**Co-ensiling of cattle manure before biogas production: effects of fermentation
stimulants and inhibitors on biomass and methane preservation**

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Abstract

The impact of formic acid and glucose addition on the co-ensiling of cattle manure with straw was assessed during 4 months at laboratory scale. Feedstock deprived of additives lost 67% of its methane potential for prolonged ensiling. This was mainly due to the lack of water-soluble carbohydrates and to the high methanogenic activity of cattle manure. The use of co-substrates enhanced biomass and energy conservation during ensiling. The best storage performance was obtained for co-ensiling of cattle manure with glucose (100 g/kg of feedstock). For this condition, lactate production was extensive, which allowed biomass acidification, suppressed ammonia emissions and led to full preservation of methane potential after 4 months. Therefore, in field-scale storage, co-ensiling with a high easily fermentable sugar content co-substrate appears to be the most resourceful method to optimize cattle manure preservation. Application of this promising technique will have a major impact on the methane yield of agricultural biogas plants where cattle manure has to be stored for long periods.

Keywords: Cattle manure; Additives; Storage; Ensiling; Anaerobic digestion; Methane potential

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Highlights

- Ensiling of cattle manure with straw led to 67% methane potential losses
 - Ammonia emissions during ensiling were minimized with the use of co-substrates
 - Formic acid addition limited energy losses to 25% during ensiling
 - Full preservation of methane potential occurred for co-ensiling with 10% of glucose
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Abbreviations

AA, acetic acid; AD, anaerobic digestion; ADF, acid detergent fiber; ADL, acid detergent lignin; BMP, biochemical methane potential; BU, butyric acid; CEL, cellulose; COD, chemical oxygen demand; FA, formic acid; HEM, hemicellulose; LA, lactic acid; LAB, lactic acid bacteria; LIG, lignin; NDF, neutral detergent fiber; NH₃-N, ammonia nitrogen; TKN, total Kjeldahl nitrogen; TS, total solids; VS, volatile solids; WSC, water soluble carbohydrates; VFA; volatile fatty acids

1. Introduction

Cattle manure is a major input for agricultural biogas plants, especially in countries with intensive livestock systems. In France there are currently 267 of these installations [1] and nearly 87 Mt of cattle manure annually produced that may be mobilized for anaerobic digestion (AD) in the near future [2]. However, there are still several constraints slowing down the valorization of cattle manure. This includes the storage requirements and the consequences on the methane potential of cattle manure that it may entail. On the one hand, biogas plants should operate continuously and feedstock should be available throughout the year. Yet, cattle manure, such other agricultural wastes and crops, undergoes production fluctuations, which periodically leads to the need of storage during long periods.

The main issues related to the storage of cattle manure are that the current field-scale practices are not optimized for energy production purposes and scientific studies about this subject are scarce. Cattle manure is typically stored outdoors in contact of air, in order to carry out a simple and low-cost operation. This has several drawbacks: it leads to leachate generation, ammonia and odor emissions [3] and energy losses. Recently, Teixeira Franco *et al.* [4] evidenced that up to 74% of cattle manure's biochemical methane potential (BMP) may be lost during 4 months of open-air storage. Nevertheless, the authors showed that anaerobic storage, or ensiling, may limit energy losses to a maximum of 46% after 4 months.

Traditionally used as fodder conservation method, ensiling is a biochemical process that mainly rely on anaerobic conditions and biological acidification of the feedstock [5,6]. This biomass acidification consists in lowering the pH around 4.0 by organic acid production (especially lactic acid), which will impede further significant fermentative activity. The main reactions involved in carbon degradation during ensiling are represented in Figure 1.

Figure 1 – Simplified diagram of carbon degradation during ensiling, including microorganisms, substrates and reactions. Efficient energy conservation will occur if the two last reactions are inhibited, especially the methanogenesis.

While applied to specific crops, ensiling leads to full BMP conservation, even after 1 year [7]. However, fresh cattle manure does not satisfy the chemical requirements for an efficient ensiling, whether due to its high moisture, low water soluble carbohydrates

(WSC) content, strong basic buffering capacity or endogenous microflora. For instance, without WSC or another source of easily fermentable sugars, hetero or homo-lactic fermentation cannot occur and lactic acid cannot be rapidly produced in a significant extent, so as to abruptly lower the pH value and inhibit further bacterial activity.

To the best of our knowledge, few authors reported work on cattle manure storage. Teixeira Franco *et al.* [4] have approached the optimization of cattle manure silage before biogas production. The authors evidenced that co-ensiling with wheat straw may enhance methane conservation of cattle manure to more than 86% after 4 months. The decrease of moisture content and the alteration of buffering properties should be in the origin of this improvement. On the one hand, considering the actual market value of bio-methane, the additional revenue for this higher energy conservation rate (vs. open-air storage) was estimated as 24-26 €/t per 4-months batch. On the other hand, the expected cost increase by silo construction is around 57 €/t. This shows that improved energy conservation should clearly overcome in less than a year the additional cost of silo construction if an efficient ensiling of cattle manure is carried out [4]. However, since few authors have worked on cattle manure storage and since there may exist a variability among biochemical properties of different manures, *e.g.* concerning the bacterial populations [8,9], these conclusions may not be generalized for all types of manure so far.

The use of additives is considered as a resourceful method to ensure a good silage quality. Fermentation stimulants and inhibitors are two of the most important silage additives categories among the wide range of biological and chemical products commercially available. The role of fermentation stimulants is to encourage lactic fermentation either by supply of substrate (*e.g.* fermentable sugars), lactic acid bacteria

(LAB) or enzymes [5]. In contrast, inhibitors (acids) are also used to prevent biological activity of the degrading microorganisms by lowering the pH of the feedstock. These two types of silage additives are serving as a basis for most of ensiling research in recent years [7,10–17]. Nevertheless, none of these co-storage studies was performed with cattle manure.

In order to assess the effects of additives on the ensiling of cattle manure with straw, laboratory experiments were conducted for up to 4 months. A fermentation stimulant (glucose) and an inhibitor (formic acid) were used as model molecules of co-substrates for ensiling. It is important to underline that glucose was used as a representative molecule of simple sugars that are conventionally consumed during the first steps of ensiling, such as other non-structural compounds (sucrose, fructose, xylose, starch, etc.). In real applications, glucose should be replaced by a substrate containing easily accessible sugars such as maize, sugar beet pulp or another fermentable sugar-rich substrate. A similar reasoning applies in the case of formic acid, by using low-pH wastes as the one assessed by Lianhua *et al.* [18]. Besides the evaluation of BMP during storage, the monitoring of fermentative profiles, organic matter losses and potential gas emissions were included in our work. Our objective is to contribute to the improvement of cattle manure management before energy production, which will have a major impact on the ammonia emissions and on the methane yield of agricultural biogas plants.

2. Material and methods

2.1. Feedstocks

Fresh cattle manure (10% of total solids (TS) content) was collected on June 1st, 2016 from an agricultural site in the Rhône-Alpes region of France (Gaec Béreyziat, Les Teppes, 01340 Béréziat, France) and it was stored at 4 °C before further use. A mixture

of fresh cattle manure and wheat straw was prepared at the laboratory and it was tested for ensiling. Wheat straw with 0.10 m maximum length was mixed with cattle manure in order to achieve a final TS content of 19%. The TS content of cattle manure with straw was chosen according to the recommendations of Teixeira Franco *et al.* [4] for the optimization of cattle manure storage.

Besides the control assay (*i.e.* ensiling without silage additives), the following additives were added to cattle manure with straw at the beginning of each experiments (Table 1): formic acid, 8.2% of volatile solids (VS) content; glucose, 16.6% VS and; glucose, 40.2% VS. The amount of each additives was selected through a short-term preliminary screening performed at the laboratory, in which the impact of the additive on the pH evolution was examined [19].

Table 1 – Ensiling conditions and treatments applied to cattle manure with wheat straw (% of total sample weight)

Ensiling condition	Additive		TS content
	Molecule	Content	
Control	-	-	19.2±0.1
Formic acid	Formic Acid	2.0	20.4±0.1
Glucose 4%	Glucose	4.0	22.3±0.5
Glucose 10%	Glucose	10.0	24.9±1.4

2.2. Experimental approach

Laboratory trials were performed in 3.5 L airtight round plastic storage drums. In order to enable biogas output and at the same time minimizing headspace, silos were filled up to 2.55 L with raw material at packing density of 0.7 kg/L, the remaining volume being filled with gravel, using a geotextile membrane to separate it from biomass. Proper plastic lid and rubber ring were used for silo sealing and its airtightness was reinforced with silicone sealant. Then, silos were weighed and placed in a controlled-temperature

room at 25±2 °C. Storage duration varied between 7, 15, 30 and 120 days. A total of 16 (4 operating conditions x 4 storage durations) ensiling assays were performed.

2.3. Chemical analysis

For each sample time, one silo (per tested condition) was sacrificed. It was opened and weighed. Then, biomass was homogenized and two samples were taken, which followed the experimental procedure illustrated in Figure 2.

Figure 2 – Flowchart of the experimental procedure, including phase separation and chemical analysis

The first sample was used for direct analyses on the crude material and the other one was mixed with water in order to get two fractions: a water-soluble phase and a

particulate phase. This leaching test was performed with a 10:1 water/TS ratio during 2 h under constant bottle rotation. Phase separation was achieved by centrifugation (5000 G; 10 min) followed by 0.7 μm particle size filtration. Finally, the particulate phase was dried at 70 °C until constant weight and ground at 2 mm theoretical length. Crude material/water-soluble and particulate samples were stored at 4 °C and -20 °C, respectively, until analysis.

Crude material was analyzed for its TS/VS content and BMP. For the water-soluble phase, besides TS/VS content, pH, WSC, volatile fatty acids (VFA), chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN) and ammonia nitrogen ($\text{NH}_3\text{-N}$) fractions were determined. Particulate solid was analyzed for its TS/VS, COD, TKN and cell wall constituents. Feedstocks were not analyzed for COD, TKN and $\text{NH}_3\text{-N}$ content.

TS was measured by oven drying at 105 °C during 24 h and VS was subsequently burned for 2 h at 550 °C. Since TS/VS contents are underestimated due to the loss of volatile compounds during the drying tests [20], the data were corrected according to the volatilization coefficients at 100 °C suggested by Porter and Murray [21]. pH was measured by a Consort C3020 device with a SP10B pH-electrode. WSC, lactic acid and formic acid contents were determined with high performance liquid chromatography (LC Module 1 plus, Waters) equipped with a Supelcogel™ C-610H column (300 x 7.8 mm, Sigma-Aldrich), both refractive index (RID) and UV detectors and operating with H_3PO_4 0.1% v as solvent (flow rate of 0.5 mL/min). WSC content was estimated as the sum of glucose, xylose, galactose, mannose, arabinose and cellobiose and was determined using the UV detector (210 nm). Lactic acid and formic acid contents were obtained with the RID detector. Acetic, propionic, butyric, valeric and caproic acids

content were analyzed by gas chromatography (Shimadzu Corp.) equipped with a HP-FFAP fused silica capillary column (30 m x 0.25 mm, Agilent Technologies), a flame ionization detector and using H₂ as carrier gas. Total VFA was calculated as the sum of lactic, formic, acetic, propionic, butyric, valeric and caproic acids. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed through Van Soest and Wine [22] modified extractions method, based on FD U44-162 standard [23]. Hemicellulose content was calculated as NDF minus ADF; cellulose as ADF minus ADL and; lignin as equal to ADL. TKN and NH₃-N were determined through the procedure described in the NF EN 25663 standard [24]. COD of water-soluble phase was determined through the colorimetric HACH procedure (method 8000). COD of particulate phase was measured by a Walkley and Black [25] modified method, based on the NF ISO 14235 international standard [26].

The interest of our experimental procedure is to assess the composition and BMP based on the initial mass of product used, since the weight loss is measured. The results for the chemical analysis will thus be presented in two ways: based on %VS_{added} or %VS_{original}. VS_{added} relates to the organic matter of the sample analyzed. The results based on VS_{original} take into account the loss of volatile solids during storage and allow the study of the results based on the VS of the initial material.

2.4. Biochemical methane potential tests

Batch anaerobic digestion tests were conducted in a temperate room at 35 °C using 2L glass reactors. Vessels were filled with 5 gVS of sample, inoculum in way to keep a substrate/inoculum VS ratio of 0.5 and a certain volume of a mineral solution to achieve 60% of the total volume of the vessel. The inoculum used (TS 2.3-3.3%wt; VS 1.5-2.2%wt) was a digested sludge originating from the wastewater treatment plant of La

Feyssine, Lyon, France. The sludge used met the criteria for a good inoculum quality (*e.g.* pH, VFA, NH_4^+) suggested by the international task group on the harmonization of BMP protocols [27]. The mineral solution, which contains essential elements to microbial growth and also gives the solution a buffer able to control any pH adjustments, was prepared according to the recommendations of ISO 11734 international standard [28]. Once filled, reactors were purged with a N_2/CO_2 mixture (80/20% v) for about 5 minutes, sealed and equilibrated at 35 °C. Blanks with only inoculum and mineral solution were performed for each batch series in order to correct the BMP from residual methane production of the inoculum. All tests were performed in triplicates.

Biogas production was determined by pressure measurement using a Digitron precision manometer. Biogas was released when the pressure exceeded 1200 hPa. Gas composition was analyzed using an Agilent 3000 micro gas chromatography with thermal conductivity detector (GC-TCD). Molsieve 5A (14 m length; pore size: 5 Å) and PoraPlot A (10 m length; 0.320 mm ID) columns were used as stationary phases for GC-TCD, with Argon and Helium as carrier gases, respectively. Biogas production and composition were analyzed at least 8 times during the incubation and BMP was considered achieved when daily vessel overpressure of controls equalized the sample ones. The BMP tests followed the recommendations provided by Holliger *et al.* [27].

3. Results and discussion

3.1. Feedstock characterization

Chemical properties of feedstocks are gathered in Table 1 and Table 2. Considering the pH value (8.3) and the TS content (19.2%) of fresh cattle manure with wheat straw, AD microflora should have proliferated during storage. However, energy sources of raw

material were mostly structural carbohydrates. Indeed, cellulose and hemicellulose contents of cattle manure were estimated as 36.1 and 30.6% of organic matter, respectively, while WSC fraction was negligible. This means that biomass acidification during storage should be preceded by hydrolysis of polymers, as in biogas production process [29]. Since hydrolysis reactions take several weeks to occur in substantial extent under ensiling conditions [30], fermentation of sugars will occur slowly. In the meantime, hydrogen-consuming reactions may have occurred, together with proteins mineralization and subsequent $\text{NH}_4^+/\text{NH}_3$ equilibrium. Besides, it was detected a rather high VFA content in cattle manure before storage. The fact that high pH and VFA content co-existed in the raw material evidenced that cattle manure had strong basic buffer compounds. Generally speaking, the biochemical properties of cattle manure make it not suitable for ensiling, due to the absence of easily fermentable sugars and the elevated pH.

A proportional increase of TS content was observed with the use of co-substrates. This was expected since additives were used as pure compounds and not in solution. Since the activity of silage bacteria is at least in part vulnerable to changes in TS [5,31,32], this may have influenced the ensiling process. In the case of formic acid addition, the pH of the feedstock was affected as well. Indeed, it decreased to 3.5, which is a considerable lower value than the ones recommended by Kalač [33] for lactate silages (4.10-4.20 for TS content of 15-20%) and by Teixeira Franco *et al.* [4] for anaerobic stabilization of cattle manure with wheat straw (pH value of around 5.4-5.6).

Concerning raw material with glucose addition, WSC content ascended to 16.6% VS (14.8% TS) and 40.2% VS (36.5% TS) for Glucose 4% and Glucose 10% conditions, respectively. Under these conditions, the WSC contents were quite superior than the

ones found in the literature for feedstock successfully acidified during ensiling (3-14%TS) [10,16,34–37]. Thus, efficient acidification should occur for cattle manure treated with a co-substrate containing fermentable sugars if a proper amount of LAB is present.

Table 2 - Chemical properties of biomass over ensiling duration (% VS_{added}/original).

Assay	Period (days)	VS losses (%)	Chemical characteristics ^a									
			pH	LA ^b	AA ^b	BA ^b	FA ^b	VFA ^b	WSC ^b	HEM ^c	CEL ^c	LIG ^c
Control	0	-	8.3	0.0	3.7±0.0	0.0	0.0	5.5±0.1	0.0	30.6±0.5	36.1±2.3	8.3±0.5
	7	2.5±0.0	7.1	0.0	3.4±0.1	0.4±0.0	0.0	5.7±0.1	0.0	28.5±0.4	34.1±5.0	8.1±1.1
	15	5.5±0.0	7.7	0.0	2.4±0.0	0.6±0.0	0.0	5.0±0.1	0.0	28.3±1.4	31.3±1.2	8.5±0.0
	30	14.8±0.1	8.2	0.0	0.2±0.0	0.5±0.0	0.0	4.2±0.1	0.0	25.1±0.4	31.1±4.2	9.1±1.1
	120	42.0±0.2	8.4	0.2±0.0	0.0	0.0	0.0	0.2±0.0	0.0	14.6±1.0	18.7±3.3	9.2±1.3
Formic acid	0	-	3.5	0.0	1.5±0.0	0.0	8.2±0.1	10.0±0.1	0.0	32.1±0.5	32.2±3.2	7.3±0.6
	7	0.4±0.0	3.6	0.4±0.0	1.2±0.0	0.1±0.0	8.8±0.2	10.9±0.3	0.0	30.5±0.6	31.5±1.7	6.8±0.3
	15	0.6±0.0	3.9	0.5±0.0	1.2±0.1	0.0	8.6±0.4	10.5±0.5	0.0	29.5±0.4	32.0±1.3	6.9±0.2
	30	0.7±0.0	3.8	0.6±0.0	1.1±0.1	0.1±0.0	9.8±0.4	11.9±0.4	0.0	29.1±1.5	32.7±8.8	6.2±1.6
	120	13.4±0.1	6.9	0.1±0.0	5.6±0.1	1.5±0.0	0.0	9.1±0.1	0.0	23.2±1.4	29.6±2.4	7.7±0.2
Glucose 4%	0	-	7.9	0.0	1.8±0.1	0.0	0.0	2.6±0.1	16.6±0.5	27.3±1.1	30.9±4.6	10.5±1.1
	7	1.9±0.1	4.0	13.5±0.4	2.0±0.1	0.5±0.0	0.0	17.0±0.6	4.1±0.1	27.1±1.5	27.8±1.7	6.2±0.1
	15	2.5±0.1	4.1	15.2±0.4	2.0±0.1	0.7±0.0	0.0	18.9±0.5	2.0±0.1	24.8±1.3	26.2±1.9	5.0±0.2
	30	3.4±0.1	4.1	10.0±0.2	2.1±0.0	1.8±0.0	0.0	15.2±0.2	0.0	26.1±1.2	27.9±1.6	5.4±0.1
	120	9.0±0.3	5.4	0.0	2.2±0.1	3.2±0.1	0.0	10.9±0.3	0.0	25.6±0.3	29.6±0.5	6.1±0.1
Glucose 10%	0	-	7.9	0.0	1.3±0.2	0.0	0.0	1.9±0.3	40.2±4.2	21.9±0.6	23.9±2.7	4.6±0.4
	7	1.5±0.2	4.0	11.2±0.2	1.8±0.0	0.3±0.0	0.0	14.1±0.2	34.0±0.5	18.2±0.6	19.4±1.5	4.0±0.2
	15	2.3±0.3	4.0	14.5±0.2	1.8±0.0	0.4±0.0	0.0	17.9±0.3	30.1±0.5	17.3±0.4	19.9±1.1	3.6±0.2
	30	2.7±0.3	3.8	15.1±0.1	2.0±0.0	0.6±0.0	0.0	18.2±0.8	24.2±0.2	17.4±0.8	19.2±0.8	3.5±0.0
	120	4.6±0.6	3.7	19.5±0.6	3.9±0.1	1.0±0.0	0.0	25.9±1.8	18.5±0.6	15.5±0.3	18.2±1.2	3.5±0.2

^a LA stands for Lactic Acid, AA for Acetic Acid, BA for Butyric Acid, FA for Formic Acid, HEM for Hemicellulose, CEL for Cellulose and, LIG for Lignin; ^b results based on % VS_{added}; ^c results based on % VS_{original}

3.2. Effects of storage method on biomass preservation

3.2.1. Fermentation profiles

Anaerobic storage of cattle manure with wheat straw addition only (Control) did not promote a biomass acidification in the long-term, Table 2. In the first 7 days of storage, pH of control decreased to 7.1. Then, it constantly increased until the end of the storage period, reaching a value of 8.4 after 4 months. This can be explained by the evolution of VFA concentrations along ensiling duration. Indeed, there was a slight VFA accumulation in cattle manure during the first days of storage, mainly due to the short

production of butyric acid. In contrast, the acetate initially present in fresh cattle manure was almost totally lost during the first month of storage. This evidences that the methanogenic activity was already present at the beginning of storage and became predominant between 7 and 15 days of storage, which impeded biomass stabilization. This was confirmed by one-off composition measures of the gas produced during these experiments (results not shown): CH₄/CO₂ ratio was already 0.26 after 1 day and 0.61 after 10 days of storage. Furthermore, since cattle manure was successfully ensiled with straw in our previous study [4], this suggested that methanogens concentration may vary from case to case. In fact, it is known that bacterial populations can be influenced by the characteristics of manure [8] which, according to Marañón *et al.* [9], may depend on the type of cattle, animal's diet, as well as, on the time of the year. In any case, silage stabilization was not achieved for control assay and only trace concentrations of organic acids were detected after 4 months of storage.

In addition, structural carbohydrates content regularly decreased for control silage. After 4 months of storage, hemicellulose and cellulose concentrations were 14.6 and 18.7% of original VS, respectively. This represented a degradation of around 50% of the total original hemicellulosic and cellulosic compounds. Since no accumulation of WSC nor VFA was recorded during the same period, major organic and energy losses have occurred. Regarding organic matter, losses ascend to 14.8% after 30 days and 42.0% at the end of the storage. An average of 0.35% of organic matter was lost per day during this period.

Co-ensiling with formic acid improved the conservation of cattle manure, especially for short periods of storage. During the first 30 days, formic acid was fully conserved and pH persisted below 4.0. In parallel, VFA content increased, which was supposed to be

partially caused by lactic acid production. This should be a result of acid hydrolysis of hemicelluloses by the existing organic acids in silage [5,38]. As a matter of fact, hemicellulose content of formic acid silage decreased to 29.1% VS_{original} after 30 days, while cellulose fraction was stable. However, between 30 and 120 days of ensiling the fermentation profiles reversed and both formic and lactic acids were consumed. At the same time, other organic acids were produced, notably acetate and butyrate. Consequently, VFA content decreased from 11.9% VS_{added} after 30 days to 9.1% VS_{added} at the end of storage. Due to that and to the fact that the VFA produced were weaker acids than formate and lactate, the pH value increased to 6.9 after 4 months of ensiling. One hypothesis that can be suggested for the fermentation shift is that the low pH of silage was only delaying the growth of methanogens. This would explain the slow pH increase in the first month of storage. Then, as pH increased, microbial growth became important and formic acid was further degraded into biogas, which also had an impact on the pH. Consequently, lactic acid must have been converted, *e.g.*, into butyric acid by clostridial fermentation, which is a typical mechanism in poor quality silage [5]. Furthermore, the increased pH should have allowed the enzymatic hydrolysis of structural polymers for prolonged silage periods [38]; and the subsequent fermentation into VFA. This is corroborated by the accentuated decline of structural carbohydrates that was verified in the last 90 days of storage. Indeed, after 4 months of ensiling, hemicellulose content decreased to 23.2% of original VS. Finally, the two different phases of fermentation had an impact on the conservation of organic matter. After 1 month, VS losses were lower than 1%, which ascended to 13.4% at the end of the storage.

Co-ensiling of cattle manure with glucose underwent a strong acidification in the early days of storage. Indeed, pH value of silage was 4.0 for both Glucose 4% and Glucose 10% conditions after 7 days, which was due to the conversion of WSC into lactic acid (Table 2). This shows that the concentration of LAB in the original raw material was significant. Consequently, the lack of easily fermentable sugars probably blocked the conservation of cattle manure through the ensiling process of control trials. Silage acidification slowed down LAB activity but it was not completely stopped. For this reason, WSC continued to be consumed during the remaining ensiling period. After 15 days of storage, lactic acid concentration was around 15% VS_{added} for both co-silages with glucose addition. After this period, two distinct fermentation pathways were observed for the experiments with glucose. On the one hand, for Glucose 10% condition, WSC was in large excess and lactic fermentation persisted until the end of the storage. As a result, after 4 months, pH value of Glucose 10% silage was 3.7 and its lactate concentration rose to nearly 20% VS_{added}. On the other hand, WSC of Glucose 4% assay was extinguished between 15 and 30 days of ensiling. Coupled to that, there was a degradation of lactic acid and a significant increase of butyric acid content during this period. This suggests that the acid pH value (4.1) was unable to avoid clostridial activity since there was no more available substrate for lactate production. This clostridial proliferation led to a secondary fermentation that was mainly based on lactic acid consumption for the production of butyrate, carbon dioxide and hydrogen [5]. Between 30 and 120 days, clostridial fermentation led to full degradation of lactic acid on Glucose 4% silage. Besides butyric acid, other VFA were found, such as caproic and valeric acids (results not shown). Moreover, after 4 months of storage, the pH value of Glucose 4% increased to 5.4. This is explained by the fact that butyrate is a weaker acid

that lactate and because this reactional mechanism involves the consumption of two moles of lactic acid for the production of one mole of butyric acid.

Additionally, co-ensiling of cattle manure with glucose enhanced the preservation of hemicellulosic and cellulosic compounds. Regarding Glucose 4% silage, degradation of structural carbohydrates was not significant. For Glucose 10%, around 27% of the sum of initial hemicellulose and cellulose fractions were missing. This degradation should be linked with an acid hydrolysis by the VFA produced during ensiling, as already mentioned by other authors [5,38]. This was not observed in the condition Glucose 4%, since the pH was higher. Nevertheless, the degradation of the structural carbohydrates for Glucose 10% did not led to energy losses since biomass was extremely well conserved under acidic conditions. Therefore the hydrolysis products were likely conserved either under the form of non-structural oligomers, simple sugars or organic acids. Consequently, VS losses were limited to 9.0% and 4.6% after 4 months for Glucose 4% and Glucose 10% assays, respectively. The poor conservation of organic matter for Glucose 4% silage should be connected to the secondary fermentation.

3.2.2. Chemical oxygen demand balance

Effects of ensiling and co-substrates on the COD of crude material and its water-soluble and particulate fractions are exposed in Figure 3. The evolution along ensiling time is related to VS_{original} in order to account for biomass weight loss in the COD balance.

The most significant results were obtained for the ensiling of cattle manure without additives. Before storage, around 93% of the total COD was in the particulate phase. This is linked to earlier statements, evidencing that in cattle manure the majority of the energy sources were hardly bio-accessible. Second, there was a clear decline of

particulate COD during the experiments, which is explained by the loss of structural carbohydrates. Furthermore, no accumulation of their degradation products in the water-soluble COD was observed. Thus, global COD in the control (without additives) decreased with ensiling time, which surely led to large methane potential losses.

The use of co-substrates for the ensiling of cattle manure improved the conservation of COD. For this reason, the fluctuations of its composition along duration were minimal, which made it difficult to establish long-term trends in co-ensiling. Regarding formic acid assays, despite the important VS losses recorded for prolonged durations, COD of crude material was stable until 120 days of storage. This can be in part explained by the fact that formic acid has low COD content (0.35 go₂/g), so that its degradation did not cause significant COD decrease. Furthermore, there was an important solubilization of particulate COD in the last 3 months of ensiling. This should correspond to the hydrolysis of structural carbohydrates and subsequent conversion into VFA, as it was seen for the fermentation profiles (subsection 3.2.1). In addition to the solubilization effect and the potential kinetic gain in AD conditions, this may have caused an amplification of carbohydrates accessibility.

(A)

(B)

Figure 3 – Evolution of COD related to VS_{original} along ensiling period for: (A) Control; (B) Formic acid; (C) Glucose 4%; (D) Glucose 10%. Feedstocks were not analyzed. Error bars represent the standard deviation of triplicates.

Ensiling of cattle manure with glucose addition had little impact on COD conservation (Figure 3). For Glucose 4%, both particulate and water-soluble fractions of COD were stable until the end of the 4 months. This was expected for the particulate phase, since fibers content did not suffer any significant degradation during storage. However, a degradation of soluble COD should have been recorded between 30 and 120 days of storage, since clostridial fermentation led to hydrogen gas formation. The reasons for this inconsistency remain unclear. Concerning Glucose 10% condition, there was a

375 persistent solubilization of particulate COD along the experiment. This can be attributed
376 to the acid hydrolysis of hemicelluloses by the VFA produced during ensiling.

377 3.2.3. Total Kjeldahl nitrogen balance

378 Anaerobic storage of cattle manure with straw (Control) led to constant TKN loss
379 during the experiments, Figure 4. This can be explained through the analysis of TKN
380 fractions along the storage period. On the one hand, there was a decrease of organic
381 nitrogen in both particulate and water-soluble phases after 4 months of about 12% and
382 39%, respectively. This loss represented 19% of the total organic nitrogen. This
383 suggests that nitrogen mineralization occurred through two successive mechanisms
384 typically found in anaerobic conditions [5,29]: hydrolysis of proteins into amino acids
385 and subsequent fermentation into ammonia nitrogen. Therefore, $\text{NH}_3\text{-N}$ concentration
386 should increase under such conditions. However, for control assays ammonia content of
387 cattle manure declined with storage time. This can be explained by the fact that pH
388 interruptedly increased to values near the pK_a (9.25) of $\text{NH}_4^+/\text{NH}_3$. Therefore, although
389 ammonia was produced, its chemical equilibrium led to ammonia losses through gas
390 emissions. After 4 months, around 23% of original TKN was lost for control
391 experiment. If we hypothesize that the TKN was lost by ammonia emissions, around
392 $8.2\text{L}_{\text{NH}_3}/\text{kgVS}_{\text{original}}$ were expected to be released into the atmosphere during the 4
393 months of control experiments.

394 Regarding the formic acid tests, 11% of particulate TKN content was degraded in the 4
395 months of storage. This may be linked with acid hydrolysis in the first 30 days; and due
396 to the proteolytic clostridial activity (for higher pH values) in the last 3 months of
397 ensiling. Nevertheless, since pH was always below 7.0, the mineralized nitrogen was

partially conserved as water-soluble ammonia. At the end of formic acid assays, less than 10% of original TKN was lost into the atmosphere.

Figure 4 – Evolution of TKN related to VS_{original} along ensiling period for: (A) Control; (B) Formic acid; (C) Glucose 4%; (D) Glucose 10%. Feedstocks were not analyzed. Error bars represent the standard deviation of triplicates.

For both conditions of cattle manure with glucose addition, no decrease of TKN was recorded until 4 months of ensiling. Yet, as for fermentation profiles, TKN composition of the two glucose experiments evolved differently during storage. In the case of

Glucose 10%, there was no degradation of particular TKN and $\text{NH}_3\text{-N}$ was constant. This shows that silage was extremely stable with glucose addition and that no secondary fermentation occurred. For Glucose 4%, the same trend was observed in the early days of ensiling. However, in the last 3 months there was a decrease of around 18% concerning the particulate TKN and an increase of around 39% for the soluble TKN. This indicates that proliferation of proteolytic clostridia occurred for prolonged storage duration. Hence, the amount of co-substrate used in Glucose 4% was not enough to fully stabilize long-term silage of cattle manure with straw.

3.3. *Effects of storage method on methane potential*

3.3.1. Initial conditions

Before storage BMP was 229 ± 8 $\text{L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ for fresh cattle manure, 250 ± 8 $\text{L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ for cattle manure + Formic acid, 239 ± 26 $\text{L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ for cattle manure + Glucose 4% and 276 ± 8 $\text{L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ for cattle manure + Glucose 10%. Formic acid and glucose had a clear impact on the BMP of the feedstock. The effect of Glucose 4% addition was not significant (within the BMP accuracy limits). Considering the theoretical BMP of glucose ($373 \text{ L}_{\text{STP}}/\text{kgVS}_{\text{added}}$) and the amount of co-substrate used, a BMP of $287 \text{ L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ was expected for Glucose 10% before storage. Furthermore, glucose contribution to the total BMP should be around 52% in this case. This evidences that glucose added was fully degradable under anaerobic conditions and that it was responsible for the initial BMP value increase of Glucose 10% mixture. In opposite, the additional methane production of Formic acid feedstock cannot be attributed to the co-substrate, due to its low BMP value ($122 \text{ L}_{\text{STP}}/\text{kgVS}_{\text{added}}$). In fact, the theoretical BMP of this feedstock mixture should be around $221 \text{ L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ with a 5% contribution of formic acid for these value. Therefore, it can be pointed out an

improvement of the biochemical accessibility of cattle manure after being treated with formic acid (before ensiling). This might be caused by an increase of accessible surface area of biomass, hemicellulose removal and alteration of lignin structure, which are typical features of dilute acid pretreatments for biomass [39]. However, it is worth mentioning that these treatments are performed at high temperatures, which was not the case in this study.

3.3.2. Evolution of the BMP during storage

Control trials showed BMP values of 128-229 $L_{STP}/kgVS_{added}$ and 75-229 $L_{STP}/kgVS_{original}$ during storage, Figure 5. In the first 7 days of control assays, both methane potential based on VS_{added} and $VS_{original}$ were stable. This evidences that neither the energy content nor the biochemical accessibility of cattle manure with straw changed during short-term ensiling, which should be linked with the small fermentation activity and pH decrease that occurred in this period. However, for longer storage durations, the BMP of control was significantly damaged. Indeed, after 30 and 120 days of ensiling, 24% and 67% of original BMP was lost, respectively. This obviously was a consequence of the extensive degradation of structural carbohydrates (without any VFA accumulation), due to the high pH levels.

Co-ensiling with formic acid limited the harmful impact of storage on the methane potential of the raw material. In this case, BMP values were 192-250 $L_{STP}/kgVS_{added}$ and 189-250 $L_{STP}/kgVS_{original}$ during the 4 months. The major variation of methane potential for formic acid silage occurred in the first 7 days of storage. In this period, around 21% of both BMP related on VS_{added} and $VS_{original}$ was lost. It is important to mention that the energy conservation rates of co-ensiling conditions are related to the initial BMP of the mixture manure/additive and not only to fresh cattle manure. As previously discussed

for formic acid assays, organic matter losses were negligible and no noticeable modification of chemical characteristics was observed during the first week. Therefore, this indicates that formic acid addition had an impact on the biodegradability of the biomass in the first days of storage. After that, BMP based on $VS_{original}$ remained constant and global energy losses were limited to 25% at the end of the 4 months. Yet, BMP related to VS_{added} increased by 14% in the last 3 months of storage. Two conclusions can be deduced from these results. First, there was an increment of biochemical accessibility, which can be attributed to the production of VFA from cell wall constituents. In fact, structural carbohydrates are not fully biodegradable in mesophilic AD conditions [40]. Therefore, their hydrolysis and fermentation during ensiling may explain the BMP increase in a VS_{added} basis. Second, this gain of accessibility countered the formic acid loss in the last 90 days, so that BMP related to $VS_{original}$ persisted unaltered until the end of the storage.

Figure 5 - BMP evolution of crude material over storage duration. (A): methane potential based on VS_{added} ; (B): methane potential based on $VS_{original}$, therefore considering storage losses. BMP for 15 days of ensiling was not determined. Error bars represent the standard deviation of triplicates.

Higher stability of methane potential was achieved through the co-ensiling of cattle manure with glucose. Indeed, Glucose 4% silage presented BMP values of 228-241 $L_{STP}/kgVS_{added}$ and 208-238 $L_{STP}/kgVS_{original}$ along the 4 months of storage. In parallel, for Glucose 10% condition, BMP values were 276-302 $L_{STP}/kgVS_{added}$ and 276-288 $L_{STP}/kgVS_{original}$. During the first month of storage, both experiments with glucose had constant methane potential. These results are in line with the data from the fermentation profiles and illustrate the efficiency of lactate production on the energy conservation during ensiling. Concerning Glucose 10% assay, this trend persisted and no loss of BMP was observed until the end of the 4 months. An eventual positive impact on the methane potential could be seen (probably due to the hemicellulose hydrolysis and fermentation), but it was not significant within the precision limits. In contrast, in the remaining 90 days of ensiling, BMP of Glucose 4% progressed differently. For this condition, 13% of the methane potential related to $VS_{original}$ was lost after 4 months. This BMP damage can be explained by the secondary fermentation that led to lactic acid degradation and nitrogen mineralization.

4. Conclusions

Ensiling of cattle manure with straw at 19% TS led to extensive methane potential losses. The use of co-substrates enhanced biomass and energy conservation during ensiling. In particular, co-ensiling with glucose (100 g/kg of feedstock) encouraged an extensive lactate production. This allowed biomass stabilization, suppressed ammonia emissions and led to full preservation of methane potential after 4 months. Therefore, co-ensiling with a high easily fermentable sugar content substrate appears to be the most resourceful method to optimize cattle manure preservation before biogas production. At

full scale application this may include a starch-rich residue, or any other sugar-rich agro product.

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