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

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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) (10 g/L, 30 g/L, 50 g/L); and particle sizes (Ps) (~0.15 mm, ~0.575 mm, ~1 mm). The most favourable conditions of Ps = 0.575 mm and Bc of 30 g/L, allowed BMP values to go from 0.23 m^3 CH_4/kg VS_{add} to 0.34 m^3 CH_4/kg VS_{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. Introduction

Anaerobic digestion (AD) is a biological process in which different populations of microorganisms intervene. 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-Rodríguez 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., 2012; 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, 2017; Akindolire et al., 2022). Aside, an anaerobic codigestion (ACoD) process

Abbreviations: AD, anaerobic digestion; ACoD, anaerobic co-digestion; Bc, biochar concentration; BMP, biomethane potential; CM, cattle manure; COD, chemical oxygen demand; CW, cheese whey; *e*, Euler's constant; K_{max} (m^3 CH_4 kg/VS_{add} days), the maximum methane production rate; P_0 (m^3 CH_4 kg/VS_{add}), the ultimate methane yield; P(t) (m^3 CH_4 kg/VS_{add}), cumulative methane production; Ps, particle size; qPCR, Quantitative Polymerase Chain Reaction; R^2, coefficient of determination; RMSE, root mean square error; t (days), time; TS, total solids; tVFA, total volatile fatty acids; VFA, volatile fatty acids; VS, volatile solids; VS_{add}, volatile solids added; wt, weight; λ (days), adaptation period (lag-phase).

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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 respect to 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 (20 °C), finding similar final specific methane production with or without biochar addition, but faster production when biochar is added. The above 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 (20 °C), 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 20 °C, 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 °C 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 >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 consequence of this lack of knowledge, 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 °C.

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.

2. Materials 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.

2.1. Substrates, support, and inoculum

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°0100.0700 W73°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*day and 0.056 g COD CH_4/g VS*day at 15 °C and 35 °C, respectively. The biochar was obtained from the gasification of recycled pine wood in a 40 L fixed bed equipment with ascendant air flux (450 L/min on average). The gasification was conducted in batches (10–15 kg pine wood/load) at temperatures between 500 and 600 °C. Then, biochar was grinded and sieved to obtain desired particle size (0.15–1 mm) and dried at 105 ± 2 °C for 24 h prior to use. Some characterizations of biochar used in this study are pore size of 11.45 μm, pH of 9.17; electrical conductivity of 57.4 mS/mm; and elemental content of carbon and oxygen of 81.75 wt% and 13.89 wt%, respectively.

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 (the best BMP value) 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. In order 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 subsequent discussions within this paper, 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):

$$y = \alpha_0 + \alpha_1 * Ps + \alpha_2 * Bc + \alpha_3 * Ps^2 + \alpha_5 * Ps * Bc + \alpha_6 * Bc^2 \quad (1)$$

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 1 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 <1 % of the total produced. 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 <0.05 considered significant.

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).

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

where, P(t) (m^3 CH_4 kg/VS_{add}) is the cumulative methane production at time t (days), P_0 (m^3 CH_4 kg/VS_{add}) is the ultimate methane yield, K_{max} (m^3 CH_4 kg/VS_{add} day) is the maximum methane production rate, *e* is the Euler's constant, and λ (days) 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.

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

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 above to establish the efficiencies of hydrolysis, acidogenesis, acetogenesis, and methanogenesis based on those proposed by Niu et al. (2014):

$$Hydrolysis\ (\%) = \frac{COD_{soluble} - COD_{soluble,in} + COD_{methane}}{COD_{total} - COD_{soluble,in}} \quad (3)$$

$$Acidogenesis\ (\%) = \frac{COD_{tVFA} - COD_{tVFA,in} + COD_{methane}}{COD_{total} - COD_{tVFA,in}} \quad (4)$$

$$Acetogenesis\ (\%) = \frac{COD_{acetate} - COD_{acetate,in} + COD_{methane}}{COD_{total} - COD_{acetate,in}} \quad (5)$$

$$Methanogenesis\ (\%) = \frac{COD_{methane}}{COD_{total}} \quad (6)$$

$COD_{soluble,\ in}$, $COD_{tVFA,\ in}$, 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.

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 analyzed 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

Table 1
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

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 2.

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.

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, 2005). 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). Total and individual VFA concentration (C2–C6: acetic, propionic, butyric, isobutyric, valeric, isovaleric, 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.

3. Results and discussion

3.1. Biochemical methane potential boosted by biochar

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

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

In psychrophilic conditions

$$BMP = 0.22740 + 0.05663 \bullet Ps + 0.00434 \bullet Bc - 0.06459 \bullet Ps^2 - 0.00058 \bullet Ps \bullet Bc - 0.00004 \bullet Bc^2 \quad (7)$$

$$tVFA = 468.48 + 727.423 \bullet Ps - 38.6102 \bullet Bc + 216.747 \bullet Ps^2 - 22.8 \bullet Ps \bullet Bc + 0.64225 \bullet Bc^2 \quad (8)$$

In mesophilic conditions

$$BMP = 0.6909 - 0.5716 \bullet Ps + 0.0023 \bullet Bc + 0.35982 \bullet Ps^2 + 0.001470 \bullet Ps \bullet Bc - 0.00006 \bullet Bc^2 \quad (9)$$

$$tVFA = 14.0043 + 31.2354 \bullet Ps - 2.34433 \bullet Bc + 125.364 \bullet Ps^2 - 4.01414 \bullet Ps \bullet Bc + 0.05864 \bullet Bc^2 \quad (10)$$

Table 3 presents the ANOVA analysis for each coded Eqs. (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 explain most of the experimental results. Nonetheless, there is no significant influence on all responses regarding the independent variable's interaction (Bc•Ps) and quadratics effects.

Under psychrophilic conditions, the variable with the highest effect on BMP and tVFA is biochar concentration (p-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 Fig. 1), thus, a higher methane generation.

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 ± 0.00 m^3 CH_4/kg VS_{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 unfavorable temperature conditions, as are considered psychrophilic ones, 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).

Fig. 2 clarifies the interaction impact of Ps and Bc at 15 °C (a) and 35 °C (b). In summary, 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.

When performing simultaneous optimization to maximize BMP and

Table 2
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	GGYTWYGAAGTNCGHGACGTDCA	460 bp	Ogier et al. (2019)
		Univ_rpoB_R	TGACGYTGCATGTTBGMRCCCATMA		
Total Archaea	16S rRNA (V6–V8 regions)	Arch915F	AGGAATTGGCGGGGGAGCAC	120 bp	Yu et al. (2005)
		Arch1059R	GCCATGCACCWCCTCT		
Total methanogens	Methyl coenzyme M reductase (mcrA)	MLfF	GGTGGTGTMGGATTCACACARTAYGC	470 bp	Luton et al. (2002)
		MLfR	TTCATTGCRTAGTTWGGRTAGTT		

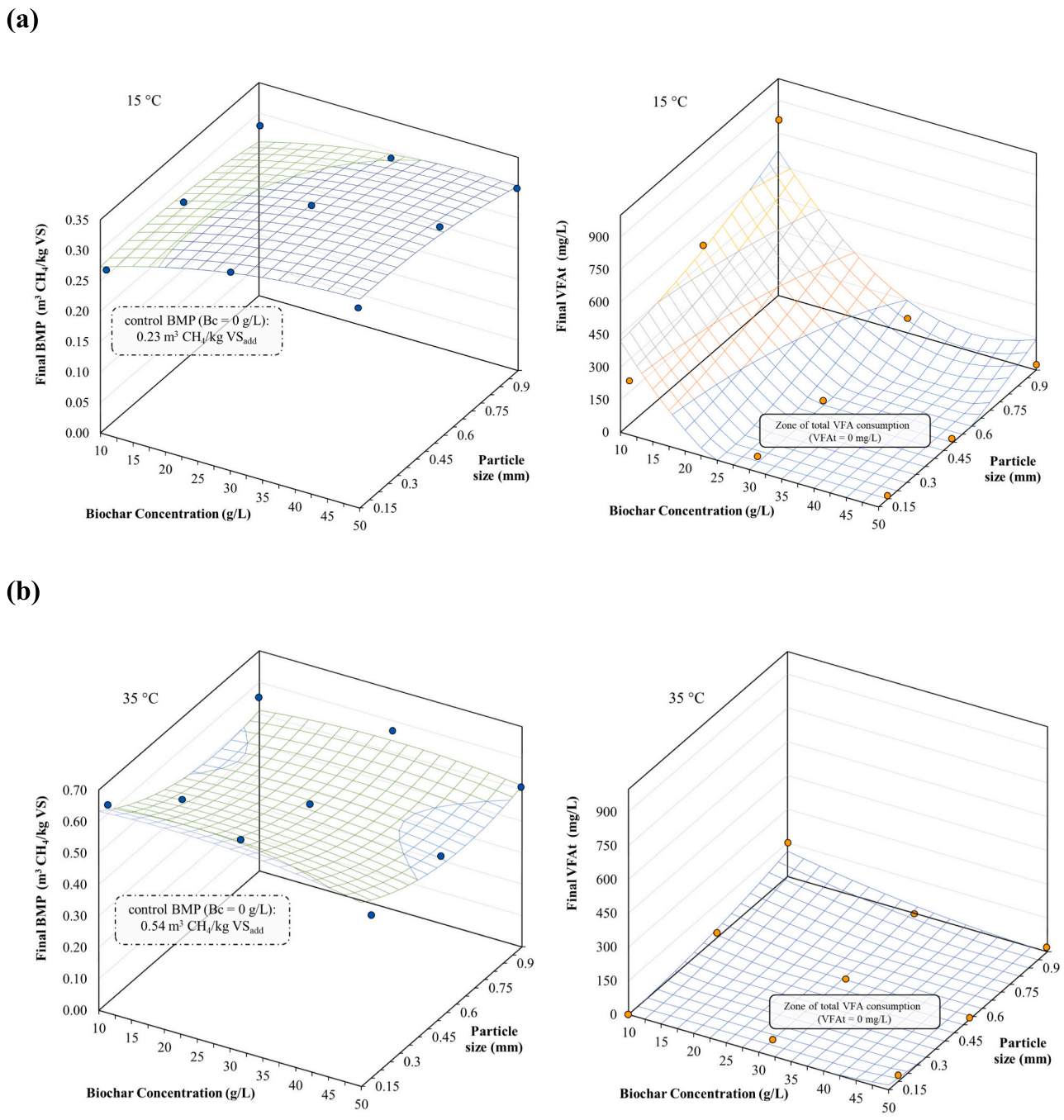

Fig. 1. 3D plots for BMP and VFA as functions of biochar concentration and particle size at 15 °C (a) and 35 °C (b).

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.

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

As validation, psychrophilic BMP under the most favourable conditions was replicated. Fig. 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).

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

Table 3
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[a]
	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[b]	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[a]
	Ps	9.99E+04	1	9.99E+04	0.0972
	Bc^2	1.24E+05	1	1.24E+05	0.0767
	Bc•Ps[b]	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[b]	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[b]	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

[a] Effects considered as significant.
[b] Independent variable's interaction.

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 4. 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.

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. Fig. 4a and b shows the changes through time for the psychrophilic (15 °C) and mesophilic (35 °C) AD metabolic stages with and without biochar.

As starting point, for day 0, there were no efficiencies. 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. 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 and 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). In other words, biochar favours the acetoclastic route while acids such as propionic or butyric are reduced. Compared to mesophilia, psychrophilic acidogenesis reaches similar (even higher) percents of COD metabolized, but it is deaccelerated by temperature influence on metabolism. As shown in Fig. 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 well-carried-out 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). Following the above, 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. 3a and b, 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. 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

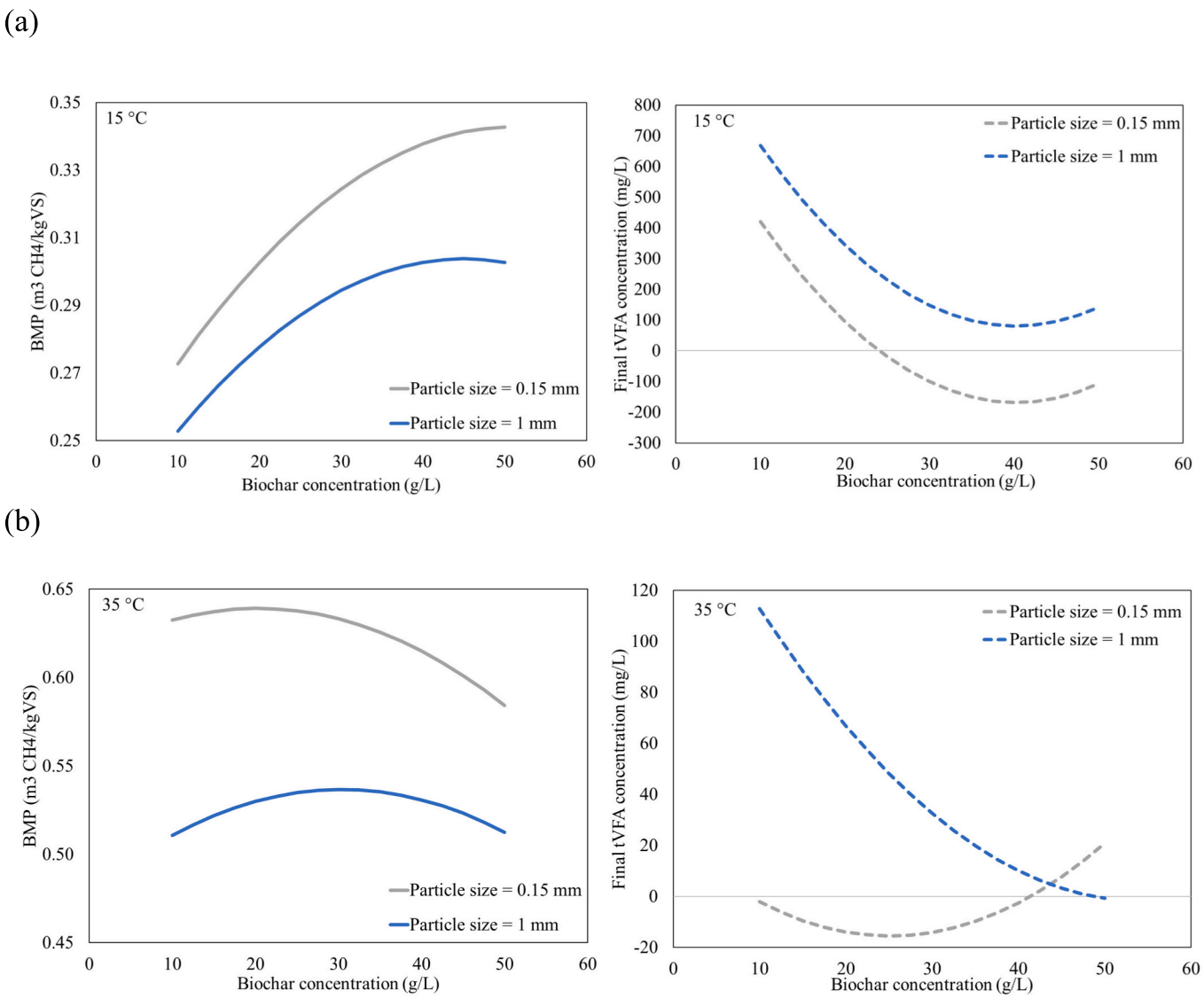

Fig. 2. Interaction plots between independent variables (Bc and Ps) with the output responses (BMP and final tVFA) at 15 °C (a) and 35 °C (b).

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.

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 Fig. 5.

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 6.99×10^7 to 2.15×10^8 copies/μL), and the archaeal populations had a two-fold increase (from 1.5×10^9 to 3.3×10^9 copies/μL). 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 psychotropic 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 1.5×10^9 to 5.14×10^9 copies/μL in archaeal and 6.99×10^7 to 1.27×10^9 copies/μL in methanogenic populations while bacterial abundance decreased from 7.99×10^8 to 5.58×10^7 copies/μL, after 40 days. The above results suggests 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

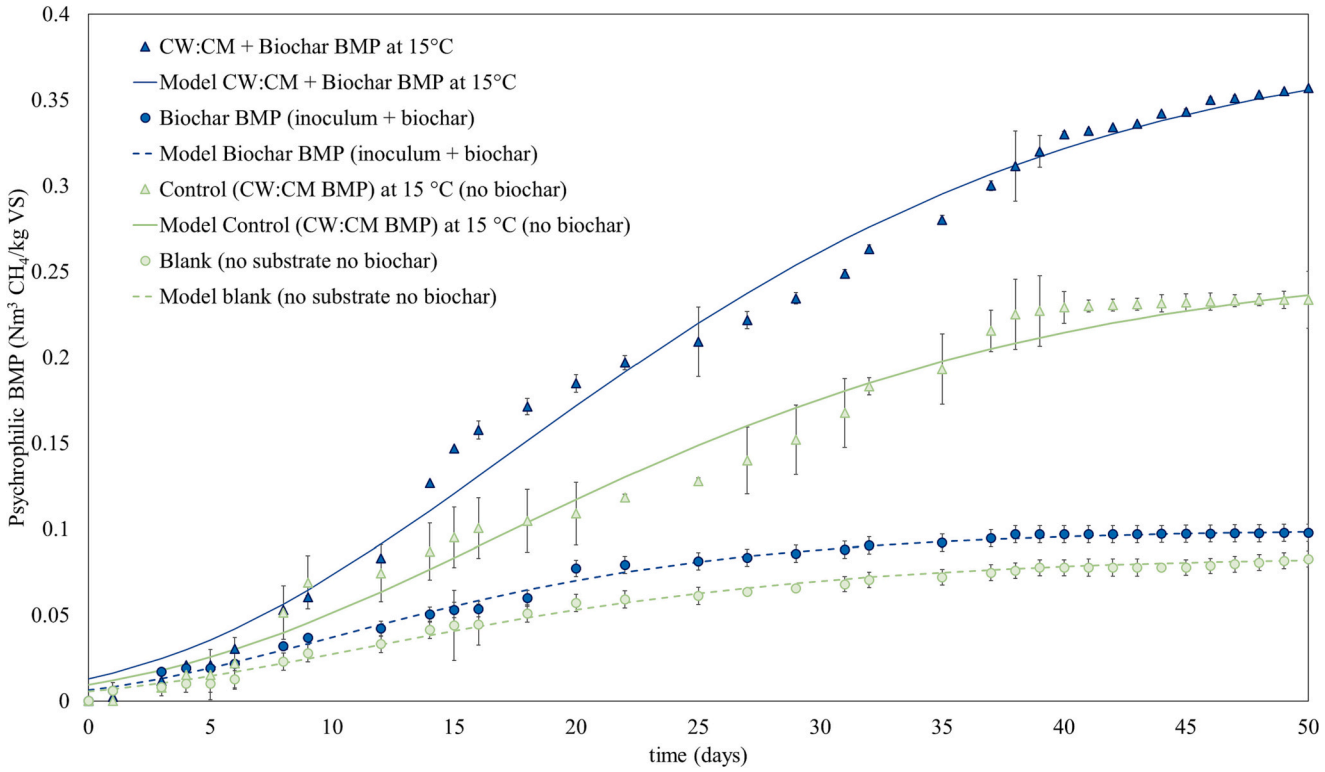

Fig. 3. Psychrophilic biomethane production kinetics and their respective Gompertz modelled data for CW:CM anaerobic co-digestion + biochar.

Table 4
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/$kgVS_{add}$ day)	λ (days)	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

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 6.89×10^8 to 5.50×10^7 copies/μL), and a significant increase (p-value <0.0001) in the archaeal (2.74×10^9 to 4.44×10^9 copies/μL) and methanogenic (from 1.76×10^8 to 1.17×10^9 copies/μL) 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 (Fig. 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., 2018; 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 + gasification is a viable alternative for organic residue treatment and energy and fertilizer generation. Biochar addition enhances the biomethane production and

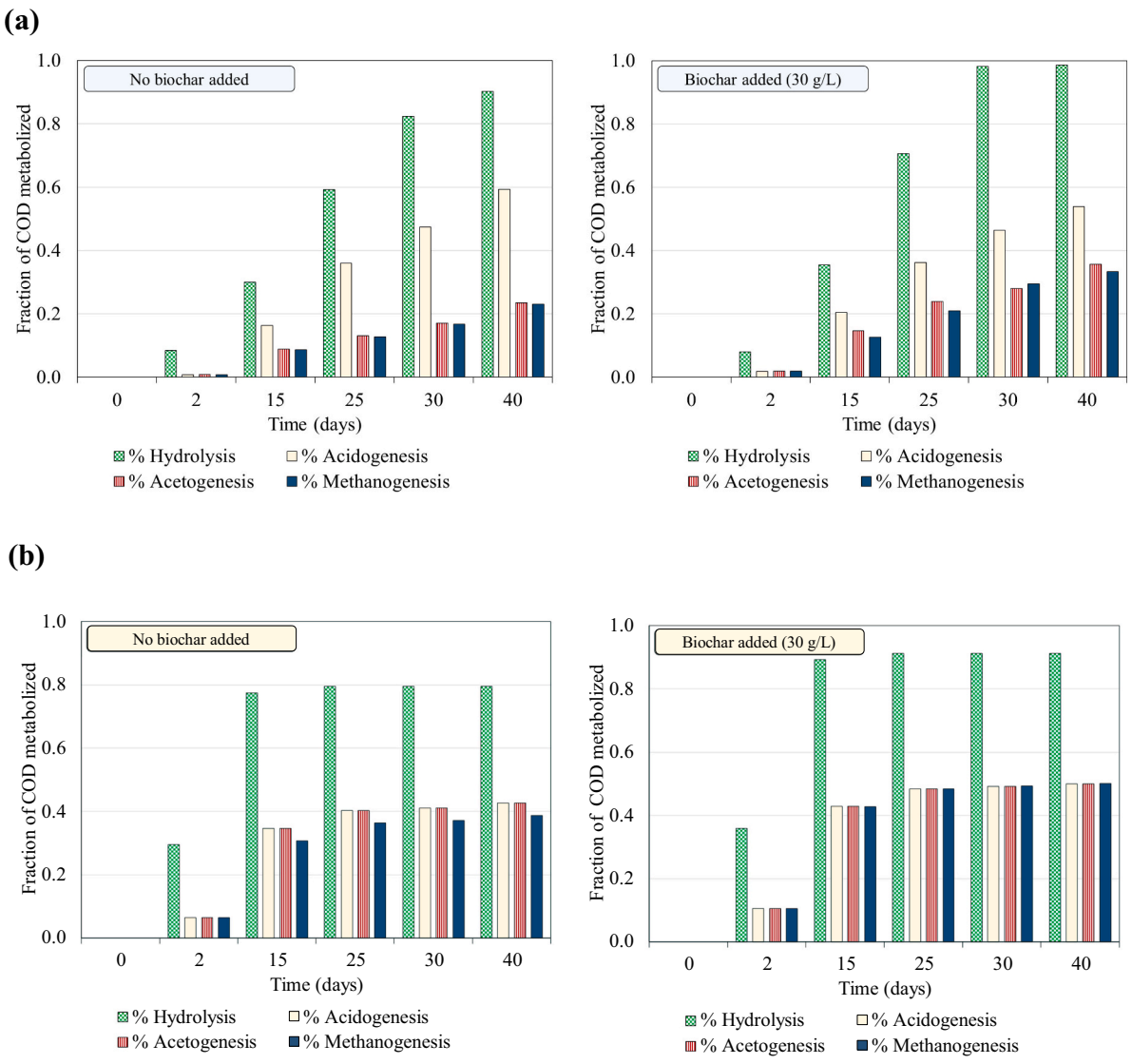

Fig. 4. Evolution of anaerobic digestion metabolic efficiencies modulated by biochar addition (30 g/L; Ps = 0.575 mm) during BMP assay at 15 °C (a) and 35 °C (b). Dotted bars for hydrolysis, clear bars for acidogenesis, striped bars for acetogenesis, and dark bars for methanogenesis.

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 above-mentioned 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 + gasification 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).

4. Conclusions

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

Kinetic improvement: 30 g/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 favours 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

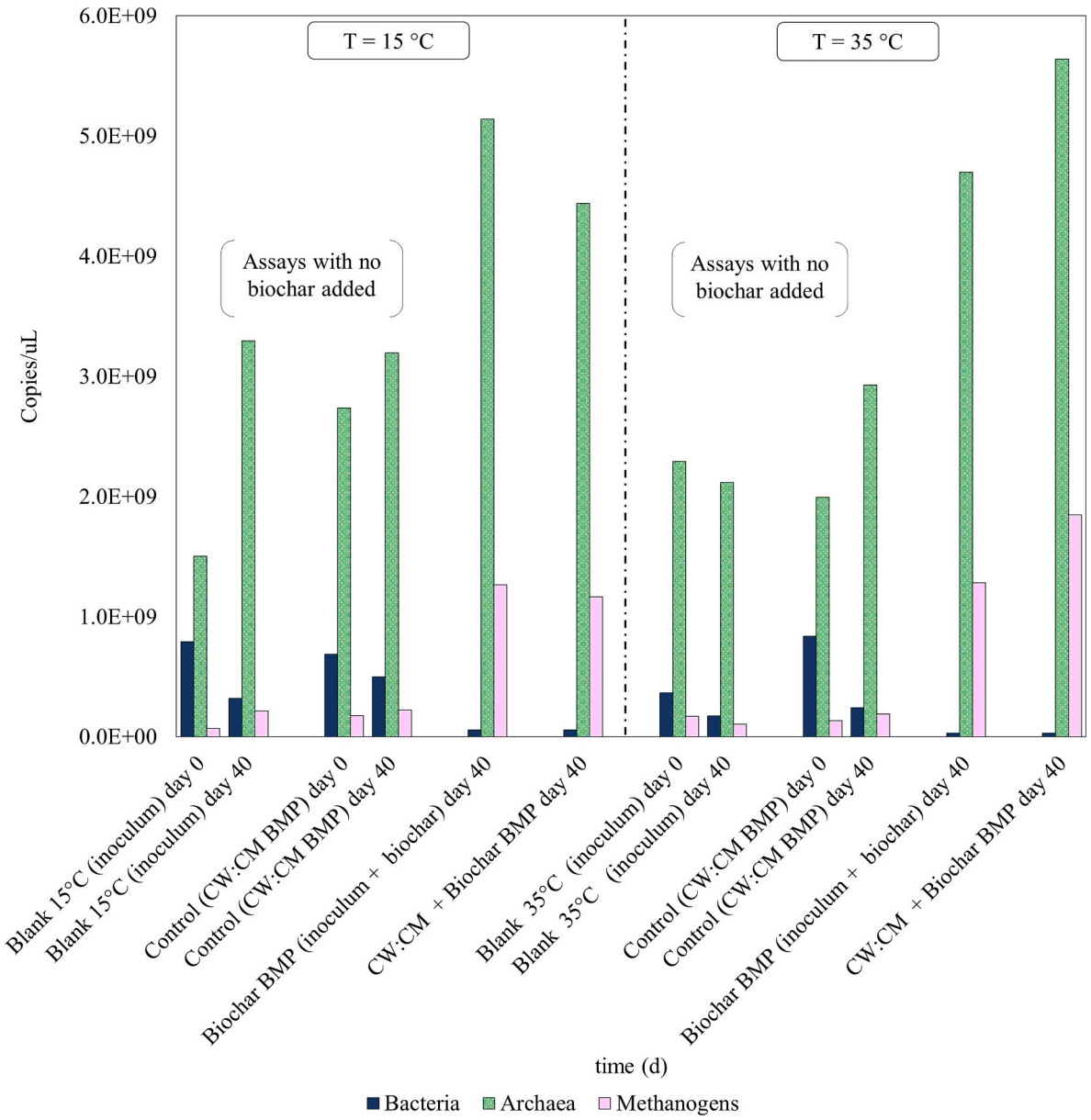

Fig. 5. Quantification of total bacteria (dark bars), archaea (stippled bars), and methanogens (light bars) from the different assays.

suggested approach in continuous systems and full-scale scenarios, making the bioprocess more attractive to be implemented in low-temperature zones.

CRediT authorship contribution statement

Jaimes-Estévez, J.: Conceptualization, methodology, investigation, visualization, writing - review & editing. Martí-Herrero, J.: Conceptualization, formal analysis, writing - review & editing. Poggio, D.: Funding acquisition, formal analysis, review. Zafra, G.: microbiological analysis – writing, editing & review. Gomez, K.: microbiological analysis – writing. Escalante H.: Resources, conceptualization, review, supervision & Editing. Castro L.: Funding acquisition, supervision, conceptualization, writing & editing.

Declaration of competing interest

The authors declare that no financial interests or personal relationships could influence the work reported in this paper.

Data availability

Data will be made available on request.

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