

A theoretical optimal experiment for accurate parameter estimation of a dynamic rumen model representing the *Asparagopsis taxiformis* effect on *in vitro* methane production

R. Muñoz-Tamayo ^a, M. Eugène ^b

^a *Université Paris-Saclay, INRAE, AgroParisTech, UMR Modélisation Systémique Appliquée aux Ruminants, 91120, Palaiseau, France*

^b *INRAE, ASSET, Centre Antilles-Guyane, Domaine Duclos, Prise d'Eau, 97170 Petit-Bourg, Guadeloupe, France*

Corresponding author: Rafael Muñoz-Tamayo. Email: Rafael.munoz-tamayo@inrae.fr

Abstract

Reducing methane (CH₄) emissions from ruminants is a major target for the livestock sector. Synergy between *in vitro* studies and mathematical models can be useful to provide high quality data enabling to quantify the mode of action of anti-methanogenic additives and thus design robust mitigation solutions. The objective of this work was to conduct a theoretical optimal experiment design (OED) to determining optimal experimental conditions allowing an accurate estimation of key parameters of a model we previously developed representing the impact of the anti-methanogenic macroalgae *Asparagopsis taxiformis* on rumen fermentation and CH₄ under *in vitro* continuous conditions. *A. taxiformis* contains the anti-methanogenic compound bromoform which inhibits directly the growth rate of methanogens. CH₄ inhibition results in hydrogen (H₂) accumulation. H₂ exerts thermodynamic control on

the fermentation *via* the flux allocation towards volatile fatty acids production. We applied the OED framework to 5 model parameters related to the mode of action of *A. taxiformis*: k_1 (inhibitor bromoform constant) and p_1, p_2, p_3, p_4 (parameters of affine functions representing flux allocation factors). After conducting a structural parameter identifiability analysis to guarantee that the parameter identification problem was well-posed, we set the OED problem for parameter estimation using the D-optimality criterion which maximizes the determinant of the Fisher information matrix (FIM) using as decision variables the bromoform content in the feed and five sampling times for a run duration of 24 h. The OED problem was solved for four feed intake patterns. The accuracy of the parameter estimates was determined by the calculation of the standard deviations (s.d.) and compared with the s.d. obtained with a classical equidistant sampling protocol. The resulting optimal solutions were shown to be specific to each feed distribution pattern. The gain in accuracy of the OED protocol with respect to the equidistant sampling depended on the pattern of feed intake. For the highest gain, the s.d. by the OED protocol were in average 57% of the s.d. of the equidistant sampling protocol. For the lowest gain, the s.d. of the OED protocol were in average 92% of the s.d. of the equidistant sampling protocol. These results demonstrate the potential of the OED approach for obtaining accurate parameter estimates, and highlight the importance of selecting an optimal supply of feed additives. Using the OED approach can be instrumental for conducting experiments that produce data for building robust rumen fermentation dynamic models that can help the design of CH₄ mitigation strategies.

Keywords: optimal experiment design, parameter identification, macroalgae, methane inhibition, rumen model

50

51 **Implications**

52 The use of antimethanogenic feed additives (AMFA) is a potent strategy to reduce
53 methane emissions from ruminants. Mathematical models can be instrumental for
54 designing optimal strategies of AMFA supply. To achieve this goal, models must be
55 robust. Here, we used the optimal experiment design framework in a dynamic
56 mathematical model representing the impact of the antimethanogenic macroalgae *A.*
57 *taxiformis* on *in vitro* rumen fermentation and methane production. We identified
58 theoretical experimental conditions with high informative content for accurately
59 estimating key model parameters. This approach has a great potential for the
60 development of robust models that could be useful for designing methane mitigation
61 strategies.

62 **Introduction**

63 Reducing methane (CH₄) emissions from ruminants is a major target for the livestock
64 sector. Agricultural CH₄ emissions must be decreased by 11 to 30% of the 2010 level
65 by 2030 and by 24 to 47% by 2050 to meet the 1.5°C target of the Paris Agreement
66 (IPCC, 2022). CH₄ mitigation can be achieved by the supply of antimethanogenic
67 feed additives (AMFA) (Arndt et al., 2022). Mechanistic modelling can be
68 instrumental to relate AMFA efficacy to fundamental elements of the rumen
69 fermentative system and for optimizing AMFA supply (Pressman & Kebreab, 2024;
70 Dijkstra et al., 2025).. To date, only two mechanistic models represent the effect of
71 AMFA on rumen fermentation and methane. The model of van Lingen et al. (2021)
72 represents the effect of 3-nitroxypropanol (3NOP) under *in vivo* conditions. The

model of Muñoz-Tamayo et al. (2021) represents the effect of bromoform from the macroalgae *Asparagopsis taxiformis* under *in vitro* conditions. In this work, we focus on *A. taxiformis* as AMFA. This red macroalgae contains the anti-methanogenic compound bromoform (Machado et al., 2016a). The effect of *A. taxiformis* is dose dependent with methane reductions from 75 to 99% *in vitro* (Huang et al., 2023; Machado et al., 2016b) and from 33 to 98% *in vivo* (Romero et al., 2024; Roque et al., 2021; Kinley et al., 2020). As discussed by Belanche et al. (2025), the elucidation of the mode of action of AMFA requires a multidisciplinary approach covering microbiology and animal metabolism and dedicated experiments both *in vitro* and *in vivo*. The synergy between experimentation and mathematical models offers a great opportunity for the design of informative experiments with high quality data enabling to quantify the AMFA mode of action and provide robust models with capabilities for the design of mitigation strategies. However, such a synergy is rarely exploited since model construction and parameter estimation is often conducted as downstream process (after data collection). On the contrary, an upstream approach could be adopted to identify optimal experimental conditions that guarantee the identifiability of parameters and produce experimental data allowing accurate estimation of model parameters (Muñoz-Tamayo and Tedeschi, 2023) and characterisation of key mechanisms. By applying a dynamic sensitivity analysis using the Shapley effects, Blondiaux et al. (2025) showed the importance of considering the dynamics for an accurate quantification of the mechanisms driving rumen fermentation and CH₄ production under methane inhibited conditions. The work identified as perspective the application of the OED framework to identify optimal sampling times and experimental conditions to provide data with high informative content for accurately estimating model parameters. Accurate parameter estimates

are central for the construction of robust models with high predictability capabilities. Accordingly, the objective of this work was to conduct a theoretical OED providing information on optimal sampling times and AMFA doses for attaining accurate estimates of key parameters of a model representing the impact of the *A. taxiformis* on rumen fermentation and CH₄ under continuous *in vitro* conditions. Following Open Science practices to promote accessibility and reproducibility (Muñoz-Tamayo et al., 2022), the mathematical developments and the scripts that support this work are freely available at Muñoz-Tamayo (2026) and in the link: https://github.com/rafaelmunoztamayo/OED_rumen.

Material and methods

Mathematical model

We used the mathematical model developed by Blondiaux et al., (2025) that represents rumen microbial fermentation under methane inhibition conditions provided by the supply of the *A. taxiformis*. The model represents *in vitro* continuous conditions similar to a RUSITEC system. The operational conditions were taken from the setting used in Belanche et al. (2017) with the volume of the liquid phase $V_l = 0.74$ L. The dynamics of the dry matter intake DMI (g/h) was represented by the following equation:

$$DMI(t) = \sum_{j=1}^{n_r} \frac{\omega_j \cdot DM_{total}}{n_r} \cdot e^{-k \cdot t} \quad (1)$$

With DM_{total} the daily total dry matter ingested, set to 11.25 g. The parameter k is the intake kinetic rate constant, set to 0.015 h^{-1} . The parameter n_r is the number of feed distributions per day, ω_j is the fraction of the total DM supplied in the distribution j . We studied four conditions of feed distribution to explore different feeding patterns:

1) $n_r = 1$.

2) $n_r = 2$ with equal DM supply across the feed distributions ($\omega_j = 0.5$).

3) $n_r = 2$ with the first feed distribution accounting for 70% of the total DM ($\omega_1 = 0.7$; $\omega_2 = 0.3$).

4) $n_r = 4$ with equal DM supply across the feed distributions ($\omega_j = 0.25$).

Figure 1 shows an example of the *DMI* pattern with two distributions ($n_r = 2$) and $\omega_1 = 0.7$, $\omega_2 = 0.3$.

The model extended the model previously developed by Muñoz-Tamayo et al. (2021) under *in vitro* batch conditions, whose parameters were estimated using *in vitro* experimental data from Chagas et al. (2019). The model is derived from mass balance equations, following the structure of the rumen fermentation model developed by Muñoz-Tamayo et al. (2016). Figure 2 displays the flow transport of the system. Figure 3 shows the macroscopic representation of the rumen fermentation process and the impact of *A. taxiformis* on the methanogenesis and on the fermentation.

The mathematical model is described in compact way as

$$\frac{d\xi}{dt} = \mathbf{S} \cdot \boldsymbol{\rho}(\xi, \mathbf{p}) - \mathbf{g}(\xi, \mathbf{p}) \quad (2)$$

Where ξ is the vector of 19 state variables (compartments in Fig. 3), defined by $\xi = (\mathbf{z}, \mathbf{s}, \mathbf{x}, \mathbf{s}_g)$, where $\mathbf{z} = (z_{\text{ndf}}, z_{\text{nsc}}, z_{\text{pro}})$ is the vector of concentrations of the polymer components (neutral detergent fiber (z_{ndf}), non-structural carbohydrates (z_{nsc}) and proteins (z_{pro}); g/L), $\mathbf{s} = (s_{\text{su}}, s_{\text{aa}}, s_{\text{ac}}, s_{\text{bu}}, s_{\text{pr}}, s_{\text{IN}}, s_{\text{IC}}, s_{\text{H}_2}, s_{\text{CH}_4}, s_{\text{br}})$ is the vector of concentrations of the soluble components (sugars (s_{su}), amino acids (s_{aa}), acetate (s_{ac}), butyrate (s_{bu}), propionate (s_{pr}), inorganic nitrogen (s_{IN}), inorganic carbon (s_{IC}), hydrogen (s_{H_2}), CH_4 (s_{CH_4}); mol/L and bromoform (s_{br}); g/L), $\mathbf{x} = (x_{\text{su}}, x_{\text{aa}}, x_{\text{H}_2})$ is the

vector of concentrations of the microbial functional groups (sugars utilizers (x_{su}), amino acids utilizers (x_{aa}) and hydrogen utilizers (x_{H_2}); mol/L), $\mathbf{s}_g = (s_{g,CO_2}, s_{g,H_2}, s_{g,CH_4})$ is the vector of concentrations in gas phase (carbon dioxide (s_{g,CO_2}), hydrogen (s_{g,H_2}) and CH_4 (s_{g,CH_4}); mol/L). $\mathbf{p}(\cdot)$ 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 (i.e., input/output fluxes, liquid–gas transfer), and $\mathbf{p}$ is the vector of the model parameters. The model is detailed in Blondiaux et al. (2025). The implementation of the model is available in the R software (Blondiaux, 2024). Few modifications were done in the original model for constructing the OED approach. The modified model was implemented in Matlab R2019b. In the scripts, state variables in capitals refer to quantities in mass or molar units. For example $S_{ac} = s_{ac} \cdot V_l$. Table 1 shows the model parameters. In this work, we focused on the parameters associated to the effect of *A. taxiformis* on the rumen fermentation and methane production. *A. taxiformis* contains the anti-methanogenic compound bromoform which inhibits directly the growth rate of methanogens. The dynamics of bromoform concentration s_{br} follows

$$\frac{ds_{br}}{dt} = F_{br,in} - k_{br} \cdot s_{br} - F_{br,out} \quad (3)$$

Where $F_{br,in} = \frac{w_{br} \cdot DMI}{V_l}$ is the input flux of bromoform concentration (g/(L h)) with w_{br} the fraction of bromoform in the diet of the animal. We assumed a bromoform content in the macroalgae of 0.684% in the DM (Chagas et al., 2019), therefore $w_{br} = 0.00684$. w_{AT} , where w_{AT} is the fraction of *A. taxiformis* in the feed (in DM). The

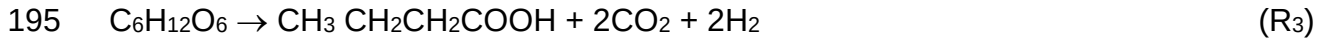
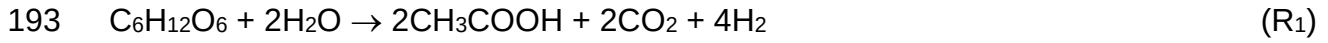
parameter k_{br} is the kinetic rate constant of bromoform utilization (h^{-1}) and $F_{br,out} = D \cdot s_{br}$ is the output flux of bromoform concentration ($\text{g}/(\text{L h})$), with D the dilution rate constant set to $D = 0.035 \text{ h}^{-1}$ (Bayat et al., 2011) to represent conditions close to the *in vivo* situation. The direct inhibition of bromoform is integrated in the kinetic rate of hydrogen utilization (ρ_{H_2}) by the methanogens:

$$\rho_{H_2} = I_{br} \cdot I_{IN} \cdot k_{m,H_2} \frac{s_{H_2}}{K_{s,H_2} + s_{H_2}} x_{H_2} \quad (4)$$

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} ($\text{mol}/(\text{mol h})$) is the maximum specific utilization rate constant of hydrogen, K_{s,H_2} (mol/L) is the Monod affinity constant of hydrogen utilization, and I_{IN} is a nitrogen limitation factor. The factor I_{br} represents the inhibition caused by the bromoform on the growth of methanogens growth and follows the non-competitive inhibition equation

$$I_{br} = \frac{k_{I,br}}{k_{I,br} + s_{br}} \quad (5)$$

With $k_{I,br}$ the bromoform inhibition constant. It should be noted that in the previous model versions (Blondiaux et al., 2025; Muñoz-Tamayo et al., 2021), I_{br} was represented by a sigmoid function. In the present work, we modified the representation to the rational Eq. (5) to facilitate the identifiability analysis which can become difficult when using exponential functions (see Muñoz-Tamayo and Tedeschi, 2023 for discussion on parameter identifiability of exponential functions). An indirect effect of bromoform on the fermentation takes place due to H_2 accumulation under CH_4 inhibition conditions. The carbon flux allocation towards the production of volatile fatty acids (VFA) from sugars is driven by the partial pressure of hydrogen (p_{H_2}) which increases when methane production is inhibited. Sugars utilizers convert glucose into VFA following the following macroscopic reactions:



196 The flux allocation is determined by the model parameters $\lambda_1, \lambda_2, \lambda_3$ that represent
 197 the molar fraction of glucose utilized to produce acetate, propionate and butyrate,
 198 respectively. They follow $\lambda_1 + \lambda_2 + \lambda_3 = 1$. The allocation parameters are
 199 represented by the following linear equations

200 $\lambda_1 = p_1 - p_2 \cdot p_{H_2}$ (6)

201 $\lambda_2 = p_3 + p_4 \cdot p_{H_2}$ (7)

202

203 ***Parameter identifiability***

204 We focused on the five key model parameters described above representing the
 205 effect of *A. taxiformis* on the rumen fermentation and methane production:

206 $k_{I,br}, p_1, p_2, p_3, p_4$. Firstly, we tested their structural identifiability, that is the theoretical
 207 ability to recover uniquely their values from measurements given by an experimental
 208 setup. The structural identifiability is relevant for the construction of models whose
 209 purpose is to predict system behaviour (Muñoz-Tamayo et al., 2018). The interested
 210 reader on parameter identifiability is referred to Muñoz-Tamayo and Tedeschi (2023)
 211 which provides information on available software tools for identifiability analysis with
 212 examples in animal science. We used the software toolbox GenSSI 2.0 (Ligon et al.,
 213 2018) to assess the structural identifiability of the five model parameters. GenSSI
 214 couples the generating series approach with identifiability tableaux.

215 We assumed that the available measurements were the concentrations of VFA
216 (s_{ac}, s_{bu}, s_{pr}), gas concentrations ($s_{g,CO_2}, s_{g,H_2}, s_{g,CH_4}$) and the concentration of
217 bromoform (s_{br}).

218 ***Optimal Experiment Design for parameter identification***

219 OED aims at obtaining the maximum information to be collected in an experiment on
220 the basis of the optimization of a suitable criterion (Walter and Pronzato, 1990). In
221 our case, the goal of the OED is to provide the conditions of an experiment whose
222 data will be used to obtain accurate parameter estimates of a model representing our
223 system under study. By this, we follow the upstream approach advocated in Muñoz-
224 Tamayo and Tedeschi (2023) to provide high informative data for the construction of
225 robust models. It should be noted that while structural identifiability addresses a
226 qualitative analysis, the OED follows a quantitative approach aiming at improving the
227 practical identifiability of the parameter estimates. That is, to improve their accuracy
228 or reduce their uncertainty. The interested reader on parameter identifiability is
229 referred to Lam et al., (2022) and Wieland et al., (2021). For the OED problem, we
230 used the D-optimality criterion which maximizes the determinant of the Fisher
231 Information Matrix (FIM):

$$232 \min_{\boldsymbol{\varphi}} -\det(\text{FIM}(\mathbf{p}, \boldsymbol{\varphi})) \quad (8)$$

233 Where $\mathbf{p}$ is the parameter vector and $\boldsymbol{\varphi}$ is the design vector that defines the
234 experimental set up. Maximizing the determinant of the FIM is equivalent to minimize
235 the volume of the confidence intervals. We followed the local design approach where
236 the OED is set using the vector of nominal values $\mathbf{p}_0$ for the parameters ($\mathbf{p} = \mathbf{p}_0$).
237 The FIM is calculated at $\mathbf{p}_0$ as follows

$$\text{FIM}(\mathbf{p}_0, \boldsymbol{\varphi}) = \sum_{i=1}^{n_t} \left[\frac{\partial \mathbf{y}_m}{\partial \mathbf{p}} \right]_{(t_i, \mathbf{p}_0)}^T \boldsymbol{\Sigma}^{-1} \left[\frac{\partial \mathbf{y}_m}{\partial \mathbf{p}} \right]_{(t_i, \mathbf{p}_0)} \quad (9)$$

Where n_t is the number of sampling (observation) times and $\boldsymbol{\Sigma}$ is the covariance matrix of the observed variables. We assumed that the standard deviations of the observed variables were the same and equal to 5×10^{-4} . The matrix $\frac{\partial \mathbf{y}_m}{\partial \mathbf{p}}$ contains the first-order sensitivities of the observed variables $\mathbf{y}_m$ with respect to the parameters $\mathbf{p}$. The sensitivity equations were obtained by symbolic manipulation in Matlab using the IDEAS toolbox (Muñoz-Tamayo et al., 2009) which is available at <http://genome.jouy.inra.fr/logiciels/IDEAS>. The calculation of the sensitivities for $n_p = 5$ parameters results from the resolution of an extended model with $19 + 19 \times n_p = 114$ state variables. The scripts with the extended model and the auxiliary functions were generated by IDEAS.

We defined the decision vector of the experimental setup as the bromoform content in the feed as DM, and five sampling times for a run of 24 h: $\boldsymbol{\varphi} = [w_{br}, t_1, t_2, \dots, t_5]$. We set a minimal difference of 30 min between two sampling times to place our theoretical exercise in a practical experimental implementation. Accordingly the k th sampling time was set as $t_k = t_{k-1} + \Delta_k + 0.5$. The parameters Δ_k are to be estimated in the optimization.

The initial conditions at t_0 were assumed to be known. The maximization of the determinant of the FIM (Eq. 8) was done using firstly the Matlab function `fminsearch` (Nelder-Mead Simplex Method). Then, we used the resulting estimates as initial guess for a global optimization using the MEIGO Matlab software (Egea et al., 2014). We used the metaheuristic enhanced scatter search method (Egea et al., 2010) implemented in MEIGO.

Results and discussion

The assessment of structural identifiability with GenSSI 2.0 (Ligon et al., 2018) led to the conclusion that the five model parameters are structurally globally identifiable under the experimental setup. The computational time for the identifiability testing was of 1.9s on a laptop with a 2.40 GHz CPU. Table 2 shows the design vector obtained after the maximization of the determinant of the FIM for each feed distribution pattern. We observed that the optimal solutions are specific to each pattern. If we analyse this outcome in the context of an animal trial in which the animals exhibit different feeding patterns, we can infer that there will be an optimal sampling protocol for each animal. However, for practical reasons, it would be preferable to have a single sampling protocol that could provide accurate measurements for all animals. Such a sampling protocol could be estimated in advance by applying the OED approach to an extended mathematical model that takes into account *in vivo* conditions and incorporates the individual characterisation of animal feeding behaviour.

Figure 4 shows the theoretical response of the model under the OED conditions for the distribution with $n_r = 2$, $\omega_1 = 0.7$; $\omega_2 = 0.3$. Table 3 shows the ratio of the standard deviations (s. d.) of the parameters by the OED compared to a design with a classical equidistant sampling with *A. taxiformis* content of 0.5% DM ($w_{br} = 0.0034$) for the four feed distribution patterns. We observed that the gain in accuracy of the OED protocol with respect to the equidistant protocol depends on the dynamic pattern of feed intake. The highest gain was obtained for the feed intake with $n_r = 2$, $\omega_1 = 0.7$, $\omega_2 = 0.3$. The standard deviations (s.d.) of the parameters by the OED protocol were in average 57% of the s.d. of the equidistant sampling protocol. The lowest gain was obtained for the feed intake with scenario $n_r = 4$, $\omega_j = 0.25$. Here, the s.d. of the OED protocol were in average 92% of the s.d. of the equidistant sampling

287 protocol. Table 3 also shows that determinant of the FIM for each feeding pattern.

288 The OED protocol for the feeding pattern with $n_r = 1$ has the highest determinant,

289 and thus it provides the smallest confidence intervals for the estimates. The

290 determinant of the FIM of the OED protocol for the feeding pattern with $n_r = 2$, $\omega_1 =$

291 0.7 , $\omega_2 = 0.3$ is 13 higher to that from the equidistant sampling. These results

292 demonstrate the potential of the OED approach for obtaining accurate parameter

293 estimates, and highlight the importance of selecting an optimal supply of feed

294 additives.

295 The research on structural identifiability is considered to have reached a high degree

296 of maturity (Wieland et al., 2021) supported by the development of a variety of

297 software toolboxes for structural identifiability analysis (Barreiro and Villaverde,

298 2023). On the other hand, practical identifiability analysis requires further

299 developments at the conceptual and technical level (Heinrich et al., 2025a; Wieland

300 et al., 2021). In our work, we used an approach based on the FIM to address the

301 practical identifiability problem because its simplicity of implementation by means of

302 symbolic manipulation via the IDEA toolbox (Muñoz-Tamayo et al., 2009). Here, we

303 focused on a few number of parameters, but the approach can be extended to a

304 larger set of parameters. Considering a large number of parameters however can

305 make difficult the computation of the FIM. One alternative strategy is to partition the

306 full OED problems into smaller sub-problems targeting a couple of parameters

307 (Muñoz-Tamayo, 2014). It must be kept in mind, however, that the use of the

308 approximation of the confidence intervals of the parameters using the FIM is only

309 valid asymptotically under the following conditions (Walter and Pronzato, 1997): *the*

310 *number of data points is large, the nonlinearity of the model with respect to its*

311 *parameters is mild and the measurement errors are independently distributed and*

have small magnitudes. When these conditions are far from being satisfied, the use of the FIM must be considered with caution. Alternatively, the profile likelihood method (Raue et al., 2009) offers an accurate approach for the assessment of practical identifiability that circumvents the shortcomings of the FIM approach. However, its implementation is computationally expensive (Heinrich et al., 2025b; Villaverde et al., 2022). It would be useful to implement further the profile likelihood approach to our case study and compare it with the results provided by the FIM approach. An example of application of the profile likelihood approach in animal science is presented by Vargas-Villamil et al. (2020) for a sheep nutrition model. To sum up, we showed that the consideration of parameter identifiability within an upstream approach (Muñoz-Tamayo and Tedeschi, 2023) provides a useful resource to improve the identifiability of model parameters and build robust models with capabilities for the prediction of the impact of AMFA on rumen fermentation and methane production. Firstly, by the structural identifiability analysis, we guaranteed that the parameter optimization problem has a unique solution. Secondly, by the design of an optimal experimental scheme, we provided conditions for producing data with high informative content for obtaining accurate parameter estimates. Such an approach allows to get the most out of experimental data and has the potential of minimizing costs of an experimental setup (Muñoz-Tamayo et al., 2018). In the broad context of measuring methane, the OED approach developed here aligns with studies aiming at identifying the best optimal sampling times in devices such as the GreenFeed to obtain accurate measurements (e.g., Van Lingen et al., 2023). The OED approach could also be useful to determine optimal supply of single AMFA and winning combination of additives allowing to address potential drawbacks of impaired synchronization when using combination of additives (Muñoz et al., 2024). To exploit

the potential of parameter identifiability and OED, a strong synergy between experimentalist and modellers is required.

The next step of this work is to extend the approach to an ongoing modelling project aiming at representing the synergetic effect of combining *A. taxiformis* with the hydrogen acceptor phloroglucinol under *in vitro* conditions (Roger et al., 2025). This strategy aims at reducing methane while improving rumen fermentation. A further application of the OED approach to *in vivo* conditions would be instrumental to improve the connection between *in vitro* and *in vivo* results and better assess the impact of AMFA on the animal. By combining modelling and dedicated experiments, we expect to enhance our understanding on the rumen microbial ecosystem and provide tools to inform the design of strategies for sustainable ruminant production. This approach aligns with the 3Rs (Replacement, Reduction, and Refinement) principle in setting optimal conditions for refining experiments and building *in silico* models that can reduce, in the future, the use of animal experiments for evaluating methane mitigation strategies.

Ethics approval

Not applicable.

Data and model availability statement

The scripts of the OED implementation and identifiability testing are available at Muñoz-Tamayo (2026) and in the link:
https://github.com/rafaelmunoztamayo/OED_rumen.

359 **Declaration of generative AI and AI-assisted technologies in the writing**
360 **process**

361 We used the software DeepL to correct the English.

362 **Author ORCIDs**

363 Rafael Muñoz-Tamayo: <https://orcid.org/0000-0002-9266-4132>

364 Maguy Eugène: <https://orcid.org/0000-0002-2111-0597>

365 **Declaration of interest**

366 None.

367 **Acknowledgements**

368 We thank Paul Blondiaux and Tristan Senga Kiessé (INRAE, France) for helpful
369 discussions on sensitivity analysis applied to our rumen fermentation model.

370 **Financial support statement**

371 Rafael Muñoz-Tamayo acknowledges funding from the ANR project H2Rumen (ANR-
372 24-CE20-7802).

373 **CRedit Author contributions**

374 Rafael Muñoz-Tamayo: Conceptualization, Funding acquisition, Investigation,
375 Methodology, Software, Writing – original draft.

376 Maguy Eugène: Conceptualization, Methodology, Writing – review & editing.

377 **References**

378 Arndt, C., Hristov, A.N., Price, W.J., McClelland, S.C., Pelaez, A.M., Cueva, S.F., Oh, J.,
 379 Dijkstra, J., Bannink, A., Bayat, A.R., 2022. Full Adoption of the Most Effective
 380 Strategies to Mitigate Methane Emissions by Ruminants Can Help Meet the 1.5 °C
 381 Target by 2030 but Not 2050. *Proceedings of the National Academy of Sciences of*
 382 *the United States of America* 119. doi:10.1073/PNAS.2111294119
 383 Barreiro, R.X., Villaverde, A.F., 2023. Benchmarking tools for a priori identifiability analysis.
 384 *Bioinformatics* 39, btad065. doi:10.1093/bioinformatics/btad065
 385 Bayat, A.R., Rinne, M., Kuoppala, K., Ahvenjärvi, S., Huhtanen, P., 2011. Ruminant large and
 386 small particle kinetics in dairy cows fed primary growth and regrowth grass silages
 387 harvested at two stages of growth. *Animal Feed Science and Technology* 165, 51–60.
 388 doi:10.1016/J.ANIFEEDSCI.2011.02.018
 389 Belanche, A., Bannink, A., Dijkstra, J., Durmic, Z., Garcia, F., Santos, F.G., Huws, S.,
 390 Jeyanathan, J., Lund, P., Mackie, R.I., McAllister, T.A., Morgavi, D.P., Muetzel, S.,
 391 Pitta, D.W., Yáñez-Ruiz, D.R., Ungerfeld, E.M., 2025. Feed additives for methane
 392 mitigation: A guideline to uncover the mode of action of antimethanogenic feed
 393 additives for ruminants. *Journal of Dairy Science* 108, 375–394.
 394 doi:10.3168/JDS.2024-25046
 395 Belanche, A., Newbold, C.J., Lin, W., Rees Stevens, P., Kingston-Smith, A.H., 2017. A
 396 systems biology approach reveals differences in the dynamics of colonization and
 397 degradation of grass vs. hay by rumen microbes with minor effects of vitamin E
 398 supplementation. *Frontiers in Microbiology* 8. doi:10.3389/fmicb.2017.01456
 399 Blondiaux, P., 2024. Implementation of a dynamic sensitivity analysis of a mathematical
 400 model describing the effect of the macroalgae *Asparagopsis taxiformis* on rumen
 401 fermentation and methane production under in vitro continuous conditions.
 402 doi:10.5281/ZENODO.12167243
 403 Blondiaux, P., Kiessé, T.S., Eugène, M., Muñoz-Tamayo, R., 2025. Dynamic sensitivity
 404 analysis of a mathematical model describing the effect of the macroalgae
 405 *Asparagopsis taxiformis* on rumen fermentation and methane production under in

406 vitro continuous conditions. Peer Community Journal 5, e126.
407 doi:10.24072/pcjournal.644

408 Chagas, J.C., Ramin, M., Krizsan, S.J., 2019. In vitro evaluation of different dietary methane
409 mitigation strategies. Animals 9, 1120. doi:10.3390/ani9121120

410 Dijkstra J, Bannink A, Congio GFS, Ellis JL, Eugène M, Garcia F, Niu M, Vibart RE, Yáñez-
411 Ruiz DR, Kebreab E (2025) Feed additives for methane mitigation: Modeling the
412 impact of feed additives on enteric methane emission of ruminants—Approaches and
413 recommendations. Journal of Dairy Science, 108, 356–374.
414 <https://doi.org/10.3168/jds.2024-25049>

415 Egea, J.A., Henriques, D., Cokelaer, T., Villaverde, A.F., MacNamara, A., Danciu, D.P.,
416 Banga, J.R., Saez-Rodriguez, J., 2014. MEIGO: An open-source software suite
417 based on metaheuristics for global optimization in systems biology and
418 bioinformatics. BMC Bioinformatics 15, 136. doi:10.1186/1471-2105-15-136

419 Egea, J.A., Martí, R., Banga, J.R., 2010. An evolutionary method for complex-process
420 optimization. Computers and Operations Research 37, 315–324.
421 doi:10.1016/j.cor.2009.05.003

422 Heinrich, M., Arutjunjan, R., Timmer, J., 2025a. On the different flavours of practical
423 identifiability. Current Opinion in Systems Biology 42, 100556.
424 doi:10.1016/j.coisb.2025.100556

425 Heinrich, M., Rosenblatt, M., Wieland, F.-G., Stigter, H., Timmer, J., 2025b. On structural and
426 practical identifiability: Current status and update of results. Current Opinion in
427 Systems Biology 41, 100546. doi:10.1016/j.coisb.2025.100546

428 Huang, R., Romero, P., Belanche, A., Ungerfeld, E.M., Yanez-Ruiz, D., Morgavi, D.P.,
429 Popova, M., 2023. Evaluating the effect of phenolic compounds as hydrogen
430 acceptors when ruminal methanogenesis is inhibited in vitro – Part 1. Dairy cows.
431 animal 17, 100788. doi:10.1016/J.ANIMAL.2023.100788

432 IPCC, 2022. Global Warming of 1.5°C. IPCC Special Report on Impacts of Global Warming
433 of 1.5°C above Pre-industrial Levels in Context of Strengthening Response to Climate

434 Change, Sustainable Development, and Efforts to Eradicate Poverty. Cambridge
 435 University Press. doi:10.1017/9781009157940

436 Kinley, R.D., Martinez-Fernandez, G., Matthews, M.K., de Nys, R., Magnusson, M., Tomkins,
 437 N.W., 2020. Mitigating the carbon footprint and improving productivity of ruminant
 438 livestock agriculture using a red seaweed. *Journal of Cleaner Production* 259,
 439 120836. doi:10.1016/j.jclepro.2020.120836

440 Lam, N.N., Docherty, P.D., Murray, R., 2022. Practical identifiability of parametrised models:
 441 A review of benefits and limitations of various approaches. *Mathematics and*
 442 *Computers in Simulation* 199, 202–216. doi:10.1016/J.MATCOM.2022.03.020

443 Ligon, T.S., Fröhlich, F., Chiş, O.T., Banga, J.R., Balsa-Canto, E., Hasenauer, J., 2018.
 444 GenSSI 2.0: multi-experiment structural identifiability analysis of SBML models.
 445 *Bioinformatics* 34, 1421–1423. doi:10.1093/BIOINFORMATICS/BTX735

446 Machado, L., Magnusson, M., Paul, N.A., Kinley, R., de Nys, R., Tomkins, N., 2016a.
 447 Identification of bioactives from the red seaweed *Asparagopsis taxiformis* that
 448 promote antimethanogenic activity in vitro. *Journal of Applied Phycology* 28, 3117–
 449 3126. doi:10.1007/s10811-016-0830-7

450 Machado, L., Magnusson, M., Paul, N.A., Kinley, R., de Nys, R., Tomkins, N., 2016b.
 451 Dose-response effects of *Asparagopsis taxiformis* and *Oedogonium* sp. on in vitro
 452 fermentation and methane production. *J. Appl. Phycol.* 28, 1443–1452.
 453 doi:10.1007/s10811-015-0639-9

454 Muñoz, C., Muñoz, I.A., Rodríguez, R., Urrutia, N.L., Ungerfeld, E.M., 2024. Effect of
 455 combining the methanogenesis inhibitor 3-nitrooxypropanol and cottonseeds on
 456 methane emissions, feed intake, and milk production of grazing dairy cows. *animal*
 457 18, 101203. doi:10.1016/j.animal.2024.101203

458 Muñoz-Tamayo, R, 2026. Optimal Experiment Design of a rumen model representing the
 459 effect of *Asparagopsis taxiformis* on in vitro fermentation.
 460 doi:10.5281/ZENODO.19696662

Muñoz-Tamayo, R., Chagas, J.C., Ramin, M., Krizsan, S.J., 2021. Modelling the impact of the macroalgae *Asparagopsis taxiformis* on rumen microbial fermentation and methane production. *Peer Community Journal* 1, e7. doi:10.24072/PCJOURNAL.11

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. doi: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. doi:10.3182/20090706-3-FR-2004.0211

Muñoz-Tamayo, R., Martinon, P., Bougaran, G., Mairet, F., Bernard, O., 2014. Getting the most out of it: Optimal experiments for parameter estimation of microalgae growth models. *J. Process Control* 24, 991–1001. doi:10.1016/j.jprocont.2014.04.021

Muñoz-Tamayo, R., Nielsen, B.L., Gagaoua, M., Gondret, F., Krause, E.T., Morgavi, D.P., Olsson, I.A.S., Pastell, M., Taghipoor, M., Tedeschi, L., Veissier, I., Nawroth, C., 2022. Seven steps to enhance open science practices in animal science. *PNAS Nexus* 1, pgac106. doi:10.1093/pnasnexus/pgac106

Muñoz-Tamayo, R., Puillet, L., Daniel, J.B., 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. doi:10.1017/S1751731117002774

Muñoz-Tamayo, R., Tedeschi, L.O., 2023. ASAS-NANP symposium: Mathematical Modeling in Animal Nutrition: The power of identifiability analysis for dynamic modeling in animal science: a practitioner approach. *Journal of Animal Science* 101, 1–13. doi:10.1093/JAS/SKAD320

Pressman EM, Kebreab E (2024) A review of key microbial and nutritional elements for mechanistic modeling of rumen fermentation in cattle under methane-inhibition. *Front. Microbiol.*, 15, 1488370. <https://doi.org/10.3389/FMICB.2024.1488370>

488 Raue, A., Kreutz, C., Maiwald, T., Bachmann, J., Schilling, M., Klingmuller, U., Timmer, J.,
 489 2009. Structural and practical identifiability analysis of partially observed dynamical
 490 models by exploiting the profile likelihood. *Bioinformatics* 25, 1923–1929.
 491 doi:10.1093/bioinformatics/btp358

492 Roger, A., Popova, M., Yáñez-Ruiz, D., Muñoz-Tamayo, R., 2025. A conceptual model of the
 493 synergetic effect of combining the anti-methanogenic macroalgae *Asparagopsis*
 494 *taxiformis* with the hydrogen acceptor phloroglucinol: a strategy for methane
 495 mitigation with fermentation co-benefits for ruminants. *Animal - Science proceedings*
 496 16, 482–483. doi:10.1016/j.anscip.2025.07.315

497 Romero, P., Ungerfeld, E.M., Popova, M., Morgavi, D.P., Yáñez-Ruiz, D.R., Belanche, A.,
 498 2024. Exploring the combination of *Asparagopsis taxiformis* and phloroglucinol to
 499 decrease rumen methanogenesis and redirect hydrogen production in goats. *Animal*
 500 *Feed Science and Technology* 316, 116060. doi:10.1016/j.anifeedsci.2024.116060

501 Roque, B.M., Venegas, M., Kinley, R.D., De Nys, R., Duarte, T.L., Yang, X., Kebreab, E.,
 502 2021. Red seaweed (*Asparagopsis taxiformis*) supplementation reduces enteric
 503 methane by over 80 percent in beef steers. *PLoS One* 16, e0247820.
 504 doi:10.1371/JOURNAL.PONE.0247820

505 Van Lingen, H.J., Fadel, J.G., Kebreab, E., Bannink, A., Dijkstra, J., Van Gastelen, S., 2023.
 506 Smoothing spline assessment of the accuracy of enteric hydrogen and methane
 507 production measurements from dairy cattle using various sampling schemes. *Journal*
 508 *of Dairy Science* 106, 6834–6848. doi:10.3168/jds.2022-23207

509 van Lingen, H.J., Fadel, J.G., Yáñez-Ruiz, D.R., Kindermann, M., Kebreab, E., 2021.
 510 Inhibited methanogenesis in the rumen of cattle: microbial metabolism in response to
 511 supplemental 3-nitrooxypropanol and nitrate. *Frontiers in Microbiology* 12, 2026.
 512 doi:10.3389/FMICB.2021.705613

513 Vargas-Villamil LM, Tedeschi LO, Medina-Peralta S, Izquierdo-Reyes F, Navarro-Alberto J,
 514 González-Garduño R (2020) A multi-inverse approach for a holistic understanding of

515 applied animal science systems. *Animal*, 14, s238–s249.

516 <https://doi.org/10.1017/S1751731120000877>

517 Villaverde, A.F., Raimundez, E., Hasenauer, J., Banga, J.R., 2022. Assessment of Prediction
518 Uncertainty Quantification Methods in Systems Biology. *IEEE/ACM Transactions on*
519 *Computational Biology and Bioinformatics*. doi:10.1109/TCBB.2022.3213914

520 Walter, E., Pronzato, L., 1997. Identification of Parametric Models from Experimental Data.
521 Springer, London.

522 Walter, E., Pronzato, L., 1990. Qualitative and quantitative experiment design for
523 phenomenological models—A survey. *Automatica* 26, 195–213. doi:10.1016/0005-
524 1098(90)90116-Y

525 Wieland, F.G., Hauber, A.L., Rosenblatt, M., Tönsing, C., Timmer, J., 2021. On structural
526 and practical identifiability. *Current Opinion in Systems Biology* 25.
527 doi:10.1016/j.coisb.2021.03.005

528

529

530

531

532

533

534

535

536

537

538

539

540

541 **Table 1**

542 Model parameters. The parameters used in the Optimal Experiment Design are
 543 marked with *.

Mathematical symbol	Definition	Units	Value
$k_{I,br}^*$	Bromoform inhibition constant	g/L	5.07e-05
λ_1	Molar fraction of the sugars utilized to produce acetate	mol/mol	$p_1 - p_2 \cdot p_{H_2}$
λ_2	Molar fraction of the sugars utilized to produce acetate	mol/mol	$p_3 + p_4 \cdot p_{H_2}$
λ_3	Molar fraction of the sugars utilized to produce acetate	mol/mol	$1 - (\lambda_1 + \lambda_2)$
p_1^*	Intercept of λ_1		0.36
p_2^*	Slope of λ_1		0.64
p_3^*	Intercept of λ_2		0.38
p_4^*	Slope of λ_2		0.12
$\sigma_{ac,aa}$	Stoichiometry coefficient of acetate production from amino acids utilization	mol/mol	0.67
$\sigma_{bu,aa}$	Stoichiometry coefficient of butyrate production from amino acids utilization	mol/mol	0.24
$\sigma_{pr,aa}$	Stoichiometry coefficient of propionate production from amino acids utilization	mol/mol	0.062
$\sigma_{H_2,aa}$	Stoichiometry coefficient of hydrogen production from amino acids utilization	mol/mol	0.82
$\sigma_{IC,aa}$	Stoichiometry coefficient of inorganic carbon production from amino acids utilization	mol/mol	0.88
f_{H_2}	Fraction of hydrogen utilized for product formation	mol/mol	$1 - 10 \cdot Y_{H_2}$
f_{su}	Fraction of glucose utilized for product formation	mol/mol	$1 - \frac{5}{6} \cdot Y_{su}$
$f_{ch,x}$	Mass fraction of carbohydrates in the microbial cells	g/g	0.20
$f_{pro,x}$	Mass fraction of proteins in the microbial cells	g/g	0.55
k_{br}	Kinetic rate constant of bromoform utilization	h ⁻¹	0.095

k_d	Death cell rate constant	h^{-1}	8.33e-04
$k_{hyd,ndf}$	Hydrolysis rate constant of cell wall carbohydrates	h^{-1}	0.024
$k_{hyd,nsc}$	Hydrolysis rate constant of non-structural carbohydrates	h^{-1}	0.06
$k_{hyd,pro}$	Hydrolysis rate constant of proteins	h^{-1}	0.09
$k_{m,aa}$	Maximum specific utilization rate constant of amino acids	mol/(mol h)	2.00
k_{m,H_2}	Maximum specific utilization rate constant of hydrogen	mol/(mol h)	16
$k_{m,su}$	Maximum specific utilization rate constant of glucose	mol/(mol h)	1.00
$K_{s,aa}$	Monod constant associated with the utilization of amino acids	mol/L	6.40e-03
K_{s,H_2}	Monod constant associated with the utilization of hydrogen	mol/L	5.84e-06
$K_{s,su}$	Monod constant associated with the utilization of glucose	mol/L	9.00e-03
$K_{s,IN}$	Nitrogen limitation constant	mol/L	2.0e-04
Y_{aa}	Microbial biomass yield factor of amino acids utilizers	mol/mol	0.31
Y_{H_2}	Microbial biomass yield factor of hydrogen utilizers	mol/mol	0.006
Y_{su}	Microbial biomass yield factor of glucose utilizers	mol/mol	0.16
$Y_{ac,su}$	Yield factor of the acetate during utilization of glucose	mol/mol	$f_{su} \cdot (2 \cdot \lambda_1 + \frac{2}{3} \cdot \lambda_2)$
$Y_{bu,su}$	Yield factor of the butyrate during utilization of glucose	mol/mol	$f_{su} \cdot (\lambda_3)$
$Y_{pr,su}$	Yield factor of the propionate during utilization of glucose	mol/mol	$f_{su} \cdot (\frac{4}{3} \cdot \lambda_2)$
$Y_{H_2,su}$	Yield factor of the hydrogen during utilization of glucose	mol/mol	$f_{su} \cdot (4 \cdot \lambda_1 + 2 \cdot \lambda_3)$
$Y_{IN,su}$	Yield factor of the inorganic nitrogen during utilization of glucose	mol/mol	$-Y_{su}$
$Y_{IC,su}$	Yield factor of the inorganic carbon during utilization of	mol/mol	$f_{su} \cdot (2 \cdot \lambda_1 + \frac{2}{3} \cdot \lambda_2 + 2 \cdot \lambda_3)$

$Y_{\text{CH}_4, \text{H}_2}$	glucose Yield factor of the methane during utilization of hydrogen	mol/mol	$f_{\text{H}_2} \cdot \left(\frac{1}{4}\right)$
$Y_{\text{IC}, \text{H}_2}$	Yield factor of the inorganic carbon during utilization of hydrogen	mol/mol	$-\left(\left(\frac{1}{4}\right) \cdot f_{\text{H}_2} + \left(\frac{5}{10}\right) \cdot (1 - f_{\text{H}_2})\right)$
$Y_{\text{IN}, \text{H}_2}$	Yield factor of the inorganic nitrogen during utilization of hydrogen	mol/mol	$-Y_{\text{H}_2}$
$Y_{\text{i}, \text{aa}}$	Yield factor of the component i during utilization of amino acids	mol/mol	$(1 - Y_{\text{aa}}) \cdot \sigma_{\text{i}, \text{aa}}$
$Y_{\text{IN}, \text{aa}}$	Yield factor of the component inorganic nitrogen during utilization of amino acids	mol/mol	$N_{\text{aa}} - Y_{\text{aa}} \cdot N_{\text{mb}}$
$K_{\text{a}, \text{CO}_2}$	Equilibrium constant of bicarbonate		5.13e-07
$K_{\text{a}, \text{NH}_4}$	Equilibrium constant of ammonium		1.44e-09
$K_{\text{a}, \text{VFA}}$	Equilibrium constant of VFA		1.74e-05
K_{w}	Equilibrium coefficient of water		2.75e-14
$k_{\text{L}} a$	Liquid-gas transfer constant	h^{-1}	8.33
$K_{\text{H}, \text{CO}_2}$	Henry's law coefficient of carbon dioxide	M/bar	2.46e-02
$K_{\text{H}, \text{CH}_4}$	Henry's law coefficient of methane	M/bar	1.10e-03
K_{H, H_2}	Henry's law coefficient of hydrogen	M/bar	7.23e-04
P	Pressure	bars	1.01325
T	Temperature	K	312.15
w_{aa}	Molecular weight of average amino acid	g/mol	134
w_{ac}	Molecular weight of acetate	g/mol	60.05
w_{bu}	Molecular weight of butyrate	g/mol	88.10
w_{mb}	Molecular weight of microbial cells	g/mol	113
w_{pr}	Molecular weight of propionate	g/mol	74.1
w_{su}	Molecular weight of glucose	g/mol	180.16
V_{g}	Volume of the gas phase	L	0.06
V_{l}	Volume of the liquid phase	L	0.74

544

545

Table 2

Optimal design vectors obtained after maximization of the determinant of the Fisher Information Matrix for four feed distribution patterns. The daily dry matter intake pattern is determined by the number of feed distributions n_r and the fraction ω_j of the total DM supplied in the distribution j .

$n_r = 1$					
w_{AT} (% DM)	t_1 (h)	t_2 (h)	t_3 (h)	t_4 (h)	t_5 (h)
0.25	9.8	10.7	11.2	23.5	24
$n_r = 2 \ \omega_j = 0.5$					
w_{AT} (% DM)	t_1 (h)	t_2 (h)	t_3 (h)	t_4 (h)	t_5 (h)
0.51	3	13.5	14.2	21.4	22
$n_r = 2 \ \omega_1 = 0.7 \ \omega_2 = 0.3$					
w_{AT} (% DM)	t_1 (h)	t_2 (h)	t_3 (h)	t_4 (h)	t_5 (h)
0.30	8.7	9.2	18	23.4	24
$n_r = 4 \ \omega_j = 0.25$					
w_{AT} (% DM)	t_1 (h)	t_2 (h)	t_3 (h)	t_4 (h)	t_5 (h)
0.60	8.3	13	13.5	23.5	24

558 **Table 3**

559 Ratio of the standard deviation (s. d.) of the parameter estimates from the OED to the
560 s. d. from a design with equidistant sampling (ES) and *A. taxiformis* content of 0.5%
561 DM for four feed distribution patterns. The daily dry matter intake pattern is
562 determined by the number of feed distributions n_r in one day and the fraction ω_j of
563 the total DM supplied in the distribution j .

564

		k_1	p_1	p_2	p_3
$n_r = 1$	$100 \frac{\text{s. d. OED}}{\text{s. d. equidistant sampling}}$	75	47	67	54
$\frac{\det(\text{FIM}) \text{ OED}}{\det(\text{FIM}) \text{ ES}} = \frac{3.5087e + 20}{4.0332e + 19} = 8.70$					
$n_r = 2$	$100 \frac{\text{s. d. OED}}{\text{s. d. equidistant sampling}}$	97	69	69	72
$\omega_j = 0.5$					
$\frac{\det(\text{FIM}) \text{ OED}}{\det(\text{FIM}) \text{ ES}} = \frac{2.7881e + 18}{5.6142e + 17} = 5.0$					
$n_r = 2$	$100 \frac{\text{s. d. OED}}{\text{s. d. equidistant sampling}}$	103	37	53	35
$\omega_1 = 0.7$					
$\omega_2 = 0.3$					
$\frac{\det(\text{FIM}) \text{ OED}}{\det(\text{FIM}) \text{ ES}} = \frac{1.3714e + 19}{1.0377e + 18} = 13.22$					
$n_r = 4$	$100 \frac{\text{s. d. OED}}{\text{s. d. equidistant sampling}}$	99	110	74	100
$\omega_j = 0.25$					
$\frac{\det(\text{FIM}) \text{ OED}}{\det(\text{FIM}) \text{ ES}} = \frac{1.1883e + 19}{2.7790e + 18} = 4.30$					

565

Figure captions

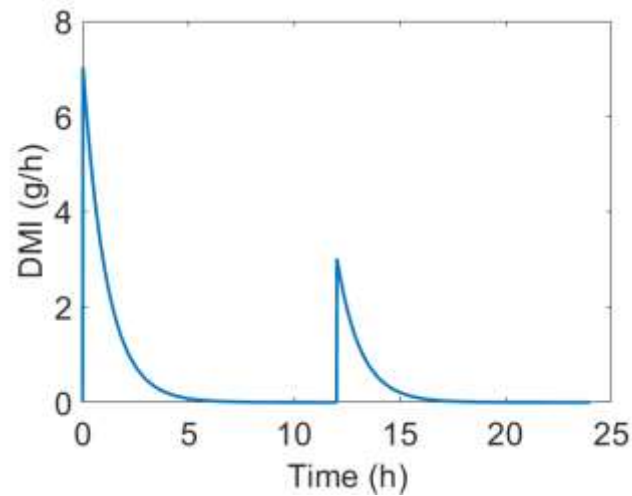


Fig. 1. Daily dry matter intake pattern with two distributions ($n_r = 2$) with the first distribution accounting for 70% of the total DM ($\omega_1 = 0.7$; $\omega_2 = 0.3$).

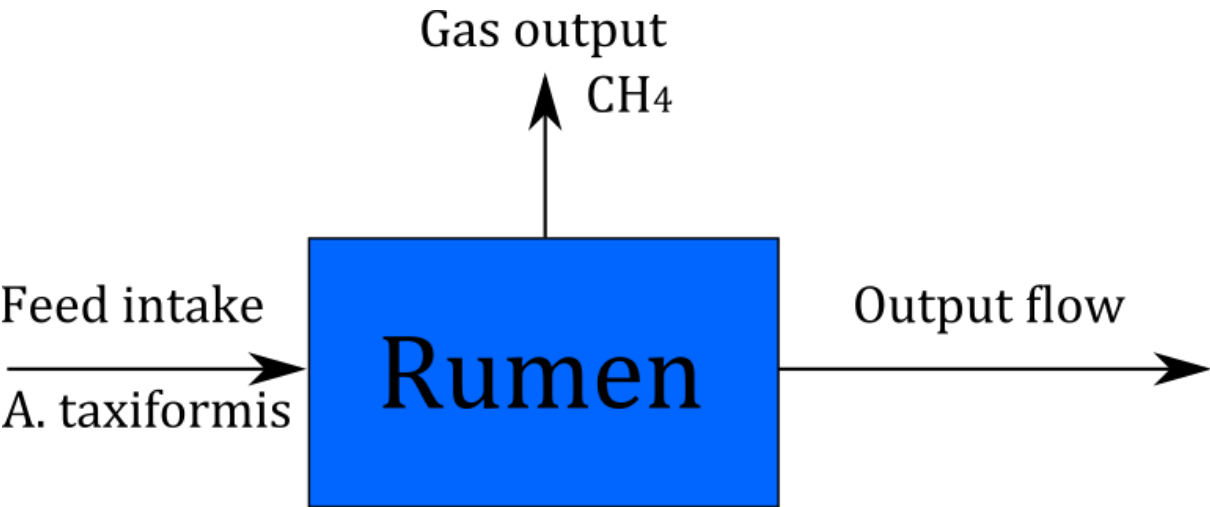


Fig. 2. Representation of the flow transport of the *in vitro* rumen fermentation system.

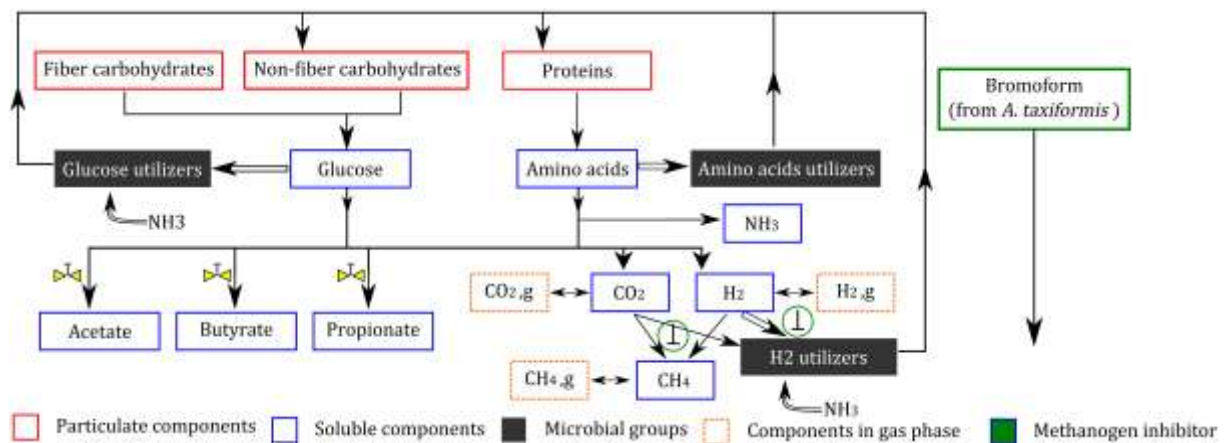
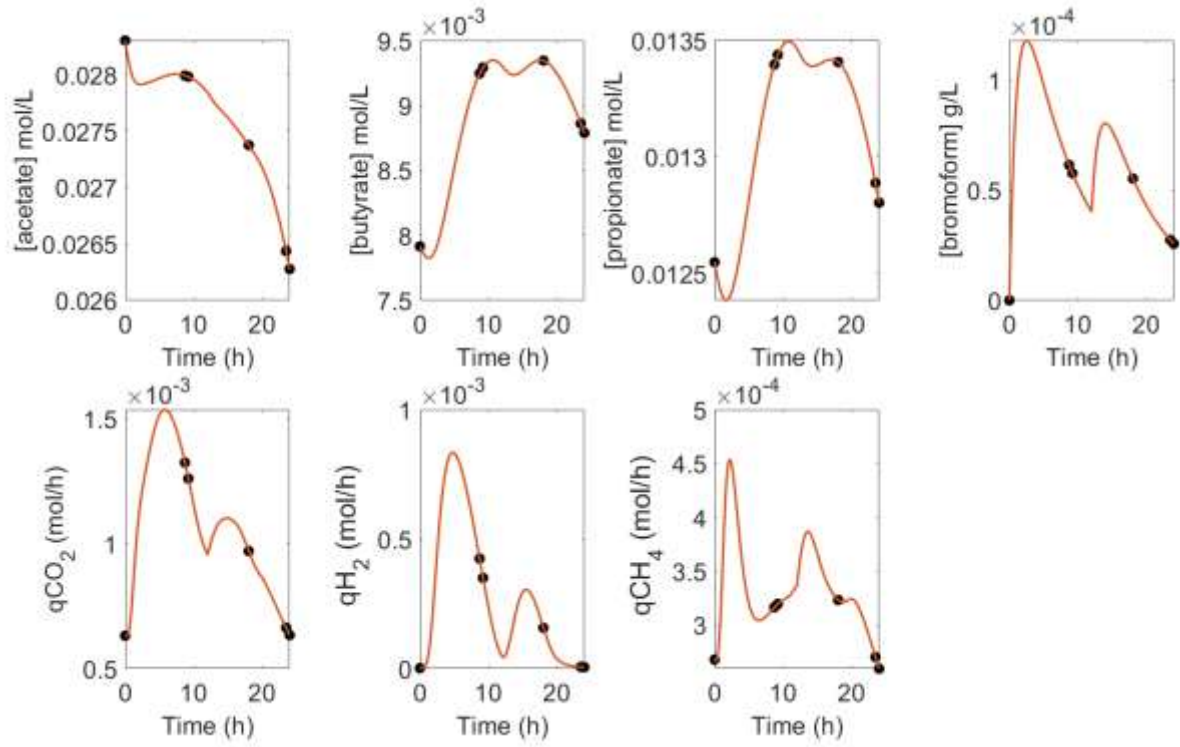


Fig. 3. Macroscopic representation of the rumen fermentation process and the impact of *A. taxiformis* on the methanogenesis and on the fermentation from (Muñoz-Tamayo et al., 2021). Hydrolysis of carbohydrates (fiber and non-fiber) and proteins releases respectively sugars and amino acids soluble monomers which are utilized by specific functional microbial groups. Substrate utilization results in product formation (single arrows) and microbial biomass (double arrows). 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  indicates the direct effect of the bromoform on the methanogenesis. Hydrogen exerts a thermodynamic control on the flux allocation towards volatile fatty acids production. The symbol  indicates the hydrogen control effect on the rumen fermentation.



589

590 **Fig. 4.** Theoretical dynamic response of the model variables under the Optimal
 591 Experiment Design conditions for two feed distributions at 70% and 30% of the total
 592 DM ($n_r = 2$, $\omega_1 = 0.7$; $\omega_2 = 0.3$). The circles • are the optimal sampling times.