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# Mechanistic modelling of *in vitro* fermentation and methane production by rumen microbiota

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## Abstract

Existing mechanistic models of the rumen ecosystem have proven to be useful to better understand and represent rumen fermentation. Opportunities for improving rumen fermentation models include a better representation of the microbiota, hydrogen dynamics and a mechanistic description of pH. The objective of this work was to include such aspects in the development of a mathematical model of rumen fermentation under *in vitro* conditions. The developed model integrates microbial metabolism, acid base reactions and liquid-gas transfer. Model construction was based on an aggregated representation of the hydrolysis of carbohydrates and proteins, and the further fermentation of soluble monomers. The model is a differential algebraic equation model with 18 compartments. One of the main contributions of the model developed here resides in the mechanistic description of pH, the use of biochemical reactions and partition rules to define the stoichiometry of fermentation, the representation of hydrogen metabolism, and the representation of the rumen microbiota into functional groups associated with the utilization of hexoses, amino acids and hydrogen. The model was calibrated with published data from a 2×2 factorial experiment devoted to assessing the relative importance of the type of inoculum and substrate on the fermentation pattern. The treatments were the level of concentrate in the substrate (low concentrate vs. high concentrate), and the inocula type (obtained from goats fed at low or high concentrate). The model was implemented in Matlab. The code is available on request for academic purposes. Model evaluation was performed by regression analysis and the calculation of statistical indicators using the model predicted values and observed values. The model was capable to represent in a satisfactory fashion the dynamics of the fermentation, that is the pH, the individual

29 volatile fatty acids and the gas compounds, namely methane, hydrogen and carbon diox-  
30 ide. The model predictions exhibited high concordance correlation coefficients (CCC) .  
31 For the pH and the CH<sub>4</sub>, the CCC was of 0.91 and 0.93 respectively. For the other vari-  
32 ables CCC>0.96. The model developed was instrumental to quantify the differences of the  
33 fermentation pattern between the treatment combinations. These differences were mainly  
34 captured by parameters related to the flux distribution and were found to be dependent  
35 mainly on the type of inoculum. For instance, the flux towards butyrate production from  
36 sugars utilization for the microbiota of the inoculum adapted to high concentrate was  
37 about 30% higher than that for the inoculum adapted to low concentrate. This result,  
38 however, requires further validation with new data. Further developments are needed to  
39 incorporate physiological *in vivo* factors into our model. Nevertheless, the structure devel-  
40 oped here appears to be a promising approach for enhancing the mechanistic description  
41 of the rumen microbial ecosystem.

42 *Keywords:* anaerobic digestion; hydrogen; methane; pH; stoichiometry; volatile fatty  
43 acids

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## 44 1. Introduction

45 The design of optimal nutritional strategies for ruminants with the target of maximiz-  
46 ing animal performance and efficiency while reducing enteric methane emissions necessi-  
47 tates a thorough understanding of rumen fermentation. Mathematical models of rumen  
48 fermentation can be effective tools to contribute to the development of these feeding strate-  
49 gies. Naturally, to exploit such usefulness, the models must be able to represent the real  
50 system with an adequate degree of reliability. Mechanistic modelling provides a rational  
51 way of integrating knowledge and exploiting information for predicting system function.  
52 The development of mechanistic models of rumen fermentation has been a longstanding  
53 research activity in animal nutrition (*e.g.*, Baldwin et al. (1987); Dijkstra et al. (1992);  
54 Lescoat and Sauvant (1995); Bannink et al. (2006); Serment and Sauvant (2011); Mills  
55 et al. (2014)). However, the assessment of the limitations of current mechanistic models  
56 suggests that there is still room for model improvement to better predict the dynami-  
57 cal pattern of rumen fermentation, including H<sub>2</sub>, CH<sub>4</sub> and VFA production (Offner and

58 Sauvant, 2004; Ellis et al., 2008; Alemu et al., 2011; Bannink et al., 2011; Vetharanim  
59 et al., 2015). Some of the features to be tackled for improving the predictive capabilities of  
60 current mechanistic models include a better representation of the rumen microbiota and  
61 of hydrogen dynamics. The concentration of hydrogen in the rumen affects the pattern of  
62 fermentation (Janssen, 2010). Hydrogen utilization by microbes is central to maintaining  
63 an hydrogen level that allows the thermodynamic feasibility of the fermentation pathways  
64 (Offner and Sauvant, 2006; Janssen, 2010). Moreover, hydrogen is the main substrate for  
65 methane production. Additionally, progress on predicting ruminal pH is required since  
66 it is a central indicator of rumen status and function (Kohn and Boston, 2000; Dijkstra  
67 et al., 2012). Few works have actually addressed the mechanistic modelling of ruminal  
68 pH. In this respect, following the work of Kohn and Dunlap (1998), Imamidoost and Cant  
69 (2005) developed a model based on acid-base reactions, which provided satisfactory pH  
70 predictions. However, the model of Imamidoost and Cant (2005) underpredicted the con-  
71 centration of organic acids. A similar approach was used by Serment and Sauvant (2011)  
72 to describe the pH under *in vitro* conditions. The model of Serment and Sauvant (2011)  
73 could predict with accuracy the pH dynamics only during the first hours of the fermen-  
74 tation. Another attempt at pH modelling within a thermodynamical framework of the  
75 rumen was presented by Offner and Sauvant (2006) but the accuracy of the predictions  
76 was unsatisfactory.

77 In this context, the objective of our work was to develop a mathematical model that con-  
78 tributes to a better mechanistic representation of rumen fermentation by closely coupling  
79 biological and physicochemical phenomena. The mathematical model herein developed  
80 extends the mechanistic model developed by Serment and Sauvant (2011), which was cal-  
81 ibrated with experimental data resulting from an *in vitro* fermentation study of pea flour  
82 (Maaroufi et al., 2009) and assessed against *in vitro* experimental data from a wide range  
83 of feeds (Giger-Reverdin et al., 2014). The model developed by Serment and Sauvant  
84 (2011) was adequate in capturing the dynamical pattern of ammonia and gas production  
85 across a large variety of substrates. In contrast, model predictions of total VFA were less  
86 accurate and finally predictions of pH were rather unsatisfactory. The extensions that we  
87 pursued in the present work results in an alternative model structure compared to exist-

ing models of rumen fermentation. Our model development has the following features: *i*) it proposes an alternative representation of the rumen microbiota by the incorporation of three theoretical microbial functional groups responsible for the utilization of hexoses, amino acids and hydrogen, *ii*) it includes a mechanistic description of the pH by incorporating acid-base reactions, *iii*) it includes liquid-gas transfer phenomena for hydrogen, methane and carbon dioxide, and *iv*) it separates sugars and amino acids metabolism and the partition rules are defined by using knowledge on the main biochemical reactions of the fermentation.

## 2. Material and methods

### 2.1. Experimental case study

The model here developed was built on the basis of an *in vitro* experimental study devoted to quantify the effect and interaction of both inocula and substrates on ruminal fermentation (Serment et al., 2016). Two substrates differing in the proportion of concentrate were evaluated: a low ( $L_s$ ) concentrate substrate (350 g  $\text{kg}^{-1}$  dry matter (DM) concentrate) versus a high ( $H_s$ ) concentrate substrate (700 g  $\text{kg}^{-1}$ ). The substrate was composed of grass hay, dehydrated alfalfa and concentrate as described by Serment (2012). The ingredient and analytical composition of these diets is presented in Table 1. Ruminal contents from adult goats in mid-lactation were used as inoculum. Two types of inocula were evaluated. The first inoculum ( $L_i$ ) was composed of rumen fluids from three goats adapted to the  $L_s$  diet. The second inoculum ( $H_i$ ) was composed of rumen fluids from three goats adapted to the  $H_s$  diet. The *in vitro* experiment was therefore defined by a  $2 \times 2$  factorial design where the factors were the inoculum type (with levels  $L_i$  vs.  $H_i$ ) and the substrate type (with levels  $L_s$  vs.  $H_s$ ). The resulting four treatment combinations were  $L_iL_s$ ,  $L_iH_s$ ,  $H_iL_s$ ,  $H_iH_s$ .

The experimental technique used in the work of Serment et al. (2016) is an adaptation of the syringe gas test (Menke et al., 1979) developed by Maaroufi et al. (2009) that allows to measure the fermentation dynamics. The fermentation took place during 24 h at constant temperature of 39°C. It was monitored by measurements of pH, acetate, butyrate, propionate, branched-chain volatile fatty acids, valerate and  $\text{NH}_3$  at  $t = 0, 3, 6, 9, 12, 24$

117 hours. The production of gas and its composition ( $\text{CH}_4$ ,  $\text{H}_2$  and  $\text{CO}_2$ ) were measured for  
118 the time intervals: 0-3h, 3-12h, 12-24h. Experimental data are means of eight replicates.  
119 These data were used for estimating the parameters of the model.

## 120 2.2. Model development

121 The mathematical model was built on the following assumptions:

122 *i)* Two pools of carbohydrates are considered, namely cell wall (structural) carbohy-  
123 drates, expressed as neutral detergent fibre (NDF), and non-structural carbohydrates  
124 (NSC). Following previous models of rumen fermentation, this distinction is made  
125 to account for the observed differences in degradation rates of feed carbohydrates.  
126 In our model, NSC does not include monosaccharides since they are represented  
127 separately as described below.

128 *ii)* Hydrolysis is an extracellular process that depends only on the concentration of  
129 polymers, *i.e.* carbohydrates and proteins. It is known that hydrolysis processes  
130 are carried out by multiple steps and are mediated by the enzymatic action of the  
131 microbes. However, it has been proposed that first-order kinetics reflect accurately  
132 the cumulative effect of the various processes involved in the hydrolysis (Waldo et al.,  
133 1972; Batstone et al., 2002). At this stage of our model development, we adopted  
134 the same premise.

135 *iii)* Hydrolysis of carbohydrates releases hexose monomers that are collectively repre-  
136 sented by a unique compartment of glucose.

137 *iv)* Hydrolysis of proteins releases amino acids that are collectively represented by an  
138 average pool of amino acids treated as a unique compartment. Formation and hydroly-  
139 sis of peptides are aggregated into the process of protein hydrolysis. The molecular  
140 formula of the average amino acid ( $\text{C}_5\text{H}_{9.8}\text{O}_{2.7}\text{N}_{1.5}$ ) was calculated using the mean  
141 values of amino acids composition of dehydrated alfalfa obtained from Feedipedia  
142 (<http://www.feedipedia.org/>). The nitrogen content of the average amino acid  
143 was of 13.4%. The molecular formula was corrected to set the nitrogen content to  
144 the standard value of 16% used in ruminant nutrition (see Appendix A).

- 145 *v)* The rumen microbiota is represented by three theoretical functional groups. A func-  
146 tional group is determined by the microbial utilization of a soluble substrate in the  
147 fermentation pathway. The substrates available for microbial utilization are sugars  
148 (represented by glucose), amino acids (average) and hydrogen. Therefore, three mi-  
149 crobial groups are accounted for, namely: sugars utilizers ( $x_{su}$ ), amino acids utilizers  
150 ( $x_{aa}$ ) and hydrogen utilizers ( $x_{H_2}$ ). The approach of representing the microbiota by  
151 several functional groups was not applied in the model of (Serment and Sauvant,  
152 2011). However, it has been used to model the fermentation in the human colon  
153 (Muñoz-Tamayo et al., 2009) and in anaerobic reactors (Batstone et al., 2002). It  
154 should be noted that this representation differs from the traditional way of represent-  
155 ing the rumen microbiota as fibre degraders and starch degraders (Dijkstra et al.,  
156 1992).
- 157 *vi)* Ammonia is assumed to be the sole source of nitrogen for sugars utilizers. This  
158 assumption is a simplification of the biochemistry of the fermentation, since sugars  
159 utilizers are able to use both  $NH_3$  and amino acids as nitrogen source of microbial  
160 protein (Wallace et al., 1997). Our assumption is however consistent with microbio-  
161 logical studies with both cellulolytic and noncellulolytic ruminal bacteria that have  
162 shown that under ruminal physiological conditions,  $NH_3$  provides in average 80% of  
163 microbial N (Atasoglu et al., 2001). In our model, the positive effect of amino acid  
164 on microbial growth of sugars utilizers is thus represented by the supply of  $NH_3$  from  
165 deamination.
- 166 *vii)* Soluble substrates are utilized towards product formation and microbial growth.  
167 Substrate utilization depends on the concentration of the respective substrate and  
168 the microbial functional group.
- 169 *viii)* Dead microbial cells are recycled as source of proteins and carbohydrates.
- 170 *ix)* Acetate, propionate and butyrate are assumed to be the only VFAs produced during  
171 fermentation.
- 172 *x)* Acid-base reactions are considered as instantaneous.
- 173 *xi)* Microbial maintenance was not explicitly represented in our model, although some  
174 works assume that decay rates (as the death cell rate in our model) can take into

175 account maintenance (Xu et al., 2011; Sari and Harmand, 2016). Thus, microbial  
176 maintenance requirements were assumed to be negligible.

177 The model integrates metabolic conversions, liquid-gas transfer and acid-base reac-  
178 tions. The model comprises 18 ordinary differential equations resulting from mass balance  
179 equations for a batch system and algebraic equations representing the acid-base reactions.  
180 The resulting model is therefore a differential algebraic equation (DAE) model. The no-  
181 tation used in the model and the list of abbreviations used is shown in Table 2. In the  
182 next sections, model equations are detailed. We adopted the formalism used in mathe-  
183 matical models developed to describe the anaerobic digestion in reactors for waste water  
184 treatment (Batstone et al., 2002) and fermentation in the human colon (Muñoz-Tamayo  
185 et al., 2010). We have borrowed modelling concepts from the model developed by Bat-  
186 stone et al. (2002) (the Anaerobic Digestion Model No.1 (ADM1)) to define our model  
187 by considering the specificities of rumen fermentation. For example, the ADM1 includes  
188 reactions of VFA oxidation and acetoclastic methanogenesis. These reactions were not  
189 included in our model given that they are not relevant for the rumen ecosystem.

### 190 *2.2.1. Microbial metabolism and liquid-gas transfer*

191 To keep a relative simple structure, our model aggregated the metabolic pathways of  
192 rumen fermentation into a limited number of macroscopic reactions, which have been doc-  
193 umented in the literature for a long time (Wolin et al., 1997). A scheme representing the  
194 mass fluxes of the fermentation process is shown in Fig. 1. The utilization of substrates in  
195 the fermentation drives two reaction processes namely product formation and microbial  
196 growth. In addition to these biological reactions, mass transfer phenomena of  $H_2$ ,  $CH_4$   
197 and  $CO_2$  between the liquid and gas phases occur.

198 The model has 18 state variables (compartments in Fig. 1) that correspond to the con-  
199 centrations of polymer components ( $z_i$ , g/L), soluble components ( $s_i$ , mol/L), microbial  
200 functional groups ( $x_i$ , mol/L) and to the amount of components in the gas phase ( $n_{g,i}$ ,  
201 moles). Polymer components are cell wall carbohydrates ( $z_{ndf}$ ), non-structural carbohy-  
202 drates ( $z_{nsc}$ ) and proteins ( $z_{pro}$ ). Soluble components are sugars ( $s_{su}$ ), amino acids ( $s_{aa}$ ),  
203 hydrogen ( $s_{H_2}$ ), acetate ( $s_{ac}$ ), butyrate ( $s_{bu}$ ), propionate ( $s_{pr}$ ), inorganic nitrogen ( $s_{IN}$ )



204 and inorganic carbon ( $s_{\text{IC}}$ ). The inorganic carbon concentration  $s_{\text{IC}}$  is the sum of the  
 205 concentrations of soluble carbon dioxide ( $s_{\text{CO}_2}$ ) plus bicarbonate ions ( $s_{\text{HCO}_3^-}$ ). Similarly,  
 206 the inorganic nitrogen concentration  $s_{\text{IN}}$  is the sum of the concentrations of ammonia  
 207 ( $s_{\text{NH}_3}$ ) plus ammonium ions ( $s_{\text{NH}_4^+}$ ). For the sake of readability, these relationships are  
 208 not depicted in Fig. 1 but are further explained after the definition of equations (12) and  
 209 (13). Microbial functional groups are sugars utilizers ( $x_{\text{su}}$ ), amino acids utilizers ( $x_{\text{aa}}$ ) and  
 210 hydrogen utilizers ( $x_{\text{H}_2}$ ). Moles in the gas phase correspond to hydrogen ( $n_{\text{g,H}_2}$ ), carbon  
 211 dioxide ( $n_{\text{g,CO}_2}$ ) and methane ( $n_{\text{g,CH}_4}$ ).

212 The breakdown and fermentation of feedstuffs by the rumen microbiota was repre-  
 213 sented in the model through nine biological processes: hydrolysis of cell wall carbohy-  
 214 drates ( $\rho_{\text{ndf}}$  (g/(L·h)), hydrolysis of non-fibre carbohydrates ( $\rho_{\text{nsc}}$  (g/(L·h)), hydrolysis of  
 215 proteins ( $\rho_{\text{pro}}$  (g/(L·h)), utilization of sugars ( $\rho_{\text{su}}$  (mol/(L·h)), utilization of amino acids  
 216 ( $\rho_{\text{aa}}$  (mol/(L·h)), utilization of hydrogen ( $\rho_{\text{H}_2}$  (mol/(L·h)) and the death of the three mi-  
 217 crobial groups ( $\rho_{x_{\text{su}}}, \rho_{x_{\text{aa}}}, \rho_{\text{H}_2}$ ) expressed in mol/(L·h). The mass transfer phenomena of  
 218  $\text{H}_2$ ,  $\text{CH}_4$  and  $\text{CO}_2$  between the liquid and gas phases are represented by the kinetic rates  
 219  $\rho_{\text{T,H}_2}, \rho_{\text{T,CH}_4}, \rho_{\text{T,CO}_2}$ , and are given in mol/(L·h).

220 The microbial process associated with utilization or hydrolysis of the component  $j$  is  
 221 represented by the kinetic rate  $\rho_j$ , which is given in g/(L·h) or mol/(L·h). Hydrolysis and  
 222 microbial death were described by first-order kinetics. Substrate utilization was described  
 223 by Monod (Michaelis-Menten) kinetics. The Monod equation is the most widely used  
 224 expression to describe microbial growth rate. The kinetic rate of substrate utilization  
 225 ( $\rho_j$ ) by Monod kinetics is

$$\rho_j = k_{\text{m},j} \frac{s_j}{K_{\text{s},j} + s_j} x_j \quad (1)$$

226 Where  $s_j$  and  $x_j$  represent respectively the substrate and the microbial biomass con-  
 227 centrations. The kinetic expression is defined by two parameters namely the maximum  
 228 specific utilization rate constant of substrate ( $k_{\text{m},j}$ ) and the Monod constant ( $K_{\text{s},j}$ ). The  
 229 maximum specific utilization rate constant of substrate relates to microbial activity, and  
 230 the Monod constant relates to substrate affinity of the microbes. These parameters will  
 231 be defined in the parameter estimation strategy, as it will be explained later. To account  
 232 for nitrogen limitation on the rates of sugars and hydrogen utilization, and additional

233 factor was included in the respective kinetic expressions (see Table 3). Monod kinetics  
 234 is a comprehensive equation that has the advantage of being defined by parameters with  
 235 biological meaning.

236 The formation or consumption of the component  $j$  in the process described by the  
 237 kinetic rate  $\rho_j$  is defined via the yield factors as summarized in Table 3. This way of rep-  
 238 resentation, sometimes denoted as the Petersen matrix (Petersen, 1965), is well suited for  
 239 displaying the stoichiometry of the fermentation through the yield factors ( $Y_j, Y_{i,j}$ ). This  
 240 type of approach has been already applied at the elementary level of rumen fermentation  
 241 (Reichl and Baldwin, 1975) and allows to reducing substantially the number of model  
 242 parameters to be estimated. Referring to the Table 3, one mole of substrate  $s_j$  utilized  
 243 produces  $Y_j$  moles of microbial biomass. The amount of moles of the compound  $s_i$  that  
 244 is either produced or consumed during the substrate utilization of  $s_j$  is given by the yield  
 245 factor  $Y_{i,j}$ . The link between yield factors and stoichiometry will be explained in the next  
 246 section.

247 On the basis of the previous statements on model structure, we proceed to detail the  
 248 equations of the model. By applying mass balances, we obtain the following equations.

*For polymer components*

$$\frac{dz_{\text{ndf}}}{dt} = -\rho_{\text{ndf}} \quad (2)$$

$$\frac{dz_{\text{nsc}}}{dt} = -\rho_{\text{nsc}} + (f_{\text{ch},x} \cdot w_{\text{mb}}) \cdot (\rho_{x_{\text{su}}} + \rho_{x_{\text{aa}}} + \rho_{x_{\text{H}_2}}) \quad (3)$$

$$\frac{dz_{\text{pro}}}{dt} = -\rho_{\text{pro}} + (f_{\text{pro},x} \cdot w_{\text{mb}}) \cdot (\rho_{x_{\text{su}}} + \rho_{x_{\text{aa}}} + \rho_{x_{\text{H}_2}}) \quad (4)$$

249 Where  $\rho_{\text{ndf}}, \rho_{\text{nsc}}, \rho_{\text{pro}}$  are the hydrolysis rates of the polymers, described by first-order  
 250 kinetics with respect to the substrate (g/(L·h)). The second terms in the right-hand side  
 251 of equations (3) and (4) represent the recycling of dead microbial cells. The rate of dead  
 252 of microbial cells is determined by the parameter  $k_d$  (1/h) (Table 3). The parameters  
 253  $f_{\text{ch},x}, f_{\text{pro},x}$  are the fractions of carbohydrates and proteins by weight of microbial cells.  
 254 The molecular weight of microbial cells is  $w_{\text{mb}}$  (Table 2).

For soluble components

$$\frac{ds_{\text{su}}}{dt} = \rho_{\text{ndf}}/w_{\text{su}} + \rho_{\text{nsc}}/w_{\text{su}} - \rho_{\text{su}} \quad (5)$$

$$\frac{ds_{\text{aa}}}{dt} = \rho_{\text{pro}}/w_{\text{aa}} - \rho_{\text{aa}} \quad (6)$$

$$\frac{ds_{\text{H}_2}}{dt} = Y_{\text{H}_2,\text{aa}} \cdot \rho_{\text{aa}} + Y_{\text{H}_2,\text{su}} \cdot \rho_{\text{su}} - \rho_{\text{H}_2} - \rho_{\text{T,H}_2} \quad (7)$$

$$\frac{ds_{\text{ac}}}{dt} = Y_{\text{ac},\text{su}} \cdot \rho_{\text{su}} + Y_{\text{ac},\text{aa}} \cdot \rho_{\text{aa}} \quad (8)$$

$$\frac{ds_{\text{bu}}}{dt} = Y_{\text{bu},\text{su}} \cdot \rho_{\text{su}} + Y_{\text{bu},\text{aa}} \cdot \rho_{\text{aa}} \quad (9)$$

$$\frac{ds_{\text{pr}}}{dt} = Y_{\text{pr},\text{su}} \cdot \rho_{\text{su}} + Y_{\text{pr},\text{aa}} \cdot \rho_{\text{aa}} \quad (10)$$

$$\frac{ds_{\text{CH}_4}}{dt} = Y_{\text{CH}_4,\text{H}_2} \cdot \rho_{\text{H}_2} - \rho_{\text{T,CH}_4} \quad (11)$$

255 To illustrate the structure of the model, let us consider the dynamics of sugars con-  
 256 centration ( $s_{\text{su}}$ ), which has a molecular weight of  $w_{\text{su}}$ . Sugars are produced from the  
 257 hydrolysis of cell wall carbohydrates ( $z_{\text{ndf}}$ ) and non-structural carbohydrates ( $z_{\text{nsc}}$ ). The  
 258 hydrolysis rates are represented by  $\rho_{\text{ndf}}$  and  $\rho_{\text{nsc}}$  respectively. Sugars are further fermented  
 259 (following the kinetic rate  $\rho_{\text{su}}$ ) and produces, among other metabolites, acetate and  $\text{H}_2$   
 260 with a stoichiometry given by the yield factors  $Y_{\text{ac},\text{su}}$  and  $Y_{\text{H}_2,\text{su}}$  respectively. Hydrogen in  
 261 the liquid phase is utilized by the microbes following the kinetic rate  $\rho_{\text{H}_2}$  and participates  
 262 in a mass transfer phenomena with the hydrogen pool of the gas phase. This transfer is  
 263 represented by the kinetic rate  $\rho_{\text{T,H}_2}$ .

264 The concentrations of individual VFAs are the sum of the concentrations of the ionic  
 265 (conjugate base) and free (undissociated) forms. So, instead of modelling the individual  
 266 acid and base forms, we model the total VFA and one of the acid-base components. This  
 267 choice was made to facilitate the implementation of the model (Rosen et al., 2006). This  
 268 same reasoning was applied for the couples  $s_{\text{CO}_2} \leftrightarrow s_{\text{HCO}_3^-}$  (gathered in the variable  $s_{\text{IC}}$ )  
 269 and  $s_{\text{NH}_3} \leftrightarrow s_{\text{NH}_4^+}$  (gathered in the variable  $s_{\text{IN}}$ ). The dynamics of  $s_{\text{IC}}$  and  $s_{\text{IN}}$  are defined  
 270 as follows.

$$\frac{ds_{\text{IC}}}{dt} = Y_{\text{IC},\text{aa}} \cdot \rho_{\text{aa}} + Y_{\text{IC},\text{su}} \cdot \rho_{\text{su}} + Y_{\text{IC},\text{H}_2} \cdot \rho_{\text{H}_2} - \rho_{\text{T,CO}_2} \quad (12)$$

$$\frac{ds_{\text{IN}}}{dt} = Y_{\text{IN},\text{aa}} \cdot \rho_{\text{aa}} + Y_{\text{IN},\text{su}} \cdot \rho_{\text{su}} + Y_{\text{IN},\text{H}_2} \cdot \rho_{\text{H}_2} \quad (13)$$

For microbial functional groups

$$\frac{dx_{\text{su}}}{dt} = Y_{\text{su}} \cdot \rho_{\text{su}} - \rho_{x_{\text{su}}} \quad (14)$$

$$\frac{dx_{\text{aa}}}{dt} = Y_{\text{aa}} \cdot \rho_{\text{aa}} - \rho_{x_{\text{aa}}} \quad (15)$$

$$\frac{dx_{\text{H}_2}}{dt} = Y_{\text{H}_2} \cdot \rho_{\text{H}_2} - \rho_{x_{\text{H}_2}} \quad (16)$$

271 It should be noted that the fermentation reactions have been defined on the basis  
 272 of the kinetic rate of substrate utilization ( $\rho_j$ ). In some works, the growth rate ( $\mu_j$ ) is  
 273 rather used. If maintenance requirements are negligible as presently stated, the growth  
 274 and substrate utilization rates are related by

$$\mu_j = Y_j \cdot \rho_j \quad (17)$$

275 The maximal growth rate occurs when the substrate utilization is maximal. It is important  
 276 to note that in the model structure used here, the microbial yields  $Y_j$  are actually linked  
 277 to the ATP derived from the reactions of product formation as it will be explained in the  
 278 next section.

Now, for the gas phase, we have

$$\frac{dn_{\text{g,H}_2}}{dt} = V_l \cdot \rho_{\text{T,H}_2} \quad (18)$$

$$\frac{dn_{\text{g,CO}_2}}{dt} = V_l \cdot \rho_{\text{T,CO}_2} \quad (19)$$

$$\frac{dn_{\text{g,CH}_4}}{dt} = V_l \cdot \rho_{\text{T,CH}_4} \quad (20)$$

The term  $\rho_{\text{T},i}$  represents the liquid-gas transfer rate (at non-equilibria), described as

$$\rho_{\text{T,H}_2} = k_{\text{L}}a \cdot (s_{\text{H}_2} - K_{\text{H,H}_2} \cdot p_{\text{g,H}_2}) \quad (21)$$

$$\rho_{\text{T,CO}_2} = k_{\text{L}}a \cdot (s_{\text{CO}_2} - K_{\text{H,CO}_2} \cdot p_{\text{g,CO}_2}) \quad (22)$$

$$\rho_{\text{T,CH}_4} = k_{\text{L}}a \cdot (s_{\text{CH}_4} - K_{\text{H,CH}_4} \cdot p_{\text{g,CH}_4}) \quad (23)$$

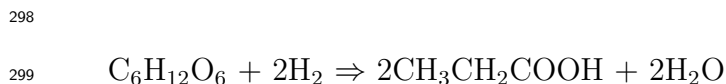
279 Where  $k_{\text{L}}a$  ( $\text{h}^{-1}$ ) is the mass transfer coefficient,  $K_{\text{H},i}$  ( $\text{M}/\text{bar}$ ) is the Henry's law coeffi-  
 280 cient and  $p_{\text{g},i}$  (bars) is the partial pressure of component  $i$ . For simplification, we assumed  
 281 that the total pressure in the gas phase was constant. Its value was set to  $P = 1.01325$   
 282 bars. Therefore, the partial pressure of component  $i$  satisfies

$$p_{\text{g},i} = \frac{n_{\text{g},i}}{\sum_{i=1}^3 n_{\text{g},i}} P \quad (24)$$

283 *2.2.2. Stoichiometry of the fermentation*

284 The yield factors defined in the model can be estimated experimentally or via param-  
 285 eter estimation. In our case, we have capitalized on the knowledge of the stoichiometry  
 286 of certain well known reactions to favour a mechanistic representation of metabolism and  
 287 to reducing the dimension of unknown parameters. For the microbial utilization of sugars  
 288 (glucose) and hydrogen, the reactions accounted for by the model are given in Table 4.  
 289 The microbial fermentation profile is defined by the partitioning of fluxes between product  
 290 formation and microbial growth reactions. To define the stoichiometry of the microbial  
 291 growth reaction, it was assumed that the microbes have an average molecular formula of  
 292  $C_5H_7O_2N$  (Batstone et al., 2002).

293 To illustrate how the yield factors were calculated, let us consider the utilization of  
 294 sugars described in the model by reactions  $R_1$ - $R_4$  in Table 4. Firstly, it should be noted  
 295 that reaction  $R_2$  associated with propionate formation also produces acetate. Some works  
 296 on rumen stoichiometry consider as possible pathway for propionate formation the reac-  
 297 tion:



300  
 301 However, propionate production by rumen microbes concomitantly occurs with ac-  
 302 etate production as identified in the fermentation pathway of the predominant propionate  
 303 producer *Selenomonas ruminantium* (Wolin et al., 1997). For these reasons, we decided  
 304 to use the reaction  $R_2$  (Table 4) as the main pathway of propionate formation.

305 As previously mentioned, substrate conversion is directed to reactions of product for-  
 306 mation and microbial growth. The balance of these two processes is coupled to the cofactor  
 307 ATP/ADP. In our model, the partition of substrate is parameterized as follows. The frac-  
 308 tion of glucose utilized for microbial growth is defined by the microbial yield factor  $Y_{su}$   
 309 (which is linked to the ATP available from microbial synthesis). Let  $f_{su}$  be the fraction  
 310 of sugars that is utilized for product formation. From the reaction  $R_4$  of the Table 4, it  
 311 follows that

$$f_{su} = 1 - \frac{5}{6}Y_{su} \quad (25)$$

Now, the utilization of sugars for product formation occurs only via the reactions R<sub>1</sub>-R<sub>3</sub>. Let  $\lambda_k$  denote the fraction of the sugars utilized via reaction  $k$  of Table 4, such that  $\sum_{k=1}^3 \lambda_k = 1$  to respect mass conservation. The yield factors for sugars utilization can thus be defined as follows

$$Y_{ac,su} = f_{su} \cdot (2 \cdot \lambda_1 + 2/3 \cdot \lambda_2) \quad (26)$$

$$Y_{bu,su} = f_{su} \cdot (\lambda_3) \quad (27)$$

$$Y_{pr,su} = f_{su} \cdot (4/3 \cdot \lambda_2) \quad (28)$$

$$Y_{H_2,su} = f_{su} \cdot (4 \cdot \lambda_1 + 2 \cdot \lambda_3) \quad (29)$$

$$Y_{IN,su} = -Y_{su} \quad (30)$$

$$Y_{IC,su} = f_{su} \cdot (2 \cdot \lambda_1 + 2/3 \cdot \lambda_2 + 2 \cdot \lambda_3) \quad (31)$$

312 Note that by using the stoichiometry of the reactions, we only need three parameters  
 313 to be estimated ( $Y_{su}, \lambda_1, \lambda_2$ ) to define all the yield factors for sugars utilization. Without  
 314 including the stoichiometry, we would have to estimate seven parameters.

315 Applying the same principles for hydrogen utilization, we obtain the following rela-  
 316 tionships for determining the yield factors.

$$f_{H_2} = 1 - 10Y_{H_2} \quad (32)$$

$$Y_{CH_4,H_2} = f_{H_2} \cdot (1/4) \quad (33)$$

$$Y_{IC,H_2} = -((1/4) \cdot f_{H_2} + (5/10) \cdot (1 - f_{H_2})) \quad (34)$$

$$Y_{IN,H_2} = -Y_{H_2} \quad (35)$$

317 For amino acids utilization, on the basis of amino acid composition from alfalfa, a  
 318 theoretical reaction of the overall fermentation was derived (R5 in Table 4). This overall  
 319 reaction was obtained by selecting the main fermentation reactions of individual amino  
 320 acids for anaerobic bacteria. Following the procedure proposed by Ramsay and Pullam-  
 321 manappallil (2001), the overall reaction results from weighing the individual reactions by  
 322 the molar composition of the feed. The procedure for deriving the overall fermentation  
 323 reaction is detailed in Appendix A. The yield factor of component  $i$  ( $Y_{i,aa}$ ) is defined by  
 324 the following equation

$$Y_{i,aa} = (1 - Y_{aa}) \cdot \sigma_{i,aa} \quad (36)$$

325 Where the parameter  $\sigma_{i,aa}$  is the stoichiometric coefficient of the overall product for-  
 326 mation reaction.

327 To respect the elementary balance of nitrogen, we set the following relationship:

$$Y_{IN,aa} = N_{aa} - Y_{aa} \cdot N_{mb} \quad (37)$$

328 with  $Y_{aa}$  (mol/mol) and  $Y_{IN,aa}$  (mol/mol) the yield factors of microbial cells and inorganic  
 329 nitrogen during amino acids utilization.  $N_{mb}$  is the molar fraction of nitrogen of the  
 330 microbial cells,  $N_{aa}$  the nitrogen molar fraction of the average amino acid.

331 It should be stressed that using biochemical reactions to defining the stoichiometry  
 332 allows the automatic maintaining of elementary balances and reduces the number of un-  
 333 known parameters of the model.

### 334 2.2.3. Acid-base reactions, charge balance and pH

Bases and acid compounds are made up by acid-base pairs. It follows that

$$s_{IC} = s_{HCO_3^-} + s_{CO_2} \quad (38)$$

$$s_{IN} = s_{NH_4^+} + s_{NH_3} \quad (39)$$

$$s_{ac} = s_{ac^-} + s_{hac} \quad (40)$$

$$s_{bu} = s_{bu^-} + s_{hbu} \quad (41)$$

$$s_{pr} = s_{pr^-} + s_{hpr} \quad (42)$$

335 For the VFAs, for instance acetate,  $s_{ac^-}$  and  $s_{hac}$  are the concentrations of the VFA in  
 336 ionic and free forms respectively.

By using the acid-base equilibrium equations, the concentrations of the ions are for-

337 mulated as functions of the concentration of the hydrogen ion  $s_{H^+}$

$$s_{HCO_3^-} = \frac{K_{a,CO_2} s_{IC}}{K_{a,CO_2} + s_{H^+}} \quad (43)$$

$$s_{NH_4^+} = \frac{s_{IN} s_{H^+}}{K_{a,NH_4} + s_{H^+}} \quad (44)$$

$$s_{ac^-} = \frac{K_{a,ac} s_{ac}}{K_{a,ac} + s_{H^+}} \quad (45)$$

$$s_{bu^-} = \frac{K_{a,bu} s_{bu}}{K_{a,bu} + s_{H^+}} \quad (46)$$

$$s_{pr^-} = \frac{K_{a,pr} s_{pr}}{K_{a,pr} + s_{H^+}} \quad (47)$$

$$s_{OH^-} = \frac{K_w}{s_{H^+}} \quad (48)$$

337 Since the equilibrium constants (and pKa values) for the VFA are very close (see, *e.g.*,  
 338 Kohn and Dunlap (1998)), we decided to simplify by aggregating the three acid-base  
 339 reactions of VFA into a single one, that is

$$s_{VFA^-} = \frac{K_{a,VFA} s_{VFA}}{K_{a,VFA} + s_{H^+}} \quad (49)$$

340 With  $s_{VFA^-} = s_{ac^-} + s_{bu^-} + s_{pr^-}$  and  $s_{VFA} = s_{ac} + s_{bu} + s_{pr}$ . This approach favours the  
 341 mathematical treatment of the equations and does not have significant implications on  
 342 the model output.

343 The pH of the medium is determined by the charge balance between the cations and  
 344 anions from the acid-base reactions. It follows that the sum of cations minus the sum of  
 345 anions must be equal to zero. Therefore

$$s_{cat^+} + s_{NH_4^+} + s_{H^+} - (s_{HCO_3^-} + s_{VFA^-} + s_{OH^-}) = 0 \quad (50)$$

346 In Equation (50),  $s_{cat^+}$  represents the balance of cations and anions of the medium that  
 347 are not modelled. They referred to metallic ions. For the medium solution, we only  
 348 considered bicarbonate in the model. However, the medium is actually composed of buffer  
 349 and mineral solutions that contains components such phosphate (Menke et al., 1979) that  
 350 have an effect on the pH. Using experimental data at  $t=0$ , we calculated the concentration  
 351 of  $s_{HCO_3^-}$  needed to make the charge balance equal to zero. Without including  $s_{cat^+}$ , the  
 352 bicarbonate concentration was negative, which has no physical meaning. Thus, it was



353 essential to include the metallic ions  $s_{\text{cat}+}$ . We assumed that  $s_{\text{cat}+}$  remains constant over  
354 the fermentation.

355 By inserting equations (43),(44),(48) and (49) into equation (50), we obtain a poly-  
356 nomial of degree five with  $s_{\text{H}+}$  as independent variable. The structure of the polynomial  
357 makes that it has a unique real positive root. At each time step, the polynomial is solved  
358 to find  $s_{\text{H}+}$ . The pH is therefore determined by  $\text{pH} = -\log_{10}[s_{\text{H}+}]$ .

359 The model was implemented in Matlab<sup>®</sup> following guidelines for models of anaerobic  
360 digestion in reactors for waste water treatment (Batstone et al., 2002; Rosen et al., 2006).  
361 The Matlab code is available on request for academic purposes. The resulting model  
362 presents multiple time scales. For instance, while hydrolysis processes are characterized  
363 by time constants that can be of the order of days, acid-base equilibrium reactions take  
364 place almost instantaneously. In numerical computation, this property is also referred to  
365 as stiffness. Model simulation was carried out with the Matlab ODE solver ode23s. The  
366 algorithm used in ode23s is based on a modified Rosenbrock formula of order 2, adapted  
367 to stiff models (Shampine and Reichelt, 1997).

### 368 *2.3. Parameter estimation and statistical analysis*

369 For the parameter estimation routine, we aimed at reducing the number of parameters  
370 to be estimated. Accordingly, some parameters were set to be known and common to  
371 all the treatment combinations. The values of these parameters were extracted from the  
372 literature. Most of the physicochemical parameters were fixed to known values reported by  
373 Batstone et al. (2002) and extracted from the operational conditions of the experimental  
374 study of Serment et al. (2016). The values reported by Batstone et al. (2002) for the CO<sub>2</sub>  
375 and bicarbonate system are very similar to values reported for the rumen (Hille et al.,  
376 2016). Only  $k_{\text{La}}$  and  $s_{\text{cat}+}$  were estimated.

377 The biological parameters that were fixed were the hydrolysis rate constant of cell wall  
378 carbohydrates, the death cell rate constant (assumed to be the same for all microbes in  
379 all experimental conditions), the fraction of carbohydrates and protein of the microbial  
380 biomasses, the stoichiometric coefficients for amino acid fermentation, the nitrogen limita-  
381 tion constant, and the affinity Monod constants for the utilization of sugars, amino acids

382 and hydrogen. The hydrolysis rate constant for cell wall carbohydrates was set constant  
 383 because different studies have shown that digestibility of NDF appears to be unaffected by  
 384 the level of concentrate (Nagadi et al., 2000; Serment et al., 2011). Setting the values of  
 385 the affinity constants reduces the difficulties associated with practical identifiability prob-  
 386 lems (*e.g.* high correlation between the parameters of Monod kinetics). Table 5 shows  
 387 the primary sources used to extract the parameter values. Estimation was therefore per-  
 388 formed for the hydrolysis rate constants of non-structural carbohydrates and protein, the  
 389 maximum specific utilization rate constants of substrates, the microbial yield factors and  
 390 the parameters related to the flux distribution for sugars and amino acid utilization. The  
 391 parameter estimation was performed independently for each treatment combination. At  
 392  $t_0$ , on the basis of microbiological data (Krause and Russell, 1996; Morgavi et al., 2010),  
 393 the composition of the microbiota was set as 94% of sugar utilizers, 5 % of amino acids  
 394 utilizers and 1 % of methanogens. The initial guess for the total microbial concentration  
 395 was extracted from reported values of *in vitro* rumen fermentation (Nagadi et al., 2000).  
 396 In the study of Serment et al. (2016), the incubation of blanks (inoculum without addi-  
 397 tion of substrate) showed that the inoculum was biologically active and that it contained  
 398 enough energy sources to allow the fermentation to proceed towards VFA and methane  
 399 production. Therefore, the parameter estimation was set to consider the contribution of  
 400 NSC and protein of the inoculum. The initial concentration of sugars were set to 0.67 mM  
 401 which is the range reported for the rumen (Janssen, 2010). Initial conditions for dissolved  
 402 hydrogen and methane were taken from Wang et al. (2016a) and were set to 2  $\mu$ M and  
 403 0.7 mM for hydrogen and methane respectively. Initial guess values used for the estima-  
 404 tion were taken from studies of rumen and anaerobic microbial ecosystems (Robinson and  
 405 Tiedje, 1982; Baldwin et al., 1987; Batstone et al., 2002).

406 Parameter estimation was performed with the Matlab<sup>®</sup> toolbox IDEAS (Muñoz-  
 407 Tamayo et al., 2009), which is freely available at  
 408 <http://www.inra.fr/miaj/public/logiciels/ideas/index.html>.

409 We used the maximum likelihood estimator that minimizes

$$J(\boldsymbol{\theta}) = \sum_{k=1}^{n_y} \frac{n_{t,k}}{2} \ln \left[ \sum_{i=1}^{n_{t,k}} [y_k(t_{i_k}) - y_{m_k}(t_{i_k}, \boldsymbol{\theta})]^2 \right] \quad (51)$$

410 With  $n_y$  the number of measured (observed) variables and  $n_{t,k}$  the number of observation  
411 times for the  $k$ th variable. The observed variables are  
412  $\{s_{ac}, s_{bu}, s_{pr}, s_{IN}, n_{g,H_2}, n_{g,CO_2}, n_{g,CH_4}, pH\}$

413 Equation (51) applies to the case of asynchronous measurements (see Walter and  
414 Pronzato (1997)). This equation is adapted to our experimental case study. The Nelder-  
415 Mead Simplex method (Lagarias et al., 1998) was used as the optimization algorithm.  
416 Upper and lower bounds were imposed to force the parameters to lie on the interval of  
417 values reported in the literature.

418 The model was evaluated by regression analysis of observed values against predicted  
419 values as suggested by Piñeiro et al. (2008). The root mean squared error (RMSE)  
420 was calculated as a statistics of model performance. The agreement between observed  
421 values and predicted values was assessed by the concordance correlation coefficient (CCC)  
422 proposed by Lin (1989). Statistical analyzes were performed with Matlab<sup>®</sup>.

### 423 3. Results

#### 424 3.1. Dynamical description of *in vitro* fermentation and model evaluation

425 Figures 2 and 3 display the *in vitro* data obtained from Serment et al. (2016) and the  
426 response of the calibrated model. A common scale is used to facilitate the comparison  
427 between the responses of the two inocula. The model satisfactorily captures the dynamics  
428 of all variables. It is worth noting that the model is able to represent the inflexions of the  
429 pH dynamics. This dynamic behaviour of the pH is also observed *in vivo* after feeding.  
430 Figure 4 summarizes the results by plotting the observations against model predictions.  
431 Table 6 summarizes the results of the regression analysis. For all variables, the intercept  
432 constants were not significant different from zero ( $p$ -value > 0.05) and the slopes of the  
433 regression lines were not different from one (with the exception of the regression curves  
434 for propionate, CH<sub>4</sub> and CO<sub>2</sub>). The regression line for CO<sub>2</sub> had the slowest slope (0.86)  
435 while the regression line of CH<sub>4</sub> had the highest one (1.23). This means that the model  
436 predictions of the amount of CH<sub>4</sub> are about 81% of the experimental data and the amount  
437 of CO<sub>2</sub> are about 1.16% of the experimental data. We will need to challenge the model  
438 against a new set of data to identify if the underprediction of CH<sub>4</sub> and overprediction of

439 CO<sub>2</sub> are related to the experimental device analyzed or whether the model parameters  
440 require further refinement.

441 The pH exhibited the lowest determination coefficient ( $r^2 = 0.83$ ) (Table 6), whereas  
442 for the other variables  $r^2 \geq 0.97$ . The model predictions exhibited high concordance cor-  
443 relation coefficients (CCC). For the pH and the CH<sub>4</sub>, the CCC was of 0.91 and 0.93 respec-  
444 tively. For the other variables  $CCC \geq 0.96$ . The pH had the lowest coefficient of variation  
445 of the RMSE (CV(RMSE)). The components in the gas phase had the higher CV(RMSE)  
446 (11%-25%), while the components in the liquid phase had in average a CV(RMSE) of  
447 4.9%.

448 Figure 5 displays the predicted dynamics of the non measured variables for all the treat-  
449 ment combinations. The concentration of carbohydrate and protein polymers decreased  
450 in time as result of the hydrolysis. Sugars followed a monotonic decreasing dynamics,  
451 implying that the rate of sugars utilization was always faster than the rate of hexose  
452 release from carbohydrate hydrolysis. It is observed that the dynamics of carbohydrates,  
453 protein and sugars is very similar among the treatment combinations and the curve re-  
454 sponses tend to overlap. For non-structural carbohydrates, it is observed that its initial  
455 concentration is higher for the experiments with the inoculum H<sub>i</sub> than for the inoculum  
456 L<sub>i</sub>.

457 For the average amino acid, it was observed that at the beginning of the fermentation,  
458 the production rate of amino acids from protein hydrolysis was higher than the amino  
459 acid utilization rate. After 2 h, microbes utilized amino acids at a higher rate than  
460 the protein hydrolysis rate. During the experimentation time all the microbial groups  
461 exhibited a growth rate higher than the decay rate. The dynamics of the microbial groups  
462 for the experiments with the same type of inoculum almost overlap. It is noted that the  
463 concentration of microbial biomass for the inoculum adapted to the high concentrate is  
464 higher than that of the inoculum adapted to the low concentrate, which is in agreement  
465 with the measurements reported by Nagadi et al. (2000). At the end of the fermentation,  
466 the microbiota was composed in average by 91.7% of sugars utilizers, 7.3% of amino acids  
467 utilizers and 1.0% of hydrogen utilizers.

468 Overall the predicted concentrations of sugars, amino acids, dissolved hydrogen and

469 methane are in agreement with values reported in the rumen literature (Atasoglu et al.,  
470 2001; Wang et al., 2016a; Janssen, 2010).

### 471 3.2. Model parameters

472 Table 5 shows the numerical values of parameters. The model has in total 9 physico-  
473 chemical parameters and 23 biological parameters (operational parameters such as pres-  
474 sure ( $P$ ) and temperature ( $T$ ), and the molecular weights of compounds are not counted).  
475 Figure 6 displays the estimated parameters for each treatment combination. Only two  
476 physicochemical parameters ( $k_{La}$ ,  $s_{cat+}$ ) were estimated. Their estimated values did not  
477 exhibit significant differences among the treatments, which was expected. The liquid-gas  
478 transfer coefficient  $k_{La}$  is specific to the *in vitro* system utilized and  $s_{cat+}$  is mainly deter-  
479 mined by the buffer solution used in the medium, which has the same concentration for  
480 all experiments.

481 From the set of biological parameters, 13 parameters were set as known. In defining  
482 these biological parameters, we assumed a constant composition of biomass in terms of  
483 proteins and carbohydrates for all microbial groups. Additionally, we set the death cell  
484 rate to be the same for all microbes in all experimental conditions. We assumed that  
485 the substrate affinity for sugars, amino acids and hydrogen by the rumen microbiota  
486 are similar among the four treatment combinations. The affinity constants for sugars  
487 and amino acids were taken from (Baldwin et al., 1987). For hydrogen utilizers, the  
488 affinity constant was set as an average value reported for rumen microbes (Robinson and  
489 Tiedje, 1982). Stoichiometric coefficients for amino acid fermentation were derived from  
490 biochemical knowledge (see Appendix A).

491 Given the limited number of experiments analyzed in this work, it will be incautious  
492 to provide general statements about the validity of the estimated parameters to repre-  
493 sent rumen fermentation in a broader context than the experimental study analyzed here.  
494 In spite of these limitations, the following analysis can be derived from the results. In  
495 Table 5, it is shown that the non-structural carbohydrates exhibited very similar hydroly-  
496 ysis rate constants among the experiments. The hydrolysis rate constants of NSC are  
497 higher than the hydrolysis rate constant of NDF (Table 5, which is biologically consis-

498 tent). Concerning protein hydrolysis, the hydrolysis rate constants are of the same order  
499 of magnitude than those for non-structural carbohydrates. The inoculum  $H_i$  adapted to  
500 the high concentrate diet appears to have a slightly higher hydrolysis rate constant of  
501 proteins than that of the inoculum  $L_i$ .

502 Concerning sugars utilization, the maximum specific utilization rate constant of sugars  
503 was the same for all experiments. The microbial yield for sugars utilization has an average  
504 value of 0.16 mol/mol. This value has the same order of magnitude of yield factors re-  
505 ported for utilization of glucose by rumen microbes (Russell and Wallace, 1997). There is  
506 not a clear dependency of the microbial yield factors with respect to either the inoculum  
507 or substrate.

508 Concerning amino acid utilization, the maximum specific utilization rate constant and the  
509 yield factors appear to be clustered with respect to the type of the inoculum. The group  
510 of amino acid utilizers of the inoculum  $L_i$  adapted to the low concentrate diet appears to  
511 have a higher growth than the microbial group of the inoculum  $H_i$ .

512 For hydrogen utilization, the microbial yield factors are the same for all the treatment  
513 combinations. The value corresponds to the lower bound used for constraining the opti-  
514 mization. This was done to allow the parameters to fall in a biological plausible space.  
515 The maximum specific utilization rate constant has an average value of 13.93 mol/(mol·h),  
516 which is in agreement with values reported for methanogens in reactors (Batstone et al.,  
517 2002).

518 From these results, we suggest that overall, the average values of the kinetic parame-  
519 ters ( $k_{m,j}, Y_j, Y_{i,j}$ ) can be used to represent the fermentation, with the exception of the  
520 parameters associated to amino acid utilization. However, since neither amino acids  
521 nor the microbial group related to their utilization were measured, new experiments are  
522 needed to confirm the specificity of amino acid utilization with respect to the substrate  
523 and inoculum. We suggest that the differences of the fermentation pattern between the  
524 treatment combinations are mainly due to the parameters associated to flux distribution  
525 ( $\lambda_1, \lambda_2$ ), which seem to depend to the type of the inoculum (Fig. 6). For example, in the  
526 experiments with the inoculum  $H_i$ , the direction of fluxes towards butyrate production  
527 from sugars utilization ( $\lambda_3$ ) is about 30% higher than that for the inoculum  $L_i$  (Table 5).

528 However, further validation with new data is required to confirm this hypothesis.

#### 529 **4. Discussion**

530 The objective of this work was to develop a mathematical model of rumen *in vitro* fer-  
531 mentation by taking into consideration some aspects that have been identified as central  
532 for the improvement of rumen fermentation models. These aspects include a better repre-  
533 sentation of the microbiota, hydrogen dynamics and a mechanistic description of pH. The  
534 resulting model was effective in describing data of four *in vitro* treatment combinations,  
535 which indicates the strengthen of its structure to represent fermentation.

536 Initially, our strategy of model construction used mainly as conceptual basis the model  
537 developed by Serment and Sauvant (2011). The extensions that we pursued resulted in a  
538 mathematical model that partially differs to the model of Serment and Sauvant (2011). A  
539 major difference of our approach compared to the model of Serment and Sauvant (2011)  
540 is the representation of the microbiota as three functional groups associated with the  
541 specific utilization of sugars, amino acids and hydrogen. Other differences relate to the  
542 incorporation of macroscopic biochemical reactions to define the yield factors that deter-  
543 mine the fermentation pattern, including the individual VFA. While Serment and Sauvant  
544 (2011) related methane production rate to the global fermentation rate of the soluble car-  
545 bohydrates compartment, we incorporated a hydrogen compartment that enabled us to  
546 explicitly represent the methanogenesis reaction and define partition rules of hydrogen.  
547 This approach will be helpful to further developments for describing the determinants of  
548 hydrogen fluxes in the rumen. In relation to the pH, our model is based on the compu-  
549 tation of the hydrogen ion concentration needed to balance the charges in the acid-base  
550 equilibrium reactions, while in the model of Serment and Sauvant (2011), the pH was  
551 determined by the equilibrium state of an acid-base reaction that aggregates the bicar-  
552 bonate and the total VFAs. The improvement in fitting capabilities of our model suggests  
553 that the model developments performed here are useful.

554 In terms of model structure, we have borrowed modelling concepts from the Anaero-  
555 bic Digestion Model No.1 (ADM1) (Batstone et al., 2002). The ADM1 was developed by  
556 international experts in the domain of anaerobic digestion processes for waste water treat-

557 ment. The ADM1 model is the result of a consensus of the state of the art in anaerobic  
558 digestion models, and it has been consolidated as a common modelling platform in the  
559 engineering community. The ADM1 has been used in a large number of applications and  
560 has also enriched the discussion between scholars for identifying key aspects for model  
561 improvement. Moreover, The ADM1 has been used as a basis for model developments  
562 of the fermentation in the human colon (Muñoz-Tamayo et al., 2010; Motelica-Wagenaar  
563 et al., 2014). The fermentation pathway as described in the original structure of ADM1  
564 does not apply to the rumen ecosystem and thus our work has focused on developing  
565 a model that accounts for the specificities of the fermentation performed by the rumen  
566 microbiota. To expand the capabilities of our model, it is of course needed to provide a  
567 mathematical description of the rumen environment and of the input and output fluxes  
568 of the rumen as it has been included in existing models (Baldwin et al., 1987; Dijkstra  
569 et al., 1992). We think that the mathematical structure developed here can facilitate the  
570 standardization of rumen models, which is central to boost further modelling progresses  
571 and favour exchange between rumen modellers. In addition, incorporation of new aspects  
572 on rumen function into mechanistic models can complexify existing rumen models. Given  
573 the different time scales of processes involved in rumen metabolism and their non-linear  
574 nature, adequate and robust software is required to provide numerically stable simula-  
575 tions. Matlab<sup>®</sup> and the free software Scilab (<http://www.scilab.org/fr>) are excellent  
576 options due to their numerical and functional capabilities.

#### 577 *4.1. Parameter estimation strategy*

578 The strategy that we used to estimate the parameters of the model aimed to reduce  
579 the number of parameters specific to each treatment combination to render possible the  
580 identification of the most sensitive parameters. A large body of knowledge on rumen  
581 fermentation was incorporated into the strategy via the setting of prior values or by  
582 relationships such as the biochemistry of sugars and amino acids fermentation. This  
583 approach facilitates the numerical issues related to the optimization problem. In addition,  
584 we avoid known difficulties associated with high correlation between the parameters of  
585 the Monod kinetics. This correlation is reflected by the fact that is often possible to



586 accurately estimate the ratio  $k_{m,j}/K_{s,j}$ , but not to accurately estimate the individual  
587 parameters (Holmberg, 1982).

588 The strategy used is not only suitable for handling numerical issues but also produces  
589 outcomes that have a clear biological interpretation. For instance, the experimental study  
590 of (Serment et al., 2016) identified the effect of the inoculum as the most influential  
591 factor of the fermentation pattern. This effect is reflected by the results of the parameter  
592 estimation. The estimated parameters of each treatment combination indicated that the  
593 fluxes distribution for sugars utilization depended mainly on the inoculum. However, it is  
594 important to keep in mind that the effect of the inoculum is modulated by the substrate  
595 via microbial adaptation (Broudiscou et al., 2014).

#### 596 *4.2. Stoichiometry and pathways of rumen fermentation*

597 Stoichiometry is a central aspect of any fermentation model. Empirical approaches  
598 have been essentially developed to describe in particular the stoichiometry of VFA in  
599 the rumen by deriving equations from the analysis of databases that link the proportion  
600 of VFA with respect to measured values of composition and digestion of various diets  
601 (Friggens et al., 1998; Nozière et al., 2011). For instance, Nozière et al. (2011) used  
602 *in vivo* measurements of ruminal VFA production rate, rates of duodenal and faecal  
603 digestion to establish empirical equations that predicts the proportion of VFA by using  
604 as predictor variables dry matter intake, digestible organic matter, digestible NDF and  
605 rumen starch digestibility. By including biochemical stoichiometric relationships, the  
606 work of Murphy et al. (1982) established an important basis to estimate empirically  
607 the stoichiometric coefficients of VFA produced during rumen fermentation for distinct  
608 substrates. By following the work of Murphy et al. (1982), Bannink et al. (2006) developed  
609 a stoichiometry model with the attempt of improving the accuracy of prediction of VFA  
610 molar proportions in the rumen of lactating cows.

611 The approach that we have used in the present model aimed to improve the mech-  
612 anistic representation of the stoichiometry. The formalism used here is similar to the  
613 approach used by Bannink et al. (2006) with certain differences related to the detail of  
614 the biochemical description of the fermentation and the calculation of the yield factors of

615 the individuals VFA. For example, in the model developed by Bannink et al. (2006), the  
616 production of VFAs were set dependent on the rates of utilization of different types of  
617 substrates (including polymers). In our model, extracellular hydrolysis and fermentation  
618 reactions that occur at intracellular level are split. With this approach, VFA production  
619 is dependent only on the utilization of monomers (either sugars or amino acids), which  
620 is more biologically consistent, allowing us to explicitly include well known macroscopic  
621 reactions of rumen fermentation (Wolin et al., 1997) (Table 4). Further, amino acids uti-  
622 lization is not aggregated into carbohydrate utilization. This particularity of the model  
623 allows to consider the specific metabolism of carbohydrates and proteins by the rumen  
624 microbiota, which occur via different pathways (*e.g.*, Embden-Meyerhof-Parnas pathway  
625 for hexoses, and Stickland or non-Stickland reactions for amino acids). These pathways  
626 exhibit different thermodynamic responses with respect to hydrogen concentration as dis-  
627 cussed by Janssen (2010). Taking together, by incorporating a microbial group of amino  
628 acids utilizers, our model structure should facilitate the study of alternatives for reducing  
629 amino acid deamination, which is of great interest to favour protein utilization by the  
630 host animal. It should be said however that the stoichiometry for amino acid fermenta-  
631 tion used in our model is an approximation and further refinements are needed to improve  
632 the representation of amino acid fermentation. For example, we considered alfalfa as the  
633 only source of amino acids in the calculations. However, as it can be seen from Table 1  
634 other components of the feed are source of amino acids and should also be considered. In  
635 addition to the weight associated with the amino acid composition of the feed (Ramsay  
636 and Pullammanappallil, 2001), the overall stoichiometry and kinetics are weighing by the  
637 relative activity of the proteolytic microbes and their substrate affinity (Monod constant).  
638 In our model, it is implicitly assumed that the microbiota do not have a particular pref-  
639 erence for the amino acids. However, rumen microbes do exhibit different affinities with  
640 respect to individual amino acids (Wallace et al., 1997; Bach et al., 2005). As discussed by  
641 Wallace et al. (1997), amino acid fermentation could be carried out by either numerically  
642 abundant bacteria with low deaminative activity or by few species with high deaminative  
643 activity. In our model, we elaborated on the latter option. However, it will be interesting  
644 in the future to support our choice on microbiological data of abundance and activity of

645 amino acids utilizers. With respect to sugars utilization, in our model the stoichiometry is  
646 based on the metabolism of hexoses. To consider the metabolism of monosaccharides with  
647 five carbon atoms, a generalised method has been proposed by García-Gen et al. (2013).  
648 The approach consists in expressing the stoichiometry of the alternative substrate (*e.g.*,  
649 xylose) in terms of equivalent glucose fermentation, and assign to the sugars-utilizers  
650 group the capability of growing with multiple substrates. The kinetic parameters and mi-  
651 crobial yields are set to be dependent on each type of substrate. The kinetics of utilization  
652 include a competitive inhibition term among the multiple substrates.

653 With respect to hydrogen and methane, existing rumen models based on the work  
654 of Baldwin et al. (1987) and Dijkstra et al. (1992), use the same approach for predicting  
655 methane production. This approach consists in calculating stoichiometrically the methane  
656 from the net surplus of hydrogen in the rumen, which is obtained from subtracting the  
657 hydrogen used for microbial growth and biohydrogenation to the hydrogen production  
658 from fermentation. This approach does not consider the effect of the methanogen pop-  
659 ulation nor the effect of liquid-gas transfer phenomena. By including these aspects, our  
660 model contributes to the mechanistic representation of ruminal hydrogen and methane.  
661 Moreover, our model complements recent modelling attempts to improve the representa-  
662 tion of hydrogen metabolism and methane production in the rumen (Vetharaniam et al.,  
663 2015; Wang et al., 2016).

664 By incorporating fermentation reactions, we are not only able to predict  $H_2$  and  $CH_4$   
665 as other models do (Mills et al., 2001), but also to maintain elementary balances of  
666 nitrogen and carbon. Furthermore, by taking into account  $NH_3$  and  $CO_2$ , our model allows  
667 linking of fermentation products with the pH via the definition of acid-base reactions,  
668 as it will be discussed later on. In our approach, the yield factors are parameterized  
669 to be dependent on the flux distribution (defined by the parameters  $\lambda_i$ ) across the set  
670 of reactions defined in the model. In the current version of the model, we used fixed  
671 stoichiometric yields. Nevertheless, the yields (and the repartition of fluxes) depends  
672 partly on thermodynamics and may vary. For instance, hydrogen concentration is linked  
673 to the fermentation pattern and shift of reactions (Offner and Sauvant, 2006; Janssen,  
674 2010). This thermodynamic driving force results in a variable stoichiometry (Rodríguez

675 et al., 2006). Including thermodynamic control into the model is indeed a challenging  
676 perspective. It should be a useful approach for tackling aspects related to the individual  
677 variability of rumen fermentation patterns. We expect that the structure of the model  
678 will enable us to include in the future such aspects.

679 Fermentation was represented in aggregated form. It may be also interesting to relax  
680 the level of aggregation to consider for instance lactate metabolism and address aspects  
681 concerning lactic acidosis state (Mills et al., 2014). In this direction, the kinetic model  
682 developed by Muñoz-Tamayo et al. (2011) to describe lactate utilization by human colonic  
683 microbiota can be integrated in the model here developed.

684 Currently, our representation of the fermentation pathway includes three theoretical  
685 functional groups of microbes with fixed chemical composition. This approach differs  
686 from the one often used in rumen models where the microbiota is represented either by  
687 a single microbial group (Baldwin et al., 1987) or by two microbial pools, namely fibre  
688 degraders and starch degraders (Dijkstra et al., 1992). In our model, the functional groups  
689 are defined solely in terms of the monomers sugars, amino acids and hydrogen used as  
690 substrates. This approach is a clearly simplified representation of the rumen microbiome,  
691 which for example ignores variation of the chemical composition of rumen microbiota  
692 that has been suggested to affect the fermentation (Dijkstra et al., 1992). In our model  
693 structure, the actions of protozoa are included in an aggregated way by considering that  
694 sugars and amino acids utilizers account for the combined activity of both bacteria and  
695 protozoa. The engulfment of bacteria is incorporated by considering a kinetics of cell  
696 death. Given the strong link between protozoa concentration and methane production  
697 (Guyader et al., 2014), it will be interesting to including explicitly this microbial group  
698 as in the model of Dijkstra (1994).

699 In the present version of our model, we do not include explicitly maintenance require-  
700 ments of rumen microbes. Under the experimental conditions here studied, maintenance  
701 requirements were assumed to be negligible. Indeed, the model performance was satisfac-  
702 tory without including this factor. However, under *in vivo* conditions, microbial mainte-  
703 nance can be very high. Including maintenance is therefore central and thus should be  
704 included in the model. It should be noted that including a maintenance term by using

705 for example the Pirt equation into our model can be done straightforward. To favour  
706 a mechanistic representation of microbial metabolism, mechanistic modelling of mainte-  
707 nance is a challenging subject that calls for re-evaluation of previous models (including  
708 the Pirt equation). An ideal model should explicitly describe the different non-growth  
709 components that constitute microbial maintenance as discussed by van Bodegom (2007).

710 It is clear that further improvements are needed to gain insight into the full picture  
711 of the rumen microbiome. However, it should be said that the approach used here and  
712 the simplifications adopted already provide a satisfactory prediction of the fermentation  
713 pattern.

#### 714 *4.3. A mechanistic modelling of pH*

715 Our model satisfactorily represented the dynamics of pH for the four treatment com-  
716 binations analyzed. Nevertheless, in this experiment the pH varied in a narrow range due  
717 to the high buffering capacity of the medium. To validate our model, it will be necessary  
718 to compare the model against experimental data exhibiting pH variations in a broader  
719 range than the one analyzed here.

720 The mechanistic description of pH is one of the hallmarks of the present model. The  
721 formulation of acid-base equilibrium reactions of the main chemical components that in-  
722 fluence the pH enhances the knowledge basis of the model. We have integrated acid-base  
723 equilibria to the liquid-gas transfer of CO<sub>2</sub>, and the fermentation pattern (VFA, NH<sub>3</sub>),  
724 extending previous attempts to model pH (Imamidoost and Cant, 2005). The model de-  
725 veloped here follows the principle of considering the bicarbonate system of the rumen as  
726 an open system (Kohn and Dunlap, 1998; Hille et al., 2016)). Although our model does  
727 not yet incorporate the role of salivary secretion, VFA absorption on rumen fermentation,  
728 and other phenomena that play a role on the buffer capacity of the rumen. It will also be  
729 important to incorporate the retroactive effect of the pH on the fermentation (Argyle and  
730 Baldwin, 1988) and microbial activities. The current model structure should facilitate  
731 such development.

732 Finally, since the model was calibrated with a small number of experiments, further vali-  
733 dation with independent data is required. In this line, the next step of our work will be to

734 challenge the model against other data set with different substrates to assess how model  
735 parameters vary with respect the substrate type. Furthermore, we will extend the devel-  
736 oped model for describing *in vitro* continuous systems (RUSITEC and chemostat) that  
737 accounts for substrate inflows and products outflows. Validation of the model structure  
738 for *in vitro* continuous systems will then support the task of modelling the rumen under *in*  
739 *vivo* conditions, which is the ultimate goal of our modelling endeavour. For this, further  
740 extensions are required to account for physiological factors such as kinetics of substrate  
741 supply, VFA and  $\text{NH}_3$  absorption. Since, the physical form of the feeds impacts transit  
742 time and nutrient availability, it will be useful to include this aspect in a further version.  
743 Incorporating the aforementioned factors into the model should improve its capabilities for  
744 both research and practical purposes such as the design of optimal nutritional strategies .

## 745 **5. Conclusions**

746 A mechanistic mathematical model of *in vitro* rumen fermentation has been developed.  
747 The model was calibrated with experimental data and represented effectively the profile  
748 of individuals VFA, pH,  $\text{NH}_3$  and gas production ( $\text{H}_2$ ,  $\text{CO}_2$ ,  $\text{CH}_4$ ). By enhancing the  
749 description of microbial metabolism, acid-base reactions (central for pH calculation) and  
750 gas-liquid transfer, this model contributes to improve mechanistic modelling of rumen fer-  
751 mentation. It also provides a strong basis for extension to modelling *in vivo* fermentation.

752

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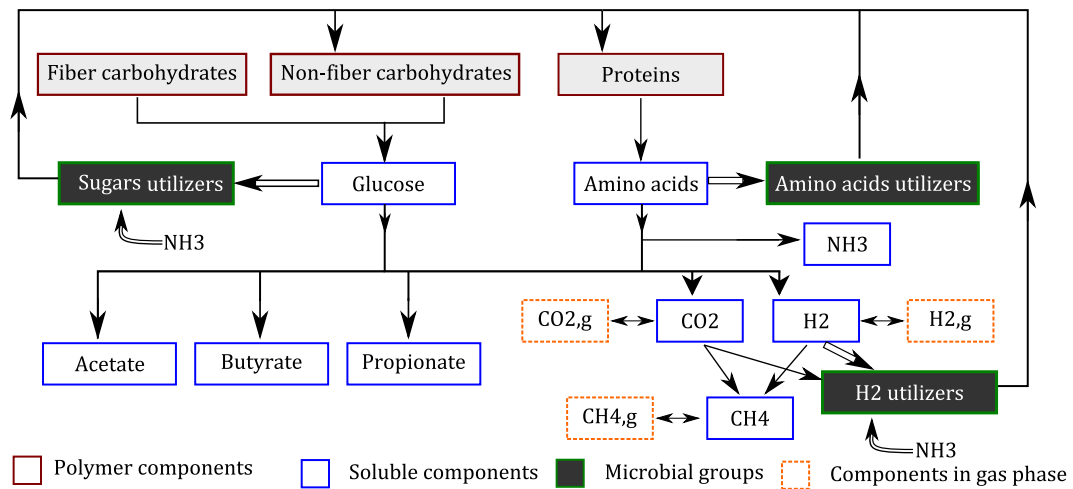


Figure 1: Fluxes representing the digestion of feedstuffs by the rumen microbiota. Hydrolysis of carbohydrates (fiber and non-fiber) and proteins releases respectively sugars and amino acids monomers, which are further utilized by the microbiota intracellularly. The utilization of substrate occurs via by two processes, namely product formation (single arrows) and microbial growth (double arrows). The metabolic fluxes of substrate utilization are thus partitioned in these two processes. The utilization of each substrate is attributed to a single microbial functional group. Microbial synthesis requires both carbon and nitrogen. Dead microbial cells are recycled as source of non-fibers carbohydrates and proteins. CO<sub>2</sub>, CH<sub>4</sub> and H<sub>2</sub> produced during fermentation participate in liquid-gas transfer phenomena.

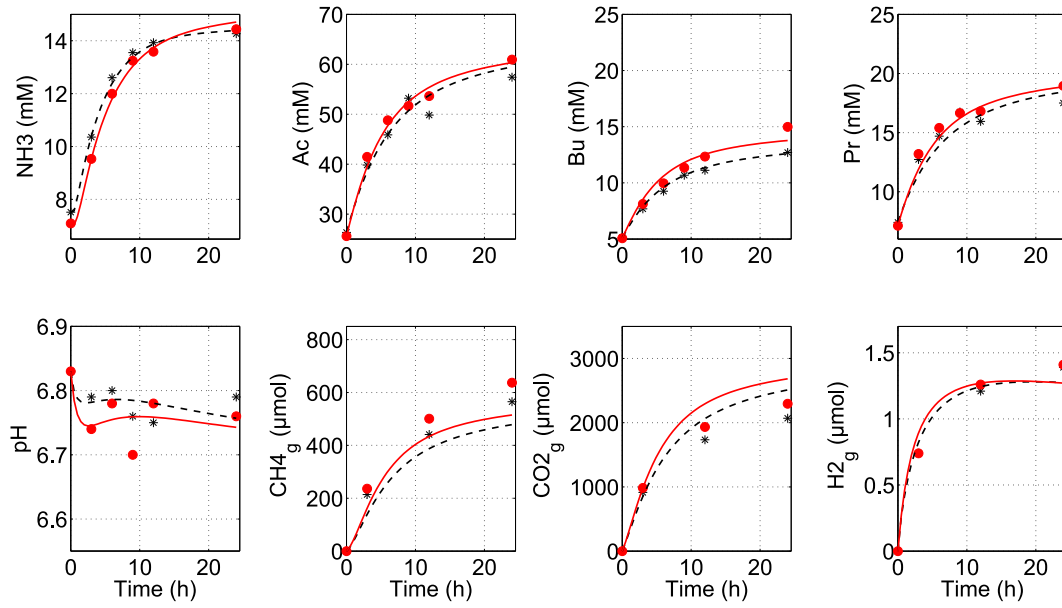


Figure 2: Model calibration with experimental data using the inoculum ( $L_i$ ) adapted to the low concentrate. The experimental data of the treatments with low concentrate substrate ( $L_i L_s$  (\*)) and high substrate concentration ( $L_i H_s$  (•)) are compared against the model responses depicted in dashed lines ( $L_i L_s$ ) and solid lines ( $L_i H_s$ ).

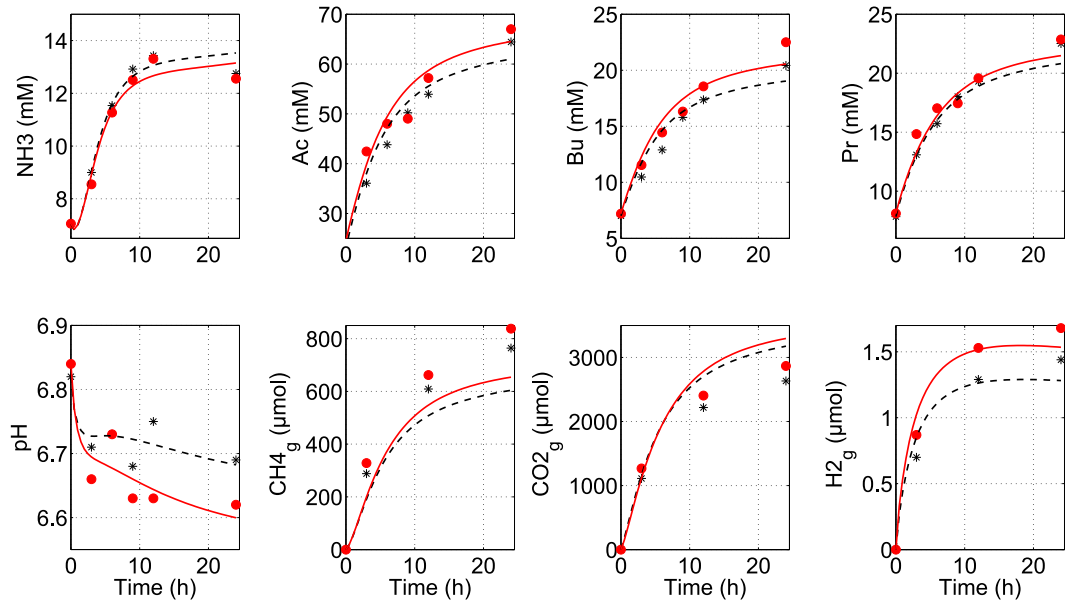


Figure 3: Model calibration with experimental data using the inoculum ( $H_i$ ) adapted to the high concentrate. The experimental data of the treatments with low concentrate substrate ( $H_iL_s$  (\*)) and high concentrate substrate ( $H_iH_s$  (●)) are compared against the model responses depicted in dashed lines ( $H_iL_s$ ) and solid lines ( $H_iH_s$ ).



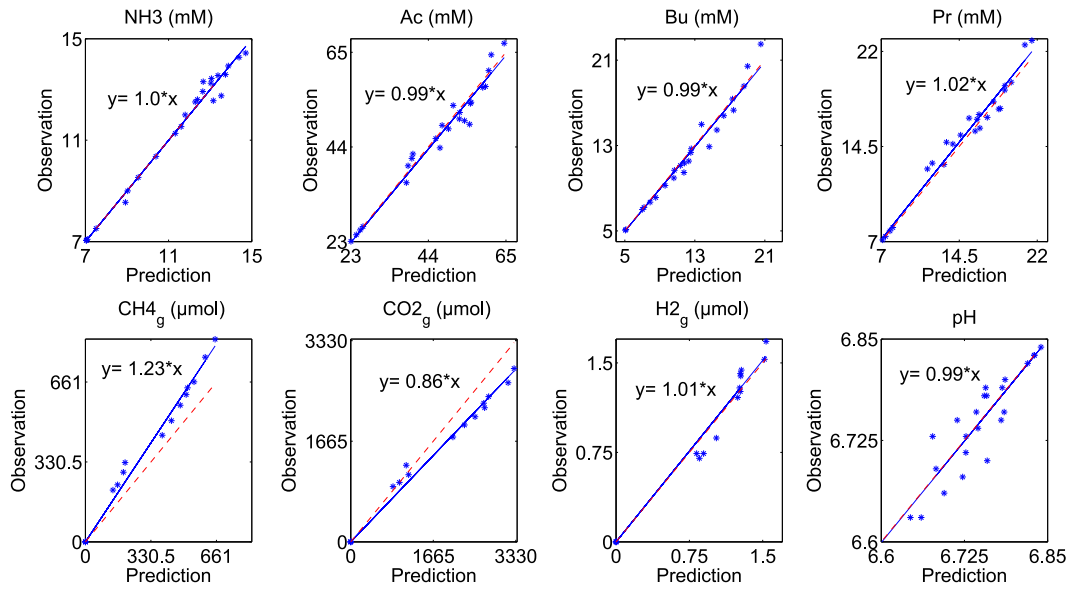


Figure 4: The observations are plot against the model predictions (\*). The linear fitted curve is displayed in solid line with the resulting regression equation. The line  $y = x$  is shown in dashed line. The linearity between model outputs and observations indicates the reliability of the mathematical model to represent the pattern of rumen fermentation.

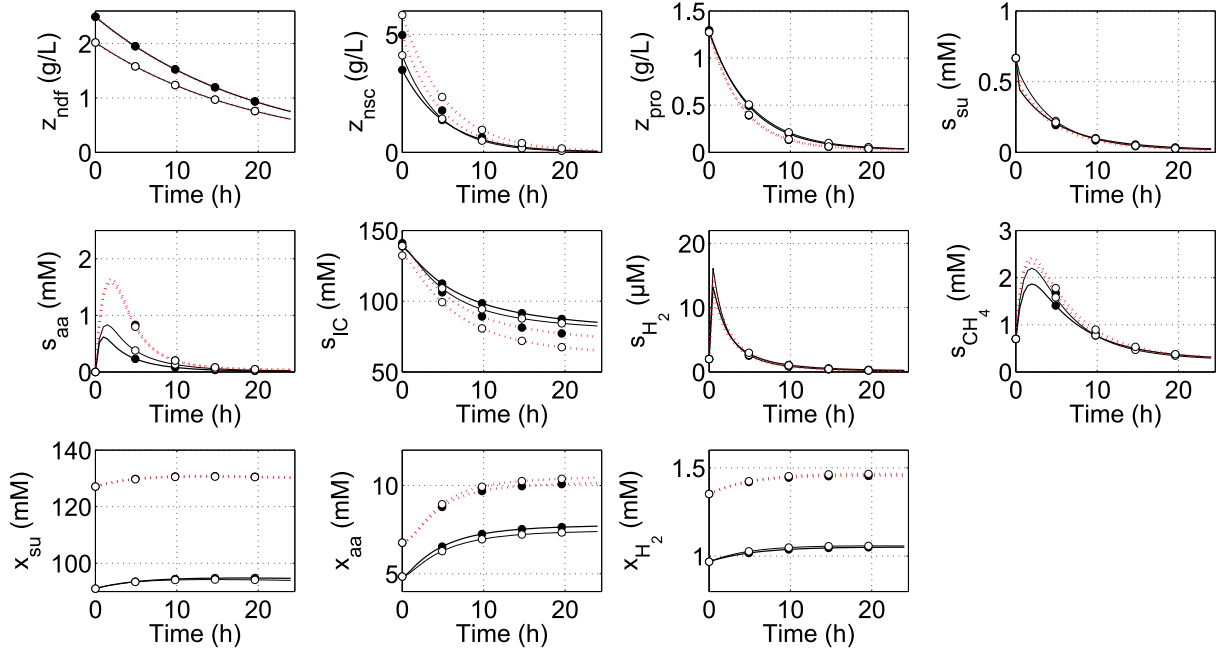


Figure 5: Predicted dynamics of non measured variables for the treatment combinations. Solid lines refer to the inoculum  $L_i$  adapted to the low concentrate diet. Dotted lines refer to the inoculum  $H_i$  adapted to the high concentrate diet. Filled circles refer to the low concentrate substrate  $L_s$ . Open circles refer to the high concentrate substrate  $H_s$ . The variables are neutral detergent fiber concentration ( $z_{ndf}$ ), non-structural carbohydrates concentration ( $z_{nsc}$ ), protein concentration ( $z_{pro}$ ), sugars concentration ( $s_{su}$ ), average amino acids concentration ( $s_{aa}$ ), concentration of inorganic carbon ( $s_{IC}$ ), dissolved hydrogen concentration ( $s_{H_2}$ ) and dissolved methane concentration ( $s_{CH_4}$ ), concentration of sugars-utilizing microbes ( $x_{su}$ ), concentration of amino acids-utilizing microbes ( $x_{aa}$ ), and concentration of hydrogen-utilizing microbes ( $x_{H_2}$ ).

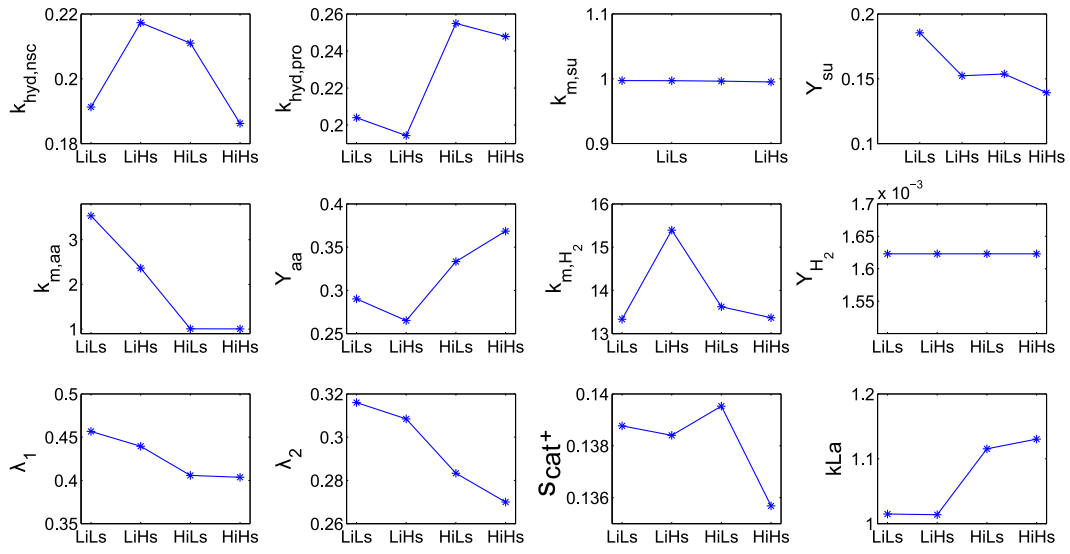


Figure 6: Estimated parameters for each treatment combination.

Table 1: Composition of the substrates (Serment, 2012)

	L substrate	H substrate
<i>Ingredients</i> (100× g/g of DM)		
Forage	65	30
Grass hay	29.9	13.8
Dehydrated alfalfa <sup>a</sup>	35.1	16.2
Concentrate	35	70
Commercial concentrate <sup>b</sup>	20	50
Sugar beet pulp silage	7	20
Calcium palm oil salt	1.4	
Soybean meal	6.6	
<i>Chemical composition</i> (100× g/g of DM)		
Crude protein	14.4	14.1
aNDFom <sup>c</sup>	40.5	32.6
ADFom <sup>d</sup>	23.0	18.0
Lignin	3.12	2.30
Starch	4.39	9.39
Ash	8.40	7.05
Fatty acids	2.35	2.55

<sup>a</sup> Rumiluz, Désialis, Paris (France).

<sup>b</sup> Agralys Aliment, Châteaudun (France): 18% maize, 14% sugar beet pulp, 12% sunflower meal, 10% wheat, 10% soybean, 9% rapeseed meal, 6% soybean meal, 4% wheat distiller, 3.5% linseed, 3% pea seed, 1% rapeseed oil, 3% molasses, 6.5% mineral and vitamin premix.

<sup>c</sup> aNDFom: Neutral Detergent Fiber assayed with a heat stable amylase and expressed exclusive of residual ash.

<sup>d</sup> ADFom: Acid Detergent Fiber expressed exclusive of residual ash.

Table 2: Model notation and abbreviations.

	Definition	Units
<b>Abbreviations</b>		
NSC	Non-structural carbohydrates	
NDF	Neutral detergent fiber	
VFA	Volatile fatty acids	
<b>Model variables</b>		
<i>Polymer components</i>		
$z_{\text{ndf}}$	Neutral detergent fiber (NDF) concentration	g/L
$z_{\text{nsc}}$	Non-structural carbohydrates (NSC) concentration	g/L
$z_{\text{pro}}$	Proteins concentration	g/L
<i>Soluble components</i>		
$s_{\text{su}}$	Sugars concentration	mol/L
$s_{\text{aa}}$	Average amino acids concentration	mol/L
$s_{\text{ac}}$	Total acetate concentration	mol/L
$s_{\text{bu}}$	Total butyrate concentration	mol/L
$s_{\text{pr}}$	Total propionate concentration	mol/L
$s_{\text{VFA}}$	Total volatile fatty acids concentration	mol/L
$s_{\text{IC}}$	Inorganic carbon concentration	mol/L
$s_{\text{CO}_2}$	Carbon dioxide concentration in the liquid phase	mol/L
$s_{\text{IN}}$	Inorganic nitrogen concentration	mol/L
$s_{\text{NH}_3}$	Ammonia concentration	mol/L
$s_{\text{H}_2}$	Hydrogen concentration in liquid phase	mol/L
$s_{\text{CH}_4}$	Methane concentration in the liquid phase	mol/L
<i>Gas components</i>		
$n_{\text{g,H}_2}$	Moles of hydrogen in the gas phase	mol
$n_{\text{g,CO}_2}$	Moles of carbon dioxide in the gas phase	mol
$n_{\text{g,CH}_4}$	Moles of methane in the gas phase	mol
<i>Microbial functional groups</i>		
$x_{\text{su}}$	Concentration of sugars-utilizing microbes	mol/L
$x_{\text{aa}}$	Concentration of amino acids-utilizing microbes	mol/L
$x_{\text{H}_2}$	Concentration of hydrogen-utilizing microbes (methanogens)	mol/L
<i>Acid-base components</i>		
$s_{\text{cat}^+}$	Concentration of metallic cations	mol/L
$s_{\text{NH}_4^+}$	Ammonium ions concentration	mol/L
$s_{\text{H}^+}$	Hydrogen ions concentration	mol/L
$s_{\text{HCO}_3^-}$	Bicarbonate concentration	mol/L
$s_{\text{ac}^-}$	Acetate ions concentration	mol/L
$s_{\text{bu}^-}$	Butyrate ions concentration	mol/L
$s_{\text{pr}^-}$	Propionate ions concentration	mol/L
$s_{\text{VFA}^-}$	Total volatile fatty acids ions concentration	mol/L
$s_{\text{hac}}$	Acetate concentration in free form	mol/L
$s_{\text{hbu}}$	Butyrate concentration in free form	mol/L
$s_{\text{hpr}}$	Propionate concentration in free form	mol/L

Table 2: Model notation and abbreviations.

	Definition	Units
$s_{\text{hVFA-}}$	Total volatile fatty acids concentration in free form	mol/L
$s_{\text{OH-}}$	Hydroxide ions concentration	mol/L
<b>Rates and parameters</b>		
<i>Rates</i>		
$\mu_j$	Growth rate of the microbial group $j$	mol $j$ /(L·h)
$\rho_j$	Kinetic rate of microbial process $j$	mol (or g) $j$ /(L·h)
$\rho_{x_j}$	Death cell rate of microbes $j$	mol $j$ /(L·h)
$\rho_{\text{T},j}$	Liquid-gas transfer rate of component $j$	mol $j$ /(L·h)
<i>Biochemical parameters</i>		
$\lambda_k$	Molar fraction of the sugars utilized via reaction $k$	mol/mol
$\mu_{\text{max},j}$	Maximum specific growth rate constant of the microbial group $j$	$\text{h}^{-1}$
$\sigma_{i,\text{aa}}$	Stoichiometric coefficient of component $i$ from amino acids utilization	mol/mol
$f_j$	Fraction of the substrate $j$ utilized for product formation	mol/mol
$f_{\text{ch},x}$	Mass fraction of carbohydrates in the microbial cells	g/g
$f_{\text{pro},x}$	Mass fraction of proteins in the microbial cells	g/g
$k_{\text{d}}$	Death cell rate constant	$\text{h}^{-1}$
$k_{\text{hyd},\text{ndf}}$	Hydrolysis rate constant of cell wall carbohydrates	$\text{h}^{-1}$
$k_{\text{hyd},\text{nsc}}$	Hydrolysis rate constant of non-structural carbohydrates	$\text{h}^{-1}$
$k_{\text{hyd},\text{pro}}$	Hydrolysis rate constant of proteins	$\text{h}^{-1}$
$k_{\text{m},\text{aa}}$	Maximum specific utilization rate constant of amino acids	mol/(mol · h)
$k_{\text{m},\text{H}_2}$	Maximum specific utilization rate constant of hydrogen	mol/(mol · h)
$k_{\text{m},\text{su}}$	Maximum specific utilization rate constant of amino sugars	mol/(mol · h)
$K_{\text{s},\text{aa}}$	Monod constant associated with the utilization of amino acids	mol/L
$K_{\text{s},\text{H}_2}$	Monod constant associated with the utilization of hydrogen	mol/L
$K_{\text{s},\text{su}}$	Monod constant associated with the utilization of sugars	mol/L
$K_{\text{s},\text{IN}}$	Nitrogen limitation constant	mol/L
$Y_j$	Microbial biomass yield factor	mol/mol
$Y_{i,j}$	Yield factor of the compound $i$ during utilization of substrate $j$	mol/mol
<i>Physicochemical parameters</i>		
$K_{\text{a},\text{CO}_2}$	Equilibrium constant of bicarbonate	
$K_{\text{a},\text{NH}_4}$	Equilibrium constant of ammonium	
$K_{\text{a},\text{VFA}}$	Equilibrium constant of VFA	
$K_w$	Equilibrium coefficient of water	
$k_{\text{L},\text{a}}$	Liquid-gas transfer constant	$\text{h}^{-1}$
$K_{\text{H},\text{CO}_2}$	Henry's law coefficient of carbon dioxide	M/bar
$K_{\text{H},\text{CH}_4}$	Henry's law coefficient of methane	M/bar
$K_{\text{H},\text{H}_2}$	Henry's law coefficient of hydrogen	M/bar
$P$	Pressure	bars
$T$	Temperature	K
$w_j$	Molecular weight (MW) of component $j$	g/mol

Table 2: Model notation and abbreviations.

	Definition	Units
$w_{aa}$	Molecular weight of average amino acid	g/mol
$w_{mb}$	Molecular weight of microbial cells	g/mol
$w_{su}$	Molecular weight of sugars	g/mol
$V_l$	Volume of the liquid phase	L

Table 3: Petersen matrix representing rumen fermentation. The microbial utilization of substrate  $j$  is described by the kinetic rate  $\rho_j$ . The elements of the matrix describe the stoichiometry of the fermentation via the yield factors. The utilization of one mole of substrate  $j$  produces  $Y_j$  moles of microbial cells.  $Y_{i,j}$  is the amount of moles of component  $i$  that is either consumed or produced by the utilization of one mole of substrate  $j$ .

Component $\rightarrow i$		1	2	3	4	5	6	7	8	Kinetic rate
$j$	Microbial process $\downarrow$	$z_{\text{ndf}}$	$z_{\text{nsc}}$	$z_{\text{pro}}$	$s_{\text{su}}$	$s_{\text{aa}}$	$s_{\text{H}_2}$	$s_{\text{ac}}$	$s_{\text{bu}}$	
1	Hydrolysis of NDF carbohydrates	-1			$1/w_{\text{su}}$					$\rho_{\text{ndf}}$
1	Hydrolysis of NSC carbohydrates		-1		$1/w_{\text{su}}$					$\rho_{\text{nsc}}$
2	Hydrolysis of proteins			-1		1				$\rho_{\text{pro}}$
3	Utilization of sugars				-1		$Y_{\text{H}_2,\text{su}}$	$Y_{\text{ac},\text{su}}$	$Y_{\text{bu},\text{su}}$	$\rho_{\text{su}}$
4	Utilization of amino acids					-1	$Y_{\text{H}_2,\text{aa}}$	$Y_{\text{ac},\text{aa}}$	$Y_{\text{bu},\text{aa}}$	$\rho_{\text{aa}}$
5	Utilization of hydrogen						-1			$\rho_{\text{H}_2}$
6	Death of sugars utilizers		$f_{\text{ch},x} \cdot w_{\text{mb}}$	$f_{\text{pro},x} \cdot w_{\text{mb}}$						$\rho_{x_{\text{su}}}$
7	Death of amino acids utilizers		$f_{\text{ch},x} \cdot w_{\text{mb}}$	$f_{\text{pro},x} \cdot w_{\text{mb}}$						$\rho_{x_{\text{aa}}}$
8	Death of hydrogen utilizers		$f_{\text{ch},x} \cdot w_{\text{mb}}$	$f_{\text{pro},x} \cdot w_{\text{mb}}$						$\rho_{x_{\text{H}_2}}$

Component $\rightarrow i$		9	10	11	12	13	14	15	Kinetic rate
$j$	Microbial process $\downarrow$	$s_{\text{pr}}$	$s_{\text{IN}}$	$s_{\text{IC}}$	$s_{\text{CH}_4}$	$x_{\text{su}}$	$x_{\text{aa}}$	$x_{\text{H}_2}$	
1	Hydrolysis of NDF carbohydrates								$\rho_{\text{ndf}} = k_{\text{hyd,ndf}} \cdot z_{\text{ndf}}$
1	Hydrolysis of NSC carbohydrates								$\rho_{\text{nsc}} = k_{\text{hyd,nsc}} \cdot z_{\text{nsc}}$
2	Hydrolysis of proteins								$\rho_{\text{pro}} = k_{\text{hyd,pro}} \cdot z_{\text{pro}}$
3	Utilization of sugars	$Y_{\text{pr},\text{su}}$	$Y_{\text{IN},\text{su}}$	$Y_{\text{IC},\text{su}}$		$Y_{\text{su}}$			$\rho_{\text{su}} = k_{\text{m,su}} \frac{s_{\text{su}}}{K_{\text{s,su}} + s_{\text{su}}} x_{\text{su}} \cdot I_{\text{IN}}$
4	Utilization of amino acids	$Y_{\text{pr},\text{aa}}$	$Y_{\text{IN},\text{aa}}$	$Y_{\text{IC},\text{aa}}$			$Y_{\text{aa}}$		$\rho_{\text{aa}} = k_{\text{m,aa}} \frac{s_{\text{aa}}}{K_{\text{s,aa}} + s_{\text{aa}}} x_{\text{aa}}$
5	Utilization of hydrogen		$Y_{\text{IN},\text{H}_2}$	$Y_{\text{IC},\text{H}_2}$	$Y_{\text{CH}_4,\text{H}_2}$			$Y_{\text{H}_2}$	$\rho_{\text{H}_2} = k_{\text{m,H}_2} \frac{s_{\text{H}_2}}{K_{\text{s,H}_2} + s_{\text{H}_2}} x_{\text{H}_2} \cdot I_{\text{IN}}$
6	Death of sugars utilizers					-1			$\rho_{x_{\text{su}}} = k_{\text{d}} \cdot x_{\text{su}}$
7	Death of amino acids utilizers						-1		$\rho_{x_{\text{aa}}} = k_{\text{d}} \cdot x_{\text{aa}}$
8	Death of hydrogen utilizers							-1	$\rho_{x_{\text{H}_2}} = k_{\text{d}} \cdot x_{\text{H}_2}$
									$I_{\text{IN}} = \frac{s_{\text{IN}}}{s_{\text{IN}} + K_{\text{s,IN}}}$



Table 4: Stoichiometry of reactions represented in the model.

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<b>Sugars (glucose) utilization</b>	
$C_6H_{12}O_6 + 2H_2O \Rightarrow 2CH_3COOH + 2CO_2 + 4H_2$	(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> )
<b>Amino acid utilization</b>	
$C_5H_{9.8}O_{2.7}N_{1.5} \Rightarrow (1 - Y_{aa}) \cdot \sigma_{ac,aa} CH_3COOH + (1 - Y_{aa}) \cdot \sigma_{pr,aa} CH_3CH_2COOH + Y_{IN,aa} NH_3 +$ $(1 - Y_{aa}) \cdot \sigma_{bu,aa} CH_3CH_2CH_2COOH + (1 - Y_{aa}) \cdot \sigma_{IC,aa} CO_2 + (1 - Y_{aa}) \cdot \sigma_{H_2,aa} H_2 + Y_{aa} C_5H_7O_2N$	(R <sub>5</sub> <sup>*</sup> )
<b>Hydrogen utilization: methanogenesis reaction</b>	
$4H_2 + CO_2 \Rightarrow CH_4 + 2H_2O$	(R <sub>6</sub> )
$10H_2 + 5CO_2 + NH_3 \Rightarrow C_5H_7O_2N + 8H_2O$	(R <sub>7</sub> )

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\*. The molecular formula of the average amino acid was calculated from the amino acid composition of alfalfa obtained from Feedipedia (<http://www.feedipedia.org/>). On the basis of the amino acid composition, a theoretical reaction was derived by following the procedure proposed by (Ramsay and Pullammanappallil, 2001). The overall reaction results from weighing the fermentation reactions of the individual amino acids by their molar fraction in the feed. For this overall reaction, the stoichiometric coefficients are  $\sigma_{ac,aa} = 0.67$ ,  $\sigma_{pr,aa} = 0.06$ ,  $\sigma_{bu,aa} = 0.24$ ,  $\sigma_{IC,aa} = 0.88$ ,  $\sigma_{H_2,aa} = 0.84$ . See Appendix A for further details on the derivation of the stoichiometry.

Table 5: Numerical values of the model parameters.

$\theta$	Value				Reference	
<i>Constants and physicochemical and operational parameters</i>						
$K_{a,CO_2}$	$5.13 * 10^{-7}$				(Batstone et al., 2002)	
$K_{a,NH_4}$	$1.44 * 10^{-9}$				(Batstone et al., 2002)	
$K_{a,VFA}$	$1.74 * 10^{-5}$				(Batstone et al., 2002)	
$K_{H,CO_2}$	$2.46 * 10^{-2}$				(Batstone et al., 2002)	
$K_{H,CH_4}$	$1.10 * 10^{-3}$				(Batstone et al., 2002)	
$K_{H,H_2}$	$7.23 * 10^{-4}$				(Batstone et al., 2002)	
$K_w$	$2.75 * 10^{-14}$				(Batstone et al., 2002)	
$P$ (bars)	1.01325				(Serment et al., 2016)	
$T$ (K)	312.15				(Serment et al., 2016)	
$V_l$ (L)	0.030				(Serment et al., 2016)	
$w_{aa}$ (g/mol)	134				<a href="http://www.feedipedia.org/">http://www.feedipedia.org/</a> see Appendix A	
$w_{ac}$ (g/mol)	60.05				<a href="https://en.wikipedia.org/">https://en.wikipedia.org/</a>	
$w_{bu}$ (g/mol)	88.10				<a href="https://en.wikipedia.org/">https://en.wikipedia.org/</a>	
$w_{mb}$ (g/mol)	113				(Batstone et al., 2002)	
$w_{pr}$ (g/mol)	74.08				<a href="https://en.wikipedia.org/">https://en.wikipedia.org/</a>	
$w_{su}$ (g/mol)	180.16				<a href="https://en.wikipedia.org/">https://en.wikipedia.org/</a>	
$\theta$	$L_iL_s$	$L_iH_s$	$H_iL_s$	$H_iH_s$	Mean $\pm$ standard deviation	Reference
<i>Estimated physicochemical parameters</i>						
$k_{La}$ ( $h^{-1}$ )	1.01	1.01	1.11	1.13	$1.07 \pm 0.06$	
$s_{cat+}$ (mol/L)	0.14	0.14	0.14	0.13	$0.14 \pm 0.0017$	
<i>Parameters associated with hydrolysis and cell lysis processes</i>						
$f_{ch,x}$ (g/g)	0.20					(Reichl and Baldwin, 1975)
$f_{pro,x}$ (g/g)	0.55					(Reichl and Baldwin, 1975)
$k_d$ ( $h^{-1}$ )	$8.33 * 10^{-4}$					(Batstone et al., 2002)
$k_{hyd,ndf}$ ( $h^{-1}$ )	0.05					(Serment et al., 2011)
$k_{hyd,nsc}$ ( $h^{-1}$ )	0.19	0.22	0.21	0.19	$0.20 \pm 0.015$	
$k_{hyd,pro}$ ( $h^{-1}$ )	0.20	0.19	0.25	0.25	$0.22 \pm 0.03$	

Table 5: Numerical values of the model parameters.

$\theta$	$L_iL_s$	$L_iH_s$	$H_iL_s$	$H_iH_s$	Mean $\pm$ standard deviation	Reference
<i>Parameters associated with sugars utilization</i>						
$K_{s,su}$ (mol/L)		9.0 * 10 <sup>-3</sup>				(Baldwin et al., 1987)
$k_{m,su}$ (mol/(mol · h))	0.99	0.99	0.99	0.99	0.99 $\pm$ 9.0*10 <sup>-4</sup>	
$K_{s,IN}^*$ (mol/L)		2.0 * 10 <sup>-4</sup>				(Baldwin and Denham, 1979)
$Y_{su}$ (mol/mol)	0.19	0.15	0.15	0.14	0.16 $\pm$ 0.02	
$\lambda_1$	0.46	0.44	0.41	0.40	0.43 $\pm$ 0.03	
$\lambda_2$	0.32	0.31	0.28	0.27	0.29 $\pm$ 0.02	
$\lambda_3$	0.22	0.25	0.31	0.32	0.28 $\pm$ 0.05	
<i>Parameters associated with amino acids utilization</i>						
$K_{s,aa}$ (mol/L)		6.4 * 10 <sup>-3</sup>				(Baldwin et al., 1987)
$k_{m,aa}$ (mol/(mol · h))	3.53	2.36	1.0	1.0	1.98 $\pm$ 1.22	
$Y_{aa}$ (mol/mol)	0.29	0.27	0.33	0.37	0.31 $\pm$ 0.05	
$\sigma_{ac,aa}$		0.67				See Appendix A
$\sigma_{bu,aa}$		0.24				See Appendix A
$\sigma_{pr,aa}$		0.062				See Appendix A
$\sigma_{H_2,aa}$		0.82				See Appendix A
$\sigma_{IC_2,aa}$		0.88				See Appendix A
<i>Parameters associated with hydrogen utilization</i>						
$K_{s,H_2}$ (mol/L)		5.84 * 10 <sup>-6</sup>				(Robinson and Tiedje, 1982)
$k_{m,H_2}$ (mol/(mol · h))	13.33	15.40	13.62	13.37	13.93 $\pm$ 0.98	
$Y_{H_2}$ (mol/mol)	0.0016	0.0016	0.0016	0.0016	0.0016 $\pm$ 0	

\* This parameter is also associated with hydrogen utilization.

Table 6: Statistics for model evaluation

	NH <sub>3</sub>	Acetate	Butyrate	Propionate	CH <sub>4</sub>	CO <sub>2</sub>	H <sub>2</sub>	pH
	(mM)	(mM)	(mM)	(mM)	( $\mu$ mol)	( $\mu$ mol)	( $\mu$ mol)	
Slope	1.0	0.99	0.99	1.02	1.23	0.86	1.01	0.99
$r^2$	0.99	0.97	0.97	0.97	0.99	0.99	0.97	0.83
RMSE*	0.29	2.29	0.82	0.82	96.50	302.84	0.10	0.03
$100 \times CV(RMSE)^\diamond$	2.6	4.9	6.8	5.4	25.4	21.6	11.2	0.4
CCC *	0.99	0.98	0.98	0.98	0.93	0.96	0.98	0.91

\* Root mean squared error (RMSE).

$\diamond$  Coefficient of variation of the RMSE (CV(RMSE)).

\* Concordance correlation coefficient (CCC) proposed by Lin (1989)

## 936 **Appendix A. Stoichiometry of amino acids fermentation**

937 The second column of Table A.7 shows the average amino acid composition of dehy-  
938 drated alfalfa obtained from Feedipedia (<http://www.feedipedia.org/>). The molecular  
939 formula for alfalfa was calculated from this information and the elementary composition  
940 of the individual amino acids. The resulting molecular formula was  $C_{4.8}H_{9.4}O_{2.6}N_{1.2}$  which  
941 has 13.4% of nitrogen content. After correcting this value to 16% and rounding, we ob-  
942 tained the final molecular formula of the average amino acid  $C_5H_{9.8}O_{2.7}N_{1.5}$ . Table A.7  
943 shows average stoichiometric coefficients for amino acid fermentation by anaerobic bacte-  
944 ria. These coefficients were extracted from the work of Ramsay and Pullammanappallil  
945 (2001) who selected dominant amino acid fermentation reactions from an inventory of  
946 reactions for different bacterial species.

947 The overall stoichiometry was obtained by multiplying the stoichiometric coefficients  
948 of each reaction by the molar fractions of the individual amino acids for alfalfa. It should  
949 be noted that other compounds such as minor VFA are also produced during amino acid  
950 fermentation, but they are not accounted for in the current overall reaction.

Table A.7: Overall stoichiometry for amino acid fermentation.

	Molar composition (%)	Acetate (mol/mol AA)	Propionate (mol/mol AA)	Butyrate (mol/mol AA)	CO <sub>2</sub> (mol/mol AA)	H <sub>2</sub> (mol/mol AA)
Arginine	4.0	0.5	0.5	0	1	-1
Histidine	1.5	1	0	0.5	1	0
Lysine	5.0	1	0	1	0	0
Tyrosine	2.8	1	0	0	1	1
Tryptophan	0.9	0	0	0	1	2
Phenylalanine	4.3	0	0	0	1	2
Cysteine	1.9	1	0	0	1	0.5
Methionine	1.3	0	1	0	1	1
Threonine	4.1	1	0	0.5	0	-1
Serine	6.2	1	0	0	1	1
Leucine/Isoleucine	13.5	0	0	0	1	2
Valine	8.3	0	0	0	1	2
Glutamine	10.2	1	0	1	1	0
Aspartate	11.9	1	0	0.5	2	2
Glycine	9.3	1	0	0	0	-1
Alanine	8.9	1	0	0	1	2
Proline	5.8	0.5	0.5	0	0	-1
Overall stoichiometry		0.67	0.06	0.24	0.88	0.84