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ORIGINAL ARTICLE

The effect of dietary *Chlorella vulgaris* inclusion on goat's milk chemical composition, fatty acids profile and enzymes activities related to oxidation

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Summary

The impact of dietary supplementation with microalgae on goat's milk chemical composition, fatty acids (FA) profile and enzymes activities related to antioxidant mechanism has not been well documented. Thus, this study aimed to investigate the effects of dietary inclusion of *Chlorella vulgaris* on the following: (i) milk yield, chemical composition and FA profile, (ii) the activities of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione transferase (GST) and glutathione peroxidase (GSH-Px) in blood plasma and (iii) the activities of SOD, GR and lactoperoxidase (LPO) in milk of goats. Furthermore, the oxidative stress indicators for measuring total antioxidant and free radical scavenging activity [ferric reducing ability of plasma (FRAP) and 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays] and oxidative stress biomarkers [malondialdehyde (MDA) and protein carbonyls (PC)] were also determined in blood plasma and milk of the animals. For this purpose, 16 cross-bred goats were divided into two homogenous groups. Each goat of both groups was fed individually with alfalfa hay and concentrates separately. The concentrates of the control group (Control) had no microalgae, while those of the Chlorella group were supplemented with 10 g lyophilized *Chlorella vulgaris*/kg concentrates (Chlorella). Thus, the average intake was 5.15 g *Chlorella vulgaris*/kg DM. The results showed that the dietary inclusion of *Chlorella vulgaris* had not noticeable impact on goat's milk yield, chemical composition and FA profile. Significantly higher SOD (by 10.31%) and CAT (by 18.66%) activities in the blood plasma of goats fed with *Chlorella vulgaris* compared with the control were found. Moreover, the dietary supplementation with *Chlorella vulgaris* caused a significant increase in SOD (by 68.84%) activity and a reduction in PC (by 24.07%) content in goat's milk. In conclusion, the *Chlorella vulgaris* inclusion in goat's diets improved the antioxidant status of both animals and milk.

Keywords microalgae, antioxidant, dairy ruminants, caprine milk

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Introduction

Microalgae, despite being more popular for biofuel production (Medipally et al., 2015), have gained significant attention as a source of biomolecules such as n-3 polyunsaturated fatty acids (PUFA) (Lum et al., 2013; Yaakob et al., 2015). Thus, the inclusion of microalgae in ruminant's diets has been shown as an effective nutritional strategy to enrich cow's (Glover et al., 2012) and goat's (Kouřimská et al., 2014) milk with PUFA.

Microalgae contain also natural antioxidant compounds such as phenols, flavonoids, carotenoids and chlorophyll (Goiris et al., 2012; Lum et al., 2013; Yaakob et al., 2015) which enhance the antioxidant defence system. Indeed, an improvement in the antioxidant status of humans (Panahi et al., 2013), mice (Aliahmat et al., 2012) and fattening lambs

(EL-Sabagh et al., 2014) has been observed when their diets supplemented with microalgae. Among microalgae, *Chlorella vulgaris* is one of the most popular and its cultivation is easy and relatively cheap, while recently has been proven its high antioxidant capacity (Goiris et al., 2012).

In ruminants, several environmental, physiological and dietary factors can cause an imbalance between reactive oxygen species (ROS) production and neutralizing capacity of antioxidant mechanisms which lead to oxidative stress (Sies, 1991). The inclusion of feedstuffs in animal's diets rich in natural antioxidant compounds could protect them from possible oxidative stress and satisfy consumer concerns about safety. The oxidative stress is faced normally by the body with a wide range of antioxidant mechanisms that can be divided into enzymatic and non-enzymatic (e.g. metabolites) (Ye et al., 2015). Several endogenous enzymes such as superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT) glutathione transferase (GST) and glutathione peroxidase (GSH-Px) (Miller et al., 1993; Board and Menon, 2013), found both in blood and milk, represent the main components of the intracellular antioxidant defence mechanisms which regulate ROS accumulation within tissues (Celi, 2010; Sordillo, 2013). Further to that, the enzyme lactoperoxidase (LPO) in milk is related to the oxidation of lipids (O'Connor and O'Brien, 2006). To the best of our knowledge, there is no information available about the impact of microalgae to the antioxidant defence system of dairy ruminants. Research is also needed to define the effects of dietary supplementation with microalgae on the antioxidant status of milk.

Thus, the objective of this study was to investigate the effects of dietary inclusion of *Chlorella vulgaris* on the following: (i) milk yield, chemical composition and fatty acids profile, (ii) the activities of SOD, CAT, GR, GST and GSH-Px in blood plasma and (iii) the activities of SOD, GR and LPO in goat's milk. Furthermore, the oxidative stress indicators for measuring total antioxidant and free radical scavenging activity [ferric reducing ability of plasma (FRAP) and 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays] and oxidative stress biomarkers [malondialdehyde (MDA) and protein carbonyls (PC)] were also determined in blood plasma and milk of the animals.

Materials and methods

Sixteen cross-bred dairy goats, at 90–98 days in milk, of comparable age (3–4 years old) and bodyweight (43.0 ± 2.3 kg) were maintained at the Agricultural University of Athens. Housing and care of the animals conformed to Ethical Committee guidelines of Faculty of Animal Science. The animals were divided into two homogeneous subgroups, each (n = 8), balanced for bodyweight (BW) and milk yield. Throughout the experimental period, each animal of each group was fed individually according to its energy and crude protein requirements (National Academic Press, 1981). An adaptation period of two weeks was performed between 94 and 108 days in milk where the animals were fed with the rations of the main experimental period. More specifically, the goats of both groups were fed with a ration consisted of alfalfa hay and concentrates (forage/concentrate = 53/47). The forage was provided separately from the concentrates, while both of them were offered to the animals twice a day (in two equal parts at 0800 and 1600 h). The concentrate of the control group (control) had no microalgae, while that of the treated group (Chlorella) was supplemented with 10 g lyophilized *Chlorella vulgaris*/kg concentrate. The concentrate diets were prepared every week and were formulated to be isoenergetic and isoproteic (Table 1). *Chlorella vulgaris* was added in 10 g/kg concentrate and had a negligible effect on the energy and protein content of the diet (Table 1). The quantities of feed offered to the animals were adjusted weekly according to their individual requirements based on their BW and fat-corrected milk yield. The average daily intake of alfalfa hay and concentrates was 1.4 and 1.2 kg/animal, respectively, and the average daily intake of nutrients is shown in Table 1. Diet selectivity did not occur, and no refusals of forage and/or concentrates were observed. The concentrate (g/kg as fed) consisted of maize grain, 340; barley grain, 380; soybean meal, 150; wheat middlings, 110; calcium phosphate, 15; salt, 3; mineral and vitamin premix, 2. The mineral and vitamin premix contained (per kg as mixed) the following: 150 g Ca, 100 g P, 100 g Na, 100 mg Co, 300 mg I, 5000 mg Fe, 10 000 mg Mn, 20 000 mg Zn, 100 000 mg Se, 5 000 000 IU retinol, 500 000 IU cholecalciferol and 15 000 mg α-tocopherol. The whole experimental period lasted 30 days. All animals had free access to freshwater.

Collection of samples

Animals were milked twice a day at 8 am and 6 pm by a milking machine. Individual milk samples were collected at 0, 7, 14, 21 and 28 days for chemical composition analysis and at 14 and 28 days from the beginning of the experiment for fatty acids and enzymes analysis, after mixing two samples, each one

Table 1 The chemical composition and the main fatty acids (FA) of alfalfa hay, *Chlorella vulgaris* and concentrates (Control vs. Chlorella) and the mean daily nutrients, FA and minerals intake from forage and concentrates

	Alfalfa hay	*Chlorella vulgaris*	Concentrates	
			Control	Chlorella
Chemical composition (g/kg)				
DM	879	927	910	916
OM	775	848	862	868
CP	145	677	151	155
EE	11.7	10.5	23	24
NDF	596	128	246	256
ADF	365	42	69	70
Fatty acids (% of total FA)				
$C_{16:0}$	35.50	37.10	16.70	16.63
$C_{18:0}$	7.11	1.35	3.02	2.91
cis-9 $C_{18:1}$	0.99	2.43	29.51	30.45
$C_{18:2n\text{-}6c}$	32.75	22.04	47.12	45.71
$C_{18:3n\text{-}3}$	1.15	22.10	2.13	2.09
Nutrients intake (g/animal/day)	Control diet		Chlorella diet	
DM	2323		2330	
OM	2120		2126	
CP	384		389	
EE	44		45	
NDF	1129		1142	
ADF	594		595	
NEL (MJ)	13.85		13.86	
Fatty acids intake (g/animal/day)				
$C_{16:0}$	9.67		10.09	
$C_{18:0}$	1.83		1.84	
cis-9 $C_{18:1}$	9.78		10.23	
$C_{18:2n\text{-}6c}$	19.31		19.36	
$C_{18:3n\text{-}3}$	0.83		1.10	
Minerals intake (mg/animal/day)				
Se	0.32		0.35	
Zn	144.00		130.00	
Cu	18.40		18.10	
Fe	714.00		702	
Mn	150.00		152.00	
Mo	3.80		3.70	
As	0.27		0.23	
Co	1.28		1.30	
Ni	6.53		6.50	
Sb	0.32		0.41	
Pb	0.74		0.73	

composed of 5% of the volume of milk produced during the morning and evening milking respectively. Blood samples were taken at the 14 and 28 days from the beginning of the experiment, for enzymes determination, from jugular vein into EDTA-containing tubes and subsequently centrifuged at 2700 g for 15 min to separate plasma from the cells. Both milk and blood samples were stored at −80 °C prior to analysis.

Analyses of samples

Feed samples

Individual samples from alfalfa hay, concentrates and *Chlorella vulgaris* were taken at the beginning of the experiment. The feed samples were analysed for organic matter (OM; Official Method 7.009), dry matter (DM; Official Method 7.007) crude protein (CP; Official Method 7.016) and ether extract (EE; Official Method 7.060) according to Association of Official Analytical Chemists (1984) and for neutral detergent fibre (NDF) assayed without a heat stable amylase and acid detergent fibre (ADF) expressed exclusive of residual ash according to Van Soest et al. (1991). Additionally, the feed samples were analysed for fatty acids (FA) profile according to the method of O' Fallon et al. (2007) (Table 1).

Chlorella vulgaris antioxidant capacity

Extract preparation of Chlorella vulgaris. *Chlorella vulgaris* cultivation took place in photobioreactor at the Laboratory of Molecular Biology of the Agricultural University of Athens. Two hundred mg of lyophilized *Chlorella vulgaris* biomass was grounded with 2 ml of ethanol/water (3:1) and liquid nitrogen using pestle and mortar and left for 30 minutes at dark. After centrifuge at 2000 *g* for 10 min, cells were again grounded for a second time as previously. The two extracts were then pooled into one. Three technical replicates were made for each sample. The extracts were then stored at −20 °C. With this protocol, we aimed to extract both apolar and polar compounds and total phenolic content (Goiris et al., 2012).

Protein content assay in Chlorella vulgaris. In order to estimate the protein content of each lyophilized *Chlorella vulgaris* sample, so to equilibrate the samples accordingly, Bradford photometric assay was used (Bradford, 1976). More specifically, a Bradford dense solution was prepared [100 mg Coomassie Brilliant Blue G-250, 100 ml phosphoric acid (85%w/w), 100 ml ethanol (50% v/v)] and diluted four times with distilled water. Fifty μl of each sample and 950 μl of Bradford solution were used to monitor the absorbance at 595 nm (Hitachi, U-1100 spectrophotometer, Tokyo, Japan). Bovine serum albumin (Promega, Madison, WI, USA) was used as standard (Bradford, 1976).

Ferric reducing antioxidant power (FRAP) assay in Chlorella vulgaris samples. Antioxidant compounds were monitored by using the FRAP assay (Benzie and Strain,

1996). More specifically, three solutions of 300 mM CH_3COONa, 10 mM TPTZ (2,4,6-tripyridyl-S-triazine) (Sigma-Aldrich, St. Louis, MO, USA) and 20 mM $FeCl_3$ were prepared and mixed 10:1:1 respectively. Nine hundred eighty μl of FRAP solution and 20 μl of each extract were mixed and left at 37 °C for 4 min. Absorbance was measured at 593 nm (Hitachi, U-1100 spectrophotometer). Dilutions of the samples were made accordingly. Ascorbic acid was used as standard, and samples were equilibrated according to protein content.

Phenolic content of Chlorella vulgaris samples. Phenolic content was estimated by the Folin-Ciocalteu protocol (Hajimahmoodi et al., 2010). Folin solution (Sigma-Aldrich) was diluted with distilled water 1:10 (0.2 M final concentration). Fifty μl of each sample was mixed with 450 μl distilled water and added to 2.5 ml diluted Folin solution. Vigorous vortex was applied for at least 3 min before the addition of 2 ml Na_2CO_3 (75 g/l). Absorbance was measured at 765 nm (Hitachi, U-1100 spectrophotometer) after incubation at 30 °C with vigorous shaking for 90 min. Gallic acid was used as internal standard. Samples were equilibrated according to protein content.

Milk chemical composition. Milk was analysed for fat, protein and lactose by IR spectrometry (Milkoscan 133; Foss Electric, Hllerod, Demark), after calibration according to Gerber (British Standards Institution, 1955) and Kjeldahl (International Dairy Federation, 1993).

FA determination

Milk samples were analysed for FA according to the method of Nourooz-Zadeh and Appelqvist (1998). For the determination of FA profile, an Agilent 6890 N gas chromatograph equipped with an HP-88 capillary column (60 m × 0.25 mm i.d. with 0.20 μm film thickness, Agilent) and a flame ionization detector was used. A flame ionization detector (FID) temperature was set at 260 °C and the chromatographic analysis involved a temperature programmed run starting at 120 °C and held for 1 min. Then, followed by two step-up ramp, one of 1.25 °C/ min to 230 °C, and another of 10 °C/ min to 240 °C, and held for 3 min. Helium was used as the carrier gas with a linear velocity set at 30 cm/s. Each peak was identified and quantified using a 37 component FAME mix standard (Supelco; Sigma-Aldrich). Extra standards were used for the *cis-9, trans-11* C18:2, *trans-10, cis-12* C18:2 and *trans-11* C18:1 FA (Sigma-Adrich, St. Louis, MO, USA). A tricosanoate ($C_{23:0}$) was used as an internal standard for the chromatographic analysis (Fluka, Sigma, Aldrich) based on the fact that has been proposed as appropriate standard for milk FA quantification (Stolyhwo, 2007; Simionato et al., 2010; Scerra et al., 2016). Additionally, to avoid any discrepancy caused by possible co-elution of *cis-9, trans-11* $C_{18:2}$ with other conjugated linoleic acids, such as *trans-7, cis-9* $C_{18:2}$ and *trans-8, cis-10* $C_{18:2}$, it was considered that the *cis-9, trans-11* $C_{18:2}$ is the main isomer since predominates between 75 and 90% of the total conjugated linoleic acid isomers in milk (Lin et al., 1995; Precht and Molkentin, 1995). Moreover, to restrict any confounding which occurs by possible co-elution between *trans-11* $C_{18:1}$ and *trans-10* $C_{18:1}$ FAs, it was regarded that the *trans-11* $C_{18:1}$ is the principal isomer since accounts between 68 and 86% of the total *trans* $C_{18:1}$ isomers in milk (Stolyhwo, 2007).

Enzyme assays in blood plasma and milk

Glutathione transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (97%, Sigma-Aldrich) as substrate by monitoring the formation of the conjugate of each substrate and reduced glutathione (GSH) according to Labrou et al. (2001). Glutathione reductase (GR) activity was measured according to Mavis and Stellwagen (1968). Catalase (CAT) activity was assessed using continuous spectrophotometric rate for the determination of H_2O_2 at 240 nm, based on Beers and Sizer (1952) and Stern (1937). Lactoperoxidase activity was measured using ABTS [2, 2′-azino-bis (3 ethylbenzthiazoline-6-sulphonic acid)] as a substrate according to Keesey (1987) and Pütter and Becker (1983). SOD activity was assayed using a modified method of McCord and Fridovich (1969). One unit will inhibit the rate of reduction in cytochrome c by 50% in a coupled system, using xanthine and xanthine oxidase at pH 7.8. The xanthine oxidase concentration should produce an initial (uninhibited) $\Delta A_{550\ nm}$ of 0.025 ± 0.005 per minute.

Antioxidant and free radical scavenging activities in blood plasma and milk. Ferric reducing antioxidant power (FRAP) assay was used to measure total antioxidant potential according to Benzie and Strain (1996) in blood plasma, while the ABTS radical scavenging capacity assay was based on the published methods (Pellegrini et al., 2003; Li et al., 2011). The same

protocols for FRAP and ABTS determination were used in milk samples with some modifications. In detail, 1 ml of the fresh milk was added to 10 ml of HC1 (1 N)/95% ethanol (v/v, 15/85) solution in 50-ml brown bottles and samples were shaken for 1 h at 30 °C in a rotary shaker set at 300 rpm. The mixture was then centrifuged at 7800 *g* at 5 °C for 15 min. The supernatant fluids were kept at −20 °C in the dark until further analysis of FRAP and ABTS.

Lipid peroxidation activity and protein carbonyl determination in blood plasma and milk. Lipid peroxidation was assayed by measuring malondialdehyde (MDA) according to the method of Heath and Packer (1968). Protein carbonyls (PC) were determined based on a published method (Patsoukis et al., 2004). Moreover, protein concentration was determined by the Bradford assay using bovine serum albumin (fraction V) as standard (Bradford, 1976).

Statistical analysis

A repeated-measures General Linear Model (GLM) was applied to the data for fatty acids profile in milk, enzymes activities and oxidative stress indicators in both milk and blood plasma considering the sampling day as repeated measure, with fixed effects of dietary treatments (D) (Control vs. Chlorella), sampling day (S) (14, 28 days) and the interactions among them (D × S) according to the model:

$$Y_{ijk} = \mu + D_i + S_j + (D \times S)_{ij} + A_k + e_{ijk}$$

where Y_{ijk} is the dependent variable, μ the overall mean, D_i the effect of dietary treatment ($i = 2$), S_j the effect of sampling day ($j = 2$), $(D \times S)_{ij}$ the interaction between dietary treatment and sampling day, A_k the animal's random effect and e_{ijk} the residual error. Moreover, data for milk yield and its chemical composition were analysed using a repeated-measures GLM considering the sampling day (7, 14, 21, 28 days) as repeated measure, with fixed effects of dietary treatment, sampling day and the interactions among them, goat as random effect and data recorded at the beginning of the experiment as a covariate. Kolmogorov–Smirnov test revealed that all variables followed a normal distribution. The other assumptions of repeated-measures ANOVA, such as equality of covariance matrices across groups, sphericity and homogeneity of variances were checked using Box'M, Mauchly's and Levene's tests respectively. Post hoc analyses were performed when appropriate using Tukey's multiple range test. For all tests, the significance was set at 0.05. Statistical analysis was performed using the statistical package SPSS (version 16.0).

Results and discussion

Milk yield and its chemical composition

The *Chlorella vulgaris* inclusion in goat's diets did not affect their milk yield and chemical composition (Table 2). The same has been found in goats by Moreno-Indias et al. (2014) when the animals fed 5 g *Chlorella pyrenoidosa* per day. On the contrary, Póti et al. (2015) observed a significant increase in the milk fat of goats fed with 10 g *Chlorella kessleri*/ kg DM intake compared with the control, despite the fact that the chemical composition of the two dietary treatments was the same.

Milk fatty acids profile

The dietary supplementation with *Chlorella vulgaris* had no noticeable impact on goat's milk FA profile (Table 3) although the inclusion of microalgae in ruminants diets usually enhances the PUFA content

Table 2 Mean milk yield (g/day) and chemical composition (%) of goats fed with the two diets (Control vs. Chlorella) at four sampling days

	Diets (D)		Sampling days (S)†					Effects		
	Control	Chlorella	7	14	21	28	RMSE†	D	S	D × S
Milk yield	1771	1767	1800^{ab}	1829^{b}	1733^{ab}	1712^{a}	102.19	NS	**	NS
Fat	3.26	3.60	3.43	3.38	3.54	3.36	0.148	NS	NS	NS
Protein	2.80	2.93	2.90^{b}	2.85^{ab}	2.90^{b}	2.80^{a}	0.063	NS	**	NS
Lactose	4.56	4.63	4.54^{a}	4.56^{a}	4.58^{ab}	4.70^{b}	0.053	NS	**	NS

NS, No significance.

*p < 0.05, **p < 0.01, ***p < 0.001.

Means with different superscript (a, b) in each row (between sampling days) for each parameter differ significantly ($p \leq 0.05$).

†RMSE, Root-mean-square error.

Table 3 The mean individual fatty acids (FA) (% of total FA), FA groups, SFA/UFA ratio, AI and Δ^{-9} desaturate indexes of goat's milk fed with the two diets (Control vs. Chlorella) at two sampling days

	Diets (D)		Sampling days (S)		Diets × Sampling days (D × S)					Effects		
	Control	Chlorella	14	28	Control 14	Control 28	Chlorella 14	Chlorella 28	RMSE	D	S	D × S
$C_{4:0}$	2.62	2.59	2.79	2.42	2.78^{b}	2.45^{a}	2.80^{b}	2.38^{a}	0.104	NS	***	NS
$C_{6:0}$	2.82	2.69	2.82	2.69	2.91^{b}	2.73^{a}	2.74^{ab}	2.65^{a}	0.105	NS	**	NS
$C_{8:0}$	3.19	3.16	3.24	3.11	3.40	2.97	3.07	3.25	0.270	NS	NS	NS
$C_{10:0}$	11.83	11.37	11.36	11.83	11.63^{b}	12.03^{b}	11.09^{a}	11.64^{b}	0.375	NS	***	NS
$C_{11:0}$	0.23	0.24	0.23	0.24	0.23	0.23	0.23	0.25	0.023	NS	NS	NS
$C_{12:0}$	4.74	4.35	4.39	4.71	4.57^{ab}	4.91^{b}	4.21^{a}	4.50^{ab}	0.176	NS	**	NS
$C_{13:0}$	0.08	0.12	0.09	0.11	0.08	0.09	0.11	0.12	0.026	NS	NS	NS
$C_{14:0}$	10.34	10.46	10.22	10.57	10.15	10.54	10.30	10.61	0.285	NS	*	NS
$C_{14:1}$	0.38	0.35	0.37	0.36	0.38	0.38	0.36	0.34	0.030	NS	NS	NS
$C_{15:0}$	1.03	0.98	1.02	0.98	1.03	1.02	1.01	0.95	0.046	NS	NS	NS
$C_{15:1}$	0.27	0.24	0.26	0.25	0.26^{ab}	0.28^{b}	0.26^{ab}	0.23^{a}	0.014	NS	NS	*
$C_{16:0}$	28.40	32.42	30.43	30.39	28.13^{a}	28.67^{ab}	32.74^{c}	32.11^{bc}	1.164	*	NS	NS
$C_{16:1}$	0.43	0.37	0.39	0.41	0.41^{ab}	0.46^{b}	0.38^{a}	0.37^{a}	0.024	NS	NS	NS
$C_{17:0}$	0.58	0.56	0.58	0.56	0.58	0.58	0.58	0.54	0.023	NS	NS	NS
$C_{17:1}$	0.22	0.21	0.23	0.20	0.23^{b}	0.21^{ab}	0.23^{b}	0.19^{a}	0.012	NS	***	*
$C_{18:0}$	9.03	8.40	8.80	8.63	9.06	9.00	8.54	8.26	0.665	NS	NS	NS
trans $C_{18:1}$†	0.50	0.38	0.43	0.45	0.52^{b}	0.49^{b}	0.35^{a}	0.41^{ab}	0.035	*	NS	*
trans-11 $C_{18:1}$	1.56	1.02	1.32	1.26	1.66^{b}	1.46^{ab}	0.98^{a}	1.06^{ab}	0.145	*	NS	*
cis-9 $C_{18:1}$	17.35	16.30	16.92	16.73	17.49	17.21	16.35	16.25	0.816	NS	NS	NS
$C_{18:2n\text{-}6t}$	0.30	0.33	0.28	0.25	0.32	0.28	0.29	0.30	0.017	NS	*	NS
$C_{18:2n\text{-}6c}$	2.46	2.18	2.30	2.34	2.49	2.44	2.11	2.25	0.238	NS	NS	NS
cis-9, trans-11 $C_{18:2}$	0.72	0.53	0.63	0.62	0.76^{b}	0.67^{ab}	0.49^{a}	0.56^{ab}	0.063	*	NS	NS
$C_{18:3n\text{-}3}$	0.60	0.53	0.56	0.57	0.62	0.58	0.50	0.56	0.057	NS	NS	*
$C_{20:0}$	0.12	0.11	0.12	0.11	0.12	0.12	0.11	0.11	0.005	NS	NS	NS
$C_{20:3n\text{-}3}$	0.22	0.21	0.22	0.20	0.23	0.21	0.22	0.20	0.011	NS	**	NS
SCFA‡	20.68	20.06	20.44	20.30	20.95	20.41	19.94	20.18	0.751	NS	NS	NS
MCFA§	44.59	48.33	46.16	46.76	43.94^{a}	45.23^{b}	48.38^{b}	48.28^{b}	1.279	NS	NS	NS
LCFA¶	9.73	9.07	9.49	9.31	9.76	9.70	9.23	8.91	0.685	NS	NS	NS
MUFA**	20.71	18.87	19.92	19.67	20.93	20.49	18.90	18.85	0.813	NS	NS	NS
PUFA††	4.29	3.66	3.98	3.97	4.41	4.17	3.55	3.78	0.351	NS	NS	NS
SFA‡‡	75.00	77.46	76.10	76.36	74.66^{a}	75.35^{ab}	77.55^{b}	77.37^{ab}	0.929	NS	NS	NS
UFA§§	25.00	22.54	23.90	23.64	25.34^{a}	24.65^{ab}	22.45^{b}	22.63^{ab}	0.929	NS	NS	NS
SFA/UFA¶¶	3.04	3.47	3.23	3.28	2.97^{a}	3.10^{ab}	3.49^{b}	3.45^{ab}	0.170	NS	NS	NS
AI***	3.02	3.51	3.21	3.32	2.92^{a}	3.13^{ab}	3.51^{b}	3.51^{b}	0.181	NS	NS	NS
Δ^{-9} desaturase indexes												
$C_{14:1}$ / $C_{14:0}$	0.37	0.34	0.36	0.34	0.37	0.36	0.35	0.32	0.003	NS	NS	NS
$C_{16:1}$ / $C_{16:0}$	0.016	0.012	0.013	0.014	0.015^{ab}	0.016^{b}	0.012^{a}	0.011^{a}	0.001	*	NS	NS
cis-9 $C_{18:1}$ / $C_{18:0}$	1.94	2.03	1.98	1.99	1.95	1.93	2.02	2.05	0.170	NS	NS	NS
cis-9, trans-11 $C_{18:2}$/ *trans-11* $C_{18:1}$	0.47	0.52	0.49	0.50	0.47	0.47	0.51	0.53	0.042	NS	NS	NS

RMSE, Root-mean-square error; NS, No significance.

*p < 0.05, **p < 0.01, ***p < 0.001.

Means with different superscripts (a, b, c) in each row for the interaction means of each fatty acid differ significantly ($p \leq 0.05$)

†trans $C_{18:1}$ = trans-6 $C_{18:1}$ + trans-7 $C_{18:1}$ + trans-8 $C_{18:1}$ + trans-9 $C_{18:1}$

‡SCFA: Short-chain saturated fatty acids = $C_{4:0}$ + $C_{6:0}$ + $C_{8:0}$ + $C_{10:0}$ + $C_{11:0}$,

§MCFA: Medium-chain saturated fatty acids = $C_{12:0}$ + $C_{13:0}$ + $C_{14:0}$ + $C_{15:0}$ + $C_{16:0}$

¶LCFA: Long-chain saturated fatty acids = $C_{18:0}$ + $C_{20:0}$

**MUFA: Mono-unsaturated fatty acids = $C_{14:1}$ + $C_{15:1}$ + $C_{16:1}$ + $C_{17:1}$ + cis-9 $C_{18:1}$ + trans $C_{18:1}$ + trans-11$C_{18:1}$

††PUFA: Poly-unsaturated fatty acids = cis-9, trans-11 $C_{18:2}$ (CLA) + $C_{18:2n\text{-}6c}$ + $C_{18:2n\text{-}6t}$ + $C_{18:3n\text{-}3}$ + $C_{20:3n\text{-}3}$

‡‡SFA: Saturated fatty acids = SCFA + MCFA + LCFA

§§UFA: Unsaturated fatty acids = PUFA + MUFA

¶¶SFA/ UFA: Saturated/unsaturated = (SCFA + MCFA + LCFA)/(PUFA + MUFA)

***AI: Atherogenicity index = ($C_{12:0}$ + 4 × $C_{14:0}$ + $C_{16:0}$) / (PUFA + MUFA)

in their milk (Glover et al., 2012; Kouřimská et al., 2014).

However, exceptions were the proportions of *trans* $C_{18:1}$ (*trans-6* $C_{18:1}$ + *trans-7* $C_{18:1}$ + *trans-8* $C_{18:1}$ + *trans-9* $C_{18:1}$), trans-*11* $C_{18:1}$, and *cis-9, trans-11* $C_{18:2}$ which were significantly reduced, while that of $C_{16:0}$ was increased in milk fat of Chlorella goats compared with the control (Table 3). The negligible effect of *Chlorella vulgaris* on milk FA profile is likely due to the low ether extract content of *Chlorella vulgaris* which was used in this study. Indeed, only small differences were seen on the ether extract content and FA profile among the two dietary treatments (Table 1). In accordance with our findings, no differences in goat's milk FA profile were found by Moreno-Indias et al. (2014) when the animals fed with a diet supplemented daily with 5 g *Chlorella pyrenoidosa,* but no information was given about its FA profile. On the other hand, Kouřimská et al. (2014) observed a significant reduction in the $C_{16:0}$ and an increase in the *cis-9* $C_{18:1}$, $C_{18:2n-6c}$ and $C_{18:3n-3}$ content in the milk fat of goats fed with a diet supplemented with 10 g *Chlorella vulgaris* contained (% of total FAs): $C_{16:0}$ (14.42), $C_{18:0}$ (1.57), *cis-9* $C_{18:1}$ (17.62), $C_{18:2n-6c}$ (11.97), $C_{18:3n-3}$ (15.79) as main FAs. Regardless the FA profile of *Chlorella vulgaris* which were used in the study of Kouřimská et al. (2014), no information was provided about the ether extract that it contains. Significantly higher concentrations of trans-*11* $C_{18:1}$, $C_{18:2n-6c}$, $C_{18:3n-3}$, $C_{20:3n-3}$, $C_{20:4n-6}$ $C_{20:5n-3}$, $C_{22:6n-3}$, mono unsaturated FA, polyunsaturated FA and n-3 FA were found by Póti et al. (2015) in the milk fat of goats fed with a diet supplemented with 10 g *Chlorella kessleri*/ Kg DM intake even no differences existed on the FA profile among the dietary treatments used. Moreover, the main FAs (% of total FAs) of *Chlorella* kessleri used in the study of Póti et al. (2015) were the following: $C_{16:0}$ (21.3), $C_{18:0}$ (1.0), *cis-9* $C_{18:1}$ (11.2), $C_{18:2n-6c}$ (14.3), $C_{18:3n-3}$ (37.4). Finally, significant interactions between diets (D) and sampling days (S) for the $C_{15:1}$, $C_{17:1}$, *trans* $C_{18:1}$ (*trans-6* $C_{18:1}$ + *trans-7* $C_{18:1}$ + *trans-8* $C_{18:1}$ + *trans-9* $C_{18:1}$), trans-*11* $C_{18:1}$, *cis-9, trans-11* $C_{18:2}$ and C18:3n-3 FA in goat's milk were found (Table 3).

Antioxidants in blood plasma and milk

The inclusion of *Chlorella vulgaris* in goat's diet resulted a significant increase in SOD (by 10.31%) and CAT (by 18.66%) activities in their blood plasma (Table 4). SOD is the first enzyme involved in the conversion of oxygen radicals to peroxides, while CAT is involved in the second step of removing these peroxides and converting them into O_2 (Yu, 1994). The enhancement in the antioxidant defence mechanism of goats, fed the Chlorella diet, may be explained by the presence of antioxidant compounds in *Chlorella vulgaris.* Indeed, the total antioxidant capacity (measured by FRAP assay) and the phenolic content of *Chlorella vulgaris,* which was used in this study, were

Table 4 Enzymes activities (units/ml), total antioxidant capacity, malondialdehyde (μM TMP) and protein carbonyls (nmol/mg protein) content in blood plasma of goats fed the two diets (Control vs. Chlorella) at two sampling days

	Diets (D)		Sampling days (S)		Diets × Sampling days (D × S)					Effects		
	Control	Chlorella	14	28	Control 14	Control 28	Chlorella 14	Chlorella28	RMSE	D	S	D × S
Superoxide dismutase	9.99	11.02	9.81	11.20	10.00^{a}	9.99^{a}	9.62^{a}	12.41^{b}	0.302	*	***	***
Catalase	80.53	95.56	78.49	97.60	72.53^{a}	88.53^{ab}	84.44^{a}	106.67^{b}	4.624	*	**	NS
Glutathione reductase	0.053	0.046	0.051	0.048	0.052	0.054	0.050	0.042	0.004	NS	NS	NS
Glutathione transferase	0.088	0.096	0.093	0.091	0.089	0.086	0.097	0.095	0.004	NS	NS	NS
Glutathione peroxidase	0.20	0.20	0.18	0.23	0.19^{ab}	0.21^{ab}	0.18^{a}	0.23^{b}	0.025	NS	*	NS
Ferric Reducing Ability of Plasma (μmol ascorbic acid)	0.46	0.49	0.42	0.53	0.38^{a}	0.53^{b}	0.45^{ab}	0.54^{b}	0.037	NS	**	NS
2,2′-azino-di(3-ethylbenzthiazoline-6-sulphonic acid) (% inhibition)	25.12	25.10	28.21	22.02	27.17^{bc}	23.07^{ab}	29.25^{c}	20.96^{a}	1.274	NS	***	NS
Malondialdehyde	0.24	0.22	0.23	0.24	0.21	0.23	0.22	0.24	0.020	NS	NS	NS
Protein carbonyls	0.054	0.054	0.053	0.055	0.051	0.057	0.056	0.053	0.008	NS	NS	NS

RMSE, Root-mean-square error; NS, No significance.

*p < 0.05, **p < 0.01, ***p < 0.001.

Means with different superscripts (a, b, c) in each row for the interaction means of each parameter differ significantly (p ≤ 0.05).

Table 5 Enzymes activities (units/ml), total antioxidant capacity, malondialdehyde (μM TMP) and protein carbonyls (nmol/mg protein) content in milk of goats fed the two diets (Control vs. Chlorella) at two sampling days

	Diets (D)		Sampling days (S)		Diets × Sampling days (D × S)					Effects		
	Control	Chlorella	14	28	Control 14	Control 28	Chlorella 14	Chlorella 28	RMSE	D	S	D × S
Superoxide dismutase	29.80	50.30	45.14	34.95	29.51^{a}	30.08^{a}	60.78^{b}	39.82^{ab}	7.366	*	NS	NS
Glutathione reductase	0.05	0.05	0.05	0.05	0.04	0.06	0.06	0.047	0.012	NS	NS	NS
Lactoperoxidase	0.65	0.66	0.66	0.65	0.65	0.64	0.66	0.65	0.033	NS	NS	NS
Ferric Reducing Ability of Plasma (μmol ascorbic acid)	0.53	0.52	0.58	0.47	0.69^{b}	0.37^{a}	0.47^{ab}	0.57^{ab}	0.065	NS	*	**
2,2′-azino-di(3-ethylbenzthiazoline-6-sulphonic acid) (% inhibition)	27.65	28.14	34.25	21.53	33.89^{b}	21.41^{a}	34.62^{b}	21.65^{a}	2.857	NS	**	NS
Malondialdehyde	0.67	0.66	0.66	0.67	0.67	0.66	0.66	0.67	0.031	NS	NS	NS
Protein carbonyls	0.054	0.042	0.050	0.046	0.061^{b}	0.047^{ab}	0.038^{a}	0.045^{ab}	0.005	*	NS	*

RMSE, Root-mean-square error; NS, No significance.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Means with different superscripts (a, b) in each row for the interaction means of each parameter differ significantly ($p \leq 0.05$)

4787 ± 232.1 μM ascorbic acid eq/g dry weight and 0.04 ± 0.001 mg gallic acid eq./ g dry weight respectively. Moreover, other studies have also been suggested that the high antioxidant capacity of *Chlorella vulgaris* is attributed to its content in specific compounds such as polyphenols, carotenoids, chlorophyll, vitamins, β-1,3-glucan and minerals (Wu et al., 2005; Li et al., 2007; Geetha et al., 2010; Goiris et al., 2012). In agreement with our results, a significant increase either in CAT and SOD activities in blood erythrocytes (Lee et al., 2010) or in the total antioxidant measurements in serum (Panahi et al., 2013) of humans were found when their diets supplemented with *Chlorella vulgaris.* Significantly higher GSH-Px activity in the blood erythrocytes of mice, fed with a diet supplemented with *Chlorella vulgaris,* has also been observed (Aliahmat et al., 2012). Accordingly, EL-Sabagh et al. (2014) found significantly higher GSH-Px activity in the blood of fattening lambs supplemented with *Spirulina platensis*. Moreover, significantly higher SOD and GSH-Px activities in broilers meat have been found by Delles et al. (2014) when a commercial algae-based product included in their diets.

In the milk of Chlorella goats, a significant increase in SOD (by 68.84%) activity was found (Table 5). It should be pointed out here that to the best of our knowledge, the impact of microalgae supplementation on the antioxidant enzymes activities of milk has not been studied so far. However, no effect on the total antioxidant capacity (measured by FRAP and/or ABTS assays) in milk of the Chlorella goats, compared with the control, was found (Table 5). The dietary supplementation with DHA-rich microalgae in cow's milk had, also, no effect on its oxidative stability measured with TBARS (thio barbituric acid reactive substances) method as well as on its antioxidant capacity using FRAP assay (Glover et al., 2012). Additionally, feeding cows with a DHA-rich microalgae supplement tended to increase the TBARS in butter derived from their milk (Glover et al., 2012).

Moreover, in this study, the dietary inclusion of *Chlorella vulgaris* further to the increase in SOD activity caused also a significant decline in the PC content in goat's milk (Table 5). Proteins are the molecules most susceptible to oxidative damage in cells because they are often catalysts rather the stoichiometric mediators (Dalle-Donne et al., 2005; Frohnert et al., 2011). Thus, these observations may show commercial importance because oxidation of dairy products reduces their nutritional quality, organoleptic properties and shelf-life stability (Havemose et al., 2006). Besides, the quality of milk should be extended taking into account the amounts of antioxidants that it contains (Castillo et al., 2013; Chauhan et al., 2014).

Conclusions

The inclusion of *Chlorella vulgaris* at 5.15 g/kg DM intake on goat's diets did not affect their milk yield, chemical composition and FA profile, possibly due to its low fat content. However, the respective response of the *Chlorella vulgaris* on SOD and CAT activities in goat's plasma suggests an improvement in the antioxidant defence system of the animals, which may show beneficial health implications. Additionally, the

significantly higher SOD activity and the lower PC content in the milk of Chlorella goats may cause a higher shelf-life stability and better organoleptic characteristics through the reduction in undesirable oxidative reactions.

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