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1	The effect of Spirulina supplementation in ewes' oxidative status and milk quality
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20	
21	Abstract
22	Spirulina (SP) is rich in bioactive compounds (β -carotene, γ -linoleic acid, vitamins, etc.) with
23	antioxidants properties. However, its impact on the oxidative status of ewes' organism and milk,
24	as well as on milk's quality has not been extensively studied. Forty-eight dairy Chios ewes were
25	divided into four homogenous groups ($n = 12$) and were fed individually. The concentrate of the
26	control group (CON) had no SP, while in the concentrates of the treated groups, SP was added to
27	obtain a daily supply of 5 (SP5), 10 (SP10), and 15 (SP15) g per animal. The milk yield and
28	chemical composition were not affected by the addition of SP. The proportion of short-chain fatty
29	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 30 proportion of polyunsaturated fatty acids and reduced the thrombogenicity index in milk, while its 31 32 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 33 activities of catalase (CAT), glutathione peroxidase (GSH-Px), and glutathione transferase in the 34 35 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 36 stability of ewes' milk improved by the dietary addition of SP as indicated by the rise in the 37 38 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 39 milk. In conclusion, the highest amount of SP improved ewes' organism oxidative status as well 40 as their milk quality and its oxidative stability. 41

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

43 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 44 with a heat-stable amylase; AI, atherogenicity index; CAT, catalase; CP, crude protein; DM, dry 45 46 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 47 48 peroxidase; GST, glutathione transferase; HPI, health-promoting index; LCFA, long-chain fatty 49 acids; MCFA, medium-chain fatty acids; MDA, malondialdehyde; MUFA, monounsaturated 50 fatty acids; OM, organic matter; PC, protein carbonyls; PUFA, polyunsaturated fatty acids; SCC, 51 somatic cell counts; SCFA, short-chain fatty acids; SFA, saturated fatty acids; SOD, superoxide 52 dismutase; SP, Spirulina; TI, thrombogenicity index; UFA, unsaturated fatty acids.

53 **1. Introduction**

54 Over the past decades, targeted nutrition responded to the increasing consumer demands for 55 functional and highly nutritional dairy products. Supplementing ruminant diets with microalgae is 56 a direct way to promote animal health as well as enrich dairy products with bioactive compounds, 57 such as polyunsaturated fatty acids (PUFA) and antioxidants.

58 Spirulina (SP) is an edible blue-green microalga, a filamentous spiral-shaped cyanobacterium, 59 and is considered as feedstuff with high nutritional potential and has been mentioned as "food of 60 the future". SP contains up to 70% protein and has a remarkably balanced amino acid profile 61 (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 62 essential phytochemical properties it is considered a potent immunostimulant (Wu et al., 2016). 63 Interestingly, of the different SP production systems, a second sorting product may arise, which is 64 destined for usage in animal diets. Notwithstanding, high genetic merit dairy animals are 65 66 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 67 remarkable results regarding the oxidative status of ruminants (Tsiplakou et al., 2017a; Tsiplakou 68 69 et al., 2017b; Tsiplakou et al., 2018; Mavrommatis et al., 2018; Mavrommatis and Tsiplakou, 70 2020). For this purpose, the inclusion of SP in ruminant diets is expected not only to be beneficial 71 toward developing dairy products with strong shelf-life longevity but also in fortifying animals' 72 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.

87 **2. Materials and methods**

88 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 \pm 6.0 kg), fat corrected (6%) milk yield (FCM_{6%}; 1.85 \pm 0.3 kg/d), days in milk (67 \pm 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

99 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 100 101 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, 102 103 barley, wheat middling, sunflower meal, soybean meal, and mineral and vitamin premix (Table 1). 104 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 105 106 SP. In particular, the concentrate of the control group (CON) had no inclusion of SP, while in the 107 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 108 109 as the main fatty acids (g/100 g total fatty acids) of the forages (alfalfa hay and wheat straw), of 110 the concentrate, and of the SP are presented in Table 2. The daily nutrients (g/ewe/day), and main 111 fatty acids (g/ewe/day) intake are presented in Table 3. All the animals had free access to fresh 112 water. The whole experimental period lasted 60 days.

113 2.2. Sample collection

114 At the beginning of the trial as well as at every time a new concentrate batch was produced, 115 feed samples from alfalfa hay, wheat straw, concentrate, and SP were collected and were subjected 116 to chemical analysis. Ewes were milked twice per day at 07:00 and 17:00 h by a milking machine. 117 At 0, 15, 30, 45, and 60 experimental days, milk samples were collected individually from each 118 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) 119 120 were collected, stored at - 80°C, and later subjected to FA, antioxidant enzyme activity, antioxidant 121 capacity, and oxidative stress biomarkers analysis. Individual blood samples (n = 192) were

122 collected at the same intervals from the jugular vein of each ewe after the milking and before 123 feeding time. Approximately 10 mL of whole blood were directly transferred to heparin-containing 124 tubes (170 units heparin; BD Vacutainer, Plymouth, UK). Afterward, the blood samples were 125 centrifuged (SL16R, Thermo Fisher Scientific, Waltham, MA, USA) at 2500 rpm for 15 min at 126 4°C to separate plasma from the cells. Blood plasma samples were also stored at - 80°C, before 127 FA, antioxidant enzyme activity, antioxidant capacity, and oxidative stress biomarkers analysis.

128 2.3. Sample analysis

129 *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).

137 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%:

143

144 $FCM_{6\%} = (0.28 + 0.12 \times \text{milk fat concentration (\%)}) \times \text{milk yield (kg/d)}$

145

146

Energy corrected milk (ECM) yield:

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

147 (%) + 0.2224). 2.3.3. Fatty acid (FA) determination 148 Blood plasma fatty acid (FA) analysis was carried out in individual samples following the 149 150 method of Bondia-Pons et al. (2004). Furthermore, FA analysis in individual milk samples were 151 performed following the method described by Mavrommatis and Tsiplakou (2020). For this 152 purpose, an Agilent 6890 N gas chromatograph equipped with an HP-88 capillary column (60 153 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 154 Christodoulou et al. (2021). The identification and quantification of each observed peak was 155 followed using a 37 component FAME mix standard (Supelco, Sigma-Aldrich Co., St. Louis, MO, 156 157 USA). Extra standards were used for the C_{18:2} cis-9, trans-11, and C_{18:1} trans-11 FA (Sigma-Aldrich Co., 158 St. Louis, MO, USA). Finally, a tricosanoic acid ($C_{23:0}$) and a tridecanoic acid ($C_{13:0}$) were used as 159 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 160 161 were calculated as: Short Chain Fatty Acids (SCFA) = $C_{4:0} + C_{6:0} + C_{8:0} + C_{10:0} + C_{11:0}$ 162 163 Medium Chain Fatty Acids (MCFA) = $C_{12:0} + C_{14:0} + C_{15:0} + C_{16:0}$ 164 Long Chain Fatty Acids (LCFA) = $C_{17:0} + C_{18:0} + C_{20:0} + C_{22:0}$ Monounsaturated Fatty Acids (MUFA) = $C_{14:1} + C_{15:1} + C_{16:1 n-7} + C_{17:1 n-7} + C_{18:1 trans} + C_{18:1 trans}$ 165

167	Polyunsaturated Fatty Acids (PUFA) = $C_{18:2 cis-9}$, trans-11 + $C_{18:2 n-6 cis}$ + $C_{18:2 n-6 trans}$ + $C_{18:3 n-3}$ +
168	$C_{18:3 n-6} + C_{20:3 n-3}$
169	Saturated Fatty Acids (SFA) = SCFA + MCFA + LCFA
170	Unsaturated Fatty Acids (UFA) = PUFA + MUFA Saturated/Unsaturated (SFA/UFA) = (SCFA
171	+ MCFA + LCFA)/(PUFA + MUFA)
172	The atherogenicity index (AI) was defined as: $AI = (C_{12:0} + 4 \times C_{14:0} + C_{16:0})/(PUFA + MUFA)$
173	The thrombogenic index (TI) as: TI = $(C_{14:0} + C_{16:0} + C_{18:0})/(0.5 \times MUFA) + (0.5 \times \omega - 6 PUFA) + (0.5 \times \omega - 6 PUFA)$
174	$(3 \times \omega - 3 \text{ PUFA}) + (\omega - 3 \text{ PUFA}/\omega - 6 \text{ PUFA})$ as described by Ulbricht and Southgate (1991).
175	The health promoting index (HPI) as: HPI = $(\omega-6 \text{ PUFA} + \omega-3 \text{ PUFA} + \text{MUFA})/(C_{12:0} + 4 \times C_{14:0})$
176	+ C _{16:0})
177	The Δ -9 desaturase activity indexes were calculated by the following ratios:
178	C _{14:1} /C _{14:0}
179	$C_{16:1}/C_{16:0}$
180	$C_{18:1}/C_{18:0}$
181	C _{18:2 cis-9} , trans-11 / C _{18:1 trans-11} .
182	2.3.4. Antioxidant enzyme activities and oxidative status indicators
183	The followed assays for the determination of antioxidant enzyme activities, the total antioxidant
184	capacity, as well as the oxidative stress biomarkers were performed using a UV/V
185	spectrophotometer (GENESYS 180, Thermo Fisher Scientific, Waltham, NA, USA). A detailed
186	description of the assays that were followed for the determination of the antioxidant activity and

188 status indicators, malondialdehyde (MDA) was determined according to Nielsen et al. (1997) with

187

total antioxidant capacity is provided in Tsiplakou et al. (2017c). Finally, regarding the oxidative

modifications being previously described by Tsiplakou et al. (2017c), and the protein carbonyls
(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
- 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 ($D \times S$) according to the model:

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

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 = 3), 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.

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

- 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 $(D \times S)$ according to the model:

216
$$Y_{ijk} = \mu + D_i + S_j + A_k + (D \times S)_{ij} + e_{ijk}$$

- 217 where Y_{ijk} is the dependent variable, μ the overall mean, D_i the effect of dietary treatment (i = 4),
- 218 S_i the effect of sampling day (j = 4), A_k is the animal's random effect, and (D×S)_{ij} the interaction
- 219 between dietary treatment and sampling day and e_{ijk} the residual error. Post hoc analyses were
- 220 performed using Tukey's multiple range tests.
- 221 The significance threshold was set at P < 0.05.
- 222

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213

223 **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.

230 *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).
- 233 3.3. Blood fatty acid (FA) profile

234 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 235 hand, the proportions of $C_{18:0}$ in the SP5 (P=0.001), the $C_{18:1 \text{ cis-9}}$ in both SP10 and SP15 (P=0.013) 236 and the C_{18:1 trans-11} in all the SP ewes (P=0.001) declined. Moreover, the proportions of C_{18:3 n-6} 237 (P < 0.001) and C_{22:6 n-3} (P < 0.001) increased while that of C_{18:3 n-3} (P < 0.001) decreased in the blood 238 239 plasma of SP fed animals. A significant rise in the proportions of $C_{20:3 n-3}$ (P = 0.042) and $C_{18:2 n-6}$ cis (P=0.033) in the blood plasma of SP5 and SP15 ewes respectively was observed. The 240 241 proportions of $C_{16:0}$ (P<0.001) was reduced, while that of $C_{18:0}$ (P=0.020), $C_{18:2 n-6 cis}$ (P<0.001), 242 and $C_{18:3 n-3}$ (P<0.001) were increased through the experimental period.

243 3.4. Milk fatty acid (FA) profile

The concentrations of SCFA increased in the milk of SP5 and SP10 fed ewes compared with 244 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 245 246 (Table 6). On the contrary, a reduction in the proportion of $C_{16:0}$ (P<0.001) and consequently in 247 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' 248 milk, although increased the C_{22:0} (P=0.006) and C_{18:1 trans} FAs (P<0.001) contents which belong 249 250 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 251 252 highest inclusion level (SP15). The increase in the proportions of C_{18:2 n-6 cis} in the SP10 and SP15 253 fed ewes (P=0.034), that of C_{20:3 n-3} in all the SP fed animals (P=0.003), and the trend for increase of the $C_{18:3 n-3}$ content in the milk of SP15 fed ewes (P=0.054) explain these findings. 254

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

260 3.5. Ewes blood plasma oxidative status

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

270 *3.6. Ewes' milk oxidative stability*

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

280 281 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. 282 More specifically, the milk yield and chemical composition of cows were not affected when 40 g 283 SP were incorporated daily in the concentrates (Christaki et al., 2012). The same was found, when 284 285 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 286 consumed 200 g of SP daily (Kulpys et al., 2009). A rise in milk yield, fat, protein, and lactose 287 288 content was also observed in cows when 2 g of fresh weed SP were added in a forage-based diet (Simkus et al., 2007). Further to that, a decrease in milk fat content has been also reported in cows 289 290 consumed 7.4 g of dried SP/Kg DMI (Póti et al., 2015). The SP's chemical composition (protein, 291 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 292 provided with the diet in the different trials, might be responsible for these contradictory findings. 293

294 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 15th experimental day has been found, but this difference disappeared at the following intervals (35th and 45th days), becoming consistent with our findings (Christaki et al., 2012).

300 On the other hand, the dietary inclusion of SP (40 g/day/cow) increased PUFA content in cow's 301 milk (Christaki et al., 2012) in agreement with our results concerning the highest inclusion level. 302 More specifically, Christaki et al. (2012) found a significant rise in the proportion of $C_{18:2 n-6 cis}$ in

the milk of SP fed cows at the 45th experimental day in accordance with what was observed for 303 304 this FA in both blood and milk of the ewes fed with the highest SP inclusion level. From PUFA, the C_{18:3 n-6} is a typical FA of SP (Madeira et al., 2017) which can explain the increment of this FA 305 content in the blood plasma of treated ewes. Interestingly, this increment was not recovered in the 306 307 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 C_{18:1 trans} FAs content which are still controversially discussed (de Souza et al., 2015). Despite that, 312 this rise in the proportion of the *trans* $C_{18:1}$ FAs can be further eliminated by the unaffected content 313 314 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 revealed by the findings of Kouřímská et al. (2014) who, in accordance with our results, found a 317 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 318 319 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).

326 *4.3. Oxidative status of both organism and milk*

The antioxidant properties and therapeutic effects of SP are due to its proteins, polysaccharides, 327 328 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 329 330 that SP inhibits lipid peroxidation and increases SOD, CAT, and GSH-Px activities in various cell 331 types after exposure to oxidative stress (Wu et al., 2016). In fact, it has been reported a linear 332 increase in the activities of SOD, CAT, and peroxidase by increasing the H_2O_2 concentrations in 333 the medium of cultivated cells with SP, which was also accompanied by a rise in the amounts of 334 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 335 hydroperoxides decreased while the activities of SOD, GSH, and GST increased in the serum of 336 337 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 338 339 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 340 found in fattening lambs when they received SP at a rate of 1 g/ 10 kg BW/day (El-Sabagh et al., 341 342 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.

356 Microalgae such as *Chlorella* and SP have been also used to improve the nutritional value and 357 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 358 increased even its total oxidative capacity did not change when soybean was partially substituted 359 360 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 361 362 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, 363 when the animals consumed 6.18 g of low-fat *Chlorella vulgaris* daily (Tsiplakou et al., 2017b). 364 365 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 366 367 detoxification of H_2O_2 , and CAT is secondarily involved in removing the peroxides and converting 368 them into O₂ (Yu, 1994). However, it should be mentioned here that the highest dietary inclusion 369 level of SP in ewes had the most beneficial impact on the oxidative parameters of milk indicating 370 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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382

383 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.

388

389 Declaration of Conflict of Interest

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

391

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

556

	Dietary treat	ments ^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

^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.

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

562 concentrate, and *Spirulina* (SP)

563

Chemical composition (g/kg DM	A)			
	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	Alfalfa harr	Wheat strang	CD	CON
(g/100 g total fatty acids)	Allalla 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
С _{20:3 п-6}	-	-	-	0.40
C _{22:0}	1.48	3.99	-	-
$C_{24:0}$	2.71	1.94	-	0.27

564 $\overline{^{a}}$ CON = control treatment.

565 ^b SP = Spirulina.

566 $^{\circ}$ DM = dry matter.

567 ^d CP = crude protein.

568 e EE = ether extract.

569 ^f aNDFom = ash free neutral detergent fiber.

 g ADFom = acid detergent fiber.

572 Daily nutrients intake (g/ewe/day), and main fatty acids intake (g/ewe/day) from ewes fed diets (CON, SP5, SP10, SP15) with

573 different levels of *Spirulina* (5, 10, and 15 g of concentrate) throughout the experimental period

574

	Dietary tr	eatment (D) ^a			Sampling t	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 ^в	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	
\mathbf{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 tr	eatment (D) ^a			Sampling t	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-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	
C18:2 n-6 cis	20.05	20.06	20.06	20.06	0.177	20.077 ^в	19.824 ^A	20.276^{B}	0.31	1.000	0.003	0.762	
C _{18:3 n-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-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-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-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-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	

- 576 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
- 578 with 15 g Spirulina.
- 579 ^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
- 581 analyzed by ANOVA using a general linear model (GLM) for repeated measures, and post-hoc analysis was performed with appropriate use 582 of Tukey's multiple range test.
- $^{\circ}$ DM = dry matter.
- 584 $^{\rm f}$ CP = crude protein.
- 585 g EE = ether extract.
- 586 h aNDFom = ash free neutral detergent fiber.
- 587 i ADFom = acid detergent fiber.

589 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)

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	Dietar	y treatme	nt (D) ^a			Sampling time (S)						Effect ^c		
	CON	SP5	SP10	SP15	SEM ^b	1 st day	15 th day	30 th day	45 th day	60 th day	SEM ^b	D	Т	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 [°]	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

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593 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

595 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

599 of Tukey's multiple range test.

600 ^d Fat corrected milk yield in 6%.

601 ^e Energy corrected milk yield.

602 ^f Somatic Cells Count.

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)

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	Dietary	Treatme	ents (D) ^a			Sampling	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 ^в	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 ^в	20.19 ^в	21.15 ^в	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-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-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	1.81ª	1.73 ^a	1.80^{a}	0.04	1.66 ^A	1.93 ^b	2.42°	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 ^в	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
C24:1 n-9	8.24	9.67	9.85	8.56	0.27	9.15 ^{AB}	8.69 ^A	8.46 ^A	10.17 ^в	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

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609 Means with different superscript letters (a, b, c) between dietary groups and (A, B, C, D) between sampling time points differ significantly. t 610 = tendency towards statistical significance with values ranging between 0.05 and 0.10 (0.05 < t < 0.10).

= 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

612 with 15 g Spirulina.

613 ^b SEM: Standard error of the means.

- ⁶¹⁴ ^c Effect: The dietary treatment (D), sampling time (S), and the interaction between dietary treatment×sampling time (D×S) effects were
- 615 analyzed by ANOVA using a general linear model (GLM) for repeated measures, and post-hoc analysis was performed with appropriate use 616 of Tukey's multiple range test.

618 The mean individual fatty acids (FA) (% of total FA), grouped FA, FA health indices, and Δ -9 desaturase indices in the milk of

619 ewes fed diets (CON, SP5, SP10, and SP15) with different levels of *Spirulina* (5, 10, and 15 g of concentrate) throughout the

620 experimental period (15th, 30th, 45th, and 60th experimental days)

	Dietary	/ treatmen	nt (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 _{4:0}	4.23 ^b	4.30 ^b	4.25 ^b	3.97ª	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}	9.53ª	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°	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 ^в	0.177	0.584	0.004	0.830		
C14:1	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		
C16:1 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-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} <i>cis-9</i>	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 ^в	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} <i>cis-9, trans-11</i>	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		
C20:3 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-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-9. trans-11/ C _{18:1} trans-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 ^в	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

622

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

624 = 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*.

627 ^b SEM: Standard error of the means.

⁶²⁸ ^c Effect: The dietary treatment (D), sampling time (S), and the interaction between dietary treatment×sampling time (D×S) effects were

629 analyzed by ANOVA using a general linear model (GLM) for repeated measures, and post-hoc analysis was performed with appropriate use

630 of Tukey's multiple range test.

632 Enzyme activities (Units/mL), total antioxidant capacity, and oxidative status biomarkers in blood plasma and milk of ewes fed

633 diets (CON, SP5, SP10, SP15) with different levels of Spirulina (5 g, 10 g, and 15 g of concentrate) throughout the experimental 634 period (15th, 30th, 45th, and 60th experimental days)

635

	Dietary Treatment (D)					Sampling	g Time (S		Effect ^b				
	CON	SP5	SP10	SP15	SEM ^b	15	30	45	60	SEM ^b	D	S	D×S
Blood Plas	ma												
\mathbf{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	19.37ª	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
\mathbf{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^1	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 ^t	° 150.80 ^t	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}	1.58ª	0.04	1.87 ^C	1.57 ^B	1.41 ^A	2.02 ^c	0.07	0.034	< 0.001	< 0.001

636

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

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^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

643 of Tukey's multiple range test.

644 ^d SOD: Superoxide dismutase.

645 ^eCAT: Catalase.

646 ^fGSH-Px: Glutathione peroxidase.

- 647 ^g GR: Glutathione reductase.
- ^hGST: Glutathione transferase. 648
- 649
- 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. 650
- 651 ^k MDA: Malondialdehyde as µM MDA.
- ¹PC: Protein carbonyls as nmol/mL. 652