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1 The effect of Spirulina supplementation in ewes' oxidative status and milk quality

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3 C. Christodoulou^a, B. Kotsampasi^b, V. Dotas^c, M. Simoni^d, F. Righi^d, E. Tsiplakou^{a,*}

4
5 ^a *Department of Animal Science, Agricultural University of Athens, Iera Odos 75, GR-11855 Athens,*
6 *Greece*

7
8 ^b *Research Institute of Animal Science, ELGO-DIMITRA, 58100 Giannitsa, Greece*

9
10 ^c *Department of Animal Production, School of Agriculture, Aristotle University of Thessaloniki, 54124*
11 *Thessaloniki, Greece*

12
13 ^d *Department of Veterinary Science, University of Parma, Via del Taglio, 10, 43126 Parma, Italy*

14
15 * Corresponding author. E-mail address: eltsiplakou@aua.gr (E. Tsiplakou), full postal address:

16 Agricultural University of Athens, Iera Odos 75, Athens, 11855, Greece, telephone: +30 210 529
17 4435

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19 Submitted to Animal Feed Science and Technology in June 2022

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

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

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

43 *Abbreviations:* ABTS, 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid; ADFom, acid
44 detergent fibre expressed exclusive of residual ash; aNDFom, neutral detergent fibre assayed
45 with a heat-stable amylase; AI, atherogenicity index; CAT, catalase; CP, crude protein; DM, dry
46 matter; ECM, energy corrected milk yield; FA, fatty acids; FCM_{6%}, fat corrected (6%) milk
47 yield; FRAP, ferric reducing ability of plasma; GR, glutathione reductase; GSH-Px, glutathione
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
62 γ -linolenic acid, which have well-known health benefits (Howe et al., 2006), while owing to its
63 essential phytochemical properties it is considered a potent immunostimulant (Wu et al., 2016).
64 Interestingly, of the different SP production systems, a second sorting product may arise, which is
65 destined for usage in animal diets. Notwithstanding, high genetic merit dairy animals are
66 susceptible to an oxidative imbalance due to their greater energy level requirements (Wullepit et
67 al., 2009). The supplementation of ruminant diets with microalgae was previously linked with
68 remarkable results regarding the oxidative status of ruminants (Tsiplakou et al., 2017a; Tsiplakou
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.

73 Several studies investigated the effect of SP in ruminant’s performance (Kulpys et al., 2009;
74 Bezzera et al., 2010; Shimkiene et al., 2010), productivity (Šimkus et al., 2007; Kulpys et al., 2009;
75 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; Manzacchi 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

99 every two weeks and administered twice per day, after milking at 07:00 and 17:00 h (Table 1).
100 Each ewe was fed individually based on its maintenance and lactating requirements and the
101 average amount of the concentrates, alfalfa hay, and wheat straw were 1.5, 1.0, and 0.2 kg/ewe/day,
102 respectively, independently from the groups (Table 1). The concentrates consisted of maize grain,
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
105 individual feeding, ewes were offered concentrates with the inclusion of three different levels of
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,
108 10, and 15 g, per day, respectively (Table 1). Chemical composition (g/kg dry matter; DM) as well
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
119 composition analysis. Furthermore, at 15, 30, 45, and 60 days, individual milk samples (n = 192)
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*

130 Feed samples were analyzed for dry matter (DM; Official Method 934.01), ash (Official Method
131 942.05), and ether extract (EE; Official Method 920.39) according to AOAC (1984), and for crude
132 protein (CP; Official Method 988.05) according to AOAC (2001). They were also analyzed for
133 neutral detergent fibre (aNDFom), assayed with a heat-stable amylase and acid detergent fibre
134 (ADFom), expressed exclusive of residual ash according to Van Soest et al. (1991) (Table 2).
135 Samples were also collected for the determination of FA profile according to the method of O'
136 Fallon et al. (2007) (Table 2).

137 *2.3.2. Milk chemical composition*

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

143 Fat corrected milk (FCM) in 6%:

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

145 Energy corrected milk (ECM) yield:

146 $ECM = \text{milk yield (kg/d)} \times (0.071 \times \text{milk fat concentration (\%)} + 0.043 \times \text{milk protein concentration}$
 147 $(\%) + 0.2224)$.

148 2.3.3. Fatty acid (FA) determination

149 Blood plasma fatty acid (FA) analysis was carried out in individual samples following the
 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 \times 0.25 mm i.d. with 0.20 μ m film thickness, Agilent) and a flame ionization detector (FID) was
 154 used. The steps and the conditions adopted in the method are comprehensively described in
 155 Christodoulou et al. (2021). The identification and quantification of each observed peak was
 156 followed using a 37 component FAME mix standard (Supelco, Sigma-Aldrich Co., St. Louis, MO,
 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
 160 (Fluka, Sigma Aldrich Co., St. Louis, MO, USA). The different groups of FA as well as the indexes
 161 were calculated as:

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

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}

165 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-}
 166 11 + C_{18:1 cis-9}

167 Polyunsaturated Fatty Acids (PUFA) = $C_{18:2 \text{ cis-9, trans-11}} + C_{18:2 \text{ n-6 cis}} + C_{18:2 \text{ n-6 trans}} + C_{18:3 \text{ n-3}} +$
 168 $C_{18:3 \text{ n-6}} + C_{20:3 \text{ n-3}}$

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: $TI = (C_{14:0} + C_{16:0} + C_{18:0}) / (0.5 \times MUFA) + (0.5 \times \omega\text{-6 PUFA}) +$
 174 $(3 \times \omega\text{-3 PUFA}) + (\omega\text{-3 PUFA} / \omega\text{-6 PUFA})$ as described by Ulbricht and Southgate (1991).

175 The health promoting index (HPI) as: $HPI = (\omega\text{-6 PUFA} + \omega\text{-3 PUFA} + MUFA) / (C_{12:0} + 4 \times C_{14:0}$
 176 $+ C_{16:0})$

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

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
 187 total antioxidant capacity is provided in Tsiplakou et al. (2017c). Finally, regarding the oxidative
 188 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, D_i the effect of dietary treatment ($i = 4$),
 210 S_j 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.

212 In addition, data for milk FA profile as well as antioxidant enzyme activity, antioxidant capacity,
213 and oxidative stress biomarkers were analyzed using also GLM for ANOVA, considering S as a
214 repeated measure, with fixed effects the D (CON vs SP5 vs SP10 vs SP15), S (15, 30, 45, 60 days),
215 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_j the effect of sampling day ($j = 4$), A_k is the animal's random effect, and $(D \times S)_{ij}$ 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

223 **3. Results**

224 *3.1. Daily nutrients intake*

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

230 *3.2. Milk yield and its chemical composition*

231 The dietary inclusion of SP in ewes' diets induced only a numerical increase in milk yield,
232 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}
 235 in the blood plasma of SP15 compared with the CON ewes was found ($P = 0.090$). On the other
 236 hand, the proportions of C_{18:0} in the SP5 ($P=0.001$), the C_{18:1 cis-9} in both SP10 and SP15 ($P=0.013$)
 237 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}
 238 ($P<0.001$) 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
 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}
 240 *cis* ($P=0.033$) in the blood plasma of SP5 and SP15 ewes respectively was observed. The
 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

244 The concentrations of SCFA increased in the milk of SP5 and SP10 fed ewes compared with
 245 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
 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.
 248 The inclusion of SP in ewes' diets did not affect the proportions of LCFA and MUFA in ewes'
 249 milk, although increased the C_{22:0} ($P=0.006$) and C_{18:1 trans} FAs ($P<0.001$) contents which belong
 250 to the respective FAs groups. The SP dietary supplementation of ewes enhanced the PUFA
 251 ($P=0.027$) and ω -3 ($P=0.010$) contents in their milk, but the results were significant only for the
 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
 254 of the C_{18:3 n-3} content in the milk of SP15 fed ewes ($P=0.054$) explain these findings.

255 Additionally, the highest inclusion level of SP (SP15) tended to reduce the AI ($P=0.093$) and
 256 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

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

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

294 *4.2. Milk and blood plasma FA profile*

295 The incorporation of SP in cows' diet did not change the proportions of C_{14:0}, and C_{16:0} in their
296 milk (Manzocchi et al., 2020), in contrast to what was observed for the C_{16:0} and consequently
297 MCFA content in ewes' milk. A significant decline in the proportion of C_{14:0} in the milk of SP-fed
298 cows at the 15th experimental day has been found, but this difference disappeared at the following
299 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

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).

326 4.3. Oxidative status of both organism and milk

327 The antioxidant properties and therapeutic effects of SP are due to its proteins, polysaccharides,
328 PUFA, vitamins, carotenoids, and other bioactive compounds (phenols, chlorophyll, etc.) with
329 antioxidant action (Liestianty et al., 2019; Han et al., 2021). Several *in vitro* studies have shown
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 H₂O₂ 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
335 from the *in vivo* studies are also in the same line. More specifically, the MDA content and the lipid
336 hydroperoxides decreased while the activities of SOD, GSH, and GST increased in the serum of
337 chronic obstructive pulmonary disease patients that were receiving SP (Ismail et al., 2014).
338 Accordingly, SP consumption enhanced the activities of GSH-Px, GSH, and GR and inhibited the
339 lipids peroxidation in the liver of rabbits, which were previously fed with a high-cholesterol diet
340 (Kim et al., 2010). A rise in the blood GSH activity and a decline in the serum MDA content was
341 found in fattening lambs when they received SP at a rate of 1 g/ 10 kg BW/day (El-Sabagh et al.,
342 2014).

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

349 oxidative stress. In accordance with our findings, an increase in the activities of GSH-Px and SOD
350 by 240 and 60% respectively was shown in healthy rats treated with SP (Guldass et al., 2021). These
351 findings might show the beneficial effects of SP, in animal's organism to meet future challenges
352 including stressors factors. Moreover, since the most intense effects of SP in the oxidative balance
353 of ewes' organism were observed with the highest supplementation dose, its dietary inclusion level
354 needs to be defined in relation to animals' physiological stage and conditions to ensure its
355 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
358 (Beheshtipour et al., 2012; Barkallah et al., 2017). Indeed, the β -carotene content of cows' milk
359 increased even its total oxidative capacity did not change when soybean was partially substituted
360 by SP (5%) in a hay-based diet (Manzocchi et al., 2020). On the other hand, an improvement of
361 the total antioxidant capacity, determined by DPPH and FRAP methods, in yogurts in which SP
362 powder was added at 0.25% has been observed (Barkallah et al., 2017). Moreover, in accordance
363 with our results a rise in SOD activity and a decline in the PC content of goats' milk was found,
364 when the animals consumed 6.18 g of low-fat *Chlorella vulgaris* daily (Tsiplakou et al., 2017b).
365 It is well documented that SOD is the first line of defense against ROS, and the first enzyme to
366 convert oxygen radicals to peroxides. In sequence, CAT and GSH-Px are involved in the
367 detoxification of H_2O_2 , and CAT is secondarily involved in removing the peroxides and converting
368 them into O_2 (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.

371

372 **5. Conclusion**

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

378

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383 **CRediT authorship contribution statement**

384 C. Christodoulou: Investigation, Data curation, Methodology, Formal analysis, Visualization,
385 Writing - original draft. B. Kotsampasi: Participated in the experiment; V. Dotas: Participated in
386 the experiment; M. Simoni: Investigation; F. Righi: Methodology, editing the draft. E. Tsiplakou:
387 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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- 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*.

560 Table 2

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)				
	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

564 ^a CON = control treatment.565 ^b SP = *Spirulina*.566 ^c DM = dry matter.567 ^d CP = crude protein.568 ^e EE = ether extract.569 ^f aNDFom = ash free neutral detergent fiber.

570 g ADFom = acid detergent fiber.

571 Table 3
 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 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
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
C _{18:2 n-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-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.

577 ^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.

580 ^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.

583 ^e DM = dry matter.

584 ^f CP = crude protein.

585 ^g EE = ether extract.

586 ^h aNDFom = ash free neutral detergent fiber.

587 ⁱ ADFom = acid detergent fiber.

588 Table 4
 589 Milk yield and chemical composition from ewes fed diets (CON, SP5, SP10, SP15) with different levels of *Spirulina* (5, 10, and 15
 590 g of concentrate) throughout the experimental period (0, 15th, 30th, 45th, and 60th experimental days)
 591

	Dietary treatment (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	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

592
 593 Means with different superscript letters (A, B, C, D) between sampling time points differ significantly.

594 ^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*.

596 ^b SEM: Standard error of the means.

597 ^c Effect: The dietary treatment (D), sampling time (S), and the interaction between dietary treatment×sampling time (D×S) effects were
 598 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.

603 Table 5

604 The mean individual fatty acids (FA) (% of total FA) in the blood plasma of ewes fed diets (CON, SP5, SP10, and SP15) with
 605 different levels of *Spirulina* (5, 10, and 15 g of concentrate) throughout the experimental period (15th, 30th, 45th, and 60th
 606 experimental days)
 607

Fatty Acids	Dietary Treatments (D) ^a					Sampling Time (S)					Effect ^c		
	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-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	1.81 ^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

608

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).

611 ^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 ° 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.

617 Table 6

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)
 621

Fatty Acids	Dietary treatment (D) ^a					Sampling time (S)					Effect ^c		
	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	3.97 ^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-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-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-9, trans-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-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	24.35 ^B	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

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).

625 ^a CON = control treatment; SP5 = dietary treatment with 5 g *Spirulina*; SP10 = dietary treatment with 10 g *Spirulina*; SP15 = dietary treatment626 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.

631 Table 7

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 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
GST ^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

636

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

638 ^a CON = control treatment; SP5 = dietary treatment with 5 g *Spirulina*; SP10 = dietary treatment with 10 g *Spirulina*; SP15 = dietary treatment
 639 with 15 g *Spirulina*.

640 ^b SEM: Standard error of the means.

641 ^c Effect: The dietary treatment (D), sampling time (S), and the interaction between dietary treatment×sampling time (D×S) effects were
 642 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 ^e CAT: Catalase.

646 ^f GSH-Px: Glutathione peroxidase.

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