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In vitro screening of natural feed additives from crustaceans, diatoms, seaweeds and plant extracts to manipulate rumen fermentation

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Running title: Alternative feed additives to manipulate rumen function

Abstract

BACKGROUND: Eight natural products from animal, unicellular algae, brown seaweed and plant origins were chosen according to their theoretical anti-microbial activity: Diatomaceous earths (DE), insoluble chitosan (ICHI), soluble chitosan (CHI), seaweed meal (SWM), *Ascophyllum nodosum* (ASC), *Laminaria digitata* (LAM), Neem oil (NOIL) and an Ivy fruit extract rich in saponins (IVY). Dose-response incubations were conducted to determine their effect on rumen fermentation pattern and gas production, while their anti-protozoal activity was tested using ^{14}C-labelled bacteria.

RESULTS: DE, SWM, NOIL and ICHI had very small effects on rumen function when used at inclusion rate up to 2g L^{-1}. ASC had anti-protozoal effects (up to -23%) promoting a decrease in gas production and methanogenesis (-15%). LAM increased VFA production (+7%) and shifted from butyrate to acetate. CHI also shifted fermentation towards propionate production and lower methane (-23%) and protozoal activity (-56%). IVY decreased protozoal activity (-39%) and ammonia concentration (-56%), as well as increased feed fermentation (+11% VFA concentration) and shifted from acetate to propionate production.

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CONCLUSIONS: ASC, LAM, CHI and IVY showed promising potential *in vitro* as feed additives to improve rumen function, thus more research is needed to investigate their mode of action in the rumen microbial ecosystem.

Keywords: brown seaweed, chitosan, Diatomaceous earth, ivy fruit saponins, neem oil

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INTRODUCTION

The manipulation of the rumen microbial ecosystem to increase the efficiency of nutrient use by the animal, or to decrease its environmental impact, has long been a goal for nutritionists and rumen microbiologist. A number of chemical feed additives, antibiotics, methane inhibitors, defaunating agents and plant extracts have been shown to improve rumen metabolism and animal growth[1, 2]. However, concerns over the presence of chemical residues in animal products, the development of bacterial resistance to antibiotics and the excessive toxicity and cost of some plant extracts have limited their utilization in animal nutrition[3]. As a consequence, the scientific community is still actively seeking alternative feed additives that could improve rumen function.Several types of natural compounds have anti-microbial properties and could theoretically be used to manipulate the rumen microbial ecosystem:

Diatomaceous earth is the fossilised remains of diatom shells (unicellular protists). After quarrying, crushing and milling, a fine light silica dust is obtained with certain abrasive properties and the ability to absorb lipids[4]. As a result of its abrasive action, DE has been successfully used for ecto- and gastrointestinal parasite control in poultry[5] and ruminants[6]. However its effect in the rumen as an anti-protozoal agent has not yet been investigated.

Chitosan (N-acetyl-D-glucosamine polymer) is a natural biopolymer derived through the deacetytalion of chitin, the major component of the crustacean's exoskeleton. As a non-toxic, biodegradable carbohydrate polymer, chitosan has received much attention for diverse potential applications in medicine and food preservations due to its antimicrobial properties against bacteria, moulds and yeast[7]. The name "chitosan" does not indicate a single compound but rather a family of compounds in which each product differs in molecular weight and degree of acetylation [8]. Chitosan deacetylation increases its solubility and presumably its activity [9], thus the anti-microbial activity of different chitosan types needs to be assessed prior its utilization as a feed additive.

Seaweeds are naturally rich in minerals, trace elements and amino acids and have therefore been proposed as feed additives to maintain health and performance in livestock fed poor quality diets[10]. Among all seaweeds, brown seaweeds are the only ones able to produce high levels of phlorotannins

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as a result of phloroglucinol polymerization[11]. Phlorotannins differ from the two major classes of tannins found in terrestrial forages, such as condensed and hydrolysable tannins[12]. Although the effect of terrestrial tannins have been studied extensively with regard to their benefits on ruminant nutrition, especially in relation to protein metabolism by rumen microflora[13], the effect of phlorotannins on rumen function is still unknown.

Neem (*Arzadirachta indica*) has frequently been used in traditional medicine due to its anti-microbial properties[14]. Neem oil is extracted from seeds or fruits of the Neem tree and predominantly contains glycerides, free fatty acids and a number of bioactive compounds such as triterpenoids (i.e. nimbin, nimbindin and azadirachtin) and sterols[15]. Some of these compounds have demonstrated to have certain antibacterial, antifungal, antimalarial, antiparasitic, anti-inflamaty and inmunomodulatory properties[16]. However, there is very little information of effects of Neem oil on the rumen function[17].

Saponins are group of plant secondary compounds which form a stable foam in aqueous solutions like soap, hence the name "saponins". Chemically, saponins include compounds that are glycosylated steroids, triterpenoids and steroid alkaloids. Some of these saponins have been shown to modify rumen fermentation and enhance animal production[3]. So far, most of the studies have been mainly focused on saponins extracts obtained from *Yucca schidigera*, *Quillaja saponaria*, *Acacia auriculiformins*, *Sapindus saponaria*, *Sesbania sesban* and *Medicago sativa*[18]. However, no studies have yet described the effect of saponins from ivy fruit (*Hedera helix*) as feed additive.

Therefore, this paper aims to expand knowledge of potential feed additives by investigating the activity of diatomaceous earths, two chitosan types with different degrees of deacetylation; two brown seaweeds, a commercial seaweed meal and two plant extracts (Neem oil and Ivy fruit saponins). Two dose-response assays were conducted to investigate the effect of these additives on rumen fermentation and on protozoal activity *in vitro*.

MATERIALS AND METHODS

Feed additives

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A total of 8 different natural feed additives were evaluated: i) insoluble chitosan (**ICHI**) with 80% deacetylation degree and viscosity 50 mPas in 10mL L^{-1} acetic acid solution at 25ºC; ii) soluble chitosan (**CHI**) with >85% deacetylation degree, viscosity 140 mPas in 10mL L^{-1} acetic acid solution at 25ºC (Nitta Gelatin India Ltd. Cocin, Kerala, India) and iii) Diatomaceous Earths (**DE**) (Diature™, Natural Feeds & Fertilizers Ltd, Aberystwyth, UK); iv) a commercial seaweed meal (**SWM**) (Glenside Group, Fertility Farming Systems, UK); v) the brown seaweed *Ascophyllum nodosum* (**ASC**); the brown seaweed *Laminaria digitata* (**LAM**); Neem oil (**NOIL**) (Neem Biotech Ltd, Cardiff, UK); and an ivy fruit extract (**IVY**) rich in saponins (350g kg^{-1} DM). This later product was obtained by extraction of raw organic compounds from ivy fruit meal using ethanol (kindly supplied by Dr. Dave Preskett, Bangor University, UK). Seaweeds ASC and LAM were collected from the coast in Aberystwyth (UK), frozen and freeze-dried (kindly supplied by Michael McMonagle).

***In vitro* batch incubations**

A dose-response experiment was conducted to identify the effects of the different feed additives on gas production and fermentation pattern. The experimental design was: 8 feed additives × 3 doses (0.5, 1 and 2g L^{-1}) × 4 replicates plus 4 controls (dose 0g L^{-1}) and 4 blanks (diluted rumen fluid without feed), giving a total of 104 bottles. Animal procedures were carried out according to the Home Office Scientific Procedures, Act 1986 (PLL 40/3653; PIL 40/9798). Procedure was conducted in quadruplicate using rumen fluid from 4 barren rumen-cannulated Holsten-Frisian cows fed at maintenance level (diet composed of perennial ryegrass hay and concentrate at 67:33 on DM basis). Rumen contents were sampled before the morning feeding, filtered through a double layer of muslin, diluted 2:1 with incubation solution(19) and anaerobically dispensed to 120-mL Wheaton bottles (50mL per bottle) containing 500mg DM of mixed diet. This basal diet was composed by alfalfa hay 300g kg^{-1}, grass hay 200g kg^{-1}, barley 300g kg^{-1}, corn 120g kg^{-1}, soya bean meal 77g $kg^{-1}$and mineral-vitamin premix 3g kg^{-1} in DM (Rumins Cattle GP; Rumeco Ltd, Burton-on-Trent, Staffs, UK. Declared composition: Ca 240, P 20, Mg 50, Na 80, Se 0.03, Co 0.09, I 0.4, Mn 3, Zn 4 and Cu 1.5 g kg^{-1}, retinol $4×10^5$, cholecalciferol $8×10^4$ and alphatocopherol 10^3 IU kg^{-1}); and its chemical composition

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was: OM 949 g kg^{-1}, CP 128 g kg^{-1}, NDF 370 g kg^{-1} and ADF 211 g kg^{-1} in DM. Diet and feed additives were dried at 60ºC for 48h and ground using a hammer mill with $1mm^2$ sieve pore size.

Bottles were sealed and held stable in an incubator at 39ºC receiving a gentle mix before each sampling time. Fermentation pattern, in terms of pH, ammonia, VFA and methane emissions, were determined after 24h incubation: after gas pressure excess was released a gas sample (0.5mL) was taken from the headspace using a sample-lock glass syringe. This gas was immediately injected in a gas chromatograph (ATI Unicam 610 Series, UK) to determine methane concentration. A sample representing 5% of the bottle liquid content was taken by aspiration using a 14G needle and divided in two subsamples: the first subsample (1.6mL) was diluted with 0.4mL deproteinizing solution (200mL L^{-1} orthophosphoric acid containing 10 mM of 2-ethylbutyric acid as an internal standard) for VFA determination using Gas Chromatography [20]. The second subsample (0.8mL) was diluted with 0.48mL of trichloroacetate (25 g L^{-1}) for ammonia analysis using a colorimetric method (21). Gas production was measured at 2, 4, 6, 9, 12, 24, 48, 72 and 96h using a semi-automated pressure transducer (Bailey & Mackey Ltd. Birmingham, UK).

Measurement of protozoal activity

The effect of the same additives on *in vitro* protozoal activity was measured from the breakdown of ^{14}C-labelled bacteria(22) by the rumen protozoa. To prepare labelled bacteria, a pure culture of *Streptococcus bovis* was incubated at 39ºC for 24h in medium number two[23] containing ^{14}C-leucine (7 kBq mL^{-1} in 8 mL tube). Labelled bacteria were harvested from the culture by centrifugation (3,000×*g* for 15 min). Supernatant was discarded and bacterial pellet was washed with simplex type salt solution (STS)[24] containing^{12}C-leucine (5 mmol L^{-1}) and centrifuged at the same speed. This wash and centrifugation cycle was repeated to prevent reincorporation of released ^{14}C-leucine by bacteria. The resulting bacterial suspension was used as bacterial inoculum and one sample was taken to determine its initial radioactivity. Incubation was conducted in quadruplicate using rumen fluid from 4 cannulated-cows. Rumen fluid was filtered, diluted in STS (1:1) and distributed anaerobically in Hungate tubes (7.5mL per tube) containing ^{14}C-labelled bacteria (0.5mL) and feed additive at 0, 0.5, 1 and 2g L^{-1}. Moreover, rumen fluid used as inoculum was sampled (1 mL of

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sample in 1mL of formalin at 92.5 mL L^{-1} and NaCl 90 g L^{-1}) for protozoal quantification by optical microscopy(25). Incubation tubes were held stable in a water bath at 39ºC with manual mixing of them every 20min. Tubes were sampled at 0, 1, 2, 3 and 4h using a syringe with a 23G needle. Samples (0.5mL) were acidified with 0.125ml of trichloroacetic acid (at 250g L^{-1}) and centrifuged (13,000× *g* for 5min). Supernatants (200 µL) were diluted with 2mL of scintillation fluid and the amount of radioactivity released was determined by liquid-scintillation spectrometry (Packard 1900 CA, Berkshire, UK). Bacterial breakdown at each incubation point was expressed as the percentage of acid-soluble radioactivity released relative to the total radioactivity present in the initial labelled bacteria.

Calculations and statistical analysis

In the first experiment, fermentable OM (FOM) was stoichiometric calculated[26]. For gas production (GP), pressure measurements were adjusted for the amount of headspace available and corrected for the background GP from blanks (bottles with no substrate). These pressure measurements were converted to units of volume (mL) using the ideal gas law, then cumulative GP data were fitted to the predictive equation described by France *et al.,*[27]: $Y = A (1 - e^{-ct})$ where Y (mL) is the cumulative GP at time t (h), A is the asymptotic or potential GP (mL) and c is the GP rate (µL h^{-1}).

In the second experiment, a simple linear regression was conducted for each tube to model the relationship between the percentage of radioactivity released (with respect to the ^{14}C-bacterial inoculum) and the time (from 0h to 4h), as well as its correlation coefficient. The slope of this trend-line indicated the bacterial degradation rate (as % h^{-1}) by the rumen protozoa and ultimately their activity. Finally trend-line slopes were analysed statistically. In order to determine the minimum effective dose, defined as the lowest concentration of a given compound which significantly differs from the control, data from each feed additive were analysed according to the following model: $Y_{ij} = \mu + D_i + e_{ij}$; where Y_{ij} is the dependent, continuous variable, μ is the overall population mean, D_i is the fix effect of the dose (D_i = 0, 0.5, 1, 2 g L^{-1}) and e_{ij} is the residual error. When significant effects were detected across the different doses, means were compared by Fisher's protected LSD-test. Polynomial contrasts were also used to determine linear (L) and/or quadratic (Q) responses to the

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feed additives (Genstat 15th Edition, VSN International, UK). Significant effects were declared at $P < 0.05$ and tendency to differences at $P < 0.1$. Finally, responses (in %) were calculated as the variation in the values of one parameter induced by the highest dose (unless stated) of a given additive respect to the control (0 g L^{-1}) as follows: % Response = 100 x (Value highest dose – Control) / Control

RESULTS

In vitro batch cultures

Inclusion of either DE or ICHI in fermenters at levels up to 2g L^{-1} had no effect on rumen fermentation and GP (Table 1). Nonetheless, inclusion of CHI had a greater impact on the rumen function with a strong shift from butyrate to propionate production and a linear and/or quadratic decrease in methane emissions and FOM. CHI minimum effective dose was 1g L^{-1} for methane emissions and 2g L^{-1} for propionate, butyrate and FOM.

(Table 1 here)

The three seaweed based products tested had diverse effects on rumen function. Addition of increasing amounts of SWM to fermenters had no effect on rumen fermentation and GP (Table 2). On the contrary addition of specific brown seaweed species (i.e. ASC and LAM) promoted substantial changes in the rumen function. Both species promoted a linear decrease in the molar proportion of branched-chain VFA (BCVFA, i.e. iso-butyrate and iso-valerate) tended to decrease methane concentration in the headspace of the bottles. ASC decreased the GP rate and methane emissions, while LAM promoted an increased FOM and total VFA production as well as a moderated shift from butyrate to propionate production. ASC minimum effective doses were 1g L^{-1} for BCVFA and 2g L^{-1} for GP rate, while for LAM they were 0.5g L^{-1} for total VFA and FOM concentration and 1g L^{-1} for butyrate and BCVFA.

(Table 2 here)

The two plant extracts used had different impacts on rumen function. NOIL had a moderated influence on rumen fermentation characterized by a linear and/or quadratic decrease in the BCVFA concentration and GP rate. NOIL also promoted a quadratic decrease in ammonia concentration

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with the lowest value registered at 1g L^{-1}. Addition of IVY in the incubation bottles promoted the strongest effect of all compounds tested: GP rate, propionate and total VFA concentration progressively increased, whereas those of acetate, BCVFA, ammonia and pH decreased as the amount of IVY increased. For IVY all effects followed a linear and quadratic response and the minimum effective doses were 0.5g L^{-1} for ammonia, propionate and GP rate, 1g L^{-1} for pH, total VFA, BCVFA and FOM and 2g L^{-1} for acetate.

(Table 3 here)

Protozoal activity

Rumen fluid used to assess protozoal activity came from experimental cows with a type B protozoal population with an average concentration of $1.9 \times 10^6 \pm 0.9 \times 10^6$ cells mL^{-1}. The amount of bacteria degraded by protozoa increased linearly ($R^2 > 0.99$) over the incubation time considered (4h) in all experimental units. The rate of bacterial degradation ranged from 3.6 to 10.8% h^{-1} across treatments, except for NOIL which gave erratic values and data were not further analysed. Addition of DE, SWM and LAM into incubation tubes at concentrations up to 2g L^{-1} had no effect on the protozoal activity. On the contrary, inclusion of CHI, ASC and IVY promoted a strong decrease in the protozoal activity with 1g L^{-1} being their minimum effective dose. The anti-protozoal effect of ICHI was less intense and its minimum effective dose wastwo times higher (2g L^{-1}).

(Table 4 here)

Minimum effective dose

Across all feed additives explored in this experiment (Table 5), IVY was found to be the most active additive since it was able to significantly impact 11 out of the 15 (11/15)parameters studied with a minimum effective dose of 0.5 to 1 g L^{-1}. CHI was the second most active feed additive (7/15 parameters at doses of 1 to 2 g L^{-1}). LAM and ASC showed similar impact on the rumen function (4/15 parameters), but with a lower minimum effective dose required for the former (0.5 to 1 g L-1) than the latter (1 to 2 g L-1). NOIL and ICHI had a smaller impact (3/15 and 1/15 parameters, respectively), having both 2g L^{-1} as minimum effective dose. Finally, DE and SWM had no effect on any of the parameters tested.

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(Table 5 here)

DISCUSSION

Diatomaceous earths

Certain anti-microbial properties have been described for DE due to its abrasive action and ability to absorb lipids[4]. As a result, addition of DE in to the diet (20g kg^{-1}) has been used to control the loads of gastrointestinal parasites, such as helminths and coccidiosis in ruminants[6] and laying hens[5]. Our *in vitro* results showed that DE had neither effect on rumen protozoa activity nor on the fermentation pattern when used at concentrations up to 2g L^{-1}, so no minimum effective dose was observed (Table 5). This observation disagrees with previous findings in which bentonite (an adsorbent aluminium phyllosilicate) reduced rumen protozoal numbers *in vitro*[28], suggesting a different mode of action of these silicates. Because DE is indigestible, its concentration along the digestive tract progressively increases as other digestible nutrients get absorbed. This increase in concentration in the intestine may explain the discrepancy in the effects of DE on eukaryotic cells with similar structure (i.e. rumen protozoa vs coccidian) depending on their location (rumen vs intestine).

Chitosan

Chitin is a long-chain biopolymer present in cell walls of fungi and the exoskeletons of crustaceans and insects. In its pure, unmodified form, chitin is resilient and insoluble; however when chemically treated with alkali sodium hydroxide, chitin is deacetylated to chitosan. This deacetylation process increases chitosan's solubility and presumably its bio-activity[29]. Thus, in this experiment we tested the effect of two types of chitosan which differed in molecular weight and deacetylation degree, on rumen fermentation. Our results indicated that ICHI had very little effect of rumen function; only a small decrease in protozoal activity was observed when used at high doses (2g L^{-1}). On the contrary, CHI decreased rumen fermentation in terms of FOM (up to -7%). In a previous study in which different chitosan compounds were incubated at 0.75g L^{-1} [29], a decreased *in vitro* OM digestibility from -19% to -30% was also observed. This study reveals for the first time that CHI has a strong anti-protozoal effect, promoting a decrease in protozoal activity up to -56% when incubated at 2g L^{-1}. This

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anti-protozoal effect could be responsible for the decrease in feed degradation and fermentation rate[30]). In our study, CHI also promoted a strong decrease in the butyrate production (-24%) which was compensated by an increase in propionate (+28%) when CHI was incubated at 2g L^{-1}. According to the stoichiometry calculations[31], this shift in the fermentation pattern should result in a moderated decrease in H_2 production, and ultimately in methane emissions (-12%). However, the observed decrease in methane emissions was double that predicted by stoichiometry (-28% with CHI at 2g L^{-1}). Three possible reasons could explain the decrease in methane emissions: i) lower H_2 production as a result of a lower protozoal activity and/or shift in the bacterial community[32]), ii) H_2 rechannelling towards non-methanogenic compounds (i.e. succinate, propionate and lactate), and iii) specific CHI anti-microbial effect against methanogens. In agreement with our results, it has been reported that chitosan may improve *in vitro* energy efficiency because chitosan reduced feed disappearance without decreasing total VFA production[33]. Although the antimicrobial mechanism of action of chitosan polymers have not yet been fully elucidated, the most feasible hypothesis is a change in cell permeability due to interactions between the polycationic chitosan and the electronegative charges on the microbial surfaces[34, 35]. It was observed that chitosan caused a reduction in the GP after 6-10h post-inoculation *in vitro*[29], and a slightly decrease in NDF digestibility in sheep[36], suggesting that chitosan could exert an predominant antimicrobial action against fibrolytic microbes (i.e. protozoa and fibrolytic bacteria). Our results indicated that this later hypothesis could be true, but more research is needed to fully understand the mode of action of CHI in the rumen microbial ecosystem.

Seaweed

Seaweeds are frequently accumulated in coastal zones and are considered a public health hazard. In the Northern Atlantic Ocean *Ascopyllum* and *Laminaria* species are particularly common algae and thus their use as non-conventional feed for animal nutrition may contribute to solving environmental problems[37]. These brown seaweed are naturally rich in minerals (ash concentration: 230, 224 and 262 g kg^{-1} for SWM, ASC and LAM, respectively) and phlorotannins (phenolic secondary metabolites with an adaptive role in the defence against herbivory). As a result, phlorotannins

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content is greater in those brown seaweeds grown on mid-tide shore, such as ASC (50-70 g kg^{-1}), than those grown on subtidal zones, such as LAM (1-2 g kg^{-1})[38]. In this experiment, the effect of these seaweeds species, together with a commercial seaweed meal, on rumen function was investigated. SWM, a commercial mixture of brown seaweeds harvested from the Northern Atlantic Ocean, showed no effect on *in vitro* rumen function neither on rumen protozoal activity when used at concentrations up to 2g L^{-1}. The reasons for this lack of effect are unknown but could be due to: i) the inclusion of seaweed species with low antimicrobial activity as the concentration of phlorotannins is highly variable among different seaweed taxa and geographical areas[39]; ii) the further inactivation of the antimicrobial compounds during their industrial processing due to the sensitivity of phlorotannins to high temperatures[40]; or iii) the diet used in this experiment may not be challenging enough the rumen fermentation (i.e. promoting rumen acidosis).

Ascophillum nodosum (ASC) has been shown to decrease the prevalence of *Escherichia coli* in ruminant's faeces with no further improvements in animal performance[41]. Moreover, its antioxidant properties seems to enhance the immune system in cattle fed endophyte-infected Tall Fescue [42]. The mode of action of ASC as an anti-microbial is not yet fully understood but could be due to high levels of phlorotannins[38]. Our study demonstrated that ASC had a moderated effect on *in vitro* rumen fermentation. ASC showed also anti-protozoal properties when incubated *in vitro* decreasing protozoal activity (-23%) and GP rate (-11%) when used at 2g L^{-1}. Similar decreases in protozoal numbers and GP rate were observed when condensed tannins from *Leucaena* were incubated with rumen fluid[43] suggesting comparable anti-protozoal activities for phlorotannins and condensed tannins. A specific effect of condensed tannins on methanogen communities based on a decrease in total methanogens numbers and a shift from *Methanobacteriales* to *Methanomicrobiales* has been reported[43]. Interestingly, ASC also promoted a similar decreased in methane emissions per FOM (-15% when used at 2g L^{-1}). This observation seems to suggest that a specific anti-methanogenic effect could also be true for ASC, since no further modifications in the fermentation pattern were observed, suggesting no major changes in the bacterial community and metabolic pathways.

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Laminaria digitata (LAM) is a brown seaweed traditionally used as fertiliser as a result of its high potash content. The use of *Laminaria* as substrate to produce methane by anaerobic digestion has recently been investigated[44]. Moreover it has been observed that North Ronaldsay sheep have a rumen microbiota to particularly adapted to digest this seaweed which represents a high percentage of their diet[45]. Our data showed that LAM, in contrast to ASC, had no effect on rumen protozoal activity and GP rate. This observation suggests that LAM had no negative antimicrobial effect in the rumen. On the contrary, LAM boosted VFA production (up to +6%) and FOM concentration (up to +6%), with this effect achieved at concentrations as low as 0.5g L^{-1}. Moreover, LAM, in contrast to ASC, modified rumen fermentation pattern promoting a linear increase in propionate (+6% at 2g L^{-1}) at the detriment of butyrate. The only similarity between ASC and LAM is that both seaweeds tend to decrease rumen pH and molar concentration of BCVFA. Taking into account that BCVFA are considered as end-products of the protein degradation, it seems possible that the phlorotannins of these seaweeds could have similar anti-proteolytic properties to those described for plant tannins[13, 46, 47]. If this is true, more *in vivo* research is needed to elucidate whether or not brown seaweeds could be used as a strategy to increase the rumen bypass protein in high producing animals.

Neem oil

Several studies have investigated the effect of water extracts of Neem leaves or seeds on ruminal digestibility[17], but there is very little information on the effects of NOIL in ruminants. Our *in vitro* data suggested that NOIL has a limited effect on rumen fermentation when used in concentrations up to 2g L^{-1}. This effect consisted mainly of a moderated increase in the GP rate (up to +9%). However, NOIL did not promoted further modifications of the fermentation pattern in terms of VFA and methane production. A similar lack of effect of NOIL supplementation on rumen concentration and patterns of VFA production has been reported using continuous cultures[17]. This later study also showed some negative effects of NOIL supplementation on feed digestibility, in terms of OM, NDF and CP, suggesting a depression in rumen microbial activity. Our data are in line with this observation since the decrease in ammonia (-34% at 1 g L^{-1}) and BCVFA (-6% at 2 g L^{-1}) seems to suggest an anti-proteolytic effect of NOIL when supplemented at high doses. Unfortunately, we

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could not measure the effect of NOIL on protozoal activity due to methodical constrains; nevertheless previous reports showed discrepancies to this respect depending on the oil extraction method used and its solubility in water[48, 49].

Ivy fruit saponins

To our knowledge, this study is a pioneer in evaluating the effect of Ivy fruit saponins on rumen function. Our findings indicated that IVY, similarly to other saponins sources, had a strong anti-protozoal effect and promoted a decrease in protozoal activity (-11% at 1g L^{-1}). This anti-protozoal effect derives from the saponins ability to forming complexes with sterols in the protozoal membrane surface[50]. In an extensive review [3] it was concluded that most saponins decrease protozoal activity *in vitro*. The anti-protozoal effect of saponins *in vivo* is however more controversial and frequently trends to disappear after 7-14 days as a result of the adaptation of the rumen microbial population[18]. Thus further research to assess the anti-protozoal effect of IVY *in vivo* after prolonged periods of time is required.

Our *in vitro* data also revealed an effect of IVY on the rumen fermentation: the observed linear decrease in ammonia concentration (-56% at 2 g L^{-1}) could be due to a combination of a lower protozoal activity and lower protein degradation which ultimately lowered the BCVFA concentration. These results support previous observations which suggested the use of the ammonia-binding ability of saponins extracted from *Yucca shidigera* to lower ruminal ammonia concentrations[51]. Our study also showed that IVY at 2 g L^{-1} promoted a linear increase in the VFA concentration (+11%), FOM and GP rate (+19%) suggesting that IVY has a potential to boost feed fermentation and bacterial activity. Nevertheless, the main effect of IVY on rumen fermentation was the shift of VFA pattern towards an increased proportion of propionate and a decreased of acetate. Interestingly, this shift towards a more energetically efficient fermentation was not accompanied by a decrease in the methane emissions, possibly because it was partially compensated by a greater VFA production. This shift in the fermentation pattern may be the result of a lower activity of those microbes which produce acetate and butyrate as main fermentation products, such as protozoa and fibrolytic bacteria[3]. Saponins can affect the structure of the bacterial and methanogens communities in the rumen[18],

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thus a change in the bacterial community favouring the proliferation of propionate-producing bacteria in presence of IVY cannot be ruled out.

CONCLUSIONS

This *in vitro* study demonstrated that IVY and CHI are able to greatly improve rumen fermentation and to decrease protozoal activity. ASC and LAM also showed a moderate ability to modify rumen function. On the contrary NOIL and ICHI had very limited effects on rumen fermentation and protozoa activity, while DE and SWM had no effect when used at concentrations up to 2g L^{-1}. This paper describes preliminary work and conclusions should be interpreted with caution; thus more research is required to assess the effects of IVY, CHI, ASC and LAM across multiple feed types using "omics" technologies to fully understand their mode of action on the rumen microbial ecosystem.

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Table 1. Effect of different doses of Diatomaceous earths (DE), insoluble chitosan (ICHO) and soluble chitosan (CHI) on *in vitro* rumen fermentation.

Dose (g L^{-1})	0	0.5	1	2	SED	P-value	Contrasts
DIATOMACEOUS EARTHS							
pH	6.43	6.45	6.44	6.45	0.014	ns	ns
NH_3-N (mg L^{-1})	17.8	17.7	14.0	17.4	3.35	ns	ns
Total VFA (mmol L^{-1})	78.7	80.7	80.7	78.6	2.05	ns	ns
VFA (mmol mol^{-1})							
Acetate	693	690	698	693	4.35	ns	ns
Propionate	145	145	143	143	2.38	ns	ns
Butyrate	125	128	123	127	2.93	ns	ns
BCVFA	21.9	21.7	21.3	21.3	0.41	ns	ns
Asymptotic GP (mL)	139	134	136	134	3.47	ns	ns
GP rate (µL h^{-1})	46.9	50.1	48.3	48.3	1.45	ns	ns
FOM (mg)	347	357	355	347	8.87	ns	L†
Methane (mL L^{-1})	145	137	146	142	3.60	†	ns
Methane (mL d^{-1})	13.6	12.6	13.5	12.8	0.56	ns	ns
Methane (mL $gFOM^{-1}$)	39.2	35.4	38.2	36.8	1.34	†	ns
INSOLUBLE CHITOSAN							
pH	6.43	6.44	6.43	6.46	0.013	ns	L*,Q†
NH_3-N (mg L^{-1})	18.1	10.8	11.7	18.7	3.87	ns	Q†
Total VFA (mmol L^{-1})	78.7	79.9	80.0	81.2	1.63	ns	ns
VFA (mmol mol^{-1})							
Acetate	693	695	691	693	3.83	ns	ns
Propionate	109	146	146	147	2.66	ns	ns
Butyrate	125	122	126	123	2.81	ns	ns
BCVFA	21.9	21.4	21.8	21.7	0.58	ns	ns
Asymptotic GP (mL)	139	132	136	135	3.73	ns	ns
GP rate (µL h^{-1})	46.9	45.5	45.0	45.0	1.85	ns	ns
FOM (mg)	347	351	353	357	7.17	ns	ns
Methane (mL L^{-1})	145	146	145	144	3.00	ns	ns
Methane (mL d^{-1})	13.6	12.8	13	12.6	0.52	ns	ns
Methane (mL $gFOM^{-1}$)	39.2	36.4	36.7	35.4	1.53	ns	L*,Q†
SOLUBLE CHITOSAN							
pH	6.43	6.45	6.46	6.44	0.016	ns	ns
NH_3-N (mg L^{-1})	18.1	12.1	18.6	14.7	4.56	ns	ns
Total VFA (mmol L^{-1})	78.7	77.6	76.6	75.2	1.77	ns	L*
VFA (mmol mol^{-1})							
Acetate	693	692	691	687	4.40	ns	ns
Propionate	145^{b}	151^{b}	154^{b}	186^{a}	4.73	***	L***,Q***
Butyrate	125^{a}	120^{a}	118^{a}	94.7^{b}	3.58	***	L***,Q***
BCVFA	21.9	21.3	21.4	19.9	0.78	ns	L*,Q†

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Asymptotic GP (mL)	139	132	129	125	6.39	ns	L^{*},$Q^{\dagger}$
GP rate (µL h^{-1})	46.9	45.0	43.2	45.7	1.85	ns	ns
FOM (mg)	347^{a}	340^{a}	336^{ab}	324^{b}	7.28	*	L^{**},Q^{*}
Methane (mL L^{-1})	145^{a}	139^{a}	134^{ab}	122^{b}	6.00	*	L^{***},Q^{**}
Methane (mL d^{-1})	13.6^{a}	11.9^{ab}	10.8^{bc}	9.67^{c}	0.95	**	L^{***},Q^{**}
Methane (mL $gFOM^{-1}$)	39.2^{a}	35.1^{ab}	32.3^{b}	30.0^{b}	3.07	*	L^{**},Q^{*}

[a-c] Means with different superscript differ (n=4). *** P<0.001; ** P<0.01; * P<0.05; † P<0.1; ns, not significant; L, linear response; Q, quadratic response.

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Table 2. Effect of different doses of seaweed meal (SWM), *Ascophyllum nodosum* (ASC) and *Laminaria digitata* (LAM) on *in vitro* rumen fermentation.

Dose (g L^{-1})	0	0.5	1	2	SED	P-value	Contrasts
SEAWEED MEAL							
pH	6.43	6.43	6.44	6.42	0.014	ns	ns
NH_3-N (mg L^{-1})	14.9	17.0	15.6	15.8	2.79	ns	ns
Total VFA (mmol L^{-1})	78.7	80.6	81.5	79.0	1.50	ns	ns
VFA (mmol mol^{-1})							
Acetate	693	692	697	687	4.18	ns	ns
Propionate	145	149	146	151	2.55	ns	ns
Butyrate	125	123	122	126	3.05	ns	ns
BCVFA	21.9	21.2	20.6	20.7	0.59	ns	$L^{†}$,$Q^{†}$
Asymptotic GP (mL)	139	138	136	141	0.76	ns	ns
GP rate (µL h^{-1})	46.9	45.0	47.2	47.1	2.12	ns	ns
FOM (mg)	347	355	359	349	6.41	ns	ns
Methane (mL L^{-1})	145	144	138	140	2.79	ns	ns
Methane (mL d^{-1})	13.6	13.0	12.8	13.3	0.65	ns	ns
Methane (mL $gFOM^{-1}$)	39.2	36.7	35.7	38.1	1.87	ns	ns
ASCOPHYLLUM NODOSUM							
pH	6.43	6.42	6.42	6.39	0.015	ns	L^{*},Q^{*}
NH_3-N (mg L^{-1})	14.9	16.8	14.3	14.9	1.99	ns	ns
Total VFA (mmol L^{-1})	78.7	79.7	80.6	82.1	2.40	ns	ns
VFA (mmol mol^{-1})							
Acetate	693	691	698	694	4.22	ns	ns
Propionate	145	148	149	151	2.80	ns	ns
Butyrate	125	126	119	123	2.43	ns	ns
BCVFA	21.9^{a}	20.9^{a}	19.4^{b}	17.8^{c}	0.60	***	L^{***},Q^{***}
Asymptotic GP (mL)	139	141	139	140	3.44	ns	ns
GP rate (µL h^{-1})	46.9^{a}	46.8^{a}	45.8^{a}	41.6^{b}	1.39	**	L^{***},Q^{**}
FOM (mg)	347	352	354	363	10.8	ns	ns
Methane (mL L^{-1})	145	140	137	134	3.62	†	L^{**},Q^{*}
Methane (mL d^{-1})	13.6	13.4	12.9	12.2	0.51	†	L^{**},Q^{*}
Methane (mL $gFOM^{-1}$)	39.2^{a}	38.2^{a}	36.3^{ab}	33.5^{b}	1.70	*	L^{**},Q^{**}
LAMINARIA DIGITATA							
pH	6.43	6.43	6.41	6.40	0.012	ns	L^{*},$Q^{†}$
NH_3-N (mg L^{-1})	14.9	14.6	13.5	13.4	2.88	ns	ns
Total VFA (mmol L^{-1})	78.7^{b}	81.9^{a}	81.5^{ab}	83.9^{a}	1.44	*	L^{**},Q^{*}
VFA (mmol mol^{-1})							
Acetate	693	693	698	694	3.85	ns	ns
Propionate	145	150	150	153	2.51	†	L^{**},Q^{*}
Butyrate	125^{a}	122^{ab}	118^{b}	120^{b}	2.10	*	$L^{†}$,Q^{*}
BCVFA	21.9^{a}	20.9^{ab}	19.7^{bc}	18.9^{c}	0.54	***	L^{***},Q^{***}

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Asymptotic GP (mL)	139	139	139	145	4.00	ns	ns
GP rate (µL h^{-1})	46.9	46.8	45.7	46.5	1.56	ns	ns
FOM (mg)	347^{b}	360^{a}	358^{ab}	369^{a}	6.22	*	L^{***},Q^{**}
Methane (mL L^{-1})	145	141	141	137	2.79	†	L^{*},Q^{*}
Methane (mL d^{-1})	13.6	13.3	13.2	13.3	0.70	ns	ns
Methane (mL $gFOM^{-1}$)	39.2	36.8	36.8	36.0	1.82	ns	ns

[a-c] Means with different superscript differ (*n*=4). *** *P*<0.001; ** *P*<0.01; * *P*<0.05; † *P*<0.1; ns, not significant; L, linear response; Q, quadratic response.

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Table 3. Effect of different doses of Neem oil and Ivy fruit saponins on *in vitro* rumen fermentation.

Dose (g L^{-1})	0	0.5	1	2	SED	P-value	Contrasts
NEEM OIL							
pH	6.43	6.46	6.43	6.42	0.020	ns	ns
NH_3-N (mg L^{-1})	18.1^{ab}	13.7^{bc}	12.0^{c}	18.6^{a}	2.17	*	Q**
Total VFA (mmol L^{-1})	78.7	79.4	79.9	81.7	1.75	ns	L†
VFA (mmol mol^{-1})							
Acetate	693	694	688	687	5.06	ns	ns
Propionate	145	146	147	149	2.33	ns	L†
Butyrate	125	123	128	128	3.10	ns	ns
BCVFA	21.9^{a}	21.2^{ab}	21.3^{ab}	20.6^{b}	0.36	*	L**,Q*
Asymptotic GP (mL)	139	137	138	134	2.73	ns	ns
GP rate (μL h^{-1})	46.9^{b}	49.0^{ab}	48.1^{b}	51.0^{a}	1.18	*	L**,Q*
FOM (mg)	347	349	353	361	7.75	ns	L*
Methane (mL L^{-1})	145	138	145	141	3.70	ns	ns
Methane (mL d^{-1})	13.6	12.9	13.5	13.3	0.56	ns	ns
Methane (mL $gFOM^{-1}$)	39.2	36.9	38.2	36.8	1.81	ns	ns
IVY FRUIT SAPONINS							
pH	6.43^{a}	6.42^{a}	6.36^{b}	6.30^{c}	0.013	***	L***,Q***
NH_3-N (mg L^{-1})	18.1^{a}	9.38^{b}	8.55^{b}	7.94^{b}	2.922	*	L†,Q*
Total VFA (mmol L^{-1})	78.7^{c}	81.9^{bc}	82.9^{b}	87.6^{a}	1.85	**	L***,Q***
VFA (mmol mol^{-1})							
Acetate	693^{a}	692^{a}	689^{a}	669^{b}	4.06	***	L***,Q***
Propionate	145^{d}	154^{c}	160^{b}	182^{a}	2.19	***	L***,Q***
Butyrate	125	121	120	118	3.05	ns	ns
BCVFA	21.9^{a}	20.9^{a}	19.4^{b}	17.8^{c}	0.60	***	L***,Q***
Asymptotic GP (mL)	139	135	139	139	3.26	ns	ns
GP rate (μL h^{-1})	46.9^{c}	50.2^{b}	50.3^{b}	56.0^{a}	1.41	***	L***,Q***
FOM (mg)	347^{c}	361^{bc}	366^{b}	386^{a}	8.36	**	L***,Q**
Methane (mL L^{-1})	145	135	143	141	4.26	ns	ns
Methane (mL d^{-1})	13.6^{bc}	12.6^{c}	13.9^{ab}	14.8^{a}	0.52	**	L*,Q*
Methane (mL $gFOM^{-1}$)	39.2^{a}	34.8^{b}	38.1^{a}	38.3^{a}	1.38	*	ns

$^{a-c}$ Means with different superscript differ (*n*=4). *** *P*<0.001; ** *P*<0.01; * *P*<0.05; † *P*<0.1; ns, not significant; L, linear response; Q, quadratic response.

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Table 4. Effect of different doses of feed additives on rumen protozoa activity assessed *in vitro* as the amount of ^{14}C-labeled bacteria broken down by rumen protozoa (% of the initial radioactivity released per hour).

Dose (g L^{-1})	0	0.5	1	2	SED	P-value	Contrasts
Diatomaceous earths	9.60	9.92	9.83	9.84	0.452	ns	ns
Insoluble chitosan	9.60[a]	9.56[a]	9.69[a]	7.33[b]	0.368	**	L**,Q**
Soluble chitosan	9.60[a]	8.86[a]	5.93[b]	4.23[c]	0.489	***	L***,Q***
Seaweed meal	9.60	9.71	9.83	9.79	0.454	ns	ns
Ascophillum nodosum	9.60[a]	8.91[ab]	7.94[bc]	7.36[c]	0.457	***	L***,Q***
Laminaria digitata	9.60	9.15	9.05	9.16	0.393	ns	ns
Neem oil[¥]	ND	ND	ND	ND			
Ivy fruit saponins	9.60[a]	9.13[ab]	8.57[b]	5.83[c]	0.463	***	L***,Q***

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Table 5. Minimum effective doses of feed additives on *in vitro* rumen fermentation and protozoal activity.

Minimum effective dose [a]	DE	ICHI	CHI	SWM	ASC	LAM	NOIL	IVY
pH	ND	ND	ND	ND	ND	ND	ND	(-)1g L^{-1}
NH_3-N (mg L^{-1})	ND	ND	ND	ND	ND	ND	(-)1g L^{-1}	(-)0.5g L^{-1}
Total VFA (mmol L^{-1})	ND	ND	ND	ND	ND	(+)0.5g L^{-1}	ND	(+)1g L^{-1}
VFA (mmol mol^{-1})								
Acetate	ND	ND	ND	ND	ND	ND	ND	-2g L^{-1}
Propionate	ND	ND	(+)2g L^{-1}	ND	ND	ND	ND	(+)0.5g L^{-1}
Butyrate	ND	ND	(-)2g L^{-1}	ND	ND	(-)1g L^{-1}	ND	ND
BCVFA	ND	ND	ND	ND	(-)1g L^{-1}	(-)1g L^{-1}	(-)-2g L^{-1}	(-)1g L^{-1}
Asymptotic GP (mL)	ND	ND	ND	ND	ND	ND	ND	ND
GP rate (µL h^{-1})	ND	ND	ND	ND	(-)2g L^{-1}	ND	(+)2g L^{-1}	(+)0.5g L^{-1}
FOM (mg)	ND	ND	(-)2g L^{-1}	ND	ND	(+)0.5g L^{-1}	ND	(+)1g L^{-1}
Methane (mL L^{-1})	ND	ND	(-)2g L^{-1}	ND	ND	ND	ND	ND
Methane (mL d^{-1})	ND	ND	(-)2g L^{-1}	ND	ND	ND	ND	(-)0.5g L^{-1}
Methane (mL $gFOM^{-1}$)	ND	ND	(-)1g L^{-1}	ND	(-)2g L^{-1}	ND	ND	(-)0.5g L^{-1}
Anti-protozoal activity [b]	ND	(-)2g L^{-1}	(-)1g L^{-1}	ND	(-)1g L^{-1}	ND	ND	(-)1g L^{-1}

[a] Minimum effective dose was defined as the lowest concentration of a given additive which significantly increased (+) or decreased (-) the mean respect to the control ($P<0.05$). ND, not detected.

[b] Rumen protozoa activity was described as the amount of ^{14}C-labeled bacteria broken down by rumen protozoa (expressed as the % of the initial radioactivity released per hour).

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