

Anaerobic cometabolism of fruit and vegetable wastes using mammalian fecal inoculums: Fast assessment of biomethane production



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ABSTRACT

Anaerobic digestion (AD) is a biological process which produces biomethane as energy source, using waste as substrate. Cometabolism is a novel way to enhance liquid AD, via augmenting the biodiversity of inoculums, especially in the hitherto little-studied 96-h initiation period critical to AD startup. Dog, horse and sheep feces were used as sources of inoculum and mixed with fruit and vegetable waste (FVW) and water. Thermophilic (55 °C), mesophilic (39 °C) and psychrophilic (25 °C) AD conditions were tested. As inoculum and water quantities alter the abundance and diversity of the anaerobic communities, 1:1 and 1:4 ratios of FVW feedstock to inoculum solutions were compared. Live *Saccharomyces cerevisiae* was supplemented as probiotic in the anaerobic reactors. Biogas, CO₂ and CH₄ were measured as well as digestion of FVW. Results showed consistently more CH₄ production under cometabolism, higher inoculum density and thermophilic conditions; higher CH₄, pH and digestion simultaneously occurred in the mixed-inoculum reactors. A strong relationship between biogas and its CO₂ and CH₄ main components was found. Horse inoculum in the mixed reactors enhanced CH₄ production; sheep inoculums improved digestibility; and the dog inoculum seemed to neutralize pH. *S. cerevisiae* may have improved cellulolytic activity in FVW digestion at 25 and 39 °C, and provided an energy and nutrient source at 55 °C.

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1. Introduction

The anaerobic digestion (i.e., AD) is an alternative to landfilling dog feces and fruit and vegetable waste (FVW). The global amount of the latter is immense (>650 million tonnes per year; FAO, 2013), difficult to estimate (Parfitt et al., 2010) and certainly rising (Hall et al., 2009). As to domestic animal feces they are egressed by nearly 1 billion dogs worldwide (Gompper, 2014) as well as 50 billion head of livestock (Goodland and Anhang, 2009).

Compared to landfills, AD of animal and food waste reduces greenhouse gas emissions (CO₂ and CH₄), landfill fires, acidic leachates and ensuing (ground)water contamination, proliferation of pathogens and their vectors, and mixing of antibiotic multi-resistance genes. The AD of water and animal waste at

thermophilic temperatures is feasible and effective in inactivating pathogens (WHO, 2012), reducing odors and effluent solids (Metcalf & Eddy, 2003).

In addition, AD generates energy as CH₄, CO₂ (used in green chemistry and fungi production) and a low-cost fertilizer without aerobic pathogens. Biomethane can be used to increase the temperature of the AD reactors optimizing the process and reducing energy costs. Methane is safe if contained in a sealed environment. As to biomethane combustion, it is deemed carbon-neutral CO₂, since its carbon emissions balance out a recent atmospheric CO₂ uptake by plants (Ward et al., 2008). Biomethane combustion can substitute fossil fuels and biofuels, thus reducing, among others, polycyclic aromatic hydrocarbons, organometal(loid)s, radionuclides, and smog and acid rain compounds. AD of herbivore and food waste has been previously studied but information on the usefulness of inoculum from dog feces is needed, or on the possible thermophilic inactivation of pathogens which afflict monogastric mammals living in close contact, such as humans and dogs.

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The 30–90 day startup is the most critical step of an AD system (Zhu et al., 2014) during which adaptation and regeneration of the inoculated microbial communities take place (Shen et al., 2013). However, the first hours (initiation) of AD have seldom been researched.

Here, AD improvements tried to emulate the highly diverse mammalian digestive tract. The process started with the collection of feces from (ruminant and non-ruminant) mammals and the extraction of inoculum to study cometabolism, or biodiversity-augmented microbial digestion. In cometabolism, a consortium of different species transforms organic compounds faster than individual species, because one species produces intermediate byproducts that are needed by others (Islas-Espinoza et al., 2012). Therefore, a consortium of three different anaerobic microbiomes supplemented with *S. cerevisiae* was tested to evaluate AD cometabolism. Sheep, horse, and dog inoculums were expected to bring in biodiversity from ruminant and non-ruminant intestinal microbiomes, to try and cope with rapid acidification brought about by FVW, which inhibits methanogenesis (Shen et al., 2013). In addition to inoculum biodiversity, its quantity is essential, since low abundance of methanogens causes accumulations of volatile fatty acids which can inhibit methanogenic growth and activity, hence a protracted startup (Zhu et al., 2014).

Live yeast (*S. cerevisiae*) used as feed additive has shown multiple benefits in the rumen: increased acetogenic bacteria, fungi and protozoa (Mao et al., 2014; Hristov et al., 2010), supply of microbial growth factors like organic acids, lipids, proteins, enzymes and vitamins (Chaucheyras et al., 1995; Erasmus et al., 1992), increased cellulolytic activity (Kumar et al., 2013), increased nutrient digestibility and fermentation of fiber (Elghandour et al., 2014; Salem et al., 2015), and oxygen scavenging with improved anaerobiosis (Chaucheyras-Durand et al., 2008).

Liquid AD takes place below 15% total solids content and has higher reaction rates hence shorter retention times than solid-state AD, agitation is less energy-intensive, and contact is better between microbes and feedstock. Essential to AD is the feedstock to inoculum (F:I) ratio because high F:I lead to overproduction of volatile fatty acids, acidification and inhibition of methanogens (Zhu et al., 2014).

Accordingly, the goal of this research was to assess the effects of cometabolism on key AD parameters (gas production, pH, and digestion).

2. Material and methods

2.1. Sampling

Fresh samples of dog, sheep and horse feces (1.5 kg per species) were collected once a week in the morning at the State of Mexico University laboratory vivarium and farm to ensure sufficient inoculum quantities. The dogs were 13 kg mean body weight, 3-year-old beagles; the horses 425 kg BW, 13-year-old quarter; and the sheep 70 kg BW, 1-year-old finnsheep. None of the animals had received vaccines, antibiotics or antiparasitics at the time of collection of feces. To minimize exposure of the inoculum to air oxygen and litter material, composite dog feces were collected immediately upon defecation following the morning meal. Horse manure was collected from the intestine with a palpation glove. Sheep morning feces were collected immediately upon defecation. Inoculum density was defined as the amount of fresh microbial biomass extracted from 10 or 25 g of fresh mammal feces. Inoculum density was used to explore F:I effects. The fecal samples (10 or 25 g w/w) were stirred in (pH 6.9) distilled water to obtain a 50 mL dilution; from this homogenate the inoculums were extracted by filtration through gauze layers to obtain a fluid fraction free of

particles larger than 1 mm.

The feedstock was a mixture of kitchen waste from three university canteens (vegetables and fruits only). The mixture was oven-dried for 48 h at 50 °C to avoid volatilization of nitrogen compounds and ground to 1 mm particle size, in a 0.25 HP dry matter (DM) General Electric mill, at 1725 rpm.

2.2. Processing

Each 150 mL bioreactor contained a 50 mL mixture of 10 g:39 mL:1 g or 25 g:24 mL:1 g diluted inoculum, distilled water and FVW, respectively. Headspace to contain biogas was 100 mL. Each bioreactor also received 2.0 mg of live *S. cerevisiae* yeast (Procreatin 7, Safmex/Fermex, Toluca, Mexico, in powder containing 1×10^{10} cells/g). Each sample was flushed with CO₂ for 8 min to diminish the oxygen concentration and mix FVW and inoculum, and then transferred to the bioreactors closed with Vacutainer stoppers.

The bioreactors (n = 72) were divided among eight treatments with three replicates: four inoculum types (dog, sheep, horse and an equal-parts mixture), and two inoculum densities. The procedure was performed at three temperatures (25, 39 and 55 °C).

The bioreactors were transferred to a temperature-controlled PolyScience model 28 L water bath and incubated for 96 h. Total biogas pressure was measured with a needle-equipped manometer (407,910 differential pressure manometer, Extech Instruments, Waltham, MA, USA) every 2 h until hour 12, and then at 18, 24, 36, 48, 72 and 96 h, while CH₄ and CO₂ were extracted with a (5 mL) syringe and introduced into an industrial gas detector (Gas-Pro Analyzer, Crowcon, Abingdon, UK). After each gas measurement, all remaining gas was purged from each bottle to minimize disturbance of the fermentation process by dissolution of gases into the liquid. At the end of each experiment, pH was measured to assess possible inhibition or continuation of AD.

Following 96 h, the fermentation process was stopped, the bottles unstoppered and pH measured using a pH meter (pH15 Model, Conductronic, Puebla, Mexico); the contents of each bottle were filtered out, dried at 65 °C for 24 h, and then compared with the initial DM weight. This was done to assess the biodegradability of FVW and to relate digestion.

2.3. Data analysis

Biogas, CO₂ and CH₄ measurements in PSI, percentage of CO₂ and percentage of lower explosive limit (%LEL) were transformed into IS units (mL/g DM) following El-Mashad and Zhang (2010) and Theodorou et al. (1994). The latter is a relatively simple method, which was combined here with an industrial gas detector rather than the more complex apparatus involving gas chromatography, to provide a rapid assessment method.

One-way Anova with post hoc tests and three-way Anova helped assess differences between treatments and interactions among variables (p < 0.05). Multiple linear regression was used to identify conditions leading to faster accumulation of gases, pH changes and feedstock digestion. Principal components analysis helped to identify the main sources of variance among samples and treatments and so, guide the regression analyses. These analyses were carried out using SPSS v12. Kinetic models were fitted to the time series data using Excel 2013. Part of the rapid assessment method, predictive models fitted to early observation of key parameters can inform timely operation decisions, reducing the need for posterior monitoring of a wide array of AD variables.

3. Results

The p values of the temperature main effect (i.e., 25, 39 and 55 °C) and inoculum type (dogs, horses and the sheep) or their interactions are shown in Table 1. One third (20/72) of the samples produced CH₄ in the first four days, with isolated pulses of CH₄, was the most frequent mode of emission, and flukes of intense release in the most productive treatments. CH₄ production started by hour 2; most production took place in two bursts between hours 6–12 and hours 36–96; the maximum peak was 0.9759 mL CH₄/g DM. between hours 8 and 10.

3.1. Cometabolism

The mixture of inoculums from all three mammals showed enhanced CH₄ production suggestive of cometabolism (Figs. 1a, b and 2b). A majority of CH₄-producing samples involved high inoculum densities (extracted from 25 g feces) and mixed inoculums, both of which likely augmented microbial diversity (Fig. 1a). Most CH₄-producing samples (Fig. 1a) occurred at 39 and 55 °C, while pH closer to neutrality occurred at mesophilic temperature (39 °C) typical of mammalian physiology (Fig. 1d).

Horse inoculum seemed to help increase the frequency of samples that produced CH₄ and decreased biogas (Figs. 1a and 2a), while sheep inoculums helped increase FVW digestion (Fig. 2b). As to dog inoculum, it seemed endowed with a buffering capacity (Fig. 1c and d). This was particularly noticeable with high-density dog and mixed inoculums (Fig. 1c). Furthermore, dog inoculum (samples beyond pH 5.5 in Fig. 1d) reversed the usual relationship between CO₂ and acidification, while herbivore samples without dog inoculum (53, 55–60 in Fig. 1d) led to more acidic media with lesser CO₂ production.

3.2. Optimality

Cometabolism seemed to lead to a CH₄, pH and digestion optimum. Again this hinged on higher temperatures, inoculum densities and mixed inoculums (Fig. 3a and b). The process may have benefitted in a non-linear way, since CH₄ could not be predicted based on the parameters studied here. Under these optimal conditions, one advantage of 55 °C was the potential for disinfection of several pathogens (Tchobanoglous et al., 1993– Table 2); one disadvantage could be the inactivation of *S. cerevisiae* and its possible use as carbon or nutrient source only.

3.3. Kinetics

Patterns of gas accumulation were modeled for the Grand Total of 72 samples, a 55 °C reactor consisting of 24 samples, the seven CH₄-pH optimal samples, and the sample 72 reactor with thermophilic, mixed, high-density inoculum. Based on goodness-of-fit and non-linear regression coefficients (Fig. 4a and b), biogas and CH₄ kinetics predicted maximal CH₄ volume ratios of 55–80% in the biogas between 154 and 164 h (pH-CH₄ optima; 4 samples at 39 °C, and 3 at 55 °C) and 200–210 h (55 °C samples).

Biogas accumulation could be understood as a linear curve with 2 parameters: initial value and growth rate of ln(biogas). Both biogas and CO₂ could be modeled as two linear segments: a steep slope until hour 10 and a plateau thereafter (Fig. 4a). Meanwhile, the CH₄ accumulation curve was exponential with a vertical asymptote eventually given by the remaining 35% available FVW. The non-linearity and kinks resulting from slackening and acceleration in CH₄ accumulation proved more difficult to fit.

The Grand Total accumulated biogas and CO₂ were 2–33-fold higher and 2–30-fold higher, respectively, than the other pooled reactors. Grand Total CH₄ was only 1 or 2-fold that of other pooled reactors, because fluke CH₄ production in sample 72 explained 63–77% of the other reactors. In other words, sample 72's CH₄ fraction of biogas was 16% compared to 1–3% in the other reactors. CO₂ was 25% of biogas in the Grand Total reactor and 1–13% in the other reactors.

3.4. Predicting key parameters

Final biogas accumulations were also predictable as a function of experimental (temperature, inoculum type and density) and output variables (gases, pH and digestion).

The best regression models (in terms of goodness-of-fit and compliance of assumptions) confirmed the strong relationship between biogas and its CO₂ and CH₄ components, and all the gases could be predicted from each other as they were somewhat linearly interrelated. Contrariwise, a relationship between pH and CO₂ was not found.

Two valuable models were biogas = f (temperature, pH) as well as pH = f (temperature, inoculum animal, biogas). Biogas increments of one mL/g DM were linked to 0.628 and 0.772 increments in temperature and pH, respectively. These models however did not accurately represent biogas accumulation under psychrophilic (25 °C) conditions (Fig. 5a and b). As to pH increases of one unit, they were linked to a 0.763 decrease in temperature and one-unit increase in biogas. CO₂ increments by one unit could

Table 1
Three-way Anova significant (p-values) main effects and interactions.

	Temperature	Inoculum type (mammal)	Inoculum density	Temperature x mammal	Temperature x inoculum density	Temperature x mammal x inoculum density	Mammal x inoculum density
Biogas	25 °C	dog ≠ herbivores	^d	0.000	0.000	0.000	0.000
CO ₂	all temperatures	dog ≠ horse	(1:1 ≠ 1:4) ^a		0.028		0.004
CH ₄	all temperatures ^c :						
Digestion	25 ≠ 55 °C	mix ≠ horse, mix ≠ dog		0.001			0.004
pH	39 ≠ 55 °C	All different but sheep = horse	(1:1 ≠ 1:4) ^b	0.000	0.000	0.000	0.000

Main effects were obtained from one way anova post hoc analyses and interactions from three-way anova. Anova was robust to variance heterogeneity since groups were of equal size.

^a CO₂: p value = 0.071.

^b pH: p value = 0.052.

^c In three-way anova, no CH₄ differences were due to main effects or interactions.

^d In three-way anova, i.e. simultaneously considering interactions, inoculum density differences were linked to significant differences in biogas (p < 0.000).

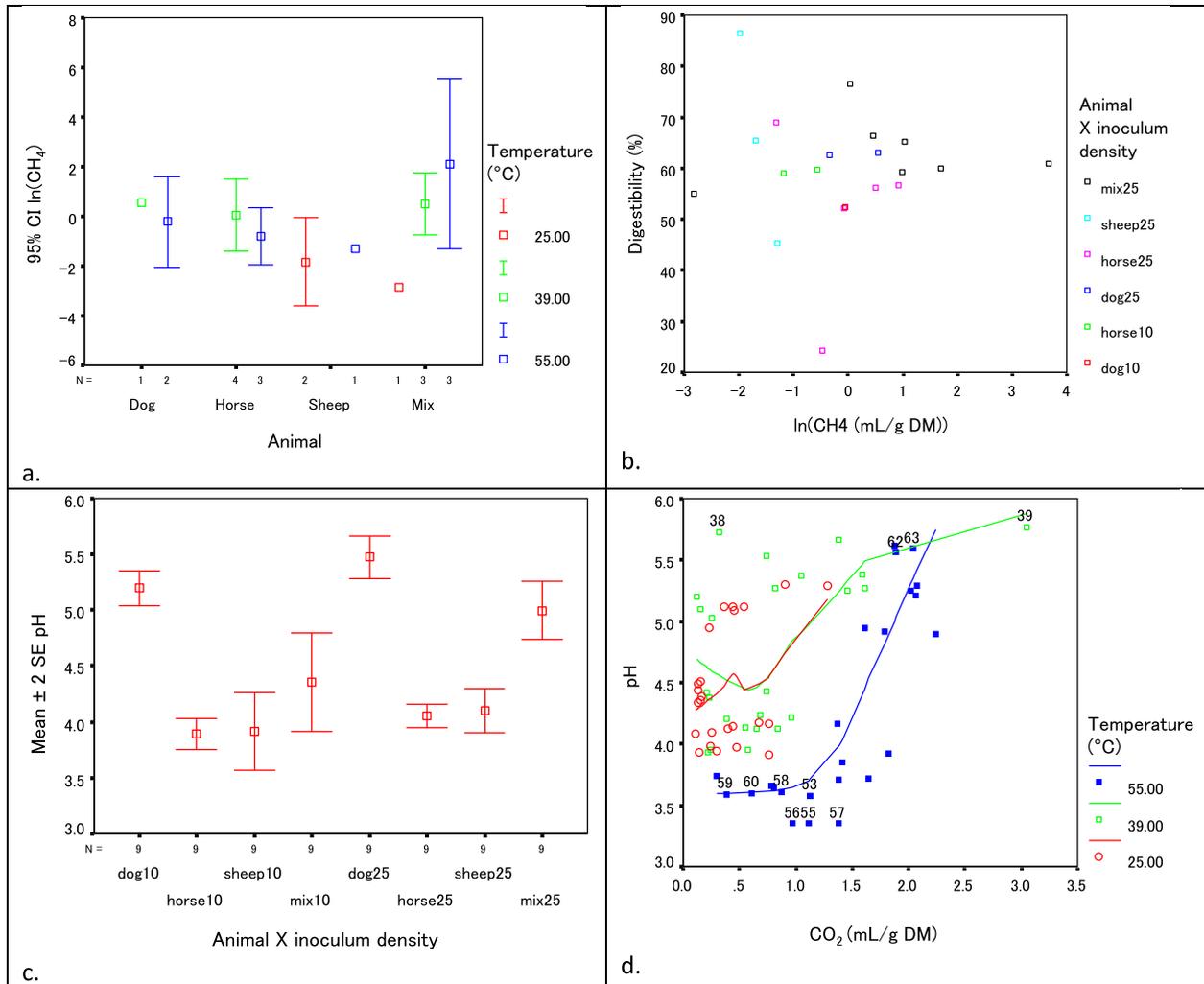


Fig. 1. a–d. Evidence of cometabolism by 96 h a. Effect of horse, dog, sheep and mixed inoculums, and inoculum density on CH₄ production (10 and 25 g stand for the mass of feces out of which inoculum was extracted). b. Effect of sheep and mixed inoculum on digestibility. c. Effect of dog and mixed inoculum on pH. d. Effect of dog inoculum on pH and CO₂ at 39 and 55 °C.

also be predicted by temperature and biogas increments of 0.383 and 0.496 respectively.

However, non-linearity and the kinks attributable to CH₄ pulses and flukes (Fig. 4b), as well as the absence of significant effects or interactions explaining CH₄ (in anova), made CH₄ more difficult to predict as a linear function of other variables (Table 1).

Turning to the role of temperature, its effects (on gases including CH₄, pH, and digestion) were significant both directly and through interactions. Furthermore, only temperature explained final CH₄ accumulation differences; other variables and interactions among temperature, density and type of inoculum did not lead to significant CH₄ accumulation differences ((Table 1).

4. Discussion

In this study, the influence of temperature, inoculum density and cometabolism on AD degradation of FVW and CH₄ generation were explored. The initiation of anaerobic reactors is critical for a reduced startup period, which can be a convincing feature toward an increased number of AD users for waste treatment and energy generation. Another factor of increased use of AD can be the timely prediction of digestion and methane generation rates via rapid assessment of key AD variables (pH and temperature).

The rapid method also yielded data on methane generation features during the initiation period (pulses, flukes or bursts, see Islas-Espinoza, 2016 online data). They were probably not artefactual since CH₄ pulses and bursts are also observable *in vivo* during eructation in ruminants and monogastric mammals. As for any method, caveats existed, such as a lower detection limit equivalent to 0.938 mL CH₄/g DM and an upper CO₂ detection limit of 9.99% of biogas which may call for sensitivity analyses of CO₂ kinetics.

4.1. Anaerobic cometabolism

Cometabolism is a hitherto little studied process. Here, inoculum diversity in combination with higher inoculum density seemed to enhance CH₄ production, pH and digestion. Inoculum selection is a key to stable AD operation and stress tolerance (De Vrieze et al., 2015) and could in future be complemented by cometabolism and high inoculum concentration (Tasnim et al., 2016).

The most intense CH₄ production events occurred with more diverse inoculum. This diversity is due to the selection of digestion genes and enzymes in different domestic animals reared and fed in consistent and narrowly defined ways (Hanning and Diaz-Sanchez, 2015). Here we assembled a diverse gastrointestinal microbiome and exposed it to FVW feed and digestion conditions different from

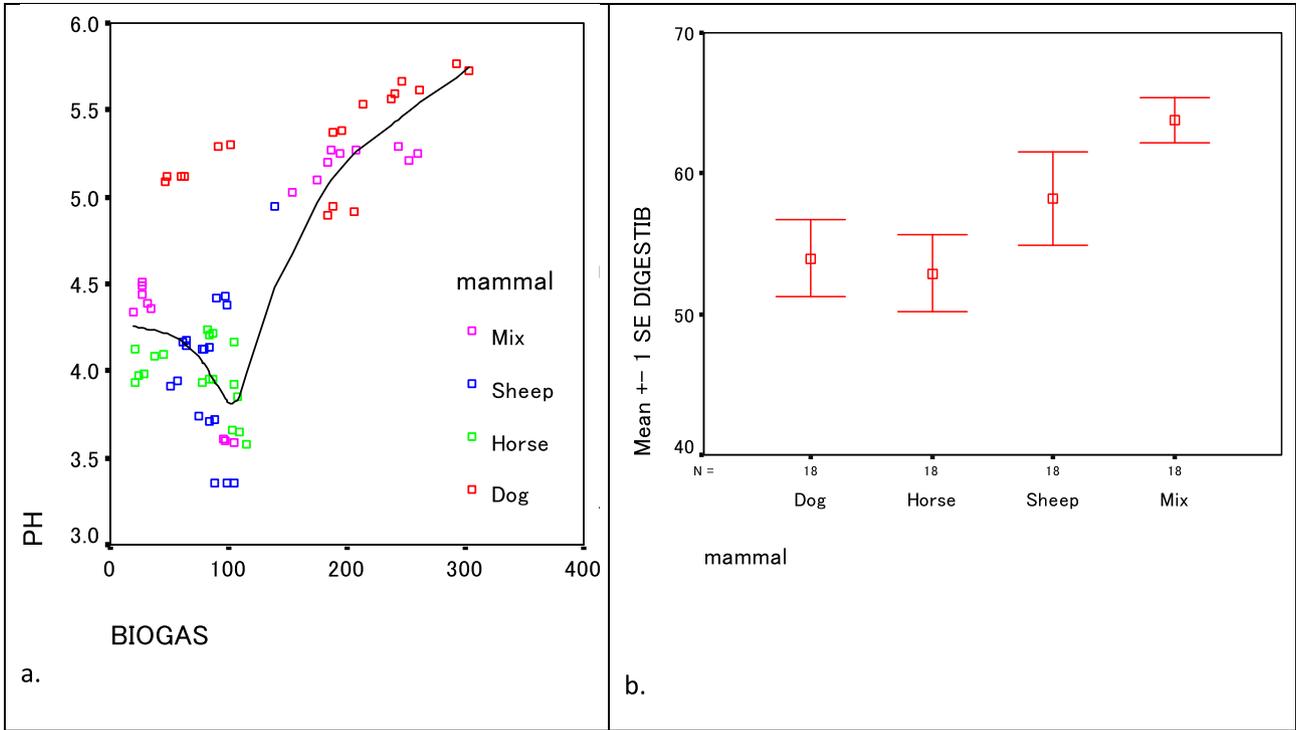


Fig. 2. a–b. Cometabolism by 96 h a. Role of herbivore (pH and biogas reduction) and dog inoculums (pH and biogas rise in mL/g DM). b. Digestibility percentage by inoculum type.

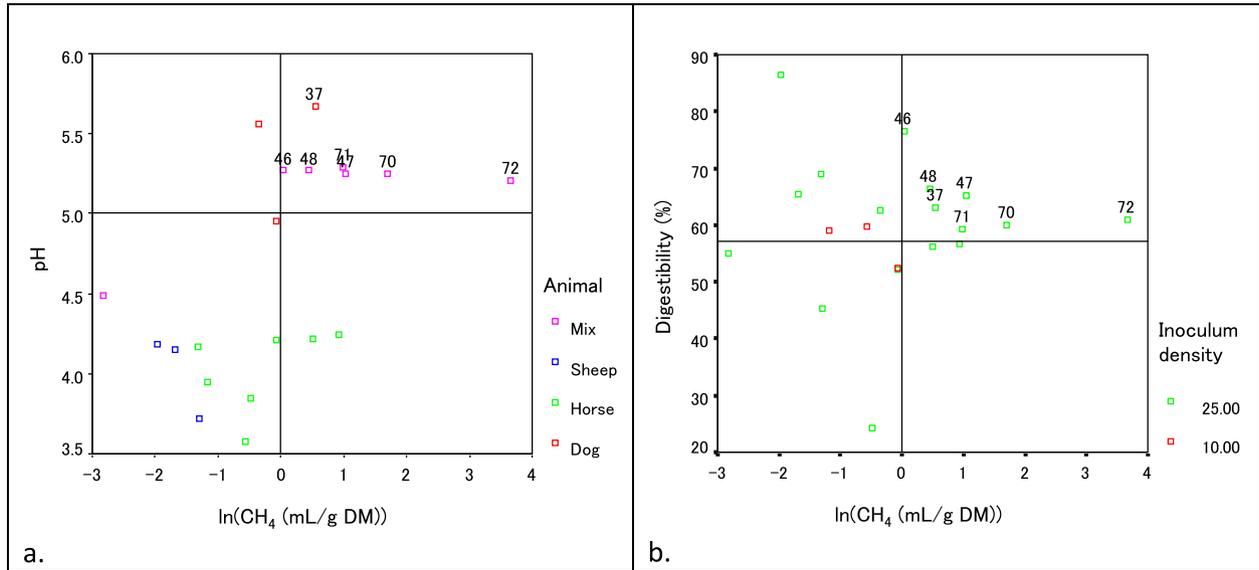


Fig. 3. a–b. Optimal pH-CH₄ samples ($5.0 \leq \text{pH}$ and $0 \leq \ln(\text{CH}_4)$) also had above average high in digestion. a. pH closer to 6 related to higher CH₄ production. b. Higher CH₄ related to higher digestibility.

in vivo studies on animal CH₄ production. This made the fibrolytic and cellulotic advantages of horse and sheep inoculum difficult to compare with other studies. As to the buffering capacity of dog inoculum, it deserves more attention; it had escaped notice despite ongoing interest dedicated to buffering capacity, which may be linked to a comparatively more alkaline digestive tract in dogs (pH 7.3). The fact that higher inoculum density did not seem to improve digestion, except when interacting with dog inoculum, confirmed the importance of the latter.

As to the role of yeast extract as carbon source in initiation of

cometabolism (Islas-Espinoza et al., 2012), it was more likely at 55 °C, a temperature which allowed for only minimal survival of yeast, since *S. cerevisiae* is mostly inactivated at 55 °C (Reveron et al., 2003). At lower temperatures, *S. cerevisiae* might have enhanced biodiversity. Other benefits from yeast might have occurred at all temperatures. In particular, Desnoyers et al. (2009) show increased pH and digestibility due to *S. cerevisiae* supplementation in a meta-analysis on ruminants.

Kinetics-wise, during the initiation period, Gompertz curves often used to model the startup period, could fit cumulative biogas

Table 2
Disinfection of several pathogens at 45–55 °C.

Organism	Required exposure (minutes)
<i>Salmonella typhosa</i>	30
<i>Salmonella</i> sp.	60
<i>Shigella</i> sp.	60
<i>Escherichia coli</i>	60
<i>Entamoeba histolytica</i> cysts	A few mn @45 °C, <1 @55 °C
<i>Taenia saginata</i>	A few mn
<i>Trichinella spiralis</i>	Quickly
<i>Brucella abortus</i>	60
<i>Micrococcus aureus pyogenes</i>	10 mn @50 °C
<i>Streptococcus pyogenes</i>	10 mn @54 °C
<i>Corynebacterium diphtheriae</i>	45
<i>Nacator americanus</i>	50 @45 °C
<i>Ascaris lumbricoides</i> eggs	60

Source: Gotaas HB, composting sanitary disposal and reclamation of solid wastes, WHO, Geneva 1956, cited in Tchobanoglous et al. (1993) Integrated solid waste management. Engineering principles and management issues, McGraw Hill, Inc. Note: The 55 °C threshold is used in particular in aerobic compost processes to reduce pathogens in US EPA (1979).

Despite an increased energy requirement to attain thermophilic conditions, this allows for disinfection of a number of known pathogens (pathogens of Table 2) in effluent water and biosolid after AD. This can entail just the inconvenient of significantly lower pH compared to mesophilic physiological temperature. In comparison, few processes seemed to benefit from a psychrophilic environment.

4.3. Acidification

Acidification was widespread in all the bioreactors, however, more temperature, inoculum and biodiverse inoculum led to optimal CH₄-pH. Explanations of the rapid acidification might hinge on the following. CO₂ flushing as premixing strategy may have accelerated the acidification process: CO₂ partial pressure produced by fermentation solubilizes CO₂ in water yielding carbonic acid which consumes alkalinity (Metcalf & Eddy, 2003). Also, if an organic compound is oxidized, two hydrogen atoms are

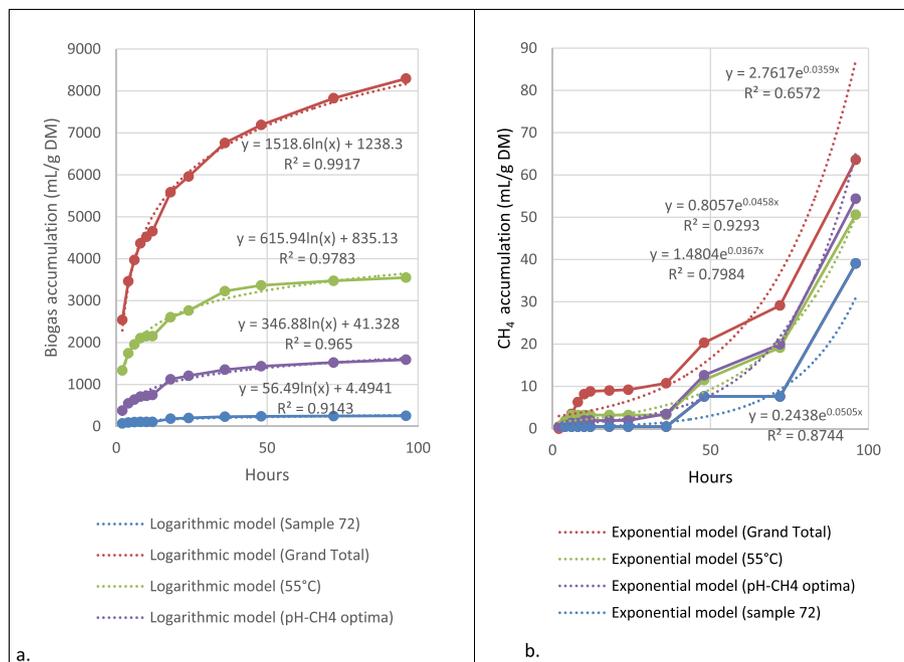


Fig. 4. a–b. Kinetics of biogas (deceleration) and CH₄ (acceleration). a. Cumulative biogas kinetics. b. Cumulative CH₄ kinetics.

kinetics but not the early onset of exponential methanogenesis. CH₄ accelerated while CO₂ decelerated suggesting that CO₂ might have been transformed directly to CH₄ by hydrogenotrophic methanogens or indirectly to acetate and then to CH₄ by acetotrophic methanogens.

4.2. Thermophilic conditions

Inoculum from mesophilic digestive-tract communities was used successfully to initiate thermophilic AD, a procedure called aggressive startup (Griffin et al., 1998). Temperatures of 25 °C and 55 °C probably were drastic treatments which modified the biodiversity prevalent at physiological temperature in mammalian intestines. In relation to CH₄ production, among the controlled variables only temperature brought about significant CH₄ accumulation differences. This is consistent with several studies where higher temperatures accelerate AD (Jain et al., 2015).

released to an electron carrier and then to a permanent electron acceptor (Gaudy and Gaudy, 1981); if an acceptor is not available, for instance due to insufficient presence of hydrogenotrophic methanogens, the hydrogen release may be a source of acidification.

However, CO₂ flushing as premixing strategy may have led to good feedstock-inoculum mixture and significantly improved AD performance (Zhu et al., 2014). In addition, 12 times faster methanogenesis is carried out by CO₂-reducing methanogens compared to other methanogens (Zinder et al., 1984). This may have coincided with the buffering capacity noticed in dog and mixed inoculum samples. *S. cerevisiae* supplementation may have removed oxygen from the reactors and improved cellulolytic activity at 25 and 30 °C, accelerating the CH₄ production.

Finally, anaerobic cometabolism with dog, horse and sheep inoculums and the use of FVW and *S. cerevisiae* seem to hold a potential for economical and sustainable methane generation as

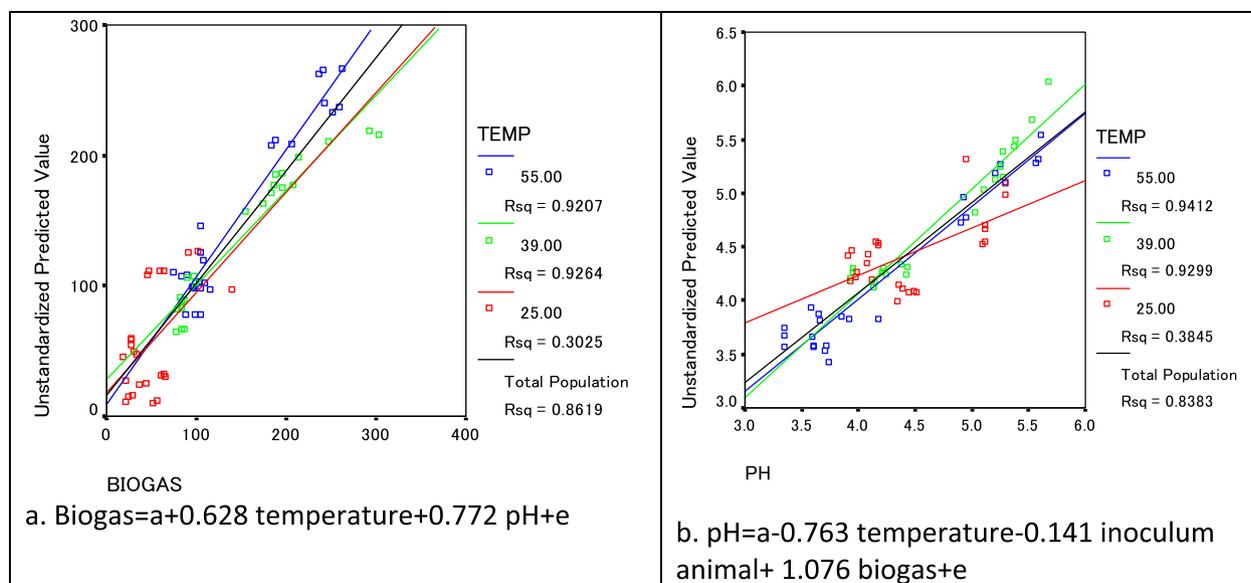


Fig. 5. a–b. Main predictors of reactors' behavior. a. Biogas predicted by temperature and pH. b. pH predicted by temperature, inoculum density and biogas. The observations were also located around the diagonal of the plots denoting neither systematic overestimates nor underestimates. Rsq is the determination coefficient.

source of energy, and a potential to reduce pollution and health-related issues.

5. Conclusions

The AD is a biological process that can produce biomethane as energy source, using FVW waste as substrate. Cometabolism proved to be a novel way to enhance liquid AD, via augmenting the biodiversity of inoculums with dog, horse and sheep feces, as well as, in the initial period, live yeast. Thermophilic and mesophilic conditions, coupled with cometabolism and higher inoculum density enhanced CH_4 generation and improved pH and digestion. Horse inoculum in the mixed reactors seemed to enhance CH_4 production; sheep inoculum to improve digestibility; and dog inoculum and *S. cerevisiae* to raise pH toward neutrality. Therefore, anaerobic cometabolism of FVW may hold a strong potential for clean energy generation and waste reduction.

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