Effect of seaweed on gastrointestinal microbiota isolated from Norwegian White sheep

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Conflict of interests

The authors declare that they have no conflict of interests.

Abstract

The effects of a commercial seaweed product and extracts collected from wild seaweeds in the Northern Norway on cultivable commensal intestinal bacterial groups isolated from Norwegian White Sheep ewes were studied *in vivo* and *in vitro*.
Bacterial counts from faeces from the ewes fed with supplement which contained seaweed meal throughout the entire indoor winter period had significantly lower lactic acid bacteria counts ($P \approx 0.05$). The screening of extracts from red and brown seaweeds showed that a number of the organic extracts had an inhibitory effect on the growth of the two *Enterococcus* sp. isolates.
The results indicate that *Ascophyllum nodosum* supplementation reduces lactic acid bacteria counts in the ewes and the lambs, and that extracts from this seaweed have an inhibitory effect on the growth of *Enterococcus* sp. isolates.

Keywords

Ascophyllum nodosum, diet supplement, *Escherichia coli*, *Enterococcus*, extracts, macroalgae

Introduction

The brown seaweed *Ascophyllum nodosum* is common along the Norwegian coast. *Ascophyllum nodosum* is harvested to be mostly used as an ingredient in livestock feeds and as fertilizer (Brattrein, 1974; Indergaard, 1983). An increasing number of reports describe the effects of seaweed products, polysaccharides purified from seaweed and seaweed extracts on different health parameters as well as on the intestinal microbiota as reviewed by O'Sullivan, L. et al., 2010. Fucoidans have for example shown to inhibit the attachment of the pathogens to the mucosa (Shibata et al., 2003). Marine seaweed produce a range of both simple and complex organohalogens and halogenated compounds with antibacterial activity for chemical defense with antibacterial effect on both Gram positive and Gram negative pathogens (Hay, 1991; Kurata et al., 1998; Brito et al., 2002; Dembitsky and Srebnik, 2002; Iliopoulou et al., 2002; Nagayama et al., 2002; Gribble, 2003; Buttler and Carter-Franklin, 2004; Cardozo et al., 2007).

100 Extracts from seaweed are reported to be effective antiviral, anticancer, antibacterial and
101 antioxidants agents, and can be used as dietary supplements for health promoting (Haugan and
102 Liaaen-Jensen, 1994; Mayer and Hamann, 2005; Dhargalkar and Verlecar, 2009; Mayer et al.,
103 2009). *Ascophyllum nodosum* is reported to contain polyphenols at concentrations that can vary
104 between 9 and 14 % of DM (Ragan and Jensen, 1978). Steers and lambs fed 20 g *A. nodosum*
105 /kg diet DM shed lower numbers of *Escherichia coli* O157:H7 and of generic *E. coli* in their
106 faeces than animals fed control diets without seaweed (Braden et al., 2004). Thus, seaweed may
107 be a beneficial diet supplement due to their antibacterial effect.
108 The microbial ecosystem in the intestine plays an important role in the animal health through
109 controlling the establishment and development of harmful bacteria. Intestinal bacteria
110 contribute as well to the development of the immune system in the early stages of life
111 (Macfarlane et al., 2006). *Escherichia coli,* lactic acid bacteria (LAB) such as *Enterococcus*
112 spp., and *Clostridium perfringens* are considered part of the normal intestinal microbiota, and
113 are often used as indicator organisms in studies of intestinal ecology. Further, *E. coli* and *C.*
114 *perfringens* are of interest because they are also potential pathogens and can cause diseases
115 outbreaks in both animals and in humans (Drasar, 1974; Erickson, 2007; Smith, 2007; Songer,
116 2010). Additionally, *E. faecalis* and *E. faecium* are most often associated with diseases in
117 humans and cause nosocomial infections with antibiotic resistance complications.
118 *Enterococcus faecalis* appears to be the most virulent (Dworkin, 2006) and induces
119 Inflammatory Bowel Disease, dysplasia and carcinoma (Balish, 2002). It has been found that
120 71 % of *E. faecium* is vancomycin resistant (Bengmark, 1998). New measures to limit the
121 formation of pathogens with different virulence elements in the animal gut are desired.
122 The aim of the present studies was to investigate how products from *A. nodosum* and other
123 seaweeds from the Arctic region affect the microbiota of the large intestine of sheep. One
124 hypothesis tested was that supplementing pregnant ewes with seaweed meal has beneficial

125 effect on the intestinal microbial balance in ewes and lambs as well as decreases the excretion
126 of potential pathogens from the animals. A second hypothesis was that seaweed extracts might
127 have inhibitory effects on the growth of *Enterococcus* sp. strains carrying antibiotic resistance
128 of animal origin.
129

130 **Materials and methods**

131 *In vivo study*

132 We performed an indoor feeding experiment at a commercial sheep farm on the island of
133 Mindland, in Nordland County, Norway (65˚46˚N, 12˚28˚E) from November 2009 to June 2010
134 with 20 1.5 - 2.5 years old Norwegian White Sheep ewes. After mating, the ewes were divided
135 into two feeding groups of 10 ewes each. The animals were then randomly allocated into two
136 pens with five animals in each. The animals were housed on deep straw litter. After lambing,
137 ewes and lambs were kept in their respective pens until the end of the experiment (pasture
138 turnout).
139 Two dietary supplements were formulated, seaweed (SW) and a control (C). The SW was based
140 on dried and ground *A. nodosum* (AlgeaFeed 3.5, Algea AS, Lødingen, Norway) and barley,
141 while the C was based on barley. Both supplements were fortified with minerals to be as similar
142 as possible with regard to mineral composition. The formulations of the experimental diet
143 supplements are given in Table 1. The level of daily supplementation was 163 g/ewe for the
144 animals on SW and 126 g/ewe for C, fed at isoenergetic level. The dietary supplements were
145 provided on pen level in the morning before being given access to forage. The animals in each
146 pen seem to eat similar amounts of the supplement. The animals had free access to grass silage
147 and water during the experiment.
148 The live weight of the ewes and their feed intake (on pen level) were recorded monthly. For the
149 lambs, weight was recorded at birth and at the end of the experiment at pasture turnout, and the

150 growth rate was defined as follows: (weight [kg] at the end of study – weight [kg] at birth) / age
151 [days] at end of study.
152 Rectal fecal samples were collected from the ewes before the starting with the experimental
153 feeds, three and six months into the indoor feeding experiment period, and once from six to
154 nine weeks old lambs. The samples were transported in a cooler, and processed in the laboratory
155 within 24 hours. Each sample was serially diluted in 0.9% saline. 0.1 ml of each diluted sample
156 was plated on selective medium. *Enterococcus* spp. were grown aerobically Merckoplate®
157 Citrate Azide Tween Carbonate at 37 °C for 24 h, and all red colonies were enumerated. Lactic
158 acid bacteria (LAB) were grown anaerobically Merck Anaerocult A on Mann-Rogosa-Sharpe
159 agar plates (Prolabo) at 37 °C for 48 h, and all the colonies were counted. The aerobic blood
160 agar counts was performed on 5% blood agar plates (Merckoplates) after aerobic incubation at
161 37°C for 24 h, and all the colonies were enumerated. Aerobic culture of *E. coli* was done on
162 Merckoplate MacConkey agar at 37 °C for 24 h, and this yielded typical large red (lactose-
163 fermenting)-colonies. *Clostridium perfringens* was grown anaerobically on Tryptose-Sulfite-
164 Cycloserine agar (Prolabo) at 37 °C for 48 h, and colonies with a black precipitate were
165 enumerated. The verification of the isolates was performed by examining the microscopic
166 image of the strains after Gram staining and by 16S rRNA sequencing. All verification work of
167 isolates in this study was performed at the Department of Food Safety and Infection Biology,
168 Faculty of veterinary Medicine, Norwegian University of Life Sciences, Oslo, Norway
169 following 16S rRNA sequencing. The general bacterial primers 27f (5’-
170 AGAGTTTGATCCTGGCTCAG-3’; Lane, 1991) and 1492r (5’-
171 GGTTACCTTGTTACGACTT-3’, Stackebrandt and Liesack. 1993) were used. Sequencing
172 was done by GATC Biotech (Constance, Germany). The Basic Local Alignment Search Tool
173 (version 2) was used for identification. Sequence similarities were ≥ 99%.

174 Due to skewed distribution, the intestinal bacterial counts were logarithmically transformed.
175 These transformed intestinal bacterial counts are the basis for the response variables used in
176 the statistical models and they are assumed to be normally distributed. The response variables
177 were modelled by a linear mixed model with type of diet supplement as fixed factor and pen
178 within type of diet supplement as random factor. The models can be expressed as

179 $$y_{ijk} = \mu + \alpha_i + P_{j(i)} + \varepsilon_{ijk} \quad (1)$$

180 where y_{ijk} is the response variable observed on ewe / lamb k for the i-th (SW and C) type of
181 diet supplement (α) and j-th pen (P) within the type of diet supplement i (two pens within
182 each type of diet supplement).
183 For ewes, the mean of the two values of the transformed intestinal bacterial counts observed
184 three and six months into the feeding trial minus the transformed intestinal bacterial counts
185 observed in the pre-treatment sample were used as the response variable in our statistical
186 model. For lambs, the transformed intestinal bacterial counts observed were used directely as
187 the response variable. The statistical calculations were done using proc mixed in SAS 9.2
188 (2002-2008; SAS Institute Inc, Cary, NC, USA).
189 The study was carried out in accordance with the laws and regulations that apply to experiments
190 using live animals in Norway and EU, and this was done under the surveillance of the
191 Norwegian Animal Research Authority.
192
193 *In vitro study*
194 The red algae *Polysiphonia lanosa*, *Corallina officinalis*, *Palmaria palmata* and *Mastocarpus*
195 *stellatus*, and the brown algae *Laminaria hyperborea* and *Ascophyllum nodosum* were
196 investigated. *Laminaria hyperborea*, *M. stellatus* and *P. palmata* were harvested in the Bodø
197 area (67°16′47″N 14°24′18″Ø). *Polysiphonia lanosa*, *C. officinalis* and *A. nodosum* were

198 harvested in tidal ponds in Oldervik at Ullsfjorden (69°45′24″N 19°40′33″Ø), Tromsø. After
199 harvesting, the thalli were cleaned and kept frozen until further processing.
200 For the production of the extracts, the samples were lyophilized and centrifuged with purified
201 water. The freeze-dried supernatant constituted the aqueous extracts. The pellets remaining
202 from the aqueous extraction were lyophilized, crushed and extracted with CH_2Cl_2/MeOH (1:1).
203 After removing the solvent with a rotary evaporator, the samples were lyophilized and
204 constituted the organic or methanolic extracts. The extracts were kept frozen until further
205 testing.
206 The bacteria strains tested were isolated from faeces samples collected from a commercial
207 sheep herd: the Gram-negative strains *E. coli* (ID MNG_12), biofilm forming *E. coli* (ID
208 MNG_09) and hemolytic *E. coli* (ID MNG_28), and the Gram-positive strains *E. faecalis* (ID
209 MNG_11) and *E. faecium* (ID MNG_03). The verification of the isolates was performed at the
210 Department of Food Safety and Infection Biology, Faculty of veterinary Medicine, Norwegian
211 University of Life Sciences, Oslo, Norway as described earlier.
212 The bacterial screening followed the minimum inhibitory concentration (MIC) principle. All
213 isolates were grown at 37˚C in Müeller-Hinton broth (Difco Laboratories, Detroit, MI, USA).
214 The test was performed in 96-well Nunc microtiter plates, in which 50 µL of the algae extracts
215 solution were incubated with 50 µL of a suspension of an actively growing (log phase) cultures
216 of bacteria diluted to a starting concentration of approximately 5 X 10^5 cells per well. The
217 aqueous extracts were dissolved in dd-H_2O. The organic extracts were dissolved in pure
218 Dimethyl sulphoxide (DMSO) and dd-H_2O. The bacterial cultures without extract present were
219 used as negative controls. The bacterial growth was monitored with a Victor plate reader
220 (Perkin-Elmer). The MIC was defined as the minimum concentration resulting in no change in
221 optical density after incubation for 24 h at 37 ˚C. The extracts were tested in duplicates at

concentrations ranging from 6.25 to 250 µg/mL for the aqueous extracts and from 6.25 to 200 µg/mL for the organic extracts (EUCAST, 2003).

The methanolic and aqueous extracts were analyzed for iodine content in a commercial laboratory following the methodology for Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

The antibiotic susceptibility of the isolates was determined by the Neo-Sensitabs® diffusion method on Müeller-Hinton agar. Tablets containing AMB (10 µg) or ITC (8 µg) or FLC (25 µg) or VRC (1 µg) were supplied by Rosco Diagnostica A/S (Taastrup, Denmark).

The following statistical model was used as a basis to describe the relation between bacterial activity (y) and extract concentration (x):

$$\ln(y) = \beta_0 + \alpha_i + \beta_1 x + \theta \ln(x+1) + (\alpha\beta)_i x + (\alpha\theta)_i \ln(x+1) + \varepsilon, \; i = 1,2,...30$$

β_0, α_i's, β_1, θ, $(\alpha\beta)_i$'s, and $(\alpha\theta)_i$'s were parameters to be estimated. For both aqueous and organic extracts there were 30 groups combining extract type and bacteria species. The parameters α_i's, $(\alpha\beta)_i$'s, and $(\alpha\theta)_i$'s allow for different relations between y and x for the 30 groups.

Based on the results from fitting the model above, the model was simplified for some of the organic extracts. For the organic extract *A. nodosum* used on the bacteria *E. faecalis* and *E. faecium* the model

$$\ln(y) = \beta_0 + \alpha_i + \beta_1 x + \theta \ln(\mathrm{x}+1) + \varepsilon, \; i = 1,2$$

was used.

For the other 28 group combinations of organic extract type and bacteria species the pure linear model

$$y = \beta_0 + \alpha_i + \beta_1 x + (\alpha\beta)_i x + \varepsilon, \; i = 1,2,...28$$

246 was used.

247 All over, a significance level of 5 % $(p \leq 0.05)$ was used.

248

249 Results

250 *Performance*

251 The dietary treatment had no effect on feed intake or live weight in the ewes or on the growth
252 rate of the lambs (data not shown).

253 *Verification of isolates*

254 (i) Citrate Azide Tween Carbonate. Isolates were identified as *E. faecalis* and *E. faecium*.

255 (ii) Mann-Rogosa-Sharpe. The isolates were identified as *E. faecium*, *Lactobacillus* sp. and
256 *E. coli*.

257 (iii) Aerobic blood agar counts. Screening of the counted colonies identified the isolates as
258 *E. coli*, *Lactobacillus* sp., *E. faecalis*, *Corynebacterium* sp., *Micrococcus* sp., *Bacillus* sp.,
259 hemolytic *E. coli* and *Pasteurella*-like bacteria.

260 (iv) MacConkey agar. The isolates were confirmed to be lactose-fermenting and non-lactose-
261 fermenting *E. coli*.

262 (v) Tryptose-Sulfite-Cycloserine. The identity of *C. perfringens* could not be confirmed
263 because the isolates were not viable. In this case, we relied on examination of the colony
264 morphology and Gram staining for verification of this species.

265 *Bacterial counts in faeces - in vivo study*

266 Table 2 shows the least squares means from the model in (1) for the faecal samples from the
267 ewes fed with SW or C. The counts of Lactic acid bacteria for ewes fed with SW decreased
268 from the pre-treatment sample (Least Squares Means = - 0.5). For ewes fed with C the Lactic
269 acid bacteria counts increased from the pre-treatment sample (Least Squares Means = 0.7).

270 The reduction for ewes fed with SW was significant different from the increase for ewes fed
271 with C (P = 0.04).
272 The aerobic blood agar counts for ewes fed with SW increased from the pre-treatment sample
273 (Least Squares Means = 0.4). For ewes fed with C the *E. coli* counts deceased from the pre-
274 treatment sample (Least Squares Means = - 0.5). The increase for ewes fed with SW was
275 significant different from the reduction for ewes fed with C (P = 0.04).
276 There was no effect of supplement on bacterial count in faeces collected from lambs (Table 3).
277 *Bacterial effect of seaweed extracts – in vitro study*
278 The results from the MIC study show that the effect of the seaweed extracts on the bacterial
279 growth varied, depending on the extract type, extract concentration and the bacteria species. In
280 general, the methanolic extracts had a growth-inhibiting effect (Table 4) whereas the aqueous
281 extracts had a growth stimulating character (Table 5). Particularly, the *Enterococcus* sp. isolates
282 were sensitive to the methanolic extracts, and extracts of *A. nodosum*, *C. officinalis* and *P.*
283 *lanosa* showed significant growth inhibitory effects on both *Enterococcus* sp. isolates. The
284 methanolic extract of *P. lanosa* also shows a significant inhibitory effect on the *E. coli* isolate.
285 The methanolic extract of the brown algea *L. hyperborea* showed a fairly strong and significant
286 antibacterial effect against *E. faecium*. The aqueous extracts from *L. hyperborea*, *M. stellatus*
287 and *P. lanosa* had a significant stimulating effect on the growth of the hemolytic *E. coli* isolate.
288 *Ascophyllum nodosum* was the only seaweed that had inhibitory growth effect from the
289 methanol and aqueous extracts and on both *Enterococcus* sp. isolates. Whereas most of the
290 observed responses were linear, the effect of methanolic extract of *A. nodosum* on bacterial
291 growth followed a nonlinear response to concentration, but where it appears to be negative
292 linear correlation between dose and response between doses 0 - 25 μ/ml with the maximum
293 inhibitory effect on the growth of both *E. faecium* and *E. faecalis* reached at a concentration of

294 25µg/ml (Fig. 1). The methanolic extracts from several of the red algae showed also a strong
295 growth-inhibiting effect on the *Enterococcus* sp. strains.
296 Our measurements of iodine concentration showed that the aqueous extracts had higher
297 concentration of iodine than the methanolic extracts, with the exception of *L. hyperborea* as
298 both extracts had high and similar levels of iodine (Table 6).
299

300 **Discussion**

301 *Bacterial counts in faeces - in vivo study*

302 The composition of the gut microbiota is strongly influenced by the diet characteristics, and
303 more specific the non-digestible carbohydrates, among other factors (Doré, 2015). The regular
304 consumption of seaweed polysaccharides is reported to act as a prebiotic promoting the
305 intestinal health and stimulating the growth of beneficial probacteria such as bifidobacteria and
306 lactobacilli (Jaspars, 2013). In our study, the effect of prolonged feeding with supplements from
307 dried *A. nodosum* meal lowered the LAB counts in the ewes in the SW treatment. Others have
308 reported similar results in farm animals indicating that the effect of feeding seaweed products
309 on different bacterial intestinal groups depends on the type and composition of the seaweed
310 product (Gardiner et al., 2008; Reilly and O'Doherty, 2008; McDonell, 2010). Our results might
311 indicate that: a) *Ascophyllum nodosum* is a poor substrate for LAB probably due to the poor
312 degradability of the alginates (Humphreys and Triffitt, 1968), b) short chain fatty acid
313 production as a result of the anaerobic fermentation of the complex polysaccharides in *A.*
314 *nodosum* might be conditioning the growth conditions for these bacterial groups (Hirshfield,
315 2003), and/or c) there might be a release of certain phytochemicals after the degradation of the
316 seaweed cells that have an antibacterial or growth inhibiting effect on this bacterial group
317 (Gupta, 2011). On the other hand, the formulation of the SW diet supplement contained half
318 the amount of barley compared to the C diet supplement. Barley (*Hordeum vulgare* L.) is a

319 cereal grain with high content in the starch, which is a substrate for LAB. This could explain
320 the significant difference between the two animal groups in relation to the LAB counts. No data
321 of the major components of the experimental diets is available.
322 An optimal balance in the commensal intestinal microbiota is beneficial for the animal health
323 among other mechanisms by controlling intestinal invasion by pathogens (Mead, 2000). Certain
324 members of the commensal microbiota can be opportunistic pathogens or can affect the
325 digestion and availability of nutrients for uptake in the intestine. The common perception is that
326 lower counts of LAB in the intestine is not a desired effect because of the regulating and
327 protecting role of these bacteria (Ljungh, 2006). However, it is reported that certain LAB like
328 *E. faecium* and *L. salivarius* commonly found in chickens, compromise lipid digestion by
329 deconjugating bile salts due to their high level of bile salt hydrolase (BSH) activity, a bacteria-
330 produced enzyme that exerts negative impact on host fat digestion and utilization causing
331 growth depression in the host (Feighner, 1987; Knarreborg, 2002, Geng & Lin, 2016).
332 Interestingly, the growth-promoting effect of the use of antibiotic growth promoters (AGP) is
333 related to a decrease in BSH activity in the gut. Dietary supplementation with seaweed products
334 as potential novel alternatives to AGP targeting LAB and inhibiting BSH might be of great
335 interest to enhance feed efficiency and body weight in production animals.
336 In our *in vivo* study, the health status of the ewes was good despite the lower LAB counts. In
337 contrast, the lambs born from ewes in the SW group had high mortality. This high mortality in
338 the lambs in the SW group is quite remarkable and is explained by inadequate levels of absorbed
339 antibodies caused by mechanisms not related to modulation in the gut microbiota adaptations
340 (Novoa-Garrido et al., 2014).
341 *Bacterial effect of seaweed extracts – in vitro study*
342 Extracts produced with different solvents are reported to have different bioactivities. Particular
343 solvents are required to extract antimicrobial substances within the algal plant with effect on a

344 specific bacteria (Cox, 2010). For antimicrobial effect, ethanol and acetone are utilized. For
345 extraction of phenolic compounds aqueous mixtures of methanol, ethanol and acetone are
346 recommended (Waterman, 1994). The MIC microtiter assay applied in this study is considered
347 to be substantially a more sensitive method to quantify effect. We applied the standard method
348 used for antibacterial effect screening in the laboratory utilizing CH_2Cl_2/MeOH (1:1) aiming to
349 maximize the dissolution of as many organic compounds as possible. In the case of *A. nodosum*
350 we observed the same effect of both the aqueous and the methanolic extracts. This inhibitory
351 effect is likely due to the high concentrations of phenolic compounds in *A. nodosum*, exceeded
352 only by *Fucus* spp. (Wang et al., 2009), but this hypothesis cannot be corroborated in this study
353 since we have no analysis of the composition of the extracts.
354 *Ascophyllum nodosum* is reported to contain high polyphenols concentrations that can vary
355 between 9 and 14 % of DM (Ragan and Jensen, 1978). Methanolic extracts from seaweed
356 contain phenolic compounds such as polyphenols and phlorotannins have antibacterial effect
357 against Gram-positive and Gram-negative bacteria (Cox, 2010; Nishiguchi, 2014), which is in
358 accordance with the effects that we have recorded from the methanolic extract on the Gram-
359 positive *E. faecalis* and *E. faecium*, and the Gram-negative *E. coli*.
360 *Ascophyllum nodosum*'s inhibiting effect on the *Enterococcus* sp. and *E. coli* strains is very
361 interesting since these bacteria are opportunistic pathogens, and in the search for remedies
362 against multi-resistant nosocomial infections caused by *E. faecalis* and *E. faecium* strains.
363 The inhibiting effect registered from the methanolic extracts from the red seaweeds *C.*
364 *officinalis* and *P. lanosa* corresponds well with other published results showing strong
365 antibacterial effect from red macroalgae species (Hellio et al., 2000).
366 Seaweeds, especially species belonging to the brown seaweed group (phylum Phaeophyceae)
367 have large concentrations of iodine (Nitschke, 2015). Iodine is known to have antibacterial
368 effect. Iodine is water-soluble and such property can explain higher iodine concentrations in

369 the aqueous extracts. Both extracts of *L. hyperborea* had high and similar concentrations of
370 iodine. This corresponds well with the fact that *Laminaria* spp. is reported to have the largest
371 iodine content among seaweed (Nitschke, 2015). As the organic extract of *A. nodosum* had
372 moderate level and much lower concentration of iodine than *L. hyperborea*, the antibacterial
373 effects observed of the organic extract from *A. nodosum* in the current study are probably not
374 correlated to the iodine content. However, our *in vitro* study did not include controls with
375 extracts without iodine and therefore we cannot exclude iodine as factor.
376 Our studies show that the seaweeds products have an effect on bacterial growth, which are
377 bacteria- and seaweed-species specific as reported by Wang, Y. *et al* (2008) (Wang et al., 2008).
378 In conclusion, the effects of seaweed extracts depend on the seaweed species, the fraction
379 extracted and the bacteria species. We found that including *A. nodosum* meal in the diet affects
380 the composition of the microbiota in the large intestine of ewes and their offspring in a bacteria
381 species dependent way with a significant reduction of the LAB counts in the faeces. Our *in vitro*
382 experiment showed that extracts from *A. nodosum* have a significant antibacterial effect with a
383 nonlinear response to concentration.
384 In general, we can expect better antibacterial effect from organic seaweed extracts than from
385 aqueous extracts. Further research is warranted to assess optimization of the extraction
386 protocols, purification and characterization of the active components will improve the potential
387 health benefits.

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536 Figure 1. *Effect of methanolic extract from Ascophyllum nodosum was tested on growth of*
537 *pure cultures of one Enterococcus faecalis strain and one Enterococcus faecium. The extract*
538 *was tested in duplicates following the minimum inhibitory concentration principle.*

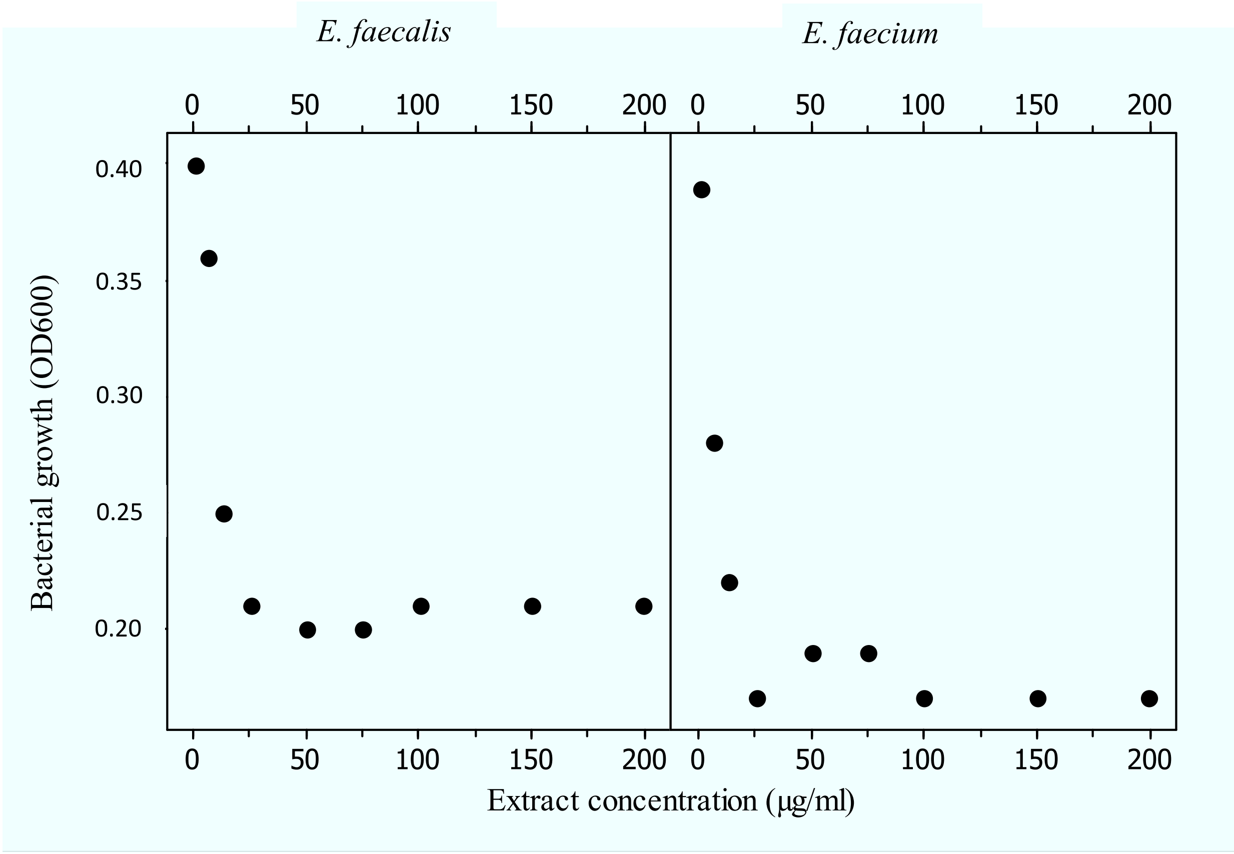
E. faecalis
E. faecium
0
50
100
150
200
0.40
0.35
0.30
0.25
0.20
Bacterial growth (OD600)
Extract concentration (μg/ml)

Table 1. Ingredients used in the composition of the two diet supplements (g.kg $^{-1}$)

	Diet supplement[1]	
	SW	C
Barley	409	881
Seaweed meal	546	0
Molasses	30.7	39.6
Vitamin E	0	0
$CaCO_3$	0	15.2
$Ca(H_2PO_4)_2 \times H_2O$	12.8	7.8
$MgHPO_4 \times 3H_2O$	0	16.2
NaCl	0	36.8
Na_2SeO_3	0.05	0.06
$ZnSO_4 \times H_2O$	0.90	1.20
$MnSO_4 \times H_2O$	0.78	0.99
$Ca(IO_3)_2$	0	0,03
$2CoCO_3 \times 3Co(OH)_2 \times H_2O$	0.01	0.01
Vitamin A, 500000 IU/g	0	0.13
Vitamin D3, 500000 IU/g	0	0.04

[1]SW = seaweed meal; C = control.

Table 2. Changes in fecal bacteria counts (colony forming units, CFU) during the experimental period in ewes supplement with seaweed meal (SW) or without (C)[1].

Supplement[a]	*Enterococcus* spp.	Lactic acid bacteria	Aerobic blood agar counts	*Escherichia coli*	*Clostridium perfringens*
SW	-0.4 (8, 0.47)	-0.5 (10, 0.34)	-0.5 (6, 0.46)	0.4 (9, 0.25)	0.4 (8, 0.36)
C	-0.4 (7, 0.50)	0.7 (7, 0.40)	-0.7 (5, 0.47)	-0.5 (7, 0.29)	-0.7 (3, 0.59)
P-value	0.97	0.04	0.77	0.04	0.16

[1] Least Squares Means (n, ± standard error of the mean) of the difference in fecal bacterial counts (Log10 CFU g^{-1}) between mean of the two values observed three and six months into the feeding trial and the pre-treatment period.

[a] n = number of ewes included in the computation. Two replicated pens per treatment.

Table 3. Fecal bacteria counts (colony forming units, CFU) at the end of the experimental period in lambs after ewes fed supplement with seaweed meal (SW) or without (C) [1].

Supplement	*Enterococcus* spp.	Lactic acid bacteria	Aerobic blood agar counts	*Escherichia coli*	*Clostridium perfringens*
SW[a]	5.7 (0.64)	8.0 (0.41)	8.0 (0.48)	7.6 (0.46)	5.6 (0.76)
C[b]	6.7 (0.59)	8.5 (0.38)	8.6 (0.44)	8.1 (0.41)	5.7 (0.71)
P-value	0.37	0.53	0.46	0.51	0.89

[1] Least Squares Means (n, ± standard error of the mean) for the response variable (Log10 CFU g^{-1}) was calculated.

[a] Two replicated pens. Number of lambs = 7

[b] Two replicated pens. Number of lambs = 9

Table 4. *In vitro* bactericidal (negative values), bacteriostatic (0) or bacterial growth stimulating effects (positive values) of methanolic extracts from two brown and four red seaweed species. Effect is shown as the difference in optical density (OD[a]) measurements[b] between the starting culture with a cell concentration of about 5 X 10^5 cells per well and the OD value resulting in no change in optical density after incubation for 24 h at 37 ˚C.

	Brown seaweeds		Red seaweeds			
	A. nodosum	*L. hyperborea*	*M. stellatus*	*P. palmata*	*C. officinalis*	*P. lanosa*
E. faecalis	-3^{*}	-3	$+1^{*}$	-2	-3^{*}	-4^{*}
E. faecium	-4^{*}	-3^{*}	-3^{*}	-3^{*}	-3^{*}	-3^{*}
Hemolytic *E. coli*	-1	-1	-1	-1	-1	-2
Biofilm forming *E. coli*	+3	0	-1	-1	-1	+1
E. coli	+1	-1	-1	-1	-1	-3^{*}

[a] OD 600

[b] 0 = no change in OD , -1 = OD reduction from -0.01 to -0.05, -2 = OD reduction from -0.06 to -0.10, -3 = OD reduction from -0.11 to -0.20, -4 = OD reduction > -0.21, +1 = OD increase from 0.01 to 0.05, +2 = OD increase from 0.06 to 0.10, +3 = OD increase from 0.11 to 0.20, +4 = OD increase > 0.21

[*] $p \leq 0.05$

Table 5. *In vitro* bactericidal (negative values), bacteriostatic (0) or bacterial growth stimulating effects (positive values) of aqueous extracts from two brown and four red seaweed species. Effect is shown as the difference in optical density (OD[a]) measurements[b] between the starting culture with a cell concentration of about 5 X 10^5 cells per well and the OD value resulting in no change in optical density after incubation for 24 h at 37 ˚C.

	Brown seaweeds		Red seaweeds			
	A. nodosum	*L. hyperborea*	*M. stellatus*	*P. palmata*	*C. officinalis*	*P. lanosa*
E. faecalis	-4*	-1	-3	-3	-2	-3
E. faecium	-3*	-2	-2	-3	-3	-3
Hemolytic *E. coli*	-2	+3*	+2*	+1	+1	+2*
Biofilm forming *E. coli*	-2	+2	0	+1	-1	+1
E. coli	-3	+2*	+2	+1	+1	+1

[a] OD 600

[b] 0 = no change in OD , -1 = OD reduction from -0.01 to -0.05, -2 = OD reduction from -0.06 to -0.10, -3 = OD reduction from -0.11 to -0.20, -4 = OD reduction > -0.21, +1 = OD increase from 0.01 to 0.05, +2 = OD increase from 0.06 to 0.10, +3 = OD increase from 0.11 to 0.20

* $p \leq 0.05$