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CONTENTS

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RESEARCH PAPER

LIVESTOCK STUDIES

Comparison of Parameters of Automatic Milking in Selected Countries in European Union and United States

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Abstract

The aim of this study was to compare selected parameters of automatic milking in various European Union countries and United States recorded between 2018 and 2020. Statistical analysis showed highly significant effect of country on all tested milking parameters. It was noted that i.e. the average number of cows per one robot, depending on country, ranged between 51.49 (the Netherlands) and 60.03 (Germany). Cows were milked on the average 2.50 (France) – 2.83 (Latvia) per day, with milking speed ranging from 2.30 kg/min (Lithuania) to 2.99 kg/min (US). Daily milk yield obtained from one cow ranged from 25.12 kg (Lithuania) to 34.11 kg (US), while milk efficiency from 1.44 (Lithuania) to 1.77 kg/min (US). Results also showed that the daily milk yield from one robot ranged from 1 351 kg (Latvia) to 1 930 kg (US). The statistical differences between the milking parameters in the compared countries may be the result of the diversified genetic potential of milked cows and the diversity of the feed base.

Introduction

Automatic milking system (AMS) that may be used to milk dairy cattle is in the use from the year 1992 – back then the first robot was launched by Lely in the Netherlands (Nixon *et al.,* 2009). Since then the interest in the automatic milking of cows is systematically growing. In 1998 an estimated number of farms with AMS worldwide was 250, in 2009 it was over 8 ths while in 2015 over 14 ths (de Koning & Van der Vorst, 2002; de Koning, 2010; Taing, 2016). Salfer *et al.* (2017) estimated that in 2017 over 35 ths AMS operated all over the world. Today, the number of only Lely robot milkers exceeds 30 ths worldwide (Lely International, 2019). The dynamics of the growing number of AMS installations, that may be observed nowadays, may result mainly from the deepening deficit of qualified employees, as well as the beneficial effect of robotization of milking on the level of milking parameters (Brzozowski *et al.*, 2018; Piwczyński *et al.*, 2020b; Sitkowska *et al.*, 2020). The results of numerous studies suggest that the increase in milk yield after the AMS installation, even up to 20%, is directly caused by the increased number of milkings performed by the cow during the day (Rotz *et al.*, 2003; Österman *et al.*, 2005). Czerniawska-Piątkowska *et al.*, (2012) noted the increase in the number of milkings from 2 to 4 times a day, which resulted in an increse of milk yiled in 305-day lactation by 1160 kg. In the previous research by the authors of this study, it was shown that cows in AMS barns in selected Eropean Union (EU) and United States (US) countries milk on average from 2.50 to 2.73 per day (Piwczyński *et al.*, 2020a). At the same time, in the latest research, conducted on a vast dataset, it was found that the change of the milking system from conventional to automatic resulted in an increase in the yield of cows in the first (+ 1 078 kg) and the second (+ 1 182 kg) standardised 305-day lactation (Piwczyński *et al.*, 2020b). The beneficial effect of changing the milking system was also demonstrated in terms of the

registered fertility traits (Brzozowski *et al.*, 2018; Piwczyński *et al.*, 2020b). It should be emphasized, however, that not in all herds covered by the study the effect of changing the milking system in terms of functional and production features was beneficial for the breeder (Brzozowski *et al.,* 2018; Piwczyński *et al.*, 2020b). Sitkowska *et al.* (2015) reported that the increase in milk yield on farms equipped with AMS is possible, but it depends on a number of factors related to the milk production process. On the other hand, Bogucki *et al.* (2014) noted that the beneficial effect of robotization of milking increased with the passage of time from the moment of AMS installation.

According to Tse *et al.* (2017), the AMS installation brings several benefits to farmers, including: higher daily milking frequency and milk production; better health and improvement of the herd's fertility rates, the possibility of better management of the herd based on collected information, lower requirement for manpower and greater work flexibility; better quality of life for breeders. AMS may record over 100 milking parameters (Lely International, 2019). Wethal and Heringstad (2019), who performed the study on Norwegian Red cattle, stated that the new parameters recorded by AMS (such as box time, average flow rate etc.) and which cannot be easily recorded in the conventional milking system (CMS), could be included in breeding programs.

The aim of this study was to compare selected parameters of automatic milking (monthly reports) in various European Union countries and United States recorded between January 2018 and October 2020.

Materials and Methods

The present study included data recorded between 1st January 2018 and 31st October 2020 by data recording system by Lely. The following data was gathered: Average number of robots per herd (no.), Number of cows per robot (no.), Daily milk yield per robot (kg), Robot's free time (%/24h), Average days in milk (days), Daily milking frequency (no./cow), Daily number of refusals (no./cow), Daily number of failed milking (no./cow), Box time – time spent by a cow in the robot during one visit (s), Milking speed (kg/min), Daily milk yield per cow (kg), Fat content (%), Protein content (%), Rumination time (min./24h) and Consumption of concentrated fodder per 100 kg of milk (kg). The accumulated data were recorded in the Czech Republic (CZ), France (FR), Germany (DE), Italy (IT), Latvia (LV), Lithuania (LT), the Netherlands (NL), Poland (PL) and the United States (US). In total, the results recorded approximately by 9 400 robots, distributed in 7 000 herds and concerning the productivity of 520 000 cows were analysed. Data was analysed with the use of two-way analysis of variance with the following linear model: $y_{ijk} = \mu + c_i + r_j + (cr)_{ij} + e_{ijk}$, where: y_{ijk} – registered value of a variable, μ – group average, c_i – effect of i^{th} country, r_j – effect of j^{th} year of milking, $(cr)_{ij}$ – country × milking year interaction, e_{ijk} – random error.

The significance of differences between countries was determined using the Scheffé test. A statistical analysis of the collected numerical material was carried out using the SAS v. 9.4 software (SAS Institute Inc., 2014).

Results and Discussion

Results showed a highly significant impact of the origin country of milked cows on all parameters recorded by AMS (Table 1), which is in accordance with the results of previous study carried out by the same authors (Waśkowicz *et al.*, 2014; Piwczyński *et al.*, 2020a). While these studies concerned analogous

Table 1. F statistic and significance (marked by *) of the impact of main factors and interactions on milking parameters.

Trait	Country (C)	Year (Y)	C×Y
Average number of robots per herd (no.)	3 845.97**	21.80**	40.33**
Number of cows per robot (no.)	200.87**	7.70**	4.11**
Daily milk yield per robot (kg)	269.79**	22.84**	2.09**
Robot free time (%/24h)	61.35**	9.45**	1.69*
Average days in milk (days)	54.96**	1.92	4.22**
Daily milking frequency (no./cow)	204.03**	4.40*	3.52**
Daily number of refusals (no./cow)	706.13**	2.24	4.85**
Daily number of failures (no./cow)	247.80**	38.88**	4.40**
Box time (s)	73.13**	10.38**	1.78*
Milking speed (kg/min)	410.82**	40.11**	1.84*
Daily milk yield per cow (kg)	366.22**	42.62**	3.14**
Milk efficiency (kg/min)	384.00**	16.01**	1.77*
Fat content (%)	148.41**	3.51*	1.05
Protein content (%)	138.19**	3.14*	0.47
Rumination time (min/24h)	98.22**	1 044.00**	3.84**
Consumption of concentrated fodder per 100 kg of milk (kg)	1 767.17**	47.80**	5.55**

*P ≤ 0.05, **P ≤ 0.01

features and countries, they spanned the period of operation from 2012-2017.

In these studies, authors suggested that the differentiation of countries in terms of controlled milk yields, was a result of a different genetic potential of dairy cows. In the present study, as well as in the previous one (Piwczyński *et al.*, 2020a) the statistical influence of milking year and the country × milking year interaction on most of the analysed features was demonstrated, with the exception of milking year on average daily days in milk and the number of rejected milkings, as well as interaction of country × year on percentage of fat and protein.

It was found that in the studied countries one farm on the average was equipped with 1.50 (in FR) - 3.32 (US) robots, with the average number of cows per one robot being at the level of 51.49 (NL) - 60.03 (DE) (Table 2). To a large extent, the observed differences can be explained by the different averages of herd sizes in countries included in this study (Piwczyński *et al*., 2020a). The study showed significant, statistical differentiation of the compared countries in terms of milk yield per robot i.e. from 1 351 kg (LT) up to 1 930 kg (US). At the same time, in 2018-2020 a general upward trend was shown in the number of robots in the herd, the number of cows per milking robot and daily milk yield per robot. This trend is in accordance with the one observed in past studies (Waśkowicz *et al.*, 2014; Piwczyński *et al.*, 2020a). It should be emphasized that a greater control over the herd, in which AMS is installed, may contribute to an increase in the number of cows per one robot without detriment to their health or their efficiency (Castro *et al.*, 2012; Tse *et al.*, 2017). Deming *et al.* (2013) found that herd performance could be increased by reducing the number of cows per robot and increasing access to the feed table and increasing the amount of provided feed. Tse *et al.* (2017) emphasize that the introduction of AMS in Canada gave breeders the opportunity to increase the number of cows in the herd (the average number of cows per robot was at the level of 52). It is very important to establish the optimal number of cows per robot. Rodenburg (2017) note that the optimal number of cows in a barn equipped with AMS should be less than 250 animals. On the other hand, Grant and Albright (2001) optimized for an even smaller herd size of less than 100 cows. In their opinion, it is good if animals recognize each other, then they can use AMS without stress. Barman *et al.* (2017) emphasize that the presence of cows in an unknown group exposes their body to stress, which results in a decrease in milk yield and weight loss. Perhaps, it is not possible to provide a universal and optimal number of cows per robot, and this number is best adjusted individually to each herd.

The results of studies by Castro *et al.* (2012) proved that the optimal free time of a robot should be about 10% of the day. In our research, it ranged from 19.00% (US) to as much as 27.42% (CZ) (Table 2). This presents a potential possibility of a significant increase in the number of cows per one robot, and then the daily milk yield and improving the profitability of production on the farm. One of the herd indicators recorded by AMS is the average lactation day (Table 2). In the present studies, the highest value of this indicator was found in NL (207.7 days), followed by PL (198.8 days), which proves that in these countries' lactation lasted the longest. On the other hand, the shortest lactations were noted in LV (181.7 days) and LT (185.3 days). In the studied herds, cows milked on average 2.71 times a day, the least frequently in FR (2.50 milkings/day), and most often in LV (2.83 milkings/day) and NL (2.82 milkings/day) (Table 2). The obtained results were generally characterized by a growing tendency in the reporting years 2018-2020, exceeding the results presented in previous studies (Waśkowicz *et al.*, 2014; Piwczyński *et al.*, 2020a). In the study by Sitkowska *et al.* (2020), who investigated primiparas performance, the importance of the average milking frequency (especially in the initial phase of the first lactation) on milk yield in its further stages was demonstrated with levels above 3.50 milkings a day considered optimal. In turn, Castro *et al.* (2012) found the range between 2.40 and 2.60 a day to be the optimal value of milking frequency per day.

It was found that in the studied countries, the milking robot software limited the animal's ability to undergo milking (refuslas milkings) on average from 1.57 (US) to 3.57 no./day (LT) (Table 3). At the same time, it was observed that the number of failed milkings ranged from 3.84 (FR) to 6.58 no./day (LT). It should be emphasized that in 2018-2020 there was a favorable tendency to reduce failed milkings (from 5.13 no./day to 4.72 no./day). Also the fact that the results presented in the present study were more favorable than those reported previously (Waśkowicz *et al.*, 2014) should also be noted. The frequency of milkings and milking time are the most common indicators of cow's maturity for full production. Castro *et al.* (2012) found that varying number of cows per robot and the milk speed had the greatest effect on the daily milk yield in AMS. Salfer *et al.* (2017) emphasised that management system in AMS equipped barns should be properly parameterized in such a way that it makes sure that cows occupy robots at the right time. In AMS, the amount of milk obtained by the robot per day is of key importance in shaping the profitability of production, which is directly related to the amount of milk collected per minute of the time cow spends in the milking stall – milk efficiency (kg/min). This feature, in turn, is directly dependent on the duration of the cow's stay in the milking stall, milking speed and milk yield, as well as time that is spend on preparing the cow for milking (including robot attachment time). Cows in the barns covered by the study spent on the average 394 s (IT) to 416 s (US) in the milking box, gave between 25.17 (LT) and 34.14 kg of milk (US), with the speed that ranged between 2.30 kg/min (LT) and 2.99 kg/min (US) (Table 3). The milk efficiency index, calculated for the entire dataset on the basis of this information, equalled 1.59 kg/min, and ranged in different counties from 1.44 kg/min (LV) to 1.77 kg/min (US).

Table 2. Milking parameters according to country and milking year.

Year	Statistics	Country									Total
		CZ	DE	FR	IT	LT	LV	NL	PL	US	
Average number of robots per herd (no.)											
2018	$\bar{x}$	2.08	1.66	1.47	1.70	2.66	1.90	1.98	1.79	3.12	2.04
2019	$\bar{x}$	2.00	1.68	1.50	1.71	2.43	1.84	2.00	1.71	3.32	2.02
2020	$\bar{x}$	1.97	1.74	1.54	1.77	2.40	1.95	2.05	1.66	3.57	2.07
Total	$\bar{x}$	2.02	1.69	1.50	1.73	2.50	1.90	2.01	1.72	3.32	2.04
		A	AB	ABC	ACD	ABCDE	ABCD EF	BCDE FG	ACEF GH	ABCD EFGH	
	CV	2.69	2.08	1.92	2.66	6.29	4.46	1.52	3.74	5.78	26.21
Number of cows per robot (no.)											
2018	$\bar{x}$	53.96	60.48	55.29	56.97	53.59	53.21	51.13	58.24	56.25	55.46
2019	$\bar{x}$	54.92	60.19	56.05	57.01	53.67	55.03	51.60	59.09	56.47	56.00
2020	$\bar{x}$	55.60	59.31	55.45	57.14	54.15	52.77	51.80	57.07	57.08	55.60
Total	$\bar{x}$	54.78	60.03	55.60	57.03	53.78	53.72	51.49	58.19	56.57	55.69
		Aa	AB	BC	ABCDb	BCDEa	BCDFa	ABCD EFG	ABCE FGHb	ABEF GH	
	CV	1.78	1.52	1.91	2.24	2.26	3.33	1.69	2.38	0.89	4.85
Daily milk yield per robot (kg)											
2018	$\bar{x}$	1525	1709	1572	1799	1277	1424	1515	1692	1908	1602
2019	$\bar{x}$	1528	1714	1587	1801	1376	1526	1529	1775	1910	1638
2020	$\bar{x}$	1588	1728	1622	1860	1411	1489	1547	1746	1979	1663
Total	$\bar{x}$	1545	1716	1592	1818	1351	1479	1529	1737	1930	1633
		Aa	AB	BCb	ABCD	ABCDE	BCDE Fa	BDEGb	ACDE FGH	ABCD EFGH	
	CV	4.08	3.04	4.70	5.25	5.82	5.74	3.12	4.10	2.64	11.33
Robot free time (%)											
2018	$\bar{x}$	28.68	21.94	26.23	27.25	28.09	22.65	26.97	20.12	18.83	24.53
2019	$\bar{x}$	27.35	21.83	25.37	27.25	24.47	23.73	25.58	19.30	19.24	23.79
2020	$\bar{x}$	26.00	21.46	24.73	26.57	23.26	22.52	24.81	19.72	18.92	23.11
Total	$\bar{x}$	27.42	21.76	25.49	27.05	25.39	22.99	25.84	19.71	19.00	23.85
		A	AB	BCa	BD	BEb	ADFab	BFG	ACDE FG	ABCDE FG	
	CV	6.97	6.74	12.22	10.76	10.98	11.61	9.45	11.36	7.33	15.84
Average days in milk (days)											
2018	$\bar{x}$	185.7	183.4	189.7	195.1	189.1	180.2	201.7	203.1	181.2	189.9
2019	$\bar{x}$	188.7	187.9	191.9	195.7	187.0	180.7	210.2	201.6	180.7	191.6
2020	$\bar{x}$	191.5	192.1	192.3	191.1	178.6	184.7	212.0	190.5	178.5	190.1
Total	$\bar{x}$	188.5	187.6	191.2	194.1	185.3	181.7	207.7	198.8	180.2	190.6
		Aa	B	Cb	D	DE	aCDF	ABCD EFG	ABEF GHb	ABCD GH	
	CV	2.16	3.17	4.86	5.19	4.72	3.79	3.14	4.48	1.35	5.76
Daily milking frequency (no.)											
2018	$\bar{x}$	2.66	2.62	2.51	2.74	2.58	2.83	2.83	2.76	2.79	2.70
2019	$\bar{x}$	2.63	2.62	2.48	2.79	2.65	2.86	2.83	2.79	2.78	2.71
2020	$\bar{x}$	2.61	2.67	2.50	2.80	2.68	2.81	2.82	2.80	2.79	2.72
Total	$\bar{x}$	2.64	2.63	2.50	2.78	2.64	2.83	2.82	2.78	2.79	2.71
		A	B	ABC	ABCDa	CDE	ABCD Eb	ABCEa	ABCEb	ABCEb	
	CV	1.84	1.80	1.55	1.55	2.62	2.42	1.76	1.39	1.29	4.40

CV – coefficient of variation (%); CZ - Czech Republic, FR – France, DE – Germany, IT – Italy, LV – Latvia, LT – Lithuania, NL – Netherlands, PL – Poland, US – United States

AA (aa) – Values that are significantly different within a variable are marked with the same letters $P \leq 0.01$ ($P \leq 0.05$)

Table 3. Milking parameters according to country and milking year.

Year	Statistics	Country									Total
		CZ	DE	FR	IT	LT	LV	NL	PL	US	
Daily number of refusals per cow (no.)											
2018	$\bar{x}$	2.33	2.46	1.77	1.65	3.64	2.05	3.61	2.09	1.55	2.35
2019	$\bar{x}$	2.39	2.49	1.70	1.69	3.33	2.12	3.53	2.01	1.61	2.32
2020	$\bar{x}$	2.22	2.49	1.66	1.60	3.78	2.10	3.29	2.01	1.56	2.3
Total	$\bar{x}$	2.32	2.48	1.71	1.65	3.57	2.09	3.49	2.04	1.57	2.32
		Aa	Ba	ABC	ABD	ABCDE	ABCDEF	ABCDFG	ABCDEGH	ABEFGH	
	CV	5.04	2.94	6.55	6.01	9.79	6.95	8.3	6.39	5.26	31.28
Daily number of failures per cow (no.)											
2018	$\bar{x}$	4.62	4.38	3.95	4.76	7.19	5.78	4.36	5.99	5.13	5.13
2019	$\bar{x}$	4.68	4.32	3.93	4.58	6.33	6.00	4.41	5.57	5.39	5.02
2020	$\bar{x}$	4.49	4.03	3.61	4.14	6.21	5.83	4.16	5.25	4.78	4.72
Total	$\bar{x}$	4.60	4.25	3.84	4.49	6.58	5.87	4.31	5.61	5.11	4.97
		Aa	aB	ABC	CD	ABCDE	ABCDEF	CEFG	ABCDEGH	ABCDEFGH	
	CV	5.67	4.42	5.29	6.43	10.96	10.25	4.85	6.18	7.32	18.81
Box time per cow per visit (s)											
2018	$\bar{x}$	413	405	424	394	388	400	403	396	419	405
2019	$\bar{x}$	413	405	427	393	399	406	405	402	413	407
2020	$\bar{x}$	418	406	430	396	397	408	410	406	415	410
Total	$\bar{x}$	415	406	427	394	395	405	406	401	416	407
		A	AB	ABC	ABCDa	ABCE	ACDEF	ACDEG	ACHa	BCDEFGH	
	CV	1.25	1.21	2.00	2.41	2.01	1.76	1.19	2.30	2.09	3.06
Milking speed (kg/min)											
2018	$\bar{x}$	2.45	2.54	2.54	2.83	2.24	2.26	2.53	2.56	2.92	2.54
2019	$\bar{x}$	2.47	2.58	2.55	2.82	2.32	2.33	2.54	2.62	3.00	2.58
2020	$\bar{x}$	2.53	2.63	2.58	2.85	2.36	2.39	2.55	2.63	3.07	2.62
Total	$\bar{x}$	2.48	2.58	2.56	2.83	2.30	2.32	2.54	2.60	2.99	2.58
		A	AB	AC	ABCD	ABCDE	ABCDF	DEFG	ADEFH	ABCDEFGH	
	CV	2.52	2.66	2.19	2.05	3.78	3.83	1.97	2.51	3.23	8.50
Daily milk yield per cow (kg)											
2018	$\bar{x}$	28.24	28.26	28.43	31.56	23.83	26.76	29.63	29.04	33.93	28.85
2019	$\bar{x}$	27.83	28.47	28.31	31.58	25.62	27.73	29.63	30.05	33.83	29.23
2020	$\bar{x}$	28.56	29.14	29.23	32.54	26.07	28.2	29.85	30.59	34.68	29.87
Total	$\bar{x}$	28.20	28.61	28.65	31.89	25.17	27.56	29.70	29.89	34.14	29.28
		A	B	C	ABCD	ABCDE	BCDEF	ABCDEFG	ABCDEFH	ABCDEFGH	
	CV	2.89	2.37	3.20	3.49	4.98	4.10	1.72	3.05	2.11	8.86
Milk efficiency (kg/min.)											
2018	$\bar{x}$	1.54	1.60	1.60	1.75	1.42	1.42	1.56	1.59	1.74	1.58
2019	$\bar{x}$	1.54	1.61	1.60	1.73	1.45	1.43	1.55	1.61	1.77	1.59
2020	$\bar{x}$	1.57	1.61	1.63	1.76	1.47	1.48	1.55	1.62	1.80	1.61
Total	$\bar{x}$	1.55	1.61	1.61	1.75	1.45	1.44	1.55	1.61	1.77	1.59
		A	AB	AC	ABCD	ABCDE	ABCDF	BCDEFG	ADEFGH	ABCEFGH	
	CV	2.19	2.09	2.19	2.16	2.95	2.96	2.30	1.87	2.49	7.09

CV – coefficient of variation (%); CZ - Czech Republic, FR – France, DE – Germany, IT – Italy LV – Latvia, LT – Lithuania, NL – Netherlands, PL – Poland, US – United States

AA (aa) – Values that are significantly different within a variable are marked with the same letters $P \leq 0.01$ ($P \leq 0.05$)

It is significant that the presented results regarding milking speed, milk yield and milk efficiency indicate a favourable upward trend in years 2018-2020, which is a continuation of the previously studied period of years 2012-2017 (Waśkowicz *et al.*, 2014; Piwczyński *et al.*, 2020a).

When analysing the chemical composition of milk, it was observed that the highest fat (4.39%) and protein (3.55%) contents were found in milk obtained from cows in NL (Table 4). On the other hand, the lowest level of fat was recorded in milk in IT (3.84%), and proteins in US (3.11%). The studies showed a significant influence of the milking year on the analysed characteristics of milk composition (Table 1), but the changes in their level in the following years did not express any clear trend. Also, the currently presented results in this respect were similar to those obtained in previous studies (Waśkowicz *et al.*, 2014; Piwczyński *et al.*, 2020a).

Salfer *et al.* (2017) noted yet another important benefit for AMS farms, namely pelleted feed supplementation in AMS box was strongly associated with body condition and thus might cause the increase in milk production. When analysing the rumination activity of cows (measured by the number of minutes per day), it was found that rumination time was the shortest in the US (468.99 minutes), and the longest in the LV (504.86 minutes) (Table 4). The amount of concentrated feed intake depends on many factors, including the palatability of the feed and the individual requirements of a cow. The analysis showed that a cow for the production of 100 kg of milk consumed from 12.96 kg (IT) to 20.05 kg (NL) of concentrate feed.

Table 4. Milking parameters according to country and milking year.

Year	Statistics	Country									Total
		CZ	DE	FR	IT	LT	LV	NL	PL	US	
		Fat content (%)									
2018	$\bar{x}$	3.95	4.04	4.09	3.83	4.18	3.95	4.37	3.91	3.87	4.02
2019	$\bar{x}$	4.00	4.10	4.17	3.86	4.11	3.96	4.41	3.92	3.90	4.05
2020	$\bar{x}$	3.96	4.09	4.16	3.84	4.14	3.95	4.39	3.97	3.89	4.04
Total	$\bar{x}$	3.97	4.07	4.14	3.84	4.14	3.96	4.39	3.93	3.88	4.04
		Aa	AB	AC	ABCD	ADE	BCDEF	ABCDEFG	BCDEG	BCEGa	
	CV	2.22	2.17	2.10	1.45	1.93	2.38	2.31	1.26	1.95	4.44
		Protein content (%)									
2018	$\bar{x}$	3.40	3.43	3.32	3.38	3.37	3.29	3.53	3.38	3.11	3.36
2019	$\bar{x}$	3.43	3.45	3.36	3.40	3.36	3.33	3.56	3.37	3.12	3.38
2020	$\bar{x}$	3.39	3.44	3.35	3.39	3.35	3.29	3.55	3.37	3.11	3.36
Total	$\bar{x}$	3.41	3.44	3.34	3.39	3.36	3.30	3.55	3.37	3.11	3.36
		Aa	B	BCa	D	BEb	ABDFb	ABCDEFG	BFGH	ABCDEFGH	
	CV	1.90	1.73	1.77	1.37	1.65	2.06	1.73	0.76	1.97	3.66
		Rumination time (min./24h)									
2018	$\bar{x}$	462.2	470.0	474.4	456.2	474.6	483.9	476.9	470.3	446.3	468.3
2019	$\bar{x}$	491.8	485.6	500.0	476.7	482.8	501.7	498.8	490.9	472.3	489.0
2020	$\bar{x}$	507.0	505.6	516.2	502.0	507.5	528.9	521.3	510.5	488.3	509.7
Total	$\bar{x}$	487.03	487.06	496.87	478.28	488.31	504.86	499.02	490.58	468.99	487.8
		A	B	ABCa	ABCD	CDE	ABCDEF	ABDEG	DFGHa	ABCDEFGH	
	CV	4.03	3.09	3.65	4.16	3.18	3.98	3.80	3.49	3.99	4.23
		Consumption of concentrated fodder per 100 kg of milk (kg)									
2018	$\bar{x}$	16.07	14.41	14.14	12.73	16.87	13.92	20.44	14.59	14.88	15.34
2019	$\bar{x}$	15.99	14.18	14.18	13.12	16.78	13.79	20.05	14.28	14.68	15.23
2020	$\bar{x}$	15.36	13.84	14.05	13.03	16.11	13.91	19.67	14.01	14.46	14.94
Total	$\bar{x}$	15.81	14.14	14.12	12.96	16.59	13.87	20.05	14.29	14.67	15.18
		A	AB	AC	ABCD	ABCDE	ADEF	ABCDEFG	ADEFGH	ABCDEFGH	
	CV	2.20	1.75	1.92	1.99	3.89	2.88	2.14	1.89	1.49	13.46

CV – coefficient of variation (%); CZ - Czech Republic, FR - France, DE - Germany, IT - Italy LV - Latvia, LT - Lithuania, NL – Netherlands, PL – Poland, US – United States

AA (aa) – Values that are significantly different within a variable are marked with the same letters $P \leq 0.01$ ($P \leq 0.05$)

The average consumption of concentrate was at the level of 15.18 kg. It should be emphasized that in the years 2018-2020, the average rumination time increased (by 41.4 minutes), while the amount of concentrated feed needed for the production of 100 kg of milk decreased (by 0.4 kg). It is particularly interesting as during this time the daily milk yield of a cow increased by more than 1 kg. This means that breeders in order to satisfy the nutritional needs of cows, instead of concentrate feed, introduced a larger amount of roughage, as evidenced by the longer rumination time. Endres and Salfer (2015), emphasized that in order to obtain the highest milk yield in AMS farms farmers, apart from introducing high-production cows and reducing box time, should also focus on providing good nutritional management.

When analysing the calculated coefficients of variation (CV) of the controlled features according to country, it should be emphasized that they showed a tendency to express low values - in most cases they did not exceed 5%. This proves a significant unification of the parameter values in the subsequent months of data reporting.

Conclusion

Summing up the results of the conducted research, it should be stated that the tested milking parameters were highly significantly influenced by the country where the milking robot was located. The presumptive reason for these differences was the differentiation of the genetic potential of milked cows and the diversity of the feed base. The research showed that the most favourable results in terms of financial efficiency of a farm (milk speed, milk yield, milk efficiency, robot yield) were found in US and IT herds. In years 2018-2020, a favourable trend was observed in terms of the above-mentioned features.

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RESEARCH PAPER

LIVESTOCK STUDIES

In Vitro Fermentation Characteristics of Camelina Meal Comparison with Soybean Meal

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Abstract

The search for new and cheap sources of protein has been increased lately. Although camelina meal has antinutritive factors; compare to soybean it can be widely useable. The objective of this study is to remove the question mark in minds about camelina meal and to determine the fermentation characteristics parameters including pH, ammonia-N level, volatile fatty acids concentration as well as total gas volume, methane proportion and the estimated degradation of camelina meal in comparison with soybean meal. Basically, we used *in vitro* gas production system according to modified Hohenheim Gas Test (HFT) to compare camelina meal and soybean meal. Rumen contents obtained from two Holstein cows. There was no significant difference of pH and ammonia-N concentration between soybean meal and camelina meal, whereas total volatile fatty acid and acetate concentration were reduced in camelina meal. Additionally, total gas production, fermentative CO_2 and estimated ME and organic matter digestibility were not altered. However, methane production decreased significantly in camelina meal fermenters. Consequently, it was concluded that camelina meal can be replaced of soybean meal, since microbial fermentation does not change and it might reduce the methane emission in which has commonly major effect on environmental pollution as a sera gas.

Introduction

Soybean meal (SBM) is commonly used in livestock nutrition as an attractive protein source of plant origin in the world, although its high price. In ruminants that costs don't compete with the humans or monogastric animals have encouraged the search alternative protein sources to replace soybean meal (Haddad, 2006; Alves *et al.*, 2016; Florou-Paneri *et al.*, 2014). In the last decades, due to its high quality protein and the search for cheaper resources, the demand for camelina seeds has increased (Russo *et al.*, 2017). *Camelina sativa* compared to soybean has low nutrient requirements, good resistance to diseases and pests (Halmemies-Beauchet-Filleau *et al.*, 2018). Camelina meal (CM), the by-product of camelina oil extraction, is an alternative protein source for livestock despite its higher antinutritive factors compared to soybean meal (Sizmaz *et al.*, 2016; Russo *et al.*, 2017). Nevertheless, CM has been considered as acceptable (Waraich *et al.,* 2013). CM in livestock diets contain glucosinolates, phytic acid, sinapine and condensed tannins. Especially glucosinolates are antinutritional factors; disrupts the thyroid activity and decreases the feed intake (Paula *et al.,* 2019). Therefore, in 2002, European Union (EU) Directive forbid the usage of *C. sativa* in livestock rations due to the presence of glucosinolates. Yet, in 2008 EU Directive, after many studies, permits the feed use of *C. sativa* and its derivatives (Colombini *et al.,* 2014). Because ruminants are more tolerant to glucosinolates compared to monogastric animals; is also a reason to put them back in the field (Vincent *et al.,* 1988).

We hypothesized that the camelina meal might be shown similar fermentation characteristics with soybean meal. Thus, the current study is conducted to investigate the *in vitro* rumen fermentation parameters including pH, ammonia-N level, volatile fatty acid concentration, estimated degradation and gas

production of camelina meal as a replacement of soybean meal.

Materials and Methods

Based on our previous study (Sizmaz *et al.,* 2016), evaluating the impact of nutrients degradation of camelina and soybean meal *in vitro*, the fermentation characteristics, gas production and fermentative methane emission were investigated in this study. The same camelina meal and soybean meal samples were used in this experiment. Thereby the nutrients of the samples were taken from our previous study (Table 1).

In Vitro Fermentation Technique

In vitro rumen fermentation was performed according to a modified HFT (Menke and Steingass, 1986). Two hundred milligrams of the camelina meal and soybean meal substrate were incubated with 30 ml of a ruminal buffered suspension (2:1; buffer solution: rumen fluid) by flushing CO_2 before was anaerobically dispensed in each syringe at 39°C. The rumen contents were obtained from two cannulated Holstein cows with a live weight of 630 ± 21.3 kg before morning feeding kept at Ministry of Agriculture and Forestry, International Center for Livestock Research and Training. Rumen fluid was immediately transferred to the laboratory to *in vitro* fermentation with preheated thermos flask. Then, rumen fluid immediately mixed with the buffer solution (Macro Element Solution: Na_2HPO_4, KH_2PO_4 and $MgSO_4.7H_2O$; Micro Element Solution: $CaCl_2.2H_2O$, $MnCl_2.4H_2O$, $CoCl_2.6H_2O$ and $FeCl_3.6H_2O$; Buffer Solution=$NaHCO_3$ and NH_4HCO_3; Resazurin Solution=Resazurin; Reductant Solution= $Na_2S.7H_2O$ and NaOH) which was bubbled with CO_2, at 39°C for 24h incubation.

Rumen Sampling and Analysis

After 24h incubation, the rumen fluid samples were collected from syringes of each group and were strained into the individual beakers with a sterile cheesecloth to stop the fermentation. The pH was measured immediately with a pH-meter (Hanna Instruments). Ammonia-N in rumen fluid was analyzed using spectrophotometry by using indophenol blue method at 546 nm according to the method described by Chaney and Marbach (1962).

Concentration of VFA were determined according to Geissler *et al.* (1976). Rumen samples were centrifuged at 4.000 rpm for 15 min at 4°C. One ml of supernatant was then transferred to an Eppendorf tube and mixed with 0.2 ml ice-cold 25% met phosphoric acid solution. Then, tubes were kept at 4°C for 30 min. Subsequently, these tubes were centrifuged again at 13.000 rpm for 10 min at 4°C and the supernatant was transferred into gas chromatography vials to determine acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid concentrations. Samples were analyzed by using gas chromatography (Shimadzu GC-2010, Shimadzu Co., Kyoto, Japan) coupled with a capillary column (TR-151035, TRB-FFAP, 30 m x 0.53 mm). The column temperature was programmed to increase gradually from 120°C to 160°C during the analysis. In addition, the injector port and flame ionization detector (FID) temperatures were fixed at 230°C and 250°C, respectively. The injection volume was set to 1 µL and analyses were performed in duplicate.

In Vitro Total Gas Volume, Methane Production and Estimated Digestion Values

After 24h of incubation, the total gas volume of each syringe was recorded. The metabolizable energy (ME) and organic matter digestibility (DOM) contents of the camelina meal and soybean meal were calculated using the equations by Menke and Steingass (1988) as follows:

$$ME\ (MJ/kg) = 2.20 + 0.136 \times Gas24h + 0.057 \times CP$$

$$DOM\ (g/kg) = 14.88 + 0.889 \times Gas24h + 0.45 \times CP + 0.0651 \times A$$

Where; Gas24 h net gas production (ml/200mg), CP; crude protein (%), A; ash content (%).

Methane production was calculated using the equations proposed by Abdl-Rahman (2010) based on the stoichiometry of Wolin (1960), as follows;

$$Fermentative\ CO_2 = A/2 + P/4 + 1.5\ B$$

$$Fermentative\ CH_4 = (A + 2\ B) - CO_2$$

A; mole of acetate, P; mole of propionate, B; mole of butyrate.

Table 1. The chemical composition of camelina meal and soybean meal used in the experiment.

Chemical Composition	Soybean Meal	Camelina Meal
DM	896.00	885.90
OM	940.00	946.10
CP	482.00	369.70
EE	16.50	14.90
CF	52.50	110.70
Ash	60.00	53.90
ME, MJ/kg	11.67	10.40

DM; Dry matter, OM; organic matter, CP; crude protein, EE; ether extract, CF; crude fiber, ME; metabolizable energy.

Statistical Analysis

Statistical analysis for the data from the rumen fermentation parameters were conducted using SPSS software (V22.0; SPSS Inc., Chicago, IL, USA). First, the Shapiro–Wilk test was adopted to check whether the distribution of the variables exhibited a normal distribution. Then, the variables that showed a normal distribution were analyzed by the independent sample t test. Significant differences were declared at $P < 0.05$; a tendency was considered for $0.05 < P \leq 0.10$.

Results

The *in vitro* fermentation characteristics of the soybean meal and camelina meal are shown in Table 2. Basically, after the results from *in vitro* fermentation; the levels of acetate and total VFA are significantly higher in soybean meal than CM ($P < 0.05$). Additionally, as it can be seen in the Table 2 the level of isovalerate and propionate tended to increase in soybean meal than CM ($0.05 < P \leq 0.10$). Soybean meal's pH level were higher than CM numerically (6.92 vs. 6.79). However, as for A/P, isobutyrate, butyrate, isovalorate and valorate levels; no significant difference was observed between soybean meal and CM ($P > 0.05$).

The *in vitro* total gas volume, fermentative CH_4, fermentative CO_2 and estimated digestibility of camelina meal and soybean meal are shown in Table 3. Initially the level of fermentative CH_4 is significantly higher in soybean meal than CM ($P < 0.05$). However, there were no differences determined in the fermentative CO_2, total gas volume, ME and DOM between soybean meal and CM ($P > 0.05$).

Discussion

In the recent years, camelina meal has been evaluated for alternative protein sources in ruminant rations. Plenty of studies have shown the potential of camelina meal to improve the degradability and few of them to modify ruminal fermentation (Moriel *et al.,* 2011; Colombini *et al.,* 2014; Lawrence *et al.,* 2016; Sizmaz *et al.,* 2016 & Brando *et al.,* 2018). Present study contributes the literatures for an *in vitro* fermentation characteristics including pH, ammonia level, VFA concentration, total gas volume and methane production of CM compared to SBM in an *in vitro* gas production system. Our study provided that pH, ammonia level and VFA were not altered while CM had less concentration of acetate and total VFA. This could be caused by the fiber content of SBM and CM and would be effective *in vivo* studies. Feeding CM at 10% of the diet to heifers did not affect ruminal pH and ammonia level and volatile fatty acids concentration (Lawrence *et al.,* 2016). Brando *et al.* (2018) reported that the ruminal pH and total VFA concentration were not affected by CM treatment at level of 50% and 100% in fermentor system. These lack of effects of CM on ruminal fermentation characteristics may be related to the lack of effects on ruminal microbial population; bacteria, fungi, and protozoa (Bayat *et al.,* 2015; Halmemies-Beauchet-Filleau *et al.,* 2016 & Paula *et al.,* 2019). Paula *et al.* (2019) stated that none of the reported studies that tested CM observed effects on total VFA concentration. Additionally, the study by Lawrence *et al.* (2016) found that the NH_3-N levels was higher in CM compared with DDGS and linseed meal fed heifers and Brandao *et al.* (2018) has shown that the ammonia level decreased by inoculation of CM to the fermenters. These results associated with the bacterial population and activity, would indicate that the protein degradation in the ration.

Because of the *in vitro* and *in vivo* conditions, protein sources, dosage of the CM, the forage:concentrate ratio and basal diet composition, the CM does not alter on overall microbial fermentation but may effect ruminal milieu as a bacterial community composition and thus change ammonia level and the VFA molar proportions.

Table 2. The *in vitro* pH, ammonia-N (mmol/l) and volatile fatty acids concentration (mM/l) of camelina meal (CM) and soybean meal (SBM).

Treatments	pH	Ammonia-N	A/P	Acetate	Propionate	Isobutyrate	Butyrate	Isovalerate	Valerate	Total VFA
SBM	6.92± 0.194	41.90± 3.119	3.05± 0.152	42.91± 1.276	14.10± 0.285	1.95± 0.111	8.17± 0.696	3.28± 0.265	2.18± 0.063	72.58± 0.67
CM	6.79± 0.147	39.04± 1.986	2.88± 0.067	35.79± 0.883	12.47± 0.595	1.75± 0.047	7.51± 1.125	2.59± 0.126	1.99± 0.059	62.11± 2.489
P	0.622	0.483	0.358	0.010	0.068	0.181	0.649	0.078	0.101	0.015

Table 3. The *in vitro* total gas volume, CO_2 and CH_4 proportion (mM/L) and estimated ME (MJ/kg) and degradation of organic matter (DOM; g/kg) of camelina meal (CM) and soybean meal (SBM).

Treatments	Fermentative CO_2	Fermentative CH_4	Total gas volume	ME	DOM
SBM	37.23±0.479	22.01±0.361	56.67±17.487	6.49±0.474	47.04±3.111
CM	32.28±2.278	18.54±0.857	71.33±32.338	6.25±0.881	44.55±5.752
P	0.101	0.020	0.710	0.820	0.723

In the present study, we evaluated the decreasing effect of CM on *in vitro* methane production. Our best knowledge is this is the first trial of CM effect on methane emission. Some studies have been conducted the effects of camelina oil on total gas volume, CO_2 and methane production (Bayat *et al.,* 2015; Ebeid *et al.,* 2020) that reported similar findings with our experiment. In these studies, the authors reported that no difference has been found in the gas volume and CO_2 concentration among treatments and a decrease in methane emission in lactating dairy cows fed with different forage:concentrate ratio and camelina oil concentrations and different basal diet compositions such as supplemented with feed additives. Camelina seeds showed the decreasing effect on methane in a ration having a roughage-to-concentrate ratio (Wang *et al.,* 2017). Therefore, the important point the effects on methane emission is the dietary form of camelina if seed, oil or meal used in the diet.

Zagorakis *et al.* (2015) and Sizmaz *et al.* (2016) reported that CM has the potential to substitute SBM, with the protein having relatively low effective degradability compared with that of SBM. Therefore, Hao *et al.* (2020) reported that the effectiveness of total diet degradation rate of CP was decreased linearly while DOM and gross energy degradation were increased in flax seed meal. On the contrary, in the trial conducted by Salas *et al.* (2019) the *in vitro* OM degradation was decreased compared with SBM. According to the study of Brando *et al.* (2018), the degradation of OM was not affected by supplemented camelina. In the present study the estimated degradation of ME and OM were not altered in the treatment groups. The digested energy of CM likewise SBM is an important reason to improve the performance in ruminants. As has been argued previously in the specific case of the comparison between CM and SBM, the form of the camelina, chemical oil extraction process and the type of degradation of CM *in vitro* or *in situ* could modify the rumen fermentation and alter the degradation.

There is lack of evidence concerning the effect of CM on ruminal OM and ME degradation as well as methane emission can be explained by the fact that just maintained *in vitro* method in the present study. One reason might be the relationship of oil extraction way in feedstuffs and the conditions during the *in vitro* trial such as bag characteristics, incubation condition in the rumen. The possible effect that there is lack of *in vitro* investigation that will support the microbial fermentation characteristics of CM cannot be excluded.

Conclusion

The results of the present study showed that replacing a proportion of SBM with CM in an *in vitro* rumen fermentation can increase the proportion of acetate and total VFA and decrease the methane production whereas the total gas volume was not affected. The other fermentation characteristics and the estimated degradation of ME and OM were not altered. Thus, we considered that CM can be replaced by SBM, when used as a main protein source in isocaloric and isonitrogenous diets. Thereby, CM could be an alternative protein source for ruminant diets.

Conflict of Interest

The authors declare no conflict of interest.

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RESEARCH PAPER

LIVESTOCK STUDIES

The Effects of Different Levels of Rosehip Fruit Added in the Rations of Laying Hens Raised Under High Altitude and Cold Stress on Some Blood Parameters, Rectal Temperature, Fertility Rate and Chick Quality

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Abstract

This study was carried out to determine the effects of different doses (5g/kg, 10g/kg, 15g/kg) of rosehip fruit, which is a source of ascorbic acid (vitamine C), on fertility rate, chick quality and some blood parameters in the feed of breeder hens and roosters raised under high altitude and cold stress. It was applied during 12-week trial. While the fertility rate was not significantly affected by the treatments, the plasma vitamine C content of the laying hens was significantly affected by the treatments ($P < 0.0001$). While the lowest plasma vitamine C content was determined as 34.54 µg/ml in the control group, it was determined as 53.23, 133.40 and 214.69 µg/ml in the groups receiving rosehip fruit, respectively. When the blood plasma values were examined, the difference among the groups was found to be significant only in terms of triglyceride values in laying hens and uric acid values in roosters ($P < 0.05$). Likewise, the difference among the groups in terms of hatching body weight values of chicks was found to be statistically significant ($P < 0.05$). As a result, it can be said that 5 g/kg rosehip fruit can be added to the diets of breeder hens and roosters exposed to high altitude and cold stress.

Introduction

The significance of rosehip fruit *(Rosa canina)* in terms of health results from its bioflavonoid and carotenoid (lycopene, zeta-carotene, beta-carotene, xanthophyll, neoxanthin and lutein) content in addition to high vitamine and minerals it includes. Beta carotene is the main rosehip carotenoid and it has a significant place in the diet as pro-vitamine A and anti-oxidant (Çınar *et al.,* 2004). In addition to its nutritious value, rosehip also has protective properties against various ailments and partially treats various diseases (Kadakal and Nas, 2004). Rosehip fruits are used in the treatment of cold, flu and mild infections (Bown, 1996). It is known that rosehip, which is used as a drug raw material in many European countries, is also used in folk medicine against kidney and bladder stones, diarrhoea, gum bleeding and chest pain (Anonim 2008). Vitamine C, which has a great significance for human health and nutrition, is found in rosehip fruit in highest amount

among fruit types in the world (Ağaoğlu *et al.,* 1987). Rosehip fruits reach the highest vitamine C rate in physiological maturity and this time generally corresponds to September-October (Anonim 2008). Although light coloured and fully ripe rosehip fruits contain more vitamine C, very ripe and dark coloured fruits have less vitamine C (Türkben, 2003). The most important feature of vitamine C is the fact that it destroys the free radicals in our body. Free radicals may cause arteriosclerosis and cancer. Vitamine C is antioxidant due to its property of neutralizing these free radicals (Saraçoğlu, 2006; Untea *et al.*, 2020). In addition to this feature, vitamine C is also used in poultry diets because of its positive effects on the immune system (Sasidhar, 2020) and as an anti-stress factor (Kutlu and Forbes, 1993; Shakeri *et al.*, 2020). Değirmencioğlu and Ak (2003) found that the application of ascorbic acid (0, 50, 100, 150 mg/kg) had no effect on nutrition performance in turkeys fed in the fall period. As a reason, they determined that animals were not exposed to low ambient temperatures. For this reason, it was concluded that new studies conducted in closer regions and at higher doses are needed to show the effects of ascorbic acid more clearly in preventing cold stress in animals.

With 70/524/EEC council directive on feed additives, European parliament and council regulations (EC) 1831/2003 defined CoE 403 numbered *Rosa canina* plant as a natural product and accepted it as a feed additive (Anonymous, 2013). Vitamine C, which is not essential for poultry and which can be synthesized by the animal, is generally not offered in the feed. However, adding vitamine C to the feed is practically applied under stress conditions that reduce the synthesis of vitamine C and increase the need for this vitamine (Kutlu, 2009). Therefore, it is hoped that the oxidative stress that will develop due to cold stress can be reduced with rosehip. The aim of this study was to find out the appropriate usage levels of natural rosehip fruit instead of commercial ascorbic acid in order to meet the vitamine C requirement in breeding hens exposed to cold stress.

Materials and Methods

Animal material of the study consists of 120 Nick Brown hens at the age of 24 weeks and 15 Nick Brown roosters at the age of 24 weeks which were required for fertilized eggs. After obtaining permission for the study with the 25.10.2018 dated and 2018/10 numbered decision of Van YYU Animal Experiments Local Ethics Committee, the trial phase was carried out in Van YYU Research and Application Farm Directorate coop (at an altitude of 1726 m). 5 groups were formed in the trial. While the group in which no additives were added constituted the control group, the groups in which 100 mg/kg ascorbic acid (vitamine C) and different doses of rosehip fruit (5g/kg, 10g/kg, 15g/kg) were added constituted the treatment groups (Table 1). Each group was divided into three subgroups with 8 hens and 1 rooster with similar average body weights and placed in cages prepared for breeding hens. The rosehip fruit used in the trials was collected in Gevaş and Edremit towns /Van in September and October when the rosehip fruit reached physiological maturity.

Each group was fed with 2850 ME (kcal/kg) basal ration including 16.75% HP prepared for breeding hens (Table 2). The hens and roosters placed randomly in cages were fed with ad-libitum feed and water. During the 12-week trial, 16 hours of light and 8 hours of dark lighting program was applied. In the trial unit, the temperature was set to 24°C for 12 hours and 14°C for 12 hours to create cold stress.

The blood required for checking blood parameters was taken from the under-wing vein. After removing the feathers on the lower surface of the wing, the vein was disinfected; 4-5 ml blood samples were taken into vacuum gel tubes by entering the vein with needle. Plasma was obtained by centrifuging blood samples at 3000 d/min and room temperature for 15 minutes. The plasma was kept at -20°C until analysis. Architect Abbott CI 16200 device and the commercial kits of this device in Van YYU Faculty of Medicine Biochemistry laboratory were used for blood analysis. Vitamine analysis in blood was made in Van YYU Central laboratory.

Nutrient Analysis of Feed

Dry matter, crude ash, crude protein, crude oil, crude fiber nutrient contents of the ration were determined by Wende analysis method (Akyıldız, 1984; AOAC, 1984). Starch, total P, Na, K, Cl and ME values are calculated according to the ration program.

Table 1. Groups in the experiment.

Group 1	Control Group	No Additive
Group 2	Ascorbic Acid (Vitamine C) Group	100 mg/kg Vitamine C
Group 3	Rosehip 1	5 g/kg
Group 4	Rosehip 2	10 g/kg
Group 5	Rosehip 3	15 g/kg

Table 2. Composition and nutrient content if experimental diet.

Raw materials	Rate(g/kg)	Analysed nutrients	(%)
Maize Corn	55.63	Dry Matter	89.62
Feed Flour (46-52)	15.000	Crude Protein	16.75
Soybean Meal (44)	10.967	Crude Celluse	3.17
Fullfat Soybean	6.346	Crude Oil	3.71
Marble Powder (GRN)	6.326	Crude Ash	10.68
Sunflower Meal (34)	3.032	Starch	44.96
DCP 18	1.636	ME-Pou	11.72
Salt	0.244	ME-Pou	2.80
Sodium Bicarbonate	0.190	Tot-P	0.61
DL-Methionine	0.170	Na	0.16
Vitamine Premix[1]	0.200	K	0.62
Mineral Premix[2]	0.100	Cl	0.22
Choline chloride- %60	0.060		
L- Threonine	0.053		
L-Lysine	0.050		

[1]: In every 2 kg mixture; 12 500 000 IU Vitamine A, 3 000 000 IU Vitamine D3, 80 000 mg Vitamine E, 5000 mg Vitamine K3, 3000 mg Vitamine B1, 12000 mg Vitamine B2, 55000 mg Niacin, 15000 mg Ca-D-Pantothenate, 4000 mg Vitamine B6, 40 mg Vitamine B12, 2000 mg Folic Acid, 250 mg D-Biotin

[2]: In every 1 kg mixture; 120000 mg Manganese, 60000 mg iron, 100000 mg zinc, 10000 mg copper, 500 mg Cobalt, 2000 mg iodine, 200 mg Selenium.

Rosehip Vitamine C Analysis

Vitamine C analysis was performed on C_{18} column (PhenomenexLuna C_{18}, 250 x 4.60 mm, 5 µ) in HPLC (high performance liquid chromatography). Column oven temperature was set to 25°C. Ultra-pure water, pH level adjusted to 2.2 with H_2SO_4, was used as mobile phase in the system at a flow rate of 1 ml/minute. The readings were performed on a DAD detector at 254 nm wave length.

L–ascorbic acid (Sigma A5960) prepared in different concentrations (50, 100, 500, 1000, 2000 ppm) was used to define vitamine C peak and to determine its amount (Demir and Özcan, 2001).

Plasma Vitamine Analyses

Vitamine A, E and C levels in plasma samples were determined with HPLC device. Vitamine A and E analyses were made according to (Zaspel and Csallany, 1983; Miller and Yang, 1985) and vitamine C analyses were made according to (Kartepe, 2004).

Vitamine A and E Plasma Extractions

200 µl plasma was taken into plastic tubes for vitamine A and E analyses. They were added 200 µl ethanol and mixed with vortex for a minute. These were added 800 µl n-hexane and vortexed again for a minute and centrifuged for 10 minutes at 2000 RPM. 600 µl was taken from the resulting hexane phase and dried under nitrogen gas. The residue was dissolved in 500 µl methanol and injected on the HPLC column (Zaspel and Csallany, 1983; Miller and Yang, 1985).

The setups were made ready for analyses by using vitamine A and E standard. 20 µl was then taken from the prepared extracts and injected into the liquid chromatography column. The diagnoses of vitamine A and E were made using DAD (diode-array detector) detector at 325 and 290 nm wavelengths. Methanol-water (98:2) was used as the mobile phase at a flow rate of 1.5 ml/min. C18 column (4.6 mm x 25 cm) was used to separate the vitamines (Kadakal and Nas, 2004; Donsbough *et al.,* 2010). The calculations were made according to peak area and concentrations of vitamine A and E standards.

Plasma Vitamine C Determination

The levels of vitamine C in plasmas were determined with HPLC-UV method as stated by Karatepe (2004). For plasma analysis, 250 µl of 0.1 M HClO4 solution was added on 200 µl plasma and vortexed for a few seconds, then 550 µl distilled water was added and after vortexing 10 minutes of centrifugation was made at 4500 RPM. Following these procedures, the supernatant was carefully removed, placed in a vial and vitamine C levels were determined

with HPLC. C18 column (25 cm x 4.6 mm) was used in the HPLC device with a mobile phase of 30 µM $KH_2PO_4^-$ methanol (82.5:17.5) at a flow rate of 1.2 ml/min. The readings were made at 250 nm wave length with UV detector. The calculations were made according to peak area and concentrations of vitamine C standards.

Fertility Rate and Chick Quality Values

Fertility rate was found by using the formula below (Türker *et al.,* 2018).

Fertility rate (%) = (The number of fertilized eggs)/(The number of eggs put in machine) X 100

Hatching weight and body length were evaluated as chick quality value. Chick body length was measured with the tip of the chick beak and the tip of the long finger stretched on the ruler (Şeremet, 2012).

Statistical Analysis

In the study conducted according to randomized plot design, SAS (2010) package program was used to analyse the data obtained. DUNCAN multiple comparison test was used to find out the difference between groups (Bek and Efe, 1998).

Results and Discussion

Vitamine C content of the rosehip fruit used in the study was determined as 2862.66 mg in 100 g (Table 3).

Table 3. Vitamine C content of rosehip fruit.

	Vitamine C (mg/100g)
Rosehip fruit	2862.66

This content was found to be higher than the vitamine C content in the rosehip fruit collected in Konya as 2365 mg/100 g and in Kastamonu as 2712 mg/100 g (Demir and Özcan, 2001). This difference can be attributed to the high altitude of the area and the higher number of sunny days in the area rosehip fruit used in the present study was grown.

When the effect of adding rosehip fruit to the ration on rectal temperature values in roosters was examined (Table 4), the difference between the means of groups was not found to be statistically significant ($P > 0.05$). While the mean rectal temperature of roosters before stress was found as 41.4°C, rectal temperature was found to decrease to 40.9°C with cold stress. It is natural for rectal temperature values of the animals to decrease with cold stress. This difference was not found to be statistically significant in the present study. Similarly, Ahmed *et al.* (2008) reported that the vitamine C supplement added in the drinking water of laying hens raised under sub-tropical conditions did not affect the rectal temperature values significantly. However, Tekeli (2014) added 0 (Control), 10, 20, 30 g/kg rosehip fruit to the ration in a study conducted with broilers and found the pre-stress and post-stress rectal temperature values as 40.8-39.8°C, 40.9-39.7°C, 41.1-39.9°C, 40.8-39.5°C and reported that this difference was statistically significant ($P < 0.05$). This result is not in parallel with the result obtained from the present study. This difference in rectal temperature values may have resulted from the animal's races, ages and differences in the ration they consumed.

In Table 5, when the effect of adding rosehip fruit in the ration on laying hen's fertility rate and the quality values of the chicks was examined, the difference between means of the groups in fertility rate and body length values was not found to be statistically significant ($P > 0.05$). When the egg fertility rates were examined, the highest value was found in the group that received 5g/kg rosehip fruit with 97.53%, while the lowest value was found in the group that received 15g/kg rosehip fruit with 92%. In a study conducted by Shit *et al*. (2012), it was reported that adding vitamine C to the ration of quail exposed to cold stress (10.47°C) had a positive effect on fertility. In the same study, while fertility rate was 72.5% in the control group, it was found as 94.57% in the group which was added 500 ppm L-ascorbic acid.

Table 4. The effect of addition of vitamine C and Rosehip fruit doses to the ration on rectal temperature values in males.

	Experiment Groups						
Parameters	1. Group (Control)	2. Group (Commercial Ascorbic Acid)	3. Group (5g/kg) Rosehip	4. Group (10g/kg) Rosehip	5. Group (15g/kg) Rosehip	SEM	P value
Pre-stress temperature (°C)	41.27	41.50	41.40	41.60	41.36	0.0560	0.2576
Post-stress temperature (°C)	41.00	41.03	40.76	41.00	40.76	0.0617	0.1819

Table 5. The effect of addition of vitamine C and Rosehip fruit doses to the ration on fertility rate and quality values of chicks hatching.

Parameters	Experiment Groups					SEM	P value
	Group 1 (Control)	Group 2 (Ascorbic Acid)	Group 3 (5g/kg) Rosehip	Group 4 (10g/kg) Rosehip	Group 5 (15g/kg) Rosehip		
Fertility rate (%)	94.87	95.12	97.53	95.06	92.00	2.1732	0.8495
Hatching body weight (g)	41.51abc	41.84ab	41.98a	40.23c	40.45bc	0.2086	0.0092
Body length (cm)	17.46	17.20	16.98	16.98	17.11	0.0523	0.0676

SEM: Standard error of difference between means.

*: The difference between the group average shown by different letters on the same line is statistically significant (P < 0.05).

In a similar study, it was reported that adding L-ascorbic acid had a positive effect on fertility in poultry under oxidative stress (Ahmadu *et al.,* 2016). The results of these studies are not similar to the results of the present study. This difference can be attributed to the difference in the form and dose of the additives used. The difference between the group means in terms of body weight at hatching was found to be statistically significant (P < 0.05).

The highest hatching body weight was found in the group that received 5g/kg rosehip fruit, similar to the fertility rate. In a study on hatching chick weight, it was reported that adding ascorbic acid obtained from 0, 200, 500, 1000 and 1500 mg/kg DM orange peels to the ration did not affect chick hatching weight significantly (Adesola *et al.,* 2013). These results are inconsistent with the results obtained in the present study in terms of hatching body weight. This difference can be attributed to the environmental conditions of the trial and the differences in forms and doses of the additives used. In addition, factors such as genetic factors, herd age, hatching egg quality, egg collection time, egg storing conditions, incubation temperature and egg weight are also reported to be effective on chick quality features such as chick weight and length (Kamanlı and Durmuş, 2014).

When Table 6 is examined, it was found that the rosehip fruit added in the rations of laying hens which were exposed to cold stress did not affect the amount of retinol and alpha tocopherol in the blood plasma of hens statistically significantly (P > 0.05). However, the difference between the groups in terms of the amount of vitamine C in blood plasma was found to be statistically significant (P < 0.0001).

Table 6. The effect of addition of vitamine C and Rosehip fruit doses to the ration on laying hens blood plasma vitamine values.

Parameters	Experiment Groups					SEM	P value
	Group 1 (Control)	Group 2 (Commercial ascorbic acid)	Group 3 (5g/kg) Rosehip	Group 4 (10g/kg) Rosehip	Group 5 (15g/kg) Rosehip		
Retinol, (µg/ml)	0.28	0.22	0.28	0.26	0.21	0.0112	0.1000
Alpha tocopherol, (µg/ml)	4.22	2.93	4.15	3.87	2.84	0.0138	0.138
Vitamine C, (µg/ml)	34.54c	47.06c	53.23c	133.40b	214.69a	6.4326	<0.0001

SEM: Standard error of difference between means.

*: The difference between the group average shown by different letters on the same line is statistically significant (P < 0.05).

While the plasma vitamine C value was 34.54 µg/ml in the control group, the highest vitamine C value was found in the group that received 15 g/kg rosehip fruit with 214.69 µg/ml. Similarly, Ahmed et al. (2008) reported that levels of vitamine C added in increasing levels in the drinking water of laying hens placed under sub-tropical conditions increased plasma vitamine C levels significantly. Exogenous vitamines such as alpha tocopherol and vitamine C protect the cells against lipid peroxidation.

Lipid peroxidation leads to the deterioration of physiological functions including immunity, growth and reproduction (Altıner *et al.,* 2017). The linear increase in the vitamine C level in blood with increasing doses of rosehip fruit can be explained with adding the rosehip fruit in the ration in increasing doses.

As can be seen in Table 7, the rosehip fruit added in the rations of laying hens exposed to cold stress significantly affected the level of triglyceride in hens' blood plasma ($P < 0.05$). In the present study, while the level of triglyceride in the control group was 1214.00 mg/dL, it was found to decrease significantly in the group that received 5 g/kg rosehip fruit and it was found as 409.60 mg/dL. High level of triglyceride in the blood has been associated with the emergence of a large number of important diseases, mainly cardiovascular diseases (Tada *et al.,* 2018). Therefore, 5g/kg rosehip supplement is evaluated as important in terms of health. Similarly, Mutlu et al. (2015) reported that gypsum extract decreased blood triglyceride level significantly in quails exposed to cold stress. Behboudi et al. (2016) reported that the use of lemon juice as a source of vitamine C significantly lowered the level of triglyceride in broilers bred under heat stress. Unlike the present study, in their study they added *Berberis vulgaris* fruit used as a source of vitamine C in laying hens' rations, Kermanshahi and Riasi (2006) reported that the value of blood triglyceride was not significantly affected. Tekeli (2014) reported that using different doses of rosehip fruit in broilers exposed to cold stress did not affect blood plasma triglyceride levels significantly. Arpat (2016) reported that rosehip fruit used in laying hens did not have a significant effect on blood triglyceride value. The inconsistency between these studies may have resulted from the differences in the source of vitamine C, type of animal, sex of the animal, purpose of breeding, conditions of breeding and the content of the basic ration used. As can be seen in Table 7, the rosehip fruit added in the rations of laying hens exposed to cold stress caused numerical differences in the amount of cholesterol, glucose, uric acid, sodium, ALT, AST and GGT in hens' blood plasma, while this difference was not found to be statistically significant ($P > 0.05$). Similarly, Tekeli (2014) reported that rosehip fruit added in the ration of broilers did not affect the glucose and uric acid levels. Arpat (2016) reported that adding different rates of rosehip (0, 0.5, 1, 2, 4 and 8%) in the rations of laying hens did not affect blood plasma, cholesterol, AST and ALT values.

Unlike the present study, in a study conducted on broilers, it was reported that adding rosehip fruit to different doses of ration (0, 10, 20, 30 g/kg) under cold stress affected the level of blood plasma cholesterol level significantly (Tekeli, 2014). In their study they added *Berberis vulgaris* fruit to the rations of laying hens as 0, 0.5, 1, 1.5 and 2% vitamine C Kermanshahi and Riasi (2006) found that the total cholesterol amount decreased significantly ($P < 0.05$). In the present study (Table 7), it was found that blood plasma GGT values were numerically lower in all treatment groups. Low GGT value is evaluated as positive in terms of liver and animal health (Kale, 2019).

Table 8 shows the effects of vitamine C and rosehip fruit added in roosters' ration on blood plasma values.

Table 7. The effect of addition of vitamine C and Rosehip fruit doses to the ration on blood plasma values in laying hens.

Parameters	Experiment Groups					SEM	P value
	Group 1 (Control)	Group 2 (Commercial ascorbic acid)	Group 3 (5g/kg) Rosehip	Group 4 (10g/kg) Rosehip	Group 5 (15g/kg) Rosehip		
Cholesterol (mg/dL)	121.00	106.40	145.20	110.33	81.25	10.6112	0.3695
Glucose (mg/dL)	223.00	232.00	212.20	235.50	239.75	3.6209	0.0942
Triglyceride (mg/dL)	1214.00a	980.33ab	409.60c	1163.83a	688.25bc	69.3159	0.0019
Uric Acid (mg/dL)	4.08	3.78	4.80	4.82	5.55	0.3526	0.4711
Sodium (mmol/L)	157.80	149.00	148.20	147.67	151.00	4.5826	0.4434

It was found that the vitamine C and rosehip fruit added in roosters' ration caused numerical differences in the amount of cholesterol, glucose, triglyceride, sodium, ALT, AST and GGT in roosters' blood plasma; however, this difference was not found to be statistically significant ($P > 0.05$). In terms of the amount of uric acid in blood plasma, the difference between the control group and the other groups was found to be statistically significant ($P < 0.05$). As can be seen in Table 7 and Table 8, while the rosehip supplement caused a difference only in triglyceride levels of laying hens, it caused statistical difference only in uric acid in roosters ($P < 0.05$).

In roosters, uric acid level which was 11.03 mg/dL in the control group decreased in groups in which rosehip was added and it was found as 7.66, 8.30, 8.50 and 8.16 mg/dL, respectively. Donsbough *et al.* (2010) reported that serum uric acid level could be used as an indicator of amino acid availability in broilers fed with sufficient and insufficient rations in terms of amino acid level. High uric acid level in plasma is considered as a risk factor for gout, renal diseases, metabolic syndrome and cardiovascular diseases (Oliveira and Burini, 2012).

Conclusion

When compared with high doses, low doses of rosehip fruit had a positive effect on hatching live weight; increased plasma vitamine C content when compared with the control group and the group which was given commercial ascorbic acid supplement; reduced the level of triglyceride and reduced the level of uric acid in roosters. Due to these results, it can be recommended to add 5 g/kg rosehip fruit instead of commercial ascorbic acid in the rations of laying hens and roosters exposed to high altitude and cold stress in order to meet their vitamine C need. Using rosehip fruit as a feed additive in livestock will be a great benefit to our country's economy in terms of utilizing our natural resources. New comprehensive studies including sperm and all incubation parameters are needed to fully reveal the effect of rosehip fruit on breeding animals exposed to cold stress.

Conflict of Interest

The authors declare no conflict of interest.

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RESEARCH PAPER

LIVESTOCK STUDIES

Effects of Environmental Factors Growth Traits of Akkaraman Sheep in Çankırı Province

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Abstract

Small ruminants are important contributors of food supply chains throughout the world. In this study, characterization of distributions of birth weight, weaning weight, average daily gain and Kleiber ratio as well as estimation of effects of certain environmental factors on these traits were aimed in Akkaraman sheep raised around Çankırı province. For this purpose, the linear mixed model was fitted to estimate the effect of factors and to obtain the least square means for the traits. The effect of all studied fixed factors on birth weight (BW), weaning weight (WW), average daily weight gain (ADWG) and Kleiber ratio (KR) were found to be significant. The male lambs have more BW and WW than female lambs. The least square mean of ADWG for female lambs was found higher than male lambs. Additionally, the least square mean of the pre-weaning growth traits of single-born lambs were found to be higher than that of twin-born lambs. The birth weights of lambs born in January and February were higher than March. In general, the study reported that pre-weaning growth traits were affected by several environmental factors, which can be used for herd management practices aiming for higher productivity in Akkaraman sheep.

Introduction

Small ruminants are important contributors of food supply chains throughout the world. They are known for their high adaptive capacity under various environmental conditions such as meagre diet and arid landscapes. Compared to large ruminants, sheep and goats require less input of feed, water and labour, which provides the species to be spread out around various environments (Joy *et al.*, 2020). Among those indigenous breeds have critical role in sustainable production and food security for various reasons such as genetic variability and high adaptation (Bingol, 2016). Nutrition of a large fraction of human population heavily depend on animal-sourced products such as meat and milk (Gül *et al*., 2020; Mondal & Reddy, 2017).

Turkey -with 55 millions of small ruminants of which majority are indigenous breeds- is among the largest producers of small ruminants around the world (TUIK, 2020). There are a wide range of indigenous fat- and thin-tailed sheep breeds in Turkey (Aksoy *et al*., 2019; Yilmaz & Wilson, 2012). As a fat-tailed and combined production purpose breed, Akkaraman has a large share of sheep population in Turkey with low productivity (Ünal, 2002; Yalcin, 1986). Even though the number of farm animals is excessively high in Turkey, low productivity leads to the reduced amount of meat production per person. Therefore, increasing growth and productivity of sheep have a great importance for sustainable food supply and food security in Turkey (Şenyüz, 2020; Yardımcı & Özbeyaz, 2001).

Similar to the other indigenous sheep, Akkaraman also suffered from the lack a systematic breeding programme until recently, with majority of the breed is still not subject to a genetic selection system. Therefore, farmers select their rams and ewes according to the morphological observations (Ceyhan *et al*., 2019). As a significant cultural component, sheep farming is majorly implemented as outdoor production based on meagre pasture. However, this approach is compromised in terms of identifying the genetic potential of animals. Recent studies showed high

phenotypic variation in terms of productivity traits which also indicates a potential background genetic variation to build upon with selection (Biçer *et al.*, 2019). A systematic selection approach initially requires characterising and ameliorating the effects of environmental factors which is followed by a genetic selection for a cumulative gain (Sönmez *et al.*, 2009).

Lamb birth weight, weaning weight, feed efficiency and average daily gain are important indicators of productivity in sheep selection programmes. Additionally, Kleiber ratio is another indicator phenotype which is long used to monitor feed efficiency (Eskandarinasab *et al.*, 2010; Jeichitra & Ramanujam, 2014; Mahala *et al.*, 2020; Supakorn & Pralomkarn, 2012). There has been a substantial progress in Çankırı with the launch of Akkaraman community-based sheep breeding programme since 2011. It is conducted as a sub-project of "National Sheep and Goat Breeding Project under Farmers Condition" implemented by the Ministry of Agriculture and Forestry in collaboration with various universities, research institutes, sheep & goat breeder associations and breeders. Birth weight, weaning weight and average daily gain of animals are routinely recorded as part of the project. Therefore, in this study, characterization of distributions of birth weight, weaning weight, average daily gain and Kleiber ratio as well as estimation of effects of certain environmental factors on these traits were aimed in Akkaraman sheep raised around Çankırı province.

Materials and Methods

Animals and Phenotype

The study was carried out on Akkaraman lambs which were born in 2014 and 2015 at 133 herds in the National Community-Based Small Ruminant Breeding Programme in Çankırı province, Turkey. Çankırı, located in the north of Central Anatolia, between Kızılırmak and the Western Black Sea main basins, is located between 40° 30' and 41'' north latitudes and 32° 30' and 34'' east longitudes. During the spring-summer period (from April to November) the animals were grazed on very poor-quality pasture, and in the winter period, they were fed with an average of 0.6 kg/day of concentrate feed per animal in the pen. The lambs were weaned after approximately 90 days of nurturing period by their dams.

Birth weight (BW), weaning weight (WW), average daily weight gain (ADWG) and Kleiber ratio at weaning (KR) of approximately 19900 observations were obtained as traits. Additionally, birth and weaning dates, sex, birth type (singlets/twins) and birth month (January/February/March) were regularly recorded. Weaning weight was interpolated weights of the animals to 90 days which is average weaning day. Average daily weight gain (ADWG) was obtained via linear statistics by using BW and WW. Kleiber ratio at weaning (KR) was also calculated from ADWG and WW ($ADWG \ / \ WW^{0.75}$). Detailed description of the data structure with the sample size after removing the outliers were presented in Table 1.

Statistical Analyses

The outliers of the observations (values exceeding mean ± 3 standard deviation) were checked. Normality of the responses were tested with Shapiro-Wilk test. Moreover, the homogeneity of variance was visually inspected by plot obtained from residual vs fitted value of the responses. Initially, the effect of environmental factors (sex, birth type, birth month and birth year in this case) were tested to build final linear mixed models. The data management and all statistical analysis were performed using "*lme4*", "*lmerTest*" and many other basic packages of R statistical environment (R Core Team, 2020).

The linear mixed models were used to estimate the effect of environmental factors after to fit final models for the traits.

Table 1. Descriptive statistics of pre-weaning growth traits.

Trait	BW (kg)	WW (kg)	ADWG (g)	KR
Number of observations	19.910	19.772	19.755	19.724
Mean	4.19	24.13	221	20.01
Standard deviation	0.93	6.64	73	2.63
Minimum	1.50	10.68	56	12.22
Maximum	6.97	44.39	438	26.82
Coefficient of Variation	0.22	0.27	0.32	0.13

BW: birth weight, WW: weaning weight, ADWG: average Daily weight gain, KR: Kleiber ratio.

The least square means of the factors was obtained from those mixed models. Herd and maternal permanent environmental effect were added the models as random factors. Subsequently, the differences between groups of the significant factors were tested with Duncan's Test. The final linear mixed models' description for the traits are given below:

Model: $\mathbf{y_{ijkl} = \mu + s_i + t_j + m_k + y_l + Z_1h + Z_2p + e_{ijkl}}$

Where $\mathbf{y_{ijkl}}$ are the observations of the dependent variables (i.e., BW, WW, ADWG and KR); μ is the intercept; s_i is the fixed effects of sex; t_j is the fixed effects of birth type (2 levels); t_j is the fixed effects of birth month (3 levels); t_j is the fixed effects of birth year (2 levels); Z_1h is the random herd effects; Z_2p is the maternal permanent environmental effects and e_{ijkl} is the residual error of observations in the models.

Results and Discussion

The effects of environmental factors such as sex, birth type, birth month, birth year, herd and maternal permanent environmental effects on BW, WW, ADWG and KR of Akkaraman lambs were investigated in this study. With this purpose, the linear mixed models were fitted to estimate the effect of factors and to obtain the least square means for the traits. The diagnostic test of the models and the interaction between factors were also implemented. Finally, the multiple comparison tests were applied to obtain significance level of differences between groups of factors.

After building the appropriate final models, all factors had significant effect on the traits (see Table 2). According to the diagnostic tests, the data showed normal distribution and homogenous variance. In general, interactions between factors were not significant.

The effect of all studied fixed factors on birth weight (BW) were found to be significant. The least square means of sex were 4.23 ± 0.03 and 3.83 ± 0.03 for male and female lambs, respectively, represented in Table 2. Moreover, the difference between male and female groups was found to be statistically important ($P < 0.001$). In a study conducted by Ünal, (2002) between 1996 and 1997 on Akkaraman lambs, the mean birth weight of male and female lambs was found to be approximately 4.69 and 4.39 kg, respectively. In another study conducted on Akkaraman lambs, corrected birth weights of male and female lambs were found to be 4.86 and 4.64 kg, respectively (Çolakoğlu & Özbeyaz, 1999). As in the present study and both studies discussed, it is seen that male lambs have 200 - 500 g more birth weight compared to female lambs. In the study, the birth weights of singlet and twin lambs were found as 4.41 ± 0.03 and 3.66 ± 0.03 kg, respectively and the differences were statistically significant ($P < 0.001$). As predicted, in many studies conducted in the same breed, it was reported that the birth weight of single born lambs was higher than that of twin born and was statistically significant (Ceyhan *et al*., 2019; Çolakoğlu & Özbeyaz, 1999; Ünal, 2002). In the study, the least square mean of the birth weights of lambs born in January, February and March were found to be 4.03 ± 0.03, 4.08 ± 0.03 and 3.98 ± 0.04 kg respectively. Moreover, the difference between the groups was found to be significant in the multiple comparison test between these months. In a study conducted by Gül *et al.*, (2020), similar results were obtained in the average birth weight of Awassi lambs in January, February and March. The seasonal pattern revealed that the birth weight of lambs born in January and February was relatively higher than that of lambs born in March. The possible reason for this situation is thought to be that the dams of lambs born in March were exposed to more feed shortness in the last trimester of pregnancy. Table 2 also illustrate that the least square means of BW of lambs born in 2014 and 2015 were 3.94 ± 0.04 and 4.13 ± 0.06 kg respectively and it was significant ($P < 0.001$). This result from the study contribute that the feeding and many other strategies applied on herds are quite important on the birth weight of lambs.

Similar to BW, all fixed effects were significant on WW of lambs in the study. The detail information and the least square means of WW were present in Table 2. The least square means of WW of male and female lambs are 23.52 ± 0.22 and 23.36 ± 0.22 kg, respectively, while the least square means of WW of single and twin lambs are 24.98 ± 0.22 and 21.89 ± 0.23 kg, respectively. Moreover, the least square mean of the lambs born in January, February and March are 20.97 ± 0.23, 24.37 ± 0.23 and 24.98 ± 0.25 kg, respectively. The effect of sex, birth type and birth month on WW were found to be significant. As a result of the multiple comparison test between the birth month groups, it was seen that the difference between the groups were significant. Similar result for the effects of sex, birth type and birth month are generally reported in the studies (Çolakoğlu & Özbeyaz, 1999; Ünal, 2002). Although there is a slight difference between the weaning weight of male lambs and female lambs, this difference was found to be statistically significant in the present study and many other studies . Additionally, in the studies reported in Akkaraman lambs, the WW of the single-born lambs were found higher than the twin-born lambs as in the study. The WW of lambs born in February and March in the study were higher than those born in the January, but in the study conducted by Gül *et al.*, (2020) in Awassi lambs the WW of the lambs born in March were lower than those born in February and January. This situation is thought to be due to the difference in breed and the seasonal conditions of the different geography and pasture where the lambs are raised.

Table 2. The least square means (±SE) of the pre-weaning traits in Akkaraman lambs.

	BW (kg)			**WW (kg)**		
Fixed Effects	n	LSM ± SE	p-value	n	LSM ± SE	p-value
Sex			***			*
Male	10104	4.23 ± 0.03[a]		10031	23.52 ± 0.22[a]	
Female	9806	3.83 ± 0.03[b]		9741	23.36 ± 0.22[b]	
Birth type			***			***
Single	14440	4.41 ± 0.03[a]		14331	24.98 ± 0.22[a]	
Twin	5470	3.66 ± 0.03[b]		5441	21.89 ± 0.23[b]	
Birth month			***			***
January	8439	4.03 ± 0.03[b]		8438	20.97 ± 0.23[c]	
February	7781	4.08 ± 0.03[a]		7733	24.37 ± 0.23[b]	
March	3690	3.98 ± 0.04[c]		3601	24.98 ± 0.25[a]	
Birth year			***			***
2014	12840	3.94 ± 0.04[b]		12724	24.63 ± 0.25[a]	
2015	7070	4.13 ± 0.06[a]		7048	22.24 ± 0.36[b]	

	ADWG (g)			**KR**		
Fixed Effects	n	LSM ± SE	p-value	n	LSM ± SE	p-value
Sex			***			***
Male	10024	213 ± 2.50[b]		10007	19.68 ± 0.08[b]	
Female	9731	216 ± 2.50[a]		9717	20.06 ± 0.08[a]	
Birth type			***			***
Single	14316	228 ± 2.45[a]		14296	20.13 ± 0.08[a]	
Twin	5439	202 ± 2.60[b]		5428	19.61 ± 0.09[b]	
Birth month			***			***
January	8438	188 ± 2.55[c]		8398	18.93 ± 0.09[c]	
February	7725	225 ± 2.53[b]		7727	20.17 ± 0.09[b]	
March	3592	232 ± 2.84[a]		3599	20.51 ± 0.10[a]	
Birth year			***			***
2014	12710	229 ± 2.83[a]		12722	20.44 ± 0.10[a]	
2015	7045	201 ± 3.98[b]		7002	19.30 ± 0.14[b]	

Notes: The mean values which have different superscript are significantly different, ***P < 0.001, **P < 0.01, *P < 0.05, SE = standard error; n = number of observations.

In Table 2 the effects of year were also illustrated, the least square means of WW lambs born in 2014 and 2015 were 24.98 ± 0.22 and 21.89 ± 0.23 kg respectively and it was significant (P < 0.001). This result from the study contribute that the different conditions of pasture in different years are crucially important factor on WW. When the dams of lambs were graze on the pasture with good conditions, their nursing ability is becoming better.

The effect of sex, birth type, birth month and birth year were found significant on ADWG similar to BW and WW. The least square means of ADWG for male lambs and female lambs were 213 ± 2.50 and 216 ± 2.50 g, while the least square means of ADWG for single-born lambs and twin-born lambs were found to be 228 ± 2.45 and 202 ± 2.60 g, respectively. The ADWG of lambs born in February were lower than those born in February and March. ADWGs by month are 188 ± 2.55, 225 ± 2.53 and 232 ± 2.84 g, respectively. Interestingly, in the present study, ADWG of female lambs was slightly higher than that of males. In the study conducted by Ceyhan *et al*. (2019) on Akkaraman lambs, it was reported that the ADWG of male lambs were higher than that of females. In the same study, ADWG of single-born lambs were higher than twin-born lambs. This result was consistent with the present study.

The least square mean of KR for BW was found 19.68 ± 0.08 and 20.06 ± 0.08 for male and female lambs, respectively. In a study conducted in Mecheri sheep, as in the current study, the KR was found to be higher in male lambs than in female lambs. The KR values in the study are 13.23 ± 0.08 and 12.91 ± 0.08 in male and female lambs, respectively (Jeichitra & Ramanujam, 2014). KR rates of single-born and twin-born lambs are 20.13 ± 0.08 and 19.61 ± 0.09, respectively in this study. In the study conducted by Mahala *et al*. (2020) on Avikalin lambs, mean Kleiber ratio was calculated as 16.8 for average daily weight gain until weaning.

Conclusion

In the study, all of the fixed effects used in the analysis were found to be very effective on pre-weaning growth parameters (BW, WW and ADWG) and Kleiber ratio (KR), which is the indicator of the feed conversion parameter. The results of the current study generally showed that male lambs have more BW and WW than female lambs. Surprisingly, for ADWG the least square mean was found higher in female lambs than males. Additionally, the least square mean of the pre-weaning growth traits of single-born lambs were found to be higher than that of twin-born lambs. The birth weights of lambs born in January and February were higher than March. It has been observed that herd and nutrition management, climate and pasture condition in different years have an effect on birth weight, weaning weight and daily live weight of lambs. The Kleiber ratio, which is an indicator of feed utilization, was higher in male and single lambs than in female and twin lambs. It was observed that the KR of lambs born in January were lower than lambs born in February and March.

In general, the study reported that pre-weaning growth traits were affected by environmental factors. Therefore, it has been observed that productivity increases can be achieved in animals by improving environmental conditions. Especially determining the birth months in accordance with the feeding strategy can provide the most effective improvement.

In order to examine the pre-weaning traits of Akkaraman sheep in more detail, the most important recommendation put forward in our study is to implement further effect size estimation studies, estimate the heritability of these traits by using full pedigree and to develop the breed genetically in addition to environmental factors.

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RESEARCH PAPER

LIVESTOCK STUDIES

The Effects of Supplementing Whole Milk with Juniper (*Juniperus oxycedrus)* Aromatic Water on Growth and General Health Parameters of Holstein Calves

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Abstract

The illness of the calves during the suckling period suppresses the growth of calves and causes them to be removed from the breeders. In the present study, it was investigated whether Juniper aromatic water (JOW) would be suitable for promoting healthy growth performance of suckling Holstein calves. Twelve newborn calves (n=3, in each group) were randomly selected and assigned to the following four treatments: Control, 50, 100 and 200 ml JOW supplemented milk per day. The supplementation of JOW showed an increase in calves' live weight and body measurements. In addition, the supplementation of whole milk with JOW decreased the frequency of occurrence of calves' diarrhea and disease. The best result was observed in the supplementation of whole milk with 50 ml JOW. The findings of the study showed that Juniper aromatic water, a by-product, can be used safely in the healthy rearing of calves.

Introduction

In newborn calves, resistance to diseases is low due to insufficient immune system. Antibiotic treatments applied during this period reduce the numbers of pathogens and also non-pathogenic bacteria. This causes the growth to stop or decline in the early period and therefore it causes economic losses (Soltan, 2009). The restriction and/or prohibition of the use of antibiotics in livestock have increased the interest in medicinal aromatic plants in recent years (Anadon, 2006).

The juniper, which is the subject of our study, has an important place in medicine manufacturing industry in Europe and many countries of the world because of its pharmacological properties medicine, due to and the extractive substances it contains. The juniper has been used as wood and material for making medicine from its fruits and leaves for many years in Anatolian geography as well as over the world. Medicines have been prepared from fruits and leaves for curing pain, cough, rheumatism, tuberculosis and it has also been used as antibacterial (Tumen and Hafizoglu, 2003). It has been determined that the JOW has selective antibacterial properties, also show antioxidant and iron-reducing properties depending on the concentration used (Isik *et al.,* 2020).

There are a few studies on the use of aromatic waters, a by-product of extracting plant extracts, in animal nutrition and breeding and their health effects. In this study, it was aimed to investigate the effects of aromatic water, which is produced as a by-product and has no economic value, on the healthy growth of calves while extracting oil from juniper (*Juniperus oxycedrus*) fruits and leaves.

Materials and Methods

The study was approved by the MAKÜ animal experiments local ethics committee with decision dated 04.10.2019 and numbered 507.

Animals and Dietary Treatments

The study was carried out in Isparta University of Applied Sciences, Faculty of Agriculture, Education,

Research and Application farm and 12 holstein calves were used, whose birth weight were the closest to each other. Power analysis method were used in determining the number of calves which was found as 3 calves in each group with the highest mean value of 4.73, the lowest mean value of 4.34 and the standard deviation of 0.3 for 95% power. In order to eliminate the colostrum effect, calves were experimented at the 4-day-old after suckling with colostrum for 3 days. Calves housed in individual boxes were provided by *ad libitum* to starter and clean water. A total of 4 l whole milk (WM) in the morning (2L) and in the evening (2L) was given to the calves in equal portions. Experimental groups were formed as follows: (CNT) control diet (4 l WM), (D1) control diet (4 l WM) + 50 ml aromatic water (1.25% JOW added milk), (D2) control diet (4 l WM) + 100 ml aromatic water (2.5% JOW added milk), (D3) control diet (4 l WM) + 200 ml aromatic water (5% JOW added milk). JOW doses were determined according to Minimum Inhibition Concentration analysis results (Isik *et al.* 2020). The chemical composition of starter and whole milk used in the study is presented in Table 1. The crude protein and ether extract ratio of starter used in the study was determined according to method 954.01 and 920.39, respectively (AOAC, 1990). Metabolic energy was calculated according to Turkish Standards Institution (TSE, 1991). Fat% (Method: IDF 141C:2000), protein%) method: IDF 141C:2000), lactose% (Methods: IDF 141C:2000) of WM were analyzed using a Bentley B150 milk analyzer (Bentley Combi 150, Bentley Instruments, Inc. Minnesota, Chaska, USA).

Sample Collection

Live weight and body measurements of calves were monitored weekly until weaning. Starter and water were drawn from the calves in the evening before the weighing, and the calves were weighed when their stomach were empty. The feed consumption of calves was recorded daily by using 1g sensitive electronic scales (TESS, Comak Tarti LTD., Turkey). In this way, calves consuming 800g of feed on consecutive days were determined and weaned.

Body measurements (Body length (BL), body depth (BD), withers height (WH), and hip height (HH) and chest girth (CG)) of calves were recorded weekly.

Blood samples were taken from vena jugularis of calves at the beginning of the experiment and at the weaning. Total cholesterol TC, Triglycerides TG, glucose GLU, Urea, Creatine CREA, Albumin ALB, Total protein TP, liver enzymes (Alanin aminotransferase ALT, Aspartate aminotransferase AST, Alkaline phoshatase ALP, Gamma-glutamyltransfarase GGT, Lactate dehydrogenase LDH) were determined in blood samples. Blood samples were centrifuged at 3000 rpm for 10 minutes, and the obtained blood serum was analyzed with Mindray BS120 Vet (Mindray Corporation, Nanshan, China).

The feces of calves were monitored daily and scored for consistency (Larson *et al.,* 1977): 1- normal, 2- soft, 3- fluid, 4- juicy. The number of days with diarrhea (NDD) and illness of calves (NID) has been recorded. When the feces score (FS) is 3 and above, the calf was registered with diarrhea. The respiratory rate (RR) and pulse rate (PR) of calves were recorded daily. Respiratory scores (RS) of calves were determined by Heinrichs *et al.* (2003): 1- normal, 2- mild cough, 3- moderate cough, 4- moderate to severe cough, 5- severe and chronic cough.

Production and Analysis of JOW

According to the method described in the European Pharmacopoeia (1975) a mixture of 200 g juniper fruits and leaves and 1.5 L tap water were placed in a Clevenger hydrodistillation device and JOW was separated from the juniper essential oil and collected in a distillation flask (5 L).

Total phenolic compounds of JOW were determined according to Singleton and Rossi (1965) using Folin-Ciocalteu colorimetric method and spectrophotometer (PG T80+ UV/VIS Spectrometer, PG Instruments Ltd. Leicestershire, UK) reading were made at 765 nm wavelength. Chromatographic analysis was used to reverse-phase high-performance liquid chromatography (Shimadzu Corp., Kyoto, Japan) for the determination of JOW components (Caponio *et al.,* 1999).

Table 1. Chemical composition of WM which supplemented JOW and without JOW, and calf starter.

Ingredients, %	CNT	D1	D2	D3	Calf starter
DM	8.90	9.00	8.80	8.60	91.26
CP	3.20	3.20	3.20	3.10	17.55
EE	3.40	3.40	3.40	3.40	3.45
Lactose	4.90	4.90	4.90	4.70	
Freezing point(-^{o}C)	0.57	0.57	0.56	0.55	
pH	6.63	6.63	6.57	6.59	
ME, kcal kg^{-1}					2848.90

* DM: Dry matter, CP: Crude protein, EE: Ether extract, ME: Metabolic energy

Antiradical activity was determined using 1,1-diphenyl-2-picrylhydrazyil (DPPH) (Shimada *et al.,* 1992). 1 mL of 0.2 mM DPPH was added to 1 mL samples (at concentrations of 50, 100, 250 ppm) and mixed well with vortex.

The readings were made at 517 nm after 30 minutes in the dark environment and room temperature. The free radical of the samples was calculated using the formula: Antiradical activity (%): [(absorbance value of control – samples absorbance value) / (absorbance value of control)] x 100.

The iron reduction capacity was determined using the method of Oyaizu (1986). Accordingly, 2.5 mL of 200 mM sodium phosphate buffer (pH: 6.6) and 2.5 mL of 1% potassium ferricyanidine were added and mixed with a 2.5 mL sample. After the samples were kept at 50 ^{o}C for 20 minutes, 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 200 rpm for 10 minutes.

A 5 mL of the upper phase was taken and 5 mL of deionized water and 1 mL of 0.1% ferric chloride were added on it. Then, the absorbance values of the samples at 700 nm wavelength were measured in the spectrophotometer. Comparison with synthetic antioxidants such as BHA (Butylated hydroxyanisole), BHT (Butylated hydroxytoluene) and Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) as high absorbance value indicates high iron reducing capacity. The iron binding strength at the samples was revealed.

Statistical Analysis

The data obtained in the study were analyzed by analysis of variance (ANOVA), and the differences between groups were examined with Tukey test (Minitab 2017 v 18.1, Minitab Ltd. UK).

Results

Components and Total Antioxidant Capacity of JOW

The components of the JOW and their percentage values are presented in Table 2. When the phenolic components of JOW were examined, it was observed that the highest component was α-cedrol (54.43%) and the lowest component was trans-pinocarveol (3.57%).

The oxidant capacity, iron reduction capacity and total phenolic content of the JOW are shown in Table 3.

The total content of JOW was obtained as 1.84 mg GAE/g. Antioxidant capacity and iron reducing capacity increased in parallel with the increase in concentration.

Table 2. Components and percentages of JOW.

Components	Percentages
α-Cedrol	54.43
Verbenone	20.16
Verbenol	14.90
Berneol	6.04
Trans-pinocarveol	3.57

Growth Performance of Calves

The effect of JOW supplementation on the growth performance of calves is shown in Table 4. The effect of JOW supplementation on the weaning age of calves was not found to be significant.

Table 3. Phenolic content, antioxidant capacity and iron reducing power of JOW.

	TPC mg GAE/g Mean±SEM	Antiradical % (50 µl/ml) Mean±SEM	Antiradical % (100 µl/ml) Mean±SEM	Antiradical % (250 µl/ml) Mean±SEM	Iron reducing (50µl/2.5ml) Mean±SEM	Iron reducing (250µl/2.5ml) Mean±SEM
JOW	1.84±0.01	29.49±0.02	45.40±0.10	70.43±0.13	0.49±0.01	4.46±0.02
Trolox	----------	85.20±0.20	91.30±0.30	97.25±0.25	3.32±0.02	10.93±0.03
BHT	----------	94.70±0.21	94.80±0.25	97.53±0.08	6.31±0.01	23.65±0.02
BHA	----------	97.30±0.23	96.75±0.15	98.70±0.20	3.62±0.02	18.52±0.02

* TPC: Total phenolic content, BHT: Butylated hyroxytoluene, BHA: Butylated hyroxyanisole

However, the trial groups tended to be weaned early compared to be CNT.

Numerical differences between initial and final live weight (LW) of calves were found to be significant ($P < 0.05$). Similarly, no significant differences were found between daily live weight gain (DLWG) and total live weight gain (TLWG) averages.

The effect of JOW supplementation on body measurements of calves was not found to be significant. However, it tended to improve the growth of calves.

The difference between the daily feed consumption (DFC) averages of groups was found to be significant ($P < 0.05$). While the highest DFC was obtained in D1, the lowest consumption was in CNT.

Healthy Parameters of Calves

The numerical difference among the averages of all values observed as health parameters of groups (Table 5) was found significant ($P < 0.05$).

Among these parameters, the highest average values for RR, PR and RT were observed in D3, while the highest RS value was obtained in CNT.

Results revealed that illness and diarrhea were less common in the calves in the trial groups compared to the CNT, and when Table 5 is examined, it can be seen that the FS, NDD and NID values were higher in the CNT than the others.

Table 4. Effects of JOW supplementation on calves' growth performance.

	CNT	D1	D2	D3	P value
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	
Weaning age, d	44.33±2.91	36.67±1.86	43.33±2.85	42.67±2.33	0.21
Initial LW, kg	42.33±5.67	42.17±1.69	41.17±1.64	40.33±1.86	0.97
Final LW, kg	56.17±6.72	56.33±1.17	57.67±2.35	54.67±4.76	0.97
DLWG, kg	0.313±0.02	0.386±0.01	0.386±0.04	0.330±0.09	0.63
TLWG, cm	13.83±1.09	14.16±0.88	14.50±0.76	14.33±4.38	0.85
TBLG, cm	7.50±1.04	9.33±0.67	11.00±1.80	9.67±1.69	0.41
TBDG, cm	3.33±0.16	3.83±0.88	5.33±1.09	6.10±0.44	0.08
TWHG, cm	5.16±0.60	8.67±1.30	7.83±1.01	6.66±0.72	0.12
THHG, cm	5.33±0.44	7.16±0.44	7.67±1.09	6.50±0.76	0.21
TCGG, cm	8.33±0.66	8.17±0.44	11.17±0.60	11.50±1.80	0.49
DFC, kg	$0.237±0.02^{B}$	$0.327±0.03^{A}$	$0.281±0.02^{AB}$	$0.246±0.03^{B}$	0.01

* LW: Live weight, DLWG: Daily weight gain, TLWG: Total live weight gain, TBLG: Total body length gain, TBDG: Total body depth gain, TWHG: Total wither height gain, THHG: Total hip height gain, TCGG: Total chest girth gain, DFC: Daily feed consumption, ^{A,B}: Shows the difference between averages on the same row

Table 5. Health parameters of calves.

	CNT	D1	D2	D3	P value
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	
RR	$79.06±0.49^{B}$	$72.89±0.63^{C}$	$70.98±1.06^{C}$	$82.03±0.32^{A}$	0.00
PR	$82.99±0.52^{B}$	$82.37±0.59^{B}$	$82.90±0.62^{B}$	$85.26±0.42^{A}$	0.00
RS	$1.15±0.03^{A}$	$1.00±0.00^{B}$	$1.05±0.02^{B}$	$1.01±0.01^{B}$	0.00
FS	$2.08±0.06^{A}$	$1.44±0.05^{B}$	$1.52±0.06^{B}$	$1.94±0.05^{A}$	0.00
RT, ℃	$38.54±0.03^{B}$	$38.39±0.03^{C}$	$38.52±0.03^{B}$	$38.82±0.03^{A}$	0.00
NDD	$10.00±2.31^{A}$	$0.33±0.33^{B}$	$3.33±3.33^{AB}$	$5.67±1.20^{AB}$	0.04
NID	$2.67±1.33^{A}$	$0.00±0.00^{B}$	$0.33±1.00^{B}$	$0.00±0.00^{B}$	0.03

* RR: Respiratory rate, PR: Pulse rate, RS: Respiratory score, FS: Feces score, RT: Rectal temperature, NDD: Number of days with diarrhea, NID: Number of illness days, $^{ABC:}$ Shows the difference between the averages in the same row.

Biochemical Blood Parameters of Calves

Blood serum biochemical values of calves are shown in Table 6. Numerical differences among observed blood serum biochemical values of all groups were not found to be significant.

Discussion

Components and Total Antioxidant Capacity of JOW

In our study, α-cedrol has the highest percentage in JOW components (Table 2) in line with Isik *et al.*'s (2020) study.

The total phenolic content of aromatic water obtained from different Juniper species shows differences. Indeed, Taviano *et al.* (2013) stated that the total phenolic contents of two different Juniper species used in their study were 5.14 and 17.89 mg GAE/g respectively, while Isik *et al.* (2020) and Miceli *et al.* (2009, 2011) reported the total phenolic contents of Juniper as 1.85 mg GAE/g and 17.64 mg GAE/g in their studies.

The impact of environmental conditions in the regions where Juniper grows may be the cause of this difference.

Many researchers reported that both essential oils and extracts obtained from Juniper species have high antioxidant properties and can be used instead of synthetic antioxidant (Elmastas *et al.,* 2006; Loizzo *et al.,* 2006; Miceli *et al.*, 2009; Lesjak 2011; Isik *et al*., 2020). Our findings revealed that JOW can be used as an alternative of synthetic antioxidants at a concentration of 250µl/ml, JOW (Table 3). Iron reduction capacity, which has an important place in determining antioxidant activity (Menconi *et al.*,

Table 6. Effects of JOW supplementation on calves blood parameters.

	CNT	D1	D2	D3	P value
	Mean ±SEM	Mean ±SEM	Mean ±SEM	Mean ±SEM	
Initial ALT	7.67±0.88	13.67±2.40	15.33±7.42	16.33±7.13	0.67
Final ALT	8.00±2.08	8.00±3.00	14.00±2.89	10.67±2.03	0.35
Initial AST	51.30±27.50	56.00±14.60	30.30±24.10	141.00±103.00	0.53
Final AST	75.00±16.80	43.70±22.40	175.00±100.00	49.70±22.20	0.33
Initial GGT	641.00±298.00	766.00±272.00	728.00±163.00	1414.00±247.00	0.19
Final GGT	35.00±8.14	56.00±18.50	38.00±4.04	44.33±4.10	0.53
Initial ALP	216.70±63.00	340.30±50.00	235.70±25.00	171.30±7.70	0.24
Final ALP	150.00±12.50	277.30±67.50	206.70±12.50	117.70±27.50	0.07
Initial GLU	125.70±11.20	113.70±16.60	116.70±16.20	115.00±3.06	0.91
Final GLU	99.00±7.00	98.00±7.64	96.62±6.36	92.33±2.33	0.87
Initial TC	60.30±28.70	59.30±39.40	41.30±23.60	83.67±2.60	0.75
Final TC	151.70±20.30	107.70±49.40	138.30±56.60	137.00±58.20	0.92
Initial CREA	1.38±0.22	1.32±0.08	1.32±0.06	1.54±0.34	0.86
Final CREA	1.20±0.08	1.40±0.06	1.50±0.10	1.22±0.15	0.24
Initial UREA	52.10±16.80	29.30±15.40	26.30±21.00	72.40±26.60	0.47
Final UREA	47.30±23.40	19.30±10.80	53.40±24.50	50.40±14.00	0.59
Initial TP	8.36±0.49	7.60±0.38	7.59±0.57	7.62±0.24	0.56
Final TP	7.72±0.33	7.45±0.07	6.89±0.26	6.96±0.35	0.18
Initial ALB	2.84±0.19	2.76±0.09	2.97±0.17	2.73±0.02	0.62
Final ALB	3.39±0.07	3.47±0.07	3.26±0.21	3.34±0.15	0.77
Initial LDH	648.00±35.70	651.30±79.70	763.00±152.00	821.00±161.00	0.68
Final LDH	579.00±275.00	713.70±57.30	943.70±88.00	822.30±19.30	0.40
Initial TG	35.00±12.70	60.00±18.20	47.67±9.74	46.70±11.70	0.64
Final TG	27.33±7.31	37.33±8.45	34.67±5.17	22.67±7.84	0.50

1995), increased in parallel with the increase in concentration of JOW (Table 3). Similarly, many researchers determined that the iron reduction capacity increases with an increase in the concentration (Elmastas *et al.*, 2006; Djeridane *et al*., 2006; Miceli *et al*., 2009; Isik *et al.,* 2020).

Growth Performance of Calves

The appetizing properties of medicinal herbs and their extracts stimulate digestion and increase feed consumption by promoting gastric and intestinal motility by increasing the release of enzymes.

Increasing feed consumption allows calves to be weaned at an early age (Tekeli *et al.*, 2006; Kehoe *et al.*, 2007; Ozkaya *et al.*, 2018).

It has been reported that the reason that aromatic waters obtained from medical herbs improve the live weight of calves may be due to the positive effect of aromatic waters on the intestinal flora (Tiihonen *et al.,* 2010; Sharma *et al.,* 2013; Ozkaya *et al.,* 2018). Chaves *et al.* (2008) have been stated that medicinal aromatic waters increase the digestibility of foods by increasing the total essential fatty acids concentration in the rumen and thus have positive effect on live weight gain. Herbal oils and extracts significantly increased the LW of calves (Ahmed *et al.*, 2009; Soltan, 2009; Ghosh *et al.*, 2010; 2011). However, in the study conducted with oregano aromatic water (OAW), Ozkaya *et al.* (2018) reported that the effect of OAW is not significant on the increase in LW of calves.

It was reported by Ozkaya *et al.* (2018) that the supplementation of OAW as a milk replacer tends to improve the body measurements of calves, but the numerical differences are not significant and supplementing JOW to milk showed similar results. Similarly, Unlu and Erkek (2013) reported that effect of oregano oil supplementation on the body measurements of calves is not significant.

Herbal extracts improve the digestion of feeds by increasing saliva, gall and enzyme activities. However, it increases the digestion and absorption capacity of the intestine by increasing the ability of epithelium cells to regenerate villi as a result of suppression pathogenic bacteria in the intestine (Maurao *et al.,* 2006). The findings obtained in the study are line with Ghosh *et al*. (2010; 2011) who reported that herbal extracts significantly increased feed consumption. However, there are studies reporting that herbal extracts and aromatic waters do not effect feed consumption (Soltan, 2009; Unlu and Erkek, 2013; Ozkaya *et al*., 2018). The difference between these results may cause systemic losses due to mucosal secretion of herbal extracts (Jamroz *et al.,* 2006).

Healthy Parameters of Calves

Supplementation oregano aromatic water (OAW) has no effect on the RR of calves (Ozkaya *et al.,* 2018). Calves have higher RRs than adults (Plumb, 2005). However, 200 ml JOW supplementation significantly increased the RR of calves (Table 5). The fact that the NID was low in the trial groups compared to the CNT indicated that the high RR was not associated with the disease. It is thought that calves being more active than adults increase RR.

It has been reported that OAW supplementation significantly increases PR of calves (Ozkaya *et al.,* 2018). Just as increase in activity increases the RR, it also increases the PR (Akgun, 1989). Calves have higher PRs than adults, just like their RRs (Plumb, 2005). For this reason, it is thought that the reason why PRs higher in trial groups compared to CNT is due to the active and excited calves rather than the disease.

Although the difference among the RT of the groups was found to be significant (Table 5), the average values remained within the normal limits (38.6-39.4 ^{o}C) for calves (Latimer *et al*., 2003). It has been reported by many researchers RT rises above 39.5 ^{o}C in both respiratory and digestive system disease (Griffin, 1997; Smith, 2000; Gunes, 2018).

Due to the antibacterial properties and iron reduction capacity of oils and extracts of aromatic plants, they are positive effects on intestinal bacterial flora and suppress pathogenic bacteria. Therefore, plant oil and extracts significantly reduce the FS (Ishihara *et al*., 2001; Goetz *et al*., 2002; Lewis *et al*., 2003; Ghosh *et al*., 2010; 2011; Ozkaya *et al*., 2018). This explains the low FS in the trial groups (Table 5). However, there are studies indicating that oil and extracts obtained from plants have no effect on the FS (Greathead *et al*., 2000; Bampidis *et al*., 2005; Unlu ve Erkek, 2013).

Digestive system diseases suppress the growth of calves and cause death (Davis and Drackley, 1998; NAHMS, 2007). The antibacterial properties of herbal extracts suppress the growth of pathogenic bacteria such as *E. coli, Coliforms* and *Enterobacteriaceae* in the intestinal flora, thereby improving the immune system and reducing the incidence of diarrhea (Ahmed *et al*., 2009; Ozkaya *et al.*, 2018). The reason for the low NDD

is thought to be the suppression of the growth of pathogenic bacteria due to the antibacterial effects of JOW (Table 5).

Oils and their extracts obtained from medicinal aromatic plants support the immune system and reduce the incidence of disease cases in calves (El-Ashry *et al.*, 2006; Ahmed *et al*., 2009; Franz *et al*., 2010; Li *et al.,* 2012a; 2012b; Wang *et al*., 2012; Sajjadi *et al*., 2014, Zeng *et al*., 2015; Ozkaya *et al*., 2018). The reason for the low NID in the trial groups may be associated with an immune system supporting effect of JOW (Table 5).

Conclusion

The supplementation of JOW tended to improve the growth performance, increased feed consumption, reduced the incidence of diarrhea and disease of calves and enabled healthy calves rearing. The fact that no negative effects were observed in blood serum biochemical variables which is an indicator of the healthy growth and good animal welfare for calves. It was concluded that applied JOW doses could be used for rearing healthy calves, but calves reared with 50 ml JOW supplemented milk (1.25% JOW supplemented milk) showed healthier growth calves compared to other groups.

It is recommended to examine the effects of JOW supplementation on fecal bacteria count, immune system and antioxidative defense parameters in order to better reveal the effects of JOW on both the growth and health of calves.

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REVIEW ARTICLE

LIVESTOCK STUDIES

Evaluation of Current Antioxidant Profile in Semen

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Abstract

The freezing and storage of the sperm are used cryopreservation of germplasm in livestock breeding, genetic improvement of indigenous species, preservation of rare races, successful tolerance to environmental changes and international germplasm exchanges. Both the freezing and thawing process causes large changes in the volume of the cell fluid. Spermatozoon removes most of its cytoplasm at differentiation stages and lacks the cytoplasmic component that contains antioxidants that counteract the harmful effect of reactive oxygen species and lipid peroxidation. Therefore, the sensitivity of spermatozoa to lipid peroxidation increases during the freezing and thawing of the sperm, which creates a significant mechanical stress on the cell membrane. Oxidative stress is caused by oxygen and oxygen-derived oxidants, commonly known as ROS, and is known as an imbalance between the ability of biological systems to easily detoxify or repair damaged reagents. Uncontrolled ROS production, which exceeds the antioxidant capacity of seminal plasma, causes oxidative stress that is harmful to spermatozoa. All cellular components, including lipids, proteins, nucleic acids, and sugars, are potential targets of oxidative stress. Antioxidants control the chemical degradation of the substrate caused by oxidation, neutralizing free radicals, thereby it is used to minimize the risk of damage to spermatozoa during cryopreservation.

Introduction

Mammalian spermatozoa have a high energy demand to function. Spermatozoa contain approximately 50 to 75 mitochondria. The production of free radicals called hydroxyl radicals (•OH), superoxide anion ($•O^{2-}$), hydrogen peroxide (H_2O_2), and nitric oxide (NO) containing reactive oxygen species occurs in spermatozoon, like any other cell that performs aerobic metabolism (Bansal and Bilaspuri, 2010a). These ROSs are highly reactive molecules since their outer shell has an unpaired electron. In addition, they have a very short half-life between nanoseconds and milliseconds. ROS is formed by natural cell activity and participates in the normal cell cycle. Gametes are often susceptible to attack by reactive oxygen species (ROS), and manipulating gametes in vitro during assisted reproductive techniques can cause ROS to be generated by cells and exposed to ROS at supraphysiological levels (Agarwal *et al*., 2014). However, oxidative stress (OS) is called when the ROS production exceeds the physiological range and oxidants become more than antioxidants. The resulting OS production causes harmful effects that result in the oxidation of lipids, proteins, carbohydrates, and nucleotides (Birben *et al*., 2012). ROS formation in spermatozoa is likewise a natural physiological process and affects essential reproductive processes such as gametes, spermatozoon-oocyte interactions, implantation, and early development of embryos.

A common and significant technique, sperm cryo-preservation provides a valuable therapeutic alternative in the field of assisted reproduction (Hezavehei *et al*., 2018). Osmotic stress that occurs during

cryopreservation results from changes in cell volume due to water movement and dissolution along the spermatozoon plasma membrane and this causes ROS formation (Ball, 2008). Numerous mitochondria, low cytoplasm, and low antioxidant content in the specific cell structure of spermatozoon and plasma membrane make spermatozoon vulnerable to damage caused by free radicals (Bollwein *et al*., 2008). Antioxidants act as the main defense factors against oxidative stress caused by free radicals (Silva *et al*., 2011). For this reason, an antioxidant is added effectively to sperm freezing diluents.

Oxidative Stress

Oxidative stress (OS) is caused by oxygen and oxidants, generally referred to as the reactive oxygen species (ROS). It is called the imbalance between the ability of biological systems to detoxify reactive intermediates easily or to repair the resulting damage easily. Uncontrolled production of ROS, which exceeds seminal plasma's antioxidant capacity, results in OS, which is harmful to sperm cells. The potential targets of oxidative stress include all cellular components including lipids, proteins, nucleic acids, and sugars (Bansal and Bilaspuri, 2010a).

The main reason for the occurrence of oxidative stress in sperms is the depletion of seminal antioxidants and the production by spermatozoon of excess free radicals (Wathes *et al*., 2007). In immature spermatozoa can produce a significant amount of ROS that negatively correlates with semen quality (Agarwal and Majzoub, 2017). Nevertheless, it is not yet clear what mechanisms increase oxidative stress in frozen-thawed sperm. Some authors attribute this to the depletion of antioxidative enzymes (Stradaioli *et al*., 2007), while others suggest that osmotic stress induces oxidative stress during sperm freezing and thawing (McCarthy *et al*., 2010). Hyperosmotic cell swelling can then induce NADPH (Nicotinamide Adenine Dinucleotide Phosphate) oxidase in somatic cells, activating membrane-bound phospholipase A2, which allows free polyunsaturated fatty acids to form as arachnoid acid, resulting in an increase in production of O_2 (Lambert *et al*., 2006).

Effects of Oxidative Stress on Spermatozoa

The adverse effects of oxidative stress on spermatozoon activity have been seen in several ways as it affects many essential molecules, including lipids, proteins, and DNA due to excessive ROS (Bollwein and Bittner, 2018). Through the presence of small molecules and the action of antioxidant enzymes in their cytoplasm, most cells may prevent oxidative stress, but through transcriptional activation of genes corresponding to these proteins, spermatozoa are exceptions. The silent nature of this cell also demonstrates that it cannot reverse the changes that affect it and in particular the damage to its genetic material. In other words, if spermatozoa are unable to protect themselves effectively against oxidative stress, death due to necrosis or apoptosis is the only option for this cell if subjected to acute stress.

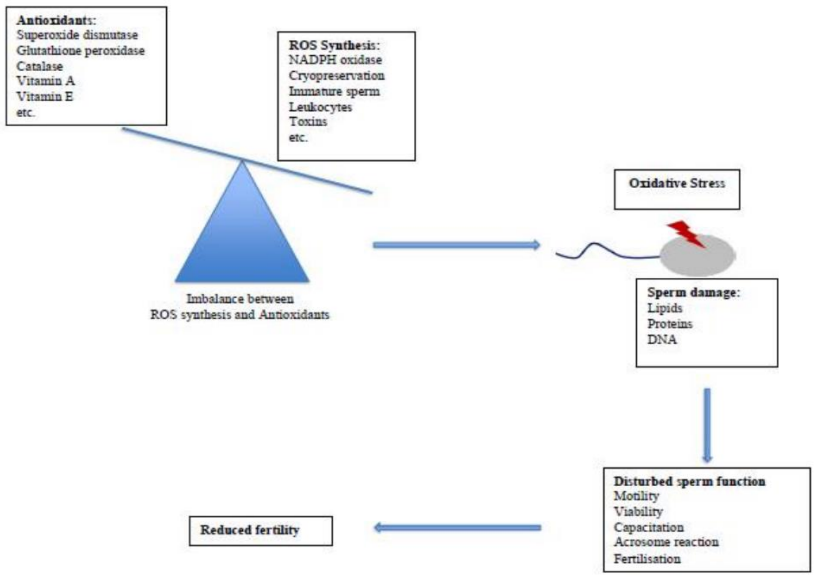

Figure 1. Oxidation sources in semen and their role in disturbances of sperm quality and male fertility (Bollwein and Bittner, 2018).

This state of fragility worsens with the unique lipid composition of the spermatozoon plasma membrane in relation to oxidative stress (Drevet and Aitken, 2020).

Lipid Peroxidation

The damage mechanism caused by ROS in spermatozoa involves an oxidative attack that leads to the initiation of lipid peroxidation (LPO) on spermatozoon membrane lipids (Aitken, 2017). Lipid peroxidation produces a variety of lipid metabolites, including lipid peroxyl radicals, alkoxyl radicals, malondialdehyde, 4-hydroxynonenal, and acrolein. Lipid peroxides are self-produced in the plasma membrane of spermatozoa and released under the action of phospholipase A2, and are also reduced by glutathione peroxidase to phospholipids. The membranes of mammalian spermatozoa are abundant in polyunsaturated fatty acids (PUFAs) and are responsive to lipid peroxidation-mediated oxygen damages. The presence of PUFAs gives the flexibility of the membranes to help the membrane enter into the fluidity-related membrane fusion events and the capacity of sperm to fertilize. Unfortunately, the presence of double bonds in these molecules makes them vulnerable to free radical attacks and LPO initiation. This leads to the impaired membrane and morphological integrity, impaired cell function, impaired sperm mobility, impaired fertility of spermatozoa with oocytes, and induction of apoptosis of spermatozoa (Bucak *et al*., 2010). In addition, lipid peroxidation is known to reduce the mobility of spermatozoa. Mechanisms include modulating the function of ion channels with changes in membrane structure (Bollwein and Bittner, 2018) and the development of lipid metabolites with flagellar axonemal proteins and proteins mediated by mitochondrial electrons. Modulation of mitochondrial proteins interferes with the transport of mitochondrial electrons and causes electrons to flow out. And these, combine with oxygen in a vicious loop to create additional ROS (Moazamian *et al*., 2015).

Free Radicals

Free radicals are short-lived chemical reactive intermediates with one or more unpaired electrons. These unpaired electrons move into nearby cellular structures so cause damage to the cells. In this case, amino acids in proteins or nucleic acids induce oxidation of the cell membrane lipids (Ifeanyi, 2018). Free radicals are also required for normal cell proliferation, differentiation, and intracellular signaling that occurs in the process of migration. Excessive free radical formation often results in a spermiogenesis error, which causes abnormally high levels of cytoplasmic retention to be released from the germinal epithelium (Sanocka and Kurpisz, 2004).

Reactive Oxygen Types (ROS)

Reactive oxygen species (ROS) contain radicals of oxygen as well as hydrogen peroxide (H_2O_2), superoxide anion (• O_2–), and hypochlorous acid (HOCl) and highly reactive forms of O_2 which do not contain unpaired (non-radicals) electrons (Bollwein and Bittner, 2018). ROS also represents a wide category of molecules indicating that radicals (hydroxyl ion, superoxide, nitric oxide, peroxyl, etc.) and non-radicals (ozone, single oxygen, lipid peroxide, hydrogen peroxide) and oxygen derivatives are collected. These molecules are unstable and try to capture a stabilizing electron, increasing the imbalance and leading to oxidation of other molecules (Drevet and Aitken, 2020).

ROS produced by sperm plays a significant role in normal physiological processes such as capacitation of spermatozoa, acrosome reaction, preservation of fertility capacity, and stabilization of the mitochondrial capsule in cattle (Desai *et al*., 2010; Gonçalves *et al*., 2010). Physiological ROS concentrations in vivo play a role in ensuring membrane fluidity, maintaining spermatozoa fertilization capability, and acrosome reaction (Bucak *et al*., 2010). Therefore, maintaining an appropriate ROS level is essential for the functionality of appropriate spermatozoon functions. The advanced antioxidant system counteracts the homeostasis of ROS and controls it.

Protein Modifications

Reactive oxygen species are thought to oxidize the side chains of amino acid residues, directly altering the proteins by supplying peptide bond splitting and covalent protein-protein cross-link formation. Oxidation may affect the conformation or activity of proteins. The thiol group is an example of this situation. Cysteine, the amino acid, contains a group of thiol and this can affect many proteins. In semen, the tyrosine phosphatase enzyme which plays an important role in the capacitation spermatozoa has a thiol group and is therefore vulnerable to ROS oxidation. Also, long-term oxidative stress can lead to excessive oxidation of thiol protamine groups, thereby causing hyper-condensation of DNA has a negative effect on function (Bollwein and Bittner, 2018).

DNA Damage

Free radicals may have many ways of damage to DNA. Double bonds of DNA bases and abstract hydrogen from deoxyribose sugar are linked to hydroxyl radicals (Cadet *et al*., 2003). Hydrogen isolation from deoxyribose carbon induces splits in the fiber and the base emissions. The ROS attack on the bases causes a large number of base changes (Bollwein and Bittner, 2018). One of the most common changes is guanine

oxidation. Depending on the intensity of oxidative stress, there may be changes in DNA fragmentation (single or double chain breaks), from simple oxidation of bases (the most sensitive bases, guanosine, and adenosine) to oxidative stress. Other oxidative DNA events can also be observed, including the creation of specific regions and crosslinking of DNA-proteins. Due to the lack of a fully functioning DNA repair mechanism in mature spermatozoa, oocyte DNA repair systems (mainly post-fertilization oocyte base excision repair pathway) are needed to correct these oxidative changes. Also, in circumstances of moderate to low oxidative stress that does not cause DNA degradation, baseline oxidation may occur and must be corrected in this case (i.e., each oxidized base must be substituted with an unoxidized base) (Drevet and Aitken, 2020).

Current Antioxidant Substances

Antioxidants can be anti-ROS antioxidants (metal chelators or binding proteins such as lactoferrin) or antioxidants that eliminate existing ROS (such as vitamins C and E) (Hussain *et al*., 2018). It is classified into two groups according to its chemical properties: enzymatic antioxidants and non-enzymatic antioxidants (Félix *et al*., 2021). Enzymatic antioxidants are known as natural antioxidants; glutathione, glutathione peroxidase, glutathione reductase, catalase, and superoxide dismutase (SOD). These all join the spermatozoa's normal antioxidant defense mechanism. Non-enzymatic antioxidants or synthetic antioxidants or nutritional supplements include vitamin C, and minerals such as vitamin E, zinc and selenium, taurine, hypotaurine, butylated hydroxytoluene (BHT), and melatonin. Non-enzymatic antioxidants are obtained from fruits or vegetables (Bansal and Bilaspuri, 2010b). Antioxidants also serve as motility-enhancing agents, thereby increasing sperm motility and the ability to fertilize. Additionally, antioxidants are not only used to preserve the spermatozoon membrane integrity but also to prevent membrane damage by pressing on lipid peroxidation and ROS development of embryos and oocytes (Uysal *et al*., 2000).

Enzymatic Antioxidants

Glutathione

Glutathione (GSH) the key protein thiol compound in mammalian cells is present in a variety of cells and has the capacity to react directly with ROS. Additionally, glutathione has a defensive role to exogenous antioxidants such as vitamin C and E active forms. (Atmaca, 2004). It also helps in reducing toxic H_2O_2 and hydroperoxides, thus shielding mammalian cells from ROS. Sulphydryl groups of GSH protect cells against oxidants, electrophiles, and free radicals (Agarwal *et al*., 2008). This function of GSH has been shown in vitro studies in which the frequency of the tail shot is maintained, LPO is decreased, and properties of the spermatozoon membrane are improved. Glutathione acts as protective agents in many types of cells against the negative effects of ROS-induced damage. In spermatozoa, it prevents the process of lipid peroxidation from damage to ROS. GSH also has a protective effect on normal acrosome integrity and also stabilizes sperm plasmalemma and improves motility (Bello *et al*., 2020).

Glutathione peroxidase (GSHPx) reduces lipoperoxides to alkyl alcohols and to hydrogen peroxide H_2O by using glutathione (Amidi *et al*., 2016). Glutathione peroxidase primarily acts as an antioxidant cleaner in the epididymis and testicles (Mora-Esteves and Shin, 2013) and helps preserve the viability and motility of spermatozoa by supplying protection in the lipid components of the spermatozoon membrane (Bello *et al*., 2020). GSH can be regenerated from the oxidized form of glutathione reductase (GSR), the function of which is inducible to oxidative stress. In pigs, the application of 1 and 5 mM GSH to semen diluent had founded beneficial effects on the quality of semen (Gadea *et al*., 2005). In ram semen, the use of GSH concentrations has had a beneficial impact on acrosome integrity between 2 and 5 mm (Silva *et al*., 2011).

Superoxide Dismutase and Catalase

SOD catalyzes the conversion of superoxide to oxygen and H_2O_2, which protects sperm from superoxide anions and thereby prevents LPO and enhances motility (Agarwal *et al*., 2008). Although SOD transforms the spontaneous superoxide anion to form (O^{2-} •) O_2 and H_2O_2, as seen in the equation below, it converts catalase H_2O_2 to O_2 and H_2O, so both SOD and catalase help eliminate ROS, which has the potential to damage spermatozoa and it plays an important role in reducing LPO (Bello *et al*., 2020).

Before the cryopreservation, dilution with extenders decreases the concentration of these components in seminal plasma and leaves sperm susceptible to oxidative stress (Martinez-Paramo *et al*., 2012). Adding SOD or CAT to the porcine sperm freezing extender, however, not only increases the sperm mobility and viability but also decreases post-thaw ROS production, which leads to an increase in the in vitro fertilization capacity of the dissolved sperm. Such results

indicate that adding CAT and SOD to the extender increases the survival and in vitro viability of liquid semen storage (Bello *et al*., 2020).

Non-Enzymatic Antioxidants

Vitamin E

Vitamin E (α-tocopherol) is a chain-breaking antioxidant present in the membrane of the spermatozoon. It works by neutralizing H_2O_2 and quenching free radicals (Bansal and Bilaspuri, 2010b). It thus prevents the chain reactions that create lipid peroxide and protects the plasma of sperm from ROS damage (Bello *et al*., 2020). This also increases the function of other oxidizing agents (Mora-Esteves and Shin, 2013), thereby helping to preserve sperm mobility as well as morphology (Agarwal *et al*., 2008). Vitamin E supplementation during cryopreservation has a beneficial effect on sperm motility, mitochondrial membrane potential, and membrane integrity, depending on the fraction of the ejaculate (Bello *et al*., 2020). It has been reported that adding vitamin E (5 µg/ Ml) in combination with 1% Nano-Se also increases sperm quality after thawing and enhances cock sperm oxidative variables (Safa *et al*., 2016).

Vitamin C

Vitamin C (Ascorbic acid) is a water-soluble chain breaker antioxidant, capable of clearing radicals of oxygen with low toxicity and high ability. Ascorbic acid secreted from seminal vesicles is the main antioxidant found in fertile men's seminal plasma, providing up to 65% of the total chain-breaking antioxidant capacity (Amidi *et al*., 2016). Vitamin C plays a significant role in the battle against oxidative stress in seminal plasma by reacting in extracellular fluid with OH-, O_2- and H_2O_2, maintaining sperm viability, stability, and preventing sperm agglutination (>65%) (Agarwal *et al*., 2008). Vitamin C can also serve as a pro-oxidant and make radicals, in the presence of transition metals, more reactive and highly destructive. The addition of 2,5 mM and 0,02-0,6 mM of vitamin C to cattle and human sperm has been shown to have a detrimental effect on sperm motility in frozen-thawed bull sperm and in samples of human normozoospermic and asthenozoospermic, however, the subsequent application of 5 mM revealed, however, an important protective effect against lipid peroxidation in frozen bovine quality semen (Amidi *et al*., 2016).

Butylated Hydroxy Toluene (BHT)

Butylated hydroxytoluene (BHT), also known as dibutylhydroxytoluene, is a non-enzymatic, synthetic analog of vitamin E. BHT is an organic soluble molecule used to stop autooxidation of the double lipid layer and spermatozoon membrane. BHT has properties of antioxidants, antivirals, and antimicrobials. Because spermatozoa are high targets for reactive oxygen species (ROS), the addition of BHT is beneficial for its role in free radical scavenging. BHT reacts with ROS and converts to hydroperoxides. It helps to avoid lipid peroxidation in biological membranes and by being used as a complement in different semen extender it increases the consistency of spermatozoa. BHT also acts as an antiviral agent and thus reduces the risk of viral disease transmission to the female animal during artificial insemination (Bello *et al*., 2020).

Farshad *et al*. (2011) demonstrated that the optimum concentration of BHT needed to optimally preserve ram semen during cryopreservation is 2-3 mM BHT. While the optimal concentration of BHT required for sperm survival varies depending on the animal species, it may range from 0.05-2.0 mM. Higher butylated concentrations of hydroxytoluene adversely affect the properties of freezing thawed semen and the process remains unclear. BHT concentrations of 0.5 and 2.0 mM/mL in buffaloes are the preferred quantities for sperm preservation, depending on the diluent and freezing stage (Bello *et al*., 2020).

Carnitine

Carnitine is an antioxidant that is water-soluble and typically obtained from dietary sources. This can contribute to sperm motility as a source of fuel by aiding the use of free fatty acids and preventing lipid oxidation (Mora-Esteves and Shin, 2013). Therefore, Carnitine protects spermatozoa's viability and motility by shielding the spermatozoon DNA and membranes from oxidative damage. L-carnitine has been shown to be efficient in protecting chicken sperm from apoptosis, loss of mitochondrial function, and DNA binding (Hussain *et al*., 2018).

Albumin

Through interacting with peroxyl radicals, albumin avoids chain reactions that produce more free radicals and thus maintains sperm motility and viability by reducing the production of ROS. Albumin is known to improve sperm and plasma membrane integrity stability and to protect the acrosomes of ram spermatozoa from temperature shock during freeze-thaw. Albumin can also increase sperm survival and fertility capacities in the female reproductive system before fertilization, and it has been shown to improve antioxidant catalase activity after frozen and thawed bull semen (Hussain *et al*., 2018).

Zinc

Zinc is one of the important trace elements that cause infertility in its deficiency, as well as controlling events such as testicular development, spermatogenesis, steroidogenesis through the secretion of gonadotropic hormones, genetic expression of steroid receptors, testosterone synthesis, and adjustment of serum cholesterol levels. In zinc-deficient animals, zinc supplementation contributes to increased fertility by increasing semen concentration, motility, and spermatozoon membrane integrity, as well as reducing spermatozoon DNA damage. Zinc also contributes to the spermatozoon chromatin stability and DNA damage repair. Zinc affects the fluidity of lipids and therefore the stability of biological membranes. It plays a function in the formation of free oxygen radicals and can play a regulatory role in capacitation and acrosome reaction processes, sperm condensation of nuclear chromatin, and acrosine activity (Dorostkar *et al*., 2014). Although the addition of higher zinc concentrations (0.576 and 1.152 mg/L) to the extender is detrimental to spermatozoa, Dorostkar *et al*. (2014) reported that the addition of zinc sulphate (0.288 mg/L) resulted in improvement in the preservation of spermatozoon quality (progressive motility, viability, membrane integrity, and total antioxidant capacity) in freezing processes, and also caused less DNA damage affecting the cell membrane after semen freeze-thaw and this provides a higher fertility rate.

Zinc-nanoparticles are also among the metal nanoparticles used successfully to improve the quality of sperm and are considered an important element of spermatogenesis since it enhances the quality of sperms. In young rams, the addition of 50 mg/kg or 100 mg/kg Zn-nanoparticle in the diet improved the efficiency of epididymal sperm, seminal anti-oxidase plasma activity, and the expression of copper-zinc superoxide dismutase (Cu-Zn SOD) (Zang *et al*., 2015). Additionally, the Zn nano-complex has improved the dose-dependent functionality of the spermatozoon plasma membranes without any detrimental effects on motility parameters. Studies have shown that in streptozotocin-induced diabetic rats, ZnO nanoparticles increase the activity of antioxidant enzymes in testicular tissue, increase sperm count and improve the properties of spermatozoa by protecting against oxidative stress (Falchia *et al*., 2018).

L-cysteine

L-cysteine (L-Cys) is a non-essential amino acid with a low molecular weight containing thiol. To engage in GSH biosynthesis both in vitro and in vivo, it achieves its effect on the cell membrane by easily penetrating the cell membrane. It protects membrane lipids and proteins by indirect scavenging of free radicals; it also acts as a membrane stabilizer and spermatozoon capacitation inhibitor (Amidi *et al*., 2016). L-cysteine has been shown to increase sperm motility, fertility, and morphology in bulls (Khan *et al*., 2021), ram (Andreea and Stela, 2010) and goats (Bucak and Uysal, 2010), and to enhance the chromatin structure and membrane integrity of pig semen stored by cooling. Çoyan *et al*. (2012) reported that cysteine improves Merino ram semen's mitochondrial activity after freeze-thaw without enhancing sperm motility. This can be metabolized to taurine after L-Cysteine passes through the cells. After a combination with a fatty acid in the plasma membrane, taurine transforms into acyl-taurine which improves surfactant properties and spermatozoon membrane osmoregulation (Amidi *et al*., 2016).

Selenium (Se)

Selenium (Se) is an important trace element well known to cells in both humans and animals. It acts as a key enzyme in the biological system in protection against oxidative stress by detoxifying free radicals by activating glutathione peroxidase and serves as a cofactor of glutathione synthetase. Selenium detoxifies the environment in the form of selenite in cell culture, to protect cells against oxidative damage (Amidi *et al*., 2016). Selenium supplementation in the diet has been reported to increase reproductive capacity in mice, sheep, and cattle (Amidi *et al*., 2016) and also to improve semen parameters after thawing Barbari goat frozen semen (Kumar *et al*., 2011). Reproductive problems and decreased semen quality resulting from selenium deficiency have been demonstrated in rats, rodents, pigs, rams, and cattle (Amidi *et al*., 2016).

The addition of selenium before freezing substantially increased the motility of bull semen (Amidi *et al*., 2016). Dorostkar *et al*. (2012) reported that diluents containing 1 and 2 µg mL^{-1} Se significantly improved sperm motility, viability, membrane integrity, and total antioxidant capacity in sperm, and less damage was caused to the DNA of spermatozoa. It was also observed that selenium effects occurred in a dose-dependent manner and harmful effects occurred on semen parameters at 4 to 8 µg mL^{-1} levels.

Many studies have used nano-selenium (SeNPs) as ROS scavengers to protect against oxidative damage in spermatozoa. The addition of Nano-Se to the semen extender improved the post-thaw efficiency and

oxidative semen variables in a study on male semen. Furthermore, the oral treatment of Se-nanoparticles preserves the quality of spermatozoa (motility, DNA integrity) and spermatogenesis from oxidative damage caused by Cisplatin, a toxic anticancer agent on male reproduction (Safa *et al*., 2016).

Melatonin (MLT)

Melatonin is an indoleamine with two side chains, 5-methoxy group and 3-amide group. This can act as an antioxidant with its molecular weight of 232.2 g/mol, as well as regulate the biological clock and seasonal reproduction. MLT can protect different biomolecules from ROS damage by acting as a direct scavenger to detoxify reactive oxygen and nitrogen species through an easy crossing of cell membranes and the blood-brain barrier (Bhattacharya *et al*., 2019). It may also have indirect effects by stimulating various antioxidant enzymes such as melatonin, glutathione peroxidase/reductase, catalase, and superoxide dismutase. MLT demonstrates its powerful non-enzymatic antioxidant properties by removing various reactive oxygen and nitrogen species in vivo and in vitro. MLT has enhanced the characteristics of goats (Samir *et al*., 2020), rats (Sönmez *et al*., 2007), boar (Rocco *et al*., 2018), rams (Sarabi *et al*., 2009) and human sperm (Ortiz *et al*., 2010), according to results from some studies. The melatonin concentration needed to exert antioxidant effects on stored semen depends on the species of the animal, and this concentration varies from 0.01 mM to 3.00 mM (Medrano *et al*., 2017).

Curcumin

Curcumin is formulated as [1,7-bis (4-hydroxy-3-methoxyphenyl) -1,6-heptadiene-3,5-dione] (CUR). Curcumin is the phytochemical that gives a yellow color to the turmeric and is now considered therapeutic. Curcumin demonstrates its antioxidant properties by scavenging different types of reactive oxygen, including radicals of superoxide anions, hydroxyl radicals, and radicals of nitrogen dioxides. Lipid peroxidation has also been shown to be inhibited in different animal models. At the same time, there are conflicting reports concerning the effects of curcumin on the parameters of male fertility. Many in vivo and in vitro studies illustrate its role in the energy-promoting and protective effects of curcumin on testicular tissue, spermatogenesis, and spermatozoa oxidative balance (Tvrda *et al*., 2015).

Conclusion

Cryopreservation of sperm is an applicable technique among assisted reproduction methods. However; some post-thaw semen parameters, including morphology, motility, viability, and DNA integrity, can be affected by cryopreservation. It is well known that antioxidants can reduce the negative effects of oxidative stress on the processing and development of the spermatozoa and embryos. The use of antioxidants has gained popularity for this reason because it prevents the formation of oxidation during semen freezing, minimizes the harmful impact of ROS and increases semen quality after thawing. Oxidative stress evaluation and the use of antioxidants are not regularly conducted in clinical practice. The explanation for this situation could be that antioxidant protective effects could not be completely identified, and their effect on pregnancy levels in the field could not be confirmed. It should also be recalled that antioxidants are costly chemicals. Therefore, further studies are required to determine an optimal freezing procedure for a species' semen and the appropriate antioxidant to be used.

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