



Improved production and valorization of volatile fatty acids in a biorefinery-type model from pig manure

A thesis

Submitted by

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May, 2024



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*in partial fulfilment of the requirements for the degree
of*

Doctor of Philosophy

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Acknowledgments

I would like to express my deepest gratitude to the following individuals and institutions who have played a critical role in supporting and guiding me through this journey:

I extend my heartfelt thanks to the Graduate Program of Environmental and Sanitary Engineering, and Universidad del Valle for providing me with the educational foundation and resources necessary for my research. I also want to thank to Minciencias for the financial support and recognition provided by the national doctoral fellowship, which made my doctoral studies possible.

Professor Janeth Sanabria: your mentorship and guidance have been invaluable throughout my studies. Thank you for your insightful feedback and constant encouragement. Professors Luz Stella Cadavid and Julia Rosa Caicedo: your expertise and support have greatly contributed to my growth as a researcher. I am grateful for the knowledge and insights you have shared.

Dany Mercedes Acevedo: your friendship, assistance, and collaboration have been essential to my work. I appreciate your kindness and dedication. I would also like to thank Nicolás Romero, Daniela González, Sebastián Torres, Nataly Alvear, and Jorsanni Diaz from BIOX-ENERGY research group - Laboratory Microbiota, for their invaluable contributions to my research. Your hard work and camaraderie have made this journey memorable.

A special thank to Professors Julien Wist and Elaine Holmes, along with David García, from Murdoch University, Australia. Your collaboration and support have been instrumental in broadening the scope of my research.

To my family, thank you for your unwavering love and support. You have been my rock throughout this journey. To all who have supported me in various ways, I thank you from the bottom of my heart. This accomplishment would not have been possible without each of you.

Abstract

The management of the wastes produced by the pork industry, particularly pig manure, is a significant challenge due to its high content of organic matter and polluting potential. The production of volatile fatty acids (VFAs) through bioprocesses is a promising alternative for the management and valorization of this organic waste. The objective of this work was to demonstrate that it is possible to enhance the production and valorization of VFAs from pig manure by applying short thermal pretreatment, at 90 °C by 10 minutes, to improve inoculum conditions, and by evaluating operational conditions of the fermentative process, such as initial pH, total solids content, and the inoculum-to-substrate ratio. Furthermore, the VFAs obtained from fermentation of pig manure were evaluated as a sustainable carbon source for biological nitrogen fixation. The results demonstrated that it is possible to improve VFAs production from pig manure by modifying inoculum characteristics through short thermal pretreatment, and setting operational conditions that favor VFAs accumulation. Moreover, it was demonstrated that pig-derived VFAs can be recovered by means of distillation and used as a promising alternative carbon source for nitrogen fixation. This work constitutes a contribution to the knowledge in the valorization of livestock waste, under the approach of process integration within the context of biorefineries.

Table of contents

Acknowledgments.....	3
Abstract.....	4
Table of contents.....	5
List of tables.....	7
List of figures.....	8
Chapter 1: Introduction.....	10
1.1. Context of pig farming and waste management.....	10
1.2. Problem statement.....	12
1.3. Objectives.....	16
1.4. General structure of the document.....	17
Chapter 2: Literature review.....	19
2.1. Anaerobic digestion.....	19
2.2. Volatile fatty acids.....	23
2.2.1. Characteristics of the VFAs.....	23
2.2.2. Acidogenic fermentation.....	25
2.3. AD-biorefineries for organic wastes.....	31
2.3.1. VFA-based biorefinery: the carboxylate platform.....	37
2.3.2. R&D trends on integral valorization of pig manure.....	42
Chapter 3: Effect of inoculum thermal pretreatment on the production of VFAs.....	45
3.1. Introduction.....	45
3.2. Materials and methods.....	47
3.2.1. Substrate and inocula.....	47
3.2.2. Pig manure fermentation.....	48
3.2.3. Analytical methods.....	50
3.2.4. Statistical analysis.....	51
3.3. Results and discussion.....	52
3.3.1. Effect of the short thermal pretreatment on the concentration of volatile fatty acids.....	52
3.3.2. Effect of thermal pretreatment on the stability of the fermentation process.....	55
3.3.3. Effect of short thermal pretreatment on microbial composition.....	56
3.4. Chapter conclusions.....	63
Chapter 4: Evaluation of operating conditions and scalability of the production and recovery of VFAs from pig manure.....	65
4.1. Introduction.....	65
4.2. Materials and methods.....	66
4.2.1. Inoculum and substrate.....	66
4.2.2. Evaluation of operating conditions.....	67
4.2.3. Demonstration of process scalability.....	68

4.2.4. Recovery of pig-manure-derived volatile fatty acids.....	68
4.2.5. Analytical methods.....	69
4.2.6. Statistic analysis.....	70
4.3. Results and discussion.....	71
4.3.1 Evaluation of operating conditions.....	71
4.3.2. Analysis of the effect of individual factors of the process.....	73
4.3.3. Analysis of the effect of the interaction between process factors.....	75
4.3.4. Demonstration of the scalability of the fermentation process.....	77
4.3.5. Recovery of pig-manure-derived volatile fatty acids.....	80
4.4. Chapter conclusions.....	86
Chapter 5: VFAs valorization in a nitrogen fixation system: Recovery of pig-derived VFAs as a sustainable carbon source.....	87
5.1. Introduction.....	87
5.2. Materials and methods.....	89
5.2.1. Bioreactors system.....	89
5.2.2. Evaluation of nitrogen fixation of the adapted inoculum with different carbon sources.....	90
5.2.3. Methods for physicochemical analysis.....	91
5.2.4. Statistical analysis.....	92
5.3. Results and discussion.....	93
5.3.1. Evaluation of nitrogen fixation of the adapted inoculum with different carbon sources.....	93
5.3.2. Microbial growth in the nitrogen fixation process with different carbon sources.....	97
5.4. Chapter conclusions.....	100
Chapter 6: General conclusions.....	101
References.....	103
Appendix.....	131

List of tables

	Page
Table 2.1. Physicochemical characteristics of some carboxylic acids	24
Table 2.2. Literature of VFA production from organic wastes	30
Table 2.3. Global demand and applications of VFAs	40
Table 3.1. Physicochemical characteristics of the substrate and inocula used in the experiment	48
Table 4.1. Characteristics of inoculum and substrate used to evaluate the operational conditions on VFAs production in the fermentation of pig manure	67
Table 4.2. Coding of variables for statistical analysis	70
Table 4.3. Concentration of individual and total VFAs (mg/L) depending on the inoculum / substrate ratio (ISR), initial pH and total solids (TS), on day 15 of pig manure fermentation	72
Table 4.4. Multicriteria analysis of recovery techniques applicable to VFAs	85
Table 5.1. Experimental design to evaluate different carbon sources on nitrogen fixation	91

List of figures

	Page
Figure 1.1. Balance of COD in the AD process.	13
Figure 1.2. General methodological scheme of experimental chapters	18
Figure 2.1. Metabolic pathways in anaerobic digestion	20
Figure 2.3. General scheme of main metabolic pathways in ideal acidogenic fermentation from complex organic wastes	27
Figure 2.4. Co-occurrence network with author keywords related to the search “biorefinery” in Scopus, limited to reviews, books and book chapters	32
Figure 2.5. Co-occurrence network with author keywords related to the search “biorefinery AND manure” in Scopus.	33
Figure 2.6. Proposed AD-based biorefinery for producing biofuels and bio-based products	36
Figure 2.7. General scheme of chemical routes to obtain chemicals products and hydrocarbon fuels in carboxylate platform	38
Figure 2.8. Bioproducts that can be obtained from pig manure, retrieved as word frequency from data processed with Biblioshiny package	43
Figure 3.1. Methodological scheme to evaluate the effect of thermal pretreatment of inocula on VFAs production	49
Figure 3.2. Concentration of individual VFAs and degree of acidification in the fermentation of pig manure with three inocula	53
Figure 3.3. pH and the VFA/alkalinity ratio in the fermentation of PM with three inocula	55
Figure 3.4. Microbiological composition at the genus level after 15 days of fermentation of pig manure, as a function of the thermal pretreatment of the inoculum	58
Figure 3.5. Diversity indexes in the fermentation of pig manure as a function of inoculum an thermal pretreatment	60

Figure 3.6. Canonical correspondence analysis (CCA) for physicochemical and microbiological composition at genus level in the fermentation of pig manure	62
Figure 4.1. Concentration of individual VFAs (mg/L) in the fermentation of pig manure as a function of the inoculum/substrate ratio (ISR), initial pH and time	71
Figure 4.2. Effect of individual operating factors on VFA production from pig manure after 15 days of fermentation	73
Figure 4.3. Effect of the interaction between factors on the production of VFAs from pig manure after 15 days of fermentation. Acids: acetic (A), isobutyric (B), isovaleric (C and D), total VFAs (E)	76
Figure 4.4. Production and distribution of VFAs in semi-continuous fermentation of pig manure	78
Figure 5.1. Historic world agricultural production and harvested area	87
Figure 5.2. Worldwide fertilizer usage in agriculture by nutrient	88
Figure 5.3. Scheme of air filtration and pumping	90
Figure 5.4. N-total concentration in bacterial biomass (A) and supernatant (B), from a nitrogen-fixing adapted consortium with different carbon sources	94
Figure 5.5. Assimilation of VFA during nitrogen-fixing adapted consortium with different carbon sources: individual VFAs (A), synthetic VFA mix (B), VFA mix from fermented pig manure (C)	95
Figure 5.6. pH of effluent from a nitrogen-fixing bacteria consortium with different carbon sources	97
Figure 5.7. Optical density (OD) of effluent from a nitrogen-fixing adapted consortium with different carbon source	98
Figure 5.8. Biochemical pathway of butyrate in nitrogen fixation	99

Chapter 1: Introduction

1.1. Context of pig farming and waste management

Pig farming is one of the oldest and most important livestock activities for food security, reaching a global production of 120.37 million tons (975.4 million heads) and positioning itself as the second most important source of animal protein, behind chicken, with a share of 33.68% in 2021. In that year, 44.78% of world pork production was concentrated in China, followed by the European Union (25.58%) and the United States (10.43%). In Latin America, Brazil and Mexico were the countries with the highest participation, with 3.63% and 1.41%, respectively. Colombia contributed 0.38% of world production (FAO, 2023a).

Although food production plays a significant role in economic development and poverty reduction, it is recognized as one of the activities with the greatest environmental impact. Specifically, livestock production is associated with deforestation and the emission of greenhouse gases (GHG) such as CH₄, CO₂ and N₂O, which represent 35%, 9% and 65% of global anthropogenic emissions. Additionally, wastewater (urine and manure) from intensive animal husbandry generates around 64% of NH₃ emissions caused by human activities (Lassaletta *et al.*, 2019; Tabassum-Abbasi *et al.*, 2016).

Pig manure is composed of feces (solid fraction) and urine (liquid fraction), it is generally mixed with food residues, dust and washing water (Noreña *et al.*, 2016). Its chemical composition presents levels of nitrogen, phosphorus and potassium above 1% each (Noreña *et al.*, 2016; J. Yang *et al.*, 2019). And its average production rate is estimated at 2.35 kg/animal-day (Mariscal, 2007), presenting variations due to the age and physiological maturity of the animal, the quantity and quality of the food ingested, the volume of water consumed and the climate (Galvis & Acevedo, 2008).

In Colombia, the pork industry registered a population of 5.5 million animals in 2022, with an average annual growth of 6.5% in the last 10 years (PorkColombia, 2023); this implies the generation of around 4,700 tons of manure in that year.

In a study carried out by the Colombian Association of Pig Farmers (ACP, 2013), in 4,595 intensive technology farms evaluated in Colombia, the application of at least one of the following treatments to solid waste was identified: drying (27.03%), composting (16.58%), vermiculture (1.63%), storage (10.79%), anaerobic digestion (AD) (7.79%), others (28.77%), none (7.42%). It is worth mentioning that this information may have changed in the last decade, given that no recent studies were found, but the most important thing is to recognize that each of these processes alone does not offer a complete solution to the problem of waste management. The cleaning operation of the pigsties must also be considered, whose water consumption ranges between 10 and 20 L/d per animal (Galvis & Acevedo, 2008), which determines the total volume of wastewater that is generated.

From this perspective, it is crucial to establish an adequate system for waste management, which prevents the generation of negative environmental and social impacts, and which minimizes the costs of collection, treatment and final disposal that may condition the sustainability of companies (Aguilar-Rivera & de Jesús Debernardi-Vázquez, 2018).

On the other hand, there is pressure from the environmental control entities related to this industry. In Colombia, there is a legal and regulatory framework that regulates the management of pig manure, including alternatives such as: i) discharge into surface waters, regulated by Resolution 631/2015 (MADS, 2015); ii) fertilization of soils, which requires studies of slurry, soils and nearby water sources, regulated by Resolution 0660-0504/2017 (CVC, 2017), and iii) reuse of wastewater, regulated by Resolution 1207/2014 (MADS, 2014). Although the primary purpose of these alternatives is, rightly, to prevent environmental pollution, they leave very little room for waste recovery. For this reason, the pork

industry still has the technical and economic challenge of finding more efficient, economical and environmentally safe options to manage its waste.

The valorization of waste involves its transformation into products with demand in the market, such as organic compounds, nutrients, energy, materials and fuels, avoiding their uncontrolled release into the environment (Havukainen *et al.*, 2020; Lü *et al.*, 2021; Nzeteu *et al.*, 2022; Ogbu & Okechukwu, 2023). The efficient use of these materials is increasingly important, along with the improvement and implementation of new techniques for the use of these widely available resources, and is in line with trends such as Zero Waste, Circular Economy and Biorefineries, which are gaining interest in the agricultural and agroindustrial sector.

Among the wide range of technologies with potential to achieve the goal of sustainability, biotechnology could occupy an important place, especially in the fields of food production, renewable raw materials and energy, pollution prevention and bioremediation (Yeung *et al.*, 2019). Currently, the main application of biotechnology in Environmental Engineering is the use of microorganisms to control pollution (Giovanella *et al.*, 2020; Pessôa *et al.*, 2019; Silva *et al.*, 2018; Strous & Sharp, 2018), which implies the acquisition of low-cost raw materials that serve as fermentable substrates, that is, rich in carbon and nitrogen (Zaharia, 2015) that do not compete with food security. The pork industry, and agribusiness in general, can provide these substrates through a large quantity and variety of byproducts.

1.2. Problem statement

The pig manure (PM) has a solid content that usually exceeds 20%, and nitrogen, phosphorus and potassium contents above 1% each (Noreña *et al.*, 2016; J. Yang *et al.*, 2019). Such characteristics give it considerable polluting power, if it is not treated properly. But these same characteristics make PM an attractive substrate for biotechnological applications such as anaerobic digestion (AD), a process carried out by microorganisms that decompose biodegradable materials in an oxygen-free environment,

producing biogas and digestate as an energy source and nutrients, respectively (Czekala, 2022), and allow the stabilization of organic contaminants (Manchala *et al.*, 2017).

As waste valorization technology, the AD process has been traditionally focused on biogas production for energy recovery as CH₄, which is considered as a promising renewable energy source in the fight against climate change. Statistics on global electricity generation from biogas shows an increase from 54,096 GWh in 2011 to 96,565 GWh in 2020, that means a growth of 78.5% in ten years (IRENA, 2022). However, according to World Bioenergy Association (WBA, 2022), biogas represented only 0,25% of the global total primary energy supply (TPES) in 2020. In Colombia, the technical potential for energy production of biogas from residual biomass is 53,354 TJ/year, which is equivalent to 25% of the national demand for natural gas in 2016 (UNAL & TECSOL, 2018). However, the lack of technical guidelines about anaerobic digestion from agroindustry wastes, and the limited infrastructure for biogas depuration in compliance to quality required for injection and thermal conversion as established at Resolution 240/2016 (CREG, 2016), makes biogas utilisation very limited. This suggests that other ways to use the AD downstreams (i.e. digestate) could also be investigated and developed.

The possibility to propose technological AD-based models for integral valorization of manure, can be understood with the balance of Chemical Oxygen Demand (COD) in the AD process (Figure 1.1), which COD total is composed of biodegradable and recalcitrant fractions. The recalcitrant fraction remains along the process, while biodegradable fraction is consumed for cellular growth, production of volatile fatty acids (VFA) and methane. At the end of the AD, cells and methane are considered as COD removed, while VFA and COD recalcitrant are considered as COD not removed (Chernicharo, 2007).

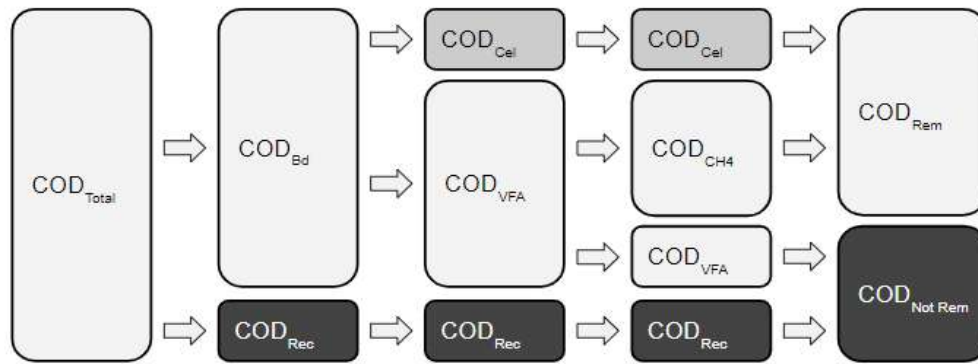


Figure 1.1. Balance of COD in the AD process. Source: (Chernicharo, 2007).

This scheme suggests that mass balance in AD can be moved towards VFA accumulation in digestate, instead of methane accumulation in biogas. This has been regarded as a promising roadmap for organic wastes stabilization and resource recovery, because, compared to methane, VFA have higher value and a wider range of applications (W. Fang *et al.*, 2020; Kleerebezem *et al.*, 2015; Yuan *et al.*, 2019). The VFA recovery is in line with recent trends such as the circular economy, and its global demand for 2020 is estimated at 18,500 kilotons (Atasoy *et al.*, 2018). In this vein, the improving VFA production through the fermentation of pork wastes could be the key for further valorization. As a reference, the value of methane produced from one ton of biomass is only one-fifth the economic value of VFAs produced from the same amount of biomass (Ross, 1998).

VFA have applications in food, pharmaceutical industries as preservatives (Lee *et al.*, 2014), production of esters, bioplastics (Kleerebezem & van Loosdrecht, 2007) and bioenergy (Uyar *et al.*, 2009), as well as in the biological removal of nutrients from wastewater as a carbon source (Agler *et al.*, 2011; X. Li *et al.*, 2011). In this sense, acidogenic fermentation has been proposed as a base for biorefineries (Agnihotri *et al.*, 2022; Sekoai *et al.*, 2021; Venkata Mohan *et al.*, 2016).

After their production, VFAs must be recovered from complex aqueous solutions. This can be achieved through a variety of physical/chemical techniques, for example: precipitation (B. Tao *et al.*, 2016), electrodialysis distillation (R. Kumar *et al.*, 2006), adsorption (Reyhanitash *et al.*, 2017), ion exchange (Rebecchi *et al.*, 2016), reactive extraction (Rasrendra *et al.*, 2011), liquid - liquid extraction (Reyhanitash *et al.*, 2016), and membranes (Outram & Zhang, 2018; Yesil *et al.*, 2014; Zacharof *et al.*, 2016). However, the VFAs extraction produced from fermentation is a bottleneck in the valorization processes, due to the low concentration of these compounds and its complexity in the fermentation broths. Moreover, the pure form of individual acids is more valuable and useful than mixed VFAs, making necessary the application of further separation processes to obtain separate added value compounds (Aghapour Aktij *et al.*, 2020).

AD has been traditionally driven to methane production to treat pork wastes, while VFAs has been reported as inhibitors of the anaerobic process (Ahring *et al.*, 1995; Ni *et al.*, 2017). Furthermore, some authors have addressed the VFA production and recovery from pig manure by means of substrate hydrolysis followed by membrane electrodialysis (Shi *et al.*, 2018) and short-term dry anaerobic digestion followed by ammonia stripping (W. Huang *et al.*, 2016). However, few studies have been performed related to VFA recovery from this substrate.

Considering these facts, the following research questions can be formulated:

- Is it possible to chemically and microbiologically modify the inoculum in an efficient way to improve the production of VFAs from pig manure?
- Which are the optimal operational conditions to increase VFA production in acidogenic fermentation of pig manure?
- Is it possible to couple pig-derived VFAs production to biological nitrogen fixation as a recovery process?

1.3. Objectives

General

To Propose a coupled system for recovery and valorization of volatile fatty acids (VFAs) by anaerobic digestion from pig manure potentially applicable to pig industry.

Specifics

- To evaluate a efficient method to modify the inoculum for improving VFAs production from pig manure
- To find the conditions to maximize VFA production in acidogenic fermentation of pig manure at laboratory and bench scales.
- To evaluate pig-manure-derived VFAs as a carbon source for biological nitrogen fixation

1.4. General structure of the document

This document is divided into chapters, as described below. In **Chapter one**, the reader is introduced to the general context of pig farming, and the environmental problems derived from the production and management of its waste, leading to the objectives of this research work. In **Chapter two**, the generalities of anaerobic digestion as a process for the management of pig manure are presented, and special emphasis is placed on acidogenic fermentation for the production of VFAs. It is also demonstrated, with the help of bibliometric tools, that anaerobic digestion is the most studied process as a platform for organic waste biorefineries, and the use of VFAs to obtain different value-added products is deepened.

Chapters 3, 4, and 5 deal with the experimental work, and these are interconnected as described below. **Chapter 3** comprises the first experiment, in which the effect of thermal pretreatment of three inocula at 90°C for 10 minutes on the production of VFAs in the fermentation of pig manure in 100 mL serological bottles was evaluated. In this experiment, the quantity and composition of VFAs and the microbiological composition were observed over a period of 15 days, allowing correlations to be found between the conditions evaluated and the resulting physicochemical and microbiological parameters. The inoculum that generated the highest production of VFAs in Chapter 3 was selected for the evaluation of operational conditions (initial pH, total solids and inoculum/substrate ratio) and the subsequent scale-up of the process, developed in **Chapter 4**. The scale-up was carried out in two semi-continuous reactors of 10 L, with an effective volume of 6L, hydraulic retention time of 10 days and organic loading rate of 10 g VS/L/d. The operation of the two 10 L reactors allowed the production of a sufficient amount of effluent rich in VFAs for the next phase of the project. Finally, **Chapter 5** deals with the valorization of the VFAs obtained from pig manure fermentation; this begins with the distillation of the VFAs present in the effluent of the 10 L semi-continuous reactors, and its subsequent use as a carbon source in a biological fixation process of atmospheric nitrogen, using a bacterial consortium. Figure 1.2 shows a general methodological scheme of experimental chapters.

Finally, **Chapter six** compiles the conclusions of all the work presented.

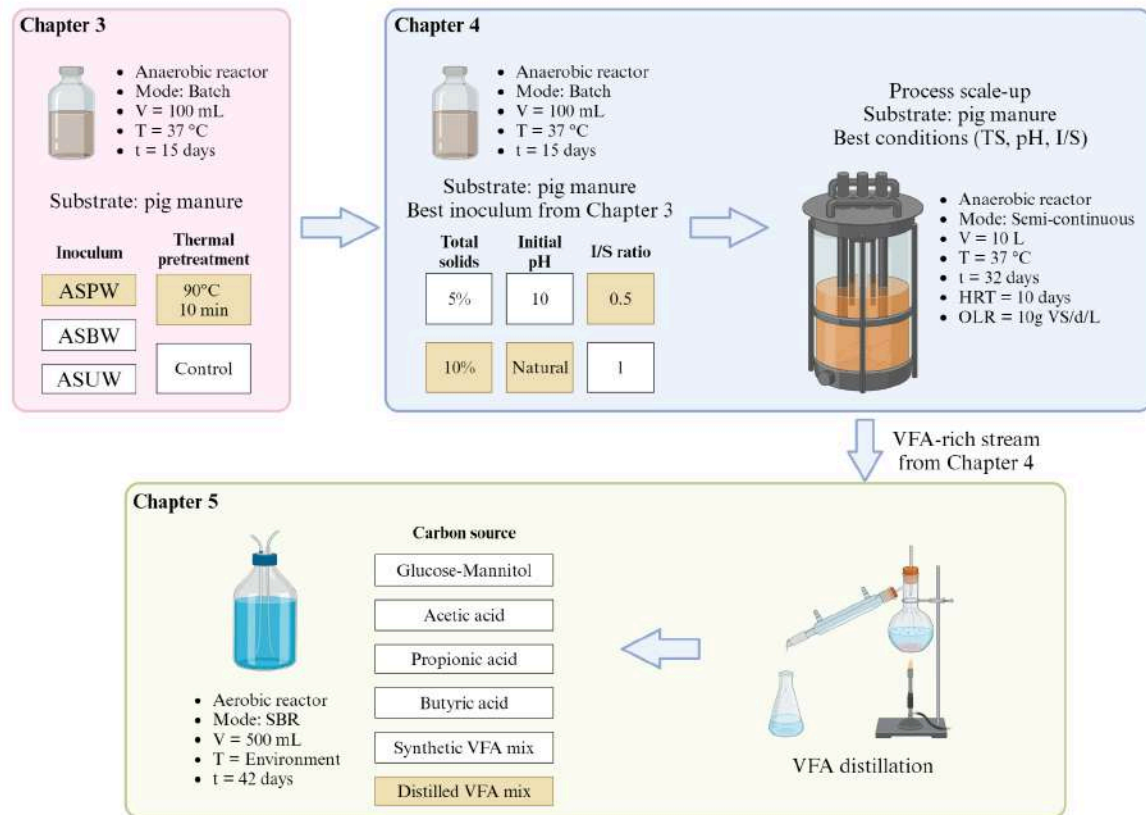


Figure 1.2. General methodological scheme of experimental chapters

Note: The orange highlighted boxes within the experimental designs represent the relevant conditions within each experiment.

Chapter 2: Literature review

2.1. Anaerobic digestion

Anaerobic digestion (AD) is a process of transformation of organic matter by the action of a diverse set of microorganisms in an molecular oxygen-free environment, mainly generating biogas rich in methane and a liquid effluent with a variable concentration of organic matter and nutrients (i.e. nitrogen, phosphorus and potassium) (Czekala, 2022). This process manages to convert between 70 and 90% of the biodegradable organic matter present in the raw material into biogas (Chernicharo, 2007), ranking among the most important options in the field of sanitary and environmental engineering for safe and efficient depuration of different organic residues such as: urban sewage (Abeyratne *et al.*, 2023; Pretel *et al.*, 2015), urban solid waste (Bautista Angeli *et al.*, 2022; Santos *et al.*, 2023), wastewater from distilleries (Gunes *et al.*, 2019; Hackula *et al.*, 2023), waste from breeding (Usman Khan & Kiaer Ahring, 2021; M. Wang *et al.*, 2023) and slaughtering (Musa & Idrus, 2020; Sánchez *et al.*, 2023) of cattle, harvest agricultural residues (Momayez *et al.*, 2019; Ribeiro & Junior, 2023), among others.

The environmental benefit of AD is not only limited to the depuration of organic pollutants. As mentioned above, the main products of this process are methane and nutrients, which can become important sources of energy and fertilizers for agricultural use (Czekala, 2022), two issues considered critical for the sustainability of humanity. Although these have been the two flagship products in AD research and development (R&D) in much of the world over the last century, it is important to mention that AD is a complex process from a biochemical point of view and that throughout the likewise, other products could be also recovered and valued, increasing the global potential benefits of waste management. Therefore, it is convenient to make a brief biochemical description of this process.

AD is divided into four biochemical steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Chernicharo, 2007; Laiq Ur Rehman *et al.*, 2019), as illustrated in Figure 2.1.

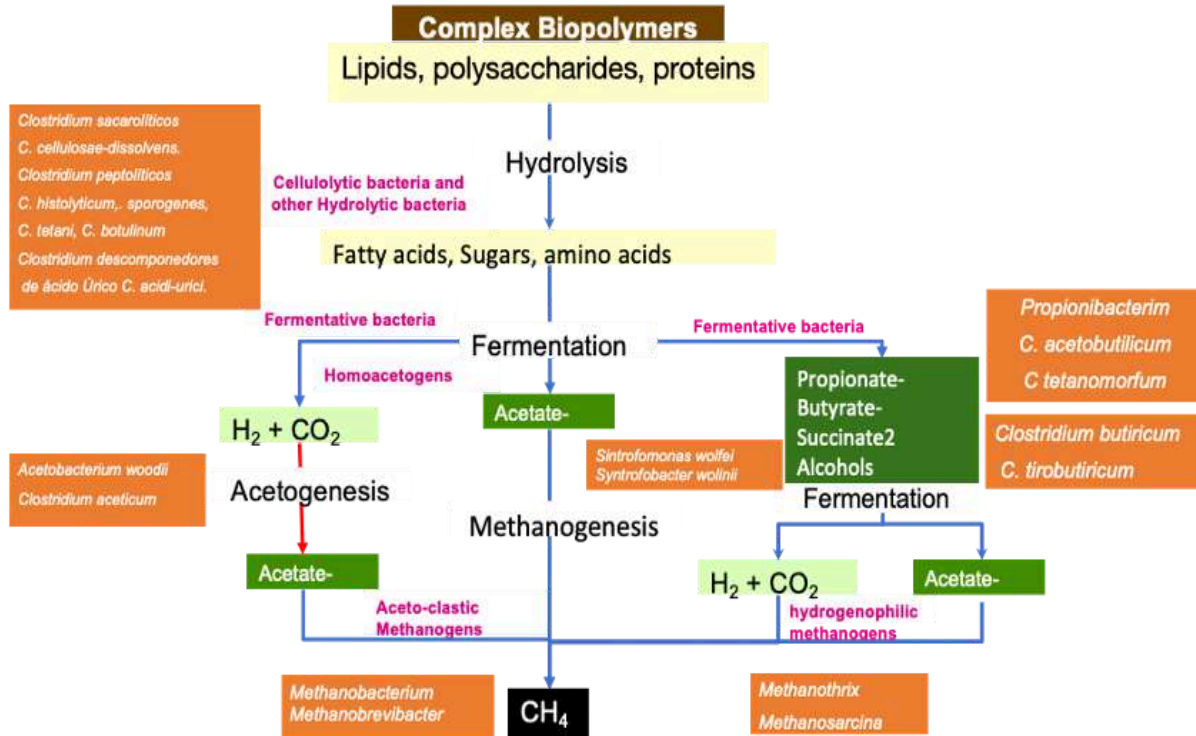
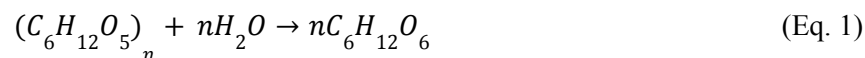


Figure 2.1. Metabolic pathways in anaerobic digestion. Adapted from (Madigan *et al.*, 2014)

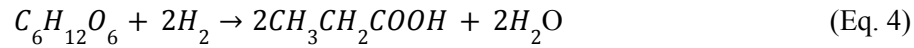
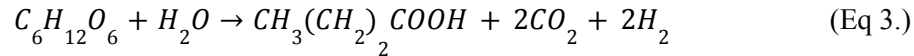
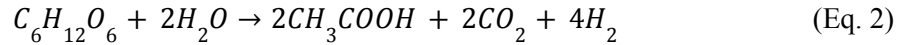
In **hydrolysis** stage, complex organic matter, made up of polysaccharides, polypeptides, and lipids, is first fractionated into soluble molecules such as monosaccharides, amino acids, free fatty acids, and alcohols, by the action of exoenzymes released by hydrolytic microorganisms (Yadav *et al.*, 2022). These microorganisms are relatively resistant to fluctuations in environmental conditions, although their action is strongly influenced by the characteristics of the substrate in terms of size, shape and surface area (Obileke *et al.*, 2021), being particularly slow when the substrate is rich in lignocellulose, becoming a bottleneck in the process (Van *et al.*, 2020). The most important bacterial genera in hydrolysis are *Streptococcus* and *Enterobacter* (Yadav *et al.*, 2022).

Polysaccharide hydrolysis reactions (Van *et al.*, 2020):



The molecules resulting from hydrolysis are used in the **acidogenesis** stage, also called the fermentative stage. Here, acidogenic microorganisms convert soluble substrates into carboxylic acids between 2 and 6 carbons and alcohols (Laiq Ur Rehman *et al.*, 2019). *Ruminococcus*, *Paenibacillus* and *Clostridium* have been identified as important bacterial genera in acidogenesis (Yadav *et al.*, 2022).

Some acidogenesis pathways using glucose as substrate can be seen in the Equations 2-5. pH and hydrogen partial pressure could determine the choice of acidogenesis pathway. For instance, when the pH is below 5.7, the metabolism shifts towards the production of alcohols instead of acids (acetic and butyric). This prevents further acidification of the system, and also lowers the partial pressure of hydrogen (Gottwald & Gottschalk, 1985; S.-Y. Li *et al.*, 2011). When the pH is below 4, fermentative reactions can stop (Obileke *et al.*, 2021).

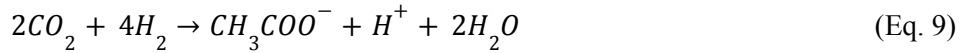
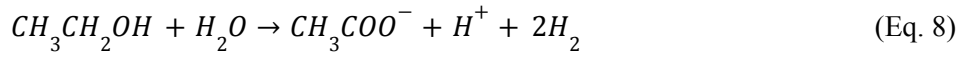
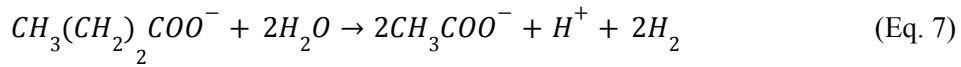
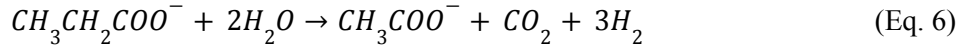


Studies have shown that in the acidogenic fermentation of protein-rich substrates, VFAs are dominant compared to lactic acid and alcohols (Cadavid-Rodriguez *et al.*, 2020; Lü *et al.*, 2008).

Then, in the acetogenesis stage, most of the products of the hydrolysis and acidogenesis are converted to acetate, hydrogen, and carbon dioxide. VFAs such as butyrate, propionate are broken down by bacteria of the genera *Syntrophomonas*, *Syntrophospora*, *Syntrophothermus*, *Thermosyntropha*, and *Pelotomaculum*. There are also bacteria that oxidize acetate into H_2 and CO_2 , belonging to the genera *Clostridium*, *Thermoacetogenium*, *Syntrophaceticus*, and *Tepidanaerobacter* (Westerholm *et al.*, 2011, 2019). These microorganisms are strictly anaerobic and work better at pH values between 6.0 and 6.2 (Van *et al.*,

2020). In addition to these conditions, most acetogenesis reactions are only thermodynamically viable at a low partial pressure of hydrogen. For this reason, it is important that hydrogen to be consumed by other bacteria in order to regulate its partial pressure, thus establishing a syntrophic relationship with methanogenesis (Obileke *et al.*, 2021).

Some examples of acetogenic reactions are presented below (Van *et al.*, 2020):



Methanogenesis is the final stage of the AD process. Here, acetate, hydrogen, and carbon dioxide are converted to methane in two ways: i) the acetoclastic pathway, where acetate is fermented to methane and CO₂, exclusive for the families Methanosarcinaceae and Methanosaetaceae and ii) the hydrogenotrophic pathway, where carbon dioxide is reduced with H₂ to form methane (Obileke *et al.*, 2021). Hydrogenotrophs are found within all methanogenic orders, except for the Methanomassiliicoccales (Enzmann *et al.*, 2018).

Some examples of methanogenic reactions are the following (Van *et al.*, 2020):



2.2. Volatile fatty acids

2.2.1. Characteristics of the VFAs

Carboxylic acids are organic compounds constituted by a carboxyl group and a substituent group (R), which can be aliphatic or aromatic (Figure 2.2) (Wade, 2012). In Table 1, the physical characteristics of some carboxylic acids are shown.

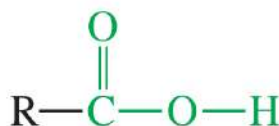


Figure 2.2. Chemical structure of a carboxylic acid. Source: (Wade, 2012)

When the R group is a long aliphatic chain, the acid is named as fatty acid. However, aliphatic acids with 8 carbons or less are also recognized as volatile fatty acids (VFA) or short chain fatty acids (SCFA), where the most important in anaerobic digestion are acetic, propionic and butyric (highlighted in blue, in Table 2.1) (Cibis *et al.*, 2016; Holtzapple *et al.*, 2022).

Carboxylic acids up to 4 carbons are soluble in water, but as the R group is longer, this solubility decreases. Furthermore, although carboxylic acids are not as strong as the most mineral acids (acid constant around 10^{-5}), they are much stronger than other organic functional groups. For example, acetic acid is about 10^{11} times more acidic than the most acidic alcohol (Wade, 2012).

Table 2.1. Physicochemical characteristics of some carboxylic acids. Source: (Wade, 2012)

IUPAC name	Common name	Formula	Melting point (°C)	Boiling point (°C)	Solubility (g/100g H ₂ O)	pKa (25°C)
methanoic	formic	HCOOH	8	101	∞ (miscible)	3.75
ethanoic	acetic	CH ₃ COOH	17	118	∞	4.74
propanoic	propionic	CH ₃ CH ₂ COOH	-121	141	∞	4.87
butanoic	butyric	CH ₃ (CH ₂) ₂ COOH	-6	163	∞	4.82
2-methylpropanoic	isobutyric	(CH ₃) ₂ CHCOOH	-46	155	23.0	
pentanoic	valeric	CH ₃ (CH ₂) ₃ COOH	-34	186	3.7	4.81
hexanoic	caproic	CH ₃ (CH ₂) ₄ COOH	-4	206	1.0	4.88
octanoic	caprylic	CH ₃ (CH ₂) ₆ COOH	16	240	0.7	4.89
decanoic	capric	CH ₃ (CH ₂) ₈ COOH	31	269	0.2	4.84
dodecanoic	lauric	CH ₃ (CH ₂) ₁₀ COOH	44		i (immiscible)	
tetradecanoic	myristic	CH ₃ (CH ₂) ₁₂ COOH	54		i	
decanoic	palmitic	CH ₃ (CH ₂) ₁₄ COOH	63		i	
octadecanoic	stearic	CH ₃ (CH ₂) ₁₆ COOH	72		i	

The main ways for global production of VFA are chemical synthesis, such as methanol carbonylation, propanal air-oxidation and oxidation of butyraldehyde (Dwidar *et al.*, 2012; Yoneda *et al.*, 2001). However, this causes negative health and environmental effects. For instance, reported greenhouse gases (GHG) emissions from acetic acid production in the petrochemical industry are 3.3 t CO₂ eq./t for cradle-to-grave (Atasoy *et al.*, 2018).

The above suggests that the use of biological processes could be a promissory alternative for VFA production since it also allows to recover value added products from organic wastes used as substrates. In this scenario, fermentation is the central process for obtaining VFAs. Since this process usually develops at pH values above the pKa of most VFAs, these are usually found mainly in their carboxylated form. For this reason, it is important to identify the nomenclature commonly used in the literature (Holtzappple *et al.*, 2022):

- *Carboxylic acids*: monocarboxylic acids made up of 1 to 8 carbons, with a linear or branched hydrocarbon chain.
- *Carboxylate salts*: salts of carboxylic acids, formed when the pH of the solution is higher than the pKa of the corresponding acid, and there are cations such as Na^+ , K^+ , or NH_4^+ .
- *Volatile fatty acids (VFAs)*: is the mixture of carboxylic acids and their corresponding carboxylic salts, in a proportion depending on the pH of the solution.
- *Short-chain FAs*: FAs made up of 1 to 4 carbons.
- *Medium-chain FAs*: VFAs made up of 5 to 8 carbons.
- *Long-chain FAs*: VFAs made up of 8 or more carbons.

2.2.2. Acidogenic fermentation

As demonstrated in section 2.1, VFAs play a fundamental role as intermediaries in AD. When the objective of the process is the production of methane, there must be a balance between the generation and consumption of VFAs to maintain a stable operation. Excessive accumulation of VFA is common, particularly propionate, since its oxidation has the highest Gibbs free energy compared to other VFAs (Mu *et al.*, 2023). As a reference, when the VFA/alkalinity ratio is above 0.3-0.4, indicates that the process is becoming acidic, negatively affecting methanogenic microorganisms (Chernicharo, 2007).

Nevertheless, VFAs can also be a desired product of the AD process. For this purpose, the AD process can be separated into hydrolytic/acidogenic and methanogenic stages, in order to optimize conditions for each group of reactions. This is an emerging approach for valorizing various kinds of organic wastes, allowing the extraction of value added products, specifically VFA and biohydrogen in the first stage, and methane in the second stage (Cadavid-Rodríguez & Horan, 2014; Srisowmeya *et al.*, 2020). Two stage-AD has been evaluated using different substrates such as landfill leachate (Begum *et al.*, 2018), expired food (Tsigkou *et al.*, 2020), palm oil wastewater (Lim *et al.*, 2020), agroindustrial wastes (Sakarika *et al.*, 2020), among others.

The fermentation process optimized for the production of fatty acids is known as “acidogenic fermentation” (AF), and refers to the subprocess comprised by the hydrolytic/acidogenic stages of AD, where the solubilization of organic matter and the subsequent fermentation of soluble organic compounds into volatile fatty acids, alcohols and hydrogen occur (Dahiya *et al.*, 2023; Ramos-Suarez *et al.*, 2021; Reis *et al.*, 2011).

From a metabolic point of view, acidogenic fermentation takes advantage of the microbiome present in the system, made up of hydrolytic, acidogenic and acetogenic bacteria (methanogenic organisms are inhibited). Carbohydrates are enzymatically hydrolyzed to simple sugars (i.e. glucose), which fate is glycolysis via the Embden-Meyerhof-Parnas pathway, where pyruvate is the main product. The proteins are enzymatically hydrolyzed to amino acids, which are then catabolized 90% by the Stickland pathway (coupled oxidation-reduction reaction). Lipids are degraded by the β -oxidation pathway to Acetyl-CoA. The primary metabolites resulting from fermentation of these three macromolecules are acetate, propionate and butyrate, accompanied by other degradation products such as H₂ and CO₂ (Dahiya *et al.*, 2023). Additionally, if reducing conditions exist in the process (provided by ethanol, lactate, or an adequate supply of electrical current), primary VFAs can be elongated to >C₅ VFAs (Holtzapple *et al.*, 2015). Figure 2.3 summarizes the acidogenic fermentation process of complex organic wastes.

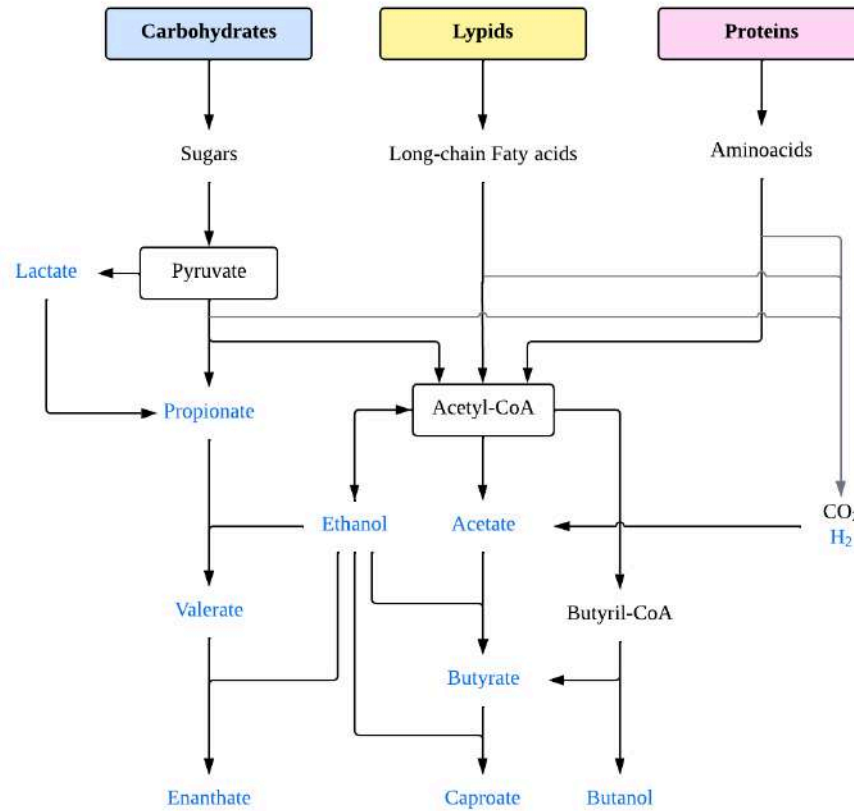


Figure 2.3. General scheme of main metabolic pathways in ideal acidogenic fermentation from complex organic wastes. Glucose is shown as an example of a monomer from polysaccharides. Main products are shown in blue. Source: (Dahiya *et al.*, 2023; Holtzapple *et al.*, 2015, 2022; Madigan *et al.*, 2014; Sangavai *et al.*, 2019)

As mentioned before, acidogenic fermentation requires suppression of methanogenesis to not convert VFA and hydrogen to methane, for that reason this process is also called methane-arrested anaerobic digestion (MAAD) (Holtzapple *et al.*, 2022). Different methods have been reported in the literature to improve the production of VFAs through the suppression of methanogenesis, as described below:

- i) **High pH.** pH 9 enhances acidogenesis, while pH 11 inhibits methanogenesis, resulting in higher VFA production (Zhao *et al.*, 2018), also at pH 10 optimal VFA production has been

reported (X. Huang *et al.*, 2018). pH 8.9 promotes growth of Clostridia (acidogenic), while Euryarcheota (methanogenic) decreases (Chen *et al.*, 2017).

ii) Thermal treatment. Thermal treatment of substrate and inoculum at 70 - 90 °C promote VFA production (Lazaro *et al.*, 2014; Liao *et al.*, 2016; J. Wu *et al.*, 2017). Thermophilic fermentation led to 10 times more VFAs production compared to mesophilic conditions without pH adjustment (Hao & Wang, 2015). Thermophilic conditions increase hydrolysis rate compared to mesophilic conditions, resulting in higher production of VFA (P. Zhang *et al.*, 2009).

iii) High solid content in substrate and short retention time. Several authors have reported that the shorter the retention time and the higher the solids content, the lower the production of methane and the higher the production of VFAs. Results vary depending on substrate characteristics and process conditions (Hu *et al.*, 2019; Jankowska *et al.*, 2015; Slezak *et al.*, 2017; Yin *et al.*, 2014; Zhao *et al.*, 2015).

iv) The use of protein-rich feedstocks, which are transformed into ammonia that inhibits methanogens and causes a build-up of VFA (Chen *et al.*, 2008).

v) The use of additives, such as zero-valent iron (J. Cao *et al.*, 2019; Y. Yang *et al.*, 2019), or alkyl-polyglucose (Q. Wang, Xin, *et al.*, 2023), are capable of inducing changes in microbial communities, favoring acidogenesis

vi) Mixed-culture fermentation, which is another interesting focus to improve the production of mixed VFAs (Lee *et al.*, 2014; Yuan *et al.*, 2019).

These strategies are usually complemented with other methods aimed to the solubilization of fiber-rich substrates to promote hydrolysis (limiting stage of the overall process):

vii) The use of lignocellulolytic cultures, that allows a better carbon availability from lignocelulosic feedstocks (Tuesorn *et al.*, 2013).

viii) The use of additives, such as: choline chloride (W. Cao *et al.*, 2023), urea hydrogen peroxide (S. Wang, Jiang, *et al.*, 2023), iron persulfate (Cheng *et al.*, 2023), lysozyme + cation exchange resins (Pan *et al.*, 2023), hydrolytic enzymes (S. Yu *et al.*, 2013), or free nitrous acid pretreatment (Zhao *et al.*, 2015).

Regarding **pig manure**, the work carried out at the Chinese Academy of Agricultural Sciences stands out, where different strategies were evaluated to improve the production of AGVs and study their relationship with the microbiological composition, combining thermal pretreatment (TP) of inoculum (90°C, 3h) with other process factors, such as the carbon/nitrogen ratio (Q. Cao *et al.*, 2020), the initial pH (W. Zhang *et al.*, 2020), the organic loading rate (Q. Cao *et al.*, 2021), and the micro aeration (Q. Cao *et al.*, 2022). Other authors have evaluated different strategies with this residue, which do not involve TP, based on the use of additives such as zero-valent iron (Y. Yang *et al.*, 2019) or magnetite (G. Zhang *et al.*, 2020), or in the optimization of operating conditions such as pH, temperature, solids content and retention time (W. Huang *et al.*, 2016).

In Table 2.2, some relevant studies about VFA production from pig manure and other organic wastes are summarized. It is worth highlighting that, regardless of the type of substrate, an important variation is observed in the production of VFAs when treatments, such as chemical or enzymatic additives, are applied. The operating conditions of the fermentation process, such as total solids, inoculum/substrate ratio, hydraulic retention time or temperature, also have a notable effect on VFAs production. This demonstrates the relevance of evaluating all these process variables, in order to improve the production and utilization potential of VFAs. Likewise, greater knowledge of this process helps create opportunities to improve the management of organic waste, through the creation of value.

Table 2.2. Literature of VFA production from organic wastes.

Waste stream	Best operational conditions	VFA production	References
Screenings from municipal wastewater treatment plant	pH 6, leached bed reactor, recirculation 8 l/reactor/day	213 g /kg VS	(Cadavid-Rodríguez & Horan, 2014)
Sludge from municipal wastewater treatment plant	pH 10, solid retention time (SRT) 20 d	3,163.71 mg COD/L	(Jie <i>et al.</i> , 2014)
Raw pig manure	55 °C, pH 11, 20% TS, SRT 8 d	79.1 mg COD /g VS	(W. Huang <i>et al.</i> , 2016)
Poultry litter	pH 5.5, SRT 4d, 35°C	670 mg VFA /g VS	(Kuruti <i>et al.</i> , 2017)
Tuna waste	pH 8, SRT 32 d, 35 °C	30.61 mg COD /L	(Bermúdez-Penabad <i>et al.</i> , 2017)
Food wastes	Addition of zero-valent iron and persulfate, SRT 8 d	9,800 mg COD /L	(J. Cao <i>et al.</i> , 2019)
Raw pig manure	Addition of zero-valent iron, two-stage anaerobic digestion	12,279 mg VFA /L	(Y. Yang <i>et al.</i> , 2019)
Waste activated sludge	Free nitrous acid pretreatment (1.54 g L ⁻¹), pH 10, SRT 2 d	370.1 g COD /g VSS	(Zhao <i>et al.</i> , 2015)
Sludge from municipal wastewater treatment plant	Endogenous hydrolytic enzyme (amylase) pretreatment, SRT 28 h	1,719 mg VFA /L	(S. Yu <i>et al.</i> , 2013)
Pig manure	TS 23.6%, 35 °C, SRT 23 days	6,298 mg COD /L	(Hu <i>et al.</i> , 2019)
Pig manure + corn silage	C/N ratio 25, SRT 18 d, 37 °C	26,214 mg COD /L	(Q. Cao <i>et al.</i> , 2020)
Pig manure + corn silage	C/N ratio 35, 37 °C, HRT 12 d, OLR	19.1 g COD /L	(Q. Cao <i>et al.</i> , 2021)

Pig manure	35 °C, initial pH 10, SRT 8 d, micro-aeration at 1000 mL/L/d	8.21 g /L (improvement of 22.4%)	(Q. Cao <i>et al.</i> , 2022)
Waste activated sludge (WAS)	35 °C, TSS 1.54%, WAS was treated with choline chloride 20 mM ChCl	3,884 mg COD /L (improvement of 723.3%)	(W. Cao <i>et al.</i> , 2023)
Pig manure	Initial pH 10, I/S ratio 0.4, SV 4%, 37 °C, SRT 20 d	15.2 g COD/L	(W. Zhang <i>et al.</i> , 2020)

2.3. AD-biorefineries for organic wastes

A biorefinery is an integrated system that uses chemical, physical, thermochemical, and/or biochemical processes to transform organic waste into different value-added products, making the best use of available resources (Corrêa *et al.*, 2022; de Jong *et al.*, 2020; Ferreira, 2017). The focus of residual biomass transformation has mainly been on lignocellulosic compounds from the agri-food industry (Bastidas-Oyanedel & Schmidt, 2019); however, any type of organic waste, of animal, plant or microbial origin can be transformed into value-added compounds (Ferreira, 2017).

A systematic way of classifying biorefineries is the one proposed by de Jong *et al.* (2020), where four aspects are defined: raw material (waste or crops), platform (main intermediates such as C5-C6 carbohydrates, syngas, lignin, bio-oil), products (biofuels, chemicals, materials, etc.), and processing technology (chemical, biochemical, thermochemical, physical). Specifically, three types of biorefinery for the transformation of biomass into fuels and chemicals have been characterized in detail (Holtzapfel *et al.*, 2022):

- **Thermochemical.** Chemical transformation of biomass due to the effect of high temperature in the absence of oxygen (pyrolysis) or with low oxygen concentration (gasification). Gases (CO, H₂), liquids (bio-oils), and solids (biochar) are obtained as main products.

- **Sugar.** Mainly applicable to lignocellulosic biomass. An enzymatic pretreatment is used to hydrolyze starch, cellulose, and hemicellulose into sugars, which are then fermented under controlled conditions mainly to obtain alcohols.
- **Carboxylate.** Applicable to different types of biomass. It is characterized by the use of mixed cultures that hydrolyze different macromolecules (polysaccharides, lipids, polypeptides) into their corresponding monomers, and then ferment them to obtain monocarboxylate salts between 1 and 8 carbons. This platform model will be discussed in more detail in section 2.3.2.

A recent search in Scopus with the term “biorefinery”, resulted in 14,420 documents between 1987 and 2024. When limited to reviews, books and book chapters (to see trends from a conceptual basis), the search resulted in 3,656 documents in a time span between 2002 and 2024. The most relevant authors in this field are Ashok K. Pandey and Hector Ruiz. It is observed in Figure 2.4 that the most studied raw material regarding biorefineries is lignocellulosic waste, in a separate cluster (blue). Likewise, the associated technologies (fractionation, hydrolysis, pretreatment) are closely related to the bioconversion of this type of material.

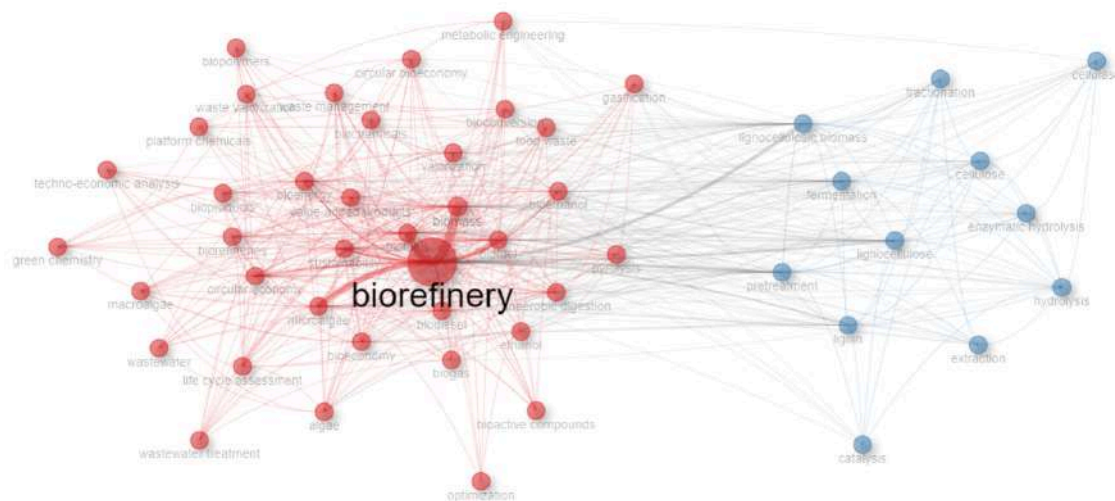


Figure 2.4. Co-occurrence network with author keywords related to the search “biorefinery” in Scopus, limited to reviews, books and book chapters. Created with Biblioshiny.

However, when the search term "manure" is included, the situation changes completely. In a search in Scopus, for the term “biorefinery AND manure” in the years 2005-2023, a total of 119 documents were found. The most relevant authors in this field are Irini Angelidaki and Ashok K. Pandey. In this case, **anaerobic digestion** emerges as the core process of the biorefinery (Figure 2.5).

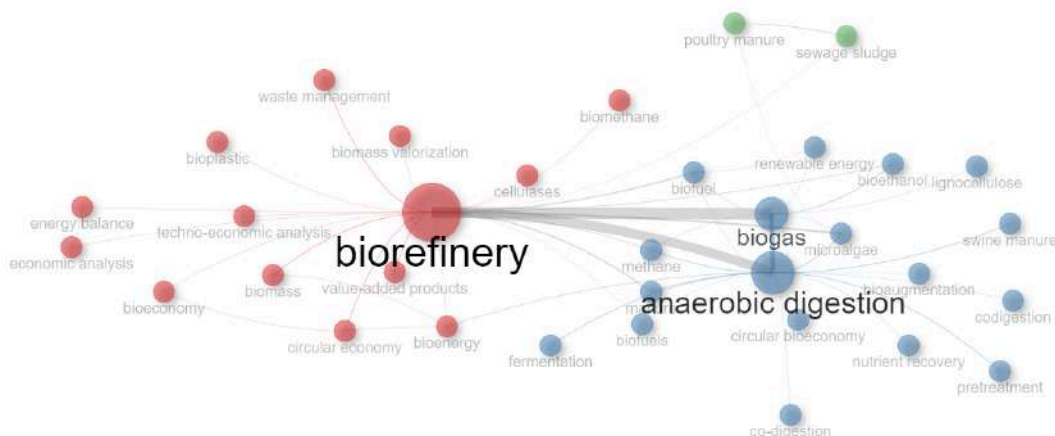


Figure 2.5. Co-occurrence network with author keywords related to the search “biorefinery AND manure” in Scopus. Created with Biblioshiny.

This change in approach ratifies that the physical and chemical characteristics of the waste to be used as substrate is decisive when designing a biorefinery, as reported in literature (Ferreira, 2017). And it also confirms that AD might be suitable as a core process for biorefinery from animal waste (i.e. pig manure). Moreover, in this scenario, biogas and nutrients stand out as main products; but the study of the organic fraction of the liquid effluent of the AD continues to be a topic little explored.

Another aspect to consider when proposing AD as a central process of a biorefinery is that, regardless its advantages as process for pollution control and waste valorization, AD alone does not guarantee a completely safe environmental treatment for products generated in the process (i.e., biogas, solid

digestate, and liquid effluent). In addition, each of these products has a chemical potential that can (should) be recovered.

The methane contained in biogas (40 – 70%), has a greenhouse effect equivalent to twenty-five times carbon dioxide effect if it is released into the atmosphere (Sawatdeenarunat *et al.*, 2016). Therefore, the recovery of methane is the best option, for which there are various applications such as: thermal conversion (e.g., lighting, heating, power generation, etc), methanol synthesis, which is the source of many high-value industrial chemicals (e.g., anhydride, ethylene, polyols and other aromatic compounds), or hydrogen production through methane reforming (Janajreh *et al.*, 2021; J. Tao *et al.*, 2020). Otherwise, burning it would be an option.

On the other hand, AD produces a liquid effluent with different amounts of nutrients and organic matter that depends on the efficiency of the process. Within the safe handling of anaerobic effluents, the use as fertilizer and/or amendment is commonly suggested, taking the necessary technical measures to avoid the risk of eutrophication in soils and water bodies. Alternative uses of this effluent include the recovery and separation of VFAs and the use of the nutrients present for the cultivation of algae, both with various options for further valorization.

Finally, 5 to 15% of incoming COD is converted into solid digestate in AD plants (Chernicharo, 2007). This stream mainly consists of suspended solids and undigested residues, which composition varies depending on the characteristics of substrate and process performance, and it needs to be utilized or disposed in an environmental-friendly fashion to minimize risks of contamination due to presence of pathogenic microorganisms and nutrient runoff (Vasco-Correa *et al.*, 2018). Although the nutrients present in the digestate can be used in soil fertilization, its sanitization is necessary to control pathogens, before its application to crops. Other safe options for using solid digestate are composting and pyrolysis, to obtain organic amendments and biochar, respectively.

In view of the above, the integration of AD with other processes not only allows minimizing the risk of environmental contamination, but also generates greater opportunities for valorization through the recovery of value-added products, contributing to the sustainability of the whole process. The processes proposed for the valorization of each of the output currents of the AD are only examples among a variety of possibilities, whose technical convenience must be evaluated in each particular case, depending on the characteristics of the substrate and the AD process. In a previous publication (Clavijo-Salinas *et al.*, 2020), a comprehensive description of the technologies that can be coupled to AD within the framework of a biorefinery is presented. A general model proposed for an AD-based biorefinery for organic wastes is shown in Figure 2.6.

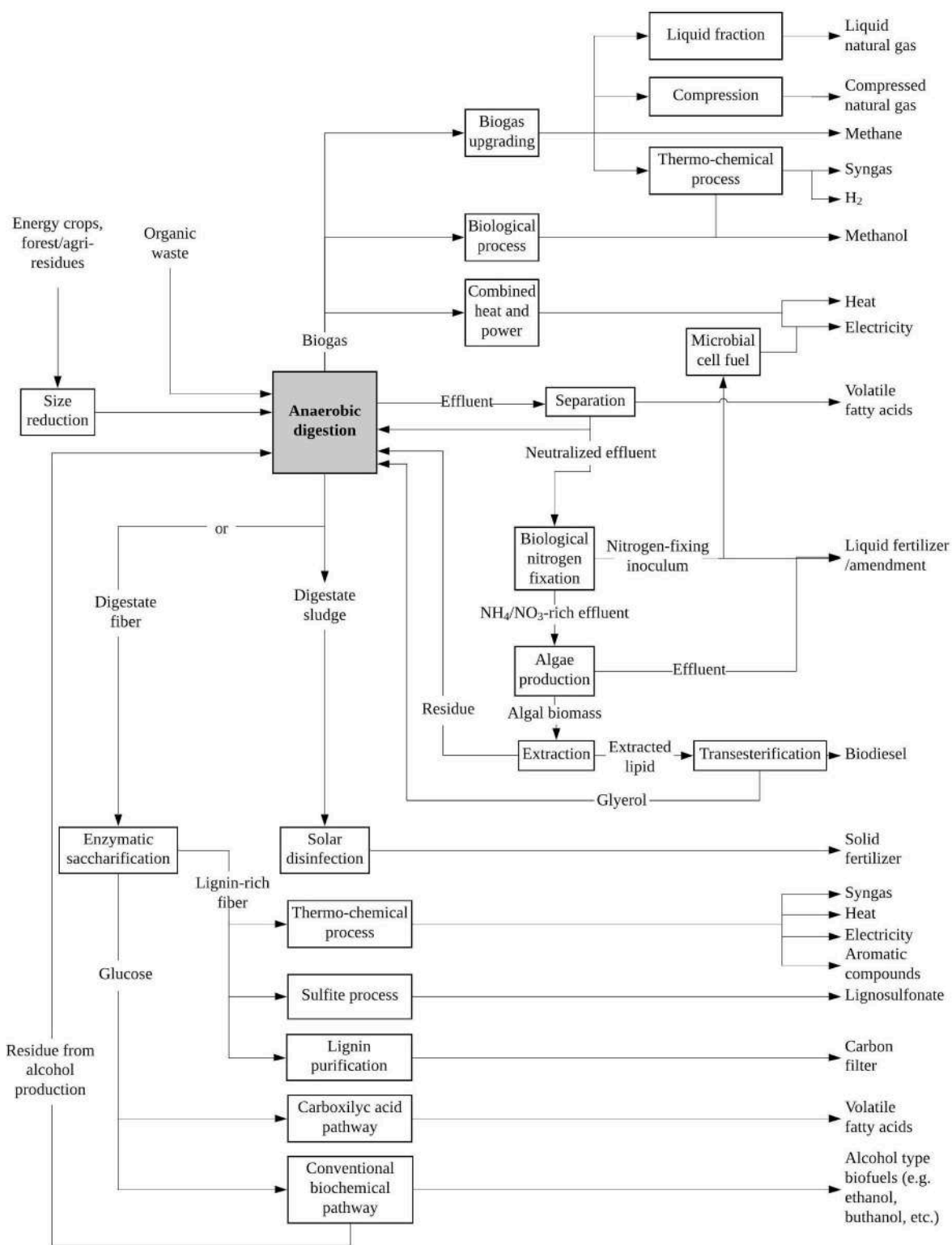


Figure 2.6. Proposed AD-based biorefinery for producing biofuels and bio-based products

(Clavijo-Salinas *et al.*, 2020), modified from (Surendra *et al.*, 2015)

2.3.1. VFA-based biorefinery: the carboxylate platform

Among all the recovery possibilities that arise from AD, the possibility of using the remaining organic fraction in the process effluent stands out, specifically the VFAs, which can be utilized as raw material to produce a wide range of organic compounds, including medium-chain fatty acids, primary and secondary alcohols, bio-based hydrocarbons, biohydrogen, bioplastics, bioelectricity, etc (Venkata Mohan *et al.*, 2016).

The idea of obtaining products from fermentation-based VFAs origin dates back to the beginning of the 20th century. Examples of this are the fermentation of kelp to obtain acetic acid, followed by its neutralization into calcium acetate, and subsequently its thermochemical conversion (pyrolysis) to acetone, to finally prepare explosives that were used in the First World War; or the use of carboxylic acids to produce mixed ketones, esters and hydrocarbons, as alternative fuels during the energy crisis of 1970s and early 80s (Holtzapple *et al.*, 2022).

The term “Carboxylate Platform” (CP) was coined in 2009, to describe an undefined-mixed-culture fermentation able to produce a mixture of VFAs, which are then used as intermediate platform chemicals to obtain complex chemicals and fuels (Holtzapple & Granda, 2009). The use of undefined mixed cultures is one of the greatest attractions of this valorization model, since it allows to tolerate the variability and complexity of the residual biomass used as a substrate (Agler *et al.*, 2011). Consequently, acidogenic fermentation and all strategies aimed at maximizing VFAs production (see Section 2.2.2) are a fundamental part of the CP.

The CP includes three possible routes for the transformation of VFAs (Holtzapple *et al.*, 2015, 2022): **i) the Kolbe electrolysis route**, which allows the conversion of carboxylic acids into long-chain hydrocarbons using low voltage (<3V); **ii) the secondary alcohols route**, where VFAs are converted into ketones, which are then hydrogenated to secondary alcohols, and finally dehydrated and oligomerized to

hydrocarbons; and **iii) the primary alcohol route**, where VFAs are reduced to primary alcohols through hydrogenation or esterification, and finally converted into hydrocarbons. Figure 2.7 shows the chemicals routes included in CP.

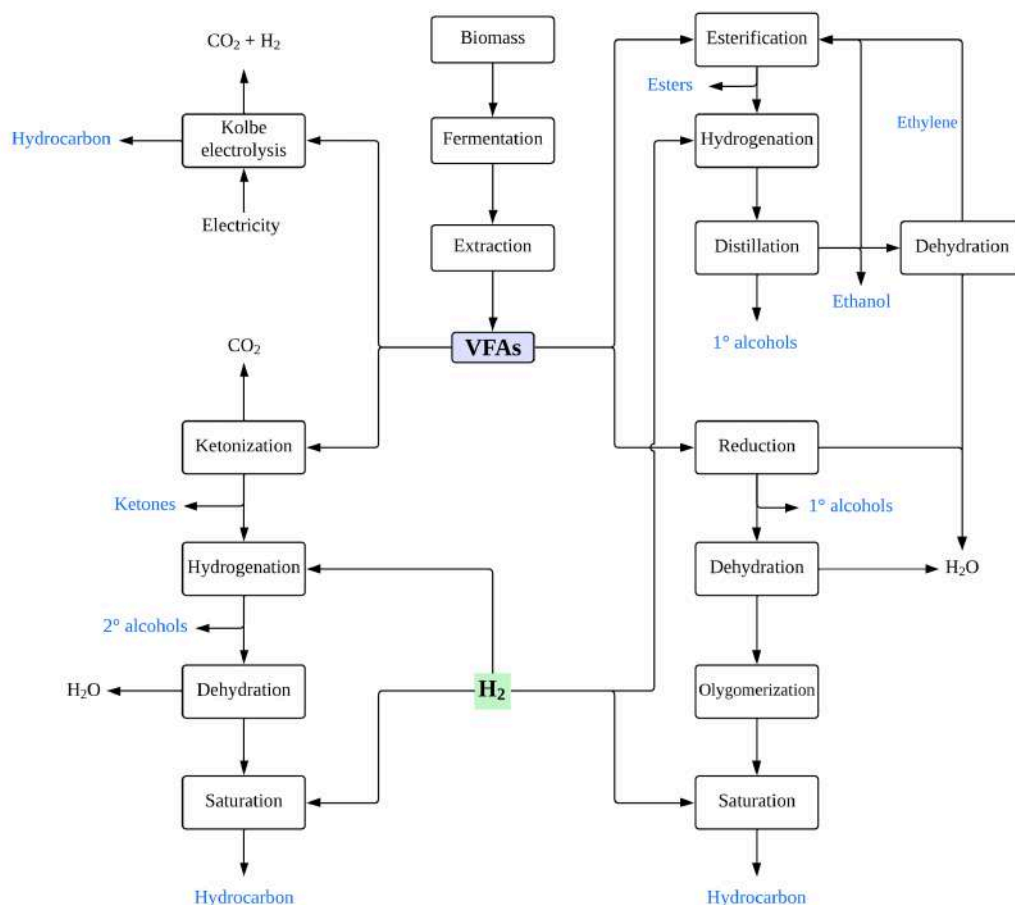


Figure 2.7. General scheme of chemical routes to obtain chemicals products and hydrocarbon fuels in carboxylate platform. Products are shown in blue. Adapted from Holtzapple *et al.* (2015, 2022)

Hydrocarbons obtained from CP can be used as synthetic biofuels, reducing dependence on fossil fuels, and helping to mitigate greenhouse gas (GHGs) emissions (Porwal *et al.*, 2020). This is also applicable to other biofuels that can be obtained from VFAs, such as biodiesel, alcohols, methane and hydrogen, or the mixture of the last two (biohythane); and bioelectricity obtained via microbial cell fuels (MCF) using VFAs as substrate (Sekoai *et al.*, 2023).

Acidogenic VFAs can also be used as a low-cost carbon source for different biotechnological processes to obtain value-added bioproducts and benefits:

- Biobased polymers, such as polyhydroxyalkanoates (PHAs), can be accumulated by some bacteria in the fermentation process under nutrient-limiting conditions of nitrogen, phosphorus, sulfur, or oxygen. VFAs can be used as alternative carbon sources for this process, reducing its cost by more than 40% (Sekoai *et al.*, 2023).
- Wastewater tertiary treatment, focused on removal of nutrients such as nitrogen and phosphorus remaining in effluents from biological wastewater treatments, can be achieved by anaerobic-anoxic-aerobic processes, using VFAs as alternative carbon source in excess. This is a necessary process to avoid the eutrophication of soils and waters (Sekoai *et al.*, 2023).
- Single-cell protein, that can be produced by synergistic metabolism of acidogenic bacteria and other microorganisms. Although it is a very young technology, it is considered promising since it does not generate environmental pollution, nor does it require large areas of land or water, as is currently the case with protein of animal origin (Sekoai *et al.*, 2023).
- Biological nitrogen fixation (BNF) is an alternative to the Haber-Bosch process for the production of nitrogen fertilizers, where VFAs could replace conventional and expensive carbon sources for diazotrophic bacteria. Although there is extensive documentation on the use of VFAs as a carbon source for other nitrogen-related biological processes (i.e. nitrification and denitrification), no reports have been found to date on the use of VFAs in biological nitrogen fixation. Chapter 5 develops a proposal consisting of the evaluation of VFAs as an alternative carbon source for BNF.

Furthermore, several biobased chemicals such as organic acids, solvents, food preservatives, pharmaceuticals, and many other, are examples of bioproducts that can be obtained from CP (Agnihotri *et al.*, 2022; Venkata Mohan *et al.*, 2016). This gives an idea of the potential of this valorization model, and how it can advance the transition from a fossil-based economy to a bioeconomy, more competitive

and sustainable. Table 2.3 summarizes the global market and applications of VFAs and derivative products, which represents the potential market of a CP biorefinery.

Table 2.3. Global demand and applications of VFAs

Acid	Global market	Sector	Application
Acetic acid	USD 19.76 billion in 2022 and it is expected to reach USD 32.65 billion by 2032 (CAGR 5.2%) (Precedentresearch.com, 2023b).	Food and beverage industry	Solvent, vinegar, preservative, acidity regulator, flavor component. Animal feed supplementation
		Chemical industry	Herbicides
			Chemical precursor of ethylenediaminetetraacetic acid (EDTA), glycine and carboxymethyl cellulose.
			Acetate esters are used as solvents for inks, paints, and coatings.
			Acetic anhydride, used in the manufacturing of perfumes, antibiotics, explosives and dyes.
Propionic acid	USD 1.51 billion in 2021 and it is expected to reach USD 2.79 billion by 2029 (CAGR 8%) (DBMR, 2022).	Food and beverage industry	Production of vinyl acetate, then polymerized to polyvinyl acetate, used in textiles, paper coatings, latex paint, adhesives, latex fibres.
			production of terephthalic acid (TPA), as precursor of polyethylene terephthalate (PET), used in fibers, clothing, plastic bottles and films.
		Chemical industry	Preservative flavors. Animal feed
		Polymer industry	Esters, herbicides
		Pharmaceutical industry	Plastics
Butyric acid	USD 374.2 million in 2022 and it is projected to reach USD 578.3 million by 2032 (CAGR 4.5%) (Precedentresearch.com, 2023a).	Food and beverage industry	Salts of butyric acid: sodium butyrate, potassium butyrate, calcium butyrate, and magnesium butyrate, used in animal feed products.
			Esters of butyric acid: methyl, ethyl, and amyl butyrate are usually aromatic, used as flavoring and fragrance agents in food, cosmetic, and beverage industries
		Polymer industry	Synthesis of cellulose acetate butyrate (CAB)
		Healthcare industry	Anticancer prodrug

Isobutyric acid	USD 173.2 million in 2022 and it is expected to reach USD 332.65 million by 2029 (CAGR 8.5%) (MMR, 2023).	Food and beverage industry	Reminiscent of apple, banana, pineapple, apricot, butter, cherry, and ginger flavor.
Valeric acid	USD 16.4 billion in 2021 and is expected to reach USD 24.3 billion by 2028 (CAGR 5.8%) (PBI, 2023).	Food and beverage industry	Esters of valerate, used in food flavors and daily flavors
		Chemical industry	Synthesis of 1,2-pentanediol, used in preparation of fungicide propiconazole. Application in the pharmaceutical, surfactant, polyester fiber, and other industries
		Pharmaceutical industry	Preparation of valeric anhydride. Intermediate in chemical synthesis, pharmaceutical raw materials and biopharmaceuticals
		Automotive industry	Preparation of lubricating oil
Isovaleric acid	USD 122.55 million in 2021 and is expected to reach USD 212.73 million by 2030 (CAGR 6.32%) (Marketsandresearch.com, 2022).	Food and beverage industry	Flavor: wine, cheese, baked food, meat products, cream, fruits, spices, condiments
		Pharmaceutical industry	Preparation of medicines. Synthesis of β -hydroxyisovaleric acid, indicator of biotin deficiency. Synthesis of bromo isovaleryl urea, used as sedative and hypnotic
Caproic acid	US\$191.8 Million in the year 2022, is projected to reach US\$358.8 Million by 2030 (CAGR 8.1%) (Researchandmarkets.com, 2023).	Food and beverage industry	Supplementary food for special needs, food preservative
		Pharmaceutical industry	Synthesis of drugs and cosmetics

Source: adapted from (Agnihotri *et al.*, 2022)

2.3.2. R&D trends on integral valorization of pig manure

Recently, members of the research group on Advanced Processes for Chemical and Biological Treatments (GAOX), Universidad del Valle, carried out a bibliometric analysis to identify R&D trends in the recovery of pig manure. A systematic search with selected terms was conducted in Scopus and Web of Science databases for scientific literature, and Orbit database for patents, up to March 2023.

The results indicate that the United States and China emerge as the leading contributors to scientific literature, accounting for 43% and 32% of research articles, respectively; this aligns with their status as the largest world pork producers. The top authors and institutions primarily hailed from China, further emphasizing its dominance in scientific knowledge and technological advancements. Europe also showed significant scientific production, with Spain and Italy leading the way.

Latin America contributes 9% of research articles and 7% of review articles, suggesting limited knowledge generation and proposed developments regarding pig manure management under the concept of biorefineries. Nevertheless, this region exhibited the highest annual growth rate of research articles, indicating a growing concern for sustainable pig manure management. There is a need for greater international collaboration and recognition of experts in the region.

The examination of co-occurrence of terms associated with bioproducts allowed for the identification of key technologies and trends in the valorization of pig waste (Figure 2.8). Six distinct groups of bioproducts emerged: bioenergy (biofuels and energy), gas stream (CH_4 , H_2 , CO_2), solid stream (compost, amendments, fertilizers), nutrients (C, N, P, K, NH_4^+ , NO_3^-), algae culture, and VFAs. Notably, in Latin America, the concept of bioenergy and gas streams stood out at the proposal level of reviews, indicating the region's recognition of the potential in this technological approach. However, at the research level, the focus shifted towards the importance of nutrients and gas streams across all three country categories. This shift may be attributed to the increased co-occurrence of terms related to anaerobic digestion (CH_4 , CO_2 ,

and H₂) at the research level, where volatile fatty acids (VFA) are also produced and can be utilized (Q. Cao *et al.*, 2020; Lin *et al.*, 2021). At the patent level, while the significance of nutrients observed in the articles remained, there was also an increasing proportion of co-occurring terms associated with the solid stream. This suggests a growing emphasis on the invention of systems and processes that harness the mineral component resulting from the bioconversion of pig manure (Guillard, 2020; Hodgkinson *et al.*, 2006). It is crucial to assess the economic feasibility and environmental sustainability of these bioproducts within the framework of a circular economy.

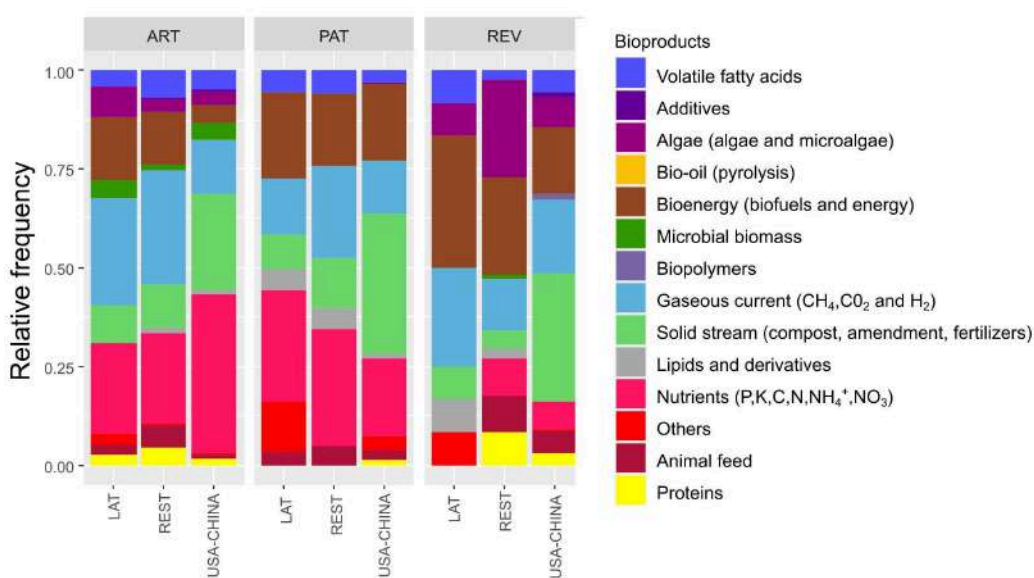


Figure 2.8. Bioproducts that can be obtained from pig manure, retrieved as word frequency from data processed with Biblioshiny package. ART: research articles PAT: Patents, REV: review articles, LAT: Latin America, REST: other countries excluding USA, China and Latin America

Finally, significant progress has been made in the valorization of pig manure, but there is still room for improvement, especially in Latin America. Greater collaboration, knowledge sharing, and adoption of biorefinery approaches can lead to more sustainable management of pig manure and contribute to environmental preservation and resource efficiency.

Publications related to the section 2.3:

- Clavijo-Salinas, J.C., Fuertez, J., Cadavid-Rodríguez, L.S., Sanabria, J. (2020). “*Compatible Technologies to Anaerobic Digestion for the Integral Valorization of Organic Waste*”. In: Zakaria, Z., Boopathy, R., Dib, J. (eds) Valorisation of Agro-industrial Residues – Volume I: Biological Approaches. Applied Environmental Science and Engineering for a Sustainable Future. Springer, Cham. https://doi.org/10.1007/978-3-030-39137-9_9
- Clavijo-Salinas, J.C, González-Payán, D., Rodriguez-Romero, N., Torres-Lucas, J.S., Sanabria, J. “*Integration of key bioprocesses in a biorefinery model for the valorization of pig waste: a bibliometric-based proposal*” (In revision process).

Chapter 3: Effect of inoculum thermal pretreatment on the production of VFAs

3.1. Introduction

Pork is one of the main sources of protein for human nutrition; with a production of 120 million tons (975.4 million heads), it ranks as the second most consumed meat in the world in 2021 (FAO, 2023a). This production is expected to rise in the near future, mainly in Asia, and to a lesser extent in Latin America and Africa (OECD & FAO, 2023). The rapid expansion and intensification of the pork industry come along with the generation of multiple environmental challenges, including greenhouse gas emissions, land use, and land change, and an increased risk of contamination of soil, air, and water sources (W. Yu & Jensen, 2022).

One of the main wastes generated by this industry is pig manure (PM). This waste is usually mixed with urine, food waste, and washing water in intensive exploitation systems (Galvis & Acevedo, 2008; Roa *et al.*, 2020). Moreover, it has a solids content that usually exceeds 20%, an organic matter content of around 70%, and nitrogen, phosphorus, and potassium contents of above 1% each (Noreña *et al.*, 2016; J. Yang *et al.*, 2019). This high concentration of nutrients makes pig manure not only a potential environmental hazard but also an attractive substrate for biotechnological processes such as anaerobic digestion (AD). Valuable energy products such as methane, and nutrients such as nitrogen, phosphorus and potassium can be recovered after the biological transformation of these wastes (Czekala, 2022). Other valuable products of AD are volatile fatty acids (VFAs), which are produced in the acidogenic stage of this process. VFAs are considered “chemical building blocks” for a wide range of industries, including chemicals, pharmaceuticals, food and agriculture (den Boer *et al.*, 2020; Ramos-Suarez *et al.*, 2021), which show a greater potential for applications compared to methane. However, barriers remain for waste-based VFA production to be competitive on a commercial scale, such as the lack of control over

metabolic activities and the persistence of methanogenic organisms that consume VFAs (Atasoy *et al.*, 2018; Dahiya *et al.*, 2023). Nowadays, the global market of VFAs is almost completely dominated by the petrochemical industry, whose environmental impact has been broadly discussed previously (Belahmadi *et al.*, 2023; L. Fang *et al.*, 2022; Xiang *et al.*, 2023).

Multiple strategies have been explored to modify AD and promote the biological production of VFAs and H₂ in a process called acidogenic fermentation or dark fermentation (Dahiya *et al.*, 2023). To do this, it is necessary to suppress methanogenesis within the AD process, which can be achieved through different strategies such as pH modification (Q. Wang, Xin, *et al.*, 2023; Zhao *et al.*, 2018), the addition of alkylpolyglucose (Q. Wang, Xin, *et al.*, 2023), or the thermal pretreatment (TP) of the inoculum (Lazaro *et al.*, 2014). In addition, these treatments are usually complemented with additives that aid the solubilization of fiber-rich substrates to promote hydrolysis, which is a limiting stage of the global process. These additives include choline chloride (W. Cao *et al.*, 2023), urea hydrogen peroxide (S. Wang, Jiang, *et al.*, 2023), iron persulfate (Cheng *et al.*, 2023), or lysozyme and cation exchange resins (Pan *et al.*, 2023), among others.

In the particular case of PM, the TP of the inoculum has shown promising results in enhancing the production of VFA during the AD of this waste. Previous works have provided insights into the effect of combining the TP of the inoculum with the modification of other process factors, such as the carbon/nitrogen ratio (Q. Cao *et al.*, 2020), the initial pH (W. Zhang *et al.*, 2020), the organic load rate (Q. Cao *et al.*, 2021), and micro-aeration (Q. Cao *et al.*, 2022). Other authors have evaluated strategies that do not involve TP, including the optimization of operating conditions such as pH, solids content and retention time (W. Huang *et al.*, 2016) and the use of additives such as zero-valent iron (Y. Yang *et al.*, 2019), and magnetite (G. Zhang *et al.*, 2020). However, the TP of the inoculum is an attractive option because: i) no addition of any chemical substance is required, ii) it has the ability to suppress methanogenic organisms without significantly affecting hydrolytic or fermentative microorganisms capable of producing VFAs (Dahiya *et al.*, 2023), and iii) heat also improves the rate of hydrolysis, by

facilitating the degradation and solubilization of the fibers present in the substrate (Q. Wang, Xin, *et al.*, 2023).

One of the main drawbacks of the TP is the high energy consumption that is required for heating the inoculum. Indeed, the duration of this treatment is not standardized and varies across different works ranging from 3 h to 5 h (Q. Cao *et al.*, 2020, 2021, 2022). However, previous studies on the valorization of waste from yeast production (Ospina-Betancourth *et al.*, 2022) and ethanol production (Lazaro *et al.*, 2014) have shown that a TP of 90 °C for 10 minutes is enough to promote the production of VFAs. This is an alternative that has not yet been explored in the context of pig manure fermentation. In this work, we evaluated the effect of a short TP for enhancing the production of VFAs using PM as a substrate. Also, we compared the effect of this treatment on the microbial communities of three distinct inocula. This research provides valuable insights into the feasibility of implementing a short TP for a more energy-efficient and profitable recovery of VFAs from the acidogenic fermentation of PM.

3.2. Materials and methods

3.2.1. Substrate and inocula

Three inocula were used in this experiment: i) anaerobic sludge from a pig wastewater treatment plant (ASPW), located in Palmira, Colombia; ii) anaerobic sludge from a brewery wastewater treatment plant (ASBW), located in Yumbo, Colombia, and iii) anaerobic sludge from an urban wastewater treatment plant (ASUW), located in Jamundí, Colombia. All inocula were stored in the laboratory at room temperature until their use in the experiment. The substrate used was fresh pig manure (PM) obtained from a full-cycle pig farm located in Palmira, Colombia. A full-cycle pig farm is defined as a farm that includes all the age stages of the pig, namely, breeding, lactation, pre-fattening, and fattening. The PM was stored at -20 °C until its use in the experiment. The physicochemical characteristics of the substrate and inocula are shown in Table 3.1.

Table 3.1. Physicochemical characteristics of the substrate and inocula used in the experiment

Parameter	Units	PM	ASPW	ASBW	ASUW
Total solids	%	29.60	17.63	20.51	11.86
Volatile solids	% of total solids	75.46	67.97	51.36	75.35
Total carbon	% dry basis	42.36	N.D.	N.D.	N.D.
Total nitrogen	% dry basis	3.05	N.D.	N.D.	N.D.
Volatile fatty acids	mg/L	5,085.24	0	0	0
Chemical oxygen demand	mg O ₂ /L	167,409.46	6,845.85	13,212.21	3,602.51

Pig manure (PM), anaerobic sludge from a pig wastewater treatment plant (ASPW), anaerobic sludge from a brewery wastewater treatment plant (ASBW), anaerobic sludge from an urban wastewater treatment plant (ASUW). N.D.: not determined

3.2.2. Pig manure fermentation

The fermentation experiment was carried out in the Environmental Microbiology and Biotechnology Laboratory of the Universidad del Valle, Santiago de Cali, Colombia (3.377518698678618, -76.53277436292304), located at 1,018 meters above sea level.

The PM was fermented with three inocula (ASPW, ASBW, ASUW) subjected to TP at 90 °C for 10 minutes, with the objective of suppressing methanogenic microorganisms and thus promoting the accumulation of VFAs (Lazaro *et al.*, 2014; Ospina-Betancourth *et al.*, 2022). Controls with the same mixtures without TP were included. The experimental unit consisted of 100 mL amber glass bottles, with

a working volume of 40 mL, a total solids content of 10% and a volatile solids ratio between inoculum and substrate equal to 1. The bottles were hermetically sealed with a butyl rubber stopper and aluminum cap, and air displacement was carried out with N₂/CO₂ 80:20. Three replicates of each treatment were performed for each sampling time at 0, 3, 8, and 15 days, for a total of 72 experimental units. The bottles were kept at 37 ± 2 °C in an incubator (Precision Thelco, model 6).

The experimental setup and sample handling for physicochemical and microbiological analyzes is summarized in Figure 3.1.

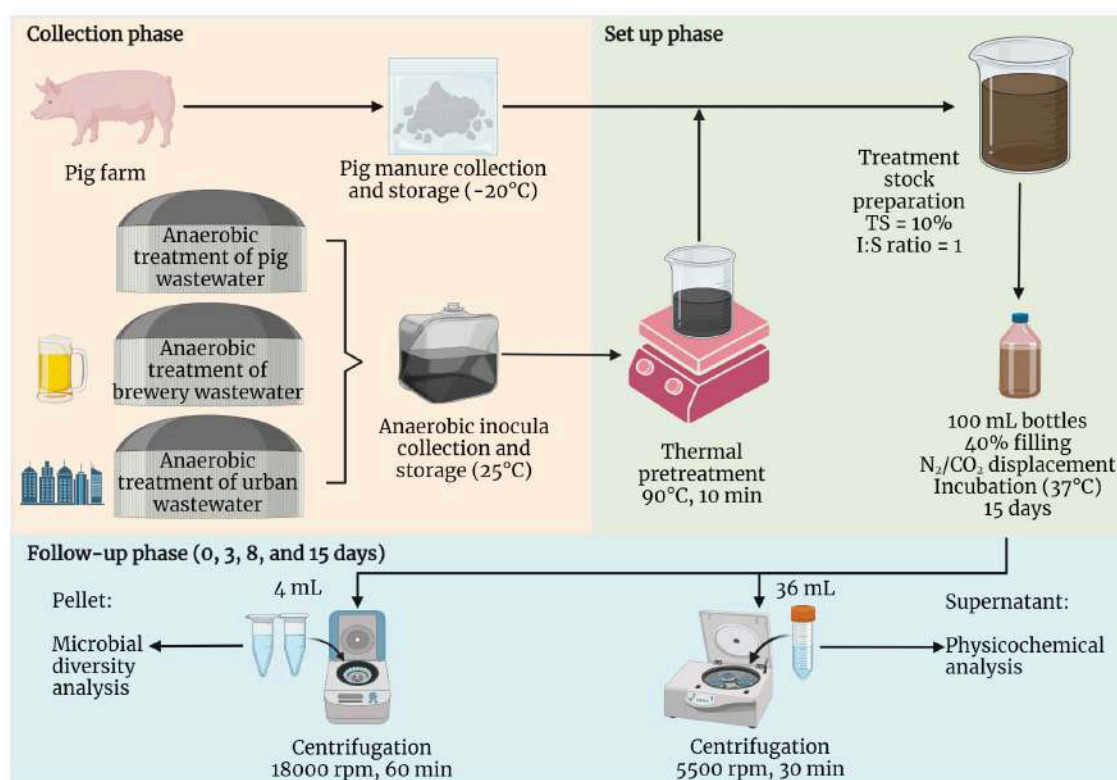


Figure 3.1. Methodological scheme to evaluate the effect of thermal pretreatment of inocula on VFAs production. Created with BioRender

3.2.3. Analytical methods

3.2.3.1. Physicochemical analysis

The total solids (TS) and volatile (VS) content were determined according to standard methods 2540 B and E, respectively (APHA, 2017). Carbon and nitrogen contents were measured according to standard method D5373-21, A (ASTM, 2021). At each sampling time, the contents of 3 bottles (triplicate) of each treatment were extracted and centrifuged at 5,500 rpm for 30 minutes. The supernatant was recovered and preserved for physicochemical analysis. The pH was measured with the potentiometric method (Hanna, HI 98160); alkalinity was measured by titration with HCl solution (Honeywell Fluka, Austria) standardized with Na₂CO₃ 99% (Merk, Germany), according to standard method 2320B; and the chemical oxygen demand (COD) was determined by the closed reflux method, colorimetric, according to the standard method 5220 D (APHA, 2017). The determination of VFAs was carried out in a gas chromatograph (Perkin Elmer Clarus 590), with an elite FFAP column 30 m x 0.25 mm ID x 0.25 mm DF and a flame ionization detector (FID). Nitrogen was used as carrier gas at 15 psi with a split ratio of 120 mL/min. The temperatures of the oven, injector, and detector were maintained at 140, 250, and 250 °C, respectively. To carry out the calibration curve, a standard mixture of 10 mM VFAs (Sigma-Aldrich, USA) was used, which includes acetic (HAc), propionic (HPr), isobutyric acid (iHBu), n-butyric acid (HBu), isovaleric (iHV_a), n-valeric (HV_a), 4-methyl valeric (4methHV_a), caproic (HCa), and enanthic (HEn) acids.

3.2.3.2. Microbial composition analysis

Aliquots of 2 mL of the effluent from each reactor were taken at the beginning and at the end of the experiment and centrifuged at 18,000 rpm for 1 hour. The supernatant was discarded, and the pellet was used for DNA extraction with a NucleoSpin Soil kit (Macherey–Nagel GmbH, Germany). The standard instructions of the kit were followed (lysis buffer SL1 and without Enhancer SX) with some modifications: after adding the lysis buffer SL1, the tubes were sonicated for one minute (ELMA TS 540,

USA) with a frequency of 35 kHz, subsequently, a mechanical lysis time of 30 minutes was used. Finally, the elution of the DNA was carried out twice in the same tube, the first time with 30 μ L of elution buffer and the second time with 20 μ L. The quality and concentration of the DNA were measured with a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA.). DNA was sent to Molecular Research (USA) to be sequenced using the Illumina platform for the v3 and v4 regions of the 16S rRNA gene (NovaSeq 6000 PE150).

The quality of the raw reads was evaluated using the FASTQC program, and samples were demultiplexed using sabre (v 1.0). Processing of the sequencing read was performed using dada2 (10.1038/nmeth.3869). Barcode sequences and primers were removed, and a quality filter (maxN = 0) was applied. The length of the reads was truncated to 280 pb for the forward reads, and 260 for the reverse reads. Error rates were calculated, and the samples were denoised using the dada algorithm. Sequences were de-replicated, and high-resolution amplicon sequence variants (ASVs) were obtained. Chimeraes were removed, and taxonomy was assigned using the function assignTaxonomy within dada2 using the SILVA 16S bacteria v132.1 database as a reference. The raw sequencing data was uploaded to the European Nucleotide Archive under project accession number PRJEB68159.

3.2.4. Statistical analysis

The results of pH, alkalinity, COD, and VFAs corresponding to day 15 of fermentation were subjected to an analysis of variance ($p < 0.05$) and then to a post hoc Tukey test ($p < 0.05$) to evaluate the effect of thermal pretreatment on the different inoculums. To evaluate the assumptions of normality and homogeneity of variances, the Shapiro-Wilks ($p < 0.05$) and Levene ($p < 0.05$) tests were used, respectively. The microbial composition data and alpha diversity of the samples were visualized using phyloseq (v 1.44.0) and ampvis2 (v 2.8.6). Finally, a canonical correspondence analysis (CCA) was performed to relate the abundance of species to the physicochemical parameters that were monitored using the package vegan v2.6-4. All the analyses were performed in R (v 4.3.1), and reproducible code is available at https://github.com/jclavijos/O_VFA.

3.3. Results and discussion

3.3.1. Effect of the short thermal pretreatment on the concentration of volatile fatty acids

From the three evaluated inocula, only ASPW and ASBW showed a significant increase in the concentration of VFAs after the short TP (Figure 3.2). After 15 days, the short TP of ASPW significantly increased the total VFAs concentration to 13,030.07 mg COD/L, compared to only 4,105.53 mg COD/L in the control (Figure 3.2A). These VFAs were dominated by acetic (51.12%) and n-butyric (21.57%) acids. The highest production of VFAs achieved with ASPW is expected, considering that this inoculum is already adapted to the substrate, which has been widely recognized to be beneficial for fermentative processes (Awati *et al.*, 2005; Bianchini *et al.*, 2022). Interestingly, although ASBW (Figure 3.2B) was not previously adapted to PM, the PT caused a drastic and significant enhancement in the VFA concentration, going from 79.76 mg COD/L in the control to a maximum value of 11,919.86 mg COD/L. Moreover, the overall composition of the VFAs was similar to that observed in ASPW being mainly acetic (47.00%), propionic (11.13%), and n-butyric (20.78%) acids. In previous studies, this type of inoculum has shown good performance in the AD of extremely high and complex organic loads, such as wastewater derived from petroleum production (Z. Huang *et al.*, 2021). In contrast to the other two inocula, fermentation with ASUW (Figure 3.2C) did not show a significant increase in the concentration of VFAs with TP, reaching the lowest concentration of all the treatments of only 5,934.18 mg COD/L of total VFAs, mainly composed by acetic (51.04%), propionic (14.66%), n-butyric (13.06%), and isovaleric (11.79%) acids.

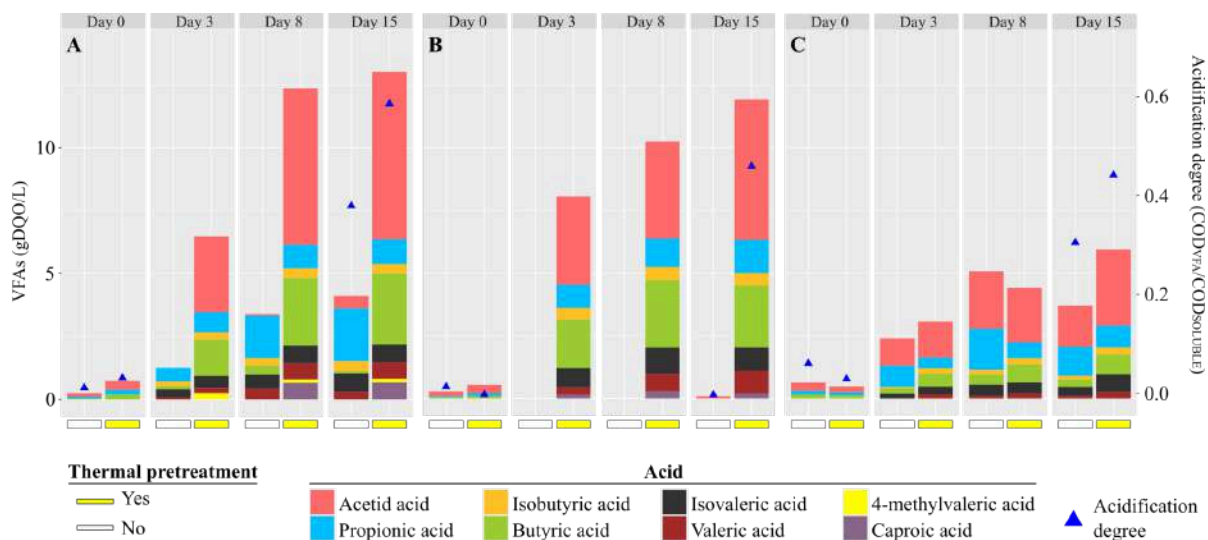


Figure 3.2. Concentration of individual VFAs and degree of acidification in the fermentation of pig manure with three inocula: A) anaerobic sludge from a pig wastewater treatment plant (ASPW), B) anaerobic sludge from a brewery wastewater treatment plant (ASBW), C) anaerobic sludge from an urban wastewater treatment plant (ASUW), as a function of thermal pretreatment (TP) of the inoculum and time (days).

These results are consistent with other studies that confirm the beneficial effect of TP on the production of VFAs. A key precedent is provided by Y.-Y. Wang *et al.* (2011), who evaluated the fermentation of glucose, using ASPW as inoculum. They found that a short TP (80 °C for 15 minutes) increased the concentration of total VFA from 9,026 to 12,305 mg COD/L, composed mainly of acetic (72%) and n-butyric (20%) acids, also noting that TP with higher temperature and longer time, causes a slight decrease in the concentration of VFAs. Moreover, the concentration of VFAs reached in our study is not so far from other studies concerning AD of PM with a more intensive TP on inoculum (75-85 °C, 3-5 h), and further combined with other strategies to boost VFAs. For instance, Q. Cao *et al.* (2020) observed that VFAs concentration in co-digestion of PM and corn silage decreased from 24.1 to 17.5 g COD/L, when the C/N ratio increased from 12.5 to 25. W. Zhang *et al.* (2020) evaluated the initial pH at 6.0, 8.0, and 10.0, reaching a maximum VFAs concentration of 9.9, 11.4, and 15.2 g COD/L, respectively. Q. Cao *et al.*

(2022) observed a maximum VFAs concentration of 8.21 g/L (an increase of 22.4%) when micro-aeration (1 L/L/d) was used in the fermentation of PM. The above suggests that a short PT could be enough to achieve a substantial increase in VFA production, without the need to spend more time and energy to completely eliminate methanogenic microorganisms. An example of this was the detection of archaea species (methane producers) in inoculum samples even after TP at 104 °C for 12 h (Bhattad *et al.*, 2017).

Alternatively, the effect of TP on the chemical composition of the sludge, and its subsequent impact on microorganisms, should also be considered. For instance, the thermal hydrolysis process (160 °C, 30 min) of waste activated sludge leads to a release of biodegradable proteins, which may increase the release of ammoniacal nitrogen (particularly free ammonia nitrogen - FAN) during AD, which is widely recognized as inhibitory for acetotrophic methanogenic microorganisms (Pavez-Jara *et al.*, 2023; Rajagopal *et al.*, 2013). Similarly, if it comes to the composition of the substrate, TP seems to give better results than other methods to improve the production of VFAs. For instance, Y. Yang *et al.* (2019) achieved a VFAs concentration of 4,577.74 mg/L (an increase of 32.1% with respect to control) with the addition of zero-valent iron (ZVI) in the AD of PM, and G. Zhang *et al.* (2020) obtained 3,933.0 mg/L (an increase of 57.32% with respect to control) under the effect of ZVI combined with magnetite in the two-stage AD of PM.

On the other hand, the acidification degree represents the proportion of organic matter (in this case expressed as soluble COD) that has been transformed into total VFAs (also expressed in terms of COD). It is observed that after 15 days there is an increase in the acidification degree in all treatments. It is also observed that the TP exerts a positive effect on this variable compared to the control in the three inocula, presenting values of 0.39 to 0.59, from 0.01 to 0.46, and from 0.31 to 0.45, for ASPW, ASBW, and ASUW, respectively.

3.3.2. Effect of thermal pretreatment on the stability of the fermentation process

The pH and the VFA/alkalinity ratio were selected as explanatory variables of the stability of the fermentation process. Figure 3.3 shows these two variables as a function of time, inoculum, and TP. In general, the pH was stable throughout the process, showing values between 5.92 and 7.48. Moreover, the TP caused a slight decrease in the pH in the treatments where ASPW and ASBW were used as inocula. Statistical comparisons on day 15 in fermentations with ASPW showed significant differences ($p < 0.05$) between the pH values of the TP (5.92) and the control (6.96) (Figure 3A). A similar trend was observed for ASBW (Figure 3B). On the other hand, there were no significant differences in the pH of both groups when ASUW was used as inoculum, maintaining almost the same values during the experiment (Figure 3C). The drop in pH observed with ASPW and ASBW may be caused by the accumulation of VFAs during acidogenesis and a loss of buffer capacity (Parra Huertas, 2015). Furthermore, pH values below 6.5 are harmful to methanogenic microorganisms, which causes VFAs to continue accumulating and acidifying the system (Raja Ram & Nikhil, 2022).

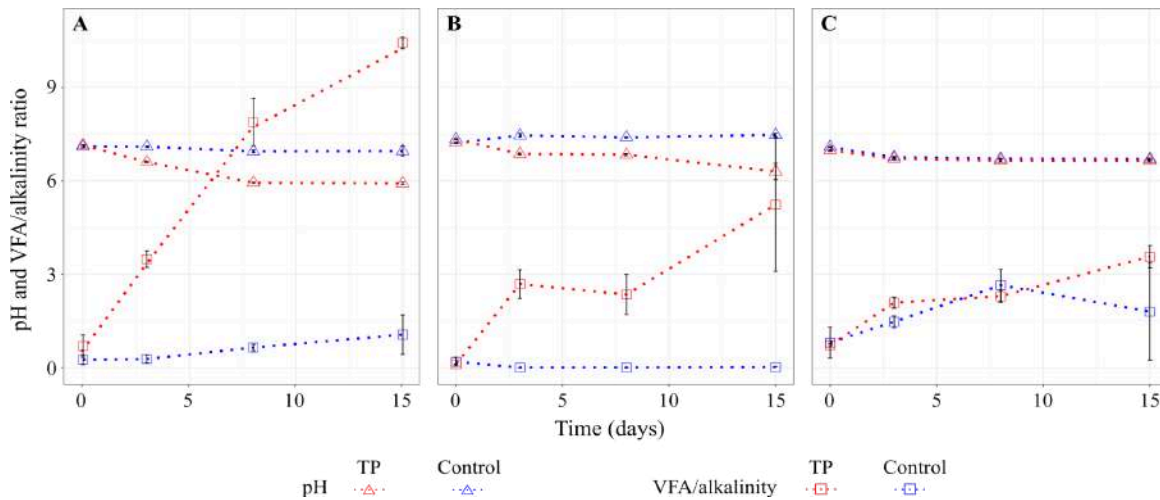


Figure 3.3. pH and the VFA/alkalinity ratio in the fermentation of PM with three inocula: A) anaerobic sludge from a pig wastewater treatment plant (ASPW), B) anaerobic sludge from a brewery wastewater treatment plant (ASBW), C) anaerobic sludge from an urban wastewater treatment plant (ASUW), as a function of thermal pretreatment (TP) of the inoculum and time.

The VFAs/alkalinity ratio, calculated as the quotient between the sum of all the individual VFAs (in mg/L) and the total alkalinity (in mg/L), showed a significant increase with the TP of the inocula ASPW and ASBW (Figure 3.3). The statistical comparison on day 15 showed significant differences ($p < 0.05$) between the TP and the control for the ASPW inoculum, with values of 10.43 and 1.06, respectively. This trend was also observed for the ASBW inoculum, with values of 5.24 and 0.01, respectively. In contrast, this ratio did not follow this trend for the ASUW inoculum. Nevertheless, all the ratios obtained in all the treatments were well above the recommended range for methanogenesis (0.3 - 0.4), indicating that the system has become acidic, and confirming the loss of the buffer capacity (Raja Ram & Nikhil, 2022). For further reference, Appendix 3.1 presents the mean, standard deviation, and statistical tests for all the monitored physicochemical parameters.

3.3.3. Effect of short thermal pretreatment on microbial composition

3.3.3.1. Changes in microbial composition as a function of inoculum and thermal pretreatment

The initial microbial composition of the ASBW and ASPW inocula was dominated by methanogens such as *Methanosaeta*, which accounted for up to 52.2 and 54.7% of the relative abundance of the microbial community, respectively. In contrast, the community composition of ASUW showed a high relative abundance of *Clostridium sensu stricto 1* (21%). An appreciable number of ASVs was detected from unknown taxa, especially in ASBW and ASPW, representing 22.9 and 15.7% of the total relative abundance, respectively. Other methanogens, such as *Methanobacterium*, *Methanolinea*, and *Methanobrevibacter*, were also detected in the inocula samples. The composition of the microbial community did not show major changes for ASBW and ASPW immediately after TP, except for a slight decrease observed in *Methanosaeta*.

Compared to the other two inocula, ASUW presents a higher initial relative abundance in genera such as *Clostridium sensu stricto 1*, *Romoutsia*, *Terrisporobacter*, *Caproiciproducens*, and *Methanobrevibacter*.

Moreover, the heating of the ASUW inoculum caused notorious shifts in the initial microbial composition, increasing, even more, the relative abundance of *Clostridium sensu stricto 1* and *Romboutsia*, and decreasing for *Methanosaeta* and *Caproiciproducens* (See Appendix 3.2).

After 15 days of fermentation of PM, the overall composition of the samples was different between inocula. Moreover, TP induced compositional shifts within each inoculum (Figure 3.4). The sequencing results suggest that selective pressure caused by TP did not affect the presence of methanogens in fermentation with ASBW, but showed a positive effect on the growth of *Clostridium sensu stricto 1*, *Caproiciproducens*, *Methanobacterium*, and *Syntrophobacter*; and a negative effect on *Ruminofilibacter*, *Proteiniphilum*, and *Syntrophomonas*. In the case of ASPW, the TP showed a positive effect on the relative abundance of *Clostridium sensu stricto 1*, *Terrisporobacter*, *Caproiciproducens*, and *Methanobrevibacter*; while *Methanosaeta*, *Mobilitalea*, and *HN-HF0106* showed a decrease in their relative abundance. In fermentations with ASUW, TP promoted the growth of *Methanobacterium*, *Acetomicrobium*, *Escherichia-Shigella*, and *Enterococcus*; but showed drastic decreases in the relative abundance of *Methanosaeta* and *Proteiniphilum* (See Appendix 3.4).

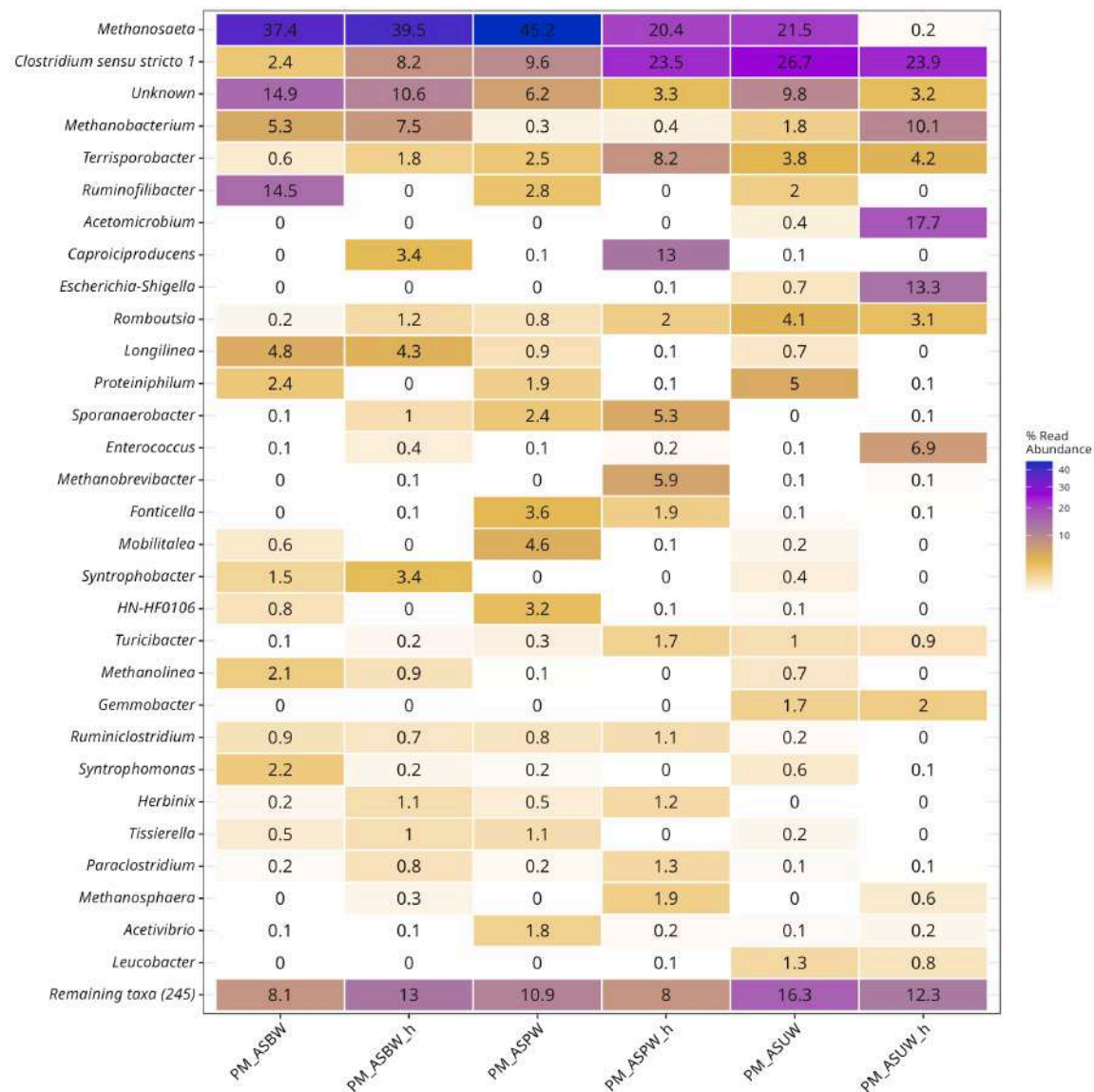


Figure 3.4. Microbiological composition at the genus level after 15 days of fermentation of pig manure, as a function of the thermal pretreatment of the inoculum. Treatments: pig manure with anaerobic sludge from pig wastewater treatment (PM_ASPW); pig manure with anaerobic sludge from brewery wastewater treatment (PM_ASBW); pig manure with anaerobic sludge from urban wastewater treatment (PM_ASUW); thermal pretreatment applied to inoculum (_h). The data shown is the average of three replicates.

TP was expected to mainly affect the growth of non-sporulating microorganisms, resulting in spore-forming bacteria increasing their relative abundance in the first days of fermentation (Blasco *et al.*, 2020). However, on day 15 of fermentation, a greater relative abundance of non-sporulating genera such as *Caproiciproducens*, *Escherichia-Shigella*, *Syntrophobacter*, and *Longilinea* was observed in treatments including TP. Spore-forming genera, such as *Clostridium sensu stricto 1* and *Ruminiclostridium*, increased their relative abundance in most treatments involving TP, as expected. However, other spore-forming bacteria decreased their abundance after TP, such as *Syntrophomonas*. On the other hand, *Archaea* members have demonstrated to be resistant to heat. In a previous study, archaeal species were still detected in an inoculum sample treated at 104 °C for 12 h, identifying increasing resistance in the following order: *Methanospirillum* > *Methanosaeta* > *Methanoculleus* (Bhattad *et al.*, 2017).

Considering the previous discussion, it is unlikely that the microorganisms have suffered direct damage from the application of a short TP, but they could have been indirectly affected by chemical changes caused by this pretreatment (Pavez-Jara *et al.*, 2023; Rajagopal *et al.*, 2013).

3.3.3.2. Changes in diversity indexes

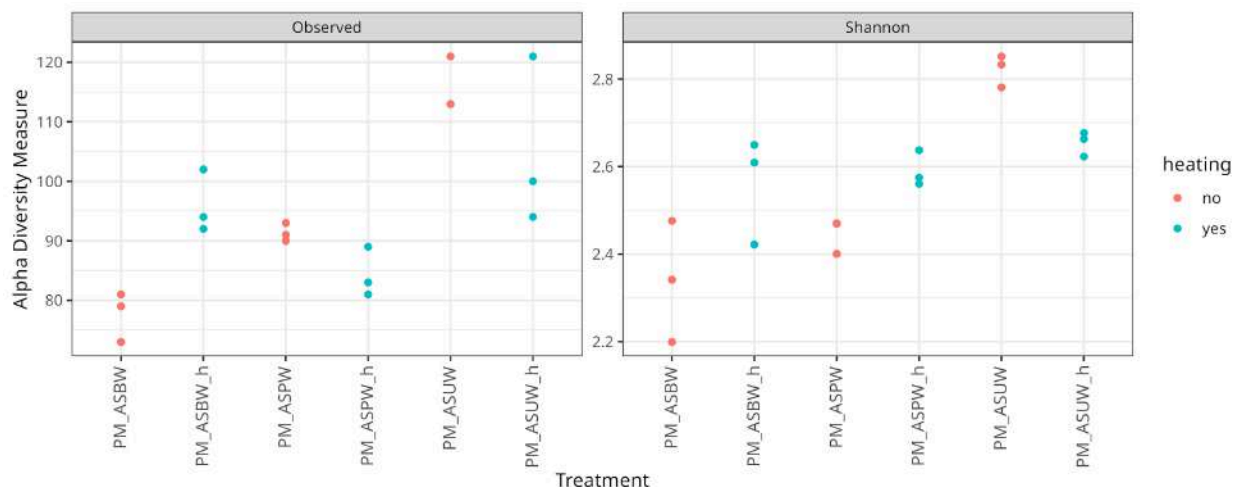


Figure 3.5. Diversity indexes in the fermentation of pig manure as a function of inoculum and thermal pretreatment. Treatments: pig manure with anaerobic sludge from pig wastewater treatment (PM_ASPW); pig manure with anaerobic sludge from brewery wastewater treatment (PM_ASBW); pig manure with anaerobic sludge from urban wastewater treatment (PM_ASUW); thermal pretreatment applied to inoculum (_h)

3.3.3.3. Relationship between physicochemical parameters and dominant bacteria genera

Correlations between VFAs concentration and microbial composition after 15 days of fermentation of pig manure were identified in the treatments evaluated (Figure 3.6). The genera *Mathenobrevibacter*, *Caproiciproducens*, *Sporanaerobacter*, and *Terrisporobacter* showed a strong positive correlation with acetic and n-butyric acids, followed by a moderate correlation with isobutyric, isovaleric, and valeric acids. These variables are also closely related to the treatment involving PM_ASPW_h but oppositely correlated to PM_ASPW (control), where genera such as *Methanosaeta*, *Mobilitalea*, and *Syntrophobacter* are abundant.

Other genera such as *Ruminofilibacter* and *Longilinea* were found to be closely related to the treatment PM_ASBW (control) and showed strong negative correlations with VFAs concentration, but positive correlations with higher values of alkalinity and pH. When TP is applied, the graphical location of PM_ASBW_h treatment displaces towards a closer correlation with VFAs. At this point, it is notorious that the treatments involving ASPW and ASBW showed graphical displacement in the same direction of VFAS when TP was applied, explaining the increase observed in this variable in both cases.

On the other hand, the genera *Escherichia-Shigella*, *Enterococcus*, and *Acetomicrobium* showed a poor correlation with VFAs, but a close correlation with the treatment PM_ASUW_h. Moreover, the graphical displacement of this inocula when TP is applied, occurs in a transversal direction to the trajectory of the VFAs, which could explain the poor change in this variable.

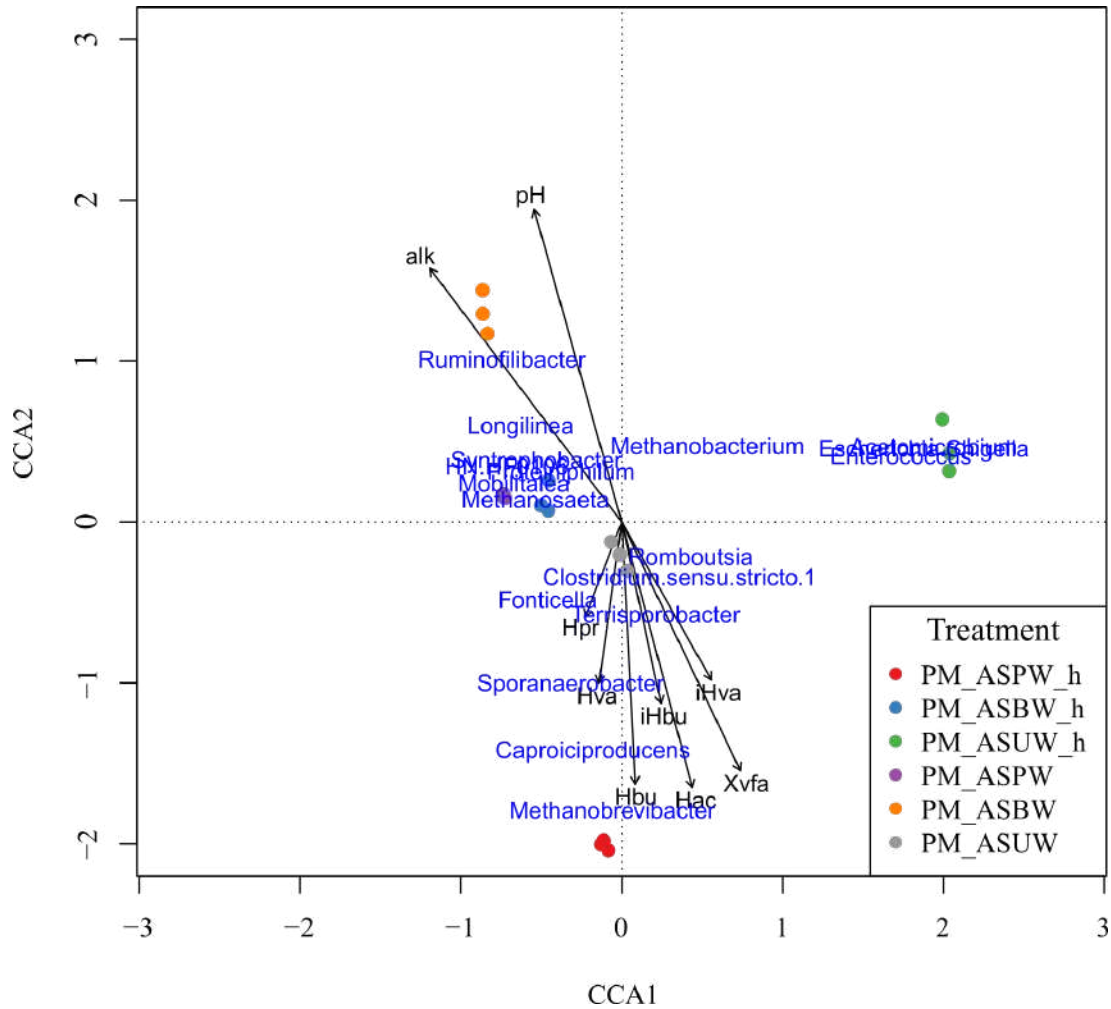


Figure 3.6. Canonical correspondence analysis (CCA) for physicochemical and microbiological composition at genus level in the fermentation of pig manure. Treatments: pig manure with anaerobic sludge from pig wastewater treatment (PM_ASPW); pig manure with anaerobic sludge from brewery wastewater treatment (PM_ASBW); pig manure with anaerobic sludge from urban wastewater treatment (PM_ASUW); thermal pretreatment applied to inoculum (_h). Physicochemical variables: alkalinity (alk); acetic acid (Hac); propionic acid (Hpr); isobutyric acid (iHbu); butyric acid (Hbu); isovaleric acid (iHva); total VFAs (Xvfa).

Although *Clostridium* showed a weak correlation with VFAs production, this genus has been widely recognized as one of the most important in this matter, particularly for n-butyric acid production (Q. Cao *et al.*, 2021; J. Huang *et al.*, 2023; R. Wang, Gu, *et al.*, 2023). Other studies have also found a positive correlation between *Caproiciproducens* and VFAs, which may be due to their ability to produce butyric and caproic acids, using lactic acid as an electron donor (Feng *et al.*, 2018; J. Huang *et al.*, 2023).

Regarding methanogenic microorganisms, changes in their abundance after TP could have a different effect on the accumulation of VFAs, depending on their type of metabolism. *Methanosaeta* species, for instance, are negatively correlated to VFAs because they are strictly dependent on acetate (Patil *et al.*, 2021), while *Methanobrevibacter* is a hydrogenotrophic methanogen, consuming the excess of H₂/CO₂ produced during VFAs degradation, alleviating the inhibition of syntrophic acetogenesis (R. Wang, Gu, *et al.*, 2023) and showing a positive correlation with the concentration of acetic acid. However, other hydrogenotrophic methanogens, such as *Methanobacterium*, showed a negative correlation to VFAs probably because they also can compete against acetogens for H₂ and CO₂, using these substrates to produce methane instead of acetate (Dueholm *et al.*, 2023).

A weak correlation has been confirmed between VFAs production and genera such as *Romboutsia* and *Fonticella*, in co-fermentation of dairy cattle manure and corn straw silage (C/N ratio around 25) (Lian *et al.*, 2022). *Escherichia-Shigella* members are associated with enteric diseases in humans, which could explain their presence in ASUW. This genus has shown negative correlations with the production of propionic and butyric acids in previous fermentative assays, further suggesting that the presence of this genus may hinder the growth of other VFA-producing bacteria (Pi *et al.*, 2024).

3.4. Chapter conclusions

Short TP applied to inoculum increased the VFAs concentration up to 13,030.07 mg COD/L (217%) in the anaerobic digestion of pig manure, which is comparable to other more energy-intensive or chemical-demanding pretreatments. The increase in VFAs concentration was proportional to the loss of

stability of the process, verified as a decrease in pH and an increase in the VFA/alkalinity ratio. Short TP promoted changes in microbial composition in all the inocula, likely by means of other mechanisms than direct cellular damage. Relevant genera such as *Methanobrevibacter*, *Caproiciproducens*, and *Terrisporobacter* showed a positive correlation with VFAs production.

The results obtained in this chapter allowed us to validate an efficient method for conditioning the microbiome present in anaerobic inocula, increasing the potential for valorization of VFAs from pig manure. From this validation, it will be possible to study other aspects of the engineering of the fermentation process, such as the evaluation of operating conditions and scaling, which will be presented in the next chapter.

Chapter 4: Evaluation of operating conditions and scalability of the production and recovery of VFAs from pig manure

4.1. Introduction

Nowadays, pig farming is an important component of food security. In 2021, this industry produced 120 million tons of meat and was the second source of animal protein (FAO, 2023a), generating economic benefits in many regions of the world (Smith *et al.*, 2022). Despite its importance, this industry also faces environmental challenges due to the high generation of manure, whose production rate is estimated at 2.35 kg/animal/day, which represents about 837 million tons per year (Mariscal, 2007). In addition, manure has nitrogen, phosphorus and potassium contents above 1% each (Noreña *et al.*, 2016; J. Yang *et al.*, 2019), for these reasons, the suitable management of this waste has been identified as a critical issue in the environmental agenda of this industry (Andretta *et al.*, 2021).

One of the most important technological options for the treatment and valorization of pig manure (PM) is anaerobic digestion (AD), a process of depuration of organic matter in the absence of oxygen, which allows the recovery of energy (methane) and nutrients (nitrogen, phosphorus and potassium) (Czekala, 2022). However, the remaining liquid fraction of AD also offers other valuable products such as volatile fatty acids (VFAs), which are considered “chemical building blocks” for a wide range of industries including chemicals, pharmaceuticals, food and agriculture (den Boer *et al.*, 2020; Ramos-Suarez *et al.*, 2021). For these reasons, the recovery of VFAs from pig manure using AD can be an opportunity to increase the recovery options for this type of waste, and at the same time avoid its uncontrolled emission into the environment (Clavijo-Salinas *et al.*, 2020).

Several investigations have explored the production of VFAs from pig manure using AD, evaluating factors related to the biochemistry of the process, such as the carbon/nitrogen ratio (Q. Cao *et al.*, 2020), co-digestion with carbon-rich substrates (Xu *et al.*, 2020), or the use of additives such as zero-valent iron

(Y. Yang *et al.*, 2019) or magnetite (G. Zhang *et al.*, 2020). However, to achieve a profitable production of pig-derived VFAs on a larger scale it is necessary to optimize the operating conditions of the AD process. Several authors have made contributions in this aspect, evaluating the effect of individual factors such as temperature, pH, total solids content or the inoculum/substrate ratio (ISR) (W. Huang *et al.*, 2016; Y. Li *et al.*, 2022; Mao *et al.*, 2017; W. Zhang *et al.*, 2020). However, for the optimization to be efficient and to avoid uncontrollable variations (noise) in the measurements, it is advisable to first carry out a screening design to evaluate the significant effects associated with the relevant factors of the process, as well as the antagonistic or synergisms that may exist between them (Snee & Bailey, 2023).

In this sense, the objective of this work was to carry out an evaluation of the individual and combined effect of operating factors such as pH, TS, and the ISR, on the production of VFAs in the AD of pig manure, on a laboratory scale. Subsequently, the most appropriate operating conditions were selected to scale up the process to a volume of 6 liters. This work is expected to contribute to knowledge for the efficient management of pig manure with new lines of recovery from VFAs and, in the future, to improve the environmental sustainability of this industry.

4.2. Materials and methods

4.2.1. Inoculum and substrate

The inoculum used in this experiment was anaerobic sludge from a biodigester that treats pig wastewater, while the substrate was fresh pig manure. Both substances were collected in a pig farm dedicated to the breeding and sale of piglets, located in the municipality of Palmira, department of Valle del Cauca, Colombia. The inoculum was stored at room temperature, and the substrate was stored at -20 °C until use. Since the TS of the inoculum as collected was less than 1%, it was necessary to concentrate it by settling for 24 hours. For the treatments evaluated at TS 10%, it was necessary to make an additional concentration of the settled inoculum, by centrifugation at 4,227 RCF for 30 minutes (MPW-260R, rotor

reference 11213). The characterization of the pH, TS and volatile solids (VS) content of the substrate and the inoculum (settled and centrifuged) are shown in Table 4.1.

Table 4.1. Characteristics of inoculum and substrate used to evaluate the operational conditions on VFAs production in the fermentation of pig manure

	Settled inoculum	Centrifuged inoculum	Substrate
pH	8.70 ± 0.00	7.97 ± 0.06	6.77 ± 0.15
Total solids (%)	4.77 ± 0.01	18.27 ± 0.20	36.35 ± 0.68
Volatile solids (% of TS)	62.77 ± 0.19	64.18 ± 0.27	82.24 ± 4.12

n = 3

4.2.2 Evaluation of operating conditions

The experiment was carried out in the Environmental Microbiology and Biotechnology Laboratory at Universidad del Valle, Santiago de Cali, Colombia, located at 1,018 meters above sea level. A complete factorial experimental design was carried out to evaluate the effect of the initial pH (natural and 10), TS (5 and 10%), and ISR (0.5 and 1), on the production of VFAs in the AD of pig manure.

The inoculum was subjected to a thermal pretreatment that consisted of heating at 90 °C for 10 minutes, with the objective of inhibiting methanogenic microorganisms and thus promoting the acidogenesis stage, accumulating VFAs (Lazaro *et al.*, 2014). Treatments with pH 10 were adjusted with 1M NaOH. The experimental unit consisted of 100 mL amber glass bottles with an effective volume of 40%, sealed with a butyl rubber stopper and aluminum cap, and placed in anaerobiosis by displacement with a 80/20 N₂/CO₂ gas mixture. The bottles were incubated at a temperature of 37 ± 2 °C (Precision Thelco, model 6) for 15 days.

4.2.3. Demonstration of process scalability

From the analysis of the significant effects of the different factors and their interactions, the operational conditions and the monitoring and control scheme of the scale-up bioprocess were established. The process was carried out in two identical reactors, used as observational units, with a total volume of 10 L and an effective volume of 6 L. The TS content was set at 10%, the ISR was set at 0.2, and the initial pH was not modified. The temperature was set at 37 ± 2 °C. The reactors were operated in batch mode for 10 days, then changed to semi-continuous mode for a period of 32 days, with a hydraulic retention time (HRT) of 10 days and an organic load rate (OLR) of 10 g SV/L/day, which is equivalent to 12.16 g ST/L/day. The monitoring variables were VFAs concentration and pH. The steady state was considered to be reached when the total VFAs concentration varied less than 5% during a period of time equal to a theoretical residence time (Arroyave G. *et al.*, 2010).

4.2.4. Recovery of pig-manure-derived volatile fatty acids

In this work, the selection of the VFAs extraction method was carried out through a multicriteria analysis (MA), involving the following steps (Juanpera *et al.*, 2022): i) a set of criteria was defined, ii) the selected criteria was weighted according to their relative importance, iii) the alternatives were assessed according to each criterion, and iv) a total numerical score was calculated by aggregating all the criterion for every alternative evaluated.

The alternatives in MA corresponded to recovery techniques identified from literature. Relevant criterion related to VFAs extraction were defined, according to their proposed application as carbon source for biological nitrogen fixation. Each criterion was weighted in a scale from 0 to 1, so that the sum of the weights of all criteria is equal to 1. Moreover, each criterion was assigned a rating from 1 to 5 for each of the alternatives evaluated, as follows:

- Extraction efficiency: defined as the quotient between the mass of VFAs extracted and the mass of VFAs present in the fermentative effluent. The higher the extraction efficiency, the higher the score to be assigned. Weight assigned: 0.3
- Operating costs: corresponds to an estimate of the energy consumption, inputs, and costs in general, necessary for the operation of the extraction technique. The higher the operating costs, the lower the score to be assigned. Weight assigned: 0.25
- Need for pH modification: this is considered a decision criterion, since it determines the quality of the effluent resulting from the extraction, and the viability of its use in other biotechnological processes. If the extraction technique requires pH modification, the score to be assigned will be low. Weight assigned: 0.2
- Ease of installation at laboratory or bench scale: refers to the availability or accessibility of this technique in most laboratories and its simplicity of installation and set-up. The greater the ease of installation of the technique, the higher the score to be assigned. Weight assigned: 0.25

4.2.5. Analytical methods

The total solids (TS) content was determined by drying the sample at 103-105 °C until constant weight, and the volatile solids (SV) content was determined by igniting the dried sample at 550 °C for 1 hour, in accordance with standard methods 2540 B and E, respectively (APHA, 2017).

In the monitoring phase, a liquid sample of 40 mL was extracted from each experimental unit and the pH was immediately measured with the potentiometric method (Hanna, HI 98160). Then the sample was centrifuged at 4,227 RCF for 30 minutes (MPW-260R, rotor 11213) and an aliquot of 5 mL of the supernatant was preserved, which was acidified with fuming HCl (Honeywell Fluka, Austria) and stored at a temperature of - 20 °C for the determination of VFAs. At the time of analysis, the samples were thawed at room temperature and filtered with 0.45 µm PTFE.

The analysis of the AGVs was carried out by gas chromatography (Perkin Elmer Clarus 590), with a column elite FFAP 30m x 0.25 mm ID x 0.25 μ m DF, and a flame ionization detector. Nitrogen was used as carrier gas at 15 psi with a split ratio of 120 mL/min. The temperatures of the oven, injector, and detector were maintained at 140, 250, and 250 °C, respectively. The calibration curve was made from a standard mixture of 10 mM VFAs (Sigma-Aldrich, United States) including acetic, propionic, isobutyric, n-butyric, isovaleric, n-valeric, 4-methylvaleric, hexanoic, and heptanoic acids.

4.2.6. *Statistic analysis*

Statistical analysis was performed in R statistical software (v 4.3.1) using the SixSigma (v 0.11.0) and nparMD (v 0.2.1) packages. The levels for each treatment were coded as shown in Table 4.2. For each data group, the average and standard deviation were calculated. Subsequently, the normality of the data was evaluated using the Shapiro-Wilk test and the homogeneity of variances with the Levene test. For the response variables that met the assumptions of normality and homogeneity of variances, significant differences between the means were evaluated by means of an analysis of variance (ANOVA). On the contrary, for data groups that did not meet these criteria, the non-parametric or Kruskal-Wallis test was used. Additionally, the effect sizes and interactions of all treatments were represented graphically using the ggplot2 package (v 3.4.2).

Table 4.2. Coding of variables for statistical analysis

Factor	Level	Coding
Inoculum to substrate ratio (ISR)	0.5	-
	1	+
Total solids (%)	5	-
	10	+
pH	natural (7.56 ± 0.29)	-
	10	+

4.3. Results and discussion

4.3.1 Evaluation of operating conditions

By increasing the TS from 5 to 10%, a general increase in the production of total VFAs was observed, for all pH and ISR levels evaluated (Figure 4.1). The data also showed an increasing trend over the time evaluated, much clearer for the set of treatments with 10% TS. The initial pH and the ISR showed variable effects, a product of their interaction with the other factors of the experiment, as can be seen in Figure 4.1.

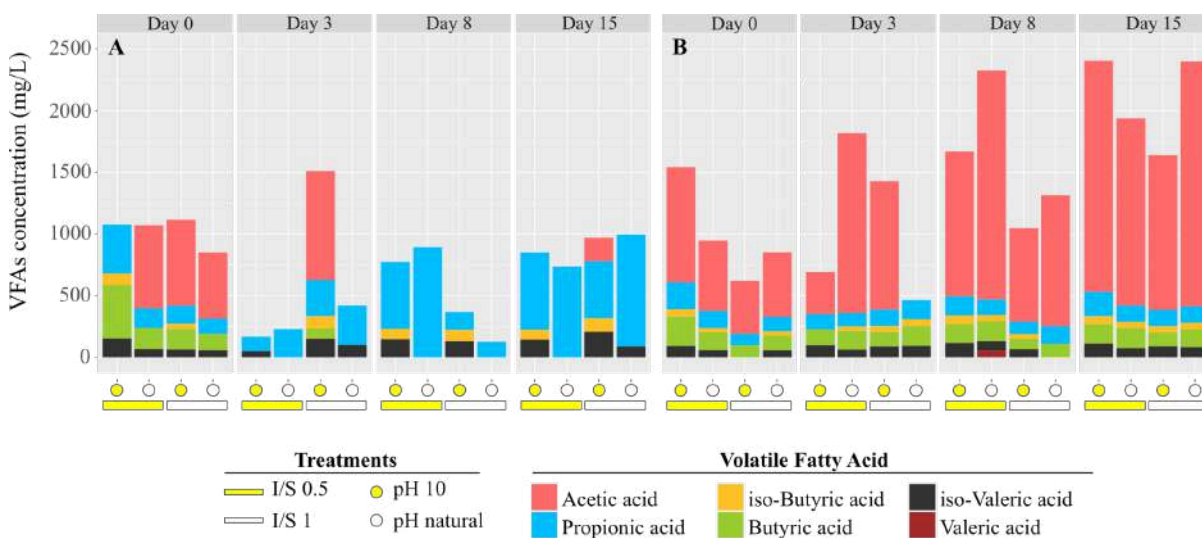


Figure 4.1. Concentration of individual VFAs (mg/L) in the fermentation of pig manure as a function of the inoculum/substrate ratio (ISR), initial pH and time. A and B represent a total solids (TS) content of 5% and 10%, respectively.

To examine the effects of the treatments, a statistical analysis was performed on the data obtained on day 15 of fermentation. The changes in VFA production for each combination of variables are summarized in Table 4.3. Under the same pH and ISR conditions, a maximum increase of 183.65% was observed when the TS content was doubled. Acetic and n-butyric acids showed the greatest increases, reaching maximum concentrations of 1,985.62 and 162.57 mg/L, respectively; while propionic acid decreased by up to 567.29% with the increase in TS. The majority proportion of acetic acid in VFAs could be based on the

important role that this acid plays as an intermediate in anaerobic metabolism (Pan *et al.*, 2021). It is worth mentioning that the combination of factors where the highest concentration of total VFAs was produced was ISR: 0.5, TS: 10% and pH: 10, which reached a concentration of VFAs equal to 2,403.94 mg/L. On the other hand, the combination of ISR 0.5, natural pH (close to neutrality) and TS of 5% obtained the lowest VFAs concentration among all treatments (733.55 mg/L).

Table 4.3. Concentration of individual and total VFAs (mg/L) depending on the inoculum / substrate ratio (ISR), initial pH and total solids (TS), on day 15 of pig manure fermentation

ISR	pH	TS	Acetic acid	Propionic acid	Isobutyric acid	Isovaleric acid	Butyric acid	Total VFAs
-	-	-	0 ± 0	733.75 ± 108.68	0 ± 0	0 ± 0	0 ± 0	733.75 ± 108.68
+	-	-	0 ± 0	906.92 ± 189.3	0 ± 0	87.2 ± 15.31	0 ± 0	994.12 ± 197.28
-	+	-	0 ± 0	625.68 ± 128.09	79.32 ± 28.9	142.5 ± 51.17	0 ± 0	847.51 ± 52.64
+	+	-	192.02 ± 44.61	460.8 ± 37.11	110.65 ± 7.67	205.56 ± 14.01	0 ± 0	969.03 ± 91.25
-	-	+	1517.45 ± 621.1	134.01 ± 23.7	47.99 ± 4.89	74.83 ± 5.74	162.57 ± 78.04	1936.84 ± 729.22
+	-	+	1985.62 ± 570.25	135.91 ± 29.28	51.96 ± 8.53	81.53 ± 10.05	143.77 ± 26.87	2398.79 ± 644.8
-	+	+	1873.46 ± 309.44	198.92 ± 22.51	65.63 ± 4.58	109.48 ± 7.7	156.44 ± 14.46	2403.94 ± 336.85
+	+	+	1253.19 ± 431.89	130.98 ± 44.53	51.97 ± 13.69	88.57 ± 21.41	112 ± 33.87	1636.72 ± 544.59

Note: the concentration of propionic acid met the assumptions for the comparison of means using the analysis of variance (ANOVA), the other acids were analyzed with the non-parametric Kruskal-Wallis test. 4-Methylvaleric, hexanoic and heptanoic acids were not detected on day 15 for any treatment.

4.3.2. Analysis of the effect of individual factors of the process

When analyzing each factor individually, it was observed that its effect was different for each VFA. For example, increasing the initial pH produced significant increases ($p < 0.05$) in the production of isobutyric and isovaleric acids. The increase in TS caused a significant increase in the levels of acetic acid, isovaleric acid, and total VFAs; however, the effect was negative for propionic acid. Finally, the ISR did not show a significant effect on the production of any of the VFAs under the conditions evaluated (Figure 4.2).

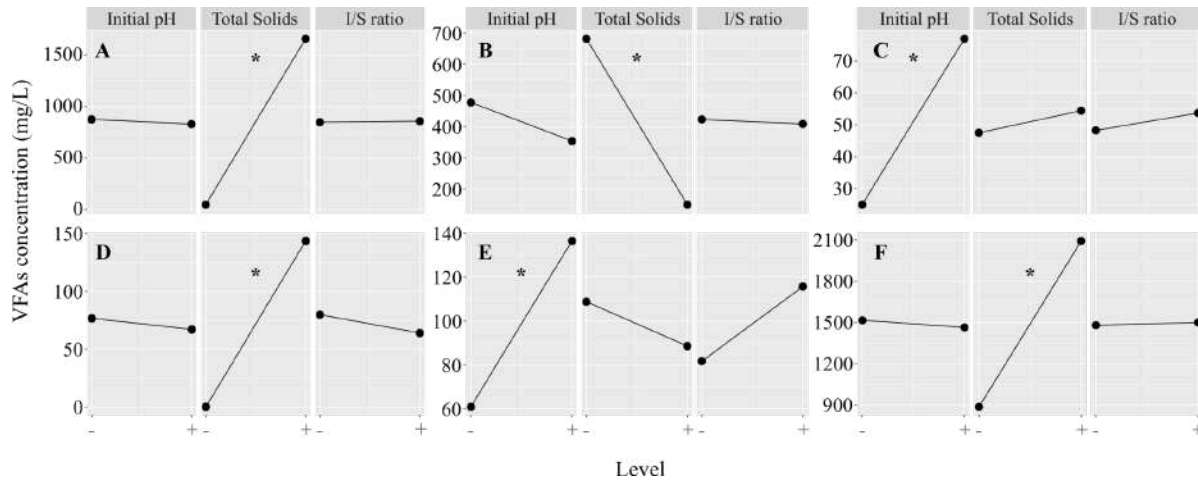


Figure 4.2. Effect of individual operating factors on VFA production from pig manure after 15 days of fermentation. Acids: acetic (A), propionic (B), isobutyric (C), n-butyric (D), isovaleric (E), total VFAs (F). Factors marked with * indicate statistically significant differences

The positive correlation found between TS and the production of total VFAs has also been reported by other authors who studied the fermentation of pig manure. S. Wang and collaborators (S. Wang, Chai, *et al.*, 2023), found optimal conditions for the production of VFAs by increasing the TS content from 5 to 15%, the same did W. Zhang *et al.* (2022) by modifying the VS content from 2 to 6%, and Xu *et al.* (2020) reported an increase in the concentration of total VFAs by increasing the TS from 4 to 10% in the

batch co-digestion of pig manure with sewage sludge (C/N of the mixture=13.47), also finding a lower proportion of propionic acid/acetic acid as the TS content increased, something that was also evident in the present study. W. Huang *et al.* (2016), on the other hand, found a negative correlation between TS and VFAs in the thermophilic anaerobic digestion of pig manure; However, the range of TS evaluated in their study was 20 to 35%, suggesting that there is possibly an inflection point in the maximum concentration of VFAs around 20% of TS, in the fermentation of pig manure. In the literature it has been reported that an increase in the TS content in the substrate is associated with a higher concentration of available organic matter, which generally results in an acidification of the anaerobic process (W. Li *et al.*, 2022; Pastor-Poquet *et al.*, 2018). This is because acidogenic microorganisms adapt faster than methanogenic ones to the shock caused by the increase in load, leading to an accumulation of VFAs and low methane production (Y. Zhang *et al.*, 2023).

Regarding the increase in the initial pH, the acids that were favored with this change were isobutyric and isovaleric. However, these were minor acids in relation to the rest of the VFAs, whose total concentration was not significantly affected by the change in initial pH. In the literature there are different results about the effect of pH on the production of VFAs, for example Mao *et al.* (2017) found a negative correlation between the concentration of VFAs and the increase in the initial pH in the 70/30 co-digestion of pig manure (C/N 18.75) with corn straw (C/N 82.26). This contrasts with the study by W. Huang *et al.* (2016), who reported an increase in the concentration of VFAs from 50.5 to 79.1 mg COD/g SV when increasing the initial pH from 8 to 11 in thermophilic anaerobic digestion of pig manure. Other studies, such as that of T. Zhang *et al.* (2015), reported a VFA/alkalinity relationship without significant changes, when increasing the pH from 6 to 8, in the 70/30 anaerobic co-digestion of pig manure with corn stalks. The majority of enzymes associated with the metabolism of methanogenic microorganisms are highly sensitive to extreme pH values, without this significantly affecting the metabolism of acidogenic microorganisms (Qiu *et al.*, 2023).

Finally, although in this study a significant effect was not found associated with the change in the ISR, it has been reported in the literature that low values in this variable are associated with higher concentrations of VFAs. This has been demonstrated with different organic waste such as pig manure (Rangel *et al.*, 2020), wastewater from olive oil extraction (da Fonseca *et al.*, 2023), food waste (Y. Li *et al.*, 2022), among others. This is because the ISR is a parameter that affects the stability of the fermentation: if it is too low it can cause toxicity and irreversibly acidify the reactor; but if it is too high, it can affect the production of enzymes important for the process (Xing *et al.*, 2020).

The factors evaluated in this experiment can also be analyzed from the point of view of practical application. The first factor to manage in a pig farm would be the TS content that is fed to the anaerobic process, since this variable is directly related to the water consumption in the pigsty cleaning operation, which can vary between 10 and 20 L/ day (Galvis & Acevedo, 2008). The TS in the feed stream modifies the ISR, which could be placed at a second level of complexity, since controlling this factor would require modifying the amount of inoculum inside the reactor. Finally, modifying the pH of the process would require the introduction of a chemical input (an acid or a base) to the system, which could increase the operational costs and the risk of releasing inorganic contaminants into the environment.

4.3.3. Analysis of the effect of the interaction between process factors

Although the analysis of the effect of individual factors provides important information about the process, it is also important to evaluate how the factors interact with each other, to identify possible synergistic or antagonistic effects that may have repercussions when optimizing and scaling the process (Snee & Bailey, 2023).

The analysis of the interaction between the factors showed that for acetic acid, when the initial pH is low, the increase in the ISR has a positive effect on its concentration (Figure 4.3A). On the other hand, when the initial pH is raised to 10, the increase in the ISR produces a completely opposite effect. The

concentration of total VFAs (Figure 4.3E) presents an identical behavior, which is expected given the majority proportion of acetic acid with respect to the other VFAs. This antagonistic interaction indicates that, in a 15-day acidogenic fermentation of pig manure, caution should be taken when using low values for ISR and pH. It is also striking that for both acetic acid and total VFAs, no significant interactions with TS have been identified; this may be due to the fact that the effect of this factor on the concentration of VFAs is much greater compared to the pH and the ISR, as shown in Figure 4.1.

In a study carried out with artisanal fishing waste, Cadavid-Rodriguez *et al.* (2020) found that by decreasing the pH from 9 to 7, and decreasing the ISR from 0.20 to 0.05, the production of VFAs increased in a period of 15 days, indicating a correlation that differs from the result of this study, which can be attributed to the difference in the chemical nature of the substrate.

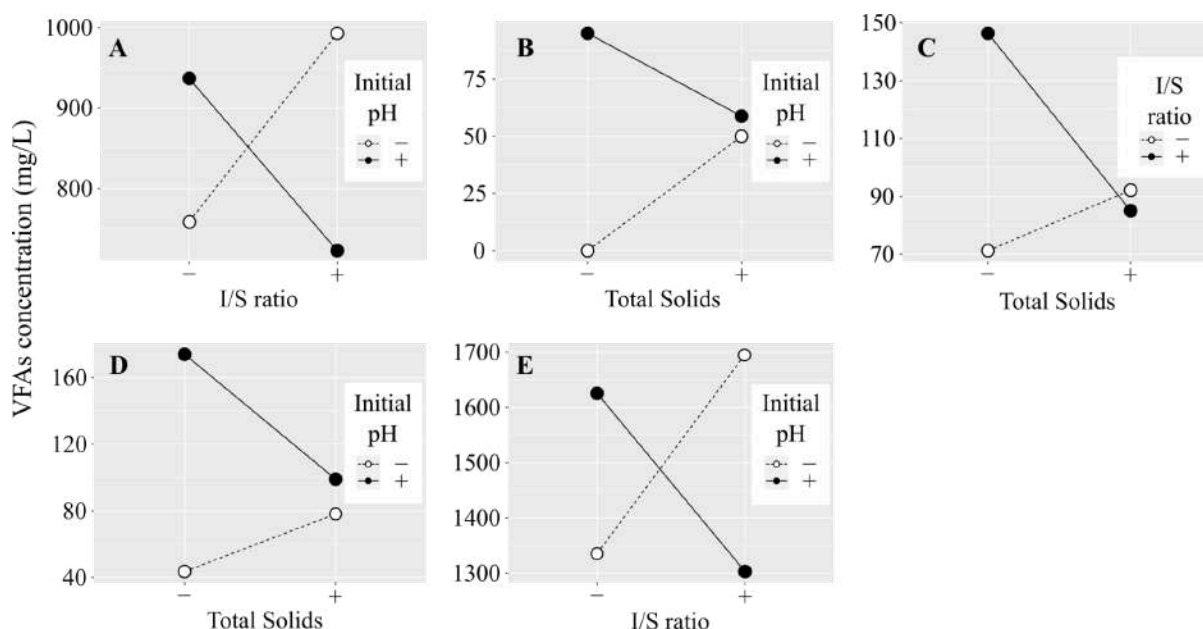


Figure 4.3. Effect of the interaction between factors on the production of VFAs from pig manure after 15 days of fermentation. Acids: acetic (A), isobutyric (B), isovaleric (C and D), total VFAs (E).

For isobutyric acid (Figure 4.3B), an interaction between TS and initial pH was presented. At low pH, the increase in TS has a positive impact on the concentration of this acid. But as the pH increases, the

opposite behavior is observed. The same interaction between TS and initial pH was also observed for isovaleric acid (Figure 4.3D). It is worth highlighting the practical convenience of this interaction for these two acids, since without the need to modify the initial pH of the process, a positive effect would be obtained by increasing the TS concentration.

Another interaction that could have a practical advantage is that observed between TS and the ISR, in isovaleric acid (Figure 4.3C). In this case, there is a positive effect in the increase of TS on the acid concentration when the ISR is low, this implies that the fermentation system could receive a greater amount of TS in the substrate without needing to modify the amount of inoculum, when the objective is to increase the concentration of this acid. The results show how the variability of inoculum conditions induces changes in metabolic pathways, and raise the need to carry out specific treatability studies to achieve process optimization.

4.3.4. Demonstration of the scalability of the fermentation process

To establish the conditions for scaling the process, the analysis of the effect of the factors of the fermentation process was taken into account, where it was shown that the TS content is the most influential factor, with 10% being the most appropriate level to maximize the VFAs production. Even a higher percentage of TS could be proposed (for example 15 or 20%) (W. Huang *et al.*, 2016), but operational aspects such as the need for a greater supply of manure and a greater power for the agitation system would have to be considered. In this sense, an TS content of 10% was defined to carry out an inoculum adaptation period for 10 days, and subsequently, an organic loading rate of 10 g SV/L/d and a hydraulic retention time (HRT) of 10 days were established, for semi-continuous operation. Regarding the initial pH, since no significant effect was found on VFAs production under the conditions evaluated, and since there was no clear trend in the literature, it was decided to leave it unchanged (7.56 ± 0.29). This would also be advantageous from a practical point of view, because, as explained in the previous section, it would eliminate the need to introduce chemical inputs to the process. Finally, the literature indicates

that low ISR promotes the production of VFAs, which is why it was decided to set a value even below the conditions evaluated, in this case 0.2.

Once these conditions were established, the installation and start-up of the fermentation process was carried out as described in section 4.2.3, to demonstrate its scalability. During the monitoring time, both reactors showed relatively homogeneous behavior in terms of VFAs production and pH. This last variable presented a stable behavior throughout the observed period, with an average of 6.5 ± 0.26 .

Figure 4.4 shows the production and distribution of VFAs from the two reactors evaluated. Total VFAs distribution data are presented separately (reactors a and b), while the VFAs distribution is presented as an average of the two reactors, since no significantly variation was observed between them. The concentration of total VFAs reached a maximum peak of 8,783 mg/L in reactor a (14 days), and 7,868 mg/L in reactor b (32 days). The concentration of total VFAs ranged from 6,442 to 7,286 mg/L in the last four days of the experiment for both reactors. Steady state could not be verified in any of the reactors. A decrease in the concentration of total VFAs in both reactors was observed between days 32 and 38, this could be related to the entry of a new manure feedstock that was collected in that week, to continue feeding the reactor. On the other hand, the VFAs composition remained constant for most of the time, represented mainly by acetic, n-butyric and propionic acids, with average percentages of 79.40, 9.45 and 5.58%, respectively.

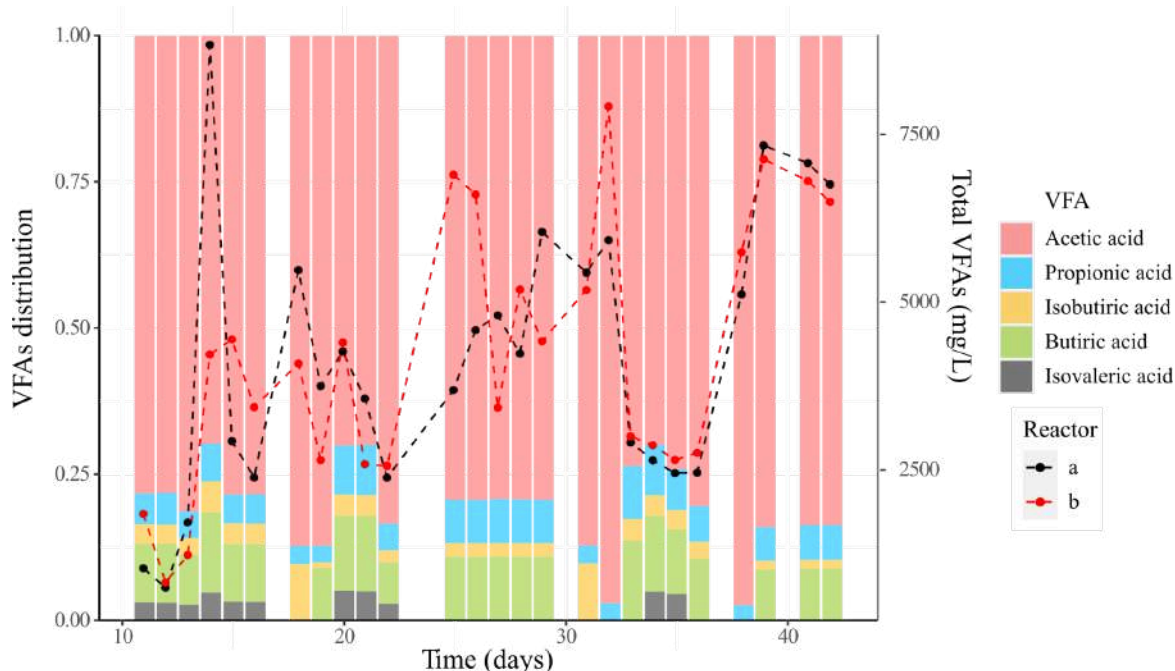


Figure 4.4. Production and distribution of VFAs in semi-continuous fermentation of pig manure

These results are comparable with the experiment of Z. Wang *et al.* (2022), who obtained a stable VFA production of 6,065 mg COD/L from pig manure, in a 2-liter bioreactor operated at a temperature of 30 °C and initial pH of 10, with a feed COD of 7,660 mg/L and a HRT of 1.5 days (OLR estimated at 3.8 g SV/L/d). Another similar experiment was that of Q. Cao *et al.* (2020), who evaluated the co-fermentation of pig manure and corn silage, in a 10 L bioreactor, at a temperature of 37°C and a HRT of 12 days. The substrate/inoculum ratio was 9:1 (estimated ISR = 0.11), and the initial pH was adjusted to 6.0 ± 2 . They reported a maximum concentration of VFAs of 19.1 g COD/L (acetic, n-butyric and hexanoic acids added up to 80%), using a OLR of 3.5 g SV/L/day. In both cases the use of low OLR stands out compared to the present study, but the higher result of Cao and collaborators could be explained by the use of a co-substrate rich in carbon, which has already been demonstrated to be beneficial for the production of VFAs (Xu *et al.*, 2020).

By the other hand, when comparing the results before and after scaling up the fermentation process, it is observed that the change in volume and mode of operation resulted in an increase of more than 200% in the concentration of total VFAs. This response has also been reported in fermentation of other organic wastes such as primary sludge from wastewater treatment (Shylaja Prakash *et al.*, 2024). A possible explanation comes from the fact that changing the operating mode from batch to semi-continuous modifies the concentration of substrate and products inside the reactor. In the batch mode, the substrate is consumed and the products accumulate as the retention time elapse, while in the semi-continuous mode, new substrate enters and the products are removed from the reactor periodically, avoiding their accumulation. An example of this occurs with H_2 and CO_2 : in batch mode, these fermentation products increase their partial pressure in the gas phase, increasing their solubility in the liquid phase (Henry's law), which modifies the pH and affects the metabolism of microorganisms. While in the semi-continuous mode the pressure of the system remains constant, since there is a constant release of the liquid and gaseous effluents that are produced (Charalambous *et al.*, 2023).

It is also important to highlight that the scaling of any bioprocess requires attention to several aspects. For the specific case of VFAs production, H. Wu *et al.* (2023) highlight factors such as the composition and pretreatment of the substrate, pH, alkalinity, temperature, HRT and OLR. On the other hand, González *et al.* (2023) point out that guaranteeing a stable supply (in quantity and quality) of the raw material, carrying out the relevant technical-economic analysis, and a detailed analysis of actors in the value chain, are key aspects for the scaling of biorefineries in Latin America.

4.3.5. Recovery of pig-manure-derived volatile fatty acids

4.3.5.1. Description of methods for recovery of VFAs

There are various technologies for recovery of VFAs; however, technical and economic barriers may limit their implementation in specific contexts, typically their low concentration and the chemical complexity of

the fermentation broth (Dahiya *et al.*, 2023). Selecting the optimal recovery process involves careful consideration of both the intended use of the final product and the level of purity necessary for its application (Asunis *et al.*, 2022). The separation of VFAs typically comprises multiple stages: (1) initial recovery stage, which removes VFAs from the fermentation broth, and (2) subsequent purification stage aimed at refining the VFAs and concentrating them, either for potential sale in the market or for further upgrading (Giduthuri & Ahring, 2023).

The alternatives considered for multicriteria analysis consisted of techniques for VFAs extraction reported in literature, as described below:

- **Precipitation:** calcium-based salts are added to the VFA-rich stream to produce carboxylate salts, which are usually concentrated by evaporation, and followed by **crystallization** or other method to separate the salts (Aghapour Aktij *et al.*, 2020) This technique is well established, and allows high production yields with high purity; nevertheless, the solubility of the desired products might put the efficiency of the process at risk, and aspects such as the high energy demand and the generation of solids wastes must be carefully considered (Dahiya *et al.*, 2023).
- **Adsorption:** this technique consists of passing the VFAs-rich stream through a solid absorbent matrix, mainly ion exchange resin. The VFAs' carboxylate group interacts with active groups present in the resin, remaining attached to them while the rest of the effluent follows along. When the resin becomes saturated, it must be regenerated by means of a countercurrent flow with a solvent. The main advantages of adsorption are given by its technological maturity and ease of operation, while the main drawbacks includes the high resin cost, the high energy consumption for resin regeneration, the low absorption capacity, and low selectivity (Dahiya *et al.*, 2023). Within traditional methods, ionic resins and liquid extractants stand out as well-established technologies extensively employed for the industrial-scale extraction of organic acids.
- **Distillation:** this technique allows the extraction of VFAs from the fermentation effluent by means of the differences in the vapor pressure of the components of the mixture. For this, a heat

source is needed, and the VFAs must be in non-ionic form, so pH must be adjusted. An alternative methodology is to neutralize VFAs with ammonia, then the ammonium carboxylates are mixed with alcohol to produce esters, which can be separated by distillation (Aghapour Aktij *et al.*, 2020). Distillation is recognized as a well established process, relatively easy to installation, that allows the obtaining of highly pure products. Nevertheless, its high energy consumption can put its technical and economic viability at risk (Giduthuri & Ahring, 2023). Low-cost energy sources could contribute substantially to the sustainability of this technique (Nzeteu *et al.*, 2022).

- **Solvent extraction:** this technique is based on the affinity of the desired product (VFAs) with a solvent. Although solvent extraction has demonstrated recovery efficiency over 80%, an additional stage consisting of vacuum evaporation is needed to recover the solvent used in the extraction, which is high energy demanding (Giduthuri & Ahring, 2023). Moreover, the use of organic solvents is controversial in terms of toxicity and sustainability, since most of them are fossil-fuel derived chemicals (Nzeteu *et al.*, 2022).
- **Non-pressure-driven membrane separation techniques**
 - **Electrodialysis:** by means of a difference of voltages, VFAs in their ionic form are forced to migrate through a membrane towards an electrode with opposite charge. This makes pH adjustment unnecessary, and allows to obtain a high concentration of the desired product. However, this technique is high-energy demanding, and the membrane is prone to clogging due to the presence of impurities (Dahiya *et al.*, 2023).
 - **Membrane contactor:** a microporous hydrophobic membrane allows contact between liquid and gas phases, promoting mass transfer of desired solutes (i.e. VFAs) without phase dispersion within one another. This technique can also be used with vapour, in which case, the difference in partial pressures on the sides of the membrane is the driving force for the desired solute to migrate into the gas phase. Membrane contactor is considered a promising alternative for waste valorization, because of its high

performance, wide range of applications, low energy consumption and ease to scale up.

The main drawbacks are the membrane fouling and clogging, and the lack of studies on complex waste systems (Dahiya *et al.*, 2023).

- **Forward osmosis (FO):** water is drawn from a feed stream (VFA-rich fermentation broth) by means of the osmotic pressure given by the concentration gradient with respect to a draw stream (a saturated salt solution), which are separated by a semipermeable membrane, resulting in the increase of VFAs concentration in the feed stream up to 60%. The main drawbacks are the need for pH modification of the feed solution, and the need for energy and chemical inputs for the draw solution (Aghapour Aktij *et al.*, 2020).
- **Non-porous membrane extractrion (NPM):** unlike FO, the NPM technique is an *in situ* product recovery (ISPR) that consist of a membrane made of non-porpus material (i.e. silicone) that allows VFAs to migrate from the feed stream to the draw solution (water) by a difference of concentration, separating them from the fermentative effluent (Outram & Zhang, 2018). This approach stands out because of its low cost of installation and operation and good performance with high-solids content feed streams such as fermentation broth, but its efficiency is limited by the low concentration of VFAs in the feed stream. Moreover, a pH lower than the pKa of the VFAs is required for successful mass transfer (Placido & Zhang, 2018; Yesil *et al.*, 2014).
- **Membrane distillation:** this thermally-driven membrane technique utilizes heat to concentrate or separate the desired product (i.e. VFAs) from the feed stream, so that the volatile components tend to become gaseous and pass through a hydrophobic membrane, and then are condensed in a cold permeate side. The main drawback is the high energy consumption (Aghapour Aktij *et al.*, 2020).
- **Pervaporation:** this is a separation process based on membranes that leverages variations in the solubility and diffusivity of distinct components as they pass through a dense membrane. In pervaporation (PV), the driving force arises from the chemical potential

gradient across the membrane. This gradient can be established by employing a vacuum or gas purge on the permeate side, ensuring that the permeate vapor pressure remains lower than the partial pressure of the feed liquid. Although pervaporation is a promising technique. Although pervaporation is a promising technique for the recovery of VFAs in situ, without affecting the microorganisms, its installation is complex and its technology is under development (Aghapour Aktij *et al.*, 2020).

- **Pressure-driven membrane separation techniques:**

- **Microfiltration** (MF) and **ultrafiltration** (UF) can be employed to clarify the fermented effluent, thereby reducing its fouling potential in further separation methods. **Nanofiltration** (NF) and **reverse osmosis** (RO) can be further used to effectively recover VFAs from other components in aqueous solutions. These techniques are influenced by temperature and pH of the feed solution, achieving good performance in short times. The main drawbacks are the high energy consumption to generate pressure gradient, and the risk of the membrane becoming clogged, so a sequence of filtration is recommended (Aghapour Aktij *et al.*, 2020).

4.3.5.2. Multicriteria analysis to select a method for VFAs recovery

A comparative multicriteria analysis of different recovery techniques applicable to VFAs from organic wastes is presented in Table 4.4.

Table 4.4. Multicriteria analysis of recovery techniques applicable to VFAs

Assigned Weight	0.3	0.25	0.2	0.25	1
Criterion	Recovery efficiency	Operating costs	Need for pH modification	Ease of installation and scalability	Total
Precipitation	4	2	3	3	3.05
Adsorption	4	2	3	4	3.3
Distillation	3	3	2	5	3.3
Solvent extraction	5	1	4	2	3.05
Membrane separation (includes pressure and non-pressure driven methods)	5	2	3	2	3.1

The multicriteria analysis showed distillation and adsorption as the most appropriate techniques according to the evaluated criteria. However, for the proposed recovery process it is feasible to feed a carbon source at concentrations below 3,000 mg/L, and taking into account the ease of installation, distillation was selected as a recovery technique for pig-derived VFAs.

4.3.5.3. Distillation tests at atmospheric pressure

Effluent obtained from bench-scale fermentation of pig manure (section 4.3.4), with a VFAs concentration of 6,759 mg/L was distilled at atmospheric pressure in a lab-scale glass distillator, following the sample preparation method for VFAs determination in accordance with Standard Method 5560 C (APHA, 2017).

The total VFAs final concentration after distillation was averaged at 2,745.69 mg/L, which represents an estimated recovery efficiency of 40.62%.

4.4. Chapter conclusions

The TS content was identified as the most influential factor in the fermentation of pig manure, promoting a maximum increase of 183.65% in the concentration of total VFAs by increasing the ST from 5 to 10%, under the conditions of pH and ISR evaluated.

The analysis of the interaction effect between factors revealed that at low pH, both the TS and the ISR present positive correlations with the VFAs concentration, which is considered beneficial for an eventual implementation of the process in a relevant environment.

The fermentation of manure in 10 L semi-continuous bioreactors, with the selected conditions for pH (7.56), OLR (10 g SV/L/d), HRT (10 days), and ISR (0.2), reached a maximum concentration of total VFAs of 8,783 mg/L and ranged from 6,442 to 7,286 mg/L in the last four days of the experiment for both reactors, maintaining an average pH of 6.5 ± 0.26 . This demonstrates the scalability of the evaluated methodology; however, it is necessary to advance in the optimization of all the relevant technical and economic factors of the process to ensure efficient scaling.

Multicriteria analysis allowed to select distillation as VFAs recovery method under the experimental conditions. The total VFAs final concentration after distillation was averaged at 2,745.69 mg/L, which represents an estimated recovery efficiency of 40.62%.

The establishment of operational conditions for the production of VFAs on a larger scale, and their subsequent recovery, are key aspects to evaluate the valorization potential of these VFAs in other applications, as will be demonstrated in the next chapter.

Chapter 5: VFAs valorization in a nitrogen fixation system: Recovery of pig-derived VFAs as a sustainable carbon source.

5.1. Introduction

In an era where global agricultural production plays a pivotal role in securing the world's food supply, the demand for essential nutrients, particularly nitrogen, has reached unprecedented levels. According to the FAOSTAT's latest data in 2023, the world's agricultural production soared to an astounding 18.6 billion tons in 2021, continuing a consistent growth pattern of 1.54% annually over the past decade (Figure 5.1). This expansion in agriculture, while essential for feeding the growing global population, presents a significant challenge – the ever-increasing need for land, water, and fertilizers, with a particular focus on nitrogen-based chemical fertilizers.

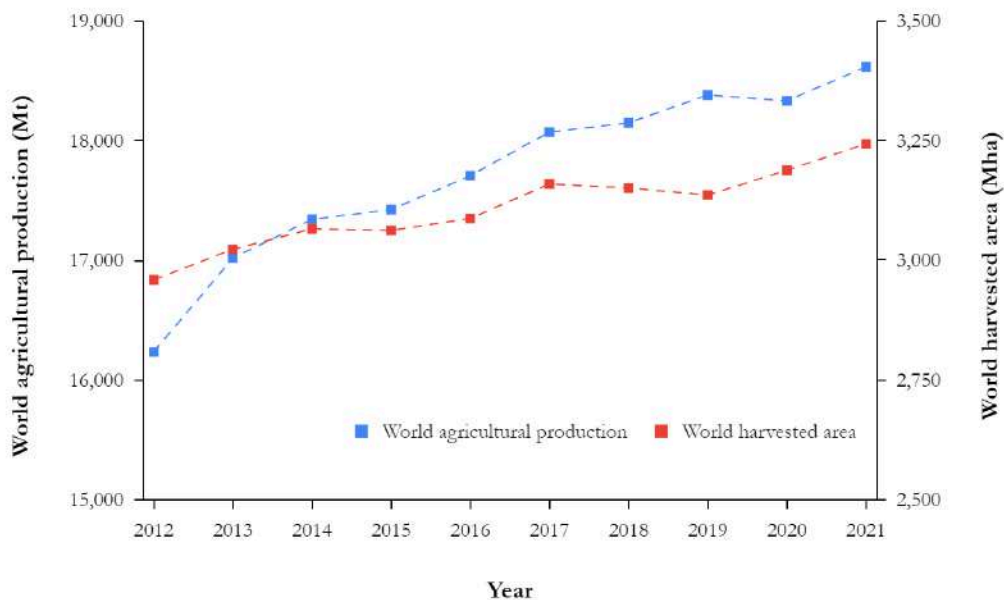


Figure 5.1. Historic world agricultural production and harvested area (FAOSTAT, 2023).

In 2021 alone, the global consumption of agricultural fertilizers, specifically those providing nitrogen, phosphorus, and potassium, reached staggering figures of 108.7 billion tons, 46.3 billion tons, and 40.1 billion tons, respectively (Figure 5.2). The production of ammonia (NH_3), a key ingredient in nitrogen fertilizers, surpasses 175 million tons annually, with approximately 85% dedicated to nitrogen fertilizer production, as revealed in recent studies (Kong *et al.*, 2023; MacFarlane *et al.*, 2020).

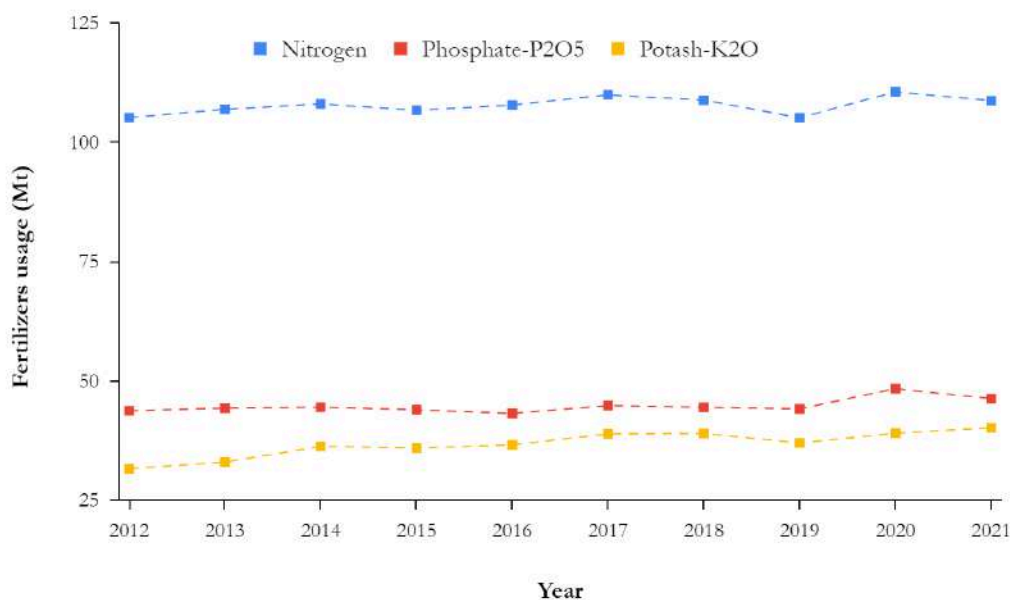


Figure 5.2. Worldwide fertilizer usage in agriculture by nutrient (FAO, 2023b)

Note: Straight nitrogen fertilizers: urea, ammonium sulfate, ammonium nitrate, calcium ammonium nitrate, sodium nitrate, ammonia, and others. Straight phosphatic fertilizers: phosphate rock, superphosphates, other. Straight potassic fertilizers: potassium chloride, potassium phosphate, other. Compound fertilizers: NPK, monoammonium and diammonium phosphate, potassium nitrate, other NK and PK compounds.

The cornerstone of modern nitrogen fertilizer production, the Haber-Bosch (HB) process, has revolutionized agriculture, but it comes with a significant environmental cost. This process requires massive amounts of energy to break the formidable triple bond of N_2 molecules, demanding 941 kJ/mol. Consequently, the HB process is responsible for consuming roughly 2% of global energy and contributing

1.6% of global CO₂ emissions (Chowdhury *et al.*, 2023). The energy-intensive HB process consumes 8–10 MWh per ton of NH₃ and emits 2 tons of CO₂ per ton of NH₃ (MacFarlane *et al.*, 2020).

The indiscriminate application of nitrogen fertilizers poses severe environmental risks, ranging from pollution and greenhouse gas emissions to threats to food security, water contamination, and even human health issues such as respiratory and immunogenic diseases (Penuelas *et al.*, 2023). To address these challenges and promote agricultural sustainability, innovative practices such as the utilization of microorganisms to enhance nutrient availability, phytohormone production, and nutrient cycling in the soil have gained prominence (M. Tahat *et al.*, 2020).

In this chapter, the critical issue of worldwide nitrogen demand for agriculture and the associated problems with chemical fertilizers is deepened. Moreover, alternative nitrogen sources, particularly nitrogen-fixing bacteria, and the importance of carbon sources, including the potential of utilizing waste-derived volatile fatty acids (VFAs), is explored. Additionally, we investigate ongoing studies involving N-fixing bacteria and the use of carbon sources from waste materials, shedding light on their limitations and the promising prospect of coupling pig manure fermentation with biological nitrogen fixation.

5.2. Materials and methods

5.2.1. Bioreactors system

The bioreactors used in the experiments consisted of 500 mL glass bottles, with a working volume of 250 mL, operated at room temperature, and covered with aluminum foil to prevent light from entering. The nitrogen source was atmospheric air, filtered (0.22 µm) and permanently driven into the bioreactors with an air pump at a flow rate of approximately 1 L/min (Figure 5.3). The continuous supply of air also provided full mixing of the reactor contents.

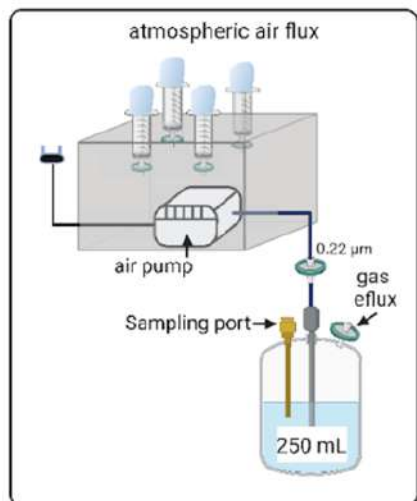


Figure 5.3. Scheme of air filtration and pumping

The composition of the culture medium used for the inoculum adaptation and the nitrogen fixation experiment with different carbon sources is: (g/L) 0.5 K_2HPO_4 , 0.1 NaCl , 0.2 MgSO_4 , 0.2 KOH , 0.02 CaCl_2 , 2 mL/L micronutrients solution, and 4 mL/L Fe-EDTA solution.

All details about isolation and adaptation of the inoculum used in nitrogen fixation experiments is reported in the working paper by Nicolas Rodriguez and collaborators (Appendix 5.2, section 2.2).

5.2.2. Evaluation of nitrogen fixation of the adapted inoculum with different carbon sources

The inoculum that showed the best performance in the adaptation phase was selected to evaluate the nitrogen fixation process in a completely randomized experimental design depending on the carbon source. 7 treatments were established, each corresponding to a different carbon source, as shown in Table 5.1. In this section, effluent from the acidogenic fermentation of pig manure, centrifuged and distilled (PMIX) was included as a carbon source, in order to demonstrate its potential for use in the biological nitrogen fixation process.

Table 5.1. Experimental design to evaluate different carbon sources on nitrogen fixation

Factor	Level	Description
Carbon source	GLU	Aqueous solution of glucose and mannitol, 8 g/L each
	AC	Aqueous solution of acetic acid, 830 mg/L
	PRO	Aqueous solution of propionic acid, 830 mg/L
	BUT	Aqueous solution of butyric acid, 830 mg/L
	SMIX	Synthetic VFA mix solution prepared with a concentration of 600, 100, and 130 mg/L for acetic, propionic and butyric acid, respectively; simulating VFA mix obtained from fermented pig manure
	PMIX	Distilled effluent from pig manure fermentation. Characterization showed an average concentration of 463.1 ± 8.65 , 75.66 ± 4.03 , 40.63 ± 1.59 , 123.4 ± 4.29 , 64.16 ± 2.63 , and 29.31 ± 5.03 mg/L for acetic, propionic, isobutyric, butyric, isovaleric and valeric acid, respectively.
	WC	Without adding any carbon source

n = 3

The experimental setup was the same as described in section 5.2.1, adding sterile distilled water as needed, to maintain the working volume at 250 mL. The reactors were operated in semibatch mode, with a hydraulic retention time of 7 days, replacing 99% of the reactor content with fresh culture medium, once every week. Before each feeding, aliquots were taken to analyze pH, optical density, ammonia, nitrate, total nitrogen and VFAs.

5.2.3. *Methods for physicochemical analysis*

The optical density was read at 600 nm in by spectrophotometry (Hoefer SP-2001), and pH was determined by potentiometry (Hanna Instruments HI-98160). Total nitrogen was measured by spectrophotometry (Hanna Instruments), with reagent kits HI93767A-50, HI93764A-25, and HI93766-50, respectively. In this experiment, nitrogen fixation was measured as the concentration of total nitrogen in both the bacterial biomass and the supernatant of the reactor contents.

The concentration of VFAs was determined in a gas chromatograph (Perkin Elmer Clarus 590) equipped with a column elite FFAP 30m x 0.25 mm ID x 0.25 μ m DF, and a flame ionization detector (FID). Nitrogen was used as carrier gas, with a pressure of 15 psi and a split ratio of 120 mL/min. The temperatures of the oven, injector, and detector were maintained at 140, 250, and 250 °C, respectively. The samples for VFA analysis were acidified with 37% HCl (Honeywell Fluka, Austria), filtered with 0.45 μ m PTFE, and stored at -20 °C until analysis. A standard mixture of 10 mM VFAs (Sigma-Aldrich, USA) was used for the calibration curve, which includes acetic, propionic, isobutyric, butyric, isovaleric, valeric, 4-methyl valeric, hexanoic, and heptanoic acids.

5.2.4. Statistical analysis

Statistical analysis and visualizations were performed in R statistical software (v 4.3.1). The assumptions of normality were tested using the Shapiro Wilks test ($p < 0.05$) and Homogeneity of variances using the Levene test ($p < 0.05$) for each of the variable response measures. Subsequently, analysis of variance and comparisons of means were performed using Tukey's test ($p < 0.05$).

5.3. Results and discussion

5.3.1. Evaluation of nitrogen fixation of the adapted inoculum with different carbon sources

The use of pig-derived VFAs (PMIX) as a carbon source resulted in the highest concentration of total nitrogen in bacterial biomass after 28 days of operation, among all carbon sources evaluated (Figure 5.4 A). Synthetic VFA mixture (SMIX) yielded the second-highest nitrogen concentration in biomass at the same time. It is also observed that, in the case of individual VFAs, nitrogen fixation is higher with butyric acid, followed by propionic acid, and finally acetic acid at 7, 14, and 21 days.

Interestingly, the positive control with glucose-mannitol (GLU) as a carbon source produced the lowest concentration of total nitrogen species in biomass among all the evaluated conditions; and the negative control (without carbon source - WC) yielded more nitrogen in biomass than the control with GLU. This may indicate that, besides organic carbon sources added to the culture media, this microbial community may take carbon from inorganic sources. For instance, members of the genus *Azospirillum* are recognized nitrogen fixers with broad metabolic diversity in terms of nitrogen and carbon sources, and some species of this genus such as *A. lipoferum* and *A. brasilense* can grow autotrophically in the presence of H₂ and CO₂ (Tilak *et al.*, 1986).

Regarding the supernatant of the bioreactor content (Figure 5.4 B), total nitrogen concentrations were observed between two and three times lower than those found in the bacterial biomass after 28 days, in the case of the mixtures of pig and synthetic VFAs, and the individual VFAs. This suggests that fixed nitrogen has been used primarily for microbial growth.

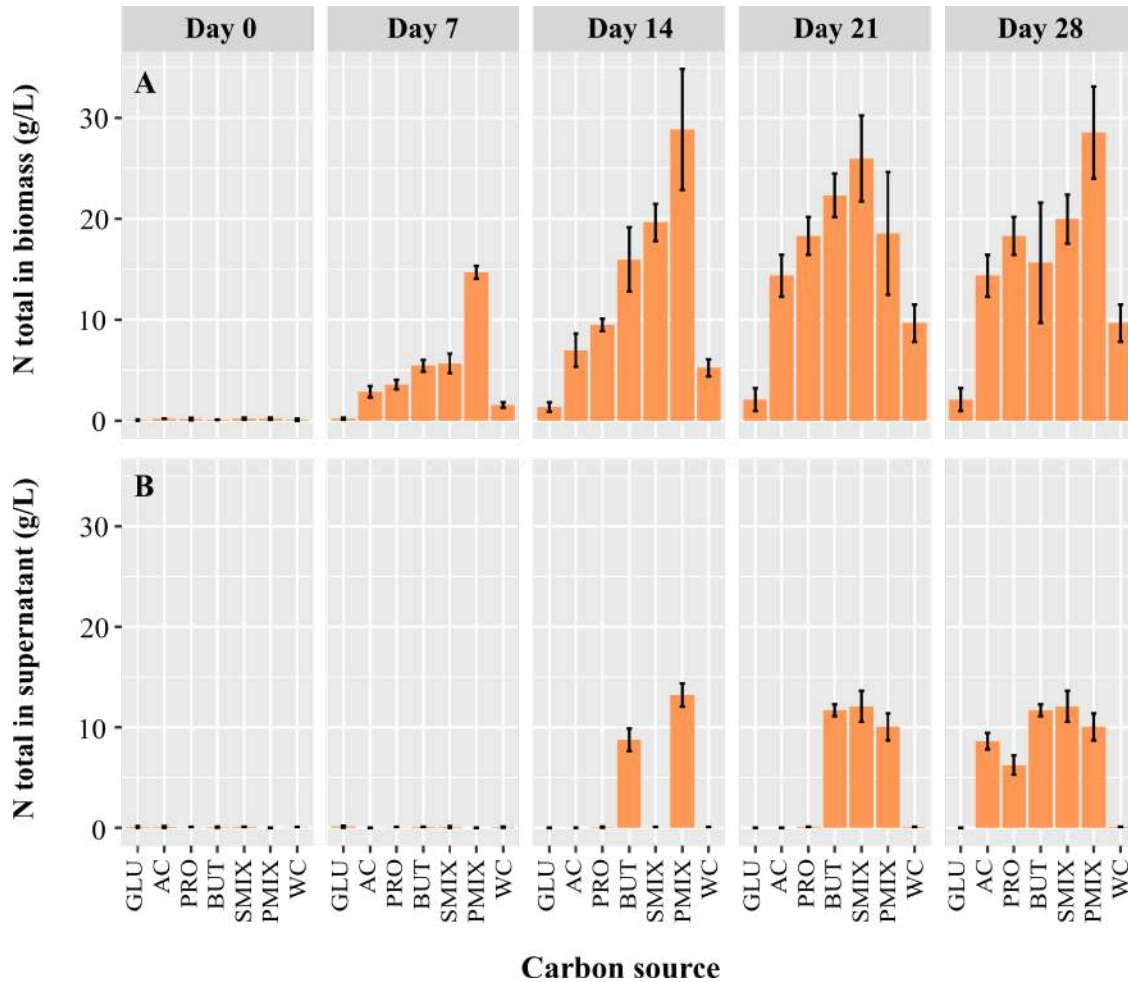


Figure 5.4. N-total concentration in bacterial biomass (A) and supernatant (B), from a nitrogen-fixing adapted consortium with different carbon sources: glucose (GLU), acetic acid (AC), propionic acid (PRO), butyric acid (BUT), synthetic VFA mix (SMIX), VFA mix from fermented pig manure (PMIX), and without carbon source added (WC).

The evaluation of alternative carbon sources for nitrogen fixation has been the subject of study by other researchers; for instance, the sugars obtained from the thermal hydrolysis of soybean hulls (Roesler *et al.*, 2024), or lignocellulosic waste directly as a substrate for cellulolytic nitrogen-fixing microorganisms (Harindintwali *et al.*, 2022). However, the molecule that enters the cellular energy metabolism in these examples is still a sugar. Therefore, the first reason that could explain the differences obtained in nitrogen

fixation within this experiment would be the structural difference between sugars (glucose and mannitol) and carboxylic acids (acetic, propionic, and butyric acids).

An aspect that draws attention to VFAs is that their observable assimilation rate by nitrogen-fixing microorganisms seems to be directly proportional to the size of each VFA, with the highest for butyric acid, followed by propionic acid, and finally acetic acid (Figure 5.5. A). When using pig-derived VFAs as a carbon source, rapid consumption of these compounds is observed during the first 15 days of operation (Figure 5.5. C); a similar behavior is observed for the synthetic VFA mixture (Figure 5.5. B), following the same order of assimilation for individuals VFAs in both cases.

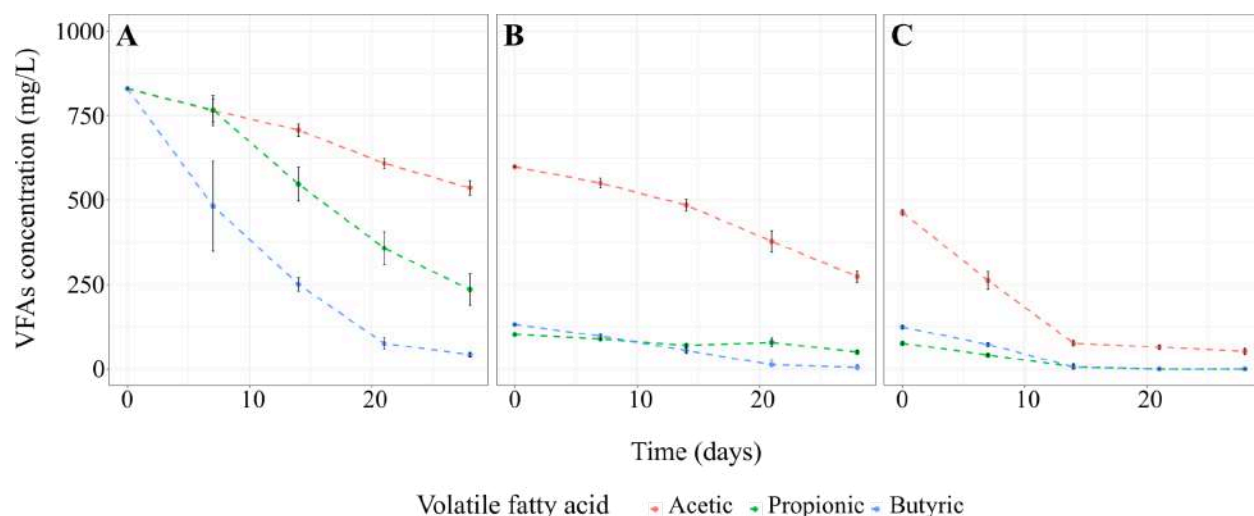


Figure 5.5. Assimilation of VFA during nitrogen-fixing adapted consortium with different carbon sources: individual VFAs (A), synthetic VFA mix (B), VFA mix from fermented pig manure (C)

This assimilation order has also been observed in other complex microbial ecosystems, such as the microbiota present in the digestive tract of ruminants, where higher assimilation rates have been reported for butyrate, followed by propionate, and finally acetate (Fernández & Bacha, 2022). However, in a study carried out with mixotrophic algae, the opposite behavior was observed in the individual VFAs assimilation rate (acetate > propionate > butyrate) (Su *et al.*, 2021). This may be due to that acetyl-CoA

synthesis from acetic acid is less energy-demanding, and requires a shorter metabolic pathway compared to propionic and butyric acids, resulting in a higher acetic acid assimilation efficiency under the heterotrophic algae cultivation (Zheng *et al.*, 2012).

The above suggests that individual VFAs assimilation rate may vary as a function of the cultivation system. And since there is no precedent in the use of VFAs as a carbon source for nitrogen-fixing bacteria cultivation, more studies are needed to clear the metabolic pathways under this condition.

Another aspect related to the use of VFAs as a carbon source is the pH response. When glucose was used as a carbon source, the pH decreased significantly to values below 3, after 21 days of operation. While, in the case of VFAs, the pH remained around neutral throughout the experiment, whether individually or in a mixture (Figure 5.6).

By using glucose-mannitol as a carbon source, the metabolic pathway of glycolysis is privileged (Madigan *et al.*, 2014). It has been reported that under aerobic conditions lactate is also produced in this route (Matus Ortega *et al.*, 2020), whose pKa (3.86) is lower than that of the VFAs evaluated, which ranges between 4.74 and 4.82 (Wade, 2012). However, since there were no bicarbonate precursors in the culture medium, a buffering effect would not be achieved in the system, which could have caused the drop in pH observed in the GLU treatment.

On the other hand, VFAs used as a carbon source have a weaker dissociation constant compared to that of lactic acid. These acids could have reacted with the KOH present in the medium forming water and acid salts. This could explain the little variation in pH observed in the treatments that involved VFAs.

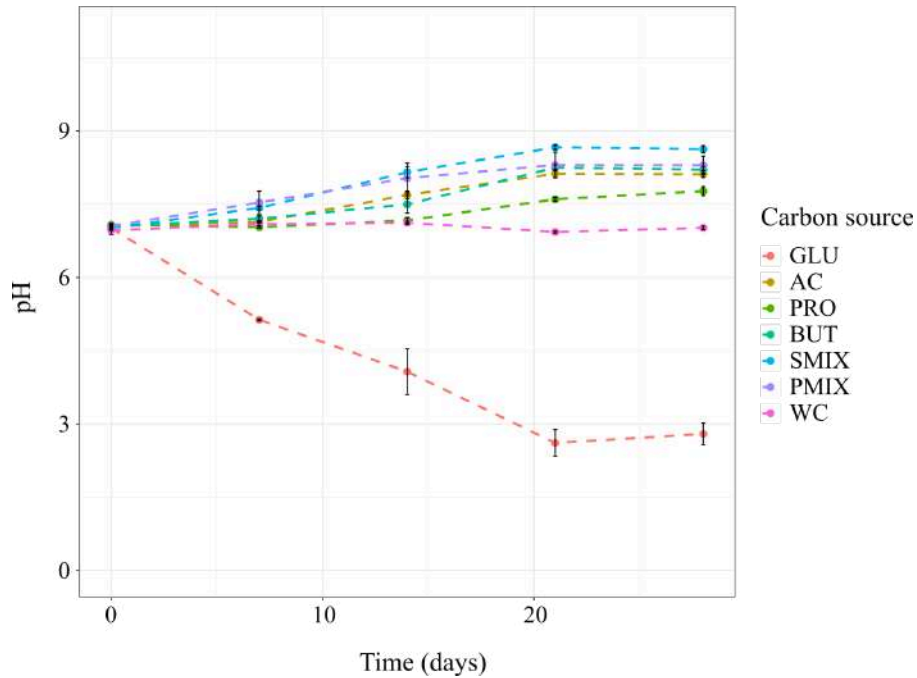


Figure 5.6. pH of effluent from a nitrogen-fixing bacteria consortium with different carbon sources: glucose (GLU), acetic acid (AC), propionic acid (PRO), butyric acid (BUT), synthetic VFA mix (SMIX), VFA mix from fermented pig manure (PMIX), and without carbon source added (WC).

The pH changes described above could also have an impact on nitrogen fixation. Representative genera of nitrogen fixers, such as *Azotobacter*, grow by fixing molecular nitrogen at pH between 7.0 and 7.5 (Espín, 2002). Given that the GLU treatment presented a significantly low pH throughout the experiment, this fact may explain the low biomass nitrogen concentration observed in this treatment.

5.3.2. Microbial growth in the nitrogen fixation process with different carbon sources

The optical density was selected as an indirect measure of microbial growth in the nitrogen fixation process. The use of a synthetic mixture of VFAs promoted the highest optical density, observed at 28 days of operation. A similar result was obtained with the VFA mixture derived from the fermentation of pig

manure. Close behind were the individual VFAs, in the order of butyric, propionic, and acetic acids, and finally, the lowest growth was obtained with glucose as a carbon source (Figure 5.7).

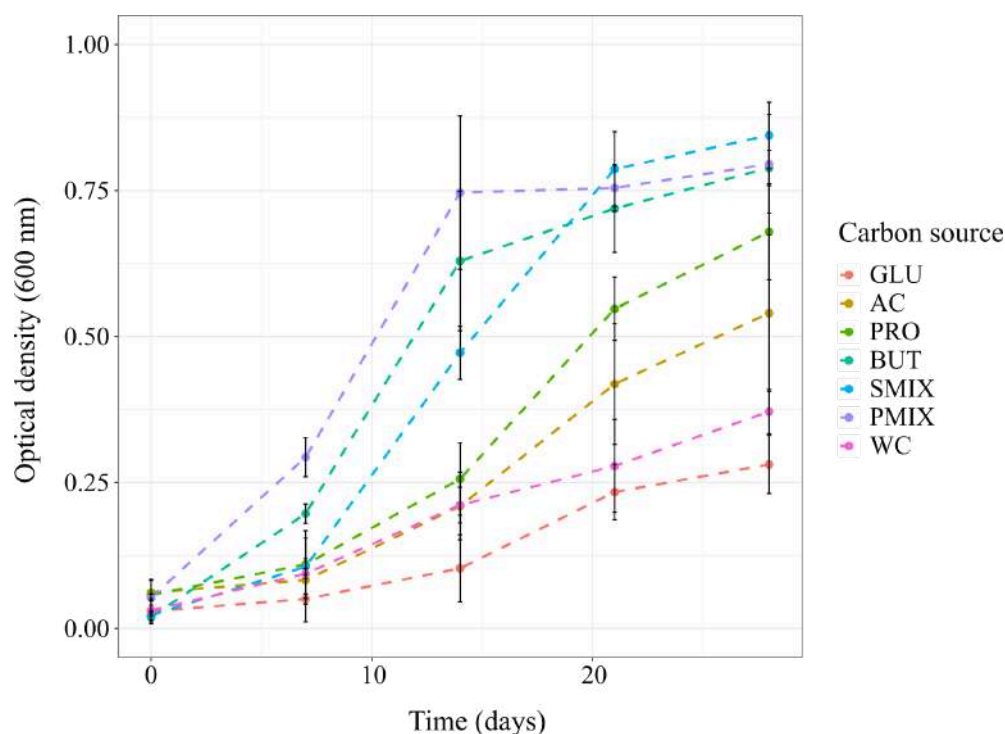


Figure 5.7. Optical density (OD) of effluent from a nitrogen-fixing adapted consortium with different carbon sources: glucose (GLU), acetic acid (AC), propionic acid (PRO), butyric acid (BUT), synthetic VFA mixture (SMIX), VFA mixture from fermented pig manure (PMIX), and without carbon source added (WC).

Observing the behavior of microbial growth with individual VFAs as carbon sources, as well as their consumption, it is noteworthy that the higher the molecular weight of the volatile fatty acid, the greater the microbial growth and the greater the acid consumption.

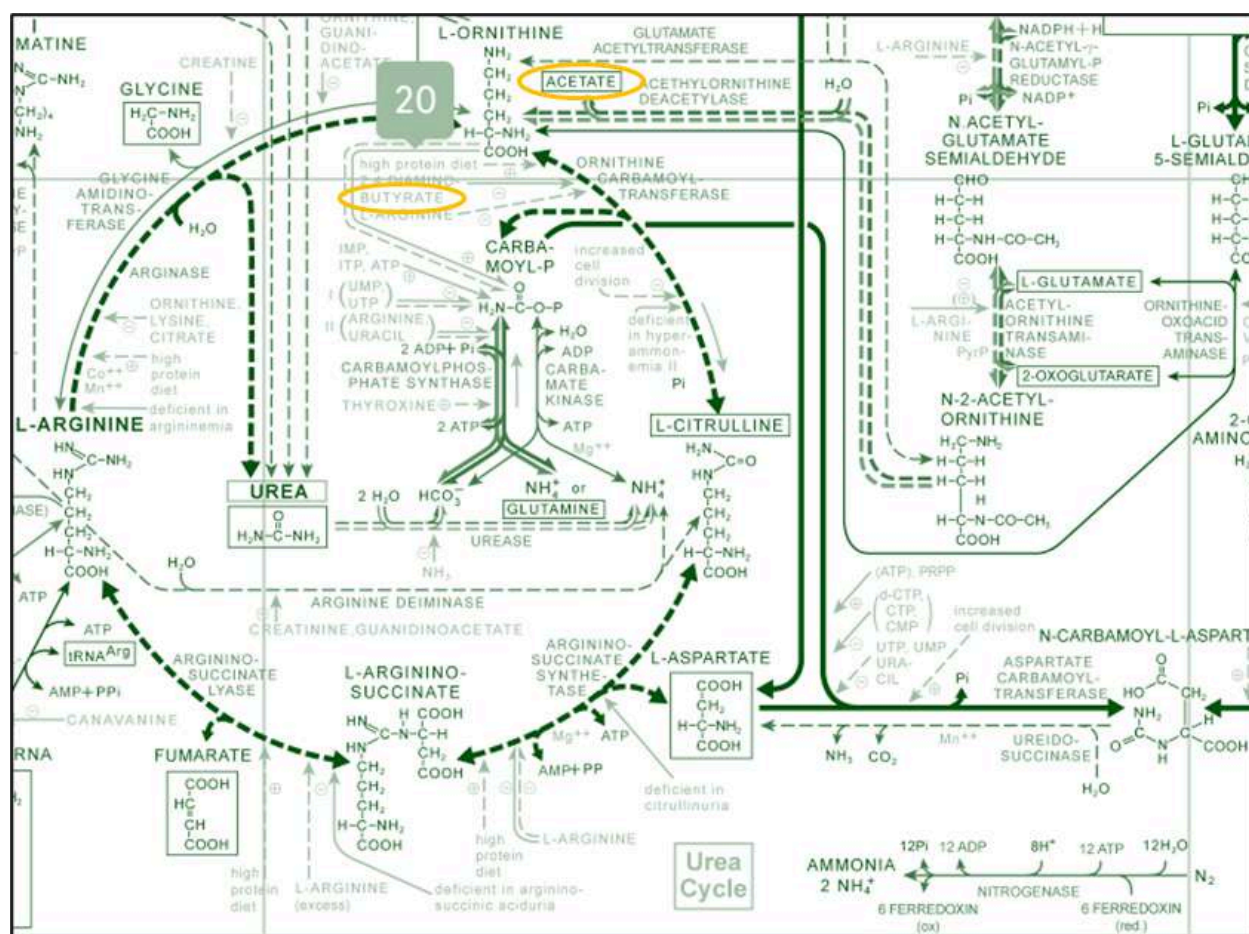


Figure 5.8. Biochemical pathway of butyrate in nitrogen fixation.

Source: <https://biochemical-pathways.com/>

In the proposed metabolic map by Roche (Figure 5.8), in the section concerning the urea cycle, it is observed that butyrate is intricately linked to nitrogen transformations. Additionally, the incorporation of acetate into this same cycle is evident. This observation may explain the higher affinity of butyrate and, consequently, a greater assimilation of butyrate followed by acetate.

It is also important to keep in mind that the carbon source affects the type of metabolism that the microorganisms will use to obtain energy. While glucose takes the catabolic pathway of glycolysis to obtain acetyl Co-A and then enters the Krebs cycle, VFAs can enter this cycle directly, which can be more

energetically efficient. This is an important aspect of nitrogen fixation since this process requires 16 ATP molecules to fix one N_2 molecule (Madigan *et al.*, 2014).

The literature highlights the critical role of optimizing carbon sources to enhance the sustainability and efficiency of nitrogen-fixing biotechnologies. For instance, as mentioned in Bashan & de-Bashan (2015), the successful application of microbial technologies in agriculture depends significantly on the choice of carbon source, which influences microbial growth and activity levels.

Moreover, the environmental benefits of using VFAs from pig manure, as discussed by Tilman *et al.*, (2002), include not only waste valorization but also the reduction of reliance on synthetic fertilizers, which are associated with high energy costs and environmental pollution. These benefits, added to the increasing availability of strains capable of improving crop yields at a lower cost, have boosted their positioning in the market in recent years (Sharma *et al.*, 2023). This also aligns with global sustainability goals aimed at reducing greenhouse gas emissions and enhancing the nutrient cycling in agricultural systems.

A summary of all the physicochemical variables from this experiment is provided in Appendix 5.1. This chapter is part of a larger ongoing research project related to the production of nitrogen-fixing bioinoculants from pig-derived VFAs, and their further use in a tomato crop (see Appendix 5.2).

5.4. Chapter conclusions

This study revealed that utilizing pig-derived VFAs as a carbon source for nitrogen fixation led to the highest concentration of nitrogen species in bacterial biomass after 28 days, comparable to the synthetic VFA mixture. Notably, individual VFAs showed varying nitrogen fixation rates, with butyric acid demonstrating the highest, followed by propionic and acetic acids. Glucose as a carbon source produced the lowest nitrogen concentration in biomass. The rapid consumption of VFAs during the initial 15 days,

observed with both porcine-derived VFAs and the synthetic VFA mixture, underscores their effective utilization. Additionally, the assimilation rate of individual VFAs correlated with their size, with butyric acid exhibiting the highest rate. Furthermore, the pH decrease when using glucose as a carbon source highlights its distinct impact compared to VFAs. The bacterial growth pattern, measured indirectly through optical density, indicated that a mixture of VFAs from pig manure fermentation and the synthetic VFA mixture supported the highest growth, followed closely by individual VFAs, while glucose resulted in the least bacterial growth. These findings underscore the complex interplay of carbon sources on nitrogen fixation and microbial growth in the studied system.

Chapter 6: General conclusions

The short thermal pretreatment (TP), at 90°C for 10 minutes, has proven to be an efficient method for conditioning the microbiome present in anaerobic sludge, increasing the production of VFAs by up to 217% in the fermentation of pig manure. With a maximum concentration of VFAs of 13,030.07 mgCOD/L over a period of 15 days, these results are comparable to those achieved with more energy-intensive or chemical-demanding pretreatments. This boost on VFAs production is correlated with a decrease in process stability, evidenced by pH reduction and an elevated VFA/alkalinity ratio, moving away from the recommended conditions for methanogenesis, and promoting acidogenesis.

Regarding microbiome conditioning, 16s sequencing allowed to verify microbial composition changes induced by short TP. Contrary to what was expected, some species of archaea were detected after the application of the TP, suggesting that there may be other mechanisms of microbiome alteration beyond direct heat damage, under the evaluated conditions. Key genera like *Methanobrevibacter*, *Caproiciproducens*, and *Terrisporobacter* were found to positively correlate with VFAs production.

The evaluation of operational conditions showed that the Total Solids (TS) content emerged as a crucial operational factor influencing pig manure fermentation, yielding a remarkable 183.65% increase in total VFAs concentration when increasing the TS from 5% to 10%, leaving the natural initial pH of the substrate, and setting the inoculum-to-substrate ratio (ISR) at 1. The interaction effect analysis indicated positive correlations between TS, ISR, and VFAs concentration at low pH, suggesting potential benefits for applications in relevant environments.

The volume increase of bioreactor from 120 mL to 10 L, and the change in operation mode from batch to semi-continuous, demonstrated the scalability of this methodology, achieving a maximum total VFAs concentration of 8,783 mg/L. Distillation tests allowed a recovery rate of 40.62% of VFAs from

fermentation effluent. However, further optimization of technical and economic factors is essential for efficient scaling and recovery of VFAs and further valorization.

Finally, the pig-derived VFAs emerges as a promising carbon source for cultivation of nitrogen-fixing microorganisms, unveiling significant impacts on microbial growth, nitrogen concentration, and assimilation rates, showcasing the intricate influence of carbon sources on the studied system's dynamics.

This work demonstrates that it is possible to improve the acidogenic fermentation of pig manure and subsequently recover the VFAs produced to use them as a carbon source in biological nitrogen fixation, within a biorefinery model. The results obtained represent an advancement in the understanding of biological processes to fully valorize livestock waste, generating environmental and economic benefits that contribute to the sustainability of the food industry.

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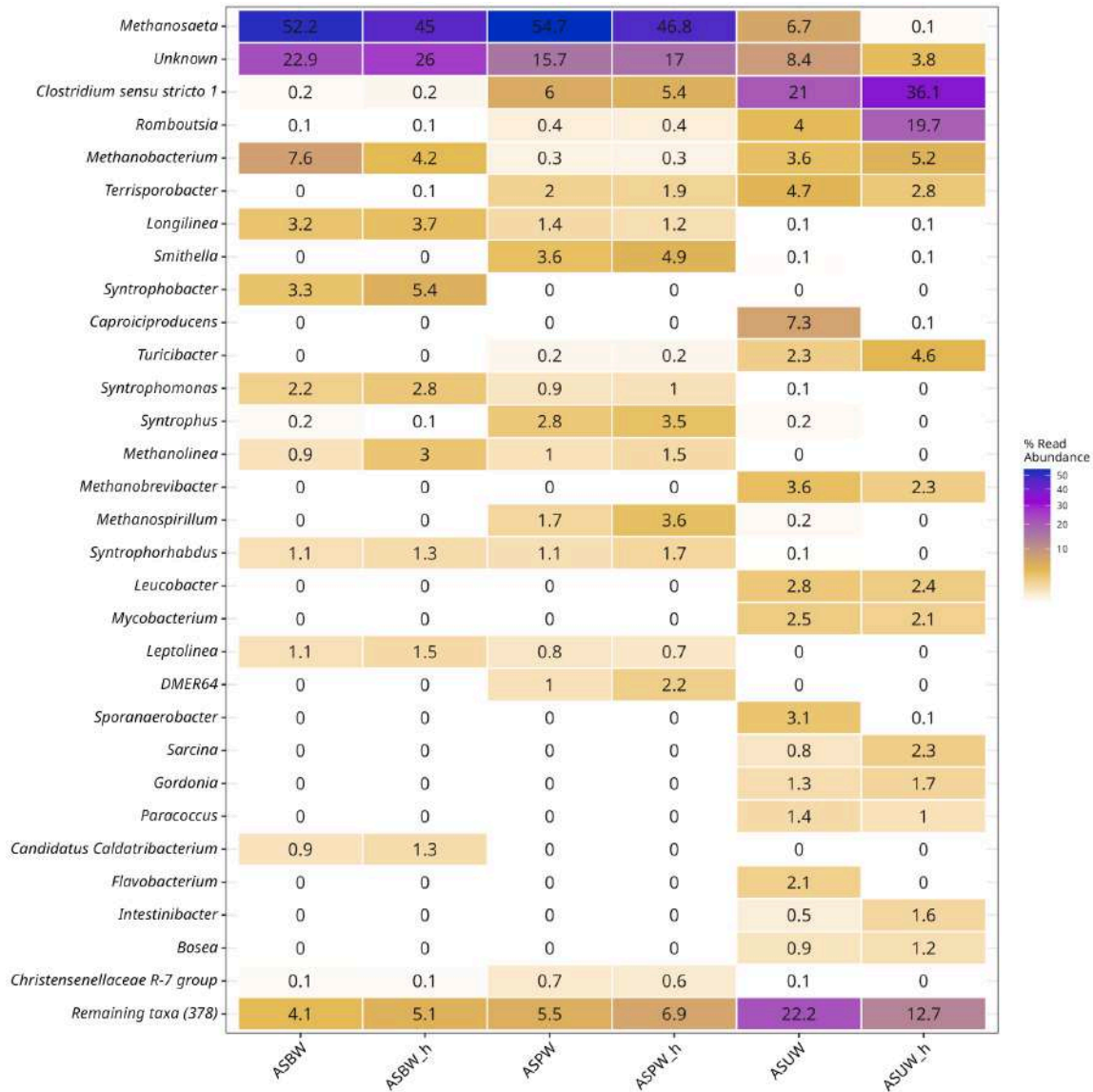
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Appendix

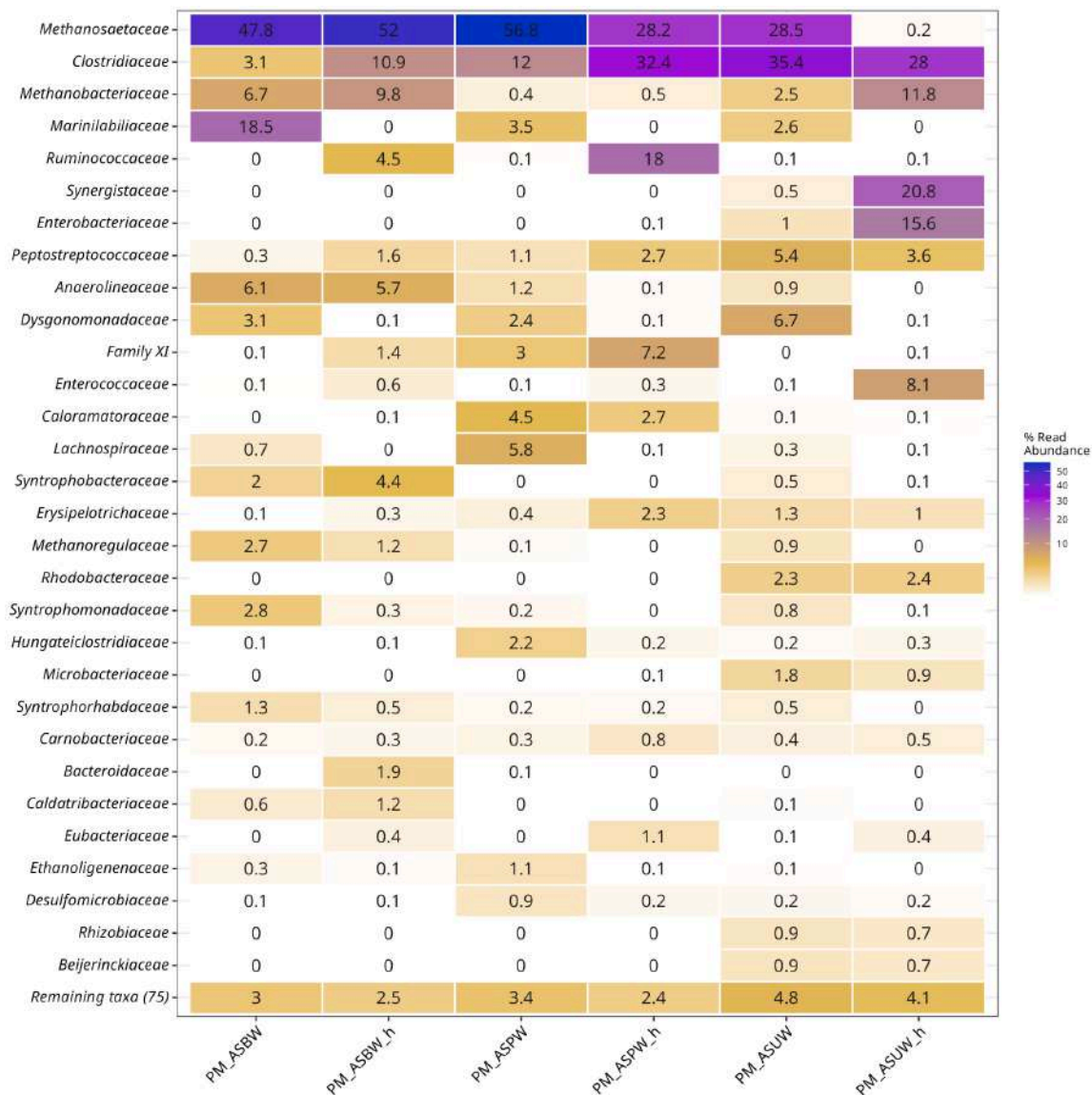
Appendix 3.1. Means, standard deviation, and statistical tests for physicochemical variables after 15 days of fermentation of pig manure, as a function of inoculum and thermal pretreatment

Variable	PM_ASPW		PM_ASBW		PM_ASUW	
	Control	TP	Control	TP	Control	TP
pH	6.96 ± 0.16 ^b	5.92 ± 0.04 ^d	7.48 ± 0.05 ^a	6.3 ± 0.26 ^c	6.7 ± 0.03 ^b	6.65 ± 0.04 ^{bc}
Alkalinity (mg*L ⁻¹)	2634.61 ± 389.96 ^b	929.63 ± 61.2 ^d	5395.72 ± 74.51 ^a	1783.84 ± 434.35 ^c	1457.56 ± 305.37 ^{cd}	1255.29 ± 20.36 ^{cd}
Chemical Oxygen Demand (mg*L ⁻¹)	10649.61 ± 432.81 ^c	22202.53 ± 2448.93 ^b	10555.98 ± 1027.14 ^c	25688.17 ± 928.42 ^a	12038.05 ± 970.31 ^c	13314.37 ± 105.96 ^c
Acidification degree (COD _{VFA} /COD _{soluble})	0.39 ± 0.15 ^{ab}	0.59 ± 0.03 ^a	0.01 ± 0.01 ^b	0.46 ± 0.05 ^{ab}	0.31 ± 0.28 ^{ab}	0.45 ± 0.04 ^{ab}
VFAs/alkalinity	1.06 ± 0.64 ^{cd}	10.43 ± 0.18 ^a	0.01 ± 0.02 ^d	5.24 ± 2.15 ^b	1.8 ± 1.56 ^{cd}	3.57 ± 0.36 ^{bc}
Acetic acid (mgCOD*L ⁻¹)	497.07 ± 764.77 ^c	6660.49 ± 348.85 ^a	79.76 ± 138.15 ^c	5602.71 ± 1059.88 ^a	1635.47 ± 1454.18 ^{bc}	3028.85 ± 211.73 ^b
Propionic acid (mgCOD*L ⁻¹)	2102.86 ± 383.74 ^a	989.03 ± 72.94 ^{abc}	0 ± 0 ^c	1326.61 ± 166.7 ^{ab}	1170.63 ± 1031.79 ^{abc}	869.87 ± 119.23 ^{bc}
Isobutyric acid (mgCOD*L ⁻¹)	387.93 ± 61.4 ^{ab}	390.24 ± 55.58 ^{ab}	0 ± 0 ^d	483.55 ± 15.39 ^a	149.47 ± 130.87 ^{cd}	302.12 ± 42.99 ^{bc}
Butyric acid (mgCOD*L ⁻¹)	82.34 ± 142.62 ^{bc}	2810.9 ± 363.1 ^a	0 ± 0 ^c	2476.56 ± 420.32 ^a	295.85 ± 260.52 ^{bc}	775.24 ± 118.77 ^b
Isovaleric acid (mgCOD*L ⁻¹)	736.44 ± 159.91 ^{ab}	716.14 ± 108.72 ^{ab}	0 ± 0 ^c	926.32 ± 30.73 ^a	362.76 ± 315.15 ^{bc}	699.59 ± 105.88 ^{ab}
Valeric acid (mgCOD*L ⁻¹)	298.9 ± 262.66 ^{ab}	647.57 ± 71.49 ^a	0 ± 0 ^b	914.13 ± 451.77 ^a	90.7 ± 79.18 ^b	258.51 ± 43.41 ^{ab}
4-methylvaleric acid (mgCOD*L ⁻¹)	0 ± 0	66.92 ± 59.12	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Caproic acid (mgCOD*L ⁻¹)	0 ± 0	303.22 ± 80.3	0 ± 0	86.21 ± 149.31	0 ± 0	0 ± 0

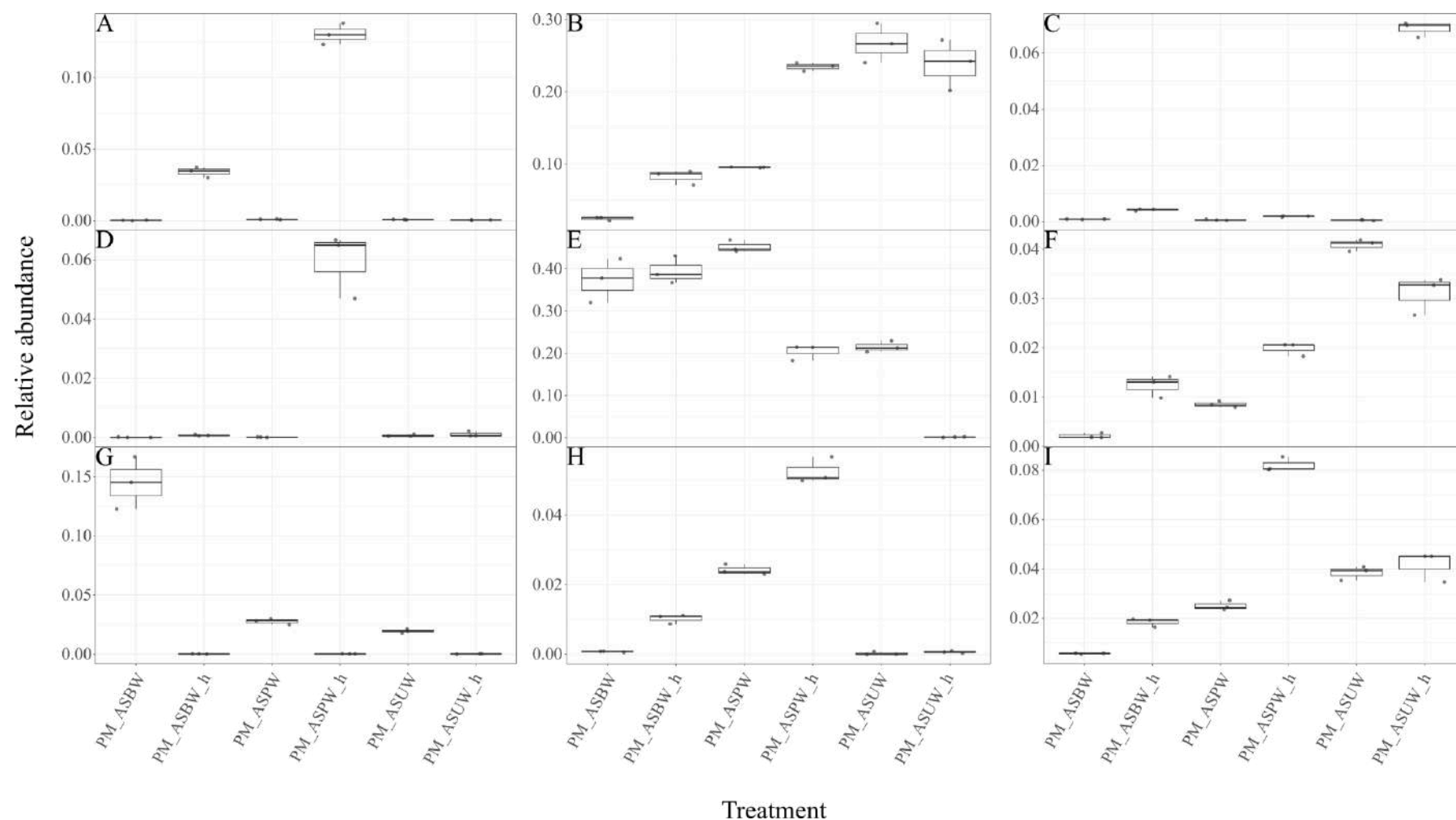
Note: Treatments: pig manure with anaerobic sludge from pig wastewater treatment (PM_ASPW); pig manure with anaerobic sludge from brewery wastewater treatment (PM_ASBW); pig manure with anaerobic sludge from urban wastewater treatment (PM_ASUW). Different letters indicate significant differences between treatments.



Appendix 3.2. Initial microbiological composition, at the genus level, of the different inocula used in the experiment before and after the thermal pretreatment. Treatments: pig manure with anaerobic sludge from pig wastewater treatment (PM_ASPW); pig manure with anaerobic sludge from brewery wastewater treatment (PM_ASBW); pig manure with anaerobic sludge from urban wastewater treatment (PM_ASUW); thermal pretreatment applied to inoculum (_h). The data shown is the average of three replicates



Appendix 3.3. Microbiological composition, at the family level, after 15 days of fermentation of pig manure as a function of the thermal pretreatment of the inoculum. Treatments: pig manure with anaerobic sludge from pig wastewater treatment (PM_ASPW); pig manure with anaerobic sludge from brewery wastewater treatment (PM_ASBW); pig manure with anaerobic sludge from urban wastewater treatment (PM_ASUW); thermal pretreatment applied to inoculum (_h). Data shown is the average of three replicates.



Appendix 3.4. Boxplots of relative abundance of relevant genera, as a function of inoculum and thermal pretreatment, after 15 days of fermentation. Genera: *Caproiciproducens* (A), *Clostridium sensu stricto 1* (B), *Enterococcus* (C), *Methanobrevibacter* (D), *Methanosaeta* (E), *Romboutsia* (F), *Ruminofilibacter* (G), *Sporanaerobacter* (H), *Terrisporobacter* (I). Treatments: pig manure with anaerobic sludge from pig wastewater treatment (PM_ASPW); pig manure with anaerobic sludge from brewery wastewater treatment (PM_ASBW); pig manure with anaerobic sludge from urban wastewater treatment (PM_ASUW); thermal pretreatment applied to inoculum (_h)

Appendix 5.1. Summary of physicochemical variables at 28 days of cultivation of nitrogen-fixing adapted consortium with different carbon sources

Variable	GLU	AC	PRO	BUT	SMIX	PMIX	WC
pH	2.8 ± 0.22 ^e	8.11 ± 0.07 ^{bc}	7.77 ± 0.09 ^c	8.2 ± 0.07 ^b	8.62 ± 0.07 ^a	8.3 ± 0.19 ^{ab}	7.01 ± 0.04 ^d
DO	0.28 ± 0.05 ^d	0.54 ± 0.13 ^{bc}	0.68 ± 0.08 ^{ab}	0.79 ± 0.03 ^a	0.84 ± 0.06 ^a	0.8 ± 0.08 ^a	0.37 ± 0.04 ^{cd}
Biomass:							
Total nitrogen (g/L)	2.13 ± 1.12 ^d	14.4 ± 2.08 ^{bc}	18.33 ± 1.85 ^{bc}	15.67 ± 5.97 ^{bc}	20 ± 2.43 ^{ab}	28.57 ± 4.57 ^a	9.7 ± 1.85 ^{cd}
Supernatant:							
Total nitrogen (g/L)	0 ± 0 ^d	8.63 ± 0.81 ^{bc}	6.27 ± 0.97 ^c	11.73 ± 0.59 ^a	12.1 ± 1.54 ^a	10.07 ± 1.36 ^{ab}	0.1 ± 0 ^d

Note: Carbon sources: glucose (GLU), acetic acid (AC), propionic acid (PRO), butyric acid (BUT), synthetic VFA mix (SMIX), VFA mix from fermented pig manure (PMIX), and without carbon source added (WC). Treatments with the same letter are not significantly different.

Appendix 5.2. Working paper

Estrategia innovadora para la producción de bioinoculante fijador de nitrógeno utilizando fuentes de carbono y nitrógeno de bajo costo.

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Resumen

La agricultura actual emplea aproximadamente 100 millones de toneladas de fertilizantes nitrogenados anualmente para satisfacer la demanda alimentaria mundial, causando impactos ambientales significativos. Además, la producción de alimentos genera millones de toneladas de residuos agroindustriales cada año. Se han propuesto soluciones a estas problemáticas que se basan en la teoría ecológica de comunidades microbianas, la ingeniería de bioprocesos y la valorización de residuos agroindustriales para la producción de biofertilizantes. En este estudio, nuestro objetivo fue utilizar la bioprospección dirigida centrada en microbiomas, para cultivar comunidades microbianas diazótroficas. Esto se logró mediante el enriquecimiento de muestras de suelo en biorreactores que suministran nitrógeno mediante bombeo de aire y son alimentados con ácidos grasos volátiles como fuente de carbono. Los resultados muestran que a través de un proceso de selección microbiana se puede obtener una comunidad microbiana diazótropa capaz de fijar 2,7 veces más nitrógeno que el inóculo de partida. Dicha comunidad es capaz de crecer usando ácidos grasos volátiles provenientes de residuos como fuente de carbono. Durante el proceso de cultivo se pueden obtener formas inorgánicas de nitrógeno en el sobrenadante en concentraciones que alcanzan 12,7 mg*ml⁻¹. La comunidad microbiana diazótropa crecida en biorreactores, cuyos tres géneros más abundantes son *Sinirhodobacter* (44,4%), *Aureimonas* (17,7%) y *Taibaiella* (12,4%); es capaz de promover el crecimiento de plantas de tomate tanto en hidroponía como en suelo. Alcanzando una altura y producción igual a la obtenida usando fertilizantes nitrogenados de síntesis química. Se evidencia el potencial de la metodología para hacer crecer una comunidad microbiana diazótropa con potencial biofertilizante. La sencillez de funcionamiento del reactor y la capacidad de operar con fuentes de carbono provenientes del tratamiento de residuos agroindustriales hace que su aplicación sea prometedora para los países en desarrollo con bajo progreso tecnológico.

Palabras clave: bioeconomía, valorización de residuos, bioprospección dirigida.

1. Introducción

En 2022, la producción mundial de alimentos alcanzó los 9,6 billones de toneladas (FAOSTAT, 2023), y se proyecta que para 2050 esta cifra aumente en un 70% (FAOSTAT, 2023). Los fertilizantes nitrogenados son esenciales para la producción agrícola, representando el principal insumo. Se estima que cerca del 80% del nitrógeno utilizado en la agricultura proviene de la síntesis química mediante el proceso de Haber-Bosch (HB) (Galloway *et al.*, 2021), que, para 2021, generó alrededor de 108 millones de toneladas de fertilizantes nitrogenados (FAOSTAT, 2023). HB requiere entre 20 y 30 GJ*ton NH₃-1 de energía no renovable, lo que equivale al 1,6% de la energía global disponible (Fúnez Guerra *et al.*, 2020), y contribuye aproximadamente al 2% de las emisiones globales de gases de efecto invernadero (Galloway *et al.*, 2021), haciéndolo insostenible a largo plazo.

La fijación biológica de nitrógeno por microorganismos se ha explorado como una alternativa para mitigar los impactos ambientales de HB. Aunque el mercado de biofertilizantes basados en microorganismos fijadores de nitrógeno está en crecimiento, su participación global es del 5% (Aloo *et al.*, 2021). Esto se atribuye a varias razones, incluida la limitación de los biofertilizantes nitrogenados a cultivos específicos, como las leguminosas, y la eficacia limitada de otros tipos de biofertilizantes basados en cultivos puros a nivel de campo (Joshi & Gauraha, 2022). Además, la producción industrial de *Rizhobium*, *Azospirillum* y

Azotobacter usa fuentes de carbono como sacarosa, ácido málico, manitol o glucosa que representan aproximadamente el 54% de los costos variables de producción (Amaresan *et al.*, 2023; Sethi & Adhikary, 2012), haciendo que este tipo de productos sigan siendo costosos en el mercado.

Ante esta situación, considerando las bases teóricas de la ecología de comunidades microbianas, la ingeniería de bioprocesos para cultivos microbianos y el creciente interés por los microbiomas terrestres, se propone la producción en bioreactores de comunidades microbianas autoensambladas, utilizando diferentes tipos de presión selectiva (Gutierrez *et al.*, 2020). Una de las ventajas principales de este enfoque es su capacidad para aprovechar la diversidad y la sinergia entre diferentes microorganismos, lo que conduce a una mayor estabilidad y resistencia a condiciones ambientales cambiantes en comparación con los cultivos puros (Gutierrez *et al.*, 2020). A nivel experimental, se han obtenido comunidades autoensambladas fijadoras de nitrógeno usando ácido cítrico como fuente de carbono. Estas comunidades, una vez inoculadas en plantas de tomate, han mostrado un crecimiento igual al obtenido con fertilizantes nitrogenados de síntesis química (Gutiérrez *et al.*, 2022). Sin embargo, su aproximación práctica sigue siendo costosa debido a las fuentes de carbono que se usan. En este sentido, es conveniente explorar otras fuentes de carbono, como puede ser moléculas que se puedan obtener a partir de residuos orgánicos. Así, se puede pensar en la producción de biofertilizantes como una estrategia de valorización de residuos.

Anualmente se generan 55 billones de toneladas de residuos de origen animal (Giroto & Cossu, 2017). Este tipo de residuos merecen atención especial debido a que por su alto contenido de humedad liberan CO₂ (3,5 billones de toneladas de CO₂eq en 2018), amoníaco (60 millones de toneladas en 2018) y otros contaminantes, contribuyendo al efecto invernadero y contaminación de fuentes de agua (FAO, 2020). El uso de la digestión anaerobia como opción de manejo y valorización de este tipo de residuos se ha venido usando desde hace muchos años enfocada en la producción de biogas, dejando de lado la fase sólida y la fase líquida del efluente que normalmente se convierten en un desecho más (Kranert *et al.*, 2012). La fase líquida puede llegar a contener concentraciones considerables de moléculas orgánicas con alto potencial de valorización como los Ácidos Grasos Volátiles (AGVs) (Wainaina *et al.*, 2019). Los AGVs provenientes del tratamiento anaerobio de residuos se han usado como materia prima para la obtención de diferentes tipos de bioproductos como polihidroxialcanoatos, biocombustibles, biogás, hidrógeno y otras moléculas como ésteres, cetonas y alcanos (Agnihotri *et al.*, 2022). La fijación de nitrógeno se ha propuesto como otra opción de valorización de los AGVs (Clavijo-Salinas *et al.*, 2020), sin embargo, a nivel experimental no existen trabajos que demuestren que este tipo de moléculas pueden ser útiles para cultivar microorganismos diazotróficos y producir biofertilizantes.

Teniendo en cuenta lo anteriormente dicho, el presente trabajo busca evaluar una estrategia de valorización de ácidos grasos volátiles provenientes de residuos de la industria porcícola para la producción de inoculantes diazótrofos basados en microbiomas con potencial biofertilizante.

2. Materiales y métodos

2.1. Aislamiento del inóculo inicial

Se tomó como inóculo inicial el suelo rizosférico de plantas de yuca, plátano, sábila y pasto brachiaria de la finca “cañabravas” vereda San José, municipio del Darién, Valle del Cauca, Colombia (3°53'12.9"N, 76°22'9.2"W). Corresponde a una finca de agricultura familiar sin historial de aplicación de fertilizantes nitrogenados de síntesis química. El objetivo de usar diferentes especies de plantas corresponde tener una amplia diversidad microbiana de partida para el enriquecimiento y selección de la comunidad autoensamblada. El suelo rizosférico fue recolectado siguiendo la metodología de Barillot y colaboradores (2013). Brevemente, se retiraron del suelo 3 plantas de cada especie, se agitaron vigorosamente para desprender el suelo de soporte y se seleccionaron las raíces jóvenes con pelos radicales, las cuales fueron cortadas, almacenadas en bolsas plásticas Ziploc a 4°C para su transporte al laboratorio. Para cada planta se pesaron 100 g de raíces, se mezclaron las raíces de las 4 especies de plantas en un frasco estéril de 1 L, el cual se llenó hasta la mitad con solución salina al 0.85 % y se añadió tween 80 al 0.01 %. Posteriormente, se agitó durante 90 minutos a 300 rpm para desprender el suelo rizosférico y las bacterias del rizoplaneo. La fase líquida se centrifugó a 150 g durante 10 minutos, el sobrenadante se filtró (1 mm) y se transfirió a otro frasco estéril. Finalmente, este filtrado se utilizó como inóculo de partida para el proceso de enriquecimiento y selección (figura 1A).

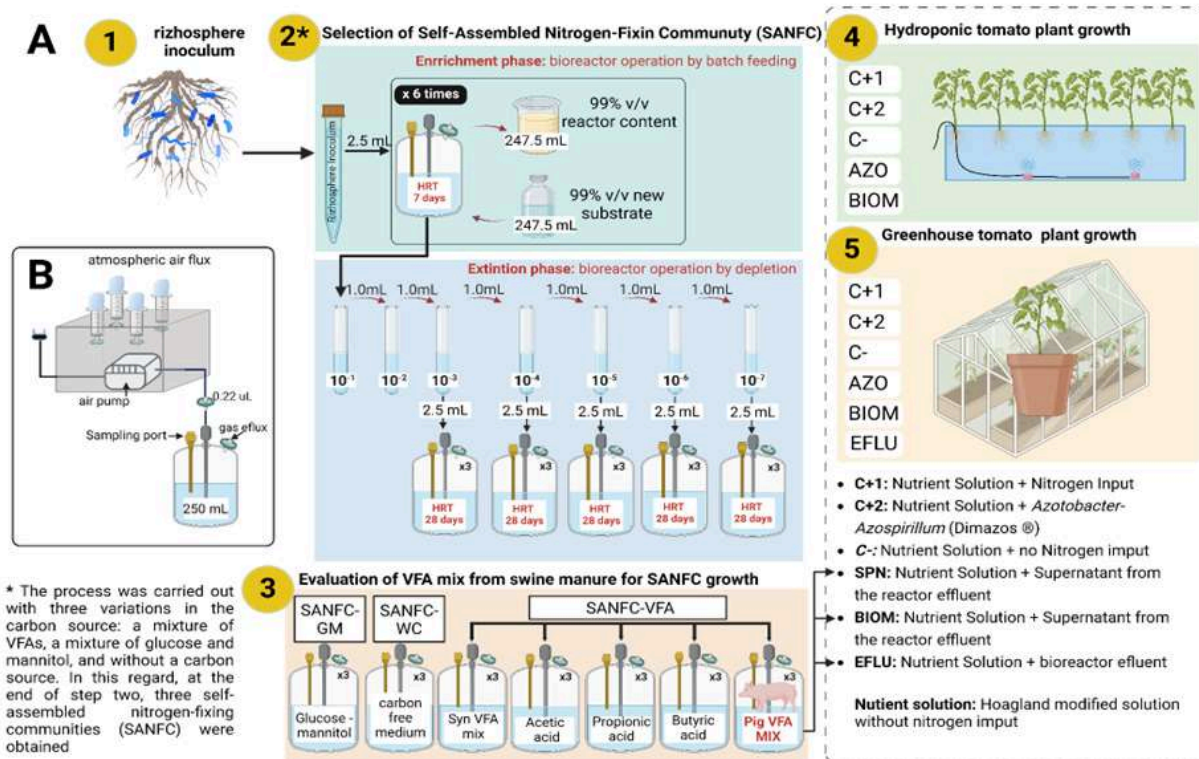


Figura 1. A. Montaje experimental (1) Aislamiento del inóculo rizosférico. (2) Proceso de selección de una comunidad autoensamblada fijadora de nitrógeno (CAFN) en dos fases: fase de estimulación, proceso de alimentación por lotes, con inyección de nitrógeno atmosférico y mezcla completa; fase de extinción realizada por agotamiento mediante diluciones en serie. (3) Comparación de diferentes formas de carbono sobre la fijación de nitrógeno por las CAFN. (4) Comparación del crecimiento de plantas de tomate en hidroponía e invernadero, usando por separado, la fase sólida (celulas bacterianas resuspendidas en solución salina), la fase líquida del efluente del reactor o el efluente directamente sin ningún tratamiento. B. Detalle de la inyección de aire al bioreactor. Realizado con Biorender. SANFC-GM: comunidad autoensamblada fijadora de nitrógeno obtenida usando como fuente de carbono una mezcla de glucosa y manitol, SANFC-WC: comunidad autoensamblada fijadora de nitrógeno obtenida sin usar ninguna fuente de caarbono, SANFC-VFA: comunidad autoensamblada fijadora de nitrógeno obtenida usando como fuente de carbono una mezcla de ácidos grasos volátiles, Syn VFA mix: mezcla de ácidos grasos sintéticos, Pig VFA mix: mezcla de ácidos grasos obtenidos por digestión anaerobia de estiércol de cerdo.

2.2. Selección de una comunidad autoensamblada fijadora de nitrógeno (CAFN)

Se utilizó la metodología propuesta por Díaz-García y colaboradores, (2021) para la obtención de la comunidad autoensamblada. Se realizaron algunas modificaciones, usando reactores aerobios en semibatch (figura 1B). Para la fase de estimulación (figura 1A-2), el inóculo rizosférico se cultivó en medio NFB (Rennie, 1981) evaluando tres variaciones en las fuentes de carbono (tabla 1): la primera fue una mezcla de ácido acético (Sigma-Aldrich, USA), ácido propiónico (Sigma-Aldrich, USA) y ácido butírico (Sigma-Aldrich, USA); la segunda fue una mezcla de glucosa y manitol; y en la tercera sin fuente de carbono. En ningún tratamiento se adicionaron vitaminas. El pH fue ajustado a 7 para los tres casos. El volumen efectivo de cada reactor fue de 250 mL. Se realizó una alimentación por lotes con un tiempo de retención hidráulica de 7 días, reemplazando el 99% del contenido del reactor con medio de cultivo estéril. El proceso de alimentación se realizó 6 veces (figura 1A-2). Previo a cada alimentación, se tomaron alícuotas para medición de variables de seguimiento.

Para la fase de extinción (Figura 1A-2), se realizaron diluciones en serie del efluente del reactor obtenido en la sexta alimentación. Se usó 1 mL del contenido del reactor en 9 mL de solución salina estéril (0.85%). Se tomaron 2,5 mL de las diluciones 10⁻³, 10⁻⁴, 10⁻⁵, 10⁻⁶ y 10⁻⁷ y se inoculaban en reactores con medio NFB estéril con las tres variaciones en la fuente de carbono registradas en la tabla 1. Los reactores se incubaron paralelamente durante 28 días a temperatura ambiente con entrada de aire permanente (1 L*min⁻¹) (figura 1A-3). Cada siete días se tomaron alícuotas para medición de variables de seguimiento.

Tabla 1. Medio NFB con fuentes de carbono modificadas.

Fuente de carbono	Mezcla AGVs sintéticos *	Sin carbono *	Glucosa - manitol *
Ácido acético (g*L ⁻¹)	7,45	0	0
Ácido propiónico (g*L ⁻¹)	2,25	0	0
Ácido butírico (g*L ⁻¹)	1,85	0	0
Glucosa (g*L ⁻¹)	0	0	8
Manitol (g*L ⁻¹)	0	0	8

*Adicional a las fuentes de carbono, se añadieron otros nutrientes en las siguientes cantidades para 1 L de medio de cultivo: 0.5g K₂HPO₄, 0.1g NaCl, 0.2g MgSO₄, 0.2g KOH, 0.02g CaCl₂, 2 mL de solución de micronutrientes y 4 mL de solución Fe-EDTA. Solución de micronutrientes para 200 mL: 0.02g Na₂MoO₄ · 2H₂O, 0.235g MnSO₄ , 0.280 g H₃BO₃ , 0.008 g CuSO₄ · 5H₂O y 0.0024 g ZnSO₄ · 7H₂O. Solución de Fe-EDTA para 100 mL: 0.8258 g FeSO₄ · 7H₂O y 0.556 g Na₂EDTA·2H₂O. Estas cantidades se añadieron de igual forma a todos los tratamientos. El pH del medio fue ajustado a 7.

2.3. Evaluación de una mezcla de AGVs proveniente de estiércol de cerdo como fuente de carbono para la CAFN

Se usó un diseño completamente al azar con siete tratamientos, cada tratamiento corresponde a una fuente de carbono diferente. Todos los tratamientos fueron operados con medio de cultivo NFB con las fuentes de carbono modificadas (Tabla 2). Para cada tratamiento se usaron tres réplicas. Cada réplica consistió en el montaje descrito en la sección 2.4. Los reactores fueron inoculados con 2,5 mL del inóculo adaptado y operados en semibatch durante 28 días. Cada semana se tomaron muestras para medición de pH, densidad óptica (600 nm), nitrógeno total y concentración de AGVs. Se agregó agua destilada estéril para mantener un volumen efectivo de 250 mL durante el tiempo de operación.

Tabla 2. Concentraciones de fuentes de carbono

Fuente de carbono (mg*L ⁻¹)	AGVs_S *	AGVs_C*	AA *	AP *	AB *	SC *	GM *
Ácido acético	600	463,10	830	0	0	0	0
Ácido propiónico	100	108,33	0	830	0	0	0
Ácido butírico	130	123,4	0	0	830	0	0
Ácido isobutírico	0	40.63	0	0	0	0	0
Ácido isovalérico	0	64,16	0	0	0	0	0
Ácido valérico	0	29.29	0	0	0	0	0
Glucosa	0	0	0	0	0	0	8g/L
Manitol	0	0	0	0	0	0	8g/L

*Adicional a las fuentes de carbono, se añadieron otros nutrientes en las siguientes cantidades para 1 L de medio de cultivo: 0.5g K₂HPO₄, 0.1g NaCl, 0.2g MgSO₄, 0.2g KOH, 0.02g CaCl₂, 2 mL de solución de micronutrientes y 4 mL de solución Fe-EDTA. Solución de micronutrientes para 200 mL: 0.02g Na₂MoO₄ 2H₂O, 0.235g MnSO₄, 0.280 g H₃BO₃, 0.008 g CuSO₄ .5H₂O y 0.0024 g ZnSO₄ .7H₂O. Solución de Fe-EDTA para 100 mL: 0.8258 g FeSO₄ .7H₂O y 0.556 g Na₂EDTA.2H₂O. Estas cantidades se añadieron de igual forma a todos los tratamientos. El pH del medio fue ajustado a 7. AGVs_S: mezcla de AGVs sintéticos, AGVs_C: mezcla de AGVs proveniente de estiercol de cerdo, AA: ácido acético, AP: ácido propiónico, AB: ácido butírico, SC: sin fuente de carbono, GM: mezcla de glucosa y manitol.

2.4. Sistema de reactores

Se usaron reactores en semibatch con mezcla completa (figura 1B). Cada reactor se compone de una botella de vidrio de 500 mL con una entrada de aire permanente (1 L*min⁻¹) inyectada por una bomba de aire. La bomba está dentro de una caja hermética de plástico que tiene 4 entradas de aire del ambiente que es filtrado por cilindros de plástico rellenos de algodón antiséptico. Cada bomba inyecta aire a través de una manguera de plástico en los reactores. Antes de ingresar al reactor, cada manguera se conecta a un filtro de 0.22 µm para garantizar condiciones asépticas dentro del reactor. El burbujeo generado por la manguera mantiene el medio de cultivo en mezcla constante. Los reactores se cubrieron con papel de aluminio para evitar la entrada de luz.

2.5. Variables de seguimiento de los reactores

En condiciones estériles, se desconectó cada reactor de la fuente de aire y se tomaron alícuotas para medir densidad óptica a 600 nm (Hoefer SP-2001), pH (Hanna Instruments HI-98160), nitrógeno total (Hanna Instruments HI93767A-50) y concentración de AGVs. Los AGVs se midieron mediante cromatografía de gases (Perkin Elmer Clarus 590), con una columna elite FFAP 30m x 0.25 mm ID x 0.25 µm DF y un detector de ionización de flama. Se utilizó nitrógeno como gas de arrastre a 15 psi con un split ratio de 120 mL/min. Las temperaturas del horno, el inyector y el detector se mantuvieron en 140, 250, y 250 °C, respectivamente. Las muestras para el análisis de AGVs se acidificaron con HCl al 37% (Honeywell Fluka, Austria), se filtraron con PTFE 0.45 µm, y se almacenaron a -20 °C hasta su análisis. Se utilizó una mezcla estándar de AGVs 10 mM (Sigma-Aldrich, USA) para la curva de calibración, que incluye los ácidos acético (HAc), propiónico (HPr), iso butírico (iHBu), butírico (HBu), iso valérico (iHV), valérico (HV), 4 metil valérico (4metHV), hexanóico (HHex), y heptanóico (HHep).

2.6. Evaluación del potencial biofertilizante de la CAFN sobre plantas de tomate en hidroponía y suelo

Para el experimento en hidroponía, se usó un sistema tipo “deep-water” culture o balsa flotante (Son *et al.*, 2020) (figura 1A) para evaluar el efecto del sobrenadante y de la biomasa obtenidas de la CAFN alimentada con una mezcla de AGVs de cerdo. Se usaron semillas de tomate de la variedad UNAPAL-Maravilla (Estrada Salazar *et al.*, 2004). Las semillas fueron puestas a germinar en cajas de petri en oscuridad durante 4 días a una temperatura de 25°C y posteriormente fueron transplantadas al sistema

hidropónico. En cada tanque se usó un volumen de trabajo de 12 L, con agua de grifo. La oxigenación de la solución nutritiva se realizó usando una bomba de aire con flujo constante manteniendo un porcentaje de oxígeno disuelto del 2%. Las condiciones de la sala de crecimiento fueron temperatura promedio de $26\text{ }^{\circ}\text{C} \pm 3\text{ }^{\circ}\text{C}$, $70\% \pm 4$ de humedad relativa y fotoperiodo de 12 h suministrado por luces LED (densidad de flujo de fotones fotosintéticos $600\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$). Se usó un diseño completamente al azar con cinco tratamientos como se describe en la tabla 3. Cada tanque corresponde a un tratamiento diferente y se usaron 6 plantas por tanque (figura 1a).

Tabla 3. Descripción de los tratamientos y forma de aplicación para el experimento en hidroponía

Tratamiento	Características	Aplicación
BIOM*	El efluente del reactor es centrifugado a 5082 g por 30 minutos, el pellet es resuspendido en solución salina 0,85% y ajustado a una densidad óptica de 0,3 OD 600 nm (Hoefer Vision SP-2001).	Se aplicó semanalmente 10 mL del producto dentro del tanque, durante tres semanas.
SOBRE*	El efluente del reactor es centrifugado a 5082 g por 30 minutos, el sobrenadante es colectado, se determinó nitrógeno total (Hanna Instruments HI93767A-50) ($9,8\text{ mg}\cdot\text{mL}^{-1}$) y fue esterilizado.	Se aplicó 257 mL del sobrenadante esterilizado y se completó a 12 litros con agua del grifo para lograr una concentración final de 210 ppm de nitrógeno total en el tanque.
AZO*	Biofertilizante comercial Dimazos® a base de <i>Azotobacter chroococum</i> y <i>Azospirillum sp.</i>	Se aplicó semanalmente 10 mL del producto dentro del tanque, durante tres semanas siguiendo la dosis recomendada por el fabricante.
NITRO	Fertilización nitrogenada con solución de Hoagland	Se aplicó una sola vez, con las concentraciones finales al 100% (Kaur <i>et al.</i> , 2016)
C-*	Solución de Hoagland modificada sin aporte de nitrógeno	Se aplicó una sola vez, con las concentraciones finales al 100%, sin aporte de nitrógeno (Gutiérrez <i>et al.</i> , 2022)

*Los macro y micronutrientes fueron aportados con solución de Hoagland (100%) modificada sin aporte de nitrógeno como lo describe Gutiérrez *et al.*, 2022.

Para el experimento en invernadero, se usó suelo como sustrato. Plántulas de tomate de la variedad UNAPAL-Maravilla (Estrada Salazar *et al.*, 2004) de 20 días de germinadas fueron adquiridas en una plantuladora comercial ubicada en Candelaria, Valle del Cauca. Las plántulas fueron trasplantadas a bolsas plásticas con 3 Kg de suelo cada una. El suelo fue recolectado de los primeros 20 cm de un bosque con más de 30 años en proceso de restauración natural, situado junto a la Estación Experimental en el campus de la Universidad del Valle ($3^{\circ}22'23''\text{N}$, $76^{\circ}31'51''\text{W}$; Cali, Colombia). El experimento fue realizado en el invernadero de entomología de la Universidad del Valle con temperatura promedio de $23\text{ }^{\circ}\text{C} \pm 4\text{ }^{\circ}\text{C}$, $70\% \pm 3$ de humedad relativa y fotoperiodo de 12 h. Se utilizó un diseño completamente al azar con seis tratamientos como se describe en la tabla 4. Para cada uno de los tratamientos se usaron 8 réplicas, cada réplica consistió en una planta de tomate sembrada en una bolsa plástica con 3 Kg de suelo.

Tabla 4. Características y forma de aplicación de los 6 tratamientos evaluados en el experimento de invernadero

Nivel	Fuente de N - Descripción	Aplicación
BIO*	El efluente del reactor es centrifugado a 5500 rpm por 30 minutos, el pellet es resuspendido en solución salina 0,85% y ajustado a una densidad óptica de 0,3 OD 600 nm (Hoefer Vision SP-2001)	Se aplicó semanalmente 10 mL del producto en drench a cada planta durante cuatro semanas.
SOBRE*	El efluente del reactor es centrifugado a 5082 g por 30 minutos, el sobrenadante es colectado, se determinó nitrógeno total (Hanna Instruments HI93767A-50) (9,8 mg*mL ⁻¹) y fue esterilizado.	Se aplicaron 265 mL semanalmente a cada planta durante 4 semanas. Esta cantidad se calculó para lograr un aporte de 12g de nitrógeno por planta, atendiendo a la demanda de nitrógeno para esta variedad, también se tomó en cuenta la cantidad de nitrógeno inicialmente disponible en el suelo utilizado.
AZOTO*	Biofertilizante comercial Dimazos® a base de <i>Azotobacter chroococum</i> y <i>Azospirillum sp.</i>	Se aplicó semanalmente 7,5 mL del producto en forma de drench a cada planta semanalmente durante cuatro semanas siguiendo la dosis recomendada por el fabricante.
NITRO*	Fertilización nitrogenada con solución de Hoagland	Se aplicaron 12,37 mL de solución al 100% semanalmente a cada planta durante 4 semanas. Esta cantidad se calculó para lograr un aporte de 12g de nitrógeno por planta, atendiendo a la demanda de nitrógeno para esta variedad, también se tomó en cuenta la cantidad de nitrógeno inicialmente disponible en el suelo utilizado.
C-	Solución de Hoagland modificada sin aporte de nitrógeno (Gutiérrez <i>et al.</i> , 2022)	Se aplicaron 12,37 mL de solución al 100% sin aporte de nitrógeno semanalmente a cada planta durante 4 semanas
EFLU*	El efluente del reactor es ajustado a una densidad óptica de 0,3 OD 600 nm (Hoefer Vision SP-2001)	Se aplicó semanalmente 10 mL del producto en drench a cada planta durante cuatro semanas.

*Los macro y micronutrientes fueron aportados con solución de Hoagland (100%) modificada sin aporte de nitrógeno como lo describe Gutiérrez *et al.* (2022).

Para las plantas crecidas en hidroponía, a los 45 días después de trasplante (fase de crecimiento vegetativo) se midieron variables agronómicas como longitud del tallo, número de hojas, longitud de la raíz, peso fresco y clorofilas totales. Adicionalmente, se determinó la composición del microbioma rizosférico (sección 2.7). Para las plantas crecidas en invernadero, se midió altura de la planta y número de hojas semanalmente hasta el día 70 después de trasplante (DDT). Se midió la producción en peso fresco del fruto para cada tratamiento a los 70 DDT. Adicionalmente, se determinó la composición del microbioma rizosférico (sección 4.3.3) a los 35 DDT (fase de crecimiento vegetativo).

2.7 Extracción de ADN, secuenciación y análisis bioinformático

Se realizó una secuenciación dirigida al gen 16sRNA con el fin de determinar la composición taxonómica en diferentes etapas del montaje experimental. Así, determinó la composición taxonómica del inóculo rizosférico inicial (IR), de las diluciones de la etapa de extinción alimentadas con AGVs (10-3 - 10-6), de los reactores alimentados con mezcla de AGVs sintéticos y mezcla de AGVs de cerdo () y rizosfera de los tratamientos AZO, BIOM, y C- tanto para el experimento en hidroponía como en invernadero.

Para el caso de los reactores, 50 mL del efluente fueron centrifugados a 10000 g por 30 minutos (UNIVERSAL 320 - Hettich), se descartó el sobrenadante y el pellet fue usado como material de partida para la extracción de ADN. Para las plantas crecidas en hidroponía, los microorganismos rizosféricos fueron recolectados siguiendo la metodología de (Barillot *et al.*, 2013) con algunas modificaciones. Brevemente, se retiraron del sistema 3 plantas de cada tratamiento, las raíces (10 g de peso fresco), se colocaron en tubos estériles de 50 ml al cual se añadió 25 mL de solución salina al 0.85 % y se añadió tween 80 al 0.01 % (v/v). Posteriormente, se agitó durante 90 minutos a 300 rpm para desprender las bacterias adheridas a la raíz. La fase líquida se centrifugó a 150g durante 10 minutos, el sobrenadante se filtró (1 mm), se transfirió a otro frasco estéril y fue centrifugado 10000 g por 30 minutos (UNIVERSAL 320 - Hettich), se descartó el sobrenadante y el pellet fue usado como material de partida para la extracción de ADN. Para el caso de las plantas crecidas en suelo, cada planta se agitó vigorosamente para eliminar los agregados grandes del suelo. El suelo que permaneció en las raíces se consideró como el suelo rizosférico y fue colectado siguiendo el protocolo de Lundberg *et al.* (Lundberg *et al.*, 2012) con algunas modificaciones. Brevemente, las raíces (10 g de peso fresco), con el suelo de rizosfera asociado, se colocaron en tubos estériles de 50 ml y se trataron con 30 ml de buffer fosfato 1X más Tween 20® al 2 % (v/v); luego, cada tubo se agitó durante 10 min. Luego, las suspensiones se centrifugaron durante 5 minutos a 7200g, se descartó el sobrenadante y el pellet fue usado como material de partida para la extracción de ADN.

Se usó el kit NucleoSpin Soil (Macherey–Nagel GmbH, Germany) siguiendo las recomendaciones del fabricante (solución de lisis SL1). Se midió la calidad y cantidad del ADN extraído mediante electroforesis en gel de agarosa y espectrofotometría usando Nanodrop 2000 (Thermo Scientific, USA). Las regiones del gen V3-V4 16S rRNA (341F - 805R) fueron secuenciadas por MrDNA Lab (Molecular Research LP Co, TX, USA) utilizando una plataforma Illumina MiSeq. Los datos de secuenciación fueron analizados utilizando QIIME2 v 2023.9 (<https://qiime2.org>) (Bolyen *et al.*, 2019). La eliminación de ruido, la desreplicación de las secuencias y el filtrado de quimeras con el método de consenso se realizaron utilizando DADA2 (Callahan *et al.*, 2016) dentro de QIIME2. La asignación taxonómica de las variantes de secuencia de amplicones (ASV) (97% de identidad de secuencia) se realizó utilizando el complemento “fit-classifier-naïve-Bayes”, frente a la base de datos de referencia SILVA 138. La visualización de abundancias e índices de diversidad se realizó usando el paquete Phyloseq (McMurdie & Holmes, 2013) de R (R Core Team, 2022)

2.8. Análisis estadístico

Para las variables de seguimiento de los bioreactores se realizó un análisis de varianza ANOVA ($p < 0,05$), se probaron los supuestos de normalidad mediante prueba de Shapiro-Wilks ($p < 0,05$) y homogeneidad de varianzas mediante prueba de Bartlett ($p < 0,05$). Se realizaron análisis de comparaciones de medias usando Tukey ($p < 0,05$). Todos los análisis fueron realizados usando el programa R (R Core Team, 2022). Se realizó una correlación de Pearson entre las variables fisicoquímicas de los reactores y las abundancias relativas a nivel de género.

3. Resultados

3.1. La CAFN es capaz de consumir AGVs y fijar nitrógeno en cantidades significativamente mas altas que el inóculo rizosférico

Los resultados muestran que al iniciar la fase de enriquecimiento (figura 2) se obtuvo un crecimiento significativamente menor usando AGVs como fuente de carbono con respecto de la glucosa. Al finalizar la alimentación 5 y 6 se obtiene un crecimiento significativamente mayor usando AGVs (figura 2). La oxidación de los AGVs genera un aumento significativo del pH mientras que la de la glucosa genera una disminución (figura 2). La fase de extinción mostró que usando AGVs se obtiene una concentración significativamente mayor de nitrógeno total para la dilución 10-6 con respecto del inóculo sin ningún proceso de enriquecimiento o selección (figura 3). Para el caso de la glucosa, en la fase de selección se obtienen cantidades de nitrógeno total significativamente menores con respecto al inóculo inicial. Adicionalmente, se evidenció una reducción significativa de la concentración de ácido butírico y ácido propiónico para cada una de las diluciones (figura 3) con respecto a un control no inoculado.

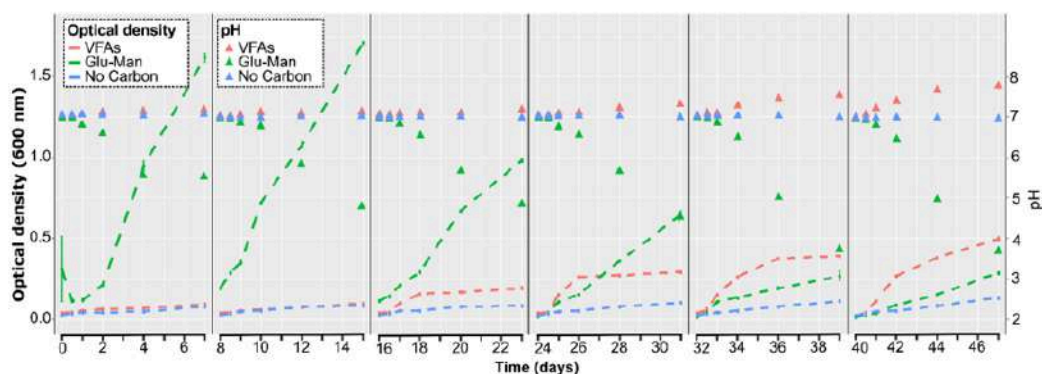


Figura 2. Densidad óptica (curvas discontinuas) y pH (triángulos) para cada una de las 6 alimentaciones sucesivas en la fase de enriquecimiento. VFAs: ácidos grasos volátiles, Glu-Man: mezcla de glucosa y manitol. n=3.

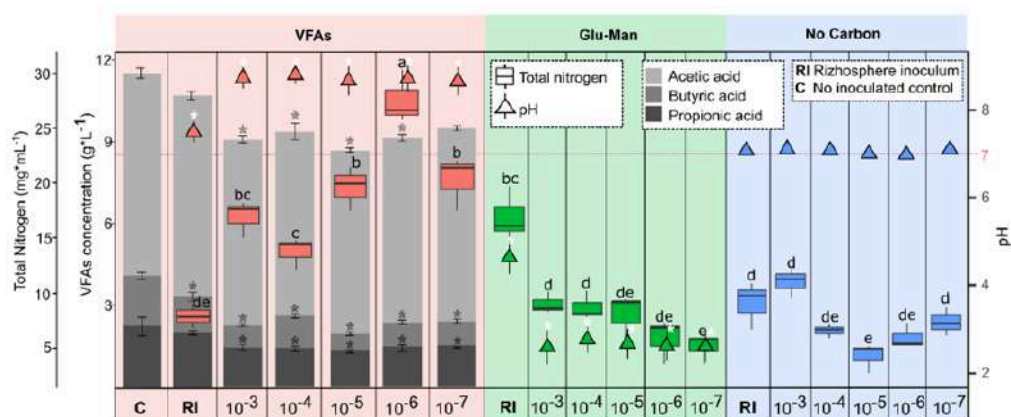


Figura 3. Variables fisicoquímicas en la fase de selección medidas a los 28 días de operación de los reactores. VFAs: ácidos grasos volátiles, Glu-Man: mezcla de glucosa y manitol. Letras diferentes muestran diferencias estadísticamente significativas de nitrógeno total entre las diluciones (ANOVA -Tukey $p < 0,05$). * grises indican diferencias estadísticamente significativas en concentración de AGVs respecto al control no inoculado (ANOVA-Dunnett $p < 0,05$). * blancos indican diferencias estadísticamente significativas en pH respecto a pH 7 (ANOVA-Dunnett $p < 0,05$). n=3.

3.2. La CAFN fija nitrógeno usando como fuente de carbono una mezcla de AGVs provenientes de estiércol de cerdo

Se obtuvo un crecimiento significativamente mayor cuando se usó una mezcla de ácidos grasos ya sea sintética o proveniente del estiércol de cerdo (Figura 4A). Usar solamente ácido butírico también genera un crecimiento significativamente mayor al que se obtiene usando solamente ácido acético o ácido propiónico (Figura 4A). Nuevamente los resultados muestran un aumento significativo del pH cuando se usan ácidos grasos ya sean separados o en mezcla y una disminución significativa cuando se usa glucosa-manitol (Figura 4B).

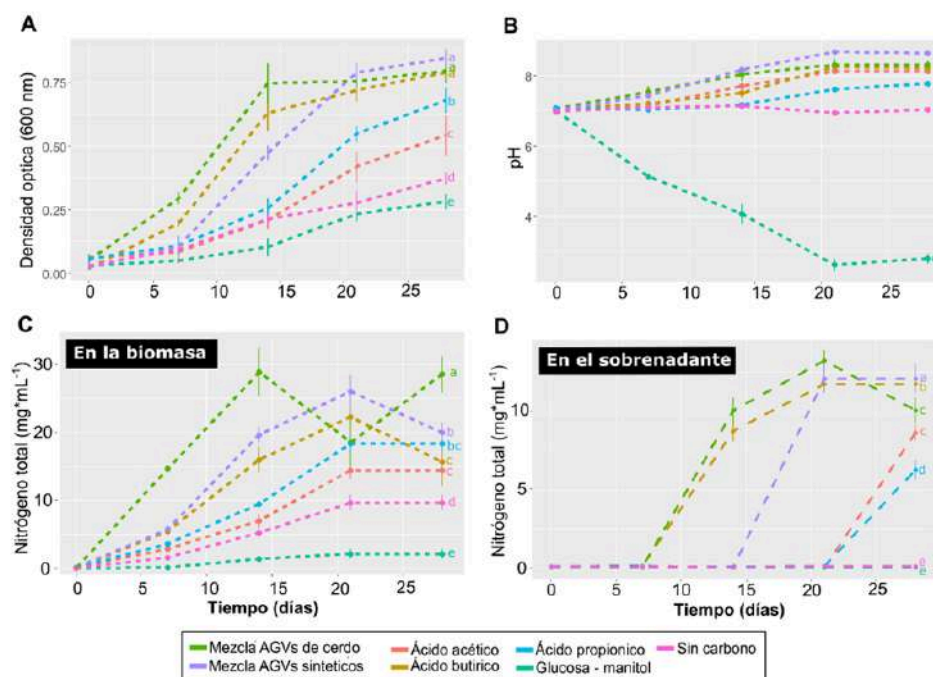


Figura 4. Variables fisicoquímicas medidas durante 28 días. A: densidad optica, B: pH, C: Nitrógeno total en la biomasa, D: Nitrógeno total en el sobrenadante. Letras diferentes muestran diferencias estadísticamente significativas en el día 28 (ANOVA-Tukey $p < 0,05$).

Se midió nitrógeno total en biomasa y sobrenadante. Se obtuvo un pico de nitrógeno total en la biomasa para el tratamiento alimentado con una mezcla de AGVs de cerdo (figura 4C). Este pico coincide con el inicio de la fase estacionaria a los 14 días de operación. Igualmente, en el sobrenadante se obtiene nitrógeno a partir de los 14 días para los tratamientos con ácido butírico y mezcla de AGVs de estiércol de cerdo y a los 21 días para la mezcla de AGVs sintéticos (figura 4D); también coincide con el inicio de la fase estacionaria. Todos los tratamientos partieron de la misma concentración total de AGVs; sin embargo, se observa un consumo diferencial. Se aprecia un consumo por encima del 90% para la mezcla de AGVs de cerdo y para ácido butírico (Figura 5). El menor consumo corresponde al ácido acético, representado en un 35,28% de la cantidad inicial.

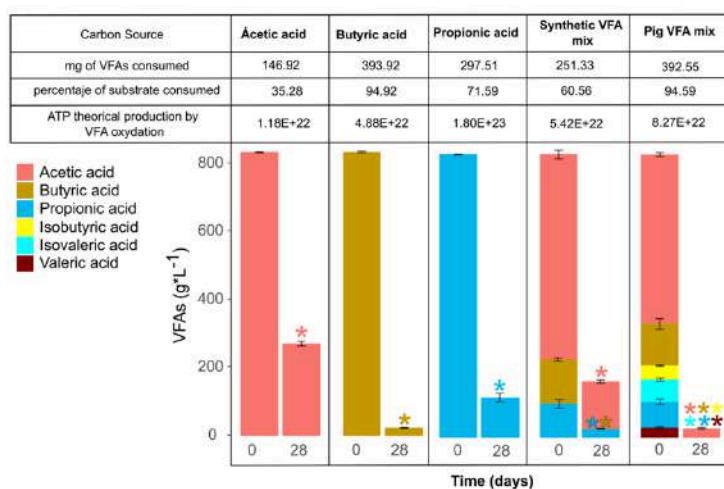


Figura 5. Concentración de AGVs en el día 0 y el día 28. Letras diferentes muestran diferencias estadísticamente significativas en el día 28 (ANOVA-Tukey $p < 0,05$).

3.3. La CAFN inoculada en plantas de tomate genera un crecimiento y producción significativamente igual al que se obtiene usando nitrógeno de síntesis química

Plantas de tomate cultivadas en hidroponía mostraron que la aplicación de la biomasa microbiana en la solución nutritiva logra un crecimiento significativamente igual al que se obtiene con una fertilización usando nitrógeno (Figura 6). El uso del producto comercial con *Azotobacter* y *Azospirillum*, si bien mostró un crecimiento significativamente mayor al control negativo (C-), no fue suficiente para igualar el control positivo con nitrógeno (NITRO). Estos resultados se pueden apreciar en las fotografías de las plantas de tomate (figura S1) donde es evidente un mayor crecimiento aéreo y desarrollo de raíz en las plantas de los tratamientos BIOM y NITRO. Por otra parte, las plantas de los tratamientos AZO y SOBRE presentan un bajo desarrollo foliar, poco crecimiento de la raíz y síntomas de clorosis. Las plantas del control, negativo (C-) solamente desarrollaron un par de hojas verdaderas, evidenciando la importancia del nitrógeno. La figura S2 muestra fotografías microscópicas de raíces de tomate del tratamiento BIOM y C-, donde se aprecia la formación de biopelículas sobre la superficie de la raíz en las plantas del tratamiento BIOM.

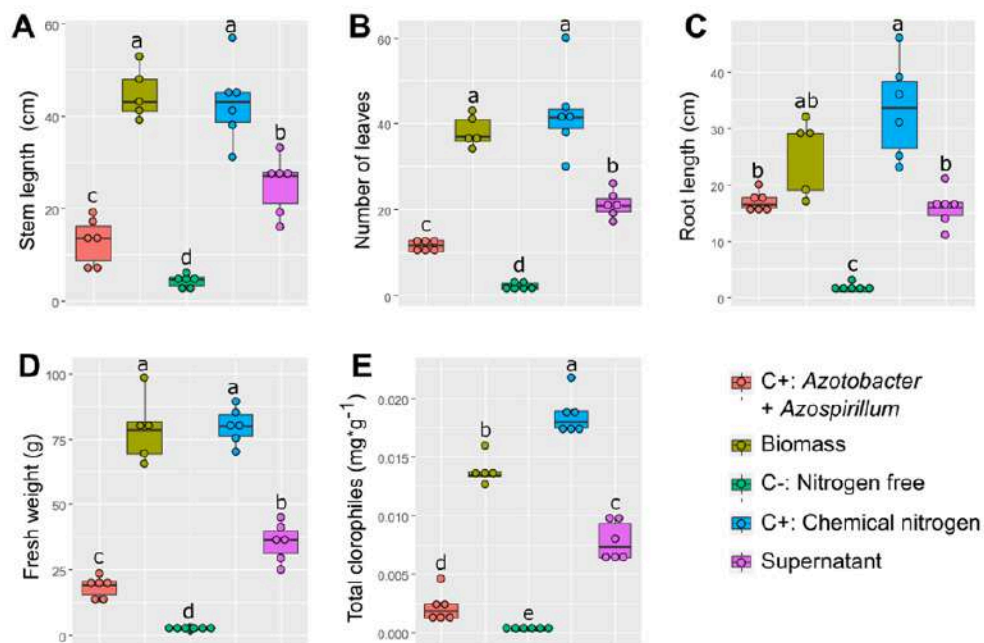


Figura 6. Crecimiento de plantas de tomate en hidroponía. Mediciones tomadas a los 36 días después de siembra. A: Longitud del tallo, B: Número de hojas, C: Longitud de la raíz. D: Peso fresco. E: Clorofilas totales. Letras diferentes muestran diferencias estadísticamente significativas (ANOVA-Tukey $p < 0.05$).

Los resultados muestran que la esterilización del suelo afecta significativamente la producción para el control positivo (NITRO) para el sobrenadante (SOBRE) y para el control negativo (C-) comparado con suelo no esterilizado (figura 7). Este efecto no se evidencia en los demás tratamientos que tienen aplicación de microorganismos (AZO, BIOM y EFLU). Se observa que se alcanza una producción igual al control con nitrógeno químico usando directamente el efluente del reactor tanto en suelo esterilizado como en suelo sin esterilizar.

La figura S3 muestra fotografías de plantas de tomate de cada tratamiento a los 35 DDS, allí se aprecia de forma visual diferencias en el crecimiento. La figura S4A muestra el crecimiento en términos de altura de la planta durante el tiempo que duró el experimento. Se aprecia un menor crecimiento en las plantas sembradas en suelo esterilizado, sin embargo las diferencias no son tan contundentes como se aprecia en la producción (figura 7).

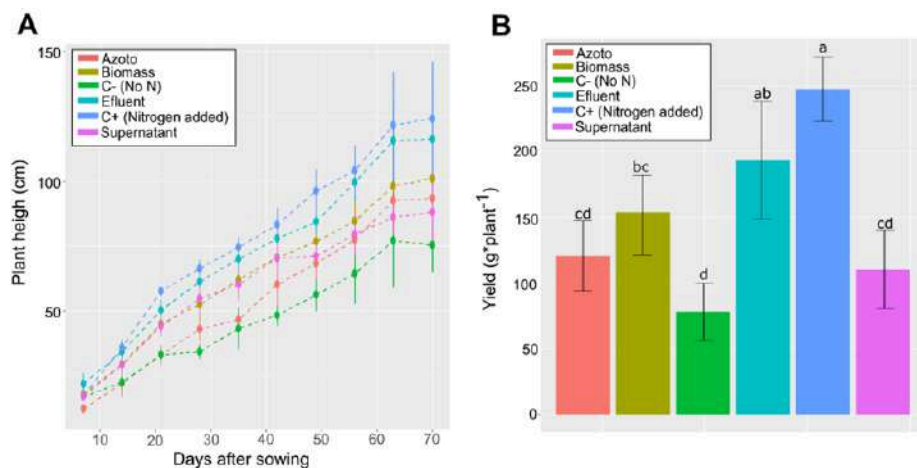


Figura 7. Crecimiento y producción de plantas de tomate inoculadas con la comunidad autoensamblada fijadora de nitrógeno. A: Altura de la planta. B: Rendimiento en peso fresco de los frutos de tomate a los 45 DDS. Letras diferentes muestran diferencias estadísticamente significativas (ANOVA-Tukey $p < 0,05$).

3.4. La composición taxonómica de la CAFN revela taxones clave para la fijación de nitrógeno y promoción del crecimiento de las plantas

Al final de la fase de extinción, se obtiene una composición taxonómica similar en todas las diluciones, donde *Sinorhodobacter* es el taxón más abundante seguido por *Gordonia* (Figura 8). Para la comunidad obtenida en la dilución 10-6, *Sinorhodobacter*, *Gordonia* y *Gluconoacetobacter* en conjunto representan el 84,2% de la abundancia total. Cuando la comunidad autoensamblada obtenida en 10-6 es alimentada con una mezcla de AGVs provenientes de estiércol de cerdo, *Sinorhodobacter* se mantiene como el taxón más abundante, sin embargo *Gordonia* y *Gluconoacetobacter* tienden a desaparecer y por el contrario *Aureimonas* y *Taibaiella* tienen un aumento significativo en cuanto a abundancia (Figura 8). Los índices de diversidad muestran ...

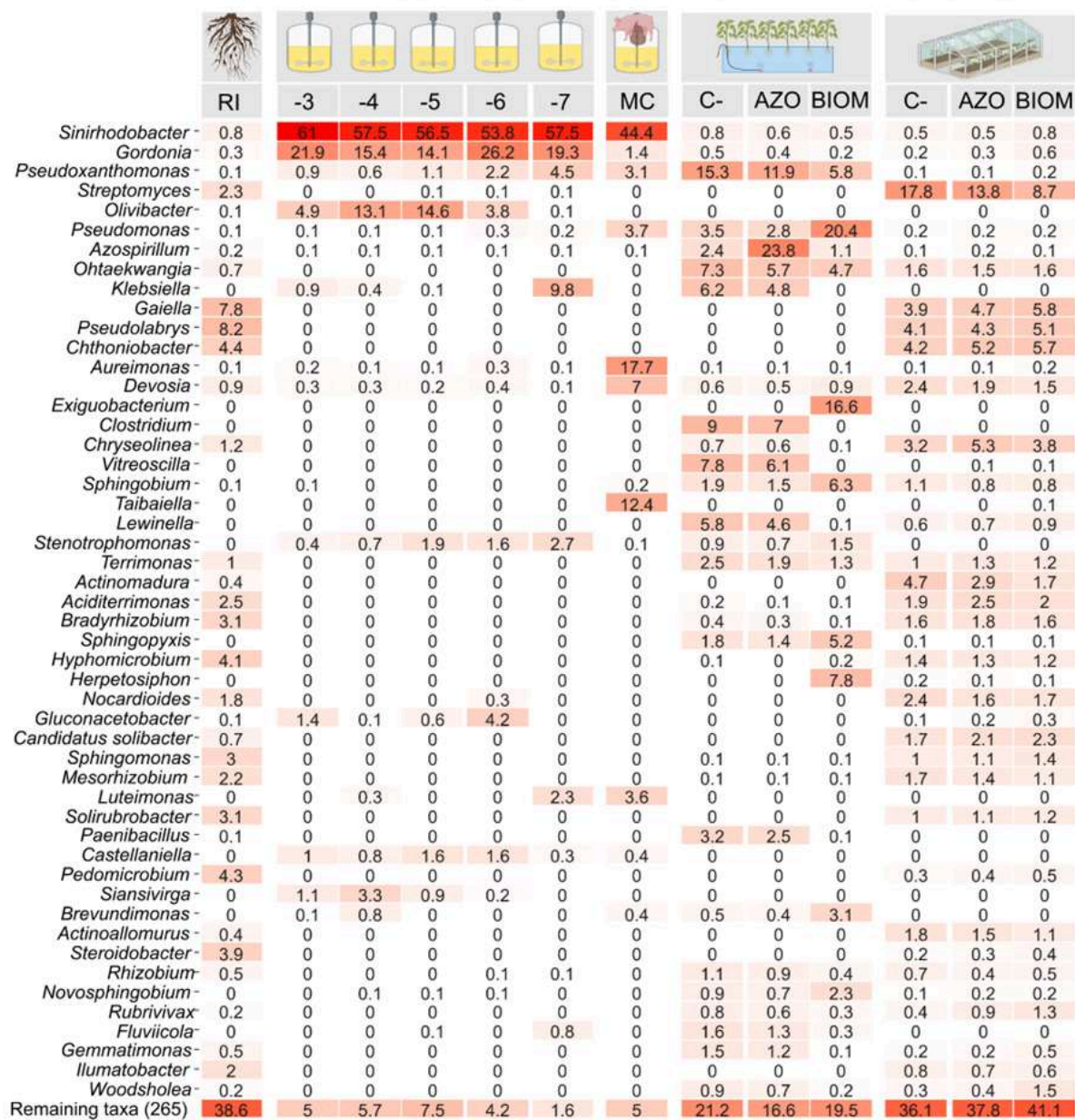


Figura 8. Grafico de calor con los valores de abundancia relativa para los 30 generos más abundantes de cada tratamiento analizado. RI: inóculo rizosferico, MC comunidad autoensamblada alimentada con una mezcla de AGVs proveniente de estiércol de cerdo. C-: control negativo sin aporte de nitrógeno. AZO: comunidad microbiana de la rizosfera de plantas de tomate inoculadas con un producto comercial a base de *Azotobacter chroococum* y *Azospirillum* sp. BIOM: comunidad microbiana de la rizosfera de plantas de tomate inoculadas con la biomasa microbiana de la comunidad microbiana fijadora de nitrógeno alimentada con una mezcla de AGVs provenientes de estiércol de cerdo.

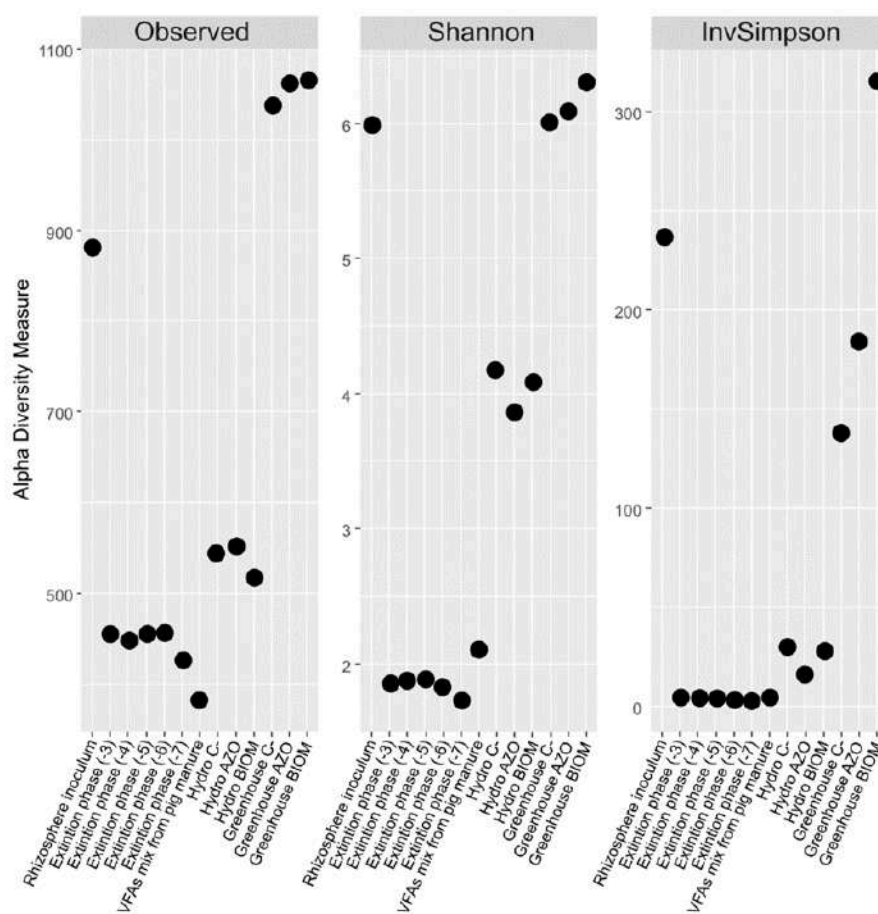


Figura 9. Índices de alfa diversidad para las muestras analizadas. A: Índice ObservedFeatures, B: Índice de Shannon, C: Índice de Simpson invertido.

4. Discusión

Este trabajo muestra que se puede usar ingeniería de microbiomas para obtener una comunidad autoensamblada fijadora de nitrógeno usando como factores de selección la fuente de nitrógeno y la fuente de carbono. La comunidad obtenida en la dilución 10-6 es capaz de fijar una cantidad significativamente más alta de nitrógeno con respecto a la comunidad original y a las demás diluciones. Además puede usar ácidos grasos de cadena corta como fuente de carbono para el proceso. Los resultados sugieren que un ligero aumento del pH está correlacionado con la fijación de nitrógeno (Figura 8) e incluso podría usarse el pH como indicador de la oxidación de los ácidos grasos en este bioproceso en particular.

En la fase de enriquecimiento se aprecia que los reactores alimentados con glucosa-manitol disminuyen paulatinamente su crecimiento en cada alimentación (figura 2). Además, al final de la fase de selección, usando glucosa-manitol como fuente de carbono se obtienen cantidades de nitrógeno total incluso menores a las obtenidas sin usar ninguna fuente de carbono (figura 3). Este comportamiento puede ser debido a los valores de pH que se alcanzan al final de cada alimentación, esta condición ácida puede hacer que se inhiba la fijación de nitrógeno o que se restrinja la diversidad de microorganismos que son capaces de soportar esas condiciones. Por lo dicho anteriormente, es posible que al final de cada alimentación, la comunidad microbiana esté cada vez más empobrecida. Por otra parte, las condiciones ácidas pueden estar causando una desnitrificación acelerada, haciendo que el nitrógeno fijado se pierda rápidamente en forma de gas. Estos resultados remarcan la necesidad de usar sustancias amortiguadoras de pH y condiciones controladas en este tipo de sistemas cuando se usan azúcares simples como fuente de carbono.

Pocas investigaciones han usado el aire como fuente de nitrógeno para cultivar microorganismos fijadores de nitrógeno en bioreactores. En el trabajo reciente de Gutiérrez y colaboradores (2022) lograron obtener una comunidad autoensamblada fijadora de nitrógeno donde los géneros más abundantes fueron *Sphingobium*, *Pseudoxanthomonas* y *Xanthobacter*. En este trabajo, los

géneros más abundantes en la comunidad autoensamblada que presentó mayor fijación de nitrógeno fueron *Sinirhodobacter* (53,8), *Gordonia* (26,2) y *Gluconoacetobacter* (4,2). Pocos estudios han investigado el papel de *Gordonia* como fijador de nitrógeno (Kayasth *et al.*, 2014). Se ha explorado el potencial de especies de este género para degradar contaminantes o producción de biopolímeros (Sowani *et al.*, 2018). Es un microorganismo que se ha encontrado como endofito en plantas y ha sido aislado del suelo también; por lo que tenerlo en una alta abundancia dentro de un bioreactor puede hacer potencialmente aplicable como inoculante. Los resultados muestran una correlación significativa entre la abundancia de *Gordonia* y nitrógeno total (figura suplementaria 1) evidenciando su papel como fijador de nitrógeno.

Sinirhodobacter es otro género que no se menciona frecuentemente dentro de los fijadores de nitrógeno, K. Keerthana-Ponni, (2021) caracterizó una cepa de *Sinirhodobacter* sp demostrando su capacidad de fijación de nitrógeno. Esta cepa antes mencionada y otras dos cepas (Xi *et al.*, 2017; Young *et al.*, 2020) han sido aisladas de efluentes de digestores anaerobios. Los resultados muestran que este género presenta una correlación significativa en cuanto al consumo de AGVs, confirmando su capacidad para usar AGVs como fuente de carbono. Posiblemente *Sinirhodobacter* consume los AGVs y de alguna forma le pasa la energía a *Gordonia* que es el que fija nitrógeno. Estos dos géneros presentan una correlación entre sí, indicando probablemente una codependencia para poder sobrevivir dentro del bioreactor. Sin embargo, son necesarios experimentos adicionales que permitan confirmar o rechazar esta hipótesis.

Como se esperaba, a medida que se aumentaron las diluciones hay una tendencia a disminuir la diversidad (Figura 7). Sin embargo, se mantienen los mismos taxones como los más abundantes. Esto indica que en este caso el ensamblaje de la comunidad está siguiendo un patrón determinístico, es decir que se obtienen comunidades similares con unos factores de selección determinados. Estudios recientes hablan de que este tipo de ensamblaje de la comunidad obedece a algo llamado convergencia funcional que refleja una auto organización metabólica como una propiedad emergente de las comunidades microbianas (Chang *et al.*, 2023; Estrela *et al.*, 2022). Por otra parte, este trabajo evidencia que hay una diversidad de microorganismos fijadores de nitrógeno poco conocidos que podrían tener un alto potencial para desarrollar bioinoculantes.

La fase de selección usada en este trabajo, también conocida como dilución a extinción ha sido ampliamente usada para la obtención de cultivos puros. Solamente trabajos recientes han mostrado su uso para inducir la formación de comunidades microbianas con funciones deseadas, como Díaz-García y colaboradores (2021) que lograron obtener una comunidad microbiana con capacidad para la degradación de compuestos lignocelulósicos, o Kang y colaboradores (2020) que obtuvieron un consorcio para degradar compuestos recalcitrantes. Sin embargo, esta es la primera vez que se usa esta metodología para obtener una comunidad diazotrofa. La importancia de este proceso radica en que en primer lugar induce una composición microbiana sencilla, es decir, pocos taxones que en comunidad son capaces de llevar a cabo la función deseada. Y en segundo lugar, revela taxones poco conocidos que sería difícil obtenerlos en cultivos puros, porque posiblemente la única forma que puedan crecer es en asociación con otros microorganismos.

Es bien sabido que el complejo enzimático de la nitrógenasa es inhibido irreversiblemente por el oxígeno (Einsle & Rees, 2020). Se han descrito varios mecanismos por los cuales los microorganismos pueden proteger el complejo enzimático de la nitrógenasa como la microaerofilia, aumento en las tasas de respiración, producción de células especializadas, polisacáridos extracelulares o colonización de nódulos (Zuberer, 2021). Todos estos mecanismos se han elucidado a partir de cultivos puros y dejan la pregunta de que otros mecanismos pueden usar cuando se trata de una comunidad microbiana en un reactor aerobio con inyección constante de aire como es el caso de este trabajo.

Este es el primer trabajo que muestra la fijación biológica de nitrógeno como opción de valorización de AGVs que se puedan obtener a partir de residuos agroindustriales. Los resultados muestran que usar una mezcla de AGVs funciona mejor a usar AGVs separados. Es importante mencionar que aparentemente los microorganismos prefieren AGVs de cadena más larga, como es el caso del ácido butírico, ya que se observa un mayor consumo con respecto al acético o propiónico. En la mezcla de AGVs de cerdo se tiene además ácido valérico e isovalérico y son los que se consumen en su totalidad. Los microorganismos convierten los AGVs en acetil Co-A por la ruta de la B-Oxidación y posteriormente se produce ATP por el ciclo de Krebs (Clark & Cronan, 2005). El tener una cadena carbonada más larga hace que teóricamente se pueda producir una mayor cantidad de ATP que es el recurso más limitante para la fijación de nitrógeno (Zuberer, 2021).

Recientemente se han enfocado esfuerzos en obtener cada AGV por separado a partir de la mezcla de AGVs y evaluar formas de valorización como fuente de carbono para diferentes bioprocesos. Sin embargo, obtener cada AGV por separado siendo poco eficiente y costoso (Atasoy *et al.*, 2018). En este sentido la evaluación de formas de valorización que usen la mezcla de AGVs puede ser más aplicable. Nuestros resultados muestran que la mezcla funciona mejor que usar ácido acético o ácido propiónico por separado. Es importante mencionar que es posible que durante el proceso de destilación para la obtención de los AGVs, existan otros compuestos que no se determinaron y que estén favoreciendo el crecimiento de la comunidad autoensamblada.

Un aspecto importante de los resultados obtenidos es que se logra obtener nitrógeno en el sobrenadante, es decir, que las células bacterianas están liberando el nitrógeno. Normalmente la liberación del nitrógeno en microorganismos diazotróficos está bajo un riguroso control genético (Bueno Batista & Dixon, 2019), puesto que al ser tan costoso en términos energéticos, no resulta viable liberar el nitrógeno fijado. En cultivos puros de *Azotobacter vinelandii* se ha logrado una concentración de nitrógeno extracelular de 20 mM de amonio (Ambrosio *et al.*, 2017). En este caso, al llegar a fase estacionaria, se observa liberación de nitrógeno en el sobrenadante de 15 mg*mL⁻¹ de nitrógeno total. Como sucede con las asociaciones simbióticas entre rizobios y leguminosas, algunos estudios demuestran que microorganismos diazotótrofos de vida libre pueden liberar nitrógeno cuando están en asociación con otros microorganismos o plantas (Ambrosio *et al.*, 2017; Ortiz-Marquez *et al.*, 2012, 2014). Por otra parte, esta liberación de nitrógeno puede estar relacionada también con la disponibilidad de fuentes de carbono en el medio de cultivo (Batista *et al.*, 2021). En cualquier caso, poder entender mejor estos mecanismos dentro del bioreactor podría significar la producción de nitrógeno reactivo que puede ser usado directamente como biofertilizante o como fuente nutricional para otros bioprocesos. En este sentido, se han propuesto las biofábricas fijadoras de nitrógeno como alternativa a Haber Bosch (Ortiz-Marquez *et al.*, 2014).

Desde el punto de vista de la operación de los reactores, se podría pensar en optimizar las condiciones de cultivo para poder maximizar la fijación de nitrógeno. Se pueden evaluar diferentes concentraciones de AGVs, tiempos de retención, temperatura, concentración de oxígeno, entre otras. Un aspecto importante de este trabajo es que se ajusta muy bien dentro de un contexto de economía circular y biorefinerías, en donde se agrega valor a los residuos para obtener bioproducto mediante la fijación de nitrógeno, y que a su vez se pueden conectar otros bioprocesos para aprovechar el nitrógeno fijado.

Los resultados muestran que tanto en hidroponía como en suelo, la inoculación de las plantas de tomate con la comunidad autoensamblada ya sea la biomasa centrifugada o directamente el efluente, permite una mayor producción y crecimiento con respecto a un control negativo y se alcanzan valores iguales a los obtenidos con una fertilización química convencional.

Es interesante que en el tratamiento AZO, no aparece *Azotobacter* en los análisis de diversidad. *Azospirillum* a pesar de que aparece con una abundancia del 24,7%, la fijación de nitrógeno no parece ser suficiente para mantener un crecimiento normal de la planta. La desaparición de *Azotobacter* podría ser debida a múltiples causas como no adaptación al hospedante, poca capacidad de competencia en la rizosfera, falta de adaptación al sistema de cultivo, entre otras. Por otra parte, *Azospirillum* se caracteriza por tener un metabolismo oxidativo microaerófilico que es óptimo a 0,4% de oxígeno disuelto (Chemotaxis in *Azospirillum* | SpringerLink, s. f.). Además, es capaz de detectar gradientes de oxígeno (aerotaxis) para identificar y moverse hacia espacios con baja concentración de oxígeno (Chemotaxis in *Azospirillum* | SpringerLink, s. f.). Estas dos características hacen que posiblemente esta bacteria no sea recomendable como fijador de nitrógeno en sistemas hidropónicos con alta concentración de oxígeno disuelto. Sin embargo, se ha reportado su capacidad de promoción de crecimiento cuando se aplica nitrógeno en la solución nutritiva (Benavides *et al.*, 2023).

Para el tratamiento BIOM en hidroponía los resultados sugieren que hay algunos taxones que la planta recluta a partir de la comunidad inoculada como *Pseudomonas* (20,7%), y *Sphingobium* (6,8%). Aparece también *Exiguobacterium* (11,9%) como el segundo más abundante, sin embargo, tiene una abundancia de cero en la comunidad autoensamblada (MC). Podría pensarse que es reclutado a partir del ambiente, pero no aparece en los otros dos tratamientos. La otra posibilidad es que estaba en una muy baja abundancia y la resolución del 16s no lo detectó, pero a pesar de estar poco abundante, fue capaz de prosperar en la rizosfera y alcanzar un 11,9%. La literatura muestra que microorganismos pertenecientes a *Pseudomonas* tienen la capacidad de fijar nitrógeno y han sido identificados en múltiples especies de cultivos de interés comercial (Islam *et al.*, 2013; Li *et al.*, 2017). Por otra parte, no hay reportes que muestren que *Exiguobacterium* ni *Sphingobium* son capaces de fijar nitrógeno, sin embargo, si es bien sabido de su capacidad como promotores de crecimiento por múltiples mecanismos como producción de ácido indol-acético, solubilización de fosfatos o formación de sideróforos (Boss *et al.*, 2022; Kasana & Pandey, 2018; Pandey, 2020).

Adicionalmente, para el experimento de hidroponía, los resultados muestran que hay otros taxones que son abundantes en el C- y AZO pero que desaparecen en BIOM, como *Clostridium sensu stricto* 1. Un comportamiento similar ocurre con *Pseudoxantomonas* y *Paenibacillus*, los cuales disminuyen su abundancia en BIOM con respecto al control no inoculado (C-). Esto sugiere que la aplicación de la comunidad autoensamblada no solamente aporta fijadores de nitrógeno, sino que posiblemente están involucrados otros mecanismos de promoción de crecimiento e interacciones ecológicas que en su conjunto hacen que la planta tenga un buen crecimiento. En este sentido, es vital avanzar en técnicas que permitan poder entender todas estas interacciones.

Es importante recalcar que a pesar de que las plantas usadas inicialmente para obtener el inóculo de inicio para todo el proceso de enriquecimiento y selección no eran tomate, y a pesar de que la comunidad fue cultivada con fuentes de carbono que no son las más comunes en la rizosfera, los microorganismos son capaces de responder a las plantas de tomate. Esto remarca la versatilidad metabólica de una comunidad autoensamblada en términos de poder adaptarse no solo a las condiciones de cultivo de la planta, sino también a la misma planta.

Usar el sobrenadante en hidroponía, si bien genera un crecimiento significativamente mayor al control negativo sin nitrógeno, se esperaba que alcanzara unos resultados similares a usar nitrógeno químico, teniendo en cuenta que ambos tratamientos en principio tienen la misma cantidad de nitrógeno aplicado. Sin embargo, para el sobrenadante la medición que se realizó fue solamente nitrógeno total, y con base en ello se realizó el cálculo del aporte final. Las plantas solamente asimilan nitrógeno en forma de nitrato o amonio, y la proporción de estos también es importante, por lo que además de la medición de nitrógeno total, es conveniente medir las formas de nitrógeno presentes en el sobrenadante. Es posible también que dentro de ese nitrógeno total hayan formas orgánicas del nitrógeno. Por otra parte, la medición de nitrógeno total en el sobrenadante fue realizada antes de esterilizar, es posible que durante el proceso de esterilización se haya perdido nitrógeno por volatilización. Finalmente es importante mencionar que en el sobrenadante aún se mantienen AGVs que no fueron consumidos, y hasta el momento es desconocido el efecto que estas moléculas presentes en la solución nutritiva puedan tener sobre el crecimiento de las plantas. Las razones anteriormente expuestas podrían explicar el hecho de que el sobrenadante lo generara los efectos esperados, sin embargo, es un primer punto de partida para seguir trabajando en las metodologías que permitan mejorar los resultados.

Los hallazgos obtenidos en este estudio indican la presencia de una sorprendente diversidad de microorganismos posiblemente fijadores de nitrógeno, algunos desconocidos, que podrían albergar un potencial biotecnológico significativo. La aparición de microorganismos no clasificados sugiere la dificultad de obtenerlos mediante cultivos puros. Sin embargo, el presente trabajo destaca la viabilidad de cultivar estos microorganismos en biorreactores, lo que sugiere que las condiciones propicias para su desarrollo podrían depender de las asociaciones que puedan formar con otros organismos en un entorno controlado. Este fenómeno resalta la importancia de adoptar un enfoque basado en la teoría ecológica de comunidades microbianas en donde la bioprospección no se hace a partir de la identidad de los microorganismos sino a partir de la función que son capaces de llevar a cabo en un contexto de comunidad microbiana.

La valorización de los ácidos grasos volátiles (AGVs) derivados de residuos porcinos se revela como una opción altamente prometedora para el cultivo de microorganismos fijadores de nitrógeno en biorreactores. Más allá de la mera producción de biomasa microbiana, este enfoque ha demostrado la obtención de nitrógeno combinado en el sobrenadante, sugiriendo la posibilidad de emplear los biorreactores fijadores de nitrógeno como biofábricas de nitrógeno combinado. Además, este proceso puede integrarse de manera eficiente en un contexto de biorefinería al aprovechar los AGVs generados a través de la digestión anaerobia de residuos porcinos. Este estudio representa un enfoque pionero en esta área emergente, proporcionando valiosa información inicial. Sin embargo, se reconoce que el camino hacia la optimización del proceso aún se encuentra en sus primeras etapas. Aspectos cruciales, como la evaluación de diferentes concentraciones de AGVs y modos de operación de los biorreactores, necesitan una atención más detallada. La variabilidad en la composición y cantidad de AGVs podría influir directamente en la eficiencia del cultivo microbiano y la producción de nitrógeno combinado, por lo que es esencial investigar a futuro cuidadosamente estas variables para maximizar los beneficios del sistema. Además, se vislumbra la oportunidad de explorar otros bioprocesos conectados a la fijación de nitrógeno. La posibilidad de considerar la integración con procesos como el cultivo de microalgas o la utilización de otros microorganismos de interés industrial amplía aún más el potencial de este enfoque.

Este estudio propone la utilización de inoculantes compuestos por una variedad de microorganismos como una alternativa a los biofertilizantes convencionales que se formulan a partir de cultivos puros. Aunque esta alternativa demuestra su eficacia a nivel experimental, se presentan desafíos legales que podrían restringir su aplicación. En el contexto colombiano, existe un requisito mínimo de pureza del microorganismo en los productos formulados, y cumplir con este criterio se vuelve más complejo en el caso de inoculantes compuestos. Para abordar este problema, sería esencial incorporar técnicas de secuenciación de nueva generación, las cuales permiten determinar la diversidad de especies presentes y su abundancia en el inoculante. No obstante, la implementación de estas técnicas conlleva desafíos adicionales, ya que no son pruebas de rutina y requieren la disponibilidad de equipos especializados y personal altamente calificado. Esto no solo añade una barrera logística, sino también un costo adicional a la aplicación de los inoculantes compuestos. En este contexto, resulta imperativo revisar las normativas existentes para adecuarlas a la realidad de estas nuevas tecnologías agrícolas. La revisión de las normativas sería fundamental para fomentar la adopción de inoculantes compuestos en la práctica agrícola, ya que permitiría la flexibilización de los requisitos de pureza y la integración de métodos avanzados de caracterización microbiana. Además, se podría considerar la creación de protocolos específicos que faciliten la implementación de técnicas de secuenciación de nueva generación, promoviendo la accesibilidad y la estandarización de estas herramientas.

4. Conclusión

Sección en construcción.

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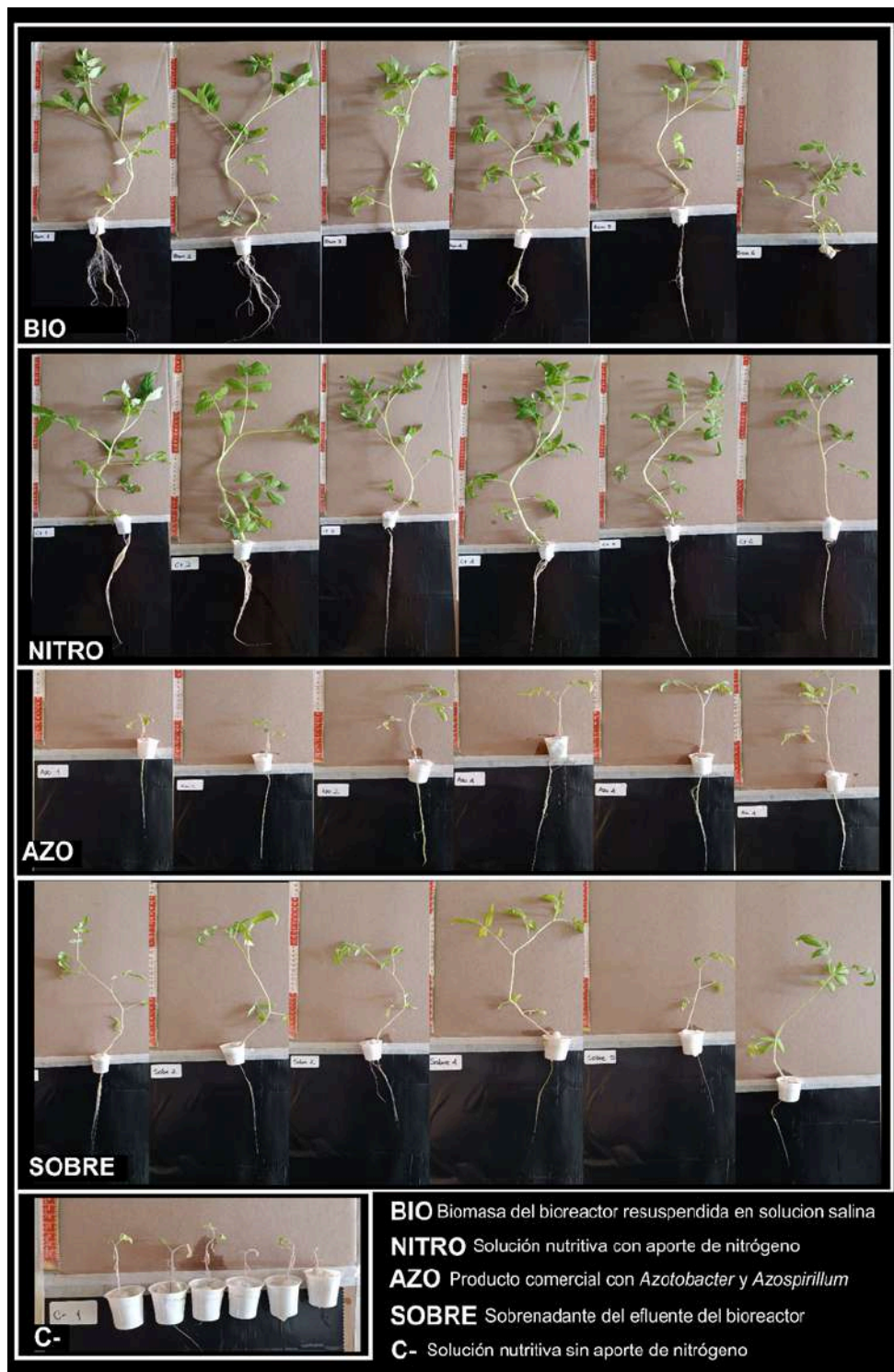


Figura S1. Fotografías de plantas de tomate de 45 días de crecimiento en hidroponía.

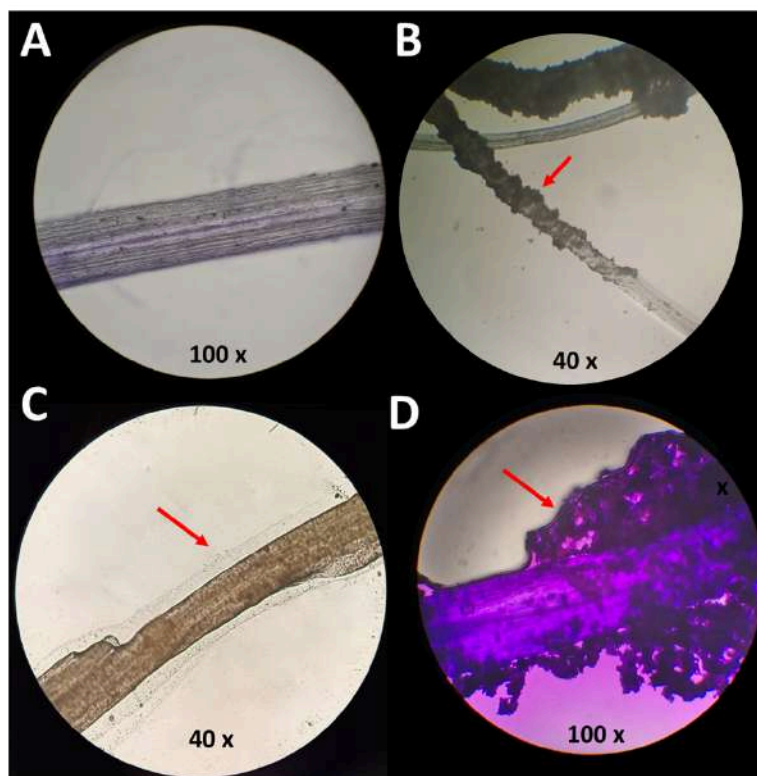


Figura S2. Fotografías microscópicas de raíces de plantas de tomate del control negativo sin aporte de nitrógeno (A) y plantas inoculadas con la biomasa resuspendida en solución salina (B-C-D).



Figura S3. Fotografías de plantas de tomate de los diferentes tratamientos crecidas en invernadero a los 35 DDS. AZO: aplicación de un producto comercial a base de *Azotobacter* y *Asospirillum*, BIOM: aplicación de la biomasa centrifugada del reactor de fijación de nitrógeno, SOBRE: aplicación del sobrenadante obtenido del reactor fijador de nitrógeno, C-: control negativo sin aporte de nitrógeno, EFLU: aplicación del efluente del reactor sin centrifugar, NE: suelo no esterilizado, E: suelo esterilizado.

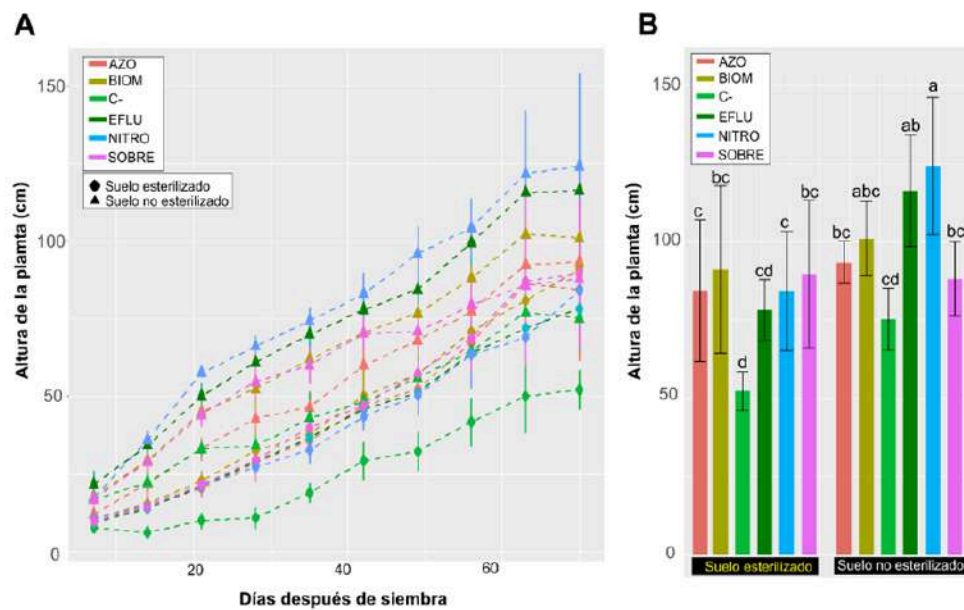


Figura S4. Altura de la planta durante el tiempo de evaluación del experimento (A) y a los 70 DDS (B). Letras diferentes muestran diferencias estadísticamente significativas (ANOVA-Tukey $p < 0.05$). AZO: aplicación de un producto comercial a base de Azotobacter y Asospirillum, BIOM: aplicación de la biomasa centrifugada del reactor de fijación de nitrógeno, SOBRE: aplicación del sobrenadante obtenido del reactor fijador de nitrógeno, C-: control negativo sin aporte de nitrógeno, EFLU: aplicación del efluente del reactor sin centrifugar.