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Evaluation of different ensiling methods for *Saccharina latissima* preservation: influence on chemical composition and *in vitro* ruminal fermentation

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Abstract

Saccharina latissima is a brown seaweed that could be used in ruminant feeding, but its fast deteriorating and seasonal growth nature limit their utilization in the practice. Ensiling could be used as a preservation method, but information of its effects on the nutritional value of the seaweed is limited. This study evaluated the *in vitro* ruminal fermentation of different *S. latissima* silages using ruminal inoculum either from goats fed a mixed diet (60:40 oat hay:concentrate) or from sheep fed a high-forage diet (90:10 alfalfa hay:concentrate) to simulate different small ruminant production systems. *S. latissima* was ensiled in vacuum bags without additives (Control), with formic acid (4 g/kg seaweed; FA), with lactic acid bacteria (LAB), or with LAB after a pre-wilting treatment to reach a seaweed dry matter (DM) content of 30% (30LAB). Ensiling *S. latissima* decreased ($P < 0.05$) the content in DM, neutral detergent fibre, and total extractable polyphenols, but nitrogen and fat content were unaffected. For both ruminal inoculums, ensiling decreased ($P < 0.05$) the asymptotic gas production after 120 h of fermentation (excepting for FA silage with goats' inoculum), but total volatile fatty acid (VFA) production was unaffected. The VFA profile shifted towards greater ($P < 0.05$) acetate and lower ($P < 0.05$) propionate proportions in all silages compared with the pre-ensiling *S. latissima*. When goats inoculum was used, greater ($P < 0.05$) CH_4 production compared with pre-ensiling *S. latissima* was observed in all silages, excepting the Control one, which led to greater ($P < 0.05$) CH_4/total VFA ratio. In contrast, no differences among samples ($P > 0.05$) in either CH_4 production or CH_4/total VFA ratio were observed when sheep' inoculum was used. Fermentation of all samples started earlier with goats' inoculum than with sheep' inoculum, which was attributed to the different diet fed to the animals. These results suggest that ensiling *S. latissima* with either formic acid or lactic acid bacteria could be a viable conservation method to preserve the nutritive value.

1. Introduction

Seaweeds production has increasingly grown over the last decade and currently is greater than 30 million tones worldwide (Yen et al., 2022), but seaweeds still remain as an underutilized resource for either human or animal nutrition. In recent years, the interest in using seaweeds as food or feed has increased in Europe, as it might contribute to fulfill objectives related to 'Blue Growth' and climate and food security and to alleviate the shortage of feedstuffs (Barbier et al., 2019). Moreover, utilization of seaweeds in animal feeding is of special interest, as it might also contribute to the sustainability of the livestock sector. Seaweeds containing high proportions of fibre might be more adequate for small ruminants (sheep and goats) than for other animals species, as they ferment fibre more efficiently (Van Soest, 1994). However, its practical implementation as common feeds in the livestock sector is difficult as decomposition processes start shortly after harvest due to their high-water content (Novoa-Garrido et al., 2020). Therefore, it is necessary to utilize conservation and storage methods capable of preserving the nutrients and bioactive compounds present in seaweeds.

Silage is a low cost and energy efficient storage method commonly used with terrestrial crops which could be utilized for preserving seaweeds; however, some characteristics of seaweeds might be troublesome to adapt this conservation method. Seaweeds have high pH, and high content in water, ashes, and non-fermentable carbohydrates and other polysaccharides, which might limit the lactic acid fermentation required for this process (Magnusson et al., 2016). Nevertheless, when the silage process is challenging, fermentation of water-soluble carbohydrates and other easily fermentable fractions can be enhanced with inoculants or/and additives but information on their efficacy in seaweeds silages is limited (Yen et al., 2022).

Saccharina latissima is the most produced brown seaweed in Europe because of its large biomass yields, broad geographical distribution, early availability of kelp production protocols and potential nutritional value for either humans or animals (Araújo et al., 2021). It contains a wide range of bioactive compounds, such as phlorotannins and pigments (Holdt and Kraan, 2011), and complex carbohydrates like laminarin, mannitol and alginate (Horn, 2009). For these reasons, *S. latissima* could become a resource of interest for the animal feed industry. The potential ensilability of *S. latissima* and the effect of various additives on silage quality have been evaluated in previous works (Cabrita et al., 2017; Campbell et al., 2020; Novoa-Garrido et al., 2020; Yen et al., 2022), but information about the effect of ensiling *S. latissima* on its ruminal fermentation is inexistent. Therefore, the objective of this study was to evaluate the *in vitro* ruminal fermentation of different silages of *S. latissima*. The diet of the donor is one of the main factors affecting *in vitro* fermentation parameters (Martínez et al., 2010a; Mateos et al., 2013), and therefore the *in vitro* trials were conducted using as inoculum ruminal fluid from small ruminants fed either medium-forage or high-forage diets. Diets were formulated to be representative of different practical feeding conditions, such as those for dairy goats (medium-forage diet) and low-producing sheep (high-forage).

2. Material and methods

2.1. Seaweed samples and experimental procedure

Biomass of *Saccharina latissima* was obtained from commercial stocks at Lofoten (Lofoten Blue Harvest AS, Lofoten, Norway) in June 2019, when seaweeds is expected to contain high amounts of total nitrogen (N) and low amounts of neutral detergent fibre (NDF; de la Moneda et al., 2019). The *S. latissima* biomass was stored in tanks with running seawater and processed within two days after collection. The biomass was washed in three sequential water baths with decreasing salinity (seawater, 7:3 mixture of

seawater and fresh water, and fresh water), the excess water was drained by hand, and the biomass was cut into pieces of approximately 2 x 3 cm. Two samples of the biomass (pre-ensiling seaweed) were randomly collected and frozen (-40ºC) until further analysis.

The experiment was conducted as a completely randomized design in which the treatment was the only factor analyzed. Five treatments (pre-ensiling seaweed and four silage treatments) were evaluated. Four silage treatments were tested: ensiling without additives (Control), with formic acid (FA; 4 g/kg seaweed), with lactic acid bacteria (LAB; *Lactobacillus fermentum* at $2.5x10^9$ CFU (colony forming unit)/kg seaweed and *Lactobacillus plantarum* at $2.5x10^9$ CFU/kg seaweed), and with LAB inoculants after a pre-wilting treatment of the seaweed until reaching a dry matter (DM) content of 30% before ensiling (30LAB).

To prepare the Control, FA and LAB silages, 2 kg of fresh chopped *S. latissima* were introduced into each of 18 vacuum bags (Lavezzini, Fiorenzuola d'Arda, Italy; dimensions 30 × 60 cm). The precise quantity of the additive required for each silage treatment were prepared (weighed or measured) and they were added to each bag of the corresponding treatment (6 bags / silage treatment; no additive was added in the Control silage). The content of each bag was thoroughly mixed by hand until the additive was evenly distributed in the whole seaweed biomass. Finally, the air inside the bags was extracted by using a vacuum-packing machine (model Elix; Lavezzini, vacuum pump 20–24 l/min), and bags were heat-sealed.

For preparing the 30LAB silages, 2 kg of *S. latissima* biomass were weighed into each of 6 netting bags that were placed into an air-forced oven at 37 ºC. The weight of the bags was controlled every 2 h until reaching the expected DM content of 30%. The targeted weight was calculated based on the DM content of seaweed measured immediately after biomass arrival to the laboratory. Once the expected weight was reached, the biomass

was quantitatively transferred to vacuum bags and bags were processed as described for the LAB silage treatment.

All silage bags were stored in darkness at 16ºC. After three months of storage, the bags were opened and the content of 3 bags from each treatment were randomly pooled to obtain 2 samples per silage treatment. Samples were then frozen and stored frozen (- 40 ºC) until further analysis. Frozen samples of the pre-ensiling seaweed (2 samples) and of the silages (8 samples; 2 samples / silage treatment) were freeze-dried and ground to pass a 1 mm sieve (ZM 200 mill, Retsch GmbH, Haan, Germany) before chemical composition analyses and *in vitro* incubations.

2.2. Donor animals and feeding

Four Murciano-Granadina female goats (51.5 ± 6.15 kg body weight (BW)) and four Lacaune female sheep (64.2 ± 1.95 kg BW), each provided with a permanent ruminal cannula, were used as donors of ruminal fluid. Animals were individually housed in pens with free access to drinking water, and they were fed different diets (Table 1). Goats were fed a mixed diet composed of 60% oat hay and 40% concentrate, whereas sheep were fed a diet composed of 90% alfalfa hay and 10% concentrate (both in fresh matter basis). Animals were fed twice daily in two equal portions at 45 g DM/kg $BW^{0.75}$ to prevent feed selection. The care of the animals and ruminal fluid extraction were carried out by trained personnel in accordance with the Spanish guidelines for the protection of animals used for experimentation or other purposes. The experimental procedures with goats were approved by the Animal Welfare Committee at the Zaidín Experimental Station of the Spanish National Research Council (Approval number: 05/24/2016/091), and those with sheep were approved by the Institutional Animal Care and Use Committee of the Comunidad Autónoma de Madrid of Spain (Approval number PROEX 035/17).

Table 1 near here

2.3. In vitro trials

In vitro trials were conducted using as inoculum the ruminal fluid from each group of rumen-fistulated animals. Within each group of donor animals, the ruminal fluid from each individual animal, was used independently as inoculum to obtain four replicates per analyzed sample. Two different *in vitro* trials were carried out using the same methodology. The goal of the first trial was to determine the gas production kinetics of the samples and lasted for 120 h, whereas the second trial was carried out to assess the main fermentation parameters and CH_4 production after 24 h of incubation.

The *in vitro* trial aimed to measure gas production kinetics was performed following the recommendations of Rymer et al. (2005). The ruminal content of each animal was collected before the morning feeding and immediately transported to the laboratory in thermal flasks pre-warmed at 39 ºC. Ruminal contents were filtered through four layers of surgical gauze and the fluid of each animal (sheep or goat) was independently mixed with a pre-warmed (39 ºC) buffer solution (Goering and Van Soest, 1970; without trypticase) in a proportion 1:4 under a flow of CO_2. Five hundred mg of DM of each sample were weighted in 120 mL vials (4 vials per sample), which were filled with 50 mL of the ruminal fluid-buffer mixture and sealed with rubber stoppers before being incubated at 39 ºC for 120 h. Gas production was measured at 2, 4, 6, 9, 12, 24, 30, 48, 54, 72, 96 and 120 h, using a pressure gauge scope (Sper Scientific LTD, Scottsdale, AZ, USA) and a calibrated plastic syringe (Ruthe®, Normax Marinha Grande, Portugal), releasing the gas produced at each measurement time. Additionally, two vials without substrate per inoculum were incubated to correct for the gas produced by endogenous substrates added with the inoculum.

The *in vitro* incubations for measuring the main fermentation parameters were performed following the same methodology, with the exception that incubations lasted for 24 h and the parameters were determined at the end of the incubation period. Gas production was measured as described before and about 10 mL of gas was sampled through the vial caps with a syringe and stored in a vacuum tube (Terumo Europe N.V., Leuven, Belgium) for CH_4 concentration analysis. Vials were then uncapped, their content was homogenized, the pH was measured (Crison Basic 20 pH-meter, Crisson Instruments, Barcelona, Spain) and fermentation was stopped by placing the vials into cold water. Two mL of the vials' contents were mixed with 2 mL of 0.5 M HCl for volatile fatty acids (VFA) and NH_3-N analyses, and the samples were frozen (−20 ºC) until analysis.

When performing the *in vitro* trials an additional sample of the forage fed to donor animals (either oat or alfalfa hay) was included in the incubations to be used as reference feed.

2.4. Chemical analyses

The DM content of pre-ensiling *S. latissima* and the silages was determined by freeze-drying and subsequent drying of the freeze-dried material in an oven at 103 ºC for 24 h. Ash (ID 048.13), ether extract (EE; ID 945.16) and total starch (ID 996.11) contents were determined according the AOAC procedures (AOAC, 2005). Total nitrogen (N) content was analyzed according to the Dumas method using a TruSpec CN equipment (Leco Corp., St. Joseph, MI, USA). The NDF content was determined following the procedure of Van Soest et al. (1991) with α-amylase and using an Ankom 220 Fiber Analyzer unit (Ankom Technology Corp., Macedon, NY), whereas both acid detergent fibre (ADF) and lignin were determined as described by Robertson and Van Soest (1981). All results were expressed ash-free. Non-structural carbohydrates (NSC) content was calculated as [1000 - (NDF + crude protein + EE + ash)]. For this calculation the crude protein level was

197 estimated as (4.79 x N content), as Bikker et al. (2020) reported conversion factors
198 ranging from 4.69 to 4.89 for *S. latissima* samples.

199 The content in total extractable polyphenols (TEP) was determined using the
200 methodology described by Julkunen-Tiito (1985), and soluble sugars content was
201 determined by the Anthrone method (Yemm and Willis, 1954). The gross energy (GE)
202 was analyzed using a calorimeter (6400 Automatic Isoperibol Calorimeter, Parr
203 Instruments, Moline, IA). The concentration of N-NH_3 was determined following the
204 colorimetric method described by Weatherburn (1967) using a spectrophotometer
205 (Thermo Scientific, Genesys 10 uV Scanning, Madison, WI, USA) and those of VFA by
206 gas chromatography using a HP 5890 Series II gas chromatograph (Hewlett Packard,
207 Waldbronn, Germany) equipped with a flame ionization detector and an HPINNOWAX
208 cross linked polyethylene glycol column (25 m x 0.2 mm x 0.2 µm; Teknokroma, Madrid,
209 Spain) as described by de la Moneda et al. (2019). Determination of CH_4 concentration
210 in the gas produced was performed by gas chromatography as described by Martínez et
211 al. (2010a).

212 *2.5. Calculations and statistical analysis*

213 The data of gas production were adjusted to the model proposed by Schofield et al.
214 (1994): Gas = $A/(1 + \exp^{(2 + 4*c*(lag-t))})$ in which A is the asymptotic gas production, *c* is
215 the gas production rate, *lag* is the delay at the start of gas production, and *t* is the time of
216 gas measurement. Parameters A, *c* and *lag* were estimated using an iterative least-square
217 procedure with the NLIN procedure of SAS (version 9.4. SAS Inst. Inc., Cary, NC, USA).
218 The average gas production rate (AGPR, mL/h) was calculated as AGPR = *A***c*/[2*(ln2
219 + *c***lag*)] and it represents the average rate of gas production between the start of the
220 incubation and the time at which half of A is reached.

Data on chemical composition of samples were analyzed using the proc GLM of SAS (2017), considering the treatment as the main effect. Data of both gas production kinetics and fermentation parameters were analyzed independently for each donor animal group (goats or sheep) as a mixed model using the proc MIXED of SAS (2017). The treatment was considered a fixed effect and the inoculum was considered as a random effect. Significance was declared at $P < 0.05$ whereas $0.05 \leq P \leq 0.10$ indicates a trend. When a significant effect of the treatment was detected, the differences between the means were tested using the Tukey's multiple comparison test.

3. Results

3.1.Chemical composition of S. latissima and silages

The DM of the pre-ensiling *S. latissima* was greater ($P < 0.05$) than that of Control, FA and LAB silages, but lower than 30LAB silage (Table 2). There were no differences between treatments ($P > 0.05$) in N and EE content, though the ash content was on average 22.8 g/kg DM greater ($P < 0.05$) in 30LAB silage compared to the rest of the samples. Samples differed ($P < 0.05$) in NDF, and ADF content, but ensiling without additives did not change the content of these fractions compared with the pre-ensiling seaweed. In contrast, the content of both fractions was lower ($P < 0.05$) in FA and 30LAB silages. Lignin content in FA and 30LAB silages was greater ($P < 0.05$) than in the pre-ensiling *S. latissima* and LAB silage, while Control silage showed intermediate values. Ensiling increased ($P < 0.05$) starch content of the pre-ensiling *S. latissima* excepting for 30LAB. In contrast, soluble sugars content was lower ($P < 0.05$) in the silages compared with the pre-ensiling *S. latissima* with the exception of FA silage that showed greater ($P < 0.05$) content. Similarly, differences ($P < 0.05$) were observed in the content of NSC, which was on average 102 g/kg DM greater in silages compared to the fresh seaweed. Although differences were observed in the chemical fractions analyzed, no differences between

246 samples (P = 0.806) were observed in the GE content. Finally, the TEP content in all
247 silages was lower (P < 0.05) than that observed in the pre-ensiling *S. latissima.*

248 Table 2 near here

249 *3.2. In vitro trials*

250 The parameters of gas production kinetics of the samples when the donor goats received
251 medium-forage diets are shown in Table 3. The asymptotic gas production (A) decreased
252 (P < 0.05) when *S. latissima* was ensiled, with the exception of FA silage that showed no
253 change. On the other hand, ensiling had no effect on the fractional rate of gas production
254 (*c*) with the exception of 30LAB silage that showed an increased (P < 0.05) *c* value. Both
255 Control and 30LAB silages showed the greatest (P < 0.05) *lag* values, whereas the pre-
256 ensiling seaweed and the FA silage had the greatest (P < 0.05) AGPR values.

257 Fermentation parameters after 24 h of *in vitro* incubation using rumen inoculum from
258 goats fed medium-forage diets are shown in Table 4. No differences among samples (P =
259 0.310) were observed for gas production, but control and LAB silages had greater pH
260 values (P < 0.05) than the rest of samples. On the contrary, no differences (P > 0.05) were
261 detected in total VFA production. All silage treatments increased (P < 0.05) the molar
262 proportions of acetate but decreased (P < 0.05) those of propionate, being this effect most
263 noticeable in Control and 30LAB silages. Both Control and 30LAB silages had the
264 greatest (P < 0.05) butyrate and minor VFA molar proportions and acetate/propionate
265 ratios. Control silage also showed the greatest (P < 0.05) NH_3-N concentration, whereas
266 FA and 30LAB silages had the greatest (P < 0.05) values of both CH_4 production and
267 CH_4/VFA ratio.

268 Tables 3 and 4 near here

Similarly to that observed in the *in vitro* incubations with the inoculum from the goats fed medium-forage diets, A parameter decreased ($P < 0.05$) when *S. latissima* was ensiled, and the effect was more marked in Control and 30LAB silages (Table 5) when the donor sheep were fed a high-forage diet. Differences ($P < 0.05$) among samples were also observed in *c* and *lag* values. Compared with the pre-ensiling seaweed, only Control and LAB silages had lower ($P < 0.05$) *c* values, whereas FA, LAB and 30LAB silages had lower *lag* values. Both *lag* and *c* values were numerically greater for the inoculum from high-forage fed sheep than those observed when medium-forage fed goat inoculum was used. Pre-ensiling seaweed and FA and 30LAB silages had the highest ($P < 0.05$) AGPR, and Control silage the lowest one ($P < 0.05$), with LAB silage showing an intermediate value.

As previously observed with medium-forage fed goats' inoculum, there were differences among treatments ($P < 0.001$) in pH, with Control and LAB silages having greater values than the rest of the samples. Differences ($P = 0.014$) were also observed in gas production, with FA silage having greater gas production than control silage. On the contrary, no differences ($P > 0.05$) in total VFA production were detected (Table 6). However, ensiling caused shifts in the molar proportions of individual VFA. All silage treatments led towards a more acetic and less propionic fermentation ($P < 0.05$), increasing ($P < 0.05$) the acetate/propionate ratio, and increased proportions of minor VFA, excepting for FA silage that showed no differences with the pre-ensiling seaweed. The LAB silage showed greater ($P < 0.05$) NH_3-N concentration than the Control silage, but no differences ($P > 0.05$) among samples were detected in either CH_4 or CH_4/total VFA ratio.

Tables 5 and 6 near here

4. Discussion

293 The characteristics of the *S. latissima* silages have been reported previously by Yen et al.
294 (2022), who evaluated different silage treatments of *S. latissima*. As reported by Yen et
295 al. (2022), the silages tested in the present study had pH below 4.6 (4.56, 3.58, 3.69 and
296 4.38 for Control, FA, LAB and 30LAB, respectively), either low (1.45 and 0.24 g/kg DM
297 for Control and 30LAB silages, respectively) or undetected (FA and LAB silages)
298 butyrate content, and low NH_3 content (0.175, 0.114, 0.126 and 0.049 g/kg DM for
299 Control, FA, LAB and 30LAB, respectively). As the characteristics of the silages were in
300 the range recommended for forage silages (Van Soest, 1994), these silages were selected
301 for testing their *in vitro* ruminal fermentation.

302 The diet fed to donor animals has been identified as one of the main factor affecting *in*
303 *vitro* fermentation parameters (Martínez et al., 2010a; Mateos et al., 2013). Therefore, all
304 samples were incubated with ruminal fluid from goats fed a medium-forage diet and from
305 sheep fed a high-forage diet. These diets were formulated to simulate practical feeding
306 conditions in dairy goats and in low-producing (forage-based) sheep systems,
307 respectively.

308 *4.1. Chemical composition of samples*

309 Dry matter content of pre-ensiling *S. latissima* was considerably lower than that reported
310 in other studies (Cabrita et al., 2017; Bikker et al., 2020; Campbell et al., 2020; Novoa-
311 Garrido et al., 2020), which may be due to the relatively short growing period of 8 months
312 in comparison to the wild harvested biomass used in other studies. In addition, DM
313 decreased when *S. latissima* was ensiled without pre-wilting (Control, LAB, and FA
314 silages).

315 The ash and N content of *S. latissima* was similar to that reported in previous studies (de
316 la Moneda et al., 2019; Bikker et al., 2020; Campbell et al., 2020; Novoa-Garrido et al.,

317 2020). In agreement with Novoa-Garrido et al. (2020) and Cabrita et al. (2017), ash
318 content was not significantly affected by ensiling, although Campbell et al. (2020)
319 observed that ensiling *S. latissima* wihout additives decreased its ash content. Neither
320 Novoa-Garrido et al. (2020) nor Cabrita et al. (2017) reported changes in N content after
321 ensiling *S. latissima,* which agrees well with our results.

322 The content of NDF and ADF of *S. latissima* was greater than that previously reported
323 for *S. latissima* samples harvested in different seasons (Bikker et al., 2020; Novoa-
324 Garrido et al., 2020), although Campbell et al. (2020) reported similar values for *S.*
325 *latissima* samples collected in Northern Ireland in July. Seasonal variation has been
326 reported as one of the main factors which might affect chemical composition and
327 nutritional value of seaweeds (de la Moneda et al., 2019), but many other factors can also
328 influence chemical composition (Handå et al., 2013; Schiener et al., 2014). Sharma et al.
329 (2018) observed high variations in the content of some monosaccharides in cultivated *S.*
330 *latissima* due to both season and growing depth.

331 Campbell et al. (2020) observed that the NDF and ADF content decreased after ensiling,
332 although no changes in fibre fractions have been reported in other studies (Cabrita et al.,
333 2017; Novoa-Garrido et al., 2020). In contrast, lignin content increased in some silages,
334 which suggest the formation of Maillard products that were recovered in the lignin
335 analysis and can be considered artifacts. Previous studies have reported that the starch
336 and soluble sugars contents in *S. latissima* are low or even negligible (Bikker et al., 2020;
337 Campbel et al., 2020). Although these fractions might be important for ensiling, the low
338 values observed in the pre-ensiling *S. latissima* indicate that probably their effect on
339 silages' quality and nutritive value was scarce.

340 As reported previously, EE content in *S. latissima* is low (Cabrita et al., 2017; Bikker et
341 al., 2020) and the lack of changes in EE content due to ensiling is in agreement with

342 previous results (Cabrita et al., 2017). The TEP content was greater than that previously
343 reported by others for *S. latissima* (Campbell et al., 2020), but it decreased after ensiling.
344 Piekarska-Radzik and Klewicka (2020) observed that *Lactobacillus spp.* can degrade
345 phenolic compounds, which could help explain the loss of TEP during the silage process.

346 *4.2. In vitro trials*

347 The shape of gas production curves was similar for goats and sheep' inoculum, which
348 suggests that the pre-ensiling *S. latissima* and the silages were fermented in a similar
349 pattern by both inoculums (Figure 1). Compared with our results, Novoa-Garrido et al.
350 (2020) observed greater asymptotic gas production (A) values when *S. latissima* was
351 fermented *in vitro* using inoculum from sheep fed a 2:1 grass hay:concentrate diet, and
352 gas data were fitted to an exponential model. However, de la Moneda et al. (2019)
353 reported A values similar to those of the present study for *S. latissima* harvested in
354 autumn, but much lower values when the seaweed was harvested in spring. Such disparity
355 of results could be related to differences in the chemical composition of *S. latissima* tested
356 in the different studies.

357 The lower A values observed in the silages compared with the pre-ensiling *S. latissima*
358 agree well with the hypothesis of losing easily fermentable carbohydrates like laminarin
359 and mannitol during the silage fermentation (Horn et al., 2000), as the gas produced
360 during *in vitro* fermentations is directly related to the amount of organic matter fermented
361 by rumen microorganisms (Menke et al., 1979) and fibre fractions (NDF and ADF) are
362 less fermentable than non-structural or water-soluble carbohydrates (Van Soest, 1994).
363 For both ruminal inoculums, the FA and LAB silages had the greatest gas production.
364 Other studies have reported that ADF content decreased when adding formic acid to
365 silages of forages and other terrestrial crops (Wei et al., 2021) which might help explain
366 these results. On the other hand, the use of lactic acid bacteria as additives for ensiling

terrestrial plants has produced controversial results (Wei et al., 2021), and previous studies observed no changes in the amount of DM degraded *in vitro* when adding lactic acid bacteria to *S. latissima* silages (Cabrita et al., 2017; Campbell et al., 2020).

Lag values were considerably lower for the inoculum of the goats fed the medium-forage diet compared with that from high-forage fed sheep, which was probably related to the different diets fed to each animal species. Mixed diets can stimulate the growth of ruminal microorganisms compared with high-forage diets (Ramos et al., 2009), and therefore the inoculum from ruminants fed mixed diets can contain more diverse microorganisms. Moreover, the amount of concentrate was greater in the diet fed to goats than in that for sheep, and this might have promoted greater concentrations of microorganisms in the inoculum. Finally, for both inoculums, the FA and 30LAB silages showed higher AGPR than that of the Control silage, probably due the lower ADF content.

For both inoculums, *S. latissima* and its silages produced less gas than the reference feeds, and A values were about half of those observed for oat and alfalfa hay. Similarly, the AGPR values of *S. latissima* and its silages were about 0.26 and 0.20 of those observed for oat and alfalfa hay, respectively. These results indicate that both the pre-ensiling seaweed and all silages were less fermented in the rumen than the reference forages. Nevertheless, previous studies (Novoa-Garrido et al., 2020) have reported that the nutritive value of *S. latissima* and different seaweed silages can be similar to that of a medium-quality hay. The large variability observed between samples of the same seaweed can explain these discrepancies. In fact, De la Moneda et al. (2019) observed that VFA profile in the *in vitro* 24-h ruminal fermentation of *S. latissima* samples considerably differed depending on harvest season, but the average values of the individual VFA proportions reported were similar to those in the present study for pre-ensiling *S. latissima*. Greater propionate proportions are often associated with the ruminal

392 fermentation of easily-fermentable substrates, whereas fibre fermentation is associated
393 with greater acetate and butyrate proportions (Van Soest, 1994). Therefore, the greater
394 acetate and lower propionate proportions of silages compared with the pre-ensiling *S.*
395 *latissima* observed with both inoculums may reflect a more intensive fibre fermentation
396 in the silages. Greater proportions of minor VFA can be indicative of increased protein
397 degradation in the rumen (Van Soest, 1994). During the ensiling process, protein can
398 suffer alterations which might facilitate its degradation by ruminal microorganisms; this
399 could help to explain the greater minor VFA proportions and NH_3-N concentration
400 observed in Control and LAB silages compared with the pre-ensiling *S. latissima*. Formic
401 acid has been reported to decrease protein degradation during the ensiling of terrestrial
402 crops (Wei et al., 2021) and our results seem to confirm this effect for *S. latissima*, as
403 NH_3-N concentrations for the FA silage were similar to those observed in the pre-ensiling
404 *S. latissima* with both inoculums.

405 In the rumen, acetate and butyrate production from glucose is associated with the net
406 production of hydrogen that can be utilized by methanogens to reduce CO_2 to CH_4
407 (Janssen, 2010). Therefore, the greater CH_4 production observed for all silages compared
408 with the pre-ensiling *S. latissima* when the ruminal fluid from goats fed a medium-forage
409 diet was used as inoculum is consequent with the greater acetate proportions of the
410 silages. Moreover, higher TEP concentrations in the fresh seaweed can decrease CH_4
411 production by reducing fibre degradation and thus acetate production (Vasta et al., 2019).
412 Indeed, the CH_4 / total VFA ratio for the pre-ensiling seaweed was lower than that
413 observed for the oat hay (Table 4), whereas values of the silages were similar to that of
414 the oat hay, which might indicate that TEP in fresh seaweed could reduce methane
415 production. The lack of differences among fresh seaweed and silages in CH_4 production
416 when using the high-forage fed sheep' inoculum is difficult to explain, but in agreement

with that observed for goats inoculum the CH_4 / total VFA ratio for both the fresh seaweed and its silages was lower than that for the alfalfa hay used as reference feed (Table 6).

The VFA profile of the silages was similar to that observed for the oat hay when samples were incubated with ruminal fluid from medium-forage fed goats (Table 4), but fermentation of fresh seaweed resulted in lower acetate and greater propionate proportions as discussed above. When the ruminal fluid from sheep fed a high-forage diet was used, the VFA profile of the silages was also similar to that of the alfalfa hay (especially for FA and LAB silages), and only small shifts in individual VFA proportions were observed for both the fresh seaweed and the rest of silages, thus reflecting a typical forage ruminal fermentation pattern. Despite these differences, acetate/propionate ratios for both fresh seaweed and silages were in the range of those reported in vitro for ruminants fed forage based diets (Martínez et al., 2010b; Mateos et al., 2013).

5. *Conclusions*

Ensiling decreased the *in vitro* gas production with the two inoculums used in this study and the fermentation pattern of the silages was shifted towards more acetate and less propionate proportions compared with the pre-ensiling seaweed, which was probably related to changes in the chemical composition of the silages. These changes were less noticeable when either formic acid or lactic acid bacteria were used as silage additives. The use of formic acid or lactic acid bacteria, either without or with a pre-wilting treatment, is recommended for ensiling *S. latissima.*

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Disclosure statement

The authors report there are no competing interests to declare.

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Table 1. Chemical composition [g/kg dry matter (DM) unless otherwise stated] of oat hay, alfalfa hay and the concentrates fed to goats and sheep used as donors of ruminal fluid for the *in vitro* incubations.

Item	Alfalfa hay	Oat hay	Concentrate (goats)	Concentrate (sheep)
DM [g/kg feed]	909	901	897	890
Ash	104	45.9	48.2	55.1
Nitrogen	26.9	9.82	25.9	26.1
Neutral detergent fibre	433	680	195	224
Acid detergent fibre	317	372	88.4	78.2
Lignin	70.3	41.4	15.1	17.0
Ether extract	25.0	4.36	37.5	39.1
Total extractable polyphenols [mg tannic acid/g DM]	4.91	9.22	5.81	5.79

563 **Table 2.** Chemical composition [g/kg dry matter (DM) unless otherwise stated] of pre-ensiling *Saccharina latissima* and different *S. latissima* silages

564 (n = 2)[1].

Item	Pre-ensiling *S. latissima*	Control	FA	LAB	30LAB	SEM	P value
DM [g/kg feed]	78.6^{b}	61.4^{a}	64.8^{a}	66.3^{a}	333^{c}	1.81	<0.001
Ash	225	222	231	227	257	8.6	0.057
Nitrogen	15.8	16.7	16.1	16.1	16.1	0.94	0.763
Neutral detergent fibre (NDF)	379^{b}	307^{ab}	245^{a}	243^{a}	245^{a}	20.0	0.004
Acid detergent fibre	263^{b}	254^{b}	204^{a}	248^{b}	205^{a}	11.6	0.009
Lignin	38.2^{a}	50.9^{abc}	63.9^{bc}	42.2^{a}	65.2^{c}	4.43	0.005
Ether extract (EE)	4.97	4.98	4.34	4.69	4.39	1.204	0.912
Starch	0.84^{b}	2.63^{c}	6.42^{d}	2.72^{c}	0.06^{a}	0.176	<0.001
Soluble sugars	9.55^{b}	4.70^{a}	15.7^{c}	3.25^{a}	4.65^{a}	0.436	<0.001
Non-structural carbohydrates[2]	315^{a}	374^{ab}	439^{b}	445^{b}	409^{ab}	27.1	0.022
Gross energy [MJ/kg DM]	11.8	11.0	11.3	11.3	11.3	0.65	0.806
Total extractable polyphenols [mg tannic acid/g DM]	6.09^{b}	3.55^{a}	3.12^{a}	3.17^{a}	3.31^{a}	0.600	0.038

565 $^{a\text{-}c}$ Within each chemical fraction, average values for each sample not sharing the same superscript differ ($P < 0.05$).

566 [1] Control: ensiled without any additive; FA: ensiled with 4 g of formic acid per kg seaweed; LAB: ensiled with *Lactobacillus fermentum* ($2.5x10^{9}$ CFU/kg

567 seaweed) and *Lactobacillus plantarum* ($2.5x10^{9}$ CFU/kg seaweed); 30LAB: seaweed pre-wilted to 30% of dry matter and ensiled as LAB.

568 [2] calculated as [1000 - (NDF + crude protein + EE + ash)]; crude protein content was estimated as (4.79 x N content), as suggested by Bikker et al. (2020)

569 for *S. latissima* samples.

570 **Table 3**. Parameters of gas production kinetics (A, c, lag and AGPR) of pre-ensiling *Saccharina latissima*, different *S. latissima* silages and oat hay

571 (used as reference feed) after *in vitro* fermentation with ruminal fluid from goats fed a mixed diet composed of 60% of oat hay and 40% of concentrate

572 (n = 8)[1].

Item[2]	Pre-ensiling *S. latissima*	Control	FA	LAB	30LAB	SEM	P value	Oat hay
A [mL/g dry matter]	155^{c}	128^{a}	149^{bc}	141^{b}	129^{a}	3.2	<0.001	293
c [% h^{-1}]	1.61^{a}	1.72^{a}	1.69^{a}	1.57^{a}	2.01^{b}	0.076	<0.001	3.07
lag [h]	1.15^{ab}	5.00^{c}	0.00^{a}	0.44^{a}	3.69^{bc}	0.959	<0.001	0.00
AGPR [mL/g dry matter]	1.73^{c}	1.41^{a}	1.82^{c}	1.56^{ab}	1.70^{bc}	0.058	<0.001	6.79

573 [a-c] Within each parameter, average values for each sample not sharing the same superscript differ (P < 0.05).

574 [1] Control: ensiled without any additive; FA: ensiled with 4 g of formic acid per kg seaweed; LAB: ensiled with *Lactobacillus fermentum* ($2.5x10^{9}$ CFU/kg

575 seaweed) and *Lactobacillus plantarum* ($2.5x10^{9}$ CFU/kg seaweed); 30LAB: seaweed pre-wilted to 30% of dry matter and ensiled as LAB.

576 [2] A: asymptotic gas production; *c:* rate of gas production; *lag*: time before fermentation starts; AGPR: average gas production rate.

Table 4. Fermentation parameters after 24 h of *in vitro* incubation of pre-ensiling *Saccharina latissima*, different *S. latissima* silages and oat hay (used as reference feed) with ruminal fluid from goats fed a mixed diet composed of 60% of oat hay and 40% of concentrate (n = 8)[1].

Item[2]	Pre-ensiling *S. latissima*	Control	FA	LAB	30LAB	SEM	P value	Oat hay
Gas [mL/g]	37.2	27.7	44.8	37.8	42.3	2.36	0.310	106
pH	6.86^{a}	6.97^{b}	6.86^{a}	6.90^{b}	6.86^{a}	0.022	0.009	6.75
Total VFA[mmol/g DM]	4.04	4.08	4.24	4.06	4.03	0.092	0.680	6.62
Molar proportions [mol/100 mol]								
Ac	64.3^{a}	70.9^{c}	68.0^{bc}	66.6^{b}	69.0^{bc}	0.23	<0.001	69.8
Pr	24.5^{c}	15.5^{a}	19.8^{b}	20.7^{b}	17.2^{a}	0.29	<0.001	19.7
But	6.12^{a}	7.21^{b}	6.39^{a}	6.59^{a}	7.51^{b}	0.042	<0.001	7.89
Minor	5.08^{a}	6.39^{d}	5.81^{b}	6.11^{c}	6.29^{cd}	0.023	<0.001	2.60
Ac/Pr [mol/mol]	2.66^{a}	4.60^{c}	3.44^{a}	3.24^{b}	4.05^{c}	0.014	<0.001	3.54
NH_3-N [mg/l]	133^{a}	186^{c}	151^{ab}	163^{b}	150^{ab}	7.7	<0.001	141
CH_4 [mL/g DM]	11.8^{a}	13.7^{b}	17.3^{c}	14.8^{b}	16.3^{c}	0.73	<0.001	24.4
CH_4/VFA [mL/mmol]	2.92^{a}	3.36^{b}	4.07^{c}	3.64^{b}	4.05^{c}	0.221	0.002	3.70

[a-d] Within each parameter, average values for each sample not sharing the same superscript differ (P < 0.05).

[1] Control: ensiled without any additive; FA: ensiled with 4 g of formic acid per kg seaweed; LAB: ensiled with *Lactobacillus fermentum* ($2.5x10^9$ CFU/kg seaweed) and *Lactobacillus plantarum* ($2.5x10^9$ CFU/kg seaweed); 30LAB: seaweed pre-wilted to 30% of dry matter and ensiled as LAB.

[2] VFA: volatile fatty acids; Ac: acetate; Pr: propionate; But: butyrate; Minor: sum of isobutyrate, valerate and isovalerate.

583 **Table 5**. Parameters of gas production kinetics (A, c, *lag* and AGPR) of pre-ensiling *Saccharina latissima* and different *S. latissima* silages and
584 alfalfa hay (used as reference feed) after in vitro fermentation with ruminal fluid from sheep fed a diet composed of 90% of alfalfa hay and 10% of
585 concentrate (n = 8)[1].

Item[2]	Pre-ensiling *S. latissima*	Control	FA	LAB	30LAB	SEM	P value	Alfalfa hay
A [mL/g dry matter]	143^{d}	115^{a}	129^{bc}	130^{c}	120^{ab}	2.5	<0.001	198
c [% h^{-1}]	2.40^{c}	2.00^{ab}	2.22^{abc}	1.95^{a}	2.34^{bc}	0.110	0.002	5.19
lag [h]	13.6^{b}	15.3^{b}	8.31^{a}	9.31^{a}	7.00^{a}	1.437	<0.001	0.54
AGPR [mL/g dry matter]	1.68^{c}	1.16^{a}	1.65^{c}	1.44^{b}	1.61^{c}	0.051	<0.001	6.96

586 [a-c] For each parameter, average values for each sample not sharing the same superscript differ ($P < 0.05$).

587 [1] Control: ensiled without any additive; FA: ensiled with 4 g of formic acid per kg seaweed; LAB: ensiled with *Lactobacillus fermentum* ($2.5x10^{9}$ CFU/kg
588 seaweed) and *Lactobacillus plantarum* ($2.5x10^{9}$ CFU/kg seaweed); 30LAB: seaweed pre-wilted to 30% of dry matter and ensiled as LAB.

589 [2] A: asymptotic gas production; *c:* rate of gas production; *lag*: time before fermentation starts; AGPR: average gas production rate.

590 **Table 6.** Fermentation parameters after 24 h of *in vitro* incubation of pre-ensiled *Saccharina latissima* and different *Saccharina latissima* silages
591 and alfalfa hay (used as reference feed) with ruminal fluid from sheep fed a fed a diet composed of 90% of alfalfa hay and 10% of concentrate (*n* =
592 8)[1].

Item [2]	Pre-ensiling *S. latissima*	Control	FA	LAB	30LAB	SEM	P value	Alfalfa hay
Gas [mL/g]	49.0^{ab}	41.1^{a}	58.6^{b}	45.8^{ab}	53.4^{ab}	1.26	0.014	96.0
pH	6.95^{a}	7.01^{b}	6.95^{a}	6.99^{b}	6.93^{a}	0.014	<0.001	6.99
Total VFA[mmol/g DM]	4.49	4.51	4.65	4.47	4.60	0.241	0.931	9.13
Molar proportions [mol/100 mol]								
Ac	62.0^{a}	66.0^{c}	63.8^{b}	63.4^{b}	65.6^{c}	0.38	<0.001	64.5
Pr	21.7^{d}	16.1^{a}	19.1^{c}	18.6^{c}	17.2^{b}	0.29	<0.001	19.7
But	10.4^{a}	11.0^{ab}	10.7^{ab}	11.2^{b}	10.6^{ab}	0.22	0.008	9.24
Minor	5.90^{a}	6.90^{b}	6.40^{ab}	6.80^{b}	6.60^{b}	0.200	<0.001	6.28
Ac/Pr [mol/mol]	2.89^{a}	4.18^{d}	3.38^{b}	3.45^{b}	3.89^{c}	0.074	<0.001	3.29
NH_3-N [mg/l]	148^{ab}	166^{bc}	158^{abc}	174^{c}	145^{a}	6.1	<0.001	284
CH_4 [mL/g DM]	11.9	11.2	12.0	12.7	12.9	0.84	0.270	34.7
CH_4/VFA [mL/mmol]	2.71	2.54	2.70	2.98	3.01	0.236	0.231	3.80

593 [a-d] For each parameter, average values for each sample not sharing the same superscript differ ($P < 0.05$).

594 [1] Control: ensiled without any additive; FA: ensiled with 4 g of formic acid per kg seaweed; LAB: ensiled with *Lactobacillus fermentum* ($2.5x10^9$ CFU/kg
595 seaweed) and *Lactobacillus plantarum* ($2.5x10^9$ CFU/kg seaweed); 30LAB: seaweed pre-wilted to 30% of dry matter and ensiled as LAB.

596 [2] VFA: volatile fatty acids; Ac: acetate; Pr: propionate; But: butyrate; Minor: sum of isobutyrate, valerate and isovalerate.

597

Figure 1. Cumulated gas production [mL/g dry matter] over a 120 h incubation period using ruminal fluid from goats fed a medium-forage diet (a) and sheep fed a high-forage diet (b). Error bars show the mean square error at each point (n = 8). Treatments: *Sacharina latissma*: seaweed before ensiling (Residual standard deviation (RSD) = 5.77 and 3.65 mL for goats and sheep respectively); Control: ensiled without any additive (RSD = 5.00 and 4.05 mL); FA: ensiled with 4 g of formic acid per kg seaweed (RSD = 3.22 and 3.08 mL); LAB: ensiled with *Lactobacillus fermentum* ($2.5x10^9$ CFU/g seaweed) and *Lactobacillus plantarum* ($2.5x10^9$ CFU/g seaweed) (RSD = 4.15 and 3.26 mL); 30LAB: seaweed pre-wilted to 30% of dry matter and ensiled as LAB (RSD = 4.92 and 4.90 mL).