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# **Hydrogenotrophic methanogens of the mammalian gut: functionally similar, thermodynamically different - A modelling approach**

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8

9 **Running title: Thermodynamic and kinetic modelling of gut methanogens**

10

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14

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16

17

## 18 **Abstract**

19 Methanogenic archaea occupy a functionally important niche in the gut microbial ecosystem of  
20 mammals. Our purpose was to quantitatively characterize the dynamics of methanogenesis by  
21 integrating microbiology, thermodynamics and mathematical modelling. For that, *in vitro* growth  
22 experiments were performed with pure cultures of key methanogens from the human and ruminant gut,  
23 namely *Methanobrevibacter smithii*, *Methanobrevibacter ruminantium* and *Methanobacterium*  
24 *formicum*. Microcalorimetric experiments were performed to quantify the methanogenesis heat flux.  
25 We constructed an energetic-based mathematical model of methanogenesis. Our model captured  
26 efficiently the dynamics of methanogenesis with concordance correlation coefficients of 0.94 for CO<sub>2</sub>,  
27 0.99 for H<sub>2</sub> and 0.97 for CH<sub>4</sub>. Together, experimental data and model enabled us to quantify metabolism  
28 kinetics and energetic patterns that were specific and distinct for each species despite their use of  
29 analogous methane-producing pathways. Then, we tested *in silico* the interactions between these  
30 methanogens under an *in vivo* simulation scenario using a theoretical modelling exercise. *In silico*  
31 simulations suggest that the classical competitive exclusion principle is inapplicable to gut ecosystems  
32 and that kinetic information alone cannot explain gut ecological aspects such as microbial coexistence.  
33 We suggest that ecological models of gut ecosystems require the integration of microbial kinetics with  
34 nonlinear behaviours related to spatial and temporal variations taking place in mammalian guts. Our  
35 work provides novel information on the thermodynamics and dynamics of methanogens. This  
36 understanding will be useful to construct new gut models with enhanced prediction capabilities and  
37 could have practical applications for promoting gut health in mammals and mitigating ruminant  
38 methane emissions.

39

## 40 **Introduction**

41 Methanogenic archaea inhabit the gastro-intestinal tract of mammals where they have established  
42 syntrophic interactions within the microbial community (1–3) playing a critical role in the energy  
43 balance of the host (4,5). In the human gut microbiota, the implication of methanogens in host  
44 homeostasis or diseases is poorly studied, but of growing interest (6). *Methanobrevibacter smithii*  
45 (accounting for 94% of the methanogen population) and *Methanosphaera stadtmanae* are specifically  
46 recognized by the human innate immune system and contribute to the activation of the adaptive  
47 immune response (7). Decreased abundance of *M. smithii* was reported in inflammatory bowel disease  
48 patients (8), and it has been suggested that methanogens may contribute to obesity (9). In the rumen,  
49 the methanogens community is more diverse though still dominated by *Methanobrevibacter* spp.,  
50 followed by *Methanomicrobium* spp., *Methanobacterium* spp. (10) and *Methanomassillicoccus* spp  
51 (11). However, the proportion of these taxa could vary largely, with *Methanomicrobium mobile* and  
52 *Methanobacterium formicium* being reported as major methanogens in grazing cattle (12). Though  
53 methanogens in the rumen are essential for the optimal functioning of the ecosystem (by providing  
54 final electron acceptors), the methane they produce is emitted by the host animal, contributing to global  
55 greenhouse gas (GHG) emissions. The livestock sector is responsible for 14.5% of the anthropogenic  
56 GHG emissions, of which enteric methane from ruminants contributes to more than 40% of total  
57 emissions (13). Methanogens can be separated in two groups based on the presence or absence of  
58 cytochromes, which are membrane-associated electron transfer proteins providing energetic  
59 advantages for microbial growth (14). However, in the gastrointestinal tract of mammals, the only  
60 methanogens with cytochromes are the *Methanosarcinales* which are minor members of the  
61 community (4,15). Major rumen methanogens (16) and the dominant human archaeon *M. smithii* (17),  
62 are hydrogenotrophic archaea without cytochrome. Cytochrome-lacking methanogens exhibit lower  
63 growth yields than archaea with cytochromes (14). However, this apparent energetic disadvantage has  
64 been counterbalanced by a greater adaptation to the environmental conditions prevailing in the  
65 gastrointestinal tract (15), and by the establishment of syntrophic interactions with feed fermenting

66 microbes. This syntrophic cooperation centred on hydrogen allows the anaerobic reactions of substrate  
67 conversion to proceed close to the thermodynamic equilibrium (18,19) (that is with Gibbs free energy  
68 change close to zero).

69 To our knowledge, the impact of thermodynamics on human gut metabolism has been poorly addressed  
70 in existing mathematical models (20–23). For the rumen, thermodynamic principles have been  
71 incorporated already into mathematical research frameworks because of their important role in feed  
72 fermentation, (24–29). Despite these relevant efforts, much work remains to be conducted for attaining  
73 a predictive thermodynamic-based model that allows for quantitative assessment of the impact of the  
74 thermodynamics on fermentation dynamics. Theoretical frameworks have been developed to calculate  
75 stoichiometric and energetic balances of microbial growth from the specification of the anabolic and  
76 catabolic reactions of microbial metabolism (30,31), and advances have been done to link  
77 thermodynamics to kinetics (32–34). These works constitute a solid basis for tackling the  
78 thermodynamic modelling of gut metabolism. In this respect, new knowledge on the extent of  
79 methanogenesis could help to improve existing gut models. Accordingly, our purpose was to  
80 quantitatively characterize the dynamics of hydrogen utilization, methane production, growth and heat  
81 flux of three hydrogenotrophic gut methanogens by integrating microbiology, thermodynamics, and  
82 mathematical modelling. We investigated the rate and extent of methanogenesis by performing *in vitro*  
83 experiments with three methanogenic species representing major human and ruminant genera: *M.*  
84 *smithii*, *M. formicium* and *Methanobrevibacter ruminantium*. To interpret and get the most out of the  
85 resulting data, a mathematical model with thermodynamic basis was developed to describe the  
86 dynamics of the methanogenesis. Our findings allowed to quantify metabolism kinetics and energetic  
87 patterns that were specific and distinct for each species despite their use of analogous methane-  
88 producing pathways and their common belonging to the group of cytochrome-lacking methanogenic  
89 archaea.

90

## 91 **Material and Methods**

### 92 ***In vitro* growth experiments**

#### 93 **Archaeal strains and growth media**

94 Archaeal strains used in the study were *M. ruminantium* M1 (DSM 1093), *M. smithii* PS (type strain  
95 DSM 861), and *M. formicium* MF (type strain DSM 1535). The growth media was prepared as  
96 previously described (35) and composition is summarized in Table S1 of the Supplementary material.

#### 97 **Experimental design and measures**

98 Starter cultures were grown until reaching optical density at 660 nm ( $OD_{660}$ ) of  $0.400 \pm 0.030$ . Optical  
99 density was measured on a Jenway spectrophotometer (Bibby Scientific). Then, 0.6 ml were used to  
100 inoculate one experimental tube. Commercially prepared high purity  $H_2/CO_2$  (80%/20%) gas mix was  
101 added to inoculated tubes by flushing for 1 min at 2.5 Pa. Mean initial  $OD_{660}$  and pressure values are  
102 summarized in Table S2 of the Supplementary material. Growth kinetics for each strain were followed  
103 over 72 h. The experiment was repeated twice. Each kinetics study started with 40 tubes inoculated at  
104 the same time. At a given time point (3 h, 5 h, 6.5 h, 24 h, 26 h, 30 h, 47h, 72 h post inoculation), two  
105 tubes with similar  $OD_{660}$  values were sampled. The tubes were used for measuring gas parameters:  
106 pressure was measured using a manometer and composition of the gas phase was analysed by gas  
107 chromatography on a Micro GC 3000A (Agilent Technologies, France). One of the tubes was  
108 centrifuged 10 min at 13 000 g. The microbial pellet was weighed and stored at  $-20^\circ C$  in 2 ml screw-  
109 cap tubes containing 0.4 g of sterile zirconia beads (0.3 g of 1 mm and 0.1 g of 0.5 mm).

## 110 **DNA extraction and qPCR quantification of 16S rRNA genes**

111 One ml of lysis buffer (50mM NaCl, 50 mM TrisHCl pH 7.6, 50 mM EDTA, 5 % SDS) was added  
112 directly to the frozen microbial pellet before homogenizing for  $2 \times 30$  s at 5100 tours/min in a Precellys  
113 bead-beater (Bertin Instruments). Samples were centrifuged for 3 min at 14 000 g and the liquid phase  
114 transferred to a new tube before adding 600  $\mu$ l of phenol–chloroform–3-methyl-1-butanol (25:24:1)  
115 solution. After centrifugation at 14 000 g for 3 min, the aqueous phase was transferred to a fresh tube  
116 and 500  $\mu$ l of chloroform were added. The chloroform-washing step was repeated twice with  
117 centrifugation at 14000 g for 3 min between steps. The final volume of the aqueous phase was measured  
118 and DNA precipitation was initiated by adding 70% of the volume of isopropanol 100% and 10% of  
119 the volume of sodium acetate 3M. Sedimentation at 14 000 g for 30 min was again performed and the  
120 resulting DNA pellet was washed with 500  $\mu$ l of ethanol 70% and dissolved in 50 $\mu$ l of molecular  
121 biology quality water. The extraction yield was checked on a Nanodrop 1000 Spectrophotometer  
122 (Thermo Fisher Scientific, France) and extracts run on a FlashGel System (Lonza, Rockland, Inc) to  
123 check integrity.

124 Copies of 16S rRNA genes were quantified using a qPCR approach. Primers used are those of Ohene-  
125 Adjei et al (36) ; reaction assay and temperature cycles were as described previously (37) . Triplicate  
126 qPCR quantification was performed on 20 ng of extracted DNA. Amplifications were carried out using  
127 SYBR Premix Ex Taq (TaKaRa Bio Inc., Otsu, Japan) on a StepOne system (Applied Biosystems,  
128 Courtabeuf, France). Absolute quantification involved the use of standard curves that had been  
129 prepared with gDNA of *Methanobrevibacter ruminantium* DSM 1093. PCR efficiency was of 103%.  
130 Results were expressed as copy numbers per ng of extracted DNA per g of microbial pellet. *M. smithii*  
131 and *M. ruminantium* strains used in this study possess two copies of 16S rRNA genes in their genomes.  
132 The number of cells was computed by dividing 16S copy numbers by 2.

133

## 134 **Microcalorimetry**

135 Microcalorimetric experiments were performed to determine the heat flux pattern of each methanogen.  
136 Metabolic activity and microbial growth were monitored by using isothermal calorimeters of the heat-  
137 conduction type (A TAM III, TA Instruments, France) equipped with two multicalorimeters, each  
138 holding six independent minicalorimeters, allowed continuous and simultaneous recording as a  
139 function of time of the heat flux produced by 12 samples. The bath temperature was set at 39°C; its  
140 long-term stability was better than  $\pm 1 \times 10^{-4} \text{°C}$  over 24h. Each minicalorimeter was electrically  
141 calibrated. The specific disposable 4 mL microcalorimetric glass ampoules capped with butyl rubber  
142 stoppers and sealed with aluminium crimps were filled with 1.75 mL of Balch growth media and  
143 overpressed with 2.5 Pa of H<sub>2</sub>/CO<sub>2</sub> 80%/20% gas mixture for 30 s. There was no significant difference  
144 in pressure at the beginning of the study. They were sterilized by autoclave and stored at 39°C until the  
145 beginning of the microcalorimetric measurements. Actively growing cultures of methanogens (OD<sub>660</sub>  
146 of 0.280±0.030 for *M. smithii*, 0.271±0.078 for *M. ruminantium* and 0.142±0.042 for *M. formicium*)  
147 were stored at -20°C in order to diminish microbial activity before inoculation. Cultures were thawed  
148 for 30 min at ambient temperature and inoculation was carried out by injecting 0.25 mL of the culture  
149 through the septum of the overpressed microcalorimetric ampoules just before inserting them into the  
150 minicalorimeters. Samples took about two hours to reach the bath temperature and yield a stable zero  
151 baseline. Blank experiments were also carried out by inserting ampoules that were not inoculated and,  
152 as expected, no heat flux was observed confirming the medium sterility. Each experiment was repeated  
153 thrice.

154 The heat flux  $\left(\frac{dQ}{dt}\right)$ , also called thermal power output  $P$ , was measured for each methanogen and blank  
155 samples with a precision  $\geq 0.2 \mu\text{W}$ . The heat flux data of each sample were collected every 5 minutes



156 during more than 10 days. The total heat  $Q$  was obtained by integrating the overall heat flux time curve  
157 using the TAM Assistant Software and its integrating function (TA Instruments, France).

158 Classically, the heat flux-time curve for a growing culture starts like the S-shaped biomass curve (a lag  
159 phase followed by an exponential growth phase) but differs beyond the growth phase, the heat flux  
160 being then modulated by transition periods (38). Heat flux data can be used to infer the microbial  
161 growth rate constant provided the existence of a correlation between isothermal microcalorimetry data  
162 and microbiological data (e.g., cell counts) at early growth (39). During the exponential growth phase,  
163 microbial growth follows a first-order kinetics defined by the specific growth rate constant  $\mu_c$  ( $\text{h}^{-1}$ ).  
164 Analogously, the heat flux follows an exponential behaviour determined by the parameter  $\mu_c$  as  
165 described by (38,39).

166 
$$\frac{dQ}{dt} = \mu_c \cdot Q \quad (1)$$

167 The growth rate constant  $\mu_c$  can be determined by fitting the exponential part of the heat flux-time  
168 curve using the fitting function of the TAM Assistant Software. In our case study, careful selection of  
169 the exponential phase of heat flux dynamics was performed to provide a reliable estimation of the  
170 maximum growth rate constant from calorimetric data.

## 171 **Mathematical model development**

### 172 **Modelling *in vitro* methanogenesis**

173 The process of *in vitro* methanogenesis is depicted in Figure 1. The  $\text{H}_2/\text{CO}_2$  mixture in the gas phase  
174 diffuses to the liquid phase. The  $\text{H}_2$  and  $\text{CO}_2$  in the liquid phase are further utilized by the pure culture  
175 to produce  $\text{CH}_4$ . Methane in the liquid phase diffuses to the gas phase.

176 Model construction was inspired by our previous dynamic models of human gut (20) and rumen *in*  
177 *vitro* fermentation (40) followed by certain simplifications. The model considers the liquid-gas transfer  
178 of carbon dioxide. Due to the low solubility of hydrogen and methane (41), the concentration of these  
179 two gases in the liquid phase was not modelled. We assumed that the dynamics of concentrations in  
180 the gas phase are determined by kinetic rate of the methanogenesis. To incorporate thermodynamic  
181 information, instead of using the Monod equation in the original formulation, we used the kinetic rate  
182 function proposed by Desmond-Le Quémener and Bouchez (33). The resulting model is described by  
183 the following ordinary differential equations

$$184 \quad \frac{dx_{H_2}}{dt} = \mu_{\max} \cdot \exp\left(-\frac{K_S \cdot V_g}{n_{g,H_2}}\right) \cdot x_{H_2} - k_d \cdot x_{H_2} \quad (2)$$

$$185 \quad \frac{ds_{CO_2}}{dt} = -\frac{-Y_{CO_2} \cdot \mu_{\max}}{Y} \cdot \exp\left(-\frac{K_S \cdot V_g}{n_{g,H_2}}\right) \cdot x_{H_2} - k_L a \cdot (s_{CO_2} - K_{H,CO_2} \cdot R \cdot T \cdot n_{g,CO_2}/V_g) \quad (3)$$

$$186 \quad \frac{dn_{g,H_2}}{dt} = -\frac{\mu_{\max}}{Y} \cdot \exp\left(-\frac{K_S \cdot V_g}{n_{g,H_2}}\right) \cdot V_L \cdot x_{H_2} \quad (4)$$

$$187 \quad \frac{dn_{g,CO_2}}{dt} = V_L \cdot k_L a \cdot (s_{CO_2} - K_{H,CO_2} \cdot R \cdot T \cdot n_{g,CO_2}/V_g) \quad (5)$$

$$188 \quad \frac{dn_{g,CH_4}}{dt} = \frac{Y_{CH_4} \cdot \mu_{\max}}{Y} \cdot \exp\left(-\frac{K_S \cdot V_g}{n_{g,H_2}}\right) \cdot V_L \cdot x_{H_2} \quad (6)$$

189 where  $s_{CO_2}$  is the concentration (mol/L) of carbon dioxide in the liquid phase and  $x_{H_2}$  is the biomass  
190 concentration (mol/L) of hydrogenotrophic methanogens. The numbers of moles in the gas phase are  
191 represented by the variables  $n_{g,H_2}, n_{g,CO_2}, n_{g,CH_4}$ . The gas phase volume  $V_g = 20$  mL and the liquid  
192 phase volume  $V_L = 6$  mL. Liquid-gas transfer for carbon dioxide is described by a non-equilibria  
193 transfer rate which is driven by the gradient of the concentration of the gases in the liquid and gas  
194 phase. The transfer rate is determined by the mass transfer coefficient  $k_L a$  ( $h^{-1}$ ) and the Henry's law  
195 coefficients  $K_{H,CO_2}$  (M/bar).  $R$  ( $bar \cdot (M \cdot K)^{-1}$ ) is the ideal gas law constant and  $T$  is the temperature (K).

196 Microbial decay is represented by a first-order kinetic rate with  $k_d$  ( $\text{h}^{-1}$ ) the death cell rate constant.  
197 Microbial growth was represented by the rate function proposed by Desmond-Le Quéméner and  
198 Bouchez (33) using hydrogen as the limiting reactant

$$199 \quad \mu = \mu_{\max} \cdot \exp\left(-\frac{K_s \cdot V_g}{n_{g, \text{H}_2}}\right) \quad (7)$$

200 where  $\mu$  is the growth rate ( $\text{h}^{-1}$ ),  $\mu_{\max}$  ( $\text{h}^{-1}$ ) is the maximum specific growth rate constant and  $K_s$  ( $\text{mol/L}$ )  
201 the affinity constant. Equation (7) is derived from energetic principles following Boltzmann statistics  
202 and uses the concept of exergy (maximum work available for a microorganism during a chemical  
203 transformation). The affinity constant has an energetic interpretation since it is defined as

$$204 \quad K_s = \frac{E_M + E_{\text{dis}}}{v_{\text{harv}} \cdot E_{\text{cat}}} \quad (8)$$

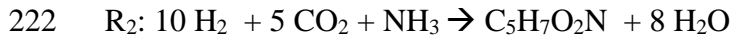
205 where  $E_{\text{dis}}$  ( $\text{kJ/mol}$ ) and  $E_M$  ( $\text{kJ/mol}$ ) are the dissipated exergy and stored exergy during growth  
206 respectively.  $E_{\text{cat}}$  ( $\text{kJ/mol}$ ) is the catabolic exergy of one molecule of energy-limiting substrate, and  
207  $v_{\text{harv}}$  is the volume at which the microbe can harvest the chemical energy in the form of substrate  
208 molecules (33).  $E_{\text{cat}}$  is the absolute value of the Gibbs energy of catabolism ( $\Delta G_{r,c}$ ) when the reaction  
209 is exergonic ( $\Delta G_{r,c} < 0$ ) or zero otherwise. The stored exergy  $E_M$  is calculated from a reaction (destock)  
210 representing the situation where the microbe gets the energy by consuming its own biomass.  $E_M$  is the  
211 absolute value of the Gibbs energy of biomass consuming reaction ( $\Delta G_{r,\text{destock}}$ ) when the reaction is  
212 exergonic ( $\Delta G_{r,\text{destock}} < 0$ ) or zero otherwise. Finally, the dissipated exergy  $E_{\text{dis}}$  is the opposite of the  
213 Gibbs energy of the overall metabolic reaction, which is a linear combination of the catabolic and  
214 destock reactions. This calculation follows the Gibbs energy dissipation detailed in Kleerebezem and  
215 Van Loosdrecht (31).

216 In our model, the stoichiometry of methanogenesis is represented macroscopically by one catabolic  
217 reaction ( $R_1$ ) for methane production and one anabolic reaction ( $R_2$ ) for microbial formation. We

218 assumed that ammonia is the only nitrogen source for microbial formation. The molecular formula of  
219 microbial biomass was assumed to be  $C_5H_7O_2N$  (41).



221



223

224 In the model, the stoichiometry of the reactions is taken into account *via* the parameters  $Y, Y_{CO_2}, Y_{CH_4}$ ,  
225 which are the yield factors (mol/mol) of microbial biomass,  $CO_2$  and  $CH_4$ . The microbial yield factor  
226  $Y$  was extracted from literature. We assumed that *M. smithii* and *M. ruminantium* have the same yield  
227 (being both *Methanobrevibacter*). This yield factor was set to 0.006 mol biomass/mol  $H_2$ , using the  
228 methane-based molar growth yield of 2.8 g biomass/mol  $CH_4$  estimated for *M. smithii* (42) and the  
229 equations (9) and (11). Similarly, the yield factor for *M. formicum* was set to 0.007 mol biomass/mol  
230  $H_2$  using the methane-based molar growth yield of 3.5 g biomass/mol  $CH_4$  reported by Schauer and  
231 Ferry (43). The fraction of  $H_2$  utilized for microbial growth (reaction  $R_2$ ) is defined by the yield factor  
232  $Y$  (mol of microbial biomass/mol of  $H_2$ ). Now, let  $f$  be the fraction of  $H_2$  used for the catabolic reaction  
233  $R_1$ . Reaction  $R_2$  tells us that for every 10 moles of  $H_2$  used in  $R_2$ , we get 1 mol of microbial biomass.  
234 Hence, it follows that

235 
$$Y = \frac{1}{10} \cdot (1 - f) \quad (9)$$

236 If  $Y$  is known, the fraction  $f$  can be computed from Equation (9).

237 The yield factors of  $CO_2$  and  $CH_4$  can be expressed as functions of the the fraction  $f$ :

238 
$$Y_{CO_2} = \left(\frac{1}{4}\right) \cdot f + \left(\frac{5}{10}\right) \cdot (1 - f) \quad (10)$$

239 
$$Y_{CH_4} = \left(\frac{1}{4}\right) \cdot f \quad (11)$$

240 The model has two physicochemical parameters ( $k_{La}, K_{H,CO_2}$ ) and four biological parameters  
241 ( $\mu_{max}, K_s, Y, k_d$ ). The initial condition for  $s_{CO_2}$  is unknown and was also included in the parameter  
242 vector for estimation. The Henry's law coefficients are known values calculated at 39°C using the  
243 equations provided by Batstone et al. (41). If the article is accepted, an implementation of the model in  
244 the Open Source software Scilab (<https://www.scilab.org/>) will be made available at Zenodo  
245 (<https://doi.org/10.5281/zenodo.3271611>).

## 246 **Theoretical model to study interactions among methanogens**

247 The experimental study of microbial interactions requires sophisticated *in vitro* systems under  
248 continuous operation such as the one developed by Haydock *et al.* (44). In our work, we explored by  
249 means of mathematical modelling how the methanogens can interact under *in vivo* conditions. For this  
250 theoretical study, we elaborated a toy model based on the previous model for *in vitro* methanogenesis.  
251 Let us consider the following simple model for representing the consumption of hydrogen by the  
252 methanogenic species  $i$  under an *in vivo* scenario of continuous flow

$$253 \quad \frac{dx_{H_2,i}}{dt} = \mu_{max,i} \cdot \exp\left(-\frac{K_{s,i} \cdot V_g}{n_{g,H_2}}\right) \cdot x_{H_2,i} - D_i \cdot x_{H_2,i} \quad (12)$$

$$254 \quad \frac{dn_{g,H_2}}{dt} = q_{H_2} - \frac{\mu_{max,i}}{Y_i} \cdot \exp\left(-\frac{K_{s,i} \cdot V_g}{n_{g,H_2}}\right) \cdot V_L \cdot x_{H_2,i} - b \cdot n_{g,H_2} \quad (13)$$

255 where  $q_{H_2}$  (mol/h) is the flux of hydrogen produced from the fermentation of carbohydrates. The kinetic  
256 parameters are specific to the species  $i$  ( $x_{H_2,i}$ ). The parameter  $D_i$  ( $h^{-1}$ ) is the dilution rate of the  
257 methanogens and  $b$  ( $h^{-1}$ ) is an output substrate rate constant. Extending the model to  $n$  species with a  
258 common yield factor  $Y$ , the dynamics of hydrogen is given by

$$259 \quad \frac{dn_{g,H_2}}{dt} = q_{H_2} - \frac{V_L}{Y} \sum_{i=1}^n \mu_{max,i} \cdot \exp\left(-\frac{K_{s,i} \cdot V_g}{n_{g,H_2}}\right) \cdot x_{H_2,i} - b \cdot n_{g,H_2} \quad (14)$$

260 where the sub index  $i$  indicates the species. In our case study,  $n = 3$ .

## 261 **Parameter identification**

262 Before tackling the numerical estimation of the model parameters, we addressed the question of  
263 whether it was theoretically possible to determine uniquely the model parameters given the available  
264 measurements from the experimental setup. This question is referred to as structural identifiability (45).  
265 Structural identifiability analysis is of particular relevance for model whose parameters are biologically  
266 meaningful, since knowing the actual value of the parameter is useful for providing biological insight  
267 on the system under study (46). Moreover, in our case, we are interested in finding accurate estimates  
268 that can be further used as priors in an extended model describing the *in vivo* system.

269 We used the freely available software DAISY (47) to assess the structural identifiability of our model.  
270 Physical parameters ( $k_L a, K_{H,CO_2}$ ) were set to be known. The model was found to be structurally  
271 globally identifiable. In practice, however, to facilitate the actual identification of parameters and  
272 reduce practical identifiability problems such as high correlation between the parameters (48), we fixed  
273 some model parameters to values reported in the literature. The transport coefficient  $k_L a$ , the Henry's  
274 law coefficient  $K_{H,CO_2}$ , and the dead cell rate constant  $k_d$  were set to be known and were extracted from  
275 Batstone et al. (41). Therefore, only the parameters  $\mu_{max}$ ,  $K_s$  and initial condition of  $s_{CO_2}$  were set to  
276 be estimated. To capitalize on the calorimetric data, we further assumed that  $\mu_{max}$  was equal to the  
277 specific rate constant  $\mu_c$  estimated from the heat flux-time curve. By this, only the affinity constant for  
278 each strain and the initial condition of  $s_{CO_2}$  were left to be estimated.

279 The parameter identification for each methanogen was performed with the IDEAS Matlab® toolbox  
280 (49), which is freely available at <http://genome.jouy.inra.fr/logiciels/IDEAS>. The measured variables

281 are the number of moles in the gas phase (H<sub>2</sub>, CH<sub>4</sub>, CO<sub>2</sub>). The Lin's concordance correlation coefficient  
282 (CCC) (50) was computed to quantify the agreement between the observations and model predictions.

## 283 **Results**

### 284 **Methanogens biomass**

285 Archaea-specific primers targeting the 16S rRNA gene were used to enumerate microbial cells in each  
286 pure culture. Three hours post inoculation microbial numbers varied from  $7.62 \times 10^7$  to  $2.81 \times 10^8$  and  
287 reached  $10^9$  after 72 hours of incubation. Table S3 summarizes microbial numbers at different sampling  
288 times.

### 289 **Calorimetric pattern of methanogens**

290 Figure 2 displays a representative isothermal calorimetric curve for each methanogen. The three  
291 measured heat flux dynamics of each methanogen were found to follow similar energetic patterns. *M.*  
292 *smithii* and *M. formicium* exhibited a lag phase of a few hours, while *M. ruminantium* was already  
293 metabolically active when introduced into the minicalorimeter though several attempts were made to  
294 obtain a lag phase by changing storage conditions and thawing the culture just before inoculating the  
295 microcalorimetric ampoules. The pattern of heat flux for all tested methanogens is characterized by  
296 one predominant peak which was observed at different times for each methanogen. *M. smithii* exhibited  
297 a second metabolic event occurring at 60 h with an increase of heat flux. The same phenomenon was  
298 observed for *M. formicium* but at a lower intensity that started at 140 h. One possible explanation for  
299 this event is cell lysis (39). The process was considered completed when the heat flux ceased marking  
300 the end of the metabolic activity. It is noted that *M. formicium* produced a small peak at 14 h (Fig. 2).  
301 A similar peak, but of much smaller size, was observed on the other curves obtained with this  
302 methanogen. *M. smithii* also exhibits a small peak (occurrence of 3 out of 3) at 7.4 h shown in the inset

303 of Figure 2. For *M. ruminantium*, we do not know whether the small peak exists since the initial part  
304 of the curve is missing. This small peak translates in a metabolic activity that remains to be elucidated.  
305 The total heat ( $Q_m$ ) produced during the methanogenesis process that took place under the present  
306 experimental conditions was, on average,  $-5.5 \pm 0.5$  J for the three methanogens (for *M. ruminantium*,  
307 the missing initial part of the heat flux-time curve was approximately estimated by extrapolating the  
308 exponential fit). As we shall see below, this experimental value is consistent with the theoretically  
309 expected value.

### 310 **Estimation of thermodynamic properties**

311 We defined two macroscopic reactions to represent the catabolism (R1) and anabolism (R2) of the  
312 methanogenesis (see Modelling in vitro methanogenesis section). All thermodynamic properties result  
313 from the contribution of both catabolic and anabolic reactions. In the Supplementary Material, the  
314 calculations of total heat ( $Q_m$ ), enthalpy ( $\Delta H_m$ ), Gibbs energy ( $\Delta G_m$ ) and entropy ( $\Delta S_m$ ) of the  
315 methanogenesis are detailed. The estimated overall heat produced during the methanogenesis process  
316 under our experimental conditions was in average  $Q_m = -5.66$  J. This heat results from the sum of  
317 the heat of the catabolic reaction ( $Q_c$ ) and the heat of the anabolic reaction ( $Q_a$ ). From the total heat  
318 of the methanogenesis, the anabolic reaction contributes to 7% of the metabolic heat for *M. smithii* and  
319 *M. ruminantium*. For *M. formicium*, the contribution of the anabolic reaction to the metabolic heat is  
320 9%. It is also interesting to note that there is a very good agreement between the theoretical value  
321 calculated above and the overall heat experimentally determined by microcalorimetry ( $-5.5 \pm 0.5$  J).

322 Table 1 shows the thermodynamic properties per mole of biomass formed during methanogenesis of  
323 *M. ruminantium*, *M. smithii* and *M. formicium* on  $H_2/CO_2$ . These properties are compared with values  
324 found in the literature for other methanogens grown on different substrates.



## 325 **Dynamic description of *in vitro* kinetics**

326 The developed mathematical model was calibrated with the experimental data from *in vitro* growth  
327 experiments in Balch tubes. Table 2 shows the parameters of the dynamic kinetic model described in  
328 Equations 2-6. The reported value of  $\mu_{\max}$  for each methanogen corresponds to the average value  
329 obtained from three heat flux-time curves. From Table 2, it is concluded that *M. smithii* exhibited the  
330 highest growth rate constant, followed by *M. ruminantium* and finally *M. formicium*. In terms of the  
331 affinity constant  $K_s$ , while *M. smithii* and *M. ruminantium* are of the same order, the affinity constant  
332 for *M. formicium* is lower in one order of magnitude.

333 Figure 3 displays the dynamics of the compounds in the methanogenesis for the three methanogens.  
334 Experimental data are compared against the model responses. Table 3 shows standard statistics for  
335 model evaluation. The model captures efficiently the overall dynamics of the methanogenesis.  
336 Hydrogen and methane are very well described by the model with concordance correlation coefficients  
337 (CCC) of 0.99 and 0.97 respectively. For carbon dioxide, CCC = 0.94.

338 Figure 4 displays the dynamics for the methanogens as measured by the 16S rRNA gene, as well as the  
339 dynamics of biomass as predicted for the model. As observed, the microbes follow a typical Monod-  
340 like trajectory.

## 341 **Discussion**

342 Our objective in this work was to quantitatively characterize the dynamics of hydrogen utilization,  
343 methane production, growth and heat flux of three hydrogenotrophic methanogens by integrating  
344 microbiology, thermodynamics and mathematical modelling. Our model developments were  
345 instrumental to quantify energetic and kinetic differences between the three methanogens studied,  
346 strengthening the potentiality of microcalorimetry as a tool for characterizing the metabolism of

347 microorganisms (51). This modelling work provides estimated parameters that can be used as prior  
348 values for other modelling developments of gut microbiota.

### 349 **Energetic and kinetic differences between methanogens**

350 Methanogenesis appears as a simple reaction with a single limiting substrate ( $H_2$ ). The  
351 microcalorimetry approach we applied revealed that this simplicity is only apparent and that  
352 hydrogenotrophic methanogens exhibit energetic and kinetic differences. Methanogenesis is indeed a  
353 complex process that can be broken down in several stages. The dominant metabolic phase is  
354 represented by one peak that occurs at different times. The magnitude of the peak differs between the  
355 methanogens and also the slope of the heat flux trajectories. The return time of the heat flux to the zero  
356 baseline was also different. The energetic difference is associated with kinetic differences that translate  
357 into specific kinetic parameters, namely affinity constant ( $K_s$ ) and maximum growth rate constant  
358 ( $\mu_{max}$ ). Previously, energetic differences between methanogens have been ascribed to the presence or  
359 absence of cytochromes (14). These differences are translated into different yield factors,  $H_2$  thresholds,  
360 and doubling times. The kinetic differences revealed in this study for three cytochrome-lacking  
361 methanogens indicate that factors other than the presence of cytochromes might play a role in the  
362 energetics of methanogenesis. Interestingly, calorimetric experiments showed that *M. ruminantium*  
363 was metabolically active faster than the other methanogens, characteristic that could explain the  
364 predominance of *M. ruminantium* in the rumen (52). Looking at the expression of the affinity constant  
365 (Equation (8)), the differences between the affinity constants among the methanogens can be explained  
366 by the differences between the by the harvest volume  $v_{harv}$  and the yield factors. Note that in the kinetic  
367 function developed by Desmond-Le Quéméner and Bouchez (33), the maximum growth rate did not  
368 have any dependency on the energetics of the reaction. Our experimental study revealed that  $\mu_{max}$  is  
369 species-specific and reflects the dynamics of the heat flux of the reaction at the exponential phase. This  
370 finding suggests that a further extension of the kinetic model developed by Desmond-Le Quéméner

371 and Bouchez (33) should include the impact of energetics on  $\mu_{\max}$ . Since our study is limited to three  
372 species, it is important to conduct further research on other methanogens to validate our findings.

373

## 374 **Thermodynamic analysis**

375 Regarding the energetic information for different methanogens summarized in Table 1, it is observed  
376 that the thermodynamic behaviour of the three methanogens is analogous to that observed for  
377 *Methanobacterium thermoautotrophicum* (53). The values reported in Table 1 show indeed that the  
378 methanogenesis on H<sub>2</sub>/CO<sub>2</sub> is characterized by large heat production. The growth is highly exothermic,  
379 with a  $\Delta H_m$  value that largely exceeds the values found when other energy substrates are used. The  
380 enthalpy change  $\Delta H_m$ , which is more negative than the Gibbs energy change  $\Delta G_m$ , largely controls the  
381 process. Growth on H<sub>2</sub>/CO<sub>2</sub> is also characterized by a negative entropic contribution  $T\Delta S_m$  which, at  
382 first sight, may look surprising since entropy increases in most cases of anaerobic growth (54).  
383 However, this can be understood if one remembers that  $T\Delta S_m$  corresponds in fact to the balance  
384 between the final state and the initial state of the process, that is

$$385 \quad T\Delta S_m = \frac{(1-10Y)}{4Y} T\Delta S_c + T\Delta S_a = \frac{(1-10Y)}{4Y} T(S_{final} - S_{initial})_c + T(S_{final} - S_{initial})_a$$

386 Methanogenesis on H<sub>2</sub>/CO<sub>2</sub> is particular because the final state of its catabolic reaction (1 mol CH<sub>4</sub> +  
387 2 mol H<sub>2</sub>O) involves a smaller number of moles than the initial state (4 mol H<sub>2</sub> + 1 mol CO<sub>2</sub>), which  
388 results in a significant loss of entropy during the process. For spontaneous growth in such a case, the  
389  $\Delta H_m$  must not only contribute to the driving force but must also compensate the growth-unfavourable  
390  $T\Delta S_m$ , which means that  $\Delta H_m$  must be much more negative than  $\Delta G_m$  (55). For this reason,  
391 methanogenesis on H<sub>2</sub>/CO<sub>2</sub>, which is accompanied by a considerable decrease of entropy and a large

392 production of heat, has been designed as an entropy-retarded process (53). More generally, von Stockar  
393 and Liu (55) noticed that when the Gibbs energy of the metabolic process is resolved into its enthalpic  
394 and entropic contributions, very different thermodynamic behaviours are observed depending on the  
395 growth type. These thermodynamic behaviours are: aerobic respiration is clearly enthalpy-driven ( $\Delta H_m$   
396  $\ll 0$  and  $T\Delta S_m > 0$ ), whereas fermentative metabolism is mainly entropy-driven ( $\Delta H_m < 0$  and  $T\Delta S_m$   
397  $\gg 0$ ). Methanogenesis on  $H_2/CO_2$  is enthalpy-driven but entropy-retarded ( $\Delta H_m \ll 0$  and  $T\Delta S_m < 0$ ),  
398 whereas methanogenesis on acetate is entropy-driven but enthalpy-retarded ( $\Delta H_m > 0$  and  $T\Delta S_m \gg 0$ ).  
399 In the present case, the highly exothermic growth of *M. ruminantium*, *M. smithii* and *M. formicium* on  
400  $H_2/CO_2$  is largely due to the considerable decrease of entropy during the process: in fact, 50% of the  
401 heat produced here serves only to compensate the loss of entropy. A proportion of 80% was found for  
402 *M. thermoautotrophicum* (53), which results from the fact that their  $T\Delta S_m$  and  $\Delta H_m$  values are,  
403 respectively, 2.7 and 1.7 times larger than ours. This difference might be due to the differences in the  
404 temperature of the studies, namely 39°C in our study vs 60°C in the study by Schill et al. (53).

405

## 406 **Do our results inform on ecological questions such as species coexistence?**

407 The competitive exclusion principle (56) states that coexistence cannot occur between species that  
408 occupy the same niche (the same function). Only the most competitive species will survive. Recently,  
409 by using thermodynamic principles, Großkopf & Soyer (34) demonstrated theoretically that species  
410 utilizing the same substrate and producing different compounds can coexist by the action of  
411 thermodynamic driving forces. Since in our study the three methanogens perform the same metabolic  
412 reactions, the thermodynamic framework developed Großkopf & Soyer (34) predicts, as the original  
413 exclusion principle (56), the survival of only one species. By incorporating thermodynamic control on  
414 microbial growth kinetics, Lynch et al (57) showed theoretically that differentiation of ATP yields can

415 explain ecological differentiation of methanogens over a range of liquid turnover rates. This theoretical  
416 work predicts that for a fixed liquid turnover rate, only one species survives. For the continuous culture  
417 of microorganisms, it has been demonstrated that at the equilibrium (growth rate equals the dilution  
418 rate) with constant dilution rates and substrate input rates, the species that has the lowest limiting  
419 substrate concentration wins the competition. From Eq. (12), the number of moles of hydrogen of the  
420 species  $n_{g,H_2,i}^*$  at the steady state is

$$421 \quad n_{g,H_2,i}^* = \frac{K_{s,i} \cdot V_g}{\log(\mu_{\max,i}/D_i)}$$

422  
423 Using the model parameters of Table 2, we studied *in silico* three possible competition scenarios,  
424 assuming a constant environment (constant dilution rate  $D$ ). Two dilution rates were evaluated:  $D =$   
425  $0.021 \text{ h}^{-1}$  (retention time = 48 h) and  $D = 0.04 \text{ h}^{-1}$  (retention time = 25 h). A retention time of 48 h  
426 corresponds to values measured in small ruminants (58) and to humans as we used in our gut model  
427 (20). For higher retention times, the results obtained for 48 h hold. For  $D = 0.021 \text{ h}^{-1}$ , we obtained that  
428  $n_{g,H_2,Ms}^* = 0.32 \text{ mmol}$ ,  $n_{g,H_2,Mr}^* = 0.68 \text{ mmol}$ ,  $n_{g,H_2,Mf}^* = 0.28 \text{ mmol}$ , where the subindex Ms, Mr, Mf  
429 stand for *M. smithii*, *M. ruminantium* and *M. formicium*. From these results, it appears that under a  
430 constant environment, *M. formicium* will win the competition. Since  $n_{g,H_2,Ms}^* < n_{g,H_2,Mr}^*$ , *M.*  
431 *ruminantium* will be extinguished before *M. smithii*. For  $D = 0.04 \text{ h}^{-1}$ , we obtained that  $n_{g,H_2,Ms}^* = 0.49$   
432  $\text{mmol}$ ,  $n_{g,H_2,Mr}^* = 1.42 \text{ mmol}$ ,  $n_{g,H_2,Mf}^* = 1.57 \text{ mmol}$ , and thus *M. smithii* wins the competition. To win  
433 the competition, *M. ruminantium* requires longer retention times than its competitors. Retention times  
434 of digesta longer than 48 h are physiologically uncommon, thus the presence of *M. ruminantium* in the  
435 gut ecosystem can be explained, for example, from known adhesion properties (both *M. ruminantium*  
436 and *M. smithii* genes encode adhesin-like proteins (59,60). To illustrate these aspects, we built a  
437 multiple-species model with the three methanogens using Eq. (12) and Eq. (14). The parameter  $b$  was

438 set to  $0.5 \text{ h}^{-1}$  and the hydrogen flux production  $q_{\text{H}_2}$  rate was set to  $0.02 \text{ mol/min}$ . Figure 5A displays  
439 the dynamics of the three methanogens for the first scenario ( $D= 0.021 \text{ h}^{-1}$ ). It is observed that at 50 d  
440 only *M. formicium* survives. This result, however, is not representative of what occurs in the rumen  
441 where the three methanogens coexist (5,61). It is intriguing that in our toy model it is *M. formicium*  
442 that wins the competition, bearing in mind that *M. ruminantium* and *M. smithii* are more abundant  
443 than *M. formicium* (5,52). Figure 5 shows that selective conditions favour the survival of one species.  
444 Similar results can be obtained for the human gut by including the effect of pH on microbial growth  
445 (23) and setting the gut pH to select one of the species. On the basis of the competitive exclusion  
446 principle, it is thus intriguing that having a very specialized function, methanogens are a diverse group  
447 that coexist. Gut ecosystems, therefore, exhibit the paradox of the plankton introduced by Hutchinson  
448 (1961) that presents the coexistence of species all competing for the same substrate in a relatively  
449 isotropic or unstructured environment (62). In the case of the rumen, our modelling work suggests that  
450 in addition to kinetic and thermodynamic factors, other forces contribute to the ecological shaping of  
451 the methanogens community in the rumen favouring the microbial diversity. Indeed, methanogenic  
452 diversity in the rumen results from multiple factors that include pH sensitivity, the association with  
453 rumen fractions (fluid and particulate material), and the endosymbiosis with rumen protozoa (5,52).  
454 For the human gut, ecological factors enable methanogens to coexist to a competitive environment  
455 where hydrogenotrophic microbes (acetogens, methanogenic archaea and sulfate-reducing bacteria)  
456 utilize  $\text{H}_2$  via different pathways (63–65). Both in the human gut and in the rumen, microbes grow in  
457 association with biofilms that form a polymer-based matrix that provides nutritional and hydraulic  
458 advantages for microbial growth and resistance to shear forces (20,66). Indeed, in our modelling work  
459 of human gut fermentation (20), we suggested that, from the different actions the mucus has on colonic  
460 fermentation, the mechanism of promoting conditions for microbial aggregation appears as the most  
461 relevant factor for attaining the high microbial density and the high level of fibre degradation

462 characteristic of the human gut. Altogether, these factors result in nonlinear behaviours, spatial and  
463 temporal variations that promote coexistence and diversity, that, as discussed in dedicated literature on  
464 microbial ecology (67–71), render the classical formulation of the competitive exclusion principle  
465 (56,72) inapplicable to gut ecosystems.

466 Finally, mathematical modelling is expected to enhance our understanding of gut ecosystems (66,73).  
467 It is then key that in addition to metabolic aspects, mathematical models of gut fermentation incorporate  
468 the multiple aspects that shape microbial dynamics to provide accurate predictions and improve insight  
469 on gut metabolism dynamics and its potential modulation. For ruminants, the development of precision  
470 livestock technologies provides promising alternatives for integrating real-time data of key animal  
471 phenotypes such as feeding behaviour with mathematical models for estimating methane emissions  
472 (74) and rumen function indicators at large scale. These tools will be instrumental to support livestock  
473 management decisions and guide timely interventions. Similarly, for humans, mathematical models  
474 coupled with electronic technologies for online monitoring of gut function (75) might facilitate the  
475 diagnosis and the design of personalized therapies for gastrointestinal diseases.

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480

## 481 **Conflict of interest**

482 No conflict.

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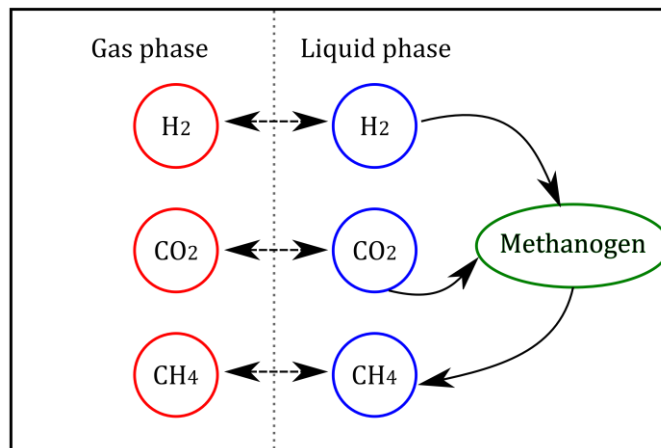
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669 **Figures**



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671 **Figure 1** Schematics of the *in vitro* methanogenesis process by hydrogenotrophic methanogens.

672 Double arrows represent fluxes due to liquid-gas transfer, simple arrows represent metabolic fluxes.

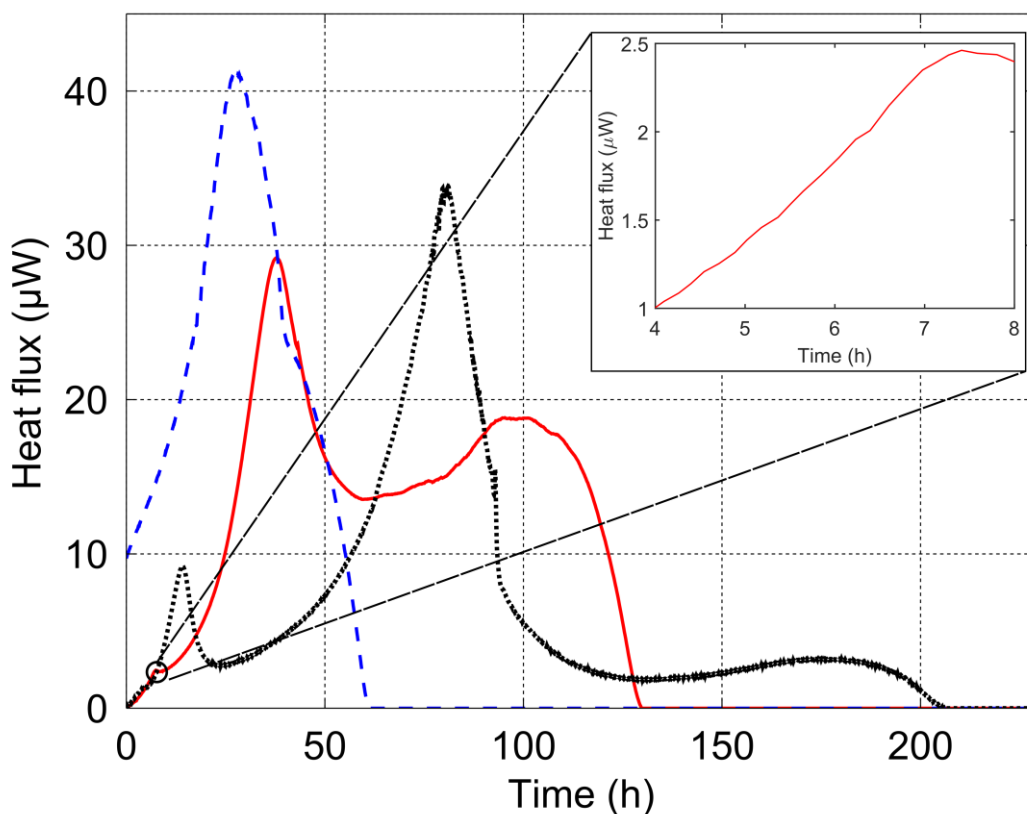
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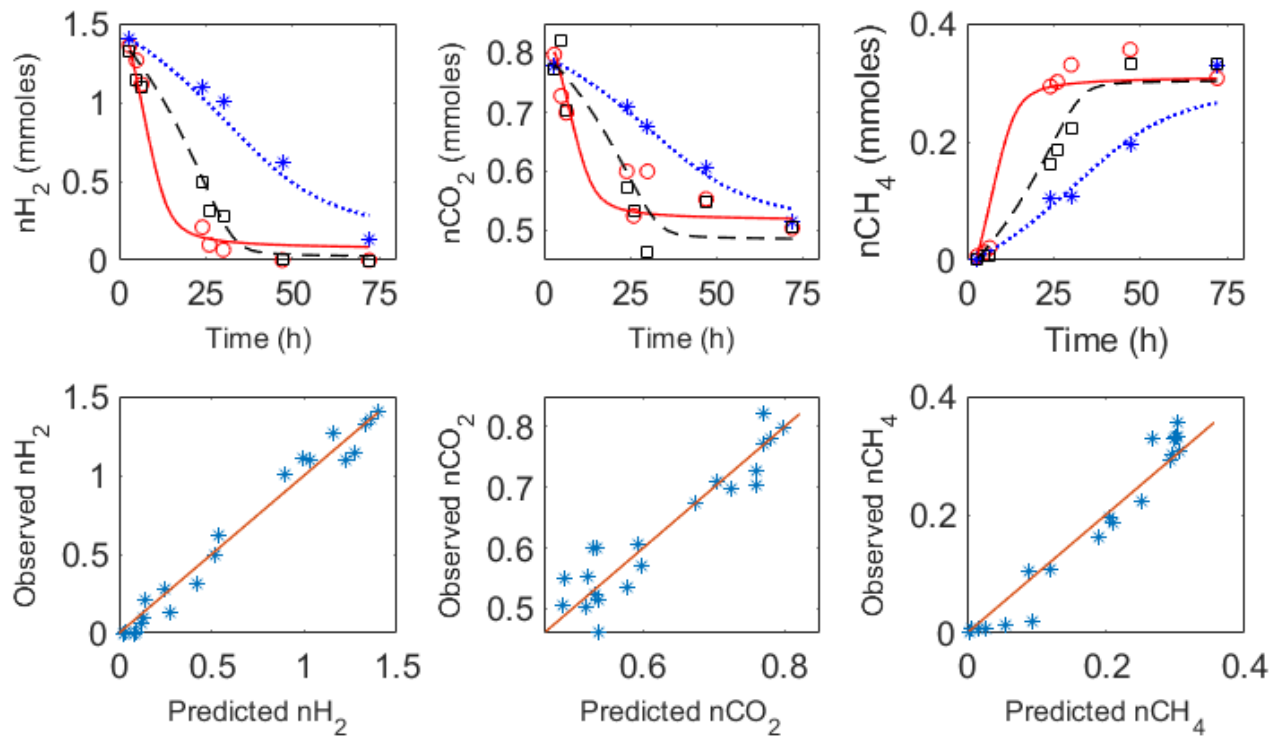
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679 **Figure 2** Example of isothermal calorimetric curves for *M. ruminantium* (dashed blue line), *M. smithii*  
680 (solid red line) and *M. formicium* (dotted black line). The dominant metabolic phase is represented by  
681 one peak. The magnitude of the peak differs between the methanogens and also the slope of the heat  
682 flux trajectories. The return of the heat flux to the zero baseline also differs between the three  
683 methanogens. The inset zoom displays the peak exhibited by *M. smithii* at 7.4 h.

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688 **Figure 3** Top plots: dynamics of methanogenesis by *M. ruminantium* (\*), *M. smithii* (o) and *M.*  
689 *formicium* ( $\square$ ). Experimental data (\*,o, $\square$ ) are compared against model predicted responses: dotted blue  
690 lines (*M. ruminantium*), solid red lines (*M. smithii*) and dashed black lines (*M. formicium*). Bottom  
691 plots: summary observed vs predicted variables. The solid red line is the isocline.

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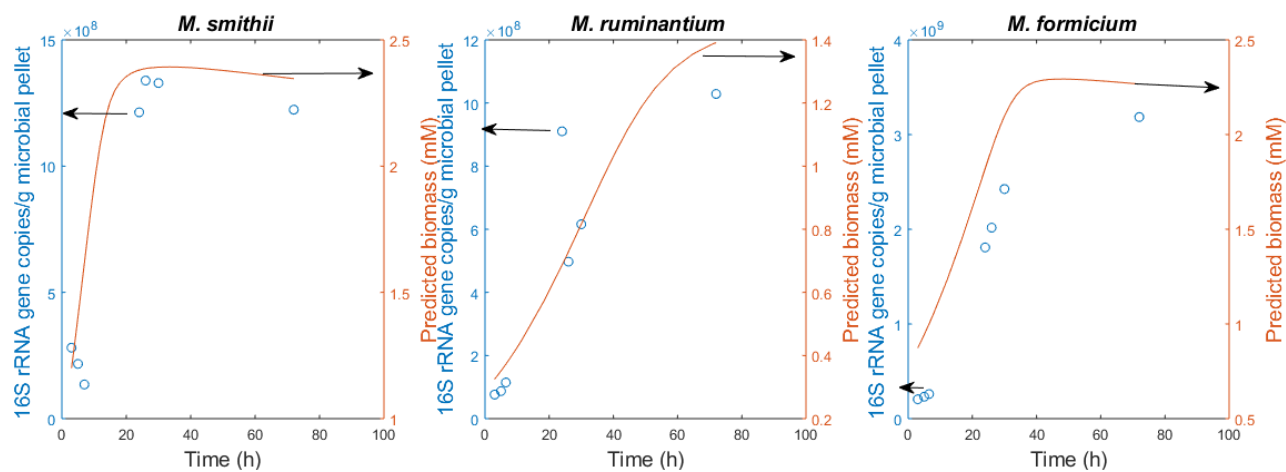
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699 **Figure 4** Dynamics of methanogens as measured by 16S rRNA gene copies (circles) and biomass  
700 concentrations (solid line) predicted by the model.

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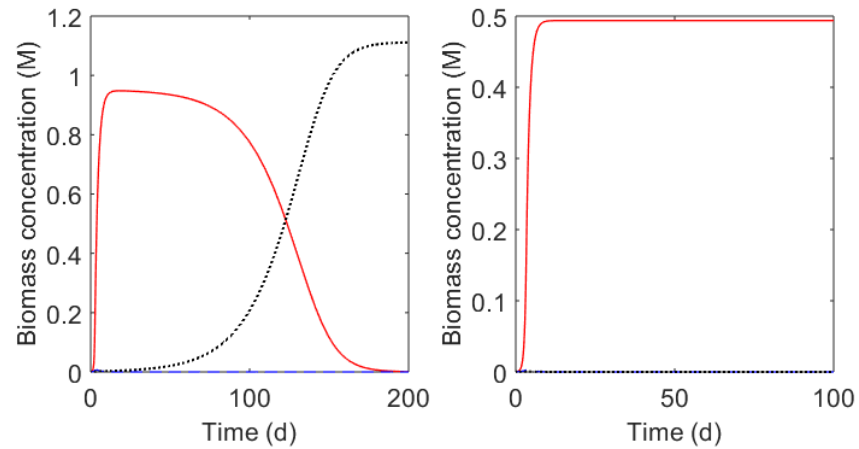
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711 **Figure 5** Possible competition scenarios between *M. ruminantium* (blue dashed line), *M. smithii* (red  
712 solid line) and *M. formicum* (black dotted line) in a hypothetical constant environment. A. At constant  
713 dilution rate of  $0.021 \text{ h}^{-1}$ , *M. formicum* displaces the other two methanogens. B. With a constant  
714 dilution rate of  $0.04 \text{ h}^{-1}$ , *M. smithii* wins the competition. At constant environmental conditions, only  
715 one species wins and displaces the other methanogens.

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729 **Tables**

730 **Table 1** Gibbs energies, enthalpies and entropies of metabolic processes involving some methanogens  
 731 growing on different energy sources

Microorganism	Energy substrate	Growth conditions	$\Delta G_m$ kJ / C- mol	$\Delta H_m$ kJ / C-mol	$T\Delta S_m$ kJ / K <sup>-1</sup> C- mol	Driving force	Reference
<i>M. ruminantium</i> , <i>M. smithii</i> ,	H <sub>2</sub> /CO <sub>2</sub>	anaerobic	-1073	-2132	-1059	Enthalpy-driven but Entropy- retarded	this work
<i>M. formicium</i>	H <sub>2</sub> /CO <sub>2</sub>	anaerobic	-801	-1605	-804	Enthalpy-driven but Entropy- retarded	this work
<i>M. thermo- autotrophicum</i>	H <sub>2</sub> /CO <sub>2</sub>	anaerobic	-802	-3730	-2928	Enthalpy-driven but Entropy- retarded	(53)
<i>M. formicium</i>	formate	anaerobic	-880	-613	+267	Enthalpy-driven	(30)
<i>M. barkeri</i>	methanol	anaerobic	-570	-420	+150	Enthalpy-driven	(30)

<i>M. barkeri</i>	acetate	anaerobic	-366	+145	+511	Entropy-driven but enthalpy- retarded	(76)
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732 **Table 2** Parameters of the model of *in vitro* methanogenesis. The value reported  
733  $\mu_{\max}$  for each methanogen is the mean value obtained from three heat flux-time curves

Parameter	Definition	Value		
$k_L a$ (h <sup>-1</sup> )	Liquid–gas transfer constant	8.33		
$K_{H,CO_2}$ (M/bar)	Henry’s law coefficient of carbon dioxide	0.0246		
$k_d$ (h <sup>-1</sup> )	Death cell rate constant	8.33x10 <sup>-4</sup>		
		<i>M. smithii</i>	<i>M. ruminantium</i>	<i>M. formicium</i>
$K_s$ (mol/L)	Affinity constant	0.028	0.042	0.011
$\mu_{\max}$ (h <sup>-1</sup> )	Maximum specific growth rate constant	0.12	0.07	0.046
$Y$ (mol biomass /mol H <sub>2</sub> )	Microbial biomass yield factor	0.006	0.006	0.007

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735 **Table 3** Statistical indicators for model evaluation

Component	CCC*	$r^2$	$CV_{RMSE}^{**}$
Hydrogen	0.99	0.97	14
Methane	0.97	0.95	18
Carbon dioxide	0.94	0.88	6

736 \* CCC: Lin's concordance correlation coefficient.

737 \*\*  $CV_{RMSE}$ : coefficient of variation of the root mean squared error.

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## 752 **Supplementary Material**

### **Hydrogenotrophic methanogens of the mammalian gut: functionally similar, thermodynamically different - A modelling approach**

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#### 761 **1. Growth media information and inoculation conditions**

762 **Table S1** Methanogens growth media composition (modified from DSMZ medium 119  
763 [https://www.dsmz.de/microorganisms/medium/pdf/DSMZ\\_Medium119.pdf](https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium119.pdf) . Growth media was  
764 distributed in Balch tubes (6 ml per tube), tubes were sealed and sterilized by autoclaving at 121°C for  
765 20 min. Media preparation and distribution was realized under CO<sub>2</sub> flushing to assure anoxic  
766 conditions. Oxygen traces from commercial gases were scrubbed using a heated cylinder containing  
767 reduced copper (35).

<b>Composition per 100 ml</b>	<b>Amount</b>
Clarified rumen fluid <sup>1</sup>	30 ml
Dipotassium phosphate (K <sub>2</sub> HPO <sub>4</sub> ) 0.06% (w/v)	5 ml
Balch Mineral solution <sup>3</sup>	5 ml
Tryptone	0.2 g
Yeast extract	0.2 g
Balch oligo-elements solution <sup>4</sup>	1 ml
Balch vitamin solution <sup>5</sup>	1 ml
Resazurin 0.1% <sup>2</sup>	1 ml

Ammonium chloride	0.05 g
Sodium acetate	0.25 g
Sodium formate	0.25 g
Sodium carbonate	0.5g
L-cystein HCl <sup>2</sup>	0.4g
Distilled water	qs 100 ml

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- 768 <sup>1</sup> Rumen fluid, that was the main constituent of the culture medium, was sampled through the rumen cannula from a grazing dairy cow  
769 prior to the beginning of the experiment. Sampled rumen contents were strained through a monofilament cloth, the filtrate was then  
770 centrifuged at 5 000 g for 15 min. The supernatant was autoclaved and centrifuged again at the same conditions as above. The clarified  
771 rumen fluid (decanted supernatant) was stored at -20°C and centrifuged again after thawing prior to media preparation.
- 772 <sup>2</sup> Media was boiled to expel dissolved oxygen, a reducing agent (L-cystein) and a redox indicator (resazurin) were added to keep a low  
773 redox potential and indicate the oxidative state of the medium, respectively.
- 774 <sup>3</sup> KH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O (0.6g), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (0.6g), NaCl (1.2g), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.12g), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.12g), distilled water qs 100 ml
- 775 <sup>4</sup> Nitrilotriacetic acid (0.15 g), MgSO<sub>4</sub>·7H<sub>2</sub>O (0.3g), MnSO<sub>4</sub>·2H<sub>2</sub>O (0.05g), NaCl (0.1g), FeSO<sub>4</sub>·7H<sub>2</sub>O (0.01g), CoSO<sub>4</sub> (0.01g),  
776 CaCl<sub>2</sub>·2H<sub>2</sub>O (0.01g), ZnSO<sub>4</sub>·2H<sub>2</sub>O (0.01g), CuSO<sub>4</sub>·5H<sub>2</sub>O (0.001g), AlK(SO<sub>4</sub>)<sub>2</sub> (0.001g), H<sub>3</sub>BO<sub>3</sub> (0.001g), NaMoO<sub>4</sub>·2H<sub>2</sub>O (0.001g),  
777 NiCl<sub>2</sub>·6H<sub>2</sub>O (0.01g), Na<sub>2</sub>SeO<sub>3</sub> (0.001g), distilled water qs 100 ml
- 778 <sup>5</sup> Biotine (0.2 mg), PABA (0.5 mg), Riboflavine (0.5 mg), Pantothenic acid (0.5 mg), Sodium ascorbate (0.5 mg), Folic acid (0.2 mg),  
779 Niacin (0.5 mg), Pyridoxine (0.10 mg), thiamine (0.05 mg), Vitamin B12 0.1mg/ml (0.1 ml), lipoic acid (0.5 mg), Choline chloride (0.5  
780 mg), Inositol (0.5 mg), Nicotinamide (0.5 mg), Pyridoxal (0.5mg), distilled water qs 100 ml

**Table S2** Summary of initial OD and pressure measured immediately after primary inoculation. Presented are means (sd) of 31 values per strain.

Strain	<i>Methanobrevibacter ruminantium</i>	<i>Methanobrevibacter smithii</i>	<i>Methanobacterium formicium</i>
Initial OD	0.054 (0.007)	0.099 (0.027)	0.046 (0.01)
Initial pressure (mbar)	2996 (39.956)	2927 (98.985)	2910 (48.883)

## 2. Quantification of methanogens

**Table S3** qPCR quantification of 16S rRNA genes

Time point (hours after inoculation)	<i>Methanobrevibacter smithii</i>						
	3	5	6.5	24	26	30	72
Microbial pellet (g) *	0.204 (±0.007)	0.16 (±0.017)	0.147 (±0.003)	0.194 (±0.002)	0.182 (±0)	0.176 (±0.012)	0.202 (±0.012)
Extracted DNA ng/g of microbial pellet*	9765.5 (±3078)	12695.1 (±838.4)	13752.8 (±2662.3)	41257.4 (±25296.9)	31983.2 (±512.5)	49386.3 (±3956.8)	28943.7 (±7300.7)
Total number of 16S rRNA gene copies in the microbial pellet	5.62x10 <sup>8</sup>	4.34x10 <sup>8</sup>	2.71x10 <sup>8</sup>	2.43x10 <sup>9</sup>	2.68x10 <sup>9</sup>	2.66x10 <sup>9</sup>	2.45x10 <sup>9</sup>
Total number of cells in the microbial pellet **	2.81x10 <sup>8</sup>	2.17x10 <sup>8</sup>	1.35x10 <sup>8</sup>	1.21x10 <sup>9</sup>	1.34x10 <sup>9</sup>	1.33x10 <sup>9</sup>	1.22x10 <sup>9</sup>
Time point (hours after inoculation)	<i>Methanobrevibacter ruminantium</i>						
	3	5	6.5	24	26	30	72
Microbial pellet (g) *	0.148 (±0.048)	0.134 (±0.006)	0.183 (±0.013)	0.161 (±0.006)	0.153 (±0.039)	0.196 (±0.004)	0.164 (±0.023)
Extracted DNA ng/g of	8764.7 (±1345.4)	10864.3 (±1325.5)	7546.3 (±1126.5)	22274.2 (±16)	19412.8 (±4976.2)	16184.1 (±578.6)	23463.5 (±2053.3)

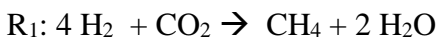
microbial pellet*							
Total number of 16S rRNA gene copies in the microbial pellet	1.52x10 <sup>8</sup>	1.74x10 <sup>8</sup>	2.29x10 <sup>8</sup>	1.82x10 <sup>9</sup>	9.95x10 <sup>8</sup>	1.23x10 <sup>9</sup>	2.06x10 <sup>9</sup>
Total number of cells in the microbial pellet **	7.62x10 <sup>7</sup>	8.71x10 <sup>7</sup>	1.15x10 <sup>8</sup>	9.10x10 <sup>8</sup>	4.97x10 <sup>8</sup>	6.16x10 <sup>8</sup>	1.03x10 <sup>9</sup>
<i>Methanobacterium formicium</i>							
Time point (hours after inoculation)							
	3	5	6.5	24	26	30	72
Microbial pellet (g) *	0.13 (±0.017)	0.167 (±0.017)	0.086 (±0.003)	0.15 (±0.064)	0.167 (±0.044)	0.195 (±0.016)	0.153 (±0.052)
Extracted DNA ng/g of microbial pellet*	19517.7 (±3588.2)	13263.9 (±875)	21530.9 (±7188.2)	63810.6 (±28842.7)	52442.7 (±21309.7)	38602.9 (±2761.3)	60968.8 (±28872.4)
Total number of 16S rRNA gene copies in the microbial pellet	2.05x10 <sup>8</sup>	2.31x10 <sup>8</sup>	2.60x10 <sup>8</sup>	1.81x10 <sup>9</sup>	2.02x10 <sup>9</sup>	2.42x10 <sup>9</sup>	3.19x10 <sup>9</sup>
Total number of cells in the microbial pellet	2.05x10 <sup>8</sup>	2.31x10 <sup>8</sup>	2.60x10 <sup>8</sup>	1.81x10 <sup>9</sup>	2.02x10 <sup>9</sup>	2.42x10 <sup>9</sup>	3.19x10 <sup>9</sup>

\* Mean and standard deviation of two observations.

\*\* *M. smithii* and *M. ruminantium* possess two copies of 16S rRNA gene in their genomes.

### 3. Calculation of thermodynamic properties of the methanogenesis

In our model, the methanogenesis is represented macroscopically by one catabolic reaction (R1) for methane production and one anabolic reaction (R2) for microbial formation. We assumed that ammonia is the only nitrogen source for microbial formation. The molecular formula of microbial biomass was assumed to be C<sub>5</sub>H<sub>7</sub>O<sub>2</sub>N (41).



The thermodynamic properties associated to the methanogenesis result from the contribution of both catabolic and anabolic reactions.

### 3.1 Thermodynamic properties of formation used for the calculations

**Table S4** Standard enthalpies ( $\Delta H_f^\circ$ ) and Gibbs energies ( $\Delta G_f^\circ$ ) of formation at 25°C of compounds involved in hydrogenotrophic methanogenesis. Values were extracted from Wagman et al. (77), with the exception of the microbial biomass that was calculated from values for *Methanosarcina barkeri* reported by Liu et al. (76)

Compound (phase)	$\Delta H_f^\circ$ (kJ/mol)	$\Delta G_f^\circ$ (kJ/mol)
H <sub>2</sub> O (l)	-285.830	-237.129
H <sub>2</sub> (g)	0	0
CO <sub>2</sub> (g)	-393.509	-394.359
CH <sub>4</sub> (g)	-74.81	-50.72
NH <sub>3</sub> (aq)	-80.29	-26.50
C <sub>5</sub> H <sub>7</sub> O <sub>2</sub> N	-511.50*	-349.50*

\* These values are five times the values reported by Liu et al. (76) since the biomass formula we used has five carbon molecules, while the biomass formula used by Liu et al. has one carbon.

### 3.2 Enthalpies

The heat produced during methanogenesis results from the contribution of both catabolic and anabolic reactions. So, first, we calculated the standard enthalpies of the catabolic and anabolic reactions using the standard enthalpies of formation given in Table S4 for the different compounds involved in methanogenesis.

The standard enthalpy of the catabolic reaction  $\Delta H_{r,c}^\circ$  was calculated as follows

$$\Delta H_{r,c}^\circ = \Delta H_{f,CH_4}^\circ + 2 \cdot \Delta H_{f,H_2O}^\circ - (4 \cdot \Delta H_{f,H_2}^\circ + \Delta H_{f,CO_2}^\circ) = -252.96 \frac{\text{kJ}}{\text{mol}}$$

A similar equation was used for the calculation of the standard enthalpy of the anabolic reaction  $\Delta H_{r,a}^\circ$

$$\Delta H_{r,a}^\circ = \Delta H_{f,C_5H_7O_2N}^\circ + 8 \cdot \Delta H_{f,H_2O}^\circ - (10 \cdot \Delta H_{f,H_2}^\circ + 5 \cdot \Delta H_{f,CO_2}^\circ + \Delta H_{f,NH_3}^\circ) = -750.31 \frac{\text{kJ}}{\text{mol}}$$

These results are at 25°C since this is the temperature of the standard enthalpies of formation reported in Table S3. A correction could be made to get results at 39°C but the heat capacities reported by Wagman et al. (77) show that the temperature correction can be neglected. Similarly, in the interest of simplicity, we assumed that the effect of pressure is negligible. Next, we considered the fact that the heat of a given reaction can be calculated at any state along the reaction pathway *via* the determination of the reaction coordinate or degree of advancement  $\varepsilon$  (78). Under our assumptions, the heat produced or consumed by a particular reaction during a given interval can be calculated as follows

$$Q = \int_{\varepsilon_0}^{\varepsilon_{t_f}} \Delta H_r^\circ d\varepsilon$$

For our two reactions, at the instant  $t$  we have

$$\varepsilon_c(t) = \frac{n_{H_2}(t) - f \cdot n_{H_{2,0}}}{-4}$$

$$\varepsilon_a(t) = \frac{n_{H_2}(t) - (1-f) \cdot n_{H_{2,0}}}{-10}$$

where  $n_{H_2}(t)$  is the number of moles of hydrogen at the instant  $t$ ,  $n_{H_{2,0}}$  is the initial number of moles of hydrogen, and  $f$  is the fraction of  $H_2$  used for the catabolic reaction. Our calorimetric experiments started with  $n_{H_{2,0}} = 8.83 \cdot 10^{-5}$  mol in all cases. At the final time  $t_f$ , all the hydrogen was consumed, so that  $n_{H_2}(t_f) = 0$ . For *M. smithii* and *M. ruminantium*, the microbial yield factor is  $Y=0.006$  (6) which implies that  $f=0.94$ . Accordingly,  $\varepsilon_c = 2.075 \cdot 10^{-5}$  mol and  $\varepsilon_a = 5.30 \cdot 10^{-7}$  mol. It thus follows that the overall heat produced during the methanogenesis process ( $Q_m$ ) can be calculated using the following equation

$$Q_m = Q_c + Q_a = \varepsilon_c(t_f) \cdot \Delta H_{r,c}^\circ + \varepsilon_a(t_f) \cdot \Delta H_{r,a}^\circ$$

where  $Q_c, Q_a$  are the heat produced during catabolism and anabolism respectively. The previous equation can also be written as

$$Q_m = n_{H_{2,0}} \left[ \frac{(1-10Y)}{4} \cdot \Delta H_{r,c}^\circ + Y \cdot \Delta H_{r,a}^\circ \right]$$

Under the experimental conditions of our study, this yields

$$Q_m = Q_c + Q_a = (-5.25) + (-0.40) = -5.65 \text{ J}$$

This result shows that the anabolic reaction contributes to only 7% of the metabolic heat.

Since the substrate was totally consumed, the enthalpy of the methanogenesis process per mole (or C-mol) of biomass formed,  $\Delta H_m$ , can be calculated as follows

$$\Delta H_m = \frac{Q_m}{n_{\text{biomass}}} = \frac{Q_m}{Y \cdot n_{\text{H}_2,0}} = \frac{(1 - 10Y)}{4 \cdot Y} \cdot \Delta H_{r,c}^\circ + \Delta H_{r,a}^\circ$$

which yields

$$\Delta H_m = -10658 \frac{\text{kJ}}{\text{mol}} = -2132 \frac{\text{kJ}}{\text{C-mol}}$$

For *M. formicium*,  $Y=0.007$  (7). Applying the same procedure, we obtained  $Q_m = -5.66$  J and

$$\Delta H_m = -1830 \frac{\text{kJ}}{\text{C-mol}}$$

The anabolic reaction contributes to 8% of the metabolic heat.

### 3.3 Gibbs energies and entropies

Following a procedure analogous to the one used above for the enthalpies, the standard Gibbs energies of the catabolic ( $\Delta G_{r,c}^\circ$ ) and anabolic ( $\Delta G_{r,a}^\circ$ ) reactions were calculated using the standard Gibbs energies of formation listed in Table S3.

$$\Delta G_{r,c}^\circ = \Delta G_{f,\text{CH}_4}^\circ + 2 \cdot \Delta G_{f,\text{H}_2\text{O}}^\circ - (4 \cdot \Delta G_{f,\text{H}_2}^\circ + \Delta G_{f,\text{CO}_2}^\circ) = -130.62 \frac{\text{kJ}}{\text{mol}}$$

$$\Delta G_{r,a}^\circ = \Delta G_{f,\text{C}_5\text{H}_2\text{O}_7\text{N}}^\circ + 8 \cdot \Delta G_{f,\text{H}_2\text{O}}^\circ - (10 \cdot \Delta G_{f,\text{H}_2}^\circ + 5 \cdot \Delta G_{f,\text{CO}_2}^\circ + \Delta G_{f,\text{NH}_3}^\circ) = -248.24 \frac{\text{kJ}}{\text{mol}}$$

The free energy of the methanogenesis process per mole (or C-mol) of biomass formed,  $\Delta G_m$ , can then be obtained from the following equation

$$\Delta G_m = \frac{(1 - 10Y)}{4 \cdot Y} \cdot \Delta G_{r,c}^\circ + \Delta G_{r,a}^\circ$$

which yields for *M. smithii* and *M. ruminantium*

$$\Delta G_m = -5364 \frac{\text{kJ}}{\text{mol}} = -1073 \frac{\text{kJ}}{\text{C-mol}}$$

Knowing that

$$\Delta G_m = \Delta H_m - T \cdot \Delta S_m$$

it follows that the entropic contribution to the methanogenesis process is equal to

$$T \cdot \Delta S_m = \Delta H_m - \Delta G_m = -5294 \frac{\text{kJ}}{\text{mol}} = -1059 \frac{\text{kJ}}{\text{C} - \text{mol}}$$

which gives, at 39°C, the following value for the entropy of the methanogenesis process per mole (or C-mol) of biomass formed

$$\Delta S_m = \frac{(\Delta H_m - \Delta G_m)}{(273.15 + 39)} = -16.96 \frac{\text{kJ}}{\text{K mol}} = -3.40 \frac{\text{kJ}}{\text{K C} - \text{mol}}$$

The same procedure applied to *M. formicium* yields  $\Delta G_m = -917 \frac{\text{kJ}}{\text{C-mol}}$ ,  $\Delta S_m = -2.92 \frac{\text{kJ}}{\text{C-mol}}$

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