



Immunostimulatory and antioxidant effects of supplemental feeding with macroalga *Sargassum* spp. on goat kids

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Abstract

Algae are a rich source of bioactive compounds and health properties that have been narrowly explored in goat production systems. The aim of this study was to determine the effect of feeding diets supplemented with *Sargassum* spp. on antioxidant status and immune parameters in goat kids. The diets were as follows: control (basal diet without alga), *Sargassum* spp. 2.5% (Ss2.5), and *Sargassum* spp. 5% (S5) fed over a 70-day period. A total of 11 body tissues, intestinal mucus, and blood serum were sampled at necropsy. Protein content, superoxide dismutase (SOD), catalase (CAT), myeloperoxidase (MPO), lysozyme, and anti-protease activities were determined, as well as immunoglobulin A (IgA) and immunoglobulin G (IgG). The results indicated that *Sargassum* spp. supplementation increased protein content in six tissues. Antioxidant activities (SOD and CAT) and immune-related (lysozyme, MPO, and anti-protease) activities were statistically higher ($P < 0.05$) in *Sargassum* spp. groups compared with control in several tissues, intestinal mucus, and serum. Immunoglobulin A levels in intestinal mucus were higher ($P < 0.05$) in *Sargassum* spp.-supplemented groups than the control group. In conclusion, diet supplementation of *Sargassum* spp. improves the antioxidant status and enhances the immune parameters in goats. *Sargassum* spp. dietary supplementation is proposed as strategy to strengthen antioxidant status and stimulate the immune system, which helps in the control of opportunistic pathogens in goats.

Keywords Ruminant · Macroalgae · Supplementation · Radical scavenging activity · Immunostimulant · Functional foods

Introduction

The increasing demand for animal protein by consumers challenges commercial goat production to face several important issues, such as climate change, emerging diseases, and food shortage. In addition, development of modern intensive farming can compromise health and welfare of livestock. The sum

of intensive management practices may lead to oxidative stress and immunosuppression in animals. Toxic reactive oxygen species (ROS) are produced in large amounts in stressed animals, so cells have developed defense mechanisms based on non-enzymatic (i.e., vitamin E, ubiquinol, and flavonoids) and enzymatic antioxidants (i.e., superoxide dismutase (SOD) and catalase (CAT)) to counteract their toxic effects (Cadenas 1997). SOD is an enzyme that decomposes the main dangerous ROS, the superoxide anion, and thus its activity is an indicator of antioxidant status (Celi 2011). Another biomarker for antioxidant status is CAT, which uses the H_2O_2 substrate as other peroxidases do (Giblin et al. 1982). Furthermore, stressed animals become immunosuppressed, making them susceptible to diseases by primary and opportunistic pathogen infections that affect meat and milk production (Griebel et al. 2014). The activities of immune-related enzymes, such as anti-proteases, lysozyme, and myeloperoxidase (MPO), are indicators of bactericidal activity mainly related to phagocytosis of microbial invaders (Meyer et al. 1991; Loria et al. 2008). Similarly, antibodies are one of the most studied biomarkers of the immune system due to their multiple functions against

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pathogens or their toxic compounds (Butler 1985). Therefore, both immune and antioxidant status are critical issues for goat well-being and production.

In this regard, the intake of natural antioxidant and immunostimulant compounds has demonstrated to induce healthier antioxidant and immune status in animals. Currently, macroalgae (also called seaweeds) are gaining interest not only for their nutritional value and positive effects on animal production (i.e., increased growth performance, increased diet digestibility) but also for their health effects (i.e., reduced shedding of pathogenic bacteria, improved stress resistance). Remarkably, the benefits of seaweeds as a feed additive are due to many antioxidant and immunostimulant compounds, such as sulfated polysaccharides, carotenoids, polyphenols (phlorotannins), vitamins, and minerals (Makkar et al. 2016). In fact, seaweed supplements have been used for enhancing immune and/or antioxidant status in several animal species, such as poultry, pigs, sheep, and cattle (i.e., Katayama et al. 2011; Al-Harthi and El-Deek 2012). Although extensive trials with chickens, pigs, sheep, and dairy cows have been reported, only one study of seaweeds in goat nutrition has been published so far (Casas-Valdez et al. 2006).

Sargassum spp. are macroalgae that grow in tropical and subtropical seas worldwide. Notably, *Sargassum* spp. are particularly abundant natural resources that are potentially harvested from the Gulf of California (183,000 t/year). This seaweed has been proposed for feed supplementation in sheep (Marin et al. 2009), cattle (Gojon-Baez et al. 1998), and goats (Casas-Valdez et al. 2006). Also, administration of *Sargassum* species or their components as supplement has a role in antioxidant status and immune responses in domestic production animals, including chickens (Zhang et al. 2011), fish (Zhu et al. 2017), and shrimp (Sivagnanavelmurugan et al. 2014). Overall, dietary supplementation with *Sargassum* spp. and their derived molecules have enhanced the antioxidant capabilities and immune responses (Zhang et al. 2011; Telles et al. 2018). Therefore, the aim of this study was to investigate the effect of *Sargassum* spp. feed supplementation on antioxidant enzymatic activities and immune parameters of goat kids.

Materials and methods

Sargassum spp. collection and processing

In March 2014, a ton and a half of fresh *Sargassum* spp. (approximately 150 kg dry matter) were collected from Piedras Coloradas beach, San Juan de la Costa, Baja California Sur (BCS), Mexico. The *Sargassum* spp. collected were immediately transported to the Sheep and Goat Research

Unit of the Universidad Autonoma de Baja California Sur (UABCS) where it was spread on a semi-shaded area and washed with plenty of fresh water. Afterwards, it was open air-dried for 3 days. The *Sargassum* spp. hay was immediately picked up and ground in a hammer mill, using a 0.63-cm screen. Experimental diets were fabricated during the same week of collection and stored appropriately in porous bags (40 l) under dark conditions at 25–30 °C.

Animals, diet formulation, and experimental design

An experiment was carried out, including a total of twelve 3-month-old Saanen × Anglo-Nubian crossbred growing goat kids (initial weight 15.4 ± 3.0 kg), which were maintained under stabled conditions. The experiment used three treatment groups ($n = 4$ goats per group) as follows: (1) control (Ctrl), goat kids that received a complete base diet, without *Sargassum* spp.; (2) Ss2.5, goat kids that received the base diet containing 2.5% (dry matter percentage) of *Sargassum* spp.; and (3) Ss5, goat kids that received the base diet containing 5% of *Sargassum* spp. The formulated diets were based on proportional increases of *Sargassum* spp. for comparison purposes, considering low (2.5–5%) *Sargassum* spp. concentrations in diets. Ingredients included in the base diet were alfalfa hay, ground corn grain, soybean meal, fish meal, and salt (Table 1). Prior to diet formulation, samples (DM weight 300 g) of each diet ingredient were ground in a Wiley mill (General Electric, USA), and chemical analyses

Table 1 Ingredient composition and nutritive value of diets supplemented with *Sargassum* spp. to feed the experimental groups of goat kids.

Ingredient (%)	Treatment group ¹		
	Ctrl	Ss2.5	Ss5
Alfalfa hay	65	62.4	56.3
Ground corn grain	30	30.1	33.8
Soybean meal	2.6	2.6	2.6
Fishmeal	1.4	1.4	1.4
Sodium chloride	0.9	0.9	0.9
<i>Sargassum</i> spp.	0.0	2.5	5.0
Nutritive value			
Crude Protein %	11.86	11.69	11.50
Metabolizable Energy ²	10.77	10.60	10.77
Calcium %	0.1	0.1	0.09
Phosphorus %	0.03	0.03	0.03

¹ Ctrl: control with no *Sargassum* spp; Ss2.5: *Sargassum* spp. supplemented at a 2.5% of dry matter; Ss5: *Sargassum* spp. supplemented at 5% of dry matter ($n=4$ goats per group). The diets were calculated to be caloric and protein-equivalent, according to requirements established by NRC (2007) for goats.

² MJ/kg of dry matter of feed.

were performed according to AOAC (1999) and Van Soest et al. (1991). Dry matter (DM), ash (A), organic matter (OM), crude protein (CP), ether extract (EE), nitrogen-free extract (NFE), lignin (LI), cellulose (CE), hemicellulose (HC), (AOAC 1999), neutral detergent fiber (NDF), and acid detergent fiber (ADF) (Van Soest et al. 1991).

Digestible energy (DE) was calculated according to the following formula (NRC 2001):

$$DE = (NFE \times 0.042) + (NDF \times 0.042) + (CP \times 0.056) + (0.094 \times (EE/2.25)) - 0.3$$

and metabolizable energy (ME) was calculated as follows:

$$ME = 0.82 DE.$$

Diet composition and nutritional values were calculated according to the requirements established by the National Research Council (NRC, 2007). Nutrient composition of the experimental diets was later confirmed by respective feed chemical analyses. The experimental diets were fed ad libitum for 70 days, including a 14-day diet adaptation period. The experiment was conducted in agreement with the local Bioethical Guidelines of UABCS and Baja California Sur State regulations to avoid causing unnecessary discomfort to experimental animals.

Sampling procedure

After the feeding period, all the goats were slaughtered in the Municipal Abattoir, La Paz, BCS, Mexico. Samples from each selected tissue (0.5 g) were taken with tweezers and scalpel and placed in sterile empty Eppendorf tubes (2 mL) in bath-ice and immediately frozen in liquid nitrogen ($-196\text{ }^{\circ}\text{C}$). A total of 12 tissues (rumen, omasum, abomasum, duodenum, jejunum, cecum, colon, liver, spleen, kidney, muscle, and intestinal mucus) were sampled. Intestinal mucus was gently removed from the mucosal surface using a plastic scraper, placed in tubes, and frozen as described above. Blood was also obtained from the jugular vein of each goat during the entire experiment (day 0–70) once every 14 days. Blood was put into Vacutainer® tubes to obtain blood serum by centrifuging (2500g at $4\text{ }^{\circ}\text{C}$ for 15 min). Thereafter, tissue and serum samples were stored at $-80\text{ }^{\circ}\text{C}$ until antioxidant and immunological analyses.

Antioxidant status

The SOD and CAT enzymatic activities were determined after grinding 100 mg of each sampled tissue in 500 μl of phosphate-buffered saline (PBS, pH 7.4) at 20,000 rpm using a Polytron grinder (PT2100, VWR International, Radnor, Pennsylvania, USA) for 10 s. Tissue homogenates were then centrifuged (10,000g at $4\text{ }^{\circ}\text{C}$ for 10 min) and supernatant was transferred to new tubes after centrifugation. Total protein was quantified by the Bio-Rad protein assay (500-0002, Bio-Rad, Hercules, CA, USA) following the manufacturer instructions.

Bovine serum albumin (500-0206, Bio-Rad, Hercules, CA, USA) was used as a standard in the reference curve.

The SOD activity was measured by the percentage reaction inhibition rate of enzyme with water-soluble tetrazolium dye (WST-1) substrate and xanthine oxidase using a SOD Assay Kit (19160, Sigma, St Louis, MO, USA) according to the manufacturer's instructions. After 20 min of reaction time at $37\text{ }^{\circ}\text{C}$, absorbance was read at 440 nm (the absorbance wavelength for the colored product of the WST-1 reaction with superoxide) in a microplate reader (Model 3550 UV, Bio-Rad, Hercules, CA, USA). The SOD activity was expressed as U mg^{-1} protein.

The CAT activity was determined using the method of Johansson and Borg (1988). Homogenized tissues were mixed with methanol and hydrogen peroxide in 250 mM KH_2PO_4 -NaOH buffer, pH 7.0 (50 μl sample, 25 μl buffer, 25 μl methanol, 5 μl hydrogen peroxide). The reaction was incubated at $20\text{ }^{\circ}\text{C}$ under gentle agitation for 20 min. Then, 25 μl of potassium hydroxide, 10 M, and 50 μl of Purpald reagent (162892, Sigma, St Louis, MO, USA) were added. After 10 min of incubation in dark conditions, 25 μl of 65.2 mM potassium periodate in 470 mM potassium hydroxide was added. A standard reference curve was generated using the sample that consisted of 0.125–1.0 $\mu\text{g/ml}$ purified CAT from bovine liver (C1345, Sigma, St Louis, MO, USA). The CAT activity was determined by measuring the absorbance at 655 nm (Model 3550 UV, Bio-Rad, Hercules, CA, USA) and expressed as U mg^{-1} protein.

Immune response parameters

Total MPO activity in intestinal mucus and blood serum samples was measured following the methods described in Quade and Roth (1997) with slight modifications. Briefly, 20 μl of intestinal mucus (diluted 1:1 in PBS, pH 7.4) and blood serum samples was placed in 96-well plates (Nunc-Immuno, MaxiSorp, 44-2404-21, Thermo Fisher Scientific, Waltham, MA, USA). Then, 100 μl of 20 mM 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB) (T3405, Sigma, St Louis, MO, USA) and 5 mM H_2O_2 were added (reagents were prepared immediately before use). The colorimetric reaction was stopped after 2 min by adding 50 μl of 2 M sulfuric acid (H_2SO_4). The optical density was read at 450 nm (Model 3550 UV, Bio-Rad, Hercules, CA, USA) and the MPO activity calculated (1 unit produces the change of one unit of absorbance).

The lysozyme activity was tested based on the method of Lange et al. (2001). On flat-bottomed 96-well microtiter plates (Nunc-Immuno, MaxiSorp, 44-2404-21, Thermo Fisher Scientific, Waltham, MA, USA), 100 μl of 0.4 mg ml^{-1} suspension of *Micrococcus lysodeikticus* (M3770, Sigma, St Louis, MO, USA) in 0.05 mol l^{-1}

PBS (pH 5.2) was added to 100 μl of samples in serial dilutions of 1/5–1/40. The OD was taken (570 nm) using an ELISA plate reader (Model 3550 UV, Bio-Rad, Hercules, CA, USA) at 0 and 60 min. A unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance of 0.001 units/min.

Total anti-protease activity was determined as indicated by the capacity of serum or intestinal mucus to inhibit trypsin activity as described by Ellis (1990). Briefly, aliquots of 20 μl of sample were incubated with 20 μl of standard trypsin solution (1000–2000 BAEE (benzoyl L-arginine ethyl ester), 5 mg ml^{-1} ; B4500, Sigma, St Louis, MO, USA) at 22 °C in Eppendorf tubes for 10 min. Then, 200 μl of 0.1 M PBS (pH 7.0) and 250 μl of 2.5% (w/v) azocasein (A2765, Sigma, St Louis, MO, USA) in PBS were added and incubated at 22 °C for 1 h. The reaction was stopped by the addition of 500 μl of 10% (v/v) trichloroacetic acid (TCA, T6399, Sigma, St Louis, MO, USA), incubated at 22 °C for 30 min, and then centrifuged at 6000g for 5 min. The supernatants (0.1 ml) were transferred to 96-well microtiter plates (Nunc-Immuno, MaxiSorp, 44-2404-21, Thermo Fisher Scientific, Waltham, MA, USA) containing 0.1-ml wells 1 of 1 N NaOH. The OD was read at 450 nm (Model 3550 UV, Bio-Rad, Hercules, CA, USA). For a positive (100%) control, buffer replaced the sample, and for a negative control, buffer replaced both sample and trypsin. The inhibitory ability of anti-protease was expressed in terms of percentage trypsin inhibition.

%Trypsin inhibition = trypsin OD

$$\times \text{sample OD/trypsin OD} \times 100$$

Enzyme-linked immunosorbent assay (ELISA) was performed to detect IgA and IgG in intestinal mucus. Briefly, 96-well plates (Nunc-Immuno, MaxiSorp, 44-2404-21, Thermo Fisher Scientific, Waltham, MA, USA) were coated with 0.1 ml (20 μg total protein) of intestinal mucus and incubated at 37 °C for 2 h. The wells were blocked for 2 h at room temperature with 5% of skim milk. After rinsing, 0.1 ml rabbit anti-goat IgA (1:500, AAI44P, Bio-Rad, Hercules, CA, USA) and IgG (1:2000, A5420, Sigma, St Louis, MO, USA) horseradish peroxidase conjugated was added to each well and incubated at 37 °C for 1.5 h. The revelation process was carried out with O-phenylenediamine dihydrochloride (OPD, P8787, Sigma, St Louis, MO, USA). The reaction was stopped with 25 ml 2 M H_2SO_4 per well, and absorbance was read at 492 nm (Nunc-Immuno, MaxiSorp, 44-2404-21, Thermo Fisher Scientific, Waltham, MA, USA). The ELISA parameters were considered when intra-assay coefficient of variation (CV) was less than 10%, inter-assay CV was less than 20%, and sensitivity was up to 90%.

Statistical analysis

To test the effects of the different levels of *Sargassum* in the diet on antioxidant status and immune responses, a complete random design was used (Steel and Torrie 1980). For this purpose, the response data were firstly tested for normality (Shapiro-Wilk W normality test) and homogeneous variance (Levene test). Then, the effects of the treatments evaluated were tested by one-way analyses of variance. The model applied was:

$$Y_{ij} = \mu + \tau_i + \varepsilon_{ij}$$

where Y was the antioxidant or immune response; μ was the general mean; τ was the level of *Sargassum* spp. in the diet (0, 2.5%, 5%); and ε was a random element of variation. Post hoc Tukey test was used to detect significant differences in means. Differences were considered significant when $P < 0.05$. The STATISTICA V10 software was used for statistical analyses.

Results

Protein content

Diet supplementation with *Sargassum* spp. had significant effects ($P < 0.05$) on protein concentration in different organs and tissues (Table 2). Protein content was higher in rumen (Ss2.5), abomasum (Ss2.5 and Ss5), jejunum (Ss2.5 and

Table 2 Total protein in 11 tissues and intestinal mucus sampled at necropsy from goat kids fed with diets including macroalgae *Sargassum* spp. at different levels.

Tissue	Treatment group ¹		
	Ctrl	Ss2.5	Ss5
Rumen	2.03±0.31 ^a	4.24±0.41 ^b	3.10±0.54 ^{ab}
Omasum	2.63±0.32 ^a	3.57±0.40 ^a	3.80±0.80 ^a
Abomasum	2.65±0.49 ^a	5.92±0.48 ^b	5.09±0.37 ^b
Duodenum	4.32±0.49 ^a	7.09±1.20 ^a	8.01±1.21 ^a
Jejunum	6.60±0.51 ^a	19.20±1.05 ^c	10.60±0.30 ^b
Cecum	4.89±0.60 ^a	9.10±0.94 ^b	7.64±0.83 ^{ab}
Colon	6.08±0.38 ^a	12.99±1.15 ^b	6.58±0.42 ^a
Liver	13.98±0.40 ^b	7.43±0.57 ^a	15.51±2.12 ^b
Spleen	9.11±1.08 ^a	12.31±1.30 ^{ab}	14.29±0.96 ^b
Kidney	6.74±0.59 ^a	8.02±1.08 ^a	7.18±0.72 ^a
Muscle	5.00±0.26 ^b	4.45±0.47 ^b	2.63±0.39 ^a
Intestinal mucus	8.24±0.70 ^b	5.61±0.53 ^a	5.85±0.18 ^a

¹ Ctrl: control with no *Sargassum* spp; Ss2.5: *Sargassum* spp. supplemented at a 2.5% of dry matter; Ss5: *Sargassum* spp. supplemented at 5% of dry matter. Concentrations are expressed as $\text{mg}\cdot\text{ml}^{-1}$ of protein, ($n=4$ goats per group).

^{abc} Different superscripts on the same row indicate statistical differences among groups ($p < 0.05$).

Ss5), cecum (Ss2.5), colon (Ss2.5), and spleen (Ss5) compared with the control group. By contrast, protein content in muscle and intestinal mucus was higher in the control group than in the *Sargassum* spp. groups. Protein concentration in other collected samples was similar among groups.

Antioxidant enzymatic activities

Overall, SOD and CAT enzyme activities were consistently stimulated in the rumen and liver of *Sargassum* spp.-supplemented goats compared with the control group. SOD and CAT activities in rumen were higher ($P < 0.05$) in Ss2.5 and Ss5 groups, respectively, compared with the control group. In the liver, SOD activity increased in the Ss5 group while CAT activity increased in both the Ss2.5 and Ss5 groups compared with the control group (Tables 3 and 4).

The SOD activity level was similar ($P > 0.05$) in omasum, duodenum, cecum, and intestinal mucus of control and *Sargassum* spp.-supplemented groups (Table 3). Moreover, the low level of *Sargassum* spp. (Ss2.5) supplementation in the diet appeared to stimulate SOD activity in goats' rumen, jejunum, and spleen. The SOD activity was higher ($P < 0.05$) in abomasum, jejunum, colon, liver, kidney, and muscle of goats supplemented with *Sargassum* spp. 5% (Ss5 group) than the control and Ss2.5 groups.

The CAT activity found in different tissues is shown in Table 4. The activity level of CAT was higher ($P < 0.05$) in

Table 3 Superoxide dismutase activity in 11 tissues and intestinal mucus sampled at necropsy from goats fed with diets containing macroalgae *Sargassum* spp. at different levels.

Tissue	Treatment-group ¹		
	Ctrl	Ss2.5	Ss5
Rumen	1.46±0.41 ^a	5.64±0.58 ^b	5.10±1.62 ^{ab}
Omasum	22.04±0.96 ^a	16.85±1.93 ^a	22.89±5.46 ^a
Abomasum	13.83±1.67 ^a	8.12±1.23 ^a	23.83±1.99 ^b
Duodenum	5.83±1.09 ^a	3.64±0.20 ^a	3.81±0.73 ^a
Jejunum	0.76±0.40 ^a	3.59±0.11 ^b	6.81±0.16 ^c
Cecum	16.31±2.44 ^a	14.49±1.87 ^a	11.35±2.58 ^a
Colon	2.07±0.33 ^a	2.57±0.10 ^a	4.74±0.78 ^b
Liver	11.00±0.72 ^a	9.89±0.67 ^a	15.84±1.11 ^b
Spleen	11.33±0.84 ^{ab}	12.25±1.09 ^b	8.41±0.38 ^a
Kidney	3.43±0.76 ^a	8.70±1.40 ^a	15.25±1.61 ^b
Muscle	17.85±0.73 ^a	22.06±2.90 ^{ab}	31.83±4.32 ^b
Intestinal mucus	1.53±0.20 ^a	6.35±1.55 ^a	6.04±1.09 ^a

¹ Ctrl: control with no *Sargassum* spp; Ss2.5: *Sargassum* spp. supplemented at a 2.5% of dry matter; Ss5: *Sargassum* spp. supplemented at a 5% of dry matter. Concentrations are expressed as U mg⁻¹ protein, (n=4 goats per group).

^{abc} Different superscripts on the same row indicate statistical differences between groups ($p < 0.05$).

Table 4 Catalase activity in 11 tissues and intestinal mucus sampled at necropsy from goats fed with diets containing macroalgae *Sargassum* spp. at different concentrations.

Tissue	Treatment-group ¹		
	Ctrl	Ss2.5	Ss5
Rumen	0.39±0.017 ^a	0.67±0.06 ^{ab}	1.16±0.15 ^b
Omasum	1.24±0.35 ^a	0.84±0.19 ^a	0.78±0.37 ^a
Abomasum	1.45±0.69 ^a	0.46±0.03 ^a	0.53±0.09 ^a
Duodenum	0.63±0.43 ^a	0.47±0.12 ^a	0.45±0.05 ^a
Jejunum	0.50±0.10 ^b	0.20±0.02 ^a	0.31±0.03 ^a
Cecum	0.57±0.04 ^b	0.34±0.09 ^a	0.41±0.12 ^{ab}
Colon	0.59±0.15 ^b	0.27±0.10 ^a	0.43±0.04 ^{ab}
Liver	0.09±0.00 ^a	0.15±0.04 ^b	0.16±0.03 ^b
Spleen	0.25±0.04 ^a	0.28±0.07 ^a	0.17±0.05 ^a
Kidney	0.68±0.06 ^a	0.51±0.09 ^a	0.62±0.10 ^a
Muscle	0.44±0.00 ^a	0.77±0.07 ^a	0.92±0.31 ^a
Intestinal mucus	0.26±0.01 ^a	0.58±0.12 ^b	0.52±0.08 ^b

¹ Ctrl: control with no *Sargassum* spp; Ss2.5: *Sargassum* spp. supplemented at a 2.5% of dry matter; Ss5: *Sargassum* spp. supplemented at a 5% of dry matter. Concentrations are expressed as U mg⁻¹ protein, (n=4 goats per group).

^{abc} Different superscripts on the same row indicate statistical differences between groups in that tissue ($p < 0.05$).

rumen, liver, and intestinal mucus in *Sargassum* spp. groups than in the control group. Otherwise, CAT activity was lower ($P < 0.05$) in jejunum, cecum, and colon of the control group than *Sargassum* spp. groups. The CAT activity in other analyzed tissues was similar among treatment groups.

Immune response parameters

Lysozyme activity

Blood serum lysozyme activity was found to have increased ($P < 0.05$) after 28 and 42 days of *Sargassum* supplementation groups, particularly in the Ss2.5 group (Fig. 1a). Thereafter, lysozyme activity in serum decreased until the end of the experiment without significant differences among groups. At necropsy, lysozyme activity was higher ($P < 0.05$) in intestinal mucus of goats from the Ss2.5 group than the control and Ss5 groups (Fig. 1b).

Myeloperoxidase activity

The serum myeloperoxidase (MPO) activity was higher ($P < 0.05$) at days 14 and 42 in goats from the Ss5 group compared with the control group (Fig. 2a). Similarly, the MPO activity was higher ($P < 0.05$) at days 42 and 70 in the Ss2.5 group compared with the control group. In addition, the MPO activity in goat's intestinal mucus was higher ($P < 0.05$)

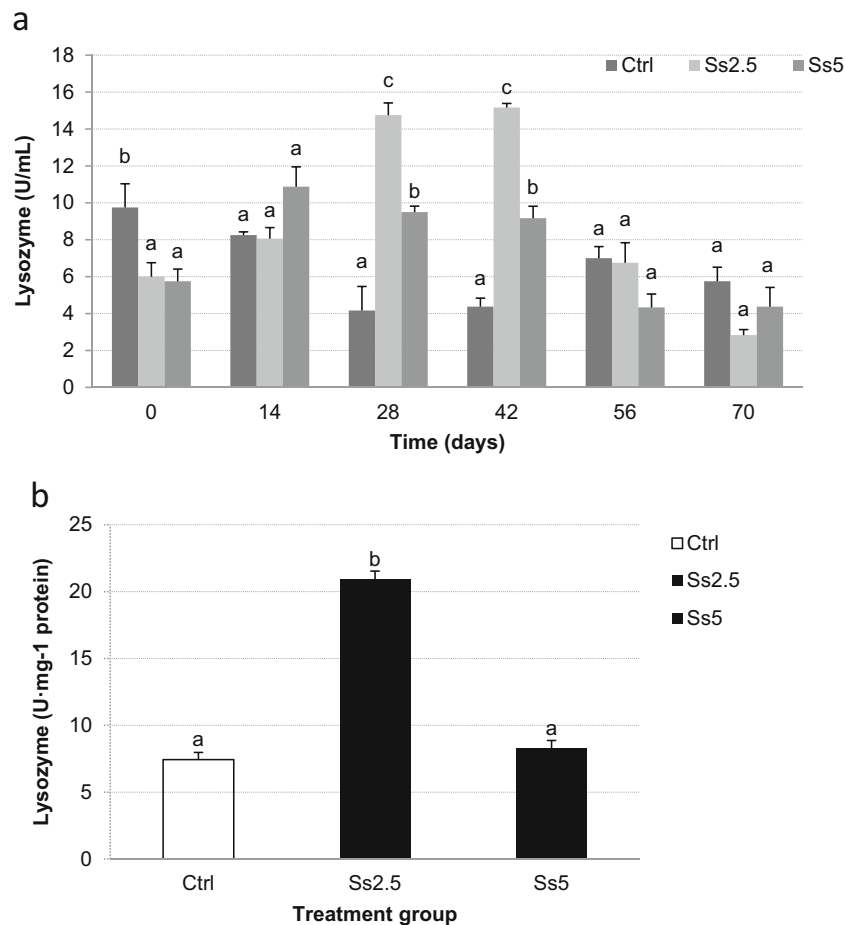


Fig. 1 Effect of diet supplementation with *Sargassum* spp. on lysozyme activity in goats' blood serum (**a**) and intestinal mucus (**b**); bars represent mean and standard error ($n = 3$), respectively. Values are expressed in

Um L^{-1} . Different superscripts on bars indicate significant differences among groups ($P < 0.05$)

in *Sargassum* spp.-supplemented groups than in the control group (Fig. 2b).

Anti-protease activity

Serum anti-protease activity was higher ($P < 0.05$) in goats supplemented with *Sargassum* spp. than in the control group at days 42, 56, and 70; in fact, anti-protease activity in the Ss2.5 group was even higher than that in the Ss5 group (Fig. 3a), and it was also higher ($P < 0.05$) in goats' intestinal mucus of groups Ss5 and Ss2.5 than the control (Fig. 3b).

Immunoglobulins A and G

The IgG production in intestinal mucus of goats supplemented with *Sargassum* spp. was similar ($P > 0.05$) to that of the control (Fig. 4a). In contrast, IgA antibody levels were significantly higher ($P < 0.05$) in both the Ss2.5 and Ss5 groups than the control group (Fig. 4b).

Discussion

Several seaweed species consumed by ruminants directly on the beach can be used as feed ingredients in commercial livestock farming (Makkar et al. 2016). In Mexico, *Sargassum* spp. are particularly abundant along the coastline of the Gulf of California where significant biomasses have been estimated (Casas-Valdez et al. 2006; Marin et al. 2009). The effects of *Sargassum* spp. or their biological compounds in the diets for husbandry animals are limited to a few studies performed in shrimp (Sivagnanavelmurugan et al. 2014), hens (Al-Harathi and El-Deek 2012), sheep (Marin et al. 2009), and goats (Casas-Valdez et al. 2006).

Protein contents in sampled tissues were determined as they are a primary constituent of all body tissues, such as hair, skin, muscle, bones, and vital organs. In this study, protein concentration in *Sargassum* spp.-supplemented goats was higher in rumen, abomasum, jejunum, cecum, colon, and spleen than in the control. These results mean that *Sargassum* spp. increase protein content in some of the

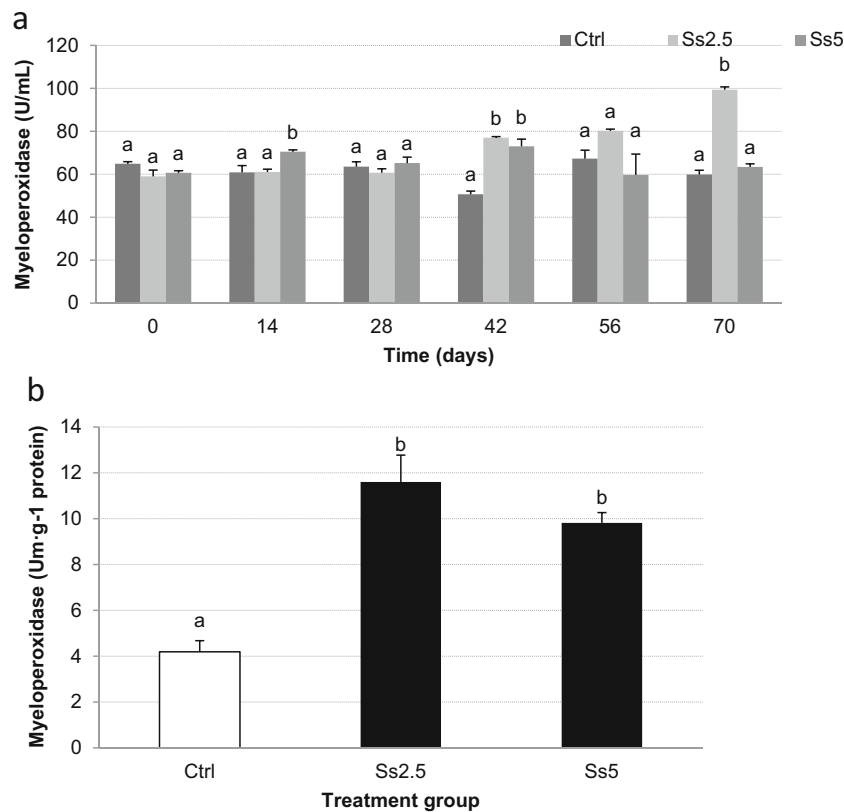


Fig. 2 Effect of diet supplementation with *Sargassum* spp. on myeloperoxidase activity in goats' blood serum (a) and intestinal mucus (b); bars represent mean and standard error ($n = 3$), respectively. Different

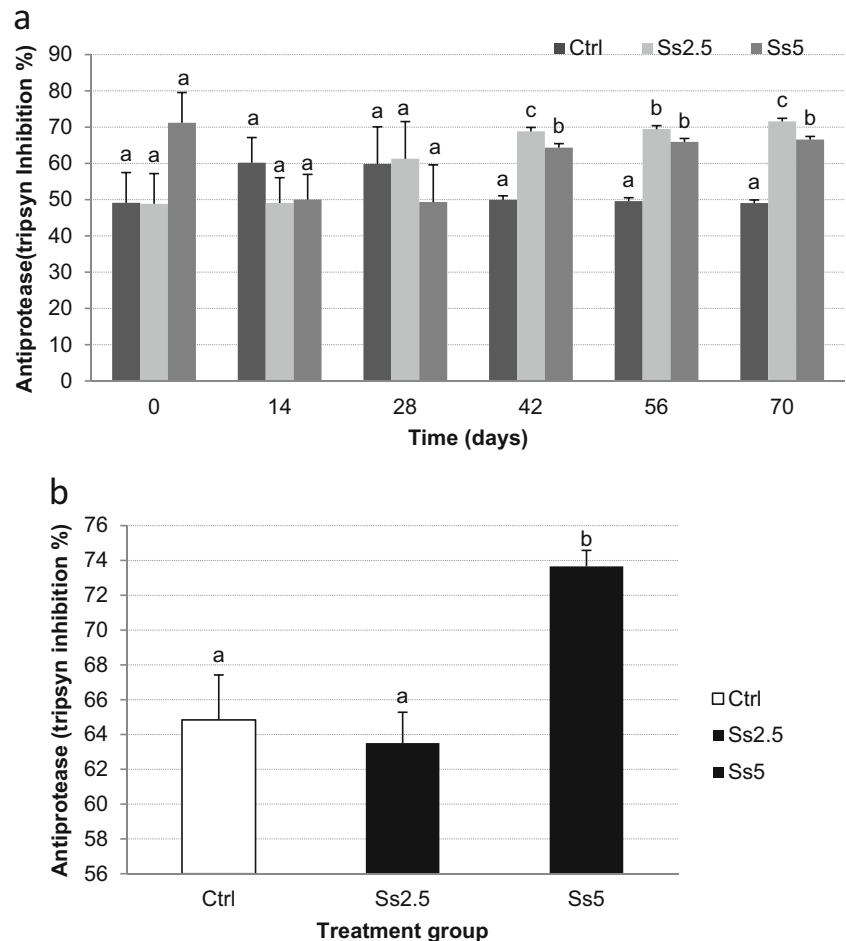
superscripts on bars indicate significant differences among groups ($P < 0.05$)

gastrointestinal tissues and organs, which could be related with a better nutrition and antioxidant status in these organs in *Sargassum* spp.-supplemented goats; it is important since proteins have relevant functions in health and nitrogen metabolism in animals. For example, supplemented lambs with other kinds of extract (pomegranate peel extract; 45 mL/kg of diet) improved nitrogen retention and antioxidant capacities (Rajabi et al. 2017). In another study, curiously, total protein in blood plasma was not affected by *Astragalus membranaceus* supplementation; however, antioxidant capacity and immune functions were improved in lambs (Zhong et al. 2012). Moreover, in in vitro assays, Kang et al. (2015) demonstrated that *Sargassum horneri* extract rescues murine skeletal muscle cells from oxidative stress-induced cytotoxicity. Surprisingly, protein content in muscle was similar and reduced in *Sargassum* spp.-supplemented goats with 2.5 and 5%, respectively, compared with the control group. On this regard, Marin et al. (2003) found that growth, which is highly dependent on protein deposition in muscle of lambs supplemented with *Sargassum* spp., was not affected. Similarly, dietary supplementation of *Sargassum fusiforme* polysaccharide extracts had no effect on growth in shrimp (Huang et al. 2006).

In this study, physiological antioxidant parameters were determined in goats supplemented with *Sargassum* spp.

Several reports have pointed out that antioxidant compounds from brown algae (i.e., *Sargassum* spp.) incorporated in diets have health benefits on the antioxidative status without affecting growth of ruminants (Cofrades et al. 2010; Wullepit et al. 2012). These effects against oxidative stress are related with increased antioxidant enzymatic activities in ruminants, such as SOD, which is an enzyme that decomposes the main dangerous ROS in living organisms (Celi 2011). In our study, SOD activity level was higher in almost all analyzed tissues and organs of *Sargassum* spp.-supplemented goats compared with the control. This SOD activity increment can be associated to the presence of certain minerals in *Sargassum* spp. including copper and zinc that are cofactors of SOD (SOD/Cu-Zn dependent; Rahman 2007). Similar to our results, increased SOD activities have been found in shrimp supplemented with fucoidan, a polysaccharide deriving from the brown seaweed *Sargassum wightii* (Sivagnanavelmurugan et al. 2014). In another study, the dietary supplementation of *Sargassum fusiforme* polysaccharides increased SOD activities in serum of immunosuppressed mice (Wang et al. 2013). Therefore, *Sargassum* spp. polysaccharides increase SOD activity in supplemented animals, as observed in our study. Remarkably, high SOD activity was found in muscle of *Sargassum* spp.-supplemented (5%) goats. This finding is

Fig. 3 Effect of diet supplementation with *Sargassum* spp. on anti-protease activity in goats' blood serum (a) and intestinal mucus (b); bars represent group mean and standard error ($n = 3$), respectively. Different superscripts on bars indicate significant differences among groups ($P < 0.05$)



important for goat meat production since color of fresh meat is a primary factor used by consumers to judge meat quality. The oxidative status of meat indicates its color stability and is directly related with the oxidation of oxymyoglobin to metmyoglobin (James et al. 2002). Another study found increased SOD activity in goats supplemented (200 g/day) with *Moringa oleifera* leaves, indicating its ability to protect the animal's body tissues and to maintain meat quality by quenching free radicals (Qwele et al. 2013). Therefore, the increases in SOD activity in muscle because of *Sargassum* spp. supplementation must cause antioxidant resistance to oxymyoglobin, making the reddish color of meat last longer; however, this effect remains to be evaluated.

The CAT is involved in the second step of removing peroxides (i.e., oxygen peroxide, H_2O_2) produced by SOD, converting them into water (H_2O) and oxygen (O_2). The CAT activity level was only higher in the rumen, liver, and intestinal mucus in both *Sargassum* spp. groups compared with the control. This effect is associated to glutathione peroxidase (GPx) and MPO activities that use the same H_2O_2 substrate. For example, CAT acts in the presence of high concentrations of H_2O_2 and GPx at low ones, which show an inverse correlation in the activity of both enzymes. In another

study, fish fed fucoidan (0.05–0.2%) supplement extracted from *Sargassum horneri* had higher CAT and SOD activities than those of the control groups (Yang et al. 2014). Altogether, high SOD and CAT activities in tissues of *Sargassum* spp.-supplemented goats are related with increased physiological protection from ROS, which are commonly generated during intensive animal production.

Maintenance of immune health status is critical for the goat farming industry since several opportunistic pathogens affect health and production. Lysozyme is an enzyme related to the innate immune system that cleaves N-acetyl-glucosamine and N-acetylmuramic acid linkage in bacterial cell wall peptidoglycan as a result of the phagocytic activity. In this study, blood serum lysozyme activity increased after days 28 and 42 in *Sargassum* spp.-supplemented groups and then decreased at the end of experiment. Similarly, lysozyme activity in the intestinal mucus of goats from the Ss2.5 was higher than in the control. As a matter of comparison, the serum lysozyme activities, as well as phagocytosis and respiratory burst activities of yellow catfish fed with fucoidan extracted from *Sargassum horneri*, were significantly higher than in the control (Yang et al. 2014). Since lysozyme in the gastrointestinal tract can modulate inflammatory responses, our results have

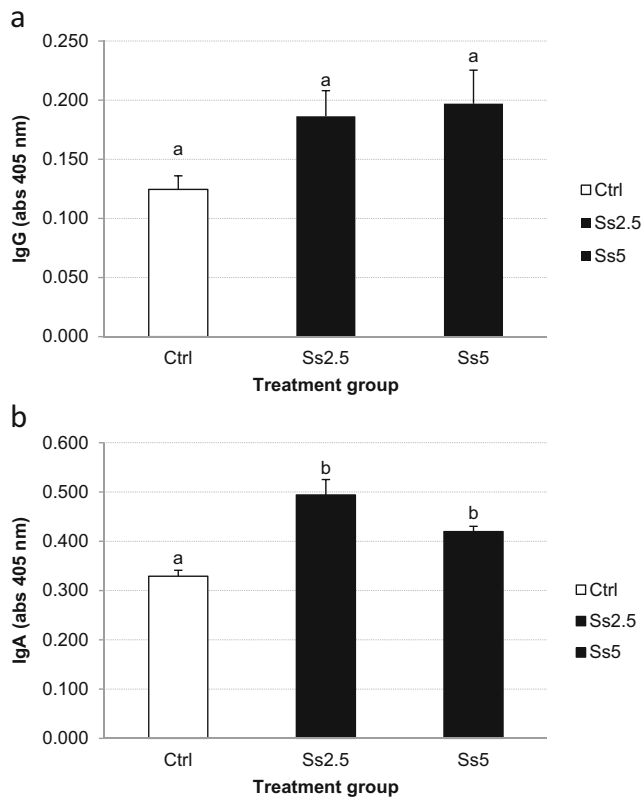


Fig. 4 Effect of *Sargassum* spp. supplementation on IgG (a) and IgA (b) in goats' intestinal mucus; bars represent group mean and standard error ($n = 3$), respectively. Values are expressed as absorbance at 405 nm. Different superscripts on bars indicate significant differences ($P < 0.05$)

demonstrated that *Sargassum* spp. supplementation affected the lysozyme activity, which is related with phagocytic and respiratory burst functions in goats.

The MPO is stored in neutrophils and macrophages and released during phagocytosis activity. This enzyme catalyzes the conversion of chloride and hydrogen peroxide to hypochlorite, which is a highly toxic compound for pathogens (Loria et al. 2008). Serum MPO activity was higher at days 14 and 42 in the Ss5 group and at days 42 and 70 in the Ss2.5 group, compared with the control group. In addition, the MPO activity in goat's intestinal mucus was higher in *Sargassum* spp.-supplemented groups than in the control group. Therefore, these results indicated that *Sargassum* spp. supplementation enhanced innate immunity by activating neutrophils and macrophages.

Anti-proteases (such as $\alpha 1$ -anti-protease, $\alpha 2$ -antiplasmin, and $\alpha 2$ -macroglobulin) are enzymes that circulate in plasma and can inhibit bacteria proteases essential for growth (Meyer et al. 1991). The serum anti-protease activity was higher in goats supplemented with *Sargassum* spp. than in those of the control group after 42 days of supplementation while in intestinal mucus, anti-protease activity was significantly higher in goats supplemented with 5% (Ss5 group). These enzymes are also related to innate immunity and can inhibit bacterial

proteases and also restrict their ability to survive. In the intestinal mucus layer, anti-protease activity is particularly critical since intestinal mucosa is the main entry for pathogens, and therefore, anti-proteases contribute to the local immune defense mechanism against pathogens. In another study using *Sargassum wightii* fucoidan, Sivagnanavelmurugan et al. (2014) found that innate immune parameters were enhanced in the hemolymph of supplemented shrimp increasing both bactericidal and phagocytic activities. Moreover, these immune parameters were related with an increased shrimp resistance to *Vibrio parahaemolyticus* infection.

Immunoglobulins are produced by B lymphocytes as part of the immune system, which can bind to pathogen-associated molecular patterns, neutralize toxins, opsonize (attach) pathogens, and activate several immune mechanisms. Among antibodies, the production of intestinal IgA and IgG was analyzed since the gastrointestinal mucosal epithelium is the first entry barrier against pathogens. IgA and IgG levels were higher in *Sargassum* spp.-supplemented goats than those in the control; however, only IgA showed statistical differences compared with the control, which means that mucosal immunity through IgA production, the main mucosal immunoglobulin, was effectively stimulated in supplemented goats. In contrast, Novoa-Garrido et al. (2014) found that serum IgG concentration was reduced in sheep supplemented with seaweed. Interestingly, the dietary supplementation of non-fermented or fermented brown seaweed (*Undaria pinnatifida*) and seaweed fusiforme (*Hizikia fusiformis*) by-products for 35 days led to increases in IgA and immunoglobulin M (IgM), but not IgG, production in broiler chickens (Choi et al. 2014). A recent study showed that *Sargassum fusiforme* polysaccharides potentiate mucosal immunity in the digestive tract of orally supplemented mice (Wang et al. 2013). In addition, plasma total immunoglobulin production was increased in fish after supplementation of extract from *Sargassum* sp., an effect that was associated with pathogen resistance (Yangthong et al. 2016). The above studies and our findings have demonstrated that dietary supplementation of algae or some of their carbohydrates can stimulate the production of immunoglobulins, which are critical players to combat pathogens. However, differences in immune responses by immunostimulants, such as a lack of mucosal immunoglobulin production, can be connected with increases of other innate immune (i.e., acute phase proteins) or endocrine parameters (Oh et al. 2017). On the other hand, natural immunoglobulins can interact with other molecules to promote immune defense and homeostasis (Panda and Ding 2015).

Diet supplementation of *Sargassum* spp. modulated the antioxidant status and enhanced the immune system in goats. *Sargassum* spp. supplementation increased protein content, antioxidant enzymatic (SOD and CAT), and immune-related (lysozyme, MPO and anti-protease) activities in tissues, intestinal mucus, and/or serum, as well as IgA production in

intestinal mucus. Therefore, *Sargassum* spp. dietary supplementation is recommended to improve antioxidant status and immune responses against opportunistic pathogens in goat production systems. Even though healthier animals mean healthier products, further studies should be focused on productive performance of goats fed *Sargassum* spp.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval The experiment was conducted in agreement with the local Bioethical Guidelines of UABCS and Baja California Sur State regulations to avoid causing unnecessary discomfort to experimental animals.

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