IMPROVEMENT OF THE PSYCHROPHILIC BIOMETANATION OF CHEESE WHEY THROUGH BIOCHAR USE AS ORGANIC SUPPORT

MEJORAMIENTO DE LA BIOMETANIZACIÓN PSICROFÍLICA DE LACTOSUERO MEDIANTE EL USO DE BIOCHAR COMO SOPORTE ORGÁNICO

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Bucaramanga, April 2024

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Doctoral Thesis

Ph.D. in Chemical Engineering

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Famille

pour toujours, á jamais, peu importe

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Overall Synthesis

Tittle: improvement of the psychrophilic biometanation of cheese whey through biochar use as organic support*

Author: Jaime Jaimes Estévez**

Key words: Biofilm; Biochemical Methane Potential; Co-digestion feasibility; microbial communities, economic feasibility; life cycle assessment.

This research addressed the challenges of anaerobic digestion (AD) under psychrophilic conditions (15 °C – 25 °C), exploring the efficacy of pine wood biochar as a support material to improve methane production and metabolic efficiency. Findings indicate that biochar at a concentration < 10 g/L (optimal 30g/L and particle size of 0.575mm) enhances biomethane potential to 0.39 m^3 CH_4/kg VS_{added}, achieving 70% of mesophilic yields. Biochar's role in promoting psychrophilic metabolism was evident through its enhancement of acetoclastic activity, resulting in improved acetic acid conversion to methane and a substantial increase in *archaea*l populations, suggesting a strengthened methanogenic community. In continuous AD processes, adding biochar mitigated inhibition risks associated with volatile fatty acids (VFA) and pH fluctuations, maintaining operational stability over 170 days and demonstrating enhanced resilience to organic overloads. Biochar's presence led to hydrolytic and methanogenic activity efficiencies of 74.09±14.44% and 78.74 ± 11.30%, respectively, for organic loading rates of 1.0 kg COD/m^3d, with marked improvements even under higher loads. Implementing biochar in an 8 m^3 household biodigester showcased substantial improvements in VFA consumption, alkalinity, and methane production, with VFA removals up to 91.86%±2.32% and increases in specific biogas and methane yields by 21.2% and 29.7%, respectively. Environmental benefits were also notable, including an 85% reduction in climate change impacts and a 93.7% decrease in fossil resource consumption. This study highlights biochar's potential to significantly enhance AD processes in colder climates, offering a promising solution for waste management, energy recovery, and sustainable rural development.

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* Doctoral Thesis

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Resumen General

Título: mejoramiento de la biometanización psicrofílica de lactosuero mediante el uso de biochar como soporte orgánico*

Autor: Jaime Jaimes Estévez**

Key words: Biopelícula; Potencial Bioquímico de Metano; Co-digestión anaeróbica; comunidades microbianas, viabilidad económica; análisis de ciclo de vida.

Esta investigación abordó los desafíos de la digestión anaerobia (DA) en condiciones psicrófilas (15 °C - 25 °C), explorando la eficacia del biochar de madera de pino como material de soporte para mejorar la producción de metano y la eficiencia metabólica. Los hallazgos indican que el biochar en una concentración <10 g/L (óptima de 30g/L y tamaño de partícula de 0.575mm) aumenta el potencial de biometano a 0.39 m^3 CH_4/kg VS_agregado, alcanzando el 70% de los rendimientos mesofílicos. El rol del biochar en promover el metabolismo psicrófilo se evidenció al mejorar la actividad acetoclástica, logrando una mejor conversión de ácido acético en metano y un incremento sustancial en las poblaciones de arqueas, lo que sugiere una comunidad metanogénica fortalecida. En procesos anaeróbicos continuos, la adición de biochar mitigó los riesgos de inhibición asociados a los ácidos grasos volátiles (AGV) y fluctuaciones de pH, manteniendo la estabilidad operacional durante más de 170 días y demostrando mayor resistencia a sobrecargas orgánicas. La presencia de biochar llevó a eficiencias en las actividades hidrolítica y metanogénica del 74.09±14.44% y 78.74 ± 11.30%, respectivamente, para tasas de carga orgánica de 1.0 kg DQO/m^3d, con mejoras significativas incluso bajo cargas mayores. La implementación de biochar en un biodigestor doméstico de 8 m^3 mostró mejoras sustanciales en el consumo de AGV, alcalinidad y producción de metano, con remociones de AGV de hasta 91.86%±2.32% e incrementos en la producción específica de biogás y metano de 21.2% y 29.7%, respectivamente. Los beneficios ambientales también fueron notables, incluyendo una reducción del 85% en impactos al cambio climático y una disminución del 93.7% en el consumo de recursos fósiles. Este estudio destaca el potencial del biochar para mejorar significativamente los procesos de AD en climas fríos, ofreciendo una solución prometedora para la gestión de residuos, la recuperación de energía y el desarrollo rural sostenible.

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* Tesis doctoral

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Introductory Framework

Anaerobic digestion (AD) is a biological process that involves different populations of microorganisms whose metabolism facilitates the reduction of a substrate (considered as the nutrient source of the process) into simpler compounds. The AD process produces biogas with an energy potential (lower heating value between 13,720 - 27,440 kJ/m^3) (Hosseini & Wahid 2013) and an effluent sludge called digestate. Biogas (primarily composed of methane and carbon dioxide) and digestate, which contains most of the original substrate's nutrients (Rivera *et al*., 2012), account for 30% and 70% (in chemical oxygen demand) of the products generated during digestion, respectively. The most common uses for biogas, an energy-interest product, are heating, electricity production (thermal uses), and cooking. Meanwhile, due to its high nutritional content, digestate has been used as an organic fertilizer.

For the anaerobic process to be carried out properly, conditions such as substrate type (availability, nutrient content), inoculum source, and operating temperature must be favourable. A substrate widely studied at the laboratory scale is cheese whey (CW), the main by-product of cheese manufacturing from milk. This by-product has a considerable content of macronutrients such as lipids (1.92%), proteins (23%), and carbohydrates (55.33%), making it an ideal substrate for biodegradation and biogas production (Rico *et al*., 2015), achieving yields of up to 0.56 m^3 CH_4/kg VS under optimal temperature conditions, (namely between 35 - 37 °C) and in batch operation (Escalante *et al*., 2018). Despite the high potential for methane production from whey, its anaerobic digestion faces inhibition problems mainly due to the accumulation of volatile fatty acids (VFAs) from the fermentation of carbohydrates, lipids, and proteins. As a solution to inhibition issues, the use of two or more substrates (Anaerobic Co-Digestion; ACoD) is presented. In this case, co-substrates minimize deficiencies or enhance the system's characteristics thanks to the synergy of the mix. Therefore, in an inhibited AD process, it is important to identify an appropriate co-substrate and a favourable mixing ratio in order to generate positive interactions (synergism), nutritional balance, and improve biogas production yields and nutrient disposal in the digestate (Jaimes-Estévez *et al.*, 2020). A co-substrate with positive synergistic effects for the co-digestion of whey is cattle manure (CM). Among its characteristics, CM provides the system with alkalinity (1,800 mg/L manure) and nutrients to balance acidity and organic load. The mixing ratio established as most favourable for the CoAD of CW:CM is 70:30, which allows obtaining yields around 0.6 m^3 CH_4/kg VS with a reduced VFA content (360 mg HAc/L).

When discussing the effect of temperature, AD in cold climates (between 5 and 25 °C) presents various challenges due to operational, environmental, physicochemical, and microbiological issues. Regarding operational challenges, there may be an increase in the viscosity of the liquid medium, leading to higher mixing energy requirements and, notably, a decrease in the diffusion of soluble compounds and contact between biomass-substrate. This results in lower biogas production yields and organic matter consumption (Dev *et al.*, 2019). Furthermore, physical and chemical changes in the properties of substrates or digestate can significantly affect the treatment system's performance. For instance, increases in the solubility of gases contained in the liquid phase, such as H_2, H_2S, and CO_2, can lower the medium's pH (Lettinga *et al*., 2001). The accumulation of these compounds in the liquid phase and low rates of organic matter removal can lead to environmental issues due to poor digestate quality, potentially causing soil eutrophication, pathogen spread, and greenhouse gas emissions (Castro *et al*., 2017).

Regards a microbiological standpoint, most methanogenic *archaea* are mesophiles responsible for acetate metabolism, with an optimal growth temperature of 37 °C. Therefore, temperatures below 25 °C hinder their microbial activity as substrate transport through the cell membrane is inhibited (Dev *et al*., 2019). This translates to lower biogas quality and accumulation of VFAs, represented respectively by (i) methane contents below 50% and an increase in H_2S content, and (ii) a decrease in the system's pH, leading to stability issues such as medium acidification. Regarding the CW:CM co-digestion, despite positive synergistic effects due to nutritional balance, yields only reach around 0.2 m^3 CH_4/kg VS when operated at low temperatures (Jaimes-Estévez *et al*., 2018). A new strategy to mitigate limitations of anaerobic digestion or co-digestion under psychrophilic conditions is the combination of increased biomass concentration and retention to enhance inoculum/substrate interactions. One possible alternative to achieve these conditions is the addition of carriers for biofilm formation within the bioreactor (Martí-Herrero *et al*., 2014; Martí-Herrero *et al*., 2018). Using carrier materials improves organic loading capacities, allows biomass increase or concentration within the reactor, enhances resistance to hydraulic or organic overloads, eliminates the need for mechanical mixing during the bioprocess, and significantly reduces the adaptation time of the microbial consortium to the substrate (Karadag *et al*., 2015).

When using a support material in AD, microorganisms adhere to the surface, forming colonies on it. Since substrate consumption is inherent in the digestion process, for process improvement to occur, diffusion of all "reactants" (organic matter to be consumed) from the liquid phase to the biofilm and the return of metabolic waste (products) to the liquid phase are necessary. Thus, a continuous change in substrate concentration between the liquid and the biofilm is facilitated (Williamson & McCarty, 1976). This behaviour can be described by Fick's law, which defines mass transfer within the biofilm per unit area, as follows:

$$\frac{\partial S_M}{\partial t} = -A_c D_c (\partial S_c / \partial z)$$

Where $\frac{\partial S_M}{\partial t}$ represents the transfer rate of a characteristic substrate S (mg/d); A_c is the biofilm area; D_c is the diffusion coefficient within the biofilm (cm^2/d), and $\partial S_c / \partial z$ symbolizes the substrate gradient perpendicular to the surface (mg/cm^4). S_c is the substrate concentration within the biofilm matrix in mg/L. On the other hand, the substrate consumption rate *S* at any point in the biofilm can be represented by the Monod equation:

$$-\frac{\partial S_c}{\partial t} = \frac{k S_c X_c}{(S_c + K_s)}$$

where $-\frac{\partial S_c}{\partial t}$ is the consumption rate of S (mg/Ld); k is the maximum substrate consumption rate (mg $_{\text{substrate consumed}}$/mg $_{\text{microorganisms}}$d); k is the Monod half-velocity coefficient (mg/L), and *Xc* is the biomass concentration within the biofilm (mg/L). This equation shows the effect of biomass concentration on substrate consumption and, therefore, on product generation. In systems utilizing biofilms, *Xc* is higher compared to conventional digestion systems (Qiu *et al*., 2019). Thus, it is imperative to mention that *Xc* can change according to operational conditions (pH, organic load), environmental factors (temperature), and the nature of the support material. Novel, low-cost, and readily available alternatives as support material for biofilm formation include biochar (particulate material). Biochar has been shown to promote the growth of microorganisms, favouring the colonization of acetogenic bacteria and methanogenic *archaea* (Qiu *et al*., 2019). He *et al*., (2020) studied the behaviour of biogas production using biochar in a batch process under mesophilic conditions, reporting that biochar utilization can maintain stable methane production during anaerobic digestion even with increasing organic loads. Additionally, the authors argue that biochar promotes the growth of specific microorganisms such as *Methanosarcina* and *Methanosaeta*, which metabolize acetate specifically for CH_4 production. Each of the described research represents an advancement in the improvement of the digestion process, mainly reflected in increased yield and biogas quality. However, these studies are limited to improve biogas production in a batch or upflow anaerobic sludge blanket digestion processes under mesophilic conditions (temperature controlled between 25 - 42 ºC). This shows there are no reports on the ACoD process of substrates such as CW under psychrophilic conditions, including the use of support materials and the study of its influence on process metabolism. Likewise, there is limited knowledge about the effect of using organic supports (such as biochar for biofilm formation) on the metabolic activities of the microbial communities involved in digestion, which impact biogas yield and quality and process stability. From this perspective, this research aims to study the effect biochar as an organic support on the biochemistry and microbiology of the anaerobic co-digestion process of CW and CM under psychrophilic conditions.

Justification. In developing countries, approximately 52% of rural areas are categorized as "non-interconnected" and are difficult to access due to their tertiary roads. In these areas, people use firewood and/or propane gas as cooking fuel. However, the use of firewood leads to environmental impacts such as deforestation, while propane presents economic (cost of gas cylinder and transportation) and technical limitations (risk in propane tank management) for fuel access. In this context, biogas production through AD is an attractive renewable energy alternative that helps mitigate the mentioned impacts and limitations. Currently, in Latin America, the most commonly used systems to carry out the anaerobic process are tubular digesters (TD). TDs consist of a plastic bag (usually made of polyethylene), PVC inlet and outlet, and a pipe to collect biogas from the digester to a reservoir. The design of TDs is characterized by: a) low construction, operation, and maintenance costs; b) on-site energy generation; and c) no agitation or heating, making them more feasible in terms of handling and installation. The benefits of TDs are associated with organic matter stabilization (mainly manure), biogas production as a renewable fuel, reduction of greenhouse gases, and agronomic use of digestate. Particularly in Colombia, there are approximately 5,000 installed digesters (Tavera-Ruiz *et al.*, 2023). These digesters carry out anaerobic mono-digestion processes and are fed mainly with CM and pig manure. The gas produced in these small and medium biogas plants is used for cooking and heating for animals. As for the digestate, it is the main product of digestion in terms of mass (70% of the final product), which is a mixture of microbial biomass and material not degraded during bioconversion that is used as fertilizer. Livestock activity is a fundamental pillar for the economy and livelihoods of rural populations, representing about 19% of the population in the country. For instance, in the department of Santander, approximately 1.7 million hectares of the total department are dedicated to livestock farming, mainly to dual-purpose cattle (meat and milk production), where 83% of bovine production is achieved through backyard and non-technified activities. This characterizes the department with the presence of 1.4 million heads generating 734 thousand litters of milk per day. The major residues generated for each purpose are CM and CW (from milk used for cheese making).

AD systems installed in rural areas are mostly fed with CM (Martí-Herrero 2019). In order to improve biogas production yields, it is common to use co-substrates. Co-digestion allows for the synergy of the mixture, minimizing deficiencies or improving the characteristics of each substrate (Sanchez *et al*., 2023). Considering the bovine production chain, whey is an ideal co-substrate due to its concentration of easily biodegradable soluble organic matter, which enhances biogas yield from bovine manure. Additionally, the performance of low-cost tubular digesters is heavily influenced by temperature. Santander Department exhibits a diversity of geographical and meteorological conditions leading to a wide range of thermal floors, allowing for the presence of psychrophilic conditions (< 25°C) to mesophilic regimes (>25°C and <45°C) throughout the year. This demonstrates the potential applicability and the need to research anaerobic digestion technology at low temperatures.

To approach the research question of the thesis, aspects as microbiology, biochemistry, and engineering were studied, enabling an understanding of the effect of biochar on psychrophilic anaerobic digestion, using CW and CM as nutrient sources. To address these topics, this doctoral thesis was written in 4 main chapters.

In Chapter I, the effect of pine wood biochar on kinetics, acid metabolism, and microbial population under psychrophilic conditions is studied through biometanization potential assays.

In the second chapter, pine wood biochar is evaluated as a modulator of metabolic efficiencies of AD in continuous bench-scale operation, varying organic load rates.

In Chapter 3, the environmental and economic impact of implementing an integrated gasification + anaerobic digestion system for CW and CM in a rural psychrophilic scenario was studied. This was done through a life cycle analysis, and capital and operational cost assessments, focusing on monitoring an 8 m^3 biodigester.

In the final chapter an outlook from an urban perspective was addressed, where the effects of biochar on psychrophilic AD of a highly biodegradable substrate such as kitchen wastewater (KWW) were evaluated, assessing the kinetics, biochemistry, and energy potential of the process.

Chapter I. The role of biochar in the psychrophilic anaerobic digestion: effects on kinetics, acids metabolism, and microbial population

1. The role of biochar in the psychrophilic anaerobic digestion: effects on kinetics, acids metabolism, and microbial population

Abstract

This study investigated the effect of biochar in the psychrophilic anaerobic co-digestion regarding biomethane production potential (BMP), metabolic efficiencies, and microbial population. BMP tests of cheese whey and cattle manure as substrates were conducted at different gasified pine wood biochar concentrations (Bc) (10g/L, 30g/L, 50g/L); and particle sizes (Ps) (~0.15mm, ~0.575 mm, ~1mm). The most favourable conditions of Ps = 0.575mm and Bc of 30g/L, allowed BMP values to go from $0.23m^3CH_4/kgVS_{add}$ to $0.34m^3CH_4/kgVS_{add}$. The study of metabolic stages showed how the biochar modulates hydrolysis and methanogenesis and favours the acetoclastic metabolism to improve methane yield even at 15°C. The biochar's positive effect is reinforced by its addition boosting the growth of methanogen psychrotrophs populations up to 520% compared with a BMP with no biochar added at 15°C. The study showed that psychrophilic AD + biochar might overcome mesophilia's energy needs by improving yields with no extra energetic requirements.

1.1. Introduction

In the AD process, temperature plays an important role, because it significantly influences metabolism performance, so, AD is classified into three types: psychrophilic, which occurs below 20 °C; mesophilic, between 20 °C and 43°C (where 35 °C – 37 °C are considered as optimal); and thermophilic, which is performed at higher temperatures as 50 °C and 60 °C (Fernández-Rodriguez *et al.,* 2016; Zhang *et al.,* 2014). At temperatures below 25 °C, biochemical reaction velocity is reduced considerably compared to higher ones (25 °C < T <

42 °C). Also, psychrophilic conditions lead to a decrease in the microorganism growth, and substrate consumption rates (Lettinga *et al.,* 2001). So, some improvements must be made in the process to enhance their application under sub-optimal temperatures. Accordingly, increasing hydraulic retention time could be profitable, but it implies a larger digester size. Another alternative is to incorporate the bioclimatic design by implementing a passive solar heating design such as insulation or a greenhouse covering the digester (Perrigault *et al.,* 2014; Jaimes-Estévez *et al.,* 2021). Moreover, biogas yield can be enhanced by utilizing cold-adapted microorganisms such as psychrophiles and cold-adaptable psychrotrophs, which can grow at temperatures below 15 °C (with an optimal temperature of 20 °C) (Feller 2015; Akindolire *et al.,* 2022). Also, an anaerobic co-digestion (ACoD) process that guarantees synergistic effects during the biodegradative process is a favourable option that improves biogas production and the economic viability of implementing the technology (McKeown *et al.,* 2012). Nevertheless, even with positive synergistic effects thanks to the nutritional balance, if the process is operated at low temperatures, the yields are reduced with regards mesophilia (Dev *et al.,* 2019; Jaimes-Estévez *et al.,* 2022b).

A new strategy to mitigate the limitations of ACoD under psychrophilic conditions is the combination of concentration increase and biomass retention to improve inoculum/substrate interactions (Tiwari *et al.,* 2021). A possible alternative to achieve these conditions is the addition of supports (carriers) for the biofilm formation inside the bioreactor (Martí-Herrero *et al.,* 2014; Martí-Herrero *et al.,* 2018; Chiappero *et al*., 2020). For example, Cruz Viggi *et al.,* (2017) assessed the impact of biochar from different materials in AD at upper limit of psychrophilia (20ºC), finding similar final specific methane production with or without biochar addition, but faster production when biochar is added. This is affirmed by the improvement in interspecies electron transfer rate between microorganisms, but, the biochar effect in other metabolic stages is not considered.

Jang *et al.,* (2018) showed the effect of the biochar in psychrophilic (20ºC), mesophilic (35°C), and thermophilic (55°C) AD conditions. Using biochar increased AD methane yield by 26.47%, 24.9 %, and 24.69% for psychrophilic, mesophilic, and thermophilic, respectively, compared with no biochar assays. Despite the inoculum used by Jang *et al.,* (2018) was not pre-acclimated to 20ºC, and the test was run for the same time than mesophilic and thermophilic BMPs (which worsen psychrophilic AD yields as suggested by Martí-Herrero *et al.,* (2022), results showed that adding biochar to psychrophilic AD can achieve better effects than in mesophilia. But also, 20 ºC is a range of temperature for AD where psychrothophic microorganisms can show maximum activity (Akindolire *et al.,* 2022). Despite the increasing number of research proving the biochar's capability to improve the anaerobic digestion process, there is a gap in the knowledge about biochar influence in metabolic activities and kinetic in a "pure" psychrophilic conditions' scenario (T < 20 °C). This is reinforced by the fact that temperature reduction exponentially affects methane production, so a five Celsius degrees reduction (from 20 °C to 15°C) can bring down more than 15% of the methanogenic activity (Lettinga *et al.,* 2001). The above makes it necessary to know favourable biochar concentration and particle size and support influence on kinetics and microorganisms growth in AD under 20 °C. As a result of this knowledge gap, the psychrophilic AD yield can be misestimated as biochar's impact on the overall process performance can be different than mesophilic. Hence, this research aims to determine biochar's incidence on methane production and the metabolic efficiencies of the anaerobic co-digestion process under psychrophilic conditions at 15 ºC. To promote the stabilization of agro-industrial wastes, the substrates employed in this ACoD process were cheese whey (CW) and cattle manure (CM). Those residues are derived from the dairy industry's productive chain, which enhances food security and serves as a significant source of employment and income for millions of small-scale farming families. This study represents a meaningful alternative for small to medium enterprises or household scenarios to manage challenging wastes such as CW.

1.2. Material and Methods

This study was developed in three stages: i) evaluation of biochar concentration and particle size on CW:CM biochemical methane potential (BMP) under psychrophilic (15 °C) and mesophilic (35 °C) conditions, ii) determination of the process metabolic efficiencies modulated by biochar, and iii) changes in microbial populations after AD.

1.2.1. Raw Materials and Inoculum Sources

CW was obtained from a dairy enterprise that treats around 1.6 m^3 of milk daily, generating nearly 1 m^3 of the substrate. Fresh CM and inoculum were recollected from a Colombian farm (latitude of N 7°0100.0700W73°08013.300 with an average temperature of 23 ± 5 °C) that produces biogas and digestate from CM treatment via AD in a 9.5 m^3 tubular digester. Further, as Martí-Herrero *et al.,* (2022) suggested, the inoculum used was pre-adapted separately at the two temperature assay conditions (15 °C and 35 °C). The acclimatization lasted for 70 days, feeding the inoculum every two weeks with an acetic acid solution (200 g/L), maintaining an inoculum/acetic acid ratio of 5 (volatile solid basis). After its use, the inoculum presented a specific methanogenic activity of 0.030 g COD CH_4/g VS*d and 0.056 g COD CH_4/g VS*d at 15 °C and 35 °C, respectively.

The biochar was obtained from the gasification (GS) of recycled pine wood in a 40 L fixed bed equipment with ascendant air flux (450 L/min on average). The GS was conducted in batches (10 - 15 kg pine wood/load) at temperatures between 500 - 600°C. Then, biochar was grinded and sieved to obtain desired particle size (0.15 – 1mm) and dried at 105 ± 2 °C for 24 h prior to use. The biochar characterization is presented in Table 1.

Table 1. Physicochemical characteristics of pine wood biochar

Analysis	**Contents**	**Units**	**Pine wood biochar**
Size[a]	Pore diameter	µm	11.45
Langmuir Parameters[b]	Adsorption maximum capacity (qeq)	mg/g	10.12
	Affinity with adsorbate (kL)	L/g	11.048
physicochemical	pH	---	9.17
	Electric conductivity	mS/cm	57.40
	Ash	%	5.98
Surface elemental composition[c]	C	Wt%	81.75
	O		13.89
	Na		0.62
	Mg		0.46
	K		1.83
	Ca		1.47

[a] Determined by Scanning Electron Microscopy (SEM).

[b] Determined by adsorption test of pollutant (CEFIC 1986)

[c] Determined by Energy-dispersive X-ray spectroscopy (EDS).

1.2.2. Multivariable optimization of multiple responses: BMP and VFA content.

A simultaneous optimization was proposed to determine the effect of biochar, guaranteeing well-conducted biomethane production and high consumption of soluble compounds. The optimization criteria were the maximization of BMP and the minimization of ultimate total volatile fatty acids (tVFA) concentration (the most significant consumption of VFA). So, the influence of particle size (Ps) and biochar concentration (Bc) on the BMP and tVFA was evaluated at 15°C using the 3^2 factorial design and analyzed using the response surface methodology. Three levels of Bc and Ps were selected: 10 g/L, 30 g/L, and 50 g/L and 0.15 mm, 0.575 mm, and 1 mm, respectively. Those values were selected to cover some ranges that have been studied in the literature (Zhao *et al.,* 2021). To meet the Ps required for the experimental design, biochar size was sorted in a vibratory sieve shaker. Biochar was sieved through mesh sizes of 200 to 100 for the low level, corresponding to particle sizes within the 0.074 mm - 0.150 mm range. For the high particle size level, the biochar was sieved through mesh sizes 20 to 18, which yielded particles within the size range of 0.841 mm - 1.00 mm. To obtain the medium Ps distribution for the experimental design (0.575 mm), two different sizes of biochar particles were mixed. This involved combining 12% from a particle size range of 0.420 mm - 0.500 mm (sieved through mesh 40-35) and 88% from a size range of 0.500 mm - 0.595 mm (sieved through mesh 35-30). In the upcoming sections of this document, these particle sizes were denoted as 0.15 mm, 0.575 mm, and 1 mm.

The desirability criteria were chosen to find the experimental conditions (factor levels) to reach, simultaneously, the optimal value for BMP and tVFA (Candioti *et al.,* 2014). The experimental results for the response variables were adjusted to the second-order expression presented in Eq. 1:

$$y = \alpha_0 + \alpha_1 * Ps + \alpha_2 * Bc + \alpha_3 * Ps^2 + \alpha_5 * Ps * Bc + \alpha_6 * Bc^2 \qquad \text{Eq. 1}$$

Where y symbolizes the response variable (either BMP or final tVFA), α_0 is a constant, α_1, and α_2 are linear coefficients, α_5 is an interaction coefficient, and α_3 and α_6 are quadratic coefficients. To compare the psychrophilic behaviour with the best temperature conditions, this set of experiments was performed at 35°C too. BMP tests were conducted considering the production of methane by substrates bioconversion, subtracting endogenous methane production by inoculum (blank assay) at psychrophilic (15 ± 2 °C) and mesophilic (35 ± 2 °C) temperature conditions. For comparison, control assays (CW:CM co-digestion with no biochar) and blank assays (inoculum without biochar neither substrates) were performed to contrast the effect of biochar on methane yield at psychrophilic conditions (Control 15°C; Blank 15°C), and optimum temperature condition (Control 35°C; Blank 35°C). Table 2 shows the conditions evaluated in the experimental design. The assays were sets of triplicates in 120 mL glass flasks, with an inoculum/substrate ratio of 2 (VS basis). The CW:CM ratio was established at 70:30 (on a volatile solids basis) to evaluate a favourable mixing ratio (Jaimes-Estévez *et al.,* 2022a). The methane production was measured daily by pressure determination and gas chromatography (Angelidaki *et al.,* 2011; Holliger *et al.,* 2016). Assays were finalized when the daily methane quantity was undetectable or less than 1% of the total produced.

Table 2. Experimental design data for the biochar effect evaluation on CW:CM BMP and final tVFA content.

Assay ID	Assay temperature °C	Inoculum	Substrate	Support	Particle size (mm)	Biochar concentration (g/L)
A1	15	Stabilized cattle manure at 15 °C	Cheese whey and cattle manure (70:30 VS basis)	Gasified pine Wood	0.15	10
A2					0.15	30
A3					0.15	50
A4					0.575	10
A5					0.575	30
A6					0.575	50
A7					1	10
A8					1	30
A9					1	50
A10	35	Stabilized cattle manure at 35 °C	Cheese whey and cattle manure (70:30 VS basis)	Gasified pine wood	0.15	10
A11					0.15	30
A12					0.15	50
A13					0.575	10
A14					0.575	30
A15					0.575	50
A16					1	10
A17					1	30
A18					1	50

Then, the tVFA was quantified by adding the individual VFA (C2-C6) concentration determined via chromatography (Raposo *et al.,* 2013). The statistical significance of the experimental results was assessed using a one-way ANOVA with a confidence level of 95%, with p-values less than 0.05 considered significant.

1.2.3. Effect of biochar on kinetic and ACoD process efficiencies:

The BMP was replied to under the most favourable conditions obtained in the previous section. The inoculum used for validation was the same of the experimental design but with

50 days of extra acclimatization. Results were modelled to validate the methane production and study biochar's effect on the kinetic parameters involved in AD. The above was done by fitting the experimental methane production data with the modified Gompertz model (Eq. 2) (Shi *et al.,* 2022).

$$P(t) = P_0 * \exp\left\{- \exp\left[\left(K_{max} * e * \frac{\lambda - t}{P_0} + 1\right)\right]\right\} \qquad \text{Eq. 2}$$

Where, P(t) (m^3 CH_4 kg/ VS_{add}) is the cumulative methane production at time t (d), P_0 (m^3 CH_4 kg/VS_{add}) is the ultimate methane yield, K_{max} (m^3 CH_4 kg /VS_{add} d) is the maximum methane production rate, *e* is the Euler's constant, and λ (d) is the adaptation period (lag-phase). The Levenberg-Marquard algorithm and non-linear regression were used to determine the numerical and kinetic parameters (Statistica 10.0 software). The coefficient of determination (R^2) and root mean square error (RMSE) were also employed to describe the adjustment level between the experimental and the predicted BMP.

1.2.4. Stability of the ACoD process: effect of biochar on metabolic stages

To establish the efficiencies of hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Eq. 3 to Eq. 6) during the validation BMP assay, total chemical oxygen demand fed (COD_{total}), soluble COD ($COD_{soluble}$), tVFA (COD_{tVFA}), and the equivalence of methane produced on COD ($COD_{methane}$) were measured periodically from six sacrifice assays (three for inoculum + biochar and three for CW:CM + inoculum + biochar, under same conditions from BMP validation). The efficiencies were determined as follows (Niu *et al.,* 2014):

$$Hydrolysis\ (\%) = \frac{COD_{soluble} - COD_{soluble,in} + COD_{methane}}{COD_{total} - COD_{soluble,in}} \qquad \text{Eq 3.}$$

$$Acidogenesis\ (\%) = \frac{COD_{tVFA} - COD_{tVFA,in} + COD_{methane}}{COD_{total} - COD_{tVFA,in}} \qquad \text{Eq 4.}$$

$$Acetogenesis\ (\%) = \frac{COD_{acetate} - COD_{acetate,in} + COD_{methane}}{COD_{total} - COD_{acetate,in}} \qquad \text{Eq 5.}$$

$$Methanogenesis\ (\%) = \frac{COD_{methane}}{COD_{total}} \qquad \text{Eq 6.}$$

$COD_{soluble,\ in}$, $COD_{tVFA,\ in}$, and $COD_{acetate,in}$, were the corresponding values measured at the beginning of the validation. Acetate was considered equivalent to acetic acid concentration from the tVFA values. $COD_{methane}$ was calculated considering the coefficient of 350 mL CH_4/g COD at pressure and temperature standard condition (101.325 kPa, 273.15 K). All values are expressed in net mass units (grams of COD), subtracting the inoculum + biochar assay respective value.

1.2.4. Microbiological Analysis

Quantitative Polymerase Chain Reaction (qPCR) assays were used to measure the effect of biochar on the microbial population counts (total bacteria, total *archaea*, and total methanogens) during BMP assays under psychrophilic conditions (15 ± 2 °C). The analysed samples corresponded to those having the highest BMP and lowest tVFA concentrations after the optimization described in section 2.2. For comparative purposes, qPCR determinations were also performed to a blank assay (inoculum) and applied for control assays at 35 °C. Once sampled from digesters, all samples were conserved at -20°C until further processing.

Total genomic DNA was extracted directly from samples using the DNeasy® PowerSoil Kit (QIAGEN®, Venlo, The Netherlands). Before extraction, liquid samples were thawed, and homogenized by vortexing and 1 mL was centrifuged at 10,000 rpm for 5 min to collect solids and microorganisms. The supernatant liquid was discarded, and the pellet was used as the starting material for DNA extraction following the manufacturer's protocol. After extraction, DNA quantity and quality were measured by agarose gel electrophoresis and by the ratio of absorbance at 260 nm and 280 nm using an Implen NP80 NanoPhotometer® (Implen GmbH). qPCR assays were carried out on a CFX96™ Touch Real-Time PCR Detection System C1000 (BIO-RAD), using the SYBR green-based Luna® Universal qPCR Master Mix kit (New England Biolabs). The abundance of bacterial, archaeal, and methanogenic communities in samples was determined individually by amplifying and quantifying three genes of interest using the primers described in Table 3.

Table 3. Primers used in qPCR assays for microbial quantitation.

Target	**Gene of interest**	**Primer**	**Sequence (5' – 3')**	**Amplicon size**	**Reference**
Total bacteria	β-subunit of bacterial RNA polymerase (rpoB)	Univ_rpoB_F Univ_rpoB_R	GGYTWYGAAGTNCGHGACGTDCA TGACGYTGCATGTTBGMRCCCATMA	460 bp	Ogier *et al.*, (2019)
Total *Archaea*	16S rRNA (V6 – V8 regions)	Arch915F Arch1059R	AGGAATTGGCGGGGGAGCAC GCCATGCACCWCCTCT	120 bp	Yu *et al.,* (2005)
Total methanogens	Methyl coenzyme M reductase (mcrA)	MLfF MLfR	GGTGGTGTMGGATTCACACARTAYGC TTCATTGCRTAGTTWGGRTAGTT	470 bp	Luton *et al.*, (2002)

Absolute gene quantifications were performed by constructing a standard curve for each of the three genes and plotting on a log-linear scale the quantification cycle (Cq) values against known amounts of the target DNA. Data were expressed as gene copy number/µL. One-way analysis of variance (ANOVA) with multiple comparisons test (Fisher-LSD test and Bonferroni test) was used to estimate any statistically significant differences between the means of microbial counts.

1.2.5. Physicochemical Analysis

The analysis of COD, total solids (TS), and volatile solids (VS) were performed according to standard methods for the examination of water and wastewater (APHA 2017). A transductor was used to measure biogas production pressure. The compositions of methane and carbon dioxide in biogas were detected by gas chromatography (Holliger *et al*., 2016). The analysis was conducted using a gas custom chromatograph (SRI Instruments) equipped with a thermal conductivity detector (TCD) for the quantification of gas components. A 6' packed column filled with HayeSep D was employed to achieve effective compounds separison. Helium was used as the carrier gas due to its superior thermal conductivity and inertness, providing enhanced sensitivity for the TCD. Total and Individual VFA concentration (C2 – C6: acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, and caproic acid) were determined according to Raposo *et al.,* (2013) using a BP21 GC capillary column (treated polyethylene glycol as packing material) coupled to a flame ionization detector. Scanning Electron Microscopy and Energy-dispersive X-ray spectroscopy determined biochar pore size and elemental composition, respectively.

1.3. Results and Discussion

1.3.1. Biochemical methane potential boosted by biochar.

Figures 1a and 1b show the response surfaces described by the second-order fit equations for BMP and final VFA at psychrophilic and mesophilic temperatures, respectively.

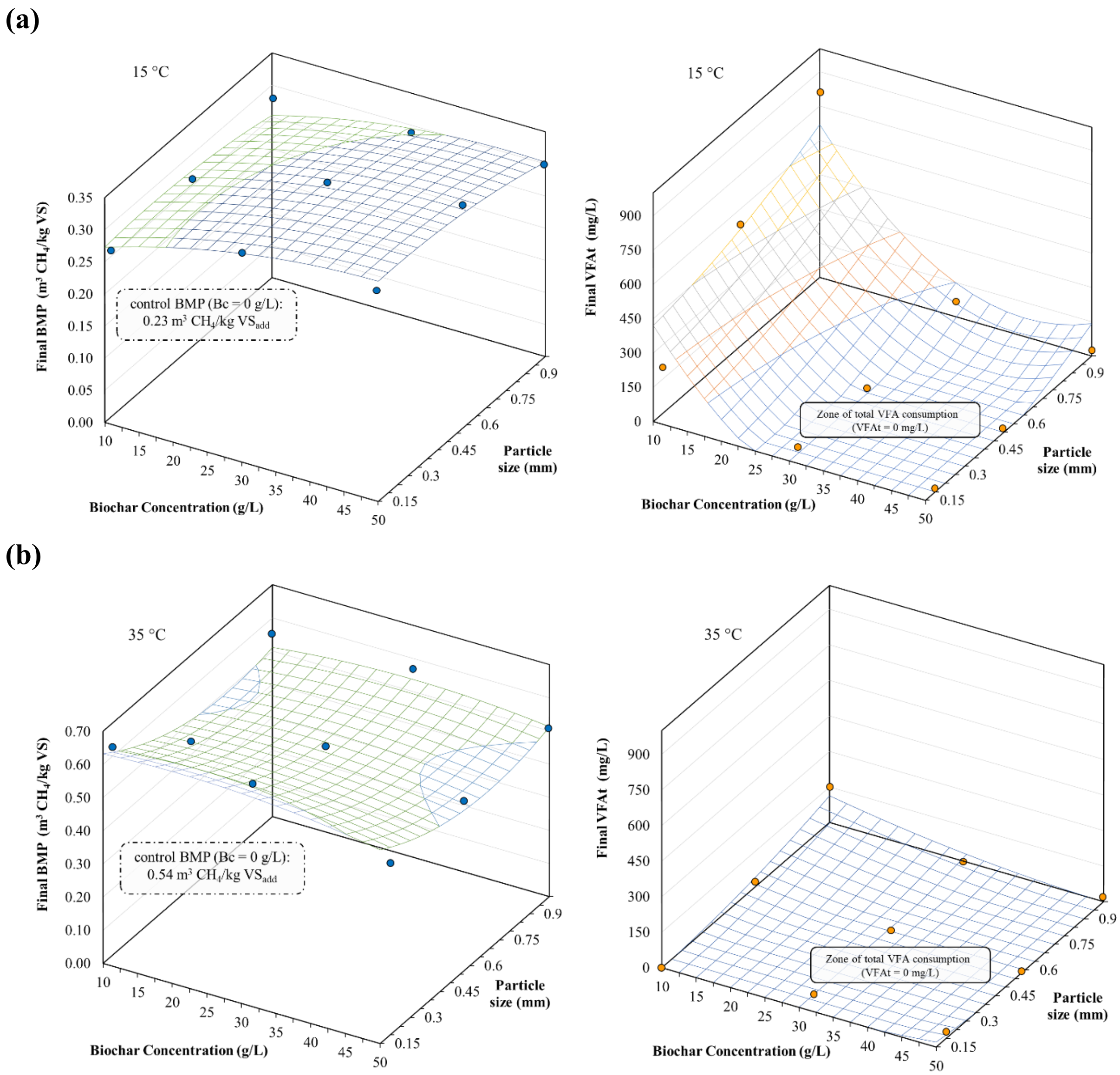

Figure 1. 3D plots for BMP and VFA as functions of biochar concentration and particle size at 15 °C (a) and 35 °C (b).

The equations were obtained based on the mathematical regression model are as follows:

In psychrophilic conditions

$$BMP = 0.22740 + 0.05663 \cdot Ps + 0.00434 \cdot Bc - 0.06459 \cdot Ps^2 - 0.00058 \cdot Ps \cdot Bc - 0.00004 \cdot Bc^2 \quad \text{(Eq. 7)}$$

$$tVFA = 468.48 + 727.423 \cdot Ps - 38.6102 \cdot Bc + 216.747 \cdot Ps^2 - 22.8 \cdot Ps \cdot Bc + 0.64225 \cdot Bc^2 \quad \text{(Eq. 8)}$$

In mesophilic conditions

$$BMP = 0.6909 - 0.5716 \cdot Ps + 0.0023 \cdot Bc + 0.35982 \cdot Ps^2 + 0.001470 \cdot Ps \cdot Bc - 0.00006 \cdot Bc^2 \quad \text{(Eq. 9)}$$

$$tVFA = 14.0043 + 31.2354 \cdot Ps - 2.34433 \cdot Bc + 125.364 \cdot Ps^2 - 4.01414 \cdot Ps \cdot Bc + 0.05864 \cdot Bc^2 \quad \text{(Eq. 10)}$$

Table 4 presents the ANOVA analysis for each coded equation (7 - 10). According to the R^2 values found from the fit, the expressions for the BMP and the VFA explained the behaviour of the experimental data in 82.67 % and 93.75 % and in 84.42% and 81.39 % at 15°C and 35°. C, respectively. The above indicates that the model equations can explain the experimental results. Nonetheless, there is no significant influence on all responses regarding the independent variable's interaction ($Bc \cdot Ps$) and quadratics effects. Under psychrophilic conditions, the variable with the highest effect on BMP and tVFA is biochar concentration ($P\text{-value} < 0.05$). Experimentally, adding an adequate biochar proportion can increase the BMP by 43 % compared to the ACoD with no biochar (control BMP at 15°C = 0.23 m^3 CH_4/kg VS_{add}). So, a $Bc \geq 30$g/L allows the total reduction of VFA (zone of total VFA consumption in Figure 1), thus, a higher methane generation.

Table 4. ANOVA table for the BMP and final tVFA as output responses for psychrophilic and mesophilic conditions.

Case	Source	Sum of Squares	df	Mean Square	p-Value
BMP at 15 °C	Bc (Biochar concentration)	5.40E-03	1	5.40E-03	0.0498*
	Ps (Particle size)	1.35E-03	1	1.35E-03	0.2181
	Bc^2	5.56E-04	1	5.56E-04	0.3924
	Bc·Ps**	1.00E-04	1	1.00E-04	0.7009
	Ps^2	2.72E-04	1	2.72E-04	0.5356
	Total error	1.68E-03	3	5.59E-04	
	Total (corr.)	9.36E-03	8	R^2	0.8267
tVFA at 15°C	Bc	4.13E+05	1	4.13E+05	0.0167*
	Ps	9.99E+04	1	9.99E+04	0.0972
	Bc^2	1.24E+05	1	1.24E+05	0.0767
	Bc·Ps**	1.50E+05	1	1.50E+05	0.0613
	Ps^2	3.42E+03	1	3.42E+03	0.689
	Total error	5.27E+04	3	1.76E+04	
	Total (corr.)	8.43E+05	8	R^2	0.9375
BMP at 35 °C	Bc	8.17E-04	1	8.17E-04	0.5201
	Ps	1.40E-02	1	1.40E-02	0.0527
	Bc^2	1.25E-03	1	1.25E-03	0.435
	Bc·Ps**	6.25E-04	1	6.25E-04	0.5702
	Ps^2	8.45E-03	1	8.45E-03	0.1015
	Total error	4.64E-03	3	1.55E-03	
	Total (corr.)	2.98E-02	8	R^2	0.8442
tVFA at 35 °C	Bc	3.08E+03	1	3.08E+03	0.2108
	Ps	3.28E+03	1	3.28E+03	0.2006
	Bc^2	1.10E+03	1	1.10E+03	0.4133
	Bc·Ps**	4.66E+03	1	4.66E+03	0.1464
	Ps^2	1.03E+03	1	1.03E+03	0.4278
	Total error	3.68E+03	3	1.23E+03	
	Total (corr.)	1.68E+04	8	R^2	0.8139

*Effects considered as significant. **Independent variable's interaction

Regarding mesophilia, the studied values showed minor changes. At 35 °C, the average BMP and final VFA removal values were 0.55 ± 0.06 m^3 CH_4/kg VS_{add}, and 97.58 ± 6.35 %, respectively. Those changes were 0.1% and 1.82 % higher than the assay with no biochar addition. So, in mesophilia, biochar addition slightly influences biomethane production, where Ps presented the highest effect (P-value = 0.0527). This behaviour was similar to that reported by Madrigal *et al.,* (2022): even with different biochar doses, the mesophilic methane trend is the same ($0.36 \pm 0.00\ m^3CH_4/kgVS_{add}$). In that case, one of the most relevant effects of biochar in a mesophilic BMP is the contribution to the buffering capacity of the system and the prevention of VFA accumulation, making viable the anaerobic mono-digestion of acids substrates as CW. This can be to the alkalinity contribution of biochar due to its high pH (9.17). The preceding indicates that by adding biochar to unfavourable temperature conditions, as are considered psychrophilic, there is a synergistic effect that increases methane production, reinforced by high consumption of VFA during the process, which can be translated into an improvement in methanogenesis. This behaviour can be accredited because biochar could stimulate a direct interspecies electron transfer (DIET) pathway between methanogens to simplify the reduction of carbon dioxide to methane, as studied by Zhang *et al.,* (2018).

Figure 2 clarifies the interaction impact of Ps and Bc at 15°C (a) and 35 °C (b). Psychrophilic BMP increases when the biochar size is reduced, and the support concentration is incremented. Final tVFA at 15 °C shows a consistent behaviour: if the biochar size is smaller than 0.15 mm and it is added above 10 g/L, the volatile organic compounds are consumed easily. In mesophilia, the lower Ps values favour methane generation, but the positive effect does not prevail if more biochar is added. In the case of tVFA, these are totally consumed due to the operational condition's favourability.

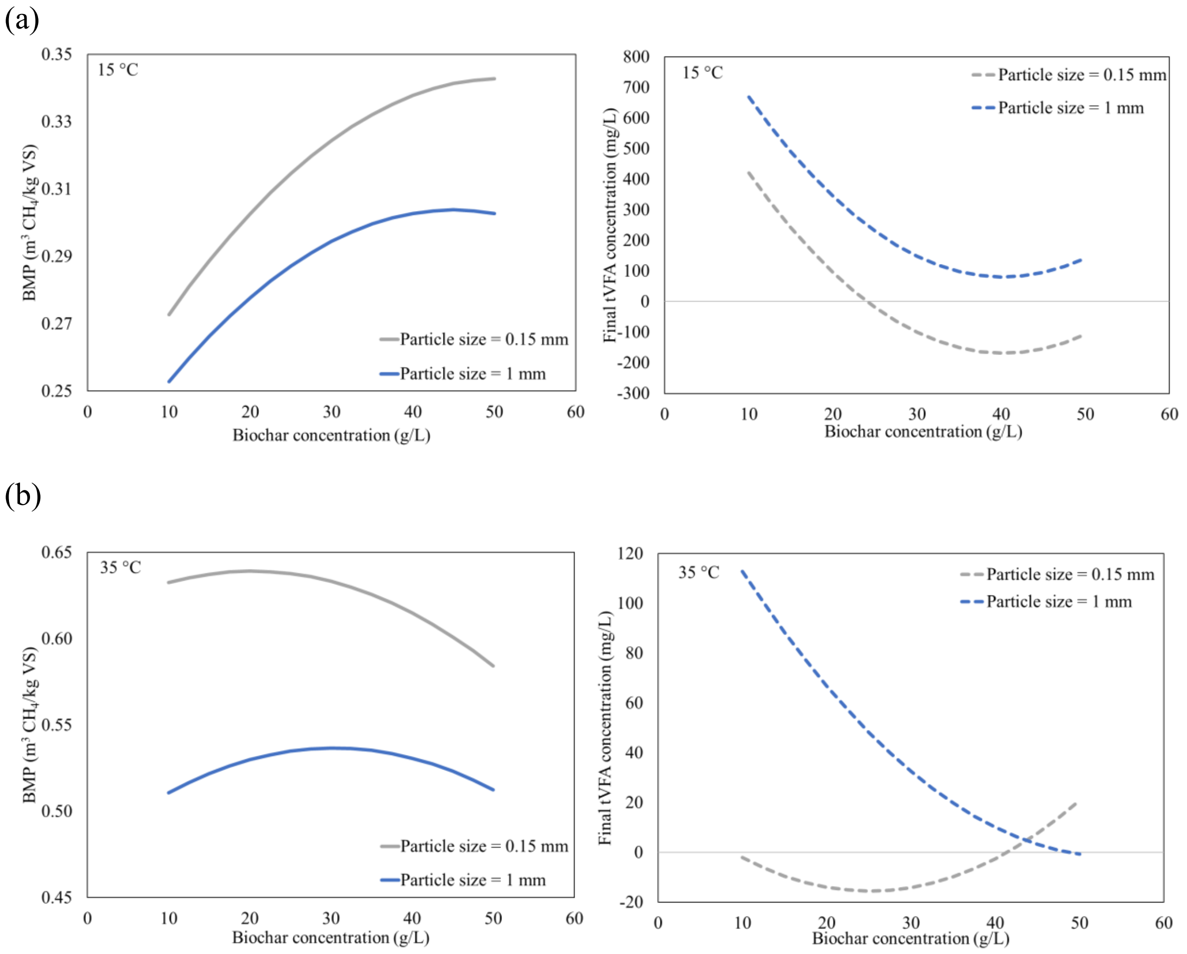

Figure 2. Interaction plots between independent variables (Bc and Ps) with the output responses (BMP and final tVFA) at 15 °C (a) and 35 °C (b).

When performing simultaneous optimization to maximize BMP and minimize VFA concentration at the end of the trial, the most favourable conditions were obtained: Ps = 0.575 mm and a Bc of 30 g/L, which is valid for both temperature regimes. The desirability achieved under these conditions was 0.99 and 1 for 15 °C and 35 °C, respectively. With these operating values, it is expected to obtain a 48 % higher BMP from 0.23 m^3 CH_4/kg VS_{add} (control) to 0.34 m^3 CH_4/kg VS_{add} (optimum value) with a total VFA consumption (final VFA concentration = 0 mg/L) at 15 °C.

1.3.2. Anaerobic digestion modulated by biochar addition: kinetic and metabolic efficiencies behaviour.

As validation, psychrophilic BMP under the most favourable conditions was replicated. Figure 3. presents the psychrophilic biomethane production kinetics, and their respective Gompertz-modelled data for optimized BMP (CW:CM + biochar BMP), control assay (CW:CM BMP with no biochar), biochar BMP (inoculum + biochar), and blank (inoculum alone; no substrate, no biochar).

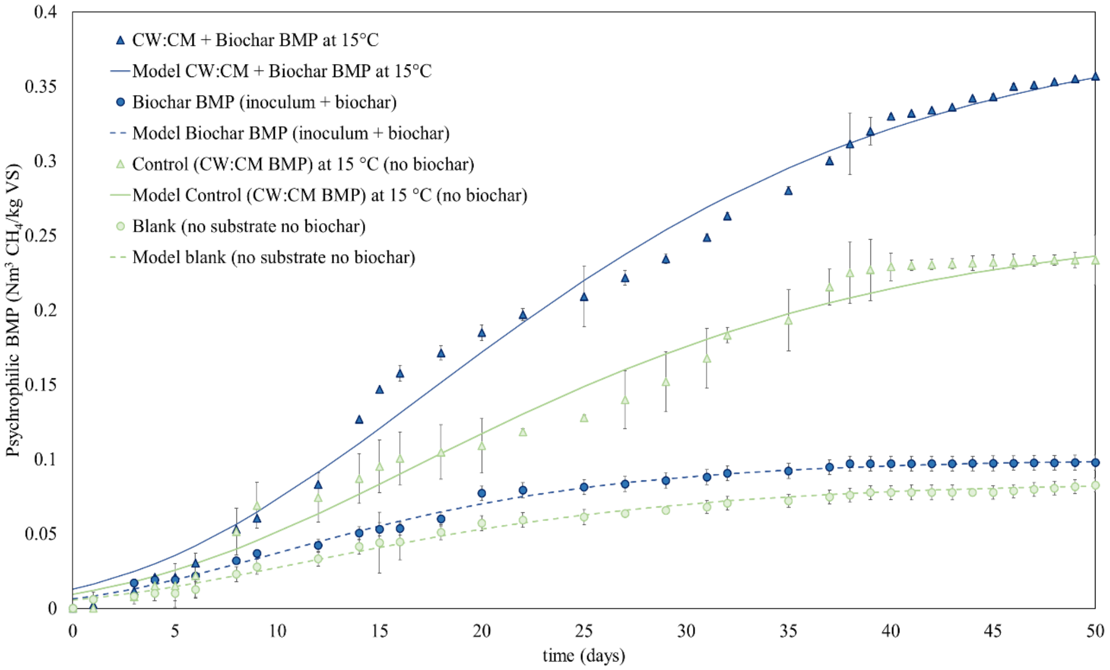

Figure 3. Psychrophilic biomethane production kinetics and their respective Gompertz modelled data for CW:CM anaerobic co-digestion + biochar.

Evidently, the assay with 30 g/L of biochar (Ps = 0.575 mm) shows a faster kinetic with an elevated BMP (0.36 m^3 CH_4/kg VS_{add}). Moreover, in 27 days, the assay with biochar reached the same value of cumulated methane production as the control BMP for 40 days. This can represent an improvement in hydrolytic activity, which means a faster production of soluble compounds to be bio-converted to methane. It is important to mention that the biochar does not act as a substrate insomuch as the biochar BMP was close to methane production due to inoculum (blank assay) endogenous production (0.09 ± 0.01 m^3 CH_4/kg VS_{add}). Those results are consistent with others reporting positive effects of using organic biochar on methane production even at mesophilic conditions. For example, Madrigal *et al.,* (2022) reported that the "doping" AD process (operating at 35 °C) with biochar from pyrolyzed cattle manure considerably increases the yields of mono-digested cheese whey reaching an ultimate methane production of 0.36 m^3 CH_4/kg $VS_{add,}$ against an inhibited BMP with no biochar.

The psychrophilic behaviour observed in this study can be represented by the kinetic parameters estimated by fitting the data of biomethane production by the modified Gompertz model. The kinetic model parameters were validated based on the values of error functions R^2 and RMSE, summarized in Table 5. For all the curves of methane production, R^2 and RSME values were higher than 0.991 and lower than 1.33E-06, respectively, indicating the best fit and high accuracy of the models to the corresponding experimental data.

Table 5. Gompertz parameters for CW:CM anaerobic co-digestion with and without biochar addition.

Assay	**Po (m^3 CH_4 /$kgVS_{add}$)**	**K_{max} (m^3 CH_4 /$kgVS_{add}$ d)**	**λ (d)**	**R^2**	**RMSE**
BMP with Biochar at 15°C	0.39	0.010	3.25	0.996	1.39E-06
Control BMP at 15 °C (no biochar)	0.26	0.007	2.78	0.991	1.33E-06
Biochar BMP (inoculum + biochar)	0.10	0.004	0.18	0.997	1.43E-08
Blank (no substrate no biochar)	0.08	0.003	0.11	0.995	1.90E-08

Regarding kinetic parameters, the maximum P_0 occurred with BMP with biochar assay (0.39 m^3 CH_4/kg VS_{add}), which was 1.5 folds of P_0 in control and 71% of the average BMP reached in mesophilic conditions (0.55 ± 0.06 m^3 CH_4/kg VS_{add}). P_0 tendency corresponds with K_{max} comportment, where the assay loaded with biochar reached a maximum methane production rate 1.42-fold higher if no support is used, even with an adaptation period 0.47 days higher (λ with biochar = 3.25 days). The preceding shows that using pine wood biochar in a favourable proportion allows psychrophilic methane production yield to be close to those obtained at better temperature conditions.

The positive affectation by biochar can be justified by studying the metabolic efficiencies along the process. Figure 4a and 4b show the changes through time for the psychrophilic (15 °C) and mesophilic (35 °C) AD metabolic stages with and without biochar. During the first 30 days of the monitoring, in the psychrophilic assay, the hydrolysis was more active permitting the solubilization of macromolecules as carbohydrates, lipids, and proteins present in substrates. Comparing assays with and without biochar addition, the organic support stimulates the hydrolysis of organics. It is indicated by the hydrolysis of all biodegradable biomass by day 30, compared to 40 days if biochar is not added.

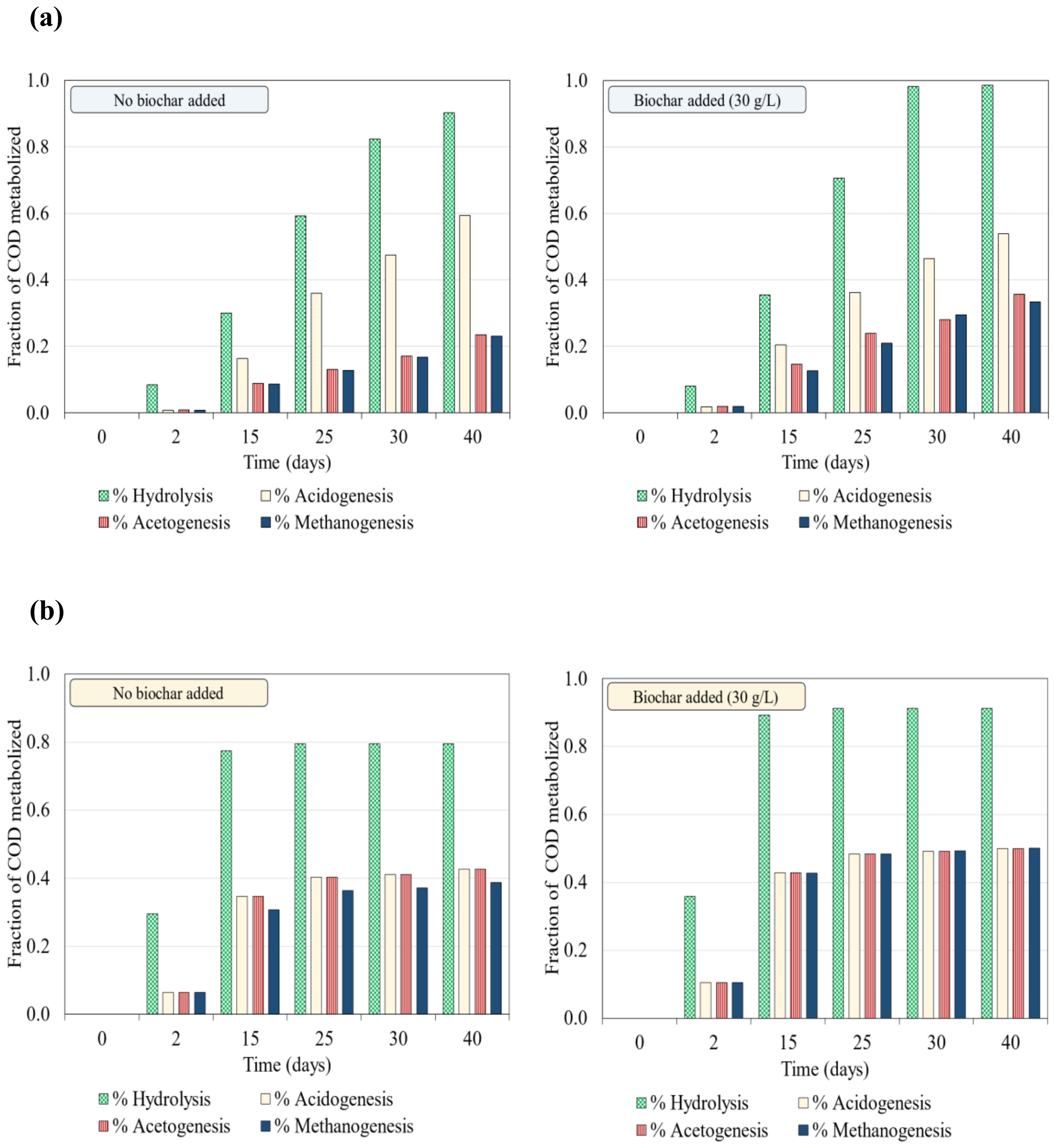

Figure 4. Evolution of anaerobic digestion metabolic efficiencies modulated by biochar addition (30 g/L; Ps = 0.575 mm) during BMP assay at 15°C (a) and 35 °C (b). Dotted bars for hydrolysis, clear bars for acidogenesis, striped bars for acetogenesis, and dark bars for methanogenesis.

Considering the total gCOD hydrolysed during monitoring, the biochar-supplemented assay reached 99 %, favouring until 19 % the generation of easier biodegradable compounds to be treated in the posterior steps. As AD is a syntrophic process, it is necessary to improve the hydrolysis as the initial step (Thygesen *et al.,* 2021), to enhance other metabolic stages to avoid an unbalance and posterior inhibition (Demirel & Scherer, 2008). This improvement is what adding biochar provokes in a psychrophilic system. In the case of mesophilic assays, biochar incremented the hydrolytic efficiency constantly during days 2 to 45 at 14.90 ± 0.20 %, but it does not seem a clear acceleration of the process. This behaviour was also reported by Zhao *et al.,* (2015): using biochar promotes the conversion of macromolecules from artificial wastewater into methane in an up-flow anaerobic sludge blanket.

Regarding acidogenesis in psychrophilic conditions, the production and consumption of VFA occur throughout the whole AD process. VFA, mostly transformed from smaller organic compounds in the acidogenesis phase, are essential carbon substrates to produce biogas during anaerobic digestion. During the first 15 days of the process, the psychrophilic formation of VFA was higher when using biochar. Even with no augmentation of acidogenesis efficiency after day 15, the formation of acetic acid was favoured. Similar findings from earlier studies have been published, demonstrating the significance of biochar in the process of VFAs breakdown and their absorption into methane (Zhu *et al.,* 2023). Hence, biochar modulates acid generation, promoting acetic acid. A controlled generation of acetic acid boosts methane generation since this is the precursor of 70% of methane generated (Hill *et al.,* 1987). To put it another way, biochar favours the acetoclastic route while acids such as propionic or butyric are reduced. Compared to mesophilia, psychrophilic acidogenesis reaches similar (even higher) percent of COD metabolized, but it is deaccelerated by temperature influence on metabolism. As shown in figure 4b, operating under 35 °C allows a constant acid formation during AD, and adding biochar facilitates its production of over 19.8%. However, biochar addition in the mesophilic acidogenesis process does not significantly affect rate nor final efficiency.

In a successfully conducted acetogenesis phase, longest-chain VFA (C3 – C6) are gradually bio-degraded to acetic, which is converted into methane via the acetyl-CoA pathway (Drake 1994). Subsequently, from day 15 to 45 of monitoring, the average acetogenesis efficiency goes from 41.48 ± 8.48 % to 66.03 ± 4.44 % if organic support is added. In particular, mesophilic acidogenesis presented a total conversion into acetic, even with no biochar addition. An aspect being highlighted is that, despite a similar total acid formation under psychrophilic and mesophilic conditions, the acetogenesis efficiency is lower at 15°C. The above can indicate that acidogenesis in psychrophilia is a limiting step, restricting the acetogenesis and hence the methane formation. So, acetic production is one of the main differences between psychrophilic and mesophilic AD.

After CH_3-COOH formation, the final step is to convert it into CH_4 during methanogenesis. So, if there is more acetic, the tendency is that more methane is formed. This behaviour is similar in the presence or absence of biochar, even at 15 °C or 35 °C. In all cases shown in Fig 4a and 4b, more of the 87% of acetic is transformed to methane. So, methanogenesis is not the limiting step, while most of the acetic is transformed in methane, independently of the temperature range and the presence of biochar. Notably, in the mesophilic process with biochar, all acids were acetic and all acetic was methane; so, the support addition facilitates the methanogenesis. Regarding 15 °C AD, the preceding behaviour is unclear, but there is a notorious augmentation of methane efficiency above 45.2%, a value very similar to the methane generation increment of 50% described in Fig 2 (see section 1.3.1). However, there are remanent organics that can be post-treated or used by giving extra time to the AD treatment; even so, the residual methane potential in an assay with biochar is lower.

As mentioned by Indren *et al.,* (2020), using biochar increases the total methane yield; in the present study, the psychrophilic methanogenesis was improved, reaching 33% of total COD conversion until day 45 due to a higher effective acetate generation and consumption, while without biochar is 23% of total COD. These results are comparable with few studies under psychrophilic temperatures. As example, Park *et al.,* (2020) reported that using a granular activated carbon increases methane yields by 17.8% at 15 °C with an acid consumption of 91 %. However, associating psychrophilic results with those obtained at 35 °C (Fig. 3b), it is evident that a temperature reduction of 20° considerably affects the anaerobic process, decreasing the reaction rates, metabolic efficiencies, and removal yields. Nevertheless, adding biochar is a clear alternative to mitigate those hindrances.

1.3.3. Dynamics of microbial populations during BMP tests

The effects described in the previous section were consistent with the quantification of total bacteria, *archaea*, and methanogens measured at the beginning and the end of the validation stage (section 3.2) which is described in Figure 5.

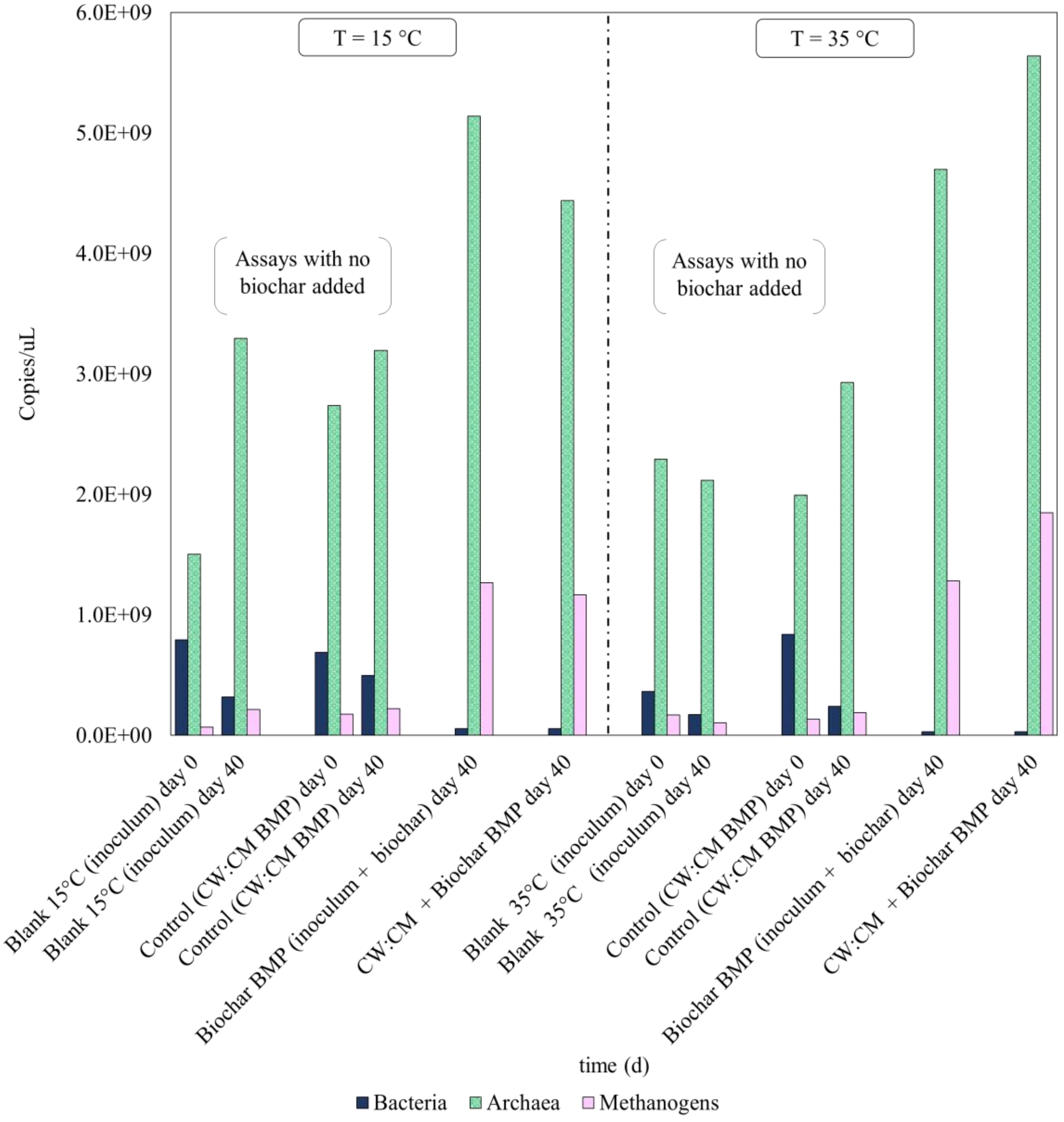

Figure 5. Quantification of total bacteria (dark bars), *archaea* (stippled bars), and methanogens (light bars) from the different assays.

In addition, the ANOVA test in this stage confirmed a statistically significant variation in the different assays' populations of bacteria, *archaea*, and methanogens. After 40 days at 15°C, the inoculum (blank assay) with no extra substrate addition presented a significant decrease in bacterial abundance. In contrast, methanogenic populations had a three-fold increase (from 6.99 x 10^7 to 2.15 x 10^8 copies/uL), and the archaeal populations had a twofold increase (from 1.5 x 10^9 to 3.3 x 10^9 copies/uL). This could indicate that the endogenous metabolism of inoculum allowed the growth of microbial populations even under psychrophilic conditions, which is consistent with the presence of psychrotrophic populations that could be modulated to boost cold temperature methanogenesis (Akindolire *et al.,* 2022). This could also explain the decrease in the overall numbers of total *archaea* and methanogenic populations in the assays carried out at 35 °C. At psychrophilic conditions, the addition of biochar to the inoculum with no extra substrates produced a positive effect on the growth of *archaea* and methanogens, inducing an increase from 1.5 x 10^9 to 5.14 x 10^9 copies/uL in archaeal and 6.99 x 10^7 to 1.27 x 10^9 copies/uL in methanogenic populations while bacterial abundance decreased from 7.99 x 10^8 to 5.58 x 10^7 copies/uL, after 40 days. The aforementioned results suggest that biochar alone could be an alternative to prepare or strengthen an inoculum for psychrophilic AD tests, or even to improve inoculum acclimatization during real scale digesters installation, favouring the growth of microbial populations directly involved in the methanogenesis phase of AD. In spite the addition of a substrate (CW) and a co-substrate (CM) produced changes in the abundance of archaeal and methanogenic populations at day 0, but by day 40, the changes in the abundance of all three microbial communities under psychrophilic (15 °C) and mesophilic (35 °C) conditions were similar to their respective blanks.

As observed with the inoculum, the addition of biochar to CW:CM BMP assays (CW:CM + Biochar BMP day 40) also induced a decrease in bacterial abundance (from 6.89 x 10^8 to 5.50 x 10^7 copies/uL), and a significant increase (p-value <0.0001) in the archaeal (2.74 x 10^9 to 4.44 x 10^9 copies/uL) and methanogenic (from 1.76 x 10^8 to 1.17 x 10^9 copies/uL) populations abundance at 15°C after 40 days. Also, comparing the final day of monitoring of the control assay with the CW:CM + Biochar BMP, the increase in methanogenic populations was about 520%. This increase suggests favourable changes for the growth of archaeal, and particularly, methanogenic populations, which could be associated with the formation of biofilm structures on biochar surfaces that boost microbial growth and methane production, being consistent with studies reporting that biochar addition could change the relative abundance of bacteria and *archaea* favouring the abundance of methanogens (Wang *et al.,* 2022a). Interestingly, the abundance of bacterial populations in all assays involving the addition of biochar decreased after 40 days, either at mesophilic or psychrophilic conditions, despite the positive evolution of hydrolysis, acidogenesis and acetogenesis efficiencies modulated by biochar addition (Figure 4), processes in which bacteria are directly involved. Biochar from different materials has been associated with promoting archaeal and methanogenic populations and bacterial growth during AD processes (Li *et al.,* 2017; Cimon *et al.,* 2020). Further improving microbial diversity and abundance, biochar stimulates cell attachment, biofilm formation/maturation, and cell survival. This is especially important considering the relevance of hydrolytic, fermentative and acetogenic bacteria during AD, as well as the syntrophic relationships between acetate-forming bacteria and methanogens to produce methane. Biochar composition could be determinant to modulate the function of microorganisms in the AD systems and could be a factor influencing the enrichment of certain microbial populations to the detriment of others. For example, a previous study on AD for biogas production reported that the addition of rice straw biochar affected the abundance of bacteria and *archaea*, selectively favouring the overall abundance of certain methanogenic populations, a decrease in acetogenic bacteria and the inhibition of carbohydrate metabolism, yet maintaining an increased biogas production during anaerobic fermentation (Wang *et al.,* 2022b). As in this study methane production yields were high in BMP assays involving the use of biochar, a plausible scenario is that under psychrophilic conditions, biochar selectively improves the growth and function of methanogenic microorganisms to the detriment of bacteria, even though this decrease does not seem to affect the overall production of methane, indicating that despite a significant decrease of bacterial abundance, biochar had no negative effects on the function of microbial populations during the AD process.

In a particular case of full-scale AD systems, applying organic support such as gasified pine wood can avoid heating, reducing cost and increasing digester efficiencies in psychrophilia. In areas with high availability of agro-industrial organic waste, AD + GS is a viable alternative for organic residue treatment and energy and fertilizer generation. Biochar addition enhances the biomethane production and fertilizing properties of the digestate. For the codigestion process of CW and CM, the results show how adding biochar improves the psychrophilic yields, which can result in a diminution in heating energetic requirements and chemical compounds for pH control. Results show adding biochar (concentrations higher than 10 g/L) can boost methane generation (better volatile fatty acids consumption) and improve the stability of AD, even in low-temperature scenarios. The data discussed reinforces the feasibility of adding biochar to the AD process. Although biochar exhibits promising potential for integration in psychrophilic AD, research should continue to be conducted to advance technology maturity further. To cover those barriers, the future perspectives of this study are the bench-scale continuous process evaluation followed by full-scale anaerobic digester implementation in a psychrophilic rural area, focusing on the energy, economic and environmental assessment. Additionally, it is necessary the evaluation of the digestate quality. In this sense, running assays in the laboratory exhibit the improvement of biofertilizer potential of digestate due to biochar (data not shown). On the other hand, AD + GS has been implemented in a rural area (average temperature: 17 °C), which counts on the availability of raw materials such as residual pine wood and CW and CM. This alternative presents significant improvement regarding daily specific methane generation and reduction of negative environmental impact (Data in the process of publication).

1.4. Conclusions

Organic support modulates the anaerobic co-digestion process counteracting the adverse effects of psychrophilic conditions in three main ways:

Kinetic improvement: 30g/L of pine wood biochar with a Ps = 0.575 mm can boost the psychrophilic process, reaching a BMP near 70% of the obtained at 35°C.

Metabolism influence: biochar favors acetoclastic metabolism improving methane yield even at an unfavorable temperature (15°C).

Microbial impact: biochar facilitates the growth of the microbial population in charge of methanogenesis, represented in about 500% more *archaea* than in no biochar AD.

This work highlights the importance of adding an organic support material to improve AD in psychrophilic conditions, linking laboratory procedures to agro-industrial residue treatment for energy production. In that sense, more research must be done on the performance of the suggested approach in continuous systems and full-scale scenarios, making the bioprocess more attractive to be implemented in low-temperature zones.

1.5. Generation of new knowledge: Research Impact

The scientific products associated with the development of Chapter I are presented below.

Publications.

Jaimes-Estévez, J., Martí-Herrero, J., Poggio, D., Zafra, G., Gómez, K., Escalante, H., & Castro, L. (2023). The role of biochar in the psychrophilic anaerobic digestion: Effects on kinetics, acids metabolism, and microbial population. Bioresource Technology Reports, 23, 101566. https://doi.org/10.1016/j.biteb.2023.101566

Madrigal, G., Huaraya, M., Sancho, T., Mendieta, O., & Jaimes-Estévez, J. (2022). Biochar from bovine manure as a sustainable additive to improve the anaerobic digestion of cheese whey. Bioresource Technology Reports, 20, 101258. https://doi.org/10.1016/j.biteb.2022.101258

Martí-Herrero, Jaime, Liliana Castro, Jaime Jaimes-Estévez, Mario Grijalva, Monica Gualatoña, María Belen Aldás, and Humberto Escalante. "Biomethane potential test applied to psychrophilic conditions: Three issues about inoculum temperature adaptation." Bioresource Technology Reports 20 (2022): 101279. https://doi.org/10.1016/j.biteb.2022.101279

Foster, W., Azimov, U., Gauthier-Maradei, P., Molano, L. C., Combrinck, M., Munoz, J., Jaimes Estévez, J., & Patino, L. (2021). Waste-to-energy conversion technologies in the UK: Processes and barriers–A review. Renewable and Sustainable Energy Reviews, 135, 110226. https://doi.org/10.1016/j.rser.2020.110226

Presentations.

L. Castro, J. Jaimes-Estévez, H. Escalante, J. Martí-Herrero, M. Romero-Guiza. When you make a psychrophilic BMP without an adapted inoculum, a low cost digester dies. 16th World Conference on Anaerobic Digestion 23-27 June 2019, Holanda.

Jaimes-Estévez, J., Castro, L., Escalante, H., Vera, E., y Arias, C. Lactosuero y estiércol bovino, co-sustratos sinérgicos para incrementar los rendimientos de energía en forma de biogás y estruvita. XI Encuentro de la RedBioLAC. Octuber 2019, Cuba.

Human Resource Development.

Co-supervision of undergraduate thesis for two Chemical Engineering students. Status: defended and approved. Title: “*Estudio teórico sobre el efecto del uso de soportes orgánicos en el proceso de digestión anaeróbica”.*

Chapter II. Biochar as metabolic efficiencies modulator during continuous psychrophilic anaerobic co-digestion

2. Biochar as metabolic efficiencies modulator during continuous psychrophilic anaerobic co-digestion

Abstract

Biochar is traditionally used as material support to improve mesophilic anaerobic digestion. This research aimed to evaluate the biochar impact on the continuous anaerobic co-digestion (ACoD) at psychrophilia to treat agroindustrial wastes such as cheese whey and cattle manure. Adding biochar improved acidogenesis and acetogenesis, balanced the production and consumption of acetic acid, promoted CH_4 formation, and avoided inhibition risks. The bioprocess efficiency under inhibitory loads was 0.35 m^3CH_4/kg VS*d, 38% higher than control. The above means a higher biomethane production based on the biodigester size by 14.28% and 38.55% for OLR of 1 and 1.5 kg COD/m^3*d, respectively. Those improvements are reinforced because biochar promotes psychrophilic biofilm formation, reaching a methanogen concentration increment of over 440%. This work demonstrated the importance of using organic materials to improve the viability of ACoD even at temperatures under 25 °C via metabolic synergism enhancement.

2.1. Introduction

Anaerobic digestion (AD) is a biological process where microorganism metabolism allows the reduction of a substrate, considered as the source of nutrients, to simpler compounds. As a result, the AD process produces biogas with energy potential (lower calorific value between 13,720 - 27,440 kJ/m^3) (Hosseini & Wahid 2013). The most common uses for biogas, a product of energy interest, are heating, electricity production (thermal benefits), and food cooking. In Latin American rural areas, the most used systems for AD implementation are tubular digesters, with bovine manure being the most frequently treated waste (Jaimes-Estévez *et al*., 2022; Lansing *et al*., 2008; Marti-Herrero *et al*., 2015; Castro *et al*., 2017). These digesters have been installed in various regions with a wide range of climates, ranging from 8 °C to 35 °C (Garfi *et al*., 2016), covering psychrophilic regimes (0°C < T < 25 °C) and thermophilic temperature ranges (25 °C < T < 42 °C). Specifically, approximately 20% of the Colombian national territory experiences temperatures below 25 °C, highlighting a high potential for implementing AD technology in cold climates. Despite the extensive territory with temperatures below 25°C, reported literature indicates that AD under mesophilic conditions achieves better yields than psychrophilic conditions (Álvarez *et al*., 2006). In other words, operating under psychrophilic conditions poses a challenge due to operational, physicochemical, and microbiological issues. Concerning operational drawbacks, there may be an increase in the viscosity of the liquid medium, which constitutes a decrease in the diffusion of soluble compounds and the contact between biomass and substrate. This scenario represents low yields in biogas production and organic matter consumption (Dev *et al*., 2019).

On the other hand, physical and chemical changes in the properties of the substrates or digestate can considerably affect the treatment system's operation. For example, increases in the solubility of gases in the liquid phase, such as H_2, H_2S, and CO_2, can occur, decreasing the pH of the medium (Lettinga, *et al*., 2001). Regarding the limitations from the microbiological point of view, the methanogenic *archaea* are mostly mesophiles in charge of metabolizing acetate, and their optimum growth temperature is 37 ºC. Therefore, temperature values below mesophilia affect their microbial activity since substrate transport through the cell membrane is inhibited (Dev *et al*., 2019). The preceding translates into low-quality biogas and accumulation of VFA, represented respectively in i) CH_4 content less than 50% and increased H_2S content and ii) the decrease in system pH, which translates into stability problems such as acidification of the medium.

A new approach for improving AD involves employing biochar as an organic carrier (support material), fostering heightened interactions between microorganisms and substrates (Khalid *et al*., 2021; Tang *et al*., 2020). The use of support materials increases of biomass concentration inside the reactor, improves resistance to organic overloads, and significantly reduces the time adaptation of the microbial consortium to the substrate (Karadag *et al*., 2015). Also, the use of biochar as a carrier in AD enhances methanogenesis through stimulated direct electron transfer, accelerates the generation and degradation of volatile fatty acids (VFA), controls ammonia, and improves acids-buffering (Pan *et al.,* 2019; Qiu *et al.,* 2019; Singh *et al.,* 2022). According to the literature, it has been demonstrated that the addition of supporting material can also shorten the methanogenic lag phase, immobilize functional microbes, and accelerate electron transfer between methanogenic and acetogenic microorganisms during CoDA (Sunyoto *et al*., 2016; Mumme *et al*., 2014; Chen *et al*., 2023). In a specific case, the study conducted by Sugiarto *et al*., (2021) revealed that the biomethane yield increased by 46.9% by adding pine sawdust biochar to the AD of food waste. Similarly, Sunyoto et al., (2016) used pine wood biochar as a support in anaerobic digestion of food waste at thermophilic temperatures (55°C), improving VFA degradation by up to 31% and increasing the maximum CH_4 production rate by 41.6%. However, several reviews on the topic have been published in the last years but focusing only on mesophilic and thermophilic conditions (Fagbohungbe *et al.,* 2017; Pan *et al.,* 2019; Qiu et al., 2019; Masebinu *et al.,* 2019; Chiappero *et al*., 2020; Tang *et al.,* 2024; Khalid *et al*., 2021; Ambaye *et al.,* 2021; Zhao *et al.,* 2021; Kumar *et al.,* 2021; Madrigal *et al*., 2022; Hoang *et al.,* 2022). From those reviews, only two publications mentioned that evaluated biochar's influence on psychrophilic AD. So, ACoD studies using organic supports focus on processes at mesophilic and thermophilic temperature conditions, most of which are operated in batch mode. The above highlights a lack of knowledge regarding the effect of using biochar in psychrophilic anaerobic digestion in stationary systems operated continuously. Considering the described scenario, this research was aimed to study and analyze the effect of biochar use on the metabolic and energy efficiencies of the psychrophilic AD process in continuous mode. The system investigated in this study was the continuous co-digestion of cheese whey (CW) and cattle manure (CM) using gasified pine wood as organic support material.

2.2. Material and Methods

This study was developed in three stages: a) the monitoring of the metabolic efficiencies of the continuous process, b) the effect of biochar addition on the biomethane generation and c) the evolution of the microorganism content during ACoD. These stages were carried out using two polyvinyl chloride tubular digesters (length of 1 m; diameter of 0.1 m; a total volume of 8 L), with an operational volume of 5.0 L. The first digester (control; D1) was used to carry out the ACoD of CW and CW, while the second one (D2) was loaded with pine wood biochar. The fed daily diet was a mixture of CW and CM in a 70:30 ratio (volatile solids basis), and the concentration of biochar added was 30 g/L, with a particle size of 0.5 mm, considered the most favourable according to Jaimes-Estévez *et al*., (2023) (Chapter I).

The organic loading rates (OLRs) evaluated in each digester were 1.0 and 1.5 kg COD/m^{3}d, maintained for at least two hydraulic retention times (HRT). Before initiating the continuous feeding stage, the digesters were inoculated with stabilized bovine manure taken from a digester located in the rural area of Santander – Colombia (latitude of N 7°01’0.07’’ W 73°08’13.3’’), which has been in operation since 2017 (Castro *et al*., 2017). This inoculum had a specific methanogenic activity of 0.054 ± 0.008 g COD CH_4/g $VS_{inoculum}$d and a hydrolytic activity of 0.10 ± 0.01 g COD/g $VS_{inoculum}$d.

2.2.1. Evaluation of the biochar effect on the metabolic efficiencies in a continuous ACoD process.

To determine the influence of biochar on the metabolic stages of the process (hydrolysis, acidogenesis, acetogenesis and methanogenesis), total chemical oxygen demand fed (COD_{total}), soluble COD ($COD_{soluble}$), total VFA (COD_{tVFA}), acetate ($COD_{acetate}$) and the equivalence of methene produced ($COD_{methane}$) were measured every fifteen days. A mass balance was applied to each bioreactor to establish the efficiencies, resulting in the derivation of Eq 11 – 14:

$$Hydrolysis\ (\%) = \frac{COD_{soluble} - COD_{soluble,in} + COD_{methane}}{COD_{total} - COD_{soluble,in}} \qquad \text{Eq 11.}$$

$$Acidogenesis\ (\%) = \frac{COD_{tVFA} - COD_{tVFA,in} + COD_{methane}}{COD_{total} - COD_{tVFA,in}} \qquad \text{Eq 12.}$$

$$Acetogenesis\ (\%) = \frac{COD_{acetate} - COD_{acetate,in} + COD_{methane}}{COD_{total} - COD_{acetate,in}} \qquad \text{Eq 13.}$$

$$Methanogenesis\ (\%) = \frac{COD_{methane}}{COD_{total}} \qquad \text{Eq 14.}$$

$COD_{soluble,\ in}$, $COD_{tVFA,\ in}$, and $COD_{acetate,in}$, were the corresponding values measured to the influent. $COD_{methane}$ was calculated considering the 350 mL CH_4/g COD coefficient at pressure and temperature standard conditions (101.325 kPa, 273.15 K). All values were expressed in grams of COD (net mass units).

2.2.2. Biomethane yields influenced by biochar addition.

To assess the effectiveness of the process in biogas production, operational efficiencies were calculated, represented by the biodigester and bioprocess efficiencies. Biodigester efficiency (η_{bd}) is determined by the ratio of the daily biomethane volume to the biodigester volume, as shown in Equation 15 (Marti-Herrero *et al.*, 2015).

$$\eta_{bd} = \frac{m^3CH_4}{m^3digester * d} \qquad \text{(Eq. 15)}$$

On the other hand, the bioprocess efficiency (η_{bp}) according to Marti-Herrero *et al.*, (2015), refers to the amount of organic matter converted into CH_4 during anaerobic digestion, as outlined in Equation 16.

$$\eta_{bp} = \frac{m^3CH_4}{kg\ SV_{in} * día} \qquad \text{(Eq. 16)}$$

Thus, (η_{bp}) relates the daily amount of CH_4 produced (m^3CH_4/d) from the digestion of the organic matter loaded into the process ($kg\ SV_{in}$).

2.2.3. Microorganism content behaviour along monitoring: Population growth and biofilm formation.

Quantitative Polymerase Chain Reaction (qPCR) assays were employed to assess biochar's impact on microbial population counts, including total bacteria, total *archaea*, and total methanogens during digesters monitoring. The methodology employed was the same proposed by Jaimes-Estévez *et al.,* (2023). qPCR determinations were conducted at the middle and the end of each OLR on D1 and D2, to establish a baseline for comparison. All samples were preserved at −20 °C upon collection from digesters until further processing. To prove the influence of biochar on biofilm formation (Fernández *et al.*, 2008), a Scanning Electron Microscope (SEM) assay (Scanning Electron Microscope -Field Emission Gun QUANTA FEG 650) was done on the dehydrated effluent samples at the end of monitoring for D1 and D2. The images were captured with a high vacuum and an acceleration voltage of 30 kV.

2.2.4. Physicochemical Analysis

The content of COD, and volatile solids (VS) were analysed according to standard methods for the examination of water and wastewater (APHA 2017). A transductor was used to measure the digesters' pressure to determine the daily biogas generation. The compositions of biomethane and carbon dioxide in biogas were detected by gas chromatography (Holliger 2016). Acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, and caproic acid concentrations were determined via gas chromatography (flame ionization detector) according to Raposo *et al*., (2013) (See section 1.2.5). The total VFA content was considered as the sum of each VFA in each sample. The data collected were analyzed for statistical significance through ANOVA, with significance determined at a p-value threshold of < 0.05.

2.3. Results and Discussion

2.3.1. The impact of biochar on metabolic efficiencies in a continuous Anaerobic Co-Digestion (ACoD) process.

Figure 6 shows the behaviour of the main AD metabolic stages over time for D1 (1a) and D2 (1b) under two different OLR. Regarding D1, its hydrolysis shows evident stability during monitoring, reaching an average value of 55 ± 0.14 %. The deviation value can be affected because the system needed at least two weeks to adapt correctly to the substrate loaded. During the first measurement, the hydrolysis showed the lowest value, reaching a 13% of COD metabolized.

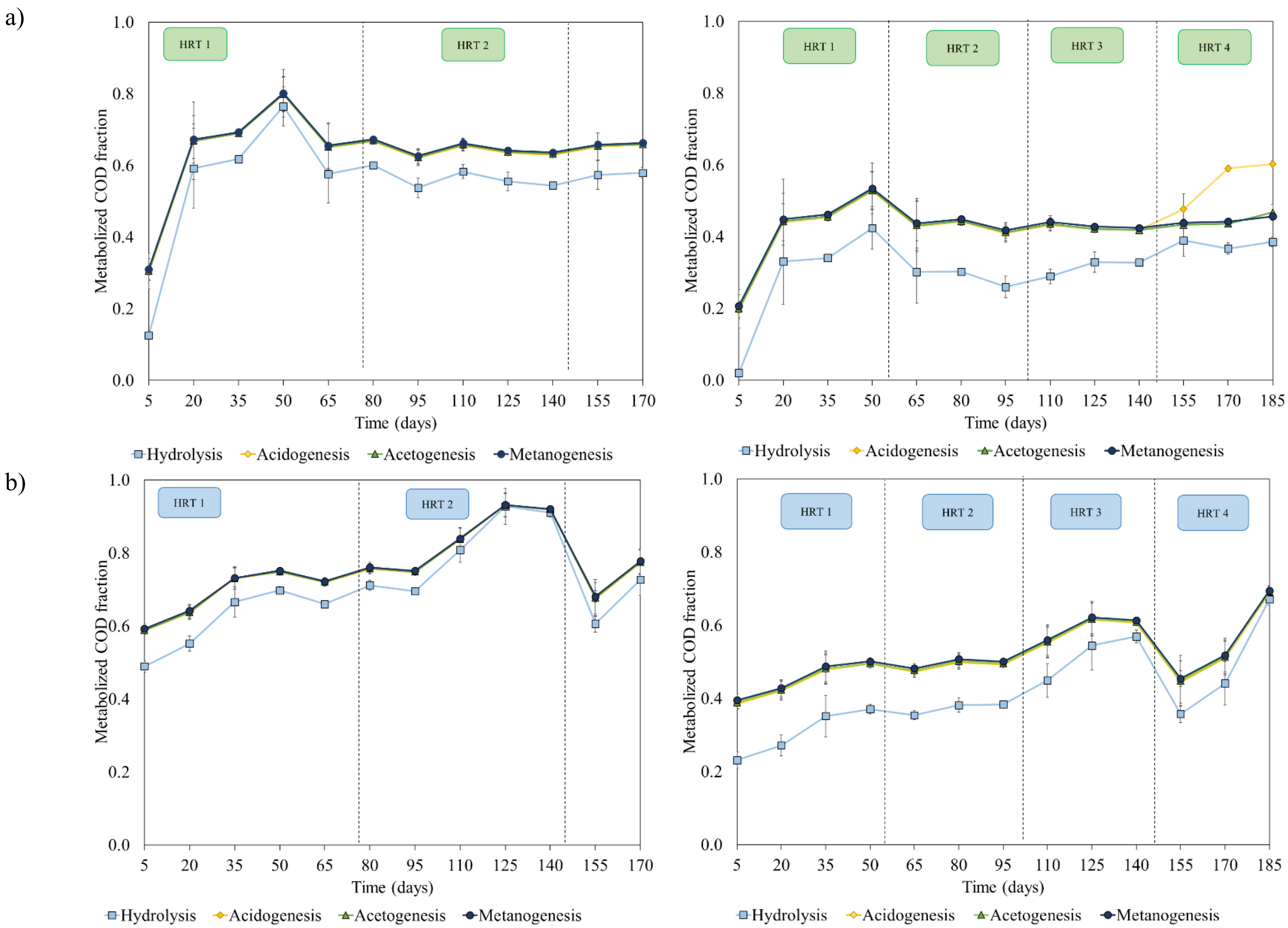

Figure 6. Metabolic efficiencies behaviour of a continuous anaerobic digestion process modulated by biochar addition. (a) Control Assay (D1) and (b) tubular digester with (30 g/L (Ps = 0.575 mm) of biochar loaded (D2).

The stability of the system can be reinforced by the fact that acidogenesis and acetogenesis were almost the same in each sampling. The aforementioned means that the most of VFA produced was acetic acid, showing a dominance of acetogenic microorganisms. The stability of the system and the good acid formation cause favourable methanogenesis that for D1 reached values between 0.63 – 0.80 of COD metabolized to produce biomethane. Comparing the methanogenic efficiency (Eq 14.) with a global organic matter remotion, D1 reached a 64.1 ± 11.3%. The traditional method of calculation AD remotion is expressed as a function of the initial and final organic matter content, and it can be overestimated. However, methanogenic efficiency can be considered more accurate due to the direct relation of product generated by total organic matter loaded. The stability of the system can be compared with the results reached previously, where an OLR of 1 kg COD/m^{3}d showed to be the most favourable for the treatment of CW and CM mixture at temperatures surrounding 25 °C (Jaimes-Estévez *et al.,* 2020).

Increasing OLR from 1 to 1.5 kg COD/m^3*d provokes a certain diminution in general metabolism. Firstly, if more substrate is fed, there is less time to process it. This is a reduction in HRT of 33.3 % (passing from 70 to 47 days) which leads to a decrease in global metabolism of around 35%. At the beginning of D1 monitoring, the higher OLR considerably reduced the hydrolysis, which was around 2%. After a stabilization period, this activity reached an average of 31.3% of substrate hydrolysed that was kept for 180 days. However, acidogenesis showed a different behaviour due to some values of metabolized COD higher than those reached by the other metabolic stages. During HRT 4, the VFA formation overcame the solubilization of organics, as the specific acetic acid formation and the biomethane generation. When an imbalance of this kind occurs, system inhibition is imminent, so acidogenesis restricts AD and becomes an indicator of process failure. This tendency is similar to the one presented by Jaimes-Estévez *et al*, (2023), where the acidogenic efficiency is the limiting step in psychrophilic AD due to the influence of temperature on the acetic acid formation.

Regarding biochar addition, the first aspect visualized in Fig 1b. is the increase of all the studied metabolic stages during monitoring. Considering D1 (with an OLR = 1 kg COD/m^3*d) as control, 30g/L of pine wood biochar can boost AD. Relating to hydrolysis, the proportion of soluble COD in D2 was increased considerably in the digestate, improving the availability of simpler compounds, reaching an average hydrolytic efficiency of 70% (27.09% higher than obtained by D1). Therefore, biochar addition enhances the solubilization of low biodegradable substrates as lignocellulosic or lipidic compounds available in CM and CW (Carlsson *et al*., 2012; Ma *et al*., 2019). As the concentration of soluble compounds increases, the production of substrates that can be efficiently utilized by methanogens (among them the acetate), too. So, the biochar also facilitates the generation of VFA, highlighting the production of acetate during the acidogenesis and acetogenesis, respectively. For D2, total VFA generation and acetic acid formation reached efficiency values from 0.59 to 0.93 of COD metabolized (under an OLR of 1 kg COD/m^3*d). As those metabolic stages are directly related to the system's stability and methene generation (Leng *et al*., 2018), the methanogenesis efficiency is clearly benefited under appropriate conditions. As all VFA formed during acidogenesis were precisely acetate due to a balanced acidogenesis, the methanogenic efficiency presented the same tendency with a maximum of 93%.

As seen in Fig 1b, the increase in organic loading from 1 to 1.5 kg COD/m^3*d caused a significant reduction in all the efficiencies. However, the first effect of biochar is the reduction of inoculum adaptation time to the system leading to a 23% hydrolysed COD; that means faster hydrolysis even under unfavourable OLR. The previous results could be supported by what Cavali *et al*., (2022) have discussed, as they argue that adding biochar facilitates hydrolysis by increasing the reaction rate through the immobilization of microorganisms. This considers that biochar can serve as organic support for the adhesion and augmentation of microorganisms, enhancing their interactions and promoting a higher hydrolysis rate. Plus, during monitoring of D2, with 1.5 kg COD/m^3*d OLR, the acetogenesis and acidogenesis metabolic stages were balanced and regulated by biochar presence, avoiding VFA overproduction. Comparing the methanogenesis stage for the two biodigesters, a decrease was also observed due to the increase in load (from 1.0 to 1.5 kg COD/m^3*d). Nevertheless, the addition of pine wood biochar resulted in an increase of 38.86%, and 24.60% in the methanogenic rate corresponding to hydraulic retention times three and four, respectively. Compared to with Sanchez *et al.,* (2021), who argued that in mesophilia the effect of biochar decreased as OLR increased, the results achieved in the present study show that biochar helps the system to lead with inhibitory loads and its effect is more pronounced. The global positive effect under OLR = 1.5 kg COD/m^3*d was 24.3 %, instead of the 14.4 % obtained at 1.0 kg COD/m^3*d. The above means that there exists a synergistic effect of biochar with substrates, which is higher at unfavourable conditions such as overloads and low temperatures. Additionally, it can be mentioned that the biochar effect can be prolonged for at least four HRT or 185 days.

2.3.2. Impact of biochar on process stability: variations in VFA levels during the monitoring period.

Figure 7 shows the digestate VFA content during the continuous ACoD with and without biochar supplementation.

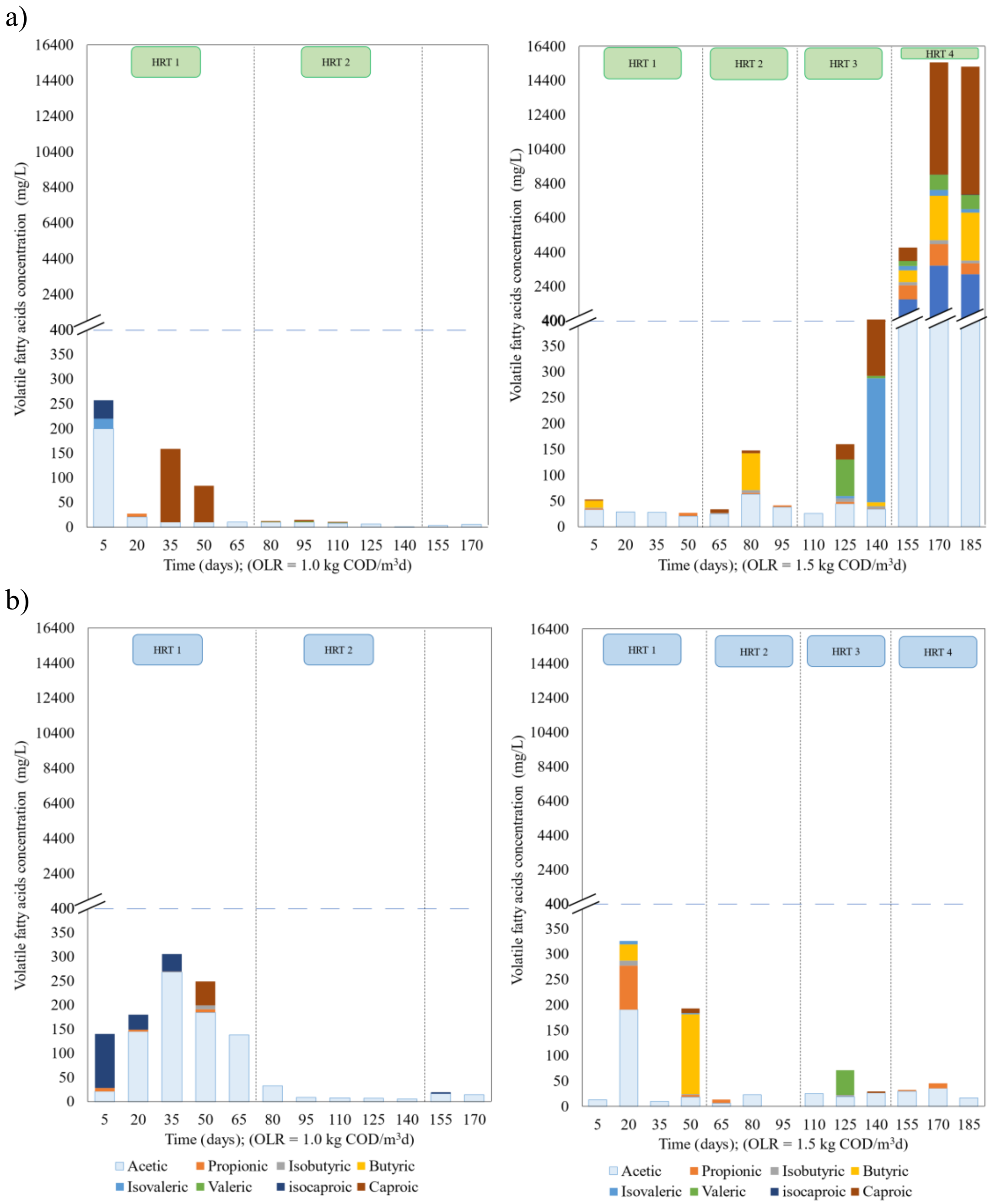

Figure 7. Behaviour of the final VFA at OLR of 1 and 1.5 kg COD/m^3*d for D1 (no biochar added) and D2 (30 g/L of pine wood biochar).

For both OLRs, the average VFA concentration for the substrate fed (inlet) was 1605.70 ± 278.13 mg/L (66.70 % acetic; 10.70% propionic; 9.53% valeric; 6.84% butyric). During the acetogenesis phase, acidogenic bacteria transform hydrolysates, such as amino acids, fatty acids, and sugars, into compounds like VFA. Acids like propionic and butyric, which pose challenges for direct conversion into CH_4, undergo additional biodegradation to become substrates more readily convertible during the acetogenesis phase (Leng et al., 2018). Under a stable OLR, the system can prioritize the acetic acid formation and its consumption for biomethane generation. Therefore, for D1 and D2 under an OLR of 1 kg COD/m^3*d, the main acid present in digestate was acetic. Also, the global remotion of VFA was 99% for both cases, confirming the "good health" of the continuous process. However, a VFA accumulation that was initialized by acidogenesis could be observed at the fourth HRT for D1 (Fig. 2a). The dynamic distribution of VFA in D1 showed an accumulation pattern from day 140. On days 155, 170, and 185, the total VFA were 4,667 mg/L, 15,468 mg/L, and 15,209 mg/L, respectively, which are above inhibition limits for a continuous process (established at 1,500 mg/L; Søndergaard et al., 2015). So, OLR above 1.5 kg COD/m^3*d inhibits the system due to VFA accumulation and unbalance in acidogenesis. In that case, the caproic acid exhibited a dominant role in VFA composition (45.60 %), which was followed by acetic acid (21.99 %) and butyric (17.49 %). Subsequently, it seemed that the transformation of acids different than acetic and its low consumption rate gradually restrict AD as mentioned by Wang et al., (2023). Also, the accumulation of VFA suggests that its consumption was reduced due to the presence of lower microbial synergism between hydrolysis, acidogenesis, acetogenesis, and final methanogenesis (Zhao et al., 2016). Noticeably, final VFA concentrations in D1 were higher than in D2, proving that the biochar effect on balancing AD metabolism. This effect is justified by the fact that pine wood biochar in psychrophilic conditions favours the formation of acetic acid over other volatile fatty acids, promoting its bioconversion to CH_4 by methanogenic microorganisms (Jaimes Estévez et al., 2023).

2.3.3. Bioprocess efficiencies as a function of biomethane production.

The changes observed in both the metabolic efficiencies (Eq. 10 - 14) and the VFAs dynamic are reflected in biomethane generation. Figures 8a and 8b depict the cumulative CH_4 production for loads of 1.0 kg COD/m^3*d and 1.5 kg COD/m^3*d for D1 and D2, respectively. The cumulative values were fitted to a linear equation where the slope represents the average CH_4 production value (NL/d) for each hydraulic retention time.

For the control (D1), the daily CH_4 generation at 1 kg COD/m^3d (Fig. 3a) was 1.53 ± 0.038 NL. During the first evaluated OLR, the addition of pine wood biochar increased CH_4 production by 14.3%. With an increase in the loading to 1.5 kg COD/m^3d (Fig. 3b), it is evident that biochar influence is more significant, favouring biomethane production. During all monitoring, D2 obtained an average output of 1.76 NL/d, 38.5% higher than control under inhibitory loads. These results contrast with those obtained by Sunyoto et al., (2016), who observed a 10% increase in CH_4 production by adding 8.3 g/L of pine sawdust biochar to food waste digestion under mesophilic conditions (35°C). Those results reinforce the affirmation that the addition of pine wood biochar under unfavourable temperature conditions (psychrophilia) has a more significant positive effect than under mesophilic conditions, supporting biomethane production.

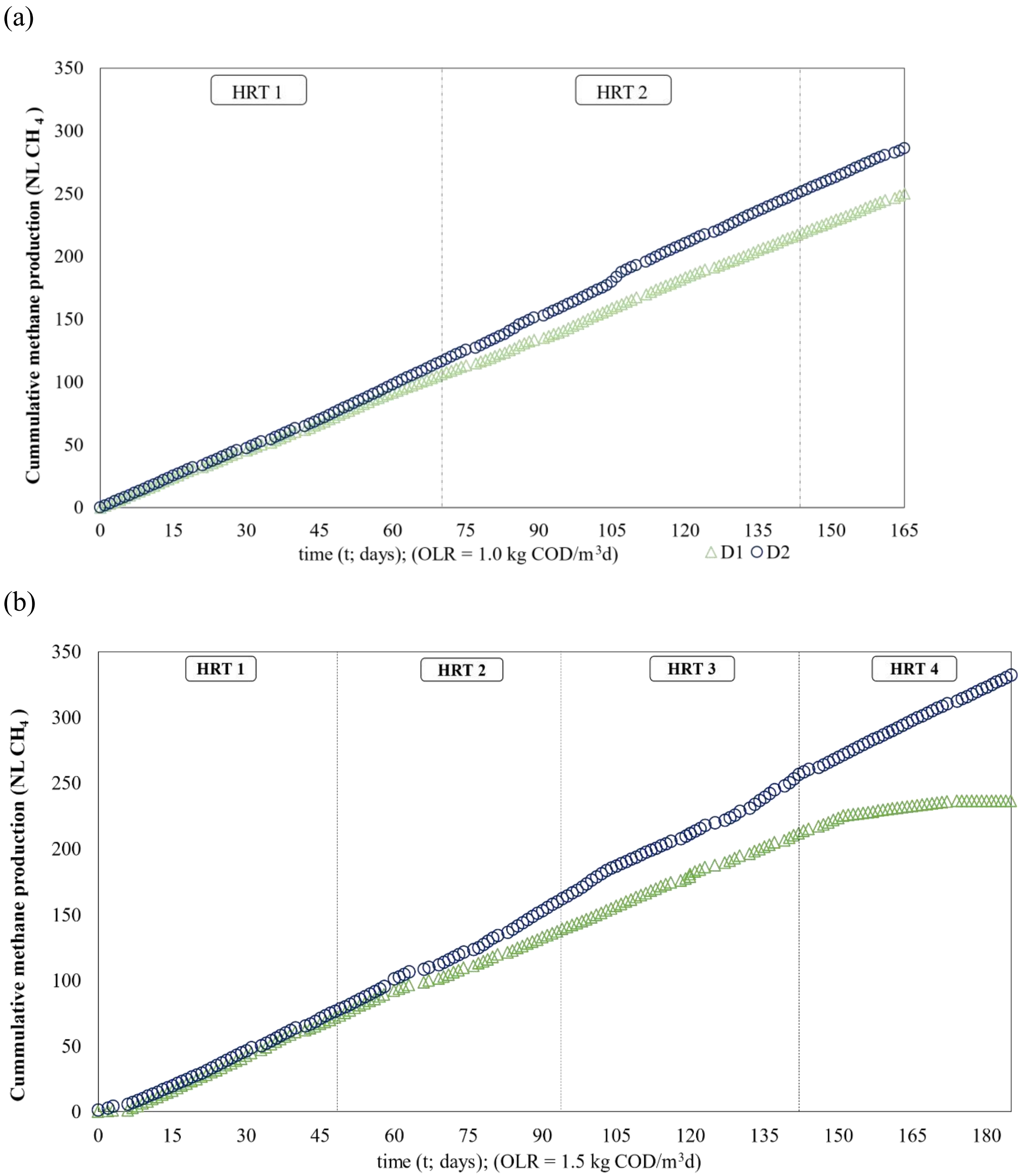

Figure 8. Variation in biomethane production by time with and without biochar addition at two different OLRs: 1.0 kg COD/m^3*d (a) and 1.5 kg COD/m^3*d (b). (D1 green triangles and D2 blue circles).

Table 6 recompiles the adjusted values for biomethane production of Fig. 3 and presents the bioprocess and biodigesters efficiencies. The linearity of the cumulative CH_4 production represents the stability of the process over time. So, a small r^2, denotes a low adjustment and hence is an indicator of underproduction and, in a continuous process case, AD inhibition. As an example, at the end of monitoring D1 was inhibited by VFA accumulation, represented in the no biomethane production at the end of HRT 4 (ORL 1.5 kg COD/m^3*d), which showed a lack of adjustment (r^2=0.857).

Table 6. Bioprocess and the biodigester efficiencies for organic loading rates of 1.0 kg COD/m^{3}d and 1.5 kg COD/m^{3}d with and without biochar addition.

Operational Conditions		**Biomethane production (Adjusted value; N L/d)**				**Bioprocess Efficiency (n_{bp}; m^{3}CH$_4$/kg VS*d)**		**Digester Efficiency (n_{bd}; m^{3}CH$_4$/m$^3_{digester}$*d)**	
OLR kgCOD/m^{3}d	HRT	D1	r^2	D2	r^2	D1	D2	D1	D2
1.0	HRT 1 (day 0 - 70)	1.51	0.999	1.62	0.998	0.34	0.37	0.30	0.32
	HRT 2 (day 71 - 140)	1.56	0.999	1.88	0.998	0.35	0.43	0.31	0.38
1.5	HRT 1 (day 0 - 47)	1.65	0.994	1.66	0.996	0.25	0.25	0.33	0.33
	HRT 2 (day 48 - 93)	1.39	0.997	1.72	0.993	0.21	0.26	0.28	0.34
	HRT 3 (day 94 - 140)	1.52	0.998	1.87	0.996	0.23	0.28	0.30	0.37
	HRT 4 (día 140 - 186)	0.51	0.857	1.78	0.999	0.08	0.27	0.10	0.36

It was observed that, in all cases, increasing the organic load resulted in a decrease in the η_{bp}. Consequently, in line with the methane production trends, a 50 % increase in the organic load (from 1.0 to 1.5 kg COD/m^3*d) leads to a decrease in the specific CH$_4$ production per organic matter loaded. However, the positive effects of adding pine wood biochar make the system capable of supporting the increase in organic loads, making it more efficient than the control. The bioprocess efficiency in D2 (OLR 1.5 = 0.35 m^{3}CH$_4$/kg VS*d) was 1.39 times higher than that determined for the control (0.25 m^{3}CH$_4$/kg VS*d). The bioprocess efficiency values achieved in this study were considerably higher than those reported by Álvarez et al., (2009), who determined efficiencies for the bioprocess of 0.19 m^3CH_4/kg VS*d for the co-digestion of cow and sheep manure under psychrophilic conditions (18 °C).

Regarding biodigester efficiency η_{bd}, it was found that adding pine wood biochar in ACoD increases biomethane production based on the biodigester size by 14.28% and 38.55% for OLR of 1 and 1.5 kg COD/m^3*d, respectively. Also, even when D2 obtained a similar methane production under the two OLRs evaluated, the use of biochar not only improves bioprocess stability, efficiencies, and yields but also reduces the size of operational volume, that is, using biochar allows OLR increase, and then HRT reduction to obtain determined yield. In other words, adding suitable organic support reduces of the operational volume required to obtain a determined biogas production.

2.3.4. Analysis of bacteria, archaeal, and methanogens content communities at different anaerobic digestion stages

Figure 9. presents the evolution of microorganism (total bacteria, *archaea*, and methanogens) content for D1 and D2 at the evaluated OLRs. The mixture of substrates loaded presented a considerably slow concentration of 4.79 x10^8 copies/uL, 4.61 x10^8 copies/uL, and 2.18 x10^7 copies/uL for bacteria, *archaea*, and methanogens, respectively. Regarding the control assay, operating with an appropriated HRT leads to a balanced metabolic process presenting a moderated increase of microorganism content over time. In 70 days, D1 presented an augmentation of bacteria, *archaea*, and methanogens of 70%, 115%, and 184%,

respectively. The previous behaviour was similar for the case with biochar addition except for 50% more bacteria.

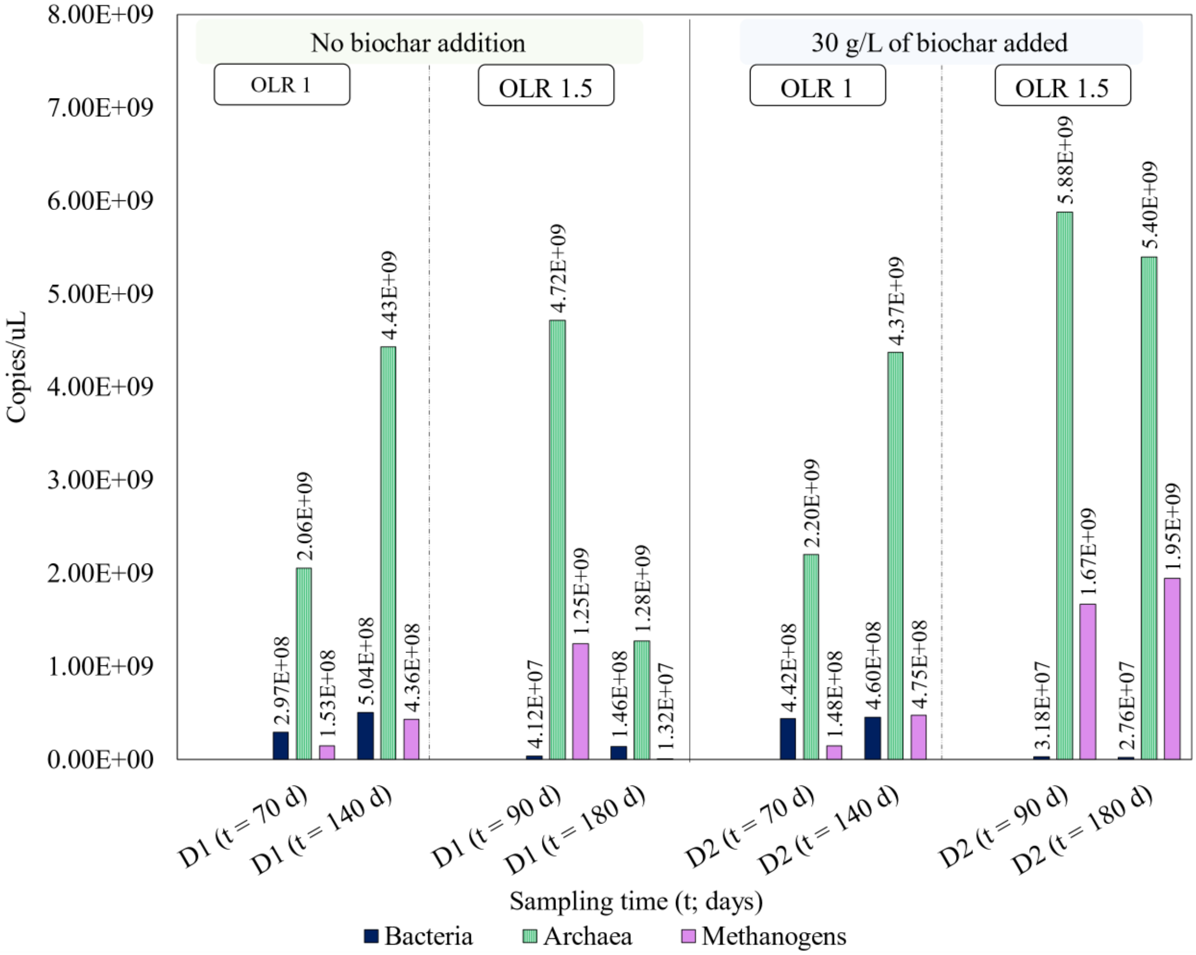

Figure 9. Changes in the total bacteria (dark bars), *archaea* (stippled bars), and methanogens (light bars) at two different OLRs with and without biochar adding.

On the other hand, increasing OLR (decreasing treatment time) affects process stability and inhibits methane generation. This can be supported by the considerable reduction of *archaea* and methanogens content from day 90 to 180, which went from 4.72 x10^9 copies/uL to 1.28 x10^9 copies/uL and from 1.25 x10^9 copies/uL to 1.32 x10^7 copies/uL. This tendency reconfirms process failure (as evidenced in figure 7) by an uncontrolled acidogenesis and then the reduction of acetic acid, considered methane precursor; as there was less acetic acid, the methanogenesis reached was weakened. Adding pine wood biochar allows a better digester performance at higher organic loading rates that were limited by the acidogenesis since the methanogens abundance was considerably increased. In this study, even at inhibitory OLR, the methanogens content in digestate reached values above 1.67 x10^9 copies/uL. This holds particular significance given the importance of the synergistic relationships between acetate-forming bacteria and methanogens for methane production (Wang et al., 2022). In the methanogenesis stage, the products of acidogenesis and acetogenesis are transformed into methane by strictly anaerobic methanogens (Li et al., 2019). With the results obtained, it is possible to mention that biochar after enriching acetic acid content during AD, also improves acetoclastic methanogenesis. Considering the behaviour during monitoring, the most abundant methanogens present in D2 could be *Methanothrix* which is an obligate acetoclastic (FitzGerald *et al.,* 2015), and has a high growth rate and tolerance against inhibition even during long operation periods (Giwa et al., 2019).

Besides enhancing microbial diversity and abundance, biochar promotes cell attachment and, hence, biofilm formation. Figure 10 presents the SEM applied to inspect the microorganism on the biochar surface and pores. The digestate from D1 at day 180 did not show a clear biofilm formation (Fig. 5b) instead it was a group of non-hydrolysed substrate.

Furthermore, For D2 after approximately 180 days of operation, a considerable number of cells were attached homogeneously to biochar. So, it is noteworthy that biochar serves as a propitious environment for the growth and enrichment of methanogens (Figure 10 c-d). Consequently, excess of loading can change the bacterial community abundance and it is proven that the presence of biochar can effectively alleviate this impact.

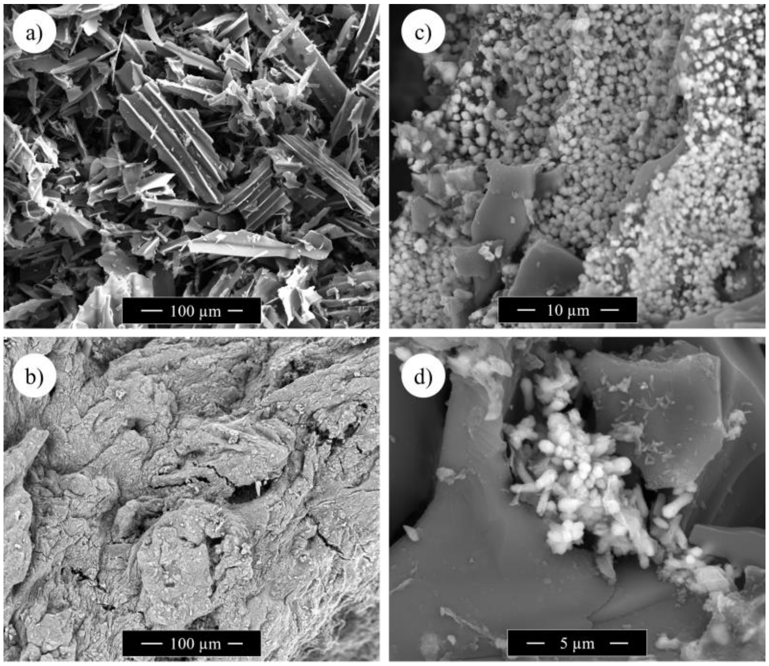

Figure 10. SEM images of raw pine wood biochar surface (a), digestate from D1(b), biofilm formation on biochar surface (c) and pore (d) from D2 digestate.

These phenomena depicted in this study align with previous findings (Jaimes-Estévez *et al.,* 2023) and advocate that the positive effect of pine wood biochar on psychrophilic AD systems can be attributed to two aspects: (i) regulation of acidogenesis and acetate metabolism, thereby optimizing the acetic acid formation and its conversion into methane and (ii) bringing a favourable platform to biofilm formation to the increment of methanogens content.

2.4. Conclusions

In the psychrophilic ACoD of acid substrates such as cheese whey, 30 g/L of biochar efficiently increases the cumulative biomethane yield via enhancement of acidogenesis-acetogenesis, prioritizing controlled acetic acid production and generating 38.55% more methane during operation. Also, biochar is a platform for biofilm formation, reaching a methanogen concentration increment of over 440%, even under inhibitory organic loads. Using biochar is a clear alternative to reducing digesters' size or increasing the volume of waste treated. This study can be considered as the precursor of the ACoD + biochar systems implementation under actual conditions in psychrophilic scenarios.

2.5. Generation of new knowledge: Research Impact

The scientific products associated with the development of Chapter II are presented below.

Publications.

Jaimes-Estévez, J., Castro, L., Sanabria, K., Rondón, Z., & Escalante, H. (2020). Metodología para la producción de biogás sin riesgos de inhibición en laboratorio: codigestión de lactosuero y estiércol bovino. www. redbiolac. org, 14, 88.

Jaimes-Estévez, J., Castro, L., Escalante, H., Carrillo, D., Portillo, S., Sotres, A., & Morán, A. (2020). Cheese whey co-digestion treatment in a tubular system: Microbiological behaviour along the axial axis. Biomass Conversion and Biorefinery, 1-10. https://doi.org/10.1007/s13399-020-00988-4

Jaimes-Estévez, J., Mendieta, O., Sánchez, Z., Castro, L. & Escalante, H. (2022). Technological alternative for the management of agro-industrial waste via anaerobic digestion: sugarcane, cheese whey, and slaughter residues. Industrial Biotechnology, 18(4), 257-261. https://doi.org/10.1089/ind.2022.0005

Presentations.

Jaimes-Estévez, J., Madrigal, G., Mendieta, O. Bovine manure biochar as sustainable stabilizer of acid substrate anaerobic digestion. 4th International Conference for Bioresource technology for bioenergy, bioproducts & Environmental sustainability. Italy may 2023

Jaimes-Estévez, J., Mendieta, O., Sánchez, Z., Castro, L. & Escalante, H. Management and valorization of agro-industrial wastes through anaerobic digestion: sugarcane, cheese whey, and slaughter residues. VII Sigera 2021.

Jaimes-Estévez, J., Mendieta, O., Madrigal, G. Digestión anaeróbica de lactosuero con biocarbón de estiércol bovino como estrategia para contribuir a la bioeconomía circular de las fábricas de queso. 1er Congreso Nacional de Ciencia, Tecnología e Innovación para el Agro – AGROCIENCIA 2022

Proyects.

Active participation in the development of a collaborative project with Northumbria University (UK) under the Royal Academy of Engineering call, titled 'Development of a small-scale combined Waste-to-BioEnergy system with solar Organic Rankine Cycle for heat and power production in rural areas of Colombia.

Human Resource Development.

Co-supervision of undergraduate thesis for two Chemical Engineering students. Status: defended and approved. Title: "*Influencia del uso de soportes orgánicos sobre el rendimiento y la eficiencia del proceso de digestión anaeróbica semicontinua en condiciones psicrófilas*".

Chapter III. Psychrophilic Anaerobic Digestion Combined with Gasification: A Sustainable Path to Energy Independence

3. Psychrophilic Anaerobic Digestion Combined with Gasification: A Sustainable Path to Energy Independence

Abstract

This investigation assessed the environmental advantages of integrating Anaerobic Digestion (AD) and Gasification through a Life Cycle Analysis (LCA) with the monitoring of digester dynamics and economic viability determination. The case studied is a farm (located in a zone with an average temperature 17.3 ± 3.3 °C) with the capacity to produce cheese. As an alternative to treat cheese whey and cattle manure, the primary wastes generated, and to generate heat, an 8m^3 anaerobic digester and a gasifier were installed. Incorporating pine wood biochar into the anaerobic digestion of CW and CM boosts VFA absorption, enhances the alkalinity of the solution, and increases methane generation. Hence, adopting an integrated AD and Gasification approach reduces climate change impacts (such as fossil resource scarcity, global warming and ozone depletion) by 85% and achieves a positive net present value (NPV) of US$ 5,133.47 when the system is implemented, positioning AD + gasification as a budget-friendly and eco-conscious option that improves AD efficiency. Adopting this energy integration alternative has resulted in a notable improvement in living standards for rural users through lowered costs for fertilizers and fuel.

3.1. Introduction

18% of the Latin American population resides in rural areas. These territories are challenging to access, and the most vulnerable families depend on firewood, coal, and vegetable charcoal (biochar) for heating and cooking (Garfí *et al.,* 2019). Remarkably, 6.5 million people in Colombia use traditional solid fuels as liquefied petroleum gas (LPG) for cooking, where rural inhabitants present a notorious lack of energy access (Pérez *et al*., 2021). In this scenario regions at temperatures below 25°C (psychrotrophic conditions) demand more energy due to the use of heating systems, electrical appliances, and transportation. Various energy technologies, such as biomass plants, photovoltaic solar plants, and wind plants, have been studied and developed to meet these needs. However, these technologies come with high installation costs and depend on environmental conditions (Roman *et al*., 2022).

Gasification (GS) and anaerobic co-digestion (ACoD) promises an economical and environmentally friendly alternative. In GS, organic matter is oxidated at high temperatures ($600 < T < 900$ °C) to generate energy, synthesis gas (syngas: CO, CO_2, H_2, and N_2), and biochar, with a yield of 10% from the biomass used (Song et al., 2021). Biochar is used for soil preparation in agriculture and as a contaminant adsorbent. On the other hand, during the AD bioprocess, microorganisms degrade waste, producing biogas (with a high methane content) and digestate (liquid phase) used as a soil conditioner. Despite the energy benefits obtained from using these technologies, the environmental impacts that may arise and the possible uses for the produced biochar, which is considered a modulating agent in the anaerobic process (Masebinu *et al*., 2019; Gutiérrez *et al*., 2022; Madrigal *et al*., 2022; Jaimes-Estévez *et al*., 2023; Zhang *et al*., 2024), remain unknown. A valuable tool for quantifying environmental impacts with and without ACoD is life cycle assessment (LCA), which allows for evaluating and comparing different scenarios throughout the entire life cycle of a product, process, or activity (ISO, 2006). Garfí *et al*., (2019) conducted an LCA on anaerobic digestion (AD) on two small-scale farm digesters under mesophilic and psychrophilic conditions. This research determined that biogas production replaces 100% of propane gas used for cooking under mesophilic conditions, while under psychrophilic conditions, 5% of propane continues to be used for daily activities. Additionally, greenhouse gas (GHG) emissions at mesophilic temperatures decreased between 1.2 and 1.5 times compared to the digester implemented under psychrophilic conditions (Garfí *et al*., 2019). On the other hand, Mendieta *et al*., (2021) conducted an LCA of AD from non-centrifugal cane sugar production waste. The authors showed that implementing a biodigester reduces soil eutrophication problems by 99%. However, to date, there are no reports of LCA studies for an integrated system of anaerobic digestion and GS under psychrophilic conditions, employing pine wood biochar as an organic additive to improve the anaerobic process. Therefore, this study aims to assess the environmental impacts, through LCA, for the waste management and valorization of agro-industrial wastes via ACoD + GS and the impact of biochar addition to AD as a metabolic modulator. As considered in previous studies, the substrates to treat are cheese whey (CW) and cattle manure (CM), the main subproducts generated in livestock farming, which is a fundamental pillar for the economy and livelihood of Colombian (and Santander) rural inhabitants. In that sense, two scenarios were considered: i) a familiar dairy farm as a study case where wastes are not treated, and propane and wood are used to cover farm energy requirements, and ii) the AD + GS scenario, in which a low-cost biodigester and a gasifier are used for organic wastes treatment producing energy and fertilizer.

3.2. Material and Methods

This study was developed in three phases: a) the study of the microbiological and biochemical behaviour of the ACoD process of CW and CM using pine wood biochar as organic support in a 8m^3 rural digester; b) the assessment of the environmental impacts of the integrated GS + ACoD process through Life Cycle Assessment (LCA), compared to a reference scenario (no digester or gasifier). and c) the financial analysis of the integrated system.

3.2.1. Study case characteristics.

A five-person family farm was selected as the study case. This farm is located in a rural psychrophilic area near San Turban moor that connects the Departments of Santander and North Santander – Colombia. The farm average temperature is around 17.3 ± 3.3 °C. The farm has thirteen heads of dual-purpose cattle, which produce 80 L/d of milk used for cheese production (1.5 tons/year). This activity generates approximately 72 L of CW and 221 kg of manure per day. Furthermore, some agricultural practices are developed on the farm, requiring synthetic fertilizers and irrigation water. On the other hand, to meet their heating and cooking needs, farm owners require 1.22x10^4 kWh/year.

3.2.1.1 Description of the Integrated System: Anaerobic Co-digestion + Gasification.

A household biodigester and a gasifier were installed as an alternative to reduce energy costs and manage CW and CM generation. The reactor was constructed from high-density polyethylene with 8 m^3 total volume (65% liquid and 35% gas phases). The biodigester is fed daily with a CW/CM mixture in a 7:3 ratio, equivalent to an organic loading rate (OLR) of 0.5 kg VS/m^{3}d and a hydraulic retention time (HRT) of 150 days. The produced biogas is stored in a 3 m^3 reservoir for subsequent use. For its part, the gasifier is a 40 L fixed bed equipment with ascendant air flux (450 L/min on average) that operates with a batch load between 10 – 20 kg of recycled pine wood and reaches temperatures between 500 and 650 °C. In that sense, the energy generated from biogas is used for heating and cooking for the farm residents. Also, the energy produced during GS is applied in the cheese production process. Furthermore, the biochar generated during GS was employed as organic support for the anaerobiosis. To analyse the influence of pine wood biochar on the AD process, the organic support was added after 90 days of continuous CW/CM blend feeding; this initial period was considered a control (monitoring with no biochar). Subsequently, 52 kg of biochar were loaded gradually into the digester until day 150 (First operative HRT), aiming to reach a concentration of 10 g biochar /L and provide a good distribution of particles along the digester.

3.2.2. Psychrophilic ACoD process: Biochemical and microbiological monitoring

The biochemical analysis was based on determining the loaded and final volatile fatty acids (VFAs) content and its respective total alkalinity (TA) and pH. Additionally, the biogas and biomethane produced were quantified to establish the process yield (SBP; Nm$^3_{\text{biogas}}$/kg VS*d), biodigester yield (BPR; Nm^3/m^{3}d), and specific methane production (SMP; Nm^{3}CH$_4$/kg VS*d) (Martí-Herrero *et al.,* 2021). Besides, the microbiological monitoring was conducted by determining the specific methanogenic activity (SMA) and the microorganisms (bacteria, *archaea*, and methanogens) content in the outlet.

3.2.3. Physicochemical Analysis

The chemical oxygen demand (COD), and volatile solids (VS) content were analysed according to standard methods for the examination of water and wastewater (APHA 2017) via spectrophotometry and thermogravimetry, respectively. A commercial gasometer installed in the digester biogas outlet was used to measure daily biogas production in situ. The compositions of methane and carbon dioxide in biogas were detected by gas chromatography (Holliger 2016). Biogas and biomethane measurements were standardized in terms of pressure and temperature (standard condition: 101.325 kPa, 273.15 K). Volatile fatty acids (Acetic, propionic, butyric, iso-butyric, valeric, iso-valeric, and caproic) concentrations were determined via gas chromatography (flame ionization detector) according to Raposo *et al.,* (2013). The total VFA content was considered as the sum of each VFA in each sample. TA and pH were measured by titration (Purser *et al*., 2014), and by potentiometric assay, respectively. The SMA was determined by quantifying the amount of acetate converted to methene in 150 mL reactors (batch assays) using the methodology proposed by Astals *et al*., (2015). The concentration of *archaea*, bacteria, and methanogens were determined via Quantitative Polymerase Chain Reaction (qPCR) assays, following the established by Jaimes-Estévez *et al*., (2023). The periodicity of measurements (except for biogas) was every two weeks, and the samples were taken and transported under refrigeration to the laboratory prior to analysis.

3.2.4. Life cycle assessment for the integrated system

The potential environmental impacts were identified through a Life Cycle Assessment (LCA) and were calculated using SimaPro® software (Pre-sustainability, 2020). This analytical tool adheres to ISO 14040 standards, which establish the principles and framework for LCA. Four stages were evaluated, encompassing the definition of objectives and scope, inventory analysis, environmental impact assessment, and interpretation of results (ISO, 2006). The characterization of impacts (impact analysis) was carried out considering the 17 impact categories established in the software. Among the most influential categories in Environmental Assessment, the most remarkable are Climate Change, Ozone Depletion, Terrestrial Acidification, Freshwater Eutrophication, Marine Eutrophication, Water Consumption, Particulate Matter Formation, and Fossil Resource Depletion.

3.2.4.1. Objective and Scope of the LCA for the Case Study

The aim of the LCA was to determine the environmental benefits of the integrated system ACoD + GS. To accomplish this, two scenarios were compared: i) a baseline scenario, where wastes (CW and CM) are not treated and are directly disposed to the environment (Figure 11a), and food cooking is done using LPG; and ii) the current scenario where the integrated system operates (Figure 11.b), utilizing energy streams (biogas, synthesis gas, and heat energy) and nutritional components (using digestate as a biofertilizer). It is important to note that the production of kilograms of cheese per year (kg_{cheese}/year) was used as the functional unit for mass and energy balances.

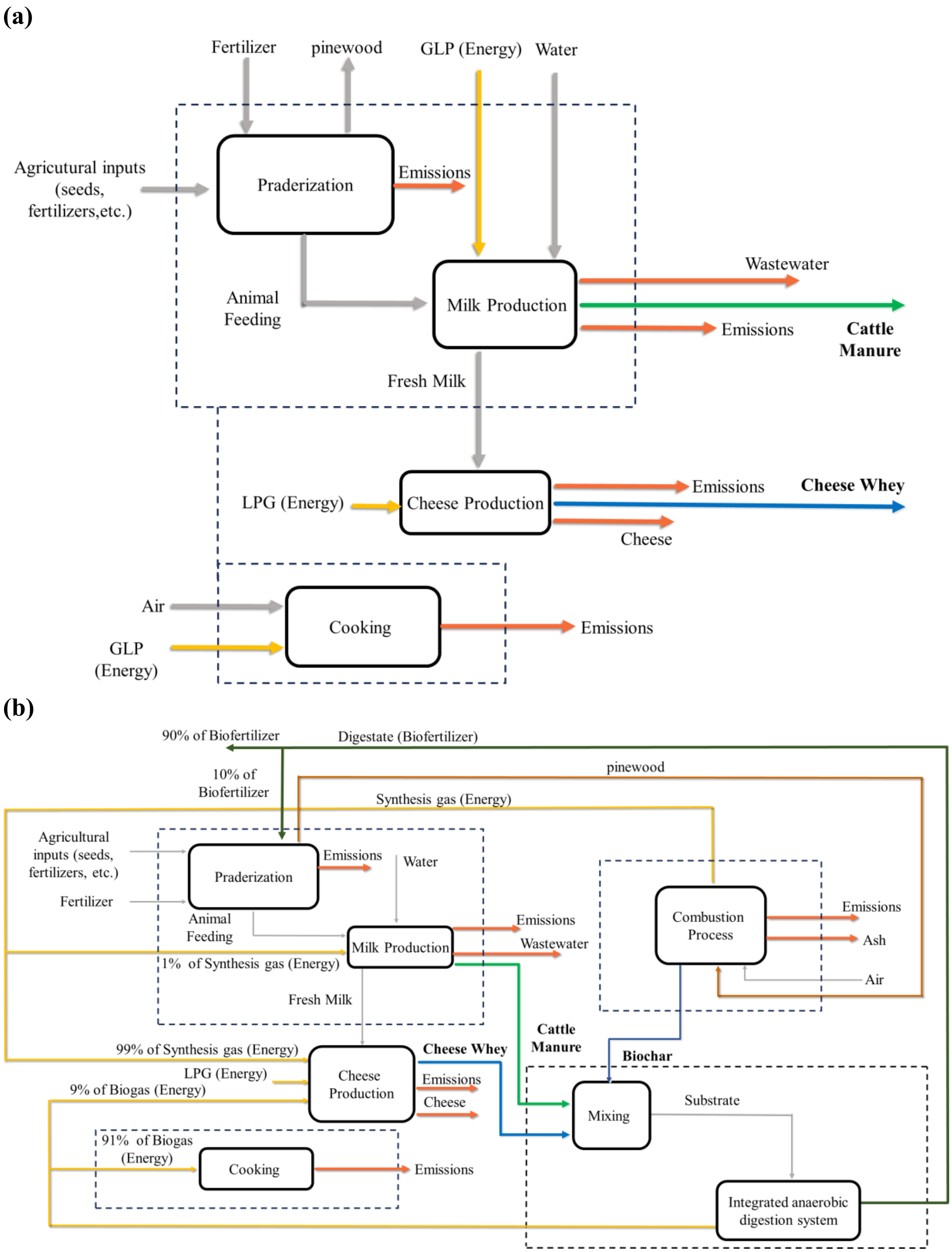

Figure 11. Process diagrams for: a) a baseline scenario, where CW and CM are no treated; and b) the integrated system scenario.

The scope of the integrated system incorporated: 1) emissions to the air and soil due to manure storage; 2) production and transportation of LPG for both scenarios; 3) atmospheric emissions from LPG combustion; 4) production and transportation of synthetic fertilizers; 5) direct emissions to the air and soil due to applying of synthetic fertilizers and digestate to the soil; 6) materials for biodigester construction and maintenance; 7) water consumption and atmospheric emissions from losses; 8) biogas combustion; 9) pine wood combustion; 10) synthesis gas combustion.

3.2.4.2. Inventory Analysis of the Base and Integrated Scenarios

For the inventory of the base scenario, a preliminary inspection of the case study was conducted along with a literature review. The observed LPG consumption in the family farm amounts to 74 m^3 per month (1 atm; 0 °C), which is used during cheese production, sterilization of milking machines, and meeting the cooking needs of five people for three hours daily. The quantity of synthetic fertilizer considered was determined based on nutrient requirements per hectare per year for the most representative crops in the case study: tomato cultivation (N: 622 kg/ha; P_2O_5: 170 kg/ha; K_2O: 994 kg/ha), grass (N: 150 kg/ha; P_2O_5: 115 kg/ha; K_2O: 120) (Jacobs and Landis 2014), and pine (N: 110 kg/ha; P_2O_5: 70 kg/ha; K_2O: 100) (Dickens *et al*., 2020). An average distance of 38 km from urban areas to the study case farm was considered for the transport of LPG and synthetic fertilizers. Milking machine cleaning was performed once a week using a washing solution of NaClO: 3.75 g/L; Na_2SiO: 3.75 g/L (Grieshop *et al*., 2011; Sfez *et al*., 2017). Direct emissions from LPG combustion were estimated considering emission rates from previous studies (i.e., CO_2 (fossil): 3085 g/kg $_{LPG}$; CO (fossil): 14.9 g/kg $_{LPG}$; CH_4 (fossil): 0.05 g/kg $_{LPG}$; Non-methane volatile organic compound NMVOC: 18.8 g/kg $_{LPG}$; NO_X: 3 g/kg $_{LPG}$; N_2O: 0.15 g/kg $_{LPG}$; PM2.5: 0.3 g/kg $_{LPG}$; PM10: 1.1 g/kg $_{LPG}$; SO_2) (Garfi *et al.*, 2019). Similarly, direct emissions to air and soil from manure storage were calculated using factors found in the literature (i.e., CH_4: 6.6 mg/kg$_{manure}$ *d) (Garfi *et al.*, 2019). Nitrogen emissions from synthetic fertilizers were calculated as 25% and 1% of the initial N content for NH_3 and N_2O, respectively (IPCC, 2006). Soil emissions were considered equivalent to those generated in manure storage, i.e., N: 0.5% and P: 0.62 % of the amount of LS produced daily (Muset & Castells, 2017). The inventory summary of the base scenario is presented in table 7.

Table 7. Summary of the inventory (inputs and outputs) for the base scenario and the integrated system

Inputs	Base Scenario	Integrated System	Units
*Construction materials for biodigester implementation**			
Accessories for biogas pipelines	---	4.67E-08	kg
Bricks	---	3.59E-07	kg
Cement	---	8.62E-08	kg
Sand	---	8.38E-06	kg
Rails	---	2.75E-08	kg
Stove	---	1.32E-08	kg
Polyethylene (biodigester and reservoir)	---	6.68E-07	kg
transport (materials)	---	1.01E-03	kg
*Construction materials for gasifier implementation***	---	2.33E+03	COP
transport (materials)	---	2.44E+05	COP km
LPG production and transport			
Production	1.34E+00	1.83E-02	kg
transport	5.09E+01	6.94E-01	kg km
Synthetic fertilizer production and transportation			
N	5.87E-01	0.00E+00	kg
P_2O_5	2.36E-01	0.00E+00	kg
K_2O	8.07E-01	0.00E+00	kg
transport	4.12E-02	0.00E+00	kg km
Washing solution for milking machines and transport			
NaClO	1.30E-04	1.30E-04	kg/L

Na_2SiO_3	1.30E-04	1.30E-04	kg/L
transport	6.55E-06	6.55E-06	kg km/L
Water consumption	4.98E+01	4.98E+01	L

Outputs

Direct emissions from LPG combustion

CO_2 (fosil)	4.13E+00	5.64E-02	kg
CO (fosil)	2.00E-02	2.72E-04	kg
CH_4 (fosil)	6.69E-05	9.14E-07	kg
NMVOC	2.52E-02	3.44E-04	kg
NOX	4.02E-03	5.48E-05	kg
N_2O	2.01E-05	2.74E-07	kg
PM2.5	4.02E-04	5.48E-06	kg
PM10	1.47E-03	2.01E-05	kg

Direct emissions to air from the application of synthetic fertilizers on crops

NH_3	1.47E-01	--	kg
N_2O	5.87E-03	--	kg

Direct emissions to soil from the application of synthetic fertilizers on crops

N	1.17E-01	--	kg
P	7.08E-02	--	kg
k	4.04E-01	--	kg

Direct air emissions from the digestate application to crops

NH_3	---	1.47E-01	kg
N_2O	---	5.87E-03	kg

Direct soil emissions from the digestate application to crops

N	---	1.17E-01	kg
P	---	7.08E-02	kg
k	---	4.04E-01	kg

Direct air emissions from cattle manure storage

NH_3	1.05E-02	8.93E-03	kg
N_2O	1.26E-03	1.07E-03	kg
CH_4	3.54E-04	3.01E-04	kg

Direct soil emissions from cattle manure storage

N	1.68E-02	1.43E-02	kg
P	5.41E-03	4.60E-03	kg
K	7.75E-02	6.58E-02	kg

Direct soil emissions from cheese production

N	4.37E-02	1.46E-02	L

P	5.42E-02	1.81E-02	L
Direct air emissions from biogas losses			
CH_4	---	7.24E-03	kg
Direct emissions from biogas combustion			
CO_2 (biogenic)	---	2.31E-01	kg
CO (biogenic)	---	3.04E-04	kg
CH_4 (biogenic)	---	1.60E-04	kg
NMVOC	---	9.60E-05	kg
NOX	---	1.44E-04	kg
N_2O	---	1.33E-05	kg
PM2.5	---	0.00E+00	kg
PM10	---	8.00E-05	kg
Direct emissions from syngas combustion			
CO_2 (biogenic)	---	2.02E+00	kg
CO (biogenic)	---	2.65E-03	kg
CH4 (biogenic)	---	1.40E-03	kg
NMVOC	---	8.38E-04	kg
NOX	---	1.26E-03	kg
N_2O	---	1.26E-04	kg
PM2.5	---	0.00E+00	kg
PM10	---	6.98E-04	kg
Direct air emissions from pine wood combustion in the gasifier			
CO_2 (biogenic)	---	7.54E+03	kg
CO (biogenic)	---	2.08E+02	kg
CH_4 (biogenic)	---	5.44E+01	kg
NMVOC (biogenic)	---	4.61E+01	kg
NOX (biogenic)	---	9.71E-01	kg
N_2O (biogenic)	---	4.85E-01	kg
PM2.5 (biogenic)	---	1.55E+01	kg
PM10 (biogenic)	---	5.15E+01	kg
SO_2 (biogenic)	---	4.13E+00	kg
N_2 (biogenic)	---	2.11E+01	kg
O_2 (biogenic)	---	1.81E+00	kg

*Calculated per kg of treated manure and kg of cheese produced

In the case of the integrated system, construction materials for the biodigester were calculated considering the characteristics and design of biogas plants. The manufacturer provided the materials and costs of the gasifier. Digestate replaced the use of synthetic fertilizers. The requirements for the washing solution for milking machines were the same as in the base scenario. Considering that a portion of LPG is still utilized, emission rates from combustion described in the previous scenario were employed. Direct emissions from biogas combustion, synthesis gas from pine wood, and the use of synthesis gas were determined using emission rates reported by Garfí et al. (2019): CO_2 (biogenic): 1,444 g/kg$_{\text{biogas}}$; CO (biogenic): 1.9 g/kg$_{\text{biogas}}$; CH_4 (fossil): 1.0 g/kg$_{\text{biogas}}$; VOC: 0.6 g/kg$_{\text{biogas}}$; NO_X: 0.9 g/kg$_{\text{biogas}}$; N_2O: 0.09 g/kg$_{\text{biogas}}$; PM2.5: 0 g/kg$_{\text{biogas}}$; PM10: 0.5 g/kg$_{\text{biogas}}$; SO_2: 0.05 g/kg$_{\text{biogas}}$. Considering that the biodigester operates under optimal conditions, a value of 5% gas production losses was taken into account. Regarding applying of digestate to crops, the rates of direct emissions to air and soil were the same as those for synthetic fertilizer (Table 1).

3.2.5. Economic potential of integrating ACoD with gasification

Phase three, which focuses on the financial analysis of the integrated system, considers that the gasifier is used to generate energy (1126.9 MJ/month) and produce the required biochar for the biodigester (350 g/d for 365 days). The price for LPG was stablished according to average actual prices in the rural community. Subsequently, the approximate price of the generated biogas was determined based on the commercial value of LPG (353.3 COP/kWh). Moreover, it was considered that the family farm partially replaces commercial fertilizers with digestate or commercialized products. So, an estimation of the biofertilizer (from digestate) price was made by comparing it with the national prices of commercial liquid fertilizers with similar content of N, P, and K. Accordingly, financial analysis was carried out by evaluating capital and operational expenses (CAPEX and OPEX). Equipment and implement costs in CAPEX were obtained through quotations, while considerations based on the available market information for fuels, raw materials, and labour near the project implementation site were taken into account for OPEX. Finally, the project's viability was assessed based on current and average consumer inflation rates (*f*) and intervention rates (*i*) over a 10-year period. Economic indicators such as Net Present Value (NPV), Internal Rate of Return (IRR), Cost/Benefit Ratio (B/C), and Payback Period (PBP) were analysed. Also, a sensitivity analysis for profitability indicators NPV and IRR was done due to the high variability in the Colombian economy.

3.3. Results and Discussion

3.3.1. Monitoring the Biochemical and Microbiological Aspects of the Psychrophilic Anaerobic Co-Digestion (ACoD) Process.

3.3.1.1. Monitoring the anaerobic digestion yields in psychrophilia

Figure 12 shows the biogas production behaviour and quality over 350 days of monitoring. It is noteworthy that the slope of each period corresponds to the daily production of biogas (Nm^3_{biogas}/d). As can be seen, during the HRT1 between the ACoD stage and ACoD + pine wood biochar, there was a 68.6% increase in biogas production. Likewise, the biogas quality improved, with the CH_4 concentration increasing from 55.4% to a maximum of 61.1%. This behaviour demonstrates that pine wood biochar boosted biogas production in the AD process under psychrophilic conditions, even in a rural scenario. Following the period of daily biochar feeding, equivalent to HRT2, the average biogas production was 1.7 m^3/d. Overall, from the start of the additive feeding to HRT3, the biogas production was 1.39 ± 0.26 Nm^3_{biogas}/d, a 27.52% increase (biogas quality $57.54 \pm 2.5\%$). This shows that operationally, the effect of biochar is more significant while it is being fed into the digester; however, this effect is sustained over a prolonged period, as evidenced on a laboratory scale (Chapter II).

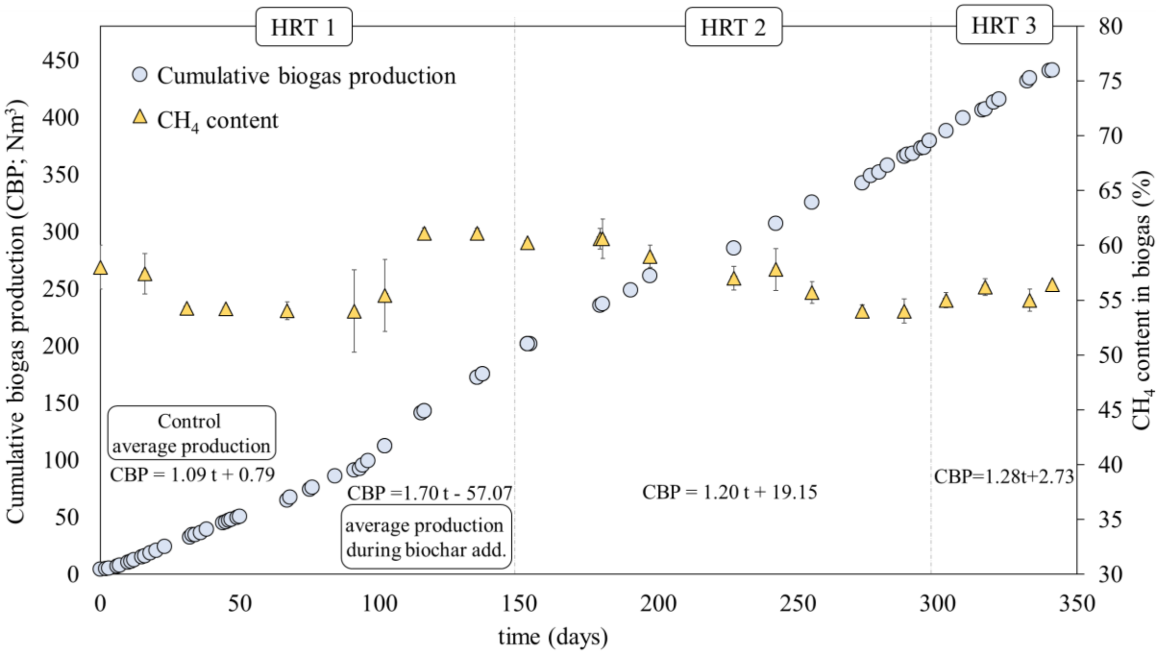

Figure 12. Cumulative biogas production tracing (blue circles) and methane content (yellow triangles) monitoring during 350 days. The “control” zone indicates no biochar addition. The linear equations represent the adjusted biogas production for each HRT, and their slopes indicate the average biogas production.

From the biogas production data and methane content, it is possible to determine the digester performance (Biogas Production Rate or BPR), bioprocess efficiency (Specific Biogas Production or SBP), and specific methane production (SMP) (Jaimes-Estévez *et al.*, 2022). Table 8 summarizes the average values of these parameters for the three monitored HRTs (considering the time range during which pine wood biochar was fed). Regarding the control event (no biochar addition during HRT1), the results obtained are comparable with studies previously conducted in the same location: the yields were 0.52 Nm^3 biogas/m^3

biodigester d, 0.72 Nm^3 biogas/kgVS*d, and 0.42 Nm^3 CH4/kgVS*d for an OLR of 1.34 kg COD/m^{3}d (Jaime Estévez et al., 2022).

Table 8. Performance characterization of biodigester during monitoring.

	HRT	**BPR (Nm^3/m$^3_{\text{digester}}$*d)**	**SBP (Nm^3/kgVS*d)**	**SMP (Nm^3/kgVS*d)**
HRT 1	**Control (no biochar added)**	0.19	0.52 ± 0.1	0.26 ± 0.037
	Biochar addition period	0.33	0.66 ± 0.021	0.37 ± 0.072
	HRT 2	0.23	0.53 ± 0.08	0.34 ± 0.08
	HRT 3	0.25	0.62 ± 0.015	0.33 ± 0.036

Evidently, the yields obtained in the reference study were higher than those found in the present research. This may be because the comparative case uses a higher OLR than the study case (0.5 ± 0.2 kg COD/m^{3}d).

The above indicates that the process can be improved in terms of yields by increasing the OLR to maximum values close to 1.5 kg COD/m^{3}d, which is possible for users in the case of more substrate availability. During the biochar feeding period (HRT 1, day 100 - day 150), there was a general increase in the evaluated parameters, close to 42.4%, 21.2%, and 29.7% for digester performance (BPR), bioprocess efficiency (SBP), and the digester methane production (SMP), respectively. After adding pine wood biochar, the system maintained its performance stable with minimal variations of 2.5 ± 0.03%. It is possible to mention that in a process at a rural scale under psychrophilic conditions, pine wood biochar can improve both the volume of biogas production and its methane content. In other words, the behaviour of substrate consumption and, therefore, product generation are significantly affected by the addition of organic support, globally improving the process performance.

3.3.1.2. Monitoring the anaerobic digestion stability: VFA, pH and TA as indicators.

Figure 13 shows the behaviour of the most representative variables of AD stability. Regarding VFA, it is possible to show that even with the increase in the concentration of acids fed to the biodigester, there are no acidification risks since the concentrations of VFA at the outlet were, on average, 235.21 ± 69.58 mg/L. These values are below concentrations considered inhibitory (>1.5 mg/L) for continuous feeding systems (Angelidaki et al., 2005). Thus, throughout the monitoring, the decrease in VFA represents a bioconversion greater than 91.46 ± 2.32%.

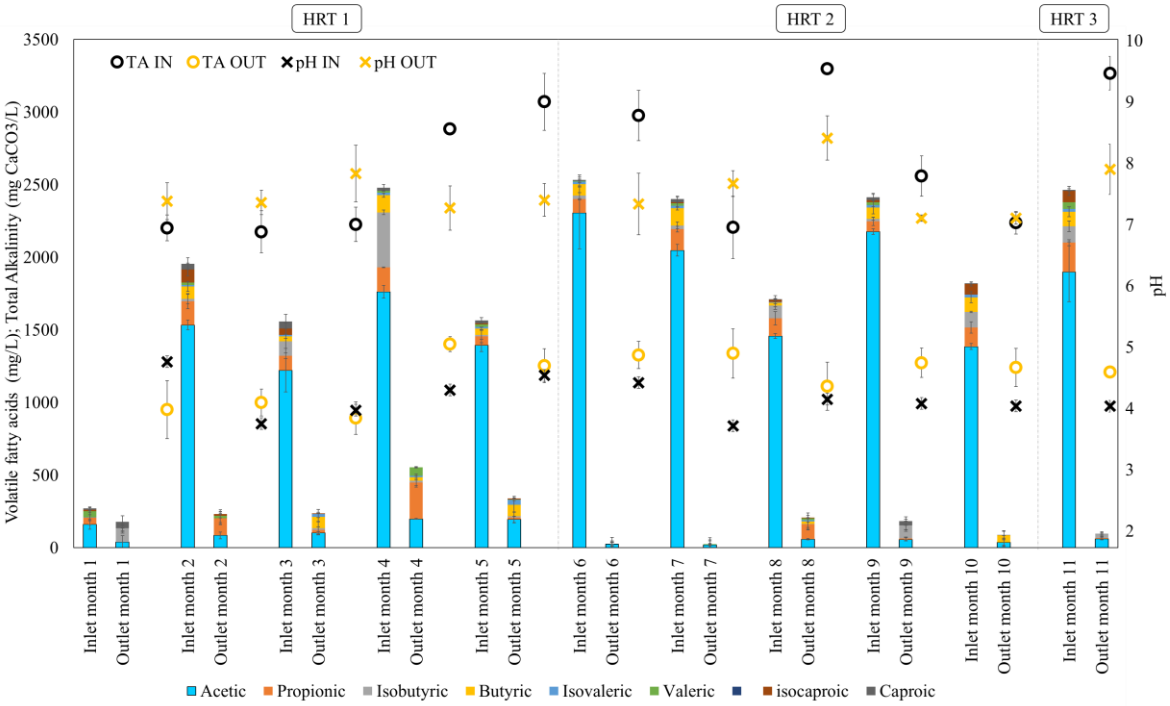

Figure 13. Stability indicators of the anaerobic digestion process on a rural scale: volatile fatty acids (C2-C6; coloured bars), total alkalinity (circles), and pH (crosses) for input and output samples.

Gas chromatography was used on a series of representative samples from the influent and effluent to evaluate the behaviour of individual acids during monitoring. The acids with the highest proportion in the effluent were acetic, propionic, and butyric acid, representing 35.65%, 22.01%, and 10.57% of the total acids, respectively. *Lettinga and Pol.,* (1991) established that the concentration of acetic acid in the effluent should not exceed concentrations of 2,000 mg/L. From the above, it is evident that in the process the concentration of acetic acid was maintained in the values of the appropriate range (83.86 ± 22.3 mg/L), since higher values can produce the inhibition of methanogenic bacteria.

Another acid that plays an important role in AD is propionic acid, since it is one of the precursors of acetate (which subsequently gives rise to the formation of CH_4) and due to its complexity of conversion to other intermediates during anaerobiosis (Hill *et al*., 1987). This statement is consistent with the results obtained in this research, since propionic acid had an average conversion of 43.48% while the rest of the acids had conversions higher than 62%. Regarding the permissible ranges of this acid, it has been shown that methanogens are inhibited at concentrations of propionic acid in the effluent greater than 1,000 mg/L (Wijekoon *et al*., 2011). For its part, some failure/stress indices have been developed to predict the state of AD. The P/A ratio (propionic acid/acetic acid) allows for establishing whether a biodigester is healthy since for values of $P/A > 1.4$, there is an accumulation of propionic acid that represents a reduction in the methane content due to the inhibition of hydrogenogenic bacteria (Bolte *et al*., 1989). The average P/A ratio of the monitoring was 0.072 and 0.61 for the influent and effluent, respectively. The above indicates that the process can produce methane from the available acetic and propionic acid without any inhibition risk. Another failure/stress index is the one proposed by Bolte *et al*., (1989), who found that under concentrations greater than 15 mg/L of isobutyric and isovaleric acid in the effluent, the bioconversion of acids to methane could be inefficient. For the case study, the average fed values of these acids were 87.88 mg/L and 14.21 mg/L. After the anaerobic process, the concentrations of these acids were 15.37 ± 5.42 mg/L and 7.10 ± 2.03 mg/L for isobutyric and isovaleric, respectively, showing the efficacy of the process more directly related to acetogenesis (acids consumption to generate acetate and so CH_4). In general, the study of individual VFAs proved to have high importance since it provides information about the state of the AD process (Wu *et al*., 2021; Jaimes Estévez *et al*., 2022). In fact, according to the parameters discussed earlier, the ACoD + biochar process had a healthy metabolism, capable of consuming organic matter with a high content of VFAs, such as cheese whey, even under actual conditions.

Regarding acidity values, a healthy AD process maintains a pH range between 6.2 and 8.2 (Fotidis *et al*., 2016). During monitoring, average values of 4.03 ± 0.46 and 7.44 ± 0.38 were obtained for the inlet and outlet, respectively. In fact, the pH of the influent was below the recommended level, which is consistent with the load of VFAs fed during the ACoD. However, the system managed to stabilize the acidity, maintaining the pH of the effluent within the recommended parameters. One important aspect is that, even with a higher percentage of CW fed, adding organic support to the influent increased its pH by 7% compared to the feed without organic support. In this way, the addition of pine wood biochar can alleviate the drop in pH caused by the increase in fed VFAs (Jang et al., 2018). For its part, due to the addition of biochar, it is possible to notice that the TA values of the food went from average values of 2,162.25 ± mg$CaCO_3$/L to values between 2,868.13 mg $CaCO_3$/L ± and 3,800 mg $CaCO_3$/L. The above represents a minimum alkalinity contribution of 705.88

mg $CaCO_3$/L by the biochar. This behaviour extended to the output; after the addition of pine wood biochar, the system TA increased by 19.72%, going from 863.13 mg $CaCO_3$/L to 1,033.33 mg $CaCO_3$/L.

3.3.1.3. Biochar impact on AD microbiology: bacteria, archaea, and methanogens content.

Figure 14 shows the behaviour of *archaea*, bacteria, and methanogens content in the psychrophilic digester during one year of monitoring. As can be seen, substrates contribute significantly to the concentration of microorganisms in the bioprocess.

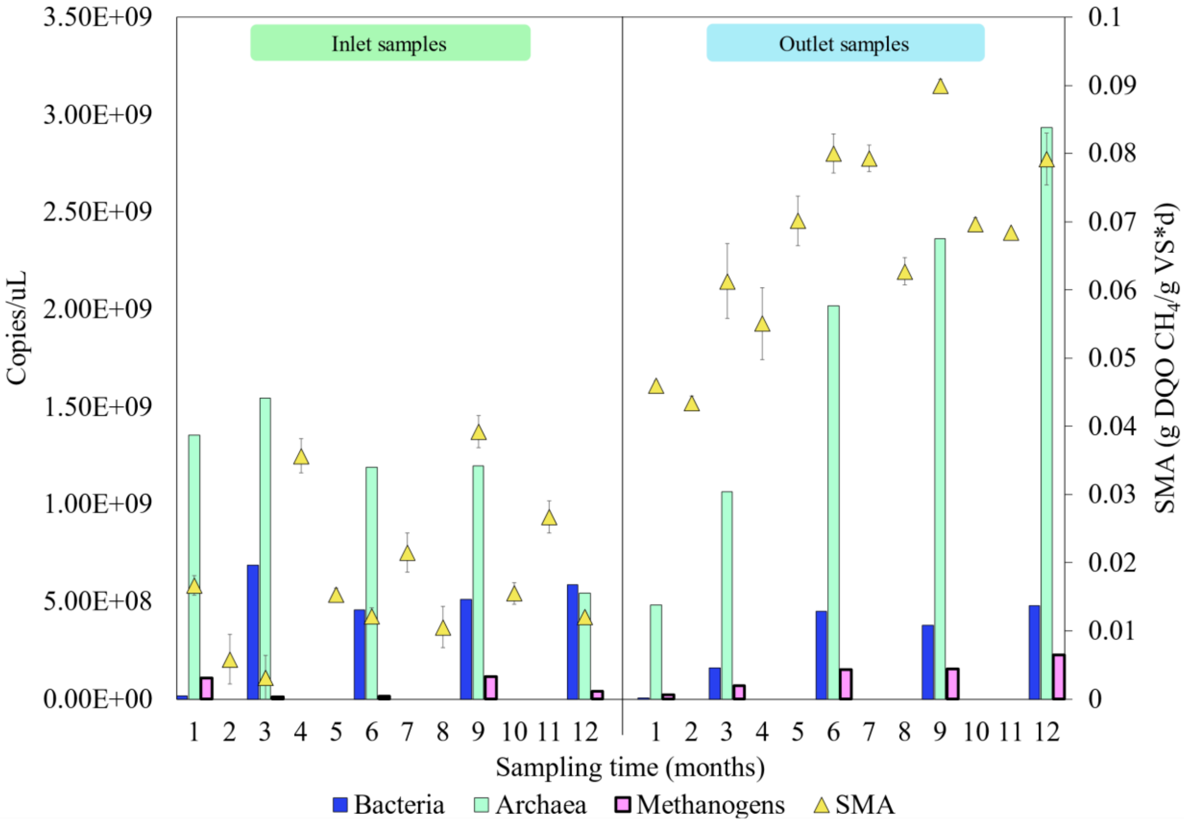

Figure 14. One year microbiology behaviour in a psychrophilic rural digester

The average content of bacteria, *archaea*, and methanogens loaded into the digester during monitoring was 4.52×10^{8}, 1.17×10^{9} and 5.74×10^{7} copies/uL, respectively. It is worth mentioning that the CW contributes 73.6% of the fed bacteria content, although it only contributes 1% of the *archaea* content. Conversely, after the first operative month, the outlet bacteria content remains constant with values around 3.76×10^{8} copies/uL.

On the other hand, the outlet qPCR findings suggest that the biochar addition favored the environment for the growth of *archaea* communities, doubling its content from month 3 to month 6. This tendency is kept during the following months of monitoring, reaching *archaea* concentration around 2.93×10^{9} copies/uL. Those values are considerably higher if compared to the obtained for an eight-year operative digester that treats pig manure (1×10^{6} copies/uL) (Jaimes- Estévez *et al*., 2020). At lower temperatures, methane production predominantly occurred through the acetoclastic pathway. Testing for acetoclastic methanogenic activity could outline optimal conditions for anaerobic systems and act as a metric for evaluating system efficacy, offering insights into the system's functioning and stability (Astals *et al*., 2015). In this study, the effluent's SMA related to acetoclastic methanogenesis was 0.067 ± 0.014 g COD CH_4/gVS*d. This value exceeds the influent average SMA (0.017 ± 0.011 g COD CH_4/g V*d). In comparison with a mono-digestion process treating cattle manure in a mesophilic region, the SMA values were 0.01 g COD CH_4/gVS*d for the influent and 0.04 g COD CH_4/g VS*d for the effluent after operating over three years (Castro *et al*., 2017). So, in this research a higher concentration of *archaea* was found in digestate, leading to an increase in SMA, which proves methane content increase due to biochar addition.

Furthermore, the type of substrate and temperature were identified as key determinants of microbial activity. The analysis of effluent SMA indicated that the bacteria and *archaea* populations had adapted to the colder temperatures and the substrates in less time than a digester with no biochar. The results highlight that biochar, at concentrations exceeding 10 g/L, enhances methane production and AD stability, even under low-temperature conditions, validating GS integration into the bioprocess.

3.3.2. Life cycle assessment for a rural scenario: integration of gasification and anaerobic digestion

The environmental impacts associated with each scenario referred to the functional unit (kg_{cheese}/year) are presented in Figure 15. As can be seen, the total emissions of the base scenario compared to the integrated system were higher in all impact categories. In particular, the combustion of LPG in activities such as cooking food, sterilization of milking machines, and cheese production had the highest influence on global warming, representing 30.70% of CO_2eq emissions in the base scenario. Concerning the integrated scenario, there was a 98% decrease in CO_2eq emissions. This is because, in the integrated scenario, LPG was replaced by synthesis gas as an energy stream in the sterilization of milking machines. So, GS as energy integration did not contribute significantly to CO_2eq emissions. However, due to GS, there was an increase of 35.3% in the formation of particulate matter due to the ashes formed in the combustion of pine wood. Also, the use of synthetic fertilizers in cultivation activities represented 76.4% of the total emissions for the base scenario in the categories analysed, significantly influencing terrestrial acidification (94%), freshwater eutrophication (82.6%), ozone depletion (96%), marine eutrophication (95.8%), particle formation (87.6%) and water consumption (66.8%).

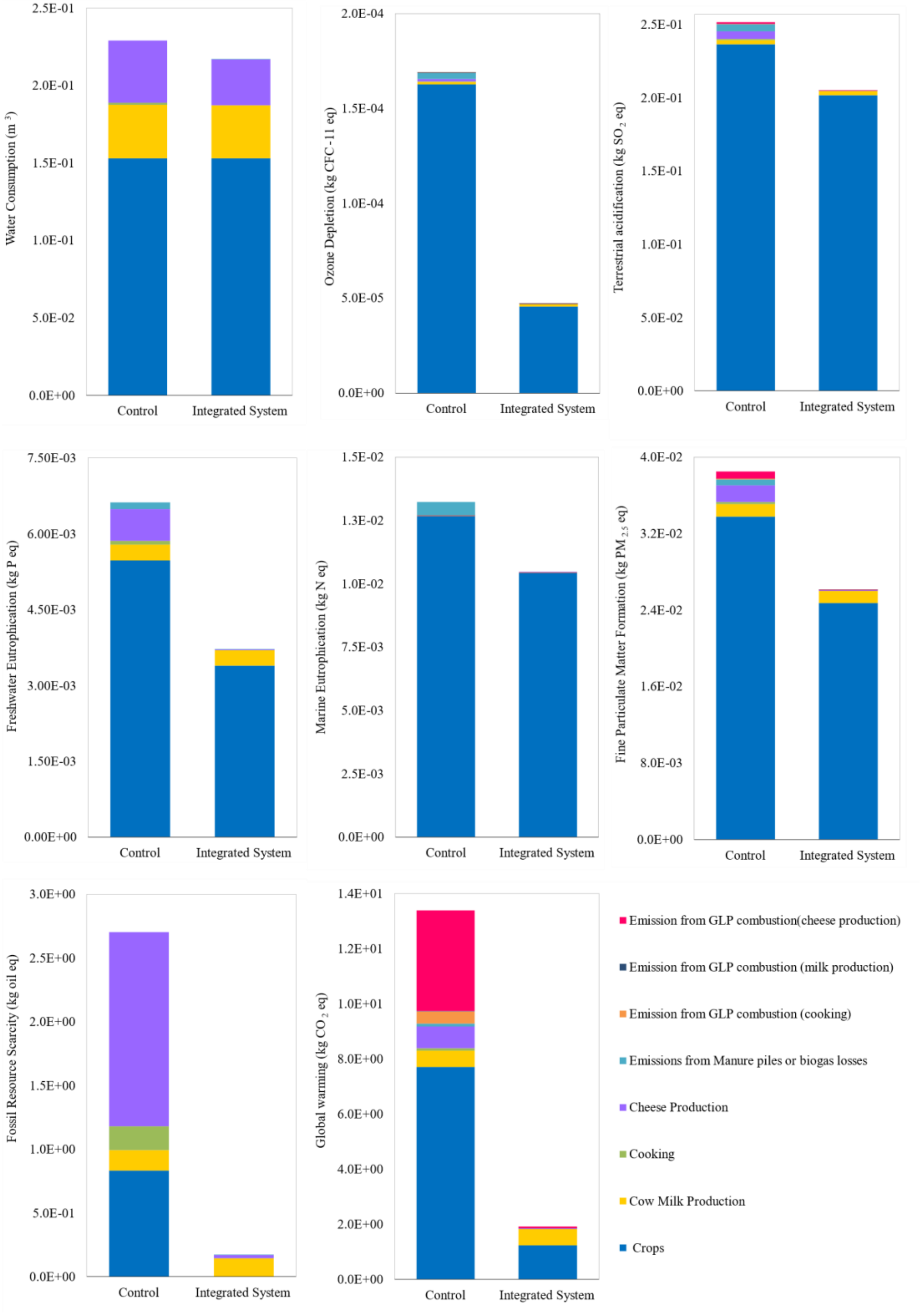

Figure 15. Environmental impacts for the control and the integrated system. Values are referred to the functional unit (kg_{cheese}/year).

These results align with the findings of Garfi *et al*., (2019), who stated that close to 70 % of the total impact was attributed to the use of synthetic fertilizers due to the high content of leached nutrients (N, P, SO_2) that it provides to the soil. Concerning the integrated scenario, there was a decrease of 29.3% due to the replacement of synthetic fertilizer with digestate.

It should be noted that although the digestate managed to completely replace the use of synthetic fertilizers, its high nutrient content represents a contribution of 80%, 51.2%, and 78.8% for terrestrial acidification and eutrophication of fresh and marine water in the integrated scenario, respectively. For its part, emissions from the accumulation of CM piles in the base scenario represented 1.5% of atmospheric emissions, which were almost zero for the integrated scenario since it was considered that all the CM was used in the anaerobic process. Other activities carried out on the farm, such as milk production, did not significantly influence on the reduction of environmental impacts; in fact, they were only reduced by 0.12% compared to the base scenario. The above is justified by the fact that in milk production, the same washing solution and the same amount of water are used in both scenarios. As a result of this activity, a significant amount of wastewater was generated that was subsequently treated in none of the cases. The disposal in the environment of this wastewater represented 4.4% ± 0.00, 4.7% ± 0.00, 3.4% ± 0.00, 5.6% ± 0.00, and 15% ± 0.00, for global warming, freshwater eutrophication, particle formation, fossil resources and water consumption for both scenarios, respectively.

Regarding cheese production, the implementation of the integrated system allowed a reduction of up to 85% in total impacts thanks to the post-treatment of the CW using AD and the use of the synthesis gas generated in the GS of pine wood. Considering that in the case study, before the installation of the integrated system, LPG was used for cooking, the use of fossil resources was the category with the most considerable influence. However, replacing

LPG with biogas in cooking generated negligible emissions (< 1% of the total impact) in the ACoD + GS scenario. In general, the environmental effects of the integrated scenario were approximately seven times lower than those of the base scenario in the categories considered, except for water consumption, which had similar environmental performance for both scenarios. In a particular case, the categories with the greatest decrease were the depletion of fossil resources, ozone depletion, and climate change. The reduction in impact due to the depletion of fossil resources was mainly due to the use of synthesis gas in farm activities. On the other hand, climate change decreased by 85.7% when the integrated system was implemented on the farm. The above is consistent with other studies where the LCA was evaluated in the management of waste generated in the production of olive oil with and without AD, where a decrease of 83.26 % in climate change was obtained for the scenario with anaerobic treatment (Batuecas *et al*., 2019).

In summary, implementing GS in the AD process promises an efficient and low-cost alternative to reduce environmental impacts and LPG consumption compared to other scenarios that only have the biodigester. The present study is a pioneer in considering LCA for an integrated anaerobic digestion and GS system on a domestic scale. For its part, although digestate replaced the use and production of synthetic fertilizers, reducing environmental loads, it still represents the most outstanding contribution to the general impact in the integrated scenario; therefore, it is suggested to study the operating conditions of the biodigester to improve the digestate quality. Finally, an alternative to reduce water consumption in the integrated scenario is to design wastewater collection and treatment. The above would reduce consumption costs and the global impact of emissions.

3.3.3. Economic potential associated with integrating ACoD and GS.

Integrating ACoD with GS also has positive economic effects. It was found that the production of biomethane + syngas could completely replace the LPG used in the study case. With a modest CH_4 production of 1.3 m^3/day, the energy potential from CW:CM ACoD reaches 2,782.75 kWh/year (CH_4 low calorific power = 9.94 kWh/m^3), and from GS is 7,610 kWh/year, which means a supply of 62.38% of the farm energy requirements. If the AD OLR is increased, and hence the biomethane yield (as reported in a scenario with no biochar surrounding 2.7 m^3/d, Jaimes Estévez *et al.,* 2022), the integrated system can cover all the necessities regarding energy. The variation of NPV with the intervention rate (*i*) and inflation (*f*) is shown in Figure 16.

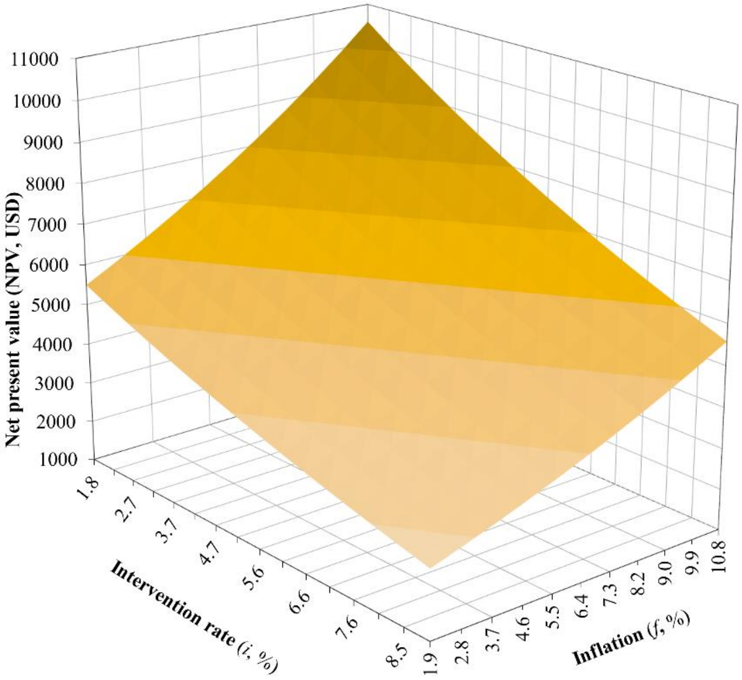

Figure 16. Variation of the NPV of implementing the integrated system as a function of the intervention rate (*i*) and inflation (*f*).

In this study, the NPV varied between US$ 2,664 and US$ 10,558. In fact, there is an inverse tendency between *i* and NPV, which means that while *i* increases, NPV decreases (I.F.A.D, 2015). However, NPV is proportional to inflation. Because f takes part in calculating income and costs, the increase in f results in higher profits over time and, consequently, an increase in NPV. It is essential to mention that, for all *i* and *f* evaluated, the NPV is positive even under unfavourable conditions, which correspond to maximum and minimum values for *i* and *f*, respectively. With these tendencies, an average NPV of US$ 5,133.47 was obtained, a value that can be considered as the average economic benefit over the 10 years of useful life established for the integrated system. For the analysed system, the ratio B/C was 2.58 with a relatively short PBP of 3.8 years. In comparison, Mendieta et al. (2020) reported an analogous tendency in the economic study regarding valorisation of waste from a non-centrifugal cane sugar mill via anaerobic co-digestion, where the bioprocess was profitable. In the case of the IRR, it is only affected by changes in inflation, having a directly proportional relationship with this variable. The minimum and maximum values of the IRR for the integrated system were 17.9% and 26.0%, respectively.

In contrast, Kabyanga et al. (2018) documented adverse NPVs, indicating that investing in a flexible balloon digester is not profitable to smallholder farmers. Conversely, Bishop and Shumway (2009) established an IRR of 17.1% for plug-flow anaerobic digesters employed in dairy manure treatment. Notably, the present research explores the synergies of combining anaerobic digestion with GS, revealing substantial enhancements in daily methane production and a mitigated environmental footprint, thereby highlighting the economic feasibility of this integrated approach. In conclusion, integrating psychrophilic anaerobic digestion with gasification represents a forward-looking approach to achieving energy independence. This combined methodology not only optimizes the production of renewable energy but also underscores a sustainable strategy for waste management. By enhancing biogas generation efficiency of and improving environmental outcomes, this research exemplifies a significant step towards a more sustainable and energy-independent anaerobic process in rural areas.

3.4. Conclusions

Results achieved in this study can be summarized into three main conclusions:

Bioprocess Effect: The addition of pine wood biochar in the AD of CW and CM positively affects the consumption of VFAs, the medium's alkalinity, and methane production.

Environmental Effect: The implementation of the integrated AD + GS system reduced the impacts associated with climate change by 85%. This study highlights GS as a low-cost, environmentally friendly alternative that enhances the AD process.

Economic Effect: Energy integration can improve the quality of life for people living in rural areas by reducing the costs associated with purchasing fertilizers and LPG. Its implementation results in an NPV of US$ 5,133.47.

3.5. Generation of new knowledge: Research Impact

The scientific products associated with the development of Chapter III are presented below.

Publications.

Jaimes-Estévez, J., Mercado, E. V., Jaramillo, J. G., Rodríguez, P., Martí-Herrero, J., Escalante, H., & Castro, L. (2022). From laboratory to farm-scale psychrophilic anaerobic co-digestion of cheese whey and cattle manure. Bioresource Technology Reports, 101168. https://doi.org/10.1016/j.biteb.2022.101168

Jaimes-Estévez, J., Zafra, G., Martí-Herrero, J., Pelaz, G., Morán, A., Puentes, A., Castro, L., & Escalante Hernández, H. (2020). Psychrophilic full scale tubular digester operating over eight years: complete performance evaluation and microbiological population. Energies, 14(1), 151. https://doi.org/10.3390/en14010151

Presentations.

Jaimes-Estévez, J., Mendieta, O., Sánchez, Z., & Escalante, H. L, Castro. The Psychrophilic Anaerobic Digestion Of Smallholder Dairy Biowastes: An Opportunity For Developing Countries (2022). 17th IWA World Congress on Anaerobic Digestion, Michigan-USA.

Jaimes-Estévez, J., Escalante, H. & Castro, L. Psychrophilic biogas plant as a viable alternative for agro-industrial wastes treatment: performance and social impact. XIV Latin

American workshop and symposium on anaerobic digestion (DAAL XIV). Querétaro – México 2023

Tavera-Ruiz, C., Martí-Herrero, J., Mendieta, O., Jaimes-Estévez, J., Gauthier-Maradei, P., Azimov, U., Castro, L. Exploring anaerobic digestion in developing countries: A case study of colombia's current landscape and future potential. XIV Latin American workshop and symposium on anaerobic digestion (DAAL XIV). Querétaro – México 2023.

Proyects.

Active participation as a doctoral researcher in the development of a collaborative project with the University of Sheffield (UK) under the Newton Fund Institutional Links call, with the project titled 'Integrated anaerobic digestion and gasification systems for sustainable farming in Colombia.

Human Resource Development.

Co-supervision of undergraduate research project for two Chemical Engineering students. Status: defended and approved. Title: "*análisis del ciclo de vida del sistema digestión anaeróbica + gasificación*".

Co-supervision of undergraduate research project for two Chemical Engineering students. Status: defended and approved. Title: "*Co-Digestión Anaeróbica en un Digestor Doméstico para el Tratamiento de Lactosuero y Estiércol Bovino Generados en el Instituto Técnico Agrícola De Cáchira*".

Prospects (Chapter IV). Enhancing Energy Generation in cold climates through Anaerobic Digestion of High-Moisture Wastes with Biochar: A Sustainable Approach for Eco-Districts

4. Enhancing Energy Generation in cold climates through Anaerobic Digestion of High-Moisture Wastes with Biochar: A Sustainable Approach for Eco-Districts

Abstract

This study investigates the impact of biochar on the psychrophilic anaerobic digestion (AD) of a highly biodegradable substrate, kitchen wastewater (KWW), focusing on process kinetics, biochemistry, and energy potential. Biomethane potential (BMP) assays with volatile fatty acids (VFAs) measurements were performed at both psychrophilic (15°C) and mesophilic (35°C) conditions, with and without biochar (30 g/L) derived from gasified pine wood. The results indicate that psychrophilic conditions favour the accumulation of VFAs, such as butyric and isovaleric, which inhibit AD. However, the addition of biochar improved metabolic activity, promoting the formation of acetic acid, a key precursor to methane production. This enhancement led to a psychrophilic methane yield of 0.34 $m^3CH_4/kgVS$, achieving 87% of the methane yield obtained at 35°C. These findings contribute to developing methodologies for assessing the biochemical and energetic viability of waste treatment through AD, offering a sustainable solution for renewable energy generation in eco-district scenarios.

4.1. Introduction

Renewable energy sources have awakened considerable interest in seeing the depletion of fossil energy. The biological process known as anaerobic digestion (AD) is widely accepted as a promising technology for waste disposal, resource recovery, and energy generation as biomethane. AD involves numerous populations of microbes that, through their metabolism, enable the breakdown of a substrate (which is regarded as the process's source of nutrients) into simpler molecules. The outcome of this is that the AD process creates biogas with energy potential (lower calorific value between 13,720 and 27,440 kJ/m^3) (Hosseini & Wahid 2013). Biomethane in biogas (which is 40% - 60 %) can be used for cooking (Jaimes – Estévez *et al.,* 2022), as fuel for combined heat and power (CHP) engine (Mohammadi *et al.,* 2021), or even be upgraded for its injection in the gas grid or as fuel for road transportation (Lodato *et al.,* 2022). So, waste treatment via AD can be translated into greenhouse gas emission reduction, fuel security, economic benefits, and pollution reduction. In addition, renewable biomethane generation supports valorisation of local low-value organic resources, increasing local energy production. The AD success can be shown by the increasing number of plants in the last years. For instance, the European Biogas Association reported that the number of biomethane-producing facilities by April 2023 across Europe increased to 1,322 (European Biogas Association, 2023).

AD performance is influenced by factors such as nutrients source (substrate) and temperature. Unfavourable conditions affect the overall metabolism of the microbial community, causing the overgeneration of volatile fatty acids (VFA) and posterior process inhibition (Jaimes Estévez *et al.,* 2022). In an urban scenario, kitchen wastewater (KWW) is a high-generation residue from domestic activities. KWW is considered a high moisture substrate with an elevated content of organic soluble compounds, making it viable for AD. Despite this, those characteristics could negatively affect due to the possibility of a rapid hydrolysis stage that would cause high production of VFA, which could inhibit the AD process. Regarding temperature, biogas production is optimal under mesophilic (30–40 °C) and thermophilic (50–60 °C) conditions (Akindolire *et al.,* 2022). Cold climates, as presented in psychrophilic zones (Temperatures below 20°C) affects directly yield and stabilization of waste treatment. So, lowering the operational temperature leads to a decrease in the maximum specific growth and substrate consumption rates (Lettinga et al., 2001). The preceding translates into low-quality biogas and accumulation of VFA, represented respectively in CH_4 content in biogas lower than 50% and the system acidification (Liu *et al.,* 2023). So, the AD of a highly biodegradable substrate at low temperatures could be deficient. The above shows a necessity to improve the process to enhance sub-optimal temperatures AD, which presents reduced methane yields compared to mesophilic treatment. In that sense, the most common alternative is heating the digester; most AD plants operate at 35°C - 37 °C or even at 55 °C. For example, thermophilic two-stage AD system (Holl *et al.,* 2022; Ramos *et al.,* 2022), and passive solar heating design (Perrigault *et al.,* 2014; Jaimes-Estévez *et al.,* 2021) have been employed. Nevertheless, the amount of energy needed to heat the bioreactors to maintain the necessary temperatures limits both the mesophilic and thermophilic processes in cold-weather countries (such as Canada), reducing the process efficiency (Rajagopal *et al.,* 2017).

A new strategy to mitigate the limitations of AD under psychrophilic conditions, avoiding inhibition, is increasing the interactions between microorganisms and substrates using support materials such as biochar (Shi *et al.,* 2022). Some authors have reported that using support materials improves organic loading capacities and maintains stable methane production during AD even with increasing organic load (Karadag *et al.,* 2015; He *et al.,* 2020). However, exist few research focused on the treatment of high moisture substrates with high inhibitory product generation and the influence of biochar during its treatment by AD and its effects on energetic generation. In that sense, the main objectives of this research were 1) to study the effects of biochar in the psychrophilic AD of a highly biodegradable substrate (as KWW); 2) to obtain the kinetic parameters of KWW AD from data modelling at psychrophilic conditions, and 3) to determine the energetic efficiency of the process. This chapter adds new knowledge by investigating the biochar effect in urban wastes AD at low temperatures, which could be considered for future implementation to energy poli-generation in urban cases.

4.2. Material and Methods

This research was developed in three steps assessed at psychrophilic (15 °C) and mesophilic (35 °C) conditions: i) the evaluation of biochar effect on yields and VFA behaviour, ii) the study of a highly biodegradable substrate kinetics, and iii) the energetic efficiency determination from KWW treatment.

4.2.1. Raw materials for the bioprocess evaluation

KWW was obtained directly from the kitchen drain of a three-people domestic scenario. The daily generation of this outflow is around 150 – 200 L/day. This high-moisture waste contains residues from food washing, soap, and food leftovers. The composition of this waste is 3,458 mg COD/L (37.7% of soluble COD) with an initial total VFA concentration of

218.99 ± 18.84 mg/L and pH of 6.2 ± 0.3. The inoculum was stabilized cattle manure obtained from a 10 m^3 rural biodigester located in a 25 ± 2 °C zone. Inoculum was pre-adapted using the alternative of temperature adaptation by time, as Martí-Herrero *et al.,* (2022) suggested. Briefly, the inoculum was preincubated for 70 days at the assay temperature conditions of 15 °C and 35°C prior to substrate addition. After acclimatization, the inoculum Specific Methanogenic Activity values were 0.036 g COD CH_4/g VS*d and 0.056 g COD CH_4/g VS*d at 15 °C and 35 °C, respectively. The biochar employed as material support was obtained from the 500 - 600°C GS of discarded pine wood in an ascendant air flux (with 40 L of capacity) gasifier. Some characteristics of biochar are a pH of 9.2, an average particle size of 117 ± 35 μm, and a carbon content of 88.34 ± 1.63 % (Chapter II)

4.2.2. Biochar effects on methane production and biochemical behaviour during the KWW AD treatment.

To evaluate the effect of support material on the psychrophilic KWW bioconversion into methane, three biochemical methane potential (BMP) test at 15 °C were carried out: The KWW + biochar BMP, the KWW BMP, as control assay (with no biochar), and a blank assay (inoculum with no substrate) to remove endogenous methane generation by inoculum. Additionally, a positive control BMP test with crystalline cellulose (97%) was conducted. Those assays were replicated at 35 °C to compare with the most favourable temperature conditions. Considering KWW presents a rapid accumulation of fermentation intermediates such as VFA, the inoculum/substrate ratio was 5 on a volatile solids basis (Holliger et al., 2016). The amount of biochar added was 30 g/L, considering a favourable support concentration for acids substrates (Jaimes Estévez *et al.,* 2023). The BMP assays were set using triplicates of 120 mL glass bottles with a 70% working volume and incubated at pre-established temperatures (15 °C and 35 °C). To guarantee anaerobic conditions, the bottles were flushed with an 80/20% N_2/CO_2 mixture and sealed with butyl rubber stoppers and aluminium caps. The methane generation was assessed periodically by biogas pressure and composition determination (Holliger *et al.,* 2016) using a transductor and gas chromatography, respectively. All BMP tests were concluded when the methane accumulated increased by less than 1% for three consecutive days. The biomethane generation was normalized and reported at pressure and temperature standard condition of 101.325 kPa, and 273.15 K.

Paralleling BMP assays, KWW AD biochemical behaviour was monitored from six extra sacrifice assays. This monitoring consisted of the individual volatile fatty acids (acids from 2 to 6 carbons) content determination via gas chromatography (Raposo *et al.,* 2013). In the initial-day, day-ten, and final-day BMP, aliquots were taken from sacrifice bottles to be centrifugated and filtered before VFA determination. All tests were conducted by triplicates. The organic matter removal was calculated as the relation between the organic matter consumed to generate methane ($g\ COD\ CH_4$) in grams of COD and the total organic matter content in the substrate ($gCOD_{KWW}$):

$$\%\ Organic\ matter\ removal = \frac{g\ COD\ CH_4}{gCOD_{KWW}} * 100 \qquad \text{Eq. (17)}$$

The above was considered due to biochar influences organic matter determination via COD measurement. $g\ COD\ CH_4$ was calculated considering the coefficient of 350 mL CH_4/g COD at standard condition (101.325 kPa, 273.15 K).

4.2.3. Analytic methods

COD and volatile solids (VS) were analysed according to standard methods (APHA 2017). The methane and carbon dioxide compositions in biogas were detected by a chromatograph coupled to a thermal conductivity detector (Holliger 2016). Total and individual VFA concentrations were determined according to Raposo *et al.,* (2013) by gas chromatography using a BP21 GC capillary column (packing material: treated polyethylene gly-col) coupled to a flame ionization detector.

4.2.4. Kinetic modelling for the KWW anaerobic treatment

BMP assays obtained in the previous section were modelled by fitting the experimental methane production data with the modified Gompertz model (Shamurad *et al.,* 2020). The application of the modified Gompertz model (Eq. (2); Shi *et al.,* 2022) assumes that methane production is a function of bacterial growth (Pan *et al.,* 2016). This is important to validate methane production and to study the effect of biochar on the kinetic parameters related to KWW AD. The Levenberg-Marquard method and non-linear regression were employed to calculate the numerical and kinetic parameters (Statistica 10.0 software). The root mean square error (RMSE) and coefficient of determination (R^2) were also used to describe the degree of adjustment between the experimental and projected BMP. In this study the biochar influence (β) on the methane generation was stablished as:

$$\beta_{15°C;35°C} = \left(\frac{P_{0\ assay\ with\ biochar}}{P_{0\ assay\ with\ no\ biochar}} - 1\right) * 100 \qquad \text{Eq. (18)}$$

Eq. 18 correlates the BMP values of an assay with biochar with its control. If $\beta < 0$, the biochar has an adverse effect, if $\beta > 0$, the biochar has a favourable effect.

4.2.5. The energetic potential and viability of an KWW AD system in an eco-district scenario

The energetic viability of AD was stablished considering the annual KWW generation of a city district (10,000 people; Rezaei et al., 2021), equivalent to 800 m^3 KWW/d. From the ultimate methane yields obtained for KWW BMP under psychrophilic and mesophilic conditions, the energy potential (Ep; kJ/m^3_{KWW}) of treating KWW by AD was determined as Eq. (19):

$$\text{Ep} = P_0 * \rho_{OM} * LCP_{CH4} \qquad \text{Eq. (19)}$$

Where P_0 is the ultimate methane potential (m^3 CH_4 kg/VS_{add}); ρ_{OM} is the KWW organic matter density (kg VS/m^3_{KWW}); LCP_{CH4} is the methane low-calorific power stablished by Li et al., (2017) as 35,800 kJ/m^3. The electrical (EE_{KWW}) and thermal (ET_{KWW}) energy production from KWW AD, was calculated considering a combined heat and power (CHP) engine, as follows:

$$EE_{KWW} = Q * E_p * \eta_E * 0.9 \qquad \text{Eq. (19)}$$

$$ET_{KWW} = Q * E_p * \eta_T \qquad \text{Eq. (20)}$$

Where Q is the caudal of KWW generated in the study case (800 m^3/d) and η_E and η_T correspond to the electrical and thermal CHP engine efficiencies, equivalent to 35% and 55%, respectively; 0.9 is a factor that represents the CHP engine's self-consumption (10% of the total electric energy generated) (Silvestre *et al.,* 2015).

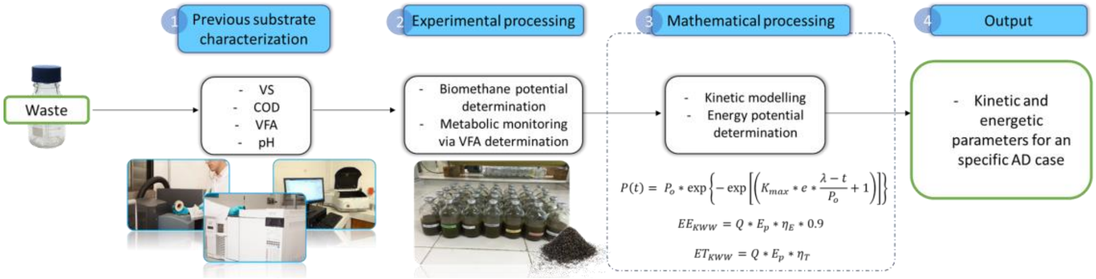

Figure 17. Acquired methodology for the biochemical and energetic viability of new waste treatment.

With this methodology the present study allows stablish an approach procedure to evaluate the biochemical and energetic viability of waste treatment via AD (Figure 17) (Jaimes Estévez *et al*., 2020b).

4.3. Results and Discussion

4.3.1. Biochar effect on high-moisture substrate biodegradability.

Figure 18. shows the experimental cumulative methane production for KWW BMP with and without biochar at 15°C to 35°C, compared with the controls and blank assays. It is notorious that endogenous methane production is similar at 15 °C than 35 °C, which can be accredited to the inoculum adaptation. It is essential to mention that the biochar does not act as a co-substrate, so its CH_4 generation is depictable. Regards to mesophilic control, the potential of methane production from KWW is 0.38 m^3 CH_4/kg VS. This value is reached by

day ten, demonstrating rapid hydrolysis and methanogenesis of the substrate due to its high percentage of soluble compounds. Comparing this behaviour with the mesophilic assay with biochar, organic support does not impact the final methane yields: the KKW BMP + biochar was 4.4% lower than its respective control. The organic matter bioconversion was similar in both cases (67% and 70% with and without biochar addition, respectively). In other words, the mesophilic AD of highly soluble substrates as KWW does not require biochar addition. In contrast, Madrigal *et al.,* (2022) found that biochar from gasified cattle manure can make viable the 35 °C methane generation of an acid substrate as cheese whey, avoiding inhibition. However, in the present study, the psychrophilic KWW with no support material obtained similar values as blank assay and even lower for cellulose BMP that rounds 0.27 m^3 CH_4/kg VS. This is because the low temperature significantly affects microorganism metabolic activities (Jaimes-Estévez *et al.,* 2023); thus, the methane production from KWW at 15°C. Additionally, this behaviour can indicate process inhibition due to the initial acid load added by KWW and a subsequent generation of VFA.

In particular, adding 30 g/L of pine wood biochar can reduce the temperature adverse effect, boosting the KWW AD: by day four, the daily methane production increases significantly compared with no biochar assay. As evidenced in Fig. 1, the supplementation with an organic additive makes the psychrophilic methane production viable to reach BMP values equivalent to 87 % of mesophilic ones.

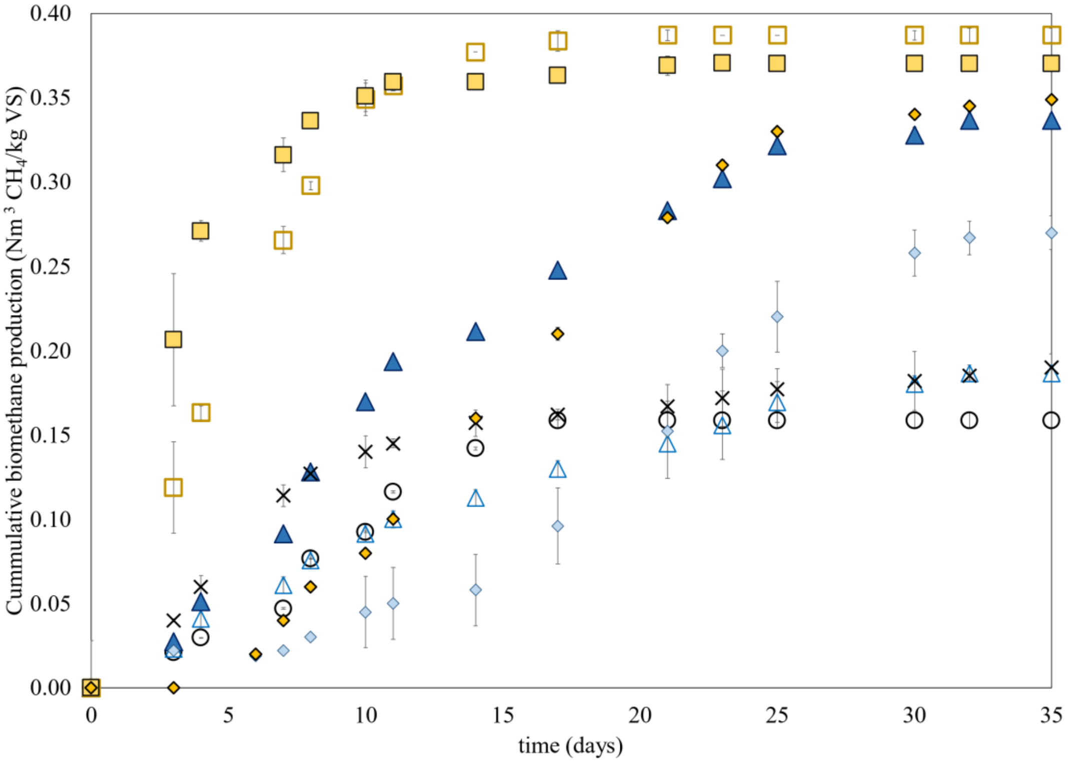

Figure 18. Cumulative biomethane production for KWW AD during monitoring. Dark and clear triangles show the experimental data for psychrophilic KWW+ Biochar BMP, and KWW BMP with no biochar, respectively. Additionally, the experimental data for mesophilic KWW AD is presented as dark (KWW BMP + Biochar at 35°C) and clear squares (KWW BMP at 35°C). The circles and "x" marks represent the Blank assay values at 15° and 35 °C, respectively. Rhombuses describe the cellulose BMP as a positive controls in psychrophilia (clear rhombus) and mesophilia (dark rhombus).

The organic matter removal was equal to the fraction of organic matter converted to methane. At the end of the psychrophilic process, the percentage of organic KWW consumed was 61% and 34% with and without biochar added, respectively. The higher removal values reached in this study are comparable with those achieved in tubular digesters operating at low ranges of mesophilic conditions (76% of removal at 25 ± 2 °C; Castro et al., 2017). The behaviour studied in this section reflects that evaluating the biodegradability of substrates via BMP assays at the concerned temperature is necessary.

4.3.2. Volatile fatty acids monitoring as AD metabolism indicator.

Volatile fatty acids behaviour during BMP assays represents the "health" of the process. Figure 19. shows the VFA profile obtained for 15°C and 35°C KWW BMP. D0 values represent the initial VFA load to start the BMP assays.

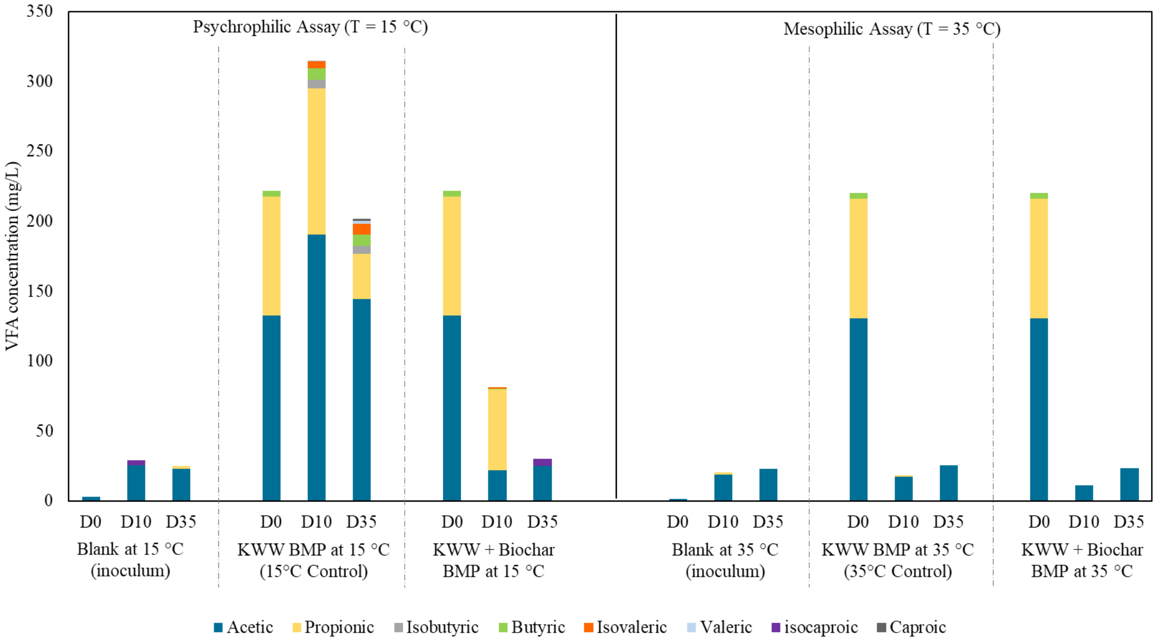

Figure 19. Individual VFA concentration changes during AD monitoring for psychrophilic and mesophilic treatment. The compounds measured were acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic, and isocaproic acids.

Regarding blanks, there is not a considerable acid content which varies from 1.36 mg/L to 28.92 mg/L, during all monitoring. So, KWW apports all the readily biodegradable organic matter to be converted into CH_4 in methanogenesis. In a no biochar psychrophilic assay, there is an evident accumulation of VFA during the first ten days and a particular generation of acids different from acetic and propionic, such as isovaleric and isocaproic. The presence of those acids can indicate the activity increment of acidifying enzymes (Bhatia & Yang, 2017). This comportment shows a possible deviation of the acidogenesis pathway and hence the limitation of acetogenesis, caused by temperature, which can affect the acetic acid generation and, subsequently, methane production (Feng *et al*., 2022).

Even with a final VFA concentration lower than 1,500 mg/L, considered inhibitory for standard BMP (at 35 °C - 37 °C; Angelidaki *et al.,* 2005), the low temperature provokes a sharper inhibition. The assay with biochar evidenced a well-conducted VFA consumption, with 83.4 % of acetic acid consumption during the first ten days. The above is in accordance with the tendency obtained during biomethane generation, whereby biochar favours acidogenesis, represented in the reduction in the formation of different acids from acetic and propionic, and methanogenesis, denoted in the easier acetic acid consumption. So, as suggested by Pan *et al.,* (2019), the presence of biochar improves the recovery rate of anaerobic microorganisms, which in this study, demonstrates this effect under psychrophilia. Regarding mesophilia, the VFA formation and consumption are equivalent in the presence or absence of organic support. On day 10, the consumption was faster, reinforcing the tendency presented in 35°C BMP assays.

4.3.3. Modelling KWW AD. From biochemical methane kinetics to energy efficiency.

4.3.3.1. highly biodegradable BMP kinetic modelling

After modelling experimental data, the mechanisms of methane production by KWW biodegradation with and without biochar addition, at psychrophilic and mesophilic conditions are shown in Figure 20.

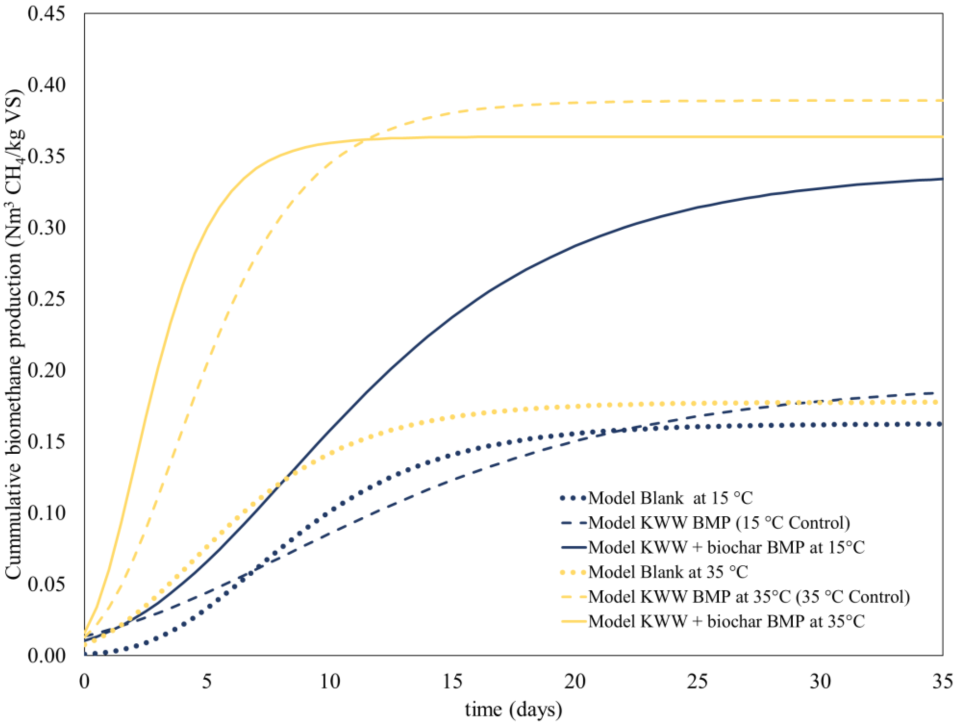

Figure 20. KWW BMP fitted data to Gompertz model. Dark and clear tendencies represent the psychrophilic (15°C) and mesophilic (35°C) methane production from KWW AD, respectively. The dotted lines are for blank assays, discontinuous lines for KWW BMP with no biochar, and continuous lines for BMP + biochar.

Blank assay tendencies presented a similar behaviour during all monitoring, reinforcing inoculum acclimatization to temperature conditions. In a psychrophilic scenario, the biomethane production was direct due to the substrate characteristics. However, the daily methane produced was reduced, which could mean an imbalance between metabolic AD stages. Even with fast hydrolysis due to high soluble COD content, the methane generation value was small (0.19 m^3 CH_4/kg VS), near than the values obtained for cattle manure (0.16 m^3 CH_4/kg VS, Castro *et al.*, 2017). In contrast, biochar addition can stabilize metabolic mechanisms improving methanogenesis. The CH_4 generation was evidently improved since day four and sowed a constant production tendency until day 14. So, applying 30 g/L of biochar enhances methane production pathway even at 15 °C. The effect of biochar under

35°C shows a slight acceleration in methane production, reducing six days for reaching ultimate methane values (from day 17 to day 11). There is three biochar mechanism that can be inferred that favours biomethane generation, as the inoculum had a strong affinity for pine wood biochar, allowing biofilm formation (Chand *et al.,* 2021), the alkalinity apport to the system (Jang *et al.,* 2018) and the modulation of acetogenesis in psychrophilia (Jaimes-Estévez *et al.,* 2023). Data modelling for the KWW BMP are described by the kinetic parameters estimated by fitting the modified Gompertz model. The kinetic model parameters were validated based on the values of error functions R^2 and RMSE (Table 9).

Table 9. Kinetic parameters from Modified Gompertz Model for KWW AD at psychrophilic and mesophilic temperature conditions

Assay	**Po (m^3 CH_4/kgVS)**	**K_{max} (m^3 CH_4/kgVS*d)**	**λ (d)**	**R^2**	**RSME**
Blank (inoculum) at 15 °C	0.16	0.014	2.76	0.994	2.03E-07
KWW BMP at 15°C (15 °C Control)	0.19	0.008	0.00	0.995	9.98E-08
KWW + biochar BMP at 15°C	0.34	0.02	1.66	1.00	9.46E-07
Blank (inoculum) at 35 °C	0.18	0.017	0.55	0.993	1.78E-06
KWW BMP at 35°C (35 °C Control)	0.39	0.048	0.68	0.998	4.18E-07
KWW + biochar BMP at 35°C	0.36	0.075	0.26	0.994	6.99E-07

For all the BMP adjustments, all R^2 and RSME values were higher than 0.994 and lower than 2.07E-07, respectively, indicating the best fit and high accuracy of the Modified Gomperz Model to the corresponding experimental values. The cumulative methane production (P_0) was the same as obtained experimentally for all cases. Regarding the maximum methane production velocity represented by *Kmax*, the 35°C assay is more rapid, producing 0.075 m^3 CH_4 kg/VS_{added}*d after an adaptation phase of 0.26 days. However, mesophilic biochar influence seems to be adverse ($\beta_{35°C} = -6.55\%$). Regarding 15°C KWW BMP, the pine wood biochar addition stabilizes the system and favours the microorganism activity to consume the organic matter present in the substrate, folding 1.8 times methane production, 2.5 times faster, compared to the assay with no biochar (going from 0.19 to 0.34 m^3 CH_4/kgVS). Biochar addition increases methane production in psychrophilia, which can be interpreted as improving methanogenesis. Thus, the effect of pine wood biochar influence on the psychrophilic AD of highly biodegradable substrates is around 77%. This modelling treatment allows obtaining a previous HRT value, to transfer a batch assay to a continuous scenario. In this case, a prior time for KWW treatment in a continuous process could be established as 21 days with no biochar addition, and 23 days when biochar is used.

4.3.3.2. *Energy potential of psychrophilic KWW biomethane generation*

Table 10 summarizes the results for the energetic balance of the established study case, where the KWW AD with and without biochar addition is compared under two temperature conditions.

Table 10. Heat and electricity potential for psychrophilic and mesophilic AD with and without biochar addition.

T (°C)	Biochar added	energy potential (Ep; kJ/m^3_{KWW})	KWW electrical energy potential kJ/m^3_{KWW}	KWW thermal energy potential kJ/m^3_{KWW}	electrical energy production (EE_{KWW}) MJ/d	thermal energy production (ET_{KWW}) MJ/d
15	no	14,964.40	4,713.79	8,230.42	3,771.03	6,584.34
	yes	26,778.40	8,435.20	14,728.12	6,748.16	11,782.50
35	no	30,716.40	9,675.67	16,894.02	7,740.53	13,515.22
	yes	29,141.20	9,179.48	16,027.66	7,343.58	12,822.13

The energy produced during an anaerobic process depends on the environmental temperature and the system microbiological conditions Biochar switches the psychrophilic energetic yield to a suitable value, leading to a daily energy potential from 14,964.40 kJ/m^3_{KWW} to 26,778.40 kJ/m^3_{KWW}. If all flux of waste were treated, an electrical and heat potential around 6,748.16 and 11,782.50 MJ/d could be reached. So, adding biochar to the system provides 87.2% of the energy than a conventional system in mesophilia, which in a eco-district scenario would reduce heat and electricity use. Adding a low-cost organic support material such as biochar from recycled biomasses enhances the energy generation in zones with unfavourable temperatures. In that sense, to improve energy generation and water recovery, wastewater treatment could be designed and developed locally (Xu *et al.,* 2019; Mohammadi *et al.,* 2021), based on the diversity of domestic wastewater streams like KWW. With growing concerns about the energy crisis but with high domestic wastewater generation and biomass wastes availability, using AD + organic support to treat those residues and produce renewable gas is a sustainable alternative. The methodology assessed in this research contains a previous characterization of substrates and their posterior treatment via BMP assays at a desired temperature. This is necessary due to the differences in substrate composition and facility to be processed by microorganisms involved in anaerobiosis. Furthermore, to strengthen the results via biochemistry evaluation, monitoring VFA benefits the understanding of process metabolism. Above developments in the mathematical modelling of experimental data to obtain kinetic parameters essential to feed models of biomethane generation and process design and a final obtainment of bioprocess implementation viability. In that sense, it is necessary to research the performance of the suggested approach considering different variables such as the temperature gains due metabolism, and the economic viability in continuous systems.

4.4. Conclusions

Organic support improves metabolic stages in the anaerobic digestion of high-moisture wastes, counteracting the adverse effects of psychrophilic conditions. This effect can be noted in the enhancement of acidogenesis and methanogenesis during BMP tests and hence, biomethane production kinetic. Psycrhophilic BMP + biochar equals 87% obtained in the 35°C AD process. Adding biochar from gasified pine wood avoids energy heating requirements and boosting the process yields. So, adding biochar is an alternative to unlock the viability of psychrophilic anaerobic digestion. This study proposes a methodology for assessing the biochemical and energy feasibility of waste treatment through anaerobic digestion, presenting it as a sustainable option for renewable energy generation in an eco-district.

4.5. Generation of new knowledge: Research Impact

The scientific products associated with the development of Chapter IV are presented below.

Publications:

Tavera-Ruiz, C., Martí-Herrero, J., Mendieta, O., Jaimes-Estévez, J., Gauthier-Maradei, P., Azimov, U., ... & Castro, L. (2023). Current understanding and perspectives on anaerobic digestion in developing countries: Colombia case study. Renewable and Sustainable Energy Reviews, 173, 113097. https://doi.org/10.1016/j.rser.2022.113097

Arango, J. G. J., Cortés, P. R., Jaimes-Estévez, J., Molano, L. C., & Hernández, H. E. (2021). Efecto del diseño bioclimático sobre el comportamiento térmico: caso de estudio dos digestores operando bajo condiciones psicrofílicas. Revista RedBioLAC, 5(1), 4-8.

Presentations:

Jaimes-Estévez, J., Martí-Herero, J., Escalante, H. & L, Castro. (2024). Design challenges in developing small scale high-rate cold Anaerobic Digestion. Renewable energy from anaerobic digestion in remote and off-grid applications. Fairbanks, Alaska, USA.

Patiño, L., & Jaimes-Estévez, J. (2020). Mejoramiento del rendimiento de la biodigestión anaeróbica en climas fríos mediante la implementación del Solar Organic Rankine Cycle. Ciclo de webinars de la RedBioLAC y el IICA 2020; Ciclo de foros técnicos virtuales:

Gestión sustentable de residuos orgánicos agrícolas y urbanos mediante la innovación tecnológica del biodigestor.

Proyects

Active participation in the international Cold/High Anaerobic Digestion Team focused on the application of anaerobic digestion in low-temperature areas. Project titled: “Renewable Energy from Anaerobic Digestion in Remote and Off-Grid Applications”.

Participation as an Intern Researcher in the Concordia’s Canada Excellence Research Chair (CERC) in Smart, Sustainable, and Resilient Communities and Cities research group. Title: 'Energy Recovery Potential from Eco-Districts Wastewater Treatment via Psychrophilic Anaerobic Digestion.

5. Final Conclusions.

This research demonstrates the significant benefits of incorporating organic support, specifically pine wood biochar, into the anaerobic co-digestion (ACoD) process under psychrophilic conditions. By systematically addressing the challenges posed by low temperatures, this study presents a multifaceted approach to enhancing the efficiency and sustainability of bioenergy production from agro-industrial residues. The key findings are as follows:

Kinetic Improvement and Metabolic Enhancement: The addition of pine wood biochar at a concentration of 30g/L significantly boosts the psychrophilic digestion process, achieving a biomethane potential (BMP) nearly 70% of that at mesophilic temperatures (35°C). This improvement is attributed to the biochar's influence on acetoclastic metabolism, which enhances methane yield even at the unfavorable temperature of 15°C, and facilitates the growth of the methanogenic microbial population, with a noted increase of about 500% more *archaea* compared to systems without biochar.

Operational and Environmental Benefits: Implementing pine wood biochar in the AD process not only mitigates the risks associated with volatile fatty acids accumulation and pH drop but also contributes to significant environmental advantages. The study highlights an 85% reduction in impacts associated with climate change, underscoring the role of the integrated AD + gasification system (GS) as a low-cost, environmentally friendly alternative that bolsters the AD process.

Economic Impact and Energy Security: The economic analysis reveals a notable positive net present value, enhancing the quality of life for individuals in rural areas by reducing expenses related to fertilizers and liquefied petroleum gas (LPG). Furthermore, adding biochar has been shown to potentially unlock the viability of psychrophilic anaerobic digestion, leading to a methodology for evaluating waste treatment's biochemical and energetic viability as a sustainable alternative for renewable energy generation.

The outcomes of this study underscore the importance of adding organic support materials to improve anaerobic digestion under cold conditions, effectively linking laboratory findings with practical applications in waste treatment for energy production. This pioneering research not only enhances our understanding of ACoD processes but also positions the AD + biochar system as a viable option for improving energy security and sustainability, especially in rural settings. These contributions are expected to have significant implications at local, regional, national, and international levels, marking a step forward in the global pursuit of sustainable and renewable energy sources.

6. Challenges, Emerging Issues and Perspectives

Challenges and Emerging Issues

The accessibility of organic matter is fundamental to produce high-quality biochar. In some regions, it may be challenging to access adequate quantities of raw material to produce enough biochar to feed the system daily, at least to reach the recommended concentration (> 10g/L). This may be due to geographical restrictions, environmental regulations, or competition with other uses of biomass, such as energy production.

Adding biochar into the anaerobic digestion process can present operational challenges, such as the risk of clogging or saturation of the digester due to material accumulation. This can negatively affect process efficiency and require costly interventions to solve the problem. It is crucial to develop design and operation strategies to ensure stable long-term system operation.

Although biochar presents clear beneficial effects in the anaerobic digestion process, its effect may diminish over time due to gradual saturation of organic material. This represents challenges in terms of maintaining performance levels and process efficiency over time. Further research is needed to better understand the mechanisms behind this saturation and develop strategies to prolong the durability and effectiveness of biochar in the digestion process.

The development of suitable technologies for on-site biochar production is crucial to overcome associated logistical and economic challenges. As presented in this work, research and development are needed to adapt existing technologies to different contexts and local conditions, as well as to develop economically and environmentally sustainable methods.

Perspectives

The digestate amended with biochar has high potential as an organic fertilizer due to its nutritional content and ability to improve soil structure. This approach can contribute to sustainable management of organic waste while enhancing soil quality and increasing agricultural productivity. This underscores the need for further research to optimize application practices focused on ensuring environmental and food safety.

The integration of energy production into the anaerobic digestion process can improve overall system energy efficiency and sustainability. Strategies such as combined heat and power generation can maximize the use of available resources and reduce dependence on external energy sources. However, technical, and economic challenges need to be addressed to optimize the implementation of these technologies in different contexts and scales.

The use of biochar as a fixed bed within the biodigester has the potential to improve microorganism retention and increase anaerobic digestion efficiency. This can result in greater process stability and a reduction in operating costs associated with frequent material replacement. However, further research is needed to fully evaluate the benefits and limitations of this practice and develop optimized design and operation approaches.

Due to its high porosity and surface area, biochar can adsorb CO_2 and H_2S, thereby improving the methane concentration in the biogas (effect not aborded in this study). The utilization of biochar for biogas upgrading could enable more efficient biogas use in residential and industrial applications, offering a greener alternative to natural gas and supporting the transition to more sustainable energy systems. Additionally, this practice could align biogas production with quality standards required for injection into natural gas grids or use in gas vehicles, opening up new avenues for the commercialization of biogas as a renewable energy source.

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