Dynamics of bacterial communities and substrate conversion during olive-mill waste dark fermentation: prediction of the metabolic routes for hydrogen production

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Abstract

The aim of this work was to study the biological catalysts and possible substrate conversion routes in mesophilic dark fermentation reactors aimed at producing H_2 from olive mill wastewater. *Bacillus* and *Clostridium* were the most abundant phylotypes during the rapid stage of H_2 production. Chemical analyses combined with predictive

functional profiling of the bacterial communities indicated that the lactate fermentation was the main H_2-producing route. In fact, during the fermentation process, lactate and acetate were consumed, while H_2 and butyrate were being produced. The fermentation process was rich in genes that encode enzymes for lactate generation from pyruvate. Lactate conversion to butyrate through the generation of pyruvate produced H_2 through the recycling of electron carriers via the pyruvate ferredoxin oxydoreductase pathway. Overall, these findings showed the synergy among lactate-, acetate- and H_2-producing bacteria, which complex interactions determine the H_2 production routes in the bioreactors.

Keywords:, Olive-mill waste dark fermentation, Biohydrogen, Bacterial Community, Metabolic route, Lactate fermentation

1. Introduction

The cultivation of olive plants and the production of olive oil have deep roots in the history of the Mediterranean basin. Olive oil production represents a very important agro-industrial activity for many countries, primarily Spain, Italy, Greece and Portugal, followed by Turkey, Morocco, Algeria and Tunisia. The production from these countries reaches more than 3.14 billion tons in 2019/2020 (http://www.internationaloliveoil.org). Countries outside the Mediterranean region, such as Argentina, Australia, the US, South Africa and countries in the Middle East are the emerging olive oil producers. Over the last decade, olive oil has become an increasingly valuable source of antioxidant, anti-inflammatory, anti-proliferative, anti-atherogenic and essential fatty acid for the human diet, representing one of the most important dietary trends worldwide (Dermeche et al., 2013). Thus, the market for olive oil is

52 growing at a rapid race owing to the surging demand from consumers for the nutritional
53 values of olive oil.
54 However, the continuous expansion of the olive oil production is accompanied by the
55 generation of huge amount of wastes, which include 30 million m^3 per year of olive mill
56 wastewater (OMW) with a high pollutant load (Meftah et al., 2019). OMW is a
57 challenging waste to treat because of its own characteristics, such as high salt
58 concentration (EC 5-10 mS cm^{-1}), high acidity (pH 4-5), high biological and chemical
59 oxygen demand (BOD and COD of 100,000 and 220,000 mg L^{-1}, respectively), and
60 high content of polyphenols (Aharonov-Nadborny et al., 2018). The disposal of
61 untreated OMW is a major environmental issue in the Mediterranean countries, where
62 olive oil production is large and concentrated in a relatively short period. The
63 uncontrolled OMW disposal is phytotoxic and it affects microbial activity,
64 biogeochemical cycles of nutrients, pH and the salinity of both soil and water (Doula et
65 al., 2017). Therefore, new treatments for the management and disposal of this waste are
66 urgently needed. Nowadays, the valorization of OMW, while simultaneously degrading
67 pollutants and producing green energy, is receiving a great attention. Indeed, the current
68 trend is to obtain the energy directly from OMW in the form of hydrogen (H_2), rather
69 than to spend the extra energy to treat the waste. There are several routes for H_2
70 production from OMW, such as photobiological processes (Pintucci et al., 2015), dark
71 fermentation processes (Scoma et al., 2013; Ghimire et al., 2015 and 2016),
72 bioelectrochemical systems (Lin et al., 2014) and a steam reforming reaction (Rocha et
73 al., 2017). Among the aforementioned routes, dark fermentation process is attractive
74 because of the wide-range operational temperatures and pressures, the possible usage of
75 various organic wastes, and the recovery of both the energy and the fermentation by-
76 products (Ghimire et al., 2015). However, the literature data on OMW dark
77 fermentation are scarce. Scoma et al. (2013) demonstrated that the production of biofuel

78 and bio-based chemicals from de-phenolized OMW under dark fermentative conditions
79 in packed bed biofilm reactors depends on the applied hydraulic retention time. Ghimire
80 and colleagues (2015) reported that the H_2 potential of OMW was 46 mL H_2 per gram
81 of VS, and that the H_2 yield doubled when the activated sludge that had been pre-treated
82 with heat shock was used as inoculum (Ghimire et al., 2016).
83 Hydrogenases are the main enzyme that regulates the H_2 metabolism and they are
84 classified into three groups based on the number and identity of the metal in their active
85 sites, such as nickel-iron [NiFe], iron-irons [FeFe] and iron [Fe] hydrogenases (Łukajtis
86 et al., 2018). H_2-producing proteins are linked to the efficiency of dark fermentative H_2
87 production and also to the bacteria involved in the process (Kothari et al., 2017).
88 Hydrogen is produced by a wide variety of obligate anaerobic and facultative anaerobic
89 microorganisms that plays an essential role in the dark fermentation process. Among the
90 microorganisms that strictly require anaerobic conditions, *Clostridium* species are key
91 players in H_2 production due to their ability to utilize a wide range of substrates (Cabrol
92 et al., 2017). *Clostridium* are able to harvest the electrons from pyruvate oxidation and
93 to use these electrons for the oxidation of reduced ferredoxin by an [FeFe] hydrogenase,
94 generating H_2 through the pyruvate ferredoxin oxydoreductase (PFOR) pathway (Cabrol
95 et al., 2017). Facultative anaerobes such as *Enterobacter* species produce biogas
96 through pyruvate-formate-lyase (PFL) pathway, in which formate is split into H_2 and
97 CO_2 by a [NiFe] hydrogenase complex (Cabrol et al., 2017).
98 Despite few papers have dealt with H_2 production through dark fermentation of OMW,
99 none of them identified the bacteria responsible for H_2 production and their partnerships
100 with other anaerobic microorganisms. Furthermore, the main pathways for H_2
101 production in the olive-mill waste dark fermentation processes is still unknown.
102 The objective of this work was to uncover the biological catalysts (the key players of
103 the bacterial community) and possible substrate conversion routes in mesophilic dark

fermentation reactors aimed at producing H_2 from OMW. The approach adopted was twofold: i) to investigate the temporal dynamics of both bacterial communities and metabolic profiles during the batch fermentation of OMW ii) to investigate the main bacterial partnerships for H_2 production via metagenome functional prediction. To this end, high-throughput 16S rRNA gene sequencing was used to unveil the key bacteria involved in the fermentation process. The taxonomic composition of the microbial assembly, the alpha- diversity of the microflora, and the fermentation products were analyzed. Finally, fermentation products analyses and predictive functional profiling were performed to study the relationships between process performance indicators and the bacterial community activity. This is the first study reporting the possible metabolic routes responsible for H_2 production in an OMW fermentation system from a microbiological perspective.

2. Materials and methods

2.1. Olive mill waste and inoculum characterization

Olive mill waste (OMW) samples were obtained from the "Fasano oil mill" located in Salerno, Italy. The chemical oxygen demand (COD) method was used to determine the OMW carbon content. The value of 138.75 $gO_2\ L^{-1}$ was obtained and used for set-up purposes. Total solids (TS), volatile solids (VS), and total kjeldahl nitrogen (TKN) of the OMW were 8.1 $gTS\ L^{-1}$, 4.9 $gVS\ L^{-1}$, and 0.45 $g\ L^{-1}$, respectively. COD, TS, VS and TKN were measured as described in the Standard Methods (APHA, 2005). A more complete characterization of the OMW can be found in Ghimire et al. (2016), where a similar wastewater from the same oil mill was used. The substrate was stored at -20°C in lab refrigerators. The initial pH of 5.3 was measured for all OMW samples. The pH value was checked prior to each experiment and a constant trend was monitored over time.

The anaerobic digestate used as inoculum for dark fermentation tests was obtained from a local full-scale anaerobic reactor treating cow sewage (Salerno, Italy). The inoculum characterization highlighted the VS content of 47.76 gVS L^{-1}. This value was converted in soluble COD by using the empirical conversion rate of 1.48 gO_2 gVS^{-1} usually adopted in wastewater treatment for activated sludge. The pH of the inoculum was 7.8. The substrate to inoculum ratio (food to microorganisms, F/M) was fixed at 2 gCOD gVS^{-1} as operational condition to produce a significant amount of H_2 under uncontrolled pH regimen (Spasiano et al., 2019).

2.2. Dark fermentation experiments

Batch experiments of dark fermentation were carried out using airtight 500-mL transparent borosilicate glass bottles (Schott Duran, Germany), placed in a thermostat-controlled water bath at 37°C (mesophilic conditions). To limit the activity of H_2-consuming microorganisms, the inoculum was pre-treated at 105°C for 2h (Maharaj et al., 2019). The batch reactors were equipped with airtight caps fitted with sampling pipes for biogas, liquid mixtures, and organic acid. All the experiments were conducted in duplicate. Each experiment was stopped when H_2 production was no longer observed.

2.3. Analytical methods

The total volume of biogas (including H_2, CO_2, O_2 and N_2), was measured using a gas chromatograph (GC) with argon as the gas carrier (Varian Star 3400, Varian Australia Pty Ltd., Victoria, Australia) equipped with a thermal conductivity detector. The GC was also equipped with a ShinCarbon ST 80/100 column (Restek Corporation, Bellafonte, PA, USA).

The organic acids (citrate, lactate, acetate, formate, butyrate, valerate and caproate) were analyzed by High Performance Liquid Chromatography (HPLC) using a Dionex

chromatograph (Dionex Corporation, Sunnyvale, CA, USA). The system consisted of a chromatography oven (Dionex LC 25) equipped with a Metrosep Organic Acids – 250/7.8 column (Metrohm, Herisau, Switzerland) and an absorbance detector (Dionex AD25) connected to a gradient pump (Dionex GP 50). All samples were eluted with 0.5 mmol L^{-1} sulfuric acid pumped at 0.7 mL min^{-1}. The pH was measured with a lab pH-meter (HI 98190 pH/ORP, Hanna Instruments, Woonsocket, RI, USA).

2.4. Bacterial community analyses

Samples for bacterial community analyses were collected at: i) day 0 (T0), corresponding to the inoculum, ii) day 3 (T3), corresponding to the initial stage of hydrogen production, iii) day 5 (T5), corresponding to the rapid stage of hydrogen production, and iv) day 8 (T8), corresponding to the end of the dark fermentation. At least three biological replicates for each time points were taken and analyzed. Total genomic DNA was extracted according to the manufacturer's instructions from PowerBiofilm DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA). High-throughput sequencing analysis of the V3–V4 region of the bacterial 16S rRNA gene (primers CS1_341F/CS2_806R) was performed by using a MiSeq platform (Illumina) with v3 chemistry providing 2 × 300 paired-end read (Rapin et al., 2017).

Raw data were preprocessed, quality filtered, trimmed, denoised , paired, and modeled via QIIME2 (Bolyen et al., 2019) and DADA2. Chimeras were detected using DADA2 according to the “consensus” method. Sequences were clustered into Sequences Variants (SV). SVs were assigned using a Naïve-Bayes classifier trained on the SILVA database. Samples were rarefied at the minimum library size of 4464.

All sequences were submitted to the European Nucleotide Archive (EMBL-EBI) under the accession numbers ERS4861634 - ERS4861650.

182 **2.5. Data analyses**

183 Multi packages of R software were used to perform the statistical analyses (R Core
184 Team, 2017). Rarefaction curves were done using no-normalized SVs table. Alpha-
185 diversity was calculated with Richness, Shannon index and Evenness using multi
186 package of R software. The differences among samples were evaluated using ANOVA
187 followed by the Turkey (HSD) test. In case of data not normally distributed, the
188 Kruskal-Wallis test was applied and followed by the Dunn test. Co-occurrences among
189 the most abundant bacterial genera (relative abundance >1%) was assessed by the
190 Pearson correlation coefficient.

191 The modified Gompertz equation (1) was used to model biogas production during the
192 fermentation process (Ghimire et al., 2015).

193 (1) $H(t) = H_o \exp\left\{-exp\left[\frac{R\,e}{H_o}(\lambda - t) + 1\right]\right\}$

194 In equation 1, H(t) is the cumulative gas production (mL), λ is the lag-phase time (h),
195 H_o is the gas production potential (mL), R is the maximum gas production rate (mL day^{-1}),
196 t is the incubation time (day), and e is the exp (1)=2.718. Parameters were estimated
197 using the curve fit function in XLSTAT (Addinsoft, version 21.4.1).

198 H_2 yields were calculated by dividing the final cumulative H_2 produced by the amount
199 of the VS added at the start of the experiment.

200 The maximum concentration of each metabolite was converted into COD (g O_2 L^{-1}) by
201 using the COD equivalent factors reported in Moscoviz et al. (2018). The OMW
202 conversion efficiency was then calculated as a percentage by dividing this maximum
203 concentration by the initial COD in the bioreactor.

204 The principal component analysis (PCA) on the correlation matrix was performed using
205 the XLSTAT software to visualize the relationship between the dominant bacterial
206 groups and the main metabolites over time. The first two PCs were subsequently plotted
207 to visualize the results.

2.6. Functional prediction analysis

PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) that infer microbial gene content from 16S rRNA data was used to explore the potential functionality of the bacterial community (Langille et al., 2013). Functional gene prediction was obtained from KEGG-Orthology (KO). Besides, information on metabolic pathways and EC numbers involved in the dark fermentation were manually categorized based on the KEGG and BRENDA databases. Bacterial genera contributing to the genes involved in the main metabolic routes were further investigated by hidden state prediction.

3. Results and Discussion

3.1. Dynamics of the bacterial community structure and diversity

To gain insight into the dark fermentation process of OMW, the dynamics of the bacterial community structure was deeply investigated by 16S rRNA gene high-throughput sequencing.

After bioinformatic analysis, a total of 1,355,103 reads assigned as Bacteria clustered in 2,679 SVs with 99% similarity were found. Rarefaction curves showed that all the samples reached the plateau, indicating that the depth of the sampling and sequencing was sufficiently to detect a nearly maximum number of SVs.

Diversity indices for the samples at different fermentation stages are shown in Figure 1A. Richness, Shannon indices and Evenness showed stable trends over time. Since these indices are based on the DNA-sequencing results, it is reasonable to expect that any variation in the fermentation performance may result primarily from the changes in the functional activities of the established bacterial community (metabolically active

233 community), rather than the changes in alpha diversity of the total community (Klein et
234 al., 2016).
235 As shown in Figure 1B, there were sixteen genera with a relative abundance higher than
236 1% in the samples over time. The predominant phyla of the whole fermentation process
237 were Firmicutes (78.58%), Cloacimonetes (4.70%), Actinobacteria (4.27%),
238 Proteobacteria (4.11%) and Bacteroidetes (3.73%). The microbial community structure
239 changed during the hydrogen fermentation process. At the inoculation stage T0, the
240 most representative taxon was *Clostridium* (38.23%), followed by *Cloacimonadaceae*
241 W5 (12.10%), *Corynebacterium* (9.64%), *Paenibacillus* (8.03%) and *Acinetobacter*
242 (7.01%).
243 From T3, *Bacillus* became the most dominant genus with percentage values ranging
244 from 30.61% to 23.07% of the total taxa, followed by *Clostridium* genus.
245 At T5, besides *Bacillus* and *Clostridium*, two other taxa belonging to the Cloacimonetes
246 and Actinobacteria phyla were also dominant, namely *Cloacimonadaceae* W5 and
247 *Corynebacterium, respectively.* At the end of the dark fermentation (T8), there was an
248 enrichment of other genera, such as *Lysinibacillus* (6.51%), *Ruminiclostridium* (6.29%),
249 *Corynebacterium* (6.16%) and *Caproiciproducens* (5.26%), as well as *Bacillus*
250 (23.07%), *Clostridium* (17.19%) and *Cloacimonadaceae* W5 (14.65%). In this study,
251 the *Clostridium* genus is referred to *Clostridium sensu stricto,* composed by 8 clusters:
252 *Clostridium sensu stricto* 1, 3, 6, 7, 10, 12 (identified as *Clostridium butyrium*) and 13.
253 Lactic acid bacteria (LAB) such as *Turicibacter* (Ikeda-Ohtsubo et al., 2018) and some
254 species of Ruminococcaceae (Boonsaen et al., 2017) remained constant over time.
255 Overall, the findings showed the enrichment of potential HPB over time. Among HPB,
256 *Clostridium* spp. are considered the most abundant and most efficient hydrogen
257 producers in mesophilic fermentation processes. These bacteria are able to generate H_2
258 from different substrates, providing H_2 yields from 0.5 to 3 mol-H_2/mol-hexose (Yang

and Wang, 2019; Yin and Wang, 2016). In batch experiments treating palm oil mill effluent inoculated with heat-shocked sludge pit, the increase of the H_2-producing potential was strongly correlated to the microbial shift toward a *Clostridium* spp.-dominated community (Maaroff et al., 2019).

The most abundant cluster of *Clostrium sensu stricto* genus was *Clostridium sensu stricto 1.* The dominance of this genus was demonstrated also in other research of H_2 production via the dark fermentation (Yang et al., 2019; Yang and Wang, 2019). In addition, *Clostridium sensu stricto 7,* the second most-common cluster, was also found in batch experiments of sludge and flower waste co-fermentation aimed at producing H_2 (Yang and Wang, 2018).

Some spore-forming facultative anaerobic Bacillales have been reported to produce H_2 through the formate hydrogen lyase pathway, providing high yields around 2 mol-H_2/mol-hexose (Cabrol et al., 2017). Compared *to Clostridium*, *Bacillus* spp. are less sensitive to oxygen and they contribute in creating the anaerobic conditions suitable for the growth of strict anaerobic HPB. In fact, at the beginning of the batch fermentation process, the oxygen in the reactor headspace can solubilize in the bulk liquid, favoring the activities of *Bacillus* spp.

Romboutsia and *Sedimentibacter* were common taxa found in H_2 production studies using heat shock pre-treated inoculum (Yang et al., 2019; Yang and Wang, 2019). Although Proteobacteria represent a small proportion of the whole phyla, the presence of *Acinetobacter* increase during the dark fermentation process. Yang and Wang (2018) reported that this taxon has an important role in H_2 production and consumption during the dark fermentation process.

Caproiciproducens increased significantly during the dark fermentation process. According to Feng et al. (2018), the presence of *Caproiciproducens* was related to the production of acetate, butyrate and H_2. *Ruminofilibacter* (Bacteroidetes phylum) is a

285 rumen bacterium that has been detected earlier in a mesophilic anaerobic reactor. This
286 genus is considered as a degrader of lignocellulosic materials.
287 Beside the presence of well-known HPB in the mesophilic fermentative microbiota, it is
288 important to note the crucial role of auxiliary bacteria that are not necessarily involved
289 in primary H_2 production, but rather tightly interacting (either positively or negatively)
290 with HPB, resulting in variable functional outcomes. In fact, the presence of these
291 microorganisms can enhance or discourage H_2 production through different
292 mechanisms, such as cometabolism, oxygen consumption, pH regulation, substrate
293 hydrolysis and cell aggregation (Cabrol et al., 2017).
294 In order to investigate the interactive structure of the fermentative community, Pearson
295 correlations between microbial abundances was performed as shown in Figure 2.
296 The Pearson's correlation analysis between the dominant bacterial groups during the
297 dark fermentation process revealed that both *Bacillus* and *Clostridium* were positively
298 correlated to *Lachnoclostridium* ($r_{Bacillus} = 0.78$, $p_{Bacillus}: < 0.001$; $r_{Clostridium} = 0.63$,
299 $p_{Clostridium}: 0.006$), *Acinetobacter* ($r_{Bacillus} = 0.76$, $p_{Bacillus}: < 0.001$; $r_{Clostridium} = 0.51$,
300 $p_{Clostridium}: 0.036$) and *Caproiciproducens* ($r_{Bacillus} = 0.65$, $p_{Bacillus}: 0.005$; $r_{Clostridium} =$
301 0.52, $p_{Clostridium}: 0.030$). *Lachnoclostridium* and *Caproiciproducens* are two well-
302 characterized carbohydrate fermenters that used [FeFe]-hydrogenase for H_2 production
303 (Greening et al., 2018). They are also major butyrate-producing bacteria (Gutiérrez and
304 Garrido 2019), sustaining the activity of other HPB that can use a butyrate-type
305 fermentation, where only the production of butyrate ensures the pathways to shift
306 towards the H_2 production. *Acinetobacter* is a genus of Gram-negative bacteria
307 belonging to the class of Gammaproteobacteria that are involved in multiple processes
308 during the H_2 production. *Acinetobacter* not only is directly responsible for H_2
309 production (Kanchanasuta et al., 2017), but also for the degradation of hemicellulose
310 and lignocellulosic materials contained in the OMW. Due to their ability to secrete high

311 quantities of hydrolytic enzymes such as xylaneses and cellulases, *Acinetobacter* is
312 implicated in the production of xylooligosaccharides (Purohit et al., 2017), providing
313 substrate hydrolysis for other HPB. The positive correlation between *Acinetobacter* and
314 the two main HPB explains the capability of both *Clostridium* and *Bacillus* to use the
315 products of *Acinetobacter* transformation to produce H_2, thus generating a metabolic
316 synergy between hydrolyzers and HPB (de Sá et al., 2020).
317 *Lachnoclostridium, Acinetobacter* and *Bacillus* showed a positive correlation with
318 *Paenibacillus* ($r_{Lachnoclostridium} = 0.88$, $p_{Lachnoclostridium}$: < 0.001; $r_{Acinetobacter} = 0.74$,
319 $p_{Acinetobacter}$: < 0.001; $r_{Bacillus} = 0.81$, $p_{Bacillus}$: < 0.001). *Paenibacillus* is able to produce
320 large amount of exopolysaccharides by utilizing OMW as the sole carbon and energy
321 sources, with a concomitant reduction in the toxicity of the waste (Dermeche et al.,
322 2013). The reduction in OMW toxicity by *Paenibacillus* was mainly related to the high
323 phenolic-degrading activity and the production of extracellular polysaccharide-
324 degrading enzymes (Aguilera et al., 2008), which likely supported the growth of other
325 microorganisms such as *Lachnoclostridium, Acinetobacter* and *Bacillus*. In addition,
326 according to Aguilera et al. (2001), *Paenibacillus* seems to be involved in the
327 production of acids rather than biogas from many carbohydrates. This means that
328 *Paenibacillus* can contribute positively to hydrogen production in mixed culture
329 fermentations by slightly acidifying the local environment, supporting the development
330 and activity of HPB
331 The main HPBs *Clostridium* and *Bacillus* presented a negative correlation with
332 *Ruminofilibacter* and *Romboutsia* ($r_{Ruminofilibacter} = -0.83$, $p_{Ruminofilibacter}$: < 0.001; $r_{Romboutsia}$
333 = -0.55, $p_{Romboutsia}$: 0.026; $r_{Ruminofilibacter} = -0.58$, $p_{Ruminofilibacter}$: 0.015; $r_{Romboutsia} = 0.69$, ;
334 $p_{Romboutsia}$: 0.002). *Ruminofilibacter* is polysaccharide-degrading genus found in
335 anaerobic digesters for methane production (Ince et al., 2020), while *Romboutsia* is a
336 potential acetogens that grows autotrophically using H_2 as an energy source,

337 respectively (Gerritsen et al., 2014). The negative correlation with the main HPB
338 suggested that these bacteria outcompete HPB for their substrates and directly consume
339 H_2.
340
341 **3.2. Metabolic dynamics of the bacterial community**
342 The temporal successions of the cumulative biogas and the main metabolic
343 intermediates from OMW fermentation were investigated (Figure 3). Biogas production
344 parameters (the gas production potential H_o (mL), the maximum gas production rate R
345 (mL d^{-1}), the lag-phase time lambda (d) and the determination coefficient R^2) measured
346 by the modified Gompertz model were reported in Figure 3A-D. The yield of H_2
347 increased over time, showing a rapid production from T3 to T8 with a maximum H_2
348 production rate of 15 mL d^{-1} and a H_2 production potential of 60.6 mL (R^2= 0.978,
349 Figure 3A). The researchers claimed that the low H_2 yield can be due to the presence of
350 polyphenols, which might have an inhibitory effect on the reactor's microflora. The
351 total biogas trend showed a gradual increase from T1 to T8, without methane generation
352 (Figure 3B-4D). The maximum biogas production rate was 166.2 mL d^{-1} with a biogas
353 production potential of 664.6 mL (R^2= 0.992). The maximum H_2 yield of 33.8 mL H_2
354 gVS^{-1} was obtained. This value was similar to the H_2 yield of 33.1 mL H_2 gVS^{-1}reported
355 by Ghimire et al. (2016), where a similar OMW was used.
356 Figure 3E showed the trend of pH during the dark fermentation process. As reported in
357 the graph, the pH value decreased over time from 7.1 at T0 to 6.5 at T5 with the
358 formation of acidic metabolites. At the end of the dark fermentation (T8), the pH value
359 kept stable at 6.1.
360 The formation of the main soluble metabolites during the dark fermentation process was
361 also investigated (Figure 3F-I). Propionate, valerate and caproate were not detected in
362 the mesophilic reactors. The results showed that lactate and acetate were the main

363 metabolites generated during the initial stage of H_2 production (T3). Among them,
364 acetate was the dominant ones. The conversions of the initial COD to lactate, acetate
365 and formate at T3 were 8.9, 16, and 0.15 %, respectively. During the stages T5-T8, the
366 concentration of formate, butyrate and H_2 increased, while the concentration of lactate
367 and acetate decreased. Once the lactate was depleted, butyrate formation and H_2
368 production also ceased, despite the acetate availability. The conversions of the initial
369 COD to lactate, acetate, formate and butyrate at T5 were 1.25, 16.6, 0.22 and 7.66%,
370 respectively. At the end of the dark fermentation (T8), the concentrations of lactate,
371 acetate, formate and butyrate were 0, 5403, 0 and 1476 mg L^{-1}, which corresponded to
372 0%, 78%, 0% and 22% of the total detected soluble metabolites, respectively.
373 The dynamics of metabolites presented herein seems to indicate a fermentative H_2
374 production from lactate and acetate. The generation of H_2, CO_2 accompanied with
375 production of butyrate from lactate/acetate fermentation has been previously reported in
376 several studies (*inter alia* Matsumoto and Nishimura 2007; Fuess et al., 2018). Acetate
377 is not only a source of carbon and energy for many metabolic reactions, but it is also
378 required as an electron acceptor in the lactate fermentation (Duncan et al., 2004). The
379 conversion of lactate and acetate to butyrate and H_2 is an energetically favorable process
380 (Duncan et al., 2004).
381 To provide reliable data to clarify the main conversion routes established in the
382 fermentation system, the bacterial community structure was correlated with the major
383 metabolic by-products through principal component analysis (PCA) (Figure 4).
384 According to the PCA, which explained 89% of the total variability, it was possible to
385 clearly discern the four phases that characterized the fermentation process of OMW,
386 from the inoculum (T0) to the H_2 production (T8). Thus, major changes in both
387 metabolites and bacterial profiles were observed as a function of the incubation time.

388 At T0, a positive correlation with pH was exhibited by *Sedimentibacter,*
389 *Corynebacterium* and *Ruminofilbacter.* The last two genera are involved in the
390 conversion of carbohydrates and complex molecules into small organic acids that
391 lowered the pH (Jin et al., 2019).
392 The T3 phase was mainly characterized by the production of lactate, which was closely
393 matched by the presence of the LAB *Turicibacter* in the bacterial community.
394 The final stage T8 encompassed the production of H_2, where lactate, acetate and
395 formate were consumed while H_2 and butyrate were produced. In fact, lactate was
396 negatively correlated with the production of H_2 and butyrate. The observed trend
397 indicated a H_2 production through a lactate fermentation (equation 2-3). During lactate
398 fermentation electrons are transferred to either ferredoxin or NAD+, which favor H_2
399 production through proton reduction used to recycle the electron carriers (equation 2).
400 Excess NADH generated by the system is used to produce other reduced fermentation
401 products, such as butyrate (Cabrol et al., 2017). The PCA biplot showed a positive
402 correlation between butyrate and *Clostridium* genus. The ability to produce H_2 from
403 lactate and acetate seems to be widely conserved in the genus *Clostridium* and other
404 HPB capable of butyrate fermentation of carbohydrates (Matsumoto and Nishimura
405 2007; Juang et al. 2011; Tao et al., 2016).
406 It is worth noting that the presence of formate was inversely correlated to lactate. The
407 detection of formate at T5 (Figure 3H) suggested that, in the bioreactor, formate was
408 produced by more than one group of bacteria via interconversion reactions, in addition
409 to its direct conversion from glucose. This result supported the fact that lactate-based
410 metabolic pathway might indirectly produce formate, which can split into H_2 and CO_2
411 through the PFL pathways (Cabrol et al., 2017). Indeed, formate positively correlated
412 with the biogas production. Interestingly, acetate was inversely correlated with H_2 and
413 butyrate production along the F2 axes. It should be noted that acetate is a substrate and

414 an intermediate of the lactate pathway in the butyrate transformation (equation 1)
415 (Duncan et al., 2004). Several studies reported the stimulation of H_2 production by
416 lactate and acetate mixed with the substrate. Baghchehsaraee et al. (2009) showed that
417 the addition of lactate to a mixed culture grown on starch-containing medium increased
418 both the H_2 production and the butyric acid formation. By contrast, when lactate was the
419 only carbon source, the level of H_2 production was very low. Juang et al. (2011)
420 observed the use of lactate and acetate for H_2 and butyrate production by mixed-bacteria
421 culture from tapioca starch. Tao et al. (2016) claimed that exogenous acetate enhanced
422 lactate conversion to butyrate at pH 6.0, whereas when fermentable lactate was omitted
423 from the medium and acetate was used as a sole energy source, acetate was not
424 consumed and butyrate was not produced. These findings suggest that the conversion of
425 lactate and acetate into H_2 and butyrate is due to the cooperative interactions between
426 lactate- and H_2-producing bacteria.
427
428 (2) lactate + acetate ⇋ butyrate + H_2 + CO_2 + H_2O
429 (3) $H^+ \leftrightharpoons H_2$
430
431 **3.3. Predictive functional analysis of bacterial communities**
432 To deepen the understanding of the OMW fermentative ecosystems, it is crucial to
433 know which substrate conversion pathways are operated by the bacterial community
434 over time. In this study, the potential substrate conversion routes that might occur in the
435 system and the contribution of the phylotypes to the main metabolic pathways were
436 identified based on the functional genes predicted using the PICRUSt software.
437 PICRUSt compares the identified 16S rRNA gene sequences to those of known species,
438 thereby inferring the possible gene contents of the bacterial communities. Although
439 PICRUSt results offer only a prediction, their combination with the temporal dynamic

of biogas production and metabolic intermediates can provide a clear idea of the possible metabolic activities in OMW dark fermentation.

Since H_2 can be produced by several different pathways, the presence and relative abundance of genes that code for the enzymes involved in the H_2 production from butyrate, acetate, formate and lactate were investigated. The predicted relative abundances of these genes, the main metabolic routes for H_2 production and their predicted metagenome contributions are summarized in Figure 5 and 6, respectively. The fermentation process aimed at H_2 production was rich in genes that encode enzymes for lactate generation from pyruvate (L-lactate dehydrogenase (cytochrome), EC:1.1.1.27, n. 6; D-lactate dehydrogenase (cytochrome), EC:1.1.1.28, n. 3-4). The predicted main phylotypes that contribute to the presence of these lactate dehydrogenases were *Clostridium*, *Bacillus*, *Paenibacillus*, *Corynebacterium* and *Turicibacter*. The decreasing concentration of lactate during the fermentation process suggests its possible consumption through several interconversion reactions. The transformation of lactate to pyruvate was mainly operated by *Bacillus*, *Lysinibacillus*, *Rummeliibacillus* and *Corynebacterium* through the enzymes lactate dehydrogenases EC:1.1.2.4 (n. 2), and EC:1.1.2.3 (n. 5). The acetyl-CoA formed followed the metabolic pathways that produced mainly acetate and butyrate. These findings combined with the metabolite profiles reported in Figures 3 and 4 corroborate the contribution of lactate fermentation in the H_2 production. LAB have been detected in almost all of the fermentation systems aimed at producing H_2. Although many studies have indicated that LAB inhibit H_2 production due to substrate competition and/or excretion of bacteriocines (Cabrol et al., 2017), positive interactions between LAB and clostridial species with production of H_2 from lactate have also been reported (Fuess et al., 2018; García-Depraect et al., 2019). Hydrogen production from lactate fermentation represents an important strategy that enable lactate-consuming HPB to obtain energy under

466 unfavorable conditions such as low carbohydrate-availability (García-Depraect et al.,
467 2019). This is not the case of OMW, which are characterized by high chemical (COD)
468 and biochemical oxygen demand (BOD) values. However, OMW are characterized by
469 high concentrations of phenolic compounds that can be toxic for the microorganisms,
470 with negative impacts on the fermentation process (Vavouraki et al., 2019). LAB can
471 easily metabolize phenolic compound by either detoxifying or utilizing them as an
472 alternative source of energy (Filannino et al., 2014).
473 The predicted relative abundance of the genes that encode key enzymes for acetate
474 production and consumption (e.g. acetate kinase, EC:2.7.2.1, n. 14; phosphate
475 acetyltransferase, EC:2.3.1.8, n. 15) was high throughout the fermentation process. The
476 decreasing acetate concentration over time suggests that the consumption of acetate for
477 several purposes, including the generation of energy and the production of other fatty
478 acids. The main phylotypes associated to acetate production and/or consumption were
479 *Bacillus, Corynebacterium, Paenibacillus Ruminococcaceae* UCG-014 and
480 *Lysinibacillus*. These phylotypes may supply extra acetate that acts as an oxidizing
481 agent for the bioconversions of lactate into H_2 (García-Depraect et al., 2019).
482 *Bacillus*, *Caproiciproducens*, *Clostridium*, *Lysinibacillus* and *Romboutsia,* were the
483 principal phylotypes contributing to the presence of butyrate kinase (EC:2.7.2.7, n. 29)
484 and acetate CoA-transferase (EC:2.8.3.8, n. 30-31), key enzymes that catalyze the
485 conversion of butyrate phosphate and butyryl CoA to butyrate. In addition, based on the
486 high butyrate and the decreasing of lactate and acetate concentrations over time, these
487 bacteria may have produced butyrate via the lactate and acetate interconversion
488 reactions.
489 Formate production and consumption are controlled by the key enzyme formate C-
490 acetyltransferase (EC:2.3.1.54, n. 33), which belonged to the genus *Caproiciproducens*,
491 *Clostridium, Lachnoclostridium, Romboutsia* and *Ruminiclostridium*.

492 An observation that is worth noting is that the H_2 production occurred mainly through
493 the oxidation of reduced ferredoxin with a monomeric ferredoxin-dependent
494 hydrogenase (hydrogen:ferredoxin oxidoreductase, EC:1.12.7.2, n. 20). Moreover,
495 NADH was oxidized and the ferredoxin reduced by NADH:ferredoxin oxidoreductase
496 (NFOR, EC 1.18.1.3, n. 21). The reduced ferredoxin can be used by electron bifurcating
497 or monomeric ferredoxin-dependent hydrogenase to reduce protons, yielding H_2. Both
498 the above-mentioned enzymes belong to the PFOR pathway that is used by obligate
499 anaerobic microorganisms to couple H_2 production to the recycling of the electron
500 carriers ferredoxin and NADH that accumulate during the anaerobic metabolisms
501 (Cabrol et al., 2017). The only two principal phylotypes carrying both the
502 hydrogen:ferredoxin oxidoreductase and the NADH:ferredoxin oxidoreductase were
503 *Clostridium* and *Romboutsia*. The PCA graph in Figure 4 showed a positive correlation
504 between H_2 and *Clostridium*, while H_2 and *Romboutsia* are inversely related. This result
505 suggested that the gut bacterium *Romboutsia* might act as a H_2 -consuming bacterium,
506 which would contribute in explaining the low H_2 production in the bioreactors.
507 *Romboutsia* is a potential acetogens that grows autotrophically using H_2 as an energy
508 source (Gerritsen et al., 2014).
509 The PiCRUST analysis also predicted the H_2 production through the PFL pathway,
510 where the formate is split into H_2 and CO_2 by a formate-hydrogen-lyase complex that
511 contains a nickel-iron [NiFe] hydrogenase. However, the predicted abundance of the
512 genes that code for the formate-hydrogen-lyase complex (EC:1.12.1.2, n. 40) was close
513 to zero throughout the fermentation process and present in few rare bacterial species
514 with relative abundance < 0.5%. Hence, on the basis of the metabolic predictions, the
515 H_2 production through the PFL pathways was not an important metabolic reaction in the
516 OMW fermentation reactors.

Figure 6 summarizes the prediction of the principal phylotypes at genus level that contribute to the genes that code for the key enzymes involved in the production and consumption of lactate, acetate, formate, butyrate, and H_2. In the bioreactor, a collaboration between the bacteria that were involved in lactate (e.g. *Clostridium*, *Corynebacterium* and *Turicibacter*), acetate (e.g. *Bacillus*, *Paenibacillus* and *Corynebacterium*), formate (e.g. *Lachnoclostridium*, *Romboutsia* and *Caproiciproducens*), butyrate (*Bacillus*, *Clostridium* and *Caproiciproducens*) and H_2 (*Clostridium* and *Romboutsia*) metabolisms took place. The figure shows a complex ecosystem where different key players act sequentially to use the available resources and maximize their own fitness. In fact, the proliferation of LAB and acetic acid bacteria (AAB) led to the transformation of complex carbohydrates, while lowering the pH and assuring an anaerobic condition. The activity of LAB and AAB was jeopardized by the exhaustion of resources and the accumulation of by-products such as lactate and acetate, which are used as carbon and energy sources by lactate-consuming HPB, acetate-consuming HPB and butyrate-producing bacteria. The cooperative interaction between LAB, AAB and HPB has been previously observed in the gut microbiota, where the conversion of lactate to butyrate is one of the important factors for maintaining homeostasis in the gastrointestinal tract (Moens et al., 2016). The pH is a key factor for H_2 production through lactate fermentation in dark fermentation systems (Juang et al., 2011). Wu and colleagues (2012) reported the range of pH for maximizing H_2 production from lactate and acetate, which was between 5.5 and 6. The measured pH in our bioreactors was slightly higher than this range. The final pH in the bioreactors might be one of the reasons for low H_2 production in our OMW-fermentation system.

4. Conclusion

High-throughput sequencing and metabolic profile analyses revealed the substrate conversion routes that take place in an OMW-fermentation system that is designed for H_2 production. Lactate fermentation was the main H_2-producing route. During the fermentation process, lactate and acetate were consumed, while H_2 and butyrate were being produced. Lactate conversion to butyrate through the generation of pyruvate proceeded via either butyrate kinase or butyryl-CoA: acetate-CoA transferase. Overall, these findings showed the synergy among LAB, AAB and HPB, which complex interactions produced H_2 through the recycling of the electron carriers ferredoxin and NADH via the PFOR pathway.

Supplementary materials

E-supplementary data of this work can be found in online version of the paper.

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References

1. Aguilera, M., Monteoliva-Sánchez, M., Suárez, A., Guerra, V., Lizama, C., Bennasar, A., Ramos-Cormenzana, A., 2001. *Paenibacillus jamilae* sp. nov., an exopolysaccharide-producing bacterium able to grow in olive-mill wastewater. Int. J. Syst. Evol. Microbiol. 51, 1687–1692. https://doi.org/10.1099/00207713-51-5-1687
2. Aguilera, M., Quesada, M.T., del Águila, V.G., Morillo, J.A., Rivadeneyra, M.A., Ramos-Cormenzana, A., Monteoliva-Sánchez, M., 2008. Characterisation of *Paenibacillus jamilae* strains that produce exopolysaccharide during growth on and detoxification of olive mill wastewaters. Bioresour. Technol. 99, 5640–5644. https://doi.org/10.1016/j.biortech.2007.10.032
3. Aharonov-Nadborny, R., Tsechansky, L., Raviv, M., Graber, E.R., 2018. Mechanisms governing the leaching of soil metals as a result of disposal of olive mill wastewater on agricultural soils. Sci. Total Environ. 630, 1115–1123. https://doi.org/10.1016/j.scitotenv.2018.02.270
4. American Public Health Association (APHA) Standard Methods for the Examination of Water and Wastewater (21st ed.), American Public Health Association Water Works Association, American Water Environment Federation, Washington, DC (2005)
5. Baghchehsaraee, B., Nakhla, G., Karamanev, D., Margaritis, A., 2009. Effect of extrinsic lactic acid on fermentative hydrogen production. Int. J. Hydrogen Energy 34, 2573–2579. https://doi.org/10.1016/j.ijhydene.2009.01.010
6. Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C.,

586 Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F.,
587 Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L.,
588 Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H.,
589 Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler,
590 B.D., Kang, K. Bin, Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I.,
591 Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield,
592 E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver,
593 L.J., Melnik, A. V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T.,
594 Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L.,
595 Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson,
596 M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J.,
597 Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi,
598 A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-
599 Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M.,
600 Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld,
601 J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible,
602 interactive, scalable and extensible microbiome data science using QIIME 2.
603 Nat. Biotechnol. 37, 852-857. https://doi.org/10.1038/s41587-019-0209-9

604 7. Boonsaen, P., Kinjo, M., Sawanon, S., Suzuki, Y., Koike, S., Kobayashi, Y.,
605 2018. Partial characterization of phylogeny, ecology and function of the
606 fibrolytic bacterium *Ruminococcus flavefaciens* OS14, newly isolated from the
607 rumen of swamp buffalo. Anim. Sci. J. 89, 377-385.
608 https://doi.org/10.1111/asj.12927

609 8. Cabrol, L., Marone, A., Tapia-Venegas, E., Steyer, J.P., Ruiz-Filippi, G., Trably,
610 E., 2017. Microbial ecology of fermentative hydrogen producing bioprocesses:
611 Useful insights for driving the ecosystem function. FEMS Microbiol. Rev. 41,

158-181. https://doi.org/10.1093/femsre/fuw043

9. de Sá, L.R.V., Faber, M. de O., da Silva, A.S.A., Cammarota, M.C., Ferreira-Leitão, V.S., 2020. Biohydrogen production using xylose or xylooligosaccharides derived from sugarcane bagasse obtained by hydrothermal and acid pretreatments. Renew. Energy 146, 2408–2415. https://doi.org/10.1016/j.renene.2019.08.089
10. Dermeche, S., Nadour, M., Larroche, C., Moulti-Mati, F., Michaud, P., 2013. Olive mill wastes: Biochemical characterizations and valorization strategies. Process Biochem. 48, 1532–1552. https://doi.org/10.1016/j.procbio.2013.07.010
11. Doula, M.K., Moreno-Ortego, J.L., Tinivella, F., Inglezakis, V.J., Sarris, A., Komnitsas, K., 2017. Olive mill waste: Recent advances for the sustainable development of olive oil industry, Olive Mill Waste: Recent Advances for Sustainable Management. Elsevier Inc. 29-56. https://doi.org/10.1016/B978-0-12-805314-0.00002-9
12. Duncan, S.H., Louis, P., Flint, H.J., 2004. Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. Appl. Environ. Microbiol. 70, 5810-5817. https://doi.org/10.1128/AEM.70.10.5810-5817.2004
13. Feng, K., Li, H., Zheng, C., 2018. Shifting product spectrum by pH adjustment during long-term continuous anaerobic fermentation of food waste. Bioresour. Technol. 270, 180–188. https://doi.org/10.1016/j.biortech.2018.09.035
14. Filannino, P., Bai, Y., Di Cagno, R., Gobbetti, M., Gänzle, M.G., 2015. Metabolism of phenolic compounds by *Lactobacillus* spp. during fermentation of cherry juice and broccoli puree. Food Microbiol. 46, 272-279. https://doi.org/10.1016/j.fm.2014.08.018
15. Fuess, L.T., Ferraz, A.D.N., Machado, C.B., Zaiat, M., 2018. Temporal

dynamics and metabolic correlation between lactate-producing and hydrogen-producing bacteria in sugarcane vinasse dark fermentation: The key role of lactate. Bioresour. Technol. 247, 426-433. https://doi.org/10.1016/j.biortech.2017.09.121

16. García-Depraect, O., Valdez-Vázquez, I., Rene, E.R., Gómez-Romero, J., López-López, A., León-Becerril, E., 2019. Lactate- and acetate-based biohydrogen production through dark co-fermentation of tequila vinasse and nixtamalization wastewater: Metabolic and microbial community dynamics. Bioresour. Technol. 282, 236-244. https://doi.org/10.1016/j.biortech.2019.02.100

17. Gerritsen, J., Fuentes, S., Grievink, W., van Niftrik, L., Tindall, B.J., Timmerman, H.M., Rijkers, G.T., Smidt, H., 2014. Characterization of *Romboutsia ilealis* gen. nov., sp. nov., isolated from the gastro-intestinal tract of a rat, and proposal for the reclassification of five closely related members of the genus *Clostridium* into the genera *Romboutsia* gen. nov., *Intestinibacter* gen. nov., *Terrisporobacter* gen. nov. and *Asaccharospora* gen. nov. Int. J. Syst. Evol. Microbiol. 64, 1600-1616. https://doi.org/10.1099/ijs.0.059543-0

18. Ghimire, A., Frunzo, L., Pontoni, L., d'Antonio, G., Lens, P.N.L., Esposito, G., Pirozzi, F., 2015. Dark fermentation of complex waste biomass for biohydrogen production by pretreated thermophilic anaerobic digestate. J. Environ. Manage. 152, 43-48. https://doi.org/10.1016/j.jenvman.2014.12.049

19. Ghimire, A., Sposito, F., Frunzo, L., Trably, E., Escudié, R., Pirozzi, F., Lens, P.N.L., Esposito, G., 2016. Effects of operational parameters on dark fermentative hydrogen production from biodegradable complex waste biomass. Waste Manag. 50, 55–64. https://doi.org/10.1016/j.wasman.2016.01.044

20. Greening, C., Geier, R., Wang, C., Woods, L.C., Morales, S.E., McDonald,

M.J., Rushton-Green, R., Morgan, X.C., Koike, S., Leahy, S.C., Kelly, W.J., Cann, I., Attwood, G.T., Cook, G.M., Mackie, R.I., 2018. Alternative hydrogen uptake pathways suppress methane production in ruminants. bioRxiv 486894. https://doi.org/10.1101/486894

21. Gutiérrez, N., Garrido, D., 2019. Species deletions from microbiome consortia reveal key metabolic interactions between gut microbes. mSystems 4, e00185-19. https://doi.org/10.1128/msystems.00185-19

22. Ikeda-Ohtsubo, W., Brugman, S., Warden, C.H., Rebel, J.M.J., Folkerts, G., Pieterse, C.M.J., 2018. How can we define "optimal microbiota?": a comparative review of structure and functions of microbiota of animals, fish, and plants in agriculture. Front. Nutr. 5, 90. https://doi.org/10.3389/fnut.2018.00090

23. Ince, O., Akyol, Ç., Ozbayram, E.G., Tutal, B., Ince, B., 2020. Enhancing methane production from anaerobic co-digestion of cow manure and barley: Link between process parameters and microbial community dynamics. Environ. Prog. Sustain. Energy. 39. https://doi.org/10.1002/ep.13292

24. Jin, Y., Lin, Y., Wang, P., Jin, R., Gao, M., Wang, Q., Chang, T.C., Ma, H., 2019. Volatile fatty acids production from saccharification residue from food waste ethanol fermentation: Effect of pH and microbial community. Bioresour. Technol. 292, 121957. https://doi.org/10.1016/j.biortech.2019.121957

25. Juang, C.P., Whang, L.M., Cheng, H.H., 2011. Evaluation of bioenergy recovery processes treating organic residues from ethanol fermentation process. Bioresour. Technol. 102, 5394-5399. https://doi.org/10.1016/j.biortech.2010.10.069

26. Kanchanasuta, S., Prommeenate, P., Boonapatcharone, N., Pisutpaisal, N., 2017. Stability of *Clostridium butyricum* in biohydrogen production from non-sterile

food waste. Int. J. Hydrogen Energy 42, 3454–3465. https://doi.org/10.1016/j.ijhydene.2016.09.111

27. Klein, A.M., Bohannan, B.J.M., Jaffe, D.A., Levin, D.A., Green, J.L., 2016. Molecular evidence for metabolically active bacteria in the atmosphere. Front. Microbiol. 7, 1–11. https://doi.org/10.3389/fmicb.2016.00772

28. Kothari, R., Kumar, V., Pathak, V. V., Ahmad, S., Aoyi, O., Tyagi, V. V., 2017. A critical review on factors influencing fermentative hydrogen production. Front. Biosci. - Landmark 22, 1195–1220. https://doi.org/10.2741/4542

29. Langille, M.G.I., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Vega Thurber, R.L., Knight, R., Beiko, R.G., Huttenhower, C., 2013. Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. Nat. Biotechnol. 31, 814–821. https://doi:10.1038/nbt.2676.

30. Lin, H., Wu, X., Hu, B., Zhu, J., 2014. microbial electrochemical systems for agro-industrial wastewater remediation and renewable products generation: a review. Elyns J. Microbes 01, 1–20. https://doi.org/10.19104/amb.2014.101

31. Łukajtis, R., Hołowacz, I., Kucharska, K., Glinka, M., Rybarczyk, P., Przyjazny, A., Kamiński, M., 2018. Hydrogen production from biomass using dark fermentation. Renew. Sustain. Energy Rev. 91, 665-694. https://doi.org/10.1016/j.rser.2018.04.043

32. Maaroff, R.M., Md Jahim, J., Azahar, A.M., Abdul, P.M., Masdar, M.S., Nordin, D., Abd Nasir, M.A., 2019. Biohydrogen production from palm oil mill effluent (POME) by two stage anaerobic sequencing batch reactor (ASBR) system for better utilization of carbon sources in POME. Int. J. Hydrogen Energy. 44, 3395-3406. https://doi.org/10.1016/j.ijhydene.2018.06.013

33. Maharaj, B.C., Mattei, M.R., Frunzo, L., Hullebusch, E.D. va., Esposito, G.,

2019. ADM1 based mathematical model of trace element complexation in anaerobic digestion processes. Bioresour. Technol. 276, 253–259. https://doi.org/10.1016/j.biortech.2018.12.064

34. Matsumoto, M., Nishimura, Y., 2007. Hydrogen production by fermentation using acetic acid and lactic acid. J. Biosci. Bioeng. 103, 236-241. https://doi.org/10.1263/jbb.103.236

35. Meftah, O., Guergueb, Z., Braham, M., Sayadi, S., Mekki, A., 2019. Long term effects of olive mill wastewaters application on soil properties and phenolic compounds migration under arid climate. Agric. Water Manag. 212, 119–125. https://doi.org/10.1016/j.agwat.2018.07.029

36. Moens, F., Verce, M., De Vuyst, L., 2017. Lactate- and acetate-based cross-feeding interactions between selected strains of lactobacilli, bifidobacteria and colon bacteria in the presence of inulin-type fructans. Int. J. Food Microbiol. 241, 225-236. https://doi.org/10.1016/j.ijfoodmicro.2016.10.019

37. Moscoviz, R., Trably, E., Bernet, N., Carrère, H., 2018. The environmental biorefinery: State-of-the-art on the production of hydrogen and value-added biomolecules in mixed-culture fermentation. Green Chem. 20, 3159–3179. https://doi.org/10.1039/c8gc00572a

38. Purohit, A., Rai, S.K., Chownk, M., Sangwan, R.S., Yadav, S.K., 2017. Xylanase from *Acinetobacter pittii* MASK 25 and developed magnetic cross-linked xylanase aggregate produce predominantly xylopentose and xylohexose from agro biomass. Bioresour. Technol. 244, 793–799. https://doi.org/10.1016/j.biortech.2017.08.034.

39. Pintucci, C., Padovani, G., Giovannelli, A., Traversi, M.L., Ena, A., Pushparaj, B., Carlozzi, P., 2015. Hydrogen photo-evolution by *Rhodopseudomonas palustris* 6A using pre-treated olive mill wastewater and a synthetic medium

containing sugars. Energy Convers. Manag. 90, 499–505. https://doi.org/10.1016/j.enconman.2014.11.045

40. Rapin, A., Pattaroni, C., Marsland, B.J., Harris, N.L., 2017. Microbiota analysis using an Illumina MiSeq platform to sequence 16S rRNA genes. Curr. Protoc. Mouse Biol. 7, 100-129. https://doi.org/10.1002/cpmo.29
41. Rocha, C., Soria, M.A., Madeira, L.M., 2017. Steam reforming of olive oil mill wastewater with in situ hydrogen and carbon dioxide separation – Thermodynamic analysis. Fuel 207, 449–460. https://doi.org/10.1016/j.fuel.2017.06.111
42. Scoma, A., Bertin, L., Fava, F., 2013. Effect of hydraulic retention time on biohydrogen and volatile fatty acids production during acidogenic digestion of dephenolized olive mill wastewaters. Biomass and Bioenergy 48, 51–58. https://doi.org/10.1016/j.biombioe.2012.10.028
43. Spasiano, D., Luongo, V., Race, M., Petrella, A., Fiore, S., Apollonio, C., Pirozzi, F., Fratino, U., Piccinni, A.F., 2019. Sustainable bio-hydrothermal sequencing treatment for asbestos-cement wastes. J. Hazard. Mater. 364, 256–263. https://doi.org/10.1016/j.jhazmat.2018.10.025
44. Tao, Y., Hu, X., Zhu, X., Jin, H., Xu, Z., Tang, Q., Li, X., 2016. Production of butyrate from lactate by a newly isolated *Clostridium* sp. BPY5. Appl. Biochem. Biotechnol. 179, 361-374. https://doi.org/10.1007/s12010-016-1999-6
45. Vavouraki, A.I., Zakoura, M. V., Dareioti, M.A., Kornaros, M., 2019. Biodegradation of polyphenolic compounds from olive mill wastewaters (OMW) during two-stage anaerobic co-digestion of agro-industrial mixtures. Waste and Biomass Valorization. https://doi.org/10.1007/s12649-019-00887-4
46. Wu, C.W., Whang, L.M., Cheng, H.H., Chan, K.C., 2012. Fermentative biohydrogen production from lactate and acetate. Bioresour. Technol. 113, 30-6.

https://doi.org/10.1016/j.biortech.2011.12.130

47. Yang, G., Wang, J., 2018. Synergistic biohydrogen production from flower wastes and sewage sludge. Energy and Fuels 32, 6879–6886. https://doi.org/10.1021/acs.energyfuels.8b01122

48. Yang, G., Wang, J., 2019. Changes in microbial community structure during dark fermentative hydrogen production. Int. J. Hydrogen Energy 44, 25542–25550. https://doi.org/10.1016/j.ijhydene.2019.08.039

49. Yang, G., Yin, Y., Wang, J., 2019. Microbial community diversity during fermentative hydrogen production inoculating various pretreated cultures. Int. J. Hydrogen Energy 44, 13147–13156. https://doi.org/10.1016/j.ijhydene.2019.03.216

50. Yin, Y., Wang, J., 2016. Changes in microbial community during biohydrogen production using gamma irradiated sludge as inoculum. Bioresour. Technol. 200, 217–222. https://doi.org/10.1016/j.biortech.2015.10.027

784 **Figure and table Captions**

785 **Figure 1.** (A) Alpha diversity indices of the microbial communities during the
786 fermentation process. (B) Taxonomic classification of the dominant bacterial groups
787 over time in the OMW fermentation reactors. Only bacteria with relative abundances >
788 1.0% were represented. Different letters indicate statistically significant differences at p
789 < 0.05. T0: inoculum; T3: the initial stage of H_2 production; T5: the rapid stage of H_2
790 production; T8: the end of the dark fermentation.

791

792 **Figure 2.** Ecological networks based on Pearson correlation analysis between the
793 dominant bacterial groups. Shaded boxes show significant correlation at $p<0.05$.

794

795 **Figure 3.** Time-course profile of cumulative biogas (A-D), pH (E) and organic acids (F-
796 I) produced during the fermentation of OMW. Biogas production parameters (the gas
797 production potential H_o (mL), the maximum gas production rate R (mL d^{-1}), the lag-
798 phase time lambda (d) and the determination coefficient R^2) measured by the modified
799 Gompertz model are reported in panels A-D. Different letters indicate statistically
800 significant differences at $p < 0.05$. T0: inoculum; T3: the initial stage of H_2 production;
801 T5: the rapid stage of H_2 production; T8: the end of the dark fermentation.

802

803 **Figure 4.** Correlation between bacterial groups, time and process performance
804 indicators by principal component analyses (PCA). T0: inoculum; T3: the initial stage
805 of H_2 production; T5: the rapid stage of H_2 production; T8: the end of the dark
806 fermentation

807

808 **Figure 5.** (A) Heat map of the predicted relative abundances of genes that encodes the
809 key enzymes involved in lactate, acetate, formate and butyrate metabolisms. T0:

inoculum; T3: the initial stage of H_2 production; T5: the rapid stage of H_2 production; T8: the end of the dark fermentation. (B) The main metabolic routes for the H_2 production from lactate acetate, formate and butyrate. Thicker lines indicated the principal metabolic route based on relative gene aboundance.

Figure 6: The predicted genus-level phylotypes that contribute to genes encoding for key enzymes involved in the production and consumption of lactate, acetate, formate, butyrate, and hydrogen.