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Effects of Temperature and Termite' Substrate on Methane and Carbon Dioxide Emissions from *Macrotermes bellicosus* and *Microcerotermes dubius* Cultures

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Abstract: Termites are considered to be a potential natural source of greenhouse gas such as methane (CH_4) and carbon dioxide (CO_2). Although it has been taken into account of termite diet in the estimation of termites' contribution to the global greenhouse gas emission, the emission rates recorded widely varies. Indeed, the emission of gases can be influenced by ecological area and its environmental parameters which can, in return, induce a physiological change among the gut microbial communities. *Macrotermes bellicosus, Microcerotermes dubius* and their intestine microbial community were investigated in this study for CO_2 and CH_4 production *in vitro* at 30°C and 37°C and on different substrates. 37°C appeared the optimal temperature leading to more CO_2 release with both species and 30°C the best temperature for CH_4 production with *M. bellicosus*. The highest productions of CH_4 and CO_2 were obtained in the presence of food substrates. The maximum rate of CH_4 emission was recorded on mango hull (1.62 ppm/termite/day), that of CO_2 upon fungus comb (29.28 ppm/termite/h) with *M. bellicosus*; but that of CO_2 was recorded on millet stem (3.71 ppm/termite/h) with *M. dubius*. A clear CH_4 decrease was observed in the presence of termite mound soil from 11 days. The gut homogenate microflora of both termite species widely exhibited CH_4 upon formate at 30°C and CO_2 upon acetate at 37°C. In general, the best CH_4 production was noted on formate after a long incubation period at 30°C.

Key words: Carbon dioxide · methane · *M. bellicosus* · *M. dubius* · Temperature · Substrate

INTRODUCTION

The atmospheric concentrations of greenhouse gases (GHG) have tremendously increased from pre-industrial levels in response to human activities, resulting in global climate change [1]. Many natural catastrophes are recorded through the world because of this climate change. Hence, it is urgent to lead researches in this way for strategies of adaptation and attenuation of GHG emission [2]. Methane (CH_4), one of GHG, has a global warming potential (GWP) 25 times of that of carbon dioxide (CO_2) on a 100 year time horizon [1]. Atmospheric CH_4 comes from 70-80% from biological sources [3]. Two-third of CH_4 emissions originate from anthropogenic sources such as fossil fuel energy production and use, paddy rice cultivation, enteric fermentation in the guts of ruminant animals, biomass burning, landfill, animal waste and domestic sewage and one-third originates from natural sources including wetlands, CH_4 hydrates, oceans, fresh water and termites [4].

Termites are the subject of several studies in the ecological functioning of savanna ecosystems via nutrient cycling and maintaining soil structure [5] and in global GHG production [6-8]. Termites constitute 61% of total macroinvertebrate abundance in savannas [9-11] and generate 0.2–2.0% of global terrestrial CO_2 emissions [12]. They are one of the few terrestrial arthropods that emit the most CH_4 amounts [13]. The estimates of global annual CH_4 emission by termites have often been debated and vary between 5 and 40% [7, 10, 14-18]. More recently, in a south sudanian savanna of Burkina Faso, Brümmer *et al.* [19] have reported the contribution of *Cubitermes fungifaber* termite mounds in global CH_4 emission at about 0.15%.

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CH_4 and CO_2 are the metabolic end products of oxic/anoxic degradation of vegetable organic matter by symbiotic microbial communities within termite digestive tract [9] following the steps of hydrolysis, acidogenesis, acetogenesis and methanogenesis [20]. Acetate, formate, hydrogen and carbon dioxide are the main precursors of methane present in different compartments of termite gut [21, 22]. Some studies have shown the variation of CH_4 production with regard to the feeding behavior of termite, such as wood-feeding, fungus-cultivating and soil-feeding termites. It is well known that soil-feeding and fungus-growing higher termites emit more CH_4 than wood-feeding termites, whereas wood-feeding termites produce more CO_2 than the others [9]. However, the emission of both gases by termites has been only based on their diet [23-25] without taking into account of growth substrate and temperature influences. The studies undertaken by Fey and Conrad [26] have revealed the increase of methane emission with temperature (10 to 37°C) in anoxic rice soils. In addition, the fermentation of monomeric compounds into certain fatty acids and H_2 in termite gut would play a role in diversity and activity of predominant microbial population for the formation of GHG (CH_4 and CO_2).

This study focused on the variation of CH_4 and CO_2 emissions from termites (which are widespread in the center of Burkina Faso) under laboratory conditions. The objectives of this study were to quantify CH_4 and CO_2 productions from termites and from the microflora of their gut, with regard to temperature of incubation and food.

MATERIAL AND METHODS

Termite Samples Collection and Experiment Setting: The termite collection was carried out within the reserve of Somgandé (12° 24'N, 1°29'W, altitude 294 m) in Ouagadougou (Burkina Faso), on June 2010. Two higher termites' species, *Macrotermes bellicosus* (fungus-growing termites) and *Microcerotermes dubius* (wood-feeding termites) were used in this study. An overdone soil pot containing food debris was placed close to a termite mound of *M. bellicosus* for a night and then termites were captured early morning. For *M. Dubius,* termites were collected from dead wood early morning too. Samples of the two termite species were carried to laboratory and only living and healthy major worker termites were selected for the experiments.

20 worker termites were withdrawn from the overdone soil pot and put into 1 L glass flasks sealed with rubber stoppers. The glass flasks contained damp cotton in order to maintain moisture and to allow termites to take water during the period of incubation. Methane and carbon dioxide emissions were measured following incubation either at different temperatures or with different food substrates. All the experiments were prepared in triplicate.

Effect of Temperature on Methane and Carbon Dioxide Emissions: The flasks containing damp cotton with worker termites were incubated at 30, 33 and 37°C, respectively, in the dark for 18 days. The headspace gas samples were then taken with a gastight syringe and analysed for CO_2 and CH_4 by gas chromatography (GC). 1 ml headspace gas sample was analysed every hour during 6 hours for CO_2, then, at 24 h intervals during 7 days and then at 72 h intervals up to 18 days incubation period for CH_4. Three replicates were prepared for each temperature of incubation.

Effect of Food Substrate on Methane and Carbon Dioxide Emissions: To the flasks containing 20 worker termites and damp cotton described above, 5 g of a food substrate were introduced before flasks were sealed with rubber stoppers. The following food substrates were tested separately: peanut foliage, mango hull, millet stem, paper, soil of termite mound, or fungus comb. Substrate-free flasks were prepared similarly for control. Termite-free flaks containing 5 g of fungus comb and damp cotton were prepared in addition. Flasks were then incubated under room temperature (30±2°C) in the dark during 14 days. 1 ml headspace gas sample was periodically analysed for CO_2 and CH_4 by GC as described above. Gas amounts were determined every hour for 6 hours, then at each 24 h interval period for 14 days, respectively.

Medium and Culture Conditions for Microflora of Termite Gut: Hungate's anaerobic techniques were used throughout these experiments [27, 28]. The culture medium was adapted from the mineral medium described by Balch *et al.* [29] and contained per litre of distilled water: 0.3 g K_2HP0_4; 0.3 g KH_2P0_4; 0.6 g NaCl; 0.1 g $MgCl_2.6H_20$; 0.1 g $CaCl_2.2H_20$; 1 g NH_4Cl; 0.1 g KCl; 0.5 g sodium acetate; 0.5 g cystein-HC1; 1 g yeast extract; 1 g peptone; 1 ml trace element solution [30] and 1 ml 0.1 % (w/v) resazurin. The medium was adjusted to pH 7.0 with 1 N NaOH solution. The medium was boiled for 15 min and

then 9 ml aliquots were immediately distributed into Hungate tubes under a stream of O_2-free Argon gas at room temperature. Then, the tubes were autoclaved at 121°C for 15 min. In parallel, 1 M filter-sterilized anoxic substrate stock solutions (acetate and formate) and filter-sterilized anoxic H_2/CO_2 gas (80:20 % v/v, 100 KPa) were prepared, separately. Before inoculation of the termite gut homogenate at room temperature, 0.3 ml of 10 % $NaHC0_3$ (w/v), 0.1 ml of 2.4 % NaS, $9H_2O$ (w/v) and a metabolic substrate were added into the basal medium.

Preparation of Termite Homogenate and Cultivation: The insects were sterilized with 70% ethanol and washed 2-3 times with distilled water. Worker termites (1g) were put into a 125 ml sterile flask that contained 30 ml distilled water and sterile glass beads, as described by Nkounkou [31]. The flask was then sealed with rubber stopper and the headspace gas was replaced by a stream of O_2-free argon gas. The set was shaken manually for 10 min to obtain the inoculum (termite gut homogenate).

After addition of either H_2/CO_2 (80:20 %, v/v), acetate (40 mM) or formate (40 mM) in the basal medium under sterile-anaerobic conditions, 1 ml inoculum was added in the same conditions. Control tubes were inoculated with 1 ml sterilized inoculum (121°C for 15 min) for each metabolic substrate. Tests were carried out per substrate at 30 and 37°C during 21 days. 1 ml headspace gas sample was taken from tubes by a gastight pressure lock syringe for GC analysis. The sampling was performed every 24 h for 7 days, then at 72 h intervals up to 21 days incubation period for both gases (CO_2 and CH_4).

GC Analysis: CO_2 and CH_4 amounts were determined with a gas chromatograph (Girdel serie 30 cathorometer) equiped with a 80/100 Porapak Q (for CH_4 analysis) and a 100/120 Porapak Q (for CO_2 analysis) columns assembled in parallel and connected to a thermal conductivity detector (TCD) and a potentiometric recorder (SERVOTRACE type Sefram Paris de 1 mV). The injector temperature was set at 90°C, the columns at 60°C and the detector at 100°C. H_2 was used as carrier gas. Methane and carbon dioxide standards (90% and 99% purity, respectively) supplied by *Burkina Industrial Gas* allowed to establish the following regression equations from which the production of both gases during the experiments was deduced:

$$CH_4\ (\mu l) = 29.68\ X + 0.0018 \text{ with } R^2 = 0.9993$$
$$CO_2\ (\mu l) = 21.574\ Y - 0.0182 \text{ with } R^2 = 0.9994$$

X and Y are areas of methane and carbon dioxide peak, respectively.

RESULTS AND DISCUSSION

Influence of Temperature on CO_2 and CH_4 Production: Figures 1 and 2 show that temperature influenced the exhibition of CO_2 and CH_4 by termites, respectively. Indeed, both termite species clearly produced more CO_2 at 37°C than 30°C during the first 6 h incubation period (Fig. 1). Globally, CO_2 production with *M. bellicosus* increased with increasing temperature and was higher than that with *M. dubius* (Fig. 1).

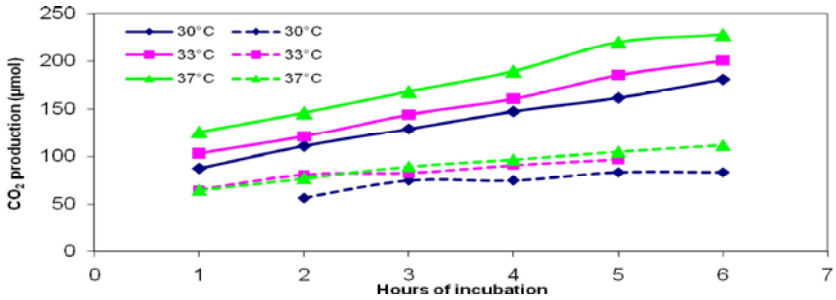

Fig 1: CO_2 production at 30, 33 and 37°C with *M. bellicosus* (line) and *M. dubius* (dash)

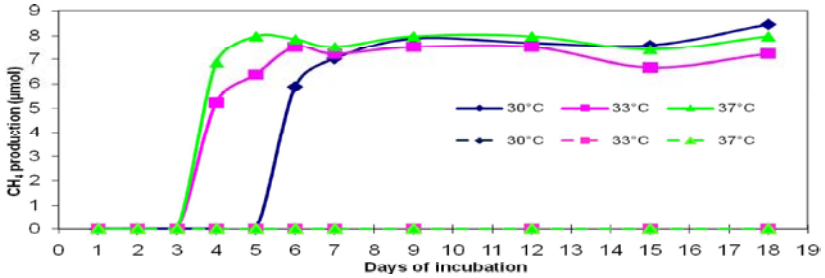

Fig. 2: CH_4 production at 30, 33 and 37°C with *M. bellicosus* (line) and *M. dubius* (dash)

Table 1: CO_2 and CH_4 emission rates (µmol/g termite dry weight/hour) with *M. bellicosus* and *M. dubius* at different temperatures compared to data from other studies. Data on other termite species are quoted from published sources

	M. bellicosus		*M. dubius*		*M. mülleri*		*M. jeanneli*		*M.serrula*	
Temperature	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2
30°C*	0.30±0.02	59.36±1.28	nd	16.64± 0.22						
33°C*	0.35±0.04	66.5± 2.02	nd	18.08±2.97						
37°C*	0.39±0.06	78.4± 5.32	nd	18.82±2.92						
26°C			0.07±0.01[c]						nd[c]	
28°C				13.45±5.38[c]						8.11[c]
29°C	0.42±0.08[a]	25.44[a]			0.35±0.09[a]	20.96[a]				
32°C							0.24± 0.02[b]	68.0± 10.16[b]		

*this study; nd: not detected; [a]Rouland *et al.* [24]; [b]Darlington *et al.* [35]; [c]Eggleton *et al.* [36]

From Figure 2 and Table 1, only *M. bellicosus* produced methane in our study. No methane production was recorded with *M. dubius* in our experiment conditions. CH_4 production was faster at 33 and 37°C than that at 30°C (after 3 and 5 days, respectively: Fig. 2). The amounts produced appeared more pronounced with the increase of temperature up to 7 days incubation period. However, from 15 days incubation period the production became higher at 30°C than that at 37°C and 33°C (Fig. 2). Globally, CH_4 production increased with increasing temperature at beginning then continued to increase slightly at lower temperature (30°C) up to 18 days. Our results agree with those obtained by Fey and Conrad [26] and Nozhevnikova *et al.* [32] from anoxic rice soils and lake sediment slurries, respectively. It was demonstrated that homoacetogenic bacteria outcompete hydrogenotrophic methanogenic bacteria at lower temperature, thus leading to an increase of acetate production to the detriment of methane production [26]. However, it was shown that the contribution of acetotrophic methanogenesis is high at low temperature [32-34]; that could explain why this important accumulation of acetate at low temperature later favours methane production [32], as observed after 15 days of incubation (Fig. 2).

From all the data obtained in our experiment conditions, it appeared that termites released precociously and more CO_2 and CH_4 at 37°C than at 30°C; however, CH_4 production became more important at 30°C over a long incubation period.

Influence of Substrate on CO_2 and CH_4 Production: Substrates showed an effect on CO_2 emission from both termite species (Fig. 3). Whatever the substrate, *M. bellicosus* produced more CO_2 than *M. dubius* (8.57-29.28 and 0.49-10.25 ppm/termite/h, respectively). This difference of carbon dioxide emission between *M. Bellicosus* and *M.dubius* could be due to their diet and their intestinal microbial communities. Indeed, wood-feeding termites harbour an abundant homoacetogenic microflora involved in the conversion of CO_2 into acetate and a few methanogenic bacterial community, in contrast to fungus-growing termites and soil-feeding termites [23, 24, 37].

Fungus comb was the substrate on which termites exhibited important amounts of CO_2. Moreover, this substrate showed an important production of CO_2 in the absence of termite (9.15 ppm/g fungus com b/h) (Fig. 3). Apart from fungus comb, *M. dubius* produced a substantial CO_2 amount on millet stem

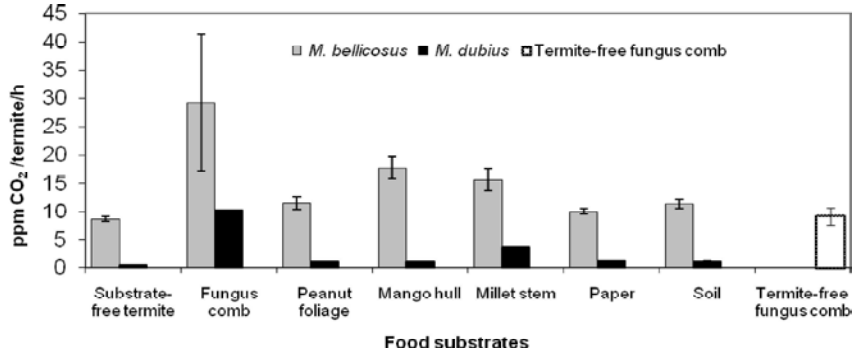

Fig 3: Carbon dioxide emission rates (ppm/termite/hour) on different food substrates with *M. bellicosus* and *M. dubius* at 30±2°C.

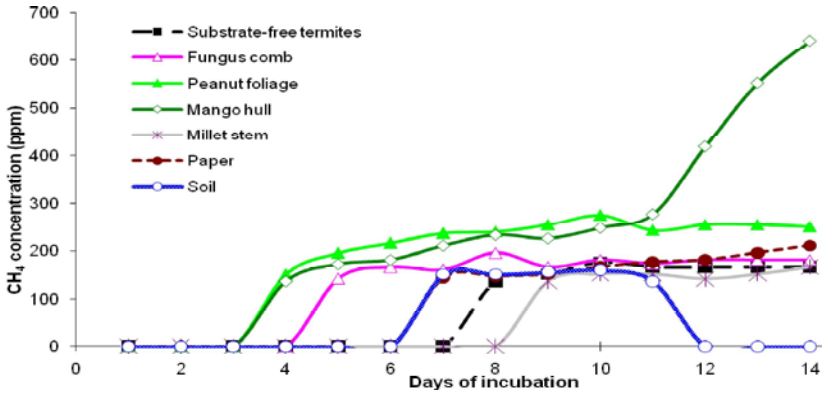

Fig. 4: Methane production on different food substrates with *M. bellicosus* at 30±2°C.

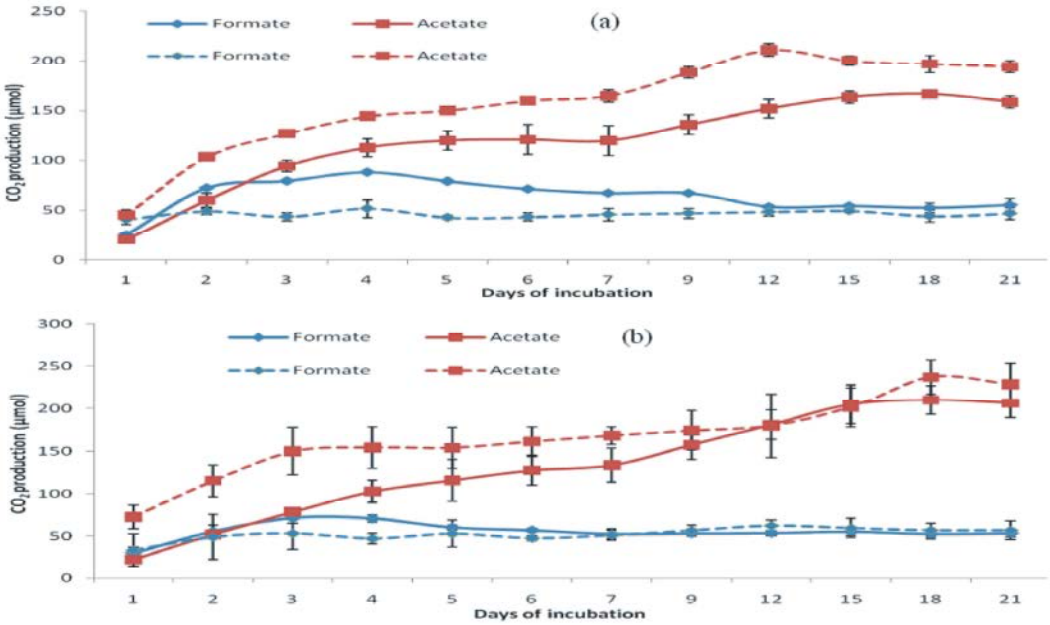

Fig 5: CO_2 produced on growth substrate by *M. Dubius* (a) *and M. bellicosus* (b) termite guts at 30°C (line) and 37°C (dash).

(3.71 ppm/termite/h). Darlington *et al*. [35] and Gomathi *et al.* [38] reported that fungus comb participates as well to CO_2 emission without affecting methane emission in termite mounds where it constitutes the basic food for *Macrotermes* termites. These termites cultivate a symbiotic fungus which aerobically digests vegetable organic matter [35], thus leading to CO_2 releasing which adds with that of termites.

As for methane, only *M. bellicosus* was capable to produce it up to 1.62 and 1.44 ppm/termite/day on mango hull and peanut foliage, respectively (Fig. 4). While methane emission was constant on other substrates, the emission rate on mango hull during the last days of incubation was more than twice of that recorded after the 11th day. It was also observed a clear CH_4 decrease (from 160.50 ppm to 0.03 ppm) with the soil of termite mound after 11 days incubation period. This result confirms the low concentration of methane in the termite mounds of *M. bellicosus* recorded in our previous study (Sawadogo *et al*., submitted for publication). Furthermore, the existence of methanotophic bacteria involved in CH_4 uptake in soils and probably that of sulfate-reducing bacteria which outcompete methanogens for substrate in termite gut were suggested [3, 15, 25, 31, 39]. These key processes might play an important role in CH_4 attenuation in ecologic ecosystems. Thus, CH_4 and CO_2 productions could be under the dependence of microbial populations of termite guts.

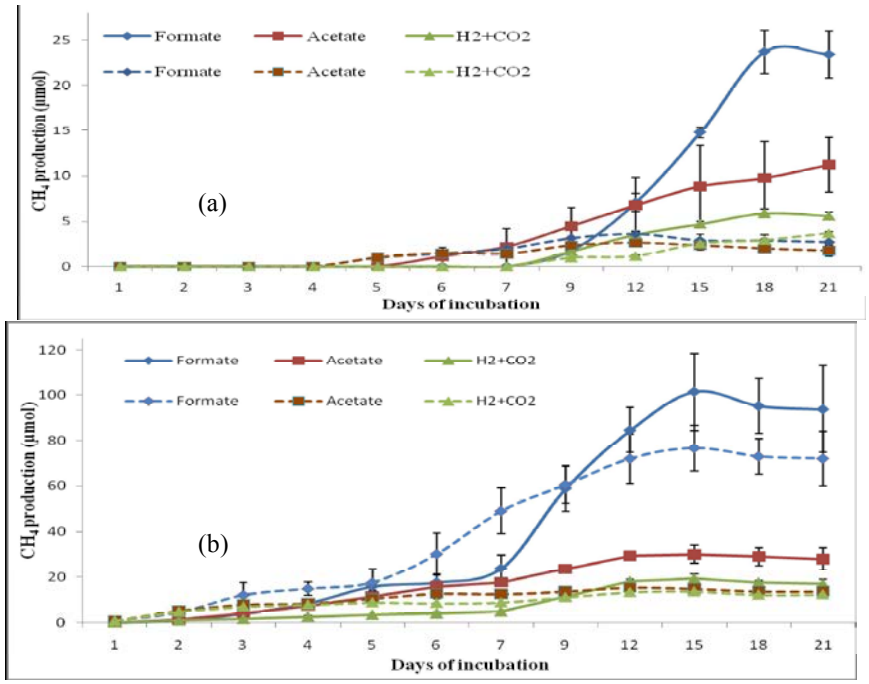

Fig. 6: CH_4 produced on growth substrate by *M. Dubius* (a) and *M. bellicosus* (b) termite guts at 30°C (line) and 37°C (dash)

Incidence of Termite Gut Homogenate Microflora on Carbon Dioxide and Methane Productions: The CO_2 production from guts of both termite species was observed at both 30 and 37°C (Fig. 5). Substrates appeared to affect CO_2 production. Indeed, optimal CO_2 productions on acetate at 30 and 37°C were 210.15 and 237.08 µmol with *M. bellicosus* gut homogenate and 166.72 and 211.16 µmol with *M. dubius* one, respectively. The CO_2 emission was more important on acetate than formate. In addition, it was noted that this production was slightly increased at 37°C on acetate and at 30°C on formate. The low concentration of CO_2 in the presence of formate could be due to the catabolism of formate into acetate by homoacetogens [40] and probably to the coexistence of homoacetogens and methanogens in termite gut [41].

Methane production was tested with acetate, formate and H_2/CO_2 from termite homogenate culture at 30 and 37°C (Fig. 6). The production varied according to the growth substrate. Formate gave a good CH_4 production whereas acetate and H_2/CO_2 led to low amounts, whatever the temperature of incubation. Schmitt-Wagner [21] also found a best emission of CH_4 with formate-utilizing methanogens in gut homogenates of *Cubitermes* spp and concluded formate as a potential substrate which stimulates more CH_4 emission than H_2 in termite hindgut. According to Garcia [42], more than 43% of methanogens ferment formate. On the other hand with H_2, high H_2 partial pressures are responsible for the predominance of homoacetogenesis over methanogenesis in termite guts [21, 43, 44]. Tolen and Brune [44] also reported that the stimulation of acetogenesis by the addition of exogenous H_2 or formate was more pronounced than that of methanogenesis, whereas the rates of methanogenesis were always significantly higher than those of reductive acetogenesis in hindgut of *Cubitermes* spp (soil-feeding higher termite). The low methane emission with acetate and H_2 could be also explained by the high affinity of sulfate-reducing bacteria for these substrates ($\Delta G^{0'}$= -60 and -172 Kj/reaction for acetate and H_2, respectively) to the detriment of methanogens ($\Delta G^{0'}$= -28 and -130.7 Kj/reaction for acetate and H_2, respectively) [26, 45]. Our results showed at 15 days of incubation, methane production rates of 0.304 and 1.021 µmol/g/h on acetate and formate, respectively, with *M. bellicosus*. These low values could be related to the trophic relationships among methanogens, homoacetogens, sulfate-reducing bacteria, methanotrophs and other trophic groups [31, 39, 45]. In contrast, Gomathi *et al*. [38] showed that a *Methanosarcina* strain isolated from *Macrotermes* were able to produce, in pure culture, up to 1596.32 µmol CH_4/g/h upon acetate.

Increasing temperature also led to precocious CH_4 production on the whole (Fig. 6). With *M. dubius*, methane was produced from 4 and 5 days of incubation at 37°C and 30°C, respectively (Fig. 6a). Likewise, with *M bellicosus*, it appeared from 1 and 2 days of incubation at 37°C and 30°C, respectively (Fig. 6b). However, 9 days later globally, methane production became higher at 30°C than at 37°C whatever the substrate (Fig. 6a, 6b), except that on formate at 37°C which always remained greater than that on acetate and H_2/CO_2 at 30°C (Fig. 6b). The trends of our results agree with those of Morabandza [46] which showed that methanogens are activated from 35°C. On the other hand, certain methanogens group could be able to adapt under conditions of temperature and substrate available. For instance, Gomathi *et al*. [38] revealed a maximum production of methane at 30°C compared to 37°C with a *Methanosarcina* strain isolated from *Macrotermes* sp gut. Furthermore, Fey and Conrad [26] found that *Methanosarcinaceae* decreased whereas *Mehanosaetaceae* increased with increasing temperature (from 10 to 37°C) on acetate. *Methanosarcinaceae* and *Methanobacteriaceae* were two families detected by probes in termite guts [9]. Although *Methanosarcinaceae* were found to be dominant in termite gut homogenate culture [22, 37, 38], that not signifies really their activity was predominating in this environment. Indeed, using probes targeting the 16S rRNA of methanogens, Brauman *et al*. [47] revealed that the members of *Methanobacteriaceae* were more abundant in the gut community of many termites. Furthermore, these latter use much more formate and H_2/CO_2 as growth substrate, energy source and electron donor for methane production in termite guts [21, 38, 48]. Hence, substrate and temperature could be limiting factors contributing to the diversity, physiological change and interaction among microbial populations in termite guts.

This study allowed to understanding carbon dioxide and methane emissions throughout cultivation of termites and their intestinal microflora in relation to diet variation, food substrate and temperature. Methane and carbon dioxide amounts were greater with *Macrotermes bellicosus* than with *Microcerotermes dubius*, whatever temperature and growth substrate. Both gases were more and precociously produced at 37°C than 30°C, but generally, maximum methane and carbon dioxide productions were recorded at 30°C and 37°C as well, over a long incubation period. According to culture conditions (*i.e*. temperature and substrate), the exhibition of gas depends on microbial communities of termite gut and their adaptation to anoxic environments. The microflora of termite homogenates emitted high methane amount upon formate at 30°C and high carbon dioxide amount upon acetate at 37°C. A phenomenon of competition and/or coexistence involved in CH_4 and CO_2 production could exist between different microbial communities. Therefore, it appears of great importance to get more insight the principal microbial groups inhabiting termite guts (through molecular approaches) and the relations among them leading to CO_2 and CH_4 emissions with respect to some ecological factors *i.e*. temperature and substrate.

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