Dear Dr. Luis Tedeschi,

We would like to thank the reviewers for their critical and constructive evaluation of our manuscript that helped to improve our paper. We have made explicit our acknowledgment in the manuscript (L454-457).

Below, we addressed the comments of reviewers. We decided to include in the title of the manuscript "methane production". Modifications were highlighted in red in the updated version uploaded in the PCI platform.

Reviewed by anonymous reviewer, 2020-12-21 18:48

A mathematical model would be important for understanding at a mechanistic level how this inhibition occurs. The model developed here could have been valuable, but unfortunately it is too preliminary. The model was calibrated with data from a single study (Chagas et al. 2019) (see L40-1). Further, it was evaluated with the same data as for calibration (see Fig. 3-4 and Table 2). It is not clear why the authors limited themselves to these data, given the number of in vitro and in vivo studies in the literature. To make the conclusions generalizable, more than one study needs to be used. Further, data for calibration and evaluation need to be independent.

Response: thanks for your comment. Our original fermentation model (Muñoz-Tamayo et al., 2016) was calibrated with data from the study of (Serment et al., 2016). This information was added in L87-88. The developments presented in the current manuscript using the data of (Chagas et al., 2019) and our previous work strengthen the capabilities of our model to predict the *in vitro* fermentation pattern and methane production from rumen microbiota. With respect to the elements accounting for the impact of *A. taxiformis*, we have acknowledged the need of external data to validate our model as discussed in L250-255, L391-398. Although there exist published *in vitro* studies on *A. taxiformis*, a robust validation requires experiments with simultaneous dynamic data of bromoform, VFA, H_2, CH_4. Such studies are to our knowledge lacking.

Reviewed by Alberto Atzori, 2021-01-15 04:51

Few comments are below highlighted following the sections of paper structure: Background is mainly focused on biological effects of the Macroalgae and bromoform on ruminal environment explaining biological basis and implication of its use with proper quotes. Lines 25 and 26 might be integrated expliciting if negative long term effects can impair the algae use on animal diets (see also line 348).

Response: many thanks for the overall assessment of our work. Additional information on negative terms was included in L28-30.

A very little part of introduction is dedicated to importance of rumen modeling, which seems a not secondary focus of the paper, especially for microbiological based models. it could represent a frontier in ruminant nutrition. In this sense the paper objective might be integrated with the generic objective of enriching rumen modeling, adaptation and testing and especially accounting for effects of bioactive compounds (as further stated by the authors in line 272) on fermentation complexity.

Response: we have added some information in L38-41.

Line 61 refers to four groups then in parenthesis and in Figure 1 only three groups are presented, it should be clarified.

Response: there was indeed a typo. The right number of microbial groups is three. Thanks for the attentive reading.

Among discussions of hydrogen control, an additional sentence can emphasize effects on energy harvesting. In fact, it is quite debated in literature the fact that methane reduction not always lead to higher energy efficiency use, by microbial groups at rumen level or for the animal, whit not significant difference in production performances.

Response: information was added in L364-380.

Reviewed by anonymous reviewer, 2020-12-07 14:12

The materials and methods are written succinctly but leave out some important details that would help the readers more clearly understand and judge their work.

1) For instance, what was the diet of the two Swedish Red cows? Was it the same as used for the incubations? It is not clear.

Response: many thanks for your evaluation. The diet cows were fed was a forage based diet with concentrate, the ratio was about 60:40. The substrates used for the *in vitro* studies was not exactly the same diet cows were fed with, but included proportions of forage and concentrate. Generally, this should not have any effect on the results since different diets (additives) were screened using the same rumen fluid taken from the cannulated cows. The paragraph explaining the experimental protocol of (Chagas et al., 2019) was rephrased (L50-63) to improve clarity.

2) Were the cows cannulated or was the rumen fluid collected via stomach tube or ruminocentesis?

Response: yes, they were cannulated cows (L58).

3) What was the buffer used for the incubations and was it of sufficient buffering capacity to maintain the pH during incubation at or near your assumed pH 6.6 value?

Response: the buffer used is a standard buffer used for *in vitro* studies, as described by the original *in vitro* methodology (Ramin and Huhtanen, 2012). The buffer is basically mimicking the saliva of cows and the main role is buffering capacity. Our 10 years' experience using the *in vitro* system has showed that after 48h of incubation the pH values are not affected, demonstrating that the buffer used is able to keep the pH stable and not fluctuating to have negative affect on microbial activity.

4) How were gases, VFA, ammonia measured? Perhaps this info is in the Ramin and Huhtanen (2012) citation but if so please describe it that way.

Response: Information was added (L64-75).

5) On lines 109-110, did you really calculate numbers of hydrogen-utilizing microbes as mol/L?

Response: yes. We assume that microbial biomass has the molecular formula C5H7O2N (L173-1175). The use of the units mol/L is needed to close the elementary balances. In our previous work, we showed that predicted dynamics of methanogens (mol/L) follow observed dynamics of methanogens measured by 16S rRNA gene copies (Muñoz-Tamayo et al., 2019).

6) Figure 1 presents the model as a closed system, is this true for your incubations or was there venting of gases? If truly a closed system I suspect increasing gas pressures would affect gas solubility.

Response: this is indeed an important remark. The reviewer is right in pointing out that the model consider the system as closed. The system is not fully closed since gas samples need to be drawn for characterisation. Clarification information was added in L107-109. In the system, the pressure was always lower than the atmospheric pressure.

You do not need to address this but I am curious to know if you had foaming in your incubations which could potentially be an unaccounted electron sink? I am thinking most fermentation balances do not generally get such good agreement between reducing equivalents consumed and produced as presented here. If you buffer was sufficient to maintain pH at or near 6.6 then that is fine, otherwise I would have liked to see pH measurements.

Response: no foaming was observed in the incubations

Overall, this is a good piece of work but I think it would be better if the authors make the effort to make it more broadly readable.

Response: we recognize the challenge of making our manuscript accessible to the animal science community. Accordingly, we have provided freely access to the code of our model to facilitate the understanding of our model developments.

Reviewed by Henk van Lingen, 2020-12-30 19:40

The overall framework and model simulations seem relevant and worth publishing in the light of sustainable production from ruminants and metabolic implications of macroalgae supplementation to ruminant diets. However, various aspects of the present study need further clarification or reconsideration.

First of all, A. taxiformis does not appear in the flow chart (Fig. 1), which I recommend the authors to add. Second, glucose utilizers, amino acid utilizers and hydrogen utilizers appear to contribute to input of non-fiber carbohydrates and proteins, for which no physiological description is given. Fiber carbohydrates appear without any influx. I would like the authors to provide more details.

Response: many thanks for the very relevant comments. Figure 1 was updated to include the effect of *A. taxiformis* through bromoform. Information about the recycling of dead cells as source of proteins and non-fiber carbohydrates is indicated in L90-92. We assumed that microbial cells are formed only by proteins and non-fiber carbohydrates. This is why there is not flux from the recycling of microbial cells to fiber carbohydrates

Third, it is shown that glucose utilization, and acetate, butyrate and propionate are controlled by A. taxiformis as is the production of methane. However, based on the equations, glucose utilization and the production of the three volatile fatty acids appear controlled by hydrogen rather than A. taxiformis. Certainly, I understand the indirect control on VFA production, but still methane production is controlled directly and indicated by the same symbol. Please correct.

Response: the legend of Figure 1 was corrected discriminating the direct and indirect effects L100-105

Fourth, three metabolic pathways for volatile fatty acid production are shown in Table 1 (R1, R2 and R3), of which R2 shows a zero hydrogen balance, and R1 and R3 result in net hydrogen production. Then, there is a λ1 and a λ2 equation that seem to correspond to R1 and R2. As expected, λ1 is inversely related to hydrogen, whereas λ2 appears positively related to hydrogen, despite R2 showing a zero

hydrogen balance. As such, the latter choice may be confusing to the reader of the present paper. Therefore, should the authors not come up with λ1 and λ3 that are both positively related to hydrogen, and next compute λ2 by subtracting λ1 and λ3 from 1? This will still result in a greater propionate proportion in response to elevated hydrogen.

Response: this is a very interesting remark. Our model was built on the basis of the regulation of NADH/NAD+ as proposed by (Mosey, 1983). Under the assumption that the oxidation/reduction reaction of NAD is in equilibrium, Mosey suggested that the couple NADH/NAD+ is linearly proportional to the H2 partial pressure. Accordingly, we have used the H2 partial pressure as proxy of the NADH/NAD+ couple. Since, it has been widely demonstrated that feed additives that reduce methane production induced a concomitant reduction in the acetate:propionate ratio, we have decided to keep the parameterization on λ1 and λ2 which can be directly interpreted by the reader as a formulation that describes the expected reduction of the acetate:propionate ratio for high levels of *A. taxiformis* (due to high H2 accumulation). We have improved the explanation of our parameterization L187-203 and discussion L383-389.

Fifth, the proposed modeling framework accounts for inhibition of glucose utilization by hydrogen (Eq. 6). I encourage the authors to defend this choice by a reference and discuss if this is actually the case in the rumen. For example, Van Gastelen et al 2020 (https://doi.org/10.3168/jds.2019-17936) in their in vivo study observed increased digestibility in response to higher doses of 3-NOP/higher hydrogen in the rumen. Therefore, it might be questionable if increased hydrogen result in decreased microbial activity.

Response: we incorporated the inhibition of glucose utilization by hydrogen as suggested by (Mosey, 1983) L154-157. Incorporating the inhibition allowed us to account for the decrease of VFA for high levels of supplementation of *A. taxiformis* observed in (Chagas et al., 2019). We enhanced the discussion in (L352-363) with the support of references.

It seems the same data were used for model calibration and evaluation. This means that the model evaluation statistics are biased and likely overestimate the model performance. These practises should be avoided and I the authors should perform a more independent model evaluation. The requires either independent data, or a cross-validation for which the data is split into multiple subsets. If the verdict of the authors is that both of these options are not practically feasible, I strongly recommend to remove Table 2 from the manuscript and include in the discussion that the present model yet requires independent evaluation.

Response: the reviewer is right in his assessment. However, we think that the Table 2 provides to the reader information about the model performance even if the evaluation is biased. For example, poor statistic indicators with the calibration data would suggest that the model structure poorly describes the fermentation and thus modifications of the model are required for improvement. Although the lack of validation of our model with external data, our results indicate that the model structure is adequate to capture the dynamics of fermentation. We have added a sentence expressing the caution that needs to be taken with the indicators following the comment of the reviewer. L250-255.

I suggest the authors perform a global sensitivity analysis to evaluate if model output is largely affected by the present set of parameters that was optimized, or parameters that were not optimized, but simply assigned a certain value.

Response: this an interesting subject but it is not in our scope to perform global sensitivity analysis in this paper. Such an analysis will be of great relevance when new data on bromoform and hydrogen dynamics become available to validate the functions of the regulation of fermentation.

I also wonder if the authors considered an identifiability analysis before they performed their model calibration procedure.

Response: we did not. To perform an identifiability analysis using for example the software DAISY (Bellu et al., 2007), a parameterization work is needed to render the sigmoid functions in rational functions.

What are the implications of setting the pH at 6.6? Has the impact of this choice been explored?

Response: in the study of (Chagas et al., 2019), the pH was set to 6.6.. We did not explore the impact of the pH on our results.

Figure 2: I recommend to show the entire dose-response picture for all metabolites as done in for hydrogen in Fig 6.

Response: we preferred to focus the attention on the measured metabolites and hydrogen due to its effect on the fermentation. However, by providing the code, the reader can look at the dynamics of the different metabolites.

The mechanism of methanic inhibition by A. taxiformis is considered bromoform in this paper. Do unsaturated fatty not play any role in methanogenic inhibition by macroalgae (it seems some papers suggest this)?

Response: we did not consider this aspect.

Fig 6: Please be more specific about hydrogen. Is this hydrogen in the headspace, dissolved hydrogen or hydrogen emission? Furthermore, results of this Figure are not described in the results section of the paper. I suggest to add this and also discuss whether these simulated values are in line with observations in other studies.

Response: the hydrogen refers to the headspace. This information was added in the legend of the Figure and in L299. The results of the figure are described in L299-301 and discussed in L335-338.

Specific minor comments

Abstract: "…adverse effect of…" and "…identifying optimal conditions on…". Adverse effects of A. taxiformis and optimal conditions on its use are barely discussed. I would suggest to reword these phrases in the abstract or to extend the discussion section of the present manuscript.

Response: the discussion was enhanced L364-380

Abstract: "…multi-experiment estimation approach…". It looks like multiple treatments from one experiment to me, not multi-experiment. Please clarify or reword.

Response: the multi-experiment term is used in the parameter estimation domain when data from different experiments are used to simultaneously estimate the parameters (Balsa-Canto and Banga, 2011)

Abstract: Penultimate sentence of the abstract "We are … in vivo conditions" does not seem a conclusion to me. Suggest to remove.

Response: we removed the sentence

Line 14-16: Why not be more specific and staying closer to Beauchemin et al: "The safety of feeding bromoform-containing macroalgae to livestock will also need to be investigated, as bromoform can be toxic to the environment (i.e. ozone depletion) and can impair human health"

Response: text modified L16-17

Lines 23-24: "fermentation profile" and "structure of the rumen microbiota". Please be more specific. Do the authors refer to dynamic profiles of certain metabolites and the relative abundance of microbiota at a certain taxonomic level, respectively?

Response: text modified L25-26

Lines 32: "metabolic hydrogen" appears only once in this paper. From other papers I understand this means atomic hydrogen [H], but I still wonder what the physiological relevance is of metabolic hydrogen. Suggest to remove "metabolic".

Response: word removed

Line 43: "basal diet" instead of "basal"?

Response: text modified L52. Control diet is used

Line 50-51: Was the in vitro system set up as a batch culture? If so, please state.

Response: yes, the text was modified L48,61

Line 52-53: Suggest to replace "along the fermentation" by "throughout the incubation period".

Response: done L65

Line 61: Three instead of four microbial groups?

Response: corrected L81

Line 103: Not sure if detailed is a verb.

Response: yes, it is a transitive verb

Line 159-165: My understanding is the present model could be considered a slightly more empirical approach than one in which the NAD is incorporated. I recommend the authors state this in a few words/sentences.

Response: modifications were done L187-203

Line 249: "Methanogenic" not "Methanogenesis". Incorporate throughout.

Response: the inhibition applies to the methanogens and thus to the methanogenesis

Line 250: Not sure if distribution is the right word here as there is no uncertainty estimate (i.e. standard deviation or variance). Would partitioning not be more appropriate?

Response: distribution should be read here as allocation

Line 310: "the linearity … be valid". What is the evidence for stating this?

Response: the argument is based on the study of (De Kok et al., 2013) but not experimental evidence on the rumen is available.

Line 311-315: To be crystal clear, stating that VFA proportions are unaffected within the rumen physiological range of hydrogen partial pressure only makes sense if you look at the plain glucose fermentation pathways, i.e. when staying away from NAD dynamics.

Response: the section 4.1 was modified to improve clarity

Lines 329-331: See previous comments about identifiability analysis. Have the authors performed an identifiability analysis? If so, the authors could simple state whether or not this was the case.

Response: information was added in L395-396

Line 338-339: What is the rationale behind incorporating the impact of hydrogen on amino acid fermentation? Improved prediction of methane production?

Response: we modified the sentence by pointing the potential effect of *A. taxiformis* on amino acids fermentation L403-407

Line 364-365: See comment on penultimate sentence of abstract

Response: text was modified

Line 365-367: This sentence is not a conclusion drawn from simulation results or model development. Suggest to remove. Including this in the discussion is fine, though.

Response: the sentence was removed and included in the discussion L429-431.

Fig 1: Suggest to show all numbers as subscript.

Response: done

Fig 2: bar, not bars; incorporate throughout.

Response: done

Fig 3: intercepts for 4 of the plots are missing. Do the points represent all the different macroalgae inclusion rates?

Response: it is normal that intercepts do not appear in the figures since they correspond to the initial conditions of the VFAs

References: Dijkstra et al. 1992 is missing.

Response: reference was included

References

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De Kok S, Meijer J, Van Loosdrecht MCM and Kleerebezem R 2013. Impact of dissolved hydrogen partial pressure on mixed culture fermentations. Applied Microbiology and Biotechnology 97, 2617–2625.

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.

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