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Metabolic route and bacterial community changes induced by inactivation of indigenous bacteria in dark fermentation

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ABSTRACT

Biohydrogen production by dark fermentation (DF) can be achieved using only indigenous bacteria from substrates or by adding an external inoculum. This study aims to provide new insights on the role of indigenous bacteria in DF process operation by investigating DF performances and microbiological aspects. DF tests are performed with only indigenous bacteria, with indigenous and exogenous bacteria and with only exogenous bacteria by inactivating indigenous bacteria by gamma irradiation. Sorghum irradiation reduces DF performances for both hydrogen (17.8 ± 12.8 versus 45.2 ± 1.7 mLH_2/gVS_{added}) and total metabolite (0.22 ± 0.01 versus 0.30 ± 0.01 $gCOD/gVS_{added}$) yields. In contrast, no difference is observed with the organic fraction of municipal solid waste, suggesting a distinct role of indigenous bacteria for both substrates. Indigenous bacteria inactivation strongly modifies the metabolic routes and final bacterial community composition. This study proves the key role of indigenous bacteria in influencing metabolite production and bacterial composition.

1. Introduction

Biohydrogen production represents a highly promising and environmentally friendly opportunity in the goal of energy decarbonization [1,2]. Food waste [3] and lignocellulosic biomass [4] are particularly interesting feedstocks. Among typical biological methods to produce biohydrogen (dark fermentation (DF), photo-fermentation, bio--photolysis), dark fermentation presents several advantages such as the capacity of using a wide range of raw materials, including organic wastes, and its cost efficiency as it does not require light energy [5]. In addition, it presents the potential for its integration in existing energy infrastructure such as biogas plants [1]. However, it has not been scaled-up to commercial production yet, a main limitation of the process being low biohydrogen yields, below the theoretical maximum of 4 mol H_2 per mole of glucose [5]. Other limitations are the presence of competition pathways (production of lactic or propionic acids) or hydrogen consuming pathways (such as methanogenesis or homoacetogenesis) [6]. As reviewed by Zhao et al. (2024) [5], current research on enhancing biohydrogen production focuses on different strategies such as reactor design and process optimization, metal addition during fermentation, substrate pretreatments to release sugars and make them accessible, and research on inocula, either pure cultures by engineering strains or management of consortia. However, in the context of industrial applications using wastes and complex feedstocks, consortia are more relevant than improved strain cultures, in reason of their higher metabolic and genetic pools, robustness to environmental stress and no need of sterile conditions, which reduces processing costs [7].

Hydrogen production from biomass by dark fermentation is usually performed after addition of a thermally pretreated microbial inoculum [8]. DF can also be carried out by the sole indigenous bacteria in a self-fermentation concept. This was shown for the codigestion of coffee wastes composed of husk and processing wastewater [9], for the dark fermentation of coffee silverskin [10]. In the case of food waste, the low hydrogen yield (4.4 $mLH_2/g\ VS_{added}$) was significantly increased after food waste alkali pretreatment (50.9 $mLH_2/g\ VS_{added}$), acid pretreatment (89.5 $mLH_2/g\ VS_{added}$) and the best results (96.9 $mLH_2/g\ VS_{added}$) were obtained after thermal pretreatment at 90 °C for 20 min [11]. This was explained by the dominance of hydrogen producing bacteria in thermally pretreated food waste whereas lactic acid bacteria were the most abundant species in untreated food waste. In contrast, the sterilization of organic fraction of municipal solid waste (OFMSW) (121 °C–15 min) led to the decrease in the H_2 yield and biomass overall bioconversion from 70 mLH_2/gVS and 0.08 gCOD/gVS to 57 mLH_2/gVS and 0.06 gCOD/gVS [12]. These results may be attributed either to the inactivation of indigenous bacteria or the release of inhibitory

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compounds such as furfural or 5-HMF [13].

Very scare studies compared DF performances with and without the addition of microbial inoculum. François et al. (2021) reported the same high DF performances (around 2.1 molH_2/mol consumed sugar) for inoculated and non-inoculated DF of heat-treated biomass (at 70 °C for 1h) Chardonnay (or Pinot Gris) must grape deposits. Dauptain et al. (2020) [14] reported that substrate endogenous consortium led to hydrogen yields as high as the ones obtained after addition of an external thermally pretreated inoculum. This was demonstrated for various organic substrates such as sorghum, corn silage or organic fraction of municipal solid waste (OFMSW) [14]. Indeed, hydrogen yields of 47 ± 10 mLH_2/gVS_{added} and 60 ± 3 mLH_2/gVS_{added} were achieved from sorghum in biological hydrogen potential (BHP) batch tests performed with or without inoculum, respectively [14]. Nevertheless, it is still unknown why the hydrogen yields are similar for experiments performed with the sole indigenous bacteria or with indigenous and exogenous bacteria. To better decipher the exact roles of indigenous bacteria and their possible interactions with exogenous bacteria, investigations can be performed by inactivating indigenous bacteria. Such experiments are designed to compare the DF process performed with the sole indigenous bacteria and with the sole exogenous bacteria and highlight possible ecological interactions between those bacteria.

To inactivate indigenous bacteria, biomass pretreatment is required. Thermal pretreatments are not always sufficient to remove completely indigenous bacteria and more especially sporulating bacteria such as *Clostridium* species. Gamma irradiation is an efficient technique that inactivates efficiently all types of microbial populations. This technology was applied to grass waste to solubilize polysaccharides but high energy conditions (150 kGy) were likely sufficient to inactivate indigenous bacteria [15]. Dark fermentation of irradiated biomass using a thermally pretreated inoculum resulted in a slight increase in hydrogen production from 29.1 mLH_2/gVS to 35.8 mLH_2/gVS but no change in the total amounts of metabolites produced (around 0.21 gCOD/gVS).

To sum up, very few studies highlighted the high efficiency of indigenous bacteria to produce bioH_2 by performing dark fermentation without external inoculum. Nevertheless, the impact of indigenous bacteria on dark fermentation carried out with external inoculum still needs to be deciphered. Thus, the present study aims to provide new insights on the exact role of indigenous bacteria in DF process operation and in overall DF performances. As a novelty, BHP batch tests with the sole indigenous bacteria (no external inoculum), exogenous and indigenous bacteria (addition of heat pretreated external inoculum) and with the sole exogenous bacteria (inactivation of indigenous bacteria by gamma irradiation) were carried out for two substrates. DF performances (hydrogen and overall metabolite production) as well as the orientation of metabolic pathways and final bacterial community composition are discussed.

2. Materials and methods

2.1. Substrate selection and preparation

OFMSW and sorghum were selected in this study as significant hydrogen yields were observed in previous experiments carried out with the sole indigenous bacteria of these substrates [14]. The OFMSW were freshly prepared by mixing well defined individual components (meat, yoghurt, potatoes, frozen carrots, coffee grounds, rice, rusk and grass), some of them were cooked, and further frozen at −20 °C. The same substrate preparation was used for the irradiated and non-irradiated experiments with a TS content of 0.655 ± 0.001 g/g and a VS content of 0.514 ± 0.009 g/g. Sorghum collected in Saint Thibery (south of France) in 2011 [16] was also stored at −20 °C to avoid any biological degradation between the experiments (TS = 0.379 ± 0.007 g/g, VS = 0.359 ± 0.016 g/g). Prior to use, both OFMSW and sorghum were shredded to obtain a homogenous mixture (Moulinex Masterchef 5000 shredder). OFMSW were composed of pieces up to 1 cm long, and sorghum fiber size was limited to 5 cm. More details about composition and biomass characteristics can be found elsewhere [14].

2.2. Biomass irradiation to inactivate indigenous bacteria

Before irradiation, the biomass was aliquoted in hermetic plastic containers to avoid contamination during handling. OFMSW and sorghum were then stored at −20 °C to avoid organic matter degradation during transportation. The irradiation site was located at Ionisos (Dagneux, France). Both biomasses were sterilized by gamma irradiation with an average radiation dose of 45 kGy. Such dose was considered as perfectly sufficient to inactivate indigenous bacteria complex biomass without affecting the substrate structure [17,18]. Condón-abanto et al. (2018) [18] reported that radiation doses lower than 10 kGy were sufficient to inactivate spores, while Gautam et al. (2015) [19] noticed that 1.5 kGy was suitable to completely eliminate pathogenic as *K. pneumonia* in food.

2.3. Exogenous microbial inoculum

For inoculated experiments, a freeze-dried inoculum (activated sludge) originated from Narbonne wastewater treatment plant (France) was stored at room temperature according to Dauptain et al. (2021) [20]. No impact of inoculum storage on DF performances using complex biomass after using a freeze-drying storage procedure was previously shown [10]. Set of experiments, named S1 or S2 in all tables, were performed at two different times using a same stored inoculum. Prior to use in fermentation, the inoculum was thermally pretreated at 90 °C for 15 min to select more specifically hydrogen-producing bacteria [8]. Inoculum was added to reach a substrate to inoculum ratio (S/X) of 12 (gVS/gVS) for both irradiated and non-irradiated experiments.

2.4. Inoculated and non-inoculated BHP tests performed with non-irradiated biomass

BHP batch tests were conducted in quadruplicates, in 550 mL glass bottles (200 mL working volume), at 37 °C using a water bath, with a starting pH adjusted to 6.0 (if needed) and with no stirring. Each BHP flask contained 2 gVS of substrate as well as macronutrients, trace elements, Milli-Q water and MES buffer (100 mM) whose concentrations or volumes are detailed elsewhere [14]. Finally, and only for inoculated experiments, the pretreated and freeze-dried inoculum was added. Then, headspace was purged with nitrogen gas to ensure the anaerobic conditions and a rubber stopper and an aluminum screw were used to seal the glass bottles. BHP tests were stopped when hydrogen production ceased (constant pressure).

2.5. BHP tests performed with irradiated biomass

Compared to BHP prepared with non-irradiated biomass, some measures were taken to avoid bacterial contamination during BHP preparation with irradiated biomass. First, the liquids (Milli-Q water, macronutrients, 4-morpholineethanesulfonic acid buffer (MES) and trace elements) were added into the BHP flask. A rubber stopper and an aluminum screw were used to seal glass bottles to avoid losses during autoclaving. All BHP flasks and required material for BHP weighing and microbiological sampling such as pipette tips were sterilized by autoclaving at 130 °C for 20 min. The pH was adjusted to 6 before sterilization to avoid contamination. Then, the BHP were prepared under a microbiological safety cabinet. For inoculated experiments, the inoculum was added just before sealing the glass bottles. All possible remaining bacteria on the packaging were removed thanks to UV radiation (30 min) and cleaning with ethanol (95%). If needed and before closing the BHP flask, the inoculum was added as previously explained. During headspace purging, a 0.20 μm air filter and sterile needles were

used to avoid contamination from the nitrogen gas circuit. Lastly, the needles from the μ-GC were cleaned with ethanol before being inserted in the septum. An uninoculated and irradiated control was also added in order to verify that indigenous bacteria were correctly inactivated.

2.6. Metabolite analyses

Prior to analysis, samples were centrifuged at 13 000 g for 15 min followed by filtration at 0.2 μm of the supernatant. Soluble metabolic products of DF effluents were measured either by gas chromatography (GC) or by high performance liquid chromatography (HPLC) running at 35 °C with a flow rate of 0.3 mL min^{-1} (0.004 mol/L of H_2SO_4). Metabolite detection was carried out at 45 °C by a Water R410 refractive index detector. The GC was equipped with a Alltech-FFAP column (N_2 at 6 mL/min) coupled to a FID running at 280 °C. Hydrogen production was determined by pressure measurements and monitored every 2 h using a micro-gas chromatography and a micro-thermal conductivity detector. More details regarding each device characteristics are described elsewhere [14].

2.7. Microbial community analysis

Final bacterial composition of one replicate among each quadruplicate was determined by sequencing at the Genotoul life science facilities located in Toulouse (France). Only one replicate was chosen as previous works reported similar final bacterial community composition in each BHP of a same quadruplicate [14]. BHP test with the closest hydrogen yield to the quadruplicate average was chosen for microbiological analyses. Before sequencing, 1.6 mL of DF effluents was centrifuged for 15 min at 13 000 g. Pellets were then stored at −20 °C until DNA extraction. After amplification of the 16S rRNA gene (V3 region), PCR products were sequenced by an Illumina MiSeq sequencer in Toulouse, France (get.genotoul.fr). The V3 region was amplified using universal primers for bacteria, as mentioned by Carmona-Martinez et al. (2015) [21]. CTTTCCCTACACGACGCTCTTCCGATCTTACGGRAGGCAGCAG and GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT were used as forward and reverse primers. After applying a bioinformatics procedure as described by Carmona-Martinez et al. (2015) [21], the nucleotide sequences were gathered into operational taxonomic units (OTUs, 97% similarity). Mothur (version 1.35.0) was used to assemble forward and reverse sequences according to a slightly modified operation procedure for MiSeq data given by Kozich et al. (2013) [22]. If required, nucleotide sequences were blasted using NCBI database. More information about all previously mentioned procedures are detailed elsewhere [14]. Results were registered in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the bioproject accession number PRJNA1157776.

2.8. Statistical analyses

To determine if two quadruplicate averages (hydrogen yield or total metabolite amount) were significantly different, a Tukey test was performed using glht function of the R software (quadruplicate multiple comparison tests). Before performing a Tukey test, the data normality was verified using the Shapiro test. In case of non-normality, the data of the quadruplicate were withdrawn from the comparison. In all tables below, a, b, c letters indicate Tukey test results. Two conditions sharing a same letter are not statistically different.

2.9. Kinetic parameters

Kinetic parameters were determined by fitting the experimental data to the modified Gompertz model (Eq. (1)), where P is the maximum cumulative hydrogen production in mLH_2/gVS_{added}, λ the lag time in days and Rm the maximum hydrogen production rate in mLH_2/gVS_{added}/day [23]. T_{95} represents the time where 95% of the maximum cumulative hydrogen production was reached and was calculated according to Eq. (2). Variable PT_{95} represents the time where 95% of the maximum hydrogen production was reached after removing the lag phase, as defined by Eq. (3).

$$P(t) = P \times \exp\left\{ -\exp\left[\frac{R_m \times e^1}{P} \times \right) (\lambda - t) + 1\right]\right\} \quad \text{Eq. 1}$$

$$T_{95} = \lambda - \frac{P}{R_m \times e^1}[\ln(-\ln(0.95) - 1] \quad \text{Eq. 2}$$

$$PT_{95} = T_{95} - \lambda \quad \text{Eq. 3}$$

3. Results and discussion

Table 1 summarizes the Dark Fermentation performances including the hydrogen production yield and the total biomass bioconversion for both non-inoculated and inoculated experiments performed with non-irradiated or irradiated biomass and using sorghum or OFMSW. Consequently, DF performances were compared with the sole indigenous bacteria (so-called “not inoculated”), with both indigenous and exogenous bacteria (so-called “inoculated”) and with the sole exogenous bacteria (so-called “irradiated”), as referred in Table 1. A control test (so-called “control”) with no inoculum and irradiated biomass was also carried out in order to verify that indigenous bacteria were correctly inactivated. For both sorghum and OFMSW, no hydrogen production was observed and negligible amount of metabolites accumulated, confirming that indigenous bacteria were efficiently inactivated and the procedure prevented external bacterial contamination. Kapoor et al. (2017) [17] studied the irradiation impact of sugarcane bagasse from 100 kGy to 2000 kGy. The authors observed low impacts on cellulose, hemicellulose and lignin content even at 100 kGy. It was therefore assumed that 45 kGy radiation dose did not affect the substrate structure in the present study but was sufficient to effectively remove indigenous bacteria.

When using non-irradiated substrates, not inoculated and inoculated experiments led to very similar hydrogen and total metabolite yields for both sorghum and OFMSW (Table 1). This is consistent with the results using a thermally pretreated inoculum for various substrates (sorghum, OFMSW but also food waste, dates and corn silage) [14]. The non-impact of inoculation on total microbial activity was also observed on Pinot Gris grape must deposit [24].

3.1. DF performance after inactivation depends on the type of biomass

3.1.1. Lower DF performances of irradiated sorghum

Hydrogen and carbon dioxide production profiles are given in Supplementary material. Although the experiments were initially inoculated, sorghum irradiation was detrimental to H_2 production with a decrease of the H_2 yield from 45.2 ± 1.7 mLH_2/gVS_{added} to 17.8 ± 12.8 mLH_2/gVS_{added}. Irradiation was also highly detrimental to total metabolite production with a reduction from 0.30 ± 0.01 $gCOD/gVS_{added}$ to 0.23 ± 0.02 $gCOD/gVS_{added}$. Lowering DF performances by inactivating the indigenous bacteria is consistent with Favaro et al. (2013) who reported a decrease from 70.1 mLH_2/gVS to 56.5 mLH_2/gVS of the H_2 yield and a decrease in soluble metabolites from 0.52 gCOD to 0.42 gCOD with sterilized OFMSW (121 °C–15 min). Furthermore, a significant increase in the hydrogen production variability (standard deviation) was observed for the experiments inoculated and operated with irradiated sorghum. This suggests that some interactions could occur between indigenous and exogenous bacteria for their co-occurrence, contributing to the reproducibility of the metabolic pathways. Rafrafi et al. (2013) [25] reported that subdominant bacteria at the end of DF could impact the ecosystem metabolic network and the hydrogen production by interacting with their environment. Those

Table 1
Hydrogen yield and total bioconversion for non-irradiated and irradiated sorghum or the OFMSW (quadruplicate average).

Name	H_2 (mL/gVS_{added})	Metabolites ($gCOD/gVS_{added}$)
	Sorghum	
S1 – No inoculum	36.9 ± 9.2 *	0.23 ± 0.02 *
S1 – Inoculated	37.7 ± 2.0 b	0.25 ± 0.01 b
S2– Inoculated	45.2 ± 1.7 b	0.30 ± 0.01 c
S2 – Inoculated – Irradiated	17.8 ± 12.8 a	0.22 ± 0.01 a
S2 – No inoculum – Irradiated	0.0 ± 0.0	0.02 ± 0.01
	OFMSW	
S1 – No inoculum	38.3 ± 4.9 *	0.23 ± 0.02 *
S1 – Inoculated	35.2 ± 1.1 a	0.22 ± 0.01 a
S2 – Inoculated – Irradiated	33.9 ± 8.2 a	0.23 ± 0.00 a
S2 – No inoculum – Irradiated	0.2 ± 0.1	0.07 ± 0.06

S1/2: set of experiments gathering the same inoculum for inoculated experiments.
a, b, c indicate Tukey test results. Two conditions sharing a same letter are not statistically different. Red/blue values illustrate a statistically higher/lower production than other conditions. * values were excluded from the Tukey test in order to compare inoculated experiments performed with irradiated and non-irradiated biomass.

interactions could be (1) trophic interactions with the dependence of one bacteria to another one for the degradation of a specific substrate or molecule [26]. (2) Negative interactions with the production of inhibitory compounds as bacteriocins [27]. (3) Intra and extracellular mechanisms as molecule or electron exchanges by cytoplasmic connections or nanotubes [28]. The existence of trophic and non-trophic interactions between indigenous bacteria and exogenous bacteria is the most likely hypothesis to explain the low hydrogen yields after indigenous bacteria inactivation. Indeed, the low DF performances could be explained by the presence of indigenous hydrogen-producing bacteria specifically well-adapted to their substrate or by the inactivation of some indigenous bacteria that positively contribute by providing more suitable conditions for hydrogen production (lowering the pH or reducing the oxygen concentration in the bulk) [29]. This result suggests that irradiation affected more particularly the indigenous hydrolytic bacteria that were well adapted to their substrate, by lowering the global microbial activity even though after inoculum addition. Some authors mentioned elsewhere the significant influence of indigenous bacteria in the hydrolysis of complex biomass [12,30].

3.1.2. *Low impact of indigenous bacteria on DF performance of OFMSW*

For irradiated OFMSW and with inoculation, the hydrogen production was very similar to the BHP tests performed with non-irradiated substrate, with H_2 yields of 33.9 ± 8.2 mLH_2/gVS_{added} and 35.2 ± 1.1 mLH_2/gVS_{added}, respectively. In addition, the total metabolite production from OFMSW remained the same between the irradiated and non-irradiated substrates with yields of 0.23 ± 0.00 $gCOD/gVS_{added}$ and 0.22 ± 0.01 $gCOD/gVS_{added}$ This is well in agreement with Yang and Wang (2018) [15], who observed similar total metabolite accumulation for raw and irradiated grass waste (150 kGy) with values of 0.20 gCOD/gVS and 0.21 gCOD/gVS, respectively but this is not consistent with the results obtained for sorghum. The difference might be due to the synthetic nature of the OFMSW made from industrial food ingredients, having low bacterial contamination, or by the cooking (thermal treatment) that probably limited the amount and diversity of indigenous bacteria with less biomass-adapted bacteria compared to sorghum.

This low diversity at T0 is confirmed by the bacterial community composition given in Fig. 1. Indeed, for not inoculated BHP tests performed with non-irradiated OFMSW, an unclassified *Enterobacteriaceae* (92%) and a *Streptococcus* sp. (6%) were the most predominant bacteria at the initial state. Moreover, the group "others" (genera with a proportion lower than 3%) represents less than 2% in non-inoculated BHP, showing the very low initial diversity in experiments performed with OFMSW and without inoculum. This initial diversity is very low compared to sorghum experiments performed with no inoculum where *Weisela* and the group others were the main genera at T0 with proportions of 41% and 27%, respectively.

Contrary to the present results obtained with irradiated OFMSW, Yang and Wang (2018) [15] noticed a slight increase in hydrogen production from 29.1 mLH_2/gVS to 35.8 mLH_2/gVS after grass waste irradiation. However, part of the increase may be attributed to an improvement in polysaccharide solubilization (+44.8%) due to the high radiation dose (150 kGy) and to an enrichment in *Clostridium* species. This radiation dose was 3 times higher than the one used in this study.

Interestingly, and similarly to the observations made with sorghum, inactivation of indigenous bacteria was detrimental to the process reproducibility with a significant increase in the hydrogen yield standard deviation (Table 1). It might be due to a more variable bacterial selection from initial bacterial communities as indigenous bacteria did not influence bacterial selection (no interaction between indigenous and exogenous bacteria). The ability of indigenous bacteria to degrade biomass further (as for sorghum) and probably faster could participate in the emergence of specific bacterial communities during DF leading to more reproducible pathway and low hydrogen production variability. Consequently, after indigenous bacteria inactivation, this selection did not occur, leading to the emergence of opportunistic bacteria that could be detrimental to hydrogen production.

The low initial diversity at the initial state for the OFMSW might explain the differences observed for hydrogen and total metabolite production for experiments performed with irradiated substrates. Indeed, indigenous bacteria from sorghum are much more adapted to their substrate with a great diversity giving them a strong advantage in comparison to exogenous bacteria by being able to degrade faster and further the substrate by indigenous hydrolytic bacteria. The way of preparation greatly influenced the initial diversity as sorghum were

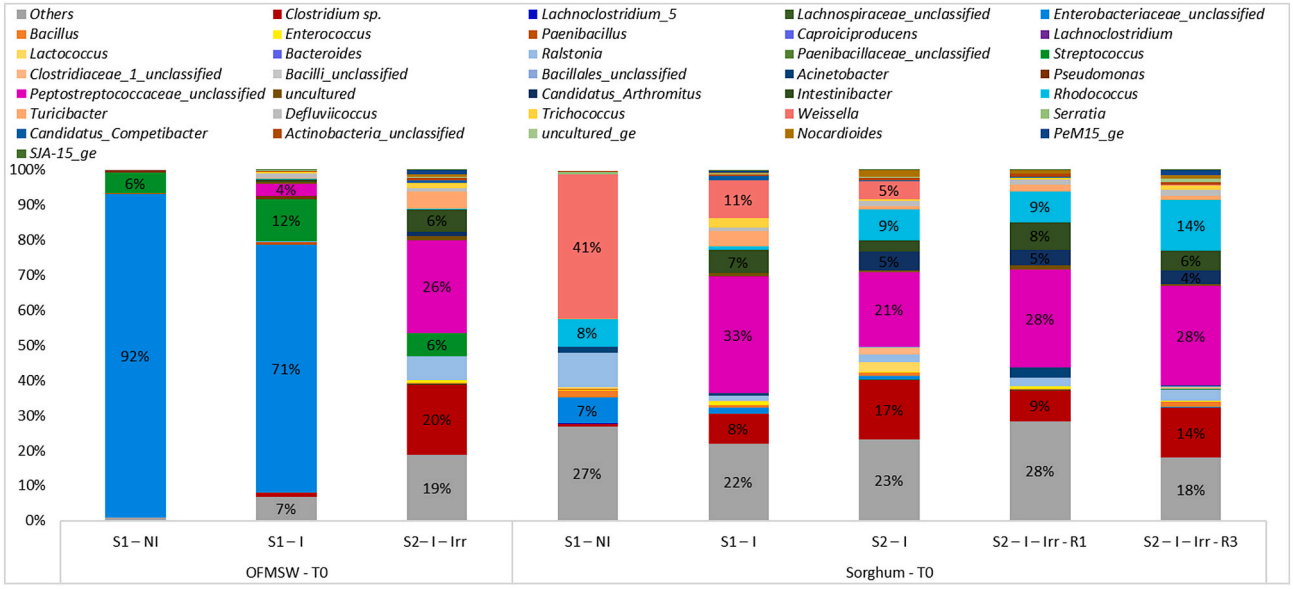

Fig. 1. Bacterial community proportions at the genus level before DF (T0) in percent for non-irradiated and irradiated sorghum or OFMSW.
NI = no inoculum, I = inoculated, Irr = irradiated, S1/2 = set of experiments number 1/2 (experiments performed at the same time with a same inoculum for inoculated experiments), R = replicate. *Only genera detected for at least one condition with a percentage > 3% are displayed.*

harvested from crops and OFMWS were reconstituted in the lab using dry of frozen industrial food with a low microbial diversity.

The nature of the substrate may also have greatly influenced the needs to have more specific hydrolytic bacteria in particular for sorghum. Indeed, as reported by Sambusiti et al. (2013) [16], the sorghum used was rich in lignin (24.1%), hemicelluloses (18.0%) and cellulose (31.1%) compared to OFMSW presenting a lower lignin (10%) and hemicelluloses content (9%) but a higher cellulose content (45%) [31]. As reported by Monlau et al. (2013) [32], the association of lignin and hemicellulose through covalent bounds as well as lignin removal considerably limit lignocellulosic biomass degradation. Consequently, sorghum biodegradation by initial hydrolytic bacterial is harder compared to OFMSW and required more specific hydrolytic bacteria to improve the overall degradation of sorghum. This shows the great importance of indigenous bacteria to produce hydrogen for lignocellulosic biomasses as sorghum.

In conclusion, the inactivation of indigenous bacteria by gamma irradiation was highly detrimental to hydrogen production metabolic routes as well as the global microbial activity for sorghum. For the OFMSW, the nature of the substrate as well as the heat treatment of most ingredients might explain the low role of indigenous bacteria. However, these results cannot be transferred to real OFMSW from waste management process, which is richer in indigenous bacteria, due to waste storage and possible auto-fermentation.

3.2. Kinetic parameters

Kinetic parameters obtained from Gompertz model are summarized in Table 2 for non-irradiated and irradiated sorghum and OFMSW. Inoculation only slightly impacted kinetic parameters for both biomasses with a decrease in lag time from 0.55 day to 0.43 day compared to uninoculated OFMSW (non-irradiated biomass). The time to produce 95% of the maximum hydrogen yield (T95) was also similar with values of 1.10 ± 0.09 days and 0.93 ± 0.05 day for not inoculated and

Table 2
Kinetic parameters from Gompertz model for non-irradiated and irradiated sorghum or OFMSW (quadruplicate average).

	Rm ($mLH_2/gVS_{added}/d$)	Lag time (days)	P (mLH_2/gVS_{added})	T95 (days)	PT95 (days)
			OFMSW		
S1 – NI	107 ± 24 [a]	0.55 ± 0.02 [b]	39 ± 4 [a]	1.10 ± 0.09 [ab]	0.54 ± 0.10 [a]
S1 – I	112 ± 7 [a]	0.43 ± 0.04 [a]	38 ± 1 [a]	0.93 ± 0.05 [a]	0.50 ± 0.02 [a]
S2 – I – Irr	93 ± 12 [a]	0.69 ± 0.03 [c]	37 ± 9 [a]	1.28 ± 0.12 [b]	0.59 ± 0.15 [a]
			Sorghum		
S1 – NI	68 ± 6 [a]	0.85 ± 0.08 [a]	36 ± 9 [b]	1.63 ± 0.14 [a]	0.78 ± 0.22 [a]
S1 – I	45 ± 3 [a]	0.35 ± 0.02 [a]	42 ± 2 [b]	1.72 ± 0.13 [a]	1.37 ± 0.14 [b]
S2 – I	69 ± 24 [a]	0.82 ± 0.05 [a]	44 ± 2 [b]	1.82 ± 0.27 [a]	1.01 ± 0.27 [ab]
S2 – I – Irr	65 ± 53 [a]	1.46 ± 0.51 [b]	18 ± 13 [a]	1.99 ± 0.67 [a]	0.53 ± 0.26 [a]

NI = no inoculum, I = inoculated, Irr = irradiated.
S1/2: set of experiments gathering the same inoculum for inoculated experiments.
T95 = time to produce 95% of the maximum hydrogen yield
PT95= Production time (T95 – lag phase).
a, b, c indicate Tukey test results. Two conditions sharing a same letter are not statistically different.
Red/blue values illustrate a significantly higher/lower proportion than other conditions.

inoculated OFMSW (not irradiated). The lag time considerably increased after irradiation of sorghum and OFMSW with external inoculum, probably due to the higher adaptation of indigenous bacteria to the biomass. For instance, the lag time increased from 0.82 ± 0.05 day to 1.46 ± 0.51 days after sorghum irradiation and from 0.43 ± 0.04 day to 0.69 ± 0.03 day after OFMSW irradiation.

Nevertheless, T95 was not significantly different for irradiated (1.99 ± 0.67 days) and non-irradiated sorghum (1.82 ± 0.27 days) and may be explained by the much lower hydrogen production after sorghum irradiation. For OFMSW, T95 increased after irradiation from 0.93 ± 0.05 day to 1.28 ± 0.12 days due to the increase in the lag time (similar production time of 95% of the hydrogen).

These results are not consistent with the ones of Yang and Wang (2018) [15], as these authors noticed a reduction in the lag time from 1.5 h to 0.1 h as well as in T95 from 10.1 h to 5.4 h after irradiation, using grass waste and a thermally pretreated inoculum. This could be explained by a higher initial soluble sugar concentration (contrary to the present study) due to more severe irradiation conditions as reported by the authors.

This section shows that the presence of indigenous bacteria was favorable for a faster start of hydrogen production, probably due to a higher adaptation of microorganisms to the substrate but they do not play a role in the time to obtain the maximum cumulative hydrogen production. Similarly to metabolite distribution, the variability of some kinetic parameters considerably increased after irradiation. For instance, the standard deviation of lag time increased from 0.05 day to 0.51 day. This confirms that indigenous bacteria are able to degrade the substrates faster and could be involved in the DF process control leading to a bacterial selection and reproducible metabolic pathways and DF performances.

To conclude, indigenous bacteria inactivation did not impact most kinetic parameters and especially the time to produce most of hydrogen from the biomass, excepted the lag time which was highly impacted.

3.3. Metabolic pathways and bacterial communities

Tables 3 and 4 present the final metabolite distribution and bacterial composition of the BHP tests operated with non-irradiated and irradiated sorghum and OFMSW. Interestingly, inactivation of indigenous bacteria led to metabolic and bacterial composition changes compared to experiments performed with non-irradiated biomass for both substrates.

3.3.1. Non-irradiated substrates

As shown in Table 3 for non-irradiated sorghum and despite similar hydrogen yields, metabolic pathways were modified with external inoculum with a decrease in butyrate proportions (48.2%/24.4%) in favor of acetate (19.9%/30.5%), ethanol (20.5%/28.6%) and succinate (0%/5.9%) for not inoculated and inoculated BHP, respectively. This is in accordance with François et al. (2021) [24] who noticed a change from butyrate to ethanol pathway after inoculum addition using thermally pretreated Pinot Gris grape must deposit as substrate (70°C-15min). Those metabolic changes can be attributed to a bacterial shift from the *Enterobactiaceae*, *Serratia* sp. (57.6%/0.0%) and *Lachnoclostridium* genus (20.5%/8.9%) to an unclassified *Enterobacteriaceae* identified as closely related to *Enterobacter cloacae* (0.0%/70.3%). Dinesh et al. (2019) [33] classified *Serratia* sp. as efficient hydrogen producer, and *Enterobacter cloacae* is also well known to produce H_2 by fermentation. Thus, *Serratia* sp. probably produced hydrogen through the butyrate pathway and *Enterobacter cloacae* was probably involved in acetate, butyrate, hydrogen, and ethanol production as previously reported by Khanna et al. (2011) [34].

Compared to sorghum, non-irradiated OFMSW showed a different behavior. The DF performances were highly similar both in terms of H_2 production and metabolite distribution for not inoculated and inoculated experiments (Table 2). These results are in accordance with Dauptain et al. (2020) [14] who noticed similar proportions in main metabolites using thermally pretreated OFMSW. Furthermore, *Clostridium* sp. (29.7%/58.5%) and *Enterobacter cloacae* (63.7%/36.5%) were predominant at the end of DF for both not inoculated and inoculated experiments, respectively (Table 3). Consequently, final bacterial community composition is consistent with similar DF performances and metabolic distribution.

3.3.2. Indigenous bacteria inactivation of sorghum and OFMSW impacts DF metabolic pathways and final bacterial composition

For experiments performed with sorghum and external inoculum (set of experiments "S2" in Table 2), irradiation led to different metabolic profiles. A shift from butyrate (71.0 ± 4.9 %/30.0 ± 24.7 %) and hydrogen (10.9 ± 0.4 %/5.7 ± 4.1 %) proportions to ethanol (9.6 ± 2.6 %/15.0 ± 3.1 %) and lactate (0 ± 0 %/34.9 ± 27.7 %) was shown for non-irradiated and irradiated sorghum, respectively. The high production of metabolites produced through non-hydrogen producing pathways such as lactate supported the low H_2 yield observed for irradiated sorghum. Theses metabolic changes induced by irradiation were in accordance with bacterial population shifts from *Enterococcus* sp. (18.3%/0.0%), *Caproiciproducens* sp. (19.8%/0.0%) and *Lachnoclostridium* sp. (13%/0.0%) to *Clostridium* sp. (38.1%/81.1% for replicate R3) or *Bacillus* sp. (6.4%/91.5% for replicate R1) for non-irradiated and irradiated sorghum, respectively. Indeed, Esquivel-Elizondo et al.

Table 3
Metabolite distribution of non-irradiated and irradiated sorghum/OFMSW in percent (gCOD/$gCOD_{ini}$).

Name	Metabolite proportions (% in $gCOD/gCOD_{ini}$)								
	Acetate	Butyrate	Ethanol	H_2	Lactate	Propionate	Succinate	Formate	Caproate
Sorghum									
S1 –NI	19.9 ± 2.1	48.2 ± 8.7	20.5 ± 5.3	11.4 ± 3.9	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
S1 –I	30.5 ± 1.2	24.4 ± 2.5	28.6 ± 0.7	10.6 ± 0.6	0 ± 0	0 ± 0	5.9 ± 0.8	0 ± 0	0 ± 0
S2 – I	7.0 ± 3.0	71.0 ± 4.9	9.6 ± 2.6	10.9 ± 0.4	0.9 ± 1.7	0 ± 0	0 ± 0	0.6 ± 0.7	0 ± 0
S2 – I – Irr	12.1 ± 7.4	30.0 ± 24.7	15.0 ± 3.1	5.7 ± 4.1	34.9 ± 27.7	0 ± 0	0 ± 0	2.3 ± 2.1	0 ± 0
OFMSW									
S1 –NI	32.4 ± 4.8	40.1 ± 4.4	13.5 ± 3.7	11.8 ± 1.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2.2 ± 4.4
S1 –I	34.1 ± 7.5	39.1 ± 6.0	14.4 ± 4.0	11.5 ± 0.4	0 ± 0	0 ± 0	0.9 ± 1.8	0 ± 0	0 ± 0
S2 – I – Irr	23.3 ± 7.6	49.6 ± 4.9	15.5 ± 3.6	10.4 ± 2.5	0 ± 0	0 ± 0	0 ± 0	1.3 ± 1.6	0 ± 0

NI = no inoculum, I = inoculated, Irr = irradiated, S1/2 = set of experiments number 1/2 (experiments performed at the same time with a same inoculum for inoculated experiments). Red/blue values illustrate a significantly higher/lower proportion compared to other conditions.

Table 4
Final Simpson's diversity indexes (OTU and Clostridiales) and bacterial community proportions at the genus level at the end of DF in percent for non-irradiated and irradiated sorghum/OFMSW.

Substrate	Sorghum					OFMSW		
Genus level (%)	S1 – NI	S1 – I	S2 – I	S2 – I – Irr - R1	S2 – I – Irr - R3	S1 – NI	S1 – I	S2 – Irr – I
Total *Clostridium sp.*	18.4	16.5	38.1	1.0	81.1	29.7	58.5	72.9
Clostridium_sensu_stricto_1	8.0	5.4	16.0	0.4	81.0	10.5	51.7	66.2
Clostridium_sensu_stricto_5	0.8	1.9	10.7	0.0	0.0	5.2	1.0	0.0
Clostridium_sensu_stricto_7	0.0	0.0	0.0	0.0	0.0	0.3	0.1	0.3
Clostridium_sensu_stricto_8	5.9	8.0	0.0	0.0	0.0	1.9	0.9	0.0
Clostridium_sensu_stricto_10	0.0	0.3	3.5	0.0	0.0	4.3	1.7	0.3
Clostridium_sensu_stricto_11	0.7	0.2	6.6	0.6	0.1	0.3	0.1	0.1
Clostridium_sensu_stricto_12	3.0	0.6	1.3	0.0	0.0	7.2	3.0	6.0
Lachnoclostridium_5	20.5	8.9	13.0	0.0	0.0	0.0	0.0	0.0
Enterobacteriaceae_un	0.0	70.3	0.0	0.0	0.0	63.7	36.5	0.0
Bacillus	0.0	0.0	6.4	91.5	18.5	0.0	0.1	18.5
Enterococcus	0.3	0.0	18.3	0.0	0.0	0.0	0.0	0.0
Paenibacillus	0.7	0.4	0.9	5.9	0.0	3.6	0.8	2.8
Caproiciproducens	1.2	3.3	19.8	0.0	0.0	0.0	0.0	0.0
Bacillales_un	0.0	0.1	2.2	0.1	0.3	0.1	0.0	2.7
Serratia	57.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Others	1.2	0.4	1.2	1.4	0.2	2.9	4.1	3.0
Diversity - Simpson - OTU Tf	0.61	0.48	0.87	0.45	0.32	0.58	0.72	0.81
Diversity - Simpson - Clostridiales Tf	0.68	0.78	0.81	0.71	0.00	0.81	0.59	0.71

NI = no inoculum, I = inoculated, Irr = irradiated, R = replicate number, Un = unclassified.
S1/2: set of experiments gathering the same inoculum for inoculated experiments.
Red/blue values illustrate a significantly higher/lower proportion than other conditions.
Only genera detected for at least one condition with a percentage >2% are displayed.

(2021) identified *Caproiciproducens* sp. as an acetate or lactate producer and Mugnai et al. (2021) reported *Clostridium* sp. and *Lachnoclostridium* sp. as butyrate and hydrogen producers [35,36]. *Bacillus* sp. and *Clostridium* sp. were also reported to be hydrogen producers as well as lactate producers [28,37,38]. The high lactate production in irradiated experiments and especially for the third replicate (R3) is consistent with its bacterial composition dominated by *Bacillus* genus. The bacterial variability observed for several replicates of the same irradiated quadruplicate will be discussed later.

The high *Clostridium* sp. content (Table 3) was consistent with the high butyrate proportions observed for experiments performed with non-irradiated sorghum. These results suggest that metabolic routes were strongly influenced by ecological interactions between indigenous and exogenous bacteria rather than a bacterial shift. In particular, after irradiation of sorghum, the hydrolytic activity was significantly lowered hence the lowest total metabolite amount 0.22 $gCOD/gVS_{added}$ compared to 0.30 $gCOD/gVS_{added}$ for non-irradiated sorghum (Table 1). Hydrolysis was performed by hydrolytic bacteria issued from the external inoculum which is probably less efficient than indigenous bacteria that are likely more adapted to the type of substrate. Moreover, the microbial diversity was also lowered in the irradiated biomass, with Simpson's index values of 0.32 for the irradiated replicate (R3) and 0.87 for non-irradiated sorghum. This result suggests a lower ability of the community to hydrolyze a wide range of organic compounds.

The inactivation of OFMSW indigenous bacteria also caused metabolic and bacteria community changes when compared to the experiments performed with non-irradiated OFMSW and an external inoculum. A metabolic shift from acetate (34.1%, 23.3%) to butyrate (39.1%, 49.6%) after OFMSW irradiation was observed (Table 2). The changes in metabolic pathways after irradiation were attributed to a shift in bacterial dominance (Table 3) from *Enterobacter cloacae* (36.5%, 0.0%) to *Bacillus* sp. (0.0%, 18.5%) and an enrichment in *Clostridium* sp. (58.5%, 72.9%), using non-irradiated and irradiated OFMSW, respectively. In particular, *Clostridium* sensus stricto 1, closely related to *Clostridium butyricum (*98.5% similarity*),* commonly reported as H_2 producer, was the main *Clostridium* species (Table 3). Detman et al. (2019) [39] associated *Clostridium butyricum* with concomitant production of butyrate and hydrogen, from acetate and lactate. This is consistent with the high proportions in butyrate and the presence of *Clostridium butyricum* in experiments performed with both non-irradiated and irradiated OFMSW (inoculated experiments).

To sum up, indigenous bacteria play a key role to achieve high DF performances, especially for sorghum due to their interactions with exogenous bacteria. However, the related mechanisms are still unclear. Moreover, the lower impact of indigenous bacteria using OFMSW could be attributed to the synthetic nature of the substrate and the cooking of some ingredients. Interestingly, indigenous bacteria inactivation induced metabolic and bacterial composition changes, especially for sorghum. For irradiated OFMSW, metabolic changes occurred between metabolites that can be produced through hydrogen producing pathways (acetate, butyrate, ethanol), leading to similar hydrogen yields compared to experiments carried out with non-irradiated biomass.

3.4. Impact of indigenous bacteria inactivation on the DF process variability

After biomass irradiation, hydrogen yields were highly variable for sorghum with productions of 17.8 ± 12.8 mLH_2/gVS_{added} compared to non-irradiated sorghum 45.2 ± 1.7 mLH_2/gVS_{added}. Similarly to hydrogen production, proportions in some metabolites were highly variable after biomass irradiation, especially for sorghum. In particular, the standard deviation of some metabolites varied from 7.0 ± 3.0 % to 12.1 ± 7.4 % for acetate, from 71.0 ± 4.9 % to 30.0 ± 24.7 % for butyrate and from 0.9 ± 1.7 % to 34.9 ± 27.7 % for lactate after sorghum irradiation. This implies that indigenous bacteria inactivation was highly detrimental to the process reproducibility. After irradiation, emergence of various bacterial communities among the different replicates was observed, resulting in different metabolic pathways for each replicate (Table 3). For example, for the experiments carried out with external inoculum and irradiated sorghum, replicate R1 was mainly composed of *Bacillus* sp. (91.5%) contrary to replicate R3 dominated by *Clostridium* species (81.1%). This explains the higher lactate concentration in replicate R1 with 1.13 $mmol/gVS_{added}$ compared to 0.41 $mmol/gVS_{added}$ in replicate R3 as well as the lower hydrogen yield in R1 with 10.5 mLH_2/gVS_{added}compared to 22.5 mLH_2/gVS_{added} for R3. This result is consistent with Yang and Wang (2018) [15], who also reported an increase in standard deviation of acetate concentration after grass waste irradiation.

Interestingly, the dominant genera at T0 for irradiated sorghum experiments with external inoculum are highly similar for replicates R1 and R3 with the genera *Peptostreptococcaceae*, *Clostridium* (9 %/14 %) and Others (28 %/18 %), data are given in supplementary materials. Nevertheless, as previously reported for experiments using irradiated sorghum, the final bacterial composition was extremely variable. This implies that indigenous bacteria played a role in the selection of microbial communities by interacting with exogenous bacteria from the inoculum, even though they represented low proportions of the initial bacterial communities. To illustrate the high impact of some specific bacteria even if they were not the main dominant ones, Rafrafi et al. (2013) [25] reported that subdominant bacteria at the end of DF could impact the ecosystem metabolic network and the hydrogen production by interacting with their environment. Those interactions could be trophic and non-trophic interactions [26], negative interactions [27] or intra and extracellular mechanisms [28].

In addition to the influence DF performances and orientation of metabolic pathways, indigenous bacteria take part in the bacterial community selection even though they were initially not in overwhelming majority. This led to a higher variability in the production of each metabolites and in the final bacterial composition for experiments that used irradiated biomass.

4. Conclusion

In this study, it was shown that indigenous bacteria play a key role in the DF process by affecting DF performances, metabolic pathways or bacterial community composition. Indeed, indigenous bacteria inactivation by irradiation was highly detrimental to DF performances and more specifically for sorghum. Irradiation induced metabolic changes from acetate to butyrate, ethanol or lactate. Bacterial population shifts from *Enterobacteriales* to *Clostridium* sp. or *Bacillus* species were consistently observed. In addition, the inactivation of substrate indigenous bacteria led to lower reproducibility in hydrogen yield and metabolite distribution. These results are of the utmost importance for future optimization of dark fermentation process, in particular when pretreatments are applied to make organic matter of the substrate more accessible. Indeed, attention should be paid to the severity of pretreatment which should preserve the activity of indigenous beneficial microorganisms. Finally, this study shows the indigenous bacteria involvement in the final bacterial composition and in the reorientation of metabolic pathways. Further research should decipher interactions between indigenous bacteria with exogenous bacteria from the inoculum and clarify the interaction mechanisms. This may result in the proposal of certain levels to optimize the dark fermentation of complex organic waste or residues.

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CRediT authorship contribution statement

K. Dauptain: Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **H. Carrere:** Writing – review & editing, Validation, Supervision, Funding acquisition, Conceptualization. **E. Trably:** Writing – review & editing, Validation, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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