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1 Conditions for efficient alkaline storage of
2 cover crops for biomethane production
3

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9 Abstract

10 An innovative process aiming to combine storage and alkali pretreatment of cover crops was
11 investigated using lime as a low cost and environmental friendly reactant. Different lime loadings and
12 Total Solid concentrations (TS) allowed to highlight the abiotic mechanisms of deacetylation during the
13 early stages of the process. Long-term storage experiments of rye and sunflower cover crops at 100
14 g.kgTS⁻¹ lime loading allowed to evaluate the fermentation kinetics and to compare performances in dry
15 and wet conditions to classical silage storage. The dry condition allowed an efficient alkaline storage and
16 up to a 15.7% Biochemical Methane Potential (BMP) increase, while the wet condition underwent a
17 succession of fermentations with a high butyric acid accumulation and H₂ production, leading to a 13%
18 BMP loss. Silage experiments allowed an efficient preservation of the BMP, with no significant variation
19 over the 6-month storage duration.

20 **Keywords**

21 Intermediate crops; Anaerobic digestion; Silage fermentation; Pretreatment of lignocellulose; Biogas

22 1 Introduction

23 In order to face the increasing demand in renewable energies, extensive research and investments are
24 currently made to develop new modes of production of clean and sustainable energies. Resulting from
25 the degradation of a large panel of sewage, effluents, biowastes and agricultural residues, production of
26 methane by anaerobic digestion (AD) processes provides a sustainable alternative to natural gas allowing
27 significant reductions in greenhouse gases emissions when substituted to natural gas or other fossil
28 fuels.

29 AD of waste-like substrates allows to produce a renewable fuel while valorizing a large variety of by-
30 products. However, a larger amount of biomass is required to substitute the present natural gas
31 consumption in France and worldwide. For this reason, energy crops were grown in various countries to
32 provide a larger amount of biomass for anaerobic digestion. As energy crops have a high biomass yield
33 per hectare and a high CH₄ yield, they allow to secure the intake supply of biogas plants. In Germany,
34 about 78% of total energy production by AD referred to energy crops in 2016 (Daniel-Gromke et al.,
35 2018), *i.e.* 25 TWh. However, despite these advantages, energy crops compete for arable land with food
36 crops and the benefits of their use as a sustainable intake for AD are questioned. As an alternative to
37 energy crops, cover crops (CC), also called intermediate crops, allow to produce biomass for AD without

38 increasing the demand in arable land. CC are grown during the intercropping period of food or feed crop
39 rotations. In addition, their use brings agro-ecological advantages, allowing to avoid erosion and nutrient
40 leaching (Igos et al., 2016), facilitate the control of self-propagating plants (Büchi et al., 2020) and
41 accumulate organic matter in the soil (Jian et al., 2020). However, the cost-effectiveness of the
42 cultivation of CC has to be improved to ensure the economical relevance of their use for AD. If it mostly
43 depends on the harvest yield that has to compensate the cultivation costs, the potential of the crop still
44 has to be efficiently stored and preserved until its use in the anaerobic digester. The storage of wet
45 harvested crops (in opposition to haymaking) is generally performed by ensiling. This process relies on
46 the spontaneous lactic fermentation and acidification of the biomass under anaerobic conditions by the
47 action of endogenous bacteria that ferment the accessible sugars contained in the plant into lactic acid
48 and other compounds such as acetic acid and ethanol (Elferink et al., 1999). Once a low pH
49 corresponding to acidic conditions is reached, the microbial activity can be inhibited over several months
50 until the silo opening. Ensiling has been shown to be an efficient conservation method of crops for AD,
51 but its success depends on several parameters, and significant degradations can occur in non-optimal
52 conditions (Teixeira Franco et al., 2016).

53 Because of their fibrous composition, the degradability of CC into methane can however be low. The
54 accessibility of the microorganisms involved in the anaerobic digestion to the fermentable
55 carbohydrates of hemicellulose and cellulose is limited by the complex structure of the lignocellulosic
56 matrix, which lowers both yield and kinetic of biogas production from crops (Monlau et al., 2013). A large
57 variety of pretreatment technologies has been reported in the literature in order to increase the
58 degradability of lignocellulosic biomasses into methane. Due to their high efficiency to degrade lignin,
59 the alkaline pretreatment constitutes a promising option for an application on lignocellulosic biomasses
60 (Carrere et al., 2016). Significant improvements of the degradability of lignocellulosic biomasses into
61 methane were obtained, even in mild conditions with low energy and reactive requirements (Khor et al.,
62 2015; Thomas et al., 2018). In mild conditions, the extension of the pretreatment duration was shown to
63 have a beneficial impact, allowing to compensate the slower kinetic of the reaction (Thomas et al., 2018).
64 Considering the alkaline reagents, NaOH, KOH and CaO are among the most studied for alkaline
65 pretreatment. However, NaOH and KOH are expensive and NaOH can additionally cause an increase in
66 the digestate salinity, which may be harmful to the soil in the case of the use of the digestate as a
67 fertilizer (Shahid et al., 2018). In contrast, CaO is already used in agriculture to control soil acidity with
68 soil liming and presents the advantage to be less expensive. CaO is also available in high quantity as it is
69 produced from the calcination of limestone, an abundant component of the earth crust. For these

70 reasons, CaO constitutes an interesting option as a reagent for alkaline pretreatment, despite its lower
71 efficiency when compared to NaOH and KOH (Jiang et al., 2017).

72 Since CC are commonly stored by ensiling for anaerobic digestion, applying an alkaline pretreatment
73 after storage would no represent an optimized solution. Indeed, a larger amount of reagent would be
74 necessary to elevate the pH of silage, which is rich in organic acids and buffered around pH 4. In order to
75 bypass this problem, the alkaline pretreatment of crops could therefore be applied directly after
76 harvesting. By extending the pretreatment duration until several months, a combined storage and
77 pretreatment process could thus be developed, allowing to take advantage of the long term action of the
78 alkaline pretreatment. Few examples of alkaline storage of crops are reported in the literature.

79 Pakarinen et al. (2011) experimented the use of urea (30 and 60 g.kg_{TS}⁻¹) as an alkaline reagent on hemp
80 for ethanol and biogas production over a 4 and 8 months storage period. Despite a final pH of 8.7 which
81 is a low value in the context of alkaline pretreatment, significant increase of the enzymatic
82 hydrolysability (+46%) and BMP (+21%) were found. However, the same increase was measured in the
83 silage experiment with no additive and no mass balance was calculated during this alkaline storage
84 experiment, which makes difficult to interpret such energy potential variations. Digman et al. (2010)
85 studied an alkaline storage process for ethanol production using lime in anaerobic conditions on
86 switchgrass and reed canarygrass. The CaO loading varied from 14.6 to 100 g.kg_{TS}⁻¹, with storage
87 durations from 30 to 180 days. The authors found that all storage conditions allowed an efficient
88 preservation of the biomass, even if no global energy balance including mass losses was evaluated. The
89 best pretreatment efficiency was found with the highest lime loadings of 85 and 100 g.kg_{TS}⁻¹. The results
90 also showed that only these high lime concentrations allowed to maintain a high pH of 9.1 ± 0.9 and 10.1
91 ± 0.4, respectively, until the end of the storage. Final pH was highly correlated with the CaO loading (r =
92 0.97) and with the efficiency of the pretreatment (r = 0.82). Interestingly, no correlation was found
93 between the pretreatment duration and its efficiency. In addition, both chemical and microbial
94 mechanisms involved during storage were not clarified. In a recent work, Van Vlierberghe et al. (2021)
95 showed a fast pH decrease from 12 to 7 after a few days, using a relatively low CaO loadings (60 g.kg_{TS}⁻¹).
96 The pH destabilization was attributed to two possible phenomena both abiotic (chemical deacetylation
97 of hemicellulose) and biotic (microbial fermentation) phenomena. The deacetylation reaction consists in
98 the solubilization and removal of the acetyl groups that are covalently bonded to the xylan backbone of
99 hemicellulose (Chen et al., 2012), releasing acetic acid in the medium. In lignocellulosic materials like
100 corn stover, acetyl groups esterified to the hemicellulose structure represent in average 2.2% of TS and
101 were shown to increase the biomass recalcitrance (Humbird et al., 2011). Dilute alkaline pretreatments

102 were shown to be efficient at solubilizing the acetyl groups of hemicellulose in mild conditions (Chen et
103 al., 2014), making probable the occurrence of this phenomenon in alkaline storage. Considering the
104 microbial fermentation, lactic, acetic and butyric fermentations were involved (Van Vlierberghe et al.,
105 2021). However, the understanding of the contribution of both mechanisms was limited. In addition,
106 even if the methane potential of the crops was efficiently preserved, the expected positive effect of the
107 pretreatment was not achieved, which was attributed to the fact that the lignocellulosic substrate was
108 not exposed to an alkaline pH for a sufficient period of time. Maintaining a high level of pH during the
109 whole storage duration was consequently identified to be necessary for both inhibitions of undesirable
110 microbial activity and increase of the pretreatment action on lignocellulose.

111 The present work aims to give an insight of the very short and long-term mechanisms involved in alkaline
112 storage of crops with lime. In the very first stage of storage, the chemical deacetylation of hemicellulose
113 and its contribution to the pH change were studied during the first hours of alkaline storage. In addition,
114 the effect of the storage conditions on the physico-chemical characteristics of the crops were identified
115 during the whole storage duration of six months, along with the evolution of the microbial communities
116 present on the substrate which had never been studied to the authors' knowledge. A direct comparison
117 of the energy potential balance between different alkaline storage conditions and the conventional
118 silage process is discussed, in order to conclude on the relevance of alkaline storage process.

119 2 MATERIAL AND METHODS

120 2.1 Feedstock

121 Three cover crops were used as a substrate. For the first experiment which evaluated chemical
122 deacetylation, a sample of rye (*Secale cereale*, *Rye 1*) harvested at BBCH59 (end of heading stage) and
123 cultivated as a winter cover crop on an agricultural site in France (Biométhagri, Florensac, 34101,
124 Hérault, France) was used. The rye was shredded using a garden shredder (AXT 2550TC, Bosch GmbH)
125 directly after harvesting and stored at -20°C until the experiment.

126 Two other cover crops harvested on another agricultural site (Biométharn, Aiguefonde 81200, Tarn,
127 France) were used to evaluate the long-term alkaline storage during the second set of experiments. One
128 sample of rye (*Secale cereale*, *Rye 2*) cultivated as a winter cover crop, harvested at BBCH 73 (early milk
129 stage) and one sample of sunflower (*Helianthus annuus*) cultivated as a summer cover crop and
130 harvested at maturity stage BBCH 88 (end of the ripening stage). The two crops were stored overnight as

131 a whole plant at 4°C in sealed plastic bags, avoiding moisture change before use, and used directly after
132 for storage experiment.

133 2.2 Experimental set-up

134 Before all experiments, the crops were chopped using a garden shredder (AXT 2550TC, Bosch GmbH)
135 until homogeneous particle size of approx. 1-2 cm was reached and further passed through a knife
136 shredder (BB230, BLiK®) for better longitudinal cut of the crop pieces. The different experiments
137 described below were made using the following setup. 2.6L glass flasks sealed with air-tight lids
138 equipped with a rubber septum that allows pressure measurement and gas sampling were used.
139 Replicates were prepared for each storage condition to be sacrificed after the determined durations to
140 monitor the impact of different storage conditions on biomass conservation and on fermentation
141 kinetics. 700g of sample were packed into the flasks which were then flushed with N₂, sealed and stored
142 in a dark room.

143 2.3 Short-term experiment

144 The deacetylation experiment was divided into two stages. The first one aimed to evaluate the acetic
145 acid production kinetic. The TS of the crop was adjusted to 25% by oven drying at 40°C, and a lime
146 loading of 100g.kg_{VS}⁻¹ was applied. Several replicates were prepared to be sacrificed after 2, 4, 8, 24 and
147 168 hours of storage (3 replicates for each storage time). The flasks were stored at 4°C in order to inhibit
148 the microbial activity. This method was preferred to others, such as thermal treatment, in order to
149 minimize the side effects on the substrate characteristics. The second stage focused on the influence of
150 lime loading and moisture content on the acetic acid release during the first hours of the process. Four
151 conditions were tested following a 2x2 factorial design. The different conditions are summarized in Table
152 1. TS values of 10 and 40% were chosen, as these extreme values can be found for cover crops at
153 harvest time. The TS value of the crop was adjusted by adding distilled water or by oven drying at 40°C.
154 CaO loadings of 25 and 200 g.kg_{TS}⁻¹ were applied. The lower level was chosen as one of the lowest
155 amounts found in the literature. The higher level was set as a large excess of CaO whose value is usually
156 less than or equal to 100 g.kg_{TS}⁻¹ (Digman et al., 2010; Khor et al., 2015).

157

158

159 *Table 1: Pretreatment conditions applied for the second phase of deacetylation experiment and effect on pH*

TS (%FM)	Loading (g.kg _{VS} ⁻¹)	Initial pH	Final pH
10	25	10.7 ± 0.0	9.9 ± 0.1
10	200	13.2 ± 0.0	13.2 ± 0.1
40	25	9.8 ± 0.0	8.4 ± 0.1
40	200	13.1 ± 0.0	13.1 ± 0.1

160

161 2.4 Long-term experiment

162 A lime loading of 100 g.kg_{TS}⁻¹ was selected according to the results of Digman *et al.* (2010) and the
 163 deacetylation experiment presented above. Alkaline storage was applied on two different cover crops
 164 (i.e., sunflower and rye). For both crops, a “dry” alkaline storage was achieved by applying a dry powder
 165 of CaO directly, reaching a TS content of the mixture of 41 and 47% TS for rye and sunflower,
 166 respectively. For rye samples, an additional “wet” condition was experimented, as Digman *et al.* (2010)
 167 showed that a higher moisture content increased the pretreatment action. Deionized water was
 168 gradually sprayed on the mixture during homogenization in order to increase the lime diffusion into the
 169 biomass and adjust TS, until a total TS of 29% was reached. Water addition was limited to this extent
 170 because higher moisture are reported to cause undesirable effluent production in bunker silo during
 171 storage (Teixeira Franco *et al.*, 2016). Ensiling assays were conducted as controls, since silage is still the
 172 most common method for crop storage prior to anaerobic digestion. The same storage protocol was
 173 used for silage experiments, except that no chemical additives were added. For each storage condition,
 174 five replicates were prepared to be sacrificed and analyzed after 2, 7, 21, 60 and 180 days of storage. The
 175 flasks were stored in a dark place where temperature was controlled at 22°C.

176 2.5 Silo monitoring and sampling

177 After the flasks were closed, gas production and weight losses were regularly monitored until flask
 178 opening. Volumetric gas production was quantified by pressure difference. The pressure was measured
 179 using a manometer (Keller LEO® 2) and gas was released when pressure exceeded 1.2 bar, after what the
 180 flasks were immediately weighted. The volume of gas inside the flask (headspace + pore space) was
 181 calculated by subtracting the volume of added substrate to the total volume of the flask. The volume of
 182 substrate was calculated using its theoretical density calculated as follows, adapted from McNulty *et al.*
 183 (McNulty and Kennedy, 1982):

184 $\rho_t = (1 - C) [(M/\rho_l) + (1 - M)/\rho_s] + C/\rho_{CaO}$ (1)

185 where M = fractional moisture content related to fresh matter (FM); C = fractional CaO content related
186 to fresh matter; ρ_l = liquid or water density = 1000 kg/m³; ρ_s = dry matter density = 1421 kg/m³ and ρ_{CaO} =
187 CaO density = 3345 kg/m³. The composition of the gas was analyzed using a gas chromatography (Perkin
188 Elmer Clarus 580) with a Rt-U-bond column (30m x 0.32mm x 10 μ m) for CO₂ separation, and a Rt-
189 Molsieve 5Å column (30m x 0.32mm x 10 μ m) for H₂, O₂, N₂ and CH₄ separation. Argon was used as vector
190 gas (350 kPa, 34 mL/min). Injector and detector temperature was set on 200°C, and oven temperature
191 on 65°C. Detection was made by thermic conductivity. For each planned storage duration, one flask was
192 opened after final gas and weight measurement. The whole sample was then crushed using a knife mill
193 (Pulverisette 11, Fritsch). Total solids (TS) and volatile solids (VS) were measured immediately. Water
194 extraction was performed for pH, water soluble carbohydrates (WSC), volatile fatty acids (VFA) and other
195 metabolites measurements. 30 g of fresh sample were soaked in 150 mL of deionized water for 16 h to
196 20 h at 4°C in sealed plastic pots in triplicate, similarly as proposed by Porter & Murray (Porter and
197 Murray, 2001). pH measurement was made directly after extraction on the mixture. Then, the liquid
198 phase was separated by centrifugation (18750 g, 20 min, 4°C) and frozen in air-tightly closed tubes for
199 further WSC, VFA and metabolites analysis. A fraction of the samples was separated and stored at -20°C
200 for further BMP measurement. Finally, aprox. 100 g of fresh sample were freeze-dried and milled using a
201 1mm grid for fiber distribution analysis.

202 2.6 Physicochemical analyses

203 TS were measured in triplicate by drying 30 g of sample at 105°C for 24 h. Volatile Solids (VS) were then
204 measured by calcination of the dry residue (550°C, 3 h). Silage and other substrates obtained from crops
205 transformation may contain volatile fatty acids (VFA), lactic acid (LA) and diverse alcohols that evaporate
206 during oven drying causing underestimation of TS. For this reason, the TS content value measured by
207 oven drying was corrected using the equation proposed by Porter and Murray (2001):

208 $TS_C = TS_M + 0.375 LA + 0.892 VFA + 0.975 Alcohols$ (2)

209 where TS_C = corrected TS; TS_M = measured TS; LA = lactic acid concentration; VFA = total VFA
210 concentration; Alcohols = total alcohols concentration. All concentrations are in g/gFM. TS_C was further
211 used for VS calculation.

212 pH was measured in triplicate using WTW® SenTix® 41 probe on a WTW® inoLab® pH7110. WSC and
213 metabolites concentration was measured from the centrifuged liquid phase after filtering (0.2 μ m nylon

214 filter) by High Performance Liquid Chromatography on Aminex 4PX-87H column (Bio-Rad) at 45°C.
215 Sulfuric acid (0.005 M; 0.3 mL/min) was used as mobile phase. WSC content was calculated as the sum of
216 glucose, xylose, arabinose, fructose. Fiber distribution was analyzed in triplicate on previously freeze
217 dried and milled samples using Van Soest method (Van Soest and Wine, 1967). Water extract (*W.EX*),
218 neutral detergent fiber (*NDF*), acid detergent fiber (*ADF*), acid detergent lignin (*ADL*) and calcination
219 residue (*CAL*) contents were determined. Water soluble compounds (*W.SOLU*), neutral detergent soluble
220 compounds (*SOLU*), hemicellulose (*HEMI*), cellulose (*CELL*) and lignin content (*LIGN*) were calculated as
221 follow : $W.SOLU = 1 - W.EX$; $SOLU = WEX - NDF$; $HEMI = NDF - ADF$; $CELL = ADF - ADL$; $LIGN = ADL -$
222 CAL . Prior to the fiber extraction, a preliminary extraction of fat was performed on sunflower samples, as
223 recommended by Van Soest *et al.* Lipids were extracted with an ASE instrument (Dionex ASE-200) with
224 heptane:ethanol (2:1, vol:vol) for solvent, as described by Yang *et al.* (Yang *et al.*, 2019). No fat extraction
225 was made on rye samples, whose lipid content was expected to be negligible (Herrmann *et al.*, 2011).
226 The total carbon (TC) and total nitrogen (TN) were measured with an elemental analyzer (FlashSmart®,
227 Thermo Fisher Scientific®) on finely grounded freeze dried samples. TC and TN analyses were not
228 replicated. BMP measurements were made following the recommendations of Holliger *et al.* (2016) .
229 Samples were previously prepared for BMP test by freezing a certain amount of substrate containing
230 around 2 g_{TS} of sample (exact TS and VS were calculated later) soaked in NaHCO₃ buffer. The buffer was
231 added to minimize the difference of pH between ensiled and alkali-stored samples, avoiding additional
232 pretreatment effect of alkaline conditions before BMP test. Gas measurement was made using an
233 automatic batch test system (AMPTS II, Bioprocess Control, Sweden). The methane potential value was
234 expressed as the volume of methane produced per initial amount of VS estimated after taking into
235 account the mass losses that occurred during storage. This was made to evaluate the global energy
236 balance of the different storage processes. The generated sequencing datasets are registered in the
237 Sequence Read Archive (<https://www.ncbi.nlm.nih.gov/sra>) under the BioProject accession number
238 PRJNA788459.

239 2.7 Microbial community analysis

240 For each sampling time of the storage experiment, a 300 mg sample was collected and stored into a 2 mL
241 sterile Eppendorf tube and stored at -20°C until DNA extraction. DNA extraction, sequence data analysis
242 and quantitative PCR were performed as described by Dauplain *et al.* (2020). Sequencing was made at
243 the technology platform Genome and Transcriptome (GeT) of the Génopole Toulouse, France. Only OTUs
244 with a relative abundance of 1.5% or more in at least one sample were selected for further data analysis.

245 **2.8 Statistical data analysis**

246 One-way analysis of variance was performed on fiber distribution and BMP after verifying normality
247 (Shapiro-Wilk test) and variance homogeneity (Levene test) with R package “rstatix”. Pair-wise t-test
248 adjusted with Bonferroni correction was further performed to assess the significance of the difference in
249 mean between two samples. The package “ggplot2” was used for all graphical representations.

250 **3 Results and discussions**

251 **3.1 Raw material characterization**

252 The main characteristics of the different crops are presented in Table 2. The higher maturity crops (i.e.,
253 rye 2 and sunflower) were characterized by higher solid content and lower water-soluble carbohydrates
254 (WSC). Cover crops are usually characterized by a TS value lower than 30%, with an average of 17 ± 5
255 (Molinuevo-Salces et al., 2013). The TS content at harvest greatly depends on the crop maturity stage,
256 related to sowing and harvesting dates, climate and crop species (Kaiser and Piltz, 2004). WSC were
257 mostly composed of fructose and glucose, the main primary soluble carbohydrates in temperate forages
258 (Downing et al., 2008). These carbohydrates are essential for the lactic fermentation and acidification of
259 silages (Elferink et al., 1999), but also represent a high amount of easily accessible carbohydrates for
260 undesirable fermentation in alkaline storage.

261 The three crops had C/N ratios of 50, 54 and 23 for rye 1, rye 2 and sunflower, respectively, which
262 corresponds to low (rye) and medium (sunflower) values when compared to other crops (Molinuevo-
263 Salces et al., 2014).

264 Sunflower samples were characterized by a higher lignin content than in rye samples. Rye samples
265 presented a higher hemicellulose content, that is a more easily accessible fiber for biogas production
266 (Monlau et al., 2013).

267

	Rye 1	Rye 2	Sunflower
TS (%FM)	21.8 ± 0.0	37.1 ± 0.1	42.5 ± 0.5
VS (%FM)	20.4 ± 0.0	35.9 ± 0.1	40.1 ± 0.2
TN (%VS)	0.9	0.8	1.9
TC (%VS)	44.6	43.3	44.8
pH	6.2 ± 0.0	6.0 ± 0.1	6.8 ± 0.0
VFA (g.kg _{VS} ⁻¹)	< d.l.	< d.l.	< d.l.
Fructose (g.kg _{VS} ⁻¹)	79.3 ± 11.5	33.6 ± 2.8	44.8 ± 0.2
Glucose (g.kg _{VS} ⁻¹)	60.1 ± 6.5	20.4 ± 1.4	21.9 ± 0.4
WSC (g.kg _{VS} ⁻¹)	147.0 ± 13.3	54.0 ± 3.1	66.9 ± 0.7
EtOH (g.kg _{VS} ⁻¹)	< d.l.	3.1 ± 0.3	< d.l.
Lipids (g.kg _{VS} ⁻¹)	n.d.	n.d.	5.6

270 n.d. : not determined; <d.l. : below the detection limit

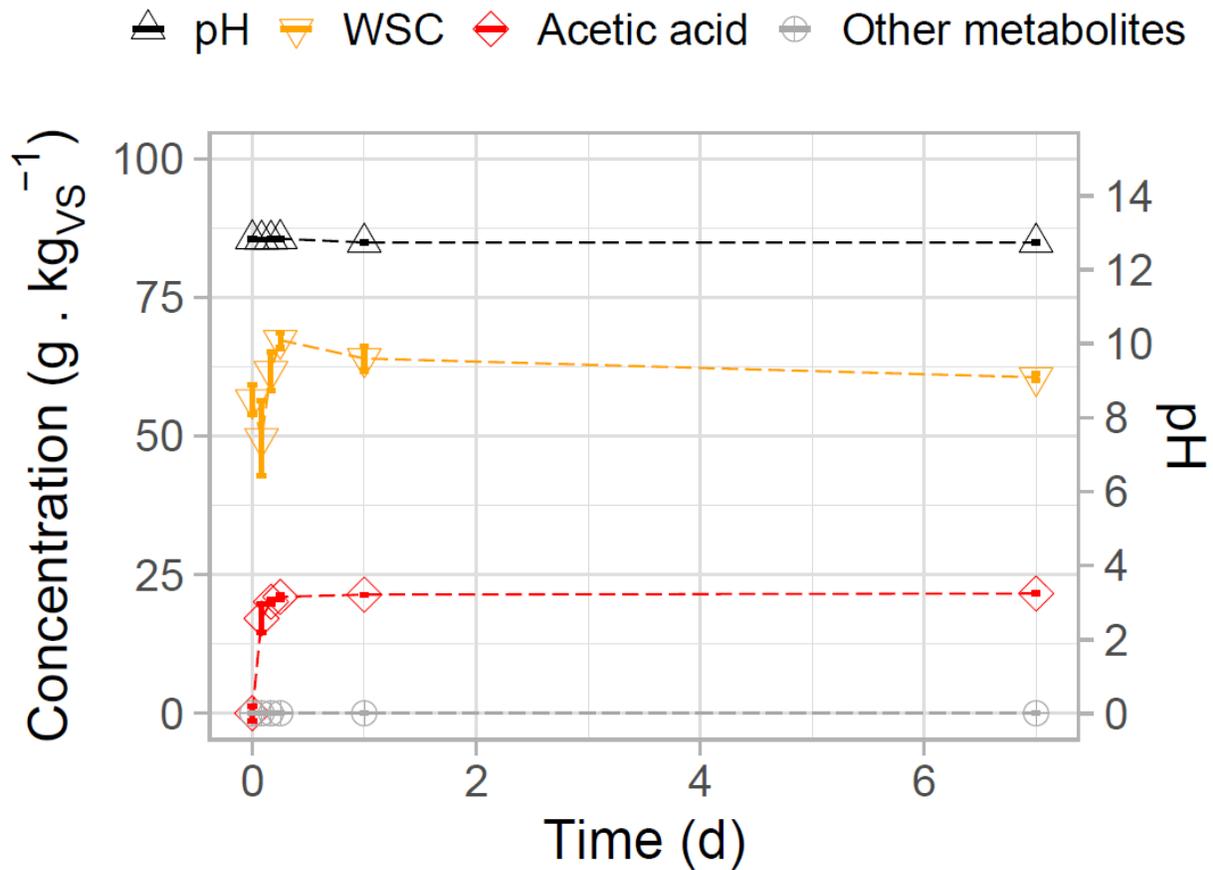
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272 3.2 Short-term experiment

273 The first short-term experiment allowed to investigate the kinetics of acetic acid production that occurs
 274 during the early stages of the alkaline storage (Figure 1). Despite the reaction temperature (4°C) which
 275 strongly limits the microbial fermentation activity, a large amount of acetic acid (AA) was produced
 276 immediately after the lime addition. After only 2 h, 80% of the final AA concentration had already been
 277 released. The maximum value of 24 g.kg_{VS}⁻¹ was reached after 4 h and remained stable for one week. In
 278 the literature, similar amounts of acetyl groups were released for lignocellulosic biomasses. Humbird et
 279 al (2011) reported an average value of 22 g.kg_{VS}⁻¹ in corn stover, while Castro et al. (2017) and Chen et al.
 280 (2014) measured acetyl content of 26 and 27 g.kg_{VS}⁻¹ in rice straw and corn stover, respectively, which
 281 suggests that a high deacetylation yield was obtained in these experiments. In addition, the AA
 282 production was not associated to WSC consumption nor fermentation gas production or other
 283 accumulation of metabolites. The pH was maintained at a value of 12.8 during this period. As a

284 consequence, the production of AA during the first hours of alkaline treatment (lime loading of 100
285 g.kg_{VS}⁻¹) is related to a chemical deacetylation mechanism.

286



287

288 *Figure 1: Variation of acetic acid, WSC and pH during the first days of alkaline storage. Dots represent averaged values of the*
289 *sacrificed triplicates. Error bars indicate standard deviation within triplicates.*

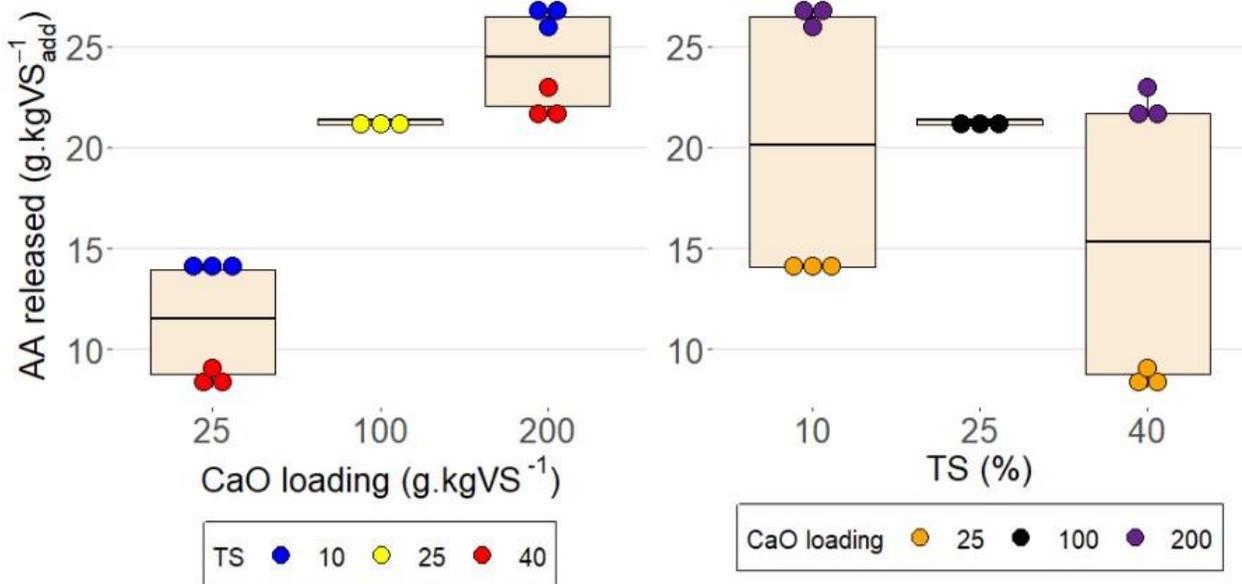
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291 The effect of lime loading and TS content on the final AA accumulation and pH was investigated in a
292 second set of experiments. The reaction duration was set at 48 h. Since deacetylation kinetics have been
293 shown to be rapid, a longer experimental time is not necessary to investigate this phenomenon. The
294 main effects of CaO and TS are shown in Figure 2. Results from the first experiment (lime loading of 100
295 g.kg_{VS}⁻¹) after 24h of reaction were also added for comparison. The CaO loading was shown to have the
296 highest effect since the AA release doubled between lime loadings of 25 and 200 gCaO.kg_{TS}⁻¹.
297 Interestingly, few additional AA was released for the highest load of 200 gCaO.kg_{TS}⁻¹ in comparison to the
298 previous experiment (100 gCaO.kg_{TS}⁻¹). This may be explained by the fact that high deacetylation yield

299 was already obtained at a lime loading of $100 \text{ gCaO.kg}_{\text{TS}}^{-1}$. Consequently, it is not expected that an
 300 increase of lime loading over $100 \text{ gCaO.kg}_{\text{TS}}^{-1}$ induces an additional effect on pretreatment efficiency,
 301 since previous studies showed that when enough lime has been added to remove acetyl groups, further
 302 lime addition is not beneficial to pretreatment action on lignocellulosic biomass (Chang et al., 1998).

303 The moisture content was also shown to have an effect on deacetylation yield, which is in agreement
 304 with the results of Digman *et al.* (2010) who observed a higher pretreatment effect in wet conditions.
 305 The higher deacetylation efficiency in wet conditions may be explained by the better solubilization and
 306 diffusion on CaO in the medium.

307 Lime loading had an important effect on initial and final pH (Table 1). At lime loading of $25 \text{ gCaO.kg}_{\text{TS}}^{-1}$,
 308 the initial pH was close to 10, while higher values of 13 were found with lime loadings of 100 and 200
 309 $\text{gCaO.kg}_{\text{TS}}^{-1}$. Most importantly, the lowest lime loading was not sufficient to ensure a high pH stability,
 310 since pH drops of 0.8 and 1.4 were observed for TS content of 10 and 40%, respectively. In such
 311 storage environments the microbial activity would not have been totally inhibited, and undesirable
 312 fermentations could start (Van Vlierberghe et al., 2021). The final pH for two highest lime loading of 100
 313 and $200 \text{ gCaO.kg}_{\text{TS}}^{-1}$ were similar and stable at a value of 13, and thus no beneficial effect on pH was
 314 found when increasing the lime loading over $100 \text{ gCaO.kg}_{\text{TS}}^{-1}$.



315
 316 *Figure 2: Effect of CaO loading and TS on acetic release after 2 days of reaction. Each dot represents a value from a single*
 317 *replicate.*

318

319 The short-term experiment confirmed the hypothesis of the chemical deacetylation of hemicellulose to
320 be responsible for the AA production at the beginning of alkaline storage. However, if the acetic acid
321 release seems to have an effect on pH at a low lime loading, no effect on pH related to this phenomenon
322 was observed for a CaO loading higher than $100 \text{ g} \cdot \text{kg}_{\text{VS}}^{-1}$ even after a week of storage at 4°C , suggesting
323 that this lime loading was sufficient to maintain a high pH in absence of microbial fermentation and to
324 reach a high deacetylation rate.

325 3.3 Long-term experiment

326 During the whole storage experiment, replicates were periodically sacrificed and analyzed in order to
327 monitor the evolution of biomass transformation over the storage period. pH, metabolites accumulation
328 and gas production (Figure 3) together with identification of the microbial populations (Figure 4) allowed
329 to understand the mechanisms involved during alkaline storages of 180 days. Depending on the cover
330 crops and storage conditions (alkali-stored and ensiled samples), three main steps can be identified and
331 are described below. In addition, the analysis of the microbial populations indicated that different
332 microorganisms were involved in each step.

333

334 3.3.1 Dynamical changes during storage

335 3.3.1.1 First phase (0 to 21 days)

336 In all alkaline storage experiments, high pH of 12.6 ± 0.1 were reached just after the addition of lime to
337 the shredded crop. These values are comparable to those observed in the previous section **Erreur !**
338 **Source du renvoi introuvable.** (Figure 1 and Table 1). By applying a CaO load of $100 \text{ g} \cdot \text{kg}_{\text{VS}}^{-1}$, pH was
339 therefore maintained in an alkaline range, since pH did not vary significantly during this phase. It is
340 noteworthy that, at a lower CaO load of $60 \text{ g} \cdot \text{kg}_{\text{VS}}^{-1}$, Van Vlierberghe *et al.* (2021) reported a significant
341 pH drop during the same period from 12 to 7 in less than one week. Acetic acid concentrations of 26 and
342 $30 \text{ g} \cdot \text{kg}_{\text{VS}}^{-1}$ were released from the hemicellulose of rye and sunflower, respectively, suggesting that a
343 high rate of deacetylation occurred. Low productions of other metabolites and fermentation gases were
344 measured, suggesting an absence or low microbial activities. In fact, during the first three weeks of
345 storage, microbial abundance remained low in the alkaline samples, due to the inhibitory action of high
346 pH (Figure 4). During the same period, for silage conditions, a lactic fermentation was responsible for the
347 acidification of the medium and the pH dropped quickly from 6.0 (rye) and 6.8 (sunflower) to 4.0. This
348 fermentation is also obviously linked to a strong production of CO_2 . Both crops underwent a
349 conventional silage fermentation pathway and presented the characteristics of high quality silages
350 according to Bureenok *et al.* (2016) with a pH lower than 4.5, a lactic acid concentration higher than 30

351 g·kg_{TS}⁻¹ and butyric acid concentration representing less than 10% of total VFA concentration. In silage
352 samples, a bacterial growth was observed and the initial abundances were multiplied by 19 and 5.2,
353 growing from 1.05·10⁹ to 2.10·10¹⁰ and from 1.08·10⁹ to 6.74·10⁹ 16S rRNA gene copy number·g_{FM}⁻¹ for
354 rye and sunflower, respectively. This bacterial growth was mostly composed of bacteria from
355 Lactobacillales and Enterobacteriales orders. Among the most represented lactic acid bacteria (LAB) after
356 2 days of fermentation, *Weissella sp.* (OTU5) and *Leuconostoc sp.* (OTU3) were dominant for rye while
357 *Weissella* and *Lactococcus* (OTU10) prevailed for sunflower. These heterofermentative bacteria are
358 known to be predominant in the epiphytic microflora of plants and to be responsible of a fast lactic acid
359 production during the early fermentation phase (Gharechahi et al., 2017). From day 2 to 21, *Weissella*,
360 *Leuconostoc* and *Lactococcus* decreased and were progressively replaced by different OTU of more acid
361 tolerant *Lactobacillus sp.*, as commonly observed in silage fermentation (Gharechahi et al., 2017).
362 Additionally, *Enterobacteriaceae* (OTU4) were observed during this early phase. *Enterobacteriaceae* are
363 commonly considered as undesirable in silage fermentation since they ferment amino acids into NH₃,
364 compete with LAB for nutrients and reduce WSC into acetic acid and components that do not decrease
365 pH. Below 4.5, pH prevented *Enterobacteriaceae* multiplication, consequently their number was
366 considerably reduced as the medium acidified. During this phase, mostly lactic acid was produced,
367 suggesting predominant homofermentation mechanisms.

368

369 3.3.1.2 Second phase (21 to 60 days)

370 After the third week of storage, silage conditions were stabilized and few additional fermentative
371 microbial activity was observed. Almost no additional accumulation of metabolite nor gas production
372 occurred. The total bacterial abundance was strongly reduced due to the acid conditions. 1.04·10⁹ and
373 1.75·10⁹ rRNA gene copy number·g_{FM}⁻¹ were measured for silage of rye and sunflower, respectively.
374 In alkali-stored conditions, two different behaviors took place. For the two experiments performed
375 under dry conditions (41 and 47 %TS for rye and sunflower, respectively), the stable state obtained in
376 phase 1 was maintained. The absence of fermentative activity can be identified by the lack of gas and
377 metabolites production, which is confirmed by the low bacterial abundance (Figure 3 and Figure 4). In
378 the wet condition however, a destabilization of the alkaline condition occurred after the third week of
379 storage. Indeed, an heterolactic fermentation suddenly started and induced a pH drop down to 7.4. In
380 this storage condition, WSC were consumed and converted into lactic acid, acetic acid, propionic acid
381 and ethanol that accumulated in the medium. Fermentation gases (CO₂ and H₂) were also released.
382 Unlike in silage conditions, a significant production of H₂ occurred. During this fermentation step in wet

383 alkaline rye, H₂ production was likely linked to acetic and propionic fermentation (Hillion et al., 2018)
384 since no butyric acid was found in the medium at this time. In storage processes prior to anaerobic
385 digestion, H₂ production has to be avoided because of the high energy potential of this molecule
386 (Kreuger et al., 2011). During this fermentation phase, the microbial concentration increased significantly
387 from 1.28·10⁸ to 9.60·10⁹ rRNA gene copy number·g_{FM}⁻¹ from day 21 to 60. The bacterial bloom was
388 mostly due to the growth of Lactobacillales and Enterobacteriales in a similar way as during the early
389 fermentation phase of silage. Clostridiales were also found in a smaller proportion. However, despite
390 similarities in the order of the most represented bacteria in the fermentation of wet alkaline and ensiled
391 samples, the actual microbial populations were different, as can be seen in Figure 4. In wet alkaline rye,
392 the bacteria from Lactobacillales order were mostly composed of *Enterococcus sp.* (OTU6), with also
393 *Carnobacterium sp.* (OTU8) present in a smaller proportion. *Enterococcus* bacteria are tolerant to pH
394 until 9.6, but not below 4.5 (Cai, 1999). *Enterococcus* are mostly known as homofermentative bacteria.
395 However high amounts of ethanol and acetic acid were found, indicating that other metabolic pathways
396 took place. Although being LAB, *Carnobacteria* grow at pH range of 7-9, and even until 10.4 for
397 *Carnobacterium maltaromaticum* that are found in the cold and alkaline tufa columns (Leisner et al.,
398 2007). This bacteria genus is mostly related to food spoilage of chilled fish, meat and dairy products
399 (Lorenzo et al., 2018). However, *Carnobacteria* can also be found in silage and are reported to participate
400 in the primary lactic acid fermentation following heterolactic mechanisms (Pahlow et al., 2003). Bacteria
401 from the Enterobacteriales order were mostly represented by a member of *Enterobacteriaceae* family
402 (OTU4). This *Enterobacteriaceae* may develop on WSC and amino acids to produce acetic acid and NH₃ in
403 destabilized alkaline storage in the same way as in silage, however the absence of further acidification
404 could not allow their inhibition. *Enterobacteriaceae* are also reported to be H₂ producers, which is
405 consistent with the observations that were made from day 21 to 60 (Cabrol et al., 2017). Additionally,
406 Clostridia from *Lachnospiraceae* family were observed (OTU41). Clostridia are highly reported as
407 undesired in silage fermentation. Clostridia proliferate mostly at pH > 5 and grow on amino acids, sugars
408 and lactic acid to produce mostly butyric acid, CO₂ and H₂; they thus cause BMP reduction (Kreuger et al.,
409 2011; Pahlow et al., 2003; Teixeira Franco et al., 2016). More specifically, *Lachnospiraceae* bacteria are
410 largely represented in the gut or rumen of mammals where pH conditions close to neutrality are met
411 (Evans et al., 1988). They are able to ferment diverse plant carbohydrates to VFA (butyrate, acetate,
412 propionate) and alcohols like ethanol (Vacca et al., 2020).

413 The transformations that occurred in wet alkaline rye during this storage phase shows the complexity of
414 inhibition mechanisms in alkaline storage. Despite a seemingly previous stable period of three weeks,

415 microbial bloom finally happened with fermentation patterns implying H₂ production and Clostridia
416 development that may lead to energy loss during the rest of storage. This shows that, in addition to CaO
417 loading and initial pH, moisture content of the medium plays a crucial role in alkaline stability.

418 3.3.1.3 Third phase (60 to 180 days)

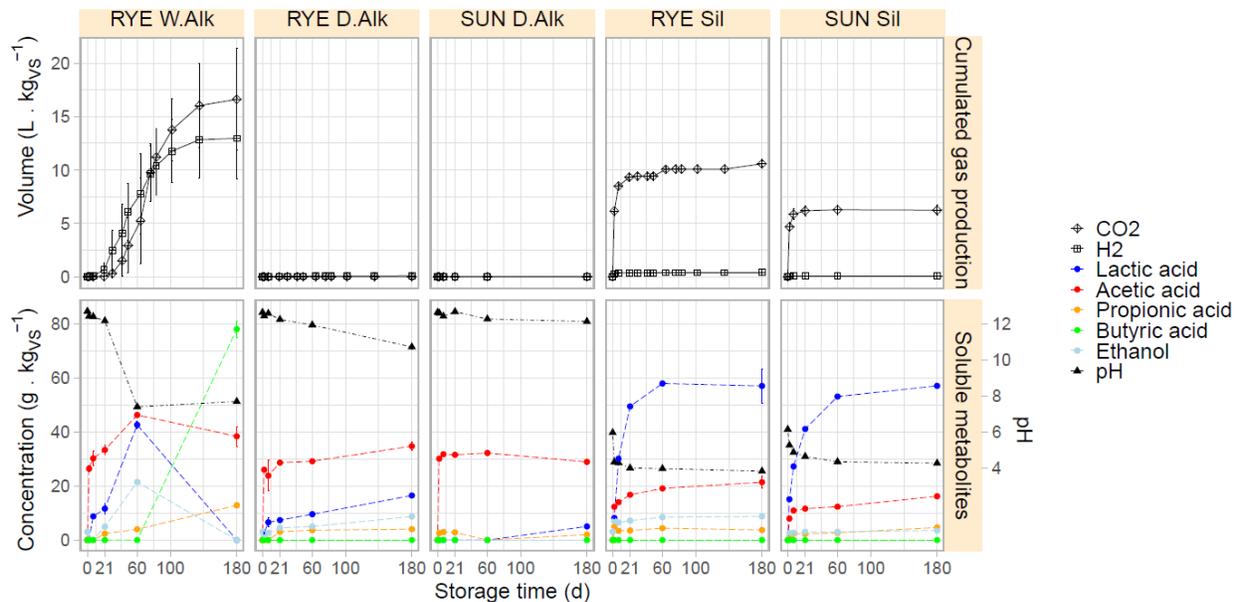
419 During this last phase of storage, no changes were observed in the silage conditions, nor in the alkaline
420 storage of sunflower in dry condition. In dry alkaline storage of rye, a slow heterolactic fermentation was
421 observed, similar to the one that was observed previously in wet alkaline rye. OTU8, OTU6 and OTU4
422 grew and were responsible for a low heterolactic fermentation, without causing a destabilization
423 comparable to the one observed in wet condition during phase 2. The bacterial development was
424 seemingly slowed down by the water availability that was lower than in wet alkaline samples. In wet
425 alkaline storage of rye, a secondary butyric fermentation took place. Most of the metabolites (i.e. lactic
426 acid, acetic acid and ethanol) released during the previous phase were converted into butyric acid until a
427 high concentration of 78 g·kg_{VS}⁻¹ was reached, similarly as observed by Van Vlierberghe et al. (2021). The
428 butyric fermentation was explained by a significant increase in the abundance of Clostridiales bacteria:
429 *Caproiciducens sp.* (OTU29) and *Clostridium sp.* (OTU13), replacing OTU41 (*Lachnospiraceae*) that was
430 found earlier. Concerning the bacteria from Lactobacillales order, no significant changes in abundance
431 nor composition of their population was observed.

432 From day 100, gas production and mass losses kinetics started to slow down in wet alkaline storage,
433 suggesting a decrease in the microbial activity. This final relative stable state could be explained by the
434 very high concentration of butyric acid that accumulated in the medium and inhibited microbial activity.
435 In wet alkaline rye, considering a pH 7.7 of the medium at this time, butyric acid was present at 99.9% in
436 the dissociated form according to the Henderson-Hasselbalch equation (Equation (1)).

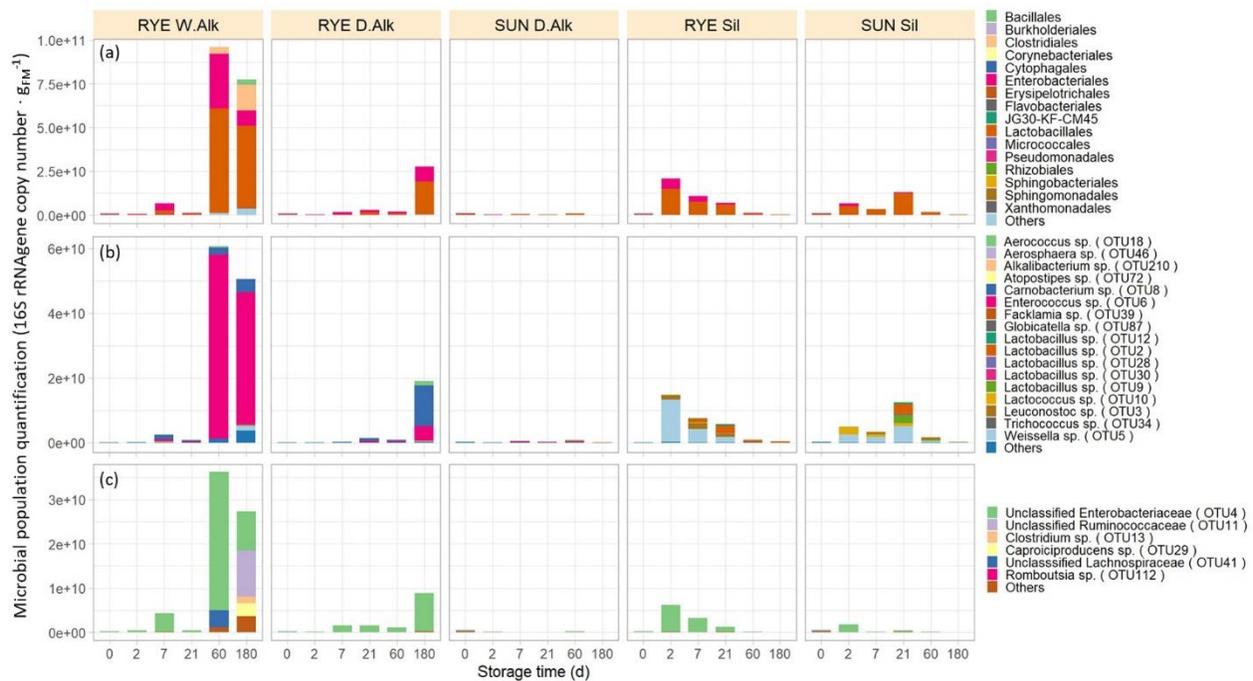
$$pH = pK_a + \log \frac{A^-}{AH} \quad (1)$$

437 where A⁻ = concentration of acid under its dissociated form, AH = concentration of acid under its
438 undissociated form. Butyric acid is known to have a strong inhibitory effect on fermentation, mostly in its
439 undissociated form that can cross the cell membrane. However, the dissociated form is also reported to
440 have an inhibitory effect from a high concentration due to the ionic strength that may result in the cell
441 lysis of hydrogen-producing bacteria (Van Niel et al., 2003). A strong inhibitory effect of butyrate on
442 fermentation for hydrogen production was reported in diverse studies. Zheng and Yu (2005) observed an
443 almost total inhibition of H₂ production with 25 g·L⁻¹ butyrate at initial pH 6.0 (93% of dissociated form).
444 Similar results were obtained in another study at 26.4 g·L⁻¹ butyrate at initial pH 7.0 (99.3% of dissociated

445 form) (Wang et al., 2008). In the present study, the final butyrate concentration of $78 \text{ g} \cdot \text{kg}_{\text{VS}}^{-1}$ is
 446 equivalent to $26.6 \text{ g} \cdot \text{L}^{-1}$ when reported to the water fraction of the samples. The high amount of
 447 butyrate present in the medium at the end of wet alkaline storage could therefore be responsible for the
 448 inhibition of the microbial activity and final stabilization of the biomass.
 449 Despite the similar initial properties of the different alkali-stored conditions, very different variations of
 450 the biomass properties occurred during storage as consecutive fermentations took place in some
 451 samples. This experiment confirmed that microbial fermentations were mostly responsible for the pH
 452 drop that can be observed in alkaline storage. Interestingly, if an elevated pH was shown to allow the
 453 inhibition of the microbial activity, the bacteria present in the medium were able to restart their activity
 454 from a high pH. As fermentation restarted, the pH decreased, which limited the inhibitory action of lime
 455 and allowed a fast bacterial growth. High pH (>12) and TS content ($>40\%$) were shown to provide the
 456 best anaerobic alkaline stability. In silage experiment, the lactic fermentation allowed an efficient
 457 acidification of the substrate that stopped the bacterial growth. As a result, no secondary fermentations
 458 occurred in silage. A third inhibition mechanism at neutral pH was observed, due to the high butyric acid
 459 concentration. This inhibition allowed to prevent further degradations of the substrate such as
 460 methanogenesis to happen, thus avoiding energy losses and greenhouse gas emissions during storage.



461
 462 *Figure 3: pH, metabolites and gaz variation during storage. CO₂ and H₂ are expressed in cumulated L.kgVS_{init}⁻¹. Metabolites are*
 463 *expressed in g.kgVS_{added}⁻¹. W.Aik, D.Aik and Sil stand for wet alkaline, dry alkaline and silage, respectively. SUN stands for*
 464 *sunflower. Error bars indicate standard deviation.*



465

466 *Figure 4: Major OTUs abundance (relative abundance >1.5% for at least one storage time) expressed in 16S rRNA gene copy.g^{FM}*
 467 *⁻¹. (a): Principal orders, (b): bacteria affiliated to Lactobacillales order, (c): bacteria affiliated to Enterobacteriales and Clostridiales*
 468 *orders. W.Alk, D.Alk and Sil stand for wet alkaline, dry alkaline, respectively. SUN stands for sunflower.*

469

470

471 3.3.2 Impact of storage conditions on biomass characteristics and methane potential

472 The impact of storage conditions on fiber distribution characteristics was evaluated according to the Van
 473 Soest and Wine method (Van Soest and Wine, 1967) after 180 days of reaction (**Erreur ! Source du**
 474 **renvoi introuvable.**). The alkaline storage had mostly a strong impact on the hemicellulose fraction, that
 475 was significantly reduced by 53, 57 and 29% for rye storage in wet and dry conditions, and sunflower in
 476 dry condition, respectively. The hemicellulose reduction could not be only explained by the deacetylation
 477 mechanisms associated with the measured acetic acid release that represents 10% of the raw material
 478 hemicellulose content in wet and dry alkaline storage, and 19% in sunflower. Consequently, an actual
 479 dissolution of hemicellulose occurred due to the pretreatment action. As the hemicellulose was
 480 solubilized, both soluble fractions W-SOLU and SOLU increased. Alkaline storage had little effect on
 481 cellulose that was significantly different only between the dry alkaline conditions and the silage of the
 482 same crop. These results are in agreement with other experiments from the literature, where lime
 483 pretreatment was shown to partly solubilize hemicellulose, while the cellulose was not degraded (Kim
 484 and Holtzapple, 2005). Considering the lignin fraction, a reduction of 10% and 21% was measured in dry
 485 alkaline storage of rye and sunflower, respectively. However, due to the high variability of the raw

486 material characteristics, these changes were not considered as significant by the pair-wise t-test adjusted
487 with Bonferroni correction. In the literature, alkaline pretreatment with lime is reported to partly
488 solubilize lignin. Delignification rates until 30-50% can be obtained with non-oxidative lime
489 pretreatments at mild temperature (25 to 55°C) (Kim and Holtzapfle, 2006; Wyman et al., 2005). The
490 limited delignification obtained in the present experiments may be explained by the low moisture
491 content applied combined with the use of CaO as a dry powder. Due to the low solubility of CaO (1.65
492 g·L⁻¹ at 20°C), only a small fraction of the added lime may have been solubilized and was actually
493 available for the pretreatment. In silage experiments, no significant changes in the share between the
494 different fractions were observed. As reported in the literature, the lignocellulosic matrix undergoes few
495 modifications in well preserved silages (Feng et al., 2018).

496 The gas production related to the different fermentation phases induced mass losses, mostly related to
497 the production of CO₂. For rye, mass losses of 7.2 ± 0.6, 0.5 ± 0.1 and 2.9 ± 0.1 % of initial VS were
498 measured in wet alkaline, dry alkaline and silage, respectively. For sunflower, the VS losses were limited
499 to 0.3 ± 0.0 and 1.9 ± 0.1 % in dry alkaline and silage, respectively. Similar VS losses from 2 to 4% are
500 normally observed in well preserved silage fermentation (Kaiser and Piltz, 2004; Kreuger et al., 2011).

501 BMP tests were performed on the fresh rye and sunflower used in storage experiment, along with
502 samples stored during 7, 60 and 180 days. The results are presented in Figure 5. In most conditions, the
503 biomass was efficiently stored and no significant BMP reduction was found after storage, excepted in
504 wet alkaline rye. The unstable wet alkaline storage of rye caused a significant BMP loss of 13%, probably
505 due to H₂ production (Kreuger et al., 2011), unlike previously observed by Van Vlierberghe et al. (2021)
506 where the gas productions did not cause any measurable BMP loss, even with a 70% higher H₂
507 production due to fermentation.

508 The stable alkaline storage experiments (e.g. dry alkaline rye and sunflower) induced a BMP increase of
509 respectively 10 and 21%. These changes were however considered as non-significant by the pair-wise t-
510 test adjusted with Bonferroni correction, probably due to the high variability of BMP test. A significant
511 increase of 15.7% was only observed between dry alkaline sunflower and sunflower silage. The results
512 show that a slight increase in BMP was obtained due to the long-term action of the pretreatment, since a
513 growing trend in BMP was only observed after 60 (sunflower) to 180 days (rye).

514 In the literature, BMP improvements of 17 to 37% (Thomas et al., 2018), 4 to 37% (Khor et al., 2015) and
515 7 to 34 % (Jiang et al., 2017) were obtained by pretreating similar lignocellulosic biomasses in mild
516 conditions. The lower methane yield obtained in this experiment could be explained by the fact that

517 drier conditions were applied, since moisture was pointed to have an impact on pretreatment efficiency
 518 (section 3.2 and (Digman et al., 2010)). TS of 29 to 47% were used here, while more wet conditions
 519 (around 10 %TS) were set in the literature. For both crops, silage storage allowed an efficient storage of
 520 the energy potential of the crop, showing the relevance of this process for crop storage for anaerobic
 521 digestion. As shown in the literature, a good management of silage allows to preserve efficiently the
 522 methane potential of crops (Teixeira Franco et al., 2016; Villa et al., 2020).

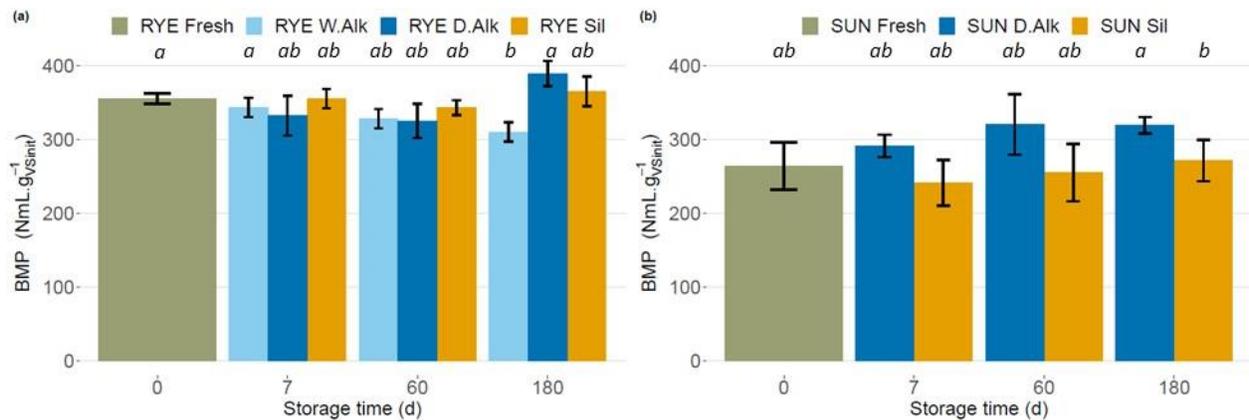
523 When stable alkaline conditions were obtained, alkaline storage showed a potential to provide a higher
 524 BMP of the crop, in comparison with storage by ensiling. However, a large amount of lime was required
 525 to obtain alkaline stability and may compromise the economical balance of the process. In a previous
 526 study, Jiang et al. (2017) found a positive net benefit of pretreatment when applying an alkaline
 527 pretreatment at 70 and 120 g Ca(OH)₂.kgTS⁻¹ (eq. 68 and 90 g CaO.kgTS⁻¹). Since alkaline stability is
 528 reached more easily at high TS, lower CaO loadings may be applied on dry crops like sunflower, which
 529 could increase the process sustainability. In order to conclude on the relevance of alkaline storage, the
 530 overall impact of lime addition on biomass valorization including aerobic stability at silo opening and
 531 continuous digestion of the crop should be investigated.

532 *Table 3: Fiber distribution of the fresh and 180 d stored crops expressed in %VSinit. Within each column, different letters (abc)*
 533 *express a statistical difference between two samples of the same crop species stored under different conditions.*

		W-SOLU	SOLU	HEMI	CELL	LIG
<i>Rye</i>	Fresh	29.5 ± 0.9 ^a	3.3 ± 1.1 ^{ac}	26.1 ± 1.5 ^a	31.6 ± 3 ^a	9.2 ± 3.6 ^a
	Wet Alkaline	29.4 ± 4.5 ^a	8.5 ± 1.3 ^b	12.2 ± 1.7 ^b	33 ± 0.9 ^a	9 ± 0.9 ^a
	Dry Alkaline	40.6 ± 7.8 ^b	5.8 ± 3.2 ^{ab}	11.2 ± 1.2 ^b	30.1 ± 0.9 ^a	8.3 ± 1 ^a
	Silage	29 ± 2.1 ^a	0.4 ± 1.3 ^c	22.8 ± 1.8 ^a	35.9 ± 3.1 ^a	10 ± 2.4 ^a
<i>Sunflower</i>	Fresh	21.4 ± 5.5 ^a	13 ± 4.6 ^{ab}	15.6 ± 0.2 ^a	27.8 ± 1.7 ^{ab}	16.5 ± 2.9 ^a
	Dry alkaline	23 ± 0.5 ^a	19.7 ± 1.2 ^a	11 ± 0.4 ^b	24.8 ± 0.1 ^a	13.1 ± 0.7 ^a
	Silage	20.7 ± 0.4 ^a	11.7 ± 0.4 ^b	13.5 ± 1.4 ^a	29.8 ± 1 ^b	15.2 ± 0.7 ^a

534

535



536

537 *Figure 5: Impact of storage condition and duration on BMP. The error bars indicate standard deviation. W.Alk, D.Alk and Sil stand*
538 *for wet alkaline, dry alkaline, respectively. SUN stands for sunflower.*

539

540

541 4 Conclusion

542 Both alkaline and acid pH related to alkaline storage and silage allowed to inhibit the microbial activity
543 over a long period. In alkaline storage, besides the initial lime loading, moisture was shown to influence
544 storage stability. Despite of a stability period of 3 weeks, the bacteria present in the wet condition were
545 able to restart their activity from an elevated pH of 12, causing a drop in pH and allowing a succession of
546 fermentations to take place and limited but significant BMP losses. In successful alkaline storage, the
547 long term pretreatment action allowed a BMP increase until nearly 16%.

548

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556 6 References

- 557 1 Büchi, L., Wendling, M., Amossé, C., Jeangros, B., Charles, R., 2020. Field Crops Research Cover
558 crops to secure weed control strategies in a maize crop with reduced tillage. *F. Crop. Res.* 247,
559 107583. <https://doi.org/10.1016/j.fcr.2019.107583>
- 560 2 Bureenok, S., Sisaath, K., Yuangklang, C., Vasupen, K., Schonewille, J.T., 2016. Ensiling
561 characteristics of silages of Stylo legume (*Stylosanthes guianensis*), Guinea grass (*Panicum*
562 *maximum*) and their mixture, treated with fermented juice of lactic bacteria, and feed intake and
563 digestibility in goats of rations based on these silages. *Small Rumin. Res.* 134, 84–89.
564 <https://doi.org/10.1016/j.smallrumres.2015.12.006>
- 565 3 Cabrol, L., Marone, A., Tapia-Venegas, E., Steyer, J.P., Ruiz-Filippi, G., Trably, E., 2017. Microbial
566 ecology of fermentative hydrogen producing bioprocesses: Useful insights for driving the
567 ecosystem function. *FEMS Microbiol. Rev.* 41, 158–181. <https://doi.org/10.1093/femsre/fuw043>
- 568 4 Cai, Y., 1999. Identification and characterization of *Enterococcus* species isolated from forage
569 crops and their influence on silage fermentation. *J. Dairy Sci.* 82, 2466–2471.
570 [https://doi.org/10.3168/jds.S0022-0302\(99\)75498-6](https://doi.org/10.3168/jds.S0022-0302(99)75498-6)
- 571 5 Carrere, H., Antonopoulou, G., Affes, R., Passos, F., Battimelli, A., Lyberatos, G., Ferrer, I., 2016.
572 Review of feedstock pretreatment strategies for improved anaerobic digestion: From lab-scale
573 research to full-scale application. *Bioresour. Technol.* 199, 386–397.
574 <https://doi.org/10.1016/J.BIORTECH.2015.09.007>
- 575 6 Castro, R.C. de A., Fonseca, B.G., dos Santos, H.T.L., Ferreira, I.S., Mussatto, S.I., Roberto, I.C.,
576 2017. Alkaline deacetylation as a strategy to improve sugars recovery and ethanol production
577 from rice straw hemicellulose and cellulose. *Ind. Crops Prod.* 106, 65–73.
578 <https://doi.org/10.1016/j.indcrop.2016.08.053>
- 579 7 Chang, V.S., Nagwani, M., Holtzapple, M.T., 1998. Lime pretreatment of crop residues bagasse
580 and wheat straw. *Appl. Biochem. Biotechnol. - Part A Enzym. Eng. Biotechnol.* 74, 135–159.
581 <https://doi.org/10.1007/BF02825962>
- 582 8 Chen, X., Shekero, J., Franden, M.A., Wang, W., Zhang, M., Kuhn, E., Johnson, D.K., Tucker, M.P.,
583 2012. The impacts of deacetylation prior to dilute acid pretreatment on the bioethanol process.
584 *Biotechnol. Biofuels* 5. <https://doi.org/10.1186/1754-6834-5-8>
- 585 9 Chen, X., Shekero, J., Pschorn, T., Sabourin, M., Tao, L., Elander, R., Park, S., Jennings, E., Nelson,
586 R., Trass, O., Flanagan, K., Wang, W., Himmel, M.E., Johnson, D., Tucker, M.P., 2014. A highly
587 efficient dilute alkali deacetylation and mechanical (disc) refining process for the conversion of

- 588 renewable biomass to lower cost sugars. *Biotechnol. Biofuels* 7. <https://doi.org/10.1186/1754->
589 6834-7-98
- 590 10 Daniel-Gromke, J., Rensberg, N., Denysenko, V., Stinner, W., Schmalfuß, T., Scheftelowitz, M.,
591 Nelles, M., Liebetrau, J., 2018. Current Developments in Production and Utilization of Biogas and
592 Biomethane in Germany. *Chemie Ing. Tech.* 90, 17–35. <https://doi.org/10.1002/cite.201700077>
- 593 11 Dauptain, K., Trably, E., Santa-Catalina, G., Bernet, N., Carrere, H., 2020. Role of indigenous
594 bacteria in dark fermentation of organic substrates. *Bioresour. Technol.* 313, 123665.
595 <https://doi.org/10.1016/j.biortech.2020.123665>
- 596 12 Digman, M.F., Shinnars, K.J., Casler, M.D., Dien, B.S., Hatfield, R.D., Jung, H.-J.G., Muck, R.E.,
597 Weimer, P.J., 2010. Optimizing on-farm pretreatment of perennial grasses for fuel ethanol
598 production. *Bioresour. Technol.* 101, 5305–5314. <https://doi.org/10.1016/j.biortech.2010.02.014>
- 599 13 Downing, T.W., Buyserie, A., Gamroth, M., French, P., 2008. Effect of Water Soluble
600 Carbohydrates on Fermentation Characteristics of Ensiled Perennial Ryegrass. *Prof. Anim. Sci.* 24,
601 35–39. [https://doi.org/10.15232/S1080-7446\(15\)30807-X](https://doi.org/10.15232/S1080-7446(15)30807-X)
- 602 14 Elferink, S.J.W.H., Driehuis, F., Gottschal, J.C., Spoelstra, S.F., 1999. Silage fermentation
603 processes and their manipulation. *FAO Plant Prod. Prot. Pap.* 17–30.
- 604 15 Evans, D.F., Pye, G., Bramley, R., Clark, a G., Dyson, T.J., Hardcastle, J.D., 1988. Measurement of
605 gastrointestinal pH profiles in normal ambulant human subjects. *Gut* 29, 1035–1041.
606 <https://doi.org/10.1136/gut.29.8.1035>
- 607 16 Feng, L., Kristensen, E.F., Moset, V., Ward, A.J., Møller, H.B., 2018. Ensiling of tall fescue for
608 biogas production: Effect of storage time, additives and mechanical pretreatment. *Energy*
609 *Sustain. Dev.* 47, 143–148. <https://doi.org/10.1016/j.esd.2018.10.001>
- 610 17 Gharechahi, J., Kharazian, Z.A., Sarikhan, S., Jouzani, G.S., Aghdasi, M., Hosseini Salekdeh, G.,
611 2017. The dynamics of the bacterial communities developed in maize silage. *Microb. Biotechnol.*
612 10, 1663–1676. <https://doi.org/10.1111/1751-7915.12751>
- 613 18 Herrmann, C., Heiermann, M., Idler, C., 2011. Effects of ensiling, silage additives and storage
614 period on methane formation of biogas crops. *Bioresour. Technol.* 102, 5153–5161.
615 <https://doi.org/10.1016/J.BIORTECH.2011.01.012>
- 616 19 Hillion, M. Lou, Moscoviz, R., Trably, E., Leblanc, Y., Bernet, N., Torrijos, M., Escudié, R., 2018. Co-
617 ensiling as a new technique for long-term storage of agro-industrial waste with low sugar
618 content prior to anaerobic digestion. *Waste Manag.* 71, 147–155.
619 <https://doi.org/10.1016/j.wasman.2017.10.024>

- 620 20 Holliger, C., Alves, M., Andrade, D., Angelidaki, I., Astals, S., Baier, U., Bougrier, C., Buffière, P.,
621 Carballa, M., De Wilde, V., Ebertseder, F., Fernández, B., Ficara, E., Fotidis, I., Frigon, J.C., De
622 Laclos, H.F., Ghasimi, D.S.M., Hack, G., Hartel, M., Heerenklage, J., Horvath, I.S., Jenicek, P., Koch,
623 K., Krautwald, J., Lizasoain, J., Liu, J., Mosberger, L., Nistor, M., Oechsner, H., Oliveira, J.V.,
624 Paterson, M., Pauss, A., Pommier, S., Porqueddu, I., Raposo, F., Ribeiro, T., Pfund, F.R.,
625 Strömberg, S., Torrijos, M., Van Eekert, M., Van Lier, J., Wedwitschka, H., Wierinck, I., 2016.
626 Towards a standardization of biomethane potential tests. *Water Sci. Technol.* 74, 2515–2522.
627 <https://doi.org/10.2166/wst.2016.336>
- 628 21 Humbird, D., Davis, R., Tao, L., Kinchin, C., Hsu, D., Aden, A., Schoen, P., Lukas, J., Olthof, B.,
629 Worley, M., Sexton, D., Dudgeon, D., 2011. *Process Design and Economics for Biochemical*
630 *Conversion of Lignocellulosic Biomass to Ethanol: Dilute-Acid Pretreatment and Enzymatic*
631 *Hydrolysis of Corn Stover, Pharmaceutical Extrusion Technology.* Golden, CO (United States).
632 <https://doi.org/10.2172/1013269>
- 633 22 Igos, E., Golkowska, K., Koster, D., Vervisch, B., Benetto, E., 2016. Using rye as cover crop for
634 bioenergy production: An environmental and economic assessment. *Biomass and Bioenergy* 95,
635 116–123. <https://doi.org/10.1016/j.biombioe.2016.09.023>
- 636 23 Jian, J., Du, X., Reiter, M.S., Stewart, R.D., 2020. A meta-analysis of global cropland soil carbon
637 changes due to cover cropping. *Soil Biol. Biochem.* 143, 107735.
638 <https://doi.org/10.1016/j.soilbio.2020.107735>
- 639 24 Jiang, D., Ge, X., Zhang, Q., Zhou, X., Chen, Z., Keener, H., Li, Y., 2017. Comparison of sodium
640 hydroxide and calcium hydroxide pretreatments of giant reed for enhanced enzymatic
641 digestibility and methane production. *Bioresour. Technol.* 244, 1150–1157.
642 <https://doi.org/10.1016/J.BIORTECH.2017.08.067>
- 643 25 Kaiser, A.G., Piltz, J.W., 2004. Successful silage.
- 644 26 Khor, W.C., Rabaey, K., Vervaeren, H., 2015. Low temperature calcium hydroxide treatment
645 enhances anaerobic methane production from (extruded) biomass. *Bioresour. Technol.* 176,
646 181–188. <https://doi.org/10.1016/J.BIORTECH.2014.11.037>
- 647 27 Kim, S., Holtzapple, M.T., 2006. Delignification kinetics of corn stover in lime pretreatment.
648 *Bioresour. Technol.* 97, 778–785. <https://doi.org/10.1016/j.biortech.2005.04.002>
- 649 28 Kim, S., Holtzapple, M.T., 2005. Lime pretreatment and enzymatic hydrolysis of corn stover.
650 *Bioresour. Technol.* 96, 1994–2006. <https://doi.org/10.1016/J.BIORTECH.2005.01.014>
- 651 29 Kreuger, E., Nges, I., Björnsson, L., 2011. Ensiling of crops for biogas production: Effects on

- 652 methane yield and total solids determination. *Biotechnol. Biofuels* 4, 1–8.
653 <https://doi.org/10.1186/1754-6834-4-44>
- 654 30 Leisner, J.J., Laursen, B.G., Prévost, H., Drider, D., Dalgaard, P., 2007. *Carnobacterium*: Positive
655 and negative effects in the environment and in foods. *FEMS Microbiol. Rev.* 31, 592–613.
656 <https://doi.org/10.1111/j.1574-6976.2007.00080.x>
- 657 31 Lorenzo, J.M., Munekata, P.E., Dominguez, R., Pateiro, M., Saraiva, J.A., Franco, D., 2018. Main
658 groups of microorganisms of relevance for food safety and stability: General aspects and overall
659 description, *Innovative technologies for food preservation: Inactivation of spoilage and*
660 *pathogenic microorganisms*. Elsevier Inc. <https://doi.org/10.1016/B978-0-12-811031-7.00003-0>
- 661 32 McNulty, P.B., Kennedy, S., 1982. Density Measurements of Grass by Toluene Displacement and
662 Air Comparison Pycnometry. *Irish J. Agric. Reserach* 21, 75–83.
- 663 33 Molinuevo-Salces, B., Fernández-Varela, R., Uellendahl, H., 2014. Key factors influencing the
664 potential of catch crops for methane production. *Environ. Technol. (United Kingdom)* 35, 1685–
665 1694. <https://doi.org/10.1080/09593330.2014.880515>
- 666 34 Molinuevo-Salces, B., Larsen, S.U., Ahring, B.K., Uellendahl, H., 2013. Biogas production from
667 catch crops: Evaluation of biomass yield and methane potential of catch crops in organic crop
668 rotations. *Biomass and Bioenergy* 59, 285–292. <https://doi.org/10.1016/j.biombioe.2013.10.008>
- 669 35 Monlau, F., Barakat, A., Trably, E., Dumas, C., Steyer, J.-P., Carrère, H., 2013. Lignocellulosic
670 Materials Into Biohydrogen and Biomethane: Impact of Structural Features and Pretreatment.
671 *Crit. Rev. Environ. Sci. Technol.* 43, 260–322. <https://doi.org/10.1080/10643389.2011.604258>
- 672 36 Pahlow, G., Muck, R.E., Driehuis, F., Elferink, S.J.W.H.O., Spoelstra, S.F., 2003. Microbiology of
673 Ensiling, in: *Silage Science and Technology*. pp. 31–93.
674 <https://doi.org/10.2134/agronmonogr42.c2>
- 675 37 Pakarinen, A., Maijala, P., Jaakkola, S., Stoddard, F., Kymäläinen, M., Viikari, L., 2011. Evaluation
676 of preservation methods for improving biogas production and enzymatic conversion yields of
677 annual crops. *Biotechnol. Biofuels* 4, 20. <https://doi.org/10.1186/1754-6834-4-20>
- 678 38 Porter, M.G., Murray, R.S., 2001. The volatility of components of grass silage on oven drying and
679 the inter-relationship between dry-matter content estimated by different analytical methods.
680 *Grass Forage Sci.* 56, 405–11. <https://doi.org/10.1046/j.1365-2494.2001.00292.x>
- 681 39 Shahid, S.A., Zaman, M., Heng, L., 2018. Soil Salinity: Historical Perspectives and a World
682 Overview of the Problem. *Guidel. Salin. Assessment, Mitig. Adapt. Using Nucl. Relat. Tech.* 43–
683 53. https://doi.org/10.1007/978-3-319-96190-3_2

- 684 40 Teixeira Franco, R., Buffière, P., Bayard, R., 2016. Ensiling for biogas production: Critical
685 parameters. A review. *Biomass and Bioenergy* 94, 94–104.
686 <https://doi.org/10.1016/j.biombioe.2016.08.014>
- 687 41 Thomas, H., Seira, J., Escudie, R., Carrère, H., 2018. Lime Pretreatment of Miscanthus: Impact on
688 BMP and Batch Dry Co-Digestion with Cattle Manure. *Molecules* 23, 1608.
689 <https://doi.org/10.3390/molecules23071608>
- 690 42 Vacca, M., Celano, G., Calabrese, F.M., Portincasa, P., Gobbetti, M., De Angelis, M., 2020. The
691 controversial role of human gut lachnospiraceae. *Microorganisms* 8, 1–25.
692 <https://doi.org/10.3390/microorganisms8040573>
- 693 43 Van Niel, E.W.J., Claassen, P.A.M., Stams, A.J.M., 2003. Substrate and product inhibition of
694 hydrogen production by the extreme thermophile, *Caldicellulosiruptor saccharolyticus*.
695 *Biotechnol. Bioeng.* 81, 255–262. <https://doi.org/10.1002/bit.10463>
- 696 44 Van Soest, P.J., Wine, R.H., 1967. Use of Detergents in the Analysis of Fibrous Feeds . IV .
697 Determination of Plant Cell-Wall Constituents. *J. Assoc. Off. Anal. Chem.* 50, 50–55.
- 698 45 Van Vlierberghe, C., Escudie, R., Bernet, N., Frederic, S., Carrere, H., 2021. Long term alkaline
699 storage and pretreatment process of cover crops for anaerobic digestion. *Bioresour. Technol.*
700 330, 124986. <https://doi.org/10.1016/j.biortech.2021.124986>
- 701 46 Villa, R., Ortega Rodriguez, L., Fenech, C., Anika, O.C., 2020. Ensiling for anaerobic digestion: A
702 review of key considerations to maximise methane yields. *Renew. Sustain. Energy Rev.* 134,
703 110401. <https://doi.org/10.1016/j.rser.2020.110401>
- 704 47 Wang, B., Wan, W., Wang, J., 2008. Inhibitory effect of ethanol, acetic acid, propionic acid and
705 butyric acid on fermentative hydrogen production. *Int. J. Hydrogen Energy* 33, 7013–7019.
706 <https://doi.org/10.1016/j.ijhydene.2008.09.027>
- 707 48 Wyman, C.E., Dale, B.E., Elander, R.T., Holtzapple, M., Ladisch, M.R., Lee, Y.Y., 2005. Coordinated
708 development of leading biomass pretreatment technologies. *Bioresour. Technol.* 96, 1959–1966.
709 <https://doi.org/10.1016/J.BIORTECH.2005.01.010>
- 710 49 Yang, Z., Piironen, V., Lampi, A.M., 2019. Epoxy and hydroxy fatty acids as non-volatile lipid
711 oxidation products in oat. *Food Chem.* 295, 82–93.
712 <https://doi.org/10.1016/j.foodchem.2019.05.052>
- 713 50 Zheng, X.J., Yu, H.Q., 2005. Inhibitory effects of butyrate on biological hydrogen production with
714 mixed anaerobic cultures. *J. Environ. Manage.* 74, 65–70.
715 <https://doi.org/10.1016/j.jenvman.2004.08.015>

