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Improving the storage of cover crops by co-ensiling with different waste types: effect on fermentation and effluent production

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1 Abstract

2 Cover crops harvested at a low maturity stage generally have a high moisture content, which
3 may generate energy losses during silage storage via effluent production and undesirable
4 fermentations. This paper investigates the use of different waste types as absorbent co-
5 substrates to be added before ensiling. The relation between the absorbent water holding
6 capacity and silage effluent volume was first studied to find an effective parameter to prevent
7 effluent production. Effluent retention was found to be proportional to the absorbent loading
8 and water holding capacity ($r^2 = 0.98$) and up to 90% of effluent production was avoided when
9 compared to control (295 L.t^{-1}). The impact of different co-substrates (including bio-waste and
10 manures) on overall ensiling performances was then investigated at an optimized absorbent
11 loading. All co-substrates allowed a total effluent retention while a 76 L.t^{-1} effluent volume was
12 reported for the control. The silage fermentation was modified or mostly unchanged depending
13 on the co-substrate chemical and microbial properties and different metabolic pathways were
14 observed (e.g. homolactic or butyric fermentation). In most conditions, the methane potential
15 of the crop was efficiently preserved over a storage of 60 days. Co-ensiling was shown to be a
16 relevant silage preparation method for biogas production.

17

18 1 Introduction

19 Cover crops, also called catch crops or intermediary crops, are receiving an increasing interest to
20 be used as substrates for anaerobic digestion. These crops are cultivated during the intercultural
21 period of main crops and bring agro-ecological services by limiting soil erosion, nitrate leaching
22 and the need for mineral fertilizers (Molinuevo-Salces et al., 2013). In agricultural biogas sector,

23 cover crops allow to produce a large amount of biomass for methane production, without
24 competing with the production of food or feed crops. Due to the management of crop rotations,
25 cover crops are usually harvested once or twice a year. Since the biogas digesters are fed
26 continuously, an efficient storage of the crops from their harvest to their final use in anaerobic
27 digesters is mandatory. The storage of fresh fodder or energy crops is commonly performed by
28 ensiling (Teixeira Franco et al., 2016). This storage process relies on the fermentation of the
29 substrate, mostly by lactic acid bacteria. In the anaerobic environment of the storage silos, the
30 water soluble carbohydrates (WSC) are fermented into lactic acid and other metabolites,
31 causing a pH drop to about 4 (Woolford and Pahlow, 1998). The combination of acidic pH and
32 anaerobic environment then ensures the inhibition of microbial activity over the whole storage
33 duration.

34 The performance of ensiling greatly depends on silo confection practices and crop
35 characteristics (Teixeira Franco et al., 2016). Among the key parameters, the total solids (TS)
36 content of the crop has a major influence on storage performances. For biogas production, a TS
37 range from 26 to 36% has been reported to be optimal (Villa et al., 2020). Silage production
38 from lower TS crops may lead to fermentation pathways dominated by *Clostridia*, characterized
39 by butyric acid and H₂ production (Woolford and Pahlow, 1998). In silage production for animal
40 feeding, clostridial fermentations are highly undesirable because of the possible development of
41 pathogenic microorganisms, poor palatability and decrease in feed value (Woolford and Pahlow,
42 1998). In silage production for biogas production, such issues are less relevant, but clostridial
43 fermentation is reported to induce energy losses due to the production of H₂ (Kreuger et al.,
44 2011). However, the overall effect of clostridial development in silage for biogas remains to be

45 investigated, since energy losses may be compensated by an increase in lignocellulose
46 hydrolysis and biomass degradability (Cui et al., 2020). Another issue with the silage of low TS
47 crops is the high production of liquid effluents (Gebrehanna et al., 2014). For TS values lower
48 than 25%, hundreds of liters of effluent can seep per ton of ensiled crop (Bastiman and Altman,
49 1986; Sutter, 1957). If not collected and valorized properly, silage effluents may cause methane
50 potential losses (Teixeira Franco et al., 2016) and surface water pollution (Holly et al., 2018). In
51 addition, silage effluents can generate significant odor nuisance (Keck et al., 2018) that greatly
52 threatens the local acceptance of biogas plants (Schumacher and Schultmann, 2017). They can
53 also cause corrosion of steel and concrete (Koenig and Dehn, 2016). As a consequence, silage
54 effluent production is reported to be highly undesirable in biogas plants.

55 Because they are grown during short periods and/or with unfavorable weather conditions,
56 cover crops are often harvested at a low maturity stage which is associated to a low TS. TS of
57 less than 20% are commonly met in cover crops (Molinuevo-Salces et al., 2013) and significant
58 volumes of effluents are expected during the storage of such crops. In order to avoid this issue,
59 various practices can be adopted, such as field wilting or the addition of an absorbent co-
60 substrate. Field wilting consists in a partial open-air drying of the freshly mowed crop prior to its
61 storage and can result in an efficient increase in TS when proper conditions are met (Borreani et
62 al., 2009). This method is however weather-dependent and unsuitable conditions may make
63 wilting inappropriate (Teixeira Franco et al., 2017). The addition of a dry co-substrate presents
64 the advantage of being independent from weather conditions and co-ensiling a wet organic
65 matter with a substrate having absorbent properties allows to immediately increase the TS of
66 the pre-silage mixture and thus avoid the effluent production (Jones and Jones, 1996). In the

67 literature, diverse co-substrates have been studied like the straw and stalks of different crop
68 species (Haigh, 1998; Razak et al., 2012), rice, barley and wheat bran (Haigh, 1999; Razak et al.,
69 2012), dried beet pulp (Fransen and Strubi, 1998; Haigh, 1999; Razak et al., 2012), ground barley
70 (Khorvash et al., 2006), dry bean hulls (Razak et al., 2012), newspaper (Fransen and Strubi,
71 1998), sodium bentonite (Fransen and Strubi, 1998; Khorvash et al., 2006; Woolford et al., 1983)
72 and various polymers (Fransen and Strubi, 1998; Healy et al., 1997), with different liquid holding
73 efficiencies. These co-ensiling experiments were mainly applied for silage production for animal
74 feeding and thus most of the tested co-substrates are edible. In the context of anaerobic
75 digestion, the strategy for selecting co-substrates is different, since as an example, high value
76 edible substrates like ground barley or dried beet pulp should be avoided. On the other hand,
77 non-edible substrates compatible with anaerobic digestion can be considered.

78 This study focuses on the use of waste-like and/or agricultural byproducts as absorbent
79 substrates in ensiling prior to anaerobic digestion. The first objective was to evaluate the
80 behavior of a well-studied absorbent substrate (wheat straw) as a co-ensiling substrate with
81 sunflower, in order to define the adequate co-substrate loading. In a second part, the influence
82 of the addition of various co-substrates (soiled paper and cardboard, wood chips, miscanthus
83 straw, chicken manure and horse manure) on the ensiling of rye was investigated. Production of
84 effluent, metabolites and gas as well as the influence on microbial community structure and the
85 preservation of methane potential were analyzed during two months of storage, in order to
86 propose a global understanding of the addition of co-substrate on silage storage.

87 2 Material and Methods

88 The experiments and physico-chemical analyses were performed at the Bio2E platform (Bio2E,
89 2018).

90 2.1 Catch crops and co-ensiling substrates

91 Two catch crops, sunflower and rye, were kindly provided by RAGT Semences and Biométhagri,
92 respectively. Sunflower cover crop was harvested in Cintegabelle (Haute Garonne, France) at
93 BBCH stage 65 (full flowering) in September of 2020. The BBCH maturity scale is described by
94 Meier (2018). Rye cover crop was collected in Florensac (Hérault, France) at BBCH 59 (end of
95 heading). These crops were hand harvested using a sickle, at cutting height of about 10 cm.
96 Sunflower was chopped using a garden shredder (AXT 2550TC, Bosch GmbH) and a knife mill
97 (BB230, BLiK®) and further frozen at -20°C until use. Rye was chopped according the same
98 protocol, but was used directly after for storage experiments.

99 Concerning the co-substrate, a wheat straw, commercialized as a bedding material, was used
100 (ZOLUX S.A.S., France). Soiled paper and cardboard (named Paper) were separated from kitchen
101 wastes. Wood chips (Wood) were collected in a sawmill that processes different wood species
102 (Ets Guille, Narbonne, Aude, France). Miscanthus stalks (*M. x giganteus Britannique*, Misc) were
103 supplied by INRAE AgroImpact (Estrées Mons experimental unit, Péronne, 80203, France).
104 Chicken manure (Chicken M) was collected on a chicken farm (GAEC d'Empare, Saint Marcel Sur
105 Aude, Aude, France). Horse manure (Horse M) was collected at a horse riding club (Narbonne
106 Equitation, Narbonne, Aude, France). Both manures contained cereal straw as a bedding
107 material.

108 2.2 Silage storage experiments

109 2.2.1 Experimental setups

110 Two different storage protocols were used, depending on the objective.

111 For the monitoring of silage effluent production, the experiments were conducted in mini-silos
112 made of PVC pipe of 100 mm inner diameter and 800 mm height (6.3 L volume), in a similar
113 configuration to that described by Savoie et al (2002). The filling was carried out by adding, in a
114 successive way, layers of approximately 200 mm of loose catch crop which were compacted
115 using a hydraulic press (20-ton capacity, KS Tools GmbH) until the targeted packing density. The
116 packing density was applied as recommended by Latsch and Sauter (2014) (Fresh matter basis –
117 FM): 800 kg.m⁻³ for 20% TS forage ; 700 kg.m⁻³ for 25% TS forage ; 650 kg.m⁻³ for 30% TS forage.
118 Packing density of intermediate levels of TS were calculated by linear interpolation. The silos
119 were closed, and two tamps allowed to measure the gas volume and composition and to collect
120 the liquid effluent at the bottom. In order to avoid air penetration, liquid effluent drainage was
121 performed at most once a day. The effluent was characterized as described in section 2.2.4. All
122 storage experiments were started on the same day and operated for 2 months. For each storage
123 condition, two replicates were prepared in independent silos. The silos and their content were
124 weighted before and after storage.

125 For the dynamic monitoring of silage fermentation, 2.6L glass bottles were used, as previously
126 described (Van Vlierberghe et al., 2021). This storage method was used in addition to storage in
127 PVC silos for the co-ensiling of rye, in order to allow a higher number of replicates for dynamic
128 monitoring of storage. For each storage condition, several replicates were prepared to be

129 sacrificed after 2, 7, 21 and 60 days of storage in order to carry out a complete physico-chemical
130 and microbial analysis of the samples during the ensiling process as described in section 2.2.5.

131 A 60-day storage period was chosen as sufficient for fine effluent and fermentation
132 characterization. Previous studies of the literature reported that the peak flow of effluent
133 production usually occurs during the first days of storage, with 90% of the total volume being
134 usually released during the 20 to 26 first days of storage (Mayne and Gordon, 1986). Concerning
135 fermentation, most of the transformations that may occur usually happen during the first weeks
136 of storage (Woolford and Pahlow, 1998).

137 2.2.2 Co-ensiling of sunflower with straw with variable absorbent loading

138 A first round of experiments was only conducted in tubular PVC silos. Five different ratios of
139 wheat straw were tested: 0, 40, 80, 150 and 250 $\text{g}\cdot\text{kg}_{\text{sunflower}}^{-1}$, on a fresh matter basis. In each
140 reactor, 3 kg_{FM} of sunflower were added, and the amount of wheat straw was adapted in order
141 to match with the desired loading. A first layer of wheat straw was added and packed, and
142 sunflower was then added on top, and packed (protocol called “bottom”). A second protocol,
143 named “mixed”, was also carried out for the ratio of wheat straw of 40 $\text{g}\cdot\text{kg}^{-1}$; sunflower and
144 straw were homogenized before filling into the silos. 6 different storage conditions were thus
145 tested.

146 2.2.3 Co-ensiling of rye with diverse co-substrates at a determined absorbent loading.

147 This storage experiment was conducted both in tubular PVC silos and bottle silos, in order to
148 evaluate the impact of the type of co-substrate on the volume of effluent produced and on the
149 fermentation and microbial community structure, respectively. Five different co-substrates

150 were used (soiled paper and cardboard, miscanthus stalks, wood chips, chicken manure and
151 horse manure) and rye was used as catch crop. One ensiling condition containing only rye was
152 also prepared as a control. The calculation of the loading of each co-substrate was based on the
153 expected cumulated effluent volume of rye ensiled alone ($\text{mL}\cdot\text{kg}_{\text{FM}}^{-1}$) and the measurement of
154 water holding capacity (WHC) (see section 2.2.6). In each PVC silo, 3 kg_{FM} of rye were added.
155 The amount of absorbent was adapted in order to match with the desired loading. In glass silos,
156 700g of mixture were added, with the same co-substrate/rye ratio as in the PVC silos.

157 2.2.4 Silage effluent characterization

158 Silage effluents from tubular silos were characterized by their volume and their chemical oxygen
159 demand (COD) at most once a day, depending on the flow rate. COD was determined by
160 colorimetric method (Aqualytic® COD Vario 0-1500 $\text{mgO}_2\cdot\text{L}^{-1}$) after 1/100 dilution.

161 2.2.5 Physicochemical analysis of solid samples

162 The cover crops, co-substrates and their mixtures were characterized as follows. Total solids (TS)
163 were determined by oven drying (105°C , 24h). Volatile solids (VS) were measured by calcination
164 of the dry residue (550°C – 3h). A TS correction method for lactic acid, VFA and alcohols
165 volatilization was applied as described by Porter and Murray (2001). For pH, ammonia, WSC,
166 and metabolites analysis, a water extraction of the solid samples was made according to the
167 protocol of Porter and Murray (2001). 30 g of freshly collected sample were soaked in 150 mL of
168 distilled water for 16 h to 20 h at 4°C in sealed plastic pots in triplicate. pH measurement was
169 made directly on the mixture after extraction. The liquid phase was then separated by
170 centrifugation (18750 g, 20 min, 4°C) and stored at -20°C until analysis. The quantification of
171 WSC, VFA and metabolites was performed on the liquid extract by HPLC, and ammonia

172 measurement was made by titration with boric acid using a Gerhardt® Vapodest 50 s® carousel.
173 The total carbon (TC) and total nitrogen (TN) were determined via an elemental analyzer
174 (FlashSmart®, Thermo Fisher Scientific®) on finely grounded freeze dried samples.

175 Biochemical methane potential (BMP) were made according to the recommendations of
176 Holliger et al. (2016). Samples for BMP tests were previously prepared by freezing a certain
177 amount of substrate containing around 2 g_{TS} of sample (exact TS and VS were calculated later).
178 BMP values were calculated following two different methods depending on the objective.

179 Methane potential expressed as the volume of methane per amount of VS added
180 (NmLCH₄.gVS_{add}⁻¹) are useful to estimate the substrate degradability before and after storage.
181 BMP reported to the initial amount of VS after taking into account the mass losses that occur
182 during storage (NmLCH₄.gVS_{init}⁻¹) are mandatory to assess the global balance of silage storage on
183 energy potential.

184 The buffering capacity (BC) of the fresh cover crops, co-substrates and mixtures was measured
185 following the method of Playne and McDonald (1966). 10 g of finely shredded samples were put
186 in 250 mL of distilled water. This mixture was first titrated under agitation to pH 3 with a
187 solution of 0.1 N HCl in order to release bicarbonate as carbon dioxide, and then titrated to pH 6
188 with a solution 0.1 N NaOH. BC was expressed as mequiv of NaOH per kg of dry matter to
189 elevate the pH from 4 to 6, after correction for the titration of a 250 mL water blank.

190 For microbial community analysis, 300 mg of fresh medium were sampled and stored at -20°C in
191 a 2mL sterile tube. DNA extraction, sequence data analysis and quantitative PCR were performed
192 as described by (Venkiteswaran et al., 2016). Sequencing was achieved at the technology

193 platform Genome and Transcriptome (GeT) of the Génopole Toulouse, France. OTUs with a
194 relative abundance of > 1.5% in at least one sample were selected for further analysis.

195 2.2.6 Water holding capacity and preliminary effluent volume estimation

196 The water holding capacities of co-ensiling substrates were characterized. WHC was measured
197 following a protocol adapted from Marsac *et al.* (Marsac et al., 2019). 10 g of shredded
198 substrate were introduced into a nylon bag (4 x 12 cm, ~30 µm pore size, FibreBag ref. 10-0127,
199 C. Gerhardt GmbH & Co. KG, Germany). The bags were soaked in tap water for 2 hours and then
200 centrifuged (200 g, 10 min) into a 500 mL bottle with a draining material in the bottom (i.e.,
201 glass balls). WHC measurements were performed in triplicate. WHC was calculated as the
202 amount of water that was hold in the sample after centrifugation, as follows:

$$WHC = \frac{m_h - m_r}{m_r} \quad (1)$$

203 with WHC the Water Holding Capacity (g.g⁻¹), m_r the mass of raw sample (g) and m_h the mass of
204 humidified sample after soaking and centrifugation (g).

205 A preliminary estimation of effluent volume to be produced by the cover crop during storage was
206 made as follows. First, effluent volume estimations from Bastiman (1986) (equation(2) and Sutter
207 (1957) (equation(3) were calculated.

$$V_{Bastiman} = 767 - 53.4 \times TS + 0.936 \times TS^2 \quad (2)$$

$$V_{Sutter} = 672 - 22.40 \times TS \quad (3)$$

208

209 With V = cumulated silage effluent volume per ton of fresh crop ($L \cdot t_{FM}^{-1}$) and TS = Total Solid
210 content ($kg \cdot kg_{FM}^{-1}$). From these values, $V_{effl\ expect}$ was calculated as the average value between
211 $V_{Bastiman}$ and V_{Sutter} , based on previous experiments (not shown). This estimation was used for the
212 calculation of co-substrates loadings in the rye storage experiment following equation(4).

$$Cosub = \frac{V_{effl\ expect}}{WHC} \quad (4)$$

213
214 where $Cosub$ represents the co-substrate loading ($g_{FM\ co-substrate} \cdot kg_{FM\ crop}^{-1}$), $V_{effl\ expect}$ the expected
215 effluent volume ($mL \cdot kg_{FM\ crop}^{-1}$) and WHC the Water holding capacity of the co-substrate ($mL \cdot g_{FM}$
216 $co-substrate^{-1}$).

217 2.3 Statistical analysis and data representation

218 One-way analysis of variance was made on BMP results after verifying both the normality
219 (Shapiro-Wilk test) and the variance homogeneity (Levene test) with R package “rstatix”. A pair-
220 wise t-test adjusted with Holm method was further realized for assessing the significance of the
221 difference in means between two samples. When data distribution could not be assumed as
222 normal, the Kruskal Wallis test was applied and Wilcoxon test was used with Holm’s p-value
223 correction in the case of pairwise analysis. The matrix of Pearson’s correlation was obtained using
224 “corrplot” package. A linear model was calculated on co-ensiling data of sunflower using lm
225 function of R. The package “ggplot2” was used for graphical representations.

226 3 Results and discussion

227 3.1 Sample characterization

228 The main characteristics of the crops and co-substrates are presented in Table 1. Sunflower and
229 rye were characterized by a low TS content of 14.6 and 21.1%, respectively. The expected
230 volume of effluent produced according to Bastiman and Altman (1986) and Sutter (1957)
231 estimations were 187 and 345 mL·kg⁻¹ for sunflower and 57 and 199 mL·kg⁻¹ for rye,
232 respectively. The average estimated volume were calculated and corresponded to 138 and 266
233 mL·kg⁻¹ for oat and rye, respectively. Co-ensiling substrates were characterized by higher TS
234 contents, with value ranging from 53 (horse manure) to 92.7 % (miscanthus stalks). The WHC of
235 the different co-substrates ranged from 0.8 (chicken manure) to 2.1 mL·g⁻¹ (soiled paper and
236 cardboard), which confirmed their ability to be used as absorbent materials. These values are
237 lower than other found in the literature. For example, WHC of 2.3 – 2.8 mL·g⁻¹ were reported for
238 miscanthus stalks (Dennery G., Dezat E., 2012) whereas they were around 3 for wheat straw
239 (Razak et al., 2012). This difference may be explained by the protocol used in the present work
240 (centrifugation with a draining material), ensuring that no water remained in the interstitial
241 spaces of the substrate. The two crops had C/N ratios of 22.2 and 22.1 g·g⁻¹ for rye and
242 sunflower, respectively, which corresponds to the medium values for cover crops (Molinuevo-
243 Salces et al., 2014). For the co-ensiling substrates, different patterns can be observed. Wheat
244 straw, soiled paper and cardboard, miscanthus stalks and wood chips had very high C/N ratios
245 ranging from 81 to 447, while horse manure had a similar C/N ratio to that of the crops. Finally,
246 chicken manure presented the highest nitrogen content, with a C/N ratio of 8.2. Both crops
247 presented high WSC of 181 and 131 g·kg⁻¹ in sunflower and rye, respectively, which are

248 sufficient to initiate the silage fermentation (da Silva et al., 2017). In all co-substrates, a
249 negligible amount of WSC was found.

250 Rye and sunflower had buffering capacities of 248 and 283 $\text{mEq}\cdot\text{kg}_{\text{TS}}^{-1}$, respectively, which are
251 considered as low values (Kaiser et al., 2004). Wheat straw, soiled paper and cardboard,
252 miscanthus stalks and wood chips had a very low buffering capacity, between 14 to 88 $\text{mEq}\cdot\text{kg}_{\text{TS}}^{-1}$
253 ¹, while that of manure was 148 $\text{mEq}\cdot\text{kg}_{\text{TS}}^{-1}$, a lower value than for rye. However, chicken
254 manure presented a high buffering capacity of 509 $\text{mEq}\cdot\text{kg}_{\text{TS}}^{-1}$, which was likely due to its high
255 nitrogen content. A high buffering capacity makes it difficult to lower the pH during silage
256 fermentation and is considered as undesirable (Teixeira Franco et al., 2016).

257 Table 1 *Fresh cover crop and co-substrate characteristics.*

258

259 3.2 Impact of co-substrate loading on co-ensiling

260 The cumulated volumes of effluent measured in the co-ensiling experiment of sunflower with
261 wheat straw are presented in Figure 1. Ensiling of sunflower alone resulted in the production of
262 295 $\text{mL}_{\text{effluent}}\cdot\text{kg}_{\text{sunflower}}^{-1}$ in average, which is comparable with the average estimations from
263 Bastiman (1986) and Sutter (1957) (266 $\text{mL}\cdot\text{kg}^{-1}$). Wheat straw addition was shown to efficiently
264 reduce the production of silage effluent. The volume of effluent produced was inversely
265 proportional to straw loading up to 150 $\text{g}\cdot\text{kg}^{-1}$. For higher straw loadings, the effluent is almost
266 totally retaining in the solid medium. A linear model was used to fit the data presented in Figure
267 1, excepted for 250 $\text{g}\cdot\text{kg}^{-1}$ straw loading, since full retention was considered to be reached over
268 150 $\text{g}\cdot\text{kg}^{-1}$. The cumulated effluent volumes from 10 different silos were consequently used for
269 the linear model definition. The regression line was forced to intercept with the effluent volume

270 value of the control. For a straw loading ranging from 0 to 150 g·kg⁻¹, 1.6 mL of effluent were
271 retained per g of wheat straw added, which corresponds to the theoretical WHC capacity of
272 wheat straw calculated previously. The straw addition method also influenced the effluent
273 retention capacity. At a loading of 40 g·kg⁻¹, mixing straw with sunflower allowed to decrease
274 the effluent production by 28 mL·kg⁻¹ compared to adding the same amount of straw as a
275 single layer at the bottom of the silo.

276 The COD of the effluent produced during the ensiling of sunflower alone was 58 gO₂·L⁻¹, which is
277 in the range of COD met in silage effluent in the literature (Gebrehanna et al., 2014). The BMP
278 of pooled effluent samples was measured and a value of 309 ± 18 NmLCH₄·gCOD⁻¹ was found,
279 which is close to the theoretical relation of 1g COD = 350 NmLCH₄, indicating that the organic
280 matter contained in the effluent is mostly biodegradable. The methane potential of the effluent
281 was consequently estimated to be 17.9 NLCH₄·L_{effluent}⁻¹. Considering the initial VS content and
282 cumulated effluent volume of sunflower, a cumulated methane potential of 40.5 Nm³·t_{VS init}⁻¹
283 flowed in the effluent when sunflower was ensiled alone. This represents 18% of fresh
284 sunflower BMP. Co-ensiling allowed to gradually reduce effluent production and thus BMP flow
285 through effluent down to until 4.2 Nm³·t_{VS init}⁻¹, i.e. 1.7% of fresh sunflower BMP as the straw
286 loading increased.

287 Straw addition had little influence on silage fermentation. In fact, very similar chemical
288 properties were found for all conditions at the end of storage, with an average pH of 4.0 ± 0.0
289 and lactic to acetic acid ratio of 4.6 ± 0.4 (ranging from 3.9 to 5.3). No significant amount of
290 other VFA than acetic acid was produced. All silages were thus considered of “high quality”
291 regarding their fermentation characteristics.

292 *Figure 1: Cumulated effluent volume after 60 days of co-ensiling (sunflower + wheat straw). Dots*
293 *indicate mean, error bars indicate maximum and minimum*

294

295 This experiment confirmed the interest of using a dry co-substrate for silage effluent retention.

296 The theoretical WHC of the co-substrate was found to be a relevant parameter for its dosing in a

297 co-ensilage and it was consequently used for the calculation of co-substrate loadings in the

298 following experiment.

299 3.3 Impact of co-substrate type on co-ensiling

300 3.3.1 Physicochemical characteristics of fresh mixtures

301 The characteristics of the different pre-silage mixtures (rye + co-substrates) are presented in

302 Table 2. Due to the loading calculation method based on WHC, the co-substrate loading varied

303 from 58.9 (R + Paper) to 182.3 g.kg_{rye}⁻¹ (R + Chicken M) on a fresh matter basis, as an effluent

304 volume of 138 mL.kg_{rye}⁻¹ was expected. The TS content of the mixtures ranged from 23.3 (R +

305 Horse M) to 29.6% (R + Chicken M). Whatever the co-substrate, the pH of the mixture was close

306 to that of rye (6.5), excepted for R + Chicken M where it was significantly higher (7.5).

307 Depending on the buffering capacity of co-substrate, those of the mixtures decreased (R+ Misc,

308 R+ Wood), were unchanged (R + Paper, R + Horse M) or increased (R + chicken M). However,

309 due to the low initial buffering capacity of rye, the buffering capacity of all mixtures remained

310 low according to the literature (Kaiser et al., 2004). For the mixtures R + Paper, R + Misc, R +

311 Wood, the C/N ratio was slightly increased, while its value dropped to 14.3 for R + chicken M.

312 The range of C/N ratios was however suitable for further anaerobic digestion, since ratios from

313 15 to 30 are recommended (Kalač, 2011). WSC content was not significantly different from

314 control in all cases excepted R+Wood whose concentration was lower. WSC content of all
315 mixtures however remained high and suitable for efficient silage fermentation (da Silva et al.,
316 2017).

317 Table 2 : *Fresh co-ensiling mixtures characteristics.*

318
319 As a consequence, the type of co-substrates influenced some key parameters of the mixture
320 that can drive the efficiency of the silage fermentation. However, the main characteristics of the
321 mixture remained in the recommended range for “adequate ensilability”, according to the
322 literature.

323 3.3.2 Silage effluent production

324 An effluent was only produced during the ensiling of rye alone. In this condition, an average
325 volume of $76 \text{ mL.kg}_{\text{CC}}^{-1}$ was measured, which was lower than the previously estimated volume
326 (138 mL.kg^{-1}) but between the estimation of Bastiman and Altman (1986) ($57 \text{ mL.kg}_{\text{CC}}^{-1}$) and the
327 one Sutter (1957) ($199 \text{ mL.kg}_{\text{CC}}^{-1}$). In addition, despite of the relatively low TS content of the
328 mixtures R + Paper, R + Wood and R + Horse M that should have led to an effluent production
329 according to Bastiman and Altman (1986) and Sutter (1957) estimations, no effluent was
330 produced. This result shows that, for co-ensiling experiments, the TS content of the mixture is
331 not a reliable parameter to estimate the effluent production, and that it is essential to consider
332 the estimation of the volume of effluent produced by the crop together with the holding
333 capacity of the co-substrate.

334 The average COD concentration in effluent collected during the storage of rye alone was 59
335 $\text{gO}_2.\text{L}^{-1}$, and a BMP of $332 \pm 13 \text{ NmLCH}_4.\text{gCOD}^{-1}$ (equivalent to $19.6 \text{ NLCH}_4.\text{L}_{\text{effluent}}^{-1}$) was found.

336 Considering the initial VS content of rye, the cumulated effluent production represented 7.6
337 $\text{Nm}^3 \cdot \text{t}_{\text{VS}_{\text{init}}}^{-1}$ of fresh rye, i.e 2 % of fresh rye BMP. Co-ensiling of rye allowed to suppress effluent
338 production and thus methane potential losses through effluent.

339 A significantly lower BMP loss due to effluent production was found during the ensiling of rye
340 alone when compared to sunflower alone, which is due to higher TS content and higher BMP of
341 fresh rye and lower effluent volume. However, the addition of absorbent co-substrates allowed
342 to drastically cut the methane potential losses due to effluent production.

343

344 3.3.3 Fermentation pathways during co-ensiling

345 *Figure 2: pH, metabolite concentration and cumulated gas production during storage.*
346 *Metabolites are expressed in $\text{g} \cdot \text{kg}_{\text{VS}_{\text{added}}}^{-1}$ of mix (cover crop + co-substrate). CO_2 and H_2 are*
347 *expressed in cumulated $\text{L} \cdot \text{kg}_{\text{VS}_{\text{init}}}^{-1}$. R stand for rye. Paper, Misc, Wood, Chicken M and Horse M*
348 *stand for soiled paper and cardboard, miscanthus stalks, wood chips, chicken manure and horse*
349 *manure, respectively*

350

351 *Figure 3: Evolution of ammonia concentration during storage. Concentrations are expressed in*
352 *$\text{g} \cdot \text{kg}_{\text{VS}_{\text{added}}}^{-1}$ of mix (cover crop + co-substrate); R stand for rye. Paper, Misc, Wood, Chicken M*
353 *and Horse M stand for soiled paper and cardboard, miscanthus stalks, wood chips, chicken*
354 *manure and horse manure, respectively*

355

356

357

358 The fermentation pathways that occurred during the different co-ensiling conditions were
359 monitored by the dynamic analysis of pH, metabolites and gases (Figure 2) and ammonia (Figure
360 3). Depending on the type of co-substrate and initial properties of the mixture, two different
361 patterns were identified. On the one hand, in all conditions except for the mixture R + Horse M,

362 secondary fermentations occurred from the second week. On the other hand, for the mixture R
363 + Horse M, a more restricted homolactic fermentation took place and led to a fast and efficient
364 acidification. The two fermentation patterns are presented in this section.

365 **Secondary fermentation patterns**

366 In Rye, R + Paper, R + Misc, R + Wood and R + Chicken MIn storage conditions, the fermentation
367 was characterized by limited lactic acid production and acidification during the first days of
368 storage. After one week, a peak in lactic acid concentration of only $30 \text{ g.kg}_{\text{VS}}^{-1}$ was reached in
369 Rye, R+ Misc, R+ Wood and R+ Chicken M. Due to this low lactic acid production, the pH did not
370 drop below 4.7. In the mixture R + Paper, lactic acid concentration was higher and reached 55
371 $\text{g.kg}_{\text{VS}}^{-1}$ but the pH value was also higher than 4.7. In the mixture R + Chicken M, the pH was
372 never lower than 6.2, which was likely due to the high ammonia concentration that was
373 measured from the very first days of storage (Figure 3). Whatever the conditions, lactic acid
374 production was also coupled with other metabolites such as acetic acid and ethanol and with
375 CO_2 production, indicating heterolactic fermentation pathways. In silage fermentation,
376 heterolactic metabolisms are mentioned to be less efficient at lowering the pH than homolactic
377 fermentation (Woolford and Pahlow, 1998). Additionally, a significant propionic acid
378 accumulation was found for rye ensiling alone. Propionic acid fermentation may be also
379 considered as undesirable for silage due to its lower acidification capacity compared to lactic
380 acid fermentation. However, acetic and propionic acids have stronger antifungal properties than
381 lactic acid and allow a better aerobic stability when the silo is opened (Pahlow et al., 2003).

382 At the end of this first phase, pH values in all conditions remained above the critical value that is
383 necessary to inhibit clostridial activity and ensure a silage stability (Pahlow et al., 2003).
384 Consequently, from day 7 to 21, clostridial fermentation started. The lactic acid that was
385 previously produced was completely consumed before the end of the 60-day storage and
386 converted into butyric acid. The secondary fermentation was coupled with the production of
387 CO₂ and H₂ which is reported to induce methane potential losses (Kreuger et al., 2011). As lactic
388 acid was converted into butyric acid, the pH increased and reached 5.3 (R + Paper, R + Misc, R +
389 Wood), 5.7 (Rye) and 6.5 (R + Chicken M). Both the pH rise and difference in pH values between
390 the different conditions may also be explained by ammonia production, that mostly took place
391 between days 2 and 21. After three weeks of storage, the highest ammonia concentration was
392 measured in the mixture R + chicken M with a value of 1.6 g.kg_{VS}⁻¹, which was equivalent to 36
393 % of initial total nitrogen (TN_{init}). For rye ensiling, 1.3 g.kg_{VS}⁻¹ (49 %TN_{init}) were measured, and
394 lower values were found in the other conditions, with 0.7 (33 %TN_{init}), 0.6 (41 %TN_{init}) and 0.8
395 g.kg_{VS}⁻¹ (27 %TN_{init}) in R + Paper, R + Wood and R + Misc, respectively. In the literature, it is
396 generally accepted that NH₃-N concentration should be lower than 10% of the total nitrogen in
397 well preserved silages (Bureenok et al., 2016). The high concentration in NH₃-N found here is an
398 indicator of the activity of enterobacteria and/or clostridia (Pahlow et al., 2003). Despite the
399 non-optimal properties of silage with high pH, stabilization was reached from day 30, since gas
400 production stopped. This stable state may be due to the fact that all WSC were consumed
401 during the first weeks of storage (data not shown). The inhibitory effect of the accumulated
402 metabolites, mostly acetic acid and butyric acid, may also contribute to the inhibition of
403 microbial activity (Wang et al., 2008).

404 **Homolactic fermentation pattern**

405 The addition of horse manure to rye had a different impact on silage fermentation. This storage
406 condition was characterized by a fast and high lactic acid production. After 7 days of storage, 92
407 g.kg_{VS}⁻¹ of lactic acid had accumulated in the medium, i.e. nearly 2 to 3 times more than in the
408 other storage conditions. This accumulation induced a significant acidification as pH dropped to
409 4.4 after 2 days and stabilized at 4.0 after one week. The fermentation was coupled to a limited
410 CO₂ production, since only 5 NL.kg_{VS}⁻¹ were measured and no H₂ was detected. The
411 fermentation characteristics suggested that mostly homolactic fermentations occurred, since
412 lactic acid was largely dominant among the other metabolites produced. Since the pH was
413 below the critical value that ensures anaerobic stability (Pahlow et al., 2003), secondary
414 fermentations did not occur. Production of NH₃-N was also lower than in the other conditions
415 (0.4 g.kg_{VS}⁻¹, 21 %TN_{init}). The silage obtained by co-ensiling rye with horse manure fulfilled the
416 criteria defining a “well preserved silage” according to the literature, with pH <4.5, lactic acid >
417 30 g.kg_{TS}⁻¹ and butyric acid < 10% of total volatile fatty acids. Only NH₃-N overpassed 10% of
418 total Nitrogen, however no other signs of undesirable microbial activity were recorded.

419

420

421 3.3.4 Microbial development during ensiling and impact of co-substrate addition

422

423 *Figure 4: (A) Microbial characterization of raw substrates (relative abundance of major orders).*
424 *Values on top represent the microbial population quantification (16S rRNA gene copy number .*

425 g_{FM}^{-1}); (B.1) Variation of total microbial abundance during storage (16S rRNA gene copy number
426 $. g_{FM}^{-1}$); (B.2) Relative composition of microbial community at the order level

427

428 In addition to modifying the physico-chemical characteristics of the mixture, the co-substrates
429 had different microbial communities that can influence the silage fermentation. Microbial
430 communities and total microbial abundance of raw substrates and their change during co-
431 ensiling storage are presented in Figure 4.

432 Among the microorganisms that are likely to play a role in silage fermentation, the microbial
433 community of fresh rye was largely dominated by enterobacteriales order, mostly from
434 enterobacteria family. Enterobacteria are commonly found on crops as epiphytic bacteria
435 (Pahlow et al., 2003). In silage fermentation, enterobacteria compete with lactic acid bacteria
436 for WSC, produce a mixture of acids and ethanol and have thus a detrimental action on silage
437 acidification. In paper, Misc and Wood, Lactobacillales, Enterobacteriales and Clostridiales were
438 found. However, the total microbial abundance of these substrates was much lower. The total
439 specific abundance of these three orders was thus lower than in rye and few modifications of
440 the initial microbial community of the related mixtures were noted (Figure 4). Paper, Misc and
441 Wood had also few influence on microbial community changes during storage when compared
442 to rye alone, which is consistent with the observations made previously on fermentation
443 metabolites. In these four storage conditions, Lactobacillales grew fast from the very first days
444 of ensiling and likely competed with Enterobacteriales, whose abundance remained high all
445 over the storage duration. From the second week of storage, the amount of Clostridiales
446 increased, which is consistent with the butyric acid and H_2 production that was observed.

447 The microbial abundances of chicken and horse manures were much higher than the other co-
448 substrates, with $2.7 \cdot 10^8$ and $1.1 \cdot 10^8$ rRNA gene copy number $\cdot g_{FM}^{-1}$, respectively. Within the
449 bacteria orders that are expected to drive silage fermentations, a high amount of
450 Enterobacteriales, Lactobacillales and Clostridiales was present. Consequently, the microbial
451 communities of the pre-silage mixtures were strongly impacted by the addition of these co-
452 substrates. The initial abundances of Enterobacteriales, Lactobacillales and Clostridiales were
453 $8.5 \cdot 10^5$, $3.9 \cdot 10^6$ and $2.4 \cdot 10^6$ in R + Chicken Manure and $9.5 \cdot 10^5$, $1.2 \cdot 10^7$ and $5.9 \cdot 10^5$ in R + Horse
454 M, respectively. The ratio between Lactobacillales and Enterobacteriales was thus reversed
455 after adding manures. Clostridiales abundance was also increased. The initial Lactobacillales to
456 Enterobacteriales seemed to still play a role all over the storage duration, since the proportion
457 in Enterobacteriales remained much lower in R + Chicken M and R + Horse M than in the 4 other
458 co-ensiling experiments. However, due to the difference in acidification kinetics, the
459 development of Clostridiales could not be avoided in R + Chicken M, which led to the
460 dominance of this bacterial order after 60 days of storage. On the contrary, Lactobacillales
461 largely dominated the microbial community of R + Horse M, as the low pH prevented the
462 development of other bacteria (Gharechahi et al., 2017). Due to the relatively similar microbial
463 communities at the order level in R + Chicken M and R + Horse M, the difference in
464 fermentation mechanisms that occurred during storage may therefore be due to different
465 chemical properties between these two co-ensiling conditions. In particular, the difference in
466 buffering capacity and nitrogen content may have played a critical role.

467 3.3.5 Impact of fresh mixture properties on fermentation and microbial populations

468 *Figure 5: Matrix of Pearson's correlation with the main bacterial orders for all ensiling and co-*
469 *ensiling experiments of rye. **: Significant with p-value <0.01, *: Significant with p-value < 0.05*

470
471 In order to assess the impact of the initial properties of the co-ensiling mixtures on
472 fermentation, a matrix of Pearson's correlations was calculated and is presented in Figure 5. The
473 impact of physico-chemical properties (TS, WSC, BC and C/N) had a weak influence on the
474 fermentations contrary to what could be expected. This may be explained by the weak
475 heterogeneity of these parameters in the various tested conditions. Interestingly, despite the
476 results the literature for the ensiling of wilted and unwilted crops, initial TS was not negatively
477 correlated with butyric fermentation nor Clostridiales growth (Borreani et al., 2009). This result
478 points out that increasing TS by adding a co-substrate may have a different impact on
479 fermentation than harvesting a crop at a high TS or applying field-wilting. Since the inhibition of
480 clostridial growth is related to the water activity (a_w) of the medium, the influence of a co-
481 substrate addition on a_w should be further studied.

482 About initial microbial properties of samples, it can be seen that the initial amount of
483 Lactobacillales was strongly correlated with lactic acid production, and a negative correlation
484 was established between initial content of Lactobacillales and the production of butyric acid and
485 CO₂. If a silage fermentation dominated by lactic acid production is expected, a co-substrate
486 with a high amount of lactic acid bacteria is thus recommended. The presence of a high
487 quantity of Enterobacteriales at the end of storage was correlated with CO₂ and H₂ production,
488 while Clostridiales were correlated with a higher concentration in acetic acid. However, the
489 study of the fermentation kinetics and microbial populations variations presented in Figure 2

490 and Figure 4 clearly shows that H₂ production coincided with the development of Clostridiales.
491 However, a large amount of Enterobacteriales was still present at the end of the storage period
492 in Rye, R + Paper, R + Misc and R + Wood where a significant H₂ production occurred, which may
493 have influenced the output of the correlation matrix.

494

495 3.3.6 Impact of fermentation on mass and methane potential preservation during co-ensiling

496 The gas production that occurred during silage fermentation caused mass losses in all
497 conditions. For Rye, R + Paper, R + Misc, R + Wood, R + Chicken M and R + Horse M, 11.3, 7.4,
498 6.4, 8.0, 9.1 and 1.9 % of initial VS of each mixture were lost during storage, respectively, mostly
499 under the form of CO₂. Since different absorbent loading were applied, the VS losses were also
500 reported to the initial VS of rye. Following this calculation method, 11.3, 9.0, 9.2, 11.2, 11.9 and
501 2.3 % of initial VS of Rye were lost. It is important to stress that mixtures with similar
502 fermentation pathways (Rye, R + Paper, R + Misc, R + Wood and R + Chicken M) led to close VS
503 losses (i.e., 9.0 – 11.9%) reported to the initial VS of rye despite different co-substrate loadings,
504 suggesting that VS from rye were mostly lost. This hypothesis is consistent with the fact that the
505 easily degradable compounds, e.g. WSC, were mainly originated from rye. For the mixture R +
506 Horse, VS losses were much lower than in any other co-ensiling condition, due to the homolactic
507 fermentation pathway that prevailed (Kreuger et al., 2011).

508 BMP tests were carried out on raw substrates and after 60 days of storage to evaluate the
509 impact of storage on the methane potential (Figure 6). In most conditions, no significant BMP
510 variations were found to take place during storage, except in R + Wood where a significant

511 decrease in BMP of 18 and 24% was observed when reporting BMP to added VS or initial VS,
512 respectively. This difference in BMP preservation between R + Wood and all other co-ensiling
513 conditions is surprising, since no difference in mass losses, fermentation properties nor gas
514 production was observed. Consequently, this difference may be due to sampling issues due to
515 the heterogeneity of the co-ensiling mixture. Since wood chips presented the lowest BMP
516 among all co-substrates, a small difference in the catch crop/co-substrate ratio in sampling for
517 BMP measurement may influence greatly the measured BMP value. Indeed, the BMP measured
518 for fresh R + Wood ($330 \pm 36 \text{ NmLCH}_4 \cdot \text{g}_{\text{VS}}^{-1}$) is higher than the theoretical BMP calculated as the
519 weighted average between rye and wood chips ($267 \text{ NmLCH}_4 \cdot \text{g}_{\text{VS}}^{-1}$). The BMP of fresh R+ Wood
520 was thus probably overestimated. Even if butyric fermentation and the related hydrogen
521 production occurred during most of storage conditions, hydrogen production did not exceed 15
522 $\text{NL} \cdot \text{kg}_{\text{VS}}^{-1}$, which theoretically induced BMP losses of about $4 \text{ NmL} \cdot \text{g}_{\text{VS}}^{-1}$. However, this value is
523 very low compared to those of the raw substrates, which explains the small difference between
524 the methane potential before and after silage.

525 Limited differences were found between BMP reported to added and initial VS in all conditions,
526 which was due to the fact that VS loss were relatively small. When higher VS losses occurred, a
527 larger difference between these two BMP values was found, as BMP was concentrated in the
528 remaining matter as WSC were converted into more energetically dense compounds like butyric
529 acid during fermentation while CO_2 was released (Kreuger et al., 2011; Van Vlierberghe et al.,
530 2021).

531

532 *Figure 6: Impact of storage under the different co-ensiling conditions on methane production per*
533 *unit of VS of mix (cover crop + co-substrate). BMP reported to added VS represent the crop*
534 *biodegradability after storage, and BMP reported to initial VS reflects the global impact of*
535 *storage on methane potential. *: significant difference two BMP values for a given storage*
536 *condition ($p < 0.05$)*

537

538 As a consequence, this study shows that the co-silage of a wet cover crop with an absorbent co-
539 substrate allows an efficient storage before anaerobic digestion, avoiding also the production of
540 a liquid effluent. In addition to the preservation of catch crops itself, co-ensiling may be
541 considered as a conservation method of manures, since this type of substrate is known to lose
542 methane potential during storage (Teixeira Franco et al., 2018). A synergistic effect can thus be
543 achieved, with the crop providing the necessary WSC for silage fermentations and the manure
544 avoiding effluent production. Teixeira Franco et al. (2018) recommended an initial WSC content
545 of $166 \text{ g.kg}_{\text{VS}}^{-1}$ for an efficient ensiling of cattle manure, which is a higher but close to initial
546 value of the present experiment (126 ± 21 and $115 \pm 5 \text{ g.kg}_{\text{VS}}^{-1}$, for R + Horse M and R + Chicken
547 M, respectively).

548 4 Conclusion

549 Co-ensiling with dryer co-substrates was shown to be an appropriate method for the ensiling of
550 wet cover crops and allowed a drastic volume reduction of energetically dense silage effluent. It
551 was found that the specific water holding capacity was an appropriate parameter for absorbent
552 dosing. Absorbent addition was shown to influence the chemical and microbial properties of
553 silage depending on whether butyric or homolactic fermentation was promoted, though neither
554 of them significantly impacted the preservation of methane potential. Co-substrate addition

555 was thus found to increase the methane potential preservation of wet crops through effluent
556 retention more than through the control of fermentation. Co-ensiling crops with agricultural
557 waste such as manures may constitute an interesting option for increasing the storage of these
558 two substrates.

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564

565 [References](#)

566

567 Bastiman, B., Altman, J.F.B., 1986. Losses at various stages in silage making. *Res. Dev. Agric.* 2,
568 19–25.

569 Bio2E, 2018. Environmental Biotechnology and Biorefinery Platform.

570 <https://doi.org/10.5454/1.557234103446854E12>

571 Borreani, G., Chion, A.R., Colombini, S., Odoardi, M., Paoletti, R., Tabacco, E., 2009.

572 Fermentative profiles of field pea (*Pisum sativum*), faba bean (*Vicia faba*) and white lupin

573 (*Lupinus albus*) silages as affected by wilting and inoculation. *Anim. Feed Sci. Technol.* 151,

574 316–323. <https://doi.org/10.1016/J.ANIFEEDSCI.2009.01.020>

575 Bureenok, S., Sisaath, K., Yuangklang, C., Vasupen, K., Schonewille, J.T., 2016. Ensiling

576 characteristics of silages of Stylo legume (*Stylosanthes guianensis*), Guinea grass (*Panicum*
577 maximum) and their mixture, treated with fermented juice of lactic bacteria, and feed
578 intake and digestibility in goats of rations based on these silages. *Small Rumin. Res.* 134,
579 84–89. <https://doi.org/10.1016/j.smallrumres.2015.12.006>

580 Cui, X., Sun, H., Sobhi, M., Ju, X., Guo, J., Dong, R., 2020. Butyric Acid Fermentation during
581 Ensiling of Wilted Maize Stover for Efficient Methane Production. *ACS Sustain. Chem. Eng.*
582 8, 6713–6721. <https://doi.org/10.1021/acssuschemeng.0c00633>

583 da Silva, T.C., da Silva, L.D., Santos, E.M., Oliveira, J.S., 2017. Importance of the Fermentation to
584 Produce High-Quality Silage, in: *Fermentation Processes*. InTech, pp. 3–22.
585 <https://doi.org/10.5772/64887>

586 Dennery G., Dezat E., R.N., 2012. Vers une gestion efficace des litières, de l’approvisionnement
587 aux techniques d’élevage avicole 48.

588 Fransen, S.C., Strubi, F.J., 1998. Relationships Among Absorbents on the Reduction of Grass
589 Silage Effluent and Silage Quality. *J. Dairy Sci.* 81, 2633–2644.
590 [https://doi.org/10.3168/JDS.S0022-0302\(98\)75821-7](https://doi.org/10.3168/JDS.S0022-0302(98)75821-7)

591 Gebrehanna, M.M., Gordon, R.J., Madani, A., VanderZaag, A.C., Wood, J.D., 2014. Silage effluent
592 management: A review. *J. Environ. Manage.* 143, 113–122.
593 <https://doi.org/10.1016/j.jenvman.2014.04.012>

594 Gharechahi, J., Kharazian, Z.A., Sarikhan, S., Jouzani, G.S., Aghdasi, M., Hosseini Salekdeh, G.,
595 2017. The dynamics of the bacterial communities developed in maize silage. *Microb.*
596 *Biotechnol.* 10, 1663–1676. <https://doi.org/10.1111/1751-7915.12751>

597 Haigh, P.M., 1999. Effluent production from grass silages treated with additives and made in
598 large-scale bunker silos. *Grass Forage Sci.* 54, 208–218. [https://doi.org/10.1046/j.1365-](https://doi.org/10.1046/j.1365-2494.1999.00172.x)
599 2494.1999.00172.x

600 Haigh, P.M., 1998. Incorporation of straw with grass at ensilage. *J. Agric. Eng. Res.* 70, 383–388.
601 <https://doi.org/10.1006/jaer.1998.0323>

602 Healy, M.G., Tighe, J.L., Allen, S.J., Smeed, M., 1997. Biosorbents for grass silage effluent
603 retention. *Stud. Environ. Sci.* 66, 223–237. [https://doi.org/10.1016/S0166-1116\(97\)80046-5](https://doi.org/10.1016/S0166-1116(97)80046-5)

604 Holliger, C., Alves, M., Andrade, D., Angelidaki, I., Astals, S., Baier, U., Bougrier, C., Buffière, P.,
605 Carballa, M., De Wilde, V., Ebertseder, F., Fernández, B., Ficara, E., Fotidis, I., Frigon, J.C.,
606 De Lacos, H.F., Ghasimi, D.S.M., Hack, G., Hartel, M., Heerenklage, J., Horvath, I.S., Jenicek,
607 P., Koch, K., Krautwald, J., Lizasoain, J., Liu, J., Mosberger, L., Nistor, M., Oechsner, H.,
608 Oliveira, J.V., Paterson, M., Pauss, A., Pommier, S., Porqueddu, I., Raposo, F., Ribeiro, T.,
609 Pfund, F.R., Strömberg, S., Torrijos, M., Van Eekert, M., Van Lier, J., Wedwitschka, H.,
610 Wierinck, I., 2016. Towards a standardization of biomethane potential tests. *Water Sci.*
611 *Technol.* 74, 2515–2522. <https://doi.org/10.2166/wst.2016.336>

612 Holly, M.A., Larson, R.A., Cooley, E.T., Wunderlin, A.M., 2018. Silage storage runoff
613 characterization: Annual nutrient loading rate and first flush analysis of bunker silos. *Agric.*
614 *Ecosyst. Environ.* 264, 85–93. <https://doi.org/10.1016/j.agee.2018.05.015>

615 Jones, R., Jones, D.I.H., 1996. The effect of in-silo effluent absorbents on effluent production
616 and silage quality. *J. Agric. Eng. Res.* 64, 173–186. <https://doi.org/10.1111/gfs.12343>

617 Kaiser, A.G., Piltz, J.W., Burns, H.M., Griffiths, N.W., 2004. Successful silage.

618 Kalač, P., 2011. The required characteristics of ensiled crops used as a feedstock for biogas
619 production: a review. *J. Agrobiol.* 28, 85–96. <https://doi.org/10.2478/v10146-011-0010-y>

620 Keck, M., Mager, K., Weber, K., Keller, M., Frei, M., Steiner, B., Schrade, S., 2018. Odour impact
621 from farms with animal husbandry and biogas facilities. *Sci. Total Environ.* 645, 1432–1443.
622 <https://doi.org/10.1016/j.scitotenv.2018.07.182>

623 Khorvash, M., Colombatto, D., Beauchemin, K.A., Ghorbani, G.R., Samei, A., 2006. Use of
624 absorbants and inoculants to enhance the quality of corn silage. *Can. J. Anim. Sci.* 86, 97–
625 107. <https://doi.org/10.4141/A05-029>

626 Koenig, A., Dehn, F., 2016. Biogenic acid attack on concretes in biogas plants. *Biosyst. Eng.* 147,
627 226–237. <https://doi.org/10.1016/j.biosystemseng.2016.03.007>

628 Kreuger, E., Nges, I., Björnsson, L., 2011. Ensiling of crops for biogas production: Effects on
629 methane yield and total solids determination. *Biotechnol. Biofuels* 4, 1–8.
630 <https://doi.org/10.1186/1754-6834-4-44>

631 Latsch, R., Sauter, J., 2014. Compactage de l'ensilage d'herbe en silo-couloir.

632 Marsac, S., Trochard, R., Delaye, N., Peu, P., Pommier, S., Brosset, D., 2019. Méthanisation par
633 voie sèche à la ferme de résidus de culture, ADEME.
634 <https://doi.org/10.3406/tiers.1960.1203>

635 Mayne, C.S., Gordon, F.J., 1986. Effect of harvesting system on nutrient losses during silage
636 making. 2. In-silo losses. *Grass Forage Sci.* 41, 341–351. [https://doi.org/10.1111/j.1365-
637 2494.1986.tb01824.x](https://doi.org/10.1111/j.1365-2494.1986.tb01824.x)

638 Meier, U., 2018. Growth stages of mono- and dicotyledonous plants, BBCH Monograph. Open
639 Agrar Repositorium. <https://doi.org/10.5073/20180906-074619>

640 Molinuevo-Salces, B., Fernández-Varela, R., Uellendahl, H., 2014. Key factors influencing the
641 potential of catch crops for methane production. *Environ. Technol. (United Kingdom)* 35,
642 1685–1694. <https://doi.org/10.1080/09593330.2014.880515>

643 Molinuevo-Salces, B., Larsen, S.U., Ahring, B.K., Uellendahl, H., 2013. Biogas production from
644 catch crops: Evaluation of biomass yield and methane potential of catch crops in organic
645 crop rotations. *Biomass and Bioenergy* 59, 285–292.
646 <https://doi.org/10.1016/j.biombioe.2013.10.008>

647 P. Savoie, A. Amyot, R. Thériault, 2002. EFFECT OF MOISTURE CONTENT, CHOPPING, AND
648 PROCESSING ON SILAGE EFFLUENT. *Trans. ASAE* 45, 907–914.
649 <https://doi.org/10.13031/2013.9937>

650 Pahlow, G., Muck, R.E., Driehuis, F., Elferink, S.J.W.H.O., Spoelstra, S.F., 2003. Microbiology of
651 Ensiling, in: *Silage Science and Technology*. pp. 31–93.
652 <https://doi.org/10.2134/agronmonogr42.c2>

653 Playne, M.J., McDonald, P., 1966. The buffering constituents of herbage and of silage. *J. Sci.*
654 *Food Agric.* 17, 264–268. <https://doi.org/10.1002/jsfa.2740170609>

655 Porter, M.G., Murray, R.S., 2001. The volatility of components of grass silage on oven drying and
656 the inter-relationship between dry-matter content estimated by different analytical
657 methods. *Grass Forage Sci.* 56, 405–11. <https://doi.org/10.1046/j.1365-2494.2001.00292.x>

658 Razak, O.A., Masaaki, H., Yimamu, A., Meiji, O., 2012. Potential water retention capacity as a
659 factor in silage effluent control: Experiments with high moisture by-product feedstuffs.
660 Asian-Australasian J. Anim. Sci. 25, 471–478. <https://doi.org/10.5713/ajas.2011.11349>

661 Schumacher, K., Schultmann, F., 2017. Local Acceptance of Biogas Plants: A Comparative Study
662 in the Trinational Upper Rhine Region. Waste and Biomass Valorization 8, 2393–2412.
663 <https://doi.org/10.1007/s12649-016-9802-z>

664 Sutter, A., 1957. Problem of waste effluent from silage making and feeding of silage. Eur. Prod.
665 Agency Organ. Eur. Econ. Co-Oper. 307, 74–82.

666 Teixeira Franco, R., Buffière, P., Bayard, R., 2018. Co-ensiling of cattle manure before biogas
667 production: Effects of fermentation stimulants and inhibitors on biomass and methane
668 preservation. Renew. Energy 121, 315–323. <https://doi.org/10.1016/j.renene.2018.01.035>

669 Teixeira Franco, R., Buffière, P., Bayard, R., 2017. Optimizing storage of a catch crop before
670 biogas production: Impact of ensiling and wilting under unsuitable weather conditions.
671 Biomass and Bioenergy 100, 84–91. <https://doi.org/10.1016/j.biombioe.2017.03.017>

672 Teixeira Franco, R., Buffière, P., Bayard, R., 2016. Ensiling for biogas production: Critical
673 parameters. A review. Biomass and Bioenergy 94, 94–104.
674 <https://doi.org/10.1016/j.biombioe.2016.08.014>

675 Van Vlierberghe, C., Escudie, R., Bernet, N., Frederic, S., Carrere, H., 2021. Long term alkaline
676 storage and pretreatment process of cover crops for anaerobic digestion. Bioresour.
677 Technol. 330, 124986. <https://doi.org/10.1016/j.biortech.2021.124986>

678 Venkiteshwaran, K., Milferstedt, K., Hamelin, J., Zitomer, D.H., 2016. Anaerobic digester
679 bioaugmentation influences quasi steady state performance and microbial community.
680 *Water Res.* 104, 128–136. <https://doi.org/10.1016/j.watres.2016.08.012>

681 Villa, R., Ortega Rodriguez, L., Fenech, C., Anika, O.C., 2020. Ensiling for anaerobic digestion: A
682 review of key considerations to maximise methane yields. *Renew. Sustain. Energy Rev.* 134,
683 110401. <https://doi.org/10.1016/j.rser.2020.110401>

684 Wang, B., Wan, W., Wang, J., 2008. Inhibitory effect of ethanol, acetic acid, propionic acid and
685 butyric acid on fermentative hydrogen production. *Int. J. Hydrogen Energy* 33, 7013–7019.
686 <https://doi.org/10.1016/j.ijhydene.2008.09.027>

687 Woolford, M.K., Pahlow, G., 1998. The silage fermentation, in: *Microbiology of Fermented*
688 *Foods*. Springer US, Boston, MA, pp. 73–102. [https://doi.org/10.1007/978-1-4613-0309-](https://doi.org/10.1007/978-1-4613-0309-1_3)
689 [1_3](https://doi.org/10.1007/978-1-4613-0309-1_3)

690 Woolford, M.K., Wilkinson, J.M., Cook, J.E., 1983. Investigations on the effect of sodium
691 bentonite and other moisture absorbents on the production of effluent from grass silage.
692 *Anim. Feed Sci. Technol.* 8, 107–118. [https://doi.org/10.1016/0377-8401\(83\)90092-5](https://doi.org/10.1016/0377-8401(83)90092-5)

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695 *Table 1: Fresh cover crop and co-substrate characteristics.*

Fresh substrates	Sunflower	Rye	Wheat straw	Soiled paper and cardboard	Miscanthus stalks	Wood chips	Horse manure	Chicken manure
TS (%FM)	14.6 ± 0.7	21.1 ± 0.2	90.4 ± 0.1	88.4 ± 0.4	92.7 ± 0.1	88.3 ± 0.1	53.0 ± 0.1	75.5 ± 0.0
VS (%FM)	12.9 ± 0.6	19.5 ± 0.1	84.2 ± 0.1	73.2 ± 1.2	69.2 ± 6.5	87.6 ± 0.8	44.7 ± 0.1	31.8 ± 0.0
pH	7.1 ± 0.0	6.5 ± 0.0	6.4 ± 0	8.2 ± 0.1	6.27 ± 0.1	5.42 ± 0.0	7.53 ± 0.1	8.59 ± 0.1
BC (mEq.kg_{TS}⁻¹)	283	248	88	73	14	23	143	509
WHC (g_{water}.g⁻¹_{FM})	n.d.	n.d.	1.6 ± 0.2	2.1 ± 0.19	1.0 ± 0.13	1.4 ± 0.09	1.4 ± 0.18	0.8 ± 0.14
C (%TS)	40.7	46.8	42.6	47.5	49.2	52.5	42.1	19.8
N (%TS)	1.84	2.11	0.52	0.21	0.23	0.11	1.43	2.43
C/N (g.g⁻¹)	22.1	22.2	81.4	223.0	216.8	477.1	29.4	8.2
VFA (g.kg⁻¹_{VS})	< d.l.	3.6 ± 0.2	4.0 ± 0.3	<d.l	<d.l	<d.l	5.7 ± 1.4	19.9 ± 0.9
WSC (g.kg⁻¹_{VS})	181 ± 13	141 ± 15	14.8 ± 0.7	<d.l	<d.l	<d.l	2.0 ± 0.3	<d.l
BMP (NmLCH₄.g_{VS}⁻¹)	n.d.	360 ± 31	n.d.	354 ± 25	181 ± 22	26 ± 16	134 ± 65	172 ± 10

696 TS: total solids; FM: fresh matter; VS: volatile solids; BC: buffering capacity; WHC: water holding capacity; VFA: volatile fatty acids;
697 WSC: water soluble carbohydrates; BMP: biochemical methane potential. Measurements that were performed in triplicates are
698 expressed as mean ± standard deviation (sd); n.d. : not determined; <d.l. : below the detection limit.

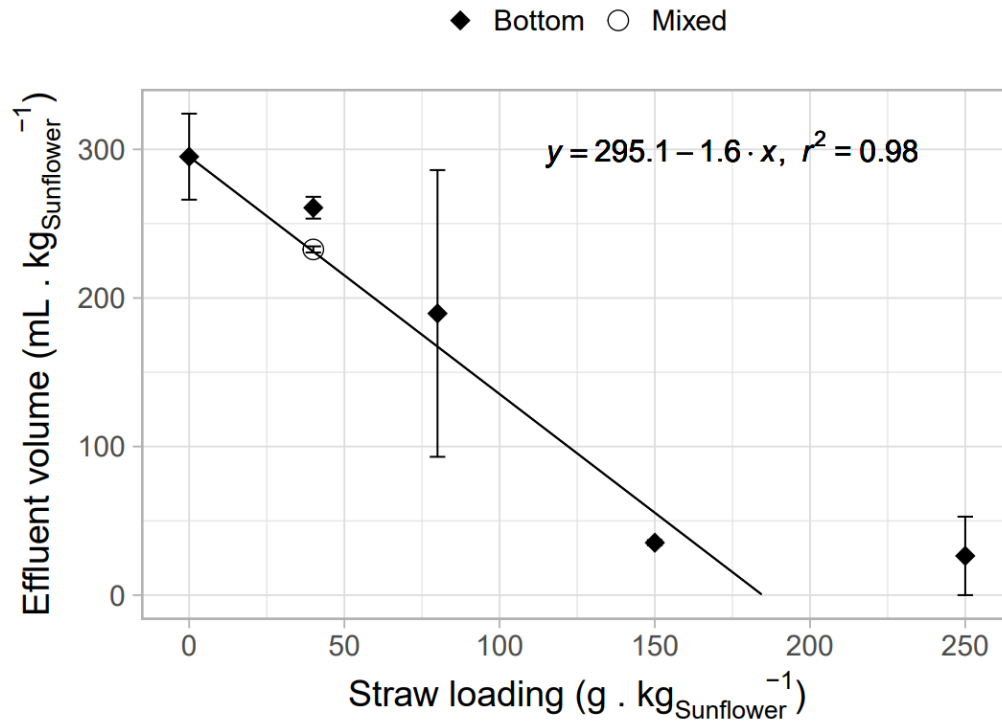
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700 *Table 2: Fresh co-ensiling mixtures characteristics.*

Fresh co-ensiling mixtures	Rye	R + Paper	R + Misc	R + Wood	R + Horse M	R + Chicken M	Pooled sd.
Co-substrate loading (g.kg⁻¹)	0	58.9	124.3	85.6	92.5	182.3	
TS (%FM)	21.1 ± 0.2 ^a	25.1 ± 0.1 ^b	29.1 ± 1.1 ^c	25.1 ± 0.3 ^b	23.3 ± 0.2 ^d	29.6 ± 0.4 ^c	0.4
VS (%FM)	19.5 ± 0.1 ^a	23.4 ± 0.1 ^b	26.4 ± 1.1 ^c	23.5 ± 0.6 ^b	20.9 ± 0.1 ^d	20.4 ± 0.6 ^{ad}	0.5
pH	6.5 ± 0.0 ^a	6.8 ± 0.0 ^b	6.3 ± 0.0 ^c	6.4 ± 0.0 ^d	6.4 ± 0.1 ^d	7.5 ± 0.0 ^e	0.0
BC (mEq.kg⁻¹)	248	246	179	214	268	314	
C (%TS)	46.8	46.1	46.7	48.3	45.7	37.6	
N (%TS)	2.1	1.7	1.9	1.9	1.9	2.6	
C/N	22.2	26.7	25.2	26.1	23.5	14.3	
VFA (g.kg⁻¹vs)	3.6 ± 0.2 ^a	3.4 ± 0.2 ^a	3.6 ± 0.6 ^a	3.3 ± 0.4 ^a	5.5 ± 0.3 ^b	6.5 ± 0.5 ^b	0.4
WSC (g.kg⁻¹vs)	141 ± 15 ^a	117 ± 4 ^{ab}	109 ± 7 ^{ab}	102 ± 17 ^b	126 ± 21 ^{ab}	115 ± 5 ^{ab}	11

701 Within a row, different letters express a statistical difference ($p < 0.05$) between two samples.
702 Measurements that were performed in triplicates are expressed as mean ± sd.

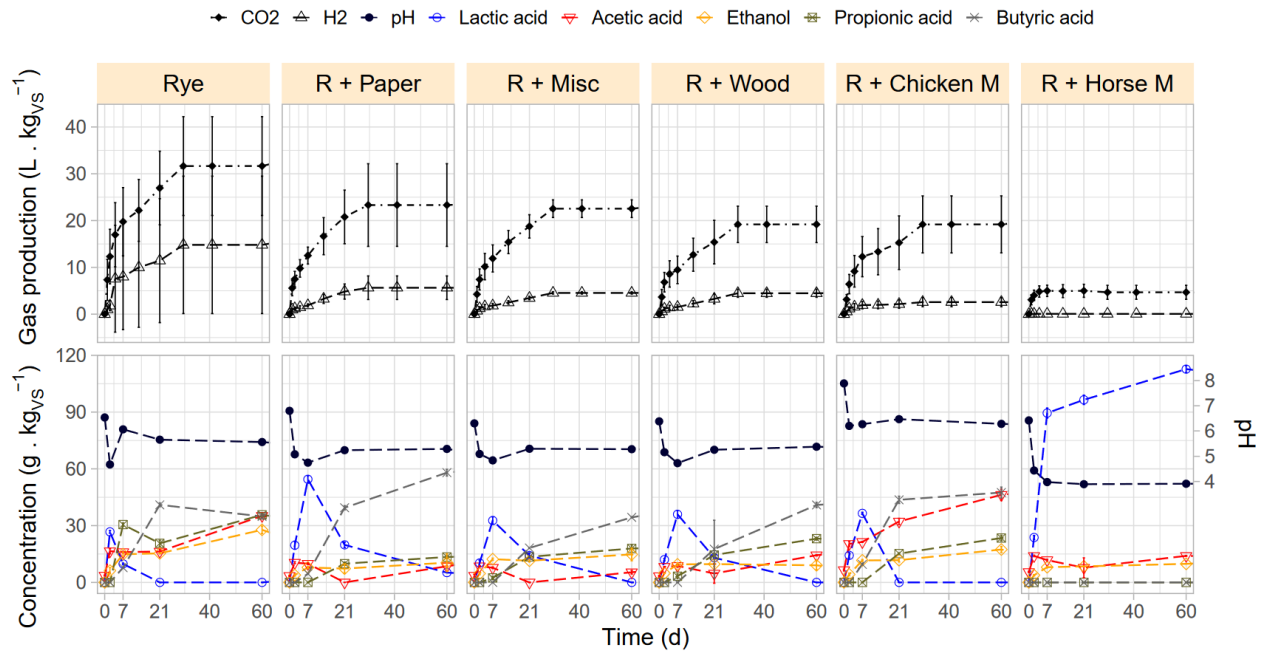
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705 *Figure 1: Cumulated effluent volume after 60 days of co-ensiling (sunflower + wheat straw). Dots*
 706 *indicate mean, error bars indicate maximum and minimum.*

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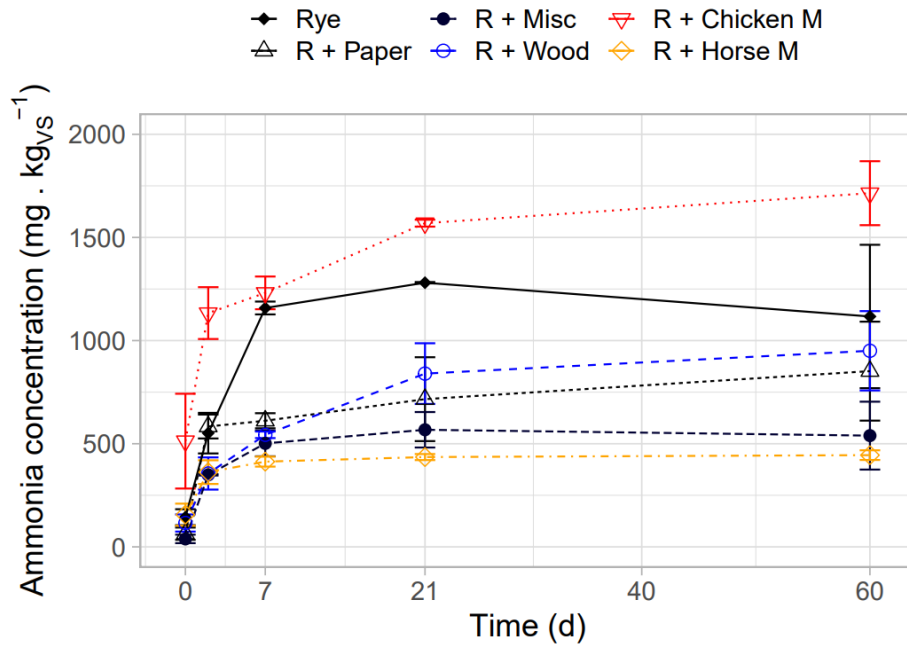


708

709 *Figure 2 : pH, metabolite concentration and cumulated gas production during storage.*
 710 *Metabolites are expressed in g.kgVS_{added}⁻¹ of mix (cover crop + co-substrate). CO₂ and H₂ are*
 711 *expressed in cumulated L.kgVS_{init}⁻¹. R stand for rye. Paper, Misc, Wood, Chicken M and Horse M*
 712 *stand for soiled paper and cardboard, miscanthus stalks, wood chips, chicken manure and horse*
 713 *manure, respectively.*

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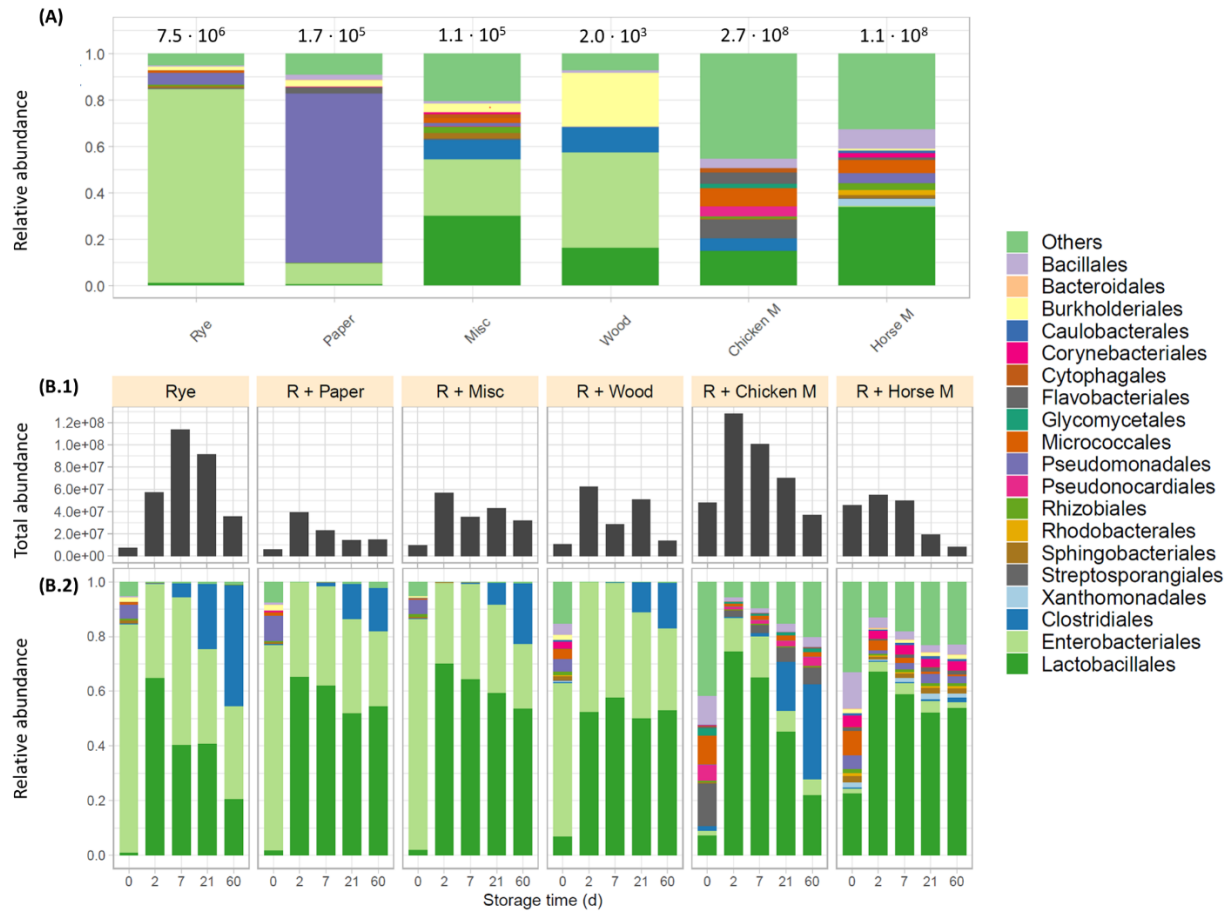
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717 *Figure 3: Evolution of ammonia concentration during storage. Concentrations are expressed in*
718 *$\text{g} \cdot \text{kg}_{\text{VSadded}}^{-1}$ of mix (cover crop + co-substrate); R stand for rye. Paper, Misc, Wood, Chicken M*
719 *and Horse M stand for soiled paper and cardboard, miscanthus stalks, wood chips, chicken*
720 *manure and horse manure, respectively.*

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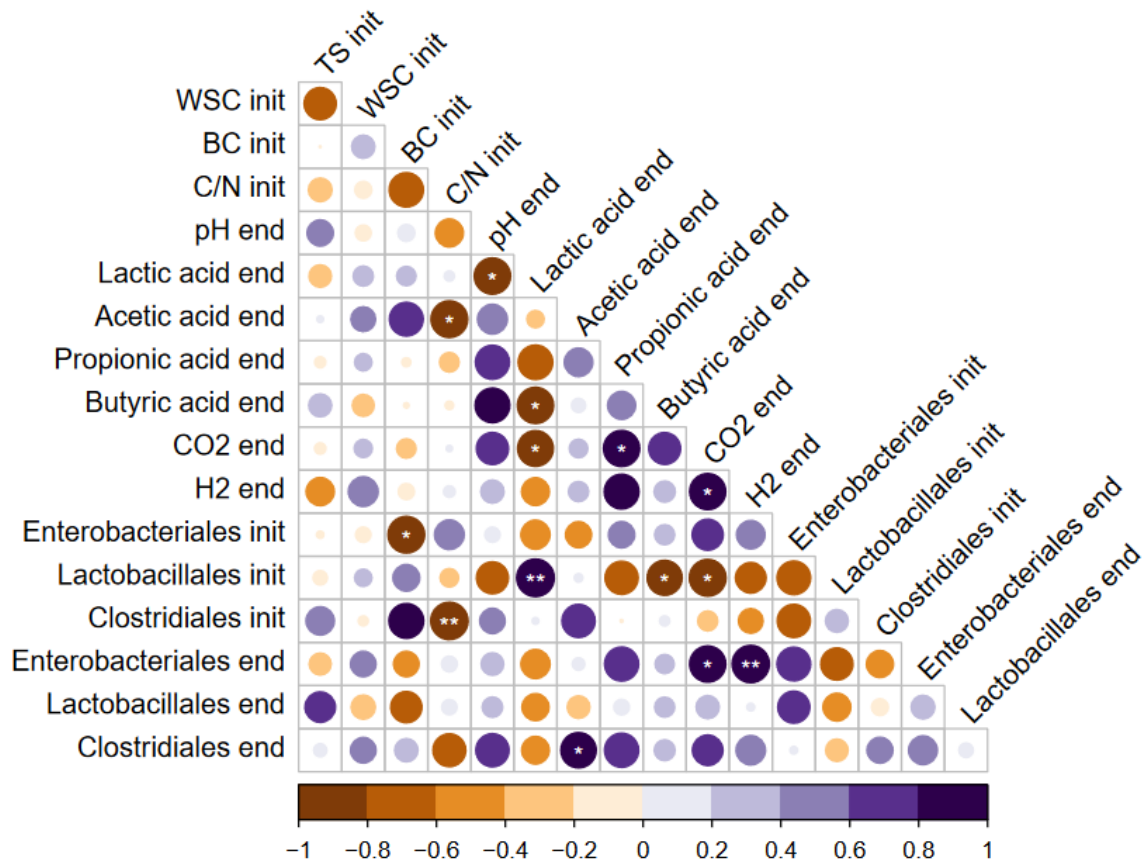


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723 *Figure 4: (A) Microbial characterization of raw substrates (relative abundance of major orders).*
 724 *Values on top represent the microbial population quantification (16S rRNA gene copy number .*
 725 *g_{FM}^{-1}); (B.1) Variation of total microbial abundance during storage (16S rRNA gene copy number*
 726 *. g_{FM}^{-1}); (B.2) Relative composition of microbial community at the order level.*

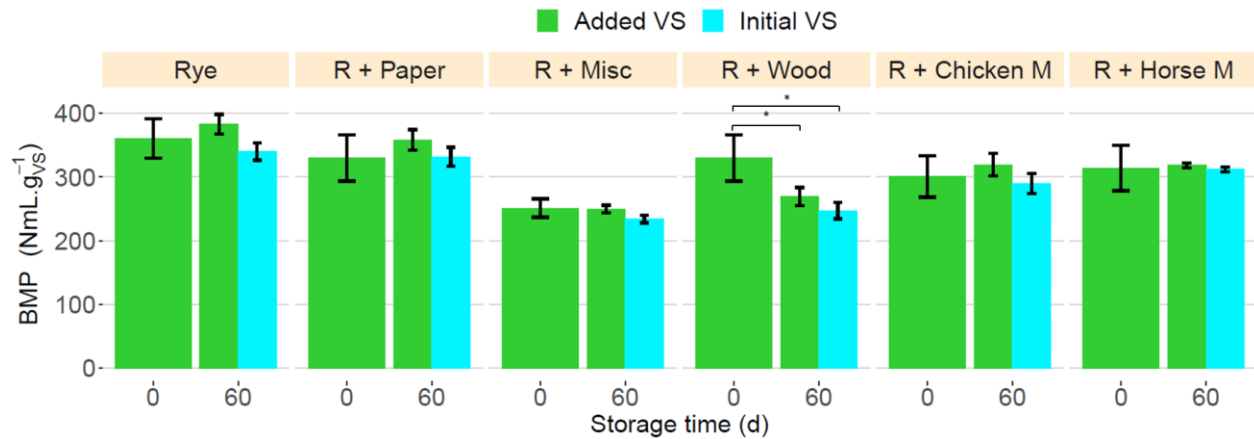
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730 *Figure 5 : Matrix of Pearson's correlation with the main bacterial orders for all ensiling and co-*
 731 *ensiling experiments of rye. **: Significant with p-value <0.01, *: Significant with p-value <0.05*
 732



733
 734 *Figure 6: Impact of storage under the different co-ensiling conditions on methane production per*
 735 *unit of VS of mix (cover crop + co-substrate). BMP reported to added VS represent the crop*
 736 *biodegradability after storage, and BMP reported to initial VS reflects the global impact of*
 737 *storage on methane potential. *: significant difference two BMP values for a given storage*
 738 *condition (p<0.05).*

739