This study also aims to define new and important selection criteria for sheep breeding by examining the relationships between milk yield parameters and plasma free amino acid levels.

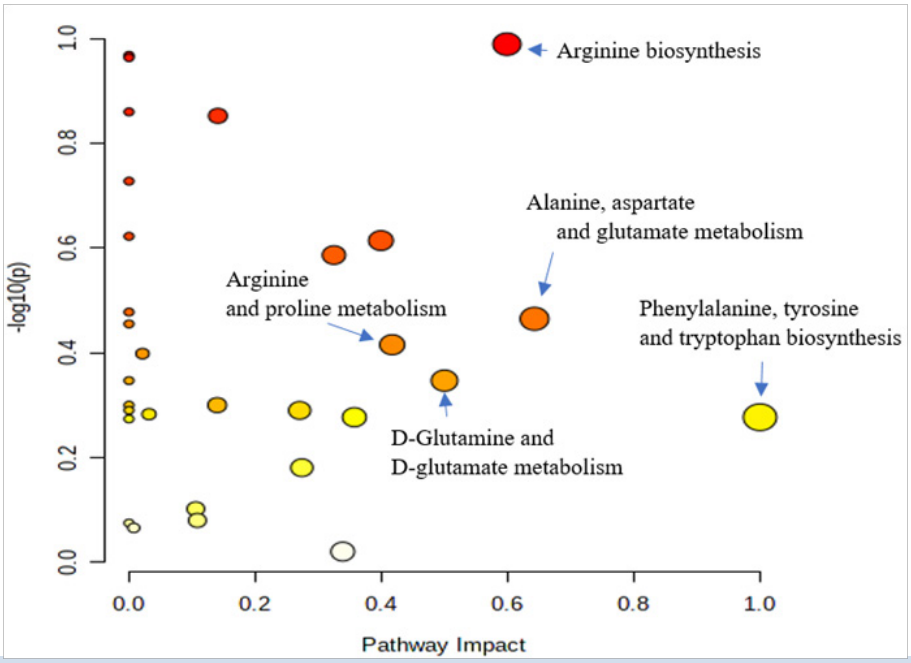

Figure 3. Summary of pathway analysis

Table 5. Impaired metabolic pathways

Pathway Name	Impact	P Value
Phenylalanine, tyrosine and tryptophan biosynthesis	1.000	0.528
Alanine, aspartate and glutamate metabolism	0.642	0.342
Arginine biosynthesis	0.598	0.102
D-Glutamine and D-glutamate metabolism	0.500	0.449
Arginine and proline metabolism	0.417	0.348
Beta-Alanine metabolism	0.399	0.243
Phenylalanine metabolism	0.357	0.528
Glycine, serine and threonine metabolism	0.338	0.953
Cysteine and methionine metabolism	0.324	0.259
Tryptophan metabolism	0.273	0.659
Histidine metabolism	0.270	0.512
Lysine degradation	0.140	0.140
Tyrosine metabolism	0.139	0.500
Glutathione metabolism	0.108	0.831
Glyoxylate and dicarboxylate metabolism	0.105	0.790

Biochemical tests are important tools for the evaluation of the physiological and health statuses of livestock. It is known that biochemical parameters vary depending on factors such as breed, age, housing conditions, hunger, environmental factors, stress, and transportation (Arfuso et al., 2016; Rathwa et al., 2017). These differences represent physiological values that can be used in the evaluation of animal production management and determination of nutritional, physiological, and health statuses of animals (Rathwa et al., 2017). The animals of both the study groups were found to have similar values in terms of biochemical parameters (Table 1). Thus, they were considered physiologically similar to each other. The results of the biochemical parameters obtained in our study are consistent with those reported by Rathwa et al. (2017). It has been reported that the change in the biochemical values of sheep blood serum during the lactation period is related to udder metabolism, lactogenesis and milk fat synthesis (Schweigert, 1990). According to the findings obtained in the study, the biochemical parameters of the sheep are within the normal physiological values.

A large variation in milk yield has been shown among ewes (Ferro et al., 2017). Milk yield chang-

es throughout the lactation period. There is a significantly high level of positive correlation between milk yield at the lactation peak and that during lactation (Ünal et al., 2008). The milk yield on day 75 in the present study was similar to the value reported by Dağ and Zülkadir (2004). The composition and physicochemical properties of sheep milk are of great importance in terms of product quality and marketing in the dairy industry (Park et al., 2007). It has been reported that total dry matter content in sheep milk varies between 14.4% and 20.7%, milk fat content between 3.60% and 9.97%, milk protein content between 4.75% and 7.20%, and milk lactose content between 4.88% and 5.51% (Ferro et al., 2017; Raynal-Ljutovac et al., 2008). However, it has been reported that milk fat content is higher in Awassi sheep than in other breeds (Ferro et al., 2017). Differences in milk yield and composition among sheep breeds have been observed by many researchers. Genetic factors, nutrition, lambing type, milking frequency, lactation period, and environmental conditions contribute to these differences (Kahraman and Yüceer Özkul 2020; Koutsouli et al., 2017; Peana et al., 2017). The somatic cells in milk are mostly cells of the immune system, such as lymphocytes, macrophages, and polymorphonuclear cells, and are part of the natural defense mechanism (Albenzio et al., 2019; Boutinaud and Jammes, 2002). Somatic cell count is an animal health status index, which is considered the standard milk quality for the production of milk and cheese. Although somatic cell count in sheep milk is different from other cattle milks, values up to 300×10^3 cells/ml are considered to be normal (Albenzio et al., 2019; Murphy et al., 2018). Somatic cell composition varies depending on many factors including animal species, breed, lactation stage, genetics, daily variation, milking interval, sampling time, sampling procedures, stress and trauma, management factors, and storage procedures (Park et al., 2013).

Glycine was the amino acid that was found at the highest level in the low- and high-milk yield groups (Table 3). It has been suggested that increased plasma glycine concentration in dairy cows is associated with the breakdown of muscle protein during lactation (Doepel et al., 2002) or the synthesis of glycine from threonine and serine (Amelio et al., 2014). The amino acid profiles evaluated within the scope of this study had low values in the group of ewes with high milk yield (Table 3). This may be due to the reduction of the limiting effect of some amino acids on milk yield (Meijer et al., 1993). This is supported by the negative correlations between amino acids and milk yield (Table 4). In addition, high milk yield and protein ratio require high amino acid concentrations. The low level of plasma amino acids observed in animals with high milk yields may be due to a decrease in amino acids resulting from their greater absorption and utilization by the mammary tissue during milk production (Swanepoel et al., 2010). Significant differences were detected between the groups in terms of aspartic acid, ornithine, anserine, and cystathionine levels. Animals used for milk production need amino acids to synthesize the proteins they need to maintain their life and yield characteristics. Each of these proteins consists of a different amino acid profile. Thus, livestock require different amounts of each amino acid in different physiological periods depending on lactation, growth, and pregnancy statuses (Tan et al., 2022). Furthermore, the marked differences of these amino acids in the plasma represent their extensive use by the mammary gland for milk protein synthesis and gluconeogenic and ketogenic precursor production (McCabe and Boerman, 2020).

Analysis of the correlations between milk yield, milk quality, and free amino acid levels in sheep (Table 4) showed a negative relationship ($P<0.05$, $P<0.01$, and $P>0.05$). There was a moderate negative correlation ($r=-0.63$) between milk yield and aspartic acid. During the lactation period, there is an increase in the calcium and magnesium requirements of farm animals for milk production. Mineral mobilization from the bone tissue occurs to maintain the mineral balance in the plasma (Goff 2008). Accordingly, aspartic acid is released by the deterioration of bone tissue; plasma concentrations of such aspartic acid may indicate the balance between bone formation and bone deterioration (Price et al., 1985). Hydroxyproline and proline are the most important amino acids which constitute collagen, the main structural protein of the connective tissues and bone (Marshall and Bangert, 2008). Anserine is a dipeptide degradation product found in the muscle tissue of many animals (Hussain et al., 2019). Since anserine is absent in human muscle, it is used as a biomarker for meat intake in humans (Mitry et al., 2019). This methylation product of histidine cannot be synthesized in humans who do not consume meat products containing anserine. In the present study, there was a moderate negative correlation ($r=-0.52$) between anserine and milk yield. It is hypothesized that the degradation of anserine in muscle tissue results in the production of 1-methylhistidine, increasing its plasma concentration (Yue et al.,

2020). Therefore, plasma anserine content may be a potential biomarker for monitoring protein mobilization in dairy animals with high milk yield (Yue et al., 2020). Although the lactation period represents only 20% of the livestock production stages, it is the most metabolically demanding production stage. During the lactation period, animals prioritize milk production for their offspring. However, most of the time, only voluntary feed intake is not sufficient for meeting the energy needs of this period, and mobilization of body fat and protein reserves is essential to support milk production in high-yielding animals (Pedersen et al., 2019, Tokach et al., 2019).

Moderate positive correlations were found between lactose and lysine (r=0.50), alpha-aminoadipic acid (r=0.52), and hydroxylysine (r=0.51). This relationship between amino acid and lactose levels may be observed due to the fact that amino acids are the precursors of alpha-lactalbumin, a protein that plays an important role in catalyzing lactose synthesis (Brew et al., 1968). There is a negative correlation between milk yield and quality. In our study, the low-quality parameters in the high-milk yield group led to positive correlations between quality parameters and amino acids. A positive moderate correlation was determined between somatic cell count and alanine, proline, and amino acids. Alanine has an effect on T cell activation; thus, it is considered to be involved in the immune system (Ron-Harel et al., 2019). Proline is structurally and functionally unique among all proteinogenic amino acids. Proteins with high proline and proline-containing peptide contents play an important biological role in metabolism, nutrition, cell recognition, and intracellular signaling processes. The high concentration of hydroxyproline and proline indicates that they are used to form collagen-containing proteins in the mammary gland, uterus, rumen, and muscle cells (Wu et al., 2011). Mastitis, a bacterial infection that causes inflammation of the mammary gland, is characterized by palpable lumps in the udder, abnormal milk, and tissue discoloration in its clinical state (Menzies and Ramanoon, 2001). The incidence of clinical mastitis is relatively low in sheep flocks (Murphy et al., 2018). Subclinical mastitis has no visual symptoms but can be diagnosed by bacterial culture or somatic cell count in sheep milk. Subclinical mastitis has a much higher morbidity rate than clinical mastitis in sheep (Arsenault et al., 2008; Murphy et al., 2018). Considering the correlations between somatic cell count and amino acids, plasma alanine and proline concentration may represent a new biomarker in subclinical mastitis.

In the present study, we identified the amino acids associated with milk yield and quality as well as the pathways in which these metabolites are involved. Based on the metabolic pathway analysis, phenylalanine, tyrosine, arginine, and tryptophan biosynthesis pathways and alanine, aspartate, glutamate, D-Glutamine, D-glutamate, arginine, and proline metabolism pathways were the most important pathways associated with metabolic changes. Phenylalanine, tyrosine, and tryptophan are the three aromatic amino acids involved in protein synthesis (Parthasarathy et al., 2018). These amino acids and their metabolism are associated with the synthesis of secondary metabolites involved in numerous anabolic pathways responsible for the synthesis of pigments, hormones, and biological polymers (Blackmore et al., 2013). Metabolites derived from these pathways mediate the transmission of nerve signals and are essential in maintaining animal health via the elimination of reactive oxygen species in the brain (Lamichhane et al., 2011). Phenylalanine is converted into tyrosine by the action of phenylalanine hydroxylase (Gilbert, 1992). Tyrosine is an essential amino acid for the production of several proteins and peptides. It is also the precursor of hormones such as thyroxine and catecholestrogens (Lemmon ve Schlessinger, 2000). Aromatic amino acids such as tryptophan, tyrosine, and phenylalanine can be converted into nicotinamide. This process results in greater energy production through glucose metabolism and hormone regulation (Pero, 2010). These characteristics may partially explain the differences observed in milk yield and quality in animals used for milk production (Tong et al., 2019). Alanine is the precursor of many compounds involved in cellular signaling, such as the aspartate and glutamate metabolism pathways, aspartate, and N-acetyl-aspartate. Additionally, it is a metabolite of the urea cycle and involved in gluconeogenesis in dairy cows (Piccioli-Cappelli et al., 2014). Studies conducted in the past years have shown that arginine, glycine, and methionine are the predominant amino acids involved in creatine synthesis in the liver (Poortmans et al., 2005). Creatine, an intermediate metabolite of energy-producing reactions, plays an important role in the regulation of negative energy balance in dairy cows and may also be a potential biomarker for heat stress (Wang et al., 2016). These findings show that amino acid profiles in sheep are not only related to yield and quality but can also be used to evaluate the health status.

CONCLUSIONS

In the present study, the low levels of some amino acids in the high-milk yield group and the correlations between milk yield, milk quality, and amino acids reveal the potential of these metabolites for use as biomarkers in the selection of high-yielding dairy animals. Correlation and pathway analyzes conducted in this study overlap with the key mechanisms involved in milk production. This study also provided better understanding of novel biochemical mechanisms underlying variations in milk yield and composition for Awassi sheep. The results may be validated by conducting studies with a large sample size and used as a preliminary screening tool for the selection of high yielding animals.

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CONFLICT OF INTEREST

The authors declared no conflict of interest.

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