

RESEARCH ARTICLE

Published  
2021-11-23

Cite as

Rafael Muñoz-Tamayo, Juana C. Chagas, Mohammad Ramin and Sophie J. Krizsan (2021)

*Modelling the impact of the macroalgae Asparagopsis taxiformis on rumen microbial fermentation and methane production*, Peer Community Journal, 1: e7.

Correspondence

Rafael.munoz-tamayo@inrae.fr

Peer-review

Peer reviewed and recommended by

PCI Animal Science,

<https://doi.org/10.24072/pci.animsci.100006>



This article is licensed under the Creative Commons Attribution 4.0 License.

## Modelling the impact of the macroalgae *Asparagopsis taxiformis* on rumen microbial fermentation and methane production

Rafael Muñoz-Tamayo<sup>1</sup>, Juana C. Chagas<sup>2</sup>, Mohammad Ramin<sup>2</sup>, and Sophie J. Krizsan<sup>2</sup>

Volume 1 (2021), article e7

<https://doi.org/10.24072/pcjournal.11>

### Abstract

**Background:** the red macroalgae *Asparagopsis taxiformis* is a potent natural supplement for reducing methane production from cattle. *A. taxiformis* contains several anti-methanogenic compounds including bromoform that inhibits directly methanogenesis. The positive and adverse effects of *A. taxiformis* on the rumen microbiota are dose-dependent and operate in a dynamic fashion. It is therefore key to characterize the dynamic response of the rumen microbial fermentation for identifying optimal conditions on the use of *A. taxiformis* as a dietary supplement for methane mitigation. Accordingly, the objective of this work was to model the effect of *A. taxiformis* supplementation on the rumen microbial fermentation under in vitro conditions. We adapted a published mathematical model of rumen microbial fermentation to account for *A. taxiformis* supplementation. We modelled the impact of *A. taxiformis* on the fermentation and methane production by two mechanisms, namely (i) direct inhibition of the growth rate of methanogens by bromoform and (ii) hydrogen control on sugars utilization and on the flux allocation towards volatile fatty acids production. We calibrated our model using a multi-experiment estimation approach that integrated experimental data with six macroalgae supplementation levels from a published in vitro study assessing the dose-response impact of *A. taxiformis* on rumen fermentation. **Results:** our model captured satisfactorily the effect of *A. taxiformis* on the dynamic profile of rumen microbial fermentation for the six supplementation levels of *A. taxiformis* with an average determination coefficient of 0.88 and an average coefficient of variation of the root mean squared error of 15.2% for acetate, butyrate, propionate, ammonia and methane. **Conclusions:** our results indicated the potential of our model as prediction tool for assessing the impact of additives such as seaweeds on the rumen microbial fermentation and methane production in vitro. Additional dynamic data on hydrogen and bromoform are required to validate our model structure and look for model structure improvements. We expect this model development can be useful to help the design of sustainable nutritional strategies promoting healthy rumen function and low environmental footprint.

<sup>1</sup>Université Paris-Saclay, INRAE, AgroParisTech, UMR Modélisation Systémique Appliquée aux Ruminants - Paris, France., <sup>2</sup>Department of Agricultural Research for Northern Sweden, Swedish University of Agricultural Sciences (SLU), Skogsmarksgränd, 90183 - Umeå, Sweden.

## 1. Introduction

Some macroalgae (seaweeds) have the potential to be used as natural supplement for reducing methane (CH<sub>4</sub>) production from cattle (Wang et al. 2008; Dubois et al. 2013; Maia et al. 2016). This anti-methanogenic activity adds value to the nutritional and healthy promoting properties of macroalgae in livestock diets (Evans, Critchley 2014; Makkar et al. 2016). The species of the red macroalgae *Asparagopsis* have proven a strong anti-methanogenic effect both *in vitro* (Machado et al. 2014) and *in vivo* (Roque et al., 2019). In particular, *Asparagopsis taxiformis* appears as the most potent species for methane mitigation with studies reporting a reduction in enteric methane up to 80% in sheep (Li et al. 2016) and up to 80% and 98% in beef cattle (Kinley et al. 2020; Roque et al. 2021). The anti-methanogenic power of *A. taxiformis* results from the action of its multiple secondary metabolites with antimicrobial activities, being bromoform the most abundant anti-methanogenic compound (Machado et al. 2016a). It should be said, however, that despite the promising anti-methanogenic capacity of bromoform, the feasibility of supplying bromoform-containing macroalgae requires a global assessment to insure safety of feeding and low environmental footprint from the algae processing, since bromoform can be toxic to the environment and can impair human health (Beauchemin et al. 2020).

Bromoform is released from specialised gland cells of the macroalage (Paul et al. 2006) in to the culture medium. The mode of action of the anti-methanogenic activity of bromoform is similar to that described for bromochloromethane (Denman et al. 2007), following the mechanism suggested for halogenated hydrocarbons (Wood et al. 1968; Czerkawski, Breckenridge 1975). Accordingly, bromoform inhibits the cobamid dependent methyl-transfer reactions that lead to methane formation. In addition to the direct effect on the methanogenesis, the antimicrobial activity of *A. taxiformis* impacts the fermentation profile (e.g., acetate:propionate ratio) and the structure of the rumen microbiota (e.g., the relative abundance of methanogens) (Machado et al., 2018; Roque et al., 2019). Fermentation changes may have detrimental effects on animal health and productivity (Chalupa 1977; Li et al. 2016). Detrimental effects might include deterioration of the ruminal mucosa and the transfer of bromoform to tissues, blood and milk. Previous studies have not detected bromoform in animal tissues (Li et al. 2016; Kinley et al. 2020; Roque et al. 2021). The positive and adverse effects of *A. taxiformis* on the rumen microbiota are dose-dependent (Machado et al. 2016b) and operate in a dynamic fashion. It is therefore key to characterize the dynamic response of the rumen microbial fermentation for identifying optimal conditions on the use of the *A. taxiformis* as a dietary supplement for methane mitigation. The development of dynamic mathematical models provides valuable tools for the assessment of feeding and mitigation strategies (Ellis et al. 2012) including developments in the manipulation of the flows of hydrogen to control rumen fermentation (Ungerfeld 2020). Progress on rumen modelling including a better representation of the rumen microbiota and the representation of additives on the fermentation is central for the deployment of predictive tools that can guide microbial manipulation strategies for sustainable livestock production (Huws et al. 2018).

Accordingly, the objective of this work was to model the effect of *A. taxiformis* supplementation on the dynamics of rumen microbial fermentation under *in vitro* conditions. We adapted a published rumen fermentation model (Muñoz-Tamayo et al., 2016) to account for the impact of *A. taxiformis* on rumen fermentation and methane production evaluated *in vitro* at six supplementation levels (Chagas et al. 2019).

## 2. Methods

### Experimental data

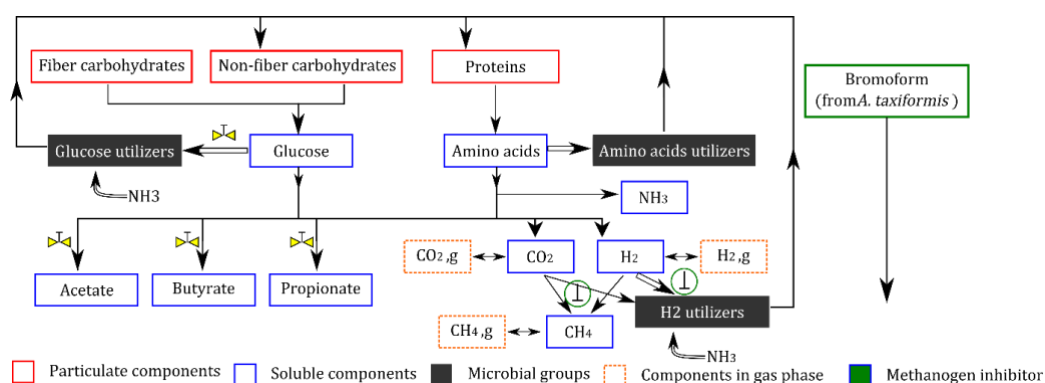
Model calibration was performed using experimental data from an *in vitro* batch study assessing the dose-response impact of *A. taxiformis* on fermentation and methane production (Chagas et al. 2019). In that study, *A. taxiformis* with 6.84 mg/g DM bromoform concentration was supplemented at six treatment levels (0, 0.06, 0.13, 0.25, 0.5, and 1.0 % of diet organic matter; OM). All experimental treatments were composed of a control diet consisted of timothy grass (*Phleum pratense*), rolled barley (*Hordeum vulgare*), and rapeseed (*Brassica napus*) meal in a ratio of 545:363:92 g/kg diet dry matter (DM) presenting chemical composition as 944 g/kg OM, 160 g/kg crude protein (CP) and 387 g/kg neutral detergent fiber (NDF). Prior to each *in vitro* incubation, dried individual ingredients milled at 1 mm were weighted into serum bottles

totalizing 1000 mg substrate on DM basis. The incubation was carried out with rumen inoculum from two lactating Swedish Red cows cannulated in the rumen, fed ad libitum on a diet of 600 g/kg grass silage and 400 g/kg concentrate on DM basis. Diet samples were incubated for 48 h in 60 ml of buffered rumen fluid (rumen fluid:buffer ratio of 1:4 by volume) as described by Chagas et al. (2019). The *in vitro* batch fermentation was run in a fully automated system that allows continuous recording of gas production (Ramin, Huhtanen 2012).

Methane production, acetate, butyrate, propionate, and ammonia were measured throughout the incubation period. Methane was measured at 0, 2, 4, 8, 24, 36 and 48 h according to (Ramin, Huhtanen 2012). Gas production was measured using a fully automated system (Gas Production Recorder, GPR-2, Version 1.0 2015, Wageningen UR), with readings made every 12 min and corrected to the normal air pressure (101.3 kPa). Methane concentration was determined with a Varian Star 3400 CX gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA, USA) equipped with a thermal conductivity detector. The volatile fatty acids (VFAs) were measured at 0, 8, 24 and 48 h and determined using a Waters Alliance 2795 UPLC system as described by (Puhakka *et al.* 2016). Ammonia was measured at 0 and 24h and analysed with a continuous flow analyzer (AutoAnalyzer 3 HR, SEAL Analytical Ltd., Southampton, UK) and according to the method provided by SEAL Analytical (Method no. G-102-93 multitest MT7). For model calibration, we only considered data until 24h, since microbial fermentation stopped around this time.

### Mathematical modelling

We adapted the mathematical model of *in vitro* rumen fermentation developed by (Muñoz-Tamayo et al. 2016) to account for the effect of *A. taxiformis* on the fermentation. This model represents the rumen microbiota by three microbial functional groups (sugar utilizers, amino acid utilizers and methanogens). Hexose monomers are represented by glucose and amino acids are represented by an average amino acid. The model is an aggregated representation of the anaerobic digestion process that comprises the hydrolysis of cell wall carbohydrates (NDF - Neutral Detergent Fiber), non-fiber carbohydrates (NSC – Non Structural Carbohydrates) and proteins, the fermentation of soluble monomers producing the VFAs acetate, butyrate, propionate, and the hydrogenotrophic methanogenesis. The original model was calibrated using *in vitro* experimental data from (Serment et al., 2016). Figure 1 displays a schematic representation of the rumen fermentation model indicating the effect of *A. taxiformis* on the fermentation. We assumed that microbial cells are formed by proteins and non-fiber carbohydrates and that dead microbial cells are recycled as carbon sources in the fermentation.



**Figure 1.** Representation of the rumen fermentation model (adapted from (Muñoz-Tamayo et al., 2016)). Hydrolysis of carbohydrates (fiber and non-fiber) and proteins releases respectively sugars and amino acids soluble monomers which are further utilized by the microbiota. The utilization of substrate is directed to product formation (single arrows) and microbial growth (double arrows). Each substrate is utilized by a single microbial functional group. The bromoform contained in *A. taxiformis* produces a direct inhibition of the growth rate of methanogens that results in a reduction of methane production and in an accumulation of hydrogen. The symbol  $\textcircled{L}$  indicates the direct effect of the bromoform on the methanogenesis. Hydrogen exerts control on sugars utilization and on the flux allocation towards volatile fatty acids production. The symbol  $\blacktriangleright\blacktriangleleft$  indicates the hydrogen control effect on the rumen fermentation.

The model is derived from mass balance equations of a closed system under the assumption that the protocol of gas sampling does not affect substantially the dynamics of methane and fermentation dynamics. Our model is described in compact way as follows

$$(1) \quad \frac{d\xi}{dt} = \mathbf{S} \cdot \boldsymbol{\rho}(\xi, \mathbf{p}) - \mathbf{g}(\xi, \mathbf{p})$$

Where  $\xi$  is the vector of state variables (metabolites),  $\boldsymbol{\rho}(\cdot)$  is a vector function with the kinetic rates of hydrolysis and substrate (sugars, amino acids, hydrogen) utilization. Hydrolysis rates are described by first-order kinetics. Substrate utilization rates are described by the Monod kinetics.  $\mathbf{S}$  is the stoichiometry matrix containing the yield factors ( $Y_{i,j}$ ) of each metabolite ( $i$ ) for each reaction ( $j$ ),  $\mathbf{g}(\cdot)$  is a vector function with the equations representing transport phenomena (liquid–gas transfer), and  $\mathbf{p}$  is the vector of the model parameters. The original model has 18 state variables (compartments in Figure. 1) and was implemented in Matlab (the code is accessible at <https://doi.org/10.5281/zenodo.4047640>). An implementation in R software is also available (Kettle *et al.* 2018). In the present work, we incorporated an additional state variable to represent the dynamics of bromoform concentration. The original model was extended to account for the impact of *A. taxiformis* on the rumen fermentation. While the original model predicts the pH, we set the pH value to 6.6.

The impact of *A. taxiformis* on the fermentation and methane production was ascribed to two mechanisms, namely the (i) direct inhibition of the growth rate of methanogens by bromoform and (ii) hydrogen control on sugars utilization and on the flux allocation towards volatile fatty acids production. These aspects are detailed below.

For the methanogenesis, the reaction rate of hydrogen utilization  $\rho_{H_2}$  (mol/(L h)) is given by

$$(2) \quad \rho_{H_2} = I_{br} \cdot I_{IN} \cdot k_{m,H_2} \frac{s_{H_2}}{K_{s,H_2} + s_{H_2}} x_{H_2}$$

where  $s_{H_2}$  (mol/L) is the hydrogen concentration in liquid phase,  $x_{H_2}$  (mol/L) is the concentration of hydrogen-utilizing microbes (methanogens),  $k_{m,H_2}$  (mol/(mol h)) is the maximum specific utilization rate constant of hydrogen and  $K_{s,H_2}$  (mol/L) is the Monod affinity constant of hydrogen utilization, and  $I_{IN}$  is a nitrogen limitation factor. The kinetic rate is inhibited by the anti-methanogenic compounds of *A. taxiformis*. The factor  $I_{br}$  represents this inhibition as function of the bromoform concentration. We used the following sigmoid function to describe  $I_{br}$

$$(3) \quad I_{br} = 1 - \frac{1}{1 + \exp(-p_1 \cdot (s_{br} + p_2))}$$

where  $s_{br}$  is the bromoform concentration (g/L) and  $p_1, p_2$  are the parameters of the sigmoid function. We included in our model the dynamics of bromoform using a first-order kinetics to take into account that the inhibition of *A. taxiformis* declines on time as a result of the consumption of anti-methanogenic compounds (Kinley *et al.* 2016). The dynamics of  $s_{br}$  is

$$(4) \quad \frac{ds_{br}}{dt} = -k_{br} \cdot s_{br}$$

where  $k_{br}$  (1/h) is the kinetic rate constant of bromoform utilization.

With regard to sugars utilization, we assumed that the effect of *A. taxiformis* is ascribed to hydrogen control due to accumulation of hydrogen resulting from the methanogenesis inhibition. Hydrogen level influences the fermentation pattern (Janssen 2010). We used the structure proposed by (Mosey 1983) to account for hydrogen control on sugar utilization and flux allocation. However, we used different parametric functions to those proposed by (Mosey 1983). The functions proposed by (Mosey 1983) did not provide satisfactory results.

In our model, the kinetic rate of sugar utilization is described by

$$(5) \quad \rho_{su} = I_{H_2} \cdot I_{IN} \cdot k_{m,su} \frac{s_{su}}{K_{s,su} + s_{su}} x_{su}$$

where  $s_{su}$  (mol/L) is the concentration of sugars,  $x_{su}$  (mol/L), is the concentration of sugar utilizers microbes,  $k_{m,su}$  (mol/(mol h)) is the maximum specific utilization rate constant of sugars and  $K_{s,su}$  (mol/L) is the Monod affinity constant of sugars utilization.

The factor  $I_{H_2}$  describes the hydrogen inhibition:

$$(6) \quad I_{H_2} = 1 - \frac{1}{1 + \exp(-p_3 \cdot (p_{H_2} + p_4))}$$

with  $p_{H_2}$  the hydrogen partial pressure ( $p_{H_2}$ ).

In our model, the rumen fermentation is represented by the macroscopic reactions in Table 1.

**Table 1.** Macroscopic reactions used in our model to representing rumen fermentation. For the anabolic reactions of microbial formation, we assume that microbial biomass has the molecular formula  $C_5H_7O_2N$ .

<i>Sugars (glucose) utilization</i>	
$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$	R <sub>1</sub>
$3C_6H_{12}O_6 \rightarrow 2CH_3COOH + 4CH_3CH_2COOH + 2CO_2 + 2H_2O$	R <sub>2</sub>
$C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$	R <sub>3</sub>
$5C_6H_{12}O_6 + 6NH_3 \rightarrow 6C_5H_7O_2N + 18H_2O$	R <sub>4</sub>
<i>Amino acid utilization</i>	
$C_5H_9.8O_{2.7}N_2 \rightarrow Y_{IN,aa}NH_3 + (1 - Y_{aa}) \cdot \sigma_{ac,aa} CH_3COOH + (1 - Y_{aa}) \cdot \sigma_{pr,aa} CH_3CH_2COOH + (1 - Y_{aa}) \cdot \sigma_{bu,aa} CH_3CH_2CH_2COOH + (1 - Y_{aa}) \cdot \sigma_{IC,aa} CO_2 + (1 - Y_{aa}) \cdot \sigma_{H_2,aa} H_2 + Y_{aa} C_5H_7O_2N$	R <sub>5</sub> *
<i>Hydrogen utilization</i>	
$4H_2 + 2CO_2 \rightarrow CH_4 + 2H_2O$	R <sub>6</sub>
$10H_2 + 5CO_2 + NH_3 \rightarrow C_5H_7O_2N + 8H_2O$	R <sub>7</sub>

\*R<sub>5</sub> is an overall reaction resulting from weighing the fermentation reactions of individual amino acids.

Table 1 shows that VFA production from glucose utilization occurs *via* reactions R<sub>1</sub>-R<sub>3</sub>. The pattern of the fermentation is determined by the flux allocation of glucose utilization through these three reactions. We denote  $\lambda_k$  as the molar fraction of the sugars utilized *via* reaction  $k$ . It follows that  $\lambda_1 + \lambda_2 + \lambda_3 = 1$ .

The fermentation pattern (represented in our model by the flux allocation parameters  $\lambda_k$ ) is controlled by thermodynamic conditions and by electron-mediating cofactors such as nicotinamide adenine dinucleotide (NAD) that drive anaerobic metabolism via the transfer of electrons in metabolic redox reactions (Mosey 1983; Hoelzle *et al.* 2014; van Lingen *et al.* 2019). In our model, the regulation exerted by the NADH/NAD<sup>+</sup> couple on the flux allocation is incorporated *via* regulation functions that are dependent on the hydrogen partial pressure ( $p_{H_2}$ ). This hybrid approach resulted by assuming a linearity between the couple NADH/NAD<sup>+</sup> and the  $p_{H_2}$  following the work of (Mosey 1983; Costello *et al.* 1991). As discussed by (van Lingen *et al.* 2019), the production of acetate *via* the reaction R<sub>1</sub> is favored at low NADH/NAD<sup>+</sup> while the production of propionate *via* the reaction R<sub>2</sub> is favored at high NADH/NAD<sup>+</sup>. Accordingly, we represented the flux allocation parameters by the following sigmoid functions:

$$(7) \quad \lambda_1 = 1 - \frac{1}{1 + \exp(-p_5 \cdot (p_{H_2} + p_6))}$$

$$(8) \quad \lambda_2 = \frac{p_7}{1 + \exp(-p_8 \cdot (p_{H_2} + p_9))}$$

Our model then predicts that high levels of supplementation of *A. taxiformis* will result in high hydrogen levels that will favor propionate production (R<sub>2</sub>) over acetate production (R<sub>1</sub>). By this parameterization of the flux allocation parameters, our model accounts for the concomitant reduction of the acetate:propionate ratio that is observed when methane production is reduced.



### Parameter estimation

We used the maximum likelihood estimator that minimizes the following objective function

$$(9) \quad J(\mathbf{p}) = \sum_{k=1}^{n_y} \frac{n_{t,k}}{2} \ln \left[ \sum_{i=1}^{n_{t,k}} [y_k(t_{i_k}) - y_{m_k}(t_{i_k}, \mathbf{p})]^2 \right]$$

Where  $\mathbf{p}$  is the vector of parameters to be estimated,  $n_y$  is the number of measured variables,  $n_{t,k}$  is the number of observation times of the variable  $k$ ,  $t_{i_k}$  is the  $i$ th measurement time for the variable  $y_k$ , and  $y_{m_k}$  is the value predicted by the model. The measured variables are the concentrations of acetate, butyrate, propionate,  $\text{NH}_3$ , and the moles of methane produced.

We used the IDEAS Matlab® toolbox (Muñoz-Tamayo *et al.* 2009) (freely available at <http://genome.jouy.inra.fr/logiciels/IDEAS>) to generate the function files for solving the optimization problem locally. Then, we used the generated files by IDEAS to look for global optimal solutions using the Matlab optimization toolbox MEIGO (Egea *et al.* 2014) that implements the enhanced scatter search method developed by (Egea *et al.* 2010) for global optimization.

We reduced substantially the number of parameters to be estimated by setting most of the model parameters to the values reported in the original model implementation and using the information obtained from the *in vitro* study (Chagas *et al.* 2019). For example, the hydrolysis rate constant for NDF was obtained from (Chagas *et al.* 2019) whereas the hydrolysis rate constants of NSC ( $k_{\text{hydr,nsC}}$ ) and proteins ( $k_{\text{hydr,pro}}$ ) were included in the parameter estimation problem. The kinetic rate constant for hydrogen utilization  $k_{\text{m,H}_2}$  was set 16 mol/(mol h) using an average value of the values we obtained for the predominant archaea *Methanobrevibacter ruminantium* and *Methanobrevibacter smithii* (Muñoz-Tamayo *et al.* 2019) using a microbial yield factor of 0.006 mol biomass/mol  $\text{H}_2$  (Pavlostathis *et al.* 1990). With this strategy, we penalize the goodness-of-fit of the model. But, on the other hand, we reduce practical identifiability problems typically found when calibrating biological kinetic-based models (Vanrolleghem *et al.* 1995). The parameter vector for the estimation is then  $\mathbf{p}: \{k_{\text{hydr,nsC}}, k_{\text{hydr,pro}}, k_{\text{br}}, p_1, p_2, \dots, p_9\}$ . The optimization was set in a multi-experiment fitting context that integrates the data of all treatments. To evaluate the model performance, we computed the determination coefficient ( $R^2$ ), the Lin's concordance correlation coefficient (CCC) (Lin 1989), the Root mean squared error (RMSE) and the coefficient of variation of the RMSE ( $\text{CV}_{\text{RMSE}}$ ). We also performed residual analysis for bias assessment according to (St-Pierre 2003).

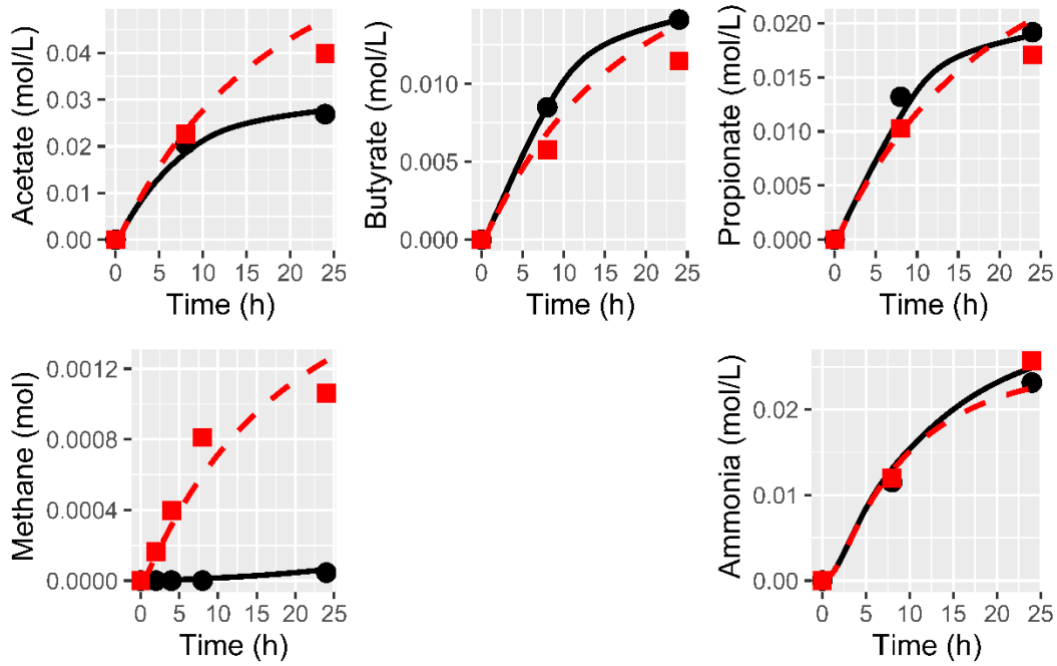
## 3. Results

### Dynamic prediction of rumen fermentation

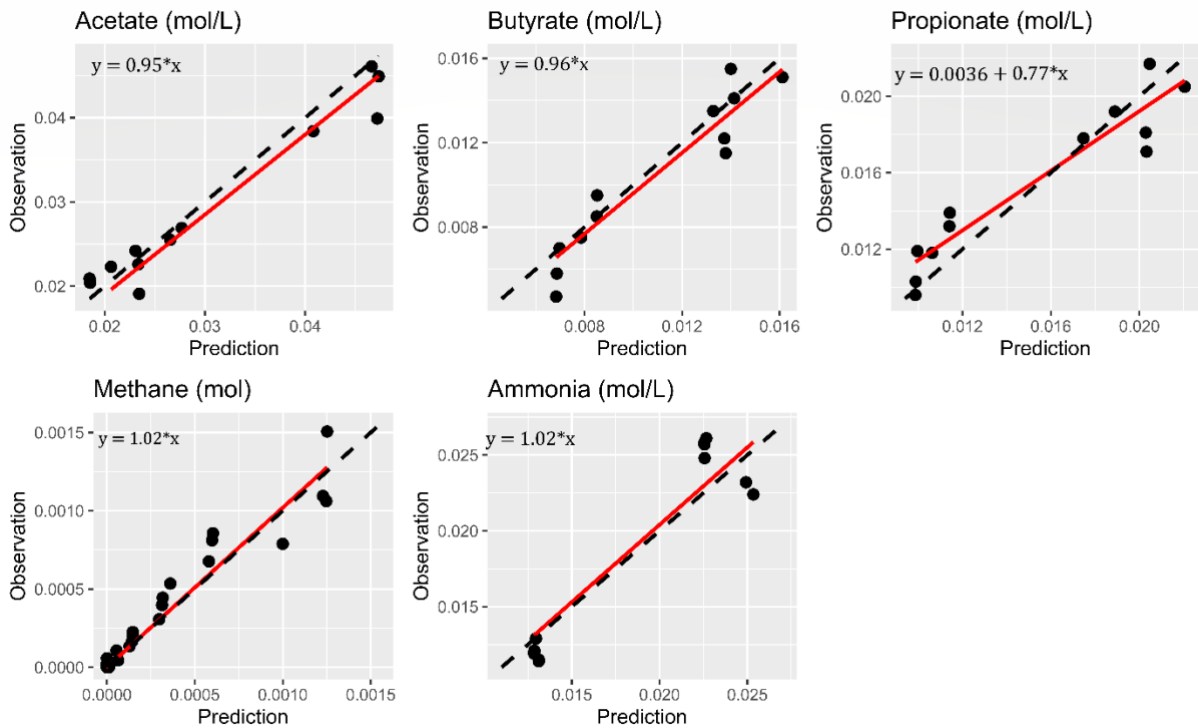
The extended model developed in the present work to account for the impact of *A. taxiformis* on the rumen fermentation is freely available at <https://doi.org/10.5281/zenodo.4090332> with all the detailed information of the model and the experimental data used for model calibration. An open source version in the Scilab software (<https://www.scilab.org/>) was made available to facilitate reproducibility since Scilab files can be opened with a text editor. The software R (<https://www.r-project.org/>) was used to plot the figures.

Figure 2 shows the dynamic data of fermentation variables for the levels of *A. taxiformis* at 0.06% and 0.5% compared against the model predicted variables. Figure 3 displays the comparison of all observations against model predictions. Figure 4 shows the residuals for all variables against centred predicted values.

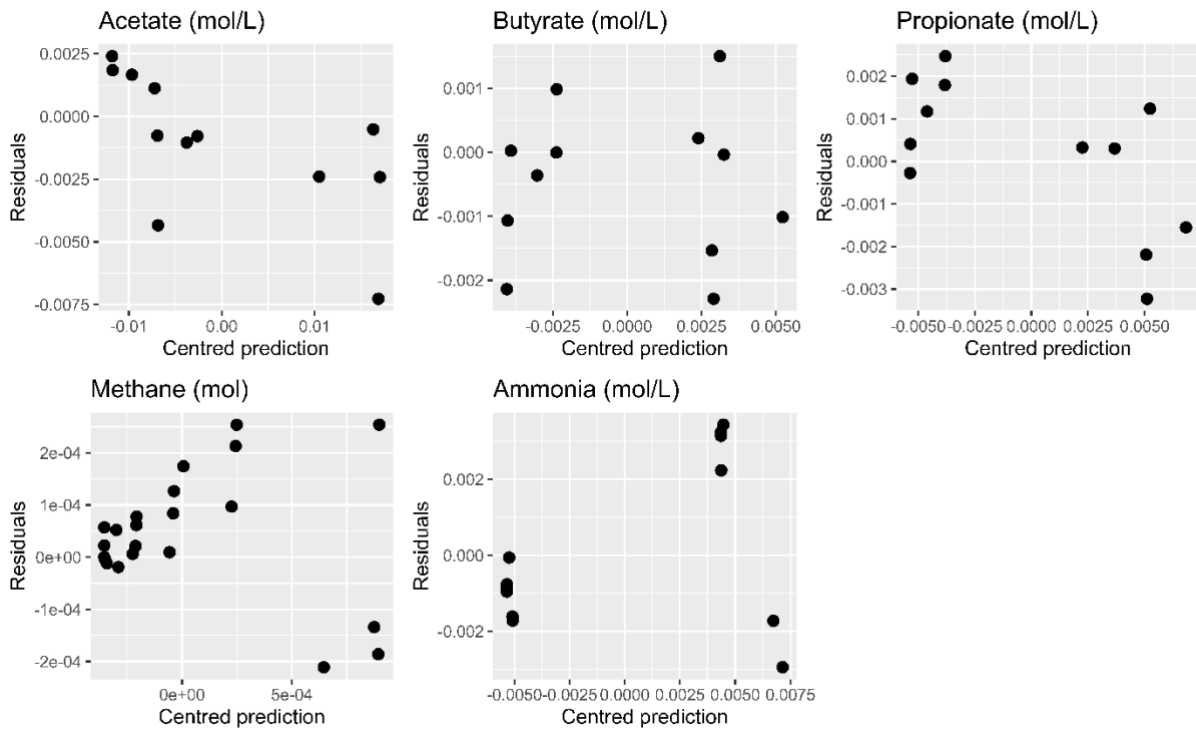
To evaluate the performance of our model and its validation, external independent data is required. Due to data limitation, we did not perform such a validation. To provide indicators of our model, we calculated standard statistical indicators of model performance which are shown in Table 2. These statistic indicators are biased and thus should be looked with caution since they are calculated using the calibration data. Nevertheless, they provide an indication of the adequacy of the model structure to represent the fermentation dynamics. For methane, butyrate and  $\text{NH}_3$  the mean and linear biases were not significant at the 5% significance level. Acetate and propionate exhibited significant linear bias. The liquid compounds have an average coefficient of variation of the RMSE ( $\text{CV}(\text{RMSE})$ ) of 11.25%. Methane had the higher  $\text{CV}(\text{RMSE})$  (31%). The concordance correlation coefficients were higher than 0.93. Propionate had the lowest determination coefficient ( $R^2=0.82$ ) while methane and the other compounds had a  $R^2$  close to 0.9.



**Figure 2.** Example of model fitting. Experimental data of fermentation variables for the levels of *A. taxiformis* at 0.5% (●) and 0.06% (■) are compared against the model predicted responses in solid black lines (for 0.5% level) and in dashed red lines (for the 0.06% level).



**Figure 3.** Summary of the model performance calibration integrating data of all treatments. Experimental data (●) are plotted against the model predicted variables. Solid lines are the linear fitted curve. Dashed lines are the isoclines. Only the intercept of the curve of propionate was different from zero at 5% significance level.



**Figure 4.** Residuals values of observed variables against centered predicted variables ( $n_{\text{CH}_4} = 24$ ,  $n_{\text{NH}_3} = n_{\text{ac}} = n_{\text{bu}} = n_{\text{pr}} = 12$ ).

**Table 2.** Statistical indicators of model performance.

	Acetate	Butyrate	Propionate	Methane	NH <sub>3</sub>
R <sup>2</sup>	0.91	0.88	0.82	0.92	0.89
RMSE <sup>a</sup>	0.0029	0.0012	0.0017	1.21x10 <sup>-4</sup>	0.002
100×CV <sub>RMSE</sub> <sup>b</sup>	10	12	11	31	12
CCC <sup>c</sup>	0.96	0.94	0.93	0.96	0.93
Residual analysis					
$residual = \alpha + \beta \cdot (predicted - mean\ predicted\ value)$					
	Acetate	Butyrate	Propionate	Methane	NH <sub>3</sub>
$\alpha$ (p-value)	-0.0010 (p= 0.14)	-0.00047 (p= 0.21)	0.00019 (p= 0.63)	4.0e-05 (p= 0.12)	0.00012 (p= 0.86)
$\beta$ (p-value)	-0.15 (p= 0.024)	0.0028 (p= 0.98)	-0.22 (p= 0.024)	-0.031 (p= 0.60)	0.15 (p= 0.23)

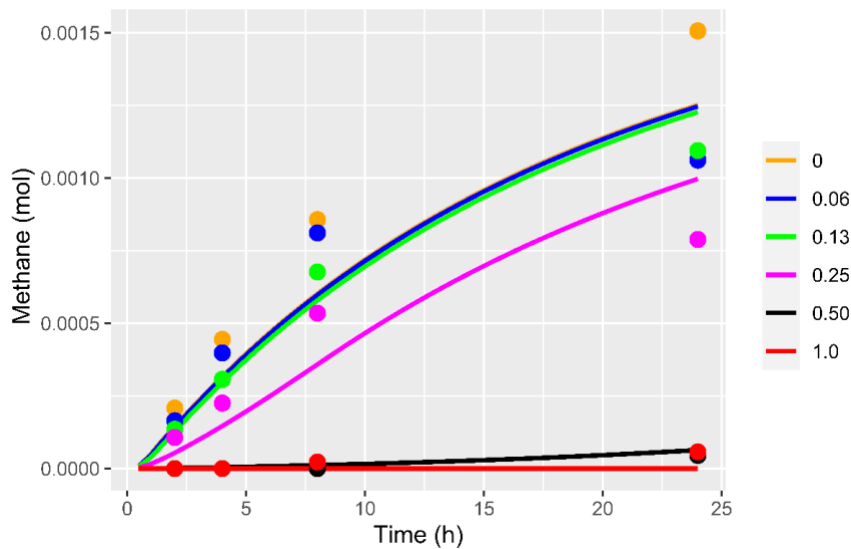
<sup>a</sup> Root mean squared error (RMSE).

<sup>b</sup> Coefficient of variation of the RMSE (CV(RMSE)).

<sup>c</sup> Concordance correlation coefficient (CCC)

Figure 5 compares specifically the experimental data of methane against the model predictions for all levels of *A. taxiformis*.

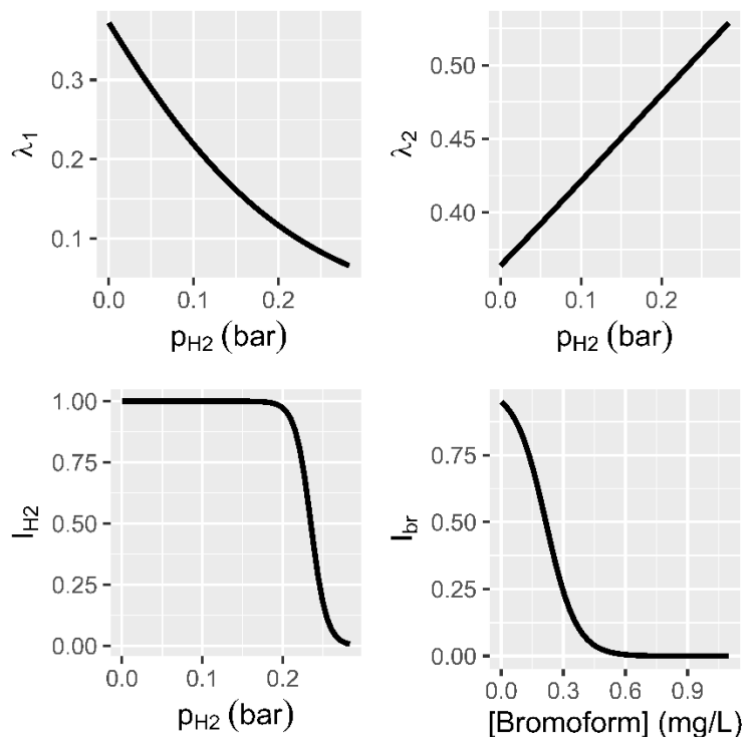




**Figure 5.** Experimental observations of methane (circles) in the headspace of the incubation system are compared against the model predicted values (solid lines). Increase of the dose of *A. taxiformis* results in a decrease of methane production.

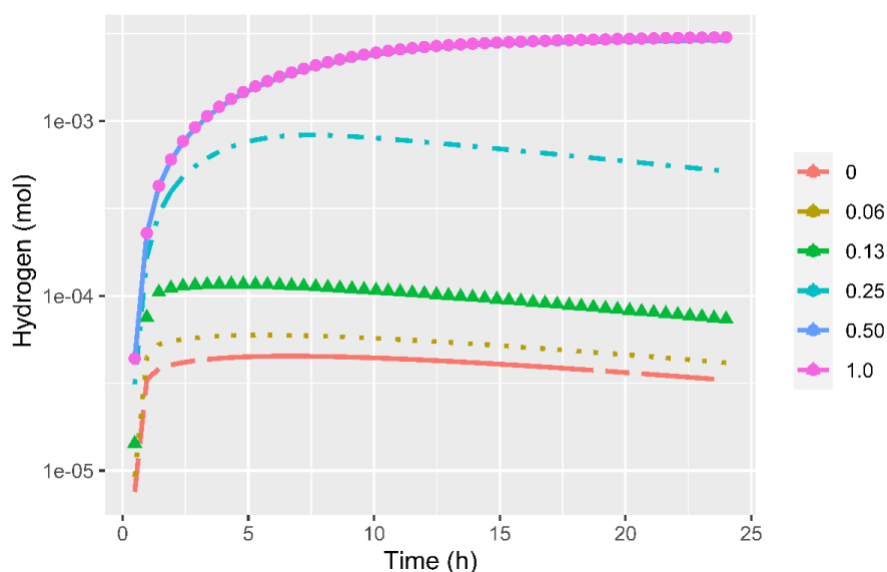
**Prediction of the factors representing the impact of *A. taxiformis* on rumen fermentation**

Figure 6 plots the factors that represent the effect of *A. taxiformis* on rumen fermentation. Direct inhibition of the methanogenesis due to the anti-methanogenic action of bromoform is represented by the factor  $I_{br}$ . Methanogenesis inhibition results in hydrogen accumulation impacting the flux allocation of sugars utilization.



**Figure 6.** In our model, the effect of *A. taxiformis* on rumen fermentation is represented by a direct inhibitory effect of bromoform ( $I_{br}$ ) on the methanogens growth rate. Methanogenesis inhibition results in hydrogen accumulation. Hydrogen control impacts sugar utilization by inhibiting the rate of sugar utilization (factor  $I_{H_2}$ ) and by regulating the flux allocation parameters ( $\lambda_1, \lambda_2$ ) towards VFA production.

Figure 7 displays the simulated dynamics of hydrogen in the headspace for all the supplementation levels of *A. taxiformis*. For supplementation levels higher than 0.25%, the methanogenesis inhibition resulted in a substantial hydrogen accumulation.



**Figure 7.** Predicted dynamics of hydrogen in the headspace for levels of *A. taxiformis*. Increase of the dose of *A. taxiformis* results in an increase of hydrogen in the incubation system.

#### 4. Discussion

The goal of this work was to model the impact of *A. taxiformis* supplementation on the rumen microbial fermentation and methane production under *in vitro* conditions using experimental data from (Chagas *et al.* 2019). Overall, our model was able to capture the dynamics of VFA, ammonia and methane production for different levels of *A. taxiformis* indicating the potential of the model structure towards the development of predictive models for assessing methane mitigation strategies in ruminants. With the exception of propionate, the slope of observed vs predicted variables is very close to one. Model limitations will be discussed further. We modelled the effect of *A. taxiformis* on rumen fermentation by two mechanisms. The first mechanism is associated to the direct inhibition of the methanogens growth rate by the anti-methanogenic compounds of *A. taxiformis* documented in different studies (Kinley *et al.*, 2016; Machado *et al.*, 2016a; Roque *et al.*, 2019). In our model, we ascribed the inhibitory effect of *A. taxiformis* only to the concentration of bromoform. The first-order kinetic rate for bromoform consumption and the inhibition factor ( $I_{br}$ ) (Fig. 6) allowed our model to account for the observed dynamic decline in methanogenesis inhibition (Kinley *et al.* 2016). It should be noted that although bromoform is the most abundant anti-methanogenic compound in *A. taxiformis*, the anti-methanogenic capacity of *A. taxiformis* is the result of the synergetic action of all halogenated products present in the macroalgae (Machado *et al.* 2016a). Accordingly, it will be useful to include further in our model other secondary compounds such as dibromochloromethane. To enhance our model, it will be central to perform new experiments to characterize the dynamics of anti-methanogenic compounds. This aspect is of great relevance to allow the model to be adapted to different applications of seaweed supplementation since it is known that the composition of halogenic compounds can vary with respect to the season, harvesting and drying methods.

The second mechanism that accounts for the impact of *A. taxiformis* on the fermentation is hydrogen control, which it is discussed below.

##### Methane inhibition and hydrogen control

The anti-methanogenic capacity of *A. taxiformis* is dose-dependent. The experimental study of (Chagas *et al.* 2019) showed that methane production was inhibited almost completely by *A. taxiformis* at a level of 0.5%. Our model predictions aligned with the experimental observations (Fig. 5). The anti-methanogenic

capacity of the macroalgae leads to hydrogen accumulation (Kinley *et al.* 2020; Roque *et al.* 2021) as predicted by our model in Fig. 7. The level of hydrogen increases as the dose of *A. taxiformis* increases. The predicted values of hydrogen levels in the headspace for low doses of *A. taxiformis* showing in Figure 7 are in agreement with *in vitro* reported values (Serment *et al.*, 2016). The level of hydrogen can impact electron-mediating cofactors such as nicotinamide adenine dinucleotide (NAD) which are important drivers of anaerobic metabolism *via* the transfer of electrons in metabolic redox reactions (Hoelzle *et al.*, 2014). van Lingen *et al.*, 2019 extended the rumen model developed by (Dijkstra *et al.* 1992) to incorporate the regulation of NADH/NAD<sup>+</sup> on the fermentation. In our model, the regulation of NADH/NAD<sup>+</sup> was incorporated *via* the control of hydrogen partial pressure assuming a linearity between the couple NADH/NAD<sup>+</sup> and the  $p_{H_2}$  and following the model structure proposed by (Mosey, 1983) with a different parameterisation for the functions describing the effect of  $p_{H_2}$  on the rate of glucose utilization and on the flux allocation. The linearity assumption between NADH/NAD<sup>+</sup> and the  $p_{H_2}$  might not be fulfilled for all values of  $p_{H_2}$  (De Kok *et al.*, 2013). In the experimental conditions used in the experiment here analysed (Chagas *et al.* 2019) and under rumen physiological conditions, the linearity between NADH/NAD<sup>+</sup> might be valid.

With regard to the hydrogen control on glucose utilization, our model predicts that the inhibition is effective at  $p_{H_2}$  higher than 0.2 bar (factor  $I_{H_2}$  in Fig. 6). In our model, the incorporation of the inhibitory effect of hydrogen was motivated to account for the decrease of the total production of VFA at high levels of supplementation of *A. taxiformis* observed by (Chagas *et al.* 2019). Such a decrease of VFA production is dose-dependent as observed in *in vitro* studies (Kinley *et al.* 2016; Machado *et al.* 2016b). *In vivo*, while insignificant changes in total VFA concentration between a control diet and diets with *A. taxiformis* supplementation were observed in Brangus steers (Kinley *et al.* 2020), inclusions of *A. taxiformis* resulted in a decrease in total VFA ruminal concentration in sheep compared with control diet (Li *et al.* 2016). Accordingly, additional studies with simultaneous measurements of VFA and hydrogen are needed to validate the relevance of the inhibitory term  $I_{H_2}$  of our model both under *in vitro* and *in vivo* conditions.

In addition to the impact of *A. taxiformis* supplementation on methane reduction, it is important to look at the effects on animal productivity. *A. taxiformis* impacts the production of VFAs, which are energy sources for the animal. Accordingly, changes in VFA production might result in changes on productivity and feed efficiency. Optimal feeding strategies should thus be designed to attain a trade-off between low methane emissions and high productivity and animal health. Studies showing the effect of *A. taxiformis* supplementation on live weight (Li *et al.* 2016), average daily weight gain and feed conversion efficiency (Kinley *et al.* 2020; Roque *et al.* 2021) are still scarce to provide a large data base for concluding on the impact of *A. taxiformis* on animal productivity and feed efficiency. However, the studies of (Kinley *et al.* 2020; Roque *et al.* 2021) suggest that feed conversion efficiency tend to increase concomitantly with the reduction of methane production induced by an adequate supplementation of *A. taxiformis*, supporting the theory of redirection of energy otherwise lost as methane (Kinley *et al.* 2020). An opportunity to enhance the action of *A. taxiformis* might be the implementation of a feeding strategy integrating macroalgae supplementation with an adequate additive allowing to redirect metabolic hydrogen towards nutritional fermentation products beneficial to the animal. Such a strategy will fulfil the objectives of reducing methane emissions while increasing animal productivity (Ungerfeld 2020).

With regard to the fermentation pattern, when the hydrogen level increases the hydrogen control operates by increasing the flux of carbon towards propionate ( $\lambda_2$ ) while the flux towards the reaction that produces only acetate ( $\lambda_1$ ) decreases (Fig. 6). Incorporating hydrogen control on the fermentation pattern in our model enabled us to predict the decrease of the acetate to propionate ratio observed at levels of *A. taxiformis* supplementation leading to substantial methane reduction both *in vitro* (Machado *et al.* 2016b; Chagas *et al.* 2019) and *in vivo* (Kinley *et al.* 2020). Our model is also consistent with *in vitro* (Kinley *et al.* 2016; Machado *et al.* 2016b) and *in vivo* (Stefenoni *et al.* 2021) studies showing the increase of butyrate level when the inclusion of *A. taxiformis* increases.

### Model limitations and perspectives

In our model, the quantification of the impact of *A. taxiformis* was ascribed by the action of bromoform on the methanogens growth rate and by the action of  $p_{H_2}$  on the fermentation pattern. However, in the experimental study of (Chagas *et al.* 2019), nor bromoform nor  $p_{H_2}$  were measured. From our bibliography

search, we did not find studies reporting dynamic measurements of bromoform. Although we did not perform an identifiability analysis, we might expect that the lack of bromoform and hydrogen data in our work might result in structural identifiability (Muñoz-Tamayo *et al.* 2018) and model distinguishability problems (Walter, Pronzato 1996). We will then require external data to validate our model. Experiments to be done within the MASTER project (<https://www.master-h2020.eu/contact.html>) will fill this gap and provide data for challenging and improving our model.

Our model aligns with the efforts of enhancing the dynamic prediction of ruminal metabolism *via* the incorporation of thermodynamics and regulation factors (Offner, Sauvant 2006; Ghimire *et al.* 2014; van Lingen *et al.* 2019). While our work focused only on hydrogen control on sugars metabolism, future work is needed to incorporate the impact of *A. taxiformis* supplementation on amino acids fermentation. The study of (Chagas *et al.* 2019) showed a decrease of branched-chain volatile fatty acids (BCVFA) with increased supplementation of *A. taxiformis*. Such a decrease of BCVFA might have a negative influence on microbial activity.

We modelled the regulation of sugars metabolism by hydrogen control following a grey-box modelling approach where the regulation factors were assigned to sigmoid functions without an explicit mechanistic interpretation. However, to enhance the understanding of rumen fermentation, it will be useful to pursue an approach incorporating the role of internal electron mediating cofactors on the direction of electrons towards hydrogen or VFA (Hoelzle *et al.* 2014; Ungerfeld 2020). Recent progress in this area (van Lingen *et al.* 2019) opens a direction for improving the prediction of rumen models.

The ultimate goal of this work is to pursue a model extension to account for *in vivo* conditions. In this endeavour, experimental data in semi-continuous devices such as the Rusitec (Roque *et al.*, 2019a) will be instrumental for model improvement. *In vivo*, in addition to the impact on fermentation, *A. taxiformis* can induce changes in rumen mucosa (Li *et al.* 2016). These mucosa changes might translate in changes on the rate of absorption of ruminal VFA. This effect on the rate of VFA absorption should be quantified and incorporated into an extended model. In our model, the pH was set constant. However, pH exhibits a dynamic behaviour that can impact the activity of the rumen microbiota. The impact of the pH on the rumen microbial groups should be then considered in a future version of the model, integrating the mechanistic calculation of pH elaborated in our previous model (Muñoz-Tamayo *et al.*, 2016).

Finally, although our model developments focused on the impact of *A. taxiformis* on rumen fermentation and methane production, we think our model structure has the potential to be applied to other additives such as 3-nitrooxypropanol (Hristov *et al.* 2015; Duin *et al.* 2016) whose action is specifically directed to inhibit methanogenic archaea, as the halogenated compounds of *A. taxiformis*. We expect these model developments can be useful to help the design of sustainable nutritional strategies promoting healthy rumen function and low environmental footprint.

## 5. Conclusions

We have developed a rumen fermentation model that accounts for the impact of *A. taxiformis* supply on *in vitro* rumen fermentation and methane production. Our model was effective in representing the dynamics of VFA, ammonia and methane for six supplementation levels of *A. taxiformis*, providing a promising prediction tool for assessing the impact of additives such as seaweeds on rumen microbial fermentation and methane production *in vitro*.

## Acknowledgements

Authors thank Henk van Lingen (Wageningen University, The Netherlands), Alberto Atzori (University of Sassari, Italy) and two anonymous reviewers appointed by Luis Tedeschi (Texas A&M University, USA) for the evaluation of a previous version of this manuscript by PCI Animal Science <https://doi.org/10.24072/pci.animsci.100006>.

## Funding

Authors acknowledge funding from the RumenPredict project funded by the Horizon2020 Research & Innovation Programme under grant agreement No 696356. Rafael Muñoz-Tamayo acknowledges funding from the MASTER project, an Innovation Action funded by the European Union's Horizon 2020 research and innovation programme under grant agreement No 818368.

## Conflict of interest disclosure

The authors of this manuscript declare that they have no financial conflict of interest with the content of this article. Rafael Muñoz-Tamayo is recommender of PCI Animal Science.

## Data, script and code availability

The datasets and scripts used in this study are available at <https://doi.org/10.5281/zenodo.4090332>

## Authors' contribution

JCC, MH and SJ produced the experimental data of the study. RMT developed the mathematical model and drafted the article. All authors contributed to the analysis and interpretation of the results. All authors read and approved the final manuscript.

## References

- Beauchemin KA, Ungerfeld EM, Eckard RJ, Wang M (2020) Review: Fifty years of research on rumen methanogenesis: Lessons learned and future challenges for mitigation. *Animal*, **14**(S1), S2–S16. <https://doi.org/10.1017/S1751731119003100>
- Chagas JC, Ramin M, Krizsan SJ (2019) In vitro evaluation of different dietary methane mitigation strategies. *Animals*, **9**, 1120. <https://doi.org/10.3390/ani9121120>
- Chalupa W (1977) Manipulating Rumen Fermentation. *Journal of Animal Science*, **46**, 585–599. <https://doi.org/10.2527/jas1977.453585x>
- Costello DJ, Greenfield PF, Lee PL (1991) Dynamic Modeling of a Single-Stage High-Rate Anaerobic Reactor- I. Model Derivation. *Water Res.*, **25**, 847–858. [https://doi.org/10.1016/0043-1354\(91\)90166-N](https://doi.org/10.1016/0043-1354(91)90166-N)
- Czerkawski JW, Breckenridge G (1975) New inhibitors of methane production by rumen micro-organisms. Development and testing of inhibitors in vitro. *British Journal of Nutrition*, **34**, 429–446. <https://doi.org/10.1017/s0007114575000499>
- Denman SE, Tomkins NW, McSweeney CS (2007) Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiology Ecology*, **62**, 313–322. <https://doi.org/10.1111/j.1574-6941.2007.00394.x>
- Dijkstra J, Neal HD, Beever DE, France J (1992) Simulation of nutrient digestion, absorption and outflow in the rumen: model description. *J. Nutr.*, **122**, 2239–2256. <https://doi.org/10.1093/jn/122.11.2239>
- Dubois B, Tomkins NW, D. Kinley R, Bai M, Seymour S, A. Paul N, Nys R de (2013) Effect of Tropical Algae as Additives on Rumen *in Vitro* Gas Production and Fermentation Characteristics. *American Journal of Plant Sciences*, **4**, 34–43. <https://doi.org/10.4236/ajps.2013.412a2005>
- Duin EC, Wagner T, Shima S, Prakash D, Cronin B, Yáñez-Ruiz DR, Duval S, Rumbeli R, Stemmler RT, Thauer RK, Kindermann M (2016) Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. *Proc Natl Acad Sci U S A*, **113**, 6172–6177. <https://doi.org/10.1073/pnas.1600298113>
- Egea JA, Henriques D, Cokelaer T, Villaverde AF, MacNamara A, Danciu DP, Banga JR, Saez-Rodriguez J (2014) MEIGO: An open-source software suite based on metaheuristics for global optimization in systems biology and bioinformatics. *BMC Bioinformatics*, **15**, 136. <https://doi.org/10.1186/1471-2105-15-136>



- Egea JA, Martí R, Banga JR (2010) An evolutionary method for complex-process optimization. *Computers and Operations Research*, **37**, 315–324. <https://doi.org/10.1016/j.cor.2009.05.003>
- Ellis JL, Dijkstra J, France J, Parsons AJ, Edwards GR, Rasmussen S, Kebreab E, Bannink A (2012) Effect of high-sugar grasses on methane emissions simulated using a dynamic model. *Journal of Dairy Science*, **95**, 272–285. <https://doi.org/10.3168/jds.2011-4385>
- Evans FD, Critchley AT (2014) Seaweeds for animal production use. *Journal of Applied Phycology*, **26**, 891–899. <https://doi.org/10.1007/s10811-013-0162-9>
- Ghimire S, Gregorini P, Hanigan MD (2014) Evaluation of predictions of volatile fatty acid production rates by the Molly cow model. *Journal of Dairy Science*, **97**, 354–362. <https://doi.org/10.3168/jds.2012-6199>
- Hoelzle RD, Viridis B, Batstone DJ (2014) Regulation mechanisms in mixed and pure culture microbial fermentation. *Biotechnology and Bioengineering*, **111**, 2139–2154. <https://doi.org/10.1002/bit.25321>
- Hristov AN, Oh J, Giallongo F, Frederick TW, Harper MT, Weeks HL, Branco AF, Moate PJ, Deighton MH, Williams SRO, Kindermann M, Duval S (2015) An inhibitor persistently decreased enteric methane emission from dairy cows with no negative effect on milk production. *Proc Natl Acad Sci U S A*, **112**, 10663–10668. <https://doi.org/10.1073/pnas.1515515112>
- Huws SA, Creevey CJ, Oyama LB, Mizrahi I, Denman SE, Popova M, Muñoz-Tamayo R, Forano E, Waters SM, Hess M, Tapio I, Smidt H, Krizsan SJ, Yáñez-Ruiz DR, Belanche A, Guan L, Gruninger RJ, McAllister TA, Newbold CJ, Roehe R, Dewhurst RJ, Snelling TJ, Watson M, Suen G, Hart EH, Kingston-Smith AH, Scollan ND, do Prado RM, Pilau EJ, Mantovani HC, Attwood GT, Edwards JE, McEwan NR, Morrisson S, Mayorga OL, Elliott C, Morgavi DP (2018) Addressing global ruminant agricultural challenges through understanding the rumen microbiome: past, present, and future. *Frontiers in Microbiology*, **9**, 2161. <https://doi.org/10.3389/fmicb.2018.02161>
- Janssen PH (2010) Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology*, **160**, 1–22. <https://doi.org/10.1016/j.anifeedsci.2010.07.002>
- Kettle H, Holtrop G, Louis P, Flint HJ (2018) microPop: Modelling microbial populations and communities in R. *Methods in Ecology and Evolution*, **9**, 399–409. <https://doi.org/10.1111/2041-210X.12873>
- Kinley RD, Martinez-Fernandez G, Matthews MK, de Nys R, Magnusson M, Tomkins NW (2020) Mitigating the carbon footprint and improving productivity of ruminant livestock agriculture using a red seaweed. *Journal of Cleaner Production*, **259**, 120836. <https://doi.org/10.1016/j.jclepro.2020.120836>
- Kinley RD, De Nys R, Vucko MJ, MacHado L, Tomkins NW (2016) The red macroalgae *Asparagopsis taxiformis* is a potent natural antimethanogenic that reduces methane production during in vitro fermentation with rumen fluid. *Animal Production Science*, **56**, 282–289. <https://doi.org/10.1071/AN15576>
- Li X, Norman HC, Kinley RD, Laurence M, Wilmot M, Bender H, De Nys R, Tomkins N (2016) *Asparagopsis taxiformis* decreases enteric methane production from sheep. *Animal Production Science*, **58**, 681–688. <https://doi.org/10.1071/AN15883>
- Lin LI (1989) A concordance correlation-coefficient to evaluate reproducibility. *Biometrics*, **45**, 255–268. <https://doi.org/10.2307/2532051>
- van Lingen HJ, Fadel JG, Moraes LE, Bannink A, Dijkstra J (2019) Bayesian mechanistic modeling of thermodynamically controlled volatile fatty acid, hydrogen and methane production in the bovine rumen. *Journal of Theoretical Biology*, **480**, 150–165. <https://doi.org/10.1016/j.jtbi.2019.08.008>
- Machado L, Magnusson M, Paul NA, Kinley R, de Nys R, Tomkins N (2016a) Identification of bioactives from the red seaweed *Asparagopsis taxiformis* that promote antimethanogenic activity in vitro. *Journal of Applied Phycology*, **28**, 3117–3126. <https://doi.org/10.1007/s10811-016-0830-7>
- Machado L, Magnusson M, Paul NA, Kinley R, de Nys R, Tomkins N (2016b) Dose-response effects of *Asparagopsis taxiformis* and *Oedogonium* sp. on in vitro fermentation and methane production. *Journal of Applied Phycology*, **28**, 1443–1452. <https://doi.org/10.1007/s10811-015-0639-9>
- Machado L, Magnusson M, Paul NA, De Nys R, Tomkins N (2014) Effects of marine and freshwater macroalgae on in vitro total gas and methane production. *PLoS ONE*, **9**, e85289. <https://doi.org/10.1371/journal.pone.0085289>
- Machado L, Tomkins N, Magnusson M, Midgley DJ, de Nys R, Rosewarne CP (2018) In Vitro Response of Rumen Microbiota to the Antimethanogenic Red Macroalga *Asparagopsis taxiformis*. *Microbial Ecology*, **75**, 811–818. <https://doi.org/10.1007/s00248-017-1086-8>



- Maia MRG, Fonseca AJM, Oliveira HM, Mendonça C, Cabrita ARJ (2016) The potential role of seaweeds in the natural manipulation of rumen fermentation and methane production. *Scientific Reports*, **6**, 32321. <https://doi.org/10.1038/srep32321>
- Makkar HPS, Tran G, Heuzé V, Giger-Reverdin S, Lessire M, Lebas F, Ankers P (2016) Seaweeds for livestock diets: A review. *Animal Feed Science and Technology*, **212**, 1–17. <https://doi.org/10.1016/j.anifeedsci.2015.09.018>
- Mosey FE (1983) Mathematical-Modeling of the Anaerobic-Digestion Process - Regulatory Mechanisms for the Formation of Short-Chain Volatile Acids from Glucose. *Water Science and Technology*, **15**, 209–232. <https://doi.org/10.2166/wst.1983.0168>
- Muñoz-Tamayo R, Giger-Reverdin S, Sauvant D (2016) Mechanistic modelling of in vitro fermentation and methane production by rumen microbiota. *Animal Feed Science and Technology*, **220**, 1–21. <https://doi.org/10.1016/j.anifeedsci.2016.07.005>
- Muñoz-Tamayo R, Laroche B, Leclerc M, Walter E (2009) IDEAS: A parameter identification toolbox with symbolic analysis of uncertainty and its application to biological modelling. In: *IFAC Proceedings Volumes*, pp. 1271–1276. <https://doi.org/10.3182/20090706-3-FR-2004.0211>
- Muñoz-Tamayo R, Popova M, Tillier M, Morgavi DP, Morel JP, Fonty G, Morel-Desrosiers N (2019) Hydrogenotrophic methanogens of the mammalian gut: Functionally similar, thermodynamically different—A modelling approach. *PLoS ONE*, **14**, e0226243. <https://doi.org/10.1371/journal.pone.0226243>
- Muñoz-Tamayo R, Puillet L, Daniel JB, Sauvant D, Martin O, Taghipoor M, Blavy P (2018) Review: To be or not to be an identifiable model. Is this a relevant question in animal science modelling? *Animal*, **12**, 701–712. <https://doi.org/10.1017/S1751731117002774>
- Offner A, Sauvant D (2006) Thermodynamic modeling of ruminal fermentations. *Animal Feed Science and Technology*, **155**, 343–365. <https://doi.org/10.1051/animres:2006021>
- Paul NA, De Nys R, Steinberg PD (2006) Chemical defence against bacteria in the red alga *Asparagopsis armata*: Linking structure with function. *Marine Ecology Progress Series*, **306**, 87–101. <https://doi.org/10.3354/meps306087>
- Pavlostathis SG, Miller TL, Wolin MJ (1990) Cellulose Fermentation by Continuous Cultures of *Ruminococcus-Albus* and *Methanobrevibacter-Smithii*. *Applied Microbiology and Biotechnology*, **33**, 109–116. <https://doi.org/10.1007/BF00170581>
- Puhakka L, Jaakkola S, Simpura I, Kokkonen T, Vanhatalo A (2016) Effects of replacing rapeseed meal with fava bean at 2 concentrate crude protein levels on feed intake, nutrient digestion, and milk production in cows fed grass silage-based diets. *Journal of Dairy Science*, **99**, 7993–8006. <https://doi.org/10.3168/jds.2016-10925>
- Ramin M, Huhtanen P (2012) Development of an in vitro method for determination of methane production kinetics using a fully automated in vitro gas system-A modelling approach. *Animal Feed Science and Technology*, **174**, 190–200. <https://doi.org/10.1016/j.anifeedsci.2012.03.008>
- Roque BM, Brooke CG, Ladau J, Polley T, Marsh LJ, Najafi N, Pandey P, Singh L, Kinley R, Salwen JK, Eloefadros E, Kebreab E, Hess M (2019) Effect of the macroalgae *Asparagopsis taxiformis* on methane production and rumen microbiome assemblage. *Animal Microbiome*, **1**:3. <https://doi.org/10.1186/s42523-019-0004-4>
- Roque BM, Salwen JK, Kinley R, Kebreab E (2019) Inclusion of *Asparagopsis armata* in lactating dairy cows' diet reduces enteric methane emission by over 50 percent. *Journal of Cleaner Production*, **234**, 132–138. <https://doi.org/10.1016/j.jclepro.2019.06.193>
- Roque BM, Venegas M, Kinley RD, Nys R de, Duarte TL, Yang X, Kebreab E (2021) Red seaweed (*Asparagopsis taxiformis*) supplementation reduces enteric methane by over 80 percent in beef steers. *PLoS ONE*, **16**, e0247820. <https://doi.org/10.1371/JOURNAL.PONE.0247820>
- St-Pierre NR (2003) Reassessment of Biases in Predicted Nitrogen Flows to the Duodenum by NRC 2001. *Journal of Dairy Science*, **86**, 344–350. [https://doi.org/10.3168/jds.S0022-0302\(03\)73612-1](https://doi.org/10.3168/jds.S0022-0302(03)73612-1)
- Stefenoni HA, Räisänen SE, Cueva SF, Wasson DE, Lage CFA, Melgar A, Fetter ME, Smith P, Hennessy M, Vecchiarelli B, Bender J, Pitta D, Cantrell CL, Yarish C, Hristov AN (2021) Effects of the macroalga *Asparagopsis taxiformis* and oregano leaves on methane emission, rumen fermentation, and lactational performance of dairy cows. *Journal of Dairy Science*. <https://doi.org/10.3168/jds.2020-19686>

- Ungerfeld EM (2020) Metabolic Hydrogen Flows in Rumen Fermentation: Principles and Possibilities of Interventions. *Frontiers in Microbiology*, **11**, 589. <https://doi.org/10.3389/fmicb.2020.00589>
- Vanrolleghem PA, Vandaele M, Dochain D (1995) Practical identifiability of a biokinetic model of activated-sludge respiration. *Water Research*, **29**, 2561–2570. [https://doi.org/10.1016/0043-1354\(95\)00105-T](https://doi.org/10.1016/0043-1354(95)00105-T)
- Walter E, Pronzato L (1996) On the identifiability and distinguishability of nonlinear parametric models. *Mathematics and Computers in Simulation*, **42**, 125–134. [https://doi.org/10.1016/0378-4754\(95\)00123-9](https://doi.org/10.1016/0378-4754(95)00123-9)
- Wang Y, Xu Z, Bach SJ, McAllister TA (2008) Effects of phlorotannins from *Ascophyllum nodosum* (brown seaweed) on in vitro ruminal digestion of mixed forage or barley grain. *Animal Feed Science and Technology*, **145**, 375–395. <https://doi.org/10.1016/j.anifeedsci.2007.03.013>
- Wood JM, Kennedy FS, Wolfe RS (1968) The Reaction of Multihalogenated Hydrocarbons with Free and Bound Reduced Vitamin B12. *Biochemistry*, **7**, 1707–1713. <https://doi.org/10.1021/bi00845a013>