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Abstract

Complex organic substrates represent an important and relevant feedstock for producing hydrogen by Dark Fermentation (DF). Usually, an external microbial inoculum originated from various natural environments is added to seed the DF reactors. However, H_2 yields are significantly impacted by the inoculum origin and the storage conditions as microbial community composition can fluctuate. This study aims to determine how the type and time of inoculum storage can impact the DF performances. Biochemical Hydrogen Potential tests were carried out using three substrates (glucose, the organic fraction of municipal solid waste, and food waste), inocula of three different origins, different storage conditions (freezing or freeze-drying) and duration. As a result, H_2 production from glucose with the differently stored inocula was significantly impacted (positively or negatively) and was inoculum-origin-dependent. For complex substrates, hydrogen yields with the stored inocula were not statistically different from the fresh inocula, offering the possibility to store an inoculum.

Keywords

Biohydrogen; Dark fermentation; Inoculum storage; Freezing; Freeze-drying

1. Introduction

In an energy transition context, dihydrogen appears as a sustainable clean fuel candidate to limit climate change. Indeed, H_2 has a clean combustion with no carbon dioxide

released and it is also an excellent energy carrier with a high energy storage capacity (33 kWh/kg) (Pudukudy et al., 2014). Considering H_2 production from renewable energies, life cycle analyses (LCA) have shown that hydrogen vehicles allowed a significant reduction of greenhouse gas emissions and other environmental parameters compared to gasoline vehicles (Ahmadi and Kjeang, 2015). However, worldwide hydrogen production mainly comes from fossil fuel technologies (>95%) and only 1% of H_2 is produced from biomass (Hosseini and Wahid, 2016). Among these techniques, biohydrogen can be produced by dark fermentation (DF) of biomass. A wide range of substrates are used in DF process, such as simple substrates as sugars (glucose, xylose, starch) (Chatellard et al., 2016), food waste (FW) (Parthiba Karthikeyan et al., 2018) and the organic fraction of municipal solid waste (OFMSW). Although some studies tackled the issue of DF without external inoculum (Dauptain et al., 2020), an inoculum originated from various natural environments such as sewage sludge or anaerobic digestate is frequently added to perform DF (Kotay and Das, 2009). Indeed, according to Ghimire et al. (2015), a seed inoculum is necessary to start the hydrogen production process. The fermentative hydrogen production by mixed microbial cultures is limited by a low substrate conversion rate into H_2 and can be significantly impacted by the inoculum origin and the sampling time as microbial community composition can fluctuate over time (Ghimire et al., 2016). Indeed, Pecorini et al. (2019) noticed that H_2 production was significantly impacted by the inoculum origin with hydrogen yield from 29 to 90 mLH_2/gVS_{sub} for FW.

Inoculum composition (TS, VS and microbial composition) may vary significantly from one inoculum to another as reported by Pecorini et al. (2019) and (Toledo-Alarcón et al., 2020), respectively. Indeed, Pecorini et al. (2019) noticed various pH (6.7 to 8.2),

TS (2.1% to 3.2%), C/N ratio (3.9 to 7.9) and light metal concentrations (as magnesium) for 4 different inocula. Microbial communities from different inocula can be significantly different as reported by Toledo-Alarcón et al. (2020) with no H_2 producers as most abundant bacteria. Indeed, the authors observed *Spirochaetaceae* (12.4%) / Rikenellaceae (18.8%) as the main family for the aerobic sludge inoculum and the anaerobic sludge inoculum, respectively. The microbial communities may also vary according to the season (temperature changes) for a same inoculum as mentioned by Flowers et al. (2013) for activated sludge. This hypothesis was also confirmed by Chatellard (2016), who noticed some changes over time in the initial microbial communities of an aerobic sludge sampled at different times (January and April). For the inoculum sampled in January /April, *Clostridiaceae* / *Enterobacteriaceae* was the main family, respectively. As microbial communities can fluctuate over time (Flowers et al. 2013) and significantly impact H_2 production (Pecorini et al., 2019), inoculum storage appears as a major issue, which may greatly influence the final hydrogen production in DF. Freezing is a common storage technique for a wide range of applications and has been extensively studied to preserve the fermentative activity of fecal inoculum for in vitro fermentation (Murray et al., 2012). Freezing and thawing has also been investigated as an inoculum pretreatment technique to select Hydrogen-Producing Bacteria (HPB) to increase H_2 yield (Kotay and Das, 2009). Freeze-drying is another storage method and has also been utilized to store fecal microbiota (Affagard et al., 2018).

As inoculum can greatly influence hydrogen yields (Pecorini et al., 2019) and final microbial communities (Toledo-Alarcón et al., 2020), inoculum storage is a topic of interest as it can be used to perform several runs of experiments staggered in time at the laboratory scale (to limit inoculum effect), help to restart a process after a failure at

larger scale, or to stabilize a process after a microbial population shift due to a process disruption. The impact of inoculum storage has been scarcely investigated for DF process. Indeed, only the work of Chatellard (2016) focused on inoculum storage for the production of hydrogen from a simple substrate (i.e., glucose). The authors stored a manure digestate inoculum and an activated sludge inoculum for 2 months at 35°C with no feeding and showed a decrease in H_2 production compared to the fresh inoculum (glucose as substrate). However, to the author knowledge, inoculum storage by freezing or freeze-drying has never been investigated in a dark fermentation context. Lack of information in inoculum storage is particularly detrimental to ensure reliable comparisons between DF experiments carried out at a different time with a same but stored inoculum. In order to increase the H_2 production, the inoculum is usually pretreated to specifically select spore-forming HPB and to limit the growth of Hydrogen-Consuming Bacteria (HCB). As a result, a large number of studies tackled the issue of inoculum or substrate pretreatment to increase hydrogen yield (Rafieenia et al., 2018). Nevertheless, comparing different pretreatment techniques (inoculum or substrate) in the literature can be controversial as the hydrogen yield is impacted by the inoculum origin. A limited number of studies have evaluated the efficiency of different pretreatments on a same substrate and with a same inoculum (Rafieenia et al., 2018). The main objective of the present work was to investigate whether the storage of an inoculum by freezing or freeze-drying, over a short (1 week) or long period (1.5 months), could impact the DF performances and the final microbial composition of various substrates (glucose, OFMSW or FW).

2. Materials and methods

2.1. Substrate preparations

Three different substrates were used to perform Biochemical Hydrogen Potential (BHP) tests (glucose, OFMSW, FW). Glucose was used as a control due to its simple structure (monomer) and the numerous uses in the literature. OFMSW and FW were considered as complex organic matter and were reconstituted each week in the laboratory to avoid time variability of an industrial substrate and to avoid substrate storage, which may affect their composition over time. Volatile solid (VS) and total solid (TS) contents were measured each time in triplicates for complex substrates and inocula according to the APHA standard method (APHA, 1999). The composition, TS and VS of synthetic complex substrates are given in supplementary material. For FW, all ingredients were bought at the supermarket. They were initially frozen. The industrial food ensured an accurate and reproducible composition (as carbohydrate content) given by the product labels. In addition to the aforementioned ingredients, OFMSW contained dry substrates with a stable composition over time, which allowed similar composition of the entire preparations. Complex substrates were selected for their complex structures (possible bacterial selection) (Chatellard et al., 2016) and for the existence of indigenous bacteria (bacteria naturally persisting on the substrate), which may strongly influence DF performances (Dauptain et al., 2020). Complex organic matter was shredded in a same manner as reported elsewhere (Dauptain et al., 2020). FW and OFMSW were thermally pretreated at 70°C for 1 h as required by the French regulation for animal by-products.

2.2. Inocula and BHP tests

Three different inocula were used for BHP tests. They corresponded to an activated sludge sampled from a wastewater treatment plant (WWTP) in Narbonne (France), a digestate from an anaerobic digestion (AD) plant treating livestock manure and industrial FW (Ennezat, France) and a leachate from a landfill in Albi (France). To

select spore-forming HPB and to increase H_2 yield, each inoculum was thermally pretreated at 90°C for 15 min before use (Parthiba Karthikeyan et al., 2018). For a same inoculum and substrate, five different conditions were evaluated: an unstored inoculum (fresh), a frozen inoculum (stored at -20°C for 1 week or 1.5 months) and a freeze-dried inoculum (stored in a closed box at room temperature for 1 week or 1.5 months). BHP tests were carried out in batch tests in quadruplicates at 37°C (water bath) and without stirring. Thereafter, the flask with the complex substrate was thermally pretreated at 70°C for 1 h (TS of 10%). Then, the BHP flasks (550 mL – 350 mL of working volume) were exactly prepared as reported in a previous work (Dauptain et al., 2020). The substrate to inoculum ratio (S/X) was set to 20 ± 5 (gVS / gVS) for complex substrates and to 10 ± 3 (gVS / gVS) for glucose. TS and VS values of the inocula were measured after the thermal pretreatment. Each BHP flask contains either 1 g_{VS} for glucose or 4 g_{VS} for complex organic matter. Initial pH was set to 6 ± 0.1 with NaOH or HCl if needed. When H_2 production remained constant (constant pressure), the BHP tests were stopped to avoid hydrogen consumption by homoacetogenesis.

2.3. Gas composition and metabolite analyses

An automatic micro-gas chromatograph (µ-GC) [SRA l-GC R3000] was used to monitor (every two hours) the gas composition (H_2, O_2, N_2, CH_4 and CO_2) and the pressure of the BHP flask head space. Volatile fatty acids were measured after fermentation by a gas chromatography. Other metabolites of the fermentative broth were measured by an HPLC (High Performance Liquid Chromatography). Prior to analysis, the samples were centrifuged 15 min at 13,000 g and filtered at 0.2 µm. All information regarding the µ-GC, the GC or the HPLC are further described in a previous work (Dauptain et al., 2020).

2.4. Microbiological analyses

For each condition, the microbial communities of one or two replicates among the quadruplicate were analyzed after fermentation. The replicate with the closest value to the quadruplicate average was chosen for analysis. All information regarding microbiological analyses (DNA extraction, PCR, sequencing, bioinformatic procedure) have been exactly performed as reported in a previous work (Dauptain et al., 2020). If needed, the nucleotide sequences of some operational taxonomic units (OTUs) were submitted to a blast search (www.ncbi. nlm.nih.gov/BLAST) in order to identify them to the closest bacterial strains.

2.5. Statistical analysis

To identify a possible statistical difference between the hydrogen production of two conditions (for a same substrate and a same inoculum), a Tukey's test was performed (glht function - multcomp R package). The correlation matrix with Pearson's coefficients were built to confirm some observations by the cor function of the corrplot R package. The same replicates as for the microbial community analyses were considered to build the correlation matrix.

3. Results and discussion

3.1 Impact of inoculum storage on DF performances of glucose

3.1.1 Impact of inoculum storage on hydrogen yield from glucose

The average and standard deviations of the maximum cumulative hydrogen productions of glucose and complex substrates are presented for all inocula (WWTP, AD, Leachate) and all storage conditions in Table 1. For glucose, the storage of WWTP inoculum led at least to similar H_2 productions compared to the fresh inoculum. Surprisingly, after a one-week-storage by freezing of WWTP inoculum, the H_2 yield (144 ± 10

mLH_2/gVS_{ini}) was higher than the fresh inoculum (112 ± 14 mLH_2/gVS_{ini}). Fig. 1 (A, B and C) presents the abundance of the main microbial communities at the order level at the end of fermentation for glucose, OFMSW and FW, respectively. Fig. 2 sums up the microbial communities at the genus level for all substrates and inocula. Fig. 3 (A and B) show the correlation matrix with the Pearson coefficients between the main microbial communities (order or genus level) and the main produced metabolites for glucose and complex substrates, respectively. According to Fig. 1A, the increase of hydrogen production can be explained by *Clostridiales* population. The relative abundance in *Clostridiales* increased from 15.4% for the fresh WWTP inoculum to 40.1% for the 1-week frozen WWTP inoculum. This increase in *Clostridiales* proportion was associated to a similar decrease in *Enterobacteriales* proportion (from 75.7% to 46.4%). This suggests that inoculum storage by freezing could be a way to pretreat the inoculum and to increase the proportions in spore-forming HPB as *Clostridiales*. Fig. 3A supports these conclusions by showing that *Clostridium sp.* are correlated to H_2 production as consistently reported elsewhere (Dauptain et al., 2020). After a 1.5-month-storage by freezing, hydrogen production was not anymore statistically different from the fresh inoculum. The similar yields are due to similar compositions in *Clostridiales*, *Enterobacteriales* and *Bacillales* (Fig. 1A). This result indicates that it is possible to store WWTP inoculum by freezing over a 1.5-month period. However, *Enterobacteriales* population increased after a longer storage, from 46.4% for short freezing to 69.5% for long freezing. This result is surprising as Murray et al. (2012) reported that bacterial cell membrane of *Enterobacteriales* (gram-negative bacteria) could be damaged by freezing and thawing. However, Kaprelyants et al. (1993) reported a dormant state for non-sporulating bacteria, which could allow some *Enterobacteriales* to

survive to stressful conditions. It seems that *Enterobacteriales* and especially *Escherichia-Shigella* (Fig. 2) can stay viable after a long freezing followed by a heat treatment.

For freeze-drying, *Enterobacteriales* population decreased with the storage time (37.1% for a 1.5-month storage and 75.7% for the fresh inocula), which is consistent with Zambon et al. (2019), who observed a decrease in the number of viable *Enterobacteriaceae* colonies after an apple slice storage (a year) by freeze-drying. For freezing, the *Enterobacteriales* relative abundance decreased to 46.4% after a short storage and increased again to 69.5% after a 1.5-month storage. This might come from interactions with minor OTU (Cabrol et al., 2017) but also due to the competition between *Enterobacteriales* and *Clostridiales* as reported by Fig. 3A (strong negative correlation). Indeed, *Clostridiales* (sporulating bacteria) probably need more time to be viable again compared to surviving *Enterobacteriales* (kinetic advantage), which might have changed according to the storage conditions. This assumption is supported by Mearls et al. (2012), who observed a longer lag time (30 hours) for *Clostridium thermocellum* (sporulating form) to reach an optical density of 0.1 compared to the non-sporulating form (20 hours). Moreover, according to the microbial communities given in supplementary material at T0 (after thermal pretreatment of the inoculum and before fermentation), the proportions in *Enterobacteriales* were very low (below 0.05%) at T0 for all samples and very high after DF of glucose (between 37.1% and 75.7%). The low *Enterobacteriales* abundance at T0 (<1%) and the high abundance after DF (>40% for OFMSW) was already observed by Dauptain et al. (2020) with complex organic substrates, which suggested a strong selection by the DF process. Despite the increase of *Clostridium sp.* abundance at T0 for the stored inoculum by freezing (3.28% - 5.31%

for short and long WWTP freezing), the final *Clostridiales* proportions were lower after a long storage, which supports the assumption that *Clostridiales* might have been outcompeted by *Enterobacteriales* for kinetic reasons, as previously mentioned. An extended exposure to stressful condition (oxygen, light, moisture) during storage might have influenced *Clostridiales* viability and the storage stability (Berninger et al., 2018), which potentially made them outperformed by other species as *Enterobacteriales*. Surprisingly, viability of bacteria belonging to the genus level *MBA03* seemed to be strongly affected by the storage as they were detected in proportions lower than 1% after fermentation for AD stored inoculum but were the main species at T0 (abundance between 34.27% and 53.37% for all AD inoculum). As a consequence, they might have been outperformed by other species.

Short freeze-drying, led to an analogous hydrogen yield compared to the fresh WWTP inoculum. However, a 1.5-month storage led to a significant increase in H_2 production 171 ± 19 mLH_2/gVS_{ini} compared to the 112 ± 14 mLH_2/gVS_{ini} of the fresh WWTP inoculum. As for short and long inoculum storage by freezing, equal / greater hydrogen yields are due to similar proportions / an enrichment in *Clostridiales*, respectively. Indeed, for the 1.5-month freeze-dried inoculum experiments, the *Clostridiales* abundance reached 46.9% compared to 15.4% for the fresh inoculum (Fig. 1A). As *Clostridiales* are sporulating bacteria (Parthiba Karthikeyan et al., 2018), long storage by freeze-drying might have favored their selection over time due to their ability to resist to stressful conditions (light, oxidative environment) during the storage. This implies that freeze-drying allowed to store an inoculum and could in addition increase the hydrogen yield by selecting some efficient HPB. It is noteworthy to mention that for short freezing and long freeze-drying, which led to higher hydrogen production

compared to the fresh inoculum, the Simpson OTU diversity was higher compared to the fresh inoculum (0.74 for 1.5-month freeze-drying, 0.66 for 1-week freezing and 0.41 for the fresh WWTP inoculum). This result is in accordance with Fig. 3A as an increase in OTU diversity is positively correlated with H_2 yield. This result is consistent with Chatellard (2016), who demonstrated a positive correlation between OTU not affiliated to the main families after DF and hydrogen production. Yang et al. (2019) also found that the heat-shock inoculum showed the highest H_2 yield (glucose as substrate) and the highest OTU Simpson's diversity. Therefore, the increase in hydrogen production might be explained by positive interactions between main bacterial communities and minor OTU.

Otherwise, the possibility to store an inoculum seems to be inoculum dependent. Indeed, according to Table 1, a short storage by freezing or freeze-drying is feasible with AD inoculum. Moreover, freezing seemed to be the most efficient technique for a 1-week storage as the hydrogen yield was again statistically higher after a short storage. This implies that the combined effect of an inoculum storage by freezing (at -20°C) over a short period followed by a thermal pretreatment at 90°C-15 min could be an efficient way to further select HPB and to increase hydrogen yields compared to a fresh heat treated inoculum. However, freeze and thaw have already been applied as an inoculum pretreatment technique leading to low H_2 productions on glucose (Wang and Yin, 2017).

For the fresh AD inoculum, the lower performances compared to the 1-week frozen inoculum could be attributed to bacteria affiliated to (*Clostridia*) *MBA03* order (according to Fig. 1A) with a relative abundance of 10.2% (<1% for stored inocula). Due to a low identification percentage, it was not possible to identify this species by a

BLAST program. Nevertheless, some authors identified *MBA03* order in biomethanation experiments (Logroño et al., 2020). As a consequence, bacteria from this order are presumably able to consume hydrogen by homoacetogenesis, which could explain the lower yield obtained with the fresh inoculum compared to the 1-week frozen AD inoculum. Moreover, this hypothesis is consistent with Table 2A. Indeed, the concentration in acetate at the end of DF for the fresh AD inoculum was higher with 3.55 ± 0.21 mmol/gVS_{ini} compared to the short frozen experiments, with 1.67 ± 0.21 mmol/$gVS_{ini.}$ This supports the assumption of a hydrogen consumption by *MBA03* order through homoacetogenic reaction. For the leachate inoculum, only freezing over a short period appears as an efficient way to store this inoculum with a H_2 yield of 184 ± 41 mLH_2/gVS_{ini} (187 ± 19 mLH_2/gVS_{ini} for the fresh inoculum). However, due to the high percentage of *Clostridiales* (96.8%), short freezing did not allow a further *Clostridiales* selection and an increase in hydrogen production as WWTP and AD inoculum.

Excepted for AD inoculum, there is a positive correlation between *Clostridiales* and hydrogen yield. This result is consistent with Dauptain et al. (2020) and with Fig. 3A. However, a 1.5-month storage was totally detrimental to hydrogen yields for AD and leachate inocula. Indeed, the hydrogen production collapsed until very low yields (<80 mLH_2/gVS_{ini}). Fig. 1A cannot explain the difference in H_2 production. According to Fig. 2, the low hydrogen yield is due to changes in microbial communities. Indeed, a clear shift was observed from *Clostridium sp.* (leachate inoculum) or *Escherichia-Shigella* (AD inoculum) to an unclassified *Enterobacteriaceae* (*Raoultella sp.*) after a long inoculum storage. For instance, the population at the end of DF of *Raoultella sp.* reached 91.4% for the 1.5-month storage by freeze-drying (65 ± 3 mLH_2/gVS_{ini}) compared to 0% for the fresh leachate inoculum (187 ± 19 mLH_2/gVS_{ini}). This could

suggest that long storage favored the emergence of such a bacterium, which was probably only present initially in AD and leachate inocula. Thanks to a BLAST program, this bacterium was identified as *Raoultella ornithinolytica* strain (accession number : NR_044799.1) with an identification percentage of 99.76%. This species is barely described in the literature. Some authors reported this species as a hydrogen producer. Marone et al. (2012) isolated *Raoultella sp.* from vegetable waste and described this species as a hydrogen producer with a high hydrogen yield (2.84 mol H_2/mol sugar). However, Zieliński et al. (2017) identified *Raoultella terrigena* as a low H_2 producer, which will be consistent with the present studies and the fact that *Raoultella ornithinolytica* is a low H_2 producer, especially with glucose as substrate. The other OTU affiliated to *Enterobacteriales* order was identified as *Escherichia-Shigella*, an efficient H_2 producer compared to other *Enterobacteriales* as consistently reported elsewhere (Cabrol et al., 2017). Moreover, the high H_2 yield of *Escherichia-Shigella* has also been proved on various complex organic substrates (Dauptain et al., 2020).

3.1.2 Impact of inoculum storage on total metabolite amount from glucose

DF performances with the stored inocula were also evaluated with the total amount of metabolites produced during DF in order to see whether the overall conversion of all substrates were impacted. Table 2 (A, B and C) sums up the average of the total metabolite amounts (biodegradability of the substrate) and the average concentration in each metabolite detected (with their standard deviations) for glucose, OFMSW and FW, respectively. In a very limited number of samples, butanol and caproate were detected in very low amounts (up to 0.22 mmol/gVS_{ini} for glucose and 0.11 mmol/gVS_{ini} for complex substrates). As a consequence, those metabolites were not mentioned in Table

2 (A, B and C) but were taken into account in total metabolite balances. For WWTP inoculum, total metabolites were not statistically different for all stored conditions compared to the fresh inoculum (Table 2A). Hydrogen, acetate, butyrate and ethanol were the main metabolites produced from glucose (all inocula). Succinate (no hydrogen producing pathway) and lactate were also detected. Excepted for the sample WWTP-Fr-1W-G, the best performances for the long freeze-dried WWTP inoculum are due to a metabolic shift from the ethanol pathway (no H_2 production) to the butyrate pathway. Moreover, this result is consistent with Fig. 3A and the literature as butyrate is often correlated with hydrogen production (Dauptain et al., 2020). As a consequence, the butyrate pathway was more used, which led to fewer metabolites produced by non-hydrogen producing pathways. This metabolic shift can also be explained by Fig. 1A. Indeed, the percentage of *Enterobacteriales* (37.1%) was lower for this experiment compared to other inoculum storage conditions and in particular to the fresh inoculum with 75.7% of *Enterobacteriales*. Indeed, this order is reported to produce ethanol (Palomo-Briones et al., 2017). For WWTP-Fr-1W-G samples, the high performances can be attributed to a high butyrate concentration and probably by an ethanol production by the acetate-ethanol pathway (same yield as butyrate pathway) (Zhou et al., 2018).

According to Table 2A, for the long-stored AD and leachate inocula, the total metabolite amount decreased significantly (as H_2 yield) compared to the fresh inoculum due to the shift from *Escherichia-Shigella* / *Clostridium sp.* to *Raoultella* sp. Moreover, no residual sugars were detected after DF (data not shown). Part of the difference might be eventually attributed to biomass growth and also due to an undetected metabolite (probably produced by *Raoultella sp.*). The better DF performances of the fresh leachate inoculum are also confirmed by a higher amount of total metabolites compared to other

inocula. For the leachate inoculum, there was a clear shift from the butyrate pathway to the ethanol pathway. It can be assumed that *Raoultella sp.* promoted ethanol production instead of butyrate production (by *Clostridium sp.).* It can be noticed that no ethanol was detected (for all inocula) when *Clostridiales order* was in overwhelming majority (fresh AD and leachate inoculum). This result is consistent with Palomo-Briones et al. (2017), who noticed that *Enterobacteriales* were associated with ethanol production. Moreover, for the samples with *Raoultella sp.* (long storage of AD and leachate inocula), and especially for freeze-dried leachate inoculum, high amounts of ethanol and low amounts of butyrate and acetate were detected. As a consequence, *Raoultella sp.* took preferentially the ethanol pathway, hence the low hydrogen yields. This result is in accordance with Marone et al. (2012), who observed high amounts of ethanol and low amounts of butyrate after DF of cellobiose, xylose and vegetable waste (*Raoultella sp.* as seed inoculum). For the AD and leachate inoculum, some other metabolites were detected in significant amounts in some samples as lactate, succinate, propionate and formate coming mainly from non-hydrogen producing pathways. However, lactate and formate can be further consumed to produce more hydrogen (Dauptain et al., 2020).

For glucose, excepted for some freeze-dried samples, inoculum storage induced only a slight increase of the lag time for WWTP and AD inoculum (data and Gompertz equation are given in supplementary material). For instance, the lag phase increased from 0.55 ± 0.02 day for the fresh WWTP inoculum to 0.74 ± 0.02 day for the 1.5-month freeze-dried WWTP inoculum. Surprisingly, the lag time considerably decreased after storage for the leachate inoculum (1.84 ± 0.02 days for the fresh inoculum and 1.09 ± 0.02 days for the long freeze-dried inoculum). However, this decrease of the lag phase was associated with a significant decrease of hydrogen yields (Table 1) coming

from a microbial shift as previously mentioned. As a consequence, it can be inferred that *Raoultella sp*. probably emerged during DF of the leachate inoculum due to a faster growth rate as suggested by Yang and Wang, (2018), for HPB. It conferred *Raoultella sp.* a kinetic advantage, compared to other HPB and allowed them to win the substrate competition to be the most abundant bacteria after DF. This kinetic advantage could be explained by the longer time for sporulating bacteria as *Clostridium sp.* to be viable again compared to *Raoultella sp.* (non-sporulating bacteria) as previously explained. However, the decrease in the lag time did not occur for AD inoculum as the bacterial shift occurred between *Escherichia-Shigella* and *Raoultella sp.*, two *Enterobacteriales* (no sporulation). Moreover, it is noteworthy to mention that the lag phase of the leachate inoculum was much higher than other inocula. This section showed that inoculum storage was possible by freezing and freeze-drying for short (1 week) and long periods (1.5 months) in order to perform DF experiments with glucose as substrate. For WWTP inoculum, the storage allowed in some cases a further selection of bacteria affiliated to *Clostridiales* order, leading to an increase of butyrate and H_2 productions. As a consequence, inoculum storage can also be seen as a complementary method to heat treatment to further pretreat an inoculum in order to increase the hydrogen yield. Further investigations are nonetheless required to better understand under which conditions an increase occurs. However, the storage is inoculum dependent and H_2 production dropped significantly for AD and leachate inoculum after a long storage. This decrease was associate with a bacterial shift from *Clostridium sp.* or *Escherichia-Shigella* to an unclassified *Enterobacteriaceae* (*Raoultella ornithinolytica).* This bacterial shift also led to a metabolite shift from butyrate to ethanol (no hydrogen producing pathway). Moreover, further investigations are required to better understand

how to avoid some low hydrogen producers as *Raoultella sp.,* which can outcompete efficient HPB after a long storage (glucose as substrate). Due to the high performances of the 1.5-month freeze-dried WWTP inoculum and the low variability of freeze-dried experiments, freeze-drying appears as a more appropriate technique to store an inoculum when working with simple sugars.

3.2 Impact of inoculum storage on DF performances of complex substrates

In general, for OFMSW, hydrogen productions for the stored inoculum were not statistically different from the fresh inoculum (WWTP and leachate inocula) as indicated by Tukey's test results (Table 1). However, in some cases, hydrogen production was higher after the inoculum storage. Indeed, for OFMSW, H_2 yield was higher after a one-week storage by freeze-drying (40 ± 3 mLH_2/gVS_{ini}) compared to the fresh leachate inoculum (33 ± 2 mLH_2/gVS_{ini}). A 1.5-month freeze-drying also led to an increase of hydrogen production (37 ± 4 mLH_2/gVS_{ini}) compared to the fresh WWTP inoculum (30.8 ± 0.2 mLH_2/gVS_{ini}). Nevertheless, short freezing of WWTP inoculum was detrimental to H_2 yield (16 ± 3 mLH_2/gVS_{ini}). In general, for AD inoculum, DF performances (H_2 and total metabolite amount) with OFMSW were similar after the inoculum storage. However, fresh AD inoculum showed a statistically significant lower hydrogen production (15 ± 1 mLH_2/gVS_{ini}). According to Fig. 1B, this lower yield is due to the abundance (19%) of *MBA03* order (HCB) (Logroño et al., 2020). The impact of inoculum storage on FW is similar to the results obtained with OFMSW. For AD inoculum, the hydrogen production of the fresh inoculum was again statistically lower (96 ± 7 mLH_2/gVS_{ini}) compared to the stored inoculum (125 ± 7 mLH_2/gVS_{ini} for long freeze-drying). For WWTP and leachate inocula, H_2 yields were similar (excepted long freeze-drying for the leachate inoculum (99 ± 6 mLH_2/gVS_{ini})).

Table 2B and Table 2C show the total metabolite amounts and the main metabolite concentrations for all inocula, for OFMSW and FW, respectively. As for glucose, the leachate inoculum presented a higher total metabolite amount for OFMSW and FW compared to other inocula. However, the hydrogen yields were similar between all inocula for complex substrates, which suggests that the difference in total metabolite amount for the leachate inoculum does not come from H_2 production. It might be due to the presence of more efficient hydrolytic bacteria (which could have allowed a further biodegradation of the substrates) or to minor OTUs and possible positive interactions with main microbial communities, as reported elsewhere (Cabrol et al., 2017). For OFMSW and WWTP inocula, total metabolite concentration was also higher after a long storage compared to the fresh inoculum, probably for the same reasons. Contrary to OFMSW, there was no statistical difference for FW between all conditions for a stored inoculum regarding the total metabolite amount.

Concerning OFMSW, H_2, acetate, butyrate and ethanol (in some cases) were the main metabolites (all inocula). For OFMSW and WWTP inoculum (Table 2B), the concentration in all metabolites were very similar, excepted for "WWTP-Fr-1W-OFMSW" samples. Its lower hydrogen production was due to a metabolic shift from the butyrate pathway (0.39 ± 0.03 mmol/g VS_{ini} - 0.62 ± 0.05 mmol/g VS_{ini} for the fresh inoculum) to the ethanol pathway (0.44 ± 0.02 mmol/g VS_{ini}), as previously observed for glucose. Surprisingly, this metabolic shift was due to a bacterial shift from *Enterobacteriales* order to *Clostridiales* order (Fig. 1B). This result is not consistent with (Palomo-Briones et al., 2017). Indeed, the authors associated *Enterobacteriales* presence to ethanol production. The ethanol production could be explained by a metabolic shift of *Clostridiales* toward solventogenesis due to stressful conditions

(Cabrol et al., 2017). For the samples "AD-FD-1W-OFMSW", low amounts of butyrate, acetate and more ethanol were detected compared to other conditions. This could be attributed to the high percentage of *Escherichia-Shigella* (37%) as reported in Fig. 2. This result is also consistent with Fig. 3B. Indeed, there are negative correlations between *Escherichia-Shigella* and butyrate or acetate. As for glucose ("AD-F-G" samples), we can infer that the statistically lower hydrogen production for "AD-F-OFMSW" and "AD-F-FW" samples is due to *MBA03* order with a relative abundance of 19% / 13.6%, respectively (Fig. 1B and Fig. 1C). The assumption of H_2 consumption to produce acetate by *MBA03* is again supported by a high level of acetate (1.85 ± 0.48 mmol/g VS_{ini} - 1.93 ± 0.11 mmol/g VS_{ini}, for OFMSW and FW, respectively), proportions much higher than other samples with an AD stored inoculum. The positive correlation between *MBA03* and acetate is also confirmed by Fig. 3B.

Surprisingly, as reported by Fig. 2, *Raoultella sp*. was detected for AD long storage with OFMSW as substrate (as for glucose), with a percentage of 14% for freezing and 39% for freeze-drying, suggesting that this bacterium was favored by the AD inoculum storage. However, contrary to glucose experiments, *Raoultella sp*. was not anymore associated with a significant decrease in H_2 yield. Contrary to glucose, with a shift toward ethanol production, no ethanol was detected in both samples. Indeed, only hydrogen, acetate and butyrate were produced for "AD-Fr-1.5M-OFMSW" and "AD-FD-1.5M-OFMSW" samples. This last result is not consistent with the results obtained on glucose (> 1.70 mmol/gVS_{ini}) and with Marone et al. (2012), who observed a 4 times higher molar concentration of ethanol compared to butyrate. This difference might be attributed to a different fermentative environment. Moreover, *Raoultella sp*. was not detected for FW and OFMSW (leachate inoculum). This could be explained by the

strong influence of indigenous bacteria on complex organic matter, as reported elsewhere (Dauptain et al., 2020). As a consequence, *Raoultella sp.* is a low hydrogen producer for glucose but an efficient H_2 producer for complex organic matter. *Raoultella sp.* is probably adapted to complex substrates and might have positively interacted with other species (Cabrol et al., 2017).

Regarding the leachate inoculum for OFMSW, H_2, acetate, butyrate and ethanol were the main metabolites. A few amounts of propionate were also detected, especially for the fresh inoculum (0.21 ± 0.01 mmol/gVS_{ini}). The inoculum storage favored ethanol production (none detected for the fresh leachate inoculum), suggesting a change regarding the metabolic pathway. However, the bacterial compositions of OFMSW experiments at the order level were very similar according to Fig. 1B (excepted for *Clostridiales* or *Enterobacteriales*). Contrary to glucose experiments, a shift from *Clostridiales* to *Enterobacteriales* was not associated with a lower hydrogen yield as reported elsewhere (Dauptain et al., 2020). This difference in metabolite concentration might come from a stress due to the inoculum storage, which led to different metabolic pathways. Indeed, according to Table 2B, the samples "Leachate-FD-1W-OFMSW" presented a higher concentration in ethanol (1.19 ± 0.27 mmol/gVS_{ini}), a significant higher hydrogen yield (40 ± 3 mLH_2/gVS_{ini}) and a lower concentration in butyrate and acetate compared to the fresh inoculum. As a consequence, ethanol was probably produced through the acetate-ethanol pathway by the freeze-dried leachate inoculum. The best H_2 yield of the sample "Leachate-FD-1W-OFMSW" might also be explained by the higher abundance in *Clostridium sp.* (Fig. 2) with a value of 82% compared to 70% for the fresh inoculum. As no *Enterobacteriales* were detected, this result is consistent with Kim et al. (2014), who observed a positive correlation between

Clostridium sp. and the hydrogen yield for FW. The lower hydrogen yield of the fresh inoculum compared to the sample "Leachate-FD-1W-OFMSW" could also be attributed to the presence of an unclassified *Lachnospiraceae*. Indeed, 15% of this genus level was detected for the fresh inoculum versus 2% for short freeze-drying. This is in accordance with Palomo-Briones et al. (2017), who reported *Lachnospiraceae* family as homoacetogenic bacteria, which could explain the high acetate concentration for the samples "Leachate-F-OFMSW" and "Leachate-Fr-1W-OFMSW" (>10% of the unclassified *Lachnospiraceae*). Moreover, the "Leachate-FD-1W-OFMSW" condition was associated with lower Simpson diversity indexes compared to the fresh inoculum with values of 0.39 / 0.68 (for the fresh) for Simpson OTU diversity and values of 0.27 / 0.66 (fresh) for Simpson *Clostridiales* diversity. This is not consistent with the results previously obtained on glucose as a higher diversity was in favor of hydrogen production. However, according to Fig. 3B, a higher OTU diversity is detrimental to hydrogen and ethanol productions for complex substrates.

For FW and WWTP inoculum, the similar DF performances are due to a very high abundance in *Clostridiales* according to Fig. 1C (>88%). This also explains the similar concentrations in each metabolite (acetate, butyrate and ethanol), excepted for the samples "WWTP-Fr-1W-FW" with a high ethanol production (1.33 ± 0.22 mmol/gVS_{ini}). Some other metabolites were also detected in significant amounts as formate. Indeed, according to Table 2C, the AD inoculum has promoted a formate pathway contrary to WWTP inoculum. The higher hydrogen production of the stored inoculum compared to the fresh AD inoculum for FW might be explained by homoacetogenesis (high acetate concentration) due to *MBA03* order as previously mentioned but also to a higher formate production. It might be inferred that the high

abundances of facultative anaerobes *as Bacillales* and *Lactobacillales* (28.2% of *Lactobacillales* for the sample *"AD-FD-1W-FW")* are responsible for a formate consumption to produce hydrogen thanks to a formate hydrogen lyase as reported elsewhere (Cabrol et al., 2017). This assumption is consistent with the lower formate concentration observed in Table 2C for the AD stored inoculum (0.25 ± 0.09 mmol/gVS_{ini} for "AD-*FD-1W-FW*" samples compared *to* 0.96 ± 0.07 mmol/gVS_{ini} for the fresh inoculum). Some ethanol was also measured in AD samples, lower proportions of acetate (less homoacetogenesis) and butyrate were also detected in those samples. However, the bacterial composition (Fig. 2) cannot explain these differences. It could be assumed that ethanol was probably only produced through the acetate-ethanol pathway instead of the butyrate pathway, which both have similar yields, hence the equal H_2 production (Zhou et al., 2018).

For the long stored leachate inoculum (FW as substrate), the high ethanol concentration observed in Table 2C is probably due to the emergence of bacteria affiliated to *Bifidobacteriales* order (spore-forming bacteria) according to Fig. 1C, which are known to break down the substrate (and especially starch) into smaller molecules (Hung et al., 2011). According to Feng et al. (2018), *Bifidobacterium sp.* are able to produce lactate, acetate and ethanol. This is in accordance with Fig. 2 and Table 2C as a significant amount of *Bifidobacteriales* (32.2%) and ethanol (2.17± 1.53 mmol/gVS_{ini}) were detected for the samples "Leachate-FD-1.5M-FW". The low amount of lactate and acetate may be explained by a further consumption by *Clostridium sp.* as *Clostridium beijerinckii* to produce butyrate (Dauptain et al., 2020). This result is consistent with a previous work (Dauptain et al., 2020) on unpretreated FW as *Bifidobacteriales* (10.10%) were detected after DF (unpretreated inoculum). Indeed, the H_2 production

was also significantly lower (79 mLH_2/gVS_{ini}) compared to the inoculated and pretreated experiments (169 mLH_2/gVS_{ini} - only *Clostridiales*). For other samples, similar abundance in all microbial communities can be observed (high percentage of *Clostridiales* for FW). As a result, the distribution of all metabolites is similar. For FW (Fig. 1C), no *Enterobacteriales* were detected contrary to glucose and OFMSW. This result is due to the inoculation and the pretreatment as reported elsewhere (Dauptain et al., 2020). The non-significant difference in H_2 yield and the similar concentration in each metabolite for complex organic matter may also be attributed to the influence of indigenous bacteria (bacteria existing on the substrate) but also to the substrate structure, which both have probably greatly influenced the final microbial composition (Dauptain et al., 2020).

In most cases, the lag phases for complex substrates were similar (or slight increase) compared to the fresh inoculum (data in supplementary material). For instance, the lag phases were not significantly different for the 1.5-month freeze-dried leachate inoculum (0.78 ± 0.02 day) and for the fresh leachate inoculum (0.81 ± 0.13 day), with OFMSW as substrate. The insignificant difference in lag phases proved that inoculum storage did not disturb bacteria growth and HPB metabolism (Yang and Wang, 2018), which is consistent with the similar metabolite concentrations between the fresh and stored inocula (for a same inoculum and substrate) given in Table 2 B and C. This emphasizes the idea of a possible inoculum storage for DF process performed with complex substrates. To conclude on complex substrates, the similar DF performances are due to a high and stable population of *Clostridiales* or *Enterobacteriales*, which led to similar metabolite concentrations and pathways. As a consequence, this work showed that inoculum storage by freezing or freeze-drying over a short (1 week) or long period (1.5

months) was feasible to perform DF experiments on complex organic matter. Freeze-drying seemed to offer better H_2 yields than freezing, probably due to a further selection of HPB of the inoculum. However, part of the results obtained with complex organic substrates could be due to the strong influence of indigenous bacteria or the complex structure of the substrate (bacterial selection). This might explain the different behavior between complex substrates and glucose regarding the impact of inoculum storage.

4 Conclusion

This study demonstrated that inocula from several origins could be stored by freezing or freeze-drying to produce H_2 by DF from complex organic substrates. Indeed, after a 1.5-month storage, hydrogen yield was statistically higher or equal to the fresh inoculum production. Generally, long-term freeze-drying offered the best performances for all inocula. This finding is particularly of interest to further investigate different parameters of DF process. However, the interactions between indigenous bacteria and inoculum-stored bacteria need to be clarified. Nevertheless, for glucose, inoculum storage had a variable impact on H_2 yield according to the inoculum origin and the storage technique.

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Supplementary data

Supplementary data of this work can be found in the online version of the article.

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Figure captions

Fig. 1 - Microbial community proportions at the order level for all inocula at the end of fermentation for glucose (A), for OFMSW (B) and for FW (C). Only orders with a

relative abundance > 5% (at least for one sample) are shown. For each experiment, one or two replicates among the quadruplicate were measured.

Fig. 2 - Microbial community proportions at the genus level for glucose, OFMSW and FW, for all inocula at the end of fermentation. Only genus levels with a relative abundance > 5% (at least for one sample) are shown. For each experiment, one or two replicates among the quadruplicate were measured.

Fig. 3 - Correlation matrix with Pearson's coefficients for Simpson's diversity indexes, main metabolites and main microbial communities at the order or genus level for glucose (A) and complex substrates (OFMSW, FW) (B). All inocula (WWTP, AD, Leachate) were considered. Only samples with microbial community analyses were considered. Only Pearson's correlations with a significant p-value are displayed ($p<0.05$).

Tables and Figures

Table 1 - Maximum cumulative hydrogen production of all substrates and inocula (quadruplicate average). Letters show Tukey's test results (for a same inoculum and substrate), i.e. if two conditions share a common letter (a, b or c), the H_2 yield is not statistically different. Underlined values indicate a significant higher or lower difference compared to the fresh inoculum. An asterisk (*) indicates that the sample was not considered in Tukey's test (too large standard deviation).

H_2 in mL / g VS_{ini}									
Substrate	**Glucose**			**OFMSW**			**FW**		
Inoculum	**WWTP**	**AD**	**Leachate**	**WWTP**	**AD**	**Leachate**	**WWTP**	**AD**	**Leachate**
Fresh	112 ± 14^{a}	111 ± 5^{b}	187 ± 19^{b}	30.8 ± 0.2^{b}	15 ± 1^{a}	33 ± 2^{ab}	115 ± 22^{a}	96 ± 7^{a}	129 ± 10^{b}
Frozen-1Week	$\underline{144 \pm 10^{bc}}$	$\underline{138 \pm 10^{c}}$	184 ± 41^{b}	$\underline{16 \pm 3^{a}}$	$\underline{32 \pm 4^{b}}$	36 ± 4^{ac}	116 ± 14^{a}	$\underline{123 \pm 2^{b}}$	128 ± 13^{b}
Freeze-Dried-1Week	125 ± 11^{ab}	102 ± 3^{b}	$\underline{78 \pm 10^{a}}$	32 ± 3^{bc}	$\underline{34 \pm 1^{b}}$	$\underline{40 \pm 3^{c}}$	120 ± 6^{a}	$\underline{122 \pm 3^{b}}$	$120 \pm 37^{*}$
Frozen - 1.5Months	130 ± 8^{ab}	$99 \pm 60^{*}$	$\underline{78 \pm 5^{a}}$	33 ± 3^{bc}	$\underline{30 \pm 2^{b}}$	31 ± 3^{a}	123 ± 10^{a}	$\underline{114 \pm 9^{b}}$	120 ± 13^{ab}
Freeze-Dried-1.5Months	$\underline{171 \pm 19^{c}}$	$\underline{58 \pm 4^{a}}$	$\underline{65 \pm 3^{a}}$	$\underline{37 \pm 4^{c}}$	$\underline{30 \pm 1^{b}}$	39 ± 3^{bc}	125 ± 7^{a}	$\underline{125 \pm 7^{b}}$	$\underline{99 \pm 6^{a}}$

Table 2 - Concentrations of main metabolites and total metabolites obtained after dark fermentation for all inocula, for glucose (A), OFMSW (B) and FW (C). The values correspond to the average of the quadruplicate with its standard deviation. F = Fresh, W = Week, M = Month, Fz = Frozen, FD = Freeze-Dried, WWTP = Waste Water Treatment Plant, AD = Anaerobic Digestate, ND= Not Detected.

A

	Metabolite concentration in mmol / g VS_{ini}								
Inoculum-Sample	H_2	Acetate	Butyrate	Ethanol	Succinate	Lactate	Propionate	Formate	Total metabolites (g COD / g VS_{ini})
WWTP-F-G	4.99±0.64	2.83±0.69	1.32±0.15	2.76±0.45	0.53±0.08	0.29±0.58	ND	ND	$0.88±0.04^{ab}$
WWTP-Fr-1W-G	6.44±0.44	1.61±0.3	1.46±0.26	4.08±0.58	0.37±0.08	0.26±0.39	ND	ND	$0.91±0.02^{b}$
WWTP-FD-1W-G	5.59±0.47	3.43±1.53	1.7±0.23	2.23±0.52	0.51±0.2	ND	0.11±0.22	ND	$0.87±0.01^{ab}$
WWTP-Fr-1.5M-G	5.81±0.36	2.04±0.55	1.16±0.35	3.42±0.71	0.63±0.05	0.71±0.37	ND	ND	$0.89±0.04^{ab}$
WWTP-FD-1.5M-G	7.63±0.83	2.05±0.35	2.7±0.78	0.72±0.97	0.27±0.1	0.6±0.46	ND	ND	$0.84±0.03^{a}$
AD-F-G	4.94±0.24	3.55±0.21	3.34±0.12	ND	ND	ND	ND	0.4±0.01	$0.85±0.03^{b}$
AD-Fr-1W-G	6.14±0.44	1.67±0.14	1.77±0.25	3.38±0.63	0.28±0.32	0.11±0.22	0.23±0.26	ND	$0.88±0.03^{b}$
AD-FD-1W-G	4.53±0.15	1.76±0.28	ND	3.35±0.38	0.67±0.05	2.96±0.64	ND	ND	$0.87±0.03^{b}$
AD-Fr-1.5M-G	4.4±2.68	3.05±1.88	0.88±1.08	1.72±1.07	ND	ND	0.09±0.18	ND	$0.58±0.14^{a}$
AD-FD-1.5M-G	2.61±0.16	3.23±2.81	ND	2.35±0.97	ND	ND	0.27±0.04	ND	$0.5±0.08^{a}$
Leachate-F-G	8.33±0.85	2.55±0.48	3.71±0.45	ND	ND	0.75±0.56	0.29±0.03	0.89±0.69	$1.01±0.03^{b}$
Leachate-Fr-1W-G	7.49±2.67	3.09±1.79	3.93±0.38	ND	ND	0.15±0.21	ND	ND	$0.96±0.03^{b}$
Leachate-FD-1W-G	3.49±0.46	0.65±0.36	0.83±0.28	3.09±0.42	0.29±0.02	ND	0.15±0.18	ND	$0.58±0.01^{a}$
Leachate-Fr-1.5M-G	3.49±0.24	4.06±2.11	0.67±0.23	1.87±1.18	0.15±0.14	ND	ND	ND	$0.62±0.07^{a}$
Leachate-FD-1.5M-G	2.92±0.1	0.52±0.12	0.52±0.12	3.24±0.32	0.07±0.08	0.96±0.35	ND	ND	$0.57±0.05^{a}$

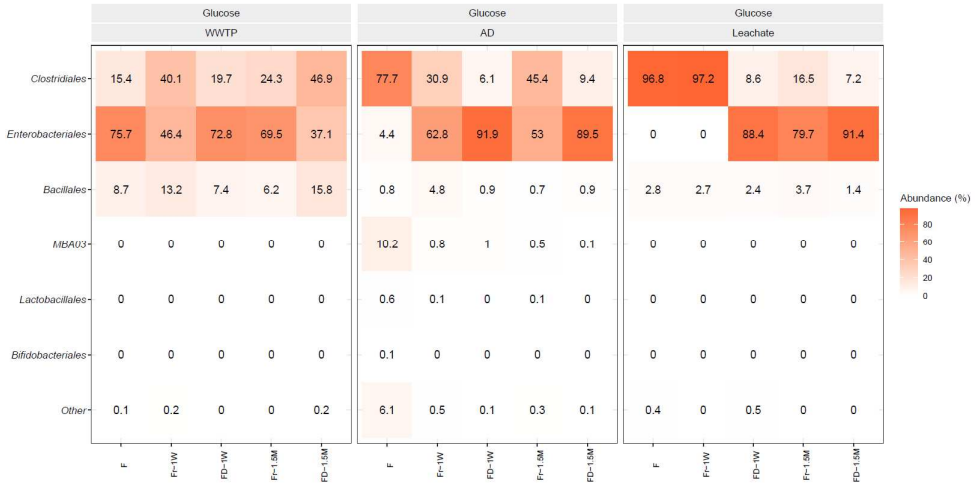
B

Inoculum-Sample	Metabolite concentration in mmol / g VS_{ini}								
	H_2	Acetate	Butyrate	Ethanol	Succinate	Lactate	Propionate	Formate	Total metabolites (g COD / g VS_{ini})
WWTP-F-OFMSW	1.38±0.01	1.03±0.14	0.62±0.05	ND	0.06±0.01	ND	ND	ND	0.19±0.01[a]
WWTP-Fr-1W-OFMSW	0.72±0.13	0.85±0.03	0.39±0.03	0.44±0.02	ND	ND	ND	ND	0.17±0.01[a]
WWTP-FD-1W-OFMSW	1.44±0.13	1.31±0.04	0.69±0.07	ND	0.13±0	ND	ND	ND	0.24±0.02[b]
WWTP-Fr-1.5M-OFMSW	1.47±0.15	1.27±0.03	0.68±0.02	ND	0.07±0.01	ND	ND	ND	0.22±0.01[b]
WWTP-FD-1.5M-OFMSW	1.66±0.16	1.11±0.05	0.79±0.03	0.05±0.09	0.06±0.01	ND	ND	ND	0.23±0.01[b]
AD-F-OFMSW	0.66±0.06	1.85±0.48	0.6±0.2	0.24±0.48	ND	ND	ND	ND	0.25±0.02[b]
AD-Fr-1W-OFMSW	1.43±0.19	1.07±0.16	0.63±0.09	0.37±0.31	ND	ND	ND	ND	0.23±0.01[b]
AD-FD-1W-OFMSW	1.51±0.05	0.53±0.12	0.43±0.03	0.57±0.15	0.1±0.02	ND	ND	ND	0.19±0.01[a]
AD-Fr-1.5M-OFMSW	1.36±0.07	1.33±0.09	0.75±0.06	ND	ND	ND	ND	ND	0.23±0.02[b]
AD-FD-1.5M-OFMSW	1.36±0.05	1.54±0.06	0.71±0.07	ND	ND	ND	0.03±0.04	ND	0.24±0[b]
Leachate-F-OFMSW	1.48±0.07	1.61±0.14	0.9±0.04	ND	ND	ND	0.21±0.01	ND	0.31±0.01[a]
Leachate-Fr-1W-OFMSW	1.62±0.18	1.5±0.35	0.8±0.23	0.64±1.27	ND	ND	0.12±0.03	ND	0.33±0.06[a]
Leachate-FD-1W-OFMSW	1.79±0.15	0.76±0.12	0.48±0.07	1.19±0.27	ND	ND	0.07±0.01	ND	0.28±0.01[a]
Leachate-Fr-1.5M-OFMSW	1.39±0.12	1.47±0.4	0.82±0.28	0.39±0.77	ND	ND	0.1±0.03	ND	0.3±0.01[a]
Leachate-FD-1.5M-OFMSW	1.73±0.11	0.92±0.27	0.6±0.14	0.9±0.61	ND	ND	0.08±0.02	ND	0.28±0.02[a]

C

Inoculum-Sample	Metabolite concentration in mmol / g VS_{ini}								
	H_2	Acetate	Butyrate	Ethanol	Succinate	Lactate	Propionate	Formate	Total metabolites (g COD / g VS_{ini})
WWTP-F-FW	5.14±0.97	1.65±0.11	1.85±0.09	0.1±0.09	0.01±0.02	0.07±0.05	ND	ND	0.52±0.01[a]
WWTP-Fr-1W-FW	5.17±0.64	1.25±0.1	1.36±0.03	1.33±0.22	0.02±0.03	ND	ND	0.09±0.08	0.52±0.05[a]
WWTP-FD-1W-FW	5.36±0.27	1.78±0.06	1.81±0.08	ND	0.12±0.01	0.01±0.03	ND	0.03±0.06	0.53±0.01[a]
WWTP-Fr-1.5M-FW	5.5±0.45	1.44±0.16	1.89±0.12	0.12±0.21	0.1±0.01	ND	ND	0.14±0.01	0.53±0.02[a]
WWTP-FD-1.5M-FW	5.59±0.33	1.63±0.05	2.13±0.1	ND	0.06±0.02	ND	ND	0.04±0.07	0.58±0.01[a]
AD-F-FW	4.28±0.31	1.93±0.11	2.14±0.08	ND	0.01±0.02	ND	0.06±0.01	0.96±0.07	0.56±0.02 [a]
AD-Fr-1W-FW	5.51±0.08	1.23±0.13	1.75±0.19	1.05±0.5	0.03±0.03	ND	0.11±0.02	0.31±0.05	0.57±0.01[a]
AD-FD-1W-FW	5.45±0.13	0.96±0.05	1.51±0.05	1.33±0.24	0.01±0.02	0.05±0.1	ND	0.25±0.09	0.53±0.02[a]
AD-Fr-1.5M-FW	5.1±0.39	1.11±0.13	1.7±0.13	0.92±0.33	0.01±0.02	0.12±0.19	0.02±0.03	0.41±0.11	0.54±0.02[a]
AD-FD-1.5M-FW	5.56±0.32	1.45±0.15	2.31±0.09	ND	0.01±0.03	0.01±0.03	ND	0.12±0.17	0.56±0.02[a]
Leachate-F-FW	5.74±0.45	2.36±0.14	2.33±0.19	ND	ND	ND	0.23±0	0.17±0.29	0.67±0.04[a]
Leachate-Fr-1W-FW	5.71±0.56	1.51±0.4	2.13±0.65	1.65±3.29	0.03±0.03	ND	0.13±0.05	0.25±0.07	0.72±0.21[a]
Leachate-FD-1W-FW	5.34±1.65	1.72±0.34	1.83±0.63	ND	0.01±0.02	0.77±1.07	0.26±0.33	0.16±0.15	0.61±0.02[a]
Leachate-Fr-1.5M-FW	5.35±0.56	0.85±0.09	0.95±0.08	3.53±0.2	ND	0.11±0.15	ND	0.17±0.11	0.65±0.02[a]
Leachate-FD-1.5M-FW	4.41±0.28	1.22±0.45	1.41±0.54	2.17±1.53	0.01±0.03	ND	0.09±0.04	0.1±0.07	0.62±0.05[a]

A


Glucose
WWTP
AD
Leachate
Clostridiales
Enterobacteriales
Bacillales
MBA03
Lactobacillales
Bifidobacteriales
Other
15.4 40.1 19.7 24.3 46.9
75.7 46.4 72.8 69.5 37.1
8.7 13.2 7.4 6.2 15.8
0 0 0 0 0
0 0 0 0 0
0 0 0 0 0
0.1 0.2 0 0 0.2
77.7 30.9 6.1 45.4 9.4
4.4 62.8 91.9 53 89.5
0.8 4.8 0.9 0.7 0.9
10.2 0.8 1 0.5 0.1
0.6 0.1 0 0.1 0
0.1 0 0 0 0
6.1 0.5 0.1 0.3 0.1
96.8 97.2 8.6 16.5 7.2
0 0 88.4 79.7 91.4
2.8 2.7 2.4 3.7 1.4
0 0 0 0 0
0 0 0 0 0
0 0 0 0 0
0.4 0 0.5 0 0
F
Fr-1W
FD-1W
Fr-1.5M
FD-1.5M
Abundance (%)
80
60
40
20
0

B


OFMSW
WWTP
AD
Leachate
Clostridiales
Enterobacteriales
Bacillales
MBA03
Lactobacillales
Bifidobacteriales
Other
55.7 84.6 51.3 64.1 68.7
35.2 3.3 40.5 27.4 26.2
8.8 11.8 7.9 8.1 5
0 0 0 0 0
0.2 0 0.2 0.1 0
0 0 0 0 0
0.1 0.3 0 0.3 0
63 82.3 59.4 80.5 55
0.1 0 37.5 13.8 40.8
4 9.2 1.8 1.5 3.9
19 4.1 0.9 2.4 0.1
1 0.3 0.1 0.2 0
0.2 0 0 0 0
12.6 4.1 0.3 1.7 0.1
96.7 95.2 91 84.1 95.4
0 0 0.1 0 0
2.1 4.5 1.9 15.5 3
0 0 0 0 0
0 0.2 7 0.1 1
0 0 0 0 0.6
1.2 0.1 0 0.3 0
F
Fr-1W
FD-1W
Fr-1.5M
FD-1.5M
Abundance (%)
80
60
40
20
0

C

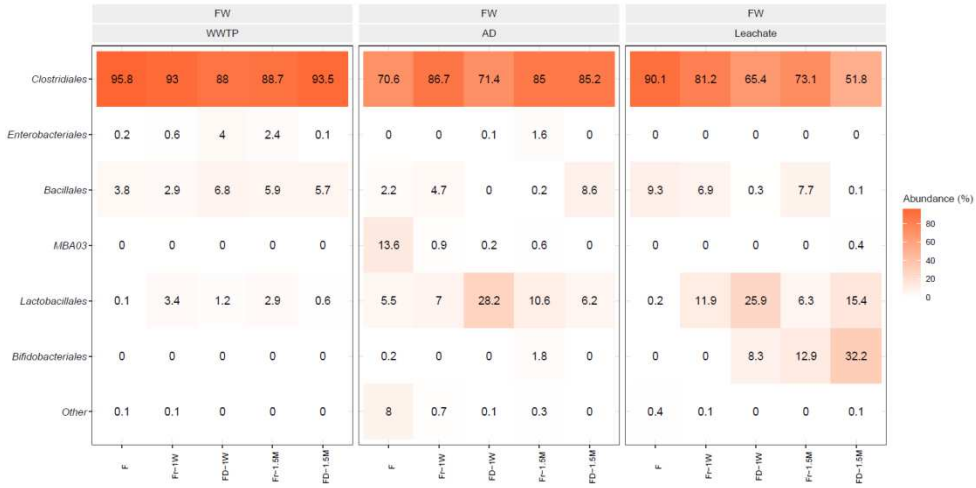

FW
WWTP
AD
Leachate
Clostridiales
Enterobacteriales
Bacillales
MBA03
Lactobacillales
Bifidobacteriales
Other
95.8 93 88 88.7 93.5
0.2 0.6 4 2.4 0.1
3.8 2.9 6.8 5.9 5.7
0 0 0 0 0
0.1 3.4 1.2 2.9 0.6
0 0 0 0 0
0.1 0.1 0 0 0
70.6 86.7 71.4 85 85.2
0 0 0.1 1.6 0
2.2 4.7 0 0.2 8.6
13.6 0.9 0.2 0.6 0
5.5 7 28.2 10.6 6.2
0.2 0 0 1.8 0
8 0.7 0.1 0.3 0
90.1 81.2 65.4 73.1 51.8
0 0 0 0 0
9.3 6.9 0.3 7.7 0.1
0 0 0 0 0.4
0.2 11.9 25.9 6.3 15.4
0 0 8.3 12.9 32.2
0.4 0.1 0 0 0.1
F
Fr-1W
FD-1W
Fr-1.5M
FD-1.5M
Abundance (%)
80
60
40
20
0

Fig. 1

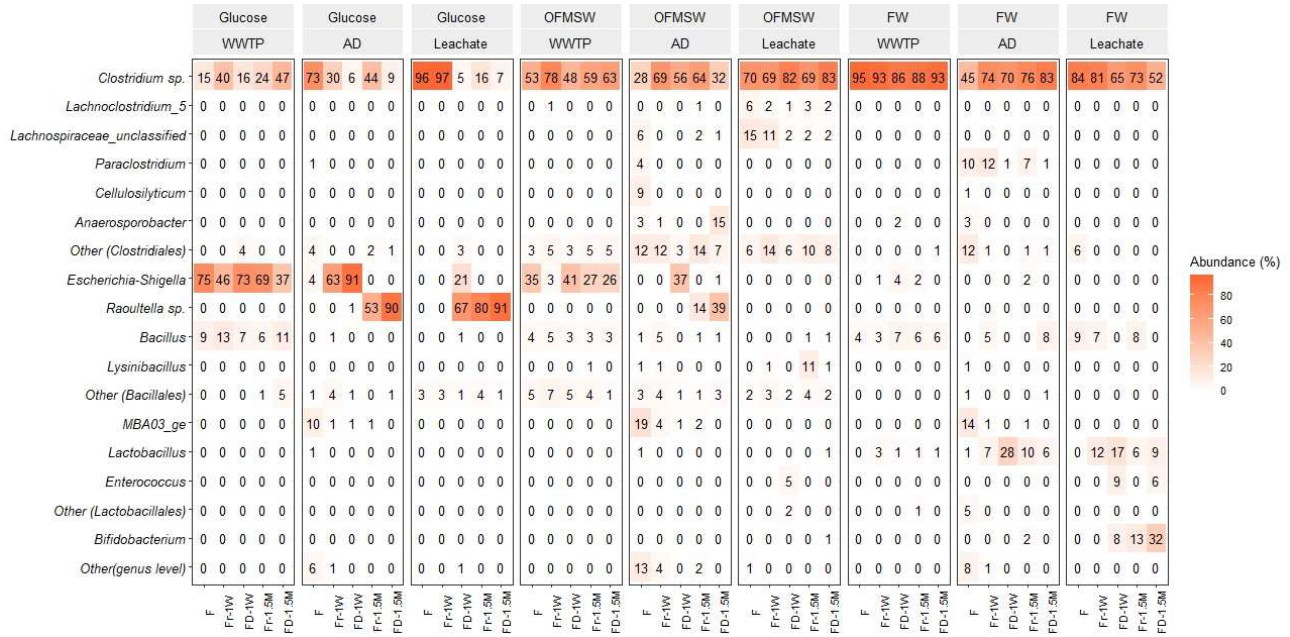
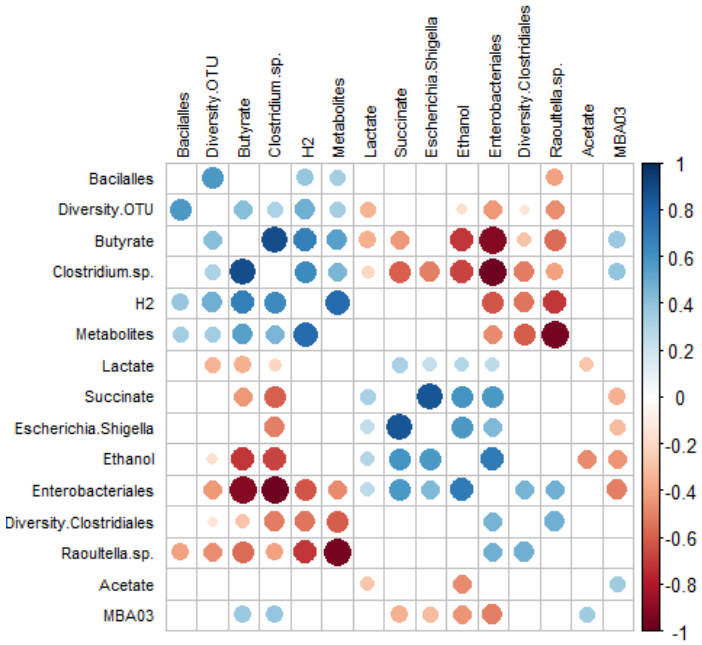

Glucose WWTP
Glucose AD
Glucose Leachate
OFMSW WWTP
OFMSW AD
OFMSW Leachate
FW WWTP
FW AD
FW Leachate
Clostridium sp. 15 40 16 24 47 | 73 30 6 44 9 | 96 97 5 16 7 | 53 78 48 59 63 | 28 69 56 64 32 | 70 69 82 69 83 | 95 93 86 88 93 | 45 74 70 76 83 | 84 81 65 73 52
Lachnoclostridium_5 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 1 0 0 0 | 0 0 0 1 0 | 6 2 1 3 2 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0
Lachnospiraceae_unclassified 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 6 0 0 2 1 | 15 11 2 2 2 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0
Paraclostridium 0 0 0 0 0 | 1 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 4 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 10 12 1 7 1 | 0 0 0 0 0
Cellulosilyticum 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 9 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 1 0 0 0 0 | 0 0 0 0 0
Anaerosporobacter 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 3 1 0 0 15 | 0 0 0 0 0 | 0 0 2 0 0 | 3 0 0 0 0 | 0 0 0 0 0
Other (Clostridiales) 0 0 4 0 0 | 4 0 0 2 1 | 0 0 3 0 0 | 3 5 3 5 5 | 12 12 3 14 7 | 6 14 6 10 8 | 0 0 0 0 1 | 12 1 0 1 1 | 6 0 0 0 0
Escherichia-Shigella 75 46 73 69 37 | 4 63 91 0 0 | 0 0 21 0 0 | 35 3 41 27 26 | 0 0 37 0 1 | 0 0 0 0 0 | 0 1 4 2 0 | 0 0 0 2 0 | 0 0 0 0 0
Raoultella sp. 0 0 0 0 0 | 0 0 1 53 90 | 0 0 67 80 91 | 0 0 0 0 0 | 0 0 0 14 39 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0
Bacillus 9 13 7 6 11 | 0 1 0 0 0 | 0 0 1 0 0 | 4 5 3 3 3 | 1 5 0 1 1 | 0 0 0 1 1 | 4 3 7 6 6 | 0 5 0 0 8 | 9 7 0 8 0
Lysinibacillus 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 1 0 | 1 1 0 0 0 | 0 1 0 11 1 | 0 0 0 0 0 | 1 0 0 0 0 | 0 0 0 0 0
Other (Bacillales) 0 0 0 1 5 | 1 4 1 0 1 | 3 3 1 4 1 | 5 7 5 4 1 | 3 4 1 1 3 | 2 3 2 4 2 | 0 0 0 0 0 | 1 0 0 0 1 | 0 0 0 0 0
MBA03_ge 0 0 0 0 0 | 10 1 1 1 0 | 0 0 0 0 0 | 0 0 0 0 0 | 19 4 1 2 0 | 0 0 0 0 0 | 0 0 0 0 0 | 14 1 0 1 0 | 0 0 0 0 0
Lactobacillus 0 0 0 0 0 | 1 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 1 0 0 0 0 | 0 0 0 0 1 | 0 3 1 1 1 | 1 7 28 10 6 | 0 12 17 6 9
Enterococcus 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 5 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 9 0 6
Other (Lactobacillales) 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 2 0 0 | 0 0 0 1 0 | 5 0 0 0 0 | 0 0 0 0 0
Bifidobacterium 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 0 | 0 0 0 0 1 | 0 0 0 0 0 | 0 0 0 2 0 | 0 0 8 13 32
Other(genus level) 0 0 0 0 0 | 6 1 0 0 0 | 0 0 1 0 0 | 0 0 0 0 0 | 13 4 0 2 0 | 1 0 0 0 0 | 0 0 0 0 0 | 8 1 0 0 0 | 0 0 0 0 0
F Fr-1W FD-1W Fr-1.5M FD-1.5M
Abundance (%)
80
60
40
20
0

Fig. 2

A


Bacilalles
Diversity.OTU
Butyrate
Clostridium.sp.
H2
Metabolites
Lactate
Succinate
Escherichia.Shigella
Ethanol
Enterobacteriales
Diversity.Clostridiales
Raoultella.sp.
Acetate
MBA03
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

B

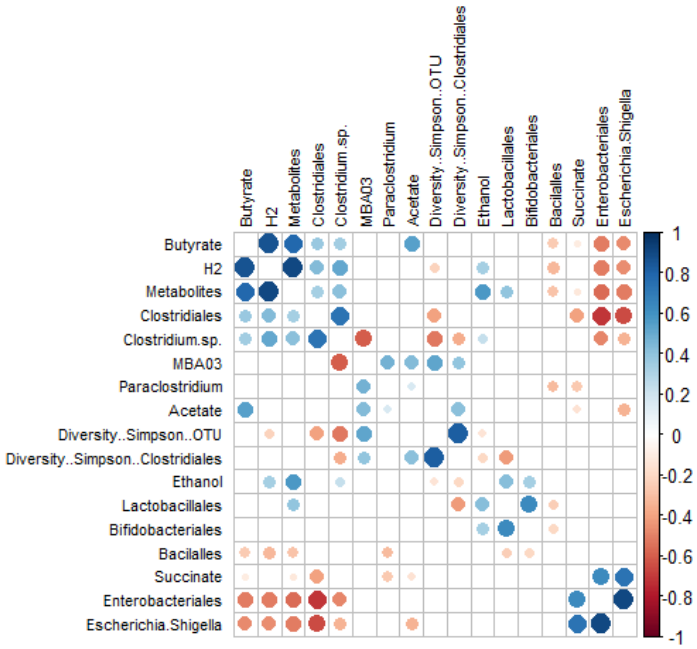
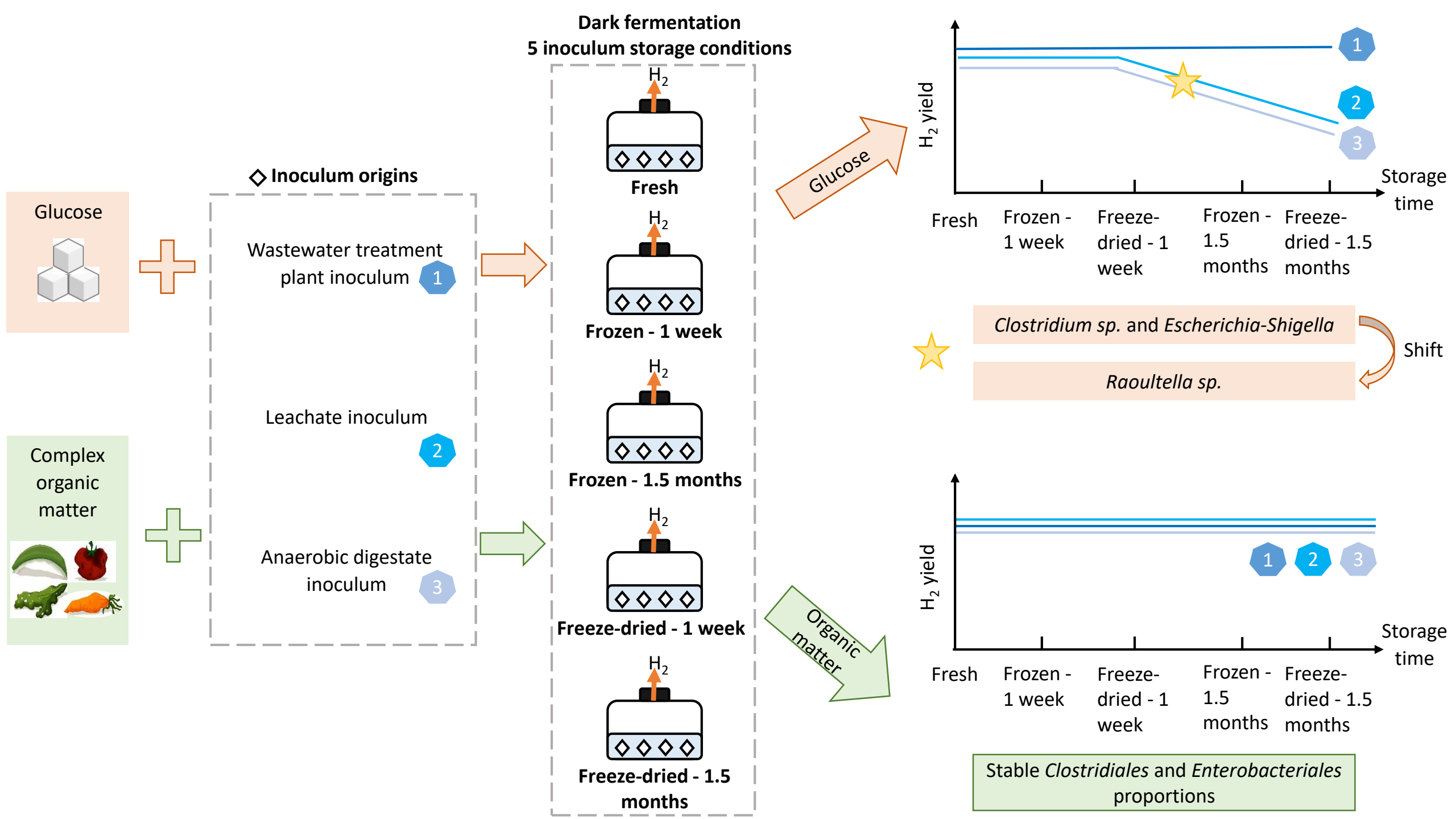

Butyrate
H2
Metabolites
Clostridiales
Clostridium.sp.
MBA03
Paraclostridium
Acetate
Diversity..Simpson..OTU
Diversity..Simpson..Clostridiales
Ethanol
Lactobacillales
Bifidobacteriales
Bacilalles
Succinate
Enterobacteriales
Escherichia.Shigella
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1

Fig. 3


Dark fermentation
5 inoculum storage conditions
Glucose
Complex organic matter
Inoculum origins
Wastewater treatment plant inoculum
1
Leachate inoculum
2
Anaerobic digestate inoculum
3
H2
Fresh
Frozen - 1 week
Frozen - 1.5 months
Freeze-dried - 1 week
Freeze-dried - 1.5 months
Glucose
Organic matter
H2 yield
Storage time
Fresh
Frozen - 1 week
Freeze-dried - 1 week
Frozen - 1.5 months
Freeze-dried - 1.5 months
Clostridium sp. and Escherichia-Shigella
Raoultella sp.
Shift
Stable Clostridiales and Enterobacteriales proportions