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Effect of a weight loss diet with or without Spirulina supplementation on serum lipids and antioxidant capacity of overweight dogs

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Obesity is a major health issue in dogs associated with disturbances in lipid metabolism and oxidative stress. Spirulina has been shown to have hypolipidemic and antioxidant effects in various animal species. No such data regarding dogs are available, however. The present study aimed to investigate the effect of a therapeutic high-protein, high-fiber weight loss diet, with or without Spirulina supplementation, on biochemical parameters of overweight dogs, with particular reference to serum lipids and plasma antioxidant capacity. Thirty-two dogs completed a double-blind randomized placebo-controlled trial in which they received either Spirulina (S) or placebo (P) tablets in a body weight-dependent amount for 12 weeks; at the same time, both groups were fed the same calorie-restricted diet. Dogs were weighed weekly and calorie restriction was adjusted accordingly to ensure a 1% body weight loss per week. Blood samples were collected at baseline (T0), after 6 weeks (T1), and after 12 weeks (T2). No difference in body weight loss (S: -11.9 ± 0.8%, P: -10.6 ± 0.8%, $p = 0.229$) was detected between groups at T2. After 6 weeks and an average weight loss of around 6% (S: -6.7 ± 0.6%, P: -5.9 ± 0.6, $p = 0.276$), significant reductions of serum total cholesterol, glucose, alkaline phosphatase, paraxonase-1 (all $p < 0.0001$) and gamma-glutamyltransferase ($p < 0.018$) were observed in both groups, regardless of supplementation. Plasma antioxidant capacity increased significantly in both groups at T2 ($p = 0.0003$). Serum triglycerides decreased significantly from T0 to T1 in the Spirulina group ($p < 0.0001$) but not in the placebo group ($p = 0.28$); as for the difference between groups, a non-significant trend ($p = 0.098$) was detected. A significantly higher percentage of dogs ($p = 0.028$) in the Spirulina group achieved a serum triglycerides reduction > 15% compared to baseline at T1 and > 30% at T2. A treatment effect ($p = 0.0416$) was found for bilirubin, which decreased only in the Spirulina group. In conclusion, a weight loss of around 6% achieved with a high-protein, high-fiber hypocaloric diet is sufficient to induce significant positive metabolic effects and improve lipid, glucose, and liver enzyme values. Plasma antioxidant capacity was tested in dogs undergoing a weight loss program for the first time, demonstrating that overweight individuals are in a deficient status and that a weight loss of around 10% is able to restore values comparable to those of healthy individuals. The results of this study suggest that Spirulina may manifest a hypotriglyceridemic effect in dogs, even if further research is needed to infer causation. The role Spirulina that supplementation plays in bilirubin metabolism and its related beneficial effect is also worth exploring.

Abbreviations

ALP	Alkaline phosphatase
ARRIVE	Animal Research: Reporting of In Vivo Experiments
BAP	Biological antioxidant potential
BCS	Body Condition Score
BIL	Bilirubin

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BW	Body weight
CHO	Total cholesterol
CONSORT	Consolidated Standards of Reporting Trials
DM	Dry matter
EDTA	Ethylenediaminetetraacetic acid
GGT	Gamma-glutamyltransferase
GLU	Glucose
HDL	High-density lipoprotein cholesterol
IBW	Ideal body weight
LDL	Low-density lipoprotein cholesterol
LS Means	Least squares means
ME	Metabolizable energy
OPBA	Organismo preposto al benessere degli animali (Body for the Protection of Animals)
P	Placebo
PHO	Phosphorus
PON-1	Paraoxonase-1
S	Spirulina
SE	Standard error
TG	Triglycerides
VTUH	Veterinary Teaching University Hospital

Obesity is the most common nutritional disorder in humans, dogs and cats, leading to both reduced life expectancy and decreased quality of life[1–4]. Obesity leads to a chronic systemic inflammatory state and to increased oxidative stress[1,5,6], and is linked to increased risk of several disorders that include hyperlipidemia[7,8], as well as insulin resistance and hyperglycemia[9].

Spirulina (*Arthrospira platensis*) is a filamentous cyanobacterium often called microalga known for its high content of essential amino acids, vitamins, chlorophylls, carotenoids, and phycobiliproteins[10]. These bioactive compounds are linked to numerous health benefits when Spirulina is used as a functional food that have been investigated in humans in recent years[11]. These benefits include hypolipidemic[12,13], antioxidant[14]and anti-inflammatory[15]effects. These properties may make Spirulina a suitable dietary supplementation for overweight individuals, even if dietary intervention promoting weight loss is the cornerstone approach[16]. Some studies conducted on people in the last decade suggest that Spirulina may also improve lipid profile and even aid weight loss[13,17,18]. The study designs adopted so far have differed widely in terms of dosage administration, intervention duration, and target individuals, however, and clinical randomized controlled trials on humans are still scarce[11]. Data on the nutraceutical supplementation of Spirulina in dogs are fewer still, even if increasing amounts of Spirulina tablets have recently been shown to be palatable and well-tolerated by dogs[19]and dry pet food containing 0.2% of spray-dried Spirulina has been observed to have immune-stimulating activity[20]. Apart from these studies, to the authors' knowledge, the use of Spirulina in dogs is remains unexplored.

The present study aimed to investigate the effect of a 12-week weight loss program (ensuring a 1% BW loss per week), during which dogs were fed a high-protein, high-fiber diet with or without Spirulina supplementation, on biochemical parameters of overweight dogs (BCS $\geq$ 7/9); in particular, we hypothesized that the 12-week weight loss diet would result in improved serum lipid profile and antioxidant capacity, and that such improvement would have been faster (detectable already at the midpoint) and greater if the diet was supplemented with Spirulina.

Results

Study population

Of the 136 dogs assessed for eligibility, 40 were recruited and randomized into two groups of 20 subjects each. The study protocol was completed compliantly by 32 of the 40 recruited dogs, 16 per group; dogs diagnosed with hypothyroidism at T1, dogs that had taken medication with hypolipidemic effects during the study period, and dogs that failed to meet compliancy (consuming less than 90% of the tablets over the 12 weeks of the trial) were excluded from per-protocol analysis (Fig. 1). Of these 32, all neutered, there were 8 males (Spirulina group: 1 Labrador Retriever, 1 Golden Retriever, 1 Flat Coated Retriever and 1 mixed breed; placebo group: 2 Labrador Retriever, 2 mixed breed). There were 24 females (Spirulina group: 6 mixed breed, 2 Labrador Retriever, 1 Golden Retriever, 1 Beagle, 1 Border Collie, and 1 Samoyed; placebo group: 4 mixed breed, 4 Golden Retriever, 2 English Cocker Spaniel, 2 Hovawart). The characteristics of the dogs in the study, divided into Spirulina and placebo groups, are shown in Table 1.

Weight loss

At T1, the mean BW loss was $-6.7 \pm 0.6\%$ in the treatment group, $-5.9 \pm 0.6\%$ in the placebo group. There was no significant difference in BW loss between groups ($p = 0.276$). Neither did the mean BW loss after 12 weeks (T2) differ between groups ($p = 0.229$), and was $-11.9 \pm 0.8\%$ in the treatment group and $-10.6 \pm 0.8\%$ in the placebo group (Fig. 2a). There was no difference between the two groups in the energy intake assumed by dogs to attain the targeted weekly BW loss either at T1 (62.0 ± 1.7 kcal*$BW^{0.75}$ in the treatment group, 64.7 ± 1.6 kcal* $BW^{0.75}$ in the placebo group, $p = 0.226$) or at T2 (55.9 ± 2.3 kcal*$BW^{0.75}$ in the treatment group, 58.2 ± 2.3 kcal*$BW^{0.75}$ in the placebo group, $p = 0.226$) (Fig. 2b).

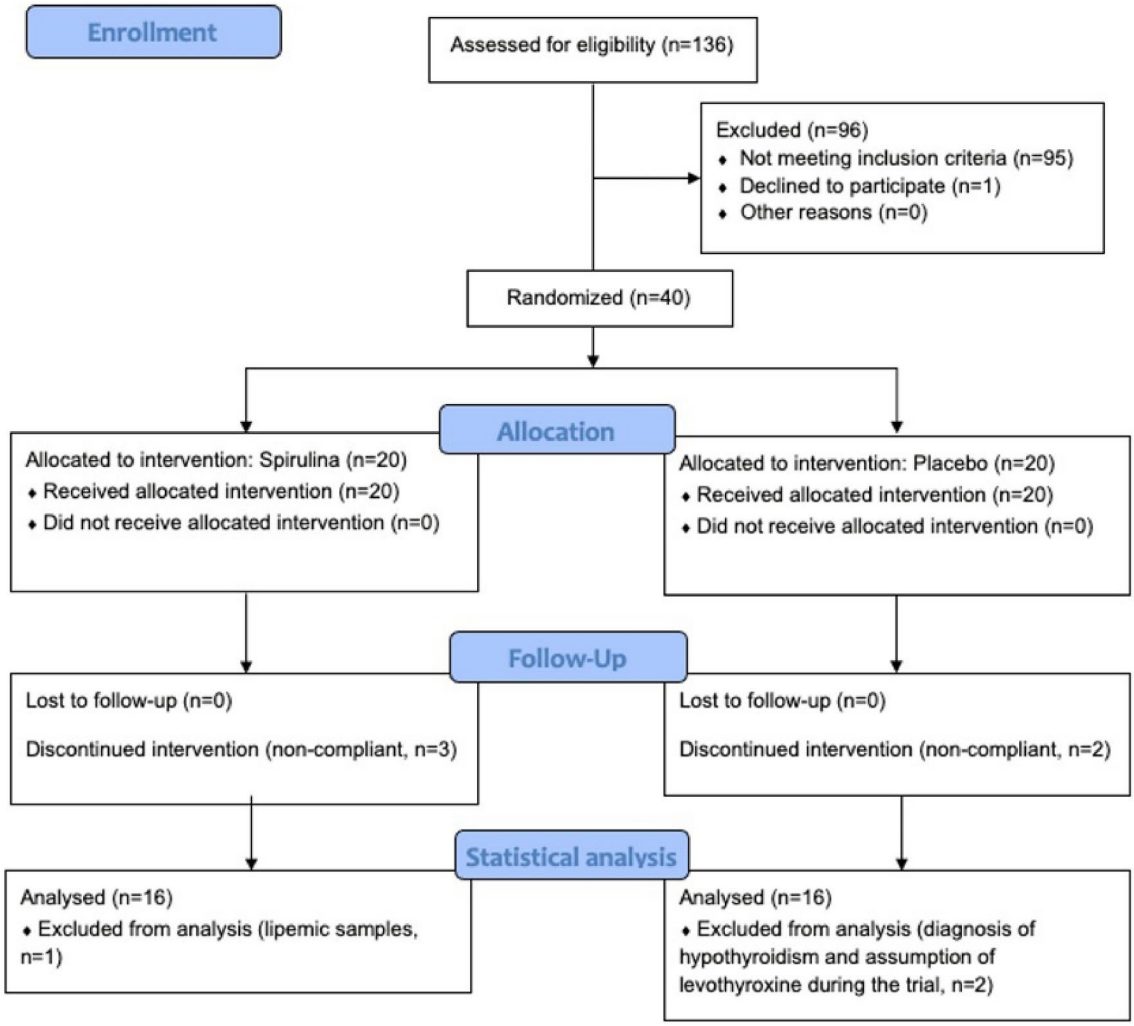

Fig. 1. Consort flow chart of study.

Dog characteristics	Spirulina group (n = 16)	Placebo group (n = 16)
Male, n	4	4
Female, n	12	12
Age, years (mean ± SD)	6.0 ± 2.4	6.9 ± 2.8
Ideal Body Weight, kg (mean ± SD)	31.9 ± 12.3	31.6 ± 11.1
Body Condition Score 7/9	10	11
Body Condition Score 8/9	5	5
Body Condition Score 9/9	1	0

Table 1. Baseline characteristics of dogs assigned to Spirulina and placebo groups that completed the study.

Primary outcome: serum lipids

Table 2 shows the average serum triglycerides (TG) and cholesterol (CHOL) in the 40 dogs at the time of recruitment in their overweight or obese condition. Serum TG and CHOL over the maximum range was observed in 47.5% and 35% of the population, respectively.

A significant reduction of serum triglycerides was found between T0 and T1 in the treatment group ($p < 0.0001$) but not in the placebo group ($p = 0.188$). The same was true for the difference between T0 and T2, in which the average serum triglycerides decreased significantly after 12 weeks of weight loss from 104.2 ± 8.9 to 65.9 ± 8.9 mg/dl in the treatment group ($p < 0.0001$) and from 83.6 ± 8.7 to 65.3 ± 8.7 mg/dl in the placebo group ($p = 0.28$) (Fig. 3). Overall, the time*treatment effect showed a non-significant trend on serum triglycerides levels ($p = 0.098$).

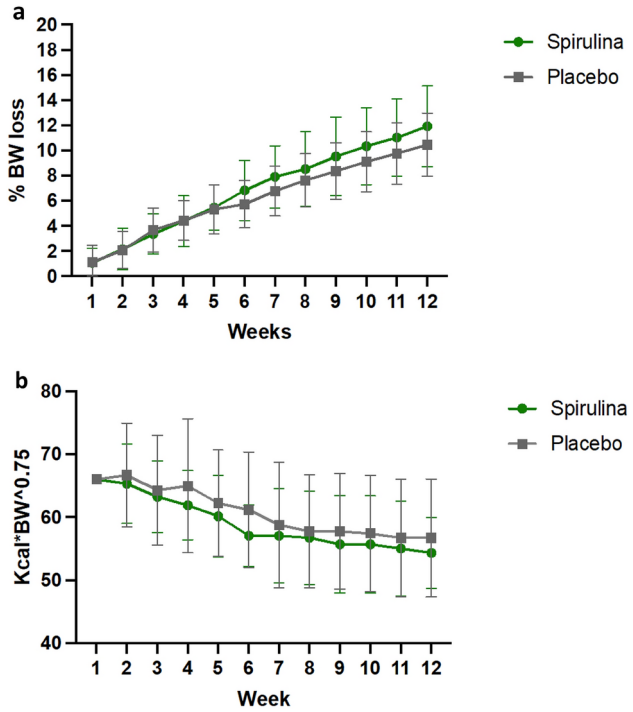

Fig. 2. **(a)** Percentage of weekly BW (body weight) loss in dogs in Spirulina and Placebo groups during the 12 week-experimental period ($p = 0.229$). **(b)** Weekly energy intake (Kcal*ideal metabolic body weight) assumed by dogs in Spirulina and Placebo groups during the 12 week-experimental period to attain the targeted weekly body weight loss.

	Within range values	Above range values	Normal range mg/dl
TG	52.5% (21/40) [65.0 ± 11.8 mg/dl]	47.5% (19/40) [153.5 ± 57.2 mg/dl]	44–80
CHOL	65.0% (26/40) [222.4 ± 30.9 mg/dl]	35.0% (14/40) [398.6 ± 123.7 mg/dl]	182–276

Table 2. Percentage of individuals ($n = 40$) falling within and above serum triglycerides (TG) and cholesterol (CHOL) range values at T0.

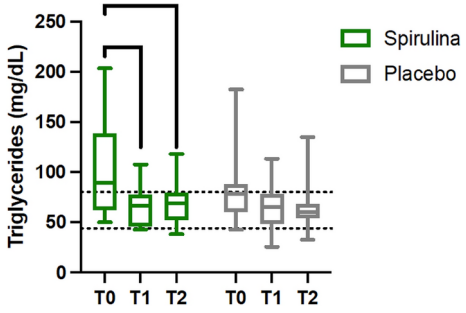

Fig. 3. Change from baseline in serum triglycerides in the Spirulina and placebo groups at T1 and T2. The dashed lines show the minimum and maximum values of the physiological range. **** indicates $p \leq 0.0001$.

Table 3 shows the percentage of individuals achieving a reduction of triglyceride levels higher than the two thresholds of −15% and −30% in the two groups.

A significant reduction of HDL and LDL cholesterol levels was found in both groups at T1 compared to T0 ($p < 0.0001$), whereas no differences between groups were detected (Table 4). NEFA were not affected by time ($p = 0.243$) or treatment ($p = 0.801$).

	T1 Spirulina	T1 Placebo	p-value	T2 Spirulina	T2 Placebo	p-value
TG reduction of at least 15%	81.3%	43.8%	***0.028***	75.0%	68.8%	0.694
TG reduction of at least 30%	50.0%	37.5%	0.476	56.3%	18.8%	***0.028***

Table 3. Percentage of individuals achieving a reduction of at least 15% and a reduction of at least 30% of serum triglycerides (TG) at T1 and T2 compared to baseline values in the two groups.

	Spirulina			Placebo			
Outcomes	T0	T1	T2	T0	T1	T2	p-value
CHO (mg/dl)	289 ± 16.8	232 ± 16.8	227 ± 16.8	231 ± 16.4	193 ± 16.4	182 ± 16.4	0.3844
HDL(mmol/L)	5.40 ± 0.27	4.76 ± 0.27	4.76 ± 0.27	4.70 ± 0.27	4.11 ± 0.27	4.02 ± 0.27	0.9107
LDL (mmol/L)	1.93 ± 0.28	1.11 ± 0.28	0.99 ± 0.28	1.19 ± 0.27	0.71 ± 0.27	0.59 ± 0.27	0.2090

Table 4. Mean values of serum total CHO (cholesterol), HDL (high-density lipoprotein cholesterol) and LDL (low-density lipoprotein cholesterol) at all time points. Data are expressed as LSmean ± SE. The p-value refers to the time*treatment effect of the ANOVA repeated mixed model.

	Spirulina			Placebo				
Outcomes	T0	T1	T2	T0	T1	T2	p-value	Normal range
GLU (mg/dl)	102 ± 3.60	88.9 ± 3.60	85.9 ± 3.60	103 ± 3.54	89.4 ± 3.54	86.8 ± 3.54	0.9857	87–106
GGT (UI/L)	5.66 ± 0.49	4.78 ± 0.49	4.73 ± 0.49	7.37 ± 0.49	5.83 ± 0.49	5.26 ± 0.49	0.3797	4.17–8.48
ALP (U/L)	272 ± 47.5	139 ± 47.5	125 ± 47.5	223 ± 46.6	123 ± 46.6	114.2 ± 46.6	0.7461	87–326
PHO (mg/dl)	4.16 ± 0.22	3.53 ± 0.22	3.46 ± 0.22	4.21 ± 0.22	3.61 ± 0.22	3.85 ± 0.22	0.2728	3.54–5.09
BIL (mg/dl)	0.35 ± 0.02^{a}	0.23 ± 0.02^{b}	0.27 ± 0.02^{b}	0.26 ± 0.02	0.24 ± 0.02	0.25 ± 0.02	***0.0416***	0.18–0.37
PON-1 (U/L)	236 ± 9.62	224 ± 9.62	211 ± 9.62	211 ± 9.42	201 ± 9.42	193.3 ± 9.42	0.762	N/A

Table 5. Mean values of secondary outcomes – glucose (GLU), gamma-glutamyltransferase (GGT), alkaline phosphatase (ALP), phosphorus (PHO), bilirubin (BIL), paraoxonase-1 (PON-1) – at all time points. Data are expressed as LSmean ± SE. Data with different superscript letters are significantly different at $p < 0.05$. The p-value refers to the time*treatment effect of the ANOVA repeated mixed model.

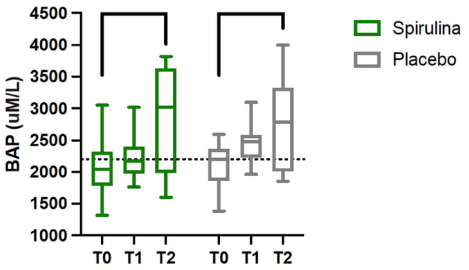

Fig. 4. Change in BAP (plasma antioxidant capacity) values from baseline at T1 and T2 in the Spirulina and placebo groups. The dashed line shows the threshold above which BAP values are considered optimal. * indicates $p \leq 0.05$.

Secondary outcomes

Serum GLU ($p < 0.0001$), GGT ($p = 0.0016$), ALP ($p < 0.0001$) and PHO ($p < 0.0001$) decreased significantly from T0 to T1 in both groups. PON-1 levels decreased in both groups from T0 to T2, and a positive correlation was found between PON-1 and HDL levels ($r = 0.81$, $p < 0.0001$). A time*treatment effect was found for bilirubin levels (BIL), which were seen to be reduced only in the Spirulina group (Table 5). The antioxidant capacity (BAP test) increased significantly from T0 to T2 regardless of treatment (Fig. 4). No other significant variation was found in the analysis performed.

Discussion

In the present study, dogs lost BW at the established average rate of about 1% of their IBW per week consistent with weight loss rates observed in previous studies[21–24]. However, in the period between T1 and T2, weight loss

was slightly lower than the loss between T0 and T1, and a higher average calorie restriction was required than in the first 6 weeks. This is consistent with the findings of other studies in which weight loss rate decreased over time when the caloric restriction remained the same[25,26]. For this reason, dog BW must be monitored and caloric amount must be adjusted to the weight loss rate in order to achieve successful weight loss[27].

This study showed that even a weight loss of 6% is sufficient for multiple positive effects on animal health to be obtained. Such weight loss was enough to achieve significant decreases in TG, CHO, HDL, LDL, GLU, ALP, and GGT in 6 weeks regardless of Spirulina supplementation. These findings are consistent with previous studies, even if similar reductions were observed after at least 2 or 3 months of diet[28–30]. All dogs at T1 still had a BCS > 6, and were therefore still overweight or obese. The relatively small weight loss achieved was sufficient to determine hypolipidemic, hypoglycemic and hepatoprotective effects, however. This fact should be taken into account in obesity management because otherwise the goal of reaching the ideal BW can often be perceived as too distant and difficult to achieve, thus demotivating owners of overweight dogs, who become more likely to fail weight loss programs[25]. For dogs with BCS of 7 or higher, a weight loss of at least 20% is required. Explaining the significant manifestation of health benefits after a 6% BW loss can motivate owners to begin a weight loss program. It is also well known that as determined by force plate analysis[31], a 6% BW also improves mobility in obese dogs with osteoarthritis. No changes in dog activity levels were applied in the present study; therefore, the weight loss was the consequence of dietary caloric restriction alone. In a randomized trial, dietary caloric restriction was proven more effective than physical activity in controlled weight loss in overweight pet dogs[32]. Physical activity can also bring other benefits desirable as part of a weight loss regimen, such as minimizing lean tissue mass loss[21]. The previous work has, however, suggested that introducing meaningful amounts of physical activity into the daily routine of an obese dog can be a challenge for owners and dogs alike[32]. The beneficial effect on metabolic status of a 6-week dietary caloric restriction alone should be emphasized to owners of obese dogs in order to prompt them to begin the diet. The findings of this study reinforce the idea that a partial weight loss program might be a valid initial target for some dogs, provided that the final target remains to reach the ideal weight[23,26].

PON-1 levels decreased from T0 to T2 in both groups and were also positively correlated with HDL levels. PON-1 is an enzyme associated with HDL that protects LDL and HDL from peroxidation and reduces the production of proinflammatory mediators[33]. PON-1 is considered a negative acute phase protein, and in human medicine the decrease in PON-1 activity in serum has been demonstrated in various diseases that include diabetes mellitus, chronic renal failure, and inflammatory bowel disease[33]. PON-1 circulating concentration was reported to be lower in human obesity, although PON-1 levels between obese and lean humans were not seen to be different in another study[34,35]. No changes in PON-1 levels have been observed in obese cats before and after weight loss, although initially lower PON-1 activity was ascribed to the failure of the weight-loss program[36]. In our study instead, a significant PON-1 reduction was detected after the weight loss. Being a negative acute phase protein, this decrease could be misinterpreted as a sign of inflammation. Instead, this finding could be explained by the contemporary reduction of HDL, given that PON-1 binds to HDL[37]. To our knowledge, this is the first study to examine PON-1 expression in canine obesity before and after weight loss.

In this study, a weight loss of more than 10% achieved in 12 weeks was a requisite in detecting a significant improvement in the dogs' antioxidant capacity, which was measured by the BAP test widely adopted to assess antioxidant status in dogs and other mammals[38–40]. Values above > 2200 µmol/L are considered optimal, while values < 2000 µmol/L indicate a deficiency status[41]. The vast majority of dogs were in the latter status at the start of this study, with BAP levels similar to those of dogs with mast cell tumors or dogs monitored immediately after intense exercise[42,43]. Although improvement was not significant after 6 weeks of diet, by the end of the study, plasma antioxidant potential rose considerably in most dogs in both groups to levels comparable to those of healthy dogs at T2[38,41]. These findings clearly show that the antioxidant capacity of overweight dogs is compromised, and that without any additional supplementation, weight loss alone is sufficient to improve antioxidant status. This demonstrates the importance of focusing first of all on dietary caloric restriction when aiming to improving the condition of chronic oxidative stress of overweight dogs. It must be borne in mind that the putative antioxidant effect of Spirulina could in this study have been masked due to the effect of weight loss on the improvement of plasma antioxidant capacity. For such reason, further studies, either on overweight dogs not undergoing a weight loss program at the same time or on dogs with compromised antioxidant status due to other reasons, are necessary to draw definitive conclusions on the antioxidant effects of Spirulina in dogs.

The biochemical profile of the dogs at T0 confirmed that hypertriglyceridemia is an important serum biochemistry finding in overweight dogs[44,45]. Given the absence of statistical significance in the time-to-treatment effect, our study does not permit causal inference on the effect of Spirulina supplementation on serum triglycerides. The trend observed and the differences between the two groups in the percentage reduction of triglycerides, however, suggest that Spirulina supplementation may be involved in the modulation of serum triglycerides, and from a clinical standpoint, the difference in the reduction of triglyceride levels appears substantial, with a significant higher number of dogs in the Spirulina group achieving a reduction of TG levels ≥ 15% at T1 and ≥ 30% at T2 compared to baseline values. The threshold of 30% was chosen based on a previous study that reported an average reduction of TG levels of 29% after a 3 months weight loss program[46]and based on a study that compared triglycerides levels in dogs with different BCS, which showed that average triglycerides levels of dogs with BCS 8–9 are 15% higher than those of dogs with BCS 6–7 and 29% higher than those of dogs with BCS 4–5, implying a correlation of + 15–30% TG levels for every 1–2 additional points of BCS[47].

The final concentration of triglycerides at T2 was the same in both groups, however, whereas the average triglyceride levels at T0 were 20 mg/dL higher in the Spirulina group than in the placebo group. Despite the randomization and stratification schemes, a treatment allocation that is unbalanced with respect to one or more baseline characteristic can still be obtained, especially when the sample size is not particularly large. In this

study, the sample size was substantial and larger than most studies published on canine obesity and nutraceutical supplementations[48–52], even if not as high as in certain human clinical trials[53]. While the triglycerides baseline difference between the groups is clinically not negligible, in the authors' interpretation, it is also not wide enough to make the two groups clearly not homogeneous. Still, it cannot be excluded that the reason why average triglyceridemia decreased from 104.2 ± 8.9 to 65.9 ± 8.9 mg/dl in the Spirulina group group (−36.8%) and from 83.6 ± 8.7 to 65.3 ± 8.7 mg/dl in the placebo group (−21.8%) is because the values at T0 in the Spirulina group were 19.7% higher than the values at T0 in the placebo group. On the other hand, we are not aware of any study that demonstrates that as triglyceridemia is higher, there is a greater possibility that the reduction is higher in the same time frame. Additionally, the placebo group could have sustained a reduction equal to that of the Spirulina group while still maintaining triglycerides value in the normal range. It should also be noted that the aim of this study was to test Spirulina in a sample of overweight dogs; however not all overweight dogs have necessarily high serum triglycerides concentrations. In this study seven dogs per each group at the per-protocol analysis had triglycerides values above the reference range at T0, with all seven in the Spirulina group and four out of seven in the placebo group having normalized triglycerides concentration inside the reference range at T2. While this was an interest finding, we could not reduce the statistical analysis to just the dogs with values above reference at T0 because this would have meant to consider a total of just fourteen dogs, which according to our sample size calculation was not enough to carry out a statistical analysis with sufficient statistical power.

Based on the results of this study and observations made in other species[12,54], we hypothesize that Spirulina may be a useful supplementation in dyslipidemic dogs with primary or secondary hypertriglyceridemia. Spirulina has also been shown to reduce total cholesterol and LDL concentrations and elevated HDL levels in humans[12]. No effect of treatment on these parameters was observed in this trial, however. The baseline condition – fewer dogs had high cholesterol at T0 compared to high triglycerides – and differences between humans and dogs in lipoprotein composition may provide a partial explanation.

Interestingly, a time-per-treatment effect was detected in serum bilirubin concentrations, which decreased significantly in the Spirulina group from T0 to T1 while remaining stable in the placebo group. The baseline values of bilirubin in the Spirulina group were closer to the upper limit of the physiological range than in the placebo group; while this may have allowed for the possibility of a higher reduction in the Spirulina group, the interpretation of the author is that the baseline difference was not clinically significant, since baseline levels were in the physiological range in both groups and since the placebo group could have sustained the same reduction that happened in the Spirulina group while maintaining bilirubin levels in the physiological range.

Among its other bioactive substances, Spirulina is rich in tetrapyrrolic compounds (phycobilins) closely related to the bilirubin molecule[55]. Phycobilins have been shown to be good substrates for biliverdin reductase, while phycobilin reduction gives rise to a set of compounds (phycorubins) that are structural analogs of bilirubin that appear likely to possess roughly comparable physiological activity[56,57]. Based on these findings, we can speculate that Spirulina supplementation may play a useful role in subjects with severe hyperbilirubinemia caused by hepatic diseases such as gallbladder mucocele , even if further research in dogs with bilirubin concentrations above the physiological range is required.

Spirulina supplementation might also be thought to induce iatrogenic Gilbert syndrome, a hereditary condition in humans and Southdown sheep in which plasma-unconjugated bilirubin levels remain mildly elevated throughout life[58,59]. Generally, Gilbert syndrome is a benign condition that has been associated with a reduced risk of cardiovascular disease, steatohepatitis, diabetes, and reduced lipoprotein oxidation and LDL concentration in humans[57,60–62]. This has prompted the suggestion that simulating Gilbert syndrome mild hyperbilirubinemia using compounds with structural analogy to bilirubin may have health benefits[63].

This study had multiple strengths, including randomization, allocation concealment, strict exclusion and inclusion criteria, a controlled weight loss that was modulated with a weekly intervention, a sample size based on power analysis and larger than many other studies on similar topics in dogs, and unlike many human studies on weight loss and nutraceuticals, the fact that all the subject were consuming the same diet without any difference besides the supplementation of Spirulina or placebo. Additionally, the study protocol allow for the absence of any ethical concern, since all the dogs recruited were privately-owned pets and, independently from which group they were randomized into, they benefited from a 12-week weight loss program.

This study had some limitations. Since Spirulina supplementation was contemporaneous with the weight loss diet, the effects of the diet may have overshadowed the potential nutraceutical effects of Spirulina. The clinical homogeneity of the baseline values of the primary outcome between the two groups is debatable, and therefore, the corresponding results are subject to interpretation. There was no adaptation period to the new diet, with the dietary change and the weight loss that started simultaneously. While this implies that the effect of improving the parameters may also have been due to the diet itself and not to weight loss, the diet used was similar to the high-protein diet used by Diez et al.[64]: in their study, they fed overweight dogs either a high-protein diet or a high-fiber diet during a weight loss program, and they observed decreases in both triglycerides and cholesterol when dogs were fed either of the 2 weight loss foods, indicating that these observed changes were not diet related but were directly related to weight loss. Still, the absence of an adaptation period represents a limitation because the fat content of the dogs' diet before the trial's beginning could have been different among different dogs, and so they could have benefited differently from the transition to a moderate-fat diet. The dogs were privately owned, and many consumed a variable diet with variable fat intake before the study, so establishing the exact fat content of their previous diet was not possible. The concentration of bioactive compounds potentially responsible for the beneficial effects of Spirulina vary in different products. The Spirulina tablets used in this study contained 16 mg/g DM of phycocyanin, which is believed to be the compound mainly responsible for Spirulina's hypotriglyceridemic and antioxidant effects[65]. In a previous study[19], the phycocyanin content of Spirulina from the same manufacturer was significantly higher (51 mg/g DM), a concentration in line with the average content (50–100 mg/g DM) of Spirulina flakes and powders available in the Italian market[66]. Variations in the content

of bioactive compounds in microalgae depend on different environmental conditions during their growth. This variability should always be taken in consideration when discussing the nutraceutical potential of ingredients like Spirulina because products marketed under the same name can have different nutrient and bioactive compound profiles that determine different nutraceutical effects. A lower content of bioactive compounds per gram of Spirulina can be compensated with a higher daily amount of supplementation. In a preliminary study carried out by our research group on healthy dogs, increasing daily amounts of Spirulina (from 0.04 g/kg/day to 0.19 g/kg/day) were administered to client-owned dogs, both males and females of different sizes and ages, and no adverse reactions were detected at any of the amounts tested[19]. The amounts used in this study were included in the 0.06–0.08 g/kg/day, so it would be possible to supplement higher amounts. However, many studies on humans on the nutraceutical properties of Spirulina do not entail a supplementation higher than 2 g per day, and in our study, 2 g was the daily amount provided to a 30 kg dog. Since dogs with different BCS were included in the study, the supplementation was based on ideal body weight to guarantee a standardization of the daily amount provided (otherwise two dogs with same ideal body weight but different BCS would have received different daily amounts). However, this entailed that the daily amount of Spirulina provided based on the initial body weight was lower, e.g., 2 g/ideal BW would translate into 1.7 g/initial BW for a 30 kg dog with 20% of extra body fat (BCS 7) or 1.5 g/initial BW for a 30 kg dog with 30% of extra body fat (BCS 8). These daily amounts are still comparatively equal or higher than those usually used for humans. Finally, dog physical activity levels were not monitored. Although owners were instructed to maintain usual physical activity levels, variations among different individuals in physical activity cannot be excluded, thus representing a covariate not accounted for in analysis.

Conclusion

In conclusion, this study showed that a 6% BW loss achieved in 6 weeks with a hypocaloric high-protein, moderate-fat, high-fiber diet is sufficient to obtain significant hypolipidemic effects in still overweight dogs, whereas a > 10% BW loss is required to improve the plasma antioxidant capacity to a level comparable to that of healthy dogs. Spirulina supplementation did not affect the weight loss rate, while it reduced endogenous bilirubin levels and may exert hypotriglyceridemic properties. Since weight loss has a substantial impact on animal metabolism, future research should focus on investigating the effect of Spirulina in dogs not undergoing a weight loss program. The role of Spirulina in bilirubin metabolism and its potential beneficial effect is also well worth exploring.

Material & methods

Trial design

This was a randomized, double-blind, placebo-controlled study conducted on overweight neutered client-owned dogs recruited among the clients of the Veterinary Teaching University Hospital (VTUH) of the University of Padova from February 2022 to June 2022. Dogs were randomly assigned to one of two parallel groups, in a 1:1 ratio, to receive either a Spirulina (*Arthrospira Platensis*) supplementation or a placebo for a period of 12 weeks, during which both groups were fed the same caloric restricted diet.

Eligible participants were neutered overweight dogs (BCS ≥ 7/9) aged between 2 and 12 years old.

Exclusion criteria included any history of chronic kidney, hepatic, cardiovascular, endocrine, autoimmune disease or cancer; any surgery (including neutering) done less than three months prior to the study; a history of infection, use of antibiotics, anti-diuretics, or glucocorticoids less than three months prior to the study; receiving drug therapy or complementary and alternative medicines that lowered the body weight (BW), blood lipid concentrations, blood glucose, or taking vitamins/minerals, functional foods or antioxidant supplements less than three months prior to the study; and lastly, a 5% or higher weight loss or any kind of controlled weight loss dietary regimen less than three months before the trial.

Participants were informed of the trial at the VTUH and through social media. Dogs were recruited in a two-step screening process: interested owners first completed an online pre-study questionnaire (Google Forms) that gathered data on their dog's signalment and clinical history. All applications were then individually screened and an interview with the owner and a clinical assessment with a physical examination at the VTUH was arranged for dogs who met the inclusion criteria. Each dog was enrolled as an individual experimental unit and only one dog per household could be enrolled. A unique study case number was assigned to each dog for the participant's identification.

The study protocol was approved by the Animal Welfare Committee of the University of Padova (OPBA, University of Padova, and Italian Ministry of Health, no. 62/2020) and followed CONSORT and ARRIVE guidelines[67]. The study was performed in accordance with all applicable national and international regulations. Each dog owner was required to sign an informed and written consent form prior to including their dog in the trial.

Intervention

Dogs enrolled in the study followed a weight loss diet with the aim of allowing them to lose 1% of their IBW per week for 12 weeks. At the beginning of the trial, all the dogs were provided with a diet containing 60% of the maintenance energy requirement (MER). The MER was calculated as 110 kcal ME (Metabolizable Energy) × ideal body weight (IBW)^0.75[68]. Each dog was then weighed weekly and initial caloric intake was adjusted according to variations of ± 5% to ± 10% to maintain the targeted weight loss rate.

In addition to the weight loss diet, the treatment group received Spirulina tablets. The control group received placebo tablets. The owners were instructed to administer all tablets to their dog with the day's first meal.

Based on previous research on dogs[19]and previous studies conducted in humans[12,13,69], an amount ranging between 0.06 g/kg/day and 0.08 g/kg/day was established for the present study. The following daily amounts based on the IBW of dogs were determined in consequence: 10–14 kg: 0.8 g/day (2 tablets); 15–20 kg: 1.2 g/day (3 tablets); 21–27 kg: 1.6 g/day (4 tablets); 28–35 kg: 2.0 g/day (5 tablets); > 35 kg: 2.4 g/day (6 tablets).

Since the tablets were not meant to be broken, and in order to avoid administering a dosage of higher than 0.08 g/kg/day, small-sized dogs (< 10 kg BW) were not recruited.

Adherence to the study protocol was verified weekly during the BW check and the overall number of tablets that had not been consumed by the dog during the week was quantified and reported by the owners. The compliance rate was set at > 90%, meaning that dogs that failed to consume at least 90% of the tablets specified during the study were considered non-compliant. The owners were asked to maintain the usual level of physical activity of their dog during the trial period.

Diet composition

Dogs were fed a high-protein, moderate-fat, high-fiber dry diet (Diusapet Alleva Care Obesity, Table 6). In order to administrate the proper amount of food to their dogs, owners were asked to weigh kibbles using a kitchen scale. Before the beginning of the trial, owners were also informed of the need to avoid feeding extra food or treats, and to use non-food-related techniques to reward their dogs. If they still wanted to use food rewards during training activities or play sessions, they were allowed to use only the kibbles provided for the trial, subtracting the amount used for such purposes from the daily amount.

Tablets composition and analysis.

Spirulina tablets (Livegreen S.r.l.) weighing 0.4 g were used in this study. Tablets of the same weight, size and color produced by the Department of Pharmaceutical and Pharmacological Sciences of University of Padua were used for the placebo.

The placebo contained microcrystalline cellulose (50%), cornstarch (47%), polybinylpyrrolidone K30 (1.5%), sodium starch glycolate (1.225%) and food colorant (0.275%). In order to obtain a green color without using green colorant of vegetal origin containing chlorophyll and other pigments that are also contained in Spirulina and might have interfered with the results of the study, a mixture of yellow (E102, E104), blue (E132, E133) and red (E124) colorants was adopted. Both Spirulina and placebo tablets were analyzed by the CNX Laboratory of the Department of Animal Medicine Productions and Health of the University of Padova for proximate analysis and fatty acid profile. Amino acid profiling and mineral quantification was performed by the La-Chi Laboratory, and pigment quantification using the same methods described in a previous research[19] was performed by the Photosynthesis and Plant Biotechnology Laboratory of the Department of Biology. Chemical composition of Spirulina and placebo tablets are available in Table 7.

Blood collection and analysis

Three blood samples (12 ml) from each dog were collected during the clinical trial at time of recruiting (T0), at day 42 (T1) and at day 84 (T2). After a 15 h-overnight fast, blood was collected from the jugular vein and placed into plastic tubes containing either K_3-EDTA or a coagulation accelerator for hematological and biochemical analysis, respectively.

The EDTA blood samples were analyzed using ADVIA 120 Hematology Systems – Siemens Healthcare equipped with Veterinary Multispecies Software version 3.18.0-MS. A blood smear was performed for each sample to confirm the ADVIA data. The hematological analyte analysis in this study included packed cell volume (PCV), platelet count (PLT), and leukocyte count (WBC), as well as the relative and absolute numbers of neutrophils, lymphocytes, monocytes, eosinophils, basophils, and large unstained cells (LUC) counts, and values for MPV, large platelet count, and platelet clumps (aggregates).

For serum biochemical analysis, coagulated samples were centrifuged (Labofuge 400, Heraeus Holding, Hanau, Germany) at 1750 g for 10 min at room temperature; serum was separated and immediately analyzed using a BT3500 automated wet chemistry analyzer. Normal and pathological internal quality controls were performed daily for the analyzer, whereas external control quality was performed once a month. The analytes analyzed were: glucose (GLU), calcium, magnesium, phosphorus (PHO), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), bilirubin, creatinine, urea, albumin, total protein, globulins, triglycerides, total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), non-esterified fatty acids (NEFA), glucose, creatine kinase, C-reactive protein (CRP) and paraoxonase-1 (PON-1). The plasma's biological antioxidant potential (BAP) was determined using a commercial kit (Diacron, Grosseto, Italy) following the manufacturer's

	Per 100 g DM	g/1000 kcal (ME)
Crude protein	42.65	119.3
Crude fat	11.14	31.2
Carbohydrate	30.54	85.4
Crude fiber	8.37	23.4
Ash	7.30	20.4

Table 6. Chemical composition of the experimental weight loss diet. Moisture content was equal to 6.47%; ME was equal to 334.5 kcal/100 g.

	Spirulina	Placebo
Moisture, %	4.5	8.48
Crude protein, %DM	65.85	2.63
Crude fat, %DM	9.83	1.74
Carbohydrate, %DM	13.15	60.19
Crude fiber, %DM	0.00	34.62
Ash, %DM	11.17	0.82
Saturated fatty acids, %DM	4.97	0.42
Monounsaturated fatty acids, %DM	1.10	1.15
Polyunsaturated fatty acids, %DM	3.77	0.17
Palmitic acid, %DM	4.27	0.27
Oleic acid, %DM	0.73	1.13
Linoleic acid, %DM	2.90	0.15
Gamma-linolenic acid, %DM	0.72	0.00
Eicosapentaenoic acid (EPA), %DM	0.00	0.00
Docasahexaenoic acid (DHA), %DM	0.00	0.00
Calcium, %DM	0.35	0.02
Potassium, %DM	2.53	0.01
Magnesium, %DM	0.41	0.03
Sodium, %DM	1.41	0.07
Phosphorus, %DM	1.60	0.01
Sulfur, %DM	0.80	0.02
Chlorophyll *a*, mg/g DM	4.84	0.00
Carotenoids, mg/g DM	0.82	0.00
Phycocyanin, mg/g DM	15.88	0.00
Allophycocyanin, mg/g DM	13.76	0.00
Phycoerythrocyanin, mg/g DM	5.08	0.00

Table 7. Chemical composition of Spirulina and placebo tablets; values are reported on dry matter (DM) basis.

instructions. The results were expressed in μmol/l of equivalent vitamin C used as an iron-reducing reference agent. The assay repeatability was expressed as intra- and inter-assay coefficients of variation that were 3.0% and 4.0%, respectively.

Outcomes

The primary outcome was represented by a change in serum lipid concentrations.

Secondary outcomes included serum parameters linked to the oxidative profile (biological antioxidant potential test, paraoxonase-1) and routine serum biochemical analysis; routine hematological analyses were also performed.

All blood parameters were assessed at baseline (T0), at midpoint (T1, day 42) and at the endpoint (T2, day 84). Dogs that had not reached at least 3% BW loss at T1 despite the caloric restricted diet were additionally submitted to a thyroid function test to exclude hypothyroidism.

Sample size

Sample size was calculated using a comparison of means test based on a study regarding the effect of antioxidant compound supplementation on serum triglycerides in overweight dogs[70]and a paired samples t-test based on a trial that investigated the use of Spirulina in overweight and obese human patients using triglycerides as primary outcomes[54]. This suggested a total of 19 and 11 dogs per group, respectively, when using an 80% statistical power with $\alpha = 0.05$. The final aim was therefore to recruit a total 40 dogs.

Randomization

Dogs were stratified into two levels by sex (males vs females) and breed (Golden and Labrador Retrievers vs other breeds), and then randomly allocated in a 1:1 ratio to either the Spirulina or the placebo group by block randomization. The randomization sequence was created using Sealed Envelope™ randomization software using a block size of 4.

Concealment and blinding

Both the Spirulina and the placebo were in tablet form and identical in appearance. They were prepacked in dark envelopes and consecutively numbered for each dog according to the randomization schedule by the investigator, who had no direct interaction with the owners (RR). Each dog was assigned an order number and each owner received the tablets in the corresponding prepacked envelopes.

In this way, the allocation sequence was concealed from the investigator (DS) assigned to participant assessment and enrollment in sequentially-numbered sealed envelopes.

The owners and the veterinarians who interacted with the dogs during the trial, the data analyst, and the laboratory staff all remained blind to allocation.

Statistical analysis

Descriptive and inferential per-protocol statistical analysis was carried out using SAS 9.4 software (SAS Institute Inc., Cary, NC, USA). The normality of the data distribution was examined using the Shapiro–Wilk test. Non-normally distributed variables (LDL, urea, alanine aminotransferase, alkaline phosphatase, sodium) were log-transformed before analysis. Following normalization through natural logarithm when necessary, the dependent variables were analyzed with an ANOVA repeated mixed model using time as the repeated effect, and using group allocation (Spirulina vs placebo), sex, race, and age as fixed effect to assess the interaction between treatment and time. Sex and age were considered potentially confounding covariates to improve the precision of estimating effects. The comparison between counting data (the percentage of individuals achieving a reduction of triglyceride levels higher than the two thresholds of −15% and −30% in the two groups) was made using the $\chi 2$ test. Pearson's correlation was computed to investigate the relationship between HDL and PON-1. Data are presented as the mean ± standard deviation and, when specified, as LSmeans ± standard error. Values of $P < 0.05$ were considered statistically significant.

Data availability

The data presented in this study are available on request from the corresponding author.

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Author contributions

D.S. and R.R. planned the study. D.S. recruited the participants and wrote the paper. D.S. and M.B. collected the samples. F.B. and D.S. conducted the laboratory work and evaluated the data. L.S. and D.S. performed the statistical analysis and analysed the data. G.G., F.B., E.F., M.C., G.M. and R.R. assisted in the early stages of the project design. R.R. supervised the execution. All authors provided input for writing the paper and reviewed it.

Declarations

Competing interests

Marco Cavazzoni is affiliated with Diusa SA, the pet food manufacturer that provided the weight loss food used in this trial. MC was not involved in sample collection, processing, or statistical analysis, and his affiliation with Diusa SA did not influence his interpretation of the present study's data. Diusa SA did not fund this research study; it only provided the food used. All other authors declare no competing interest.

Additional information

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