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Abstract

Spirulina (SP) is rich in bioactive compounds (β-carotene, γ-linoleic acid, vitamins, etc.) with antioxidants properties. However, its impact on the oxidative status of ewes' organism and milk, as well as on milk's quality has not been extensively studied. Forty-eight dairy Chios ewes were divided into four homogenous groups (n = 12) and were fed individually. The concentrate of the control group (CON) had no SP, while in the concentrates of the treated groups, SP was added to obtain a daily supply of 5 (SP5), 10 (SP10), and 15 (SP15) g per animal. The milk yield and chemical composition were not affected by the addition of SP. The proportion of short-chain fatty acids was increased in the milk of SP5 and SP10 ewes while those of medium-chain fatty acids

were reduced in the milk of SP10 compared with the CON animals. The SP addition enhanced the proportion of polyunsaturated fatty acids and reduced the thrombogenicity index in milk, while its highest level tended to decrease the milk's atherogenicity index. A rise in the activity of superoxide dismutase (SOD) in the blood plasma of SP-fed ewes was found. The same was observed for the activities of catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione transferase in the blood plasma of SP10, SP15, and SP5 fed ewes respectively. On the contrary, the protein carbonyls content (PC) in ewes' s blood plasma declined by the dietary inclusion of SP. The oxidative stability of ewes' milk improved by the dietary addition of SP as indicated by the rise in the activities of SOD, CAT, and GSH-Px and the total antioxidant capacity (measured by FRAP and ABTS assays) as well. Finally, the highest level of SP caused the sharpest drop in PC content of milk. In conclusion, the highest amount of SP improved ewes' organism oxidative status as well as their milk quality and its oxidative stability.

Keywords: Spirulina, milk, fatty acids, enzymes, antioxidants, ewes

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid; ADFom, acid detergent fibre expressed exclusive of residual ash; aNDFom, neutral detergent fibre assayed with a heat-stable amylase; AI, atherogenicity index; CAT, catalase; CP, crude protein; DM, dry matter; ECM, energy corrected milk yield; FA, fatty acids; $FCM_{6\%}$, fat corrected (6%) milk yield; FRAP, ferric reducing ability of plasma; GR, glutathione reductase; GSH-Px, glutathione peroxidase; GST, glutathione transferase; HPI, health-promoting index; LCFA, long-chain fatty acids; MCFA, medium-chain fatty acids; MDA, malondialdehyde; MUFA, monounsaturated fatty acids; OM, organic matter; PC, protein carbonyls; PUFA, polyunsaturated fatty acids; SCC, somatic cell counts; SCFA, short-chain fatty acids; SFA, saturated fatty acids; SOD, superoxide dismutase; SP, *Spirulina*; TI, thrombogenicity index; UFA, unsaturated fatty acids.

1. Introduction

Over the past decades, targeted nutrition responded to the increasing consumer demands for functional and highly nutritional dairy products. Supplementing ruminant diets with microalgae is a direct way to promote animal health as well as enrich dairy products with bioactive compounds, such as polyunsaturated fatty acids (PUFA) and antioxidants.

Spirulina (SP) is an edible blue-green microalga, a filamentous spiral-shaped cyanobacterium, and is considered as feedstuff with high nutritional potential and has been mentioned as "food of the future". SP contains up to 70% protein and has a remarkably balanced amino acid profile (Holman and Malau-Aduli, 2012). In addition, SP is rich in vitamins, minerals, antioxidants, and γ-linolenic acid, which have well-known health benefits (Howe et al., 2006), while owing to its essential phytochemical properties it is considered a potent immunostimulant (Wu et al., 2016). Interestingly, of the different SP production systems, a second sorting product may arise, which is destined for usage in animal diets. Notwithstanding, high genetic merit dairy animals are susceptible to an oxidative imbalance due to their greater energy level requirements (Wullepit et al., 2009). The supplementation of ruminant diets with microalgae was previously linked with remarkable results regarding the oxidative status of ruminants (Tsiplakou et al., 2017a; Tsiplakou et al., 2017b; Tsiplakou et al., 2018; Mavrommatis et al., 2018; Mavrommatis and Tsiplakou, 2020). For this purpose, the inclusion of SP in ruminant diets is expected not only to be beneficial toward developing dairy products with strong shelf-life longevity but also in fortifying animals' organisms with several beneficial bioactive compounds.

Several studies investigated the effect of SP in ruminant's performance (Kulpys et al., 2009; Bezzera et al., 2010; Shimkiene et al., 2010), productivity (Šimkus et al., 2007; Kulpys et al., 2009; Zhang et al., 2010) and product's quality (Šimkus et al., 2007; Kulpys et al., 2009; Christaki et al.,

2012, Póti et al., 2015; Liang et al., 2020; Manzocchi et al., 2020). More specifically, supplementing 2 g/d of SP to dairy cattle, resulted in greater average milk fat, protein, and lactose (Šimkus et al., 2007; Šimkus et al., 2008) and reduced somatic cells count (Šimkus et al., 2007). Furthermore, Christaki et al. (2012) reported decreased content of saturated fatty acids (SFA) in milk and increased monounsaturated fatty acids (MUFA) and PUFA when 40 g/d of SP were offered to crossbred Holsteins.

However, there is a lack of evidence on which extent the supplementation of different levels of SP would affect ewes' performance, oxidative status, and milk quality. In favor of the abovementioned, up to our knowledge, this is the first study that evaluates the effect of supplementing three different levels of SP in dairy ewes' milk performance, milk FA profile, as well as milk and organism oxidative stability.

2. Materials and methods

2.1. Experimental design and dietary treatments

Forty-eight dairy Chios ewes were divided into 4 homogeneous groups (n = 12) based on body weight (BW; 54.0 ± 6.0 kg), fat corrected (6%) milk yield ($FCM_{6\%}$; 1.85 ± 0.3 kg/d), days in milk (67 ± 8), and age (2 to 4 years old). Ewes were housed at the Research Institute of Animal Science, ELGO-DIMITRA (Giannitsa, Greece; 40°44′ N, 22°27′ E). Housing and care of the animals conformed to Ethical Committee guidelines of the Faculty of Animal Science (EU 63/2010; Council of the European Union 2010).

Animals were kept in a common stall, divided in different blocks for each group and at feeding time they were transferred to individual pens to achieve individual feeding. The ration consisted of alfalfa hay, wheat straw, and concentrate. The forages were provided separately from the concentrates as usually happens in traditional feeding system. The concentrates were prepared

every two weeks and administered twice per day, after milking at 07:00 and 17:00 h (Table 1). Each ewe was fed individually based on its maintenance and lactating requirements and the average amount of the concentrates, alfalfa hay, and wheat straw were 1.5, 1.0, and 0.2 kg/ewe/day, respectively, independently from the groups (Table 1). The concentrates consisted of maize grain, barley, wheat middling, sunflower meal, soybean meal, and mineral and vitamin premix (Table 1). Following an adaptation period of one week, mostly to adapt to the new environment of the individual feeding, ewes were offered concentrates with the inclusion of three different levels of SP. In particular, the concentrate of the control group (CON) had no inclusion of SP, while in the three following groups (SP5, SP10, and SP15) SP was included at the three different levels of 5, 10, and 15 g, per day, respectively (Table 1). Chemical composition (g/kg dry matter; DM) as well as the main fatty acids (g/100 g total fatty acids) of the forages (alfalfa hay and wheat straw), of the concentrate, and of the SP are presented in Table 2. The daily nutrients (g/ewe/day), and main fatty acids (g/ewe/day) intake are presented in Table 3. All the animals had free access to fresh water. The whole experimental period lasted 60 days.

2.2. Sample collection

At the beginning of the trial as well as at every time a new concentrate batch was produced, feed samples from alfalfa hay, wheat straw, concentrate, and SP were collected and were subjected to chemical analysis. Ewes were milked twice per day at 07:00 and 17:00 h by a milking machine. At 0, 15, 30, 45, and 60 experimental days, milk samples were collected individually from each ewe after mixing the evening sample with the morning one, on a 5% volume, for chemical composition analysis. Furthermore, at 15, 30, 45, and 60 days, individual milk samples (n = 192) were collected, stored at - 80°C, and later subjected to FA, antioxidant enzyme activity, antioxidant capacity, and oxidative stress biomarkers analysis. Individual blood samples (n = 192) were

collected at the same intervals from the jugular vein of each ewe after the milking and before feeding time. Approximately 10 mL of whole blood were directly transferred to heparin-containing tubes (170 units heparin; BD Vacutainer, Plymouth, UK). Afterward, the blood samples were centrifuged (SL16R, Thermo Fisher Scientific, Waltham, MA, USA) at 2500 rpm for 15 min at 4°C to separate plasma from the cells. Blood plasma samples were also stored at - 80°C, before FA, antioxidant enzyme activity, antioxidant capacity, and oxidative stress biomarkers analysis.

2.3. Sample analysis

2.3.1. Feed samples

Feed samples were analyzed for dry matter (DM; Official Method 934.01), ash (Official Method 942.05), and ether extract (EE; Official Method 920.39) according to AOAC (1984), and for crude protein (CP; Official Method 988.05) according to AOAC (2001). They were also analyzed for neutral detergent fibre (aNDFom), assayed with a heat-stable amylase and acid detergent fibre (ADFom), expressed exclusive of residual ash according to Van Soest et al. (1991) (Table 2). Samples were also collected for the determination of FA profile according to the method of O' Fallon et al. (2007) (Table 2).

2.3.2. Milk chemical composition

Individual milk samples were analyzed for fat, protein, lactose, and solids-not-fat using infrared spectroscopy (Milkoscan 6000; FOSS, Hillerød, Denmark) following the method 972.16 of AOAC (2012) as well as for somatic cell counts (SCC) using a Fossomatic 400 cell counter (FOSS, Hillerød, Denmark). Fat corrected ($FCM_{6\%}$)- and energy corrected (ECM)-milk yield were calculated using the following formulas:

Fat corrected milk (FCM) in 6%:

$$FCM_{6\%} = (0.28 + 0.12 \times \text{milk fat concentration (\%)}) \times \text{milk yield (kg/d)}$$

Energy corrected milk (ECM) yield:

ECM = milk yield (kg/d)×(0.071×milk fat concentration (%) + 0.043×milk protein concentration (%) + 0.2224).

2.3.3. Fatty acid (FA) determination

Blood plasma fatty acid (FA) analysis was carried out in individual samples following the method of Bondia-Pons et al. (2004). Furthermore, FA analysis in individual milk samples were performed following the method described by Mavrommatis and Tsiplakou (2020). For this purpose, 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 (FID) was used. The steps and the conditions adopted in the method are comprehensively described in Christodoulou et al. (2021). The identification and quantification of each observed peak was followed using a 37 component FAME mix standard (Supelco, Sigma-Aldrich Co., St. Louis, MO, USA). Extra standards were used for the $C_{18:2\ \textit{cis-9, trans-11}}$, and $C_{18:1\ \textit{trans-11}}$ FA (Sigma-Aldrich Co., St. Louis, MO, USA). Finally, a tricosanoic acid ($C_{23:0}$) and a tridecanoic acid ($C_{13:0}$) were used as internal standards for the chromatographic analysis of milk and blood samples, respectively (Fluka, Sigma Aldrich Co., St. Louis, MO, USA). The different groups of FA as well as the indexes were calculated as:

Short Chain Fatty Acids (SCFA) = $C_{4:0} + C_{6:0} + C_{8:0} + C_{10:0} + C_{11:0}$

Medium Chain Fatty Acids (MCFA) = $C_{12:0} + C_{14:0} + C_{15:0} + C_{16:0}$

Long Chain Fatty Acids (LCFA) = $C_{17:0} + C_{18:0} + C_{20:0} + C_{22:0}$

Monounsaturated Fatty Acids (MUFA) = $C_{14:1} + C_{15:1} + C_{16:1\ n\text{-}7} + C_{17:1\ n\text{-}7} + C_{18:1\ \textit{trans}} + C_{18:\textit{1 trans-11}} + C_{18:1\ cis\text{-}9}$

Polyunsaturated Fatty Acids (PUFA) = $C_{18:2\ cis\text{-}9,\ trans\text{-}11}$ + $C_{18:2\ n\text{-}6\ cis}$ + $C_{18:2\ n\text{-}6\ trans}$ + $C_{18:3\ n\text{-}3}$ + $C_{18:3\ n\text{-}6}$ + $C_{20:3\ n\text{-}3}$

Saturated Fatty Acids (SFA) = SCFA + MCFA + LCFA

Unsaturated Fatty Acids (UFA) = PUFA + MUFA Saturated/Unsaturated (SFA/UFA) = (SCFA + MCFA + LCFA)/(PUFA + MUFA)

The atherogenicity index (AI) was defined as: AI = ($C_{12:0}$ + 4×$C_{14:0}$ + $C_{16:0}$)/(PUFA + MUFA)

The thrombogenic index (TI) as: TI = ($C_{14:0}$ + $C_{16:0}$ + $C_{18:0}$)/(0.5×MUFA) + (0.5×ω-6 PUFA) + (3×ω-3 PUFA) + (ω-3 PUFA/ω-6 PUFA) as described by Ulbricht and Southgate (1991).

The health promoting index (HPI) as: HPI = (ω-6 PUFA + ω-3 PUFA + MUFA)/($C_{12:0}$ + 4×$C_{14:0}$ + $C_{16:0}$)

The Δ−9 desaturase activity indexes were calculated by the following ratios:

$C_{14:1}/C_{14:0}$

$C_{16:1}/C_{16:0}$

$C_{18:1}/C_{18:0}$

$C_{18:2\ cis\text{-}9,\ trans\text{-}11}$ / $C_{18:1\ trans\text{-}11}$.

2.3.4. Antioxidant enzyme activities and oxidative status indicators

The followed assays for the determination of antioxidant enzyme activities, the total antioxidant capacity, as well as the oxidative stress biomarkers were performed using a UV/V spectrophotometer (GENESYS 180, Thermo Fisher Scientific, Waltham, NA, USA). A detailed description of the assays that were followed for the determination of the antioxidant activity and total antioxidant capacity is provided in Tsiplakou et al. (2017c). Finally, regarding the oxidative status indicators, malondialdehyde (MDA) was determined according to Nielsen et al. (1997) with

189 modifications being previously described by Tsiplakou et al. (2017c), and the protein carbonyls
190 (PC) were assayed according to the method of Patsoukis et al. (2004).

191 *2.4. Statistical analysis*

192 Statistical analysis was performed using the IBM SPSS Statistics for Windows (IBM Corp.
193 Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY). A repeated-
194 measures general linear model (GLM) for repeated measures analysis of variance (ANOVA),
195 was applied to the daily nutrients intake (g/ewe/day), and fatty acids intake (g/ewe/day) data of
196 the ewes of the different groups (CON, SP5, SP10, SP15) throughout the experimental period,
197 considering S as a repeated measure, with fixed effects of the D (CON vs SP5 vs SP10 vs SP15),
198 S (0, 30, 60 days), and the interactions between them (D×S) according to the model:

199 $Y_{ijk} = \mu + D_i + S_j + A_k + (D \times S)_{ij} + e_{ijk}$

200 where Y_{ijk} is the dependent variable, μ the overall mean, D_i the effect of dietary treatment ($i = 4$),
201 S_j the effect of sampling day ($j = 3$), A_k is the animal's random effect, and $(D \times S)_{ij}$ the interaction
202 between dietary treatment and sampling day and e_{ijk} the residual error. Post hoc analyses were
203 performed using Tukey's multiple range tests.

204 Moreover, GLM for ANOVA was also applied to the data for milk yield and milk chemical
205 composition, considering the sampling time as a repeated measure, with fixed effects of the dietary
206 treatments (D) (CON vs SP5 vs SP10 vs SP15), sampling time (S) (0, 15, 30, 45, 60 days) and the
207 interactions between them (D×S) according to the model:

208 $Y_{ijk} = \mu + D_i + S_j + A_k + (D \times S)_{ij} + e_{ijk}$

209 where Y_{ijk} is the dependent variable, μ the overall mean, Di the effect of dietary treatment ($i = 4$),
210 Sj the effect of sampling day ($j = 5$), A_k is the animal's random effect, and $(D \times S)_{ij}$ the interaction
211 between dietary treatment and sampling day and e_{ijk} the residual error.

In addition, data for milk FA profile as well as antioxidant enzyme activity, antioxidant capacity, and oxidative stress biomarkers were analyzed using also GLM for ANOVA, considering S as a repeated measure, with fixed effects the D (CON vs SP5 vs SP10 vs SP15), S (15, 30, 45, 60 days), and the interactions between them (D × S) according to the model:

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

where Y_{ijk} is the dependent variable, μ the overall mean, D_i the effect of dietary treatment ($i = 4$), S_j the effect of sampling day ($j = 4$), A_k is the animal's random effect, and $(D \times S)_{ij}$ the interaction between dietary treatment and sampling day and e_{ijk} the residual error. Post hoc analyses were performed using Tukey's multiple range tests.

The significance threshold was set at $P<0.05$.

3. Results

3.1. Daily nutrients intake

The experimental diets did not affect the DM, ash, CP, EE, aNDFom, and ADFom intakes, while significant variations ($P=0.003$) were observed at the different sampling times. The proportion of the $C_{18:3\ n-6}$ was significantly linearly increased in the SP groups ($P<0.001$). However, there was no significant interaction between the dietary treatments and the experimental period regarding these parameters.

3.2. Milk yield and its chemical composition

The dietary inclusion of SP in ewes' diets induced only a numerical increase in milk yield, $FCM_{6\%}$, ECM, fat yield, and protein yield. (Table 4).

3.3. Blood fatty acid (FA) profile

The blood plasma FA profile is presented in Table 5. A trend for a rise in the proportion of $C_{16:0}$ in the blood plasma of SP15 compared with the CON ewes was found (P = 0.090). On the other hand, the proportions of $C_{18:0}$ in the SP5 (P=0.001), the $C_{18:1\ cis\text{-}9}$ in both SP10 and SP15 (P=0.013) and the $C_{18:1\ trans\text{-}11}$ in all the SP ewes (P=0.001) declined. Moreover, the proportions of $C_{18:3\ n\text{-}6}$ (P<0.001) and $C_{22:6\ n\text{-}3}$ (P<0.001) increased while that of $C_{18:3\ n\text{-}3}$ (P<0.001) decreased in the blood plasma of SP fed animals. A significant rise in the proportions of $C_{20:3\ n\text{-}3}$ (P = 0.042) and $C_{18:2\ n\text{-}6\ cis}$ (P=0.033) in the blood plasma of SP5 and SP15 ewes respectively was observed. The proportions of $C_{16:0}$ (P<0.001) was reduced, while that of $C_{18:0}$ (P=0.020), $C_{18:2\ n\text{-}6\ cis}$ (P<0.001), and $C_{18:3\ n\text{-}3}$ (P<0.001) were increased through the experimental period.

3.4. Milk fatty acid (FA) profile

The concentrations of SCFA increased in the milk of SP5 and SP10 fed ewes compared with the CON ones (P=0.001) due to the rise in the $C_{6:0}$ (P=0.006) and $C_{8:0}$ (P=0.001) FAs contents (Table 6). On the contrary, a reduction in the proportion of $C_{16:0}$ (P<0.001) and consequently in the MCFA (P = 0.024) in the milk of SP10 fed ewes compared with the CON ones was observed. The inclusion of SP in ewes' diets did not affect the proportions of LCFA and MUFA in ewes' milk, although increased the $C_{22:0}$ (P=0.006) and $C_{18:1\ trans}$ FAs (P<0.001) contents which belong to the respective FAs groups. The SP dietary supplementation of ewes enhanced the PUFA (P=0.027) and ω-3 (P=0.010) contents in their milk, but the results were significant only for the highest inclusion level (SP15). The increase in the proportions of $C_{18:2\ n\text{-}6\ cis}$ in the SP10 and SP15 fed ewes (P=0.034), that of $C_{20:3\ n\text{-}3}$ in all the SP fed animals (P=0.003), and the trend for increase of the $C_{18:3\ n\text{-}3}$ content in the milk of SP15 fed ewes (P=0.054) explain these findings.

Additionally, the highest inclusion level of SP (SP15) tended to reduce the AI (P=0.093) and decreased the TI (P=0.029) index in ewes' milk. Finally, the sampling time had also an effect on

257 milk FA profile. More specifically, the proportions of MCFA (P=0.016) and SFA (P=0.028), the
258 SFA/UFA ratio (P=0.015), and both the AI (P=0.001) and TI (P=0.066) indexes were increased
259 throughout the experimental period.

260 *3.5. Ewes blood plasma oxidative status*

261 The mean antioxidant activity of key studied antioxidant enzymes, oxidative stress biomarkers,
262 and total antioxidant capacity in ewes' blood plasma is presented in Table 7. The activity of SOD
263 increased (P=0.014) in the blood plasma of SP-fed ewes. The same was found for the activities of
264 CAT, GSH-Px, and GST in the SP10 (P=0.031), SP15 (P<0.001) and SP5 (P=0.026) fed ewes
265 respectively. The total antioxidant capacity measured by the FRAP assay was lower in the blood
266 plasma of SP10 fed ewes (P=0.020). Additionally, the dietary supplementation with SP reduces
267 the PC content in ewes' s blood plasma (P<0.001). On the 60th compared with the 15th
268 experimental day, the SOD activity, and the MDA content raised, while the GSH-Px, GR, and
269 GST activities declined.

270 *3.6. Ewes' milk oxidative stability*

271 The dietary inclusion of SP increased the activities of SOD, CAT, and GSH-Px in the milk of
272 ewes, with the results being significant for the SOD in the SP10 and SP15 fed ewes (P<0.007) and
273 for the CAT in the SP5 and SP15 fed animals (P<0.011) (Table 7). Additionally, an increase in the
274 total antioxidant capacity, measured either with FRAP (P<0.001) or ABTS (P<0.001) assays, was
275 observed. Finally, the GSH-Px activity raised (P<0.001) while the total antioxidant capacity
276 determined by ABTS assay declined (P<0.001) throughout the experimental period.

277

278 **4. Discussion**

279 *4.1. Milk yield and milk chemical composition*

To our best knowledge, no researches are available on the use of SP as a supplement in dairy ewes' diet, while some literature can be found concerning its use in dairy cattle feeding.

There are discrepancies about the impact of SP on cow's milk yield and chemical composition. More specifically, the milk yield and chemical composition of cows were not affected when 40 g SP were incorporated daily in the concentrates (Christaki et al., 2012). The same was found, when soybean was partially substituted (5%) by SP in a hay-based diet of cows (Manzocchi et al., 2020). On the other hand, an increase in cow's milk yield and fat content was found, when the animals consumed 200 g of SP daily (Kulpys et al., 2009). A rise in milk yield, fat, protein, and lactose content was also observed in cows when 2 g of fresh weed SP were added in a forage-based diet (Šimkus et al., 2007). Further to that, a decrease in milk fat content has been also reported in cows consumed 7.4 g of dried SP/Kg DMI (Póti et al., 2015). The SP's chemical composition (protein, fat, etc.), form (fresh, dried, etc.), and dietary inclusion levels in relation with other dietary compounds, together with animals' physiology, as well as the metabolizable energy and proteins provided with the diet in the different trials, might be responsible for these contradictory findings.

4.2. Milk and blood plasma FA profile

The incorporation of SP in cows' diet did not change the proportions of $C_{14:0}$, and $C_{16:0}$ in their milk (Manzocchi et al., 2020), in contrast to what was observed for the $C_{16:0}$ and consequently MCFA content in ewes' milk. A significant decline in the proportion of $C_{14:0}$ in the milk of SP-fed cows at the 15^{th} experimental day has been found, but this difference disappeared at the following intervals (35^{th} and 45^{th} days), becoming consistent with our findings (Christaki et al., 2012).

On the other hand, the dietary inclusion of SP (40 g/day/cow) increased PUFA content in cow's milk (Christaki et al., 2012) in agreement with our results concerning the highest inclusion level. More specifically, Christaki et al. (2012) found a significant rise in the proportion of $C_{18:2\ n\text{-}6\ cis}$ in

303 the milk of SP fed cows at the 45th experimental day in accordance with what was observed for
304 this FA in both blood and milk of the ewes fed with the highest SP inclusion level. From PUFA,
305 the $C_{18:3\ n-6}$ is a typical FA of SP (Madeira et al., 2017) which can explain the increment of this FA
306 content in the blood plasma of treated ewes. Interestingly, this increment was not recovered in the
307 milk of SP-fed ewes. Accordingly, a limited increase in the $C_{18:3\ n-6}$ milk content of SP-fed cows
308 has been also observed (Manzocchi et al., 2020), although it is considered to be desirable in
309 humans' nutrition due to its hypocholesterolemic properties (Sugano et al., 1986). However, the
310 impact of SP in both AI and TI in ewes' milk can be considered as beneficial from the human
311 health point of view (Fehily et al., 1994), while it cannot be evaluated with certainty regarding the
312 $C_{18:1\ trans}$ FAs content which are still controversially discussed (de Souza et al., 2015). Despite that,
313 this rise in the proportion of the *trans* $C_{18:1}$ FAs can be further eliminated by the unaffected content
314 of MUFA among the dietary treatments. On the other hand, Póti et al., (2015) observed higher
315 MUFA concentrations in the milk of SP-fed cows. These results might show species differences
316 between cows and small ruminants (ewes, goats). These animal species differences can be also
317 revealed by the findings of Kouřimská et al. (2014) who, in accordance with our results, found a
318 significant reduction in the $C_{16:0}$ and an increase in the $C_{18:2\ n-6\ cis}$ in the milk of goats fed with a
319 diet supplemented with 10 g of low ether extracts *Chlorella vulgaris*. Other microalgae with higher
320 ether extract content (e.g., *Schizochytrium* sp.) have a stronger impact in modulating the milk FAs
321 proportions through completely different physiological pathways. In conclusion, the milk FA
322 profile of small ruminants can be modified by the dietary supplementation with microalgae, but its
323 degree is strongly related to their ether extract content (Tsiplakou et al., 2017a; Tsiplakou et al.,
324 2017b; Mavrommatis and Tsiplakou, 2020) and inclusion levels (Mavrommatis and Tsiplakou,
325 2020).

4.3. Oxidative status of both organism and milk

The antioxidant properties and therapeutic effects of SP are due to its proteins, polysaccharides, PUFA, vitamins, carotenoids, and other bioactive compounds (phenols, chlorophyll, etc.) with antioxidant action (Liestianty et al., 2019; Han et al., 2021). Several *in vitro* studies have shown that SP inhibits lipid peroxidation and increases SOD, CAT, and GSH-Px activities in various cell types after exposure to oxidative stress (Wu et al., 2016). In fact, it has been reported a linear increase in the activities of SOD, CAT, and peroxidase by increasing the H_2O_2 concentrations in the medium of cultivated cells with SP, which was also accompanied by a rise in the amounts of cellular antioxidants compounds (lipophilic and hydrophilic) (Abd El-Baky et al., 2009). Findings from the *in vivo* studies are also in the same line. More specifically, the MDA content and the lipid hydroperoxides decreased while the activities of SOD, GSH, and GST increased in the serum of chronic obstructive pulmonary disease patients that were receiving SP (Ismail et al., 2014). Accordingly, SP consumption enhanced the activities of GSH-Px, GSH, and GR and inhibited the lipids peroxidation in the liver of rabbits, which were previously fed with a high-cholesterol diet (Kim et al., 2010). A rise in the blood GSH activity and a decline in the serum MDA content was found in fattening lambs when they received SP at a rate of 1 g/ 10 kg BW/day (El-Sabagh et al., 2014).

An increase in SOD activity and the total antioxidant capacity content in the serum and *Longissimus thoracis et lumborum* of sheep was observed when their high-energy diet was supplemented with 3 and not with 1% SP (Liang et al., 2020). Moreover, neither the 15 nor the 30 g of SP had an effect on the oxidative stress during the transition period on grazing dairy cows (Garcés et al., 2019). It should be pointed out here that the oxidative status of ewes in this study, improved with all the tested levels of SP despite the fact that the animals were not facing an

oxidative stress. In accordance with our findings, an increase in the activities of GSH-Px and SOD by 240 and 60% respectively was shown in healthy rats treated with SP (Guldas et al., 2021). These findings might show the beneficial effects of SP, in animal's organism to meet future challenges including stressors factors. Moreover, since the most intense effects of SP in the oxidative balance of ewes' organism were observed with the highest supplementation dose, its dietary inclusion level needs to be defined in relation to animals' physiological stage and conditions to ensure its effectiveness.

Microalgae such as *Chlorella* and SP have been also used to improve the nutritional value and the oxidative stability of milk (Tsiplakou et al., 2017a; Tsiplakou et al., 2017b) and yogurt (Beheshtipour et al., 2012; Barkallah et al., 2017). Indeed, the β-carotene content of cows' milk increased even its total oxidative capacity did not change when soybean was partially substituted by SP (5%) in a hay-based diet (Manzocchi et al., 2020). On the other hand, an improvement of the total antioxidant capacity, determined by DPPH and FRAP methods, in yogurts in which SP powder was added at 0.25% has been observed (Barkallah et al., 2017). Moreover, in accordance with our results a rise in SOD activity and a decline in the PC content of goats' milk was found, when the animals consumed 6.18 g of low-fat *Chlorella vulgaris* daily (Tsiplakou et al., 2017b). It is well documented that SOD is the first line of defense against ROS, and the first enzyme to convert oxygen radicals to peroxides. In sequence, CAT and GSH-Px are involved in the detoxification of H_2O_2, and CAT is secondarily involved in removing the peroxides and converting them into O_2 (Yu, 1994). However, it should be mentioned here that the highest dietary inclusion level of SP in ewes had the most beneficial impact on the oxidative parameters of milk indicating again that the inclusion level of microalgae in animals' diets should be defined.

5. Conclusion

The highest inclusion level of SP improved the quality of ewes' milk from a humans' health point of view. Moreover, the antioxidant potential of SP in ewes' organism was also justified. Finally, the reported modifications in the activity of the studied antioxidant enzymes, and in the total antioxidant capacity in the milk of SP fed ewes can be an innovation toward developing a highly nutritional product concerning consumer demands.

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CRediT authorship contribution statement

C. Christodoulou: Investigation, Data curation, Methodology, Formal analysis, Visualization, Writing - original draft. B. Kotsampasi: Participated in the experiment; V. Dotas: Participated in the experiment; M. Simoni: Investigation; F. Righi: Methodology, editing the draft. E. Tsiplakou: Conceptualization, Supervision, Project administration, Visualization, Writing - review & editing.

Declaration of Conflict of Interest

The authors declare that they are no conflict of interest to declare.

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553

554 Table 1
555 Average feed offered (g/ewe/day) and concentrate ingredients (g/kg) of the four dietary treatment groups (CON, SP5, SP10, SP15)
556

	Dietary treatments[a]			
	CON	SP5	SP10	SP15
Average feed offered (g/ewe/day)				
Wheat Straw	200	200	200	200
Alfalfa Hay	1000	1000	1000	1000
Concentrate	1500	1500	1500	1500
	Concentrates			
	CON	SP5	SP10	SP15
Ingredients (g/kg)				
Spirulina	-	5	10	15
Maize grain	344	344	344	344
Barley	200	200	200	200
Wheat middling	100	100	100	100
Sunflower meal	160	160	160	160
Soybean meal	155	155	155	155
Premix mineral and vitamins	41	41	41	41

557
558 [a] CON = control treatment; SP5 = dietary treatment with 5 g *Spirulina*; SP10 = dietary treatment with 10 g *Spirulina*; SP15 = dietary treatment
559 with 15 g *Spirulina*.

Table 2

Chemical composition (g/kg DM), and fatty acids (g/100 g total fatty acids) of the forages (alfalfa hay and wheat straw), the concentrate, and *Spirulina* (SP)

Chemical composition (g/kg DM)				
	Alfalfa hay	Wheat straw	CON[a]	SP[b]
DM[c]	894	928	902.4	931.5
Ash	93	76	60	164.3
CP[d]	200	48	200.3	571.0
EE[e]	2.8	1.6	23.6	4.2
aNDFom[f]	366	728	153	224
ADFom[g]	325	493	52.9	40
Main fatty acids (g/100 g total fatty acids)	Alfalfa hay	Wheat straw	SP	CON
$C_{14:0}$	2.35	6.16	0.49	0.18
$C_{15:0}$	0.68	0.83	-	-
$C_{16:0}$	43.53	33.38	10.06	0.22
$C_{16:1\ n-7}$	2.99	-	-	0.21
$C_{17:0}$	0.83	-	-	0.41
$C_{18:0}$	6.79	4.28	8.76	2.94
$C_{18:1\ cis-9}$	3.01	9.00	0.57	20.83
$C_{18:2\ n-6\ cis}$	16.34	26.74	18.34	54.14
$C_{18:3\ n-6}$	-	-	0.01	0.01
$C_{20:0}$	0.70	1.12	20.97	-
$C_{18:3\ n-3}$	18.59	11.22	-	-
$C_{20:1\ n-9}$	-	-	-	3.41
$C_{20:2\ n-6}$	-	-	-	0.59
$C_{20:3\ n-6}$	-	-	-	0.40
$C_{22:0}$	1.48	3.99	-	-
$C_{24:0}$	2.71	1.94	-	0.27

[a] CON = control treatment.
[b] SP = *Spirulina*.
[c] DM = dry matter.
[d] CP = crude protein.
[e] EE = ether extract.
[f] aNDFom = ash free neutral detergent fiber.

[g] ADFom = acid detergent fiber.

Table 3

Daily nutrients intake (g/ewe/day), and main fatty acids intake (g/ewe/day) from ewes fed diets (CON, SP5, SP10, SP15) with different levels of *Spirulina* (5, 10, and 15 g of concentrate) throughout the experimental period

	Dietary treatment (D)[a]					Sampling time (S)				Effects[c]		
	CON	SP5	SP10	SP15	SEM[b]	0	30	60	SEM[b]	D	S	D×S
Daily nutrients intake (g/ewe/day)												
DM[d]	2433.20	2437.71	2442.22	2446.72	21.55	2442.15^{B}	2411.39^{A}	2466.35^{B}	37.40	0.999	0.003	0.760
Ash	198.20	199.02	199.84	200.67	1.76	199.61^{B}	197.10^{A}	201.59^{B}	3.06	0.993	0.003	0.759
CP[e]	510.10	512.91	515.76	518.62	4.55	514.80^{B}	508.30^{A}	519.90^{B}	7.89	0.983	0.003	0.757
EE[f]	38.52	38.54	38.56	38.58	0.34	38.59^{B}	38.10^{A}	38.97^{B}	0.60	1.000	0.003	0.761
aNDFom[g]	740.95	742.07	743.19	744.31	6.56	743.30^{B}	733.94^{A}	750.66^{B}	11.38	1.000	0.003	0.761
ADFom[h]	502.95	503.15	503.35	503.55	4.45	503.70^{B}	497.36^{A}	508.69^{B}	7.71	1.000	0.003	0.762
	Dietary treatment (D)[a]					Sampling time (S)				Effects[c]		
	CON	SP5	SP10	SP15	SEM[b]	0	30	60	SEM[b]	D	S	D×S
Main fatty acids intake (g/ewe/day) of the total diet												
$C_{14:0}$	0.228	0.228	0.228	0.228	0.002	0.228^{B}	0.225^{A}	0.230^{B}	0.004	1.000	0.003	0.762
$C_{15:0}$	0.032	0.032	0.032	0.032	0.0003	0.032^{B}	0.032^{A}	0.032^{B}	0.0005	1.000	0.003	0.762
$C_{16:0}$	7.314	7.322	7.329	7.337	0.064	7.33^{B}	7.24^{A}	7.41^{B}	0.113	1.000	0.003	0.761
$C_{16:1\ n-7}$	0.162	0.164	0.166	0.168	0.003	0.165^{B}	0.163^{A}	0.167^{B}	0.001	0.835	0.003	0.751
$C_{17:0}$	0.023	0.023	0.023	0.023	0.0002	0.024^{B}	0.023^{A}	0.024^{B}	0.0004	1.000	0.003	0.762
$C_{18:0}$	1.041	1.043	1.044	1.046	0.009	1.044^{B}	1.031^{A}	1.055^{B}	0.02	0.999	0.003	0.760
$C_{18:1\ cis\text{-}9}$	7.602	7.602	7.602	7.603	0.067	7.609^{B}	7.513^{A}	7.684^{B}	0.49	1.000	0.003	0.762
$C_{18:2\ n\text{-}6\ cis}$	20.05	20.06	20.06	20.06	0.177	20.077^{B}	19.824^{A}	20.276^{B}	0.31	1.000	0.003	0.762
$C_{18:3\ n\text{-}6}$	0.000^{a}	0.004^{b}	0.009^{c}	0.013^{d}	0.0004	0.007^{B}	0.007^{A}	0.007^{B}	0.0007	<0.001	0.008	0.143
$C_{20:0}$	0.119	0.119	0.119	0.119	0.001	0.119^{B}	0.118^{A}	0.120^{B}	0.002	1.000	0.003	0.762
$C_{18:3\ n\text{-}3}$	1.907	1.907	1.907	1.907	0.017	1.909^{B}	1.885^{A}	1.928^{B}	0.029	1.000	0.003	0.762
$C_{20:1\ n\text{-}9}$	0.209	0.209	0.209	0.209	0.002	0.209^{B}	0.206^{A}	0.211^{B}	0.003	1.000	0.003	0.762
$C_{20:2\ n\text{-}6}$	0.163	0.163	0.163	0.163	0.001	0.163^{B}	0.161^{A}	0.163^{B}	0.003	1.000	0.003	0.762
$C_{20:3\ n\text{-}3}$	0.142	0.142	0.142	0.142	0.001	0.142^{B}	0.140^{A}	0.143^{B}	0.002	1.000	0.003	0.762
$C_{22:0}$	0.105	0.105	0.105	0.105	0.0009	0.105^{B}	0.104^{A}	0.106^{B}	0.002	1.000	0.003	0.762
$C_{24:0}$	0.203	0.203	0.203	0.203	0.002	0.203^{B}	0.200^{A}	0.205^{B}	0.003	1.000	0.003	0.762

Means with different superscript letters (A, B, C, D) between sampling time points differ significantly.
[a] CON = control treatment; SP5 = dietary treatment with 5 g *Spirulina*; SP10 = dietary treatment with 10 g *Spirulina*; SP15 = dietary treatment with 15 g *Spirulina*.
[b] SEM: Standard error of the means.
[c] Effect: The dietary treatment (D), sampling time (S), and the interaction between dietary treatment×sampling time (D×S) effects were analyzed by ANOVA using a general linear model (GLM) for repeated measures, and post-hoc analysis was performed with appropriate use of Tukey's multiple range test.
[e] DM = dry matter.
[f] CP = crude protein.
[g] EE = ether extract.
[h] aNDFom = ash free neutral detergent fiber.
[i] ADFom = acid detergent fiber.

Table 4

Milk yield and chemical composition from ewes fed diets (CON, SP5, SP10, SP15) with different levels of *Spirulina* (5, 10, and 15 g of concentrate) throughout the experimental period (0, 15th, 30th, 45th, and 60th experimental days)

	Dietary treatment (D)[a]					Sampling time (S)						Effect[c]		
	CON	SP5	SP10	SP15	SEM[b]	1st day	15th day	30th day	45th day	60th day	SEM[b]	D	T	D×S
Milk yield (kg/d)	1.71	1.74	1.86	1.85	0.03	1.97^{C}	1.84^{B}	1.84^{B}	1.61^{A}	1.68^{A}	0.05	0.486	<0.001	0.322
$FCM_{6\%}$[d] (kg/d)	1.68	1.73	1.77	1.86	0.03	1.85^{C}	1.86^{C}	1.78^{BC}	1.70^{AB}	1.63^{A}	0.07	0.529	<0.001	0.298
ECM[e] (kg/d)	1.47	1.53	1.57	1.63	0.02	1.65^{B}	1.63^{AB}	1.57^{AB}	1.47^{B}	1.44^{B}	0.04	0.510	<0.001	0.255
Fat (%)	5.89	6.02	5.66	6.07	0.17	5.57^{A}	6.07^{B}	5.72^{A}	6.48^{C}	5.72^{A}	0.11	0.342	<0.001	0.007
Fat yield (g/d)	99.76	104.01	104.38	111.79	1.48	107.95^{BC}	111.60^{C}	105.07^{B}	103.82^{B}	96.48^{A}	3.25	0.495	<0.001	0.155
Protein (%)	5.22	5.48	5.16	5.39	0.11	5.24^{A}	5.33^{B}	5.29^{AB}	5.39^{C}	5.31^{AB}	0.06	0.152	0.004	0.429
Protein (g/d)	89.15	94.69	95.81	99.54	1.30	102.81^{B}	98.28^{B}	97.23^{AB}	86.60^{A}	89.06^{A}	2.80	0.496	<0.001	0.127
Lactose (%)	4.94	5.00	5.06	5.00	0.05	5.04	4.99	5.00	4.95	5.00	0.03	0.300	0.042	0.008
SCC[f] (1000/mL)	349.7	592.8	295.4	581.5	195.4	262.8	563.1	417. 7	475.6	555.2	140.89	0.603	0.406	0.659
Total solids (%)	16.73	17.06	16.48	17.01	0.25	16.42	16.95	16.61	17.39	16.73	0.14	0.316	<0.001	0.540
Solids not fat (%)	10.84	11.04	10.82	10.94	0.10	10.85	10.89	10.89	10.91	11.01	0.07	0.411	0.207	0.002

Means with different superscript letters (A, B, C, D) between sampling time points differ significantly.

[a] CON = control treatment; SP5 = dietary treatment with 5 g *Spirulina*; SP10 = dietary treatment with 10 g *Spirulina*; SP15 = dietary treatment with 15 g *Spirulina*.

[b] SEM: Standard error of the means.

[c] Effect: The dietary treatment (D), sampling time (S), and the interaction between dietary treatment×sampling time (D×S) effects were analyzed by ANOVA using a general linear model (GLM) for repeated measures, and post-hoc analysis was performed with appropriate use of Tukey's multiple range test.

[d] Fat corrected milk yield in 6%.

[e] Energy corrected milk yield.

[f] Somatic Cells Count.

Table 5

The mean individual fatty acids (FA) (% of total FA) in the blood plasma of ewes fed diets (CON, SP5, SP10, and SP15) with different levels of *Spirulina* (5, 10, and 15 g of concentrate) throughout the experimental period (15^{th}, 30^{th}, 45^{th}, and 60^{th} experimental days)

	Dietary Treatments (D)[a]					Sampling Time (S)					Effect[c]		
Fatty Acids	CON	SP5	SP10	SP15	SEM[b]	15^{th} day	30^{th} day	45^{th} day	60^{th} day	SEM[b]	D	S	D×S
$C_{8:0}$	0.00^{a}	0.13^{b}	0.10^{ab}	0.18^{b}	0.03	0.14^{B}	0.02^{A}	0.05^{A}	0.19^{B}	0.02	0.001	<0.001	0.008
$C_{10:0}$	0.03^{a}	0.11^{ab}	0.08^{ab}	0.19^{b}	0.02	0.16^{B}	0.07^{A}	0.12^{AB}	0.08^{AB}	0.03	0.001	0.026	0.055
$C_{11:0}$	0.29	0.44^{t}	0.23^{t}	0.23^{t}	0.06	0.54^{B}	0.16^{A}	0.22^{A}	0.29^{A}	0.06	0.060	<0.001	0.209
$C_{12:0}$	0.06	0.05	0.04	0.02	0.02	0.11^{B}	0.05^{AB}	0.00^{A}	0.01^{A}	0.02	0.557	0.006	0.676
$C_{14:0}$	0.84	0.76	0.77	0.82	0.02	0.84	0.78	0.77	0.80	0.04	0.402	0.518	0.706
$C_{15:0}$	0.37	0.28	0.35	0.33	0.02	0.33	0.41	0.39	0.20	0.04	0.245	0.001	0.011
$C_{16:0}$	21.46^{t}	22.10	22.24	22.78^{t}	0.19	23.47^{B}	21.21^{A}	20.83^{A}	23.02^{B}	0.31	0.090	<0.001	<0.001
$C_{16:1\ n-7}$	1.06	1.37	1.11	1.06	0.05	1.53^{C}	1.19^{B}	0.86^{A}	1.02^{AB}	0.10	0.121	<0.001	0.100
$C_{17:0}$	2.40^{a}	1.87^{a}	2.48^{a}	3.43^{b}	0.12	2.61^{A}	2.03^{A}	2.02^{A}	3.51^{B}	0.16	<0.001	<0.001	<0.001
$C_{17:1\ n-7}$	0.02^{t}	0.09	0.09	0.11^{t}	0.01	0.09^{AB}	0.08^{AB}	0.11^{B}	0.04^{A}	0.03	0.060	0.204	0.402
$C_{18:0}$	21.10^{b}	18.17^{a}	20.72^{b}	20.38^{b}	0.38	18.44^{A}	20.59^{B}	20.19^{B}	21.15^{B}	0.61	0.001	0.020	0.010
$C_{18:1\ trans}$	0.15	0.21	0.12	0.15	0.02	0.21	0.10	0.16	0.15	0.04	0.444	0.227	0.002
$C_{18:1\ trans\text{-}11}$	1.01^{b}	0.57^{a}	0.44^{a}	0.61^{a}	0.04	0.50^{A}	0.80^{B}	0.75^{B}	0.58^{AB}	0.09	0.001	0.054	0.006
$C_{18:1\ cis\text{-}9}$	16.10^{b}	15.95^{b}	13.27^{a}	13.14^{a}	0.39	16.08^{t}	14.87	13.49^{t}	13.83^{t}	0.80	0.013	0.115	0.044
$C_{18:2\ n-6\ trans}$	0.03	0.02^{t}	0.07^{t}	0.02^{t}	0.02	0.02^{AB}	0.01^{A}	0.08^{B}	0.03^{AB}	0.02	0.080	0.037	0.029
$C_{18:2\ n-6\ cis}$	19.26^{a}	20.60^{a}	20.72^{a}	22.03^{b}	0.27	18.99^{A}	21.52^{B}	22.97^{C}	19.13^{A}	0.52	0.033	<0.001	<0.001
$C_{18:3\ n-6}$	0.11^{a}	0.40^{b}	0.34^{b}	0.46^{b}	0.02	0.30^{B}	0.36^{BC}	0.44^{C}	0.21^{A}	0.04	<0.001	<0.001	0.121
$C_{18:3\ n-3}$	2.71^{b}	1.81^{a}	1.73^{a}	1.80^{a}	0.04	1.66^{A}	1.93^{B}	2.42^{C}	1.99^{B}	0.11	<0.001	<0.001	0.115
$C_{20:3\ n-6}$	0.18	0.16	0.18	0.18	0.01	0.15^{AB}	0.21^{B}	0.21^{B}	0.10^{A}	0.04	0.938	0.080	0.257
$C_{20:3\ n-3}$	3.21^{a}	3.78^{b}	3.56^{ab}	3.63^{ab}	0.05	3.57^{A}	3.63^{B}	3.79^{B}	3.38^{A}	0.10	0.042	0.014	<0.001
$C_{22:2\ n-6}$	0.84^{b}	0.59^{ab}	0.50^{a}	0.52^{a}	0.02	0.55^{A}	0.60^{AB}	0.68^{B}	0.58^{AB}	0.05	0.005	0.168	0.022
$C_{24:1\ n-9}$	8.24	9.67	9.85	8.56	0.27	9.15^{AB}	8.69^{A}	8.46^{A}	10.17^{B}	0.54	0.200	0.124	0.035
$C_{22:6\ n-3}$	0.47^{a}	0.84^{b}	0.89^{b}	0.91^{b}	0.03	0.52^{A}	0.78^{B}	0.88^{BC}	0.90^{C}	0.05	<0.001	<0.001	0.034

Means with different superscript letters (a, b, c) between dietary groups and (A, B, C, D) between sampling time points differ significantly. t = tendency towards statistical significance with values ranging between 0.05 and 0.10 ($0.05 < t < 0.10$).

[a] CON = control treatment; SP5 = dietary treatment with 5 g *Spirulina*; SP10 = dietary treatment with 10 g *Spirulina*; SP15 = dietary treatment with 15 g *Spirulina*.

[b] SEM: Standard error of the means.

[c] Effect: The dietary treatment (D), sampling time (S), and the interaction between dietary treatment×sampling time (D×S) effects were analyzed by ANOVA using a general linear model (GLM) for repeated measures, and post-hoc analysis was performed with appropriate use of Tukey's multiple range test.

Table 6

The mean individual fatty acids (FA) (% of total FA), grouped FA, FA health indices, and Δ-9 desaturase indices in the milk of ewes fed diets (CON, SP5, SP10, and SP15) with different levels of *Spirulina* (5, 10, and 15 g of concentrate) throughout the experimental period (15th, 30th, 45th, and 60th experimental days)

	Dietary treatment (D)[a]					Sampling time (S)					Effect[c]		
Fatty Acids	CON	SP5	SP10	SP15	SEM[b]	15th day	30th day	45th day	60th day	SEM[b]	D	S	D×S
$C_{4:0}$	4.23^{b}	4.30^{b}	4.25^{b}	3.97^{a}	0.060	4.10	4.25	4.30	4.10	0.058	0.001	0.032	0.002
$C_{6:0}$	3.34^{a}	3.50^{b}	3.49^{b}	3.35^{ab}	0.041	3.36	3.43	3.45	3.43	0.040	0.006	0.427	0.402
$C_{8:0}$	3.07^{a}	3.33^{b}	3.37^{b}	3.29^{b}	0.050	3.26	3.27	3.23	3.30	0.044	0.001	0.688	0.960
$C_{10:0}$	9.53^{a}	10.45^{b}	10.37^{b}	10.23^{b}	0.177	10.04	10.22	9.98	10.34	0.142	0.003	0.208	0.959
$C_{11:0}$	0.37	0.38	0.38	0.37	0.008	0.35^{A}	0.38^{BC}	0.37^{AB}	0.40^{C}	0.008	0.859	<0.001	0.615
$C_{12:0}$	5.15^{a}	5.64^{b}	5.49^{ab}	5.49^{ab}	0.117	5.26	5.53	5.35	5.62	0.107	0.040	0.061	0.921
$C_{14:0}$	12.64	12.28	12.29	12.38	0.205	12.13^{A}	12.44^{AB}	12.07^{A}	12.95^{B}	0.177	0.584	0.004	0.830
$C_{14:1}$	0.42	0.42	0.42	0.43	0.007	0.41^{A}	0.45^{B}	0.43^{AB}	0.40^{A}	0.007	0.518	<0.001	0.014
$C_{15:0}$	0.92	0.91	0.91	0.91	0.015	0.89^{A}	0.96^{B}	0.92^{AB}	0.89^{A}	0.014	0.986	0.002	0.927
$C_{15:1}$	0.28	0.29	0.30	0.30	0.008	0.315^{B}	0.301^{B}	0.289^{AB}	0.27^{A}	0.008	0.386	0.003	0.382
$C_{16:0}$	28.55^{b}	27.12^{a}	27.13^{a}	27.17^{a}	0.230	27.17	27.59	27.72	27.50	0.248	<0.001	0.468	0.895
$C_{16:1\ n-7}$	1.07^{b}	0.97^{ab}	0.96^{a}	0.98^{ab}	0.028	0.88^{A}	1.00^{B}	1.05^{B}	1.05^{B}	0.025	0.020	<0.001	<0.001
$C_{17:0}$	0.52	0.53	0.52	0.53	0.007	0.56^{C}	0.52^{B}	0.52^{AB}	0.50^{A}	0.007	0.463	<0.001	0.010
$C_{17:1\ n-7}$	0.25	0.24	0.24	0.26	0.006	0.26^{B}	0.24^{AB}	0.25^{AB}	0.24^{A}	0.005	0.079	0.004	<0.001
$C_{18:0}$	7.68	7.73	7.65	7.67	0.140	8.13^{B}	7.44^{A}	7.74^{AB}	7.31^{A}	0.124	0.856	<0.001	0.321
$C_{18:1\ trans}$	0.52^{a}	0.69^{b}	0.73^{b}	0.69^{b}	0.011	0.69^{B}	0.75^{B}	0.60 ^{A}	0.58^{A}	0.018	<0.001	<0.001	<0.001
$C_{18:1\ trans\text{-}11}$	0.69	0.66	0.71	0.71	0.020	0.79^{B}	0.66^{A}	0.68^{A}	0.63^{A}	0.018	0.324	<0.001	0.121
$C_{18:1\ cis\text{-}9}$	16.58	16.02	16.07	16.51	0.236	16.63	16.06	16.48	16.00	0.194	0.223	0.037	0.687
$C_{18:2\ n-6\ trans}$	0.19	0.19	0.20	0.20	0.006	0.19	0.19	0.20	0.20	0.005	0.707	0.530	0.732
$C_{18:2\ n-6\ cis}$	2.70^{a}	2.78^{ab}	2.91^{b}	2.92^{b}	0.059	2.83	2.86	2.83	2.80	0.052	0.034	0.884	0.984
$C_{18:3\ n-6}$	0.01	0.02	0.01	0.01	0.007	0.031^{B}	0.002^{A}	0.006^{A}	0.020^{AB}	0.006	0.474	0.005	0.490
$C_{20:0}$	0.11	0.12	0.11	0.12	0.002	0.12^{B}	0.12^{B}	0.12^{B}	0.11^{A}	0.001	0.069	<0.001	<0.001
$C_{18:3\ n-3}$	0.54^{t}	0.57	0.58	0.60^{t}	0.015	0.57^{AB}	0.55^{A}	0.57^{AB}	0.60^{B}	0.014	0.054	0.049	0.997
$C_{18:2\ cis\text{-}9,\ trans\text{-}11}$	0.45	0.43	0.45	0.47	0.012	0.44	0.44	0.46	0.45	0.012	0.219	0.491	0.336
$C_{22:0}$	0.09^{a}	0.12^{ab}	0.13^{b}	0.13^{b}	0.009	0.13^{B}	0.11^{AB}	0.13^{AB}	0.09^{A}	0.008	0.006	0.014	<0.001
$C_{20:3\ n-3}$	0.23^{a}	0.25^{b}	0.25^{b}	0.25^{b}	0.005	0.26^{B}	0.24^{A}	0.24^{A}	0.24^{A}	0.004	0.003	0.001	0.409
Δ^{-9} Desaturase Indices													

$C_{14:1}/C_{14:0}$	0.03	0.04	0.03	0.03	0.003	0.03	0.04	0.04	0.03	0.002	0.303	0.175	0.606
$C_{16:1}/C_{16:0}$	0.04	0.04	0.04	0.04	0.001	0.03^{A}	0.04^{B}	0.04^{B}	0.04^{B}	0.001	0.361	<0.001	<0.001
$C_{18:1\ cis\text{-}9}/C_{18:0}$	2.22	2.11	2.11	2.17	0.040	2.06^{A}	2.18^{AB}	2.15^{AB}	2.21^{B}	0.034	0.196	0.012	0.824
$C_{18:2\ cis\text{-}9.\ trans\text{-}11/}\ C_{18:1\ trans\text{-}11}$	0.68	0.66	0.65	0.67	0.018	0.56	0.67	0.69	0.74	0.016	0.590	<0.001	0.001
Grouped Fatty Acids													
SCFA	20.54^{a}	21.95^{b}	21.86^{b}	21.21^{ab}	0.256	21.10	21.56	21.33	21.58	0.221	0.001	0.338	0.401
MCFA	47.28^{b}	45.97^{ab}	45.89^{a}	46.02^{ab}	0.349	45.59^{A}	46.54^{AB}	46.10^{AB}	46.95^{B}	0.319	0.024	0.016	0.796
LCFA	8.19	8.40	8.29	8.32	0.146	8.83^{B}	8.08^{A}	8.37^{A}	7.92^{A}	0.127	0.793	<0.001	0.192
MUFA	19.78	19.29	19.43	19.88	0.258	20.02^{B}	19.44^{AB}	19.77^{AB}	19.16^{A}	0.214	0.329	0.018	0.788
PUFA	4.12^{a}	4.26^{ab}	4.40^{ab}	4.45^{b}	0.079	4.34	4.28	4.31	4.30	0.071	0.027	0.948	0.907
SFA	76.01	76.33	76.04	75.55	0.289	75.51^{A}	76.17^{AB}	75.80^{AB}	76.45^{B}	0.245	0.299	0.028	0.679
UFA	23.90	23.55	23.83	24.32	0.289	24.35B	23.72^{AB}	24.07^{AB}	23.46^{A}	0.244	0.310	0.037	0.737
SFA/UFA	3.20	3.26	3.22	3.12	0.049	3.11^{A}	3.23^{AB}	3.17^{AB}	3.29^{B}	0.041	0.217	0.015	0.721
ω-3	0.77^{a}	0.82^{ab}	0.83^{ab}	0.85^{b}	0.073	0.83	0.79	0.81	0.84	0.143	0.010	0.063	0.029
ω-6	2.91^{t}	3.00	3.12	3.13^{t}	0.027	3.05	3.05	3.04	3.02	0.054	0.050	0.968	0.977
ω-6/ω-3	3.82	3.66	3.79	3.71	0.029	3.68^{A}	3.88^{B}	3.76^{AB}	3.64^{A}	0.056	0.194	0.024	0.893
Fatty Acids Health Indexes													
AI	3.56^{t}	3.55	3.46	3.39^{t}	0.263	3.35^{A}	3.52^{AB}	3.44^{A}	3.64^{B}	0.055	0.093	0.001	0.818
TI	3.52^{a}	3.43^{b}	3.38^{b}	3.31^{b}	0.022	3.33^{A}	3.44^{B}	3.40^{AB}	3.46^{B}	0.043	0.029	0.066	0.655
HPI	0.28	0.28	0.29	0.29	0.003	0.30^{B}	0.28^{AB}	0.29^{B}	0.27^{A}	0.005	0.556	0.009	0.753

Means with different superscript letters (a, b, c) between dietary groups and (A, B, C, D) between sampling time points differ significantly. t = tendency towards statistical significance with values ranging between 0.05 and 0.10 ($0.05 < t < 0.10$).

[a] CON = control treatment; SP5 = dietary treatment with 5 g *Spirulina*; SP10 = dietary treatment with 10 g *Spirulina*; SP15 = dietary treatment with 15 g *Spirulina*.

[b] SEM: Standard error of the means.

[c] Effect: The dietary treatment (D), sampling time (S), and the interaction between dietary treatment×sampling time (D×S) effects were analyzed by ANOVA using a general linear model (GLM) for repeated measures, and post-hoc analysis was performed with appropriate use of Tukey's multiple range test.

Table 7

Enzyme activities (Units/mL), total antioxidant capacity, and oxidative status biomarkers in blood plasma and milk of ewes fed diets (CON, SP5, SP10, SP15) with different levels of Spirulina (5 g, 10 g, and 15 g of concentrate) throughout the experimental period (15th, 30th, 45th, and 60th experimental days)

	Dietary Treatment (D)					Sampling Time (S)					Effect [b]		
	CON	SP5	SP10	SP15	SEM[b]	15	30	45	60	SEM[b]	D	S	D×S
Blood Plasma													
SOD[d]	14.44^{a}	16.66^{b}	17.01^{b}	17.53^{b}	16.39	14.17^{A}	14.54^{A}	18.39^{B}	18.39^{B}	16.44	0.014	<0.001	<0.001
CAT[e]	19.37^{a}	21.45^{ab}	22.30^{b}	21.32^{ab}	0.26	20.92^{AB}	20.48^{A}	21.64^{B}	21.40^{AB}	0.52	0.031	0.178	<0.001
GSH-Px[f]	0.24^{a}	0.27^{a}	0.27^{a}	0.31^{b}	0.27	0.34^{C}	0.22^{A}	0.28^{B}	0.23^{A}	0.27	<0.001	<0.001	<0.001
GR[g]	0.05	0.05	0.05	0.05	0.001	0.049^{B}	0.053^{C}	0.049^{B}	0.046^{A}	0.05	0.820	<0.001	0.001
GSTs[h]	0.15^{a}	0.18^{b}	0.17^{ab}	0.16^{ab}	0.16	0.17^{B}	0.16^{B}	0.19^{C}	0.14^{A}	0.16	0.026	<0.001	<0.001
ABTS[i]	30.10	29.10	29.80	29.34	29.64	30.69	28.62	30.00	30.04	29.64	0.464	0.002	0.001
FRAP[j]	0.93^{b}	0.90^{ab}	0.83^{a}	0.96^{b}	0.015	0.77^{A}	0.96^{C}	0.88^{B}	1.00^{C}	0.026	0.020	<0.001	<0.001
MDA[k]	0.63	0.64	0.61	0.61	0.63	0.61^{A}	0.58^{A}	0.65^{AB}	0.67^{B}	0.64	0.677	0.049	0.411
PC[l]	2.45^{b}	1.82^{a}	1.78^{a}	1.60^{a}	1.90	1.81^{AB}	2.09^{B}	1.74^{A}	2.01^{B}	1.90	<0.001	0.076	0.003
Milk													
SOD	131.49^{a}	143.13^{ab}	144.83^{b}	150.80^{b}	1.52	136.50^{A}	146.91^{B}	145.02^{AB}	141.81^{AB}	2.96	0.007	0.115	0.085
CAT	3.68^{a}	4.72^{b}	3.90^{ab}	4.60^{b}	0.13	4.22	4.29	4.37	4.02	0.26	0.011	0.736	<0.001
GSH-Px	0.28^{a}	0.55^{b}	0.58^{b}	0.62^{c}	0.02	0.29^{A}	0.35^{B}	0.75^{D}	0.65^{C}	0.02	<0.001	<0.001	<0.001
ABTS	48.04^{a}	52.73^{b}	51.62^{b}	54.86^{b}	0.60	55.48^{C}	54.39^{BC}	44.37^{A}	53.00^{B}	1.00	<0.001	<0.001	<0.001
FRAP	3.00^{a}	4.63^{b}	4.51^{b}	5.18^{b}	0.30	4.40^{B}	4.34^{B}	3.60^{A}	4.96^{B}	0.26	<0.001	0.001	0.242
MDA	0.23	0.26	0.23	0.19	0.05	0.17^{A}	0.27^{B}	0.28^{BC}	0.19^{AB}	0.03	0.114	0.001	0.285
PC	1.71^{ab}	1.90^{b}	1.65^{ab}	1.58^{a}	0.04	1.87^{C}	1.57^{B}	1.41^{A}	2.02^{C}	0.07	0. 034	<0.001	<0.001

Means with different superscript letters (a, b, c) between dietary groups and (A, B, C, D) between sampling time points differ significantly.

[a] CON = control treatment; SP5 = dietary treatment with 5 g *Spirulina*; SP10 = dietary treatment with 10 g *Spirulina*; SP15 = dietary treatment with 15 g *Spirulina*.

[b] SEM: Standard error of the means.

[c] Effect: The dietary treatment (D), sampling time (S), and the interaction between dietary treatment×sampling time (D×S) effects were analyzed by ANOVA using a general linear model (GLM) for repeated measures, and post-hoc analysis was performed with appropriate use of Tukey's multiple range test.

[d] SOD: Superoxide dismutase.

[e] CAT: Catalase.

[f] GSH-Px: Glutathione peroxidase.

[g] GR: Glutathione reductase.
[h] GST: Glutathione transferase.
[i] ABTS: 2,20-Azino-bis (3-ethylbenzthiazoline-6-sulfonic acid as % inhibition.
[j] FRAP: Ferric Reducing Ability of Plasma is expressed as μM ascorbic acid equivalents.
[k] MDA: Malondialdehyde as μM MDA.
[l] PC: Protein carbonyls as nmol/mL.