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RESEARCH ARTICLE

Altering operational conditions during protein fermentation to volatile fatty acids modifies the associated bacterial community

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Abstract

In recent years, the production of volatile fatty acids (VFA) through mixed culture fermentation (MCF) has been gaining attention. Most authors have focused on the fermentation of carbohydrates, while other possible substrates, such as proteins, have not been considered. Moreover, there is little information about how operational parameters affect the microbial communities involved in these processes, even though they are strongly related to reactor performance and VFA selectivity. Hence, this study aims to evaluate how microbial composition changes according to three different parameters (pH, type of protein and micronutrient addition) during anaerobic fermentation of protein-rich side streams. For this, two continuous stirred tank reactors (CSTR) were fed with two different proteins (casein and gelatine) and operated at different conditions: three pH values (5.0, 7.0 and 9.0) with only macronutrients supplementation and two pH values (5.0 and 7.0) with micronutrients' supplementation as well. Firmicutes, Proteobacteria and Bacteroidetes were the dominant phyla in the two reactors at all operational conditions, but their relative abundance varied with the parameters studied. At pH 7.0 and 9.0, the microbial composition was mainly affected by protein type, while at acidic conditions the driving force was the pH. The influence of micronutrients was dependent on the pH and the protein type, with a special effect on Clostridiales and Bacteroidales populations. Overall, this study shows that the acidogenic microbial community is affected by the three parameters studied and the changes in the microbial community can partially explain the macroscopic results, especially the process selectivity.

INTRODUCTION

In recent years, the production of volatile fatty acids (VFA) through anaerobic fermentation has gained attention (Atasoy et al., 2020; Strazzera et al., 2018). VFA are largely used in the chemical industry, serving as starting molecules for bioenergy production and for the synthesis of a variety of products. Thus, they present a higher market value than methane (Owusu-Agyeman et al., 2022; Vázquez-Fernández et al., 2022). In this context, VFA production can contribute to the achievement of the sustainable development goals (SDGs) of the United Nations. For example, the use of different residues to obtain an alternative to petroleum-based VFAs or even biofuels (Varghese et al., 2022) is in line with addressing SGD 6 (Clean water and sanitation), or SDG 9 (Industry innovation and infrastructure).

However, large-scale production of VFAs is hampered for several reasons, such as low process yields and high costs compared to chemical production. In addition, VFA produced by fermentation are often in mixed condition, so the physical separation of individual acids is usually required. In the case of VFA production from mono-cultures, there is a high cost associated with maintaining aseptic conditions and the need for high-quality raw materials (Varghese et al., 2022). New waste policies that promote the reuse of materials in the framework of circular economies, such as the environmental legislation in the European Union that has promoted sewage sludge valorisation, along with the instability of the biogas market could enhance the implementation of VFA production at the commercial level (Esteban-Gutiérrez et al., 2018).

Production of VFA is usually carried out via mixed culture fermentation (MCF), considering that microbial consortia show more adaptability and resilience than pure cultures (Smid & Lacroix, 2013). The most studied microorganisms able to transform complex substrates into VFA belong to the phyla Firmicutes, Proteobacteria and Bacteroidetes (Vázquez-Fernández et al., 2022). These three phyla include acidogenic microorganisms that can produce different VFAs. Firmicutes, comprised by gram-positive microorganisms, is usually the dominant phylum in acidogenic processes, with *Clostridium* and *Bacillus* as the most relevant genera. Bacteroidetes and Proteobacteria include gram-negative microorganisms, such as *Desulfovibrio* and *Geobacter*, and *Bacteroides* and *Proteiniphilum*, respectively (Leng et al., 2018).

Firmicutes and Bacteroidetes are responsible for hydrolysis and acidogenesis processes, and both are reported to degrade a wide range of substrates, with members of class Clostridia linked to amino acids fermentation. Besides, Proteobacteria phylum includes acidogenic microorganisms and shows differences at lower taxonomic levels: α-*proteobacteria* are linked to carbohydrate degradation, while β-*proteobacteria* are related to protein degradation (Iglesias-Iglesias et al., 2019).

So far, most authors have been focusing on the fermentation of carbohydrates, because they show a higher degradability and availability, neglecting other organic fractions, such as proteins (Bevilacqua et al., 2020). Proteins are one of the major components of many organic waste streams, such as sewage sludge (Liu et al., 2012), food waste (Strazzera et al., 2018) or fish-processing industries' wastewater (Ramsay & Pullammanappallil, 2001). Many authors have mentioned VFA production as an effective solution to the problem of managing the large amount of waste generated (Strazzera et al., 2018). Moreover, incorrect treatment of such waste, due to its nitrogen content, could contribute to eutrophication processes. Besides, various legislations impose increasingly restrictive discharge limits for wastewater streams. For instance, Directive 91/271/EEC establish a discharge limit of 10–15 mg/L total nitrogen for urban and certain industrial streams. Additionally, protein residues pose a greater challenge than carbohydrates due to their complex structure, composed of different amino acids (Vázquez-Fernández et al., 2022).

During anaerobic treatment of protein-containing wastewaters, amino acids are preferentially fermented via Stickland reactions (Nagase & Matsuo, 1982; Ramsay & Pullammanappallil, 2001), in which a couple of amino acids are converted to VFAs, with the generation of ATP and the release of CO_2 and ammonia:

$$\begin{gathered}R_1CHNH_2COOH + 2\,R_2CHNH_2COOH + 2\,H_2O\\ \rightarrow R_1COOH + 2\,R_2CH_2COOH + 3\,NH_3 + CO_2\end{gathered}$$

Certain amino acids, such as leucine, can serve as an electron donor and an electron acceptor. Anaerobic bacteria known for being involved in Stickland reactions belong to the genus *Clostridium*. Ramsay and Pullammanappallil (2001) proposed a fixed stoichiometry for conversion of each amino acid to specific VFAs, regardless protein composition or the operational conditions applied. However, other authors indicate that preferential consumption may occur, mainly due to bioenergetics motivation (Regueira et al., 2020).

Regarding quantification of amino acids and their degradation rates, the available information is inconsistent among studies. Some authors simply rely on literature values of well-studied proteins (Ramsay & Pullammanappallil, 2001), while others estimate from ammonification and product formation data (Nagase & Matsuo, 1982). Finally, some authors also use analytical techniques, such as high-performance anion-exchange chromatography (Rombouts et al., 2009) or high-performance liquid chromatography (Kim et al., 2020).

The microbial structure and composition is highly related to the operational conditions applied (Jankowska et al., 2017). Substrate composition (Vázquez-Fernández et al., 2022), pH (Shen et al., 2017) and micronutrients (Choong et al., 2016) are relevant parameters in acidogenic processes. However, there is little information about these parameters' effect on the microbial communities responsible for protein fermentation processes.

Substrate composition is one of the most important parameters. So far, only a few authors have been focusing on the microbial community involved in the anaerobic degradation of specific proteins. Kim et al. (2020) found differences in bacterial community structure based not only on the inoculum source but also on the protein used as substrate. In this case, gluten and gelatine were fermented in batch reactors. The most abundant microorganisms at the end of the tests belonged to different classes: γ-*proteobacteria* in the case of gluten and Clostridia in the case of gelatine. The results of Shen et al. (2017) showed that the community composition was already affected at phylum level by the aminoacidic profile of the proteins degraded. Proteobacteria was the most abundant phylum during tofu degradation, while for egg white, it was Firmicutes. Clostridia members are also usually detected in protein fermentation processes, since some *Clostridium* species are well-known protein degraders, being able to carry Stickland reactions (Ramsay & Pullammanappallil, 2001).

To the authors' knowledge, investigations related to the effect of pH on microbial community in protein fermentation cannot be found. However, there are several works studying the influence of pH in acidogenic populations for complex substrates, pointing out the importance of hydrolysis step (García-Depraect et al., 2022; She et al., 2020). All authors conclude that pH influences microbial growth rates and activity (Shi et al., 2022), as well as the diversity of the community present in the reactor. Liu et al. (2012) indicated that extreme pH values (below 5 and above 10) contributed to diversity loss, since the only microorganisms remaining in the reactor were the ones that could tolerate these conditions.

The addition of micronutrients is well-studied for anaerobic digestion, focusing on methane yield optimization. Nickel (Ni), cobalt (Co), molybdenum (Mo), iron (Fe), tungsten (W), zinc (Zn), copper (Cu) and manganese (Mn) are the main trace elements reported in these studies (Xu et al., 2018). Their positive effect can be explained by the fact that they act as cofactors for essential enzymes involved in methanogenesis. However, little information can be found about the effects of micronutrients on hydrolysis and acidogenesis steps. Firmicutes, Proteobacteria, Bacteroidetes and Chloroflexi have been identified as the more abundant bacterial phyla in anaerobic reactors supplemented with micronutrients, specifically Clostridia class (Pasalari et al., 2020). Choong et al. (2016) suggested that micronutrients could have the same positive effect on both acidogenic and methanogenic microorganisms. In this way, Yu et al. (2015) found that $FeCl_3$ could improve the anaerobic fermentation of proteins, whose supplementation was related to an increase in *Sporanaerobacter* genus relative abundance in a thermophilic reactor fed with waste-activated sludge.

This study focuses on analysing the acidogenic microbial communities in protein fermentation, a process much less studied than carbohydrate fermentation. In addition, it addresses the evolution of these communities in long-term reactor operation in response to operational parameters, such as type of protein or pH. Finally, this work aims to link the presence of certain microorganisms to specific VFA production. Thus, the object of this work were: (1) to identify the microbial communities present in protein fermentation processes, and, (2) to evaluate how three operational parameters affect these communities. For that purpose, two model proteins with different aminoacidic profiles (casein and gelatine) were fermented at three pH values (5.0, 7.0 and 9.0) without and with micronutrients' addition (Bevilacqua et al., 2021a, 2021b).

EXPERIMENTAL PROCEDURES

Inoculum

The reactors used in the present study were inoculated with fermentative biomass from an acidogenic mother reactor which, in turn, was originally inoculated with a mix of anaerobic biomass coming from a mesophilic sewage sludge digester and digestate from an anaerobic digester fed with brewery wastewaters (Bevilacqua et al., 2020). This mother reactor was fed with a mix of glucose, casein and sodium oleate, respectively accounting for 60%, 30% and 10% of the total influent chemical oxygen demand (COD), and was operated at 25°C, controlled pH (5.7 ± 0.1), an organic loading rate (OLR) of 8 g COD/L·d and a hydraulic retention time (HRT) of 2 days.

Experimental set up

Two continuous stirred tank reactors (CSTR) of 1 L of working volume were used for the experiments. They were inoculated with approximately 1.0 g VSS/L of acidogenic biomass and operated at 25°C and an HRT of 1.5 days. The reactors were fed with hydrolysed casein (A2208,0500 PanReac) and gelatine (70,951-1 KG-F Sigma-Aldrich) as the sole carbon source (Bevilacqua et al., 2020). In the first phase, the reactors were operated at 3 different pH values with only macronutrient supplementation (in g/L: NaCl 0.292; KH_2PO_4 0.780; NH_4Cl 0.530; Na_2SO_4 0.057; $MgCl_2 \cdot 6H_2O$ 0.120) for more than 400 days. Initially, they were operated at pH 7.0 for 140 days (casein reactor) and 170 days (gelatine reactor). Then, the reactors were operated at pH 9.0 for 160 days each and at pH 5 for 320 days (casein reactor) and 110 days (gelatine reactor). Before the last pH change, the reactors were acclimated at pH 7.0 for more than 30 days. In a second phase, the reactors were operated with micronutrients' supplementation (in mg/L: $FeSO_4 \cdot 7H_2O$ 3.100; $CaCl_2$ 0.600; H_3BO_4 0.1000; $Na_2MoO_4 \cdot 2H_2O$ 0.100; $ZnSO_4 \cdot 7H_2O$ 3.200; $CoCl_2 \cdot H2O$ 0.600; $CuCl_2 \cdot 2H_2O$ 2.200; $MnCl_2 \cdot 4H2O$ 2.500; $NiCl_2 \cdot 6H_2O$ 0.500; SeO_2 0.100) at two pH values, 5.0 (46 days for casein and 72 for gelatine) and 7.0 (74 days for casein and 91 days for gelatine). Further information can be found in Bevilacqua et al. (2021a, 2021b).

The operation was monitored via the measurement of the COD, the VFA and the Total Ammonia Nitrogen (TAN) three times per week, while the total and suspended solids were measured once a week. Conventional physicochemical parameters were determined according to Standard Methods (APHA, 2017). VFA were measured through gas chromatography (GLEFG1 method) with an HP 1100 equipped with an IR HP1047A detector. The column used was an AMINEX HPX-87H (300 7.8 mm) using H_2SO_4 (5 mM) as an isocratic eluent. The detection limits were for acetic and propionic acids: 600 mg/L; for iso and n- butyric acid: 800 mg/L, for iso and n-valeric acid:1000 mg/L and for iso and n-caproic: 1100 mg/L. More information on analytical methods can be found in Bevilacqua et al. (2020).

Microbial community analysis

To characterize the microbiome in each operational condition, three biomass samples were taken once the reactors had reached steady state. Steady-state periods were determined as those where the variability of the VFA molar fractions was lower than 15%. Genomic DNA from each sample was extracted using the Nucleospin™ microbial DNA extraction kit (Macherey-Nagel™) following the instructions of the manufacturer. The extracted DNA was quantified by fluorometry (InvitroGen™ Qubit™ fluorometer) and spectrophotometry (Thermo Scientific™ Nanodrop 2000C spectrophotometer).

The V3V4 hypervariable region of 16S rRNA gene was amplified and sequenced using Illumina MiSeq Platform (Unidad de Genómica, Parque Científico de Madrid). These sequence data have been submitted to the GenBank databases under accession number PRJNA1034092. The bioinformatic analysis was carried out using CLC Genomics Workbench software (QIAGEN, version 21.0.3). Raw sequences were filtered to remove low-quality reads and then clustered into operational taxonomic units (OTUs) at 97% cut-off for sequence similarity. The taxonomic classification was carried out against the version 4.8.1 MIDAS taxonomy reference (Dueholm et al., 2022). OTUs with 1% abundance or lower were not considered for further analysis. Principal Coordinate Analysis (PCoA, Bray-Curtis distance matrices) was performed to investigate changes in microbial community structure and the Shannon diversity index (*H*) was calculated to assess the effect of operational parameters on community diversity.

RESULTS AND DISCUSSION

Reactors performance

During the operation without micronutrients' supplementation (Figure 1), the acidification degree for casein was higher than for gelatine, regardless of the pH value. However, substrate conversion decreased with acidic pH values for both proteins (<35%). The effect of alkaline conditions varied with the substrate: for casein, the acidification degree was similar at neutral and alkaline conditions, while gelatine acidification was negatively affected by alkaline pH (from 40% to <30%).

Acidic pH values fostered the production of more reduced VFAs regardless of the protein fermented (Figure 1), indicating that there is an influence of this parameter on the VFA selectivity. However, this influence depends on the protein composition since the trend was more evident in the casein reactor. In this reactor, acidic conditions resulted in the production of iso-caproic acid and an increase in the production of n-butyric acid and both forms of valeric acid to the detriment of acetic acid (Bevilacqua et al., 2021a).

During the operation with micronutrients' supplementation (Figure 2A,B), no remarkable effects were observed at acidic values. However, their supplementation increased the acidification degree of both proteins at neutral pH. Again, the more relevant changes in terms of VFA selectivity were observed in the casein reactor, with an increase in the production of propionic acid and both forms of valeric acid, and the detection of n-caproic acid production (Bevilacqua et al., 2021b).

Microbial community analysis

A total number of 2108 operational taxonomic units (OTUs) were identified in this study (Tables S1 and S2).

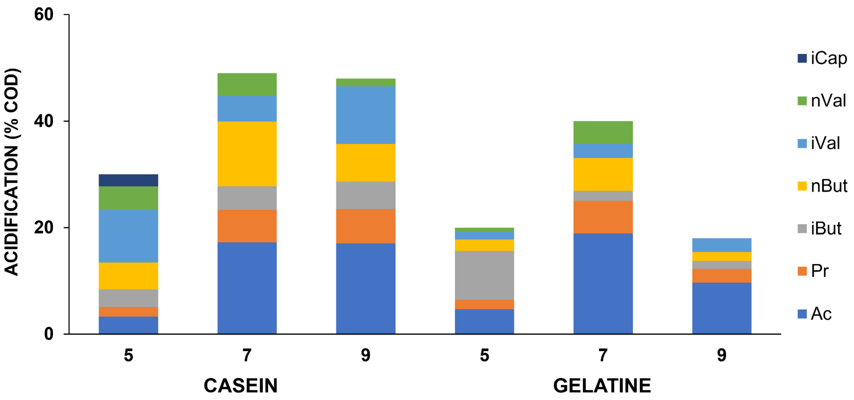

FIGURE 1 Acidification degree and VFA spectra of casein and gelatine reactors at different pH values without micronutrients' supplementation (Ac, acetic acid; iBut, iso-butyric acid; iCap, iso-caproic acid; iVal, iso-valeric acid; nBut, n-butyric acid; nVal, n-valeric acid; Pr, propionic acid).

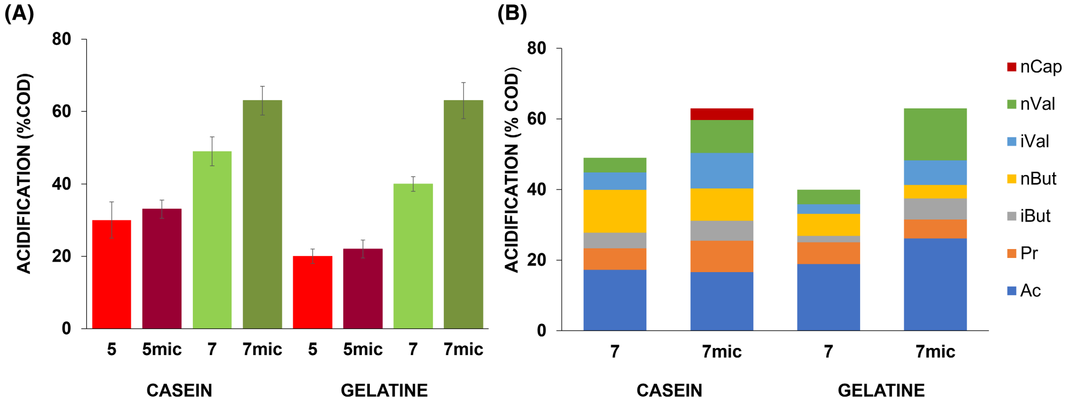

FIGURE 2 Acidification degree (A) of casein and gelatine at different pH values (red: pH 5; green: pH 7; mic states for the reactors operated with micronutrients' addition) and VFA spectra at pH 7 (B) without and with micronutrients' supplementation (Ac, acetic acid; iBut, iso-butyric acid; iVal, iso-valeric acid; nBut, n-butyric acid; nCap, n-caproic acid; nVal, n-valeric acid; Pr, propionic acid; mic states for the reactors operated with micronutrients' addition).

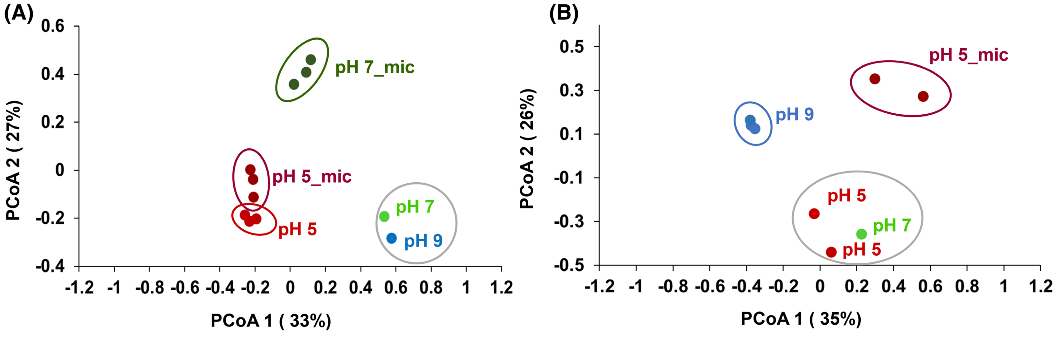

FIGURE 3 Principal component analysis (PCoA) of casein (A) and gelatine (B), showing the differences on the community composition related to the three parameters studied. Points represent each sample and are coloured according to the operational conditions: pH 5 without micronutrients (light red), pH 5 with micronutrients (red), pH 9 (blue), pH 7 without micronutrients (light green) and pH 7 with micronutrients (green).

However, some of the samples were discarded from further analysis because they did not meet the quality requirements: in casein reactor, two points of pH 7.0 and 9.0 without micronutrients' addition; in the gelatine reactor, two points of pH 7.0, one of pH 5.0 without micronutrients' addition and one of pH 5.0 with micronutrients' addition, as well as the samples of the operation at pH 7.0 with micronutrients' addition. More detailed information about the samples used for the analysis can be found in Supporting Information.

Principal Coordinate Analysis (PCoA) showed that microbial community structure was affected by all the studied parameters. In the case of the casein reactor (Figure 3A), community structure differed depending on micronutrients' addition and pH, forming four well-defined clusters. Without micronutrients' addition, community structure diverged between pH 7.0 and 5.0, but not between pH 7.0 and 9.0. The addition of micronutrients affected the structure of the community at pH 7.0, but not at pH 5.0. These results are in concordance with the macroscopic behaviour: the acidification degree without micronutrients barely varied from pH 7.0 to 9.0 (Figure 1), while their supplementation increased casein conversion only at pH 7.0 (Figure 2).

The community structure varied for gelatine as well (Figure 3B), albeit following a different pattern. In this case, only three clusters are evidenced, with the micronutrient's addition affecting microbial structure at pH 5.0, and the community structure at pH 9.0 was different than at pH 7.0 in the absence of micronutrients.

These results confirm pH as a key factor affecting bacterial populations present in anaerobic fermentation processes of complex substrates that contain proteins, such as food waste (Lv et al., 2022), sludge (Liu et al., 2012) or semi-synthetic industrial wastewater (Atasoy et al., 2020). Micronutrients' addition also affected the community structure in both proteins, which is in concordance with previous literature (Pasalari et al., 2020). Interestingly, the changes in community structure are different for each protein, which are likely related to the different aminoacidic profile of the proteins, as showed by Shen et al. (2017) and Kim et al. (2020).

Influence of pH without micronutrients' addition on microbial composition

Firmicutes, Proteobacteria and Bacteroidetes were the dominant phyla in all the samples, as reported in most literature studies (Liu et al., 2012; Xu et al., 2021). However, their relative abundance, and consequently the alpha diversity, varied with the operational parameters. This influence was even more evidenced when lower taxonomic levels were studied.

In casein reactor (Figure 4), the highest Shannon diversity index was obtained at pH 5.0 (4.44 ± 0.60), while similar values were achieved at pH 7.0 and 9.0 (2.16 and 1.92, respectively). Previous studies only have reported a decrease in diversity at extreme pH values (below 5 and above 10) (Liu et al., 2012). Also, the dominant order changed with pH. At neutral pH, Bacteroidales was the most abundant order, while Peptostreptococcales-Tissierales was the most abundant at pH 9.0 (Figure 4). Acidic values increased the relative abundance of Clostridiales and Enterobacterales, both comprising almost 80% of the microbial community in the last day of operation studied.

Therefore, pH highly influenced the composition of the microbial community, as seen in literature (Jankowska et al., 2017; Ma et al., 2019). Some microorganisms could be linked to specific pH conditions. For instance, members of Peptostreptococcales-Tissierales order are strongly correlated with alkaline pH values (Ma et al., 2019). *Bacteroides* was the most abundant genus found within Bacteroidales order and was specifically associated with the acidification of proteinaceous substrates by Shen et al. (2017). It is remarkable that this order was only abundant at neutral pH, which suggests that acidic or alkaline values does not favour its growth when fermenting proteins. In addition, the microbial composition could partially explain the macroscopic results. According to literature (Ma et al., 2019), the higher production of acetic acid at pH 9.0 could be linked to the greater abundance of microorganisms belonging to Peptostreptococcales-Tissierales order. The VFA spectra obtained for pH 5.0 are highly related to the presence of Clostridiales, since members of *Clostridium* genus are always mentioned as responsible for iso-caproic, iso and n-valeric acids through different processes. Britz and Wilkinson (1982) described the production of iso-caproic acid and iso-valeric acid from leucine by different species within this genus. Nevertheless, they studied a different pH range (between 7 and 9), but they indicate that some strains could also work at pH 5. Thus, the production of n-valeric acid is caused by chain elongation processes. The accepted mechanism for VFA chain elongation is reverse β-oxidation. This process consists of cyclic reactions that couple to the carboxylic acid chain two carbon atoms from acetyl-CoA molecules consuming reducing power. Both the acetyl-CoA and the reducing power are provided by the conversion of electron donor compounds, such as lactate or ethanol (de Cavalcante et al., 2017; den Boer et al., 2016). Bevilacqua et al. (2022) suggest that chain elongation might occur by using acetic or propionic acid. For n-valeric acid formation, different amino acids could supply reducing power and propionyl-CoA with acetic acid, or acetil-CoA for with propionic acid. Since members of Clostridia class are well-known for being involved in these processes (Angenent et al., 2016; Han et al., 2018), the presence and high relative abundance of Clostridiales could be responsible for this route.

The diversity of the community of gelatine reactor was affected by pH as well, especially when acidic conditions were used. Similarly to casein reactor, the highest Shannon index was observed at this pH (3.57 ± 0.42), but in this case, significant differences

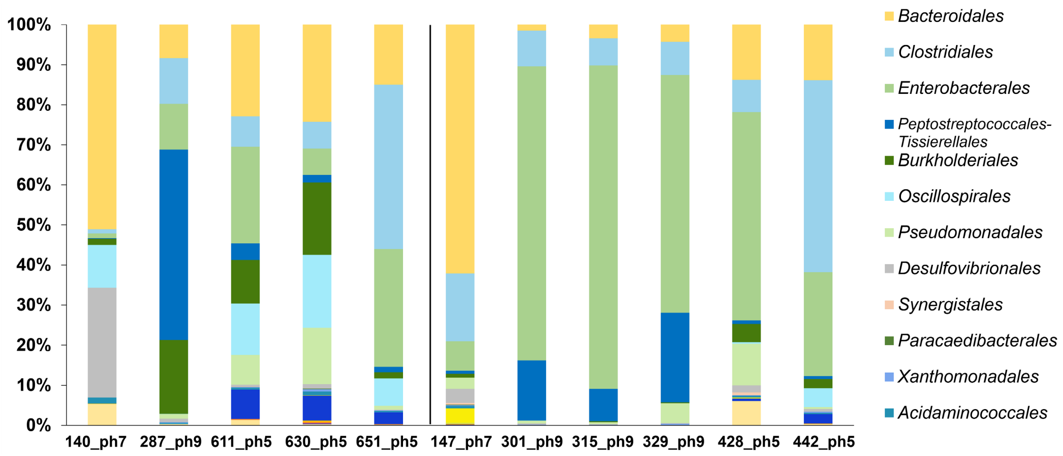

FIGURE 4 Bacterial community composition at order level for casein (left) and gelatine (right) reactors at the different pH values without micronutrients' addition. Only OTUS with total abundance higher than 1% are considered. Order abundances are coloured according to the phyla they belong to: Firmicutes (blue), Proteobacteria (green), Bacteroidota (yellow), Synergistetes, (light pink), Desulfobacterota (grey). Sample names refer to the day the sample was taken and the pH value.

were observed between pH 9.0 (2.60 ± 0.26) and pH 7.0 (3.10). The most abundant order shifted from Bacteroidales at neutral pH to Enterobacterales at pH 9.0. Most Enterobacterales present in the samples belonged to the Aeromonadaceae family, which is related to VFA production and alkaline pH values (Atasoy et al., 2020). At acidic values, a clear dominance of Clostridiales and Enterobacterales was observed as in the casein reactor, indicating that these orders are the most resistant to acidic conditions.

This convergence of communities at pH 5.0 did not correspond to a similar reactor performance, which could be attributed to the different aminoacidic profiles. Gelatine is mainly composed of glycine, proline and alanine, while casein presents a wider range of amino acids susceptible to degradation through different pathways. Kim et al. (2020) discussed the role of different microorganisms in gelatine and gluten degradation focusing on their aminoacidic profile. The most abundant genera found during gelatine degradation were Hathewaya, *Clostridium*, *Klebsiella* and *Peptoclostridium*. *Klebsiella aerogenes* has a proline oxidase that is repressed in the presence of carbohydrates but could be responsible for the degradation of this amino acid. Moreover, this genus can degrade all amino acids except three-branched amino acids, which could explain the residual protein in the gluten experiments.

Influence of micronutrients on microbial composition

Even though micronutrients did not show a clear influence on macroscopic behaviour at pH 5.0 (Figure 2), this tendency did not agree with the results obtained from the microbial community analysis (Figure 3). The diversity of the community did not change for the casein reactor, as reflected by the Shannon diversity index (4.45 ± 0.60 without micronutrients in contrast to 4.40 ± 0.19 with micronutrients' addition) while it clearly diminished for gelatine (3.57 ± 0.42 in contrast to 2.73 ± 0.18).

The microbial community composition of the casein reactor was relatively similar without and with micronutrients' addition (Figure 5), while the addition of micronutrients in the gelatine reactor promoted the substitution of Clostridiales by Oscillospirales as the predominant order within Firmicutes phylum, as well as an increase in Bacteroidales relative abundance (Figure 6). Conversely, for the casein reactor at pH 7.0 (Figure 7), the addition of micronutrients resulted in diversification of Firmicutes phylum, with a significant increase in Clostridiales order relative abundance, which is in agreement with the increase in Shannon diversity index (from 2.16 to 4.09 ± 0.11).

Once again, these results are in line with the macroscopic reactors' performance. Micronutrients act as cofactors of several enzymes, which can affect the enzymatic pathways and the production of certain acids. Since pyruvate is a reaction intermediate for several amino acids, cofactors linked to an increase in VFA production from glucose could enhance the process in the case of protein fermentation. For example, Cu and Zn have been reported to enhance n-butyric acid production from glucose (Zheng & Yu, 2004). Moreover, selenium is required for the production of glycine reductase, an essential enzyme for the conversion of this amino acid to acetic acid, and of Co^{2+} and $^{Ni2+}$ act as cofactors of biotin-methylmalonyl CoA carboxytransferase, responsible for propionic acid production (Dahiya et al., 2020). Thus, the enzymes isobutyryl-CoA mutase and isovaleryl-CoA mutase, involved in the interconversion between

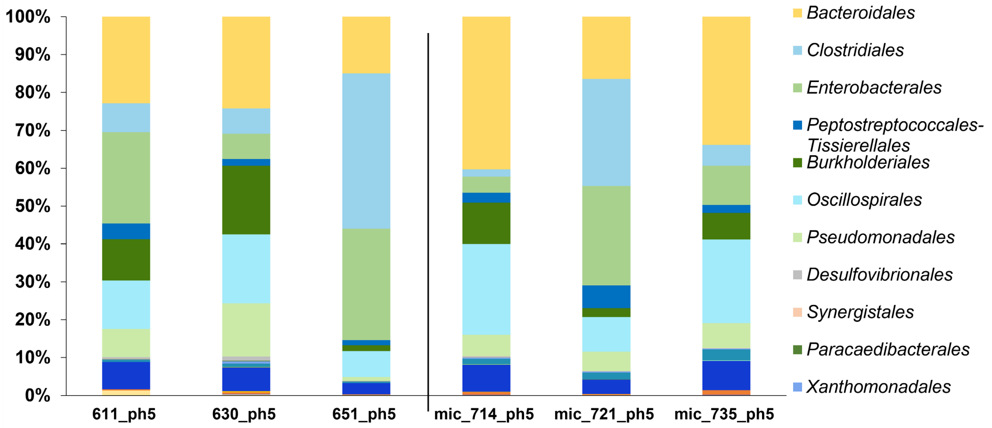

FIGURE 5 Bacterial community composition at order level for casein reactor at pH 5, without (left) and with micronutrients (right). Only OTUS with total abundance higher than 1% are considered. Order abundances are coloured according to the phyla they belong to: Firmicutes (blue), Proteobacteria (green), Bacteroidota (yellow), Synergistetes, (light pink), Desulfobacterota (grey). Sample names refer to the day the sample was taken and the pH value; mic states for the reactors operated with micronutrients' addition.

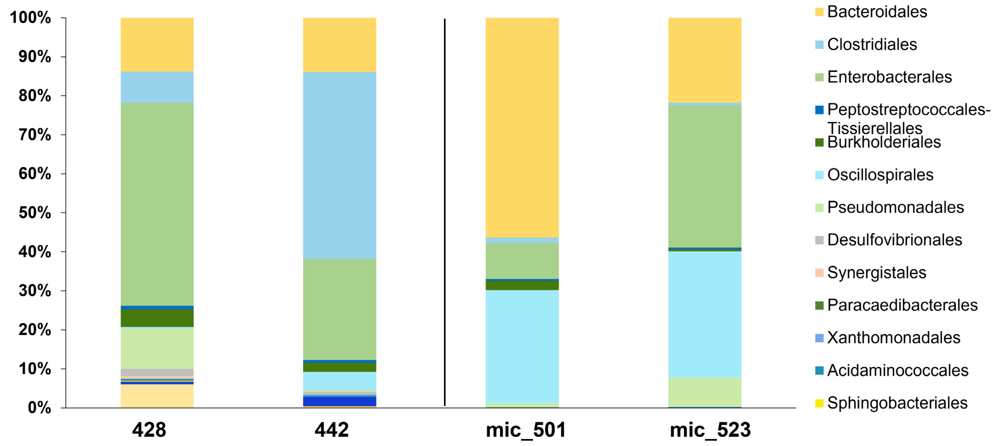

FIGURE 6 Bacterial community composition at order level for gelatine reactor at pH 5, without (left) and with micronutrients (right). Only OTUS with total abundance higher than 1% are considered. Order abundances are coloured according to the phyla they belong to: Firmicutes (blue), Proteobacteria (green), Bacteroidota (yellow), Synergistetes, (light pink), Desulfobacterota (grey). Sample names refer to the day the sample was taken and the pH value; mic states for the reactors operated with micronutrients' addition.

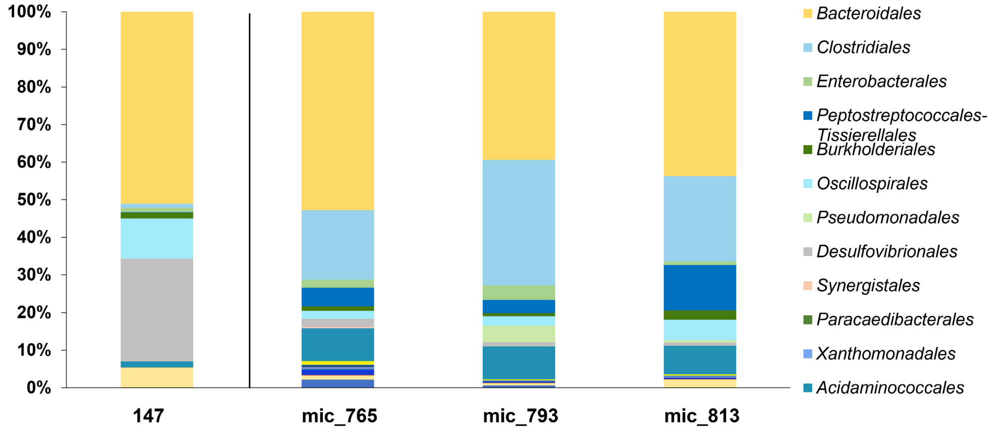

FIGURE 7 Bacterial community composition at order level for casein reactor at pH 7, without (left) and with micronutrients (right). Only OTUS with total abundance higher than 1% are considered. Order abundances are coloured according to the phyla they belong to: Firmicutes (blue), Proteobacteria (green), Bacteroidota (yellow), Synergistetes, (light pink), Desulfobacterota (grey). Sample names refer to the day the sample was taken and the pH value; mic states for the reactors operated with micronutrients' addition.

iso and n-acids, depend on Co presence (Cracan & Banerjee, 2012). Further information can be found in Bevilacqua et al. (2021b). Lastly, the production of n-caproic acid in this reactor could be linked to chain elongation processes with the addition of acetyl-CoA to acetic and, subsequently, to n-butyric acid. The microbial community of the casein reactor operated at pH 7.0 with micronutrients' addition showed a high relative abundance of Clostridiales. These results support the idea that members of this order are responsible for chain elongation processes in these reactors, as seen in literature (Angenent et al., 2016).

To sum up, this study explores the composition of the microbial communities present in anaerobic fermentation reactors and their response to changes in operational conditions. In addition, it is centred on the fermentation of proteins, a substrate for which little information is available and represents an important fraction of many industrial wastes. The conditions applied (neutral, alkaline and pH, micronutrients' addition) are known for being relevant and easily controllable parameters. In this context, the presence or absence of certain microbial populations could be used as an indicator of reactor performance. Furthermore, knowing which microorganisms are favoured by the conditions employed would allow directing production towards products of interest, such as applying acidic conditions or adding micronutrients to obtain caproic or valeric acids.

CONCLUSIONS

This work described how three different operational parameters (pH, protein aminoacidic profile and micronutrients' addition) affect microbial communities involved in protein fermentation. The microorganisms present in the reactor belong to Firmicutes, Proteobacteria and Bacteroidetes, but the dominant orders and the community structure vary with these parameters. pH plays a key role in microbial community composition and performance, and it also shapes the influence of the type of protein and micronutrients' addition. This study helps to understand the dynamics of the microbiome present in the anaerobic fermentation of proteins and its response to changes in operational parameters. Further research is needed to better comprehend these dynamics and the interactions between operational factors.

AUTHOR CONTRIBUTIONS

Carlota Vijande: Investigation; methodology; writing – original draft. **Riccardo Bevilacqua:** Methodology; writing – review and editing. **Sabela Balboa:** Conceptualization; supervision; writing – review and editing. **Marta Carballa:** Conceptualization; funding acquisition; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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