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Assessing in vivo digestibility and effects on immune system of sheep fed alfalfa hay supplemented with a fixed amount of *Ulva rigida* and *Gracilaria vermiculophylla*

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Abstract Ruminants could be the most suitable domestic animals to be supplemented with seaweeds as the rumen ecosystem might provide the animal the ability to use these feed resources by breaking down the complex polysaccharides. The objective of the present in vivo study was to determine the digestibility and the effects on the immune system of one green (*Ulva rigida*) and one red (*Gracilaria vermiculophylla*) seaweed cultivated in an integrated multitrophic aquaculture system (IMTA) and included in the diet of sheep at a supplementing level up to 25%. Both seaweeds showed lower dry matter digestibilitity than alfalfa hay, the organic matter digestibility of *U. rigida* being higher than that of *G. vermiculophylla*. The studied seaweeds had similar fiber and energy digestibility. Seaweed supplementation did not influence hematological parameters, reactive oxygen species production by neutrophils, nor lymphocytic response to T and B cells mitogens. The low fiber digestibility of selected seaweeds would be the major constraint to their use in high amounts in ruminant diets. Dietary seaweed supplementation has no deleterious effect on the immune function of cells mediating innate and acquired immunity.

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Introduction

Livestock production is increasing fast for feeding a burgeoning human population. According to the FAO, it is expected that global meat and dairy production will more than double by 2050 (Steinfeld et al. 2006). This huge increase for animal products will require increasing amounts of feed supplies; thus, the identification of novel feeds is essential for the development of the livestock sector.

Seaweeds have been used to feed livestock since immemorial times in coastal regions, in times of feed scarcity (Balasse et al. 2005), and animals will naturally consume some quantity of seaweeds if they are available, such as on coastal farms. Generally, seaweeds are markedly rich in organic minerals, complex carbohydrates, proteins and low molecular weight nitrogenous compounds, lipids, vitamins, volatile compounds, and pigments (Makkar et al. 2016). Due to the chemical diversity and complexity of polysaccharides that may account to 25–75% of algal dry weight (Jiménez-Escrig and Sánchez-Muniz 2000), herbivorous animals and especially ruminants may be well suited to be fed on seaweeds as the rumen ecosystem might provide the animal the ability to use seaweeds by breaking down the complex polysaccharides. Brown algae have been the most intensively studied and exploited in animal feeding due to their large size, ease of harvesting, and mineral profile (especially iodine) (Rey-Crespo et al. 2014), but they have lower nutritional value than red and green algae due to their lower protein content (Makkar et al. 2016). Limited data are available on the in vivo digestibility of seaweeds for ruminants (Makkar et al. 2016), and their nutritive value varies with the species, geographic area, season of the year, environmental conditions (Ito and Hori 1989), and the

nutrient content of the medium where they are cultivated (Azevedo et al. 2015).

More recently, seaweeds have been also evaluated as a prebiotic promoter (Ramnani et al. 2012) due to their content on bioactive substances with broad biological activities (Kumar et al. 2008; Santos et al. 2015). This is particularly important, as the exponential growth of the world population has contributed to the industrialization of food animal production that promoted major increases in livestock productivity largely due to the genetic progress and the development of diets tailored to specific stages of production. However, animal selection for increased production with little or no emphasis on health traits has led to a declining breeding success, increasing incidence of health problems, and declining longevity (Oltenacu and Algers 2005).

The objectives of the present study were to determine the in vivo digestibility of one green (*Ulva rigida*) and one red (*Gracilaria vermiculophylla*) seaweed cultivated in an integrated multitrophic aquaculture system (IMTA) and included in the diet of sheep up to a level of 25% (as fed), as well as to examine wether these algae may have deleterious effects on immunity status of the animal or if they could instead be considered immunity enhancers. *Gracilaria* is one of the most cultivated genera of seaweeds around the world (Yarish and Pereira 2008), *G. vermiculophylla* being a nonindigenous Asian red alga, and a dominant *Gracilaria* species in the Ria de Aveiro, Portugal, where it reproduces throughout the year and attains high rates growth success under a wide range of environmental conditions (Abreu et al. 2011b). The green algae of the genus *Ulva* are a group of edible algae widely distributed in a variety of habitats (Peña-Rodríguez et al. 2011). Culture of seaweeds increasingly contributes to supply the worldwide seaweed demand, as natural stocks of seaweeds are insufficient. The integration of seaweed culture with existing aquaculture operations (IMTA) has been successfully achieved in land-based-contained systems. These systems have potential to decrease costs as the production of seaweeds can be achieved by utilizing ammonia, phosphate, and CO_2 from aquatic animal waste water, converting them into potentially valuable biomass. Effluents can recirculate back to the fish ponds or be discharged into the environment without negative impact (Neori et al. 2004). Many seaweed species may be suitable for bioremediation of aquaculture effluents (Lawton et al. 2013). *Gracilaria* species are efficient biofilters due to their capacity to uptake N (Abreu et al. 2011a). *Ulva* species are also ideal candidates for bioremediation of aquaculture effluents due to their high growth rates, broad environmental tolerance, low susceptibility to epiphytism (de Paula Silva et al. 2008; Mata et al. 2010), and high ability to absorb inorganic phosphorous and nitrogen (Mata et al. 2010).

Seaweeds

Seaweeds used in the present experiment (*U. rigida* and *G. vermiculophylla*) were cultivated in an IMTA system by ALGAPlus (Ílhavo, Portugal) as described by Domingues et al. (2015). After harvesting, seaweeds were rinsed with freshwater to remove epiphytes, detritus, and sand, subsequently dried for 8–10 h in a drying tunnel at 25 °C, and transported to the laboratory, where they were ground at 10 mm, and kept at the room temperature until usage.

In vivo digestibility

To estimate in vivo digestibility of the two seaweed species, three male and three female cross Merino sheep weighing 46 ± 2.0 and 42 ± 4.6 kg body weight (BW), respectively, at the start of the study were used. Animals were placed in individual metabolism crates with head gates and a steel grid floor beneath which there were sloped grids that allow the separation of urine from feces, respectively collected in scree-topped plastic boxes and plastic boxes. Animals were randomly allocated within sex to one of the three experimental diets (as fed) in a replicated 3 × 3 Latin square design with 15 days per period (10 days for diet adaptation and 5 days for total feces collection): 100% ground (10 mm) alfalfa hay (AH), 75% AH and 25% *U. rigida* (ULV), and 75% AH and 25% *G. vermicullophyla* (GRA). All diets were supplemented (30 g kg^{-1} diet) with a mineral-vitamin premix (all values per kg of DM: vitamin A 226,660 IU, vitamin D3 33,330 IU, vitamin E 1330 mg, Zn 1660 mg, Mn 1000 mg, Fe 333 g, Co 3.5 mg) formulated according to the mineral composition of the seaweeds (Cabrita et al. 2016). A maximum objective of daily diet DM intake allowed was set at 20 g per kg BW. After weighing and sampling the leftovers of alfalfa hay from the previous day, if present, seaweed was fed at 0900 hours and hay at 0930 hours in order to guarantee the total consumption of the seaweed. In the afternoon, seaweed was given at 1700 hours and hay at 1730 hours when the seaweed was already eaten. Animals had free access to clean water. In the last 5 days of each experimental period, total feces voided by each animal were daily collected, dried at 65 °C in a forced air oven until constant weight, bulked, subsampled, and ground to pass a 1-mm sieve for subsequent laboratory analysis. Representative samples of feeds and refusals, when present, were daily collected and dried at 65 °C for later analysis. Animals were weighed in the first and last days of each period at the same hour.

Analysis of immunological parameters

In the last day of each experimental period, blood samples were collected from the jugular vein for hemogram and proteinogram analysis and leukocyte isolation. Polymorphonuclear (PMN) and peripheral blood mononuclear cells (PBMC) were isolated by a single-step density gradient separation procedure. Briefly, whole blood diluted (1:2) in PBS was layered on a double gradient of Histopaque 1077 over histopaque 1119 (Sigma) and centrifuged at 400×*g* for 30 min at room temperature. Peripheral blood mononuclear cells were recovered from the interface between Histopaque-1077 and the plasma, and PMN were collected from the interface between Histopaque-1119 and Histopaque-1077. Platelets were removed by low-speed centrifugation during cell washing. The success of PBMC and PMN isolation was evaluated upon standard Hemacolor staining. Briefly, cytospins of the isolated cells were methanol fixed, stained with Hemacolor Solution 2 and 3 (Merck, Germany) washed by running tap water, mounted in Entellan (Merck), and observed through a microscope.

Production of reactive oxygen species

Purified PMN (1×10^6) cells were stimulated with 10 μM phorbol-myristate acetate (PMA) for 5 min at 37 °C and 5% CO_2. Cells without stimulus were used as controls of basal reactive oxygen species (ROS) production. Reactive oxygen species production was measured by using the Superoxide Detection kit (Enzo Life Sciences) and analyzed in an EPICS XL flow cytometer (Beckman-Coulter). Flow cytometry data were analyzed with FlowJo software (version 10.1). Reactive oxygen species production was evaluated by determining the mean fluorescence intensity (MFI) of the orange fluorescence emitted due to superoxide production within cells.

Proliferation assays

Isolated PBMC were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using the CellTrace CFSE Cell Proliferation Kit, for flow cytometry (ThermoFischer Scientific). CFSE-labeled cells (5×10^4) were cultured in RPMI complete medium (RPMI 1640 supplemented with 50 U mL^{-1} penicillin, 50 μg mL^{-1} streptomycin, 1 % HEPES buffer, 10 % FCS, and 5 μM 2-mercaptoethanol, all from Sigma) in 96-well round-bottomed culture plates without stimulus or stimulated with 2.5 μg mL^{-1} Lipopolysaccharide (LPS; Sigma) or 2.5 μg mL^{-1} Concanavalin A (ConA; Sigma) for 72 h at 37 °C and 5 % CO_2 in a humidified atmosphere. Cultured nonlabeled PBMC were used to define cell's autofluorescence. Cell proliferation was measured by flow cytometry through successive halving of the fluorescence intensity of CFSE. Two parameters were used to assess cell proliferation: the percentage of cells that divided at least once and the proliferation index, defined by the equation $\frac{\sum_0^i Ni}{\sum_0^i \frac{Ni}{2^i}}$, where *i* is the generation number (0 is the undivided population) and *Ni* corresponds to the number of cells in generation *i*.

Interferon-γ measurement

The concentration of IFN-γ in 72 h-cell culture supernatants from nonstimulated and LPS- or ConA-stimulated PBMC was determined by using the Bovine IFN-γ ELISA development kit (Mabtech), according to the manufacturer's instructions. The monoclonal antibodies of the kit cross-react with IFN-γ from sheep.

Analytical methods

Ground (1 mm) samples of feeds, refusals, and feces were analyzed for DM by drying samples at 105 °C for 24 h in a forced air oven (AOAC 1990). Representative samples of each feed (bulked for period) and of feces (bulked by period and animal) were subjected to analysis of ash (ID 942.05) (AOAC 1990), ether extract (EE; ID 920.39) (AOAC 1990), and neutral detergent fiber (NDF; with α-amylase and without sodium sulfite) (Robertson and Van Soest 1981; Van Soest et al. 1991). Samples of feeds were also analyzed for Kjeldahl N (ID 954.01) (AOAC 1990), acid detergent fiber (ADF), and acid detergent lignin (ADL) (Robertson and Van Soest 1981; Van Soest et al. 1991). Crude protein (CP) was determined as Kjeldahl N × 6.25 for hay and Kjeldahl N × 5.0 for seaweeds (Angell et al. 2016). Neutral detergent fiber and ADL were expressed exclusive of residual ash. Gross energy (GE) of feeds and feces was determined in an adiabatic bomb calorimeter (Werke C2000, IKA, Germany). All chemical analyses were run in duplicate.

Calculations and statistical analysis

The apparent digestibility (g kg^{-1}) of dietary constituints (DM; organic matter, OM; NDF; and GE) was calculated according to the following equation (intake and output of nutrients in kilograms): apparent nutrient digestibility = (1 − (fecal nutrient / total nutrient intake)) × 1000. The apparent digestibility (g kg^{-1}) of the seaweeds was calculated by difference and by animal, considering the apparent digestibility of the alfalfa hay measured in each period.

Data were tested for normality using the Kolmogorov-Smirnov test. Reactive oxygen species production in nonstimulated cells and IFN-γ production under LPS stimulation were subjected to inverse transformation and PMA-

stimulated cells to square transformation to achieve a normal distribution of the data.

The experimental design was a replicated 3 × 3 Latin square. Data were analyzed using the general linear model of SPSS (IBM SPSS statistics V22.0, USA). The model was:

$$Y_{ijkl} = \mu + S_i + c_{j(i)} + p_k + D_l + e_{ijkl}$$

where Y_{ijkl} = response variable, μ = mean, S_i = the fixed effect of square, $c_{j(i)}$ = the fixed effect of animal nested within square, p_k = the fixed effect of period, D_l = the fixed effect of diet, and e_{ijkl} = the experimental error. Significance is declared at $P \leq 0.05$. Trends are discussed at $0.05 < P < 0.10$.

Results

Chemical composition

The chemical composition of the experimental feeds is listed in Table 1. Alfalfa hay presented 186 g kg^{-1} CP, 372 g kg^{-1} NDF, and 73.0 g kg^{-1} ADL (DM basis). *Gracilaria vermiculophylla* and *U. rigida* presented, respectively, 359 and 470 g kg^{-1} ash, 202 and 123 g kg^{-1} CP, 183 and 221 g kg^{-1} NDF, and 12.8 and 9.58 MJ kg^{-1} of GE (DM basis).

Feed intake and in vivo digestibility

Daily DM intake of total diet, alfalfa hay, and of each studied seaweed is given in Table 2. Animals ingested 24 and 25% more of the diet without seaweed than the diets supplemented with *G. vermiculophylla* and *U. rigida*, respectively ($P < 0.001$). With the control diet, animals achieved the maximum allowed level of intake of 20 g DM kg^{-1} of BW, but when fed diets supplemented with *G. vermiculophylla* and *U. rigida*, they were not beyond 17.6 to 17.2 g DM kg^{-1} BW, respectively. There were no differences between the intake of alfalfa hay or seaweed among diets GRA and ULV.

Table 3 presents the apparent digestibility coefficients of DM, OM, NDF, and GE of total diets and of the studied seaweeds. The DM digestibility of diets with 25% of *G. vermiculophylla* and *U. rigida* was respectively 4 and 6% lower than the control diet with no seaweed supplementation ($P = 0.009$). The digestibility of OM and GE of the AH and ULV diets were similar and higher than that of GRA diet (more 5% for OM digestibility, $P = 0.005$, and 2.5 and 1% for GE digestibility, respectively, for AH and ULV diets, $P = 0.006$). Fiber digestibility of GRA and ULV diets were respectively 6 and 9% lower than that of AH diet ($P = 0.019$).

The DM digestibility of *G. vermiculophylla* was 13% higher than that of *U. rigida*, the values for these seaweeds being, respectively, 15 and 25% lower than the DM digestibility of the alfalfa hay. Conversely, the OM digestibility of *U. rigida* was similar to that of the alfalfa hay, and 36% higher than *G. vermiculophylla*. Neutral detergent fiber and GE digestibility were not different between seaweeds and averaged, respectively, 267.8 and 522.9 g kg^{-1} DM for *U. rigida* and 290.4 and 558.3 g kg^{-1} DM for *G. vermiculophylla*.

Analysis of immunological parameters

The values of all measured hematological parameters were within the normal range for sheep. As it can be observed in Table 4, experimental treatments had no significant effects ($P > 0.05$) on the cell blood count parameters or on the concentration of serum proteins.

A putative influence of seaweed supplementation in immunological parameters characteristic of both innate and adaptive immunity was assessed using cells isolated from the peripheral blood. As shown in Fig. 1, PMA-stimulated PMN produced markedly higher amounts of superoxide anion when compared to the nonstimulated ones. Nevertheless, no differences could be observed in this parameter among animals fed with any of the diets (Table 4). Lymphocyte proliferative response to classic T and B lymphocyte mitogens ConA and LPS was assessed as a surrogate marker for adaptive immunity competence. A marked proliferative response was induced in PBMC upon stimulation with ConA. Nonetheless, it was not different among animals fed with AH, GRA, or ULV diets either when proliferation was measured as the percentage of cells that divided at least once or by the proliferation index. Stimulation of PBMC with LPS resulted in a minor proliferative effect that did not differ considerably from controls and that was similar among diet groups (Table 5 and Fig. 2). The lack of significant differences in the lymphocyte proliferative response to ConA could in part be attributed to the high variability observed, which was already reported in sheep (Wattegedera et al. 2004). The

Table 1 Chemical composition of the experimental feeds (average from three experimental periods)

	DM (g kg^{-1})	OM	Ash	EE (g kg^{-1} DM)	CP	NDF	ADF	ADL	GE (MJ kg^{-1} DM)
Alfalfa hay	881	892	108	14.8	186	372	265	73.0	18.1
Gracilaria vermiculophylla	867	625	359	2.3	202	183	95.3	31.0	12.8
Ulva rigida	845	530	470	3.2	123	221	189	71.3	9.6

Table 2 Dry matter (DM) intake of the total diet, alfalfa hay, and the seaweeds *Gracilaria vermiculophylla* and *Ulva* sp.

	AH	GRA	ULV	SEM	*P* (diet)
Total diet[a]					
g DM day^{-1}	944 a	763 b	758 b	38.8	0.016
g DM kg^{-1} body weight day^{-1}	21.0 a	17.6 b	17.2 b	0.87	0.030
g DM kg^{-1} body $weight^{0.75}$ day^{-1}	54.3 a	45.0 b	44.3 b	2.23	0.024
Alfalfa hay intake					
g DM day^{-1}	912 a	563 b	559 b	25.1	<0.001
g DM kg^{-1} body weight day^{-1}	20.3 a	13.0 b	12.7 b	0.58	<0.001
g DM kg^{-1} body $weight^{0.75}$ day^{-1}	52.5 a	33.3 b	32.7 b	1.46	<0.001
Seaweed intake					
g DM day^{-1}	–	183	174	6.6	0.448
g DM kg^{-1} body weight day^{-1}	–	4.24	3.96	0.167	0.368
g DM kg^{-1} body $weight^{0.75}$ day^{-1}	–	10.9	10.2	0.41	0.377

Means with different lowercase letters are significantly different ($P < 0.05$)

AH 100% alfalfa hay, *GRA* 75% AH + 25% *Gracilaria vermiculophylla*, *ULV* 75% AH + 25% *Ulva rigida*, *AH* alfalfa hay, *GRA Gracilaria vermiculophylla*, *ULV Ulva rigida*

[b] Including premix at 30 g kg^{-1}

amounts of the pro-inflammatory cytokine IFN-γ measured in culture supernatants of nonstimulated control cells were very low, as could be expected. Conversely, ConA-stimulated cells produced significantly higher amounts of IFN-γ than the nonstimulated cells ($P < 0.05$). However, no differences could be observed in IFN-γ production between PBMC isolated from control diet-fed animals and seaweed-supplemented diet-fed sheep, regardless of the seaweed used (Table 5). No effect was observed upon LPS stimulation, as compared to controls, and no significant differences were observed among diet groups. Altogether, these results indicated that seaweed supplementation did not influence hematological parameters, ROS production by neutrophils, and the lymphocytic response to mitogens. This provides preliminary evidence indicating that these seaweed diet supplementations have no deleterious or enhancing effects on basic immune functions of cells mediating innate and acquired immunity.

Discussion

Nutritive value

The chemical composition of the alfalfa hay used in the present study agrees with the values reported by FEDNA (2010) for a high quality alfalfa hay. Seaweeds drawn from the water are a rich source of minerals, their content being higher than those reported for edible land plants (Rupérez 2002). Ash, which broadly represents mineral content (Cabrita et al. 2016), was the greatest constituent in both seaweeds, being higher than the values observed in an earlier study (Cabrita et al. 2016) with *Ulva* sp. and *G. vermiculophylla* originated from the same IMTA system (25.0% DM for *Ulva* sp., and 27.8% DM for *G. vermiculophylla*).

Due to the high and more constant levels of nutrients in the medium, IMTA systems can improve the productivity and nutritional quality of the seaweeds, when compared to the naturally harvested ones (Abreu et al. 2009). *Ulva rigida* presented lower CP content than *G. vermiculophylla*. However, higher protein content (up to 34% in *U. lactuca*, and 49% in *Gracilaria*) has been reported in seaweed produced in IMTA systems (Schuenhoff et al. 2003; Shields and Lupatsch 2012;

Table 3 Apparent digestibility coefficients of dry matter (DM), organic matter (OM), neutral detergent fiber (NDF), and gross energy (GE) of total diets and of the seaweeds *Gracilaria vermiculophylla* and *Ulva rigida*

	Digestibility coefficient			SEM	*P* (diet)
	AH	GRA	ULV		
Diet					
DM, g kg^{-1}	628 a	605 b	592 b	5.5	0.009
OM, g kg^{-1} DM	677 a	645 b	680 a	4.8	0.005
NDF, g kg^{-1} DM	413 a	390 b	379 b	6.0	0.019
GE, MJ kg^{-1} DM	646 a	630 b	641 a	2.0	0.006
Seaweed					
DM, g kg^{-1}		534	471	8.2	0.034
OM, g kg^{-1} DM		505	686	20.4	0.025
NDF, g kg^{-1} DM		268	290	20.7	0.527
GE, MJ kg^{-1} DM		523	558	9.8	0.128

Means with different lowercase letters are significantly different ($P < 0.05$)

AH alfalfa hay, *GRA Gracilaria vermiculophylla*, *ULV Ulva rigida*

Table 4 Blood parameters from animals fed experimental diets

	AH	GRA	ULV	SEM	*P* (diet)
Leukocytes, $\times 10^3$ μL^{-1}	6.05	5.40	5.35	0.402	0.454
Total erythrocytes count, $\times 10^6$ μL^{-1}	11.35	12.03	11.45	0.289	0.310
Hemoglobin, g dL^{-1}	12.72	13.22	12.75	0.294	0.486
Hematocrit, %	42.96	45.01	42.47	1.097	0.301
Mean corpuscular volume, fL	37.73	37.53	37.05	0.346	0.383
Mean corpuscular hemoglobin, pg	11.24	11.01	11.17	0.097	0.340
Mean corpuscular hemoglobin concentration, g dL^{-1}	30.19	29.86	30.18	0.233	0.584
Erythrocytes distribution index, %	22.66	21.63	22.15	0.651	0.617
Platelets, $\times 10^3$ μL^{-1}	550	467	544	98.8	0.824
Mean platelets volume, fL	7.17	7.58	9.68	2.773	0.771
Neutrophils, $\times 10^3$ μL^{-1}	2.12	1.66	1.82	0.225	0.448
Eosinophils, $\times 10^3$ μL^{-1}	0.193	0.152	0.177	0.0207	0.478
Basophils, $\times 10^3$ μL^{-1}	0.017	0.018	0.020	0.0030	0.729
Monocytes, $\times 10^3$ μL^{-1}	0.073	0.163	0.078	0.0293	0.145
Lymphocytes, $\times 10^3$ μL^{-1}	3.64	3.39	3.24	0.224	0.472
Albumin, g dL^{-1}	2.80	2.79	2.85	0.035	0.405
Alpha, g dL^{-1}	1.68	1.53	1.62	0.117	0.730
Beta, g dL^{-1}	1.51	1.54	1.61	0.030	0.129
Gamma, g dL^{-1}	0.292	0.280	0.302	0.027	0.788
Total proteins, g dL^{-1}	6.28	6.15	6.38	0.119	0.408

AH 100% alfalfa hay, *GRA* 75% AH + 25% *Gracilaria vermiculophylla*, *ULV* 75% AH + 25% *Ulva rigida*

Silva et al. 2015). Differences among studies could be explained by the culture conditions applied. For instance, biomass production and nutrient removal from seaweeds are negatively related to the cultivation densities in the system, temperature, and light being the main environmental factors affecting the growth and nutrient removal (Abreu et al. 2011a).

The low lipid content of both seaweeds is in agreement with several studies (Khairy and El-Shafay 2013; Mouritsen et al. 2013). The high ash and the low lipid contents observed in the studied seaweeds were reflected in their low GE content, supporting the work by Hind et al. (2014) who referred that energy potential of *U. lactuca* is limited by its high mineral content. Indeed, earlier work observed higher energy content for *U. lactuca* (15.2 MJ GE kg^{-1} DM, Felix and Brindo 2014; estimated digestible energy of 10.2 MJ kg^{-1} DM, Ventura and Castañón 1998).

Ulva rigida had a higher fiber content than G. *vermiculophylla*, but lower than alfalfa hay. Unlike terrestrial plants, seaweeds have complex cell wall polysaccharides that greatly differ among seaweed classes and species (Pereira et al. 2009). Green algae are rich in soluble ulvans of the

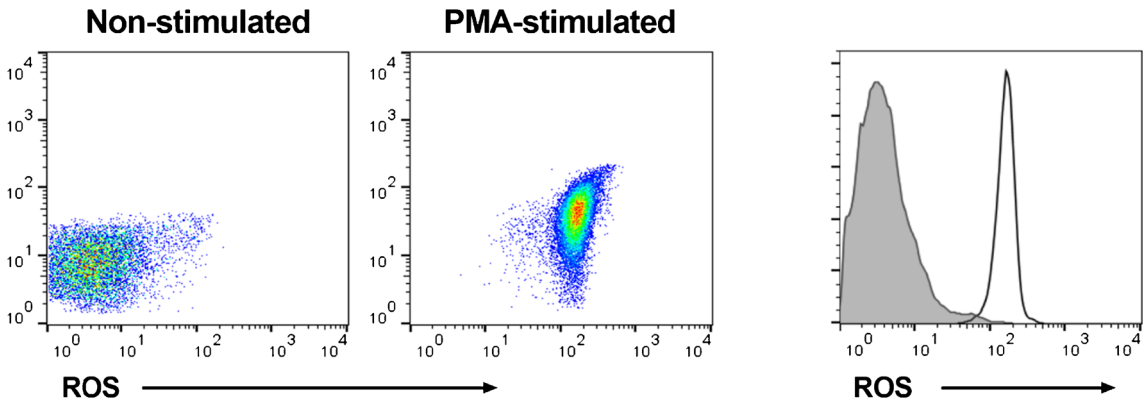

Fig. 1 Representative dot plots and overlayed histograms corresponding to flow cytometry analysis of ROS production in nonstimulated (*gray*) and PMA-stimulated (*solid line*) PMN cells. Example shown is from one animal fed with AH

Table 5 Immunological parameters from animals fed experimental diets

Immune function	Parameter	Stimulus	Diet			SEM	*P* (diet)
			AH	GRA	ULV		
ROS production	Mean fluorescence intensity	None[a]	0.159	0.180	0.189	0.0208	0.577
		PMA[b]	30,435	25,409	23,852	6023.5	0.714
Lymphocyte proliferation	Percentage of dividing cells	None	0.107	0.133	0.102	0.0172	0.473
		LPS	14.61	14.51	13.64	2.467	0.952
		ConA	46.46	46.86	42.72	4.812	0.798
	Proliferation index	LPS	1.155	1.162	1.119	0.0446	0.488
		ConA	1.702	1.851	1.560	0.2048	0.339
IFN-γ production	pg mL^{-1}	None	0.670	0.333	0.410	0.2178	0.547
		LPS[2]	2.849	1.964	2.506	0.5023	0.518
		ConA	265.6	242.9	250.9	64.43	0.969

AH 100% alfalfa hay, *GRA* 75% AH + 25% *Gracilaria vermiculophylla*, *ULV* 75% AH + 25% *Ulva rigida*

[a] Inverse

[b] Square

family of sulfated polysaccharides (Domozych et al. 2012), while carrageenans (Michel et al. 2006), agars, and porphyran (Correc et al. 2011) are the major matrix polysaccharides of red algae.

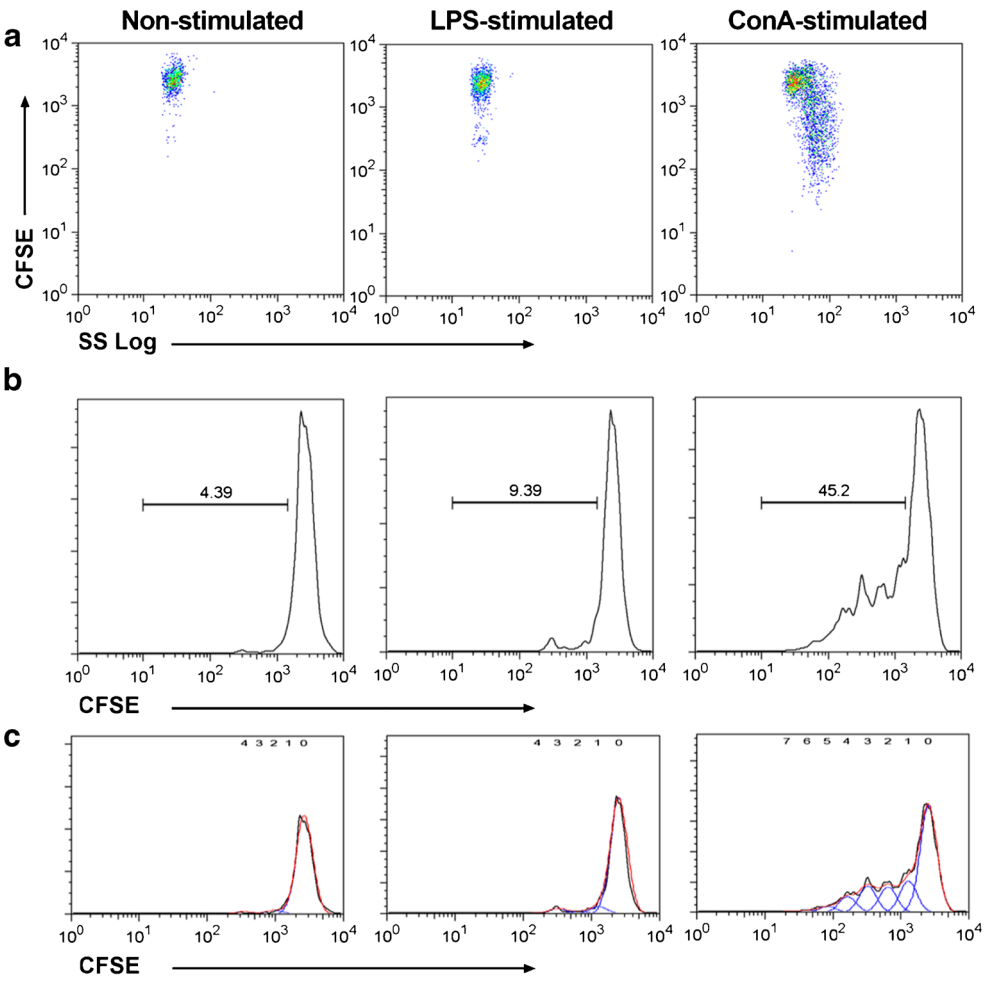

Fig. 2 Flow cytometry analysis of PBMC proliferation using CFSE-labeling. **a** Representative example showing gated CFSE-positive PBMC cells nonstimulated or stimulated with LPS or ConA, obtained from an AH diet-fed animal. **b** Histograms showing the percentage of cells that divided at least once. **c** Histograms showing the number of generations traced by CFSE fluorescence dilution. *Numbers above the peaks* represent the number of times the respective cell population divided. *Red lines* correspond to the model sum of the *blue lines* which correspond top calculated generation peaks

When fed the control diet, animals achieved the maximum allowed level of intake of 20 g DM kg^{-1} BW, but when supplemented with seaweeds, the DM intake decreased by around 20%. The lower seaweed palatability have been atributed to the existence of secondary metabolites (Cronin and Hay 1996) or to the high mineral content (Cabrita et al. 2016). This could be overcome by decreasing the level of dietary inclusion of seaweeds that in the present study was on average 24.5 and 23.7% (DM basis) respectively for *G. vermiculophylla* and *U. rigida*. Indeed, the actual low supply of these seaweeds makes lower levels of supplementation more realistic.

In the present study, in vivo digestibility was measured after 10 days for animal adaptation to the diet. This adaptation or preliminary period is designed to ensure that feed residues of the previous diet are eliminated before starting the feces collection, that a stable rumen population is established, and that the animals are eating approximately the same daily amount of feed and at the same time (Rymer 2000). The recommended length of time for the adaptation period varies from 4 to 14 days (Rymer 2000), and it is expected that its lengthening would improve the accuracy and precision of measurements. However, due to animal welfare concerns, efforts should be made to shorten the experimental periods as much as possible. When animals are fed ad libitum, where more variation in daily intake and excretion exists, the adaptation period should be at least 12 days long, but it could be shortened if following the worldwide recommendations to feed restrictively when measuring digestibility to decrease the variability of digestion and excretion processes (Farenzena et al. 2016), as done in the present study.

The digestibility values determined for alfalfa hay are in close agreement with those reported earlier (Carvalho et al. 2005). Both seaweeds presented lower DM digestibility than alfalfa hay, with the OM digestibility of *U. rigida* being similar to that of the alfalfa hay and higher than that of *G. vermiculophylla*, which was similar to the values reported for meadow hay, rice, rye, and wheat straw (Fonseca et al. 1998). A direct comparison of the values herein obtained with the literature is difficult because of the limited data regarding in vivo digestibility, *in sacco* degradability, and energy values of seaweeds for ruminants. A similar OM digestibility was obtained by Ventura and Castañón (1998) for *U. lactuca* (621 g kg^{-1} DM), but the values of OM digestibility reported in the current study were lower than that referred for a *Laminaria digitata* and *Laminaria hyperborea* mixture measured in vitro (78.3%) by Hansen et al. (2003). In the study by Arieli et al. (1993) with young rams, the in vivo energy digestibility of *U. lactuca* (60%) was similar to the value obtained in the present study, but the digestible energy value (9.1 MJ kg^{-1} DM) was higher. The results obtained suggest that the main constraint to the use of these seaweeds at high dietary inclusion rates is their low fiber digestibility and their high mineral content. As far as we know, this is the first report of seaweed in vivo fiber digestibility. Typically, seaweeds have low amounts of cellulose (around 4%) and are rich in specific polysaccharides. Despite ulvans from green algae being potentially hydrolysable to bioactive oligosaccharides (Andrieux et al. 1998), ulvan lyases have only been isolated in marine environments (Barbeyron et al. 2011) and in *Proteobacteria* species found in soil (Collén et al. 2011). Similarly, the hydrolysis of galactans from red algae requires enzymes predominantly encoded in genomes of marine microbes, but less frequent or even absent in bacteria that hydrolyze polysaccharides from land plants (Hehemann et al. 2010). These could be hydrolyzed by the rumen microbial population producing methane and acetic acid (Williams et al. 2013). However, differences on seaweed digestibility between studies could be explained not only by the composition of the algae but also by the adaptation of the animal to this particular feed (Makkar et al. 2016). Indeed, Orpin et al. (1985) found that 13% of the culturable bacteria from seaweed-fed sheep grew on alginate, 71% on laminarin, 13% on fucoidan, and 99% on mannitol, while the percentages obtained from pasture-fed animals were significantly lower (2, 32, 0, and 0%, respectively). Differences in the ability to hydrolyze mannitol between seaweed-fed or grass-fed animals were also observed in other studies (Ahmed et al. 2013). Seaweed feeding seems to change rumen microflora substantially, not including phycomycete fungi or cellulolytic bacteria, but ciliate protozoa (e.g., *Dasytricha ruminantium*, *Entodinium* species) and lactate-utilizing bacteria (e.g., *Streptococcus bovis*, *Selenomonas ruminantium*, *Butyrivibrio fibrisolvens*) (Orpin et al. 1985); thus, a stepwise increase in the levels of seaweeds in the diet may enable rumen microbes to adapt and, thus, enhance energy availability from these complex carbohydrates (Makkar et al. 2016).

Immunological parameters

Very little is known about putative effects of diet supplementation with seaweed or seaweed extracts on hematological or immunological parameters. The results presented here are in line with a previous report in which *Ascophylum nodosum* extract inclusion in the diet of goats did not affect white blood counts (Kannan et al. 2007). Evaluation of different immunological parameters also indicated that seaweed supplementation done in this study had no adverse or enhancing effects on immunity. Reactive oxygen species production is a major immune effector mechanism used by neutrophils (Amulic et al. 2012). Previous reports have shown a reduction in ROS production after ex vivo stimulation of monocytes or neutrophils with algae or their extracts (Jeon et al. 2012; Jeon et al. 2014). However, when lambs subjected to heat stress were fed with a diet supplemented with extracts of the brown alga *A. nodosum*, there was an enhancement on the ability of monocytes to produce ROS (Saker et al. 2004). Our results

did not indicate that inclusion of seaweed in the sheep diet could majorly affect ROS production by PMN. Nevertheless, minor effects in this innate immune mechanism could have been missed due to sample size limitations. Indeed, according to the results obtained in the present study, a power analysis suggested a minimum sample size per treatment group of eight to 12 to ensure a minimum margin of error.

Here, the lymphocyte proliferative response to mitogens was used as a surrogate marker of acquired immunity to evaluate whether seaweed inclusion could affect the ex vivo function of lymphocyte cells. Ciliberti et al. (2015) reported that PBMC isolated from sheep fed with a diet supplemented with *A. nodosum* had impaired T cell proliferation responses when stimulated with the T cell mitogen phytohemagglutinin (PHA). The authors attributed the effect to the high content of eicosapentaenoic acid in that brown macroalga. Contrastingly, we show here that supplementation with a green and a red algae had no negative impact on the lymphocyte proliferation response to mitogens. The low levels of this polyunsaturated fatty acid in *U. lactuca* and *G. vermiculophylla* (van Ginneken et al. 2011; Imbs et al. 2012) could be accounting for these results.

Seaweeds are particularly rich in trace minerals like zinc, copper, chromium, and selenium or in vitamins E and β-carotenes (Mišurcová 2011). It was shown that zinc diet supplementation in lambs increased lymphocyte proliferation to ConA (Nagalakshmi et al. 2009), which was in accordance with the reduced lymphocyte response to T cell mitogens reported by Droke and Spears (1993) in a zinc deficiency study. Although *G. vermicullophylla* has a relatively high zinc content (Cabrita et al. 2016), diet supplementation with this seaweed did not result in altered lymphocyte proliferation. This might agree with another study showing that in vitro lymphocyte proliferation upon PHA stimulation in PBMC from steers fed control or zinc supplemented diets was not different (Spears and Kegley 2002). Selenium (Turner and Finch 1990; Cao et al. 1992) was shown to be important for adequate lymphoproliferative responses to T cell mitogens. The macroalgae used here are enriched in selenium (Cabrita et al. 2016), yet no improved lymphocyte proliferation was found. A putative immune-enhancer effect of seaweed supplementation on lymphocyte function would nevertheless be worth assessing in a condition of selenium-poor diet.

The pro-inflammatory cytokine IFN-γ is a major effector in cell-mediated immune responses to common intracellular pathogens affecting sheep, like *Chlamydophila abortus* and *Toxoplasma gondii* (Graham et al. 1995; Esteban-Redondo and Innes 1997). Despite the importance of IFN-γ, very few studies addressed the effect of the diet on the production of this inflammatory cytokine. In accordance with the lack of effect of seaweed supplementation observed in the present study, two previous studies showed no effect of *A. nodosum* supplementation on the IFN-γ production in either stimulated or nonstimulated sheep PBMC (Ciliberti et al. 2015) and on sheep Th1 responses (Caroprese et al. 2014).

The evidence reported here indicating that *U. rigida* and *G. vermiculophylla* diet supplementation have no deleterious effect on the immune function of cells mediating innate and acquired immunity further supports the potential of seaweed as feed ingredients for ruminant feeding. Exploring the studied and other immune parameters in longer feeding periods, in different physiological conditions or environmental challenges, such as infection, would be important to more accurately ascertain the safety or potential beneficial effects of the seaweed diet supplementation.

Conclusions

Digestibility of *U. rigida* and *G. vermiculophylla* was lower than that of alfalfa hay, the results suggesting that the main constraint to use these seaweeds as feed ingredients for ruminant animals being the low fiber digestibility. On the other hand, dietary supplementation with these seaweeds at 25% (as fed) did not affect the immune function of cells mediating innate and acquired immunity, which suggests that these two species are safe ingredients for ruminant nutrition.

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Compliance with ethical standards The present experiment was conducted at the Clinical and Veterinary Investigation Center of Vairão (Vila do Conde, Portugal) from the Institute of Biomedical Sciences Abel Salazar, University of Porto (ICBAS-UP), in strict accordance with good animal practice as defined by national authorities and European Union Directive 2010/63/EU. The experimental animal procedures were approved by the Local Animal Ethics Committee of ICBAS-UP, licensed by the Portuguese Directorate-General of Food and Veterinary Medicine (Direção Geral de Alimentação e Veterinária) of the Ministry for Agriculture and Sea (Ministério da Agricultura e do Mar), and conducted by trained scientists following FELASA category C recommendations. All methods and procedures were performed following the established guidelines from these institutions.

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