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Genome-centric metagenomics revealed the effect of pH on the microbiome involved in short-chain fatty acids and ethanol production

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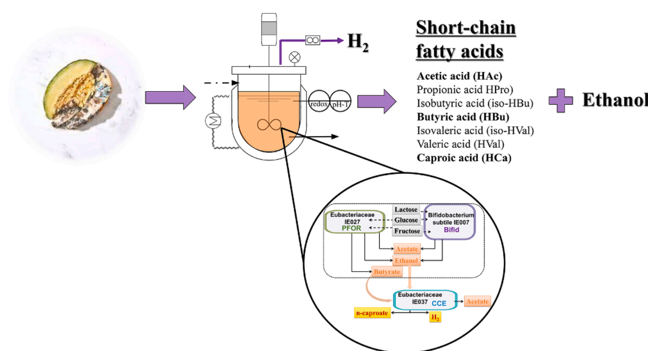
HIGHLIGHTS

- Minor pH changes resulted in similar microbiome but reshaped metabolic functions.
- High ethanol titer (37 g/L) can be reached as a co-product using open-mixed cultures.
- pH values closer to 6 enhanced carboxylic acids production.
- 25 °C was suitable to valorize carbohydrate-rich residues via anaerobic fermentation.
- *In situ* chain elongation was mediated at pH close to 6.

GRAPHICAL ABSTRACT



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ABSTRACT

Added-value chemicals production via food waste (FWs) valorization using open-mixed cultures is an emerging approach to replace petrochemical-based compounds. Nevertheless, the effects of operational parameters on the product spectrum remain uncertain given the wide number of co-occurring species and metabolisms. In this study, the identification of 58 metagenome-assembled genomes and their investigation assessed the effect of slight pH variations on microbial dynamics and the corresponding functions when FWs were subjected to anaerobic fermentation (AF) in 1-L continuous stirred tank reactors at 25 °C. The initial pH of 6.5 promoted a microbial community involved in acetate, butyrate and ethanol production, mediated by *Bifidobacterium subtile* IE007 and Eubacteriaceae IE027 as main species. A slight pH decrease to 6.1 shaped microbial functions that resulted in caproate and H_2 production, increasing the relevance of Eubacteriaceae IE037 role. This study elucidated the strong pH effect on product outputs when minimal variations take place in AF.

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1. Introduction

In the current framework of searching for renewable sources to replace the use of traditional fossil reserves, food waste (FWs) have emerged as a sustainable source for producing bioenergy and biochemicals. Over one-third of the globally produced food is lost or wasted annually, out of which 30 % corresponds to vegetables and fruits discarded in the early stage of the food supply chain (UNEP, 2021). The valorization of these wastes would contribute to mitigating the negative environmental impact related to FWs management, while promoting a circular economy model. In this context, anaerobic digestion (AD) has gained attention worldwide since organic wastes can be valorized into an energy stream using a mature and available technology. AD is a biological process wherein an open-mixed culture transforms the organic residues into biogas (CH_4 and CO_2) through four main steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis. Beyond biogas, AD has been pointed out as a potential technology to produce green chemicals and biofuels (Wang et al., 2020). During the hydrolytic and acidogenic steps (known as the anaerobic fermentation stage, AF), wastes are transformed into intermediate metabolites, such as short-chain fatty acids (SCFAs), ethanol (EtOH) and hydrogen (H_2). In conventional AD, these metabolites are methane precursors since their consumption by acetogenic bacteria and methanogenic archaea ultimately releases biogas ($CH_4 + CO_2$) as the main byproduct. However, the high industrial interest on these added-value compounds generates a turnover much higher than the biogas market price (Esparza et al., 2020). Whereas SCFAs are regarded as building blocks to produce alcohols, aldehydes or ketones (Dahiya et al., 2015), EtOH and H_2 are well-known energy carriers.

In the last decade, several studies have demonstrated AF efficiency to produce green chemicals by limiting the methanogenic step and intensifying the hydrolytic and acidogenic steps (Greses et al., 2022a; Llamas et al., 2021). More specifically, pH has been highlighted as a key factor to boost AF performance since acidic and alkaline pH promotes fermentative activity, being detrimental to methanogenic microorganisms (Zhou et al., 2018). Although acidic pH was identified as optimal for maximizing AF performance, preliminary studies revealed that slight pH variations in the acidic range (5.5–6.5) could affect the metabolites spectrum (Greses et al., 2022b). Undetermined product profile has impeded the technology scaling up since the separation and purification steps required by the subsequent processes become economically unfeasible.

Together with the chemical output, microbial characterization can bring some light to elucidate the underlying biological pathways ruling the response of open-mixed culture upon process oscillations. Previous studies have explored the metabolic exchanges in conventional AD devoted to biogas production (Campanaro et al., 2020, 2018). Some studies have been focused on direct interspecies electron transfer to enhance methane production (Zhao et al., 2020) or the effect of adding external electron donors to alter the syntrophic metabolism (Zhao et al., 2016). Nevertheless, beyond biogas, the interspecies interaction to elucidate the functional metabolisms involved in producing specific added-value compounds remains unclear in AF. To close the gap of knowledge related to the metabolic potentials involved in AF, the present study aims at elucidating the effect of minor pH variations on process performance, product profile, and functional metabolic pathways when FWs were valorized into added-value compounds using open-mixed culture to perform an AF.

2. Material and Methods

2.1. Feedstock

Given its large production in Spain, melon waste was selected as a carbohydrate-rich feedstock for revalorization (FAO, 2019). The residue was collected from markets surpluses and subsequently mashed, homogenized and chemically characterized in terms of total and soluble Chemical Oxygen Demand (TCOD and SCOD), total and volatile solids (TS and VS) and pH. The characterization included the content of carbohydrates, proteins, lipids and ash (Table 1).

2.2. Anaerobic fermentation setup

Melon waste was subjected to AF in a 1.5 L continuous stirred tank reactor (CSTR) with 0.5 L headspace. The 1L-CSTR was fully inoculated with 1 L of anaerobic sludge collected from a conventional anaerobic digester located in a wastewater treatment plant (Arroyo de El Soto, Móstoles, Spain). Table 1 shows the inoculum characterization. The homogenization was performed by magnetic stirring (Hei-PLATE Mix 20L, Heidolph, Germany), the temperature was controlled at 25 °C using a thermostatic water bath (F12-ED v2.0, Julabo, Germany) and the biogas production was continuously measured through a flow meter (Bioprocess Control, Sweden) connected to the headspace of the CSTR. AF was conducted at an organic loading rate (OLR) of 3 g VS $L^{-1}\cdot d^{-1}$, corresponding to a hydraulic retention time (HRT) of 28.3 ± 1.9 days. To do this, every day, 35.3 mL (approx.) of sludge was subtracted from the CSTR and same volume of melon waste was fed. These operational conditions were selected according to previous investigations in which, high OLR and low temperature combined with a slight acid pH have been reported to promote the intermediate metabolites accumulation (Greses et al., 2022b). To evaluate the effect of pH variations on the final products outcome, two pH conditions were sequentially tested. The CSTR was initially operated at a pH of 6.5 (1st stage) and, when the process exhibited stability, the pH was reduced to 6.1 (2nd stage) until the AF process reached the steady state again. Process stability (steady sate) was considered when the CSTR effluent showed stable composition in terms of TCOD, SCOD, TS, VS, NH_4^+ and metabolites composition. The stability was statistically confirmed by calculating the variance coefficient (VC) using STATGRAPHICS® (*Centurion XVI*) and considering 5 % as maximum value. Throughout the whole experiment, the pH was daily adjusted by manually adding NaOH (6 M). This high concentration was selected to avoid the need of large volumes of NaOH that would modify the metabolites concentration.

The CSTR was operated for 3 HRTs and considered at the steady-state when the process parameters were stable (stability period > 30 days). Once each stage reached the stable state, the efficiency of the AF was evaluated in terms of the percentage of bioconversion, acidification and organic matter removal (COD removal). These efficiency indicators were calculated according to the equations described by Greses et al. (2021):

$$\text{Bioconversion} = \frac{\text{SCFAs}_{\text{COD}}}{\text{TCOD}_{\text{influent}}}\cdot 100 \tag{1}$$

$$\text{Acidification} = \frac{\text{SCFAs}_{\text{COD}}}{\text{SCOD}_{\text{effluent}}}\cdot 100 \tag{2}$$

Table 1
Chemical characterization of melon waste used as feedstock in AF and the sludge used as inoculum (mean ± standard deviation).

	Inoculum	Melon residue
TCOD (g/L)	14.5 ± 0.1	127.5 ± 3.9
SCOD (%)	5.0 ± 0.1	88.3 ± 1.1
TS (g/L)	13.3 ± 0.2	97.2 ± 0.5
SV (% w/w)*	64.7 ± 0.2	90.9 ± 1.2
N-NH_4^+ (g N/L)	0.7 ± 0.0	0.05 ± 0.01
pH	7.3 ± 0.1	4.1 ± 0.2
Carbohydrates (% w/w)*	NA**	75.7 ± 0.2
Proteins (% w/w)*	NA**	9.8 ± 0.1
Lipids (% w/w)*	NA**	4.9 ± 0.1
Ash (% w/w)*	35.3 ± 0.2	9.6 ± 0.2

* Percentage calculated based on dry matter content.
** Not available.

$$COD_{removal} = \frac{TCOD_{influent} - TCOD_{effluent}}{TCOD_{influent}} \cdot 100 \quad (3)$$

To properly apply these equations, it should be considered that $SCFAs_{COD}$ represented the content of acetic acid (HAc), propionic acid (HPro), isobutyric acid (isoHBu), butyric acid (HBu), isovaleric acid (isoHVal), valeric acid (HVal) and caproic acid (HCa) in the CSTR effluent measured as g COD L^{-1}. $TCOD_{influent}$ was the total COD (g COD L^{-1}) of the melon waste fed to CSTR, $TCOD_{effluent}$ and SCOD were the total and soluble COD measured in the CSTR effluent (g COD L^{-1}), respectively.

2.3. Analytical methods

Melon residue characterization was performed by analyzing TCOD, SCOD, TS, VS and NH_4^+-N according to Standard Methods (APHA, 2017). The content of carbohydrates were determined following the phenol–sulfuric procedure described by Dubois et al. (1956). Total Kjeldahl Nitrogen (TKN) was measured according to Standard Methods (APHA, 2017) and this value was multiplied by 6.25 to obtain the proteins content. Lipids percentage was determined as the difference between 100 and the percentages corresponding to carbohydrates, proteins and ash.

TCOD, SCOD, TS, VS and NH_4^+-N were also analyzed in the CSTR effluent to monitor the process following the same methods. Intermediate metabolites (SCFAs and EtOH) were determined using a liquid chromatograph (1260 HPLC, Agilent) with a refractive index detector. Gas composition was analyzed using a gas chromatograph (Clarus 580 GC, PerkinElmer) with a thermal conductivity detector. The HPLC and GC analyses were performed according to the specifications described by Greses et al. (2022b). pH was daily measured in the CSTR using a pH-meter GLP 21 (Hach Lange, Germany).

The process was conducted in duplicate (2 CSTRs) to ensure process replicability. Moreover, all the analyses were performed in duplicate to calculate the average and standard deviation presented in tables and figures.

2.4. Microbial community analysis

Once the process reached the steady-state, samples were collected and immediately frozen at −80 °C. A sample from the inoculum was also stored to compare the microbiome developed in the CSTR with those microorganism present at the time zero (conventional AD). DNA extraction from Inoculum, 1st stage and 2nd stage samples was performed using a FastDNA SPIN Kit for Soil (MP Biomedicals, LCC). Nanodrop spectrophotometer (SPECTROstar Omega – BMG Labtech, DE) was used to determine the quality and concentration of extracted DNA. DNA was sequenced on Illumina Novaseq 6000 platform (2 × 150, paired end) carrying out library preparation with Nextera DNA Flex Library Prep Kit (Illumina Inc., San Diego CA). The raw sequence data for the analyzed samples was uploaded to the Sequence Read Archive (NCBI) as a part of the bioproject PRJNA907204.

Raw sequences retrieved from metagenomic sequencing (7175116 – 11,059,243 sequence reads) were initially processed using *Trimmomatic* (v0.39) and in-house developed scripts to remove low-quality sequence reads, adapters, and phiX174 contamination. Paired-end reads were merged using BBmerge program of the BBTools software suite (v38.93). The high-quality reads were assembled with Megahit (v1.1.1) with the preset "sensitive" mode (Li et al., 2015); only scaffolds longer than 1 kb were selected. The quality of the assembly was determined with QUAST (v3.1). Bowtie 2 (v2.2.4) (Parks et al., 2015) was used to align the reads onto the assembly and calculate the coverage of the scaffolds. Metagenomic binning was performed to retrieve metagenome assembled genomes (MAGs), and this step was completed with five software, namely MetaBAT (v1:2.15), MetaBAT2 (v2:2.15), Concoct (v1.1.0), Maxbin2 (v2.2.7) and VAMB (v3.0.2). dRep (v3.2.2) was afterwards used to dereplicate the MAGs with an average nucleotide identity higher than 95 %. Completeness (Cp) and contamination (Ct) were evaluated with CheckM (v1.0.3) and only high-quality MAGs (Cp ≥ 50 % and Ct ≤ 10 %) were considered for further analysis. Relative abundance of MAGs was also calculated with CheckM using as input the bam files of the reads aligned on the assembly.

The taxonomy was assigned to the MAGs using GTDBTK (v1.7.0) database (Chaumeil et al., 2020), being then converted to NCBI database taxonomy using the script gtdb_to_ncbi_majority_vote.py. PAST (v1.0.0) (Hammer et al., 2001) was used to estimate biodiversity by calculating Shannon index, microbiome dissimilarities by applying SIMPER analysis using Bray-Curtis distance metric and Principal Component Analysis (PCA) by calculating the Pearson's correlation (p-value less than 0.05).

Functional annotation was performed using METABOLIC software (Zhou et al., 2022), processing the protein-encoded genes predicted with Prodigal (v2.6.2) (Hyatt et al., 2010). The identified genes using KEGG annotation allowed predicting metabolic pathways by combining the KEGG module information (pathways completeness) with the enzymatic activity presence. Only complete or "one block missing" modules were considered.

2.5. In-silico community level simulation

The high-quality MAGs with relative abundance higher that 1 % in at least one sample (i.e. 16 out of 58), were selected for further metabolic modelling simulations. As previously described by De Bernardini et al. (2022), the genome-scale metabolic models (GSMMs) were created using gapseq (v.1.1) (Zimmermann et al., 2021) starting from the reconstructed MAGs. Briefly, the draft GSMMs were generated using the appropriate metabolic reaction universe and biomass reaction identified according to the taxonomic classification. To increase the stringency of the reconstruction, the bit score related threshold identifying core reactions was increased to 100. In the gap-filling step, default parameters were used and all reactions with a bitscore above 100 were considered as core candidate reactions. Finally, the resulting GSMMs were combined with Micom (v.0.10.1) (Diener et al., 2020) in a single community model to simultaneously infer the interaction between species and also between single species with the growth medium. For the model analyses, Cplex (v.12.8.0.0) (IBM ILOG®, USA) was adopted as the optimization solver, and the fraction of maximal community growth rate was set to 0.6. Relative abundances of the different species were used to account for the community composition during the simulations. To simulate the microbial growth, the adopted medium was defined based on the chemical characterization of the melon waste and the composition calculated with the tool "Nutrition" available at the Virtual Metabolic Human database (Noronha et al., 2019). The uptake rate boundary units were expressed in mmol/g DW/h for the internal fluxes, and in mmol/L for the growth medium fluxes. Additionally, in order to predict realistic ranges for compounds production, the experimental quantification of the relevant metabolites (i.e. HAc, HBu, HCa, EtOH, H_2 and CH_4) were used to limit the solution space.

3. Results and discussion

3.1. Effect of pH on metabolites production

Melon residue valorization into added-value compounds resulted in high SCFAs production at both pH conditions, reaching 42.2 g L^{-1} (46.3 % bioconversion) for pH 6.5 and 47.0 g L^{-1} for pH 6.1 (59.6 %) (Table 2). The bioconversion efficiency was calculated in terms of COD as described in the equation 1, using the data shown in Table 2. These values represented high process efficiencies in terms of SCFAs production when compared with previous works. For instance, Li et al. (2018) reported a SCFAs of 0.28 g SCFAs g VS^{-1}_{added} from the fermentation of carbohydrate-rich FWs at 35 °C and pH 6.5. Wainaina et al. (2019) determined 0.54 g SCFAs g VS^{-1}_{added} when performing AF of

carbohydrate-rich FWs at pH 6.0 and 37 °C. These low values compared to AF of melon residue obtained herein (0.82 and 0.84 g SCFAs g VS^{-1}_{added} for 1st and 2nd stages, respectively) revealed that 25 °C was a more appropriate temperature than 35 °C when carbohydrate-rich wastes were used as feedstock, which would increase the cost-effectiveness of the process. Carbohydrates present higher biodegradability than proteins and lipids, being not required high process temperature to promote the FWs degradation. In addition, low temperatures (25 °C) limited the growth of methanogenic archaea, reducing the metabolites consumption to produce biogas. Although both stages resulted in high SCFAs yields, 2nd stage showed higher SCFAs bioconversion, suggesting that pH values closer to 6 could be beneficial for producing these metabolites.

The implemented operational conditions were not the only factor that could explain the high process efficiencies. More specifically, the high carbohydrate content and high percentage of readily biodegradable organic matter also contributed to the high SCFAs production yields achieved. It should be stressed that remarkably low bioconversion values have been determined when proteins prevailed in the waste composition, showing efficiencies of 32.1 % (Llamas et al., 2021) or 22 % (Iglesias-Iglesias et al., 2019) even working at optimal acidogenic pH range (5.5–6.5). Process efficiencies like those reported in the present study were only observed when raw carbohydrate-rich FWs with similar features were used as feedstock under comparable operational conditions (Greses et al., 2021). These results evidenced that carbohydrate-rich waste represented a valuable resource to produce specific added-value compounds.

Although SCFAs concentration did not exhibit great difference between both pH conditions, a shift in the SCFAs distribution profile was observed. Fig. 1 shows that HAc (31.9 %) and HBu (55.6 %) dominated the carboxylic acids profile in 1st stage (pH 6.5), while the 2nd stage (pH 6.1) resulted in HAc, HBu and HCa as prevailing SCFAs. The presence of HAc and HBu has been normally reported when carbohydrate-rich substrates were used as feedstock in AF (Bolaji and Dionisi, 2017; Greses et al. ,2022c, 2020). However, the results obtained herein showed that pH values closer to neutrality (1st stage) shifted the SCFAs profile towards HBu prevalence.

Nevertheless, AF did not only transform organic matter into SCFAs but also lead to the formation of other metabolites having high industrial interest, such as EtOH. The co-occurrence of different metabolisms is a common feature in an open mixed-culture that results in an organic carbon competitiveness. As consequence, EtOH reached 37.6 g L^{-1} in 1st stage representing one of the highest EtOH titers resulting from an open mixed-culture obtained without applying any pretreatment. Investigations dealing with EtOH production via open mixed-cultures found titers between 1.3 g L^{-1} (Feng et al., 2018) and 3.0 g L^{-1} (Shi et al., 2021) when AF was conducted at 35 °C and pH 4–5. Those values were far below the values resulting from the 1st stage. The surprisingly high EtOH titer obtained might be a result of the proper combination of pH and temperature to degrade a carbohydrate-rich waste. Considering that 40 g L^{-1} was identified as the minimum EtOH titer required to develop an economically viable bioprocess (Xu et al., 2018), these results showed a promising strategy to increase FWs revenues since axenic conditions and the related costs would be avoided.

Regarding the 2nd stage, a pH decrease to 6.1 caused a HBu reduction from 55.6 % to 34.8 % at the expenses of HCa accumulation to 20.6 %. Overall, odd chain SCFAs (HBu, HCa and HAc) contributed to 92.6 % of the total carboxylates pool. An EtOH titer decrease to 19.8 g L^{-1} was also observed. The varying metabolite profile clearly showed that minor pH changes altered the metabolic mechanisms of waste transformation. Greses et al. (2021) have previously found that 25 °C and slight acid pH (5.8) were proper conditions to promote *in situ* chain elongation using carbohydrate-rich FWs, obtaining an AF effluent mainly enriched in HCa and HBu (93.8 %). This metabolism requires the use of an electron donor, such as EtOH, to elongate the carbon chain (de Cavalcante et al., 2017). The metabolite profile encountered in the 2nd stage indicated that HBu acted as electron acceptor and EtOH was consumed as electron donor, justifying the decrease of these metabolites and the HCa accumulation. Additionally, the H_2 production (37.5 mL H_2 g COD^{-1}_{fed}) confirmed that this pathway was active given that HCa release via chain elongation has been described as a hydrogenogenic process (Ding et al., 2010).

Both conditions represented an interesting strategy for wastes valorization because the 1st stage resulted in an outcome with high specificity (including high content of EtOH), which significantly increase the cost-effectiveness of the SCFAs recovery and purification technologies. On the other hand, the 2nd stage demonstrated that high content of long chain carboxylic acids can be reached by decreasing the process pH. This is also relevant because long-chain SCFAs have higher market value than the shorter ones (Nzeteu et al., 2022). These results demonstrated that the minimal pH variations can be used as a strategy to drive a specific

Table 2
Effluent of CSTR when the process exhibited stability at both pH conditions (mean ± standard deviation).

	1st Stage	2nd Stage
pH	6.5 ± 0.1	6.1 ± 0.1
TCOD (g L^{-1})	86.7 ± 3.2	93.2 ± 0.9
SCOD (%)	78.1 ± 1.3	79.4 ± 0.7
TS (g L^{-1})	70.3 ± 2.1	83.6 ± 0.4
VS (%)	54.6 ± 0.4	54.9 ± 0.1
Total SCFAs (g L^{-1})	42.2 ± 1.0	47.0 ± 0.4
HAc (%)*	31.9 ± 0.4	37.2 ± 0.7
HPro (%)*	2.0 ± 0.2	3.7 ± 1.1
HBu (%)*	55.6 ± 0.8	34.8 ± 1.4
isoHBu (%)*	0.8 ± 0.1	0.7 ± 0.1
HVal (%)*	4.5 ± 0.2	2.8 ± 0.3
isoHVal (%)*	0.7 ± 0.1	0.4 ± 0.1
HCa (%)*	4.9 ± 0.3	20.6 ± 0.2
EtOH (g L^{-1})	37.6 ± 0.4	19.8 ± 0.9
Y_{CH4} (mL CH_4 g COD^{-1}_{fed})	21.5 ± 1.6	< LD**
Y_{H2} (mL H_2 g COD^{-1}_{fed})	< LD**	37.5 ± 1.3
Bioconversion (%)	46.3 ± 1.5	59.6 ± 0.5
SCFAs yield (g SCFAs gVS^{-1}_{added})	0.82 ± 0.04	0.84 ± 0.01
COD removal (%)	5.1 ± 0.3	19.1 ± 1.3

* Calculated as SCFA/$SCFAs_{total}$ 100.
** Lower than limit of detection (LD).

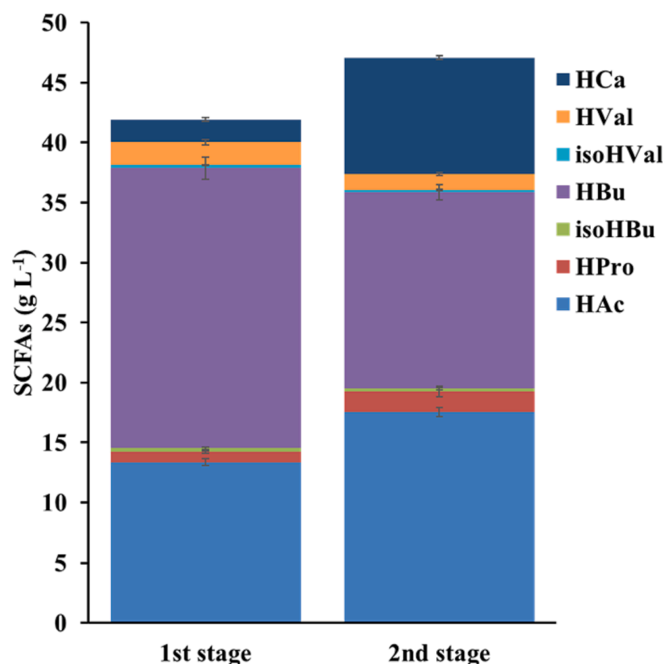

Fig. 1. SCFAs production resulting from AF of melon residue at pH 6.5 (1st stage) and pH 6.1 (2nd stage).

metabolites production, being of paramount importance to elucidate the metabolic potentials to further optimize the process.

3.2. Microbiome related to pH variation

The microbial analysis resulted in a total of 58 MAGs belonging to 13 different phyla (Fig. 2). The inoculum showed a diversified phyla relative abundance profile, whereas both stages linked to the different pH values were dominated by Actinobacteria (33.3 % in 1st stage and 34.1 % in 2nd stage) and Firmicutes (41.4 % and 41.3 % for 1st and 2nd stage, respectively). Shannon index calculated for each sample confirmed a notable biodiversity decrease (6.919 ± 0.002 for the inoculum to 2.561 ± 0.102 in the 1st stage and 2.416 ± 0.222 in the 2nd stage), evidencing the straightforward effect of pH on the microbial community. The biodiversity reduction in the CSTRs has been identified as indicator of a microbial specialization in the fermentative stage, thereby decreasing the presence of those microorganisms involved in methane production (Llamas et al., 2022, 2021). Moreover, FWs composition could have also a determinant effect on microbial diversity since the AF might be enriched with carbohydrate degraders. Hence, the growth of the microorganisms involved in the degradation of protein-rich residues was limited, such as those found in convention AD of sewage sludge.

Previous studies performing AF of carbohydrate-rich waste have also identified Firmicutes as key phylum involved in high SCFAs production yields (>80 % relative abundance), while Actinobacteria represented less than 20 % of the bacterial community (Greses et al., 2021, 2020). Thus, the results obtained herein suggested that the different product outputs were likely related to the high relative abundance of Actinobacteria (>33 %) as a possible consequence of the higher pH (6.1 and 6.5). Members of both these phyla (Firmicutes and Actinobacteria) have been described as acid-tolerant bacteria with an important role in the hydrolytic and acidogenic stages when carbohydrate-rich residues are used as feedstock (Lv et al., 2021). Nevertheless, Actinobacteria were pointed out to be more sensitive to acidic conditions (Greses et al., 2022b), confirming that this phylum can be used as pH indicator.

As it can be seen in Fig. 2, bacteria belonging to Eubacteriaceae family and the species *Acidipropionibacterium acidipropionici*, *Bifidobacterium subtile* and *Actinomyces provencensis* were the main microorganisms responsible for the differences encountered between the inoculum and the samples collected from the 1st and 2nd stages. Although both stages showed a similar microbial composition, pH shaped the relative abundance of single bacteria. According to SIMPER analysis, 28.7 % of dissimilarity was determined between the 1st and 2nd stage. Considering the microbial profile in both pH conditions, *Acidipropionibacterium acidipropionici* IE050, *Bifidobacterium subtile* IE007, Eubacteriaceae sp IE027, and Eubacteriaceae sp IE014 accounted for 55.9 % of the dissimilarity, being likely responsible of the different products.

Acidipropionibacterium acidipropionici has been reported as an acid-tolerant bacteria belonging to Actinobacteria phylum that exhibits metabolic plasticity depending on the operational conditions and commonly co-exist with some species of Firmicutes (Zheng et al., 2022). Similarly, species belonging to the *Bifidobacterium* taxon (Actinobacteria phylum) have been described as carbohydrate degraders (Carosia et al., 2017), which are also able to shift the metabolites produced depending on the operational parameters. Regarding Eubacteriaceae, some members of this family have been described as glucose fermenters (Yang et al., 2020), as well as carbon chain elongators to produce medium chain fatty acids (Scarborough et al., 2018), such as *Clostridia* (Coma et al., 2016). Overall, the metabolic versatility of the microorganism and the set of interactions co-occurring in open mixed cultures increased the complexity of the dynamics to fully comprehend the metabolisms that were promoted by slight pH variations.

A predictive functional analysis using COG revealed that the most abundant genera in both stages encoded proteins involved in ethanol synthesis (EtOH_production), fatty acids synthesis (FA _Biosynthesis) and carbon chain elongation (CCE) metabolisms. In contrast, genes related to methane production were only encountered in the 1st stage due to the presence of *Methanobrevibacter* sp IE024 among the most abundant genera (3.71 % average relative abundance). The *Methanobrevibacter* sp IE024 growth in 1st stage at expenses of *Methanolinea* sp IE033 (Inoculum) could be related to the selected temperature to perform AF (25 °C) since previous studies has been reported a similar replacement of archaea species when conventional AD was subjected to temperature oscillation (Ciotola et al., 2013). Nevertheless, methane production in AF was not detected. This fact indicated that the archaeal activity was limited, showing pH as strategic parameter to shorten conventional AD even when low HRTs are set.

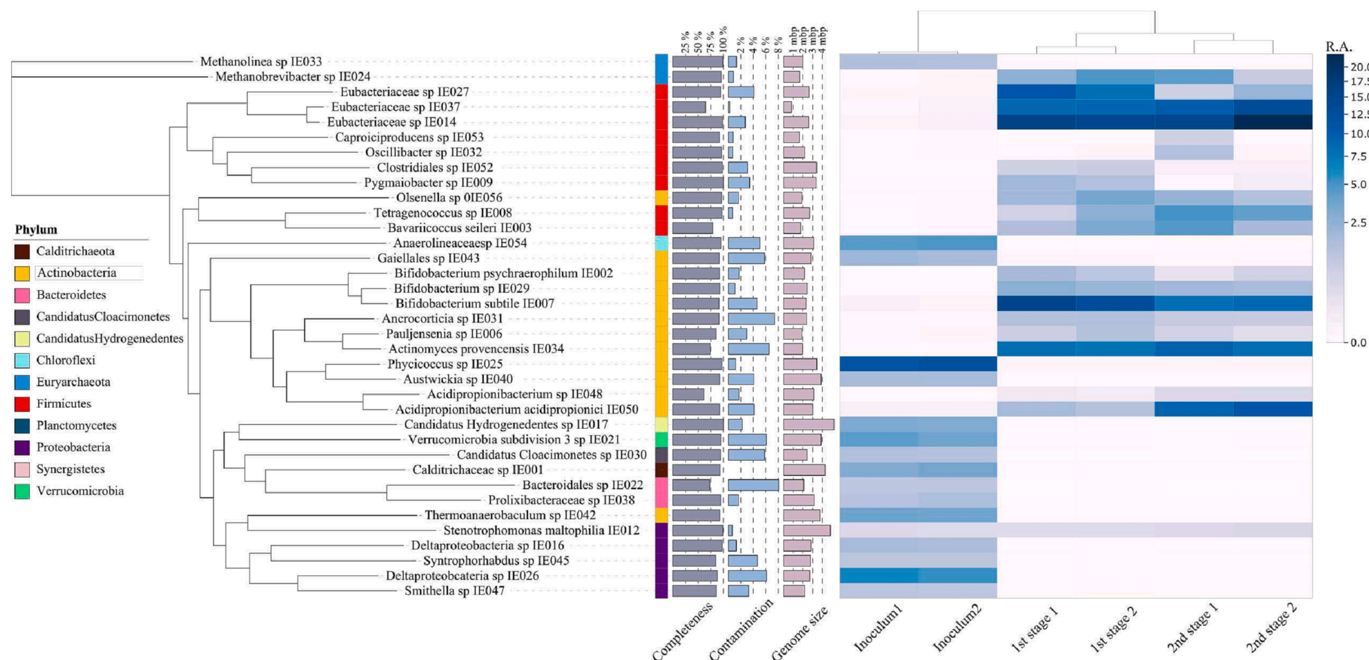

Fig. 2. Heatmap representing the relative abundance (R.A.) of the high-quality MAGs identified in the samples, including replicates named as 1 and 2.

As it can be seen in Fig. 3, high EtOH titer was significantly correlated with the gene profile of *Bifidobacteria* genera and, particularly, *Bifidobacterium subtile* IE007. This fact was of special relevance since the higher EtOH and HAc concentration registered in the 1st stage than in the 2nd stage could be explained by the 2-fold shift in relative abundance of *Bifidobacterium subtile* IE007, decreasing from 15.23 % in the 1st stage to 7.93 % the 2nd stage. Moreover, a strong correlation was also identified between FA_Biosynthesis and Eubacteriacea sp IE027 in the 1st stage. Given that some bacteria classified in Eubacteriacea family have been described as sugars fermenters producing HBu via the glycolytic pathway (Yang et al., 2020), the high relative abundance of Eubacteriacea sp IE027 (10.73 %) in the 1st stage compared to the 2nd stage (2.29 %) could be determinant for the high HBu accumulation (23.5 g L^{-1}).

An opposite trend was observed in the 2nd stage since members of Eubacteriacea (Eubacteriaceae sp IE037 and Eubacteriacea IE 014) family showed a strong correlation with CCE metabolism (Fig. 3). The significance of this correlation lies in the fact that bacteria belonging to Eubacteriaceae family have been also determined as main contributors to medium chain fatty acids production via CCE, which supported the high HCa concentration attained in the 2nd stage (10 g L^{-1} approx.) at the expenses of HBu consumption as electron acceptor.

To confirm the putative genes/pathways involved in the fermentative routes operating in both stages, functional analyses were refined using KEGG database. KEGG annotation evidenced complex carbohydrate degradation, amino acid utilization and fermentation as the most relevant pathways in 1st and 2nd stages (Fig. 4). In contrast, methanogenesis was mainly detected in the inoculum. These results confirmed that the biodiversity decrease observed at both pH stages was related to the reduction of the metabolisms associated with methanogenesis, thereby promoting intermediate metabolite accumulation resulting from the fermentation.

The presence of genes related to glycolysis (ko00010) and citrate cycle (ko00020) were identified in both pH stages, disclosing the ability of the most abundant microorganisms to perform carbohydrate fermentation. One of the main differences found between the two stages was related to the SCFAs synthesis pathways. The 1st stage was enriched with genes involved in the initial steps of FA biosynthesis pathway (M00082, ko00061) in which, the metabolites produced in the citrate and pyruvate cycles were converted into HAc (among others). However, the pathways identified in the 2nd stage included not only the initial steps of FA biosynthesis but also the elongation (M00083) and acetyl-CoA synthesis (M00086).

Regarding the 1st stage, the imposed pH (6.5) affected end-product synthesis patterns in the most dominant species. A pH lower than neutrality has been reported to be a modifier of the conventional *Bifidobacterium* metabolism from lactic acid producer to EtOH and HAc synthesizer via Bifid pathway (Xiong et al., 2019). In these conditions the Bifid pathway is more efficient and allow the degradation of sugars (lactose, glucose and fructose) to release EtOH and HAc as main products (Fig. 5). Moreover, *Bifidobacterium* co-existence with member of the Eubacteriaceae family, such as *Clostridium*, is a common feature when process pH decreased. In this regard, Eubacteriaceae IE027 encodes genes involved in pyruvate oxidation via pyruvate: ferredoxin oxidoreductase (PFOR) (k00169-k00172). PFOR is responsible for the oxidative decarboxylation of pyruvate to acetyl-CoA, generating NADH and reduced ferredoxin. These metabolites can be then oxidized to produce EtOH (Fig. 5a) and HBu (Olson et al., 2015). Thus, the high content of EtOH, HAc and HBu measured in the 1st stage was likely severely influenced by the presence of these organisms mediated by the slight pH decrease.

By contrast, the pH established in the 2nd stage (6.1) shaped the metabolic functions to promote the CCE metabolism. Several enzymes are involved in this metabolic pathway in mixed microbial cultures. In particular, alcohol dehydrogenase is required for the initial conversion of EtOH into acetyl-CoA. Thereafter, acetyl-CoA is transformed into malonyl-CoA by acetyl-CoA carboxylase, which subsequently enters the cyclic FA biosynthesis (Carere et al., 2012). The CCE metabolic pathway was identified with higher completeness mainly in Eubacteriaceae IE037, as well as in Eubacteriaceae IE014 (Fig. 5b). Moreover, the initial step of EtOH consumption to produce HAc was also identified in the metabolic pathway of *Acidipropionibacterium acidipropionici* IE050. The identification of these pathways, along with the co-occurrence of EtOH- and HBu-producers (*Bifidobacterium subtile* IE007 and Eubacteriaceae IE037), suggested that the high content of HCa and the H_2 production determined in the 2nd stage was produced via CCE using HBu as electron acceptor and malonyl-CoA as synthesizer vector.

Finally, the in-silico simulation of the microbial community interspecies interactions was performed by means of flux balance analysis. Results showed that the cross-feeding exchanges between the sixteen dominant species found in both stages exhibited the metabolic potential to transforms sugars (D-glucose, sucrose and D-fructose) into HAc, HBu

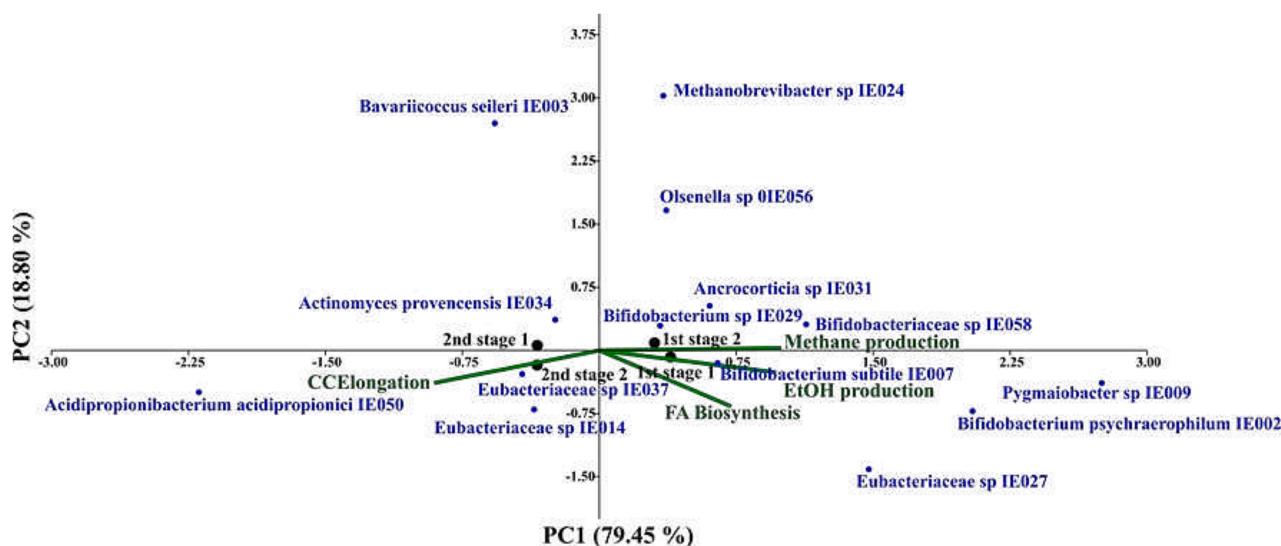

Fig. 3. Principal components (PC) analysis of protein encoding genes identified in both stages for the most abundant MAGs (>1 % relative abundance), including replicates named as 1 and 2. The green arrows represent biochemical parameters whose correlation with the gene profile of the microbiome are statistically significant ($p \leq 0.05$).

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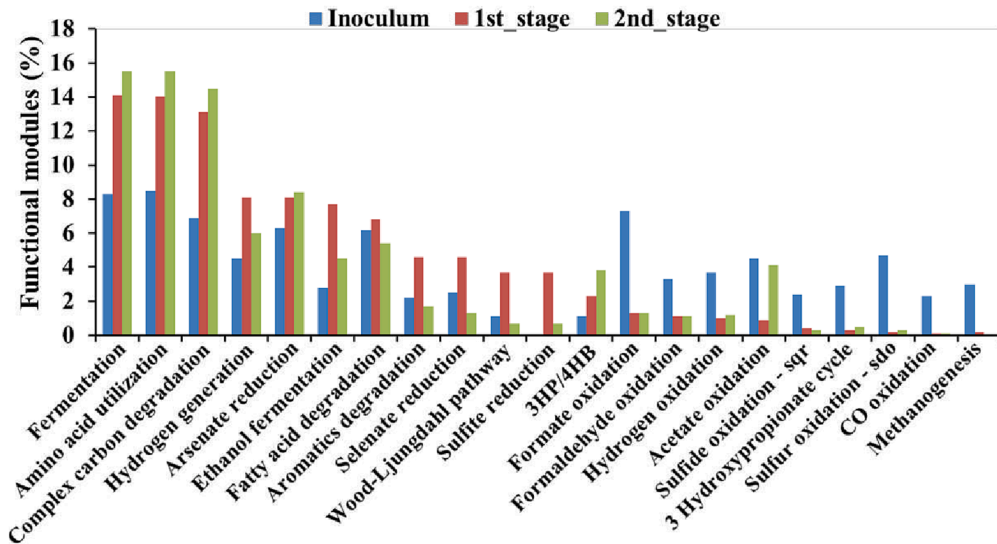

Fig. 4. Pathways dominance over the whole metabolisms found in the three different types of samples: Inoculum, 1st stage and 2nd stage. Bar graph was obtained by weighting the pathway dominance according to the relative abundance of species annotated with "complete" and "one block missing" functional modules.

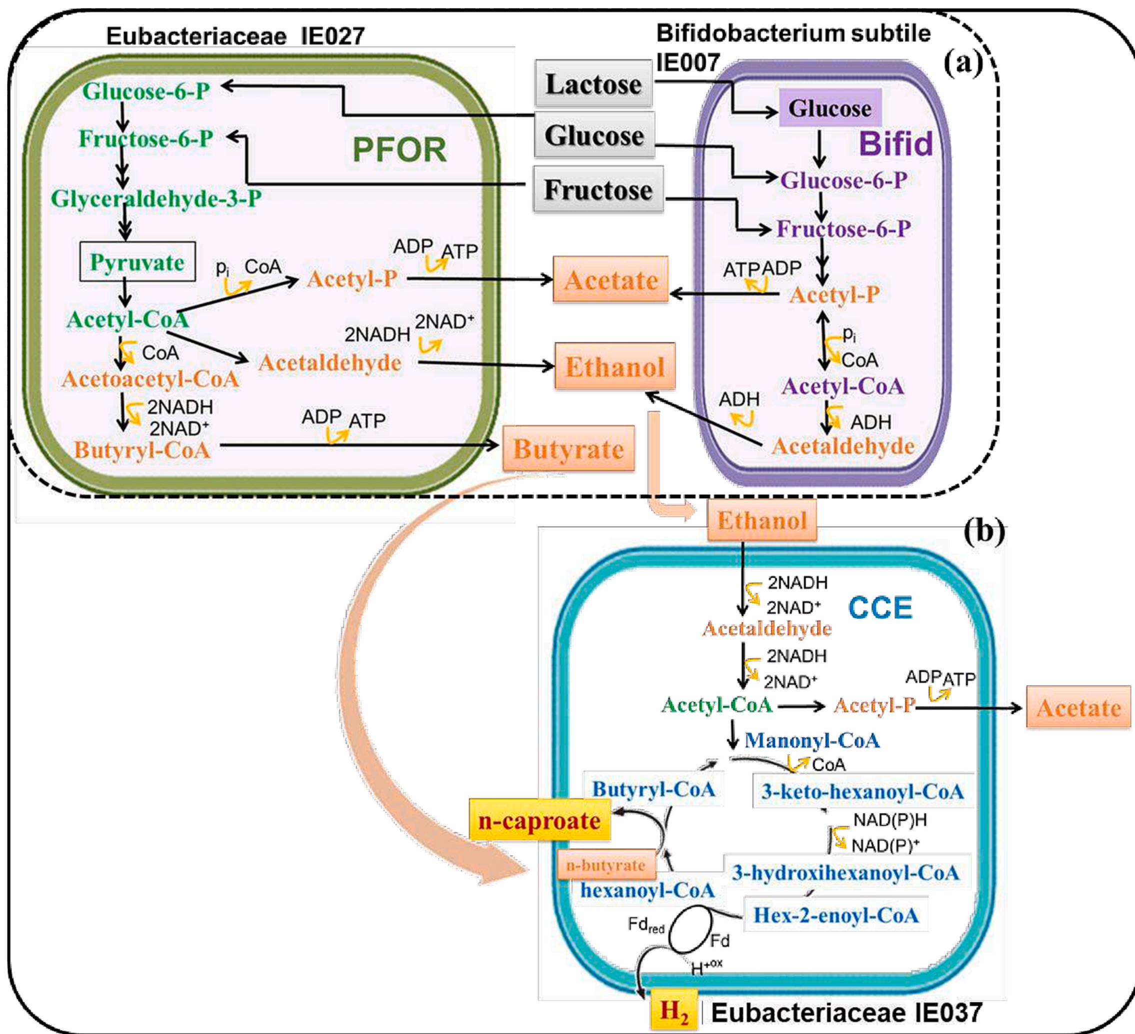

Fig. 5. Scheme of the reconstructed metabolisms of the dominant MAGs identified in the (a) 1st stage and (b) 2nd stage. Metabolic map summarizing the main metabolic pathways and putative metabolites exchanges established between Eubacteriaceae IE037, *Bifidobacterium subtile* IE007, and Eubacteriaceae IE027.

and EtOH. Nevertheless, the metabolic potential to produce EtOH was double for the 1st stage community. The microbial community modelling of the 2nd stage also included HCa and H_2 among the predicted exchanged metabolites. These results did not only support the hypothesized species role on generating the different product spectrum in both stages, but also confirmed that small pH changes can be crucial to suppress methanogenic activity. Indeed, no methane accumulation was predicted.

The model simulation and the annotated metabolic pathways of each stage evidenced that the structure and function of the microbiome were dependent on the implemented pH conditions. As a matter of fact, process pH adjustment was highlighted as a suitable strategy to modify a conventional AD, whereby targeted intermediate metabolite with higher market value than biogas can be produced.

4. Conclusions

This study demonstrated the effect of minor pH variations on added-value metabolites production via open-mixed cultures. Although the studied pHs resulted in a similar microbiome, a 0.4 pH variation can mediate the accumulation of EtOH or HCa and H_2. This was one of the first studies demonstrating the production of high EtOH titers using open-mixed culture, reaching similar levels than those resulting from pure yeast cultures without applying costly pretreatments or axenic requirements. Since microbial metabolisms can be manipulated to attaining targeted product, this investigation can be envisaged as a promising manner to develop a feasible production of metabolites useful for the chemical industry.

CRediT authorship contribution statement

Silvia Greses: Conceptualization, Investigation, Data curation, Formal analysis, Writing – original draft, Funding acquisition. **Nicola De Bernardini:** Formal analysis, Visualization, Software, Writing – review & editing. **Laura Treu:** Data curation, Writing – review & editing, Supervision. **Stefano Campanaro:** Formal analysis, Software, Writing – review & editing, Supervision. **Cristina González-Fernández:** Conceptualization, Data curation, Writing – review & editing, Supervision, Funding acquisition.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.128920.

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