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Ruben Teixeira Franco, Pierre Buffière, Rémy Bayard. Co-ensiling of cattle manure before biogas production: Effects of fermentation stimulants and inhibitors on biomass and methane preservation. *Renewable Energy*, 2018, 10.1016/j.renene.2018.01.035 . hal-01692807

HAL Id: hal-01692807

<https://hal.science/hal-01692807>

Submitted on 5 Dec 2018

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

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6 **Abstract**

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

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

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22 **Highlights**

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

27 **Abbreviations**

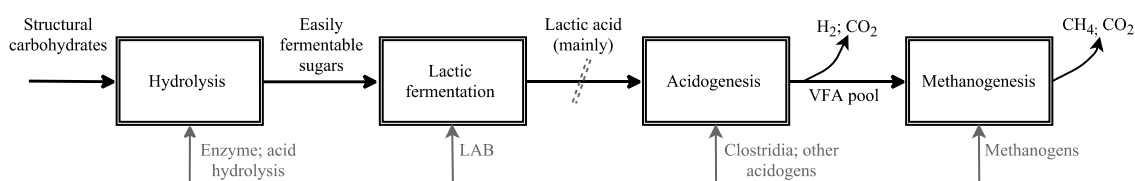
28 AA, acetic acid; AD, anaerobic digestion; ADF, acid detergent fiber; ADL, acid
29 detergent lignin; BMP, biochemical methane potential; BU, butyric acid; CEL,
30 cellulose; COD, chemical oxygen demand; FA, formic acid; HEM, hemicellulose; LA,
31 lactic acid; LAB, lactic acid bacteria; LIG, lignin;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

34 **1. Introduction**

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

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

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



60

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

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

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

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

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

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

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

110 **2. Material and methods**

111 *2.1. Feedstocks*

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

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

120 Besides the control assay (*i.e.* ensiling without silage additives), the following additives
 121 were added to cattle manure with straw at the beginning of each experiments (Table 1):
 122 formic acid, 8.2% of volatile solids (VS) content; glucose, 16.6% VS and; glucose,
 123 40.2% VS. The amount of each additives was selected through a short-term preliminary
 124 screening performed at the laboratory, in which the impact of the additive on the pH
 125 evolution was examined [19].

126 Table 1 – Ensiling conditions and treatments applied to cattle manure with wheat straw
 127 (% 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

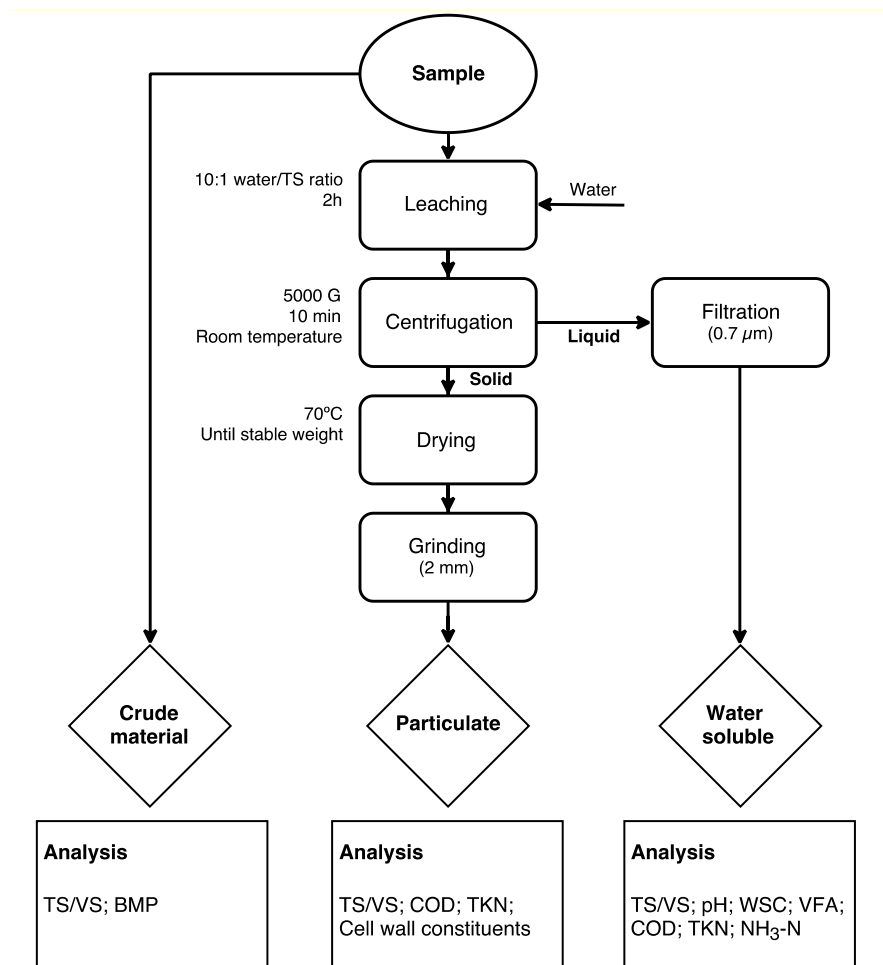
128 2.2. *Experimental approach*

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

135 room at 25±2 °C. Storage duration varied between 7, 15, 30 and 120 days. A total of 16
136 (4 operating conditions x 4 storage durations) ensiling assays were performed.

137 2.3. *Chemical analysis*

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



141

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

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

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

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

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

170 content were analyzed by gas chromatography (Shimadzu Corp.) equipped with a HP-
171 FFAP fused silica capillary column (30 m x 0.25 mm, Agilent Technologies), a flame
172 ionization detector and using H₂ as carrier gas. Total VFA was calculated as the sum of
173 lactic, formic, acetic, propionic, butyric, valeric and caproic acids. Neutral detergent
174 fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were analyzed
175 through Van Soest and Wine [22] modified extractions method, based on FD U44-162
176 standard [23]. Hemicellulose content was calculated as NDF minus ADF; cellulose as
177 ADF minus ADL and; lignin as equal to ADL. TKN and NH₃-N were determined
178 through the procedure described in the NF EN 25663 standard [24]. COD of water-
179 soluble phase was determined through the colorimetric HACH procedure (method
180 8000). COD of particulate phase was measured by a Walkley and Black [25] modified
181 method, based on the NF ISO 14235 international standard [26].

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

188 2.4. *Biochemical methane potential tests*

189 Batch anaerobic digestion tests were conducted in a temperate room at 35 °C using 2L
190 glass reactors. Vessels were filled with 5 gVS of sample, inoculum in way to keep a
191 substrate/inoculum VS ratio of 0.5 and a certain volume of a mineral solution to achieve
192 60% of the total volume of the vessel. The inoculum used (TS 2.3-3.3%wt; VS 1.5-
193 2.2%wt) was a digested sludge originating from the wastewater treatment plant of La

194 Feysine, Lyon, France. The sludge used met the criteria for a good inoculum quality
195 (*e.g.* pH, VFA, NH_4^+) suggested by the international task group on the harmonization of
196 BMP protocols [27]. The mineral solution, which contains essential elements to
197 microbial growth and also gives the solution a buffer able to control any pH
198 adjustments, was prepared according to the recommendations of ISO 11734
199 international standard [28]. Once filled, reactors were purged with a N_2/CO_2 mixture
200 (80/20%v) for about 5 minutes, sealed and equilibrated at 35 °C. Blanks with only
201 inoculum and mineral solution were performed for each batch series in order to correct
202 the BMP from residual methane production of the inoculum. All tests were performed
203 in triplicates.

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

213 **3. Results and discussion**

214 *3.1. Feedstock characterization*

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

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

231 A proportional increase of TS content was observed with the use of co-substrates. This
232 was expected since additives were used as pure compounds and not in solution. Since
233 the activity of silage bacteria is at least in part vulnerable to changes in TS [5,31,32],
234 this may have influenced the ensiling process. In the case of formic acid addition, the
235 pH of the feedstock was affected as well. Indeed, it decreased to 3.5, which is a
236 considerable lower value than the ones recommended by Kalač [33] for lactate silages
237 (4.10-4.20 for TS content of 15-20%) and by Teixeira Franco *et al.* [4] for anaerobic
238 stabilization of cattle manure with wheat straw (pH value of around 5.4-5.6).

239 Concerning raw material with glucose addition, WSC content ascended to 16.6% VS
240 (14.8% TS) and 40.2% VS (36.5% TS) for Glucose 4% and Glucose 10% conditions,
241 respectively. Under these conditions, the WSC contents were quite superior than the

242 ones found in the literature for feedstock successfully acidified during ensiling (3-
 243 14%TS) [10,16,34–37]. Thus, efficient acidification should occur for cattle manure
 244 treated with a co-substrate containing fermentable sugars if a proper amount of LAB is
 245 present.

246 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}

247 3.2. Effects of storage method on biomass preservation

248 3.2.1. Fermentation profiles

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

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

268 In addition, structural carbohydrates content regularly decreased for control silage. After
269 4 months of storage, hemicellulose and cellulose concentrations were 14.6 and 18.7% of
270 original VS, respectively. This represented a degradation of around 50% of the total
271 original hemicellulosic and cellulosic compounds. Since no accumulation of WSC nor
272 VFA was recorded during the same period, major organic and energy losses have
273 occurred. Regarding organic matter, losses ascend to 14.8% after 30 days and 42.0% at
274 the end of the storage. An average of 0.35% of organic matter was lost per day during
275 this period.

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

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

302 Co-ensiling of cattle manure with glucose underwent a strong acidification in the early
303 days of storage. Indeed, pH value of silage was 4.0 for both Glucose 4% and Glucose
304 10% conditions after 7 days, which was due to the conversion of WSC into lactic acid
305 (Table 2). This shows that the concentration of LAB in the original raw material was
306 significant. Consequently, the lack of easily fermentable sugars probably blocked the
307 conservation of cattle manure through the ensiling process of control trials. Silage
308 acidification slowed down LAB activity but it was not completely stopped. For this
309 reason, WSC continued to be consumed during the remaining ensiling period. After 15
310 days of storage, lactic acid concentration was around 15% VS_{added} for both co-silages
311 with glucose addition. After this period, two distinct fermentation pathways were
312 observed for the experiments with glucose. On the one hand, for Glucose 10%
313 condition, WSC was in large excess and lactic fermentation persisted until the end of
314 the storage. As a result, after 4 months, pH value of Glucose 10% silage was 3.7 and its
315 lactate concentration rose to nearly 20% VS_{added}. On the other hand, WSC of Glucose
316 4% assay was extinguished between 15 and 30 days of ensiling. Coupled to that, there
317 was a degradation of lactic acid and a significant increase of butyric acid content during
318 this period. This suggests that the acid pH value (4.1) was unable to avoid clostridial
319 activity since there was no more available substrate for lactate production. This
320 clostridial proliferation led to a secondary fermentation that was mainly based on lactic
321 acid consumption for the production of butyrate, carbon dioxide and hydrogen [5].
322 Between 30 and 120 days, clostridial fermentation led to full degradation of lactic acid
323 on Glucose 4% silage. Besides butyric acid, other VFA were found, such as caproic and
324 valeric acids (results not shown). Moreover, after 4 months of storage, the pH value of
325 Glucose 4% increased to 5.4. This is explained by the fact that butyrate is a weaker acid

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

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

341 3.2.2. Chemical oxygen demand balance

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

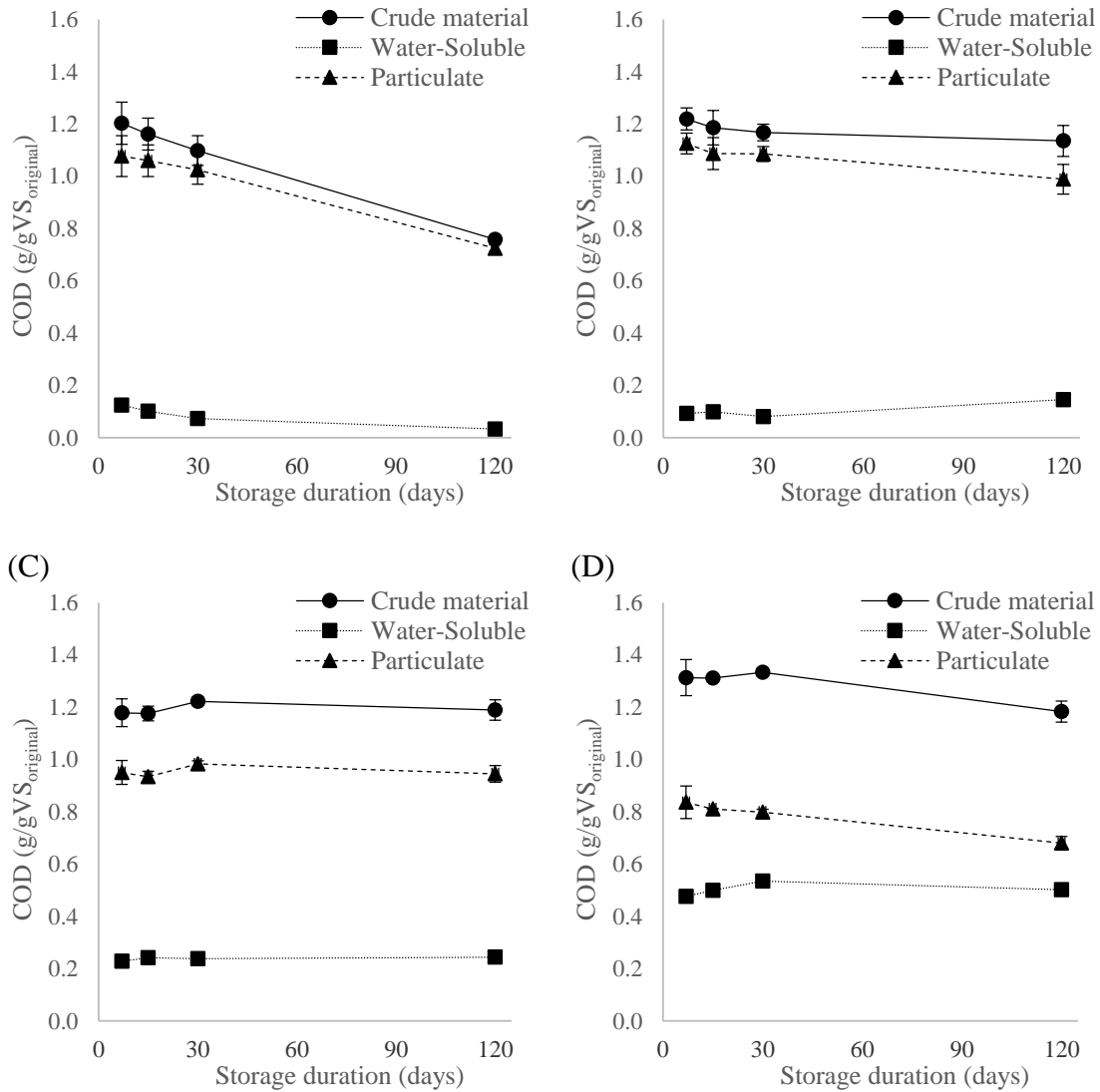
345 The most significant results were obtained for the ensiling of cattle manure without
346 additives. Before storage, around 93% of the total COD was in the particulate phase.
347 This is linked to earlier statements, evidencing that in cattle manure the majority of the
348 energy sources were hardly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

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

353 The use of co-substrates for the ensiling of cattle manure improved the conservation of
354 COD. For this reason, the fluctuations of its composition along duration were minimal,
355 which made it difficult to establish long-term trends in co-ensiling. Regarding formic
356 acid assays, despite the important VS losses recorded for prolonged durations, COD of
357 crude material was stable until 120 days of storage. This can be in part explained by the
358 fact that formic acid has low COD content (0.35 g_{O2}/g), so that its degradation did not
359 cause significant COD decrease. Furthermore, there was an important solubilization of
360 particulate COD in the last 3 months of ensiling. This should correspond to the
361 hydrolysis of structural carbohydrates and subsequent conversion into VFA, as it was
362 seen for the fermentation profiles (subsection 3.2.1). In addition to the solubilization
363 effect and the potential kinetic gain in AD conditions, this may have caused an
364 amplification of carbohydrates accessibility.

(A)

(B)



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

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

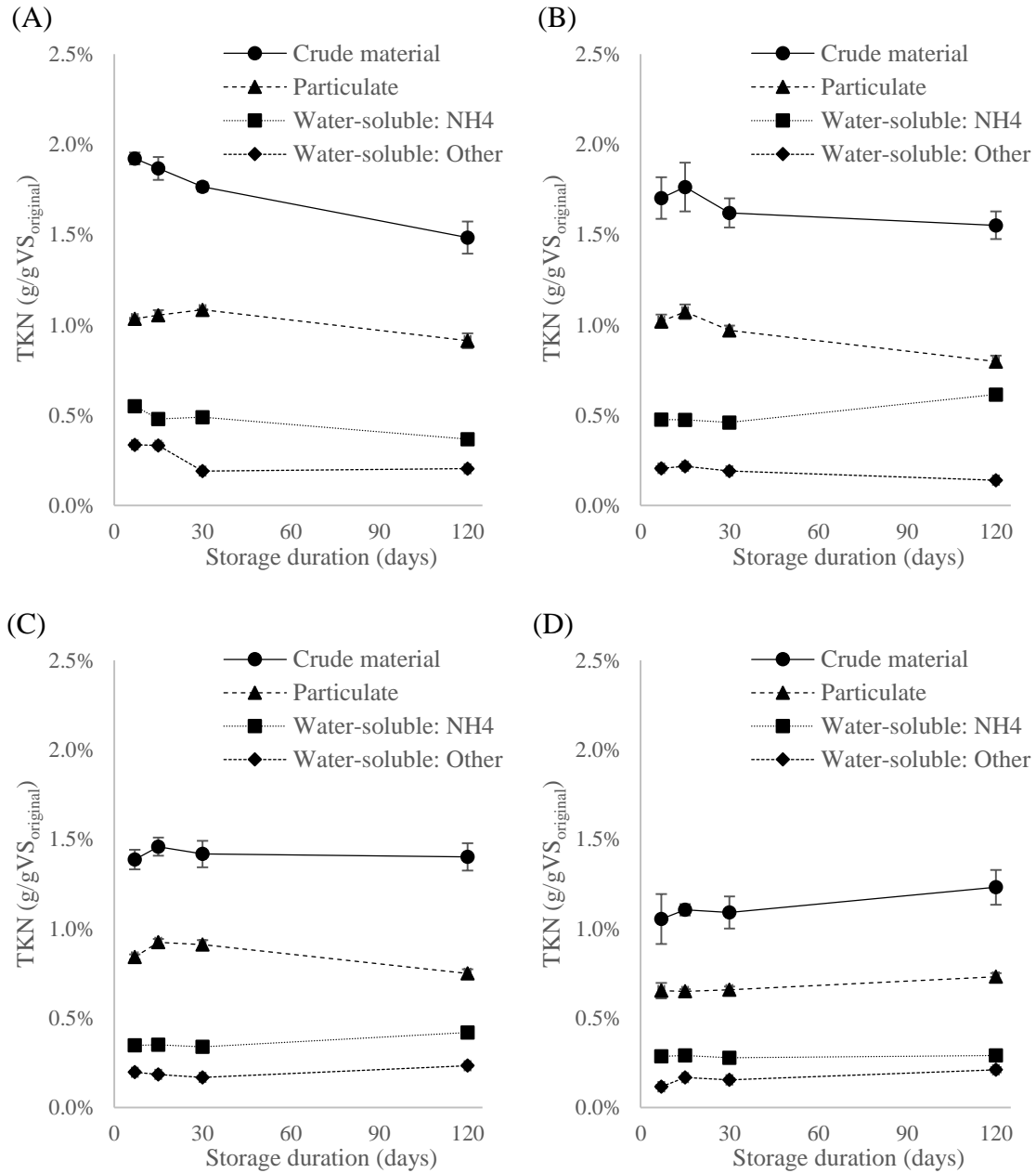
375 persistent solubilization of particulate COD along the experiment. This can be attributed
376 to the acid hydrolysis of hemicelluloses by the VFA produced during ensiling.

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 pKa (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

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



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

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

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

414 3.3. *Effects of storage method on methane potential*

415 3.3.1. Initial conditions

416 Before storage BMP was 229 ± 8 $\text{L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ for fresh cattle manure, 250 ± 8
417 $\text{L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ for cattle manure + Formic acid, 239 ± 26 $\text{L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ for cattle
418 manure + Glucose 4% and 276 ± 8 $\text{L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ for cattle manure + Glucose 10%.
419 Formic acid and glucose had a clear impact on the BMP of the feedstock. The effect of
420 Glucose 4% addition was not significant (within the BMP accuracy limits). Considering
421 the theoretical BMP of glucose ($373 \text{ L}_{\text{STP}}/\text{kgVS}_{\text{added}}$) and the amount of co-substrate
422 used, a BMP of $287 \text{ L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ was expected for Glucose 10% before storage.
423 Furthermore, glucose contribution to the total BMP should be around 52% in this case.
424 This evidences that glucose added was fully degradable under anaerobic conditions and
425 that it was responsible for the initial BMP value increase of Glucose 10% mixture. In
426 opposite, the additional methane production of Formic acid feedstock cannot be
427 attributed to the co-substrate, due to its low BMP value ($122 \text{ L}_{\text{STP}}/\text{kgVS}_{\text{added}}$). In fact,
428 the theoretical BMP of this feedstock mixture should be around $221 \text{ L}_{\text{STP}}/\text{kgVS}_{\text{added}}$ with
429 a 5% contribution of formic acid for these value. Therefore, it can be pointed out an

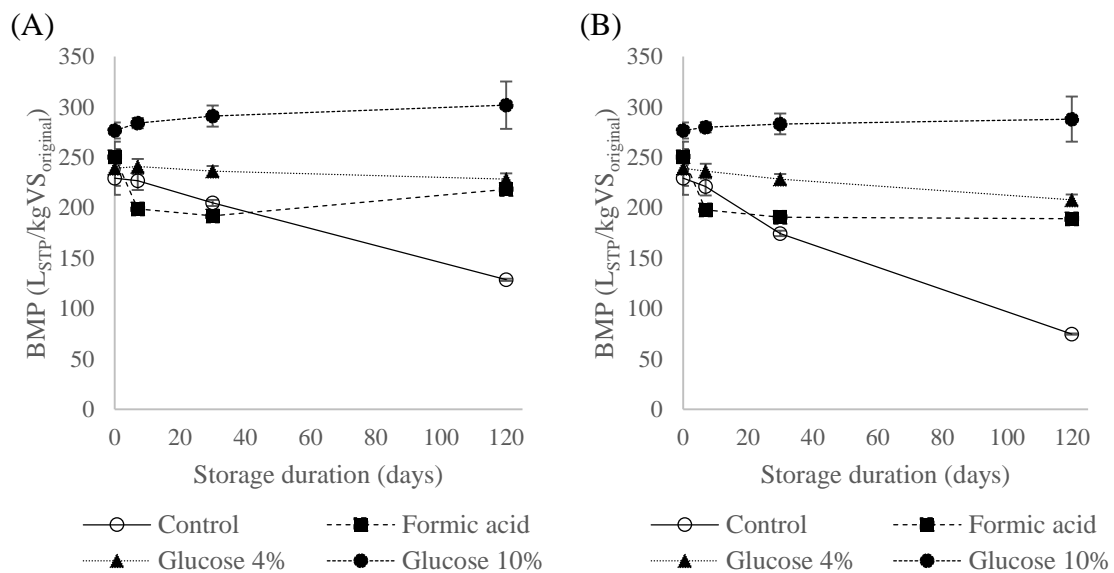
430 improvement of the biochemical accessibility of cattle manure after being treated with
431 formic acid (before ensiling). This might be caused by an increase of accessible surface
432 area of biomass, hemicellulose removal and alteration of lignin structure, which are
433 typical features of dilute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

436 3.3.2. Evolution of the BMP during storage

437 Control trials showed BMP values of 128-229 $L_{STP}/kgVS_{added}$ and 75-229
438 $L_{STP}/kgVS_{original}$ during storage, Figure 5. In the first 7 days of control assays, both
439 methane potential based on VS_{added} and $VS_{original}$ were stable. This evidences that neither
440 the energy content nor the biochemical accessibility of cattle manure with straw
441 changed during short-term ensiling, which should be linked with the small fermentation
442 activity and pH decrease that occurred in this period. However, for longer storage
443 durations, the BMP of control was significantly damaged. Indeed, after 30 and 120 days
444 of ensiling, 24% and 67% of original BMP was lost, respectively. This obviously was a
445 consequence of the extensive degradation of structural carbohydrates (without any VFA
446 accumulation), due to the high pH levels.

447 Co-ensiling with formic acid limited the harmful impact of storage on the methane
448 potential of the raw material. In this case, BMP values were 192-250 $L_{STP}/kgVS_{added}$ and
449 189-250 $L_{STP}/kgVS_{original}$ during the 4 months. The major variation of methane potential
450 for formic acid silage occurred in the first 7 days of storage. In this period, around 21%
451 of both BMP related on VS_{added} and $VS_{original}$ was lost. It is important to mention that the
452 energy conservation rates of co-ensiling conditions are related to the initial BMP of the
453 mixture manure/additive and not only to fresh cattle manure. As previously discussed

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



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

471 Higher stability of methane potential was achieved through the co-ensiling of cattle
472 manure with glucose. Indeed, Glucose 4% silage presented BMP values of 228-241
473 $L_{STP}/kgVS_{added}$ and 208-238 $L_{STP}/kgVS_{original}$ along the 4 months of storage. In parallel,
474 for Glucose 10% condition, BMP values were 276-302 $L_{STP}/kgVS_{added}$ and 276-288
475 $L_{STP}/kgVS_{original}$. During the first month of storage, both experiments with glucose had
476 constant methane potential. These results are in line with the data from the fermentation
477 profiles and illustrate the efficiency of lactate production on the energy conservation
478 during ensiling. Concerning Glucose 10% assay, this trend persisted and no loss of
479 BMP was observed until the end of the 4 months. An eventual positive impact on the
480 methane potential could be seen (probably due to the hemicellulose hydrolysis and
481 fermentation), but it was not significant within the precision limits. In contrast, in the
482 remaining 90 days of ensiling, BMP of Glucose 4% progressed differently. For this
483 condition, 13% of the methane potential related to $VS_{original}$ was lost after 4 months.
484 This BMP damage can be explained by the secondary fermentation that led to lactic acid
485 degradation and nitrogen mineralization.

486 **4. Conclusions**

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

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

496 **Acknowledgements**

497 Rúben Teixeira Franco held a doctoral fellowship from the Rhône-Alpes region. This
498 work has been undertaken within the SAM project (*Stockage Avant Méthanisation -*
499 *Storage Before AD*) funded by ADEME (# 1506C0038). The authors thank the DEEP
500 laboratory team, including David Lebouil, Hervé Perier-Camby, Nathalie Dumont and
501 Richard Poncet for the given support during the tests. We are grateful to Franck Barra
502 for his permanent availability for discussion and raw material supply. Mathilde Hardier
503 and SUEZ are also acknowledged for the inoculum provided for the BMP tests.

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