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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 and water holding capacity ($r^2 = 0.98$) and up to 90% of effluent production was avoided when 8 compared to control (295 L.t⁻¹). The impact of different co-substrates (including bio-waste and 9 10 manures) on overall ensiling performances was then investigated at an optimized absorbent loading. All co-substrates allowed a total effluent retention while a 76 L.t⁻¹ effluent volume was 11 12 reported for the control. The silage fermentation was modified or mostly unchanged depending on the co-substrate chemical and microbial properties and different metabolic pathways were 13 observed (e.g. homolactic or butyric fermentation). In most conditions, the methane potential 14 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

Cover crops, also called catch crops or intermediary crops, are receiving an increasing interest to be used as substrates for anaerobic digestion. These crops are cultivated during the intercultural period of main crops and bring agro-ecological services by limiting soil erosion, nitrate leaching 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 water soluble carbohydrates (WSC) are fermented into lactic acid and other metabolites, 30 causing a pH drop to about 4 (Woolford and Pahlow, 1998). The combination of acidic pH and 31 32 anaerobic environment then ensures the inhibition of microbial activity over the whole storage duration. 33 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

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 also cause corrosion of steel and concrete (Koenig and Dehn, 2016). As a consequence, silage 53 effluent production is reported to be highly undesirable in biogas plants. 54 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 cosubstrate. Field wilting consists in a partial open-air drying of the freshly mowed crop prior to its 60 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 wilting inappropriate (Teixeira Franco et al., 2017). The addition of a dry co-substrate presents 63 the advantage of being independent from weather conditions and co-ensiling a wet organic 64 matter with a substrate having absorbent properties allows to immediately increase the TS of 65 the pre-silage mixture and thus avoid the effluent production (Jones and Jones, 1996). In the 66

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 feeding and thus most of the tested co-substrates are edible. In the context of anaerobic 74 digestion, the strategy for selecting co-substrates is different, since as an example, high value 75 76 edible substrates like ground barley or dried beet pulp should be avoided. On the other hand, non-edible substrates compatible with anaerobic digestion can be considered. 77 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 sunflower, in order to define the adequate co-substrate loading. In a second part, the influence 81 of the addition of various co-substrates (soiled paper and cardboard, wood chips, miscanthus 82 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 propose a global understanding of the addition of co-substrate on silage storage. 86

87 2 Material and Methods

The experiments and physico-chemical analyses were performed at the Bio2E platform (Bio2E,
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 heading). These crops were hand harvested using a sickle, at cutting height of about 10 cm. 95 Sunflower was chopped using a garden shredder (AXT 2550TC, Bosch GmbH) and a knife mill 96 97 (BB230, BLiK[®]) and further frozen at -20°C until use. Rye was chopped according the same protocol, but was used directly after for storage experiments. 98 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 configuration to that described by Savoie et al (2002). The filling was carried out by adding, in a 113 114 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 packing density was applied as recommended by Latsch and Sauter (2014) (Fresh matter basis -116 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. 117 Packing density of intermediate levels of TS were calculated by linear interpolation. The silos 118 119 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 weighted before and after storage. 124

For the dynamic monitoring of silage fermentation, 2.6L glass bottles were used, as previously
 described (Van Vlierberghe et al., 2021). This storage method was used in addition to storage in
 PVC silos for the co-ensiling of rye, in order to allow a higher number of replicates for dynamic
 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. A 60-day storage period was chosen as sufficient for fine effluent and fermentation 131 characterization. Previous studies of the literature reported that the peak flow of effluent 132 133 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

A first round of experiments was only conducted in tubular PVC silos. Five different ratios of 138 wheat straw were tested: 0, 40, 80, 150 and 250 g·kg_{sunflower}-1, on a fresh matter basis. In each 139 140 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, named "mixed", was also carried out for the ratio of wheat straw of 40 g·kg⁻¹; sunflower and 143 straw were homogenized before filling into the silos. 6 different storage conditions were thus 144 tested. 145

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

This storage experiment was conducted both in tubular PVC silos and bottle silos, in order to
evaluate the impact of the type of co-substrate on the volume of effluent produced and on the
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
- 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 (mL·kg_{FM}⁻¹) and the measurement of
- 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

Silage effluents from tubular silos were characterized by their volume and their chemical oxygen
demand (COD) at most once a day, depending on the flow rate. COD was determined by

160 colorimetric method (Aqualytic[®] COD Vario 0-1500 mgO₂.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 ($550^{\circ}C - 3h$). A TS correction method for lactic acid, VFA and alcohols volatilization was applied as described by Porter and Murray (2001). For pH, ammonia, WSC, 165 166 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 distillated water for 16 h to 20 h at 4°C in sealed plastic pots in triplicate. pH measurement was made directly on the mixture after extraction. The liquid phase was then separated by 169 170 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	$(NmLCH_{4.}gVS_{add}^{-1})$ 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 (NmLCH ₄ .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 distillated 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 (Venkiteshwaran et al., 2016). Sequencing was achieved at the technology

platform Genome and Transcriptome (GeT) of the Génopole Toulouse, France. OTUs with a
 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
- 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
- amount of water that was hold in the sample after centrifugation, as follows:

$$WHC = \frac{m_h - m_r}{m_r} \tag{1}$$

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

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

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

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

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

$$Cosub = \frac{V_{effl expect}}{WHC} \tag{4}$$

213

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

217 2.3 Statistical analysis and data representation

One-way analysis of variance was made on BMP results after verifying both the normality 218 (Shapiro-Wilk test) and the variance homogeneity (Levene test) with R package "rstatix". A pair-219 wise t-test adjusted with Holm method was further realized for assessing the significance of the 220 221 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 "corrplot" package. A linear model was calculated on co-ensiling data of sunflower using Im 224 function of R. The package "ggplot2" was used for graphical representations. 225

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 volume of effluent produced according to Bastiman and Altman (1986) and Sutter (1957) 230 estimations were 187 and 345 mL·kg⁻¹ for sunflower and 57 and 199 mL·kg⁻¹ for rye, 231 respectively. The average estimated volume were calculated and corresponded to 138 and 266 232 233 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 the different co-substrates ranged from 0.8 (chicken manure) to 2.1 mL.g⁻¹ (soiled paper and 235 236 cardboard), which confirmed their ability to be used as absorbent materials. These values are lower than other found in the literature. For example, WHC of 2.3 – 2.8 mL.g⁻¹ were reported for 237 miscanthus stalks (Dennery G., Dezat E., 2012) whereas they were around 3 for wheat straw 238 239 (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 spaces of the substrate. The two crops had C/N ratios of 22.2 and 22.1 g.g⁻¹ for rye and 241 242 sunflower, respectively, which corresponds to the medium values for cover crops (Molinuevo-Salces et al., 2014). For the co-ensiling substrates, different patterns can be observed. Wheat 243 244 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, chicken manure presented the highest nitrogen content, with a C/N ratio of 8.2. Both crops 246 presented high WSC of 181 and 131 g.kg_{TS}⁻¹ in sunflower and rye, respectively, which are 247

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

Rye and sunflower had buffering capacities of 248 and 283 mEq·kg_{TS}⁻¹, respectively, which are
considered as low values (Kaiser et al., 2004). Wheat straw, soiled paper and cardboard,
miscanthus stalks and wood chips had a very low buffering capacity, between 14 to 88 mEq·kg_{TS}⁻¹
¹, while that of manure was 148 mEq·kg_{TS}⁻¹, a lower value than for rye. However, chicken
manure presented a high buffering capacity of 509 mEq·kg_{TS}⁻¹, which was likely due to its high
nitrogen content. A high buffering capacity makes it difficult to lower the pH during silage
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 295 mL_{effluent}·kg_{sunflower}⁻¹ in average, which is comparable with the average estimations from 262 Bastiman (1986) and Sutter (1957) (266 mL·kg⁻¹). Wheat straw addition was shown to efficiently 263 reduce the production of silage effluent. The volume of effluent produced was inversely 264 proportional to straw loading up to 150 g·kg⁻¹. For higher straw loadings, the effluent is almost 265 totally retaining in the solid medium. A linear model was used to fil the data presented in Figure 266 267 1, excepted for 250 g·kg⁻¹ straw loading, since full retention was considered to be reached over 150 g·kg⁻¹. The cumulated effluent volumes from 10 different silos were consequently used for 268 the linear model definition. The regression line was forced to intercept with the effluent volume 269

value of the control. For a straw loading ranging from 0 to 150 g·kg⁻¹, 1.6 mL of effluent were
retained per g of wheat straw added, which corresponds to the theoretical WHC capacity of
wheat straw calculated previously. The straw addition method also influenced the effluent
retention capacity. At a loading of 40 g·kg⁻¹, mixing straw with sunflower allowed to decrease
the effluent production by 28 mL·kgcc⁻¹ compared to adding the same amount of straw as a
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 of pooled effluent samples was measured and a value of 309 ± 18 NmLCH₄.gCOD⁻¹ was found, 278 279 which is close to the theoretical relation of $1g \text{ COD} = 350 \text{ NmLCH}_4$, indicating that the organic matter contained in the effluent is mostly biodegradable. The methane potential of the effluent 280 was consequently estimated to be 17.9 NLCH₄.L_{effluent}⁻¹. Considering the initial VS content and 281 cumulated effluent volume of sunflower, a cumulated methane potential of 40.5 Nm³.t_{VS init}-1 282 flowed in the effluent when sunflower was ensiled alone. This represents 18% of fresh 283 sunflower BMP. Co-ensiling allowed to gradually reduce effluent production and thus BMP flow 284 through effluent down to until 4.2 Nm³.t_{vS init}⁻¹, i.e. 1.7% of fresh sunflower BMP as the straw 285 286 loading increased.

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

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

294

This experiment confirmed the interest of using a dry co-substrate for silage effluent retention. The theoretical WHC of the co-substrate was found to be a relevant parameter for its dosing in a co-ensilage and it was consequently used for the calculation of co-substrate loadings in the 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

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

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

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

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

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

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

317 Table 2 : Fresh co-ensiling mixtures characteristics.

318

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

323 3.3.2 Silage effluent production

An effluent was only produced during the ensiling of rye alone. In this condition, an average 324 volume of 76 mL.kg_{CC}⁻¹ was measured, which was lower than the previously estimated volume 325 (138 mL·kg⁻¹) but between the estimation of Bastiman and Altman (1986) (57 mL.kg_{CC}⁻¹) and the 326 one Sutter (1957) (199 mL.kg_{cc}⁻¹). In addition, despite of the relatively low TS content of the 327 mixtures R + Paper, R + Wood and R + Horse M that should have led to an effluent production 328 329 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 not a reliable parameter to estimate the effluent production, and that it is essential to consider 331 the estimation of the volume of effluent produced by the crop together with the holding 332 capacity of the co-substrate. 333

The average COD concentration in effluent collected during the storage of rye alone was 59 gO₂.L⁻¹, and a BMP of 332 ± 13 NmLCH₄.gCOD⁻¹ (equivalent to 19.6 NLCH₄.L_{effluent⁻¹}) was found.

- 336 Considering the initial VS content of rye, the cumulated effluent production represented 7.6
- 337 Nm³.t_{VS 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
- 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
- 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 $g.kgVS_{added}^{-1}$ of mix (cover crop + co-substrate). CO₂ and H₂ are
- 347 expressed in cumulated L.kgVS_{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
- Figure 3: Evolution of ammonia concentration during storage. Concentrations are expressed in g.kg_{VSadded}⁻¹ of mix (cover crop + co-substrate); R stand for rye. Paper, Misc, Wood, Chicken M and Horse M stand for soiled paper and cardboard, miscanthus stalks, wood chips, chicken manure and horse manure, respectively
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- 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,

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

365 Secondary fermentation patterns

In Rye, R + Paper, R + Misc, R + Wood and R + Chicken MIn storage conditions, the fermentation 366 367 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 g.kg_{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 $g.kg_{VS}^{-1}$ 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 CO₂ production, indicating heterolactic fermentation pathways. In silage fermentation, 376 heterolactic metabolisms are mentioned to be less efficient at lowering the pH than homolactic fermentation (Woolford and Pahlow, 1998). Additionally, a significant propionic acid 377 378 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 lactic acid and allow a better aerobic stability when the silo is opened (Pahlow et al., 2003). 381

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 + Wood), 5.7 (Rye) and 6.5 (R + Chicken M). Both the pH rise and difference in pH values between 389 390 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 measured in the mixture R + chicken M with a value of 1.6 g.kgvs⁻¹, which was equivalent to 36 392 % of initial total nitrogen (TN_{init}). For rye ensiling, 1.3 g.kgvs⁻¹ (49 %TN_{init}) were measured, and 393 lower values were found in the other conditions, with 0.7 (33 %TN_{init}), 0.6 (41 %TN_{init}) and 0.8 394 g.kg_{VS}⁻¹ (27 %TN_{init}) in R + Paper, R + Wood and R + Misc, respectively. In the literature, it is 395 396 generally accepted that NH₃-N concentration should be lower than 10% of the total nitrogen in well preserved silages (Bureenok et al., 2016). The high concentration in NH₃-N found here is an 397 398 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 production stopped. This stable state may be due to the fact that all WSC were consumed 400 during the first weeks of storage (data not shown). The inhibitory effect of the accumulated 401 402 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 condition was characterized by a fast and high lactic acid production. After 7 days of storage, 92 406 g.kg_{VS}⁻¹ of lactic acid had accumulated in the medium, i.e. nearly 2 to 3 times more than in the 407 408 other storage conditions. This accumulation induced a significant acidification as pH dropped to 4.4 after 2 days and stabilized at 4.0 after one week. The fermentation was coupled to a limited 409 CO₂ production, since only 5 NL.kg_{VS}⁻¹ were measured and no H₂ was detected. The 410 411 fermentation characteristics suggested that mostly homolactic fermentations occurred, since lactic acid was largely dominant among the other metabolites produced. Since the pH was 412 below the critical value that ensures anaerobic stability (Pahlow et al., 2003), secondary 413 414 fermentations did not occur. Production of NH₃-N was also lower than in the other conditions (0.4 g.kg_{VS}⁻¹, 21 %TN_{init}). The silage obtained by co-ensiling rye with horse manure fulfilled the 415 416 criteria defining a "well preserved silage" according to the literature, with pH <4.5, lactic acid > 30 g.kg_{TS}⁻¹ and butyric acid < 10% of total volatile fatty acids. Only NH₃-N overpassed 10% of 417 418 total Nitrogen, however no other signs of undesirable microbial activity were recorded.

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

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Figure 4: (A) Microbial characterization of raw substrates (relative abundance of major orders).
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

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

Among the microorganisms that are likely to play a role in silage fermentation, the microbial 432 433 community of fresh rye was largely dominated by enterobacteriales order, mostly from enterobacteria family. Enterobacteria are commonly found on crops as epiphytic bacteria 434 (Pahlow et al., 2003). In silage fermentation, enterobacteria compete with lactic acid bacteria 435 for WSC, produce a mixture of acids and ethanol and have thus a detrimental action on silage 436 437 acidification. In paper, Misc and Wood, Lactobacillales, Enterobacteriales and Clostridiales were found. However, the total microbial abundance of these substrates was much lower. The total 438 specific abundance of these three orders was thus lower than in rye and few modifications of 439 440 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 over the storage duration. From the second week of storage, the amount of Clostridiales 445 446 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 2.7·10 ⁸ and 1.1·10 ⁸ rRNA gene copy number·g _{FM} ⁻¹ , respectively. Within the
449	bacteria orders that are expected to drive silage fermentations, a high amount of
450	Enterobacteriales, Lactobacilles 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, Lactobacilles and Clostridiales were
453	8.5·10 ⁵ , 3.9·10 ⁶ and 2.4·10 ⁶ in R + Chicken Manure and 9.5·10 ⁵ , 1.2·10 ⁷ and 5.9·10 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

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

471 In order to assess the impact of the initial properties of the co-ensiling mixtures on fermentation, a matrix of Pearson's correlations was calculated and is presented in Figure 5. The 472 impact of physico-chemical properties (TS, WSC, BC and C/N) had a weak influence on the 473 474 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 points out that increasing TS by adding a co-substrate may have a different impact on 478 fermentation than harvesting a crop at a high TS or applying field-wilting. Since the inhibition of 479 480 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 was established between initial content of Lactobacillales and the production of butyric acid and 484 CO₂. If a silage fermentation dominated by lactic acid production is expected, a co-substrate 485 486 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

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

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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, 6.4, 8.0, 9.1 and 1.9 % of initial VS of each mixture were lost during storage, respectively, mostly 498 499 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 2.3 % of initial VS of Rye were lost. It is important to stress that mixtures with similar 501 502 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 impact of storage on the methane potential (Figure 6). In most conditions, no significant BMP 509

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 conditions is surprising, since no difference in mass losses, fermentation properties nor gas 513 production was observed. Consequently, this difference may be due to sampling issues due to 514 515 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 BMP measurement may influence greatly the measured BMP value. Indeed, the BMP measured 517 for fresh R + Wood (330 \pm 36 NmLCH₄·g_{VS}⁻¹) is higher than the theoretical BMP calculated as the 518 519 weighted average between rye and wood chips (267 NmLCH₄·g_{VS}⁻¹). The BMP of fresh R+ Wood was thus probably overestimated. Even if butyric fermentation and the related hydrogen 520 production occurred during most of storage conditions, hydrogen production did not exceed 15 521 522 NL.kg_{VS}⁻¹, which theoretically induced BMP losses of about 4 NmL.g_{VS}⁻¹. However, this value is 523 very low compared to those of the raw substrates, which explains the small difference between the methane potential before and after silage. 524

Limited differences were found between BMP reported to added and initial VS in all conditions, which was due to the fact that VS loss were relatively small. When higher VS losses occurred, a larger difference between these two BMP values was found, as BMP was concentrated in the remaining matter as WSC were converted into more energetically dense compounds like butyric acid during fermentation while CO₂ was released (Kreuger et al., 2011; Van Vlierberghe et al., 2021).

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

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As a consequence, this study shows that the co-silage of a wet cover crop with an absorbent co-538 539 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 methane potential during storage (Teixeira Franco et al., 2018). A synergistic effect can thus be 542 achieved, with the crop providing the necessary WSC for silage fermentations and the manure 543 544 avoiding effluent production. Teixeira Franco et al. (2018) recommended an initial WSC content of 166 g.kg_{VS}⁻¹ for an efficient ensiling of cattle manure , which is a higher but close to initial 545 value of the present experiment (126 \pm 21 and 115 \pm 5 g.kg_{vS}⁻¹, for R + Horse M and R + Chicken 546 M, respectively). 547

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	
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695 Table 1: Fresh cover cro	p and co-substrate characteristics
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Fresh substrates	Sunflower	Rye	Wheat straw	Soiled paper and cardboard	Miscanthu s 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
рН	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
С (%ТЅ)	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< th=""><th><d.l< th=""><th><d.l< th=""><th>5.7 ± 1.4</th><th>19.9 ± 0.9</th></d.l<></th></d.l<></th></d.l<>	<d.l< th=""><th><d.l< th=""><th>5.7 ± 1.4</th><th>19.9 ± 0.9</th></d.l<></th></d.l<>	<d.l< th=""><th>5.7 ± 1.4</th><th>19.9 ± 0.9</th></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< th=""><th><d.l< th=""><th><d.l< th=""><th>2.0 ± 0.3</th><th><d.l< th=""></d.l<></th></d.l<></th></d.l<></th></d.l<>	<d.l< th=""><th><d.l< th=""><th>2.0 ± 0.3</th><th><d.l< th=""></d.l<></th></d.l<></th></d.l<>	<d.l< th=""><th>2.0 ± 0.3</th><th><d.l< th=""></d.l<></th></d.l<>	2.0 ± 0.3	<d.l< th=""></d.l<>
BMP (NmLCH ₄ .g _{VS} ⁻¹)	n.d.	360 ± 31	n.d.	354 ± 25	181 ± 22	26 ± 16	134 ± 65	172 ± 10

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.

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
рН	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 _{TS} -	248	246	179	214	268	314	
¹)							
С (%ТЅ)	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 ⁻¹ vs)	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 ⁻¹ vs)	141 ± 15ª	117 ± 4 ^{ab}	109 ± 7 ^{ab}	$102 \pm 17 {}^{b}$	126 ± 21 ^{ab}	115 ± 5 ^{ab}	11

700 Table 2: Fresh co-ensiling mixtures characteristics.

701 Within a row, different letters express a statistical difference (p < 0.05) between two samples.

Measurements that were performed in triplicates are expressed as mean \pm sd.



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



← CO2 ← H2 ← pH ← Lactic acid ← Acetic acid ← Ethanol ← Propionic acid ← Butyric acid

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.

714



717 Figure 3: Evolution of ammonia concentration during storage. Concentrations are expressed in

718 g.kg_{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.

721

39



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

 q_{EM}^{-1} ; (B.1) Variation of total microbial abundance during storage (16S rRNA gene copy number

 $. g_{FM^{-1}}$; (B.2) Relative composition of microbial community at the order level.



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



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).