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Influence of feedstock mix ratio on microbial dynamics during acidogenic fermentation for polyhydroxyalkanoates production

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A B S T R A C T

The nature of microbial populations plays an essential role in the production of volatile fatty acids (VFA) during acidogenesis, the first stage in polyhydroxyalkanoates (PHA) production using mixed cultures. However, the composition of microbial communities is generally affected by substrate alterations. This work aimed to unravel the microbial dynamics in response to a gradual change in the feedstock composition in an acidogenic reactor, with subsequent PHA production. To achieve this, co-digestion of cheese whey and brewery wastewater (BW) was carried out for the production of VFA, in which the ratio of these feedstocks was varied by gradually increasing the proportion of BW from 0 up to 50% of the organic content. Bacteria such as *Megasphaera*, *Bifidobacterium* or *Caproiciproducens* were the most abundant in the first stages of the co-digestion. However, when BW reached 25% of the organic load, new taxa emerged and displaced the former ones; like *Selenomonas*, *Ethanoligenens* or an undefined member of the *Bacteroidales* order. Accordingly, the production of butyric acid dropped from 52 down to 27%, while the production of acetic acid increased from 36 up to 52%. Furthermore, the gradual increase of the BW ratio led to a progressive drop in the degree of acidification, from 72 down to 57%. In a subsequent approach, the VFA-rich streams, obtained from the co-digestion, were used as substrates in PHA accumulation tests. All the tests yielded similar PHA contents, but with slightly different monomeric composition. The overall results confirmed that the microbiome was altered by a gradual change in the feedstock composition and, consequently, the VFA profile and the monomeric composition of the biopolymer also did.

1. Introduction

The agroindustrial sector is one of the largest in the world, supplying food to billions of people by processing natural resources. Within the agroindustrial sector, the dairy and brewing industries are among the most productive ones in Galicia, a region in north-western Spain. However, there is high concern regarding the pollutant content of some of their respective wastes, cheese whey (CW) and brewery wastewater (BW), causing serious environmental problems. CW is characterized by a high organic load, mainly composed of lactose, proteins, fats, minerals and vitamins with a chemical oxygen demand (COD) value between 50 and 102 g L^{-1} (Carvalho et al., 2013). Alike, the brewing industry is also involved in the generation of a large amount of waste, of which brewery wastewater (BW) is among the highest contributors, with a generation rate of 3–10 L per liter of beer produced (Chen et al., 2016). The organic content of BW can widely fluctuate from 2 up to 32 g COD L^{-1}, and nitrogen, phosphorous and solids do as well (Arantes et al., 2017).

In current days, anaerobic digestion (AD) of industrial by-products is gaining much interest within the circular economy framework promoted by the European Union (European Commission, 2015). The AD process consists of a cascade of 4 sequential reactions, i.e., hydrolysis, acidogenesis, acetogenesis and methanogenesis, in which higher added-value products are obtained in each of the different stages (Angelidaki et al., 2011). Accordingly, the valorization of CW and BW through AD is highly encouraged as a promising and sustainable approach for waste management. In AD, each of the reactions is catalysed by a complex and specific microbial community in which each member plays a well-defined role (Angelidaki et al., 2011; Wainaina et al., 2019). It is during acidogenesis that the production of volatile fatty acids (VFA) takes place. VFA are considered among the most promising and desirable products that can be obtained from AD as they are valuable as ready-for-application products (Atasoy et al., 2018) or as intermediates for a wide range of other bioproducts such as polyhydroxyalkanoates (PHA) (Iglesias-Iglesias et al., 2021). The specific consortium of bacteria

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involved in the production of these short chain fatty acids is known as acidogens. However, during acidogenesis, in mixed cultures, acidogens coexist with hydrolytic bacteria and methanogens. This leads to a highly complex microbial scenario with uncountable interactions, quite difficult to elucidate using traditional molecular techniques. Fortunately, next generation sequencing technologies have recently become a useful tool to unravel characteristics of these microbial interactions.

Besides, a large number of different factors cause disturbances in microbial communities during AD, with the feedstock being one of the most important. To date, the role of single substrates on the development of specific groups of bacteria during acidogenic fermentation has widely been studied (Iglesias-Iglesias et al., 2019; Lagoa-Costa et al., 2020; Teixeira et al., 2020). Nevertheless, the use of mixed substrates results into a much more complex and yet unclear scenario, especially under dynamic feedstock conditions. In this regard, the behaviour of the microbiome is important to be well understood in order to ensure a steady performance. To the best of our knowledge, only few works have studied the influence of changing the composition of the feedstock, although promoting radical changes (Carvalho et al., 2018; De Francisci et al., 2015; Fitamo et al., 2017). Accordingly, all the works experienced dramatic changes in the composition of their respective microbial communities. However, it is still unclear what would happen with the microbial community when a gradual change in feedstock composition is applied. This information can be helpful to understand either the resistance/sensitivity of a quite specialized microbial community to any substrate instability or the microbial dynamics caused by different feedstock mix ratios in industries with more than one by-product. Additionally, since any alteration in the acidogenic fermentation process usually affects the VFA distribution, the monomeric composition of the final PHA biopolymer is also disturbed. Accordingly, the present research studied the microbial dynamics linked to a gradual change in feedstock composition during an acidogenic fermentation process in order to elucidate this issue. Moreover, the production of PHA has also been studied using the VFA-rich streams obtained from the acidogenic fermentation as substrate and their influence on monomeric composition of the final biopolymer.

2. Material and methods

2.1. Experimental set-up

The entire experiment was split into two different stages: acidogenic fermentation and PHA production. The first stage, i.e. acidogenic fermentation, comprised the production of VFA through the co-digestion of CW and BW. The experimental design of this first stage consisted in four different phases in which the feedstock mix ratio was changed by increasing the proportion of BW, from 0 to 10, 25 and 50% of the total organic content. Each of these phases lasted 24–40 days, with an interphase period of 21–31 days in which the mixed culture got acclimatised to the new feedstock mix ratio. During Phase I, the acidogenic reactor was fed 100% CW. The purpose of this initial phase was to set the basis of the microbial community structure. After 34 days of operation, the feedstock mix ratio changed when BW was first introduced in the culture medium with 10% of the COD (Phase II). The remaining 90% of the organic load was composed of CW. Phase III started when the microorganisms were fed 25% of COD in the form of BW and 75% in the form of CW. Finally, in Phase IV, the proportion of BW and CW was equalled, with 50% of COD each. In order to evaluate the microbial dynamics, biomass samples were taken from the acidogenic reactor in each of the different phases when steady state was reached.

The second stage comprised PHA accumulation tests through the operation of fed-batch bioreactors in order to evaluate the VFA-rich streams on the production of PHA as well as on the final monomeric composition of the biopolymer. One PHA accumulation test was performed per each of the VFA-rich effluents obtained after the different phases of the acidogenic fermentation.

2.2. First stage: acidogenic fermentation

2.2.1. Reactor set-up

A cylindrical and glass up-flow anaerobic sludge blanket (UASB) reactor sizing 63 cm height and 7 cm of internal diameter was used to carry out the acidogenic fermentation. This corresponded to a working volume of 1.1 L. The UASB was inoculated with 0.55 L of biomass from another acidogenic reactor with a concentration of about 7 g volatile suspended solids (VSS) per liter, in which specific microbial groups for the production of VFA from CW had been previously enriched (Lagoa-Costa et al., 2020). The operational conditions mimic those in which quite good results were reached, in terms of VFA production, in a previous work (Lagoa-Costa et al., 2020). Briefly, the reactor was operated at 30 °C and the pH was maintained at 5, thanks to online measurements and a pH probe. The substrate was continuously fed to the reactor at a low flow rate of 0.55 L d^{-1}, resulting in a hydraulic retention time (HRT) of 2 days. The organic loading rate (OLR) was set at 6 g COD L^{-1} d^{-1}. Finally, a solid retention time (SRT) of 15 days was established by a daily purge. All the conditions are summarized in Table 1.

2.2.2. Substrates

Both CW and BW were sourced from regional companies in Galicia, Spain. To avoid any possible unwanted fermentation/degradation process, both by-products were stored at 4 °C in a refrigerated chamber. On the collection day, a small but representative volume of each by-product was sampled for its own characterization. CW was supplied by the cheese manufacturer company Innolact (Lugo, Spain) and it was composed of a high organic content of about 54–56 g COD L^{-1}, from which 90% was associated with lactose. The remaining 10% of the organic content was made of a small fraction of proteins and organic acids (lactic and acetic). Low (<80 mg-NH_4^+ L^{-1}) and moderate (300 mg-P L^{-1}) amounts of nitrogen and phosphorous were respectively measured. On the other hand, BW was provided by the beer manufacturer company Hijos de Rivera (A Coruña, Spain) and it was composed of a much lower organic content of around 4.8 g COD L^{-1}. About 71% of this COD (3.4 g L^{-1}) was associated with ethanol, while acetic, propionic and butyric acids accounted for the remaining 29% (1.4 g L^{-1}). Due to the low organic content, synthetic ethanol and organic acids (i.e., acetic, propionic and butyric acids) were added to BW in order to increase its organic content up to 6 g COD L^{-1}. The addition of this synthetic carbon source did exclusively take place when the UASB reactor was running in Phase IV of the acidogenic fermentation stage. Despite this external addition, the original proportion ethanol/organic acids remained constant. Finally, dissolved ammonia and phosphate concentrations were quite low and not higher than 1 and 8 mg L^{-1}, respectively.

2.3. Second stage: PHA production

Once the acidogenic fermentation ended and the effluents from each of the different phases were collected, PHA accumulation tests were

Table 1
Overview of the operational conditions of the UASB reactor at each of the different phases in the acidogenic fermentation stage.

Phase	I	II	III	IV
Time (d)	34	24	40	33
SRT (d)	15	15	15	15
HRT (d)	2	2	2	2
COD_{FEED} (g L^{-1})[a]	12	12	12	12
OLR (g COD L^{-1} d^{-1}*)*	6	6	6	6
pH	5	5	5	5
Temp. (°C)	30	30	30	30
CW (%)	100	90	75	50
BW (%)	0	10	25	50

[a] Initial concentration (in COD basis) of the feed in the UASB reactor.

performed through the operation of fed-batch bioreactors. A 2-L volume glass reactor was inoculated with 0.8 L of a mixed culture previously enriched in PHA accumulating bacteria. The selection of the mixed culture took place through the long-term operation of a sequencing batch reactor (SBR) using fermented CW as substrate under the feast and famine regime (non-published data). In order to maximize the production of PHA, the operational conditions during these assays were the same as for the selection stage of the mixed culture. The temperature was set at 30 °C and the pH was controlled in the range of 8–8.5. The air flow rate was 1 vvm (volume air/volume reactor/minute) and the stirring speed was adjusted to 500 rpm, ensuring good distribution of oxygen inside the reactor. Dissolved oxygen (DO) was an essential parameter, monitored throughout the entire assays, thanks to a DO probe. A manual pulse feeding strategy was used for the performance of the PHA accumulation tests, in which the DO values were used to decide on the introduction of new pulses of substrate to the culture medium. The PHA storing biomass was taken from the SBR at the end of the famine phase in order to minimize both dissolved ammonia and intracellular PHA. To avoid biomass growth, no extra nutrients were added to the medium.

2.4. Metagenomic analysis

In order to monitor the microbial dynamics in the UASB reactor, metagenomic analyses were performed. For that reason, samples were taken from the reactor when it reached steady state in each of the different phases of the co-digestion process. Each sample was taken in duplicate. The procedure of the sequencing and post-sequencing analysis was exactly the same as described in a previous work (Lagoa-Costa et al., 2020). Finally, raw reads were deposit in the sequence read archive of the NCBI under project PRJNA636954.

2.5. Analytical techniques

Samples from the UASB were monitored twice a week. Total suspended solids (TSS), VSS, and COD were analysed for influent and effluent characterization following Standard Methods (APHA et al., 2012). TSS and VSS were also used as a direct estimation of biomass concentration inside the reactor. Further UASB analyses included the quantification of ammonia and phosphate concentrations using a colorimetric method at 635 and 690 nm, respectively. HPLC analysis allowed the determination of water soluble products such as lactose, ethanol and VFA following the same procedure as described in Lagoa-Costa et al. (2020). Finally, the PHA content was measured by gas chromatography (Lagoa-Costa et al., 2017).

2.6. Calculations

The degree of acidification (DA) was calculated as the percentage of the organic content in the feed (in COD basis) that has been detected in the effluent in the form of VFA (in COD basis), according to Eq. (1).

$$DA = \frac{VFA_{efl}\left(g_{COD}\,L^{-1}\right) - VFA_{feed}\left(g_{COD}\,L^{-1}\right)}{COD_{feed}\left(g_{COD}\,L^{-1}\right)} \times 100 \quad (1)$$

The intracellularly accumulated PHA was calculated as the proportion of PHA with respect to the total weight of biomass (as VSS), according to Eq. (2).

$$PHA(\%) = \frac{PHA\left(mg\,mL^{-1}\right)}{VSS\left(mg\,mL^{-1}\right)} \times 100 \quad (2)$$

3. Results and discussion

The substrate is one of the most significant parameters affecting the microbiome in AD, i.e., the presence of specific populations of microorganisms is often dependant on the feed. Besides, it is known that the microbiome is also altered when feedstock dynamic conditions are applied to the system. But, to the best of our knowledge, microbial dynamics have not yet been studied when the feedstock composition is subjected to a gradual modification. In order to address this issue, the microbial community was studied in an acidogenic reactor for VFA production during the co-digestion of different proportions of CW and BW. Then, each of the VFA-rich streams were tested individually as suitable substrates for the production of PHA.

3.1. Influence of a gradual change in the feedstock mix ratio on acidogenesis

3.1.1. General considerations about the microbial community

After all the filtering steps in the post-sequencing analysis, the number of reads ranged between 60 and 100 thousand (Table 2), which is considered high enough to provide a reliable insight of the complexity of the microbial community (Campanaro et al., 2018b). At first sight, it appeared that the gradual introduction of a new substrate in the feed, i.e., BW, had a positive effect on biodiversity, since the values of the Shannon index slightly increased (Table 2). However, a decrease in microbial richness, in terms of counted operational taxonomic units (OTUs), was observed as the proportion of BW increased. De Francisci et al. (2015) reported a similar drop in microbial richness when feeding the biomass with lipids or proteins compared to glucose. They linked this observation to the inhibitory effect of lipids and proteins compared to glucose, which is an easily biodegradable carbon source that allows the growth of a wide range of different microorganisms. Accordingly, the reason why a loss of richness was observed in the present research could be due to a similar selective pressure, associated with the toxic effect of ethanol in this case. In such a way, only specific groups of bacteria would be able to survive in the presence of ethanol in the culture medium. Besides, the higher the ethanol concentration, the more intense the selective pressure. Another important factor to take into consideration is the presence of polyphenols. Polyphenolic compounds are present in beer due to the contribution of malt and hop, and their presence in BW has already been proven (Tatullo et al., 2016). Although their concentration in BW has not been measured in the present study, they could also have played an important role in the loss of population richness, as they have a proven antimicrobial activity (Barbosa-Pereira et al., 2014).

The principal coordinate analyses (PCoA) were done based on Bray-Curtis distances and it enabled to assess the microbial community composition at each of the different phases of the co-digestion process at OTU level. As observed from Fig. 1, the PCoA explained 80% of the whole microbial community variation through axis 1 (63%) and axis 2 (17%). As expected, replicates clustered close one to the other. Furthermore, the samples were staggered in three different clusters along axis 1 (Fig. 1), clearly suggesting that the gradual introduction of more BW promoted a progressive change in the composition of the microbial community. However, this staggered evolution appear to be disturbed at Phase III, in which 25% of the COD in the form of BW was fed to the system (Fig. 1). From this result, the identification of a threshold at which the microbial community suffered a remarkable variation can be concluded.

3.1.2. Microbial dynamics in response to the variation of the feedstock mix ratio

As shown in Fig. 2 A, the co-digestion of CW and BW showed a dominance of five different phyla regardless of the operational phase, with a relative abundance of almost 100%. These phyla were *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Patescibacteria* and *Proteobacteria*. All of them are widely recognized to be among the most common in AD systems thanks to their great ability to degrade a large number of different organic compounds (Campanaro et al., 2018a; He et al., 2019; Jin et al., 2019; Li et al., 2020). *Firmicutes* was, by far, the most abundant phylum

Table 2
Main results after the post sequencing analysis of raw reads at each of the different phases of the acidogenic fermentation stage. Calculations of alpha diversity (Shannon index and Observed OTU) are also included.

Sample	Replicate	Reads				Shannon index	Observed OTUs
		Raw	Filtered	Assigned (Phylum)[a]	Assigned (Genus)[b]		
Phase I	A	181001	100961	100961 (100%)	72474 (72%)	3.05	148
Phase I	B	163208	81527	81524 (100%)	55439 (68%)	3.31	158
Phase II	A	160842	93112	93103 (100%)	66185 (71%)	3.28	137
Phase II	B	164227	78157	78150 (100%)	61444 (79%)	3.19	137
Phase III	A	139565	65846	65846 (100%)	46429 (71%)	3.25	139
Phase III	B	147019	59612	59610 (100%)	42253 (71%)	3.49	148
Phase IV	A	123208	69628	69625 (100%)	44780 (64%)	3.44	114
Phase IV	B	141212	66385	66384 (100%)	46488 (70%)	3.35	113

[a] Number of assigned reads at phylum level. The percentage is given in brackets.
[b] Number of assigned reads at genus level. The percentage is given in brackets.

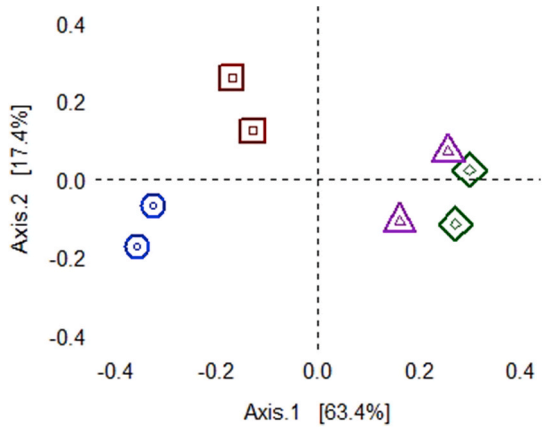

Fig. 1. PCoA plot representing microbial variations at each of the different phases during the acidogenic fermentation process: blue circles (Phase I), red squares (Phase II), violet triangles (Phase III) and green diamonds (Phase IV).

in Phase I, representing 45% of the microbial community, followed by *Patescibacteria* (16%) and *Actinobacteria* (13%). However, the gradual change in the proportion of BW affected differently to the distribution of the different phyla. *Firmicutes* showed a great resistance to the change in the feedstock mix ratio and its relative abundance scarcely varied, being the most abundant phyla regardless of the operational phase of the co-digestion process. *Actinobacteria*, *Proteobacteria* and *Patescibacteria* were sensitive to this change, suggesting they were not able to counteract the increasing concentration of BW in the feed. Finally, from all the bacterial populations, only the members belonging to *Bacteroidetes* increased with the gradual change in the feedstock mix ratio.

The vast majority of the taxonomic groups that have been identified throughout the whole co-digestion process were classified within the *Bacteria* domain. However, a small fraction of them belonged to *Archaea* (Fig. 2 B) and more specifically, to the *Euryarchaeota* phylum. They were detected only when BW was part of the feed (i.e., Phases II, III and IV). Methanogens belong to this domain, clearly suggesting that some methanogenic activity took place inside the UASB reactor although their relative abundance never reached more than 1%.

When going deeper into more specific taxonomic ranks, a reduction in the percentage of assigned reads was observed from phylum to genus level. In fact, the percentage decreased from 100% down to 70% on an average (Table 2). Consequently, an increase in the number of unclassified OTUs was registered, which is quite common in current days mainly due to a lack of information in databases corresponding to 16S rRNA. Fig. 2 C provides a heat map of the top 20 of the most abundant genera in the microbial community, including classified and unclassified ones. The results supported those obtained in the PCoA, in which the microbial community changes progressively in response to a gradual modification of the feedstock mix ratio up to a certain proportion of BW, *i.e.*, 25% of COD, at which a more radical change is observed. The dominant core of bacterial populations developed during Phases I and II was quite similar and composed mainly of *Megasphaera*, *Bifidobacterium*, *Caproiciproducens* as well as unclassified members of the *Saccharimonadales* order, the *Bacteroidales* order and the *Erysipelotrichaceae*

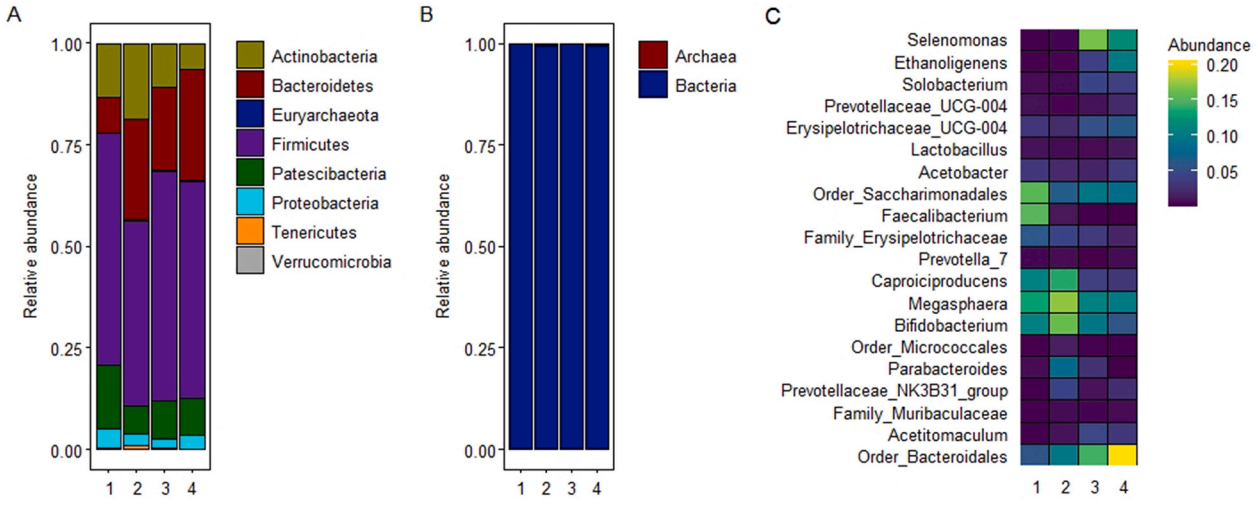

Fig. 2. Relative abundance of the different bacterial populations identified in the microbial community at each of the different phases of the co-digestion process at (A) phylum level, (B) domain level and (C) genus level of the top 20 of the most abundant genera.

family. The presence of a large number of these bacterial populations was favoured because of the seed, which was previously enriched in CW acidifying microbes (Lagoa-Costa et al., 2020). Despite large similarities, differential dynamics were observed when shifting from Phase I to Phase II (Fig. 3 A). On the one hand, the *Ruminococcaceae* family was found to be among the most negatively influenced populations. Within the *Ruminococcaceae* family, members of the *Faecalibacterium* genus reached a 15-fold reduction in their relative abundance, from being the most abundant bacterial population in Phase I (15%) to hardly reach 1% in Phase II (Fig. 2 C). In accordance with this radical change in their abundance, it may be assumed that the introduction of BW in the culture medium and, thus, of ethanol, exerted some sort of toxic effect on these bacterial populations. Interestingly, it has already been discussed that *Ruminococcaceae* and particularly, *Faecalibacterium* are strongly affected in gut microbiome by the presence of ethanol in alcoholic patients, somehow supporting the results obtained in the present work (Sarin et al., 2019). Besides downregulated taxa, upregulated taxa were also observed. *Parabacteroides* as well as one member of the *Prevotellaceae* family (*Prevotellaceae* NK3B31) suffered an 11 and 22-fold increase of their relative abundances, respectively. The former increased from 0.8 up to 8.7%, meanwhile the latter increased from 0.2 up to 4.5% on its coverage (Fig. 2 C). Consequently, these two populations showed one of the highest differential growths within the microbial community when switching from Phase I to Phase II (Fig. 3 A). Unlike *Faecalibacterium*, these gram negative bacteria belonging to *Bacteroidetes* are proven to be favoured in different ethanol-rich environments (Bluemel et al., 2020; Shan et al., 2017). However, the highest upregulated bacterial population of the whole microbial community, when shifting from Phase I to Phase II, was *Methanobrevibacter* (Fig. 3 A). Although this bacterial population had never been amongst the most abundant ones in the microbial community, it has already been reported to play an active role in hydrogenotrophic methanogenesis (Braz et al., 2019). Therefore, as previously mentioned, it may be assumed that at least a small fraction of the carbon source was diverted towards methane production during the present study, although biogas was not monitored.

As explained above, a supply of 25% of the COD in the form of BW seemed to be the threshold from which the microbial community suffered a remarkable modification of its composition (Fig. 2 C). The CW close related taxa that dominated the microbial community during Phases I and II showed a general reduction trend of their relative abundances in Phases III and IV (i.e., *Megasphaera*, *Bifidobacterium*, *Caproiciproducens* as well as the unclassified member of the *Erysipelotrichaceae* family). From all of them, *Megasphaera* was able to better counteract the entrance of higher proportions of BW, probably thanks to its ability to deal efficiently with high ethanol concentrations up to 5.5 g L^{-1} (Sakamoto and Konings, 2003). However, the main reason of considering Phase III as the threshold from which the microbial community drastically changed was due to the sudden appearance of a group of new taxa. These bacterial populations were *Selenomonas*, *Ethanoligenens*, *Acetitomaculum* and *Solobacterium* and all of them represented less than 1% coverage in the previous phases (Fig. 2 C). In the case of the first three genera it was even possible to detect a differential growth when shifting from Phase II to Phase III, with a significance value < 0.01 (Fig. 3 B). In addition to these bacterial populations, the relative abundance of unclassified members of the *Bacteroidales* order did also increase in Phases III and IV compared to Phases I and II (Fig. 2 C). Finally, when shifting from Phase III to Phase IV, only downregulated taxa were detected (Fig. 3 C).

3.1.3. Shifts in the production of metabolites

From the beginning of the experiment it was observed that the VFA were the main fermentation end-products no matter the experimental phase of the co-digestion process. The presence of these short chain fatty acids was a clear indicator that most of the bacterial populations developing in the microbial community were acidogens, as can be seen in Table 3. However, the gradual change in the feedstock mix ratio that

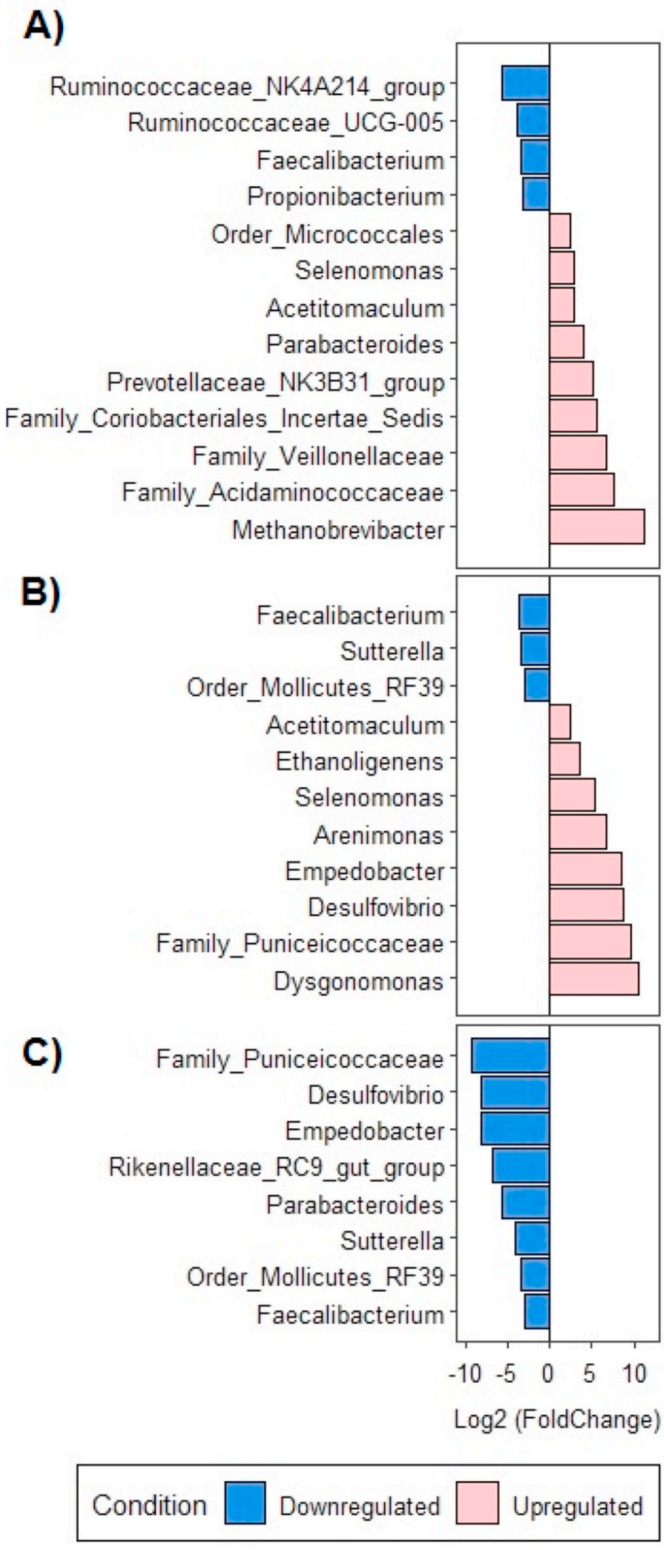

Fig. 3. Bar plot representing differential taxa analysis when changing the feedstock composition during the acidogenic fermentation process: (A) from Phase I to Phase II, (B) from Phase II to Phase III and (C) from Phase III to Phase IV. Blue bars show downregulated genera and pink bars show upregulated genera. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

was promoted in the current work caused a shift in the production of metabolites. In the first stages of the acidogenic fermentation process, Phases I and II, where lactose was by far the main substrate of the feedstock, butyrate producers were dominant in the microbial community. Bacterial populations such as *Megasphaera*, *Caproiciproducens*, *Faecalibacterium* as well as members of the *Erysipelotrichaceae* family, all of them with the ability to synthesize butyrate from carbohydrates (Table 3), were particularly enriched in the community. Besides lactose, setting the pH of the culture medium at 5 also led to butyrate producers developing in the microbial community. In accordance to this, many studies in the literature reported butyrate production under similar pH conditions using sugary substrates (Bengtsson et al., 2008a; Calero et al., 2018; Lagoa-Costa et al., 2020; Tamis et al., 2015). Therefore, butyrate became the main fermentation end-product in Phases I and II, reaching 52 and 42% of the whole VFA production, respectively (Table 4). In addition to butyrate, acetate was the other main fermentation end-product, reaching 36 and 46% of the whole VFA production in Phases I and II, respectively. Different metabolic pathways were found to be used by the microorganisms to synthesize this short chain fatty acid from lactose in these first two stages: the heterolactic fermentation, carried out by *Bifidobacterium*, and the mixed acid fermentation, which was carried out by *Caproiciproducens* as well as by unclassified members of the *Prevotellaceae* family (Table 3). Finally, in Phase II, a fraction of acetate could also have been produced through the oxidation of butyrate within a syntrophic interaction process. The presence of *Methanobrevibacter* could contribute to reduce the hydrogen partial pressure derived from the production of butyrate, making the butyrate β-oxidation process energetically favourable to the synthesis of acetate (Worm et al., 2014). As a consequence of the main bacterial populations present in the microbial community, the global production of even-chain VFA reached 88% of the whole production in Phases I and II. Conversely, the production of odd-chain VFA (i.e., propionic and valeric acids) represented only 12%, mainly due to minor abundance of microorganisms with the ability to synthesize these short chain fatty acids (Table 4).

The introduction of more BW, in Phases III and IV, caused a radical change in the basal structure of the microbial community by changing the feedstock composition. The contribution of lactose was reduced since the proportion of CW decreased, while the ethanol concentration increased in the culture medium. This caused a disturbance in the VFA profile, resulting in a final inversion in the production of butyrate and acetate (Table 4). Butyric acid fermentation decreased down to its minimum value in these phases, reaching 27–30% of the whole VFA production, mainly influenced by the reduction of CW and, therefore, CW closely-related taxa, which were also butyrate producers. Moreover, the increase in the proportion of BW benefited the appearance of new taxa such as *Selenomonas*, *Ethanoligenens*, *Solobacterium* and *Acetitomaculum*, all acetic acid synthesizing bacteria from carbohydrates, although through different metabolic pathways (Table 3). *Selenomonas* and *Solobacterium* synthesized acetate through the mixed acid fermentation, *Ethanoligenens* exhibited the acetate-ethanol type fermentation and *Acetitomaculum* is a homoacetogen microorganism. Accordingly, the production of this short chain fatty acid increased at this point of the experiment. Despite the observed inversion in butyrate and acetate, the global production of even-chain VFA did still continue to be predominant, representing 79–82%. However, a small increase in the production of propionate was also observed and it enhanced the proportion of odd-chain VFA up to 18–21% (Table 4).

Table 4
UASB reactor performance during the four different phases of the acidogenic fermentation stage.

Phase	I	II	III	IV
VFA (g COD L^{-1})[a]	8.4 (±0.3)	8.6 (±0.3)	8.2 (±0.4)	7.4 (±0.7)
Ethanol (g COD L^{-1})[b]	0.0	0.1 (±0.1)	0.3 ± (0.2)	0.2 (±0.1)
Acetate (%)	35.9 (±4.6)	45.9 (±2.4)	51.9 (±2.0)	52.4 (±2.8)
Propionate (%)	4.3 (±2.2)	8.2 (±0.6)	15.4 (±1.5)	11.9 (±0.8)
Butyrate (%)	51.9 (±3.7)	42.3 (±2.9)	26.7 (±1.4)	29.7 (±3.8)
Valerate (%)	7.9 (±2.6)	3.6 (±0.3)	6.0 (±0.7)	6.0 (±1.7)
DA (%)	72.2 (±4.5)	65.4 (±4.3)	59.3 (±5.2)	57.5 (±2.7)
VFA/COD (%)[c]	88 (±5)	100 (±5)	97 (±6)	95 (±5)

[a] VFA concentration measured in the effluent of the UASB reactor.
[b] Ethanol concentration measured in the effluent of the UASB reactor.
[c] Percentage of the soluble COD in the effluent in the form of VFA.

Besides lactose, ethanol, which considerably increased its concentration in Phases III and IV, was also an important substrate to be metabolized by the microorganisms at these stages. Ethanol, as a substrate, can be oxidized to acetate by acetate-producing bacteria, a chemical reaction that also releases carbon dioxide and hydrogen to the culture medium in the ethanol β-oxidation. However, this metabolic pathway is reversible and, when the conditions are favourable, the reverse β-oxidation can also take place (Spirito et al., 2014). During this second route, ethanol is used as an electron donor in chain elongation processes. In that sense, ethanol can first donate electrons to acetate to form butyrate and then to butyrate to form caproate in sequential reactions. Although ethanol is considered a common electron donor in chain elongation processes (He et al., 2021), the presence of hydrogenotrophs such as *Methanobrevibacter* or *Acetitomaculum*, which kept the hydrogen partial pressure low, and the non-detection of any caproate as fermentation end-product, allows to assume that ethanol β-oxidation was the leading metabolic pathway in the current work.

Apart from VFA, no other organic acids were detected as fermentation end-products in the stream of the acidogenic reactor in any of the different operational phases. The complete absence of lactate, one of the most common metabolites in the acidogenic fermentation of CW, is noteworthy. A microbial community free of lactic acid bacteria (LAB) may certainly have played an important role in the complete absence of this organic acid as end-product. *Lactobacillus* was the main LAB in the microbial community, even though its contribution in the synthesis of lactate seemed almost negligible since it barely accounted for 1% of the total relative abundance. However, lactate could still have been

Table 3
Function, substrate and end-fermentation products of the main bacterial populations identified in the UASB reactor.

Bacterial population	Function	Substrate	Final products	Reference
Bifidobacterium	Acidogenesis	Carbohydrate	Acetate and lactate	Feng et al. (2018)
Megasphaera	Acidogenesis	Carbohydrate	Butyrate	Polansky et al. (2016)
Faecalibacterium	Acidogenesis	Carbohydrate	Butyrate	Polansky et al. (2016)
Caproiciproducens	Acidogenesis	Carbohydrate	Acetate, butyrate and caproate	Kim et al. (2015)
Erysipelotrichaceae	Acidogenesis	Carbohydrate	Butyrate, lactate and formate	Si et al. (2016)
Lactobacillus	Acidogenesis	Carbohydrate	Lactate	Feng et al. (2018)
Parabacteroides	Acidogenesis	Carbohydrate	Propionate	Polansky et al. (2016)
Prevotellaceae	Acidogenesis	Carbohydrate	Acetate and succinate	Shah and Collins (1990)
Solobacterium	Acidogenesis	Carbohydrate	Acetate, lactate and butyrate	Kageyama and Benno (2000)
Ethanoligenens	Acidogenesis	Carbohydrate	Acetate and ethanol	Li et al. (2019)
Selenomonas	Acidogenesis	Carbohydrate	Acetate, propionate and lactate	Schleifer et al. (1990)
Acetitomaculum	Acetogenesis	Glucose/CO_2 + H_2	Acetate	Le Van et al. (1998)
Methanobrevibacter	Methanogenesis	CO_2 + H_2	Methane	Braz et al. (2019)

produced, though in very low amounts, either through the homolactic or heterolactic fermentation of lactose, as an intermediate product. Actually, it is considered to be a great electron donor to synthesize butyrate (Feng et al., 2018) and caproate (Brodowski et al., 2020; Contreras-Dávila et al., 2020; Iglesias-Iglesias et al., 2021) as final end-products in chain elongation processes, even though the latter, as previously mentioned, was not detected in the current work.

3.1.4. *Performance of the acidogenic fermentation process*

The development of a majorly acidogenic microbial community led to VFA becoming the predominant metabolites at the end of the fermentation process with an overall production of 8.6–7.4 g COD L^{-1} (Table 4). However, when BW was introduced as co-substrate with CW in the culture medium (Phase II, III and IV), a residual amount of ethanol was also detected in the effluent, apart from VFA (Table 4). The ethanol concentration reached values of 0.09, 0.25 and 0.17 g COD L^{-1} for Phases II, III and IV, respectively.

By changing the feedstock composition, the performance of the acidogenic fermentation got also affected. The effectiveness of the process was defined in terms of DA and this parameter reached its highest value in Phase I, when lactose reached its maximum concentration in the culture medium (72.2%) (Table 4). This result was in the same range as other DA reported in the literature (Bengtsson et al., 2008a; Duque et al., 2014; Gouveia et al., 2017; Tamis et al., 2015), even though our previous work, as well as others, suggested that there is still room for improvement (Domingos et al., 2017, 2018; Lagoa-Costa et al., 2020). Nevertheless, the introduction of more BW in the culture medium led to a progressive decrease of the DA value (Table 4). To the best of our knowledge, no other works are available on mixtures of CW and BW for the production of VFA, but, based on the experimental results, the supply of BW had a negative impact on it. Lactose, the main component of CW, is an easy biodegradable carbohydrate and, as explained above, it usually leads to a highly efficient transformation towards VFA. However, the oxidation of ethanol towards acetate, the observed metabolic route to metabolize this substrate in the current work, resulted in an overall less efficient process. Besides, the higher the ethanol concentration in the culture medium, the lower the DA, as observed. As a conclusive remark it can be said that CW has a better acidifying potential rather than BW, mainly due to its composition.

3.2. *Influence of a gradual change in the feedstock mix ratio on PHA production*

VFA, which were the main end-products throughout the whole acidogenic fermentation, are considered the main precursors for the synthesis of PHA in aerobic mixed cultures. Therefore, they were tested as substrates for PHA accumulation through the operation of fed-batch bioreactors. Four different assays were designed, in which the biomass was fed one of the VFA-rich streams collected at each of the different phases of the co-digestion process.

All the PHA accumulation tests lasted between 7 and 10 h and a variable number of 4–6 pulses were introduced in the culture medium (Fig. 4). Each of the pulses had an initial carbon concentration of around 30 Cmmol L^{-1} and the biomass was able to consume all the VFA by the end of each assay. Regardless of the effluent used in the PHA accumulation tests, the behaviour of the biomass was quite similar. When introducing the first pulses of substrate to the culture medium, the DO concentration decreased. This fact was a clear indicator that the microorganisms were consuming the substrate; to the point that, when the biomass had consumed the substrate, the DO concentration registered a sharp increase, indicating the end of the pulse and the possibility of introducing a new one (Fig. 4). Concomitant with the substrate consumption, a quick increase in the intracellular PHA concentration was also registered. As a result, the amount accumulated PHA reached half of its maximum value in less than 3 h, demonstrating fast kinetics at the beginning of the experiments (Fig. 4). As the number of pulses increased,

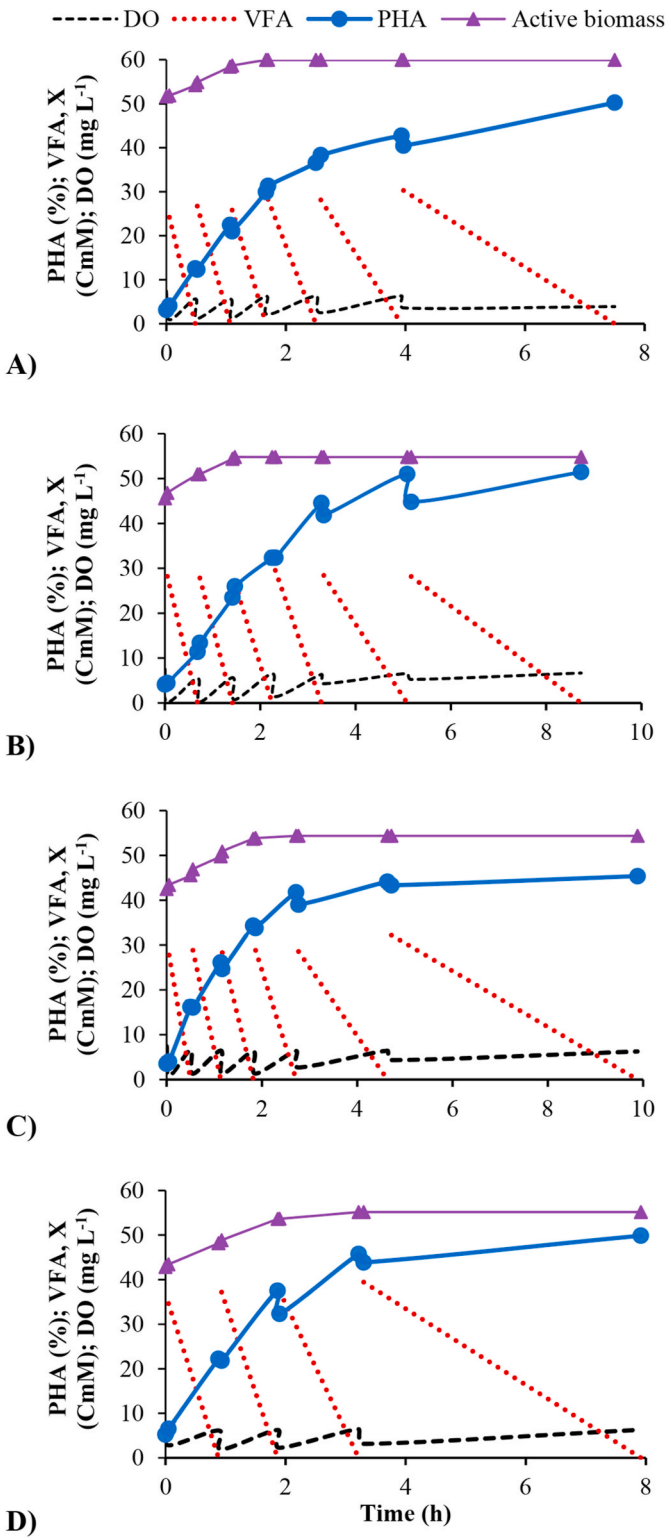

Fig. 4. Fed-batch assays for the production of PHA using as substrate each of the four different VFA-rich effluents obtained at Phase I (A), Phase II (B), Phase III (C) and Phase IV (D) of the acidogenic fermentation.

the substrate consumption rate as well as the PHA production rate slowed down, and then a plateau phase was achieved. However, this is usual when the microorganisms get saturated in terms of PHA accumulation (Johnson et al., 2010a).

All the VFA-rich streams were N-limited thanks to the low amount of free ammonia in both CW and BW. However, although no external

nutrients were added to the culture medium, a certain amount of ammonia was available for the biomass at the beginning of the PHA accumulation tests. This was due to a residual concentration of ammonia at the end of the famine cycle in the SBR previous to the inoculation of the fed-batch reactor. Nonetheless, the evolution of ammonia was monitored along all the experiments and it was possible to observe its gradual consumption as the pulses of substrate were progressively introduced in the culture medium (data not shown). Consequently, an increase in the concentration of active biomass was registered within the first 2 h of the experiment (Fig. 4). Based on these results, cell growth and PHA synthesis took place simultaneously by the time ammonia was available in the culture medium. The continuous supply of ammonia throughout the whole PHA accumulation assays has already been reported to negatively affect PHA production (Bengtsson et al., 2008b; Johnson et al., 2010b). However, when a residual amount of ammonia remains in the culture medium at the beginning of the PHA accumulation tests, like in this particular case, it is demonstrated to not affect the maximum PHA content (Jiang et al., 2012). The maximum PHA content was quite similar among all the used VFA-rich streams, with an intracellular PHA content ranging from 45 to 50% (Fig. 4). These PHA contents were in agreement to other published works in the literature when using real VFA-rich streams (Valentino et al., 2017).

These fed-batch experiments allowed to evaluate the influence of the wastewater matrix on PHA production. Since the enrichment in PHA storing bacteria of the mixed culture took place using fermented cheese whey as substrate, quite similar to that of Phase I, the wastewater matrix was varied when using the VFA-rich streams from Phases II, III and IV, when BW was introduced. Based on the similar PHA contents no matter the used VFA-rich stream (45–50%), it can be concluded that the wastewater matrix did not really influence PHA production, similar to what was observed by Albuquerque et al. (2007). Nevertheless, the wastewater matrix can truly influence PHA production when inhibitory compounds, like ethanol, are available in the feedstock (Tamang et al., 2021). Ethanol did remain in the effluents from Phases II, III and IV in the present work, but in such low amounts that no inhibitory effects were detected when performing the PHA accumulation experiments.

Although there was no evidence of the influence of the wastewater matrix on the ability of the aerobic mixed culture to synthesize PHA, the use of different VFA-rich streams, with different VFA profiles, led to a slight modification in the monomeric composition of the final biopolymer. When the feedstock mix ratio was varied by introducing more BW, the proportion of butyrate and acetate reversed. However, since both are even-chain VFA, they lead the synthesis of PHA towards hydroxybutyrate (HB), with no final influence on the monomeric composition of the biopolymer; but, as the proportion of BW increased in the culture medium, an increase in the production of propionate was also observed (Table 4). In this particular case, the increase in the production of propionate led to an increase in the amount of hydroxyvalerate (HV) monomer. The incorporation of HV up to a certain percentage is, theoretically, beneficial for the properties of the biopolymer since it reduces stiffness and brittleness, resulting in a more elastic and resistant biopolymer (Reis et al., 2011). However, the present work did not study the physical thermal properties of the obtained biopolymers and this assumption must be corroborated in future experiments. When the effluents from Phases I and II were used as substrate, the biopolymer composition was 88/12 (HB/HV). Conversely, when the effluents from Phases III and IV were used as substrate, the composition slightly changed to 76/24 (HB/HV).

4. Conclusions

The present research studied the response of the microbial community subjected to a gradual change in the feedstock mix ratio during acidogenesis for VFA production. Our results showed a progressive modification in the composition of the microbiome as the feedstock mix ratio was gradually changed, although a threshold at which the microbial community suffered a remarkable modification was observed when the proportion of BW reached 25% (Phase III). The change in the feedstock mix ratio, with a reduction of lactose and an increase of ethanol concentrations, also caused a disturbance in the VFA profile. While butyrate fermentation lost much of its importance along the process, acetic acid synthesizing bacteria prevailed. Moreover, the DA decreased as BW was progressively introduced in the culture medium, demonstrating the higher acidifying potential of CW compared to BW. Finally, all the PHA accumulation tests exhibited a quite similar performance in terms of accumulated PHA, although the different VFA profile led to slightly different monomeric compositions of the final biopolymer.

Credit author statement

BL performed the experiments; BL, MCV, CK analysed the data and contributed to the writing of the manuscript. MCV proposed the original research project and concept. All authors read and approved the final manuscript.

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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