

## SHORT COMMUNICATION

# Effects of terpenes administration on fatty acid profile and coagulation properties of ewes' milk

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## SUMMARY

The presence of lipids in animal products have attracted scientific and consumers' attention due to the health beneficial effects of mono- (MUFA) and polyunsaturated fatty acids (PUFA). Various factors may affect their concentration, while plant components, such as terpenes, can possibly modify their concentration in milk, as well as other milk characteristics. The aim of the present study was to test the effects of a mixture of three terpenes,  $\alpha$ -pinene, limonene and  $\beta$ -caryophyllene that was orally administered to ewes, on milk fatty acids profile and coagulation properties. Eight ewes were divided in two groups, control (C) and treatment (T), where the T-group received orally a mixture of the three aforementioned terpenes. Rate of firming (K20) was longer for the T-group, while other coagulation parameters were not affected. The concentration of C14:1 and C16:0 decreased significantly, while C18:0, C18:1, C18:3 increased significantly in T-group. The concentration of MUFA and PUFA increased significantly, while SFA decreased in the T-group of ewes. In conclusion, the results of the present study indicate that terpene intake could affect the proportion of FA and coagulation properties of ewes' milk.

**Keywords:** Milk; Sheep; Terpenes; Fatty acids; Coagulation properties

## INTRODUCTION

A major food component capable of providing essential nutrients for humans is lipids. In the past, epidemiological studies (Calder 2015), correlated the increased presence of saturated fatty acids (SFA) in dairy products (Noni and Battelli 2008; Revello Chion et al. 2010) with increased incidence of cardiovascular diseases (Pacheco et al. 2006). However, that fact overshadowed the benefits that lipids sub-groups such as mono- (MUFA) and polyunsaturated fatty acids (PUFA) can offer. Lately, both MUFA and PUFA have been recognized for their beneficial effect on enhancing human health (Calder 2015). Their concentration and composition in animal products is well established (Kouba and Mourot 2011; Tsiplakou et al. 2010), while various factors related to nutrition and animal genetics can also affect their presence and concentration (Tsiplakou and Zervas 2008; Vasta and Priolo 2006).

Recently, Vasta and Luciano, (2011) showed that dietary plant secondary compounds (PSCs) and more

specifically terpenes, apart from their antimicrobial and antibiotic properties (Calsamiglia et al., 2007) can affect the concentration and synthesis of FA (Bravo-Lamas et al. 2018). Terpenes, can be found in various grasses, forbs, shrubs at different growth stages, while their concentration is higher in vegetation growing under harsh conditions such as higher altitude pastures (Cornu et al. 2001). Diet is the main transfer route of terpenes to animal tissues. Their presence in animal's ration is associated with increased concentrations of PUFA in dairy products, which might be attributed to the effects of terpenes on biohydrogenation pathways (Vasta and Bessa 2012). In the study of Altomonte et al., (2019) terpenes presence in animal products was associated to the type of pasture (e.g. mountainous), season and plant species and more specifically to the genus *Lolium*.

In one of the first studies performed on cows, Noni and Battelli (2008), investigated the capacity of terpenes to alter cheese FA profile. They found that the FA concentration of the cheeses produced in two different Italian alpine

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valleys, differed significantly. They attributed that result on the different altitude of the alpine pastures, which was also associated with higher terpenes content. They also showed that values on milk coagulation properties differed significantly from the ones obtained from cows fed indoors. However, most of the publications that investigate the effect of terpenes on dairy products, refer to cows (Lejonklev et al. 2013; Zeppa et al. 2003). Thus, information on the extent at which they have the ability to modify physicochemical characteristics of milk originated from other species, is limited. Only recently in a study conducted on sheep, Basdagianni et al., (2019) associated the FA and terpene profile of sheep milk as quality indicator while they also concluded that  $\beta$ -caryophyllene could also be used as potential biomarker of grazing. Dairy sheep characterize a wide range of the livestock population in South Europe (Eurostat 2017), therefore, research findings that investigate the effect of terpenes, on quality and their ability to alter FA composition of animal products is essential. The objective of the present study was to test the ability of an orally administered terpenes mixture, consisted of  $\alpha$ -pinene, limonene and  $\beta$ -caryophyllene, to alter milk FA profile and coagulation properties of ewes' milk.

## MATERIAL AND METHODS

### Animals, diets, design

Eight adult, healthy dairy ewes (live weight 69 kg, SD 6.4 kg; crossbreed native breeds) were used for the present study. The animals were housed at the experimental unit of the Department of Nutritional Physiology and Feeding of the Agricultural University of Athens. The experimental protocol was approved by the University Institutional Committee for Animal Use and Ethics while ewes were handled in compliance with EU (Directive 2010/63/EU) and National laws for the care of animals in experimentation. During the experiment, all animals were housed and fed in group. The study design, milking and performed analyses were as described in detail by Pouloupoulou et al. (2012). Coagulation properties and fatty acids contents were recorded before the administration of terpenes (sampling time point 1), during terpenes administration (sampling time points 2-10) and after the end of the terpenes oral administration (sampling time point 11).

### Coagulation properties and fatty acids analysis

Milk coagulation properties such as, Rennet Coagulation Time (RCT): time (min) from addition of enzyme to the beginning of coagulation, K20: the interval (min) from RCT to the time at which the width of the graph attains 20 mm and A30: the extent of curd firmness (mm) 30 min after coagulant addition, were measured using Formagraph

(Foss Electric, Denmark) as described by Zoidis et al. (2018).

Milk lipids were extracted using the Rose Gottlieb method (IDF 1996), while after extraction, the lipid fraction was methylated to fatty acid methyl esters (FAME), according to IDF Standard 182:1999 (IDF 1999). Fatty acid profile was determined as described by Zoidis et al., (2018).

The different groups of FA were determined as follows:

$$\text{Saturated Fatty Acids (SFA)} = C_{4:0} + C_{6:0} + C_{8:0} + C_{10:0} + C_{12:0} + C_{14:0} + C_{15:0} + C_{16:0} + C_{18:0}$$

$$\text{Monounsaturated Fatty Acids (MUFA)} = C_{14:1} + C_{16:1} + C_{18:1}$$

$$\text{Polyunsaturated Fatty Acids (PUFA)} = \text{CLA} + C_{18:2} + C_{18:3}$$

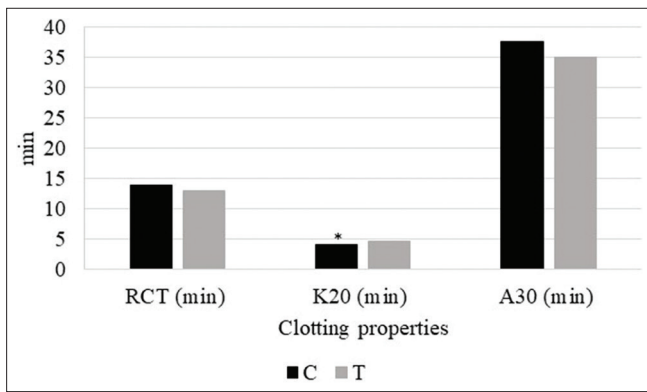
### Statistical analysis

The SPSS statistical package (version 17.0) was used for the analysis of the data. The analysis of milk FA profile was performed using a Mixed Linear Model for repeated measures with treatment and sampling time and their interactions as fixed effects, and animal as random effect. Post-hoc tests were performed for sampling time when appropriate, using Bonferoni's test. ANOVA using the general linear model (GLM) for repeated measures was used for the analysis of the coagulation properties with treatment as fixed effect and animal as random effect. Statistical significance was set at 0.05 and all data were presented as least squares (LSs) means.

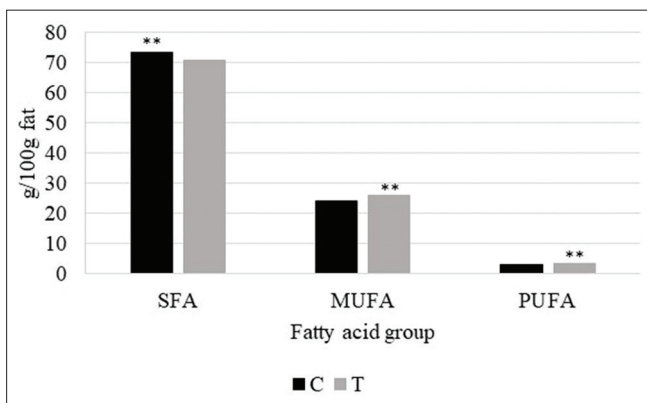
## RESULTS

K20 variable showed that milk originating from the animals that received the terpenes mixture (T-group) required longer time ( $P < 0.05$ ) to form curd, compared to that of the C-group. However, RCT and A30 did not significantly differ between the two groups (Fig. 1).

Terpenes administration significantly decreased the concentration of  $C_{16:0}$  ( $P < 0.01$ ), while the concentrations of  $C_{12:0}$  ( $P < 0.01$ ),  $C_{15:0}$  ( $P < 0.001$ ),  $C_{18:0}$  ( $P < 0.01$ ),  $C_{18:1}$  ( $P < 0.001$ ) were significantly higher in T group of animals. SFA were significantly decreased ( $P < 0.001$ ), while MUFA and PUFA were significantly increased ( $P < 0.01$ ) in the animals that received the terpenes mixture (Fig. 2). The levels of  $C_{10:0}$  ( $P < 0.01$ ),  $C_{18:0}$  ( $P < 0.01$ ),  $C_{18:1}$  ( $P < 0.001$ ),  $C_{18:2}$  ( $P < 0.01$ ),  $C_{18:3}$  ( $P < 0.05$ ) and CLA ( $P < 0.001$ ) were significantly affected (Table 1) when the sampling time point (S) was tested as effect. Concerning the FA groups it was found that SFA level decreased ( $P < 0.05$ ) until sampling point 4, slightly fluctuated from sampling point 5 to 7 and afterwards increased constantly until sampling point 10 and decreased again on the last sampling. MUFA increased ( $P < 0.05$ ) until sampling point 4 and



**Fig 1.** Coagulation properties of ewes' milk for control (C) and treatment (T) group of animals receiving a mixture of three terpenes. RCT = rennet clotting time; K20 = rate of firming; A30 = curd firmness. \* $P < 0.05$ .



**Fig 2.** Fatty acid groups of ewes' milk for control (C) and treatment (T) group of animals receiving a mixture of three terpenes. SFA = sum of saturated fatty acids, MUFA = sum of monounsaturated fatty acids, PUFA = sum of polyunsaturated fatty acid. \*\* $P < 0.01$ .

subsequently their concentration interchanged until the end of sampling period, while the same pattern was followed by PUFA ( $P < 0.05$ ). The interaction of  $T \times S$  was significant only for CLA contents ( $P < 0.05$ ).

## DISCUSSION

Research findings obtained from cows grazing on pastures rich in terpenes, reveal that dairy products' FA profile was altered compared to that of the products originated from cows fed on conventional diets (Zeppa et al. 2004). The results of the current study add information to the limited research data on this subject relating to sheep (Vasta and Luciano 2011) and indicate that terpenes administration in ewes can modify milk traits such as coagulation properties and the FA content.

The present research results on K20 are in agreement with those of Jaramillo et al., (2009) who determined higher K20 values of milk from sheep fed a diet rich in citrus

fruits, compared to the control group of animals. RCT and A30 did not show any significant differences between the two groups. That fact may be attributed to the absence of difference in the milk fat contents between the two groups (Pouloupoulou et al., 2012). However, the present finding is in contrast to that reported by Zoidis et al., (2018) who estimated a longer RCT between treatment and control groups after oral administration of a mixture rich in terpenes in dairy goats. Moreover, no differences were observed for A30 although the milk protein concentration differ significantly between the two groups (Pouloupoulou et al., 2012).

In the present study the contents of  $C_{18:0}$ ,  $C_{18:1}$ ,  $C_{18:3}$  in milk fat were higher in the T group, while time point also had an effect on  $C_{18:0}$ ,  $C_{18:1}$ ,  $C_{18:2}$ ,  $C_{18:3}$  and CLA, a result that comes in accordance with that of Revello-Chion et al., (2010). However, in that experiment cows grazed on summer pastures, which are widely known that contain plants with high terpenes concentration. Those researchers also determined the concentration of the above-mentioned FA in summer feed at significantly higher concentrations than those determined at winter feed where terpenes content was substantially lower. On the contrary, the results of the present study revealed that terpenes administration had a tendency to decrease the concentrations of some saturated FA and advance the concentration of individual MUFA and PUFA. These findings differ from those of Buchin et al., (1998), who found no significant effects on milk FA composition when cows fed on pasture with or without terpenes. Coming in contrast to those findings, Noni and Battelli (2008) found that milk originating from cows grazing upland pastures, had higher concentration in  $C_{18:3}$  compared to the milk from animals grazing at low land pastures. That observation agrees with the present results where  $C_{18:3}$  was higher for the T-group of animals. Moreover,  $C_{16:0}$  concentration changes in the same group, agreed with the results of Revello-Chion et al., (2010) who reported a decline in  $C_{16:0}$  when the milk originated from animals grazing summer pastures. However, Noni and Battelli (2008) concluded that other parameters related to the pasture or the animals ration could also affect milk FA composition, therefore they could not relate directly pasture terpenes content and milk FA profile.

Treatment group of animals showed a lower SFA concentration a result that agreed with the results of Buchin et al. (1998), who reported lower proportions of SFA in cows milk originating from pasture fed animals and higher in milk produced by animals fed on hay. Malecky et al., (2009), in a study performed with goats found no changes in the milk FA profile when animals were dosed with a blend of monoterpenes, compared to control animals, a result that is in contrast to those of the

**Table 1: Effect of treatment, sampling time point and their interaction on milk fatty acid concentration<sup>1</sup> and SFA, MUFA and PUFA for treatment and control group of sheep**

Fatty acid	Treatment		P value T	Sampling time point											SEM	P value S	P value T*S	
	No Terpenes	Terpenes		SEM	1	2	3	4	5	6	7	8	9	10				11
C4:0	2.02	1.81	0.179	0.244	2.36	2.01	1.87	1.80	1.93	1.93	1.91	1.68	1.75	1.95	1.89	0.245	0.188	0.831
C6:0	1.79	1.73	0.140	0.688	2.03	1.78	1.67	1.53	1.76	1.58	1.69	1.72	1.63	1.98	2.02	0.189	0.051	0.709
C8:0	1.51	1.78	0.185	0.142	1.86	1.69	1.50	1.35	1.57	1.63	1.48	1.60	1.58	1.92	1.95	0.240	0.128	0.928
C10:0	6.07	6.53	0.490	0.351	6.93	6.38	5.76	5.68	6.24	5.47	5.60	6.15	6.34	7.49	7.29	0.637	0.006	0.463
C12:0	2.52	3.51	0.376	0.010	3.27	3.20	2.85	2.52	2.82	2.99	2.67	3.05	2.80	3.43	3.54	0.487	0.414	0.999
C14:0	13.22	12.79	0.433	0.062	13.27	12.88	12.86	12.73	12.84	12.74	12.47	13.37	13.31	13.01	13.54	0.356	0.405	0.993
C14:1	0.59	0.53	0.033	0.048	0.57	0.56	0.54	0.59	0.58	0.53	0.54	0.55	0.54	0.61	0.56	0.043	0.782	0.999
C15:0	0.95	1.07	0.037	0.002	0.98	1.05	1.05	1.05	1.02	0.98	1.00	1.03	1.01	1.00	0.94	0.050	0.541	0.642
C16:0	36.92	32.48	0.657	0.001	34.54	34.50	34.59	34.47	34.77	35.15	35.50	34.77	35.28	34.23	33.92	0.852	0.742	0.834
C16:1	1.58	1.26	0.161	0.052	1.71	1.39	1.28	1.48	1.47	1.43	1.38	1.32	1.31	1.49	1.32	0.213	0.433	0.962
C18:0	8.05	8.93	0.305	0.005	7.93 <sup>a</sup>	8.48 <sup>ab</sup>	9.41 <sup>b</sup>	8.87 <sup>ab</sup>	8.51 <sup>ab</sup>	8.65 <sup>ab</sup>	8.54 <sup>ab</sup>	8.56 <sup>ab</sup>	8.89 <sup>ab</sup>	7.76 <sup>a</sup>	7.77 <sup>a</sup>	0.399	0.004	0.904
C18:1	21.61	24.17	0.599	0.001	21.55 <sup>a</sup>	22.89 <sup>ab</sup>	23.47 <sup>ab</sup>	24.47 <sup>b</sup>	23.07 <sup>ab</sup>	23.30 <sup>ab</sup>	23.79 <sup>ab</sup>	22.94 <sup>ab</sup>	22.26 <sup>ab</sup>	21.96 <sup>ab</sup>	22.11 <sup>b</sup>	0.806	0.001	0.636
C18:2	1.83	2.09	0.133	0.050	1.56 <sup>a</sup>	1.91 <sup>ab</sup>	1.89 <sup>ab</sup>	1.95 <sup>ab</sup>	2.09 <sup>ab</sup>	2.20 <sup>b</sup>	2.07 <sup>b</sup>	1.99 <sup>ab</sup>	2.03 <sup>b</sup>	2.02 <sup>ab</sup>	1.95 <sup>ab</sup>	0.172	0.003	0.989
C18:3	0.64	0.81	0.025	0.001	0.64 <sup>a</sup>	0.71 <sup>ab</sup>	0.70 <sup>ab</sup>	0.85 <sup>b</sup>	0.70 <sup>ab</sup>	0.73 <sup>ab</sup>	0.75 <sup>ab</sup>	0.71 <sup>ab</sup>	0.77 <sup>ab</sup>	0.72 <sup>ab</sup>	0.71 <sup>ab</sup>	0.042	0.047	0.098
CLA	0.39	0.40	0.045	0.853	0.59 <sup>a</sup>	0.37 <sup>ab</sup>	0.35 <sup>ab</sup>	0.45 <sup>ab</sup>	0.50 <sup>ab</sup>	0.52 <sup>ab</sup>	0.41 <sup>ab</sup>	0.34 <sup>ab</sup>	0.29 <sup>b</sup>	0.25 <sup>b</sup>	0.24 <sup>b</sup>	0.073	0.001	0.025
SFA	73.51	70.81	0.656	0.001	73.50 <sup>a</sup>	72.28 <sup>ab</sup>	71.88 <sup>b</sup>	70.31 <sup>ab</sup>	71.77 <sup>ab</sup>	71.42 <sup>ab</sup>	71.18 <sup>ab</sup>	72.26 <sup>ab</sup>	72.91 <sup>ab</sup>	73.09 <sup>ab</sup>	71.18 <sup>ab</sup>	0.919	0.038	0.796
MUFA	24.02	26.05	0.690	0.004	23.99 <sup>a</sup>	25.00 <sup>ab</sup>	25.46 <sup>ab</sup>	26.71 <sup>b</sup>	25.29 <sup>ab</sup>	25.42 <sup>ab</sup>	25.86 <sup>ab</sup>	24.98 <sup>ab</sup>	24.28 <sup>ab</sup>	24.23 <sup>ab</sup>	24.16 <sup>ab</sup>	0.911	0.048	0.720
PUFA	2.94	3.33	0.155	0.015	2.84 <sup>a</sup>	3.04 <sup>ab</sup>	2.99 <sup>ab</sup>	3.31 <sup>ab</sup>	3.27 <sup>ab</sup>	3.51 <sup>b</sup>	3.28 <sup>ab</sup>	3.10 <sup>ab</sup>	3.14 <sup>ab</sup>	3.05 <sup>ab</sup>	2.96 <sup>ab</sup>	0.290	0.017	0.292

SFA=sum of saturated fatty acids, MUFA=sum of monounsaturated fatty acids, PUFA=sum of polyunsaturated fatty acids, SEM=Standard error of the mean, <sup>1</sup>Percentages of fatty acids in milk fat (g/100 g fat, a, b, c: Different superscripts in a row denote significant differences (P<0.05) between different sampling points



present study. The contents of SFA were not found to evolve during the time of the trial, a result which differ to that reported by Zoidis et al., (2018) who found that terpenes oral administration on goats increased SFA contents. Moreover, MUFA contents in the present study were determined at a higher concentration for T-group compared to the control group of animals, which is in agreement to the findings of Revello-Chion et al., (2010). They found higher concentration of MUFA in cow's milk originating from summer pastures, and they associated that result with the presence of terpenes in the pasture. These researchers attributed the modifications in milk FA profile to the feed the animals consumed, while they also associated the increased supply of unsaturated FA in the ruminant's diet to an incomplete biohydrogenation in the rumen and a higher concentration of unsaturated FA in the animal products.

## CONCLUSION

In conclusion, the results of the present study show that terpenes administration can modify coagulation properties of sheep milk as well as the profile of FA in milk. Moreover, the present research findings revealed that treatment and time can modify the final FA concentrations. However, further research, with higher animal numbers, is needed in order to verify the present results and investigate the extent at which those correlations are present in products originated from e.g. mountain pastures. Thus, the determination of terpenes' mode of action in the rumen and/or the extent at which they affect animal metabolism and/or altering rumen microbial environment will be of great importance in order to achieve a better overview of the ways that the profile of FA in the final product can be modified.

## DISCLOSURE OF INTEREST

The authors report no conflict of interest.

### Author's contribution

In this research, all authors contributed effectively. Ioanna Pouloupoulou performed experiments, analyzed the data and wrote the paper; Evangelos Zoidis, Styliani Avramidou, Theofilaktos Massouras designed the experiment, performed laboratory analysis, performed data interpretation, revised manuscript and Ioannis Hadjigeorgiou supervised the project and revised the manuscript.

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