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Doctoral Program in Fundamental and Systems Biology



**Valorisation of olive mill solid waste through the
production of volatile fatty acids within a
biorefinery approach**

Department of Microbiology
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PhD Thesis, 2025

Just have a little patience
Take that

Programa de Doctorado en
Biología Fundamental y de Sistemas

Línea de Investigación
Microbiología Ambiental

**Valorisation of olive mill solid waste through the
production of volatile fatty acids within a
biorefinery approach**

**Valorización del alperujo mediante la producción
de ácidos grasos volátiles como base de
sistemas de biorrefinería**

Tesis Doctoral presentada por
Elena Jiménez Páez

Granada 2025

Valorisation of olive mill solid waste through the production of volatile fatty acids within a biorefinery approach

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Durante un apagón más que nacional

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Resumen

El objetivo de esta tesis es la valorización del alperujo mediante fermentación con cultivos mixtos para producir y recuperar ácidos grasos volátiles (AGVs). Los métodos actuales de gestión del alperujo no cumplen con los requisitos de sostenibilidad ambiental y económica que el sector del aceite de oliva exige. Por lo tanto, se ha evaluado la fermentación con cultivos mixtos del alperujo para maximizar la producción y acumulación de AGVs y su posterior recuperación utilizando un sistema de extracción electroquímica. En primer lugar, se determinó la influencia del pH operacional en la acumulación y en el perfil de AGVs obtenidos utilizando reactores alimentados por lotes. La hidrólisis del alperujo a pH neutro fue efectiva, alcanzando una biodegradación de hasta el 80%, pero no se observó acumulación de AGVs por su transformación en biogás. En condiciones a pH ácido y alcalino se obtuvieron valores de la relación sDQO/tDQO (DQO, demanda química de oxígeno) del 20% y el 50%, respectivamente, con concentraciones de AGV inferiores a $3 \text{ g} \cdot \text{L}^{-1}$. Por lo tanto, aunque la metanogénesis se inhibió con éxito mediante el control del pH, la hidrólisis y la generación de AGVs fueron limitadas. Posteriormente, se comparó en modo semicontinuo dos métodos de inhibición de la metanogénesis: uno utilizando 2-bromoetanosulfonato (BES) a pH neutro para verificar si la hidrólisis del alperujo se mantenía a pH 7, y otro operando a pH 5. Los resultados mostraron que la biodegradación anaeróbica del alperujo fue limitada a pH neutro, aunque la solubilización fue un 25% mayor que a pH 5. Esta limitación sería consecuencia de una inhibición por producto (acumulación de AGVs), cuya toxicidad es mayor a pH ácido que a pH neutro debido a una mayor disociación de los AGVs. La variación del tiempo de residencia hidráulico (TRH) en modo semicontinuo se estudió bajo condiciones de pH ácidas y alcalinas, pH 5 y 9, respectivamente. Esto permitió determinar si la hidrólisis del alperujo se vería favorecida por la adaptación de los microorganismos y, en consecuencia, obtener una mejor acumulación de AGVs. El aumento del TRH de 7 a 14 días mejoró la hidrólisis, alcanzando a un TRH de 14 días un porcentaje de alrededor del 32% y 53% para pH 5 y pH 9, respectivamente. A un TRH de 21 días, los AGV aumentaron hasta niveles que llevaron a la desestabilización del sistema (superando los $10 \text{ g DQO-AGV} \cdot \text{L}^{-1}$ en ambos pH evaluados). Esto sugiere que, entre los TRH evaluados, el TRH de 14 días puede considerarse el más adecuado para la actividad microbiana. El perfil de AGV en condiciones alcalinas (pH 9) fue menos diverso que en condiciones ácidas, siendo alrededor del 80% ácido acético. Esto podría estar relacionado con las condiciones específicas generadas a pH 9, que llevaron a una significativa presión selectiva, resultando en la selección y enriquecimiento destacado de la especie *Virgibacillus halodenitrificans*. Se ha descrito que esta especie tiene una vía para la conversión directa de azúcares simples en ácido acético. Por otro lado, a pH 5, el perfil de AGVs fue muy diverso, obteniéndose al final del tiempo experimental una composición de: 41% de ácido acético, 32% de ácido propiónico, 19% de ácido butírico y 8% de ácido valérico. Esto podría estar relacionado con una mayor diversidad y una menor dominancia de microorganismos con respecto al pH 9. Debido al impacto de la inhibición por producto (acumulación de AGV), se desarrolló un sistema de electrodialisis para recuperar los AGVs generados. La electrodialisis recuperó los AGVs de un fermento de alperujo en una matriz limpia, con una eficiencia de alrededor del

60%. Al comparar esta recuperación con una mezcla sintética de AGVs, se pudo concluir que el efecto matriz tuvo un impacto insignificante en los porcentajes de recuperación de AGVs, aunque desaceleró ligeramente el proceso. Finalmente, debido al largo tiempo requerido en el análisis de AGV individuales mediante cromatografía de gases, se evaluó la tecnología de espectroscopia de infrarrojo cercano (NIR) para una detección más rápida y sencilla de estos compuestos. Los resultados obtenidos se utilizaron para construir modelos de predicción de regresión de mínimos cuadrados parciales (PLSR) adecuados para una matriz compleja como los efluentes fermentados de alperujo. Sin embargo, el uso del NIR para una rápida determinación de los AGVs presentó dificultades en la cuantificación individual de los AGVs. Esto es debido a que las longitudes de onda de los AGVs fueron mayormente iguales, y estas bandas de absorción superpuestas pueden causar interferencia, pero dando resultados más precisos para la concentración total de AGVs.

Abstract

The objective of this thesis is the valorisation of olive mill solid waste (OMSW) by mixed culture fermentation to produce and recover volatile fatty acids (VFAs). OMSW current management methods do not match the environmental and economical sustainability requirements of the olive oil sector. Mixed culture fermentation of OMSW has been evaluated at different conditions to maximize the production and accumulation of VFAs and their subsequent recovery using an electrochemical extraction system. Firstly, the influence of operational pH on VFA accumulation and profile was determined using fed-batch reactors. The OMSW hydrolysis at neutral pH was very effective, reaching a biodegradation of up to 80%, but no accumulation of VFAs was observed due to their bioconversion into biogas. Meanwhile, acidic and alkaline pH conditions presented values of sCOD/tCOD ratio (COD, chemical oxygen demand) of 20% and 50%, respectively, and VFA concentrations lower than $3 \text{ g} \cdot \text{L}^{-1}$. Thus, although the methanogenesis was successfully inhibited by pH control, hydrolysis and VFAs generation were limited. Subsequently, two methods of methanogenesis inhibition, one using 2-bromoethanesulfonate (BES) at neutral pH to test whether OMSW hydrolysis is maintained at pH 7 and another one operating at pH 5, were comparatively tested in semi-continuous mode. The results showed that, the anaerobic biodegradation of the OMSW was limited at the neutral operational pH with BES, although the solubilisation was 25% higher than at pH 5. This observed limitation is most lightly due to by product inhibition (VFA accumulation), whose toxicity was higher at acidic pH than a neutral pH due to a higher dissociation of the VFA. Variation of the hydraulic retention time (HRT) in semi-continuous mode was studied under acidic and alkaline pH conditions, i.e. pH 5 and pH 9, respectively. That allowed determine if the OMSW hydrolysis would be favoured by the adaptation of microorganisms and, consequently, improves the accumulation of VFAs. Prolonging the HRT from 7 to 14 days increased the hydrolysis, reaching a percentage around 32% and 53% for pH 5 and pH 9, respectively, at 14-days HRT. At 21-days HRT, VFAs increased up to levels that led to system destabilisation (exceeding $10 \text{ g COD-VFA} \cdot \text{L}^{-1}$ in both pH evaluated). This suggests that, among the evaluated HRT, 14-days HRT was the most adequate for microbial organic matter hydrolysis and VFA production. The VFA profile at alkaline condition (pH 9) was less diverse than at acidic conditions, i.e. around 80% acetic acid. This could be related with the specific conditions generated at pH 9, that led to a significant selective pressure, resulting in the selection and enrichment in *S. Virgibacillus_halodenitrificans* specie. This specie has been described to have a pathway for the direct conversion of simple sugars into acetic acid. At pH 5, the VFA profile was very diverse obtaining at the end of the experimental time a composition of: 41% acetic acid, 32% propionic acid, 19% butyric acid and 8% valeric acid. This could be related with higher diversity and lower dominance of microorganisms respect to pH 9. Because product inhibition (VFA accumulation) was still not mitigated, an electrodialysis system was developed to recover the generated VFAs. Electrodialysis recovered the VFA of the OMSW ferment to a clean matrix composed with an efficiency of around 60%. Comparing this recovery with the synthetic VFA mixture, it could be concluded that the matrix effect had a negligible impact on the recovery percentages of VFAs, although it slightly decelerated the recovery process. Finally, due to the long time required in the analysis

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of individual VFAs by gas chromatography, near infra-red spectroscopy (NIR) technology was evaluated for faster and easier detection of these compounds. The results obtained were used to build partial least squares regression (PLSR) prediction models suitable for a complex matrix such as OMSW ferments. However, NIR for fast VFA determination presented difficulties for the quantification of individual VFAs, as the wavelengths of the VFAs were mostly equal and this overlapping absorption bands may cause interference, but it is highly reliable for total VFA concentration.

GENERAL INTRODUCTION

I. Anaerobic fermentation of olive mill solid waste (OMSW): an option for biorefinery processes to obtain high value-added compounds

I.1. Olive oil production and by-product generation

Virgin olive oil is one of the main components of the Mediterranean diet. Its consumption is associated with healthy physiological effects attributable to its abundant polyphenols content. These compounds, which are natural antioxidants that not only contribute to the stability of the oil, but also have anti-inflammatory and anti-atherosclerotic properties (Pamiry et al., 2024; Rubio-Senent et al., 2017). Moreover, different studies have been related the consumption of virgin olive oil with a lower incidence of coronary heart disease and certain cancers (García et al., 2016). Consequently, its consumption has been extended worldwide, being produced in many countries with adequate Mediterranean climate (Figure I.1) and reaching 2.56 million tons of olive oil worldwide production in the 2023/2024 season (InternationalOliveCouncil, 2025). The main olive oil producers are Spain, Italy, Tunisia, Greece, Portugal, Morocco and Syria (Statista, 2024). Despite of the increment in the number of producing regions, Spain remains the largest olive oil producer worldwide, reaching a production of more than 854 thousands of tons of olive oil in the season 2023/2024 (InternationalOliveCouncil, 2025). This volume means around 56% of the European Union production and 33% of the world production. Andalusia is the Spanish region that concentrate most of this olive oil production, around 80%.

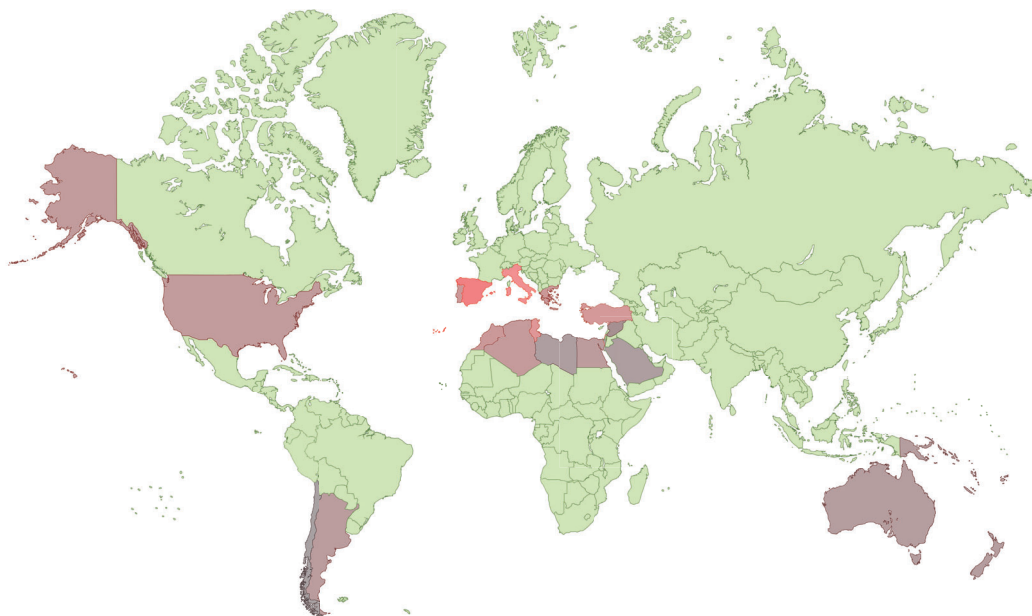


Figure I.1. Olive oil worldwide production in 2023/2024 season (created with biorender.com).

Currently, a standardized process called two-phase extraction system is widely used in the oil industrial production (Figure I.2). In fact, in Spain, 97% of olive oil produced comes from this two-phase extraction system (Serrano et al., 2021). Firstly, harvested olives are transported to the oil mill to begin the extract olive oil process. Upon arrival, the first step is cleaning and blowing the olives which removes all stems, leaves, twigs or any debris left with the olives. This is followed by a milling and a subsequent thermobeating process (Souilem et al., 2017). The milling process uses a mechanical grinder to disrupt the cellular structure of the olives, thereby facilitating the release of intracellular oil. Then, the paste generated in the grinder, is taken to the thermobeating process, where the slow and continuous kneading of the olive paste facilitate the cohesion of small oil droplets obtained during the crushing step. The ideal thermobeating temperature range has been agreed upon to be between 25°C and 30°C for 30 to 45 min (Plasquy et al., 2021). Finally, a two-outlet centrifugation is used without any water addition. This method delivers two types of streams (hence the name “two-phase extraction system”): the olive oil and a high-moisture solid waste known as olive mill solid waste (OMSW), olive pomace or *alperujo* (Dahdouh et al., 2023).

Indeed, OMSW is the most abundant byproduct generated by this process. The two-phase extraction system generates around 800 kg of OMSW per 200 kg of olive oil produced (Serrano et al., 2021). Given how Mediterranean countries produce great quantities of olive oil, disposal or use of OMSW is now considered a major challenge in these countries (Bermúdez-Oria et al., 2021; Doula et al., 2017). The challenge that implies the management of the OMSW derives not only from that the generated volume but also due to the physicochemical characteristics of this stream. OMSW is a lignocellulosic byproduct composed by vegetation water (60-70%), lignin (13–15%), cellulose and hemicellulose (18–20 %), olive oil retained in the pulp (2.5–3 %) or mineral solids (2.5%). Moreover, its organic components include sugars (3%), volatile fatty acids (C2-C7) (1%), polyalcohol (0.2%), proteins (1.5%), polyphenols (0.2-2%), and pigments (0.5%) (Rincón et al., 2008). Moreover, OMSW is characterised by acidic pH between 4.8-5.6 (Najla et al., 2022). Nowadays, most of the generated OMSW in olive oil mills is stored in open ponds. However, the uncontrolled discharge of this byproduct onto soils or into watercourses poses a range of important polluting hazards. These include soil contamination, inhibition of plant growth, leakage and contamination of underground aquifers, pollution of water sources, inhibition of natural self-purification processes, alteration of ecological equilibrium, and strong odour nuisance (Ochando-Pulido et al., 2024). Both phenolic compounds and volatile fatty acids (VFAs) content in OMSW are potential contributors to the phytotoxicity of this substrate (Kong et al., 2022; Pinho et al., 2017). Therefore, due to the properties of the OMSW and the possible impact on the environment, a proper management is required to avoid potential environmental pollution and, at the same time, to allow for the valorisation of OMSW into high value products.

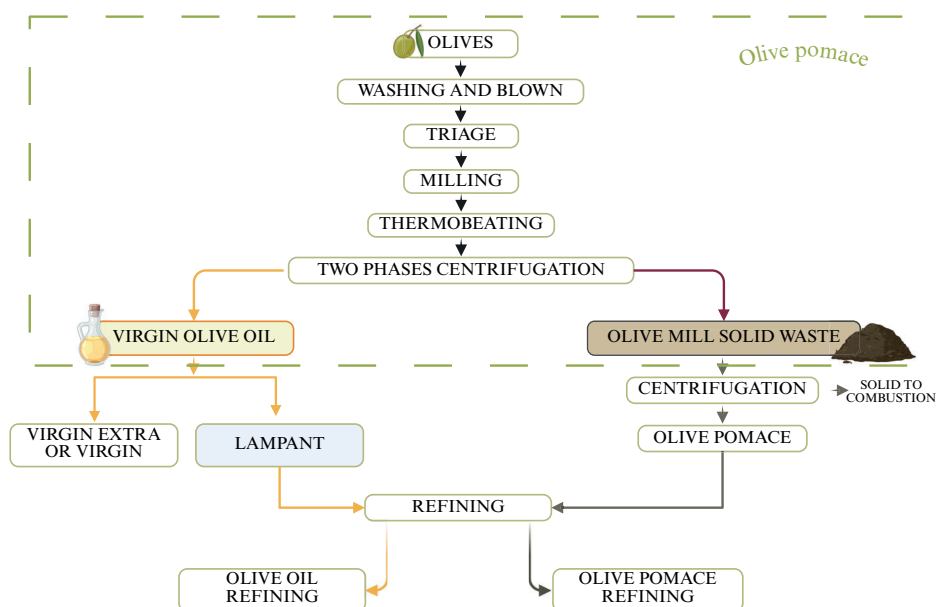


Figure I.2. Olive mill solid waste (OMSW) production from olive oil production system (created with biorender.com).

1.2. Brief description of the legislation and regulative framework

The European Green Deal, recently launched in 2019, aims to ensure the sustainability and independence of European Economy. For that, the European Green Deal highlights the necessity of favouring the market of “secondary materials”, based on the recovery and production of valuable components through the valorisation of substrates and effluents currently underused. Wastes and by-products from the agro-industrial sector has been pointed out as one of the strategic chains of value to be developed within the European Green Deal in the upcoming years.

At national level, the implementation of the above-mentioned European measures is coordinated through the Action Plan for the Implementation of the 2030 Agenda (“Reconstruir lo común: La implementación de la Agenda 2030 en España”) which establish plans and strategies that promote sustainability, circular economy and efficient use of resources. According to the latest national waste management law, Law 7/2022, concerning waste and contaminated soils for a circular economy, the responsibilities of waste producers are specified, and taxes are introduced on landfilling, incineration, and co-incineration. Furthermore, regarding bio-waste, the law emphasizes its valorisation through composting and anaerobic digestion, promoting the use of the products derived from these processes.

In the case of Andalusia, which is the main olive oil producer at world level, and, hence, the largest generator of OMSW, different strategies are being developed to improve the

sustainability of the agro-industrial sectors, with a special relevance of the olive oil sector, presenting their objectives in documents such as “Plan Integral de Residuos de Andalucía”, BOJA No. 66, of April 9, 2021 (Andalusia) (PIRec 2030) and “Estrategia Andaluza de Bioeconomía Circular”, BOJA No. 136, of July 18, 2016 (EABC).

1.3. Current management of OMSW

Currently, approximately 80% of OMSW is processed in pomace extraction plants to produce pomace oil (Figure I.3). This extraction process involves the use of organic solvents, most commonly hexane (García Martín et al., 2020). Prior to extraction, the moisture and volatile matter content of the OMSW must be reduced to 10%. This reduction is achieved through a drying phase, which constitutes the most energy-intensive step in the entire extraction process. The energy for this drying phase is typically derived from either natural gas or the combustion of the extracted pomace resulting from the pomace oil extraction process (Alonso-Fariñas et al., 2020). Consequently, OMSW has been considered a potential energy source via combustion (Serrano et al., 2021). However, this approach is not considered the most sustainable, primarily due to the use of organic solvents and the associated high carbon dioxide equivalent emissions (Ribeiro et al., 2020). To achieve the Sustainable Development Goals (SDGs) of the 2030 Agenda and the commitments of the European Green Deal, the olive oil sector must adopt measures that promote more sustainable practices, reduce its environmental impact, and optimize the use of resources. This adoption of new sustainable technologies can be also favoured by the access to Governmental aids from the European Union, designed as a chance for enhancing the competitiveness of the olive oil sector.

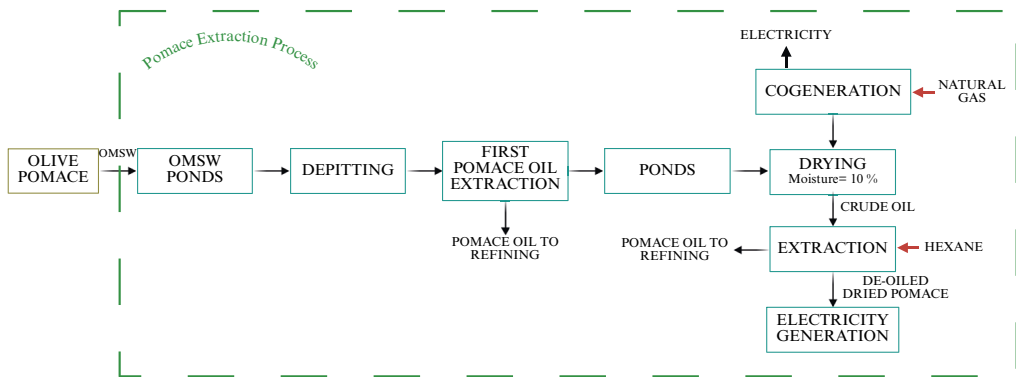


Figure I.3. Pomace extraction process to obtain pomace oil and cogeneration (created using biorender.com).

Another percentage of OMSW is being management by composting. This aerobic biological process involves the microbial degradation of organic matter, yielding a stabilized material suitable for use as a soil amendment in agriculture (Albuquerque et al., 2009). Composting is divided into two phases. The initial stage, decomposition, is characterized by microbial activity, primarily bacterial and fungal, progressing from a mesophilic phase with

initial microbial proliferation to a thermophilic phase. During the thermophilic phase, rapid organic matter degradation occurs, generating heat (60-70 °C), water vapor, and carbon dioxide (Villar et al., 2016). Subsequently, the maturation phase, where the waste stabilizes and matures, obtaining the final compost. Therefore, the temperature and microbial activity manage to sanitize the compost, eliminating potential pathogens (Villar et al., 2016). Occasionally, depending on the feedstock characteristics and processing scale, this process requires the addition of two stages of pre- and post-treatment. The adoption of OMSW composting as a waste management strategy is significantly constrained by two key factors: firstly, the extensive land area required for constructing composting windrows or piles due to the long time required for the process, which presents a critical challenge for small-scale olive oil production facilities (Noor et al., 2024); and secondly, the significant upfront capital investment necessary for infrastructure development and operational setup (Noor et al., 2024). As neither cogeneration nor composting offers an economically attractive and environmentally combined solution, alternative management methods have been proposed for OMSW where anaerobic digestion is currently gaining attention (Alonso-Fariñas et al., 2020).

1.4. Anaerobic digestion process

Anaerobic digestion (AD) presents a promising approach for solid waste treatment due to its effective waste stabilization and high energy recovery potential (Azaizeh et al., 2020). This widely studied and implemented technology facilitates organic waste valorisation through the generation of biogas as energy carrier and digestate as organic amendment. AD is a biological process in which organic matter, in the absence of oxygen and through the action of a group of specific microorganisms, is transformed into gaseous products or “biogas” (CH_4 , CO_2 , H_2 , H_2S , etc.) and digestate (Harirchi et al., 2022). AD is a complex process, due to the many microorganisms involved, such as bacteria, fungi and archaea, and to the complex nature of the concurrent biochemical reactions. AD is mainly composed by four interrelated stages (Figure 1.4A): hydrolysis, acidogenesis, acetogenesis and methanogenesis. During hydrolysis stage, bacterial and fungal consortia break down complex organic matter into soluble compounds such as carbohydrates, proteins and lipids. Subsequently, in acidogenesis stage, these products are transformed to short-chain carboxylic acids (volatile fatty acids) and alcohols bacteria consortia. Finally, during acetogenesis and methanogenesis, acetic acid is consumed or assimilates by archaea and converted into methane and carbon dioxide (Menzel et al., 2020). Nowadays, the prices of biogas from industrial waste ranges from 0.10 € to 0.48 € per cubic meter of methane (EuropeanBiogasAssociation, 2025).

AD is a well-established technology for different waste streams (agricultural, livestock, industrial, and urban waste), even though research is persevering to pursue new technological improvements and maximize its potential. The European Union is promoting biogas production and sustainable waste management through policy regulations. Notably, in January 2025, the governing council of the Andalusian Regional Government approved the Andalusian Biogas Alliance, demonstrating a commitment to the exploitation of renewable energy sources derived from organic waste. This initiative aims to optimize the utilization

of organic biomass resources through the strategic deployment of biogas facilities, thereby mitigating adverse environmental impacts associated with gaseous emissions, odour generation, and conventional waste management.

Previous research on AD of OMSW outlines some limitations. As previously described, it is a lignocellulosic feedstock, so different pretreatments are usually applied to improve the biodegradability such as hydrothermal (Fonseca et al., 2021; Lee et al., 2024), chemical, biological (Bahreini et al., 2020; Baig, 2020), mechanical (Heller et al., 2023; Lee et al., 2024) or alkaline (Lin et al., 2015), among other. Another significant limitation is the pH, as OMSW exhibits an inherently acidic pH, considerably deviating from the optimal pH range for anaerobic digestion. Consequently, substantial amounts of reagent are required to adjust the pH to a neutral value, giving a potential risk of VFA accumulation (Messineo et al., 2020). This risk of acidification can also be increased by the facility of generation of VFAs from the OMSW and the presence of inhibitors such as phenolic compounds that affect at archaea (Caroca et al., 2021), and therefore the transformation of biogas. For all these issues there are solutions, but they involve an additional cost, such as the implementation of a pretreatment step involves an additional cost of between 20-40% of the total process costs (Menzel et al., 2020); or the addition of reagents; even the oversizing of the plants to operate in co-digestion with a nitrogen-rich substrate, among others. This assumes that the interest of biogas production may not be compensated by the cost of biogas, and it is necessary to search other alternatives.

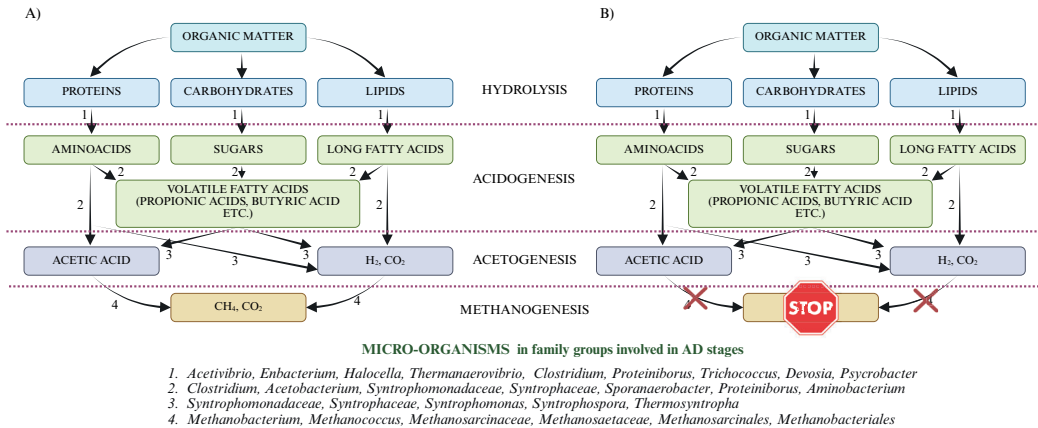


Figure I.4. A) Conventional Anaerobic digestion process. B) Anaerobic digestion process modification to inhibit methanogenesis stage. Adapted from Rodrigues et al. (2021). (Created using biorender.com).

1.5. Biorefinery concept

A biorefinery can be defined as several unitary technologies that are integrated in a multi-step system for the integral valorisation of a substrate, enabling the separation of different biomass components to produce high added-value products (Romero-García et al., 2014). Among others, it has been reported the successfully integration of different processes

including the extraction of antioxidant compounds such as hydroxytyrosol, the production of bioplastics, the generation of biogas through anaerobic digestion, bioethanol, production of hydrogen, etc. (Kourmentza et al., 2017; Romero-García et al., 2014; Serrano et al., 2017).

Originally, the first generation of biorefinery processes were based on relatively simple raw materials, such as corn, sugarcane, or cereal straw (Romero-García et al., 2014). Due to the relatively simple adaptation of these substrates to extraction processes, high yields were achieved in the production of biofuels such as bioethanol and biodiesel (Bezerra & Ragauskas, 2016; He et al., 2025). However, the price of the feedstocks limited the sustainability of the biorefinery approaches, as well as the competition in the use of these substrates with food uses. Moreover, some disadvantages have been previously reported, such as the competition, seasonal availability of crops, reduced soil fertility, production yields among others (Chandra et al., 2019). Second generation biorefinery processes were based on more complex lignocellulosic substrates, including forest or agricultural residues (He et al., 2025). This second generation of biorefineries reduces the costs associated with raw materials, as well as their independence from food crops. However, they may increase their costs due to the need for pre-treatments to increase the bioavailability of the substrate (Romero-García et al., 2014). The aim of pretreatment is to increase enzymatic hydrolysis and the yield of fermentable cellulose and hemicellulose sugars, but this represents up to one-fifth of the total cost of the system, which means searching for other technologies (Hossain et al., 2017). Due to the high cost in the second generation of biorefineries, new alternatives were sought in raw materials that involve biomass without complex and costly pretreatment, these would be the third generation biorefineries (Chandra et al., 2019). The third generation is based on the use of algae biomass to obtain high-value products such as amino acids, vitamins, biopigments, among others, in addition to electricity and other chemical products. (Bhatia et al., 2022). Despite this, although third-generation biorefineries offer great potential for the production of biofuels and other bioproducts without compromising the food production, it still presents significant technical-economic and environmental challenges. Therefore, new management processes could recover different compounds by combining with other processes for energy generation or waste stabilization to enhance the global economic and environmental sustainability of the biorefinery approach (Fermoso et al., 2018).

1.6. Shifting from biogas to volatile fatty acids: An alternative of a conventional AD

As alternative to biogas production, volatile fatty acids (VFAs) production by controlled fermentations with mixed anaerobic cultures has gained significant scientific and economic interest as a part of biorefinery processes (Figure I.4B). These versatile compounds, ranging from two to seven carbon atoms, are a key building blocks for the industry due to their role as basic molecules to obtain bioplastics, high added-value chemicals or the generation of biofuels (Annamalai et al., 2020; Fang et al., 2017). In particular, acetic acid and its derivatives find applications in different industrial processes. In food preservation, it is used as an antimicrobial agent; In the cosmetic industry, ethyl acetate is used as an aroma enhancer in different products; Likewise, in the textile industry, it is involved in different processing steps (Agnihotri et al., 2022; Bhatia & Yang, 2017; Budiman et al., 2016). Other VFAs such

as propionic, butyric or isovaleric acid are used in the food industry as preservatives and flavour enhancers (Agnihotri et al., 2022). Butyric acid, for instance, is also a precursor for the production of bio-based polymers and has potential applications in pharmaceuticals (Jiang et al., 2018). Currently reported data indicate that acetic acid is the carboxylic acid with the highest annual production, with a price of 890 USD per tonne, followed by propionic and butyric acid with a value of 2200 US\$ per tonne and 2550 US\$ per tonne, respectively (Chen et al., 2022). These values highlight the significant economic potential of VFA production from organic wastes.

As previously mentioned, although the VFA content of raw OMSW is low, these compounds are intermediate metabolites of the AD process and, therefore, could be obtained by fermentation by controlling the metabolic pathways (Figure 1.2B) (Fang et al., 2017). However, as it was above-mentioned, the high lignocellulosic content of the OMSW could struggle the acidogenic fermentation process since the lignin could limit accessibility of hydrolytic enzymes to the feedstock, thereby avoiding the bioconversion process into VFAs (Cavinato et al., 2017). Hence, for the complex organic substrates like OMSW, the hydrolysis stage often represents the rate-limiting step. Similarly to conventional anaerobic digestion, this limitation could be addressed by implementing pre-treatments, as previously described for AD. As an alternative, several authors have shown that as an alternative to the implementation of pretreatments, the control of different operational parameters such as pH, temperature, organic loading rate (OLR), hydraulic retention time (HRT), among others, can promote the hydrolysis of substrates and, thus, could enhance the VFA accumulation (Lv et al., 2022; Zhang et al., 2023). For example, pH is a crucial factor in the production, i.e. concentration and composition, of VFA. Methanogenic microorganisms, which are responsible for biogas production, are most active at neutral pH (around 7) (Niero et al., 2022). The operation out of a neutral pH range can rapidly inhibit the methanogenesis activity, avoiding the consumption of the VFA by the archaeal population. A more acidic pH might enhance the initial hydrolysis stage due to a higher activity of the hydrolytic bacteria at this pH and therefore favour the acidogenesis stage, thus improving the accumulation of VFAs. Furthermore, under alkaline conditions it can improve digestibility by partial breaking down organic matter (Sun et al., 2021). Temperature also influences VFA production. The most commonly used temperatures are psychrophilic (<25 °C), mesophilic (25-45 °C) and thermophilic (>45 °C). Within this temperature range, thermophilic favours hydrolysis and can lead to a higher amount of VFA in the system (Sun et al., 2021). However, the high energy cost associated with maintaining the thermophilic temperatures may not be compensated by the benefits of the VFA production. In contrast, mesophilic conditions, although they may result in lower total VFA accumulation, often offer a better yield of VFA per unit of waste treated, making them more attractive from an economic perspective (Sun et al., 2021). The use of moderate OLR values can improve VFA accumulation, but excessively high values of OLR can stop not only methane production but also, acidogenesis stage (Sun et al., 2021). Additionally, the selection of an appropriate HRT would allow for the growth of specific microbial populations, such as acidogenic bacteria over methanogenic archaea, enhancing the production and accumulation of VFAs (Aboudi et al., 2023). Due to the interrelation of these factors, predicting optimal values for each of them is challenging. Their combined

assessment, taking into account the specific characteristics of each, is crucial.

Therefore, the mixed culture fermentation of agro-industrial or food waste emerges as a promising biotechnological strategy, offering advantages in comparison to industrial fermentative processes with well-defined pure consortium. One of the primary advantages is the microbial diversity present in these substrates and the mixed culture used as an inoculum, in contrast to pure cultures used in the fermentation production of alcoholic beverages, dairy products, or biofuels (such as ethanol or butanol) (Formenti et al., 2014). This mixed culture implies a reduction in operational cost, as the necessity for the isolation, selection, and propagation of specific microbial strains, which are employed in current industrial fermentations, would be avoided. Moreover, pure strains are highly sensitive to variations in the operational conditions of the process, such as pH or temperature, needing sophisticated control and further increasing operational cost. Additionally, the natural adaptation of the microorganisms to the substrate and operational conditions offers greater robustness and tolerance to process variations (Chen et al., 2008).

Mixed culture fermentation of OMSW for VFA accumulation is a technology that is far to be developed for industrial implementation. It is necessary to understand the bottlenecks and challenges during the fermentation of OMSW, that might limit the accumulation of VFA. This would allow for greater insight and the development of well-targeted optimisation strategies.

1.7. VFAs recovery

Recovery of the produced VFA is as important as the mix culture fermentation (Atasoy et al., 2018). Once VFAs have been generated, their recovery and purification are still necessary to facilitate their subsequent use. Currently, the recovery of VFA from a mixing liquor is challenging from a technical and economic point of view. On the one hand, the individual separation of acids is rather difficult (Atasoy et al., 2018). While, on the other hand, the recovery of fermentation products, even in the case of fermentation processes with pure cultures, might account for 60% of the total cost of a biorefinery process and, therefore, it is necessary to search for new low-cost technologies (Bechthold et al., 2008). In addition to the recovery of VFAs for later use in the industry, it has been observed in different researchers that the accumulation of non-dissociated VFAs affects the metabolism of bacteria, affecting their growth and microbial production of VFAs (Jones et al., 2021a; Kelbert et al., 2024; Sukphun et al., 2021). Therefore, the recovery of VFAs could entail an improvement of the anaerobic fermentation process itself. Nowadays, different suitable separation methods for VFA recovery have been reported, such as precipitation, reactive extraction, resin adsorption, distillation, micro and nanofiltration, among others (Chen et al., 2022) (Table I.1). However, as it can be seen in Table I.1, the methods present different drawbacks that struggles their feasibility in the recovery of VFA.

Table I.1. Advantages and drawbacks of some different separation methods of VFAs. Adapted from Chen et al. (2022).

Process	Advantages	Drawbacks
Precipitation	Mature technology	
	No phase changes	Low selectivity
	Do not require chemical additives	
	Easy to be scaled up	
	Consumes lower energy and required lower space	High acid/alkaline consumption
	High removal of heavy metals;	
Solvent extraction	Low energy requirement	High consumption of agent
	High recovery yield	Toxicity of agent
Ion Exchange adsorption		Low selectivity for single carboxylic acids
	High affinity to carboxylic acids	High cost of resin
		High pretreatment demand
Pressure-driven membrane	Mature technology	Fouling
	Easy to operate and scale up	High scaling cost
	Lower energy consumes	Lower rates
Distillation	High product purity	
	Suitable for thermally sensitive molecule	
Forward osmosis	Lower fouling tendency	High energy consumption
	Higher fouling reversibility	
Micro-, ultra- and nano-filtration and reverse osmosis	Effective separation	Fouling/ short membrane lifetime/ decreasing transmembrane flux
	Easy to scale up	High membrane cost
	Easy operation	High operation cost
Pervaporation	High purity	
	Simultaneously concentration	
Electrodialysis (ED)	High yield	High capital costs
	High purity product	Clogging and energy loss

Among them, electrodialysis (ED) is a novel electrochemical system that is beginning to be evaluated for the VFA recovery (Pan et al., 2018; Scoma et al., 2016; Wang et al., 2022; Wei et al., 2018). ED is an abiotic process in which an electrical field is applied to induce the migration of ions across selective ion exchange membranes, resulting in the transfer of ionic compounds (Chalmers Brown et al., 2020). Despite the potential drawbacks such as high

capital cost, clogging or energy losses, the extraction with ED has also several advantages over other methods available nowadays. For example, the energy requirements are minimal, no required significant pretreatment, no chemical additives requiring post-treatment to remove them, and, the ability to adapt it to a continuous mode of operation easily, which would facilitate scaling up the process (Andersen et al., 2014; Kotoka et al., 2024).

Upon application of an electric current, a reduction reaction occurs at the cathode chamber, resulting in the release of hydroxyl ions (OH^-) and simultaneously, oxidation occurs in the anode chamber, resulting in the release of protons (H^+) (Figure I.5) (Al-Amshawee et al., 2020). Cations selectively migrate through the nearest cation-exchange membrane while being repelled by the anion exchange membrane (Al-Amshawee et al., 2020). Conversely, anions preferentially pass through the anion exchange membrane and are prevented by the cation-exchange membrane (Singh et al., 2024). This selective ion transport leads to a desalination effect in the dilute compartment, as salt ions are removed, and a corresponding concentration increase in the concentrate compartment due to ion accumulation (Figure I.5) (Singh et al., 2024). An electrolyte solution is pumped through the electrodes to provide conductivity, prevent membrane fouling and provide pH buffering for the concentrated VFA stream (El-Qelish et al., 2022).

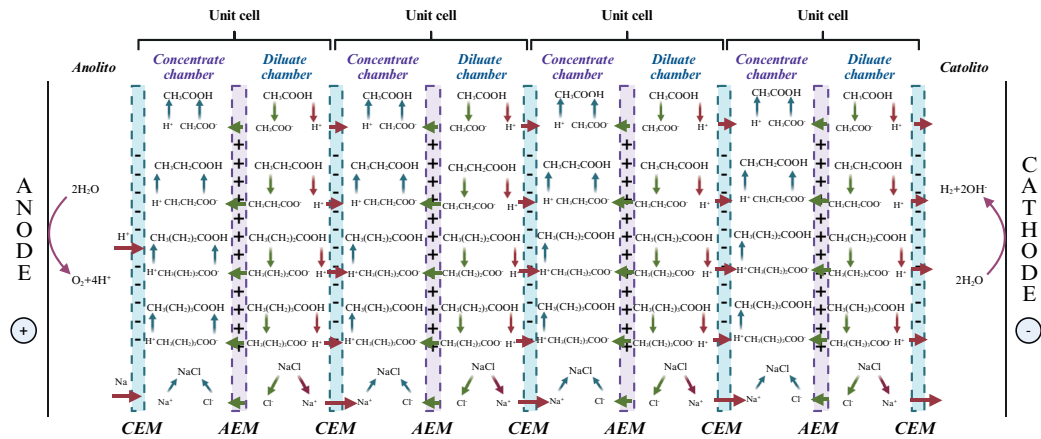


Figure I.5. Schematic representation of experimental set up employed in this research (created using biorender.com).

ED process has been mainly used for the recovery/removal of VFA, mostly in a sequential way, i.e. first the fermentation was carried out and then the treated ferment was introduced into the ED stack. Although ED has been mainly assessed for synthetic solutions (Andersen et al., 2014; Jones et al., 2015), different substrates have been also evaluated, such as grass pellet (Jones et al., 2021b; Jones et al., 2015), olive mill wastewater (OMW) (Scoma et al., 2016), municipal food waste (Jones et al., 2021a), and food waste (Kotoka et al., 2024), among others. ED has demonstrated significant success in removing VFAs. For example, Jones et al. (2015) applied ED sequentially to remove part of the VFAs from an anaerobic

digestate. First, they carried out a two-fermentation experiment using sucrose and grass pellets such a substrate to obtain two hydrogen fermentation broths. Subsequently, the digestates obtained in the reactors were introduced into an electrodialysis cell consisting of 20 pairs of unit cells. The ED cell used a titanium-coated Pt/Ir anode and a stainless-steel cathode applying a constant voltage of 18 V, achieving removal efficiencies of 94% and 96% for acetic acid and butyric acid, respectively. Scoma et al., (2016) applied ED sequentially to acidogenic fermentation operation of olive mill wastewater. They use an ED with three unitary cells applying a constant current density of $31 \text{ A} \cdot \text{m}^{-2}$ to achieving an 30-35% removal of VFA. At present, limited research evaluates the anaerobic fermentation process coupled to a continuous electrodialysis system. This coupling system aims to maximise VFA production while avoiding inhibition of the system by VFA product. Jones et al. (2021a) evaluated this continuous coupling system on a pilot scale with 100 litre reactor during 60 days fed with municipal food waste. They first operated for 35 days as a control phase where they observed a drastic decrease $<10 \text{ mg VFA} \cdot (\text{g VS} \cdot \text{d})^{-1}$ in the VFA production due to process inhibition by product. From day 35 onwards, by coupling the ED system, they observed an improvement in VFA production in situ up to $20 \text{ mg VFA} \cdot (\text{g VS} \cdot \text{d})^{-1}$, recovering in the concentrate practically the same concentration of VFA produced.

So, the application of this technology together with the fermentation of OMSW could allow obtaining concentrated VFA with a high degree of purity, making them available as basic molecules of biorefinery processes and, therefore, obtaining compounds of high interest. In this way, this hybrid management method might alleviate the accumulation of residues derived from olives processing. This alternative is very interesting due to its sustainable nature and its economic potential for the Andalusian olive oil sector.

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GENERAL OBJECTIVES

Objectives

The main objective of this thesis is the valorisation of olive mill solid waste (OMSW) by mixed culture fermentation to produce and recover volatile fatty acids (VFAs). OMSW current management methods do not match the environmental and economical sustainability requirements of the olive oil sector. Thus, mixed culture fermentation of OMSW will be evaluated at different conditions to maximize the production and accumulation of VFAs and their subsequent recovery using an electrochemical extraction system. An exhaustive pH control will be carried out during the fermentation to optimise the accumulation of VFAs by improving the hydrolysis of OMSW and, at the same time, avoiding the biodegradation of VFA into biogas. In addition, the electrochemical extraction system for the recovery of VFA is an innovative alternative that could mean a revolution in the field of the extraction and recovery of high added-value compounds, due to the low requirements of energy and solvents.

To achieve the general objective, the following specific objectives are proposed:

- Determination of the most suitable pH conditions to produce VFAs from OMSW. This determination will be based on the amount of VFAs accumulated in bioreactors operating in fed-batch mode. To optimize the process, it is proposed to carry out tests at different pH, i.e. from 4 to 10.
- Relation of the operational pH with the differences in the composition of the accumulated VFA.
- Determination of the operational parameters that maximise the accumulation of VFAs in a semi-continuous regime. During the semi-continuous regime process, operational parameters, such as hydraulic retention time, will be evaluated to optimise the VFA production without compromising the stability of the process.
- Evaluation of the synergistic dynamics between reactor conditions and microbial communities during the operating process.
- Optimisation the purification of VFAs using discontinuous electrochemical system.

Outline of this PhD Thesis

According to the previous defined objectives, this thesis aims to understand the bottlenecks and challenges during the fermentation of olive mill solid waste (OMSW) for accumulation and recovery of volatile fatty acids (VFAs). This thesis aims to provide a viable alternative for the valorisation of olive oil industry waste, still a significant challenge that struggle the sustainability of the olive sector.

In **Chapter 1**, the influence of operational pH on volatile fatty acids accumulation and profile was determined using fed-batch reactors. The OMSW hydrolysis at neutral pH was very effective, but no accumulation of VFAs was observed due to their bioconversion into biogas. Meanwhile, acidic and alkaline pH conditions limited the hydrolysis and, although the methanogenesis was successfully inhibited, VFAs generation was also limited. Based on the results of **Chapter 1**, **Chapter 2** compares two methods of methanogenesis inhibition,

one using 2-bromoethanesulfonate (BES) at neutral pH to test whether OMSW hydrolysis is maintained at pH 7 and another one operating at pH 5. In **Chapter 3** and **Chapter 4**, the hydraulic retention time was varied to determine if the OMSW hydrolysis is favoured by the adaptation of microorganisms and, consequently, improves the accumulation of VFAs. The main difference between the two chapters lays in the operational pH. **Chapter 3** was conducted at pH 5, pH of the raw OMSW, and, based on the results of **Chapter 1** and **Chapter 2**, might produce a complex VFA profile. In **Chapter 4**, the operation was forced to pH 9 since, based on the results of **Chapter 1**, it favours a high VFA productions, mainly acetic acid. In **Chapter 5** an electrodialysis system was developed for the recovery of the produced VFAs. The system was tested for both synthetic compounds and a fermented effluent obtained at pH 5 from **Chapter 3**. Finally, due to the long time required in the analysis of individual VFAs by gas chromatography, **Chapter 6** evaluated near infra-red spectroscopy (NIR) technology for faster and easier detection of these compounds. Samples of the VFAs obtained from **Chapter 3** and **Chapter 4** were used to build partial least squares regression (PLSR) prediction models suitable for a complex matrix such as the fermented effluents of OMSW.

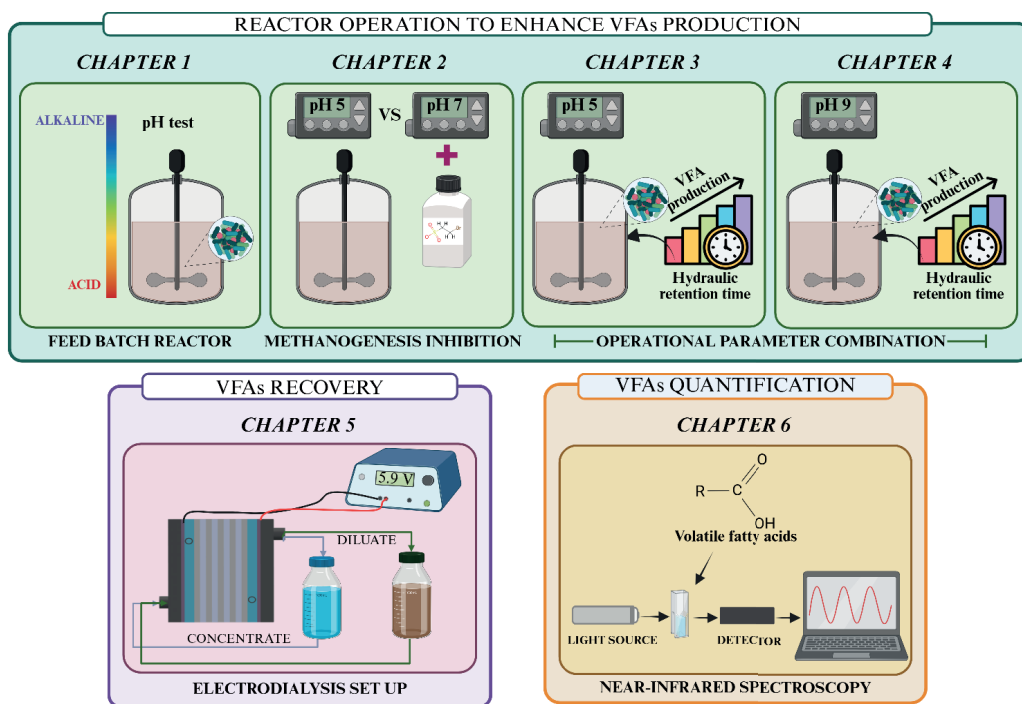


Figure 0.1. Summary outline of this PhD Thesis (created with www.biorender.com).

GENERAL MATERIALS AND METHODS

M.1. Olive mill solid waste and anaerobic inoculum collection

300 kg of olive mill solid waste (OMSW) were obtained from the "Oleícola El Tejar Ntra. Sra. de Araceli" pomace plant (coordinates in decimal degrees: 37.35809, -5.45481), located in Marchena (Seville), Spain. This volume of OMSW was carried out to ensure the availability of the same substrate throughout the entire thesis duration, avoiding a possible variability in the results due to variations in the used substrate. The collected OMSW was stored at -20 °C until further use, avoiding the degradation of the substrate.

The anaerobic inoculum used for the mixed culture fermentation experiments was always obtained from the same industrial anaerobic reactor at the "El Copero (EMASESA)" wastewater treatment plant (coordinates in decimal degrees: 37.31182, -5.98590), located in Seville, Spain. This wastewater treatment plant is designed with a capacity of 950,000 equivalent inhabitants (255.000 m³ per day).

M.2. Experimental set up

M.2.1. Fermentation reactor configuration

The capacity of the reactors was 2.0 L, with a working volume of 1.6 L (Pobel, Spain) and were initially inoculated with 10 g volatile solids per litre of fresh inoculum. The reactors were continuously stirred using a magnetic stirring system consisting in an IKA-RET basic and a magnet. The operational pH for each reactor was controlled with HP series proportional flow pumps (4-20 mA) (MYTHO technologies, Hunenberg, Switzerland) connected to a 46D - Series controller, sending signals to the pump when the pH fell below or above the pH thresholds, adding acid (HCl 3N) or base (NaOH 2N) for pH regulation. This signal is provided by an XS PRO-VP 120 pH electrode (Labprocess Distribuciones S.L, Barcelona, Spain) at each reactor. Alternatively, in Chapter 2, the regulation of pH was manually done by daily measurement of the pH (SensIONtm, Hach, Loveland, Colorado, USA) and the addition of NaOH (2N) or HCl (2N). The operation temperature (35 ± 2 °C) was maintained by means of jacketed reactors and a water bath Digiterm-TFT (J.P. Selecta, Barcelona, Spain).

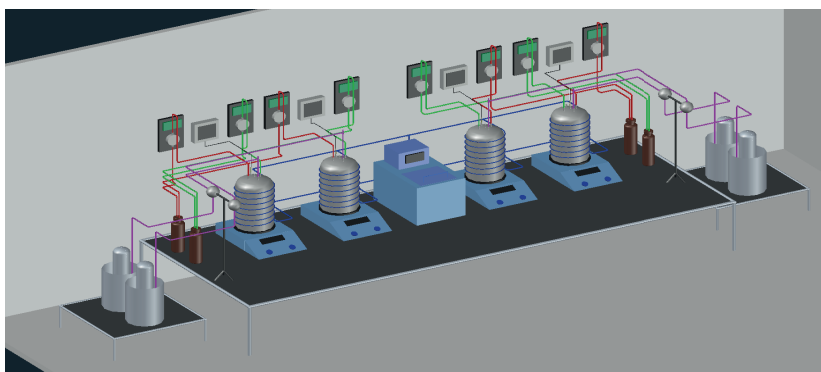


Figure M.1. Experimental system configuration. Own elaboration.

M.2.2. Electrodialysis configuration

A schematic representation of used the electrodialysis system (ED) is represented in Figure M.2. The ED was configured by 4 unit cells, thus 4 anion exchange membranes (AEMs) and 5 cation exchange membranes (CEMs), alternately disposed. The AEMs (RALEX® MEMBRANE AMHPES-MEGA-TEC, Czech Republic) and CEMs (RALEX® CMHPES-MEGA-TEC, Czech Republic) were commercial heterogeneous ion-exchange membranes. The active area of each membrane was 16 cm². Polypropylene woven spacers with 0.5 mm thick and 3 mm mesh size was used as turbulence promoters in the diluate and concentrate compartments of the ED system. Moreover, Titanium coated with iridium oxide electrode (DSE, provided by Inagasa S.A., Spain) and stainless-steel electrode (Tamesanz, Spain), were used as anode and cathode, respectively. Each diluate and concentrate compartments in ED stack had a working volume of 0.25 L. On the one hand, the diluate stream was composed of the volatile fatty acids mix solution. For the test of synthetic VFA mixture it was necessary the addition of NaCl to the dilute stream, as the conductivity provided by volatile fatty acids (VFAs) ($\sim 1 \text{ mS}\cdot\text{cm}^{-1}$) was lower in comparison to the OMSW ferment. On the other hand, the concentrate stream (i.e. draw solution) was composed of distilled water, NaCl (2.2 g·L⁻¹) and an electric conductivity of 4.45 mS·cm⁻¹ (similar to diluate stream). Finally, Na₂SO₄ 0.05 M solution was used for electrode rinse (anolyte/catholyte solution). The solutions were continuously recirculated from the corresponding reservoirs to the ED stack applying a constant flow of 40 mL·min⁻¹ (i.e. 10 mL·min⁻¹ per unit cell) and a constant voltage at room temperature. The applied voltage (RS3005D Digital Control DC, RS AMIDATA, Spain) was determined by the limiting current density (LCD), and was set at the 80% of the LCD to avoid damage in the ion-exchange membranes, as suggested by the provider.

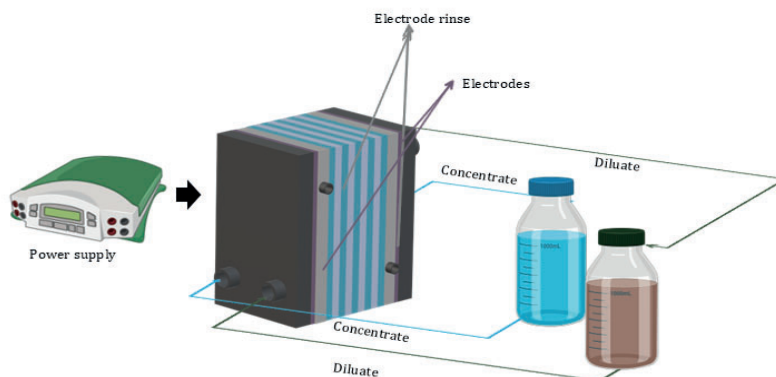


Figure M.2. Experimental electrodialysis system configuration. Own elaboration created with biorender.com.

M.3. Chemical analysis

The characterization of the anaerobic inoculum, the substrate and the routine samples taken from the reactors were determined applying per duplicate the following analytical procedures: pH, total solids, volatile solids, and total and soluble chemical oxygen demand (tCOD and sCOD), which were measured in the samples in accordance to the APHA Standard Methods (Baird et al., 2017).

M.3.1. Volatile fatty acids quantification by gas chromatograph analysis

To measure the concentration of VFAs (Acetic acid (C2), Propionic acid (C3), Iso-Butyric acid (i-C4), Butyric acid (n-C4), Iso-valeric acid and Valeric acid (n-C5)) in the samples, these were centrifuged and microfiltered with 0.45 μm nylon microfilters. The sample was prepared by adding an internal standard of 1500 $\text{mg}\cdot\text{L}^{-1}$ ethylbutyric acid with phosphoric acid (30% v/v). VFAs were determined in duplicate, injecting 0.5 μL of each sample in a gas chromatograph Shimadzu GC-2025 (Shimadzu Corporation, Kyoto, Japan) equipped. A Stabilwax-DA capillary column (RESTEK Corporation, Bellefonte, PA, USA) of 0.25 mm i.d. $\times$ 30 m and a flame ionization detector (FID) was used. The oven temperature gradually increases from 100 to 170 $^{\circ}\text{C}$ at 5 $^{\circ}\text{C}\cdot\text{min}^{-1}$. The carrier gas (nitrogen, hydrogen, air) flowed at 40 $\text{mL}\cdot\text{min}^{-1}$ at 504 kPa, with individual flow rates of 30 $\text{mL}\cdot\text{min}^{-1}$ for nitrogen, 40 $\text{mL}\cdot\text{min}^{-1}$ for hydrogen, and 400 $\text{mL}\cdot\text{min}^{-1}$ for air. The FID detector temperature was set to 250 $^{\circ}\text{C}$. Identification and quantification were performed using commercial standards by comparing retention times with the reference compound.

M.3.2. Total soluble phenols and individually phenolic compounds

Samples were previous centrifuged and microfiltered with 0.22 μm nylon microfilter. The total soluble phenolic compounds were determined by the Folin-Ciocalteu method (Singleton et al., 1965). Each sample were added with Na_2CO_3 (0.7 M) and Folin-Ciocalteu (0.2 M). After 15 min, the absorbance of the samples was recorded at 655 nm in a Bio-Rad iMark model microplate reader (Hercules, CA, USA). Finally, a calibration curve with a known gallic acid concentration was developed, and the results were expressed as milligrams of gallic acid equivalents per kilogram or per litre. The phenolic compound profile was analysed in a Hewlett-Packard 1100 series high performance liquid chromatography (HPLC), (Agilent, Barcelona, Spain), equipment coupled with a diode array detector and an Agilent 1100 automatic injector (20 μL). The chromatographic column was a Teknokroma Tracer Extrasil OSD2 with a 5 μm particle size and 25 $\times$ 0.46 mm internal diameter (Teknokroma, Barcelona, Spain). The mobile phases were Milli-Q water (Merck Millipore, Darmstadt, Germany) with an adjusted pH with 0.01% trifluoroacetic acid (A); and HPLC-grade acetonitrile (B). A 55-minute gradient elution method was used: 0-30 minutes 5% B, 30-45 minutes 25% B, 45-47 minutes 50% B, and 47-50 minutes 0% B, held until completing the run. Identification and quantification were performed using commercial standards by comparing retention times with the reference compound and recording UV spectra between 200 and 360 nm.

Results were expressed as milligrams of each phenolic compound per litre.

M.3.3. Biogas quantification and composition

In this thesis, two methods for measuring and assessing the biogas produced in the reactors were used.

On the one hand, in Chapters 1, 3 and 4, methane production was measured by liquid displacement using 0.5 L gasometers submerged in 2N NaOH solutions to remove the CO₂ from the generated gas. The biogas composition of the reactors (CH₄, CO₂, O₂, N₂, and H₂) was analysed in parallel from samples directly taken from the reactor using a gas chromatograph Shimadzu GC-2014. Each biogas sample was taken from the headspace of the reactors using 1 mL plastic syringes fitted with a special Mininert valve for Luer-Lock (Supelco) for gases. The gas chromatograph was equipped with a packed column ShinCarbon ST 100/120 (RESTEK) of 2 m × 1 mm of 1/16" OD Silco and a thermal conductivity detector (TCD) at 200 °C. The oven temperature gradually increased from 50 to 110 °C at a rate of 14 °C min⁻¹ and from 110 to 156 °C at 6.8 °C·min⁻¹, being 11.05 min, the total time of the method applied. Helium was used as carrier gas with a 10 mL·min⁻¹ flow.

On the other hand, in Chapter 2, the biogas produced of the reactors was collected in gas bags. The volume of accumulated biogas was subsequently quantified using a Ritter gasometer. Biogas concentration in the headspace samples were taken and stored using a vacuum tube. A 500 µL gaseous sample was manually injected into an HP 8860 gas chromatograph unit (Agilent, Shanghai, China) following a method modified from Araujo, et al. (2024). The equipment was equipped with a TCD detector (Agilent, Shanghai, China) and two columns, an HP-PLOT/Q (Agilent, USA), 30 m x 0.53 mm i.d. x 40 µm film thickness) and an HP-MOLESIEVE molecular sieve (Agilent, USA), 30 m x 0.53 mm i.d. x 50 µm film thickness). The initial temperature was held at 35 °C for 3 minutes, followed by a ramp to 65 °C with a ramp of 10 °C·min⁻¹, resulting in a total run time of 6 minutes. The carrier gas was N₂ at a flow rate of 10 mL·min⁻¹. The injector was operated in split mode (split ratio 2:1) and at 200 °C and pressure of 17.14 psi. The TCD detector was maintained at 250 °C with N₂ as the reference gas at a flow rate of 25 mL·min⁻¹. Compound identification and quantification was performed with retention time and peak areas after of the sample to those of increasing amounts of pure gases. The Gas chromatograph analyses were carried out at the Instrumental Technical Services of the Estación Experimental del Zaidín (SIC-EEZ), CSIC, Granada, Spain.

M.3.4. Acquisition of near infrared (NIR) spectra

The NIR spectra of the samples were acquired using a NIR portable spectrometer composed of a HL-2000-LL tungsten halogen light source (Ocean Optics B.V, Duiven, Netherlands), a cuvette holder and a Flame-NIR⁺ spectrophotometer (Ocean Optics B.V, Duiven, Netherlands) as a detector. The cuvette holder is linked by optic fibre connectors to the light source on one side and to the detector on the opposite side.

Prior to the acquisition of the spectra, the closed sample containers were placed in a thermostatic water bath at 35 °C for 30 min to bring the samples to a temperature similar

to that inside the reactors. The samples were then introduced into a 10 mm quartz cuvette and placed in the cuvette holder. Their NIR spectra from 959 to 1705 nm were acquired in transmittance mode (10 scans to average) and recorded using the OceanView software (Ocean Optics B.V, Duiven, Netherlands). The typical optical resolution of the Flame-NIR⁺ spectrophotometer is ~10.0 nm (FWHM, 25 μ m slit).

M.3.4.1. Spectral data pre-processing

M.3.4.1.1. Normalization

Spectral preprocessing techniques include a range of mathematical procedures for transforming spectral measurements before developing quantitative models (Dotto et al., 2018). Given the inherent dimensional inconsistencies among variables in many datasets, normalization becomes a critical preprocessing step in regression modelling. The concentration of VFA always differ significantly in their scales as a function of changes in pH. By normalizing the data, we ensured that all variables were on a comparable scale, allowing the model to assess each predictor equally. This step was crucial for preventing one variable from dominating due to its numeric range, ultimately enhancing the model's accuracy and fairness.

M.3.4.1.2. Spectral variable selection

After normalization, feature variable selection was conducted using competitive adaptive reweighted sampling (CARS), uninformative variable elimination (UVE), and successive projections algorithm (SPA). The application of these feature selection methods to the preprocessed spectral data significantly improved the performance of quantitative models, ensuring reliable and accurate predictions. The feature selection processes were executed using Python 3.8.1 in an Anaconda environment.

M.3.4.1.3. Spectral multivariate regression

The spectral multivariate regression involved developing partial least squares regression (PLSR) models for four types of acids. The dataset of different types of VFAs were randomly divided into calibration and validation sets with a ratio of 75% for calibration and 25% for validation. Subsequently, a grid search was performed to identify the optimal number of components (ranging from 1 to 20) and the random seed (ranging from 1 to 100) for the PLSR models. The grid search aimed to find the best model by evaluating different combinations of these parameters to maximize model performance (Mukarev & Walsh, 2012). Thus, the criteria for the grid search include maximizing the R² value, minimizing the RMSE, and ensuring that RMSEC was smaller than RMSEV to prevent underfitting, thereby identifying the optimal parameters. Following the model development, denormalization (Shin & Sanders, 2006) was applied to the predicted values to restore them to their original scale. While the normalized data was effective during the training process, the predicted outcomes were also in a normalized form. The denormalization process ensured that the

results were interpretable and directly comparable to the original measured VFA values, and the root mean square error (RMSE) was calculated using the denormalized data. The performance of the models was evaluated using the coefficient of determination (R^2), RMSE and the residual predictive deviation (RPD). R^2 indicates the proportion of variance in the dependent variable that is predictable from the independent variables, with values closer to 1 indicating better model performance. Minimizing RMSE values also indicates better model accuracy. Additionally, RPD is the ratio of the standard deviation (SD) of the reference VFAs values to the root mean square error of validation (RMSEV), providing a measure of the predictive ability of quantitative models. This parameter is very popular in NIR spectroscopy; however, it is very dependent on the range of concentrations used and the variability among samples, so the direct comparison of RPD among predictive models built with different range of concentrations is not advisable (García Martín, 2022).

All processes above were implemented using Python 3.8.1 in an Anaconda environment.

M.4. Molecular analyses of microbial communities

M.4.1. DNA extraction, amplicon sequencing & filtering

Molecular analyses were carried out to samples obtained from the digestate at the different experiments carried out throughout the thesis. Samples were centrifuged at 1000 rpm for 13 minutes to separate the solid phase from the digestate. Then, approximately 0.5 g of the wet solid phase was transferred to the dissolution matrix, immediately frozen in liquid nitrogen and stored at -80 °C. DNA was extracted from the solid phase of the digestate, using the FastDNA™ Pro Soil-Direct Kit (Biomedicals) with the FastPrep-24™ Classic bead beating system/MP Biomedicals. Amplicon sequencing was performed by the Genomic Unit at the Instituto de Parasitología y Biomedicina-López Neyra (CSIC, Granada, Spain) on the V3/V4 region of the 16S rDNA. Briefly, after DNA extracts were normalized to the same concentration, a 2-step PCR amplified and indexed the V3/V4 region [First PCR step: Primers 16S_ProV3V4_forward (5'-CCTACGGGNBGCASCAG-3') and 16S_ProV3V4_reverse (5'-GACTACNVGGGTATCTAATCC-3'), each with the adaptor (5'-CTGTCTCTTATACACATCT-3'), were used to amplify the V3/V4 region. Cycling conditions: 95 °C, 3 min; (95 °C, 30s; 55 °C, 30s; 72 °C, 30s) x 25 cycles; 72 °C, 5 min; and 4 °C, hold. Second PCR step: Sample indexing using the Nextera XT Index Kit v2, for a further 8 cycles]. Bidirectional sequencing was carried out on a MiSeq™ System (Illumina, 2x275 cycles) (Takahashi et al., 2014). Demultiplexed sequences obtained from the MiSeq™ System were processed in QIIME2 v2019.10 (Bolyen et al., 2019). Cutadapt (Martin, 2011) was used for primer removal, pair-end reads were joined using VSEARCH (maxdiff=20) (Rognes et al., 2016), and denoised with DADA2 (Callahan et al., 2016) using a quality threshold of 20, and truncating the sequence length at 400bp. The taxonomy classifier was trained using the primers above on the Greengenes database version 13.5. Taxonomic assignation at the species level was applied, and OTUs were classified according to their last known taxon.

M.4.2. RNA extraction, amplicon PCR and sequencing

Samples were centrifuged 13 minutes at 1000 rpm to separate the solid phase of the digestate. Subsequently 0.5 g wet solid phase was transferred to the lysing matrix and immediately frozen with liquid nitrogen and stored at -80 °C. RNA was extracted from the solid phase of the digestate, using the FastRNA™ Pro Soil-Direct Kit (Biomedicals) with the FastPrep-24™ Classic bead beating system/MP Biomedicals). RNA was pretreated with DNaseI, and retrotranscribed to cDNA using SuperScript™ II RT (Invitrogen) with random primers according to the manufacturer's instructions. Amplicon sequencing of the V3/V4 region of the 16S rDNA was performed by the Genomic Unit at the Instituto de Parasitología y Biomedicina-Lopez Neyra (CSIC, Granada, Spain) from the cDNA. Briefly, DNA concentration was measured using Qubit® 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and normalized to the same concentration. A 2-step PCR was performed on the DNA samples. Primers 16S_ProV3V4_forward (5'-CCTACGGGNBGCASCAG-3') and 16S_ProV3V4_reverse (5'-GACTACNVGGGTATCTAATCC-3'), each with the adaptor (5'-CTGTCTCTTATACACATCT-3'), were used to amplify the V3/V4 region with the following cycling conditions: 95 °C, 3 min; (95 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) x 25 cycles; 72 °C, 5 min; and 4 °C, hold. A second PCR step was performed to index the samples using the Nextera XT Index Kit v2, for a further 8 cycles. Bidirectional sequencing was carried out on a MiSeq™ System (Illumina, 2x275 cycles) (Takahashi et al., 2014). Demultiplexed sequences obtained from the MiSeq™ System were processed in QIIME2 v2019.10 (Bolyen et al., 2019). Primers were removed using cutadapt (Martin, 2011), pair-end reads were joined using VSEARCH (maxdiff=20) (Rognes et al., 2016), and denoised with DADA2 (Callahan et al., 2016) using a quality threshold of 20, and truncating the sequence length at 400bp. The taxonomy classifier was trained using the primers above on the Greengenes database version 13.5. Taxonomic assignation at the species level was applied, and OTUs were classified according to their last known taxon.

M.4.3. Bacterial and archaeal quantitative analysis (qPCR)

Bacterial and archaeal 16S rRNA gene quantitative PCR (qPCR) using primers 341F and 534R (Muyzer et al., 1993) and primers ARCH915 and UNI-b-rev (Yu et al., 2008) were used as proxies for the total Bacteria and Archaea abundances, respectively. Amplification reactions and PCR conditions were followed according to Correa-Galeote et al. (2021). As standards for the quantification of the genes, dilution series of cloned linear fragments developed previously in our lab were employed. Both qPCR reactions were run on a QuantStudio-3 Real-Time PCR system (Applied Biosystems, USA) by triplicate.

M.4.4. Statistical analysis

Non-metric multi-dimensional scaling (NMDS) was performed using the software PRIMER-E, v.6.0, (Plymouth, UK), and analysis of similarities (ANOSIM) was based on Bray-Curtis distance.

Pheatmap (Kolde, 2015) and RColorBrewer R packages were used to create the

heatmaps. Euclidean clustering was performed to show abundance similarity patterns on taxonomic units. Clustered OTUs at 97% and identified at the species level were labelled "s_" before their classification, the rest were labelled according to their last classification, i.e. g_genus, f_family, o_order, c_class, p_phylum and k_kingdom.

Shannon, Simpson and Chao1 were used to describe the microbial communities (Chao, 1984; Hill, 1973), with the R packages vegan (Oksanen et al., 2017) and fossil (Vavrek, 2011) using data at the ASV level. The Shannon-Wiener (H') index is a measure of the diversity, where across natural systems, values are between 0.5 and 5. Simpson index evaluates the dominance of individual species within mixed populations via evaluating the probability of two random individuals belonging to the same type, where a value of $1-D = 0$ represents complete dominance within the sample. The Chao1 index, an abundance-based coverage estimator, adjusts observed richness by adding a correction factor to obtain an estimated number of "absolute" species.

M.5. Calculations and statistical analysis

M.5.1. Reactor monitoring of organic matter transformation

Hydrolysis (Eq. O.1), acidogenesis (Eq. O.2) and soluble organic matter yield (Eq. O.3) were calculated by COD balance, based on the results experimentally determined at the different fermentation experiment.

$$\text{Hydrolysis (COD\%)} = \frac{([s\text{COD}] + [\text{COD}_{\text{CH}_4}])_{\text{effluent}} - ([s\text{COD}])_{\text{influent}}}{([t\text{COD}] - [s\text{COD}])_{\text{influent}}} \times 100 \quad \text{Eq. O.1}$$

$$\text{Acidogenesis (\%)} = \frac{[s\text{COD}_{\text{VFA}}]_{\text{effluent}}}{([s\text{COD}])_{\text{effluent}}} \times 100 \quad \text{Eq. O.2}$$

$$\text{Soluble organic matter yield} = \frac{[s\text{COD}_{\text{VFA}}]_{\text{effluent}}}{\text{VS}_{\text{effluent}}} \quad \text{Eq. O.3}$$

where $[s\text{COD}]$ = soluble COD concentration ($\text{g O}_2 \cdot \text{L}^{-1}$); $[\text{COD}_{\text{CH}_4}]$ = CH_4 concentration calculated as COD ($\text{g O}_2 \cdot \text{L}^{-1}$); $[t\text{COD}]$ = total COD concentration ($\text{g O}_2 \cdot \text{L}^{-1}$); $[s\text{COD}_{\text{VFA}}]$ = VFA concentration expressed as COD ($\text{g O}_2 \cdot \text{L}^{-1}$), $[\text{VS}]$ = volatile solids concentration stands for the effluent at the reactor ($\text{g O}_2 \cdot \text{L}^{-1}$).

M.5.2. Electrodialysis monitoring

M.5.2.1. Parameters and experimental data

To evaluate the extraction process of volatile fatty acids, the following parameters were analysed:

$$U = I \cdot R \quad \text{Eq. 0.4}$$

where U is the potential expressed in Volts (V); I is the electric current expressed in Amps (A); R is the resistance expressed in Ohms (Ω).

$$\text{VFA flow capacity to treat} = \frac{M_a}{A \cdot t_d} \quad \text{Eq. 0.5}$$

where M_a is the initial mass of VFA to treat expressed in grams (g); A is the effective membrane area expressed in m^2 ; t_d is the final operational time expressed in hours (h).

$$\text{Energy consumption (E)} = \frac{\int_0^t U(t) \cdot I(t) \cdot dt}{V_{dil}} \quad \text{Eq. 0.6}$$

where E is the energy consumption expressed in $\text{kWh} \cdot \text{m}^{-3}$; U is the electric potential applied expressed in volts (V); I is the electric current expressed in Amps (A); V_{dil} is the final volume of diluate expressed in m^3 ; t (s) is the experimental time.

$$\text{Current efficiency } (\eta) = \frac{z \cdot F \cdot \Delta n_{dil}}{N \cdot \int_0^t I(t) \cdot dt} \cdot 100\% \quad \text{Eq. 0.7}$$

$$\Delta n_{dil} = n_i(t) - n_i(0) \quad \text{Eq. 0.8}$$

where z is the valence of the transported ion; F is Faraday's constant ($96,485 \text{ C} \cdot \text{mol}^{-1}$); Δn_{dil} is the mol number of anions or cations transported into concentrate compartment during the experimental time; N is the number of unit cells; I is the electrical current expressed in Amps (A); t (s) is the experimental time; $n_i(t)$ represents the cation mol number at the final experimental time expressed in mol; $n_i(0)$ represents the cation mol number at the initial experimental time expressed in mol.

M.5.3. Statistical analysis

The results are expressed as mean values with their corresponding standard deviations from the replicate reactors. In Chapter 2 Tukey's multiple range test was used for sCOD and VFAs, while the Kruskal–Wallis test was used for total phenolic compounds in pairwise comparisons. In Chapter 4, the normal distribution of the data was confirmed through the application of the Shapiro–Wilk test. All pairwise multiple comparisons were calculated with Dunn's Method (for sCOD) and Holm–Sidak method (for sCOD and VFA). These statistical tests were done assuming normal distribution and homoscedasticity of the raw data. A

one-way analysis of variance (ANOVA) was conducted to evaluate statistical differences. All statistical analyses were performed using SigmaPlot 15.0 (Systat Software Inc., San Jose, CA, USA) with a significance threshold set at $p < 0.05$.

M.6. References

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GENERAL RESULTS AND DISCUSSION



Chapter 1

Impact on the microbial population during biological volatile fatty acid production from olive mill solid waste

Abstract

Volatile fatty acids (VFAs) revalorisation from waste products are key in achieving industrial sustainability and circular economic goals. Hence, the objective of this work was to correlate the adaptability of the microbial community in olive mill solid waste (OMSW) anaerobic fermentation processes, to the production of VFAs under different pH conditions, i.e. under acidic (pH 4 & 5), neutral (pH 6 & 7) and alkaline conditions (pH 9 & 10). At neutral conditions, anaerobic digestion exhibited minimal accumulation of VFAs, as they were primarily biotransformed to methane, where no significant changes in the microbial community were observed. At acidic conditions, a diverse profile of VFAs were present in the reactors, although the VFA production was limited to around 20% of fed OMSW. Despite the low accumulation, the VFA profile at pH 5 was more complex than those at alkaline conditions, accounting propionic acid as the main VFA compound produced at pH 5 (60% of the total VFAs). Acidic conditions entailed a shift in the microbial composition compared to the initial inoculum, although the reactors maintained similar diversity indices. At alkaline conditions, around 50% of the fed OMSW was accumulated as VFAs, mainly as acetic acid. Overall, a lower diversity and higher dominance corresponded to a less diverse VFAs profile, such as the preponderance of acetic acid correlated with a microbial diversity decrease and the increased dominance of *Tissirella*.

Keywords

Anaerobic digestion; operational pH; propionic acid; lignocellulosic material; phenolic compounds.

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1.1. Introduction

Olive oil consumption has spread worldwide; however, production is centred around Mediterranean countries. Most of the olive oil industry generates one major waste flow, i.e. the olive mill solid waste (OMSW) (Ducom et al., 2023). OMSW is mainly composed of the vegetation water, the fibrous part of the mesocarp, parts of the stone and the olives skin (Najla et al., 2022). The composition of the OMSW is water (60-70%), lignin (13-15%), cellulose and hemicellulose (18-20%), olive oil retained in the pulp (2.5-3%), and mineral solids (2.5%). Its organic components include sugars (3%), volatile fatty acids (VFAs) (C-2 to C-7) (1%), polyalcohol (0.2%), proteins (1.5%), polyphenols (0.2-2 %), and different pigments (0.5%) (Rincón et al., 2008). The olive sector generates four tons of OMSW for each ton of olive oil produced, which is creating a management challenge due to the high volume generated, but also an opportunity as it can be used as raw material for different bioprocesses. An alternative approach worth considering is obtaining VFAs from OMSW. The latter are short-chain fatty acids (from 2 to 7 carbons) and are key building blocks in the chemical industry, hence generating high value-added chemicals or biofuel generation (Fang et al., 2017). In particular, acetic acid and its derivatives are used in various industrial processes, such as food preservation (Budiman et al., 2016). In the cosmetic industry, ethyl acetate is used as an odour enhancer in different products (Bhatia and Yang, 2017), and in the textile industry, where polyvinyl acetate is used in the manufacture of synthetic fibres (Pal and Nayak, 2017). Other VFAs such as propionic, butyric or isovaleric acid are used in the food industry as preservatives and flavour enhancers (Bhatia and Yang, 2017). Other current industrial applications of VFAs include medication such as antispasmodics or cancer drugs (Dahiya et al., 2023) or the production of printer inks (Bhatia and Yang, 2017). Thus, acquiring VFAs through microbial processes from OMSW is a renewable and sustainable approach to creating chemical building blocks or oil-based by-products, otherwise obtained from fossil sources.

Although the VFAs content of OMSW is low, these compounds are intermediate metabolites of the anaerobic digestion process (AD). VFAs can be obtained from this type of fermentation via the control of operating parameters, influencing the flow of metabolic pathways, specifically increasing hydrolysis and acidogenesis, while inhibiting methanogenesis (Fang et al., 2017). Several studies have shown that controlling operational parameters such as temperature, pH, hydraulic retention time (TRH) or organic loading rate (OLR) could facilitate the accumulation of VFAs in anaerobic bioreactors (Cabrera et al., 2019; Chen et al., 2017; Garcia-Aguirre et al., 2017; Jankowska et al., 2015; Niero et al., 2022; Yuan et al., 2006). pH control during fermentation could be a highly effective strategy for VFAs accumulation, based on the microbiome involved in the AD process. Among them, microorganisms involved in methanogenesis usually have an optimum pH range of around 6.8-7.8, presenting a very limited activity outside this range. Whereas microorganisms in the hydrolytic and acidogenic stages might effectively function at more extreme pH values, thus, improving the accumulation of VFAs (Dahiya et al., 2023; Xu et al., 2021). To enhance VFAs accumulation working on such extreme pH values, the microbial community should increase the relative quantity of bacteria with hydrolytic and acidogenic activity (Zapata Martínez

et al., 2019) while decreasing the relative abundance of acetoclastic and hydrogenotrophic archaea (Jabłoński et al., 2015).

Therefore, the objective of this work was to relate the adaptability of the microbial community in anaerobic fermentation processes from OMSW for VFAs production under different pH conditions. Specifically, this work has correlated the operation of two acid pH (pH 4 and 5), two neutral pH (pH 6 and 7) and two alkaline pH (pH 9 and 10) with their microbial community.

1.2. Materials and methods

1.2.1. OMSW substrate and anaerobic inoculum

OMSW was supplied by an olive oil producing company called “Oleícola El Tejar Ntra. Sra. de Araceli”, located in Marchena (Seville), Spain. The OMSW was stored at -20 °C after collection in containers to avoid uncontrolled substrate fermentation.

The anaerobic inoculum used in the experiments was obtained from an industrial anaerobic reactor at the “COPERO” wastewater treatment plant in Seville, Spain. The main characteristics of the OMSW and the anaerobic inoculum are summarized in Table 1.1.

Table 1.1. Physicochemical characterization of olive mill solid waste (OMSW) and the anaerobic inoculum.

	OMSW	Anaerobic inoculum
pH	4.6 ± 0.1	7.3 ± 0.1
Alkalinity (mg CaCO ₃ ·L ⁻¹)	n.d.	5401 ± 154
VS/VS (%)	94.0	52.0
Moisture (%)	74.7 ± 0.3	96.0 ± 0.4
tCOD (g O ₂ ·L ⁻¹)	325.6 ± 31	27.0 ± 0.6
sCOD (g O ₂ ·L ⁻¹)	81.50 ± 0.1	0.5 ± 0.2
TOC (mg·L ⁻¹)	3795 ± 10	7150 ± 10
C/N ratio	35 ± 1.0	7 ± 1.0
Total phenols (mg gallic acid eq·L ⁻¹)	6135 ± 131	n.d.
3,4-DHPG (mg·L ⁻¹)	296 ± 6	n.d.
HT (mg·L ⁻¹)	2750 ± 65	n.d.
Ty (mg·L ⁻¹)	799 ± 11	n.d.

VS: volatile solids; TS: total solids; tCOD: total chemical oxygen demand; sCOD: soluble chemical oxygen demand; TOC: total organic carbon; 3,4-DHPG: 3,4-Dihydroxyphenilglycol; HT: Hidroxytyrosol; Ty: Tyrosol; n.d.: not determinated.

1.2.2. Batch-fed acid Fermentation Set-Up and operation

OMSW batch-fed per duplicate reactors were evaluated for each pH condition. Six pH conditions were studied, i.e., 4, 5, 6, 7, 9 and 10. The experimental design of this assay

was carried out following the procedure reported by Cabrera et al. (2019). The capacity of the reactors was 2.0 L, with a working volume of 1.6 L, and were inoculated with 10 g VS·L⁻¹ of fresh inoculum. The operational pH for each reactor was controlled with HP series proportional flow pumps (4-20 mA) (MYTHO technologies, Hunenberg, Switzerland) connected to a 46D - Series controller, sending signals to the pump when the pH fell below or above the pH thresholds, causing the addition of acid (HCl 3N) or base (NaOH 2N) for pH regulation. This signal is provided by an XS PRO-VP 120 pH electrode (Labprocess Distribuciones S.L, Barcelona, Spain) at each reactor. The operation temperature (35-37 °C) was maintained by means of jacketed reactors and a water bath Digiterm-TFT (J.P.Selecta, Barcelona, Spain). The methane generated was measured by a liquid displacement after CO₂ removal with bubblers containing a 2N NaOH solution. Batch feeding was 2 g VS OMSW·L⁻¹ in each reactor. For all pH conditions evaluated, soluble organic matter was monitored to ensure if it had stabilized prior to the next feeding. Batch feeding was performed six times throughout the 45-day experiment. The feedings took place at regular intervals of seven days, specifically on days 0, 7, 14, 21, 28, and 35.

1.2.3. Chemical analysis

The characterization of the substrate and anaerobic inoculum were determined after the following analytical tests. Solid concentrations [Total (TS), Mineral (MS), Volatile (VS)], total chemical oxygen demand (tCOD), soluble chemical oxygen demand sCOD have been determined according to APHA standard methods (Baird et al., 2017). Elemental C and N were determined through a combustion with LECO CN828 by Duma's method according to APHA. Individual VFA compounds were determined using a Shimadzu gas chromatograph (GC-2025) equipped with a 30 m × 0.25 mm (i.d.) Crossbond Carbowax polyethylene glycol column and a flame ionization detector (FID) at 250 °C. The total soluble phenols were determined by the Folin-Ciocalteu method (Trujillo-Reyes et al., 2019). The phenolic compound profile was analysed in a Hewlett-Packard 1100 series high performance liquid chromatography equipment coupled with a diode array detector and an Agilent 1100 automatic injector (20 µL samples were injected). The chromatographic column used was Teknokroma Tracer Extrasil OSD2 of 5 µL particle size and dimensions of 25 m x 0.45 mm internal diameter. The individual soluble phenolic compounds quantified were 3,4-dihydroxyphenylglycol (DHPG), hydroxytyrosol (HT), tyrosol (Ty), 4-methylcatechol (4-MC) and 4-ethylphenol (4-EP).

1.2.4. Molecular analyses of microbial communities

1.2.4.1. RNA extraction, amplicon PCR and sequencing

Three digestate samples were extracted directly from each of the reactors on day 0 (initial conditions – T0), around the mid operation time, between day 22 and day 30 (mid HRT – T1) and day 45 (the last operating day – T2). The samples were centrifuged 13 minutes at 1000 rpm to separate the solid phase of the digestate. Subsequently 0.5 g wet solid phase was transferred to the lysing matrix and immediately frozen with liquid

nitrogen and stored at -80 °C. RNA was extracted from the solid phase of the digestate, using the FastRNATM Pro Soil-Direct Kit (Biomedicals) with the FastPrep-24TM Classic bead beating system/MP Biomedicals). RNA was pretreated with DNaseI, and retrotranscribed to cDNA using SuperScript™ II RT (Invitrogen) with random primers according to the manufacturer's instructions. Amplicon sequencing of the V3/V4 region of the 16S rDNA was performed by the Genomic Unit at the Instituto de Parasitología y Biomedicina-Lopez Neyra (CSIC, Granada, Spain) from the cDNA. Briefly, DNA concentration was measured using Qubit® 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA) and normalized to the same concentration. A 2-step PCR was performed on the DNA samples. Primers 16S_ProV3V4_forward (5'-CCTACGGGNBGCASCAG-3') and 16S_ProV3V4_reverse (5'-GACTACNVGGGTATCTAATCC-3'), each with the adaptor (5'-CTGTCTCTTATACACATCT-3'), were used to amplify the V3/V4 region with the following cycling conditions: 95 °C, 3 min; (95 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) x 25 cycles; 72 °C, 5 min; and 4 °C, hold. A second PCR step was performed to index the samples using the Nextera XT Index Kit v2, for a further 8 cycles. Bidirectional sequencing was carried out on a MiSeq™ System (Illumina, 2x275 cycles) (Takahashi et al., 2014). Demultiplexed sequences obtained from the MiSeq™ System were processed in QIIME2 v2019.10 (Bolyen et al., 2019). Primers were removed using cutadapt (Martin, 2011), pair-end reads were joined using vsearch (maxdiff=20) (Rognes et al., 2016), and denoised with DADA2 (Callahan et al., 2016) using a quality threshold of 20, and truncating the sequence length at 400bp. The taxonomy classifier was trained using the primers above on the Greengenes database version 13.5. Taxonomic assignment at the species level was applied, and OTUs were classified according to their last known taxon.

1.2.4.2. Bacterial and Archaeal quantitative analysis (qPCR)

Bacterial and archaeal 16S rRNA gene quantitative PCR (qPCR) using primers 341F and 534R (Muyzer et al., 1993) and primers ARCH915 and UNI-b-rev (Yu et al., 2008) were used as proxies for the total Bacteria and Archaea abundances, respectively. Amplification reactions and PCR conditions were followed according to Correa-Galeote et al. (2021). As standards for the quantification of the genes, dilution series of cloned linear fragments developed previously in our lab were employed. Both qPCR reactions were run on a QuantStudio-3 Real-Time PCR system (Applied Biosystems, USA) by triplicate.

1.2.4.3. Statistical analysis

Non-metric multi-dimensional scaling (NMDS) was performed using the software PRIMER-E, v.6.0, (Plymouth, UK), and analysis of similarities (ANOSIM) was based on Bray-Curtis distance.

The R packages Pheatmap (Kolde, 2015) and RColorBrewer were used to create the heatmaps. Euclidean clustering was performed to show similarity patterns on taxonomic units. Clustered taxa at the species level were labelled "s_" before their classification. Unclassified clustered taxa at the species level were labelled according to their last classification, i.e. g_genus, f_family, o_order, c_class, p_phylum and k_kingdom.

Shannon, Simpson and Chao1 were used to describe the microbial communities (Hill, 1973; Chao, 1984), with the R packages *vegan* (Oksanen et al, 2017) and *fossil* (Vavrek, 2011) using data at the ASV level. The Shannon-Wiener (H') index is a measure of the diversity, where across natural systems, values are between 0.5 and 5. Simpson index evaluates the dominance of individual species within mixed populations via evaluating the probability of two random individuals belonging to the same type, where a value of $1-D = 0$ represents complete dominance within the sample. The Chao1 index, an abundance-based coverage estimator, adjusts observed richness by adding a correction factor to obtain an estimated number of "absolute" species.

1.3. Results and discussion

1.3.1. Influence of pH on organic matter solubilization during anaerobic fermentation derived from OMSW

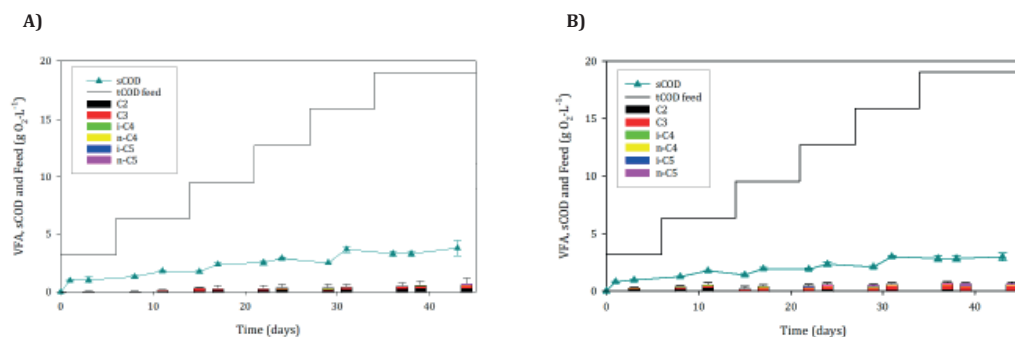
1.3.1.1. sCOD and VFAs accumulation at different pH conditions

Following each OMSW feeding, there was an immediately increase in the sCOD value in all reactors. Notably, sCOD increases slightly more at acidic (4 and 5) and alkaline (9 and 10) pH levels compared to neutral pH (6 and 7) (Figures 1.1 A-F). At pH 9 and 10, sCOD exhibited the highest accumulation at the end of the operational time, with values of $7.8 \pm 1.0 \text{ g O}_2 \cdot \text{L}^{-1}$ and $9.6 \pm 0.5 \text{ g O}_2 \cdot \text{L}^{-1}$, respectively (Figures 1.1E and 1.1F) which is around 50% of the total fed organic matter. Dahiya et al. (2015) stated that proteins and carbohydrates are favourably decomposed by the release of carboxylic groups at alkaline conditions, thus facilitating the solubilization of organic matter. Additionally, alkaline conditions have also been reported to hinder activity of acetoclastic and hydrogenoclastic methanogens, avoiding VFAs consumption (Cui et al., 2019; Dahiya et al., 2015). pH 4 and 5 also induced sCOD accumulation (Figures 1.1A and 1.1B), although to a lesser extent compared to the alkaline conditions (pH 9 and 10), approximately 20% of the total fed organic matter. These values were in the same range than the reported for other lignocellulosic substrates. For instance, in the case of the biological VFAs production from strawberry extrudate, only 55% at pH 5 and 50% at pH 9 of the fed COD was hydrolysed to sCOD (Cubero-Cardoso et al., 2022). In the case of OMSW, Cabrera et al. (2019) described that at pH 5 and 9 the hydrolysis for OMSW only reached values of 50% and 56%, respectively, in line with the present study. Despite of the previous limited hydrolysis values, at pH 7 a 90% degradation of organic matter was reported by the latest study (Cabrera et al., 2019). According to data published by Serrano et al. (2021), the biodegradability range of OMSW is between 57.3 and 93.5% at pH 7 operating at mesophilic temperature. Similarly, in the present study, at neutral pH (6 and 7) VS reduction was approximately 80-90% (Data not shown). Likewise, less than 10% of the total fed organic matter accumulated as sCOD (Figures 1.1C and 1.1D), indicating that most of the fed OMSW was transformed into biogas (methane yields coefficient at pH 6 and 7 ranged between 380 and $420 \text{ mL CH}_4 \cdot \text{g VS}^{-1} \text{ feed}$). This support that, at neutral pH conditions the methanogenic stage in the anaerobic digestion process was, as expected, favourable

(Latif et al., 2017; Sun et al., 2020).

The accumulation of VFAs showed a similar increasing trend as that observed in the accumulation of sCOD (Figure 1.1). The highest VFA accumulation was achieved at the end of the experimental time at operational pH 9 and 10, with values of 2.8 ± 0.4 g COD-VFA·L⁻¹, and 2.6 ± 0.1 g COD-VFA·L⁻¹, respectively (Figures 1.1E and 1.1F). These values of VFAs correspond with 36 % and 27 % of sCOD, respectively. At pH 4 and 5, at the end of the experimental time, VFA accumulation reached concentrations around 0.7 g COD-VFA·L⁻¹ (Figures 1.1A and 1.1B), corresponding to 19% and 23 % of sCOD, respectively. Therefore, for the two controlled acidic conditions (pH 4 and 5), approximately 75% less VFAs were obtained than at the alkaline conditions (pH 9 and 10). Operation at pH 6 and 7 did not result in a relevant accumulation of VFAs, with values below 0.3 g COD-VFA·L⁻¹ throughout the experimental time (Figures 1.1B and 1.1C). Jiang et al. (2013) operated batch reactors using organic fraction of municipal solid wastes as substrate at different pHs, i.e. 5, 6, 7 and uncontrolled pH, obtained 17.08, 39.46 and 37.09 g VFA·L⁻¹, respectively. The VFA production obtained by Jiang et al. (2013) was higher than in the present study, possibly due to the type of substrate, since OMSW is highly lignocellulosic. Despite this, at pH 5 the VFA/sCOD ratio reported by these authors was 20%, similar to the obtained in the present research, around 23%.

As shown in Figures 1.1A, 1.1B, 1.1E, 1.1F, the individual VFA profile was more diverse at acidic conditions (pH 4 and 5) than at alkaline conditions (pH 9 and 10). The major VFA at pH 5 was propionic acid representing 63% of total VFAs, whereas at pH 9 and 10 acetic acid was mainly accumulated, representing 82% and 77% of total VFAs at the end of the experimental time, respectively. Owusu-Agyeman et al. (2021) and Chen et al. (2013) reported a high abundance of propionic acid obtained at acid pH with high presence of carbohydrates in the substrate used. Khiewwijit et al. (2015) reported that, during VFA production from sewage sludge at pH 9 and 10, the VFA composition was dominated by acetic acid (58–63% of the total VFAs). VFA production also was minorly composed by propionic acid (25–30%) and butyric acid (10–15%). These VFA profiles were in line to those obtained in similar studies (Cheah et al., 2019; Jie et al., 2014).



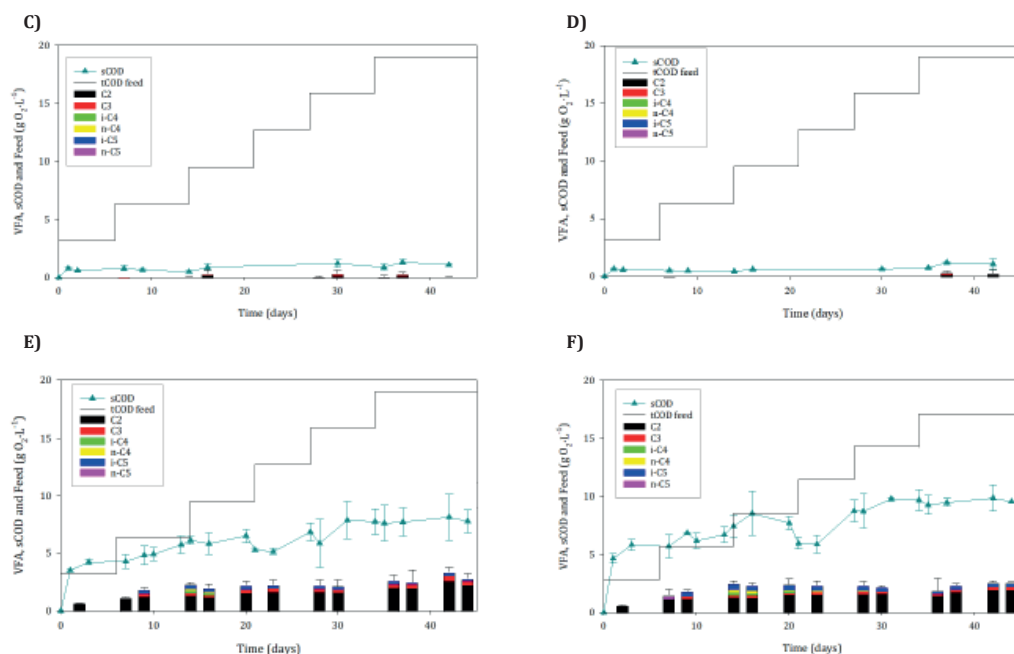


Figure 1.1. Volatile fatty acids and total organic carbon generation ($\text{g O}_2 \cdot \text{L}^{-1}$), according to the feed (expressed as $\text{g O}_2 \cdot \text{L}^{-1}$) olive mill solid waste during the experimental time. A) pH 4, B) pH 5, C) pH 6, D) pH 7, E) pH 9, F) pH 10. C2: acetic acid; C3: propionic acid; i-C4: iso-butyric acid; C4: butyric acid; i-C5: iso-valeric acid; C5: valeric acid

1.3.1.2. Evaluation of phenolic compounds in anaerobic fermentation at different pH condition

Individual and total soluble phenolic compounds were determined in the batch reactors due to the high concentration contained in the fed OMSW (Table 1.2). These compounds present high microbial toxicity that could result in the inhibition of bioprocesses (Chen et al., 2008; Poirier and Chapleur, 2018). At neutral pH (6 and 7) there is neither accumulation nor removal of phenolic compounds; in fact, approximately the same amount of total soluble phenols fed from OMSW were obtained on day 37 (Table 1.2). At both acidic and alkaline conditions phenols were accumulated (Table 1.2). The highest accumulation occurred at alkaline conditions, where at pH 10 reached concentration values up to three times the phenol concentration in the OMSW (Table 1.2). The increase of the phenolic compounds has been related to the disruption of lignocellulosic fibres (Hendriks and Zeeman, 2009), like in the hydrolysis of OMSW (Serrano et al., 2017b). The phenolic compounds are released during the breaking down of the olive pulp cell walls, whose main components are cellulose and pectic polysaccharides, and where the phenolic compounds can be found as minor components (Rubio-Senent et al., 2013). Although the concentration of phenols increased throughout the experimentation in the digesters at acidic and alkaline conditions, the values obtained were lower than the limits of inhibition for the fermentation process at the

different pH tested. According to Calabrò et al. (2018), the concentrations of phenols that can be considered inhibitory in the AD process were values greater than 2000 mg·L⁻¹.

Table 1.2. Total and individual phenolic compounds for all conditions in mg per litre of reactor.

	Time (days)	3,4-DHFG (mg·L ⁻¹)	HT (mg·L ⁻¹)	Ty (mg·L ⁻¹)	4-MC (mg·L ⁻¹)	4-EP (mg·L ⁻¹)	TP (mg gallic acid eq.·L ⁻¹)
OMSW	0	4.0 ± 0.1	37.6 ± 0.9	10.8 ± 0.1	n.d.	n.d.	82.9 ± 2.0
	3	4.2 ± 0.4	21.2 ± 0.5	traces	n.d.	n.d.	93.5 ± 4.8
	11	5.7 ± 1.8	31.4 ± 4.7	traces	n.d.	n.d.	157.9 ± 58.0
	17	5.9 ± 0.8	34.8 ± 4.7	traces	n.d.	n.d.	172.5 ± 57.0
	24	7.7 ± 0.6	41.2 ± 5.8	traces	n.d.	n.d.	188.9 ± 30.0
	31	7.8 ± 0.3	47.8 ± 5.5	traces	n.d.	n.d.	166.4 ± 2.0
	40	11.5 ± 3.3	47.3 ± 2.2	n.d.	n.d.	n.d.	196.9 ± 14.0
pH 4	3	4.4 ± 0.1	21.2 ± 0.5	n.d.	traces	n.d.	60.2 ± 5.7
	11	traces	21.1 ± 4.7	9.4 ± 1.4	traces	9.4 ± 1.4	93.0 ± 0.4
	17	1.7 ± 2.4	21.4 ± 4.7	9.8 ± 0.4	traces	9.8 ± 0.4	107.4 ± 10.3
	24	4.2 ± 0.0	24.3 ± 5.8	9.5 ± 0.9	traces	9.5 ± 0.9	169.0 ± 77.4
	31	3.9 ± 1.0	25.3 ± 5.5	9.2 ± 0.6	traces	9.2 ± 0.6	156.5 ± 26.2
	40	4.8 ± 0.2	26.8 ± 2.2	8.5 ± 0.4	traces	8.5 ± 0.4	179.2 ± 12.0
pH 5	9	3.7 ± 0.1	n.d.	n.d.	n.d.	n.d.	43.4 ± 2.0
	14	3.8 ± 2.4	n.d.	n.d.	n.d.	traces	52.5 ± 3.2
	21	4.4 ± 1.0	n.d.	n.d.	n.d.	traces	60.9 ± 3.0
	28	4.4 ± 0.7	n.d.	n.d.	n.d.	traces	74.5 ± 3.9
	37	4.7 ± 0.3	n.d.	n.d.	n.d.	traces	81.3 ± 7.0
pH 6	9	n.d.	n.d.	n.d.	n.d.	n.d.	40.8 ± 2.2
	14	n.d.	n.d.	n.d.	n.d.	n.d.	60.4 ± 70.8
	21	n.d.	n.d.	n.d.	n.d.	n.d.	67.4 ± 0.2
	28	n.d.	n.d.	n.d.	n.d.	n.d.	76.9 ± 1.0
	37	n.d.	n.d.	n.d.	n.d.	n.d.	83.7 ± 2.4
pH 7	3	n.d.	traces	traces	n.d.	n.d.	120.7 ± 24.0
	9	n.d.	traces	traces	13.6 ± 6.6	traces	179.1 ± 43.0
	16	1.8 ± 2.5	traces	traces	13.9 ± 7.5	traces	184.9 ± 75.8
	23	3.7 ± 0.1	traces	traces	14.5 ± 7.7	traces	215.8 ± 62.4
	30	3.9 ± 0.1	traces	traces	14.4 ± 8.1	traces	245.9 ± 68.8
pH 9	37	3.9 ± 0.1	n.d.	n.d.	14.3 ± 7.6	traces	256.5 ± 64.5
	3	n.d.	n.d.	n.d.	n.d.	n.d.	179.1 ± 6.0
	9	n.d.	traces	traces	23.9 ± 3.1	traces	226.3 ± 8.2
	16	n.d.	traces	traces	23.7 ± 0.2	traces	267.7 ± 19.7
	23	n.d.	traces	traces	25.1 ± 1.8	traces	297.1 ± 8.5
	30	3.6 ± 0.2	traces	traces	23.6 ± 3.9	traces	315.5 ± 10.3
pH 10	37	3.7 ± 0.9	traces	traces	22.5 ± 0.3	traces	324.7 ± 3.3

3,4-DHPG: 3,4-Dihydroxyphenilglycol; HT: Hidroxytyrosol; Ty: Tyrosol; 4-MC: 4- methylcatechol; 4-EP: 4-ethylphenol; TP: Total soluble phenols; n.d.: not detected.

The total concentration of each of the individual phenols coming from OMSW and added during the whole experimental time to each reactor were accounted by 38.34 ± 2.0 mg 3,4-DHPG·L⁻¹, 360 ± 17.0 mg HT·L⁻¹ and 103.5 ± 3.0 mg Ty·L⁻¹. Considering the measured concentration of these individual compounds in the reactors at the end of the experimental time, these phenols have been highly degraded across all pH conditions tested (Table 1.2). Remarkably, removal efficiencies exceeding 70% were achieved for all of these individual phenols with some of them reaching removal efficiencies up to 100% (Table 1.2). It is worth to notice that during anaerobic fermentation at pH 5, 9, and 10, two discernible phenolic compounds were identified which were absent in the original fed OMSW. At pH 5, the detected compound was 4-EP, while at pH 9 and 10, 4-MC was observed. In the similar study with OMSW by Cabrera et al. (2019), the authors reported the presence of 4-EP in their anaerobic fermenters at pH 5 as an outcome of their experiment. 4-EP and 4-MC have been described as intermediate metabolites in the anaerobic degradation of more complex phenols (Serrano et al., 2017a). For example, 4-EP can be generated by decarboxylation of some phenolic acids from OMSW, such as p-coumaric, ferulic or caffeic acids (Milheiro et al., 2019; Rubio-Senent et al., 2013).

1.3.3. Impact of the operational pH in the microbial populations

Three samples were extracted directly from each of the reactors on day 0 (initial conditions – T0), around the mid operation time, between day 22 and day 30 (mid HRT – T1) and day 45 (the last operating day – T2). The samples were taken for further microbial profile analysis. RNA extraction, followed by retrotranscription and 16S rDNA sequencing was performed in order to observe correlations between operational conditions, and VFA production with the activity of microbial population.

All samples at the different pH conditions at T0 had high similarity values in terms of diversity as observed in Figure 1.2 and Table 1.3. At neutral pH (6 and 7), diversity of the microbial community remained stable throughout the experimental time, showing similar values of diversity indices (Table 1.3). Only a slight change in diversity indices was observed in the neutral pH at T1 and T2 which could be explained by the adaptation of the inoculum from treating sewage sludge from a wastewater treatment plant to OMSW. The latter was also observed in Figure 1.2, where all neutral pH samples were clustered with the T0 data points. Therefore, the maintenance of a similar microbial structure can explain that methanogenic activity was maintained in the reactors operating at pH 6 and 7, thus, leading to VFA consumption (Figure 1.1 and supplementary material Table 1.4). The species' relative abundances at T0, did not present large variations in the different pH conditions studied (Figure 1.3). Species' relative abundances at neutral pH conditions for all time points, were similar to those at T0. The species with the highest relative abundances in all samples at T0, and all samples analysed for neutral pH (6 and 7) were as follows: *f_Peptostreptococcaceae*, *o_Bacillales_2*, *g_Acetobacterium*, *c_Gammaproteobacteria*, *p_Bacteroidetes* and *o_Bacteroidales*. Species *c_Deltaproteobacteria*, *p_Proteobacteria*, *c_Gammaproteobacteria*, *k_Bacteria*, whose relative abundances at neutral pH (6 and 7) were high throughout the experiment, may be involved in syntrophic methanogenesis processes (Pannekens et al., 2019).

Impact on the microbial population during biological volatile fatty acid production from olive mill solid waste

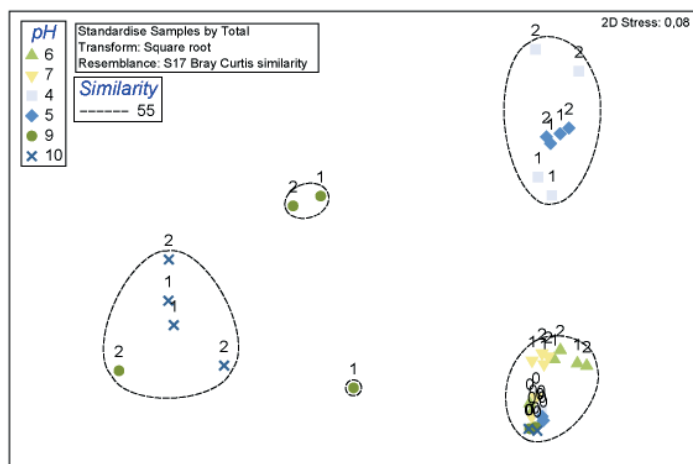


Figure 1.2. Multi-dimensional scaling (MDS) of bacterial communities at species level based on Bray-Curtis dissimilarity index. Each data point label is the time at which the sample was taken (0 – initial, 1 – mid, 2 – final experimental time points). Replicates are observed for each condition.

Table 1.3. Diversity, dominance and Species richness indices.

	Samples	Shannon Index	Simpson 1-D	Chao1
pH 4	T0	5.56 ± 0.09	0.989 ± 0.001	1068.5 ± 6.36
	T1	4.28 ± 0.1	0.967 ± 0.005	533.5 ± 28.99
	T2	3.68 ± 0.39	0.945 ± 0.01	281 ± 46.67
pH 5	T0	5.47 ± 0.09	0.987 ± 0.002	1054 ± 46.67
	T1	3.6 ± 0.05	0.901 ± 0.002	340 ± 29.7
	T2	4.11 ± 0.13	0.962 ± 0.011	349.5 ± 3.54
pH 6	T0	5.48 ± 0.07	0.986 ± 0.002	1040.5 ± 31.82
	T1	4.96 ± 0.09	0.981 ± 0.002	717 ± 26.87
	T2	4.88 ± 0.23	0.981 ± 0.007	657.5 ± 26.16
pH 7	T0	5.42 ± 0.02	0.987 ± 0.001	905 ± 32.53
	T1	5.12 ± 0.07	0.984 ± 0.003	674 ± 11.31
	T2	5.08 ± 0.04	0.986 ± 0.001	642.5 ± 9.19
pH 9	T0	5.43 ± 0.03	0.989 ± 0.001	887 ± 41.01
	T1	4.33 ± 0.6	0.959 ± 0.029	385 ± 72.12
	T2	3.42 ± 0.22	0.881 ± 0.022	248.5 ± 222.74
pH 10	T0	5.41 ± 0.1	0.988 ± 0.001	850.5 ± 21.92
	T1	2.9 ± 0.16	0.832 ± 0.058	92.5 ± 34.65
	T2	2.79 ± 0.45	0.794 ± 0.094	116.5 ± 37.48

T0: day 0 (sample initial conditions); T1: sample mid HRT; T2: sample last operational day.

Opposite trends were observed from the neutral pH reactors with acidic (4 and 5) and alkaline (9 and 10) pH reactors, such as a drop in Shannon Index at T1 and T2 (Table 1.3). The diversity reduction of the Shannon index values was accompanied by a decrease in the Simpson (1-D) and Chao1 indices. Hence, the latter indicates a decrease in species number (Chao1) and an increase in dominant species (Simpson 1-D) as a consequence of the adaptation occurring within the microbial communities in the digesters at the more extreme operational pH (Harirchi et al., 2022). This described specialization would explain the methanogenic activity cessation, usually related to neutral pH conditions (Latif et al., 2017; Sun et al., 2020), and the increment in VFA accumulation (Figure 1.1 and supplementary material, Table 1.4). The highest decrease in the Shannon and Simpson (1-D) index was observed in the reactor operating at alkaline conditions (Table 1.3). Both acidic and alkaline conditions resulted in specialization of the microbial structure for each condition, as can be observed in Figure 1.2, where each extreme pH is clustered together, especially at acidic conditions (>55% similarity) at the end of the experimental period (T2). In acidic conditions there was a marked increase in the relative abundance of *f_Peptostreptococcaceae_2*, and to a lesser extent, an increase in *s_Virgibacillus_halodenitrificans* and *f_Clostridiaceae* (Figure 1.3). The *Peptostreptococcus* genus contains coccobacillus and cocci that are producers of acetic, butyric, iso-butyric, iso-valeric and iso-caproic acids (Downes and Wade, 2006; Murdoch, 1998). This may explain the greater diversity in the VFA profile at pH 4 and 5 (Figure 1.1 and supplementary material, Table 1.4).

Although microbial populations at alkaline conditions were similar, clustering of these samples was much lower (35%) than at acidic conditions (Figure 1.2). At alkaline conditions, lower diversity and higher dominance values were observed, i.e. lower Shannon and Simpson indices values (Table 1.3). Hence, a less complex VFA profile was correlated with a lower number of microorganisms performing most of the microbial activity at these alkaline conditions. The species with the highest relative abundance were *g_Tissierella_soenggenia*, *s_Virgibacillus_halodenitrificans*, *o_Clostridiales_2* and *g_Pseudomonas_2* (Figure 1.3). The enrichment of the microbial population in *Tissierella* would explain the high production of acetic acid, since this is one of the major metabolic end products of this genus, along with butyric and iso-valeric acids (Alauzet et al., 2014; Lawson, 2019). Some species of genus *Tissierella*, such as *Tissierella creatinini*, present an optimal growth pH in the alkaline range (Farrow et al., 1995; Lawson, 2019), that would explain the enrichment in *Tissierella* observed at pH 9 and 10 (Figure 1.3).

Impact on the microbial population during biological volatile fatty acid production from olive mill solid waste

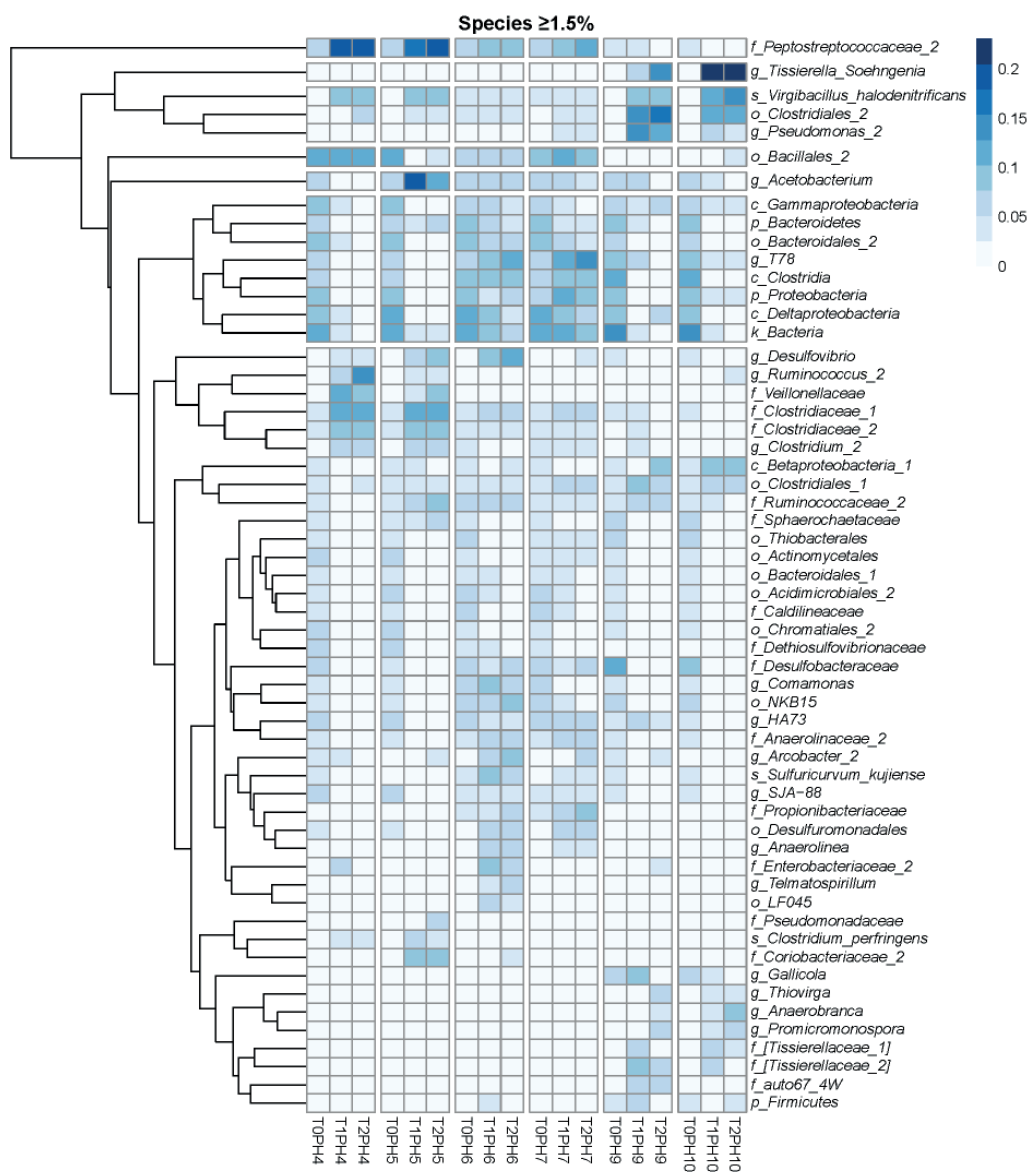


Figure 1.3. Cluster analysis of microbial species in the digestate samples at different pH conditions. The colour scale indicates the log10 of the square root of the mean relative abundance. The included species were limited to OTUs with a relative abundance of $\geq 1.5\%$, in at least 1 sample. Taxa labels are based on the last known taxon classification. Rows are clustered using Euclidean distance.

The total bacterial and archaeal abundance of the inoculum were 1.02×10^{10} and 5.99×10^7 copies per g of biomass, respectively. The variation of operational pH entailed a high change in the ration between archaea and bacteria in the reactors (Figure 1.4 and supplementary material, Table 1.5). The operation at pH 7 favoured a higher prevalence of the archaeal population respect to the number of total bacteria, reaching a archaea/bacteria ratio (x 1000) higher than 10. At pH 6 at T1, the archaea/bacteria ratio at intermediate time was similar than the obtained at pH 7, although a longer operation resulted in a decrease of the ratio (approximately 0.48 at T2). The operation at both acidic and alkaline conditions strongly affected the archaeal population, resulting in archaea/bacteria ratios (x 1000) values below 0.3 (Figure 1.4). These results were expected since the methanogenic activity has been widely associated to neutral pH (Latif et al., 2017; Sun et al., 2020). Therefore, the VFA accumulation resulted in a negative effect on the total archaeal abundances without strong impact on those of Bacteria (Figures 1.1 and 1.4), suggesting that the control of the operational pH can be a suitable strategy to inhibit the methanogenic activity and, thus, to promote the VFA accumulation.

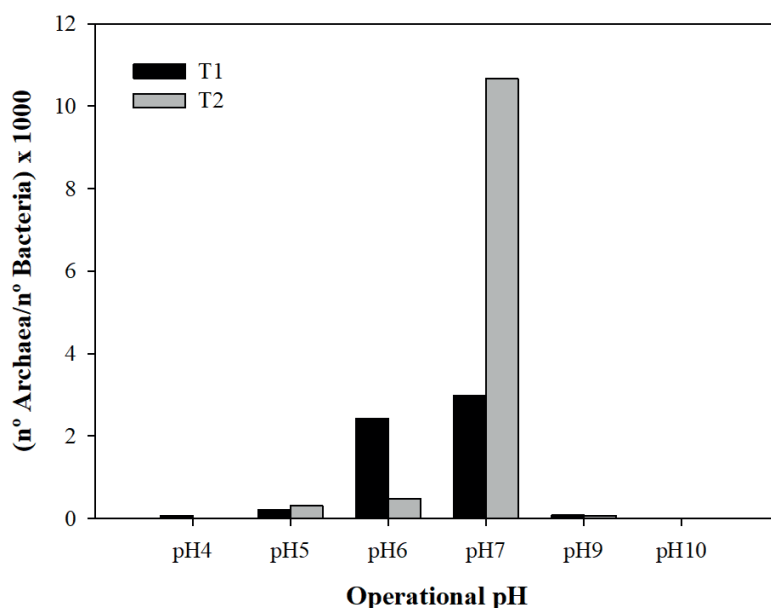


Figure 1.4. Ratio between the number of archaea and bacteria (x1000) at the intermediate and final time at each operational pH, quantified by qPCR (n=6), where T1: mid TRH sample; T2: last operational sample.

1.4. Conclusions

The anaerobic fermentation of OMSW has been investigated across a broad pH range. It has been observed that in acidic conditions a diverse profile of VFAs were present in the reactors. Alkaline condition presented the highest VFA production but with a much a less diverse VFA profile, where almost all the generated VFAs corresponded to acetic acid. Phenols were extensively removed at all the tested conditions. The differences in the microbial composition among reactors at acidic and alkaline conditions could explain the higher diversity generated in the VFA profile at acidic conditions with respect to alkaline conditions. Thus, a lower diversity and higher dominance corresponded to a less diverse VFA profile.

CRedit authorship contribution statement

E. Jiménez-Páez: Methodology, Formal analysis, Writing - Original Draft. A. Serrano: Conceptualization, Writing - Original Draft, Funding acquisition. J. Purswani: Formal analysis, Resources, Writing - Review & Editing. D. Correa-Galeote: Methodology, Resources. Juan Cubero-Cardoso: Formal analysis, Writing - Review & Editing. F.G. Fermoso: Conceptualization, Funding acquisition, Writing - Review & Editing.

Data availability

The sequence data from this study have been submitted to NCBI BioProject (<http://www.ncbi.nlm.nih.gov/bioproject>) under BioProject number PRJNA1019715.

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1.6. Supplementary material

Table 1.4. Individual VFAs concentration during the whole-time experimentation.

pH 4						pH 5						
Time (days)	C ₂ (g O ₂ ·L ⁻¹)	C ₃ (g O ₂ ·L ⁻¹)	i-C ₄ (g O ₂ ·L ⁻¹)	n-C ₄ (g O ₂ ·L ⁻¹)	i-C ₅ (g O ₂ ·L ⁻¹)	n-C ₅ (g O ₂ ·L ⁻¹)	C ₂ (g O ₂ ·L ⁻¹)	C ₃ (g O ₂ ·L ⁻¹)	i-C ₄ (g O ₂ ·L ⁻¹)	n-C ₄ (g O ₂ ·L ⁻¹)	i-C ₅ (g O ₂ ·L ⁻¹)	n-C ₅ (g O ₂ ·L ⁻¹)
0	n.d.	n.d.	n.d.	0.03 ± 0.0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
3	0.08 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	0.02 ± 0.00	0.07 ± 0.00	n.d.	0.07 ± 0.01	n.d.	n.d.
8	0.07 ± 0.03	n.d.	0.02 ± 0.03	n.d.	n.d.	n.d.	0.27 ± 0.01	0.09 ± 0.00	n.d.	0.06 ± 0.01	n.d.	n.d.
11	0.13 ± 0.04	0.05 ± 0.00	0.02 ± 0.03	n.d.	n.d.	n.d.	0.36 ± 0.04	0.15 ± 0.04	n.d.	0.12 ± 0.01	n.d.	n.d.
15	0.19 ± 0.03	0.20 ± 0.02	0.03 ± 0.02	n.d.	n.d.	n.d.	0.06 ± 0.00	0.16 ± 0.12	n.d.	0.03 ± 0.04	n.d.	n.d.
17	0.20 ± 0.15	0.09 ± 0.06	0.03 ± 0.02	0.03 ± 0.04	n.d.	n.d.	0.07 ± 0.06	0.22 ± 0.05	n.d.	0.14 ± 0.03	n.d.	0.06 ± 0.01
22	0.20 ± 0.15	0.10 ± 0.06	0.03 ± 0.02	0.03 ± 0.03	n.d.	n.d.	0.04 ± 0.01	0.25 ± 0.05	n.d.	0.13 ± 0.01	n.d.	0.06 ± 0.01
24	0.25 ± 0.15	0.12 ± 0.07	0.03 ± 0.02	0.03 ± 0.03	n.d.	n.d.	0.20 ± 0.02	0.24 ± 0.04	n.d.	0.12 ± 0.00	n.d.	0.08 ± 0.03
29	0.24 ± 0.17	0.09 ± 0.06	0.05 ± 0.01	0.03 ± 0.03	n.d.	n.d.	0.06 ± 0.04	0.27 ± 0.00	n.d.	0.11 ± 0.02	n.d.	0.07 ± 0.02
31	0.30 ± 0.15	0.12 ± 0.01	0.03 ± 0.02	0.03 ± 0.04	n.d.	n.d.	0.18 ± 0.08	0.32 ± 0.05	n.d.	0.09 ± 0.01	n.d.	0.08 ± 0.01
37	0.36 ± 0.23	0.15 ± 0.03	0.02 ± 0.01	0.04 ± 0.03	n.d.	n.d.	0.20 ± 0.03	0.38 ± 0.01	n.d.	0.10 ± 0.01	n.d.	0.10 ± 0.03
39	0.41 ± 0.29	0.18 ± 0.02	0.05 ± 0.00	0.00 ± 0.03	n.d.	n.d.	0.04 ± 0.00	0.41 ± 0.01	n.d.	0.10 ± 0.01	0.05 ± 0.00	0.11 ± 0.01
44	0.41 ± 0.30	0.19 ± 0.02	n.d.	0.05 ± 0.03	n.d.	0.06 ± 0.07	0.03 ± 0.00	0.44 ± 0.04	n.d.	0.10 ± 0.01	0.03 ± 0.02	0.10 ± 0.02

n.d.: not detected. C2: acetic acid; C3: propionic acid; i-C4: iso-butyric acid; C4: butyric acid; i-C5: iso-valeric acid; C5: valeric acid.

Continuation Table 1.4. Individual VFAs concentration during the whole-time experimentation.

pH 6						pH 7						
Time (days)	C ₂ (g O ₂ ·L ⁻¹)	C ₃ (g O ₂ ·L ⁻¹)	i-C ₄ (g O ₂ ·L ⁻¹)	n-C ₄ (g O ₂ ·L ⁻¹)	i-C ₅ (g O ₂ ·L ⁻¹)	n-C ₅ (g O ₂ ·L ⁻¹)	C ₂ (g O ₂ ·L ⁻¹)	C ₃ (g O ₂ ·L ⁻¹)	i-C ₄ (g O ₂ ·L ⁻¹)	n-C ₄ (g O ₂ ·L ⁻¹)	i-C ₅ (g O ₂ ·L ⁻¹)	n-C ₅ (g O ₂ ·L ⁻¹)
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	0.03 ± 0.01	0.04 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.04 ± 0.01	n.d.	n.d.	n.d.
9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
14	0.01 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
16	0.24 ± 0.17	0.08 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
28	0.06 ± 0.07	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
30	0.21 ± 0.20	0.13 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
35	0.07 ± 0.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
37	0.19 ± 0.16	0.10 ± 0.01	n.d.	n.d.	n.d.	n.d.	0.23 ± 0.11	0.11 ± 0.01	n.d.	n.d.	n.d.	0.33 ± 0.01
42	0.01 ± 0.02	n.d.	n.d.	n.d.	n.d.	n.d.	0.23 ± 0.24	0.04 ± 0.05	n.d.	n.d.	n.d.	0.23 ± 0.03
45	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.04 ± 0.00	0.41 ± 0.01	n.d.	0.10 ± 0.01	0.05 ± 0.0	0.11 ± 0.01

n.d.: not detected. C2: acetic acid; C3: propionic acid; i-C4: iso-butyric acid; C4: butyric acid; i-C5: iso-valeric acid; C5: valeric acid

Continuation Table 1.4. Individual VFAs concentration during the whole-time experimentation.

pH 9							pH 10					
Time (days)	C ₂ (g O ₂ ·L ⁻¹)	C ₃ (g O ₂ ·L ⁻¹)	i-C ₄ (g O ₂ ·L ⁻¹)	n-C ₄ (g O ₂ ·L ⁻¹)	i-C ₅ (g O ₂ ·L ⁻¹)	n-C ₅ (g O ₂ ·L ⁻¹)	C ₂ (g O ₂ ·L ⁻¹)	C ₃ (g O ₂ ·L ⁻¹)	i-C ₄ (g O ₂ ·L ⁻¹)	n-C ₄ (g O ₂ ·L ⁻¹)	i-C ₅ (g O ₂ ·L ⁻¹)	n-C ₅ (g O ₂ ·L ⁻¹)
0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2	0.70 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.	0.62 ± 0.05	n.d.	n.d.	n.d.	n.d.	n.d.
7	1.14 ± 0.06	n.d.	n.d.	n.d.	n.d.	n.d.	1.25 ± 0.10	n.d.	n.d.	n.d.	n.d.	0.25 ± 0.30
9	1.28 ± 0.15	0.29 ± 0.16	n.d.	n.d.	0.31 ± 0.06	n.d.	1.25 ± 0.04	0.21 ± 0.01	n.d.	n.d.	0.40 ± 0.03	n.d.
14	1.39 ± 0.15	0.26 ± 0.14	0.18 ± 0.01	0.14 ± 0.04	0.36 ± 0.07	n.d.	1.35 ± 0.08	0.21 ± 0.01	0.22 ± 0.02	0.25 ± 0.06	0.40 ± 0.03	n.d.
16	1.26 ± 0.22	0.18 ± 0.15	0.16 ± 0.02	0.13 ± 0.04	0.26 ± 0.05	n.d.	1.35 ± 0.05	0.19 ± 0.01	0.21 ± 0.02	0.23 ± 0.05	0.37 ± 0.40	n.d.
20	1.63 ± 0.22	0.31 ± 0.15	n.d.	n.d.	0.32 ± 0.05	n.d.	1.57 ± 0.08	0.22 ± 0.01	0.11 ± 0.15	0.12 ± 0.17	0.41 ± 0.04	n.d.
23	1.71 ± 0.29	0.30 ± 0.15	n.d.	n.d.	0.29 ± 0.04	n.d.	1.60 ± 0.15	0.23 ± 0.02	n.d.	0.12 ± 0.16	0.37 ± 0.04	n.d.
28	1.70 ± 0.33	0.28 ± 0.15	n.d.	n.d.	0.27 ± 0.02	n.d.	1.65 ± 0.14	0.21 ± 0.01	n.d.	0.10 ± 0.14	0.36 ± 0.04	n.d.
30	1.67 ± 0.39	0.28 ± 0.15	n.d.	n.d.	0.23 ± 0.01	n.d.	1.67 ± 0.09	0.21 ± 0.03	n.d.	n.d.	0.32 ± 0.01	n.d.
36	2.06 ± 0.46	0.31 ± 0.15	n.d.	n.d.	0.26 ± 0.18	n.d.	1.49 ± 0.77	0.20 ± 0.04	n.d.	n.d.	0.18 ± 0.26	n.d.
38	2.04 ± 0.82	0.33 ± 0.22	n.d.	n.d.	0.13 ± 0.03	n.d.	1.81 ± 0.11	0.24 ± 0.01	n.d.	n.d.	0.29 ± 0.03	n.d.
42	2.65 ± 0.47	0.42 ± 0.15	n.d.	n.d.	0.28 ± 0.20	n.d.	1.98 ± 0.13	0.28 ± 0.01	n.d.	n.d.	0.33 ± 0.01	n.d.
45	2.3 ± 0.47	0.36 ± 0.15	n.d.	n.d.	0.15 ± 0.10	n.d.	2.00 ± 0.04	0.27 ± 0.01	n.d.	n.d.	0.33 ± 0.01	n.d.

n.d.: not detected. C2: acetic acid; C3: propionic acid; i-C4: iso-butyric acid; n-C4: butyric acid; i-C5: iso-valeric acid; C5: valeric acid

Table 1.5. Gene copies/g biomass of bacteria and archaea determined by quantitative PCR (n=6) retrieved from reactors at initial, intermediate and final times at each operational pH condition.

Bacteria						
	T0		T1		T2	
	Mean	Desv.	Mean	Desv.	Mean	Desv.
pH4	1.02E+10	3.68E+09	2.75E+09	6.55E+08	2.44E+10	1.04E+09
pH5	1.02E+10	3.68E+09	1.41E+10	5.71E+09	3.35E+10	1.29E+10
pH6	1.02E+10	3.68E+09	3.56E+09	1.68E+09	4.18E+09	8.25E+08
pH7	1.02E+10	3.68E+09	6.37E+09	2.23E+09	3.95E+09	4.25E+08
pH9	1.02E+10	3.68E+09	1.44E+10	1.73E+10	4.20E+09	5.24E+09
pH10	1.02E+10	3.68E+09	8.09E+08	4.95E+08	8.78E+08	4.35E+08
Archaea						
	T0		T1		T2	
	Mean	Desv.	Mean	Desv.	Mean	Desv.
pH4	5.99E+07	1.74E+07	1.56E+05	1.94E+05	9.86E+04	1.41E+04
pH5	5.99E+07	1.74E+07	2.90E+06	1.01E+06	1.00E+07	6.89E+06
pH6	5.99E+07	1.74E+07	8.64E+06	9.27E+06	2.00E+06	7.59E+05
pH7	5.99E+07	1.74E+07	1.89E+07	2.01E+07	4.21E+07	6.71E+06
pH9	5.99E+07	1.74E+07	9.87E+05	1.14E+06	2.74E+05	8.59E+04
pH10	5.99E+07	1.74E+07	8.86E+03	4.95E+08	0.00E+00	0.00E+00

T0: day 0 (sample initial conditions); T1: sample mid HRT; T2: sample last operational day.

Impact on the microbial population during biological volatile fatty acid production from olive mill solid waste



Chapter 2

Evaluation of semi-continuous anaerobic fermentation of olive mill solid waste by methanogenesis inhibition

Abstract

Two methanogenesis inhibition strategies for enhancing volatile fatty acid (VFA) production through the anaerobic fermentation of olive mill solid waste (OMSW), a byproduct of olive oil production, were evaluated. Methanogenesis inhibition was implemented via two different approaches, one by pH adjustment to 5.0 and another one by chemical inhibition using 2-bromoethanesulfonate (BES) at pH 7. The VFA accumulation at the end of the experiment was 67% higher under the BES condition than under the pH 5 condition. Interestingly, the VFA profiles were similar under both conditions, with acetic acid as the dominant product, followed by propionic and butyric acids. The results demonstrated a 25% increase in OMSW solubilization under the BES condition, compared to under the pH 5 condition. This latest finding, together with the similar VFA profiles on both strategies, suggests that the hydrolysis step in OMSW solubilization was impacted by the pH difference. These findings highlight the effectiveness of chemical inhibition in enhancing solubilization, hydrolysis, and VFA accumulation during anaerobic fermentation of OMSW and, most importantly, the negative effect of pH 5 on the solubilization step.

Keywords

Anaerobic fermentation; pH adjustment; chemical inhibitor; carboxylate acid; phenolic compounds; semi-continuous operation.

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2.1. Introduction

The olive oil production industry is growing worldwide, achieving 2.5 million tons in the 2023/2024 season (Statista, 2024). This production is primarily concentrated in the Mediterranean basin, accounting for 90% of the total. Within the Mediterranean region, Spain is a significant contributor, responsible for 30% of the overall production (Statista, 2024). During the olive oil production process, a by-product known as olive mill solid waste (OMSW) is generated in two-phase olive oil extraction system. OMSW consists primarily of olive stones and pulp, having a composition of approximately 70-75% moisture and 35% lignocellulosic compounds (Fernández-Rodríguez et al., 2022). Different management methods are currently under consideration for OMSW, among which anaerobic digestion is a feasible option to obtain renewable carbon materials, such as biogas or a digestate (Messineo et al., 2020).

The anaerobic digestion of OMSW operates most efficiently under mesophilic conditions and within a pH range of 6.5–7.5. This process generates an average energy output from methane production comparable to the energy yield achieved through its direct combustion (Serrano et al., 2021). In fact, the biodegradability of OMSW under these operational conditions has been reported by different previous studies, with values between 60–90% (Serrano et al., 2021). Currently, the prices of biogas from industrial waste ranges from USD 0.11 to USD 0.50 per cubic meter of methane (European Biogas Association, 2025). As an alternative, volatile fatty acids (VFAs), which are intermediate metabolites produced during the fermentative stage of anaerobic digestion, have gained significant scientific and economic interest. VFAs, short-chain carboxylic acids with 2 to 5 carbon atoms, are valuable for producing bioplastics, high added value chemicals, and biofuels (Annamalai et al., 2020). Among them, acetic acid is the most produced carboxylic acid, priced at 890 USD per tonne, followed by propionic and butyric acids at 2200 USD per tonne and 2550 USD per tonne, respectively (Chen et al., 2022).

Several strategies have been proposed to transform anaerobic digestion processes from methane production to fermentative processes to produce VFA. Examples of these strategies are the thermal modification of the inoculum (Caroca et al., 2021), acid or alkali pH operation (Jiménez-Páez et al., 2023), hyperthermophilic operation (Qiu et al., 2024), or low hydraulic retention time (HRT) operation (Menzel et al., 2023), among others. Actually, there is not much research on the production and accumulation of VFAs from OMSW. The research up to date is mainly focused on the operation at different pHs. Furthermore, all these studies have been conducted on batch reactor configuration, yielding relatively low VFA production values (Cabrera et al., 2019; Cubero-Cardoso et al., 2024; da Fonseca et al., 2023; Jiménez-Páez et al., 2023). Under alkaline conditions, the highest VFA concentrations are achieved, with acetic acid being the predominant product, in combination with the highest percentage of solubilization at around 50% (Jiménez-Páez et al., 2023). In contrast, acidic conditions result in lower overall VFA concentrations as a consequence of a lower solubilization but yield a more diverse profile, with acetic, propionic, and butyric acids present in similar proportions (Cabrera et al., 2019; Jiménez-Páez et al., 2023). Additionally, these studies focused on monitoring phenolic compounds, due to their antimicrobial activity

and their increased concentration under acidic conditions (Cabrera et al., 2019; Cubero-Cardoso et al., 2024; da Fonseca et al., 2023; Jiménez-Páez et al., 2023). However, according to these same authors, under neutral pH conditions and in a complete anaerobic digestion metabolic pathway, the microorganisms were able to solubilize up to around 80% of the OMSW (Cabrera et al., 2019; Jiménez-Páez et al., 2023). These findings suggest that although operating reactors at extreme pH ranges might allow the inhibition of methanogenesis, it also affects the capacity of the bacteria to solubilize OMSW, with respect to neutral pH. In fact, certain hydrolytic bacteria are sensitive to pH. This sensitivity can result in reduced hydrolysis efficiency at an operational pH out of their optimal range, particularly when processing complex organic materials, such as OMSW.

So, pH has a crucial role in fermentation, influencing microbial community structure, metabolic pathways, and enzyme activities. This directly impacts how much a substrate can be solubilised, the accumulation of VFAs and, even, determines the favoured fermentation types and product yields (Zhou et al., 2021). Although operating reactors at extreme pH ranges allowed the inhibition of the methanogenesis, it also affected the capacity of the bacteria of solubilise the OMSW respect to neutral pH. In fact, certain hydrolytic bacteria are sensitive to pH (Amin et al., 2021). This sensitivity can result in reduced hydrolysis efficiency at operational pH out of their optimal range, particularly when processing complex organic materials such as OMSW.

The aim of this work was to compare the solubilization, as well as the production and accumulation of volatile fatty acids (VFAs), in semi-continuous reactors under two methanogenesis inhibition strategies: (1) at pH 5.0 and (2) using the chemical inhibitor archaea 2-bromoethanesulfonate (BES) at pH 7. Both strategies were applied to monitor the total soluble compounds, volatile fatty acids, and total and individual phenolic compounds as possible inhibitors of OMSW.

2.2. Materials and methods

2.2.1. Feedstock and anaerobic inoculum characterization

OMSW was obtained from the "Oleícola El Tejar Ntra. Sra. de Araceli" pomace plant, located in Marchena (Seville), Spain. The main physico-chemical characteristics of the OMSW was as follows: pH = 4.4 ± 0.1 ; Volatile solids (VS) = $252,604 \pm 2999 \text{ mg} \cdot \text{kg}^{-1}$; VS/Total Solids (VS/TS) = $93.9 \pm 0.2\%$; moisture = $71 \pm 0.5\%$; soluble chemical oxygen demand (sCOD) = $86,409 \pm 1528 \text{ mg O}_2 \cdot \text{L}^{-1}$; sCOD/ total chemical oxygen demand (tCOD) ratio = 24%; phenolic compounds = $24,744 \pm 351 \text{ mg gallic acid eq} \cdot \text{kg}^{-1}$.

The anaerobic inoculum used in the experimentation was obtained from an industrial anaerobic reactor of the wastewater treatment plant "COPERO" located in Seville, Spain. The main anaerobic inoculum characteristics was as follows: pH = 7.0 ± 0.1 ; VS = $22,607 \pm 196 \text{ mg VS} \cdot \text{kg}^{-1}$; sCOD = $443 \pm 7 \text{ mg O}_2 \cdot \text{L}^{-1}$.

2.2.2. Anaerobic fermentation experimental procedure

Duplicate glass reactors (Pobel, Spain) operating under a semi-continuous regime had a working volume of 1.6 L, and each one was inoculated with 10 g VS·L⁻¹ of fresh inoculum. The reactors were operated under mesophilic conditions (35–37 °C), applying a hydraulic retention time (HRT) of 14 days during four HRT repetitions, i.e., 57 days of operation time. This short HRT was selected to favour VFA accumulation (Battista et al., 2022). An organic loading rate (OLR) of 3 g VS of OMSW·(L·d)⁻¹ was set and maintained throughout the whole experimentation time. Methanogenesis inhibition was created on the first day through manual pH adjustment to pH 5 in duplicate reactors, while other duplicate reactors underwent chemical inhibition at pH 7. Chemical inhibition was added to the reactors at a rate of 0.5 g of 2-bromoethanesulfonate (BES) per gram of volatile solids of inoculum. The initial pH adjustment for each reactor was manually controlled using 2N NaOH and 2N HCl. In reactors maintained at pH 7 under the addition of the BES condition, sodium bicarbonate (1 g) was supplemented when the pH dropped below 7.0. Methane, carbon dioxide, and hydrogen production were collected in biogas bags.

2.2.3. Chemical analysis

The characterizations of OMSW, anaerobic inoculum, and samples obtained from the reactors were determined per duplicate following the analytical test. Total solids (TSs), volatile solids (VSs), and total and soluble chemical oxygen demand (sCOD) were measured in the samples according to the APHA Standard Methods (Baird et al., 2017).

Each analysis was carried out at least in duplicate. The standard deviation of each mean value was considered to evaluate the measurement errors.

2.2.3.1. VFA quantification by gas chromatograph analysis

To measure the concentration of VFAs (acetic acid (C2), propionic acid (C3), iso-butyric acid (i-C4), butyric acid (n-C4), iso-valeric (i-C5) acid, and valeric acid (n-C5)) in the samples, these were centrifuged and microfiltered with 0.45 µm nylon microfilters. The sample was prepared by adding an internal standard of 1500 mg·L⁻¹ ethylbutyric acid to phosphoric acid (30% v/v). VFAs were determined in duplicate, injecting 0.5 µL of each sample in a gas chromatograph equipped with a Shimadzu GC-2025 (Shimadzu Corporation, Kyoto, Japan). A Stabilwax-DA capillary column (RESTEK Corporation, Bellefonte, PA, USA) of 0.25 mm i.d. × 30 m and a flame ionization detector (FID) were used. The oven temperature gradually increased from 100 to 170 °C at 5 °C·min⁻¹. The carrier gas (nitrogen, hydrogen, and air) flowed at 40 mL·min⁻¹ at 504 kPa, with individual flow rates of 30 mL·min⁻¹ for nitrogen, 40 mL·min⁻¹ for hydrogen, and 400 mL·min⁻¹ for air. The FID detector temperature was set to 250 °C. Identification and quantification were performed using commercial standards by comparing retention times with the reference compound.

2.2.3.2. Total and soluble phenolic compounds

Samples were previously centrifuged and microfiltered with 0.22 µm nylon microfilter. The total soluble phenolic compounds were determined using the Folin–Ciocalteu method (Singleton & Rossi, 1965). For each sample, Na₂CO₃ (0.7 M) and Folin–Ciocalteu (0.2 M) were added. After 15 min, the absorbances of the samples were recorded at 655 nm in a Bio-Rad iMark model microplate reader (Hercules, CA, USA). Finally, a calibration curve with a known gallic acid concentration was developed, and the results were expressed as milligrams of gallic acid equivalents per kilogram or per litre. The phenolic compound profile was analysed in a Hewlett-Packard 1100 series high-performance liquid chromatography (HPLC) (Agilent, Barcelona, Spain) equipment coupled with a diode array detector and an Agilent 1100 automatic injector (20 µL). The chromatographic column was a Teknokroma Tracer Extrasil OSD2, with a 5 µm particle size and a 25 × 0.46 mm internal diameter (Teknokroma, Barcelona, Spain). The mobile phases were Milli-Q (Merck Millipore, Darmstadt, Germany) water with an adjusted pH with 0.01% trifluoroacetic acid (A) and HPLC-grade acetonitrile (B). A 55 min gradient elution method was used: 0–30 min of 5% B, 30–45 min of 25% B, 45–47 min of 50% B, and 47–50 min of 0% B, held until the run was completed. Identification and quantification were performed using commercial standards by comparing retention times with the reference compound and recording UV spectra between 200 and 360 nm. Results were expressed as milligrams of each phenolic compound per litre.

2.2.3.3. Biogas quantification and composition

Biogas produced during the hydraulic retention time (HRT) of the reactors was collected in gas bags. The volume of accumulated biogas was subsequently quantified using a Ritter gasometer. Biogas concentrations in the headspace samples were taken and stored using a vacuum tube. A 500 µL gaseous sample was manually injected into an HP 8860 gas chromatograph unit (Agilent, Shanghai, China) following a method modified from Araujo et al. (2024). The equipment was equipped with a TCD detector (Agilent, Shanghai, China) and two columns, an HP-PLOT/Q (Agilent, USA), 30 m × 0.53 mm i.d. × 40 µm film thickness, and an HP-MOLESIEVE molecular sieve (Agilent, USA), 30 m × 0.53 mm i.d. × 50 µm film thickness. The initial temperature was held at 35 °C for 3 min, followed by a ramp to 65 °C with a ramp of 10 °C·min⁻¹, resulting in a total run time of 6 min. The carrier gas was N₂ at a flow rate of 10 mL·min⁻¹. The injector was operated in split mode (split ratio 2:1) and at 200 °C, with a pressure of 17.14 psi. The TCD detector was maintained at 250 °C, with N₂ as the reference gas, at a flow rate of 25 mL·min⁻¹. Compound identification and quantification were performed, with retention time and peak areas of the sample being compared to those of increasing amounts of pure gases. The gas chromatograph analyses were carried out at the Instrumental Technical Services of the Estación Experimental del Zaidín (SIC-EEZ), CSIC, Granada, Spain.

2.2.4. Soluble organic matter yield and acidogenesis ratio

To evaluate the fermentation process, the soluble organic matter yield and acidogenesis rate ratio were calculated using Equation 2.1 and 2.2.

$$\text{Soluble organic matter respect solids} = \frac{([\text{sCOD}])_{\text{influent}}}{\text{VS}_{\text{effluent}}} \cdot 100 \quad \text{Eq. 2.1}$$

$$\text{Acidogenesis rate (\%)} = \frac{[\text{sCOD}_{\text{VFA}}]_{\text{influent}}}{([\text{sCOD}])_{\text{effluent}}} \cdot 100 \quad \text{Eq. 2.2}$$

where $[\text{sCOD}]_{\text{effluent}}$ = sCOD concentration stands the value of the reactor effluent ($\text{mg O}_2 \cdot \text{L}^{-1}$); $[\text{VS}]_{\text{effluent}}$ = volatile solids concentration stands for the effluent at the reactor (expressed in $\text{mg O}_2 \cdot \text{L}^{-1}$); $[\text{sCOD}_{\text{VFA}}]$ = VFA concentration calculated as COD ($\text{mg O}_2 \cdot \text{L}^{-1}$).

2.2.5. Statistical analysis

The results are expressed as mean values with their corresponding standard deviations from duplicate reactors working as biological replicates. A one-way analysis of variance (ANOVA) was conducted to evaluate statistical differences, followed by Tukey's multiple range test for sCOD and VFAs, while the Kruskal–Wallis test was used for total phenolic compounds in pairwise comparisons. All statistical analyses were performed using SigmaPlot 15.0 (Systat Software Inc., San Jose, CA, USA), with a significance threshold set at $p < 0.05$.

2.3. Results and discussion

2.3.1. Soluble compounds accumulation under anaerobic fermentation

During the first two HRTs (up to day 29), soluble chemical oxygen demand (sCOD) increased under both the pH 5 and BES conditions. Under the pH 5 condition, sCOD increased from $2034 \pm 340 \text{ mg O}_2 \cdot \text{L}^{-1}$ to $15,828 \pm 508 \text{ mg O}_2 \cdot \text{L}^{-1}$, while under the BES condition, it increased from $4328 \pm 216 \text{ mg O}_2 \cdot \text{L}^{-1}$ to $19,062 \pm 88 \text{ mg O}_2 \cdot \text{L}^{-1}$ (Figure 2.1). Steady-state conditions were reached from the third HRT repetition onwards (from day 29 to 57), with average values of $20,510 \pm 1400 \text{ mg O}_2 \cdot \text{L}^{-1}$ and $25,465 \pm 1600 \text{ mg O}_2 \cdot \text{L}^{-1}$ under pH 5 and BES, respectively (Figure 2.1). The nearly 25% increase in soluble compounds observed under the BES condition (p value < 0.001), compared to that under the pH 5 condition, may be attributed to the pH-dependent activity of hydrolytic bacteria. Specifically, the activity of certain hydrolytic bacterial populations may be limited at pH 5, while a greater diversity and potentially higher activity of these populations may be observed at a neutral pH (Amin et al., 2021; Menzel et al., 2020).

The accumulation of organic matter in the reactor, evidenced by the increase in soluble organic matter (Figure 2.1A, B), stabilized from day 28 onwards. An increase in the

percentage of sCOD/VS was observed up to this time, reaching final values of $34 \pm 3\%$ and $47 \pm 3\%$ under pH 5 and BES conditions, respectively (Figure 2.1B). Consistent with these findings, acidic pH can affect the enzymatic and metabolic activities of microorganisms, which may affect the availability of the soluble substrate and thus the hydrolytic step (da Fonseca et al., 2023; Dahiya et al., 2015).

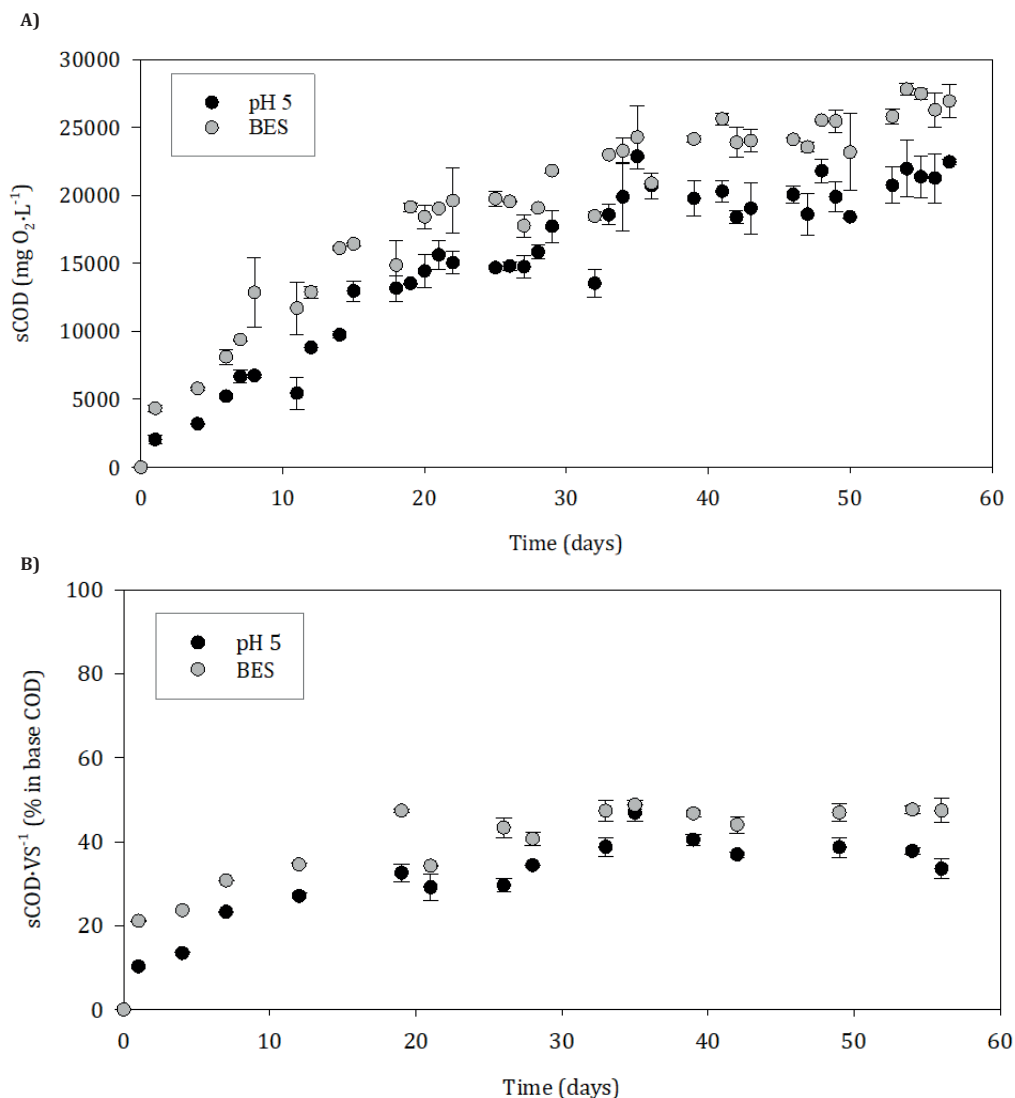


Figure 2.1. (A) Accumulation soluble compound (sCOD) values measured in milligrams of oxygen per litre over the reactor operation time under pH 5 and BES conditions. (B) Representation of the percentage of soluble organic matter in the reactor organic matter throughout the experimental time under pH 5 and BES conditions.

The composition of the biogas, including methane (CH_4), carbon dioxide (CO_2), and hydrogen (H_2), is presented in Table 2.1. In the reactor under the pH 5 condition, a high percentage of methane was observed during the first HRT, with a value of $51.35 \pm 4.77\%$ (Table 2.1), while carbon dioxide was more dominant during the second and third HRTs. The observed CH_4 percentage was substantially lower than the range of 50–70% typically achieved in complete anaerobic digestion processes (Nair et al., 2016; Zhou et al., 2016). Notably, a minimal percentage of hydrogen was detected in the biogas during the second HRT, coinciding with higher carbon dioxide composition. This composition indicated that, despite the use of pH 5 or BES conditions, the methanogenesis activity was not totally inhibited. In the reactor operated under the BES condition, methane composition was lower across all HRTs, while carbon dioxide was more predominant. Hydrogen was not detected, likely due to the unfavourable condition at pH 7. Optimal conditions for effective hydrogen production typically involve a pH of 5.5 and a temperature of 37 °C. Optimal conditions for effective hydrogen production typically involve at pH 5.5 and a temperature of 37 °C (Goren et al., 2025).

Table 2.1. Biogas composition with their standard deviation at the end of each hydraulic retention time (HRT) repetition.

	Time (days)	HRT repetitions	CH_4 (%)	CO_2 (%)	H_2 (%)	mL biogas·g VS ⁻¹
pH 5	0-13	1-HRT	51.35 ± 4.77	48.65 ± 4.77	n.d.	15.48 ± 17.29
	14-28	2-HRT	11.93 ± 5.89	87.12 ± 4.54	1.00 ± 1.00	15.79 ± 6.80
	29-42	3-HRT	27.37 ± 7.44	72.63 ± 7.44	n.d.	9.28 ± 10.12
	43-57	4-HRT	32.79 ± 15.01	67.21 ± 5.01	n.d.	2.88 ± 2.56
BES	0-13	1-HRT	8.69 ± 2.78	91.31 ± 2.78	n.d.	19.69 ± 15.84
	14-28	2-HRT	22.10 ± 9.10	77.89 ± 9.09	n.d.	25.44 ± 22.38
	29-42	3-HRT	29.59 ± 0.88	70.40 ± 0.88	n.d.	34.19 ± 14.92
	43-57	4-HRT	13.34 ± 1.73	86.66 ± 1.73	n.d.	23.47 ± 1.07

n.d.: not detected.

2.3.2. Volatile fatty acid accumulation under anaerobic fermentation

Figures 2.2 and 2.3 show the pH measurements and the individual VFAs throughout the whole experimental time. Under the pH 5 condition, pH adjustments were not required throughout the operational time. On the contrary, under pH 7 conditions, sodium bicarbonate was periodically added, as its pH dropped to 6.3. This decrease could be due to the acidification of the system by VFA accumulation (Figures 2.2 and 23).

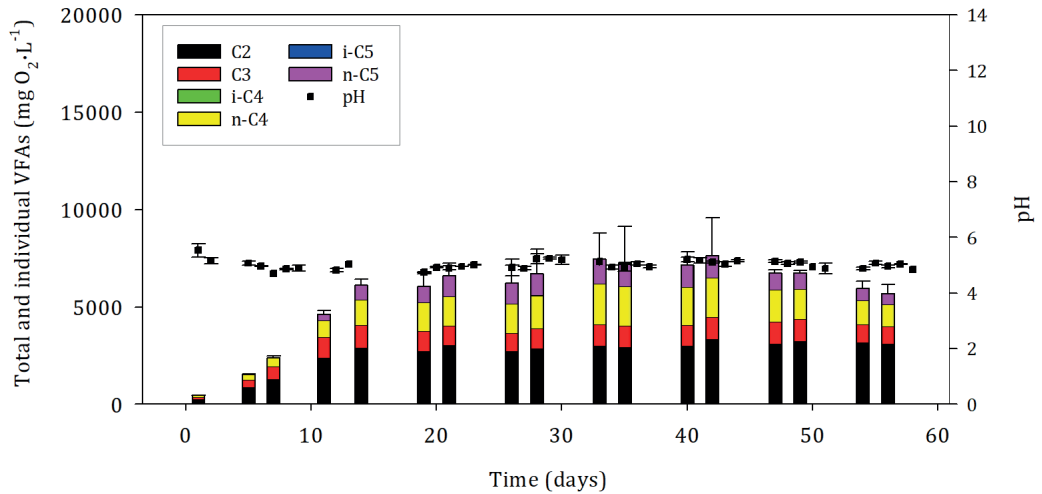


Figure 2.2. Variation of the individual VFA and pH values along operation time under the pH 5 condition (bars represent standard deviation). C2: acetic acid; C3: propionic acid; i-C4: iso-butyric acid; C4: butyric acid; i-C5: iso-valeric acid; C5: valeric acid.

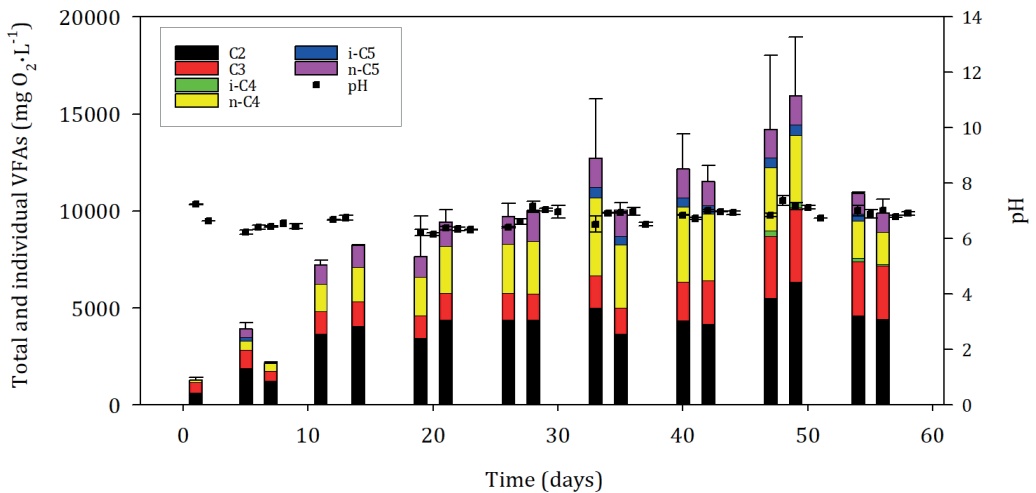


Figure 2.3. Variation of the individual VFA and pH values along the operation under the BES condition (bars represent standard deviation). C2: acetic acid; C3: propionic acid; i-C4: iso-butyric acid; C4: butyric acid; i-C5: iso-valeric acid; C5: valeric acid.

VFA concentration under the pH 5 condition increased steadily during the first HRT repetition (up to day 14). Subsequently, after a period of stabilization, the VFA concentration was observed from day 14 to day 57, with the average concentration remaining above $6000 \text{ mg} \cdot \text{L}^{-1}$ (Figure 2.2), corresponding to $33 \pm 2\%$ of the acidogenesis rate at steady-state days (from 30 to 57 days). Comparing these results with the BES condition, steady-state conditions were observed slightly later than under the pH 5 condition, concretely on day 21, accounting for total VFAs exceeding $10,000 \text{ mg O}_2 \cdot \text{L}^{-1}$, 67% higher than under the pH 5 condition (Figure 2.3). The ratio of acidogenesis (VFAs/sCOD) under the BES condition was $43 \pm 3\%$ on steady-state days (from 30 to 57 days), increasing to 10% more than the results obtained under the pH 5 condition (p value < 0.02). The increase in the percentage of acidogenesis was in agreement with the results obtained by Lu et al. (2020), in which the acidogenesis rate was higher at pH 7 than at pH 5.

The difference between pH 5 and BES conditions in VFA accumulation and transformation could be related to the dissociation of VFAs. Under the pH 5 condition, it was reported that approximately 60% of the total volatile fatty acids were dissociated, with a pK_a around 4.7–4.8. The concentration of undissociated organic acids can influence fermentation, as these forms exhibit a stronger inhibitory effect, compared to their ionized counterparts. Due to their neutral charge, undissociated acids can penetrate bacterial cells, where they dissociate internally, releasing H^+ ions and disrupting the transmembrane pH gradient (Kelbert et al., 2024; Sukphun et al., 2021). For these reasons, under the pH 5 condition, the accumulation of VFAs may have resulted in an inhibition of the fermentation stage (Sun et al., 2021). In contrast, at neutral pH, the percentage of undissociated acids was $<1\%$ and did not disrupt the transmembrane pH gradient (Holtzapple et al., 2022). Previous studies suggested that slightly acidic to neutral pH (5.5–7.0) facilitated the best VFA yield during acidogenic fermentation (Agnihotri et al., 2022). Strazzera et al. (2021) investigated the impact of pH on VFA production from various household food waste substrates in batch reactors, finding that pH 7 enhanced VFA accumulation ($8\text{--}13 \text{ g} \cdot \text{L}^{-1}$) for protein-rich, starch-rich, fiber-rich, and sugar-rich substrates, while hydrolysis limitations restricted VFA production from cellulose-rich and lipid-rich wastes. Previous studies on the anaerobic fermentation of food waste reported higher VFA concentrations under neutral and alkaline pH conditions (Cheah et al., 2019; Jiang et al., 2013). One of them, under a semi-continuous regime, achieved VFA levels of $9.8\text{--}11.5 \text{ g} \cdot \text{L}^{-1}$, with a feed concentration of $5 \text{ g} \cdot \text{L}^{-1}$ and an HRT of 3.5 days (Cheah et al., 2019). All of these studies reported difficulty in VFA accumulation at an acidic pH compared to neutral or alkaline pH, due to the dissociation of the ions.

The VFA profile showed in steady-state condition (from day 29 to 57) that acetic acid was the predominant acid under both conditions evaluated, with average concentrations of $3014 \pm 187 \text{ mg O}_2 \cdot \text{L}^{-1}$ (45 % on a COD basis) and $4378 \pm 351 \text{ mg O}_2 \cdot \text{L}^{-1}$ (39% on a COD basis) under pH 5 and BES conditions, respectively. The other most relevant VFAs were propionic acid and butyric acid, whose distributions varied according to the operational condition. Under the pH 5 condition, the second most predominant acid was butyric acid, with a value of $1643 \pm 320 \text{ mg O}_2 \cdot \text{L}^{-1}$ (25% on a COD basis), while under the BES condition, the second most dominant acid was propionic acid, reaching a value of $2347 \pm 958 \text{ mg O}_2 \cdot \text{L}^{-1}$ (21% on a COD basis). The profile concentration under the pH 5 condition diverged from the

research of fed-batch fermentation of OMSW at pH 5, wherein propionic acid emerged as the dominant acid, contributing 63% to the overall acid production (Jiménez-Páez et al., 2023). These results suggest that propionic and butyric acid fermentation pathways are not solely regulated by pH but rather depend on other factors, such as HRT (Llamas et al., 2022). Also, the observed product distribution, including the coexistence of both pathways at pH 5 with different dominances, indicates a significant influence of redox potential on the metabolic activity (Rahimieh & Nosrati, 2024). Additionally, under the BES condition, the profile was more complex than that observed under the pH 5 condition. This was evidenced by the emergence of iso-butyric and iso-valeric acids after 33 days, with concentrations of $202 \pm 94 \text{ mg O}_2\cdot\text{L}^{-1}$ (2% on a COD basis) and $453 \pm 75 \text{ mg O}_2\cdot\text{L}^{-1}$ (4% on a COD basis), respectively.

2.3.3. Phenolic compounds accumulation under anaerobic fermentation

Phenolic compounds are solubilized from OMSW during the hydrolysis and degradation steps of the organic matrix. Monitoring these phenolic compounds is crucial, as they possess inherent antibacterial properties that can potentially inhibit bacterial activity in anaerobic fermentation processes (Lenzuni et al., 2024). For this reason, total phenolic compounds and individual phenolic compounds were analysed in fermentation processes (Figure 2.4, Table 2.2). The total phenolic compounds concentration, expressed in mg gallic acid equivalents per litre, increased over the operational time similarly under both assessed conditions. Under the pH 5 condition, the mean concentration was enhanced from $62 \pm 9 \text{ mg gallic acid eq.}\cdot\text{L}^{-1}$ to $915 \pm 66 \text{ mg gallic acid eq.}\cdot\text{L}^{-1}$ during the operational time. Under the BES condition, concentrations increased from $178 \pm 10 \text{ mg gallic acid eq.}\cdot\text{L}^{-1}$ to $864 \pm 31 \text{ mg gallic acid}\cdot\text{L}^{-1}$. These values did not exhibit a significant variance between the conditions, as indicated by a p value of 0.896. Despite these increases, more than 80% of total phenolic compounds fed, provided by OMSW, were removed at the end of the operation time. Notably, the total phenolic compound concentrations observed in this study consistently remained below the inhibitory threshold for anaerobic digestion processes, which is typically around $2000 \text{ mg}\cdot\text{L}^{-1}$ (Calabrò et al., 2018).

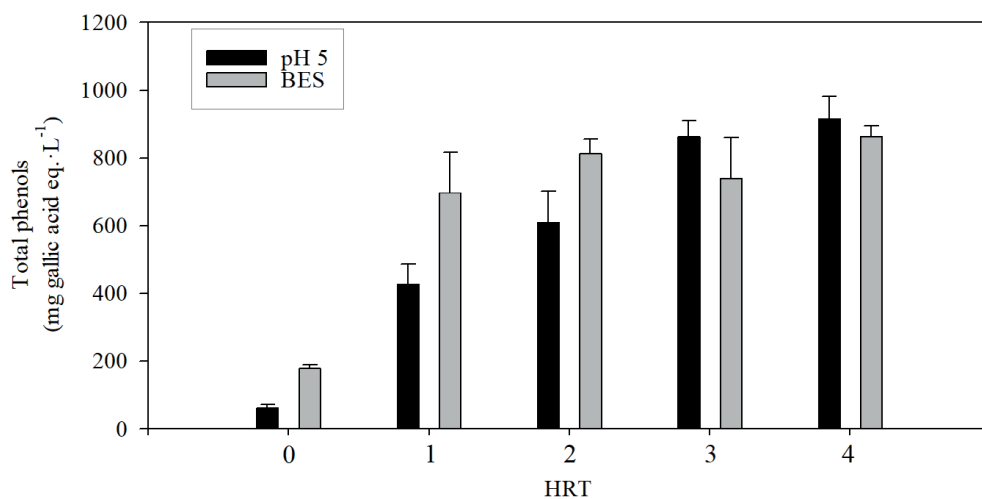


Figure 2.4. Variation of the total phenolic compound concentrations with their standard deviations at the end of each hydraulic retention time (HRT) (bars represent standard deviation).

Table 2.2 presents the concentrations of individual phenolic compounds ($\text{mg}\cdot\text{L}^{-1}$) measured under pH 5 and BES conditions at the end of each HRT repetition. The primary compounds present in OMSW and released during the anaerobic digestion process included 3,4-dihydroxyphenylglycol, hydroxytyrosol, and tyrosol (Najla et al., 2022). The concentration of 3,4-dihydroxyphenylglycol consistently increased throughout the operational time, with higher levels observed under the BES condition, reaching $168.78 \pm 27.40 \text{ mg}\cdot\text{L}^{-1}$. Hydroxytyrosol and tyrosol showed an increasing trend at pH 5, while under the BES condition, there was practically no accumulation, maintaining concentrations below $40 \text{ mg}\cdot\text{L}^{-1}$ (Table 2.2). A study on OMSW revealed that due to its high phenol content and variability, it has high antimicrobial effects. However, this antimicrobial activity comes mainly from the high concentrations of hydroxytyrosol (Caballero-Guerrero et al., 2022).

Protocatechuic acid, 4-dihydroxycinnamic acid, and 4-methylcatechol were analysed, as they are derived from lignocellulosic material, mainly from lignin, and act as indicators of solubilization during the anaerobic digestion process (Guo et al., 2022; Soares et al., 2024). Protocatechuic acid concentrations increased under both pH conditions, reaching approximately $50 \text{ mg}\cdot\text{L}^{-1}$ (Table 2.2). The 4-dihydroxycinnamic acid showed an increase under the pH 5 condition but remained at low concentrations under the BES condition. Conversely, 4-methylcatechol exhibited low concentrations under the pH 5 condition but higher concentrations under the BES condition. These results suggest that under pH 5 conditions, phenolic compounds in OMSW and the lignocellulosic material accumulate more concentration than under the BES condition, with these phenolic compounds being degraded and transformed into VFAs.

Table 2.2. Individual phenolic compound concentration with their standard deviation at the end of four repetitions of hydraulic retention time (HRT).

pH 5				
	1-HRT	2-HRT	3-HRT	4-HRT
Time (days)	0-13 days	14-28 days	29-42 days	43-57 days
3,4-dihydroxyphenylglycol (mg·L ⁻¹)	7.46 ± 1.33	7.53 ± 1.87	83.46 ± 10.34	68.04 ± 21.69
Hydroxytyrosol (mg·L ⁻¹)	56.37 ± 23.37	46.70 ± 24.14	72.47 ± 11.49	116.25 ± 11.50
Tyrosol (mg·L ⁻¹)	15.37 ± 7.19	37.57 ± 6.42	56.80 ± 16.11	38.68 ± 27.56
Protocatechuic acid (mg·L ⁻¹)	11.53 ± 9.46	48.88 ± 17.39	54.92 ± 50.24	25.81 ± 18.78
3,4-dihydroxycinnamic acid (mg·L ⁻¹)	4.80 ± 0.14	20.32 ± 20.26	38.51 ± 37.15	54.30 ± 9.03
4-Methylcatechol (mg·L ⁻¹)	8.71 ± 1.00	7.93 ± 0.69	9.03 ± 1.00	7.02 ± 1.11
BES				
	1-HRT	2-HRT	3-HRT	4-HRT
Time (days)	0-13 days	14-28 days	29-42 days	43-57 days
3,4-dihydroxyphenylglycol (mg·L ⁻¹)	4.57 ± 1.09	17.50 ± 11.41	22.33 ± 1.44	168.78 ± 27.40
Hydroxytyrosol (mg·L ⁻¹)	n.d.	n.d.	30.39 ± 1.10	25.80 ± 1.77
Tyrosol (mg·L ⁻¹)	n.d.	n.d.	17.83 ± 1.00	30.92 ± 27.40
Protocatechuic acid (mg·L ⁻¹)	8.67 ± 1.00	18.83 ± 7.04	40.93 ± 7.28	52.66 ± 2.10
3,4-dihydroxycinnamic acid (mg·L ⁻¹)	n.d.	5.59 ± 0.60	7.34 ± 0.48	6.87 ± 0.64
4-Methylcatechol (mg·L ⁻¹)	80.82 ± 7.34	108.64 ± 18.39	56.44 ± 27.45	32.68 ± 17.55

n.d.: not detected.

2.4. Conclusions

The anaerobic fermentation of OMSW was evaluated under semi-continuous conditions using two methanogenesis inhibition strategies. Under the pH 5 condition, solubilization was limited; this might be caused by the inhibitory effect of undissociated volatile fatty acids (VFAs), which penetrate the cell and disrupt the transmembrane pH gradient. In contrast,

solubilization was more efficient under the BES condition (pH 7), with a 25% increase compared to under the pH 5 condition, resulting in a higher VFA concentration (exceeding $10,000 \text{ mg O}_2 \cdot \text{L}^{-1}$, 67% higher than under the pH 5 condition). The profiles of both conditions evaluated were very similar, in which the main acid was acetic acid, followed by butyric acid under the pH 5 condition and propionic acid under the BES condition. Phenolic compound monitoring indicated concentrations below inhibitory thresholds under both conditions, with a slightly greater degradation observed under the BES condition. These findings suggest that bacterial inhibition at an acidic pH has a crucial impact on the solubilization of organic matter, affecting the hydrolytic and, subsequently, the acidogenic stages of anaerobic fermentation. Consequently, the chemical inhibition strategy that facilitates the solubilization of OMSW may be extended to other complex agro-industrial by-products to enhance VFA production.

Author Contributions

E.J.-P.: conceptualization, methodology, investigation, formal analysis, and writing—original draft preparation; J.C.-C.: conceptualization, methodology, investigation, formal analysis, and writing—original draft preparation; A.S.: conceptualization, supervision, methodology, funding acquisition, and writing—reviewing and editing; R.H.: methodology and writing—reviewing and editing; F.G.F.: funding acquisition and writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

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Chapter 3

Impact of hydraulic retention time on the production of volatile fatty acids from lignocellulosic feedstock by acidogenic fermentation

Abstract

The olive oil industry generates large quantities of olive mill solid waste, which can be used as biorefinery feedstock for the extraction of high value-added compounds. This research proposes the production and accumulation of volatile fatty acids (VFAs) through acidogenic fermentation at controlled pH 5 of olive mill solid waste. The influence of hydraulic retention time (HRT) on microbial structure dynamics during hydrolysis and acidogenesis stages was evaluated. Optimal hydrolysis was achieved with 14-day HRT, while the maximum acidogenesis was observed with a 21-day HRT, reaching $32 \pm 5\%$ and $39 \pm 3\%$, respectively. VFA concentration up to $13,421 \pm 240 \text{ mg O}_2 \cdot \text{L}^{-1}$ was achieved at 21-day HRT, mainly as acetic, butyric, and propionic acids. A positive correlation was found between the relative abundance of hydrolytic species *g_Ruminococcus* and *f_Sphaerochaetaceae*, and the hydrolysis and accumulation of VFA. Increasing HRT up to 21-days showed beginnings of destabilization, despite a further increase in the accumulation of VFA.

Keywords

Butyric acid; hydrolysis stage; long-term operation; microbial population; olive mill solid waste.

3.1. Introduction

Bioprocess technologies based on valorising organic substrates are key for implementing circular economy strategies, capable of generating bioenergy or valuable chemical products while mitigating the polluting effects of these substrates (Kumar & Verma, 2021). A widely available organic substrate in Mediterranean areas is the olive mill solid waste (OMSW), largely generated by the olive oil industry. The OMSW is a mixture of the skin, seeds and pulp of the processed olives, mainly composed by water (60–70%), lignocellulose fibres (35%), and minor compounds such as polyphenols and organic acids (Najla et al., 2022; Serrano et al., 2019). Both phenolic compounds and volatile fatty acids (VFA) content in the OMSW are potential contributors to the phytotoxicity of this substrate (Kong et al., 2022; Pinho et al., 2017). Hence, these characteristics and vast OMSW accumulation require proper management to avoid potential environmental pollution and allow for the revalorisation of OMSW into high added-value products.

Acidogenic fermentation could significantly improve the sustainability of the olive sector through the potential conversion of the OMSW into valuable chemicals like VFAs. The latter compounds have high industrial interest due to their use as a precursor molecule for bioplastics, high value-added chemicals, or biofuel (Atasoy et al., 2019; Fang et al., 2017). However, the lignocellulosic content of the OMSW could hinder the acidogenic fermentation process since the lignin could limit accessibility of hydrolytic enzymes to the substrate, thereby impeding the bioconversion process into VFAs (Cavinato et al., 2017). Hence, for complex organic substrates like OMSW, the hydrolysis stage often represents the rate-limiting step.

Previous studies have evaluated different pretreatments on lignocellulosic substrates, such as mechanical, hydrothermal or alkaline, among others, to improve their anaerobic biodegradability by facilitating the substrate hydrolysis (Bahreini et al., 2020; Fonseca et al., 2021; Hendriks & Zeeman, 2009; Serrano et al., 2023; Wang et al., 2023; Xu et al., 2021). However, the implementation of a pretreatment stage usually has some disadvantages, such as increased costs since the pretreatment accounts for 20–40% of the total process costs (Menzel et al., 2020). Furthermore, pretreatment application could release undesirable metabolites, i.e. hydrothermal pretreatment releases compound such furans and phenols (Monlau et al., 2014), whereas mechanical pretreatments can release monoterpenes, which can interfere with the growth of microorganisms (Trujillo-Reyes et al., 2023). As an alternative to the implementation of pretreatments, the optimization of different operational parameters, like temperature, pH, hydraulic retention time (HRT) or organic loading rate (OLR), during the acidogenic fermentation process can promote the hydrolysis of the substrates and, thus, favour VFA accumulation (Lv et al., 2022; Zhang et al., 2023). Jiménez-Páez et al. (2023) and García-Aguirre et al. (2017) studied the fermentation of OMSW and organic fraction of municipal solid waste (OFMSW), respectively, at acidic and alkaline pH, obtaining higher hydrolysis rates and acetic acid accumulation at alkaline pH, while at acidic conditions obtained a complex acid profile with higher concentration of propionic and butyric acids.

At present, few studies have evaluated long-term anaerobic fermentations, and those

that have, include HRT and OLR as their main experimental factors (Lim et al., 2008; Llamas et al., 2022; Swiatkiewicz et al., 2021). Both HRT and OLR seem to be crucial factors for long-term VFA operation processes because optimal VFA production requires an OLR high enough to sustain microbial activity (Jankowska et al., 2018), while HRT needs to be adequate to allow the hydrolysis of complex organic matter (Swiatkiewicz et al., 2021). Although these are microbiological processes, most studies only focus on evaluating the response of the system to the variation of a factor, offering little information about the specific effects on microbial populations.

Therefore, the aim of this research was to evaluate the impact of HRT on the long-term acidogenic fermentation of OMSW under acidic conditions to maximize VFA accumulation. The study explored the relationship between HRT variations and key process parameters, including microbial population dynamics, substrate solubilization/hydrolysis, and VFAs production maximization. The correlation of key microorganisms with the optimal accumulation of VFA at varying the experimental conditions would allow a better understanding of the process, thus facilitating its control and guaranteeing the optimization and robustness of the system in the long-term.

3.2. Material and Methods

3.2.1. OMSW and anaerobic inoculum

OMSW was obtained from the "Oleícola El Tejar Ntra. Sra. de Araceli" pomace plant, located in Marchena (Seville), Spain. The collected OMSW was stored at -20 °C until further use, avoiding the degradation of the substrate. The main physic-chemical characteristics of the OMSW were: pH = 4.6 ± 0.1 ; volatile solids/total solids ratio (VS/TS) = $94.0 \pm 0.1\%$; volatile solids = $252,604 \pm 2999 \text{ mg}\cdot\text{kg}^{-1}$; moisture = $74.8 \pm 0.3\%$; soluble chemical oxygen demand (sCOD) = $81,455 \pm 90 \text{ mg O}_2\cdot\text{L}^{-1}$; total chemical oxygen demand (tCOD) = $333,695 \pm 31,335 \text{ mg O}_2\cdot\text{L}^{-1}$; total phenols = $6135 \pm 131 \text{ mg gallic acid eq}\cdot\text{L}^{-1}$; 3,4-dihydroxyphenilglycol = $296 \pm 6 \text{ mg}\cdot\text{L}^{-1}$; hydroxytyrosol = $2750 \pm 65 \text{ mg}\cdot\text{L}^{-1}$; tyrosol = $799 \pm 11 \text{ mg}\cdot\text{L}^{-1}$.

The anaerobic inoculum used for the acidogenic fermentation experiments was obtained from an industrial anaerobic reactor at the "COPERO" wastewater treatment plant located in Seville, Spain. The main physicochemical characteristics of the inoculum were: pH = 7.3 ± 0.1 ; alkalinity = $5312 \pm 48 \text{ mg CaCO}_3\cdot\text{L}^{-1}$; VS = $22,342 \pm 41 \text{ mg VS}\cdot\text{kg}^{-1}$; sCOD = $664.50 \pm 5 \text{ mg O}_2\cdot\text{L}^{-1}$.

3.2.2. Acidogenic fermentation experimental procedure

The experiment was conducted in semi-continuous regime under pH-controlled conditions (pH: 5.0 ± 0.5) in acidogenic fermentation reactors in duplicate. Each reactor had a working volume of 1.6 L (total volume of 2.0 L), and it was initially inoculated with $10 \text{ g VS}\cdot\text{L}^{-1}$ of fresh anaerobic inoculum. Reactors were run at a mesophilic temperature range ($35 \pm 2 \text{ }^\circ\text{C}$) and continuously stirred by means magnetic stirring system consisting in an IKA-RET basic and a magnet. The impact of different HRTs on VFA production was evaluated. Specifically, HRT

of 7 days (stage I), 14 days (stage II), and 21 days (stage III) were tested (Table 1). In stages I and II, the operational conditions were maintained during the time corresponding to five HRT, while in stage III the experimental time was maintained during the time corresponding to three HRT. The OLR was maintained at 3 g VS of OMSW·(L·d)⁻¹ throughout the entire experiment, meanwhile the HRT was varied by adding distilled water to the fed OMSW. The operational pH for each reactor was controlled using HP series proportional flow pumps (4-20 mA) (MYTHO technologies, Hunenberg, Switzerland), following the procedure detailed in Jiménez-Páez et al. (2023). Methane production was measured by liquid displacement using 0.5 L gasometers submerged in 2N NaOH solutions to remove the CO₂ from the generated gas. The experiment was carried out for 176 days.

Table 3.1. Summary of the experimental conditions.

Experimental stage	HRT (d)	Duration (d)	Period (d)	OLR (g VS·(L·d) ⁻¹)
Stage I	7	36	0 - 37	3
Stage II	14	74	38 - 112	
Stage III	21	63	113 - 176	

HRT: hydraulic retention time; OLR: organic loading rate; VS: volatile solids

3.2.3. Chemical analysis

The characterization of the anaerobic inoculum, the substrate and the routine samples taken from the reactors were determined applying the following analytical procedures: pH, alkalinity, TS, VS, and total and soluble chemical oxygen demand (tCOD and sCOD), which were measured in the samples in accordance with the APHA Standard Methods (Baird et al., 2017). VFAs (C2-C5) were quantified in duplicate using a gas chromatograph Shimadzu GC-2025 equipped with a Stabilwax-DA column and a flame ionization detector (FID) (more details in Trujillo-Reyes et al. (2023). The total soluble phenols were determined by the Folin-Ciocalteu spectrophotometric method (Singleton et al., 1965). The phenolic compound profile was analysed in a Hewlett-Packard 1100 series high performance liquid chromatography (HPLC) equipment coupled with a diode array detector as described by Fernández Prior et al. (2023). The gas composition (CH₄, CO₂, O₂, N₂, and H₂) of reactors headspace was determined using a Shimadzu GC-2014 gas chromatograph previously described by Trujillo-Reyes et al. (2022). During the whole experimental time, the gas composition maintained an average composition of 84 ± 5% N₂, 15 ± 5 % CO₂, <1% CH₄.

3.2.4. Hydrolysis and acidogenesis calculations

Hydrolysis (Eq. 1) and acidogenesis (Eq.2) were calculated in base on a COD balance from the results obtained from the acidogenic fermentation as previously described by Wu et al. (2015).

$$\text{Hydrolysis ratio (\%)} = \frac{([s\text{COD}] + [\text{COD}_{\text{CH}_4}])_{\text{effluent}} - ([s\text{COD}])_{\text{influent}}}{([t\text{COD}] - [s\text{COD}])_{\text{influent}}} \times 100 \quad \text{Eq. 3.1}$$

$$\text{Acidogenesis ratio (\%)} = \frac{(s[\text{COD}_{\text{VFA}}])_{\text{effluent}}}{([s\text{COD}])_{\text{influent}}} \times 100 \quad \text{Eq. 3.2}$$

where [sCOD] = sCOD concentration ($\text{g O}_2 \cdot \text{L}^{-1}$); $[\text{COD}_{\text{CH}_4}]$ = CH_4 concentration calculated as COD ($\text{g O}_2 \cdot \text{L}^{-1}$); [tCOD] = tCOD concentration ($\text{g O}_2 \cdot \text{L}^{-1}$); $[s\text{COD}_{\text{VFA}}]$ = VFA concentration calculated as COD ($\text{g O}_2 \cdot \text{L}^{-1}$).

3.2.5. Molecular analyses of microbial communities

3.2.5.1. DNA extraction, amplicon sequencing & filtering

Samples were extracted from each of duplicate reactors on day 0, 15, 37, 65, 94, 134, 155 and 176. The samples were centrifuged at 1000 rpm for 13 minutes to separate the solid phase from the digestate. Then, approximately 0.5 g of the wet solid phase was transferred to the dissolution matrix, immediately frozen in liquid nitrogen and stored at -80°C . DNA was extracted from the solid phase of the digestate, using the FastDNATM Pro Soil-Direct Kit (Biomedicals) with the FastPrep-24TM Classic bead beating system/MP Biomedicals. Amplicon sequencing was performed by the Genomic Unit at the Instituto de Parasitología y Biomedicina-López Neyra (CSIC, Granada, Spain) on the V3/V4 region of the 16S rDNA (Jiménez-Páez et al., 2023). Briefly, after DNA extracts were normalized to the same concentration, a 2-step PCR amplified and indexed the V3/V4 region [First PCR step: Primers 16S_ProV3V4_forward (5'-CCTACGGGNBGCASCAG-3') and 16S_ProV3V4_reverse (5'-GACTACNVGGGTATCTAATCC-3'), each with the adaptor (5'-CTGTCTCTTATACACATCT-3'), were used to amplify the V3/V4 region. cycling conditions: 95°C , 3 min; (95°C , 30 s; 55°C , 30 s; 72°C , 30 s) x 25 cycles; 72°C , 5 min; and 4°C , hold. Second PCR step: Sample indexing using the Nextera XT Index Kit v2, for a further 8 cycles]. Bidirectional sequencing was carried out on a MiSeqTM System (Illumina, 2x275 cycles) (Takahashi et al., 2014). Demultiplexed sequences obtained from the MiSeqTM System were processed in QIIME2 v2019.10 (Bolyen et al., 2019). Cutadapt (Martin, 2011) was used for primer removal, pair-end reads were joined using vsearch (maxdiff=20) (Rognes et al., 2016), and denoised with DADA2 (Callahan et al., 2016) using a quality threshold of 20, and truncating the sequence length at 400bp. The taxonomy classifier was trained using the primers above on the Greengenes database version 13.5. Taxonomic assignation at the species level was applied, and OTUs were classified according to their last known taxon.

3.2.5.2. Statistical analysis

Non-metric multi-dimensional scaling (NMDS) was performed using the software PRIMER-E, v.6.0, (Plymouth, UK), and analysis of similarities (ANOSIM) was based on Bray-Curtis distance.

Heatmap (Kolde, 2015) and RColorBrewer R packages were used to create the heatmaps. Euclidean clustering was performed to show abundance similarity patterns on taxonomic units. Clustered OTUs at 97% and identified at the species level were labelled "s_" before their classification, the rest were labelled according to their last classification, i.e. g_genus, f_family, o_order, c_class, p_phylum and k_kingdom.

R packages vegan (Oksanen et al, 2017) and fossil (Vavrek, 2011) calculated diversity indices Shannon (diversity), Simpson (dominance) and Chao1 (abundance-based coverage estimator) (Hill, 1973; Chao, 1984).

3.3. Results

3.3.1. Effect of HRT changes on organic matter solubilization

3.3.1.1. OMSW solubilisation

Soluble organic matter variation was evaluated by monitoring sCOD, total and individual VFA accumulation, and total and individual phenolic compound concentrations. During stage I (7-HRT, 0-37 days), sCOD increased to a final value of $11,567 \pm 318 \text{ mg O}_2 \cdot \text{L}^{-1}$ (Figure 3.1), reaching a hydrolysis percentage of $19 \pm 1\%$ of the organic matter fed (Figure 3.2). After increasing the HRT up to 14 days (stage II, 38-112 days), sCOD raised until day 68 and remained almost constant until the end of stage II (day 112) with a mean value of $21,872 \pm 1578 \text{ mg O}_2 \cdot \text{L}^{-1}$ (Figure 3.1). This increment represented an improvement of approximately 89% in the accumulated sCOD compared to the end of stage I (7-HRT, day 37). Furthermore, the increase in sCOD accumulation was also in line with a higher hydrolysis percentage of the OMSW, which increased to $32 \pm 5\%$ during stage II (Figure 3.2). After that, the HRT was increased to 21 days (stage III, 113-176 days), where the sCOD increased again, reaching a maximum sCOD value of $40,000 \pm 4420 \text{ mg O}_2 \cdot \text{L}^{-1}$ on day 148 (Figure 1). This increment represented an improvement of approximately 45% in the accumulated sCOD compared to the end of stage II (14-HRT, day 112). Despite the increase in sCOD, hydrolysis percentage was $19 \pm 2\%$ (Figure 3.2), i.e., 41% less compared to stage II (14-HRT, day 112).

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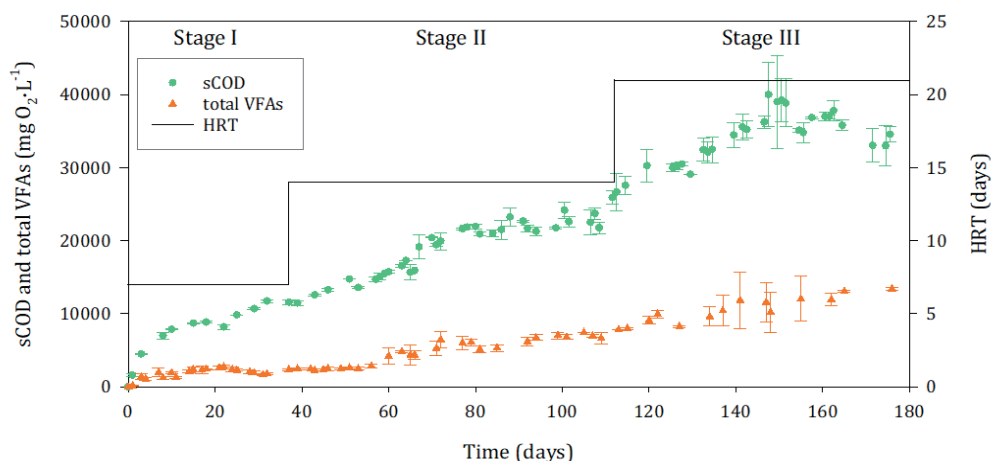


Figure 3.1. Daily variation of sCOD and total VFAs ($\text{mg O}_2 \cdot \text{L}^{-1}$) according to HRT changes at acidic pH-controlled during the experimental time (sCOD: Soluble chemical oxygen demand; VFAs: volatile fatty acids; HRT: hydraulic retention time). Error bars represent standard deviations.

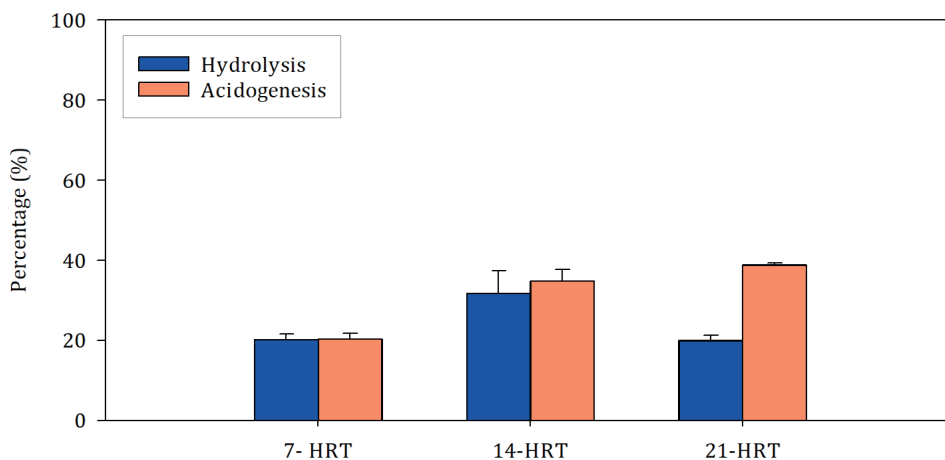


Figure 3.2. Hydrolysis and acidogenesis percentages achieved at the end of each evaluated HRT at acidic pH-controlled during the experimental time (HRT: hydraulic retention time). Error bars represent standard deviations.

3.3.1.2. Accumulation of VFA and VFA profile

During stage I (7-HRT, 0-37 days) there was almost no VFAs accumulation, i.e. only $2346 \pm 104 \text{ mg O}_2 \cdot \text{L}^{-1}$ was reached on day 37 (Figure 3.1), corresponding to $20 \pm 1\%$ of acidogenesis percentage (Figure 3.2). When HRT was extended to 14 days (stage II, 38-112 days), VFA accumulation remained constant until day 51 compared to stage I (7-HRT, 0-37 days). Subsequently, between days 52 and 112, a notable increase in the VFA accumulation was observed, reaching a maximum value at the end of this stage II of $7826 \pm 35 \text{ mg O}_2 \cdot \text{L}^{-1}$ (Figure 3.1). Compared to the end of stage I (day 37), 14-day HRT resulted in a 240% increase in VFA accumulation (Figure 3.1) and a 75 % rise in acidogenesis percentage (up to $35 \pm 3\%$) (Figure 2). Similarly, VFA production continued to increase at stage III (21-day HRT, 113-176 days), reaching a maximum concentration of $13,421 \pm 240 \text{ mg O}_2 \cdot \text{L}^{-1}$ (Figure 3.1), which means an increment of 72% respect to the maximum observed at 14-day HRT (stage II, day 112). The increase in VFA concentration also resulted in an improvement in the percentage of acidogenesis, being 12% higher to 21-day HRT compared to 14-day HRT. During the whole experimentation, despite the progressive increase in total VFA concentration, no strong variations were observed for the VFA profile, showing an average relative abundance of $41 \pm 5\%$ acetic acid, $32 \pm 5\%$ butyric acid, $19 \pm 5\%$ propionic acid, and $8 \pm 4\%$ valeric acid (Figure 3.3).

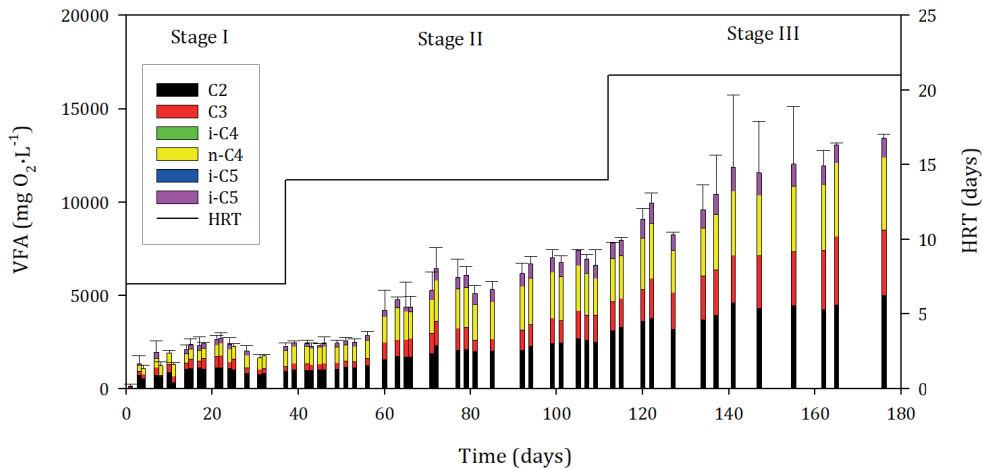


Figure 3.3. Daily variation of individual volatile fatty acids (VFA) ($\text{mg O}_2 \cdot \text{L}^{-1}$) according to hydraulic retention time (HRT) changes at acidic pH during the experimental time (C2: acetic acid; C3: propionic acid; i-C4: iso-butyric acid; n-C4: butyric acid; i-C5: isovaleric acid; n-C5: valeric acid). Error bars represent standard deviations.

3.3.1.3. Accumulation of total and individual phenolic compounds

At the end of stage I (7-HRT, day 37), $340.6 \pm 22.4 \text{ mg gallic acid eq.} \cdot \text{L}^{-1}$ of total soluble phenolic compounds were quantified (Figure 3.4). Hydroxytyrosol and 3,4-dihydroxycinnamic

acid were identified as the predominant individual phenolic compounds, reaching concentrations of $85.8 \pm 0.3 \text{ mg} \cdot \text{L}^{-1}$ and $63.7 \pm 34.0 \text{ mg} \cdot \text{L}^{-1}$, respectively (Figure 3.4). However, at the end of stage II (14-HRT, day 112), total soluble phenolic compounds increased up to $1146.6 \pm 164.7 \text{ mg gallic acid eq.} \cdot \text{L}^{-1}$ (Figure 3.4), i.e., 237% compared to the end of stage I (7-HRT, day 37). Hydroxytyrosol, 3,4-dihydroxycinnamic acid, and tyrosol were identified as the predominant phenolic compounds, whose concentrations increased 227.2 ± 106.5 , 173.7 ± 74.9 and $90.6 \pm 6.5 \text{ mg} \cdot \text{L}^{-1}$, respectively (Figure 3.4).

The highest concentration of total soluble phenolic compounds was observed at the end of stage III (21-HRT, day 176), i.e., $1320.9 \pm 64.7 \text{ mg gallic acid eq.} \cdot \text{L}^{-1}$, 15% more compared to the end of stage II (14-HRT, day 112). Hydroxytyrosol was again identified as the predominant phenolic compound, whose concentration increased up to $348.7 \pm 136.3 \text{ mg} \cdot \text{L}^{-1}$, while the concentration of the other identified phenolic compounds remained at similar concentrations than at the end of the previous stage II (Figure 3.4).

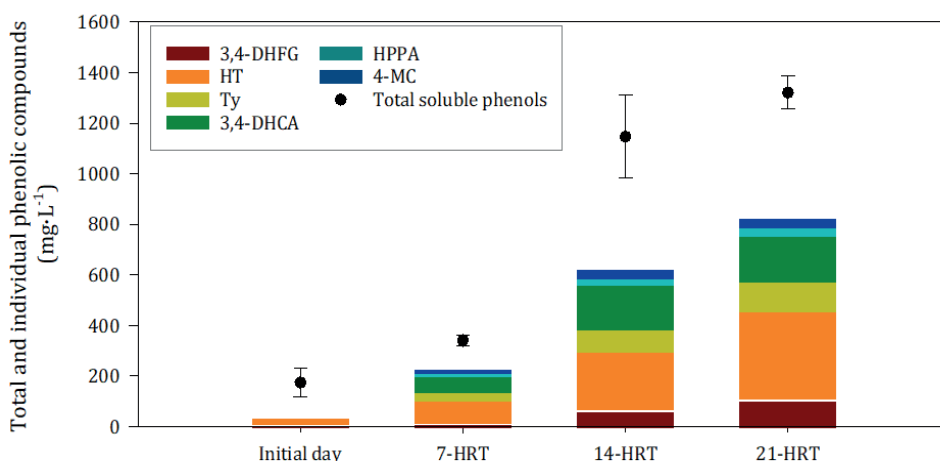


Figure 3.4. Total soluble ($\text{mg gallic acid eq.} \cdot \text{L}^{-1}$) and individual phenolic compounds ($\text{mg} \cdot \text{L}^{-1}$) on the initial and final day at the different hydraulic retention time studied (3,4DHFG: 3,4-dihydroxyphenilglycol; HT: Hydroxytyrosol; Ty: Tyrosol; 3,4-DHCA: 3,4dihydroxycinnamic acid; HPPA: 3,4-hydroxyphenyl propionic acid; 4-MC: 4-methylcatechol; HRT: hydraulic retention time). Error bars represent standard deviations.

3.3.2. Effect of HRT changes on microbial population dynamics

MDS analysis of microbial communities (Figure 3.5), showed three clear clusters of over 65%, one composed of day 15 samples of 7-HRT (stage I, 0-37 days), and a second composed of all other samples throughout the experiment. Both clusters were grouped with a similarity of 55%. The third cluster contained day 0 samples (not shown) which were highly dissimilar to the other clusters. Hence, population composition was established by the from day 37, at the end of stage I, to the end of the experimentation at day 176.

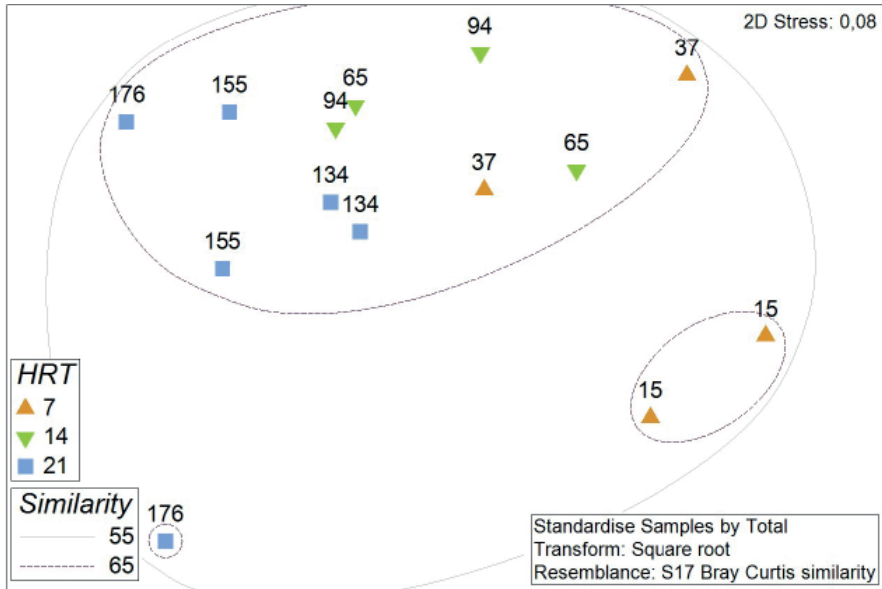


Figure 3.5. Multi-dimensional scaling (MDS) of bacterial communities at genus level based on Bray-Curtis dissimilarity index. Each data point label shows the time (days) at which the sample was taken). Replicates are observed for each condition. Day 0 samples were removed since microbial communities were too dissimilar.

Further analysis of the microbial composition revealed differences between the community structure at different HRT (Figure 3.6). The microbial profile observed at T0 corresponded to that from the inoculum, where *k_Bacteria*, *o_Bacteroidales* and *g_Acetobacterium* were predominant (T0 total rel. abundance >28). Most clusters showed clear changes in abundance from the beginning of stage I (7-HRT, T0) to T15. Most species within cluster vii had a high relative abundance (Figure 3.6). However, these drastically decreased by day 15 of operation. Clusters i-iii, v and vi increased their relative abundance during stage I (7-HRT, T0), maintaining *p_Firmicutes*, *f_Coriobacteriaceae*, *f_Lachnospiraceae* and *o_Bacteroidales*2 (clusters ii & iii) highly abundant throughout the remainder of experimentation time while *o_Bacteroidales* initial predominance stayed constant throughout ($7.3 \pm 4.7\%$). Notably, the relative abundances of species *p_Bacteroidetes*, *f_S24-7* and *s_Clostridium_perfringens* (cluster vi) were only high in stage I (7-HRT, T0). During stage II (14-day HRT, T65 and T94), relative abundance of *g_Ruminococcus*, *f_Sphaerochaetaceae*, high relative abundance was near exclusive to stage II. Finally, at stage III (21-day HRT, T134, T155 and T176), the relative abundance of *o_Bacillales* and *p_Firmicutes* increased significantly, and compared to other stages, so did species *g_Actinomyces*, *f_Ruminococcaceae*, *g_Acetobacter*, *f_Clostridiaceae*, and *g_Candidatus_Ancillula*.

Impact of hydraulic retention time on the production of volatile fatty acids from lignocellulosic feedstock by acidogenic fermentation

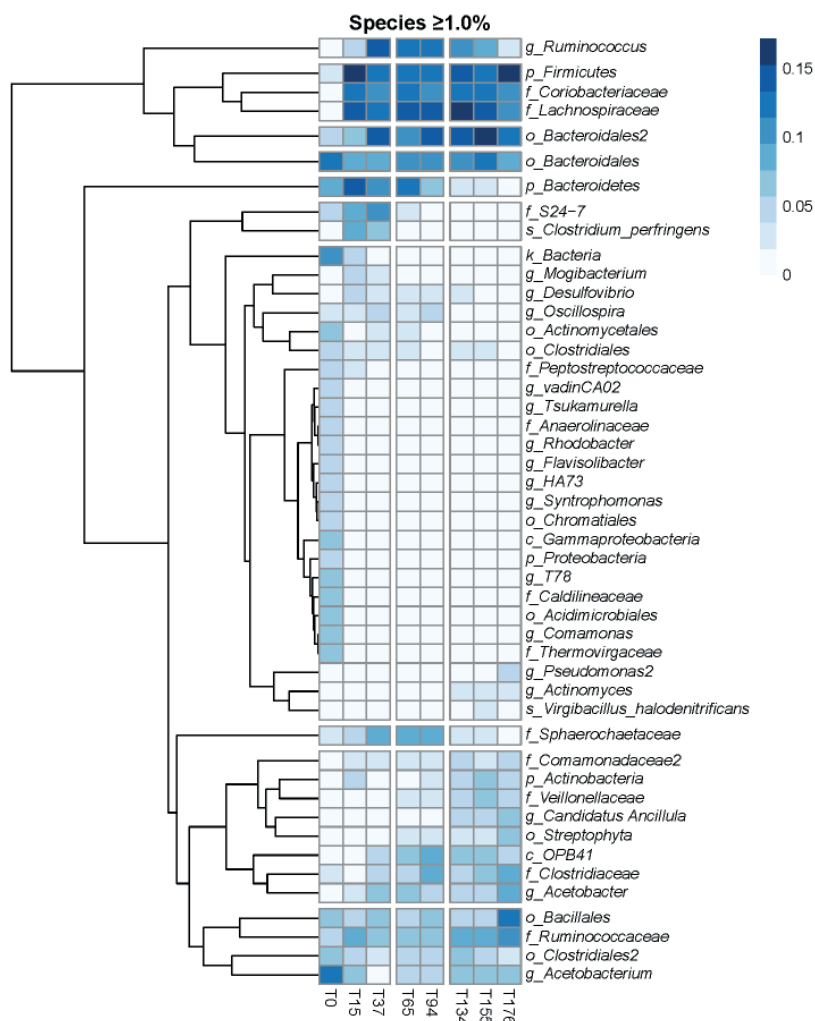


Figure 3.6. Cluster analysis of microbial species in the digestate samples at pH 5 for 176 days. The mean relative abundance was calculated for each time point, followed by data transformations taking its square root, and applying $\log_{10}$ (colour scale legend). The included species were limited to OTUs with a relative abundance of $\geq 1.0\%$, in at least 1 sample. Taxa labels are based on the last known taxon classification. Rows are clustered using Euclidean distance.

Table 2 summarizes the Shannon, Simpson 1-D and Chao1 indices. It can be observed that as HRT increases, species diversity decreases and, at the same time, a slight increment in dominance was also observed (T176, rel. abund. *p_Firmicutes* was 23%, Figure 3.6). Thus, higher microbial diversity is observed at 7-day HRT (Shannon index, Chao1) than for 14 and 21-day HRT, exhibiting specialization with a 21-day HRT (T176) (Simpson 1-D).

Table 3.2. Diversity, dominance and Species richness indices.

		Mean $\pm$ SD		
	Time (days)	Shannon Index	Simpson 1-D	Chao1
7-days HRT	0	5.73 $\pm$ 0.05	0.99 $\pm$ 0	1001 $\pm$ 107.48
	15	4.21 $\pm$ 0.24	0.96 $\pm$ 0	372.5 $\pm$ 61.52
	37	4.03 $\pm$ 0.07	0.96 $\pm$ 0.01	242.5 $\pm$ 34.65
14-days HRT	65	3.85 $\pm$ 0.08	0.96 $\pm$ 0.01	212.5 $\pm$ 34.65
	94	3.61 $\pm$ 0.25	0.94 $\pm$ 0.01	199.5 $\pm$ 37.48
	134	3.81 $\pm$ 0.05	0.95 $\pm$ 0	258.5 $\pm$ 6.36
21-days HRT	155	3.36 $\pm$ 0.18	0.91 $\pm$ 0.04	168.5 $\pm$ 2.12
	176	3.06 $\pm$ 0.15	0.90 $\pm$ 0.02	168 $\pm$ 21.21

3.4. Discussion

The operation in semi-continuous mode in the present research allowed a higher hydrolysis of OMSW in comparison to previous research in batch mode (Jiménez-Páez et al., 2023), increasing from 19% up to $35 \pm 3\%$ (Figure 3.2) from batch to semi-continuous modes. Other studies Lenzuni et al., (2024) and Rincón et al., (2008) have reported biodegradability values reaching 80-90% in semi-continuous anaerobic digestion processes for biogas production from OMSW operated at pH 7. These results suggest that the hydrolysis limitation due to the lignocellulosic composition of OMSW might be overcome by specific operational conditions, instead of a direct limitation due to the substrate composition. In general, it is considered that a longer HRT in a microbial system would increase process yield by extending the reaction time and increasing microbial diversity (Han et al., 2016). Increasing the HRT from 7 to 14 days effectively promoted organic matter solubilization, improving the hydrolysis during the fermentation process of the OMSW (Figures 3.1 and 3.2). The enhancement of the hydrolysis percentage under a 14-day HRT may be correlated with the enrichment of specific microbial populations, as evidenced by the increased relative abundance of *g_Ruminococcus* and *f_Sphaerochaetaceae* species (Figure 3.6). The genus *Ruminococcus* has been described as a cellulolytic bacterium whose function is to produce cellulolytic enzymes to degrade cellulose (Lo et al., 2009). This, in turn, facilitates the accessibility of complex carbohydrates for other members of the microbial community. For example, the cellulolytic activity of *g_Ruminococcus* would favour the growth of *f_Sphaerochaetaceae*, whose fermentative growth thrives with simple carbohydrates including pentose and hexose monosaccharides (Nazina et al., 2022). The joint increment in the relative abundance of both bacteria would indicate a symbiotic interaction between them. Nevertheless, the change from 14 to 21-day HRT did not produce an improvement in hydrolysis percentage, observing an increase in disturbances in the operation from day 140 onwards (Figure 3.1). The latter could be related to the greater accumulation of sCOD in the reactors (Figure 3.1), including substances with inhibitory potential such as VFA (Figure 3.3), which ultimately compromised the stability of the operation (Wang et al., 2020). This operational instability was reflected in the microbial

communities, where the relative abundance of the species described as key decreased during the operation at 14-day-HRT, i.e., *g_Ruminococcus* and *f_Sphaerochaetaceae* (Figure 3.6). Similarly, the relative abundance of the species *p_Bacteroidetes* (Figure 3.6), described to be involved in carbohydrate, proteins and peptides biodegradation processes (Lv et al., 2022), also decreased in 21-day HRT (Figure 3.6).

The acidogenesis rate reached for 7-day HRT was similar to the maximum acidification obtained for OMSW acidic batch operation, i.e., 20% (Jiménez-Páez et al., 2023). The increment in the hydrolysis rate observed with an increase in HRT (14- to 21 day) was in line with the results reported by Llamas et al. (2022). These authors described an improvement in the acidogenesis of microalgae from 48% to 70% from 2 to 10-day HRT. Increasing the HRT entailed a greater time for the acidification of OMSW in the reactors, as well as enough time for the microbial community to adapt and specialize to the new HRT operational conditions. Hence, an increment in the relative abundance of species *p_Firmicutes* and *f_Veillonellaceae* was observed (Figure 3.6), which was correlated with an increase in acidogenesis observed at 21-day HRT. Furthermore, the relative abundance of the species *p_Firmicutes* remained high throughout the experiment, although it increased by 21 day-HRT, corresponding to the improvement in VFA production. Species from the phylum *Firmicutes* have also been reported in acidogenic fermentation as a species with positive effects on VFA production (Zhang et al., 2020). Moreover, *f_Veillonellaceae* species have been described as producing longer chain acids, that agrees with the increase in VFA production at 21-day HRT (Strazzera et al., 2021). The accumulation of VFA was also possible due to the absence of methanogenic archaea in the reactors (Figure 3.6), which were effectively removed at operating at acidic pH 5 (Qiu et al., 2023; Zhou et al., 2023).

Despite the interest in VFA accumulation (the highest VFA concentration was reached at 21-day HRT) the latter could also be the cause of the processs destabilization at stage III. Destabilization in stage III was considered due to different factors: i) the decrease in the accumulated sCOD in the reactors (from day 148, Figure 3.1), ii) the lower hydrolysis compared to HRT 14-day (Figure 3.2), and iii) an erratic daily performance of the reactors, whose daily production of sCOD and VFA strongly varied along this stage III, which can be observed by the large error bars of these operation days (Figures 3.1 and 3.3). At low pH, carboxylic acids are found in a non-dissociated form, allowing passage into the bacteria by passive diffusion. Protons are thus released into the medium and accumulate, while the anion can accumulate in the bacteria leading to toxicity and enzyme inhibition (López-Garzón & Straathof, 2014). In parallel to the OMSW hydrolysis and VFA accumulation, phenolic compounds were also released and accumulated in the digesters. These well-known microbial inhibitors reached a maximum concentration of 1320.9 ± 64.7 mg gallic acid eq. $\cdot$ L⁻¹ (Figure 3.4), much lower than the reported inhibition limit in anaerobic digestion, i.e., 2000 mg gallic acid eq. $\cdot$ L⁻¹ (Calabrò et al., 2018). Similarly, the concentrations of the determined individual compounds also remained several orders of magnitude below the inhibition limit described in literature, i.e., furfural (4 g $\cdot$ L⁻¹), hydroxymethylfurfural (5 g $\cdot$ L⁻¹), tyrosol (<2 g $\cdot$ L⁻¹) and vanillin (>2 g $\cdot$ L⁻¹) (Caroca et al., 2021). Therefore, it is more probable that the destabilization of the process was related to the VFA accumulation instead of the phenolic compound accumulation.

The acidogenic metabolic pathway at acidic pH is crucial for the accumulation of

VFA (Zhou et al., 2018). As the present research shows (Figure 3.3), low variations in the VFA profile during the whole experimentation have also been reported for the anaerobic fermentation of kitchen waste and fruit and vegetable waste operating under acidic conditions as a consequence of HRT changes (Liu et al., 2023; Swiatkiewicz et al., 2021). These authors obtained as major VFA compounds acetic and butyric acids in approximate abundance ranges, i.e., 30-50% and 24-30%, respectively (Liu et al., 2023; Swiatkiewicz et al., 2021). The complex VFA profile throughout the entire experimental time could be related to the presence of microorganisms described as mixed acid fermenters, such as *f_Sphaerochaetaceae* or *p_Firmicutes* (Figure 3.6) and identified acetogenic species *g_Acetobacter* and *g_Acetobacterium* (Figure 3.6), which produce acetic acid from alcohols and CO/CO₂, respectively (Kremp & Müller, 2020).

3.5. Conclusions

The production of VFA was limited by the degradation of organic matter at 7-day HRT. Increasing to 14-day HRT promoted VFA accumulation by 240% relative to 7-day HRT, also improving the hydrolysis around 68%. The enhancement of the hydrolysis at 14-day HRT was correlated with the enrichment of specific hydrolytic microorganisms, i.e., *g_Ruminococcus* and *f_Sphaerochaetaceae*. 21-day HRT did not improve hydrolysis, possibly due to destabilization of the reactor and a decrease in the relative abundance of *g_Ruminococcus* and *f_Sphaerochaetaceae*. However, the increase to 21-day HRT enhanced the acidogenesis, which might be related to an enrichment of *p_Firmicutes* and *f_Veillonellaceae*.

CRedit author statement

E. Jiménez-Páez: Methodology, Formal analysis, Investigation, Writing - Original Draft. A. Serrano: Conceptualization, Writing - Original Draft, Funding acquisition. J. Purswani: Formal analysis, Writing - Review & Editing. Ángeles Trujillo-Reyes: Formal analysis, Writing - Review & Editing. Africa Fernández-Prior: Methodology, Resources. F. G. Fermoso: Conceptualization, Supervision, Funding acquisition.

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Data Availability

The sequence data from this study have been submitted to NCBI BioProject (<http://www.ncbi.nlm.nih.gov/bioproject>) under BioProject number PRJNA1106357.

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3.7. Supplementary material



Figure 3.7. Multi-dimensional scaling (MDS) of bacterial communities at genus level based on Bray-Curtis dissimilarity index. Each data point label shows the time (days) at which the sample was taken. Replicates are observed for each condition.

Impact of hydraulic retention time on the production of volatile fatty acids from
lignocellulosic feedstock by acidogenic fermentation



Chapter 4

Influence of the hydraulic retention time over the specialization of the microbial community in the alkaline fermentation of olive mill solid waste

Abstract

Anaerobic fermentation is a bioprocess applied to produce volatile fatty acids (VFA) from an organic biomass such as the olive oil byproducts (olive mill solid waste, OMSW). The hydrolysis is the limiting step of the system for lignocellulosic feedstocks so, the aim of this research was to evaluate the influence of the hydraulic retention time (HRT) on the long-term anaerobic fermentation of OMSW under alkaline conditions to maximize the production of VFAs and organic matter solubilization. This approach enabled the correlation between microorganisms as process parameters changed. Results showed that 14-day HRT yielded the highest hydrolysis rate (53%), while 21-day HRT maximized VFA accumulation ($10,035 \pm 651 \text{ mg O}_2 \cdot \text{L}^{-1}$). Acetic acid dominated the VFA profile, comprising 80-90% during the whole experimentation time. The higher relative abundance of *s_Virgibacillus halodenitrificans* when HRT were lengthened could explain the observed increment in VFA solubilisation and concentration.

Keywords

Acetic acid; anaerobic fermentation; lignocellulosic feedstock; olive mill solid waste; volatile fatty acids accumulation.

4.1. Introduction

The hydrolysis stage of the anaerobic fermentation network has been shown to be the main rate-limiting step in the biological conversion of particulate organic matter (Liu et al., 2018). This is particularly critical for lignocellulosic feedstock, where the structure of the fibres limits the action of the involved hydrolytic enzymes (Huang et al., 2018; Xu et al., 2021). Previous studies have demonstrated that applying physical, hydrothermal, chemical and/or other pre-treatments could improve the hydrolytic stage in a subsequent anaerobic fermentation process (Eskicioglu et al., 2017; Frigon et al., 2012; Olokede et al., 2022; Show et al., 2023). However, these energy-demanding pre-treatments would not be advantageous due to the generation of microbial inhibitors or insufficient biogas production improvement to compensate for the pre-treatment energy demand (Cano et al., 2015).

Anaerobic fermentation operation under alkali conditions accelerates the degradation of lignocellulosic materials since proteins and carbohydrates are decomposed by the release of carboxyl groups, facilitating the bioavailability of the substrate to microorganisms (Dahiya et al., 2015; Zhao et al., 2020), without high energy consumptions. Hence, metabolic pathways may simultaneously lead to the accumulation of VFA by inhibiting the methanogenesis due to the sensitivity of methanogenic archaea to pH variations (Zhao et al., 2020).

In addition to archaea inhibition, operational pH can also determine the amount and profile of VFA produced by determining which microbial pathways are promoted or hindered and, thus, defining the microbial community that thrives in the reactor (Chen et al., 2017; Owusu-Agyeman et al., 2022). For example, Jiménez-Páez et al., (2023) reported that for the anaerobic fermentation of 2-phased olive mill solid waste (OMSW) at acidic pH a complex profile of VFA (C2-C5) enriched in propionic and butyric acid was obtained, while, at alkaline pH (9 and 10), acetic acid was almost the only accumulated acid. Similarly, there are studies for other substrates, such as fruit and vegetable waste (Khatami et al., 2021), swine manure (Lin et al., 2015) waste activated sludge (Li et al., 2022) that report acetic acid as the predominant acid at alkaline pH fermentations. Acetic acids and its derivatives are used in various industrial processes, such as food preservation (Widawati et al., 2024), in the cosmetic industry, where ethyl acetate is used as a flavour enhancer in different products (Bhatia & Yang, 2017), or the textile industry, where polyvinyl acetate is used in paints and adhesives (Pal & Nayak, 2017). The production of VFA, such as acetic acid with a market price of 890 USD per tonne (Chen et al., 2022), would be an environmental and economic sustainable management for the valorisation of OMSW.

Previous studies have evaluated the VFA production and accumulation changes at different operational conditions such as hydraulic retention time (HRT) (Aboudi et al., 2023), pH (Jiménez-Páez et al., 2023), and/or temperature (Yuan et al., 2016) in the anaerobic fermentation for different substrates. pH and HRT were identified as the critical factors influencing the yield and composition of VFAs during anaerobic fermentation (Lv et al., 2022). There are limited studies on the microbial population dynamics that occur during long-term anaerobic fermentations, which could provide useful information about the robustness and stability of the system. For example, Liu et al. (2023) evaluated long term operation at alkaline pH, different HRT (12, 5 and 3 days) and organic loading rates (OLR) of fruit and vegetable

waste which has carbohydrate-rich correlated with the population dynamics. These authors observed that modifying the operational conditions a dominance of the genus *Lactobacillus* was correlated with a high lactic acid production. Owusu-Agyeman et al., (2022) evaluated a co-fermentation of sludge from wastewater and other organic wastes such as feedstock, applying long term operation changed the pH from 10 to 9, obtaining a higher accumulation of VFA at pH 9 which could be correlated with the increase in the relative abundance of *Dysgonomonadaceae* bacteria. Moreover, Yuan et al., (2016) evaluated a long-term alkaline fermentation of sludge at psychrophilic and mesophilic temperatures to correlate it with the microbial population. This research showed a correlation between the VFA production and the relative abundance of some bacteria such as *Paludibacter* genus, described as VFAs producers. Despite that, there is still a need for further research of long-term fermentation by modifying other crucial operational parameters and correlating it with the evolution of the microbial community. Furthermore, there is not previous long-term anaerobic fermentation studies using OMSW, a complex substrate that combines lignocellulosic fibres, fats, organic acids and microbial inhibitors such as phenolic compounds (Serrano et al., 2017).

Thus, the aim of this research was to evaluate the influence of the HRT on the long-term anaerobic fermentation of OMSW under alkaline conditions to maximize the VFA production and organic matter solubilization. This approach enabled the examination of the correlation between microorganisms, control of the reactor parameters and the outcome of the process.

4.2. Material and methods

4.2.1. Substrate and Anaerobic inoculum

OMSW was obtained from the "Oleícola El Tejar Ntra. Sra. de Araceli" pomace plant, located in Marchena (Seville), Spain, and was stored in containers at -20 °C. The main characteristics were for OMSW: pH: 4.6 ± 0.1 ; Volatile Solids/Total Solids (VS/TS): $94 \pm 0.1\%$; volatile solids: $252,604 \pm 2999 \text{ mg}\cdot\text{kg}^{-1}$; moisture: $74.8 \pm 0.3\%$; soluble chemical oxygen demand (sCOD): $81,455 \pm 90 \text{ mg O}_2\cdot\text{L}^{-1}$; total chemical oxygen demand (tCOD): $333,695 \pm 31,335 \text{ mg O}_2\cdot\text{L}^{-1}$; total phenols: $6135 \pm 131 \text{ mg gallic acid}\cdot\text{L}^{-1}$; hydroxytyrosol: $2750 \pm 65 \text{ mg}\cdot\text{L}^{-1}$; tyrosol: $799 \pm 11 \text{ mg}\cdot\text{L}^{-1}$; 3,4- dihydroxyphenilglycol: $296 \pm 6 \text{ mg}\cdot\text{L}^{-1}$.

The anaerobic inoculum used in the experimentation was obtained from an industrial anaerobic reactor of the wastewater treatment plant "COPERO" located in Seville, Spain. The main anaerobic inoculum characteristics were pH: 7.3 ± 0.1 ; alkalinity: $5312 \pm 48 \text{ mg CaCO}_3\cdot\text{L}^{-1}$; VS: $22,342 \pm 41 \text{ mg VS}\cdot\text{kg}^{-1}$; sCOD: $664.50 \pm 5 \text{ mg O}_2\cdot\text{L}^{-1}$.

4.2.2. Anaerobic fermentation experimental procedure

Duplicate experiments were conducted in a semi-continuous mode under alkaline pH conditions (pH= 9), in fermentation reactors. The reactors had a working volume of 1.6 L and each one was inoculated with $10 \text{ g VS}\cdot\text{L}^{-1}$ of fresh inoculum. The reactors were operated under mesophilic conditions (35-37 °C) applying variations in HRT. HRT of 7 days, 14 days, and 21 days were tested. In stages I and II, the operational conditions were

maintained during the time corresponding to five HRT, while in stage III the experimental time was maintained during the time corresponding to three HRT. An organic loading rate of 3 g VS of OMSW·(L·d)⁻¹ was set and maintained throughout the whole experimentation. The operational pH for each reactor was continuously controlled using HP series proportional flow pumps (4-20 mA) (MYTHO technologies, Hunenberg, Switzerland), following the procedure detailed in Jiménez-Páez et al. (2023). Methane production was measured by liquid displacement using 0.5 L gasometers submerged in 2N NaOH solutions to remove the CO₂ from the generated gas.

4.2.3. Chemical analysis

The characterization of OMSW, anaerobic inoculum and samples obtained from the reactors were determined following analytical test. TS total solids (TS), volatile solids (VS), alkalinity, and soluble chemical oxygen demand (sCOD) were measured in the samples according to the APHA Standard Methods (Baird et al., 2017). VFAs (C2-C5) were determined in duplicate using a gas chromatograph Shimadzu GC-2025 equipped with a Stabilwax-DA column (Crossbond Carbowax polyethylene glycol, RESTEK) of 0.25 mm i.d. x 30 m and a flame ionization detector (FID). The carrier gas (nitrogen, hydrogen, air) flowed at 40 mL·min⁻¹ at 504 kPa, with individual flow rates of 30 mL·min⁻¹ for nitrogen, 40 mL·min⁻¹ for hydrogen, and 400 mL·min⁻¹ for air. The FID detector temperature was set to 250 °C. The total soluble phenols were determined by the Folin-Ciocalteu method (Singleton & Rossi, 1965). The phenolic compound profile was analysed in a Hewlett-Packard 1100 series high performance liquid chromatography equipment coupled with a diode array detector and an Agilent 1100 automatic injector (20 µL) as described by Fernández Prior et al. (2023).

4.2.4. Hydrolysis and acidogenesis calculations

Hydrolysis ratio (Eq. 1) and acidogenesis ratio (Eq.2) were calculated from a COD balance in the anaerobic fermentation process, based in the expressions reported by Wu et al. (2015):

$$\text{Hydrolysis ratio (\%)} = \frac{([sCOD] + [COD_{CH_4}])_{\text{effluent}} - ([sCOD])_{\text{influent}}}{([tCOD] - [sCOD])_{\text{influent}}} \times 100 \quad \text{Eq. 3.1}$$

$$\text{Acidogenesis ratio (\%)} = \frac{(s[COD_{VFA}])_{\text{effluent}}}{([sCOD])_{\text{influent}}} \times 100 \quad \text{Eq. 3.2}$$

[sCOD]= sCOD concentration (g O₂·L⁻¹); [COD_{CH₄}] = CH₄ concentration calculated as COD (g O₂·L⁻¹); [tCOD]= tCOD concentration (g O₂·L⁻¹); [COD_{VFA}]= VFA concentration calculated as COD (g O₂·L⁻¹).

4.2.5. Statistical analysis

The results are expressed as mean values with their corresponding standard deviations from the replicate reactors. The normal distribution of the data was confirmed through the application of the Shapiro–Wilk test. All pairwise multiple comparisons were calculated with Dunn's Method (for sCOD) and Holm-Sidak method (for sCOD and VFA). These statistical tests were done assuming normal distribution and homoscedasticity of the raw data. A one-way analysis of variance (ANOVA) was conducted to evaluate statistical differences. All statistical analyses were performed using SigmaPlot 15.0 (Systat Software Inc., San Jose, CA, USA) with a significance threshold set at $p < 0.05$.

4.2.6. Molecular analyses of microbial communities

4.2.6.1. DNA extraction

Eight samples were extracted directly from each of duplicate reactors on days 0, 15, 37, 65, 94, 134, 155 and 176 to monitor the microbial community. The microbial communities of the samples were analysed for their relationship between the operating conditions with the VFA production. The samples were centrifuged at 1000 rpm for 13 minutes to separate the solid phase from the digestate. Then, approximately 0.5 g of the wet solid phase was transferred to the dissolution matrix, immediately frozen in liquid nitrogen and stored at -80 °C. DNA was extracted from the solid phase of the digestate, using the FastDNATM Pro Soil-Direct Kit (Biomedicals) with the FastPrep-24TM Classic bead beating system/MP Biomedicals. Amplicon sequencing was performed by the Genomic Unit at the Instituto de Parasitología y Biomedicina-López Neyra (CSIC, Granada, Spain) on the V3/V4 region of the 16S rDNA (Jiménez-Páez et al., 2023). Briefly, after DNA extracts were normalized to the same concentration, a 2-step PCR amplified and indexed the V3/V4 region [First PCR step: Primers 16S_ProV3V4_forward (5'-CCTACGGGNNBGCASCAG-3') and 16S_ProV3V4_reverse (5'-GACTACNVGGGTATCTAATCC-3'), each with the adaptor (5'-CTGTCTCTTATACACATCT-3'), were used to amplify the V3/V4 region. cycling conditions: 95 °C, 3 min; (95 °C, 30 s; 55 °C, 30 s; 72 °C, 30 s) x 25 cycles; 72 °C, 5 min; and 4 °C, hold. Second PCR step: Sample indexing using the Nextera XT Index Kit v2, for a further 8 cycles]. Bidirectional sequencing was carried out on a MiSeqTM System (Illumina, 2x275 cycles) (Takahashi et al., 2014). Demultiplexed sequences obtained from the MiSeq™ System were processed in QIIME2 v2019.10 (Bolyen et al., 2019). Cutadapt (Martin, 2011) was used for primer removal, pair-end reads were joined using vsearch (maxdiff=20) (Rognes et al., 2016), and denoised with DADA2 (Callahan et al., 2016) using a quality threshold of 20, and truncating the sequence length at 400bp. The taxonomy classifier was trained using the primers above on the Greengenes database version 13.5. Taxonomic assignation at the species level was applied, and OTUs were classified according to their last known taxon.

4.2.6.2. Statistical analysis applied to molecular analyses

Non-metric multi-dimensional scaling (NMDS) was performed using the software PRIMER-E, v.6.0, (Plymouth, UK), and analysis of similarities (ANOSIM) was based on Bray-Curtis distance.

Pheatmap (Kolde, 2015) and RColorBrewer R packages were used to create the heatmaps. Euclidean clustering was performed to show abundance similarity patterns on taxonomic units. Clustered OTUs at 97% and identified at the species level were labelled "s_" before their classification, the rest were labelled according to their last classification, i.e. g_genus, f_family, o_order, c_class, p_phylum and k_kingdom.

R packages vegan (Oksanen et al., 2017) and fossil (Vavrek, 2011) calculated diversity indices Shannon (diversity), Simpson (dominance) and Chao1 (abundance-based coverage estimator) (Chao, 1984; Hill, 1973).

4.3. Results and discussion

4.3.1. Organic matter solubilization and VFA dynamics

A stable concentration of sCOD, with no significant differences (p value= 0,293), was observed at 7-day HRT from day 7 to day 37, with a mean value of $11,139 \pm 2680 \text{ mg O}_2 \cdot \text{L}^{-1}$. The sCOD increased up to $14,916 \pm 160 \text{ mg O}_2 \cdot \text{L}^{-1}$ on the day 37 (7-day HRT). This increase was statistically significant (p value= 0.015) when compared to the sCOD concentration obtained up to day 7. HRT increase to 14 days resulted in a gradual raise of the accumulated sCOD until day 79 (p value<0.005), in which the sCOD stabilised at values around $28,000 \text{ mg O}_2 \cdot \text{L}^{-1}$ towards the end of the operation at 14-day HRT (day 112) (p value= 0.974) (Figure 4.1). The maximum sCOD accumulation was obtained with 21-day HRT, reaching a maximum value of $46,612 \pm 3447 \text{ mg O}_2 \cdot \text{L}^{-1}$ (day 165), 213% and 65% higher than the sCOD obtained with 7-day HRT (day 37) and 14-day HRT mean value, respectively (Figure 4.1). Operating at alkaline conditions (pH 9) could favour the degradation of cell walls in lignocellulosic materials and the accumulation of sCOD, since proteins and carbohydrates are decomposed by the release of carboxyl groups, facilitating the bioavailability of the substrate to microorganisms (Dahiya et al., 2015; Zhao et al., 2020). After this maximum observed accumulation, a decline in sCOD was observed after day 165 (Figure 4.1), suggesting a destabilisation of the reactor performance. The accumulation of this sCOD would entail that toxic compounds may accumulate at concentrations higher than the resilience limit of the microbial population (Li et al., 2024).

Considering the balance among the added substrate and the accumulation of sCOD, it was observed that the hydrolysis in the last HRT operated at 7-day HRT, i.e. from day 30 to 37, was $34 \pm 11\%$. Increasing the HRT up to 14 days, the hydrolytic percentage improved to $53 \pm 9\%$. Nevertheless, when the HRT increased from 14 to 21 days, the hydrolytic percentage was affected by the destabilisation in the reactor, decreasing to $28 \pm 6\%$. The results of the hydrolytic percentages of this research at 14-day HRT were similar than the results reported by Jiménez-Paez et al. (2023) using OMSW feedstock in batch fermentation

at pH 9, i.e. around 50%. Despite not improving the hydrolytic percentage in the present research by changing the type from batch to semi-continuous operation mode, higher sCOD concentrations were obtained ($>40,000 \text{ mg O}_2 \cdot \text{L}^{-1}$), since Jiménez-Paez et al. (2023) only reported a maximum sCOD concentration of around $8000 \text{ mg O}_2 \cdot \text{L}^{-1}$.

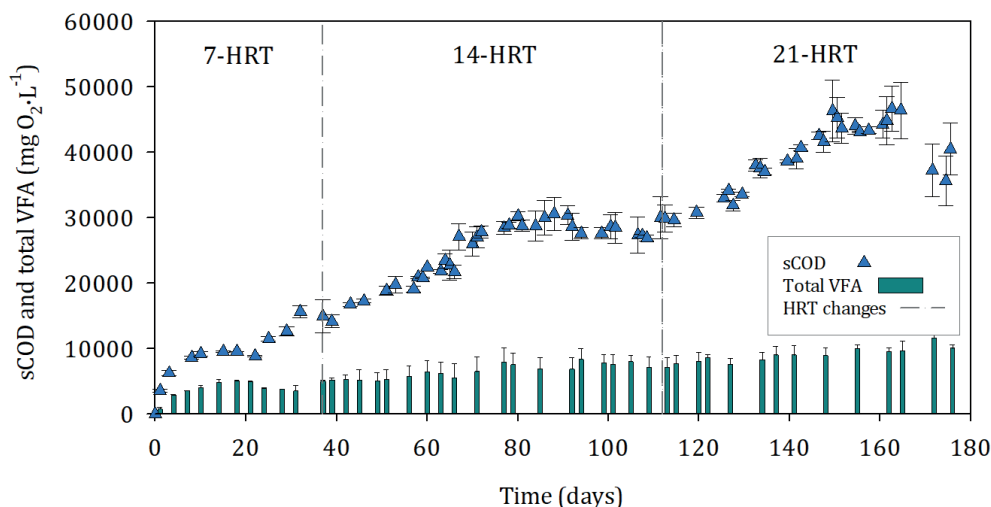


Figure 4.1. sCOD and total VFA ($\text{mg O}_2 \cdot \text{L}^{-1}$) dynamics with HRT changes at alkaline pH. sCOD: soluble chemical oxygen demand. VFAs: volatile fatty acids. HRT: hydraulic retention time.

The operation at 7-day HRT showed the lowest VFA accumulation among the HRT tested, with a value at the end of this condition (day 37) of $4977 \pm 1551 \text{ mg O}_2 \cdot \text{L}^{-1}$ (Figure 4.1), in line with the lowest sCOD accumulation previously described. When HRT increased up to 14 days, VFA concentration reached a value of $7050 \pm 840 \text{ mg O}_2 \cdot \text{L}^{-1}$ (Figure 4.1), i.e., 42% higher than 7-days HRT ($p < 0.001$). Similarly, at the end of 21-day HRT (day 176) the highest VFA accumulation was obtained with a value of $10,035 \pm 651 \text{ mg O}_2 \cdot \text{L}^{-1}$ (Figure 4.1). It is worth to noting that, in contrast to sCOD, the concentration of VFA did not show a marked drop during the last days of operation. The observed VFA increase would be expected since a longer HRT has been related to a higher resilient operation by promoting a robust microbiome (Gonçalves et al., 2024).

The produced VFA profile showed a high preponderance of acetic acid, accounting for an average relative abundance of $86 \pm 3\%$ acetic acid and $14 \pm 3\%$ propionic acid throughout the experiment (Figure 4.2). The accumulation of acetic acid at alkaline pH is likely to be attributed to the total inhibition of acetoclastic methanogenesis (Li et al., 2017). In addition, alkaline conditions could reduce microbial activity and increase the dominance of some acetic acid-producing bacteria (Chen et al., 2021; Esteban-Gutiérrez et al., 2018). These results agree with the results reported by Jankowska et al. (2017), which related the operation at pH 11 with the preponderance of acetic acid (70-90%) followed by propionic acid (5-21%) as the main products in the fermentation of lignocellulosic substrates.

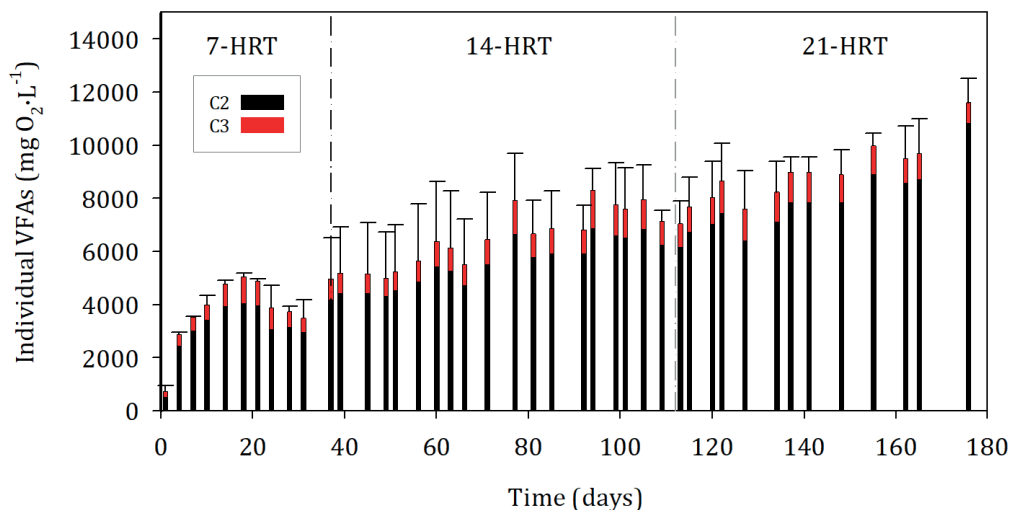


Figure 4.2. Individual volatile fatty acids (VFA) ($\text{mg O}_2 \cdot \text{L}^{-1}$) production dynamics with HRT changes at alkaline pH (C2: acetic acid; C3: propionic acid).

4.3.2. Phenolic compounds

Total soluble phenols and individual phenolic compounds were monitored in the reactors at the different assessed HRT due to the high quantity of phenolic compounds contained in the original OMSW. A positive correlation was observed between HRT and the accumulation of total soluble phenols (Figure 4.3). The total soluble phenols concentration on the final day of each experimental HRT were $888 \pm 114 \text{ mg gallic acid eq} \cdot \text{L}^{-1}$, $1322 \pm 41 \text{ mg gallic acid eq} \cdot \text{L}^{-1}$ and $1590 \pm 116 \text{ mg gallic acid eq} \cdot \text{L}^{-1}$ for 7, 14 and 21-day HRT, respectively. Despite this, the accumulated soluble phenols concentration remained notably lower than the quantity of total soluble phenols fed from the OMSW, meaning removal efficiencies of 94%, 91% and 88% at 7, 14 and 21 day-HRT, respectively. Despite the accumulation of some phenolic compounds with increasing HRT, these concentrations weren't high enough to define the inhibition observed (Tafesh et al., 2011).

Hydroxytyrosol (HT) and Tyrosol (Ty) were identified as the predominant individual phenolic compounds, reaching a maximum concentrations of $224 \pm 120 \text{ mg HT} \cdot \text{L}^{-1}$ on day 71 (14-day HRT), and $131 \pm 14 \text{ mg Ty} \cdot \text{L}^{-1}$ on day 140 (21-day HRT), respectively (Figure 4.3). On day 112 (14-days HRT), HT represented around 0.15% of the total soluble compounds (Figure 4.3). Hence, increasing HRT from 7 to 21 days, the relation of total soluble phenols that corresponds to non-identified individual phenolic compounds increased respect previous conditions (Figure 4.3). That might be a consequence of the release of non-individually identified soluble phenolic fibres during the degradation of the OMSW (Rodríguez et al.,

2009), that would also explain the slight decrease in the phenol removal efficiency at 21-days HRT. Interestingly 3,4-dihydroxyphenilglycol (DHPG) accumulated 1.5% at 14-day HRT, whereas its concentration accumulated up to 14% at 21-day HRT (Figure 4.3).

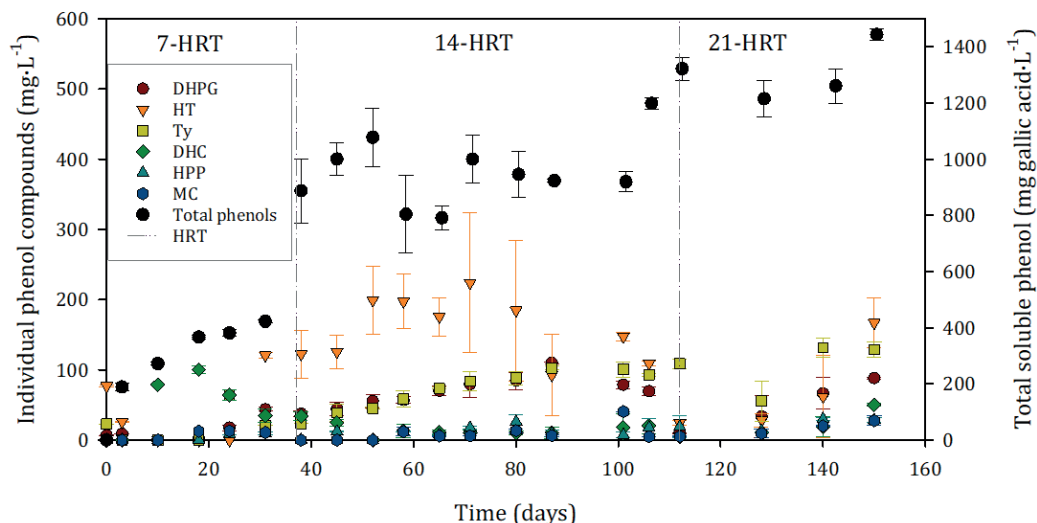


Figure 4.3. Total and individual phenolic compounds ($\text{mg}\cdot\text{L}^{-1}$) for all hydraulic retention times (HRTs) evaluated during the whole experimentation time at alkaline pH conditions (DHPG: 3,4-dihydroxyphenilglycol; HT: Hydroxytyrosol; Ty: Tyrosol; DHC: 3,4 dihydroxycinnamic acid; HPP: 3,4-hydroxyphenyl propionic acid; 4-MC: 4 methylcatechol; HRT: hydraulic retention time).

4.3.3. Microbial responses to HRT

NMDS analysis of microbial communities showed three clear clusters of over 50%: one composed by the inoculum consortia (day 0), a second composed of day 15 and 37 (7-day HRT), and a third cluster composed of all other samples throughout the experiment (14-day HRT and 21-day HRT) (Figure 4.4). Overall, diversity indices decreased with time and an increase in HRT, however a sharp decrease was observed from day 0 to day 15. At HRT 14 and 21-days, similar diversity index values were observed, i.e. Shannon index ranged from 2.5 to 3.0 and Chao1 values below 130 (Table 4.1). An increase in microbial dominance is also correlated with time and an increase in HRT (Simpson 1-D index. Table 4.1). These results were expected, as microorganisms adapt to alterations in operational conditions, resulting in a decrease in microbial diversity and a consequent increase in the dominance of specific species (Pasalari et al., 2021; Temudo et al., 2008; Zhou et al., 2016).

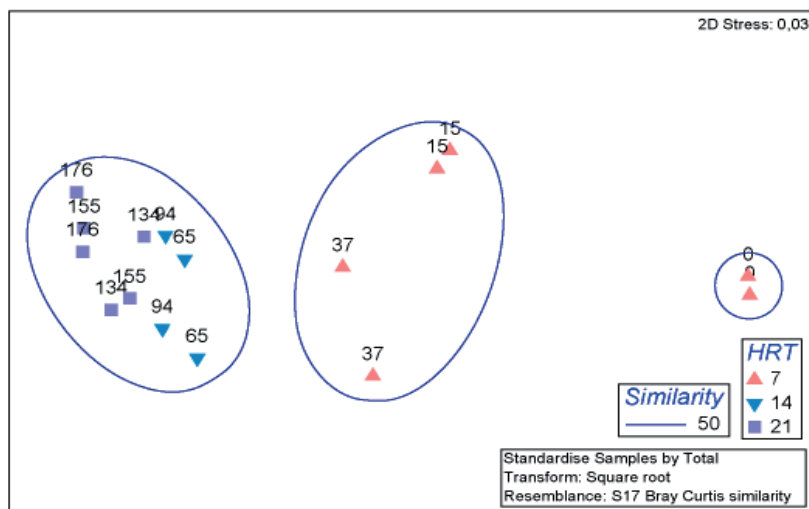


Figure 4.4. Multi-dimensional scaling (MDS) of bacterial communities at genus level based on Bray-Curtis dissimilarity index. Each data point label is the day at which the sample was taken. Replicates are observed for each condition.

Table 4.1. Diversity, dominance and Species richness indices.

		Mean $\pm$ SD		
	Time (days)	Shannon Index	Simpson 1-D	Chao1
7-days HRT	0	5.76 $\pm$ 0.19	0.99 $\pm$ 0.00	1106.50 $\pm$ 132.23
	15	3.80 $\pm$ 0.00	0.96 $\pm$ 0.00	308.00 $\pm$ 4.24
	37	3.48 $\pm$ 0.26	0.94 $\pm$ 0.02	187.50 $\pm$ 3.54
14-days HRT	65	2.99 $\pm$ 0.06	0.91 $\pm$ 0.00	124.50 $\pm$ 0.71
	94	2.77 $\pm$ 0.10	0.89 $\pm$ 0.02	112.50 $\pm$ 10.61
	134	2.58 $\pm$ 0.22	0.87 $\pm$ 0.03	88.50 $\pm$ 17.68
21-days HRT	155	2.52 $\pm$ 0.00	0.87 $\pm$ 0.00	86.50 $\pm$ 7.78
	176	2.51 $\pm$ 0.01	0.86 $\pm$ 0.02	126.00 $\pm$ 4.24

Species with greater than 1.0% relative abundance in at least 1 sample were clustered into 10 groups arbitrarily according to their relative abundance throughout the experiment (Figure 4.5). In clusters i, ii, ii, viii, ix, and x, we can observe an increase in their relative abundance in 14 and 21-day HRT compared to 7-day HRT. Hence, these species are positively correlated with an increase in VFA concentration. The others show a marked decrease with increasing HRT. As observed in Figure 4.5, the species with the highest relative abundances at 7-day HRT were *o_Bacteroidales*, *p_Firmicutes*, *g_Clostridium*, *o_Clostridiales*. The order

Bacteroidales have species described as protein and amino acid hydrolytic bacteria being able to degrade organic matter into acetic acid (Liu et al., 2025; Mei et al., 2020). Species from the phylum *Firmicutes* have also been reported in acidogenic fermentation as a species with positive effects on VFA production (Zhang et al., 2020). Moreover, *Clostridium* have been identified as acidogenic bacteria fermenting sugars to produce acetic acid (da Fonseca et al., 2023; Patakova et al., 2013), which may be related to the increase of acetic acid at this HRT.

At 14-day HRT, a decrease in the relative abundance of *g_Clostridium*, *o_Clostridiales* compared to 7-day HRT was observed (Figure 4.5). At the same time, relative abundance of *s_Virgibacillus_halodenitrificans* increased, but also of the species *g_Natronincola_Anaerovirgula*, *g_Coprococcus* and *f_Sterptomycetaceae_2* (Figure 4.5). *Virgibacillus halodenitrificans* is a halotolerant spore-forming bacterium (growth in up to 25% NaCl), previously described as having plant growth promoting traits such as nitrogen fixation, and production of hydrolytic enzymes and phytohormone indol-3-acetate (IAA) (Sharma et al., 2023). The growth of this bacteria may have been enhanced by the addition of NaOH for controlling the pH, generating a saline medium suitable for its growth. Additionally, it has been described to produce acids from a variety of sugars, such as D-galactose, D-fructose, D-glucose, D-mannose, D-trehalose, D-mannitol, maltose, D-ribose, and sucrose (Yoon et al., 2004). The growth and activity of proteases of *Virgibacillus halodenitrificans* have been observed in a varied pH range (5.8-10) (Liu et al., 2020), hence acid production from the OMSW fermentation and carbohydrates content under pH 9, may well have produced the high acid concentrations. Also, *Virgibacillus halodenitrificans* may have a pathway for the direct conversion of simple sugars into acetic acid, rather than the acetogenesis pathway as there was no accumulation of intermediate metabolites (propionic to valeric acid). These results were consistent with previous research in which strongly alkaline conditions could inhibit the activity of acidogenic bacteria despite the fixed HRT (Bermúdez-Penabad et al., 2017; Chen et al., 2013).

The increase of *f_streptomycetaceae_2* (Figure 4.5) species could correlate with the increase of the hydrolytic percentage during the 14-days HRT period, as this taxonomic group has been described as a hydrolytic enzyme-secreting bacteria involved in the degradation of complex organic compounds such as cellulose or starch to produce VFA (Rainisalo et al., 2011). Finally, taxa from *Coprococcus* and *Natronincolaanaerovirgula* have been described as acetic acid producers, and their increase at this stage could be related to the increase in the production of this VFA (Notting et al., 2023; Pikuta et al., 2006). The accumulation of acetic acid suggests that the routes for the conversion of carbohydrates into acetic acid were active, while methanogenesis pathways were inhibited. The latter was corroborated by the absence of methanogenic archaea in the microbiological analysis and the lack of biogas production.

The change from 14-days HRT to 21-days HRT resulted a decrease in the relative abundance of *o_Bacteroidales*, and *g_Natronincola_Anaerovirgula* species at the end of the experiment (176 day, 21-days HRT) compared to the previous sampling days (Figure 4.5), which could be related to the destabilisation of the process and the decrease of the accumulated sCOD and VFA observed in the last period of operation (Figure 4.1 and 4.2). However, the relative abundance of the above-mentioned relevant species at 14-days HRT does not show marked variations when shifting from 14 to 21-day HRT (Figure 4.5).

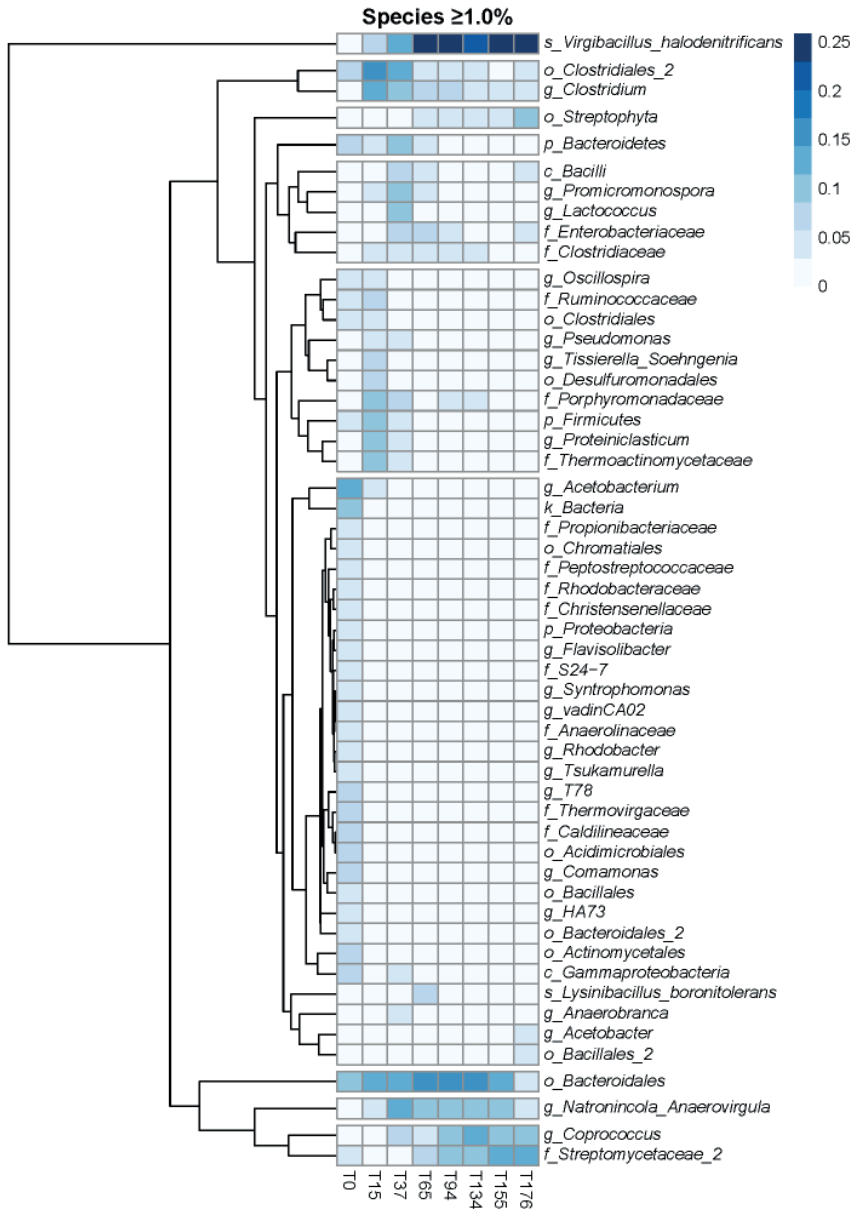


Figure 4.5. Cluster analysis of microbial species in the digestate samples at pH 9 for 176 days. The colour scale indicates the $\log_{10}$ of the square root mean relative abundance. The included species were limited to OTUs with a relative abundance of $\geq 1.0\%$, in at least 1 sample. Taxa labels are based on the last known taxon classification. Rows are clustered using Euclidean distance.

4.4. Conclusions

The highest organic matter solubilisation was obtained with 14-day HRT, with practically constant values ($53 \pm 9\%$). The improvement in solubilization resulted in a higher accumulation of VFA at 14-day HRT respect to 7-day HRT. Despite a lower solubilisation efficiency at 21-day HRT compared to 14-day HRT, the maximum VFA value was obtained at 21-day HRT with a concentration of $10,035 \pm 651 \text{ mg O}_2 \cdot \text{L}^{-1}$. However, the system did not keep a stable operation at this 21-day HRT. At 14 and 21-day HRT, microbial diversity remains similar, shown from sample clustering and diversity indices. The taxa *Virgibacillus halodenitrificans* and *Coprococcus* bacteria were correlated with the increment in the VFA accumulation. Considering all these conclusions, 14-day HRT resulted in the most interesting option for long-term OMSW operation reactors, maximising the accumulation of VFA without compromising the stability of the process.

CRedit author statement

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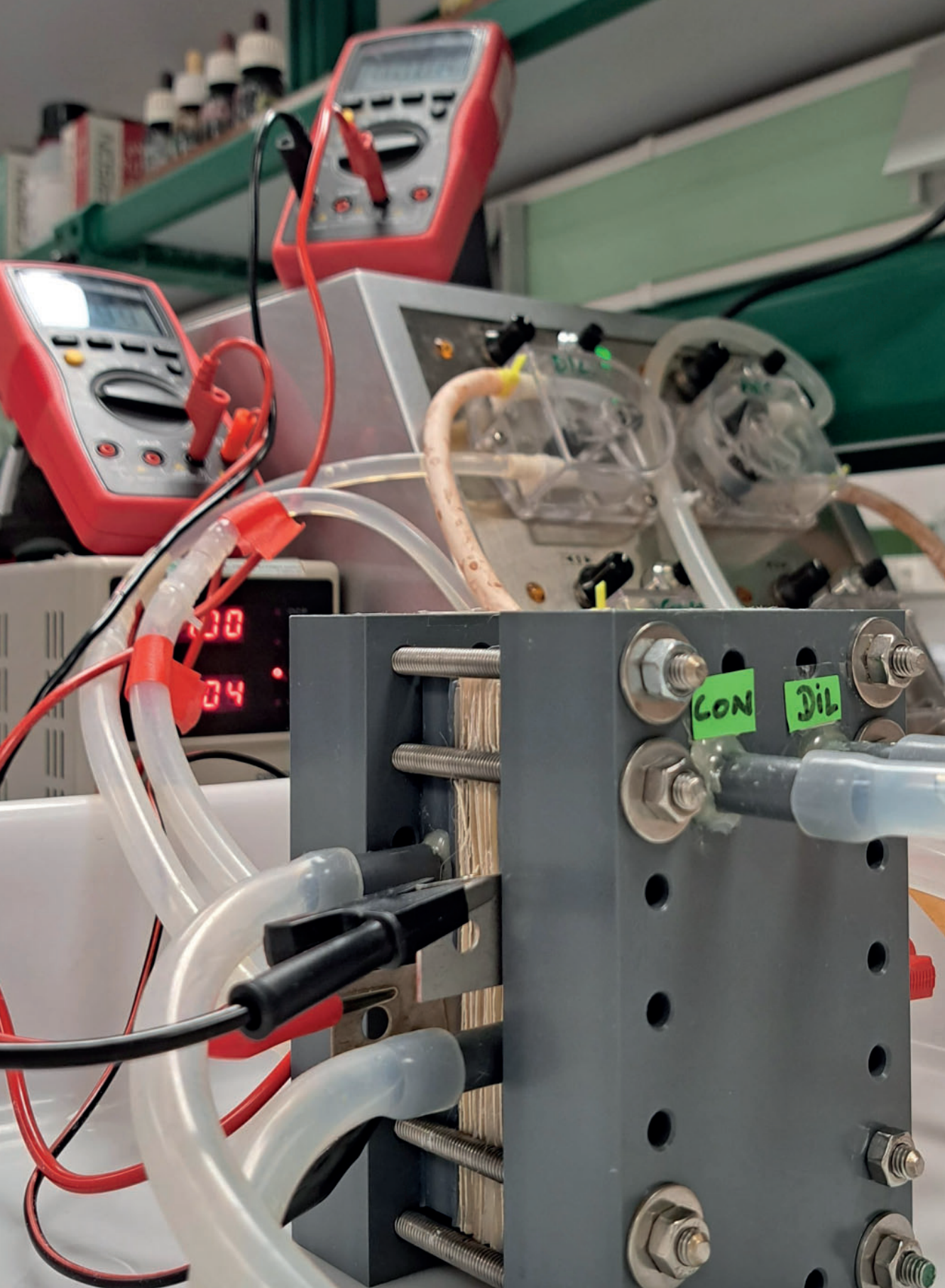
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Influence of the hydraulic retention time over the specialization of the microbial community in the alkaline fermentation of olive mill solid waste

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Chapter 5

Application of electrodialysis for volatile fatty acids recovery from olive mill solid waste ferment

Abstract

Electrodialysis has emerged as a sustainable technology for volatile fatty acids (VFAs) separation from organic streams, valued for its quick, efficient separation, low energy use, and minimal membrane pressure. This study explores the feasibility of the purification of VFAs from an olive mill solid waste (OMSW) ferment in comparison with a synthetic VFA mixture, evaluating the potential matrix effect of the OMSW ferment. The electrodialysis demonstrated to be partially effective in the extraction of VFAs from OMSW ferment, achieving a total VFA recovery of 55%. Comparing this recovery with the synthetic VFA mixture, it could be concluded that the matrix effect has a negligible impact on the recovery percentages of VFAs, although it slightly decelerates the process. It should be also taken into account that the initial concentration and molecular weight of each VFA compound influenced their specific recovery percentage as well as the migration rates, resulting in the higher efficiencies for acetic acid.

Keywords

Acidogenic fermentation; matrix effect; olive mill solid waste; power supply; purification.

5.1. Introduction

Volatile fatty acids (VFAs) are short-chain of carboxylic acids from two to six carbons, including acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and caproic acids. These compounds are widely used in pharmaceutical, cosmetic or food industry such as food preservation, odour and flavour enhancer among others (Agnihotri et al., 2022). VFAs can be also used as building blocks for oil-based chemistry, promoting the transition from a fossil fuel-based industry to a more sustainable industry based on renewable raw materials (Llamas et al., 2020). Nowadays, VFAs used in industries are mainly produced by petroleum-based chemical synthesis (Kumar et al., 2025). Alternatively, recent studies are promoting the valorisation of waste for VFA production by controlling the metabolic pathways of the conventional anaerobic digestion process, promoting the generation of VFA and avoiding their bioconversion into biogas (Cubero-Cardoso et al., 2024; Khatami et al., 2021; Sanchez-Ledesma et al., 2024; Serrano et al., 2023). This process is a mixed culture fermentation that consists of a hydrolytic stage, where complex organic matter (e.g. from waste) is degraded by microorganisms, followed by the stages of acidogenesis and acetogenesis in which VFAs and alcohols are generated as intermediate metabolites (Sun et al., 2021). Previous studies have demonstrated the feasibility of mixed culture fermentation of olive mill solid waste (OMSW) for the production of VFAs (Jiménez-Páez et al., 2025; Jiménez-Páez et al., 2023). OMSW is a by-product generated by the olive oil industry whose management is currently a challenge that is struggling the economic and environmental sustainability of the sector. Interestingly, the mixed culture fermentation of OMSW can favour the selective production of specific VFAs by manipulating the operating pH, demonstrating the potential and versatility of the process, offering an interesting option of the OMSW management (Cabrera et al., 2019; Jiménez-Páez et al., 2023). However, increasing the concentration of VFAs can negatively affect the mixed culture, inhibiting the microbial activity and reducing the production of VFA (Jiménez-Páez et al., 2025). Previous studies reported that VFAs in OMSW fermenter accounted around 30% of the soluble chemical oxygen demand (sCOD), indicating that would exist a limitation in the fermentative pathways that convert soluble compounds into simpler VFA (Jiménez-Páez et al., 2025; Jiménez-Páez et al., 2023). The extraction of VFAs from the fermentation process could be a convenient strategy for the recovery these compounds. That can simultaneously improve the fermentation process itself thanks to the reduction in the concentration of the VFAs.

Currently, there are different VFA separation processes such as precipitation, reactive extraction, resin adsorption, distillation, micro and nanofiltration, among others (Chen et al., 2022). However, the proposed separation methods have different implementation limitations, as for example high energy consumptions (distillation), no-selective separation of VFAs (resin adsorption/reactive extraction) and/or high operation costs due to cleaning of membranes (micro and nanofiltration) (Chen et al., 2022). As a novel alternative, this research proposes the electrodialysis (ED), a VFA recovery process based on electrochemical membrane systems. ED is an abiotic process in which an electrical field is applied to induce the migration of ions across selective ion exchange membranes, resulting in the transfer of ionic compounds (Chalmers Brown et al., 2020). ED has emerged as a sustainable alternative

for VFA separation from organic streams, valued for its quick, efficient separation, low energy use, and minimal membrane pressure (Bak et al., 2019). Synthetic substrates have been usually used for the optimisation and understanding of the functionality of ED processes (Haflich et al., 2024; Novalic et al., 2000). However, ED applied to VFA recovery from fermented complex organic substrates has been scarcely studied, i.e. grass pellet (Jones et al., 2021b), ferments from hydrogen production (Jones et al., 2015), dephenolised olive mill wastewater (Scoma et al., 2016), and municipal food waste (Jones et al., 2021a).

This study explores the feasibility of the purification of VFAs from an OMSW ferment for the first time. The study also includes the comparison of VFA recovery from the real stream with a synthetic VFA mixture, evaluating the matrix effect of such a complex mixture as OMSW ferment. The obtaining of a concentrated and purified VFA stream would facilitate their subsequent use in different bioprocesses, a necessary step to ensure the feasibility of biorefineries based on OMSW valorisation.

5.2. Materials and methods

5.2.1. Volatile fatty acids matrixes

The OMSW ferment was obtained from a semi-continuous OMSW acidogenic fermentation reactor. The fermentation reactor was operated at an organic loading rate (OLR) of $3 \text{ g VS of OMSW} \cdot (\text{L} \cdot \text{d})^{-1}$ (VS, volatile solids) under mesophilic temperature ($35 \pm 2 \text{ }^{\circ}\text{C}$) at pH 5. Prior to their use in ED, OMSW ferment was centrifuged at 1000 rpm for 20 min and microfiltered with $0.45 \text{ }\mu\text{m}$ nylon microfilters. Moreover, a synthetic mixture was used to mimic the VFA composition of OMSW ferment but avoiding potential matrix effects. The reagents used for synthetic VFA mixture were sodium acetate (99%), sodium propionate (99%), sodium butyrate (95%) and sodium pentanoate (97%) (Fisher scientific, S.L, Spain). The physicochemical characterization of OMSW ferment and synthetic mixture was summary in Table 5.1.

Table 5.1. Physicochemical characterisation of synthetic volatile fatty acid (VFA) mixture and olive mill solid waste (OMSW) ferment.

	Synthetic VFA mixture	OMSW ferment
pH	7.0 ± 0.2	5.0 ± 0.5
Conductivity ($\text{mS} \cdot \text{cm}^{-1}$)	4.53	4.45
Soluble chemical oxygen demand (sCOD) ($\text{mg O}_2 \cdot \text{L}^{-1}$)	1268 ± 19	2940 ± 1
Acetic acid ($\text{mg} \cdot \text{L}^{-1}$) *	425 ± 10	367 ± 8
Propionic acid ($\text{mg} \cdot \text{L}^{-1}$) *	158 ± 3	140 ± 9
Butyric acid ($\text{mg} \cdot \text{L}^{-1}$) *	216 ± 1	111 ± 10
Valeric acid ($\text{mg} \cdot \text{L}^{-1}$) *	90 ± 2	50 ± 5
VFA/sCOD (%)	100	31
Total phenols ($\text{mg gallic acid} \cdot \text{L}^{-1}$)	-	126 ± 1

5.2.2. Electrodialysis set up and start-up

5.2.2.1. Electrodialysis cell

The ED system was represented in Figure 5.1. The ED was configured by 4 unit cells, thus 4 anion exchange membranes (AEMs) and 5 cation exchange membranes (CEMs) alternately disposed. The AEMs (RALEX® MEMBRANE AMHPES-MEGA-TEC, Czech Republic) and CEMs (RALEX® CMHPES-MEGA-TEC, Czech Republic) were commercial heterogeneous ion-exchange membranes. The active area of each membrane was 16 cm². Polypropylene woven spacers with 0.5 mm thick and 3 mm mesh size was used as turbulence promoters in the diluate and concentrate compartments of the ED system. Moreover, Titanium coated with iridium oxide electrode (DSE, provided by Inagasa S.A., Spain) and stainless-steel electrode (Tamesanz, Spain), were used as anode and cathode, respectively. Each diluate and concentrate compartment in ED stack had a working volume of 0.25 L. On the one hand, the diluate stream was composed by the sample to be treated, i.e. OMSW ferment or the synthetic VFA mixture. For the test using synthetic VFA mixture, it was necessary to add NaCl to the dilute stream, as the conductivity provided by VFAs ($\sim 1 \text{ mS}\cdot\text{cm}^{-1}$) was lower in comparison to the OMSW ferment. On the other hand, the concentrate stream (i.e. draw solution) was composed of distilled water, NaCl ($2.2 \text{ g}\cdot\text{L}^{-1}$) and an electric conductivity of $4.45 \text{ mS}\cdot\text{cm}^{-1}$ (similar to diluate stream). Finally, Na_2SO_4 0.05 M solution was used for electrode rinse (anolyte/catholyte solution). The solutions were continuously recirculated from the corresponding reservoirs to the ED stack applying a constant flow of $40 \text{ mL}\cdot\text{min}^{-1}$ (i.e. $10 \text{ mL}\cdot\text{min}^{-1}$ per unit cell), a constant voltage and at room temperature. The applied voltage (RS3005D Digital Control DC, RS AMIDATA, Spain) was determined by the limiting current density (LCD), and was set at the 80% of the LCD to avoid damage in the ion-exchange membranes, as suggested by the provider.

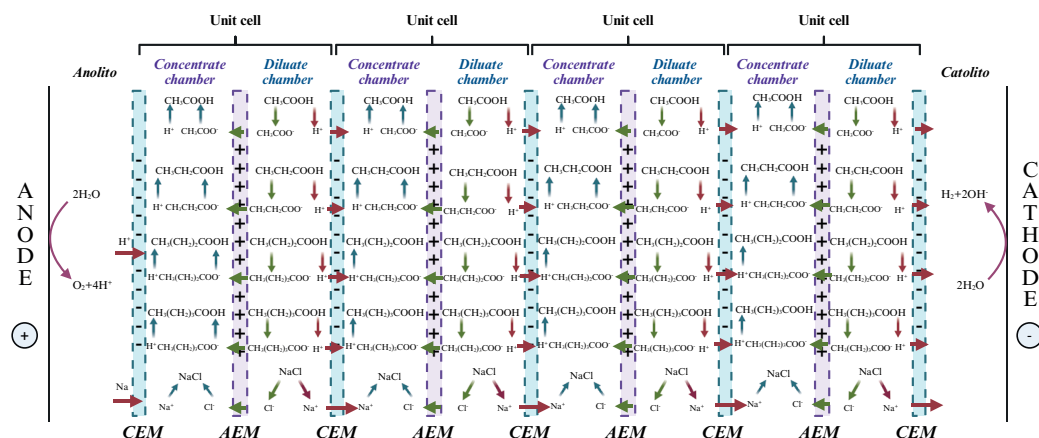


Figure 5.1. Schematic representation of experimental set up employed in this research (created using biorender.com).

5.2.1.1. Limiting current density

The Limiting Current Density (LCD) was determined by a representation of electric resistance of the ED stack (Ohm) vs $1/j$ as it was described by Lejarazu-Larrañaga et al. (2020). Two kind of samples were tested: i) the synthetic VFA mixture and ii) the OMSW ferment. All samples were analysed using an identical experimental configuration, as detailed in the preceding section. The potential was increased and measured each 30 seconds to determine the minimum resistance that needs to be used to obtain $1/j$ value. This value is an important operational parameter to determine the maximum applied potential in the ED system with no damage of membranes.

5.2.2.3. Membrane equilibration and cleaning

Membrane equilibration (i.e. ion exchange equilibrium between ion exchange membranes and solutions) was carried out by passing saline solution at the same conductivity as the OMSW ferment for 24 hours.

Additionally, a cleaning procedure was carried out at the end of each experiment. The cleaning procedure is required when membrane fouling is observed by an increased in applied voltage, a constant current operation exhibiting performance decline, or changes in current density under a constant voltage. All cleaning procedures were to be conducted at room temperature. For membranes fouled with mineral deposits, an acid cleaning using 0.1 M hydrochloric acid (HCl) for 20 minutes was carried out. For membranes fouled with organic deposits, an alkaline cleaning using 0.1 M sodium hydroxide (NaOH) for 40 minutes was carried out. In cases where fouling was attributed to a combination of mineral and organic deposits, a sequential cleaning regimen consisting of acid, alkaline, and acid treatments was done.

5.2.2. Chemical analysis

Samples were taken from the concentrate and diluate reservoirs at 15 min intervals for 180 min. A total volume of 20 mL was extracted in order to not exceeding 10% of the initial volume and to avoid interferences in the ED process due to significant volume variations. From the samples taken from OMSW ferment, the chemical oxygen demand of the initial sample, an intermediate sample and a final sample were analysed according to the APHA method (Baird et al., 2017). The conductivity change in each compartment was measured by a conductivity meter (Crison EC-Metro BASIC 30) to follow the ion migration. Ion concentrations in the dilute and concentrate compartments was analysed by Ionic Chromatography (Dionex ICS-1600 from Thermo Fisher Scientific S.A., USA). To measure the concentration of VFAs (acetic acid (C2), propionic acid (C3), iso-butyric acid (i-C4), butyric acid (n-C4), iso-valeric (i-C5) acid and valeric acid (n-C5)), samples were firstly centrifuged and microfiltered with 0.45 μm nylon microfilters. Samples were then prepared by adding an internal standard of 1500 $\text{mg}\cdot\text{L}^{-1}$ of ethylbutyric acid with phosphoric acid (30% v/v). VFAs were determined in duplicate, injecting 0.5 μL of each sample in a gas chromatograph

Shimadzu GC-2025 (Shimadzu Corporation, Kyoto, Japan) equipped. A Stabilwax-DA capillary column (RESTEK Corporation, Bellefonte, PA, USA) of 0.25 mm i.d. x 30 m and a flame ionization detector (FID) was used. The oven temperature gradually increases from 100 to 170 °C at 5 °C·min⁻¹. The carrier gas (nitrogen, hydrogen, air) flowed at 40 mL·min⁻¹ at 504 kPa, with individual flow rates of 30 mL·min⁻¹ for nitrogen, 40 mL·min⁻¹ for hydrogen, and 400 mL·min⁻¹ for air. The FID detector temperature was set to 250 °C. Identification and quantification were performed using commercial standards by comparing retention times with the reference compound.

5.2.3. Parameters and experimental data

To evaluate the extraction process of volatile fatty acids, the following parameters were analysed:

$$U = I \cdot R \quad \text{Eq. 5.4}$$

Where U is the potential expressed in Volts (V); I is the electric current expressed in Amps (A); R is the resistance expressed in Ohms (Ω).

$$\text{VFA flow capacity to treat} = \frac{M_a}{A \cdot t_d} \quad \text{Eq. 5.5}$$

Where Ma is the initial mass of VFA to treat expressed in grams (g); A is the effective membrane area expressed in m²; td is the final operational time expressed in hours (h).

$$\text{Energy consumption (E)} = \frac{\int_0^t U(t) \cdot I(t) \cdot dt}{V_{dil}} \quad \text{Eq. 5.6}$$

Where E is the energy consumption expressed in kWh·m⁻³; U is the electric potential applied expressed in volts (V); I is the electric current expressed in Amps (A); Vdil is the final volume of diluate expressed in m³; t (s) is the experimental time.

$$\text{Current efficiency } (\eta) = \frac{z \cdot F \cdot \Delta n_{dil}}{N \cdot \int_0^t I(t) \cdot dt} \cdot 100\% \quad \text{Eq. 5.7}$$

$$\Delta n_{dil} = n_i(t) - n_i(0) \quad \text{Eq. 5.8}$$

Where z is the valence of the transported ion; F is Faraday's constant (96,485 C·mol⁻¹); Δn_{dil} is the mol number of anions or cations transported into concentrate compartment during the experimental time; N is the number of unit cells; I is the electrical current expressed in Amps (A); t (s) is the experimental time; $n_i(t)$ represents the cation mol number at the final experimental time expressed in mol; $n_i(0)$ represents the cation mol number at the initial experimental time expressed in mol.

5.3. Results and discussion

5.3.1. Voltage determination by limiting current density

The LCD values determined from the applied voltage to the different assessed samples, i.e. synthetic VFA mixture and OMSW ferment, showed similar values (Figure 5.2 and Table 5.2). The similarity between the electric potential applied value obtained in LCD for synthetic VFA mixture and the OMSW ferment could suggest that the organic compounds in the OMSW ferment do not negatively interact with the ion exchange membranes, indicating a low matrix effect.

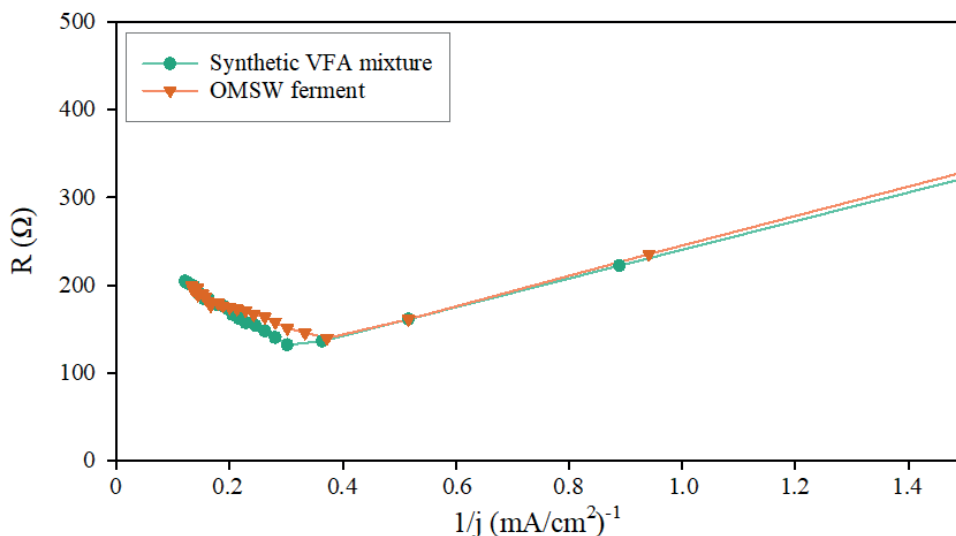


Figure 5.2. Limiting current density used for the calculation of 80% Limiting Current Density (LCD). VFA: volatile fatty acids; OMSW: olive mill solid waste; R: resistance expressed in Ohms (Ω); j: the electric current applied expressed in Milliamps (mA) respect the effective area of membranes.

Table 5.2. Voltage and resistance obtained for individual acids, synthetic volatile fatty acids (VFA) mixture and olive mill solid waste (OMSW) ferment.

		Synthetic VFA mixture	OMSW ferment
100%	1/j (mA/cm ²) ⁻¹	0.3	0.4
	LCD (mA/cm ²)	3.3	2.7
80%	LCD (mA/cm ²)	2.7	2.2
	1/j (mA/cm ²) ⁻¹	0.4	0.5
	I (mA)	42.4	34.4
	R (Ω)	138.6	153.6
	U (V)	5.9	5.3

LCD: Limiting current density; j: the electric current applied expressed in Milliamps (mA) respect the effective area of membranes; I: electrical current expressed in Amps (A); U: electric potential applied expressed in volts (V); R: resistance expressed in Ohms (Ω).

The applied voltage selected for the subsequent ED experiments was set to the value corresponding to 80% of the LCD of the synthetic mixture (5.9 V), due to its slightly higher value compared to OMSW ferments (5.3 V, 10% lower than the synthetic mixture value) (Figure 5.2 and Table 5.2). This procedure ensured uniformity in the application of the electrical potential during the comparison of both mixtures. According to Bak et al. (2019), the applied voltages have to be in the range of 0.5-1.5 V per unit cell (i.e. cell pair). In that sense, the voltage supplied in this study agreed with this assumption, applying around 1.5 V per unit cell pair. Moreover, the current density applied in this study ($2.7 \text{ mA}\cdot\text{cm}^{-2}$) was similar to that applied by Scoma et al., (2016) ($3.1 \text{ mA}\cdot\text{cm}^{-2}$) for VFA recovery from olive mill wastewater.

5.3.2. Conductivity control during the ED

During the ED of the synthetic VFA mixture and OMSW ferment for VFAs recovery process, electrical conductivity was monitored in the dilute and concentrate streams at 15 min intervals (Figure 5.3). The conductivity was measured to validate the correct functioning of the ED process and to monitor the proper migration of VFAs (and other ions). The results showed an increase in the conductivity of the concentrated stream, to reach a similar value of approximately $8 \text{ mS}\cdot\text{cm}^{-1}$ in both the synthetic VFA mixture and the OMSW ferment at the end of the ED process (Figure 5.3). Parallely, a substantial reduction in the conductivity of the diluted stream was determined, indicating the effective removal of ionic species and the achievement of a liquid solution with a lower ionic charge. Despite the similar final conductivity values, the main difference observed between the OMSW ferment and the synthetic mixture is that desalination (i.e. removal of electric conductivity in the diluate stream) is slightly slower in the OMSW ferment (Figure 5.3). Because of this, it is possible that the OMSW ferment contained some molecules that could interfere with the transfer of VFA (matrix effect) or, maybe, that also are transferred across the membrane but with a slower rate than synthetic VFA due to a higher molecular weight (Haflich et al., 2024).

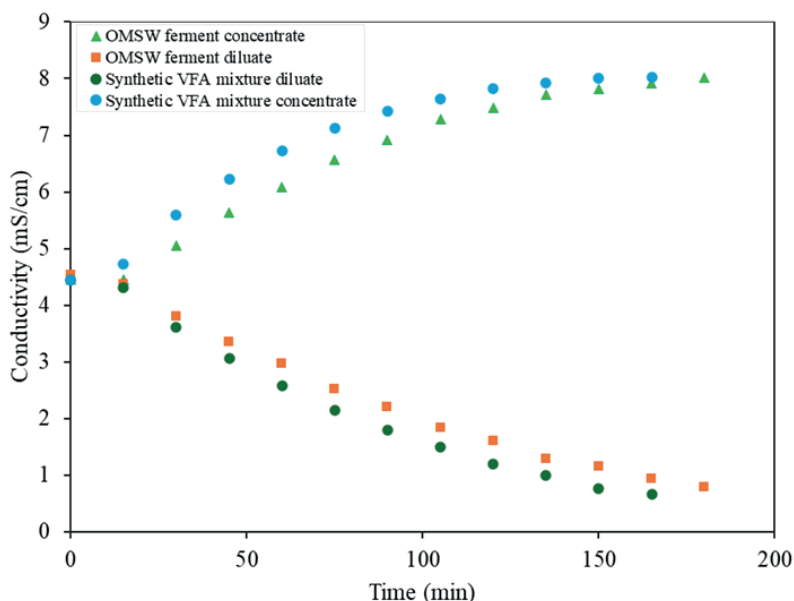


Figure 5.3. Solution conductivity during the experimental time in concentrate and diluate reservoirs measured in $\text{mS}\cdot\text{cm}^{-1}$ for synthetic volatile fatty acids (VFA) mixture and olive mill solid waste (OMSW) ferment.

5.3.3. Electrodialysis results

5.3.3.1. ED of synthetic VFA mixture

The ED results obtained of the synthetic VFA mixture are represented in Figure 5.4. The initial VFA concentration in the diluate stream was $1276 \pm 19 \text{ mg O}_2\cdot\text{L}^{-1}$, corresponding to 36% acetic acid, 19% propionic acid, 31% butyric acid and 14% valeric acid, respectively. An increase of the total VFA concentration was observed in concentrate stream during the experimental time (Figure 5.4E), reaching a final VFA recovery of 60% respect to the initial VFA concentration in the diluate stream. This percentage is higher than that obtained by Scoma et al. (2016), i.e. 35%, for a synthetic solution of a mixture of acetic acid, propionic acid and butyric acid. This difference could be explained by the different cell configuration such as the number of unit cells, i.e. 3 versus 4 unit cells in the present research, as both the initial conductivity and the applied voltage were similar in both studies. Interestingly, other authors reported an acetic acid recovery efficiency of 98% by applying 20 pairs of cells and a voltage of 18 V (Jones et al., 2015). Thus, it is worth to note that the efficiency of ED could be enhanced by adapting the operational set-up and conditions.

Individual recovery percentages of each VFA from the synthetic VFA mixture were 73% of acetic acid, 64% of propionic acid, 53% of butyric acid and 38% of valeric acid. The decrease in VFA recovery percentages observed with increasing VFA carbon chains could be related to their low initial concentrations (Table 5.1), which may have limited the efficiency

of the recovery process. The efficiency of the recovery has been reported to be affected by different factors such as the initial concentration or the size of the molecules (Haflich et al., 2024; Scoma et al., 2016). Concretely, Scoma et al. (2016) reported a clear relation between the number of carbons of the VFA and the recovery efficiency for a synthetic solution, showing values of $\sim 30\%$ for acetic acid and $\sim 20\%$ for butyric acid. From minute 15 onwards, it can be observed that there was recovery of acetic, propionic and butyric acid, but not for valeric acid, which did not start to be observed in the concentrate chamber until minute 30 (Figure 5.4). Haflich et al. (2024) determined that VFAs with longer carbon chains diffuse more slowly across the membrane due to esters and membrane deswelling.

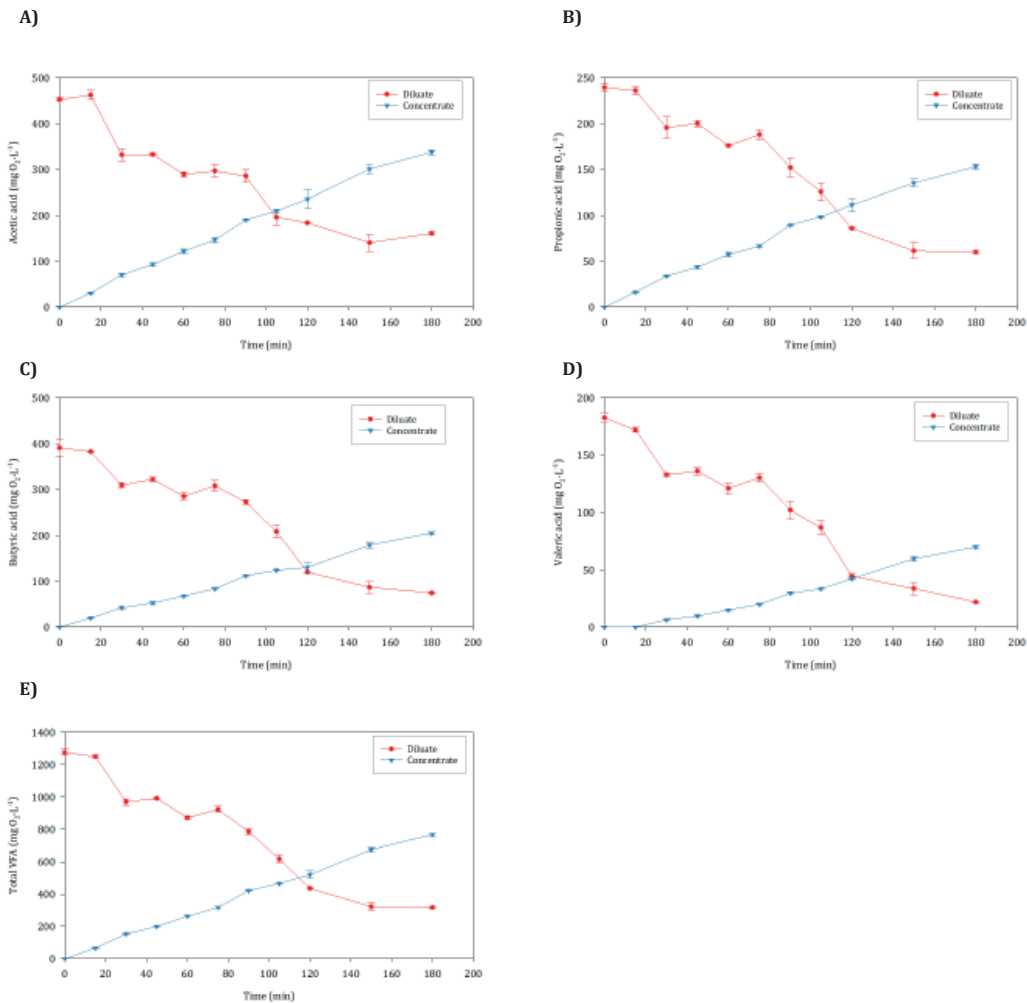
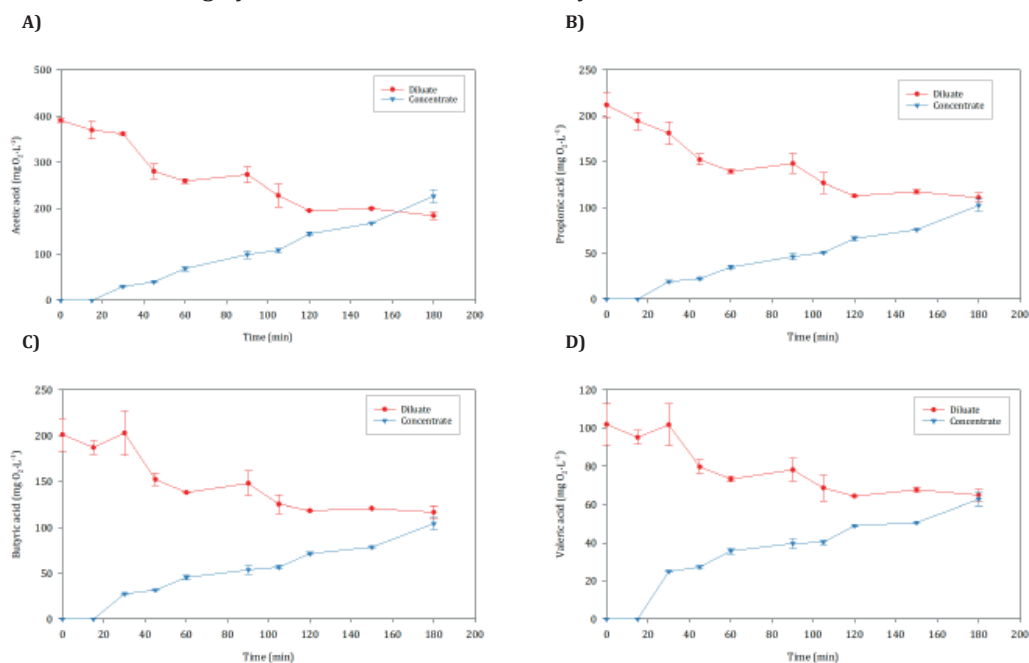


Figure 5.4. Individual and total volatile fatty acids recovery from synthetic volatile fatty acids (VFA) mixture in mg O₂ per litre during the experimental time.

The individual VFA recovery percentage from ED of OMSW ferment were 58% acetic acid, 48% propionic acid, 39% butyric acid, and 62% valeric acid (Figure 5.5). Compared to the synthetic VFA mixture, a decrease in the recovery percentages of acetic, propionic, and butyric acids was observed, whereas valeric acid recovery percentage incremented (Figure 5.4 and 5.5). Despite the variation in the recovery of individual VFA respect to the synthetic VFA mixture, the recovery percentage of the total VFA was 55% for OMSW ferment, just slightly lower than the 60% determined for the synthetic VFA mixture. This reduction could be attributed to the matrix effect of the OMSW ferment, which contains diverse short and medium-chain carboxylic acids, phenolic compounds, nutrients (NH_4^+ , PO_4^{3-} , etc), among other (Jiménez-Páez et al., 2023). Kotoka et al. (2024) described that the ED system was able to allow the transference of different nutrients contained in food waste, with efficiencies of 95.5% and 90.3% for NH_4^+ and PO_4^{3-} , respectively. The same authors achieved a VFA recovery percentage of 64-83% from food waste ferments by applying a current density of $6.25 \text{ mA}\cdot\text{cm}^2$, roughly double that used in this study.



E)

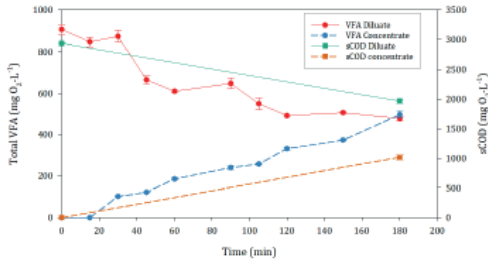


Figure 5.5. Individual and total volatile fatty acids recovery from OMSW ferment in mg O_2 per litre during the experimental time. In Figure 5.5.E it has been represented the soluble sCOD measured in initial and final point of the experimental time.

The initial sCOD in the OMSW ferment (diluate chamber) was $2940 \pm 1.0 \text{ mg O}_2 \cdot \text{L}^{-1}$, of which total VFA accounted the 30% of the measured sCOD concentration (Figure 5.4 E and Table 5.1). Following the ED process, 33% of this initial sCOD was transferred to the concentrate stream, whereas, as it was above-mentioned, the recovery efficiency for VFA was 55%. VFAs constituted 66% of the sCOD in the concentrate stream (Figure 5.5 E). Given that the total VFA analysis focused on the sum of short-chain VFAs (C2-C5), it is possible that a fraction of the unidentified 34% sCOD corresponds to unquantified short to long organic acids, potentially increasing the overall percentage of VFAs recovered. For example, OMSW fermented would contain different organic compounds derived of the partial degradation of the oleic acid, whose high molecular size would strongly influence the ED process (Bouhia et al., 2023; Lalman & Bagley, 2001). That would be in line with the differences in the kinetics observed in the variation of the conductivity between the synthetic VFA mixture and the OMSW ferment described in Figure 5.3, that were hypothesised to be explained by the transference of high size molecules with a slow transference capacity (Haflich et al., 2024).

Respect the VFA flow capacity to treat, energy consumption and current efficiency of the OMSW ferment in the ED stack configuration it has been obtained $8.7 \text{ g VFA} \cdot \text{m}^{-2} \cdot \text{h}^{-1}$, $0.97 \text{ kWh} \cdot \text{m}^{-3}$ and 86%, respectively (see Eq. 5.1, 5.2, 5.3, 5.4 and 5.5). The energy consumption has been calculated to analyse the technical feasibility of the process, resulting in a value similar than the described by Lejarazu-Larrañaga et al., (2020). This energy consumption mainly depends on the operational conditions (applied voltage), the percentage of VFA recovery (operating time) and the stream characteristics (electrical conductivity). Moreover, the current efficiency was calculated using the cations values presented in Table 5.3. Among the measured inorganic ions, Na^+ accounted for about 90% of the ionic strength in both the initial and final dilute and concentrated streams (Table 5.3). The energy consumption and current efficiency values indicated that the chosen configuration of the ED system was feasible for VFA recovery from OMSW ferment, with most of the applied energy intended to compounds transfer. Nevertheless, the observed partial VFA recovery efficiencies could make recommendable to assess new configurations of ED with a higher number of cells and/

or different applied voltages, or to test a two-stage ED, trying to reach efficiencies of 80-90% as reported in literature. Future research may be also focused on the optimisation of the energy consumption by varying the applied potential and the experimental recovery time. It would also be necessary to evaluate the possible fouling of the membranes in a real implementation of the process, especially to evaluate the operating cost (cyclic cleaning of the membranes).

5.4. Conclusions

The electrodialysis system demonstrated to be partially effective in the extracting VFAs from OMSW ferment, achieving a total VFA recovery of 55%. Comparing this recovery with the synthetic VFA mixture (60%), it could be concluded that the matrix effect has a negligible impact on the recovery percentages of VFAs, although it slightly decelerates the process. Consequently, ED allowed obtaining a clean matrix mainly composed by VFAs in the concentrate stream. Finally, it is worth noting the remarkable energy efficiency of the electrodialysis system used in this research, where 86% of the applied energy was used for ion migration.

5.5. References

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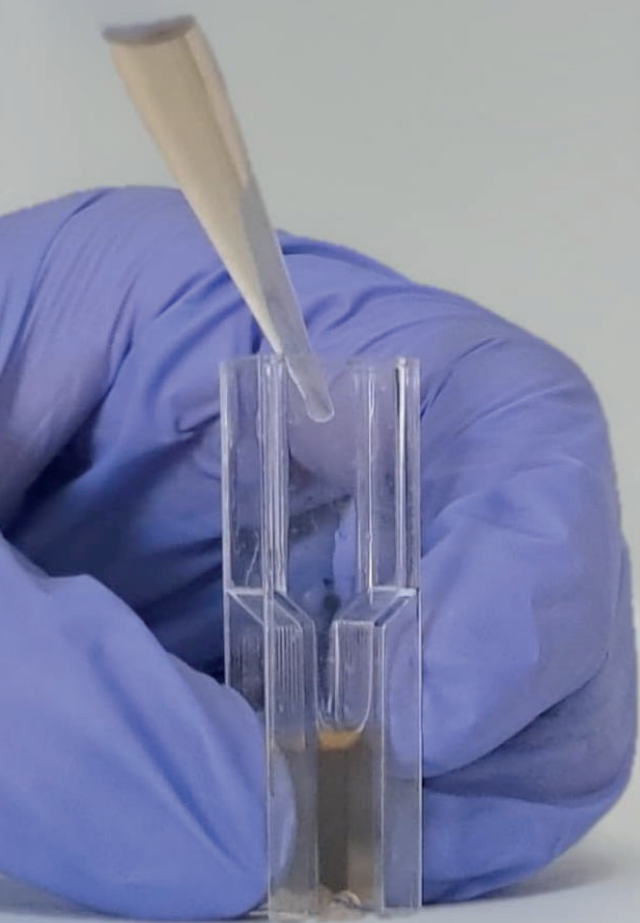
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5.6. Supplementary material

Table 5.3. Inorganic ions quantified by ionic chromatograph in mmol mixture and olive mill solid waste.

Inorganic Ions	Concentrate initial (mmol)	Concentrate final (mmol)	Diluate initial (mmol)	Diluate Final (mmol)
Na^+	4	13	10	2
NH_4^+	0	0	0	0
N-NH_4^+	n.q.	1	1	n.q.
K^+	0	0	0	0
Ca^{2+}	0	0	0	0
Mg^{2+}	5	14	11	2
F^-	0	0	0	0
Cl^-	4	15	7	1
NO_2^-	0	0	0	0
Br^-	0	0	0	0
NO_3^-	0	0	0	0
PO_4^{2-}	0	0	0	0
SO_4^{2-}	0	0	0	0



Chapter 6

Monitoring of volatile fatty acids during anaerobic digestion of olive mill solid waste by means of a hand held near infrared spectrometer

Abstract

The accumulation of volatile fatty acids (VFAs) over anaerobic digestion (AD) leads to malfunctioning of industrial reactors, hence decreasing biogas production. Real-time monitoring of VFAs is a challenge due to the complexity and high cost of current methods for their quantification. For this reason, this research evaluated the application of near infrared (NIR) spectroscopy to quantify volatile fatty acids as a tool for AD reactors monitoring. To do that, 129 samples from various AD reactors fed with olive oil pomace were taken and their NIR spectra were acquired with a hand-held spectrometer. After performing grid search, three spectral variable selection methods, namely competitive adaptive reweighted sampling, uninformative variable elimination (UVE) and successive projections algorithm, were assayed before developing PLRS models to correlate the NIR light transmittance through the samples at the wavelengths selected by those methods with their VFAs concentrations. UVE led to the best performance for all the VFAs assayed. Thus, R² of validation of UVE-PLSR models for acetic, propionic, butyric, valeric and total VFAs were 0.895, 0.622, 0.866, 0.898 and 0.871, respectively. The predictive model for total VFAs achieved the highest accuracy (RMSEV = 539.5 mg·L⁻¹), explained by the correlation between the light absorption at the wavelengths selected by UVE and the chemical characteristics of VFAs. All in all, the prediction errors achieved suggest that a portable near infrared spectrometer can be used for monitoring VFAs in AD processes.

Keywords

Anaerobic digestion; NIR spectroscopy; partial least squares regression; variable selection; volatile fatty acids.

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6.1. Introduction

In Spain, the largest olive oil producer with 766.4 thousand of tons in 2023/2024 season (Statista, 2024), most olive mills use two-outlet decanters, obtaining virgin olive oil on one hand and pomace (two-outlet pomace or olive mill solid waste) on the other. As a result, for each ton of olive oil produced, four tons of olive mill solid waste (OMSW) are generated (Serrano et al., 2019, García Martín et al., 2020). The integral use of waste and by-products from the olive oil industry such as OMSW is evolving towards the current technological concept of 'integrated biorefinery' (García Martín et al., 2020). In this context, anaerobic digestion (AD) has become one of the most extensively researched techniques for organic waste management and energy production (Balasundaram et al., 2024) due to its ability to generate both methane-rich biogas and a digestate, which can be used as fertilizer. Because of this, several olive mills in Spain are aiming at implementing industrial anaerobic digesters to manage their OMSW.

The AD process is usually clustered in four stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Under stressed operating conditions, including elevated organic loading rates, pH fluctuations, or temperature variations, AD can experience process instability (Zhou et al., 2023). This is characterized by the accumulation of volatile fatty acids (VFAs) due to the inhibition or washing out of methanogens, while other specific bacterial communities may become dominant (Peces et al., 2021). VFAs are carboxylic acids between two and five carbon atoms, namely acetic acid (AA), propionic acid (PA), iso-butyric acid (IBA), n-butyric acid (BA), iso-valeric acid (IVA) and n-valeric acid (VA).

One of the main challenges in the industrial application of AD is the real-time monitoring during the AD process to detect potential reactor malfunctioning. Conventional methodologies, including titration, spectrophotometry and distillation are used to determine total VFAs content. Moreover, individual VFAs are measured applying gas chromatography, liquid chromatography and capillary electrophoresis (Fernández et al., 2016). VFAs screening using individual methods is time-consuming and requires significant financial investment due to the complex processes involved in sample preparation and detection (Bao et al., 2023). Consequently, the search for faster methods to detect VFAs early accumulation has led to the exploration of near-infrared (NIR) spectroscopy.

NIR spectroscopy is faster and cheaper than other vibrational technologies, such as Raman and mid-infrared (MIR) spectroscopies, and traditional VFAs determination method (HPLC or GC). NIR spectroscopy is based on the absorption of electromagnetic radiation in the NIR range (780-2500 nm), providing information related to molecular vibrations of chemical bonds of organic molecules such as C-H, N-H, S-H and O-H. Coupled with chemometric tools such as partial least squares regression (PLSR), NIR spectroscopy has proven to be able to provide simultaneous determination of major chemical composition, leading to industrial applications for several types of vegetable oils such as olive oil (García Martín, 2022). NIR and MIR spectroscopy have been successfully employed to rapidly quantify VFAs during the AD process. Xu et al. (2023) developed PLSR models for AA, PA and total acid (TA) concentrations in biogas slurry using NIR spectra. Falk et al. (2015) used MIR spectroscopy to determine a wider range of VFAs (AA, PA, IBA, BA, VA and IVA) in anaerobic digesters fed

by two feedstocks, a mixture of peptone with corn starch, and wheat flour and inoculum sludge. Both research established the most accurate PLSR models for AA determination. Since NIR spectroscopy is a non-destructive tool and does not require sample preparation, these models created from NIR spectra could be integrated into automated online monitoring systems for real-time VFAs analysis. For example, by sampling over time or by implementing a flow cell for continuous measurement of the NIR light absorbed by the sample passing through the flow cell.

NIR spectra often contain overlapping absorption bands due to the complexity of samples. Predictive models using the full spectrum may include noise and background information, which can hinder accuracy (Beebe & Kowalski, 1987; García Martín, 2022). Spectral preprocessing techniques, including normalization and derivatives, and variable selection methods, such as competitive adaptive reweighted sampling (CARS), uninformative variable elimination (UVE), and successive projections algorithm (SPA), can help improve data quality by removing or reducing noise and selecting informative variables, leading to more accurate and reliable models (Acquarelli et al., 2017; García Martín, 2015; Xiaobo et al., 2010; Xu et al., 2023). In the process of optimizing the PLSR models, determining the optimal number of latent variables is critical. Excessive variables may result in underfitting, while insufficient variables may lead to overfitting. Grid search plays a crucial role in this process by systematically testing different numbers of latent variables through cross-validation. This involves repeatedly splitting the data into training and validation sets, ensuring the ability to generalize the model is thoroughly evaluated. Moreover, the impact of how the data is partitioned on the model's performance is also assessed during the grid search process, helping to build a more robust and reliable model.

The objective of the current work was to build PLSR prediction models for VFAs concentration in OMSW anaerobic digestion media by NIR spectroscopy, the ultimate aim being to develop a monitoring system for the AD of OMSW. Grid search and three spectral variable selection methods (CARS, UVE and SPA) were assayed to select only featured wavelengths, removing uninformative wavelengths from the PLRS models. To the best of our knowledge, this task has not been previously reported, and it can provide the grounds for future developments in the online analysis of VFAs and the real-time control of AD processes.

6.2. Material and methods

6.2.1. Substrate and inoculum characterization

Two-outlet OMSW, supplied by the "Oleícola El Tejar Nuestra Señora de Araceli" pomace plant (Marchena, Seville, Spain), was the substrate for anaerobic digestion. The volatile solids/total solids ratio (VS/TS) was 94% and the VS content was 25.26 wt.%. The inoculum was issued from an industrial anaerobic reactor of the urban wastewater treatment plant "Copero" (Dos Hermanas, Seville, Spain). The VS content of the inoculum was 2.23 wt.%. The OMSW was stored at -20 °C to maintain initial conditions and inhibit microbial activity and potential fermentation.

6.2.2. Anaerobic digestion

The AD process was operated in a long-term regime applying mesophilic conditions (35-37 °C). The reactors, each with a working volume of 1.6 L, were initially inoculated with a 10 g of VS·L⁻¹ from inoculum. Every day, the reactors were fed with 3 g of VS·L⁻¹ from OMSW. Two operating parameters varied at two levels to widen the variety among samples. To be specific, pH of 5 and 9 and hydraulic retention times (HRT) of 7 and 14 days were evaluated. pH selection was based on previous research by Jiménez-Páez et al. (2023), which reported significant differences in the VFAs concentration and profile at acidic and alkaline pH. In addition, the increase of HRT was used to favour the accumulation of VFAs in the reactor. Sampling was performed to monitor the performance of the reactors. To be specific, samples were taken from the reactor four days a week during the five 7-day HRT replicates and the three 14-day HRT replicates. A total of 129 samples were collected from the reactors for analysis.

6.2.3. VFAs quantification

To measure the concentration of VFAs (AA, PA, IBA, BA, IVA and VA) in the samples, these were centrifuged at 1000 rpm for 20 min and microfiltered with 0.45 µm nylon microfilters. VFAs were measured in duplicate using a GC-2025 gas chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a Stabilwax-DA capillary column (Restek Corporation, Bellefonte, PA, United States) and a flame ionization detector (FID). The carrier gas (nitrogen, hydrogen, air) flowed at 40 mL·min⁻¹ at 504 kPa, with individual flow rates of 30 mL·min⁻¹ for nitrogen, 40 mL·min⁻¹ for hydrogen, and 400 mL·min⁻¹ for air. The temperature of FID was set to 250 °C. Total VFAs concentration in a sample (TA) was simply calculated as the sum of all the VFAs found in that sample.

6.2.4. Acquisition of near infrared (NIR) spectra

The NIR spectra of the samples were acquired using a NIR portable spectrometer composed of a HL-2000-LL tungsten halogen light source (Ocean Optics B.V, Duiven, Netherlands), a cuvette holder and a Flame-NIR+ spectrophotometer (Ocean Optics B.V, Duiven, Netherlands) as a detector. The cuvette holder is linked by optic fiber connectors to the light source on one side and to the detector on the opposite side.

Prior to the acquisition of the spectra, the closed sample containers were placed in a thermostatic water bath at 35 °C for 30 min to bring the samples to a temperature similar to that inside the reactors. The samples were then introduced into a 10 mm quartz cuvette and placed in the cuvette holder. Their NIR spectra from 959 to 1705 nm were acquired in transmittance mode (10 scans to average) and recorded using the OceanView software (Ocean Optics B.V, Duiven, Netherlands). The typical optical resolution of the Flame-NIR+ spectrophotometer is ~10.0 nm (FWHM, 25 µm slit).

6.2.5. Spectral data pre-processing

6.2.5.1. Normalization

Spectral preprocessing techniques include a range of mathematical procedures for transforming spectral measurements before developing quantitative models (Dotto et al., 2018). Given the inherent dimensional inconsistencies among variables in many datasets, normalization becomes a critical preprocessing step in regression modeling. The concentration of VFA always differ significantly in their scales as a function of changes in pH. By normalizing the data, we ensured that all variables were on a comparable scale, allowing the model to assess each predictor equally. This step was crucial for preventing one variable from dominating due to its numeric range, ultimately enhancing the model's accuracy and fairness.

6.2.5.2. Spectral variable selection

After normalization, feature variable selection was conducted using competitive adaptive reweighted sampling (CARS), uninformative variable elimination (UVE), and successive projections algorithm (SPA). The application of these feature selection methods to preprocessed spectral data significantly improved the performance of quantitative models, ensuring reliable and accurate predictions. The feature selection processes were executed using Python 3.8.1 in an Anaconda environment.

6.2.6. Spectral multivariate regression

The spectral multivariate regression involved developing partial least squares regression (PLSR) models for four types of acids. The dataset of different types of VFAs were randomly divided into calibration and validation sets with a ratio of 75% for calibration and 25% for validation. Subsequently, a grid search was performed to identify the optimal number of components (ranging from 1 to 20) and the random seed (ranging from 1 to 100) for the PLSR models. The grid search aimed to find the best model by evaluating different combinations of these parameters to maximize model performance (Mukarev & Walsh, 2012). Thus, the criteria for the grid search include maximizing the R^2 value, minimizing the RMSE, and ensuring that RMSEC was smaller than RMSEV to prevent underfitting, thereby identifying the optimal parameters. Following the model development, denormalization (Shin & Sanders, 2006) was applied to the predicted values to restore them to their original scale. While the normalized data was effective during the training process, the predicted outcomes were also in a normalized form. The denormalization process ensured that the results were interpretable and directly comparable to the original measured VFA values, and the root mean square error (RMSE) was calculated using the denormalized data. The performance of the models was evaluated using the coefficient of determination (R^2), RMSE and the residual predictive deviation (RPD). R^2 indicates the proportion of variance in the dependent variable that is predictable from the independent variables, with values closer to

1 indicating better model performance. Minimizing RMSE values also indicates better model accuracy. Additionally, RPD is the ratio of the standard deviation (SD) of the reference VFAs values to the root mean square error of validation (RMSEV), providing a measure of the predictive ability of quantitative models. This parameter is very popular in NIR spectroscopy; however, it is very dependent on the range of concentrations used and the variability among samples, so the direct comparison of RPD among predictive models built with different range of concentrations is not advisable (García Martín, 2022). All processes above were implemented using Python 3.8.1 in an Anaconda environment.

6.3. Results and discussion

6.3.1. VFAs profile of anaerobic digestion samples

The gas chromatograph used, under the assayed conditions, is optimized to determine the concentrations of AA, PA, IBA, BA, VA and IVA in the AD samples. AA was the VFAs in the highest concentration found in the samples. It was present in all samples except one. That sample was taken at almost the beginning of the AD process, so that PA had not been converted yet into AA. It is well known that all VFAs degrade first to AA before degrading to methane. PA was detected and quantified in all the samples, while BA and VA were solely detected in 66 and 54 samples, respectively. Furthermore, IBA and IVA were not found in any sample. The pH affects the diversity of the VFAs profile. For example, the acid profile ranged from AA to VA at acidic pH, while the profile was not as diverse at alkaline pH, with the majority being AA (Garcia-Aguirre et al., 2017; Jiménez-Páez et al., 2023). At pH 5, AA, PA, BA and VA were obtained (54 samples), while at pH 9 only an acid profile of AA and PA was obtained (53 samples). This is in agreement with the VFAs found in samples taken over the anaerobic fermentation of lignocellulosic feedstock (Zhou et al., 2018). The concentration ranges and statistics of these VFAs in the calibration and validation sets are summarized in Table 6.1.

Table 6.1. Concentrations of acetic (AA), propionic (PA), butyric (BA), valeric (VA) and total (TA) volatile fatty acids and number of samples (n) in the calibration and validation sets.

VFA	Calibration set					Validation set				
	n	Min (mg·L ⁻¹)	Max (mg·L ⁻¹)	Mean (mg·L ⁻¹)	SD (mg·L ⁻¹)	n	Min (mg·L ⁻¹)	Max (mg·L ⁻¹)	Mean (mg·L ⁻¹)	SD (mg·L ⁻¹)
AA	96	403.5	6255.9	2467.6	1632.2	32	114.7	5252.9	2136.0	1499.1
BA	96	47.1	1186.7	397.5	228.2	33	28.2	1291.0	448.8	280.7
PA	49	156.6	1390.6	555.6	281.6	17	34.2	1243.5	524.5	342.9
VA	40	19.0	359.5	141.8	79.4	14	74.2	378.0	160.9	84.4
TA	96	47.1	7120.5	3072.9	1629.3	33	177.1	7187.4	3270.4	1568.7

SD: standard deviation.

Previous studies have reported the inhibitory effect of VFAs on anaerobic digestion. For instance, propionic acid has been shown to inhibit methanogenesis at concentrations

exceeding 3000 mg·L⁻¹ (Montañés et al., 2013). On the contrary, concentrations of 1600, 300 and 1800 mg·L⁻¹ AA, PA and BA, respectively, were proven to lead to the maximum methane yield and methanogenic bacteria concentration when using these VFAs and ethanol as the substrate for the AD (Wang et al., 2009). Additionally, the presence of butyric acid may be correlated to the suppression of acetoclastic activity, thereby hindering the methanogenic stage (Serrano et al., 2020; Van den Heuvel et al., 1988). Within the ranges of VFAs concentration of the samples (Table 6.1), no malfunctioning of the reactors was detected.

6.3.2. Spectral analysis

The transmittance spectra (Figure 6.1a) of the anaerobic digestion samples were transformed into absorbance spectra as illustrated in Figure 6.1b. The identification and assignment of absorption peaks to specific chemical structures in NIR spectra is often difficult. Notwithstanding, two noticeable absorption bands are observed. One at around 1170-1190 nm and another near 1400 nm. The first can be attributed to the second overtones of C-H and CH=CH- stretching vibrations while the second, a broader band, is probably due to the first overtone of water. The high absorption registered for the first overtone of water is logical since the AD media are mostly composed of water. These two absorption bands can be also found in the NIR spectra of AD samples of glucose to ethanol (Finn et al., 2006; Liebmann et al., 2009) and of xylose to xylitol (García Martín et al., 2023). These last authors selected the NIR region between 800 and 1850 nm from the 350–2500 nm acquired spectrum for the building of their xylose and xylitol PLSR predictive models in fermentation media, achieving good performance.

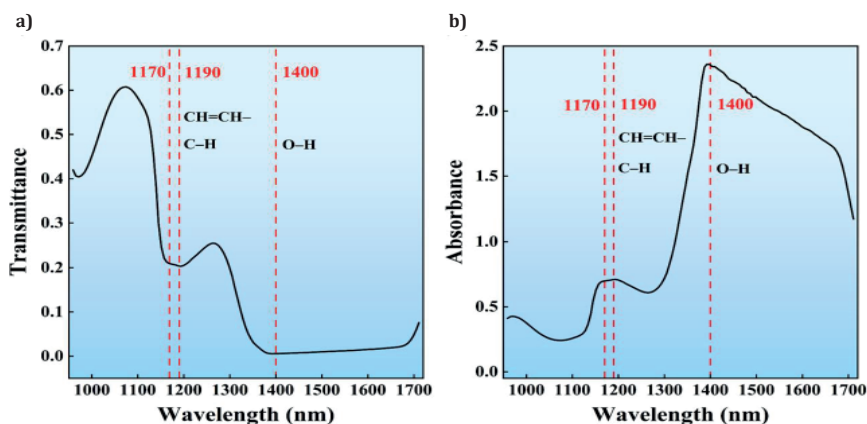


Figure 6.1. Average (A) transmittance and (B) absorbance spectra of the anaerobic digestion samples.

6.3.3. Prediction models based on variable selection methods

In this part, three distinct spectral variable selection methods were applied to the NIR

spectra of AD samples to enhance the prediction performance of the PLSR models for volatile fatty acids. Supplementary Table 6.3 highlights the characteristic wavelengths identified for each volatile fatty acid using the competitive adaptive reweighted sampling (CARS), uninformative variables elimination (UVE), and successive projections algorithm (SPA) algorithms.

The PLSR models built using uninformative variables elimination led to the best predictions for all the VFAs, according to Table 6.2. Moreover, each plot in Figure 6.2 showed the relationship between predicted and measured values for each acid separately, highlighting how well the model fits. In addition, the green areas in the figure represented the 95% confidence intervals of the predicted values, which illustrated the predictive accuracy of the model over different intervals of measurement values.

Table 6.2. Prediction results of PLSR models for the volatile fatty acids based on the NIR spectral full bands (959–1705 nm) and feature bands processed by three different variable selection methods (CARS, UVE and SPA).

Component	Variable selection	LV	Random seed	Calibration		Validation		RPD
				Rc ²	RMSEC (mg·L ⁻¹)	Rv ²	RMSEV (mg·L ⁻¹)	
AA	None	8	59	0.871	575.9	0.840	656.7	2.44
	CARS	14	91	0.886	547.6	0.871	615.8	2.60
	UVE	10	59	0.918	464.3	0.895	532.6	3.01
	SPA	16	86	0.888	544.4	0.865	608.7	2.63
PA	None	6	17	0.498	154.5	0.453	185.9	1.59
	CARS	9	17	0.589	145.5	0.543	150.9	1.61
	UVE	8	29	0.661	132.2	0.622	135.3	1.79
	SPA	9	100	0.490	162.1	0.438	185.1	1.31
BA	None	6	48	0.786	113.0	0.748	176.1	1.38
	CARS	16	7	0.895	90.3	0.829	136.9	2.16
	UVE	7	40	0.893	91.1	0.866	102.6	2.89
	SPA	9	40	0.832	114.4	0.785	129.8	2.28
VA	None	7	46	0.835	30.3	0.794	37.8	2.13
	CARS	8	8	0.888	26.3	0.881	27.2	2.96
	UVE	8	46	0.924	21.5	0.898	26.7	3.01
	SPA	7	32	0.776	37.1	0.755	38.7	2.08
TA	None	8	17	0.850	627.4	0.849	656.1	2.45
	CARS	13	82	0.899	514.7	0.845	756.8	2.13
	UVE	9	11	0.897	521.2	0.871	539.5	2.98
	SPA	10	25	0.782	756.5	0.773	783.8	2.05

LV: latent variables; Rc²: determination coefficient of calibration set; Rv²: determination coefficient of validation test (internal); RMSEC: root mean square error of calibration set; RMSEV: root mean square error of validation test (internal); RPD: residual predictive deviation of validation test (internal); CARS: competitive adaptive reweighted sampling; UVE: uninformative variables elimination; SPA: successive projections algorithm.

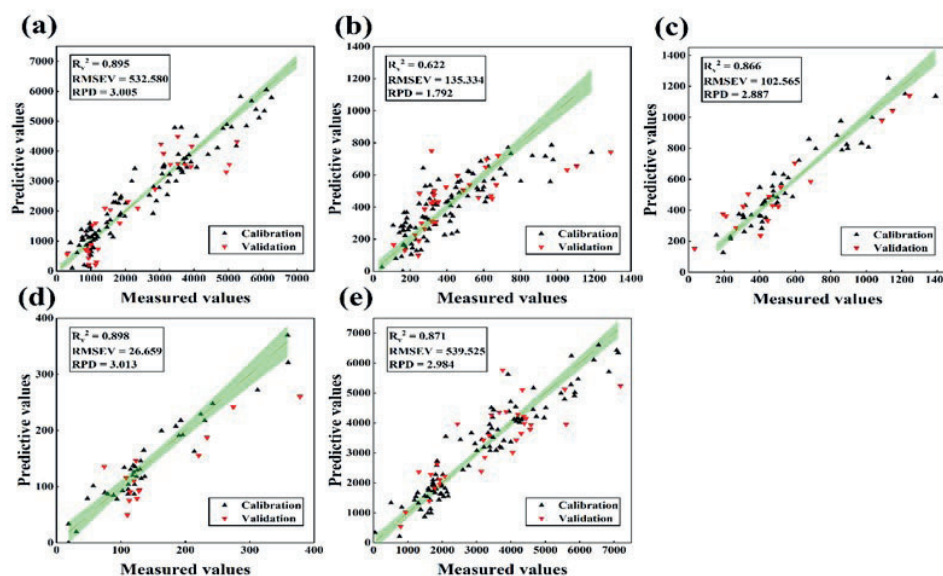


Figure 6.2. Scatter plots of volatile fatty acids concentrations ($\text{mg}\cdot\text{L}^{-1}$) prediction. a, b, c, d and e are the predictive results of the acetic acid (AA), propionic acid (PA), butyric acid (BA), valeric acid (VA) and total acids (TA), respectively. R_v^2 and RMSEV are the determination coefficient and root mean square error, respectively, of validation set. RPD is the residual predictive deviation.

For AA, the model using UVE-selected wavelengths reached R^2 of calibration and validation of 0.918 and 0.895, respectively, with a RMSEV value of $532.6 \text{ mg}\cdot\text{L}^{-1}$ (an 18.9% improvement over the PLSR model without variable selection) and RPD of 3.01. Figure 6.2a revealed a strong linear relationship between the predicted and measured values, with most data points closely clustered around the regression line. Additionally, the confidence interval was mostly narrow, indicating that the model predictions within this interval had high confidence and accuracy. However, only at low and high measured values did the confidence interval widen slightly, indicating slightly higher uncertainty in model predictions at extreme measurements.

For PA, the performance of the PLSR models were lower than for the other VFAs. The predictive model using the UVE-selected wavelengths obtained R_c^2 of 0.661 and R_v^2 of 0.622. Notwithstanding, UVE method improved PA prediction by 27.2% (RMSEPV decreased from 185.9 to $135.3 \text{ mg}\cdot\text{L}^{-1}$). Figure 6.2b illustrated a weaker linear relationship between the predicted and measured values, with data points more dispersed, particularly at measured values. The confidence interval was wide overall, and significantly wider especially in the high measurement interval, suggesting that the prediction uncertainty of PA was higher in this interval.

Even so, the performance of the AA and PA predictive models is similar to those from other studies. Thus, Fabian Jacobi et al. (2009) achieved R_c^2 of 0.69 and 0.89 for AA and PA, respectively, using samples weekly taken from an anaerobic digester of a full-scale 1-MW

biogas plant. These authors also acquired transmittance spectra in a similar wavelength range (850-1650 nm). For AA, their RMSEP was $270 \text{ mg}\cdot\text{kg}^{-1}$ for a concentration range of 70-3080 mg AA per kg fresh matter, the RPD being 1.81. For PA, the predictive performance was worse, with a RMSEP of $870 \text{ mg}\cdot\text{kg}^{-1}$ for a concentration range of 10-7470 mg PA per kg fresh matter (Jacobi et al., 2009). Notwithstanding, the RPD for PA was 2.89, higher than that for AA but with a prediction error (RMSEP) 3 times higher than that for AA. Moreover, a prediction error of $870 \text{ mg}\cdot\text{kg}^{-1}$ for PA could be regarded as too high considering that, as mentioned above, PA concentrations above $3000 \text{ mg}\cdot\text{L}^{-1}$ inhibit methanogenesis (Montañés et al., 2013). Therefore, the RMSEV achieved in the present work ($135.3 \text{ mg}\cdot\text{L}^{-1}$) seems to be more suitable for the prediction of the PA concentration, despite obtaining a lower RPD (1.79) than Jacobi et al. (2009). Hence the issues when using the parameter RPD for assessing the model performance in NIR spectroscopy pointed out in a previous work (García Martín, 2022).

Meanwhile, Xu et al. (2023) developed fast detection models of VFAs in samples of AD of corn stover, swine manure and cattle manure using NIR transmission spectroscopy combined with PLRS. These authors improved the performance of their PLSR models building them solely with the wavelengths selected by CARS and simulated annealing binary particle swarm optimization algorithm, obtaining R_c^2 of 0.985 for AA and 0.989 for PA. These results seem to be better than those of the present work. However, the VFAs concentration ranges of these authors' samples were narrower ($2049\text{-}5240 \text{ mg}\cdot\text{L}^{-1}$ for AA and $512\text{-}2714 \text{ mmg}\cdot\text{L}^{-1}$ for PA) and, more importantly, a wider NIR spectrum (from 1000-2500 nm) was used. Thus, these authors selected as featured wavelengths (used to build the PLRS models) those included in the ranges 1818-887 nm (first overtone of single bond COOH groups), 2028-2105 nm (combination band of C=O bond and single OH bond groups) and 2174-2500 nm (combination band of single bond CH_3 group of the AA). The transmittance in these wavelengths is not collected in the hand-held portable spectrometer used in the present work, hence these features could not be used for the building of the PLRS predictive models.

For BA, the UVE-selected wavelength model demonstrated notable improvements, achieving the R_v^2 of 0.866, while the full spectral range model had the R_v^2 of 0.748. The RMSEV value dramatically decreased from $176.1 \text{ mg}\cdot\text{L}^{-1}$ to $102.6 \text{ mg}\cdot\text{L}^{-1}$ (41.7% improvement), and the RPD increased from 1.38 to 2.89. Similarly, for VA, the UVE-selected wavelength model significantly outperformed the full spectral range model, with the R_v^2 of 0.898. The RMSEV value decreased to $26.7 \text{ mg}\cdot\text{L}^{-1}$ (a 29.4% improvement over the PLSR model without variable selection), and the RPD increased to 3.01, indicating a substantial enhancement in prediction accuracy. A similar strong linear relationship was shown in Figure 6.2c and Figure 6.2d for BA and VA, respectively, revealing the selected wavelengths might still align better with specific data points, reducing the overall error (lower RMSEV) (Cai et al., 2008). In terms of confidence interval, Figure 6.2c showed wider interval at both low and high measured values and narrower interval in the mid-range, indicating that the model demonstrated higher predictive accuracy in the mid-range while exhibiting increased uncertainty in predictions at the extremes. In contrast, Figure 6.2d had consistently narrower interval, particularly in the low and mid-range, reflecting high confidence in the predictions of model. Although the interval widened slightly at higher values, the overall performance remained strong.

Lastly, the regression model for TA with variable selection using the UVE method demonstrated better performance than the works conducted by other authors. Notably, the RMSEV was reduced to 539.5 mg·L⁻¹ (a 17.8% improvement over the PLS model without variable selection), lower than that achieved by Xu et al. (689 mg/L) and Jacobi et al. (820 mg·kg⁻¹) (Jacobi et al., 2009; Xu et al., 2023). Even the RMSEV achieved with the full 959-1705 nm spectrum (656.1 mg·L⁻¹) was lower. Taking into account the device used (hand-held portable spectrometer), its low typical optical resolution and the limited NIR region in which it can acquire spectra, the results for TA can be considered excellent.

However, the predictive and measured values of TA are scattered on both sides of the 1:1 line (Figure 6.2e), indicating that the predictive performance of the TA regression model could be further improved (Lin & Liu, 2020), probably with full NIR spectrometers (taking into account the potential VFAs-related information contained in the NIR spectrum above 1705 nm) or with higher FWHM. The confidence interval was generally narrow, particularly in the low and mid-range of measured value, indicating that the predictions of model in these areas were quite reliable. Similarly to Figure 6.2d, the confidence interval at higher values widened slightly.

It was evident that models developed based on variable selection through UVE outperformed those using SPA and CARS. UVE methods can effectively identify and remove redundant features with low correlation to the target variable and high uncertainty by assessing contribution of each variable to prediction stability (Xiaobo et al., 2010). This results in models that better captured the global information of feature bands, while reducing overfitting, leading to greater robustness and accuracy across different datasets. In contrast, SPA and CARS, though effective in feature reduction, rely heavily on local variable contributions (Guo et al., 2021). This can result in the omission of globally important bands or the introduction of noise, thus diminishing model accuracy. Consequently, in this study, UVE-based variable selection proved more suitable than SPA and CARS for developing regression models for VFA content during the anaerobic digestion of OMSW.

Furthermore, the predictive errors achieved for all the VFAs are suitable for AD reactor monitoring. As aforementioned, PA concentrations higher than 3000 mg·L⁻¹ inhibit methanogenesis (Montañés et al., 2013). Therefore, the predictive error of 135.3 mg·L⁻¹ is suitable for this purpose. All in all, the predictive errors of individual and total VFAs are small enough to be useful in real-world applications, especially for preventing propionic acid buildup in reactors.

5.3.4. Correlation between featured wavelengths and UVE model performance

The selected NIR wavelengths for the prediction of the various VFAs by the three assayed variable elimination methods are shown in Supplementary Table 6.3, highlighting the most informative spectral regions. Figure 6.3 illustrates the selected wavelengths by UVE method for each VFAs. Most of the selected wavelengths fall within the ranges of 1100-1250 nm and 1350-1700 nm, i.e. they are in the two main absorption bands observed in the NIR spectrum of the samples (Figure 6.1). These regions corresponded to the C-H stretch overtone and combination bands, as well as O-H and C-H combination bands, which are

highly relevant for identifying organic compounds, including volatile fatty acids. Of note is the absorption peak around 1210 nm, which is related to the second overtones of the C–H bonds stretching vibration from $-\text{CH}_3$ and $-\text{CH}_2-$ groups in aliphatic hydrocarbons (Meng et al., 2023). Meanwhile, the range from 1350 to 1700 nm, apart from comprising the first overtone of water at around 1400 nm, contains the first overtone of $-\text{CH}_3$ groups and second overtone of the C=O and $-\text{OH}$ groups (Sun et al., 2020). The presence of these bands allows for the capture of specific spectral features that are directly related to the concentration and type of fatty acids present.

To sum up, models developed based on variable selection with UVE significantly outperformed those developed with SPA and CARS by focusing on key spectral regions, particularly within the 1100–1250 nm and 1350–1700 nm ranges. These ranges correspond to the C–H overtone and combination bands, as well as O–H and C=O combination bands, which are essential for identifying organic compounds, including volatile fatty acids. The UVE method effectively captures global information from these feature bands, reducing noise and avoiding overfitting. This leads to more robust and accurate predictions for volatile fatty acids, with the exception of propionic acid, where a broader spectral range was needed to improve accuracy. Thus, the UVE approach proved more suitable for developing predictive models for VFAs in this study compared to SPA and CARS, which are more reliant on local variable contributions and may omit globally important features.

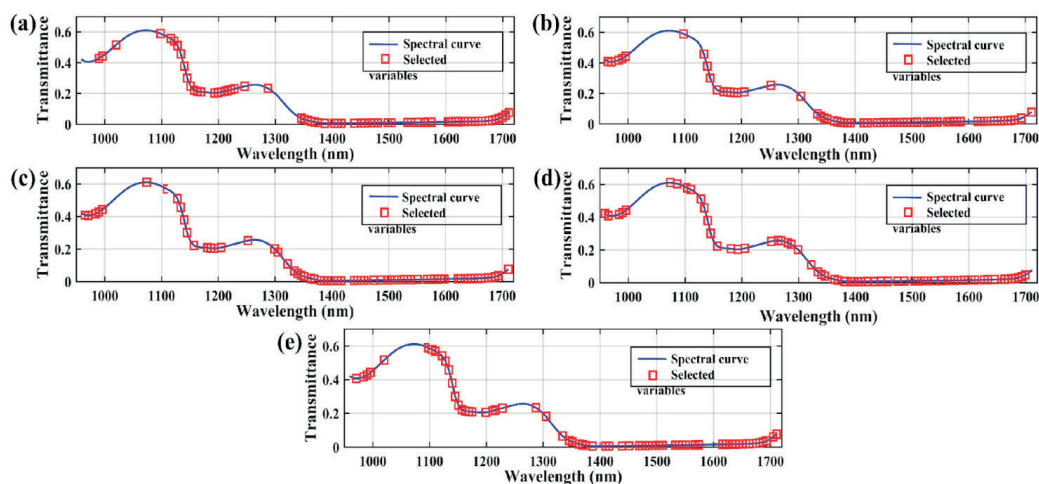


Figure 6.3. Characteristic wavelengths selected by UVE for the prediction of volatile fatty acids: (a) Acetic acid (AA), (b) propionic acid (PA), (c) butyric acid (BA), (d) valeric acid (VA) and (e) total acids (TA).

It should be noted that the main featured wavelengths selected by UVE for each type of VFAs are mostly the same, including TA. This could imply that the quantification of one VFAs is affected by the presence of another VFAs in the sample because of the overlapping of their featured absorption bands, and this would also explain the excellent performance of the PLRS model for TA.

6.4. Conclusions

The use of grid search and spectral variable selection techniques increased the predictive performance of the PLRS models by removing uninformative wavelengths and noise. Among the three spectral variable selection techniques selection assayed, UVE led to the best results.

The UVE-selected wavelength models of acetic acid (AA), propionic acid (PA), butyric acid (BA), valeric acid (VA) and total acids (TA) achieved R^2 of 0.895, 0.622, 0.866, 0.898 and 0.871, and RMSEV of 532.6, 135.3, 102.6, 26.7 and 539.5 mg·L⁻¹, respectively. Considering that instability of AD reactors occurs at concentrations of several grams of each VFAs per liter of AD medium, these errors are adequate and can meet the requirements for rapid VFAs detection.

The prediction performance of the PLRS model for TA was higher than that for AA, PA, BA and VA because the featured wavelengths selected for them by UVE were mostly the same. Hence, the prediction performance for each volatile fatty acid is affected by the presence of other VFAs, while this problem probably does not go for the quantification of TA. Besides, the use of a reduced region of the NIR spectrum (959-1705 nm) instead of the full NIR spectrum (800-2500 nm) did not affect the performance of the PLRS predictive model of total volatile fatty acids. Therefore, most of the featured wavelengths for VFAs quantification are within the 959-1705 nm region.

In conclusion, the use of a hand-held portable spectrometer can be regarded as a potential tool to monitor the production of VFAs over the anaerobic digestion of OMSW and, in general, any type of anaerobic digestion in which VFAs are produced. Looking ahead, this methodology can be further improved by integrating near-infrared spectroscopy with advanced chemometric techniques, such as deep learning, while incorporating spectroscopic methods with higher molecular structure recognition accuracy, like MIR spectroscopy. This combined approach will enable the development of more precise predictive models for VFAs.

Credit authorship contribution statement

E. Jiménez-Páez: Writing – original draft, Investigation, Formal analysis, Data curation. F. Ding: Writing – original draft, Validation, Software, Formal analysis. F.G. Feroso: Visualization, Supervision, Methodology, Funding acquisition, Conceptualization. J.F. García- Martín: Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Formal analysis, Conceptualization.

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6.5. References

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6.6. Supplementary material

Table 6.3. Variables (transmittance at a given wavelength) selected by CARS, UVE and SPA.

Component	Variable selection	Variable selection (nm)
AA	CARS	984, 990, 996, 1002, 1008, 1092, 1134, 1140, 1145, 1151, 1163, 1193, 1199, 1205, 1211, 1228, 1246, 1287, 1316, 1346, 1351, 1363, 1404, 1416, 1451, 1498, 1533, 1563, 1592, 1651, 1663, 1669, 1404, 1410, 1422, 1433, 1445, 1457, 1474, 1486, 1504, 1510, 1521, 1527, 1539, 1551, 1563, 1568, 1574, 1580, 1586, 1592, 1604, 1610, 1627, 1633, 1645, 1663, 1669, 1675, 1681, 1687, 1693, 1699
	UVE	990, 996, 1020, 1098, 1116, 1122, 1128, 1134, 1140, 1145, 1151, 1157, 1163, 1169, 1193, 1199, 1205, 1211, 1216, 1222, 1228, 1246, 1287, 1346, 1351, 1357, 1363, 1369, 1375, 1387, 1404, 1410, 1416, 1439, 1451, 1457, 1469, 1474, 1480, 1498, 1504, 1533, 1539, 1551, 1563, 1568, 1574, 1604, 1610, 1616, 1622, 1633, 1639, 1645, 1651, 1657, 1663, 1675, 1681, 1687, 1693, 1699, 1705, 1711
	SPA	959, 965, 971, 977, 984, 990, 996, 1002, 1008, 1014, 1020, 1026, 1032, 1038, 1044, 1050, 1056, 1062, 1068, 1074, 1080, 1086, 1092, 1098, 1104, 1110, 1116, 1122, 1128, 1134, 1140, 1145, 1151, 1157, 1169, 1181, 1187, 1205, 1211, 1228, 1234, 1240, 1252, 1258, 1269, 1275, 1281, 1287, 1299, 1310, 1381, 1563, 1633, 1669, 1681, 1687, 1693, 1699, 1705, 1711, 1681, 1687, 1693, 1699
PA	CARS	971, 977, 984, 990, 996, 1062, 1128, 1134, 1140, 1145, 1157, 1169, 1205, 1258, 1287, 1334, 1340, 1346, 1363, 1369, 1439, 1451, 1463, 1474, 1486, 1504, 1527, 1539, 1557, 1574, 1586, 1627, 1645, 1410, 1422, 1433, 1445, 1457, 1474, 1486, 1504, 1510, 1521, 1527, 1539, 1551, 1563, 1568, 1574, 1580, 1586, 1592, 1604, 1610, 1627, 1633, 1645, 1663, 1669, 1675, 1681, 1687, 1693, 1699
	UVE	965, 971, 984, 990, 996, 1098, 1134, 1140, 1145, 1157, 1169, 1175, 1181, 1187, 1193, 1205, 1252, 1305, 1334, 1340, 1346, 1351, 1363, 1369, 1375, 1381, 1387, 1392, 1416, 1422, 1439, 1445, 1451, 1457, 1463, 1469, 1474, 1480, 1486, 1492, 1498, 1504, 1510, 1527, 1533, 1539, 1545, 1563, 1568, 1574, 1580, 1586, 1616, 1622, 1627, 1633, 1645, 1651, 1657, 1669, 1675, 1681, 1693, 1711
	SPA	959, 965, 971, 977, 984, 990, 996, 1002, 1008, 1014, 1020, 1026, 1032, 1038, 1044, 1050, 1056, 1062, 1068, 1074, 1080, 1086, 1092, 1098, 1104, 1110, 1116, 1122, 1128, 1134, 1140, 1145, 1151, 1157, 1169, 1181, 1187, 1205, 1211, 1222, 1228, 1234, 1240, 1252, 1258, 1269, 1275, 1281, 1287, 1299, 1310, 1381, 1563, 1633, 1681, 1687, 1693, 1699, 1705, 1711, 1681, 1687, 1693, 1699
BA	CARS	990, 1014, 1020, 1056, 1074, 1104, 1110, 1128, 1140, 1181, 1205, 1211, 1252, 1281, 1287, 1299, 1316, 1322, 1334, 1363, 1416, 1457, 1504, 1539, 1551, 1574, 1610, 1539, 1557, 1574, 1586, 1627, 1645, 1410, 1422, 1433, 1445, 1457, 1474, 1486, 1504, 1510, 1521, 1527, 1539, 1551, 1563, 1568, 1574, 1580, 1586, 1592, 1604, 1610, 1627, 1633, 1645, 1663, 1669, 1675, 1681, 1687, 1693, 1699
	UVE	965, 971, 984, 990, 996, 1074, 1110, 1128, 1134, 1140, 1145, 1157, 1181, 1187, 1193, 1205, 1252, 1299, 1305, 1322, 1334, 1340, 1346, 1351, 1363, 1369, 1381, 1387, 1392, 1398, 1404, 1410, 1416, 1439, 1445, 1451, 1457, 1474, 1486, 1498, 1504, 1510, 1515, 1527, 1533, 1539, 1551, 1568, 1574, 1580, 1586, 1592, 1598, 1622, 1627, 1639, 1645, 1657, 1663, 1675, 1681, 1687, 1693, 1711
	SPA	959, 965, 971, 977, 984, 990, 996, 1002, 1008, 1014, 1020, 1026, 1032, 1038, 1044, 1050, 1056, 1062, 1068, 1074, 1080, 1086, 1092, 1098, 1104, 1110, 1116, 1122, 1128, 1134, 1140, 1145, 1151, 1157, 1163, 1181, 1199, 1205, 1211, 1228, 1234, 1240, 1246, 1252, 1258, 1264, 1269, 1293, 1299, 1310, 1410, 1563, 1610, 1633, 1681, 1687, 1693, 1699, 1705, 1711, 1681, 1687, 1693, 1699

VA	CARS	971, 990, 996, 1074, 1104, 1140, 1157, 1163, 1169, 1187, 1193, 1205, 1252, 1264, 1281, 1299, 1340, 1357, 1369, 1381, 1398, 1416, 1428, 1504, 1510, 1521, 1539, 1563, 1568, 1574, 1586, 1592, 1657, 1669, 1675, 1693, 1699, 1711, 1474, 1486, 1504, 1510, 1521, 1527, 1539, 1551, 1563, 1568, 1574, 1580, 1586, 1592, 1604, 1610, 1627, 1633, 1645, 1663, 1669, 1675, 1681, 1687, 1693, 1699
	UVE	959, 965, 984, 990, 996, 1074, 1086, 1104, 1110, 1128, 1134, 1140, 1145, 1157, 1181, 1193, 1205, 1252, 1264, 1269, 1281, 1287, 1299, 1322, 1334, 1340, 1346, 1363, 1369, 1381, 1392, 1398, 1404, 1410, 1422, 1433, 1445, 1457, 1474, 1486, 1504, 1510, 1521, 1527, 1539, 1551, 1563, 1568, 1574, 1580, 1586, 1592, 1604, 1610, 1627, 1633, 1645, 1663, 1669, 1675, 1681, 1687, 1693, 1699
	SPA	959, 965, 971, 977, 984, 990, 996, 1002, 1008, 1014, 1020, 1026, 1032, 1038, 1044, 1050, 1056, 1062, 1068, 1074, 1080, 1086, 1092, 1098, 1104, 1110, 1116, 1122, 1128, 1134, 1140, 1145, 1151, 1169, 1187, 1199, 1211, 1228, 1234, 1240, 1246, 1252, 1258, 1264, 1269, 1275, 1281, 1299, 1305, 1404, 1533, 1563, 1586, 1604, 1610, 1687, 1693, 1699, 1705, 1711, 1681, 1687, 1693, 1699
TA	CARS	959, 965, 971, 977, 984, 990, 1002, 1008, 1014, 1020, 1026, 1038, 1086, 1110, 1116, 1122, 1128, 1134, 1140, 1145, 1151, 1157, 1163, 1175, 1181, 1187, 1211, 1216, 1228, 1246, 1258, 1269, 1281, 1287, 1299, 1322, 1340, 1346, 1351, 1363, 1375, 1381, 1387, 1404, 1410, 1439, 1445, 1451, 1469, 1486, 1498, 1504, 1515, 1533, 1563, 1574, 1586, 1604, 1633, 1639, 1651, 1681, 1687, 1699
	UVE	971, 984, 990, 996, 1020, 1098, 1104, 1110, 1122, 1128, 1134, 1140, 1145, 1151, 1157, 1163, 1169, 1175, 1199, 1211, 1216, 1228, 1287, 1305, 1334, 1346, 1351, 1363, 1369, 1375, 1387, 1410, 1416, 1439, 1451, 1469, 1474, 1480, 1486, 1492, 1498, 1504, 1510, 1533, 1539, 1545, 1551, 1563, 1568, 1574, 1616, 1622, 1633, 1639, 1645, 1651, 1657, 1663, 1675, 1681, 1687, 1693, 1705, 1711
	SPA	959, 965, 971, 977, 984, 990, 996, 1002, 1008, 1014, 1020, 1026, 1032, 1038, 1044, 1050, 1056, 1062, 1068, 1074, 1080, 1086, 1092, 1098, 1104, 1110, 1116, 1122, 1128, 1134, 1140, 1145, 1151, 1157, 1169, 1181, 1187, 1205, 1211, 1222, 1228, 1234, 1240, 1252, 1258, 1269, 1275, 1281, 1287, 1299, 1310, 1381, 1563, 1633, 1681, 1687, 1693, 1699, 1705, 1711, 1687, 1693, 1705, 1711

CARS: competitive adaptive reweighted sampling; UVE: uninformative variables elimination; SPA: successive projections algorithm.

Monitoring of volatile fatty acids during anaerobic digestion of olive mill solid waste by
means of a hand held near infrared spectrometer

GENERAL DISCUSSION

This thesis focusses on the fermentation of olive mill solid waste (OMSW) as a promising technology for the production of volatile fatty acids (VFAs) as high-value-added products. The fermentation of OMSW remains challenging due to heterogeneity and lignocellulosic nature of this kind of waste. The hydrolytic stage often represents the rate-limiting step in the fermentation process of lignocellulosic based material. Pretreatments are usually proposed to break the particulate organic matter thereby increasing organic matter availability to microbial population. However, these pretreatments typically incur substantial economic costs (Menzel et al., 2020). The aim is to improve the solubilization/hydrolysis without pretreatment of OMSW to maximize the production of volatile fatty acids (VFAs), facilitating their subsequent recovery in a purified form.

In **Chapter 1**, fermentation of OMSW at neutral pH (pH 6 and 7) reached about 80% of OMSW degradation and absence of substantial VFA accumulation. Previous studies performing the valorisation of OMSW by anaerobic digestion process for biogas production observed that under pH range of 6.5-7.5 OMSW biodegradability accounts around 60-90% (Serrano et al., 2021). The absence of substantial VFA accumulation at neutral pH (pH 6 and 7) was consistent with expectations, given the consumption of VFAs by acetogenic bacteria and their subsequent conversion to methane by methanogenic archaea at these pH values (Annamalai et al., 2020). So, even though OMSW is a lignocellulosic substrate, at neutral condition it is highly degradable. However, at acidic (pH 4 and 5) and alkaline (pH 9 and 10) pH conditions methane production was not observed but VFA accumulation was achieved. The ratio of sCOD/tCOD obtained under acidic pH (pH 4 and 5) was only around 20%. This means that the OMSW fed was poorly solubilised. Under alkaline pH (pH 9 and 10) this ratio increased to 50%, clearly higher than acidic pH, but still showing a limitation in the solubilisation of the OMSW. These sCOD/tCOD ratios are in agreement with previous research reported by Cabrera et al (2019). The highest accumulation of VFA was obtained at alkaline pH (pH 9 and 10), being three times higher than at acidic pH (pH 4 and 5). However, the VFA profile was less diverse at alkaline pH (pH 9 and 10), consisting mainly of acetic acid (80% of the total sCOD-VFA), compared to acidic pH (pH 4 and 5) where none of the individual VFA reached more than 65% of the total sCOD-VFA. The dominance in the microbial communities and their diversity indexes clearly evolved at acidic and alkaline pH, in contrast to the neutral pH operation, in which microbial dominance and diversity were similar to the initial inoculum. At alkaline conditions (pH 9 and 10), low diversity and high dominance values in Shannon and Simpson indexes were observed indicating a specialisation of the microbial community. Hence, a less complex VFA profile was correlated with a lower number of microorganisms performing most of the microbial activity under alkaline conditions, being the acidogenic species with the highest relative abundance *g_Tissierella_soenggenia*, *s_Virgibacillus_halodenitrificans*, *o_Clostridiales_2* and *g_Pseudomonas_2*. Conversely, at acidic conditions (pH 4 and 5) higher diversity and lower dominance values in Shannon and Simpson indexes compared to alkaline conditions were observed. At acidic conditions it has been observed a marked increase in the relative abundance of *f_Peptostreptococcaceae_2* specie, and to a lesser extent, an increase in *s_Virgibacillus_halodenitrificans* and *f_Clostridiaceae* species. The *Peptostreptococcus* genus has been described as producers of acetic, butyric, iso-butyric, iso-valeric and iso-caproic acids (Downes & Wade, 2006; Murdoch, 1998). This may explain

the diversity in the VFA profile at pH 4 and 5.

Chapter 2 evaluated two methanogenesis inhibition strategies using semi-continuous reactors. On the one hand, due to the high biodegradability of the OMSW at pH 7, it was operated under this pH by adding an archaeal inhibitor known as 2-bromoethanesulfonate (BES), thus leading to irreversible inhibition of methanogenesis. BES has been extensively used as archaeal inhibitor due to it is similar in structure to methyl-coenzyme M (2-mercaptoethanesulfonate), which is found only in methanogenic archaea (Kosse et al., 2016). On the other hand, the system was operated at pH 5, since it is close to the pH of OMSW and, also, for its diverse VFA profile (**Chapter 1**). Contrary to expectations, the degradation of OMSW was limited under BES condition in comparison to the results in **Chapter 1** when the methane production allowed a degradation of 80%. Therefore, it is most likely not the effect of pH that affects the degradation of OMSW, but the accumulation of VFA itself, i.e. inhibition by product. BES condition improved approximately 35% the organic matter solubilisation compared to pH 5 condition. Also, BES condition also presented a VFA/sCOD ratio 10% higher than pH 5. The differences between pH 5 and BES condition in VFA accumulation and transformation could be related to the VFA dissociation and its impact on the microorganisms. At pH 5, approximately 60% of the total VFA were undissociated ($pK_a \sim 4.7-4.8$). The concentration of undissociated organic acids can influence fermentation, as these forms exhibit a stronger inhibitory effect compared to their ionized counterparts. Due to their neutral charge, undissociated acids can penetrate bacterial cells, where they dissociate internally, releasing H^+ ions and disrupting the transmembrane pH gradient (Kelbert et al., 2024; Sukphun et al., 2021). Conversely, at neutral pH, the proportion of dissociated VFA was lower than at acidic pH (Holtzapple et al., 2022) and, thus, the system at neutral pH would reach a higher total VFA concentration than at acidic pH before reaching an inhibitory level of dissociated VFA. The just slight improvement respect pH 5 would not justify this strategy due to the still limited OMSW degradation and reagents consumption, BES and pH adjustments to maintain neutral pH against reactor acidification.

So, in view of the results in **Chapter 2**, it was necessary to explore other strategies to overcome the limitations in the OMSW fermentation (**Chapters 3 and 4**). Some of the operational parameters that could be modified for enhancing the fermentation process are organic loading rate (OLR) and/or hydraulic residence time (HRT). Both HRT and OLR have been defined as crucial factors for long-term VFA operation processes, since an optimal VFA production would require a suitable OLR to sustain the microbial activity (Jankowska et al., 2018). Also, HRT needs to be long enough to allow the hydrolysis of complex organic matter (Peces et al., 2021; Swiatkiewicz et al., 2021). As the defined challenge in **Chapter 2** was the bacterial inhibition by VFA accumulation, it was proposed to implement variations in the HRT. **Chapter 3** evaluated different HRT at pH 5, because of the pH of OMSW, whereas **Chapter 4** evaluated different HRT at pH 9 because of its high production of VFA obtained in **Chapter 1**. In these **Chapters 3 and 4** it was observed that each pH evaluated have a similar trend in the accumulation of soluble organic matter (sCOD), hydrolysis and VFA accumulation. That is, prolonging the HRT from 7 to 14 days increased the hydrolysis, accounting at the end of 14-days HRT $32 \pm 5\%$ and $53 \pm 9\%$ at pH 5 and pH 9, respectively. VFA production was also enhanced, particularly at pH 5 (up to $7826 \pm 35 \text{ mg O}_2 \cdot \text{L}^{-1}$) even exceeding the production

observed at pH 9 (up to $7050 \pm 840 \text{ mg O}_2 \cdot \text{L}^{-1}$) at the end of 14-days HRT. This improvement at increasing the HRT from 7 days to 14 days was consistent with other studies that report higher VFA production at higher HRT (Bolaji & Dionisi, 2017; Jankowska et al., 2015; Llamas et al., 2022). Despite using a similar experimental set-up and design, the total VFA produced at pH 5 were 26% higher than reported in **Chapter 3**, probably due to the longer operation time that would allow a higher specialisation of the microbial population to the operational conditions. At 14-days HRT revealed that the microbial population retained their ability to generate VFAs, not reaching inhibitory levels despite the high VFA concentration reached. Nevertheless, the change from 14 to 21-days HRT did not produce an improvement in hydrolysis percentage neither at pH 5 (**Chapter 3**) nor at pH 9 (**Chapter 4**). However, 21-days HRT increase VFA up to levels that led to system destabilisation. This suggests that, among the evaluated HRT, 14-days HRT was the most adequate for microbial organic matter hydrolysis and VFA production.

Although some results followed a similar trend, some differences were also observed between pH 5 (**Chapter 3**) and pH 9 (**Chapter 4**). At pH 5 the VFA profile was diverse, obtaining at the end of the experimental time a VFA composition of acetic acid (41%), propionic acid (32%), butyric acid (19%) and valeric acid (8%). These results diverged from the research of fed-batch fermentation at pH 5 (**Chapter 1**), where in propionic acid emerged as the dominant acid, contributing 63% to the total VFA production. These results suggest that propionic and butyric acid fermentation pathways are not solely regulated by pH but rather depend on other factors, such as HRT (Peces et al., 2021). The behaviour observed during operation at pH 5 (**Chapter 3**) could be correlated by the diversity of the microbial population. For example, the enhancement and subsequent decrease of the hydrolysis percentage at 14-days HRT and 21-days HRT, may be correlated with the firstly enrichment and subsequent decrease of specific microbial populations, as evidenced by the variations in the relative abundance of *g_Ruminococcus* and *f_Sphaerochaetaceae* species. The genus *Ruminococcus* has been described as a cellulolytic bacterium whose function is to produce cellulolytic enzymes to degrade cellulose (Lo et al., 2009). This, in turn, facilitates the accessibility of complex carbohydrates for other members of the microbial community. For example, the cellulolytic activity of *g_Ruminococcus* would favour the growth of *f_Sphaerochaetaceae*, whose fermentative growth thrives with simple carbohydrates including pentose and hexose monosaccharides (Nazina et al., 2022). The joint enhancement and subsequent decrease in the relative abundance of both bacteria would indicate a symbiotic interaction between them. Conversely, at pH 9 (**Chapter 4**), the VFA profile remained consistent with **Chapter 1**, i.e. acetic acid as the predominant VFA compound. The accumulation of acetic acid may have been favoured by the chemical effects of the reagents added to maintain pH condition. This addition releases OH^- radicals, promoting the breakdown of organic matter into simpler compounds, and, thus, facilitating the bioavailability of the OMSW (Dahiya et al., 2015; Zhao et al., 2022). Furthermore, the added Na^+ (from NaOH) generated specific conditions that led to a significant selective pressure, resulting in the selection and enrichment of certain bacteria, particularly *s_Virgibacillus_halodenitrificans* specie. *Virgibacillus halodenitrificans* is a halotolerant spore-forming bacterium (growth in up to 25% NaCl) (Sharma et al., 2023). Also, *Virgibacillus halodenitrificans* may have a pathway for the direct conversion of simple

sugars into acetic acid, rather than the acetogenesis pathway as there was no accumulation of intermediate metabolites (propionic to valeric acid). These results were consistent with previous research in which strongly alkaline conditions could inhibit the activity of acidogenic bacteria despite the fixed HRT (Bermúdez-Penabad et al., 2017; Chen et al., 2013). Therefore, despite achieving relevant VFA concentrations with HRT variation, the limitation in the OMSW solubilisation and the product inhibition of the microbial community make necessary to investigate the VFA recovery from OMSW ferments to overcome these limitations.

An electrodialysis stack was developed in **Chapter 5** for VFA recovery, evaluating its application to OMSW ferment. Electrodialysis has been proposed as a complementary technology to the fermentation in order to recover the generated VFA, avoiding at the same time product inhibition in the fermentation process. This chapter evaluated VFA recovery using an electrodialysis system, comparing a synthetic VFA mixture with an OMSW ferment obtained from **Chapter 3**. VFA recovery was practically the same (~60%) using synthetic VFA mixture and OMSW ferment, with only 5% less in the OMSW ferment. These results showed that this technology was feasible to separate a complex matrix such as the OMSW ferment to a clean matrix composed mostly of VFAs and that the OMSW matrix effect did not hinder the recovery process. In line with the results reported by Scoma et al. (2016), for both the synthetic VFA mixture and the OMSW ferment, the recovery percentage of each individual acid was positively correlated with the initial concentration, and negatively correlated with the number of carbons of each compound. In addition, 86% of the energy applied in the electrodialysis system was used for ion migration, being this value similar to those reported for electrodialysis systems for water desalination (Lejarazu-Larrañaga et al., 2020). VFA recovery results were promising as they could alleviate the product inhibition of the microbial population in fermentation processes and, thus, to enhance the generation of VFAs. However, there are still many parameters to be optimised, not being cost-effective in the short term. Furthermore, a future research direction would be to apply this technology in continuous operation with a fermenter.

Due to the long-time involved in the analysis of individual VFAs by gas chromatography, **Chapter 6** assesses near infra-red (NIR) spectroscopy as an alternative for a faster and easier detection of these compounds. Samples of the VFAs obtained in **Chapters 3** and **4** were used to build a prediction model based on NIR spectra and suitable for a complex matrix such as OMSW ferments. NIR spectroscopy is based on the absorption of electromagnetic radiation in the NIR range (780-2500 nm), providing information related to the molecular vibrations of the chemical bonds of organic molecules such as C-H, N-H, S-H and O-H. However, NIR spectroscopy by itself may have limitations for detecting VFA in complex samples. To overpass that, the application of techniques to discriminate the absorption bands that are actually correlated with the presence of VFAs can allow the detection and quantification of VFAs in a reactor. The use of grid search and spectral variable selection techniques increased the predictive performance of the PLRS models by removing uninformative wavelengths and noise. Among the spectral variable techniques assayed, uninformative variables elimination (UVE) technique led to the best results for the quantification of acetic acid, propionic acid, butyric acid, valeric acid and total VFAs. Similarly, NIR was previously used for acetic acid,

propionic acid and total VFAs quantification in reactors with corn stover and cattle manure, obtaining optimal PLS regression models (Xu et al., 2023). Still, this technology presents difficulties for the quantification of individual VFAs, as the wavelengths of the VFAs were mostly equal, and this overlapping absorption bands may cause interference, but it is highly reliable for total VFA concentration.

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GENERAL CONCLUSIONS

Conclusions

- Volatile fatty acids (VFAs) production through fermentation of olive mill solid waste (OMSW) is challenging due to heterogeneity and lignocellulosic nature of this kind of waste.
- Batch anaerobic degradation of OMSW at neutral pH range reached about 80% of OMSW degradation but absence of substantial VFAs accumulation. Even though OMSW is a lignocellulosic substrate, at neutral condition it is highly degradable.
- Contrary to expectations, semicontinuous operation at neutral pH with a methanogenesis inhibitor (BES in this thesis) resulted in a limited OMSW degradation, most likely by product inhibition.
- At semicontinuous operation at acidic (pH 5) and alkaline (pH 9) conditions, methane production was not observed and VFA accumulation was achieved, with pH 5 exceeding the production observed at pH 9.
- Prolonging the hydraulic retention time (HRT) from 7 to 14 days increased the hydrolysis. However, 21-days HRT increase VFA up to levels that led to system destabilisation. This suggests that, among the evaluated HRT, 14-days HRT was the most adequate for microbial organic matter hydrolysis and VFA production.
- The VFA profile at alkaline condition (pH 9) was less diverse than at acidic conditions (pH 5). The VFA profile at pH 9 consisting mainly of acetic acid.
- At alkaline conditions (pH 9), lower diversity and higher dominance were observed indicating a specialisation of the microbial community, this is compared to acidic conditions (pH 5) with higher diversity and lower dominance values.
- Operation at pH 9 generated specific conditions that led to a significant selective pressure, resulting in the selection and enrichment in *s_Virgibacillus_halodenitrificans* specie. *Virgibacillus_halodenitrificans* has been described to have a pathway for the direct conversion of simple sugars into acetic acid.
- Electrodialysis has been proposed as a complementary technology to the fermentation to recover the generated VFA, avoiding at the same time product inhibition in the fermentation process. Electrodialysis separated the OMSW ferment, with an efficiency of around 60%, to a clean matrix composed mostly of VFAs. OMSW matrix effect did not substantially hinder the recovery process. However, it is still necessary further research to properly assess the coupling of the electrodialysis system with the mixed culture fermentation.
- Near-Infrared spectroscopy for fast VFA determination difficulties for the quantification of individual VFAs, as the wavelengths of the VFAs were mostly equal, and this overlapping absorption bands may cause interference, but it is highly reliable for total VFA concentration.

Conclusiones

- La producción de ácidos grasos volátiles (AGVs) mediante la fermentación de alperujo presenta desafíos por su heterogeneidad y naturaleza lignocelulósica.
- La degradación anaerobia a pH neutros, utilizando reactores discontinuos, alcanzó aproximadamente el 80%, pero sin una acumulación sustancial de AGVs. A pesar de que el alperujo es un sustrato lignocelulósico, en condiciones de operación de pH neutro, es altamente degradable.
- Contrario a lo esperado, la operación en régimen semicontinuo a pH neutro con adición de un inhibidor de la metanogénesis (BES en esta Tesis doctoral) resultó en una degradación limitada del alperujo, probablemente debido a una inhibición por producto.
- En la operación en régimen semicontinuo en condiciones tanto ácidas (pH 5) como alcalinas (pH 9), no se observó producción de metano significativa, lográndose acumulación de AGVs. Se observó que la producción de AGVs a pH 5 fue superior a la de pH 9.
- Aumentar el tiempo de retención hidráulico (TRH) de 7 a 14 días mejoró la hidrólisis. Sin embargo, un TRH de 21 días incrementó la concentración de AGVs hasta niveles que llevaron a la desestabilización del sistema. Por tanto, entre los TRH evaluados, el TRH de 14 días puede considerarse el más adecuado para la actividad microbiana.
- El perfil de AGVs en condiciones alcalinas (pH 9) fue menos diverso que en condiciones ácidas (pH 5). El perfil de AGVs a pH 9 consistió principalmente en ácido acético.
- En la comunidad microbiana operando a condiciones alcalinas (pH 9), se observó una menor diversidad y una mayor dominancia, lo que indica una especialización en comparación con las condiciones ácidas (pH 5), que obtuvo unos valores de mayor diversidad y menor dominancia.
- La operación a pH 9 generó condiciones específicas que llevaron a una presión selectiva significativa, lo que resultó en la selección y el enriquecimiento de la especie *Virgibacillus_halodenitrificans*. Se ha descrito que *Virgibacillus_halodenitrificans* tiene una ruta metabólica para la conversión directa de azúcares simples en ácido acético.
- La electrodiálisis se ha propuesto como una tecnología complementaria a la fermentación para recuperar los AGVs generados, evitando simultáneamente la inhibición por producto en el proceso de fermentación. La electrodiálisis separó con una eficiencia de alrededor del 60% los AGVs del fermento de alperujo en una matriz limpia compuesta principalmente por AGVs. El efecto de la matriz del alperujo no obstaculizó sustancialmente el proceso de recuperación. A pesar de esto, aún es necesaria más investigación para evaluar adecuadamente el acoplamiento del sistema de electrodiálisis con la fermentación de cultivo mixto.
- La espectroscopía de infrarrojo cercano para la determinación rápida de AGVs presenta algunas dificultades, ya que las longitudes de onda de los AGVs fueron mayormente iguales, y estas bandas de absorción superpuestas pueden causar interferencia entre la cuantificación individual de AGVs, pero dando resultados más precisos para la concentración total de AGVs.

Scientific production

Peer-review journal papers derived from this PhD Thesis

- Jiménez-Páez, E.**, Serrano, A., Purswani, J., Correa-Galeote, D., Cubero-Cardoso, J., Feroso, F.G., 2023. Impact on the microbial population during biological volatile fatty acid production from olive mill solid waste. *Environmental Technology & Innovation*, **32**, 103409. <https://doi.org/10.1016/j.eti.2023.103409>. Impact factor (JCR, 2023): 6.7. Quartile: **Q1** (JCR-Environmental sciences **44/358**).
- Jiménez-Páez, E.**, Serrano, A., Hueso, R., J., Feroso, F.G., Cubero-Cardoso, 2025. Evaluation of Semi-Continuous Anaerobic Fermentation of Alperujo by methanogenesis inhibition. *Processes*, **13**(3), 600. <https://doi.org/10.3390/pr13030600>. Impact factor (JCR, 2023): 2.8. Quartile: **Q2** (JCR- Chemical Engineering **80/170**).
- Jiménez-Páez, E.**, Serrano, A., Purswani, J., Trujillo-Reyes, Á., Fernández-Prior, Á., Feroso, F.G., 2025. Impact of hydraulic retention time on the production of volatile fatty acids from lignocellulosic feedstock by acidogenic fermentation. Under review in *Process Safety and Environmental Protection*. Impact factor (JCR, 2023): 6.9. Quartile: **Q1** (JCR- Chemical Engineering **22/170**).
- Jiménez-Páez, E.**, Candelera-Juracan, A., Purswani, J., Serrano, A., Feroso, F.G., 2025. Influence of the hydraulic retention time over the specialization of the microbial community in the alkaline fermentation of olive mill solid waste. Under review in *Bioresource Technology*. Impact factor (JCR, 2023): 9.7. Quartile: **Q1** (JCR- Biotechnology & Applied Microbiology **10/174**).
- Jiménez-Páez, E.**, Ding, F., Feroso, F.G., García-Martín, J.F., 2024. Monitoring of volatile fatty acids during anaerobic digestion of olive pomace by means of a hand held near infrared spectrometer. *Science of The Total Environment*, **955**, 176979. <https://doi.org/10.1016/j.scitotenv.2024.176979>. Impact factor (JCR, 2023): 8.2. Quartile: **Q1** (JCR- Environmental sciences **31/358**).

Other peer-review journal papers published during this PhD Thesis

- Parrilla-Lahoz, S., **Jiménez-Páez, E.**, Masteghin, M. G., Pawlak, J. J., Venditti, R. A., Bird, R., Servin, P., Odriozola, J.A., Ramirez-Reina, T., Duyar, M.S., 2025. Upcycling textile derived microplastics waste collected from washer and dryers to carbonaceous products using hydrothermal carbonization. *Waste Management*, **200**, 114740. <https://doi.org/10.1016/j.wasman.2025.114740>. Impact factor (JCR, 2023): 7.1. Quartile: **Q1** (JCR- Environmental sciences **41/358**).
- Trujillo-Reyes, Á., Jiménez-Páez, E., Serrano, A., Kassab, G., Feroso, F.G., Alonso-Fariñas, B., 2023. Comparative Life Cycle Environmental Impact Assessment of Fruit and Vegetable Waste Valorization by Anaerobic Digestion as an Alternative in a Mediterranean Market. *Processes*, **11**(12), 3397. <https://doi.org/10.3390/pr11123397>. Impact factor (JCR, 2023): 2.8. Quartile: **Q2** (JCR- Chemical Engineering **80/170**).
- Cubero-Cardoso, J., **Jiménez-Páez, E.**, Trujillo-Reyes, Á., Serrano, A., Urbano, J., Rodríguez-

Gutiérrez, G., Borja, R., Feroso, F.G., 2023. Valorization of strawberry extrudate waste: recovery of phenolic compounds by direct-hydrothermal treatment and subsequent methane production by mesophilic semi-continuous anaerobic digestion. *Waste Management*, **169**, 310-318. <https://doi.org/10.1016/j.wasman.2023.07.016>. Impact factor (JCR, 2023): 7.1. Quartile: **Q1** (JCR- Environmental sciences **41/358**).

Results derived from this PhD Thesis presented in Conferences

(*, presenter)

Jiménez-Páez, E., Cubero-Cardoso, J.*, Purswani, J., Feroso, F.G., Serrano, A. "Effects of hydraulic retention time variation on volatile fatty acids production via alkaline anaerobic fermentation". 5th Iberoamerican Congress on Biorefineries, Jaen (Spain). Poster. 2nd-4th October 2024.

Jiménez-Páez, E.* "De residuo a materia prima: la transformación de subproductos de la industria del aceite de oliva en productos de alto valor añadido." V Congreso/VII Jornadas de Investigadores en formación fomentando la interdisciplinariedad, Granada (Spain). Oral communication. 12th-14th June 2024.

Jiménez-Páez, E.*, Serrano, A., Purswani, J., Trujillo-Reyes, A., Feroso, F.G. "Anaerobic acidogenesis of a lignocellulosic feedstock: just limited by the hydrolysis step?". 18th IWA World Conference on Anaerobic Digestion, Istanbul (Turkey). Poster. 2nd-6th June, 2024, Istanbul (Turkey).

Jiménez-Páez, E.*, Purswani, J., Serrano, A., Feroso, F.G. "Influence of pH on volatile fatty acid production and microbial population from olive mill solid waste digestion". 3rd International Meeting on New Strategies in Bioremediation/Restoration Processes, Muttentz (Swiss). Oral Communication. 29th-30th June 2023

Jiménez-Páez, E., Purswani, J., Correa, D., Cubero-Cardoso, J., Feroso, F.G., Serrano, A*. "Valorización de alperujo mediante la obtención anaerobia de ácidos grasos volátiles: efecto del control operacional del pH". XXI simposium científico técnico Expoliva, Jaén (Spain). Poster. 10th-13th May 2023

Jiménez-Páez, E.*, Purswani, J., Serrano, A., Feroso, F.G. "Volatile fatty acids accumulation under controlled pH conditions". IMAB23-International Congress on Metal-microbe applications for circular economy, Porto, (Portugal). Oral Communication. 19th-21th June 2023.

Jiménez-Páez, E.*, Serrano, A., Feroso, F.G. "Recuperación de compuestos a partir del residuo generado en la industria del aceite de oliva". I edición de las Jornadas Multidisciplinares de Ciencia, Tecnología, Ingeniería y Matemáticas (CTIM) de Divulgación Científica, Seville (Spain). Poster. 14th-15th February 2023.

Jiménez-Páez, E.*, Serrano, A., Feroso, F.G. "Recuperación de compuestos a partir del residuo generado en la industria del aceite de oliva". I edición de las Jornadas Multidisciplinares de Ciencia, Tecnología, Ingeniería y Matemáticas (CTIM) de Divulgación Científica. Oral Communication. 14th-15th February 2023.

Conference Book of Abstract chapters from this PhD Thesis

- Jiménez-Páez, E.**, Cubero-Cardoso, J., Purswani, J., Feroso, F.G., Serrano, A. 2024. *“Effect of hydraulic retention time variation on volatile fatty acids production via alkaline anaerobic fermentation”*. 5th Iberoamerican Congress on Biorefineries. Universidad de Jaén, Servicio de Publicaciones, pp. 192, ISBN:978-84-9159-626-4.
- E. Jiménez-Páez**, A. Serrano, J. Purswani, Á.Trujillo-Reyes, F.G. Feroso, 2024. *“Anaerobic acidogenesis of a lignocellulosic feedstock: Just limited by the hydrolysis step”*. 18th IWA World Conference on Anaerobic Digestion, Istanbul– 2024. Pp. 1861 to 1863, ISBN: 978-975-561-673-5.

Other conference contributions

- Jiménez-Páez, E.***, Garcitua, M., Correa-Galeote, D., Zahedi-Diaz. S., Feroso, F.G., Carvajal, A., Serrano, A. “Effects of different anaerobic inoculum on the production of volatile fatty acids at acidic pH”. 18th IWA World Conference on Anaerobic Digestion, Istanbul (Turkey). Oral communication. 2nd-6th June,2024, Istanbul (Turkey).
- Serrano, A., Robledo-Mahón, Tatiana., Ángeles-De Paz, Gabriela., Muñoz-Palazón, Bárbara., Rosa-Masegosa, Aurora., Can-Ubando, Lorna., Natera, Michael., López-Rodríguez, María Del Mar, García-Toledo, María., Díaz-Moreno, Miguel Ángel., Trujillo-Reyes, Ángeles., **Jiménez-Páez, Elena.**, Dávila-Ramos, Sonia., Mattei, Luca., Hueso, Rafael., Silva-Castro, Andrea., Tamayo-Navarrete, María Isabel., Calvo, Concepción., Requena, Elena., y Aranda, Elisabet*. “Motivación de las vocaciones científicas en microbiología en alumnos de educación infantil y primaria”. XXIX Congreso Sociedad Española de Microbiología. Burgos (Spain). Poster. 25th-28th June 2023.
- Invited Speaker at the ABC Newspaper (Seville, Spain) conference entitled “El gas renovable pide pista para su despegue en Andalucía”. 29th March 2023.
- Jiménez-Páez, E.***, Trujillo-Reyes, Á., Alonso-Fariñas, B., Serrano, A., Feroso. F.G. “Análisis de ciclo de vida comparativo entre la deposición en vertedero y el tratamiento mediante digestión anaerobia de residuos agroindustriales”. XIV Congreso de la Mesa Española de Tratamiento de Aguas (META 2022), Seville (Spain). Poster. 1st-3rd June 2022.

Other Congress Book Chapter

- E. Jiménez-Páez**, M. Gacitúa, D. Correa-Galeote, S. Zahedi, F.G. Feroso, A. Carvajal, A. Serrano, 2024. *“Effects of different anaerobic inoculum on the production of volatile fatty acids at acidic pH”*. 18th IWA World Conference on Anaerobic Digestion, Istanbul– 2024. Pp. 531 to 533, ISBN: 978-975-561-673-5.

Scientific production

Scientific dissemination

- Participation as a speaker in a dissemination event for first-year Science and Technology students at the public high school I.E.S. "SIERRA SUR" in Osuna, Seville, on February 17th, 2025.
- Participation as a speaker for the International Day of Women and Girls in Science, presenting "Microbiología y tecnología ambiental" to 1st-year high school students at I.E.S. 'BAJO ARAGÓN' (Alcañiz, Spain). 12th February, 2025.
- 16th Red de Biodigestores para latino américa y el caribe (RedBioLAC) meeting, Santiago de Chile, Chile. Assistant. 21th-25th October 2024
- Participation in the Aula-COIL 2024 program for the Innovation and Entrepreneurship course (ICB 3103) within the Biochemical Civil Engineering degree program at Pontificia Universidad Católica de Valparaíso (Chile), May-June 2024.
- Participation in the 21st Edition of the Seville Science Fair as a representative of the Instituto de la Grasa. 10th-13th, May 2023.
- Participation in the Science Week Workshops at CEIP José Hurtado in Granada (Granada). 27th March 2023.
- Participation in the Jaén Provincial Council's outreach initiative "Valorizar los subproductos del olivar, estrategias para una Oleicultura Jiennense más competitiva"
- Mentored the "Encinas Robóticas" student team of I.E.S Las Encinas (Valencina de la Concepción, Seville) on their innovative project, "¿Cómo puedo aprovechar los residuos de mi entorno para obtener energía?," which received the Best Innovation Project award at the FIRST® LEGO® League 2023. Participation as a guide in the guided tours offered by the Instituto de la Grasa (CSIC) to the students of 1st Bachillerato of IES Blas Infante (El Viso del Alcor, Seville). 9th February 2023.
- Participation as a speaker with two presentations for students in 3rd and 4th ESO at IES Maestro Francisco Gallardo, Los Corrales (Seville) during the 11F International Day of Women and Girls in Science. 10th February 2023.
- Participation as a speaker for students of Dietetics (Colegio St^a Joaquina de Vedruna, Seville) at the "Jornadas de Café con Ciencia 2022" event organized by Fundación Descubre and the Scientific Culture Unit of Instituto de la Grasa (CSIC), Seville. November 2022.
- Contribution to the European Researchers' Night 2022 Conference in Seville (organized by Fundación Descubre) with the activity "Que el Sol haga una foto". 1st-2nd October 2022.
- Participation as a speaker with four presentations for kindergarten and primary school students at SAFA Blanca Paloma, Seville (Seville) during the 11F International Day of Women and Girls in Science 2022 celebration. 15th February 2022.
- Participation as a speaker with four presentations for kindergarten and primary school students at CEIP María Ana de la Calle, El Coronil (Seville) during the 11F International Day of Women and Girls in Science 2022 celebration. 11th February 2022.

