Renewable Energy 275 (2026) 126281

Contents lists available at ScienceDirect

Renewable Energy

journal homepage: www.elsevier.com/locate/renene

Optimizing specific dark fermentation strategies for enhanced bio-hydrogen production from different agricultural wastes

Davide Bertasini [a], Francesco Valentino [b], Roberto Lauri [c], David Bolzonella [a], Federico Battista [a,*]

[a] Department of Biotechnology, University of Verona, Via Strada Le Grazie 15, Verona, 37134, Italy
[b] Department of Environmental Sciences, Informatics and Statistics, Ca' Foscari University of Venice, Via Torino 155, Venezia-Mestre, 30172, Italy
[c] Inail, Department of Technological Innovations and Safety of Plants, Products and Human Settlements, Via del Torraccio di Torrenova 7, Rome, 00133, Italy

ARTICLE INFO

Keywords:
Dark fermentation
Two-stage anaerobic digestion
Agricultural residues
Biohydrogen
Biomethane potential
Co-fermentation

ABSTRACT

The integration of the dark fermentation process into existing anaerobic digestion plants, leading to the implementation of two stage processes, may enable the sequential recovery of biohydrogen and biomethane from agricultural residues. This study evaluated semi-continuous dark fermentation of cattle manure, corn residue, and soybean residue at hydraulic retention times of 3–10 days, including co-fermentation and digestate recirculation strategies. Cattle manure did not support stable biohydrogen production under the tested conditions, producing no detectable hydrogen and rapidly shifting towards methanogenesis. CR showed only transient biohydrogen peaks, followed by process instability associated with pH decrease, nutrient limitation, and methanogenic competition. Soybean residue supported stable hydrogen production at HRT 3 d, reaching 4.7 $NmLH_2/gVS$. The best performance was obtained by soybean/CR co-fermentation, which achieved 12.4 $NmLH_2/gVS$, 67.8 NmLgas/gVS, and 0.32 gCODVFA/gVS. VFA production was strongly substrate-dependent, with CR at HRT 7 d reaching the highest VFA yield among single-substrate trials. Biomethane potential tests showed that fermented effluents retained relevant residual energy potential, with fermented CR increasing biomethane recovery by up to 34% compared with the untreated substrate. Overall, the results demonstrate that substrate selection and nutrients balance are key factors for implementing two-stage anaerobic digestion of agricultural residues.

1. Introduction

Currently, more than 83,000 agricultural farms are operating in Italy. These farms are the backbone of Italy's renowned food industry but also generate substantial quantities of agricultural residues [1]. Specifically, Italian agricultural by-products amount to 18.6 Mt/year of dry vegetal waste (e.g., straws, prunings, and peels) and 103.2 Mt/year of manure and animal slurries [2]. Among these agrowaste, cattle manure (CM) and corn residue (CR) are prominent due to their widespread availability. This situation is common throughout the European Union, where corn and milk production reach 60.0 Mt/year and 151.0 Mt/year, respectively [3]. These residues, like most of agricultural by-products, are lignocellulosic in nature, which makes them inherently resistant to biodegradation. CM, for instance, contains 11.3%-26.0% hemicellulose, 3.6%-11.2% lignin, and 13.9%-26.6% cellulose [4], while CR comprises 21.4%-26.9% hemicellulose, 17.2%-21.3% lignin, and 30.1%-39.6% cellulose [5].

Some sustainable processes to valorize agricultural waste are composting and AD. Composting recovers carbon and nutrients in the form of compost, a valuable soil improver, while AD generates biogas and nutrient-rich digestate containing nitrogen (N) and phosphorus (P). Currently, Europe operates 20,800 AD plants, 1322 of which are equipped for biogas upgrading to biomethane [6]. Of these plants, 82% utilize agricultural feedstocks [7].

Abbreviations: AD, Anaerobic Digestion; BMP, BioMethanation Potential; CM, Cattle Manure; COD, Chemical Oxygen Demand; CR, Corn Residue; DF, Dark Fermentation; HRT, Hydraulic Retention Time; OLR, Organic Loading Rate; PM, Particulate Matter; SGP, Specific Gas Production; SHP, Specific Hydrogen Production; SMP, Specific Methane Production; SR, Soybean Residue; TAN, Total Ammonia Nitrogen; TKN, Total Kjeldahl Nitrogen; TS, Total Solids; TSAD, Two Stage Anaerobic Digestion; VFAs, Volatile Fatty Acids; VS, Volatile Solids; vl, Laminar burning Velocity.

* Corresponding author.
E-mail address: federico.battista@univr.it (F. Battista).

https://doi.org/10.1016/j.renene.2026.126281
Received 27 July 2025; Received in revised form 11 August 2026; Accepted 11 August 2026
Available online 12 August 2026

While, the performance of the methanogenic stage is strongly affected by substrate composition, biodegradability, feeding regime, organic loading rate, pH stability, ammonia concentration, and the balance between acetoclastic and hydrogenotrophic methanogens. Pre-treatment strategies may enhance substrate hydrolysis and improve methane recovery, particularly when recalcitrant organic matrices are treated [8]. Feeding regimes can also shape the methanogenic community by selectively enriching specific functional groups, while fluctuations in substrate composition may affect process stability and methane productivity. Therefore, when DF is proposed as the first step of a two-stage AD process, the residual biomethane potential of the fermented effluents must be evaluated together with biohydrogen production, since the VFA profile and the remaining biodegradable organic matter determine the effectiveness of the downstream methanogenic conversion ([9]).

While the biohydrogen yield from TSAD cannot compete with the efficiency of water electrolysis powered by renewable energy, it offers a pathway to partially decarbonize the existing gas grid. Biohythane, a mixture of 2%-30% biohydrogen and methane, can be injected into European pipelines (Fig. 1), provided it meets each country's hydrogen tolerance levels around but not exceeding 20% [10] as higher concentrations are currently unfeasible due to pipeline limitations, including risks of accidental leaks that could create explosive atmospheres [11].

Although TSAD has yet to reach full-scale implementation, pilot studies have reported promising results. For example, biohydrogen yields of 99.0 $NmLH_2/gVS$ were achieved using rotten fruits, potato residues, and livestock effluents [12], about 286.4 $NmLH_2/gVS$ from reducing sugars [13]. However, data on biohydrogen production from recalcitrant substrates, such as lignocellulosic materials, remain limited in literature.

This study aimed to assess the efficiency of TSAD in bio-converting agricultural residues feedstock into biohydrogen. In specific, soybean meal residue (SR), a nitrogen rich by-product, was evaluated as a potential feedstock to optimize the carbon-nitrogen ratio in the reaction medium, increasing the performance of the process. Compared with previous contributions published in *Renewable Energy* on biologically produced hydrogen and methane for gas-grid decarbonization, this study provides original experimental evidence on the semi-continuous dark fermentation of real agricultural residues. The novelty of the work lies in the substrate-specific evaluation of recalcitrant feedstocks commonly available in agricultural AD plants, combining HRT optimization, co-fermentation, digestate recirculation, VFA production, microbial community assessment, and residual biomethane potential. This approach allows the identification of practical opportunities and limitations for implementing two-stage anaerobic digestion in existing agricultural biogas plants.

2. Materials and methods

The experimental architecture of this work consisted in the following steps: i) Feedstocks and Inoculum Characterization, ii) Semi-continuous Dark Fermentation (DF) Tests, iii) Metagenomic analysis of the fermented effluents and iv) Biomethane Potential (BMP) Tests for untreated and fermented feedstocks.

2.1. Feedstocks and Inoculum characterization

CM, CR, and SR were sourced from the "Cooperativa Agricola Zootecnica La Torre," an agricultural enterprise in Isola della Scala (VR), Italy. This facility utilizes animal and crop by-products for biogas production in a 999 kW AD plant. No specific pre-treatment, such as heat shock, chemical inhibition, or acidification, was applied to the inoculum to selectively suppress methanogenic microorganisms. The use of 2 mm-filtered digestate was selected to evaluate the process under conditions closer to the possible integration of DF into existing agricultural anaerobic digestion plants.

The agricultural residues and digestate were characterized in triplicate following IRSA-CNR standard methods [14]. The parameters were analyzed in triplicate in terms of Total Solids (TS), Volatile Solids (VS), Chemical Oxygen Demand (COD) abd Total Kjeldahl Nitrogen (TKN). Table 1 summarizes the characteristics of the substrates and inoculum.

2.2. Semi-continuous dark fermentation tests

Fermentation tests were carried out in 1 L glass reactors (Duran®) with a working volume of 0.8 L. They were equipped with an orbital shaker operating at 130 rpm. The reactors were inoculated with 2 mm filtered digestate to ensure acidogens and methanogens, and the pH was adjusted to 6.5–7.0 using hydrochloric acid (37% w/w).

Daily, the reactors were opened to collect the output and add the substrate, promoting the "Venting" effect and thus reducing the partial pressure of the hydrogen, which can restrict biohydrogen production

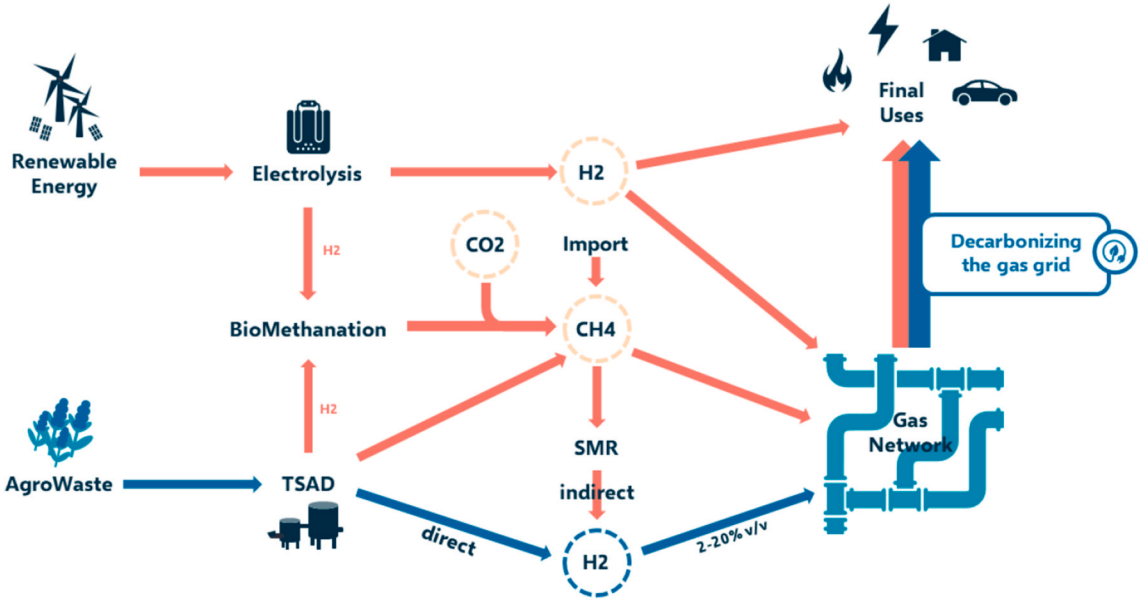

Fig. 1. Graphical representation of the potential integration of the TSAD process into the existing methane and hydrogen production chain.

Table 1
Inoculum and Substrates characterization in terms of TS, VS, COD, TKN, TAN, and C/N ratio.

	TS	VS	COD	TKN	C/N
	%	%	gCOD/KgTS	gN/KgTS	-
Digestate (Inoculum)	6.3±0.0	3.7±0.0	412±0.3	187.3 ± 11.6	2.2
CM	17.4±1.4	15.3±1.2	763±78.7	83.9 ± 4.9	9.1
CR	33.7±4.6	31.7±4.7	772±125.9	5.6 ± 1.0	137.2
SR	88.0±0.2	81.7±0.3	912±22.2	310.8 ± 12.5	2.9

[15,16]. The reactors were maintained in a thermostatic cabinet set at 38 °C, in order to operate under mesophilic conditions commonly applied in agricultural AD plants and to ensure consistency with the downstream methanogenic stage. The pH was monitored and adjusted daily between 5.5 and 6.5 using sodium hydroxide or hydrochloric acid.

Each semi-continuous fermentation test was operated for at least three times the selected HRT, corresponding to at least three theoretical reactor turnovers. This duration was selected to assess the evolution of the process under each operating condition and to determine whether the system reached stable acidogenic state or shifted towards methanogenic activity. Consequently, tests performed at longer HRTs had longer overall durations.

VFAs were quantified to track the process performance and potential inhibition events (Equation (1)). Gas samples were collected in gas bags for analysis of composition and volume, which were used to calculate the Specific Gas Production (SGP) and Specific Hydrogen Production (SHP) (Equations (2) and (3)):

$$VFAs\ Yield\left(\frac{gCOD_{VFAs}}{gVS}\right)=\frac{total\ VFAs\ produced\ in\ COD\ terms\ \left(gCOD_{VFAs}\right)}{total\ VS\ added\ as\ substrate\ (gVS)} \quad (1)$$

$$SGP\left(\frac{NmL\ Gas}{gVS}\right)=\frac{total\ gas\ produced\ (NmL\ Gas)}{total\ VS\ added\ as\ substrate\ (gVS)} \quad (2)$$

$$SHP\left(\frac{NmL\ H_2}{gVS}\right)=\frac{total\ hydrogen\ produced\ (NmL\ H_2)}{total\ VS\ added\ as\ substrate\ (gVS)} \quad (3)$$

Table 2
Summary of the trials'feedstock, HRT, OLR, Co-fermentation, and Recirculation Ratio.

Feedstock	HRT (d)	OLR (gCOD/L×d)	Co-fermentation Ratio (*)	Recirculation Ratio (°)
CM	3	12.73	-	-
	3	12.75	80% VS CR	-
	7	6.25	-	-
	10	4.37	-	-
CR	3	12.87	-	-
	3	12.87	-	86%
	3	12.42	50% VS SR	-
	5	7.72	-	-
	7	5.52	-	-
SR	3	11.98	-	-

Semi-continuous tests were conducted with varying HRTs, OLR, co-fermentation ratios, and digestate recirculation, as shown in Table 2. The selected HRT values were chosen to investigate the balance between acidogenic activity, substrate hydrolysis, and the possible onset of methanogenesis under semi-continuous conditions. An HRT of 3 days was used as the shortest condition tested, with the aim of facilitating DF and limiting the growth of slower methanogenic populations. Longer HRTs were selected according to substrate characteristics: 7 and 10 days were tested for cattle manure due to its recalcitrant lignocellulosic nature, while 5 and 7 days were tested for CR to evaluate whether a moderate increase in residence time could improve hydrolysis and VFA production. Soybean residue was tested at HRT 3 days because of its higher nitrogen content and expected higher fermentability, both as single substrate and in co-fermentation with CR.

As shown in Fig. 2, a CR fermentation test operated at an HRT of 3 days was coupled with a digestate recirculation system. The fermented effluent from the DF reactor was fed daily to a 5 L semi-continuous methanogenic reactor operated at an SRT of 15 days. This configuration was used to simulate the transition from the DF stage to the methanogenic stage. The methanogenic reactor was maintained at 38 °C using a heating jacket, without active pH control. During operation, the digestate pH ranged between 7.5 and 8.0. The reactor was equipped with a gas collector and a unidirectional valve like a fermenter. After methanogenesis, the digestate was centrifuged at 3900 rpm for 20 min to separate the liquid fraction and remove most of the methanogenic biomass. Part of this liquid fraction was then recirculated to the DF reactor, replacing water in the feedstock mixture.

The remaining fraction, including the solids, was discarded. The recirculation ratio, calculated to maintain system stability, was set at 86% using Equation (4):

$$Recirculation\ Ratio\ (\%)=\frac{Qr}{Qa}=\frac{Recirculation\ flow\ rate\ \left(\frac{mL}{d}\right)}{Input\ flow\ rate\ \left(\frac{mL}{d}\right)}\times 100 \quad (4)$$

2.3. Metagenomics analysis

Following the fermentation tests, a metagenomics analysis was performed to evaluate the selection effects of bacterial communities given by the use of different substrates and HRTs. In particular, the outputs of tests HRT 3 d CM, CR, CR with recirculation system (CR°), SR in co-fermentation with CR (SR*), and SR were the subjects of the investigation. The analysis to identify the 16s rRNA (bacteria and archaea) was

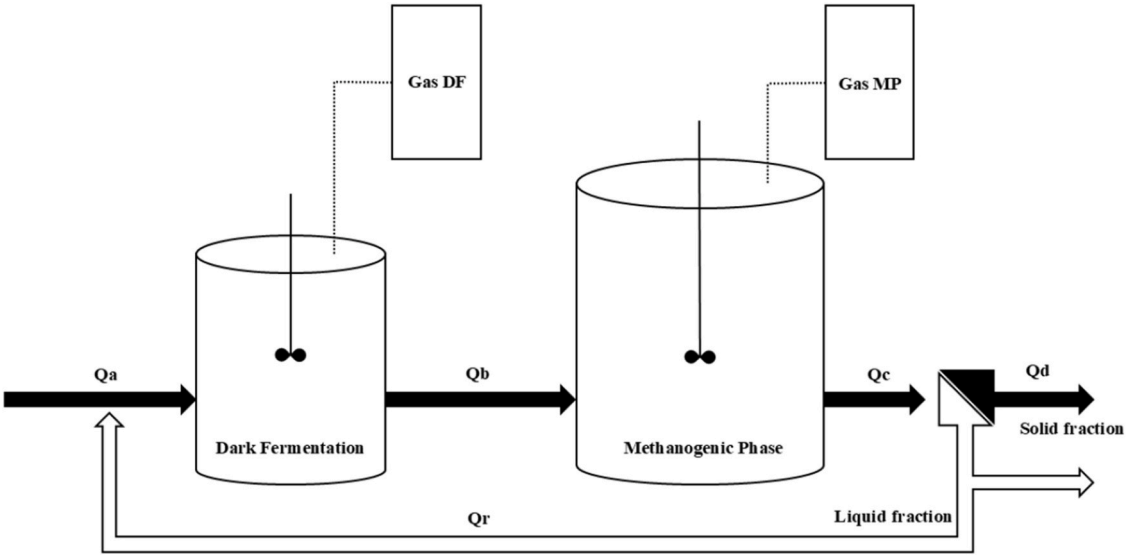

Fig. 2. Scheme of the Recirculation System of corn HRT 3 d s trial.

done through NGS sequencing by the laboratory "BMR Genomics s.r.l." (Padova, Italy).

2.4. BMP tests for untreated and fermented feedstocks

BMP tests were performed on fermented outputs and untreated feedstocks to evaluate their residual biomethane potential. The substrate-to-inoculum ratio was set at 0.25 (VS basis) [17]. Tests were carried out in 1 L glass sealed bottles with 0.5 L working volume at 38 °C for about 50 days. A blank test with only inoculum and water was conducted to account for baseline gas generation. Gas samples were collected daily by keeping the bottles manually mixed, through which accumulation curves could be constructed and Specific Methane Production (SMP) calculated (as in Equation (5)).

$$SMP\left(\frac{NmL\ CH_4}{gVS}\right) = \frac{total\ methane\ produced\ (NmL\ CH_4)}{total\ VS\ added\ as\ substrate\ \ (gVS)} \quad (5)$$

2.5. Analytical methods

From fermentations and BMP tests, solid, liquid, and gaseous samples were collected daily for a detailed analysis of the process performances.

Solid and liquid samples recovered exclusively during the fermentation tests were subjected to pH, TS, TVS, COD, TAN, and VFAs content evaluation. The pH was monitored using a pH meter "Mettler Toledo©"; the value obtained was fundamental to recognize and control the correct metabolism of the mixed culture. The gravimetric method was used to track the TS and VS content and to ensure the maintenance of 5% TS during the process; moreover, the diminution of the VS/TS ratio of the in and out feedstock made it possible to verify the organic matter conversions in gases. The total COD content was evaluated in each phase of the test to track the flow of the carbon and build the mass balance of the process; high-temperature digestion with concentrated sulfuric acid and titration with potassium dichromate and ferrous ammonium sulfate was the chosen method. The TAN content, calculated through spectrometry with the Nessler reagent, was supervised to highlight potential inhibition events. TKN was verified through the same method after digestion at high temperature with concentrated sulfuric acid, so it was possible to calculate the C/N ratio, an essential parameter for the proper growth of microorganism communities. The quantification of VFAs, from C2 to C6, was performed using an ion chromatographer "DionexTM ICS-1100 (Thermo Fisher Scientific Inc.)", operating with an IonPac ICEAS1 column, as in Ref. [18]. Gaseous samples were collected in gas bags during both the fermentation trial and BMP tests. The water displacement method made it possible to quantify the gas produced, while the gases composition was analyzed by gas chromatography through a "MicroGC Fusion ® Gas Analyzer (INFICON)" with a TCD detector.

3. Results and discussion

3.1. Substrates and Inoculum characterization

Characterizing the substrates and inoculum was crucial for understanding their composition and determining the optimal approach for the subsequent fermentation and digestion trials. In Table 1, the data for the analyzed parameters: TS, VS, COD, TKN, TAN, and the C/N ratio, are reported.

The low C/N ratios for CM and SR were attributed to their respective compositions—urea from livestock effluents in CM and the high protein content of SR [19]. These low ratios indicated a potential risk of ammonia inhibition during fermentation and digestion, typically occurring at concentrations between 3.0 and 8.0 gN/L [16]. Conversely, the high C/N ratio of CR, resulting from its lignocellulosic nature, suggested potential challenges due to nitrogen deficiency, a critical macronutrient for microbial growth.

The distinct C/N ratios of these substrates present an opportunity to explore co-fermentation strategies, such as combining CR with CM or

Table 3
Summary of fermentation test results, in terms of SGP, SHP, and VFA yield.

Feedstock	HRT	SGP	SHP	VFA yield
	d	NmLGas/gVS	$NmLH_2/gVS$	$gCOD_{VFA}/gVS$
CM	3	2.5 ± 0.6	0.0 ± 0.0	0.10 ± 0.2
	3[a]	64.3 ± 27.6	N/A	0.21 ± 0.1
	7	8.0 ± 4.5	0.0 ± 0.0	0.10 ± 0.2
	10	14.1 ± 8.5	0.0 ± 0.0	0.12 ± 0.3
CR	3	57.2 ± 20.6	N/A	0.26 ± 0.1
	3[b]	101.9 ± 56.3	N/A	0.27 ± 0.1
	3[a]	67.8 ± 23.4	12.4 ± 7.2	0.32 ± 0.2
	5	39.8 ± 27.5	N/A	0.33 ± 0.1
	7	38.8 ± 8.3	N/A	0.37 ± 0.1
SR	3	47.9 ± 11.4	4.7 ± 1.3	0.27 ± 0.1

[a] Co-fermentation.
[b] Recirculation; N/A = not applicable, discontinuous $bioH_2$ production.

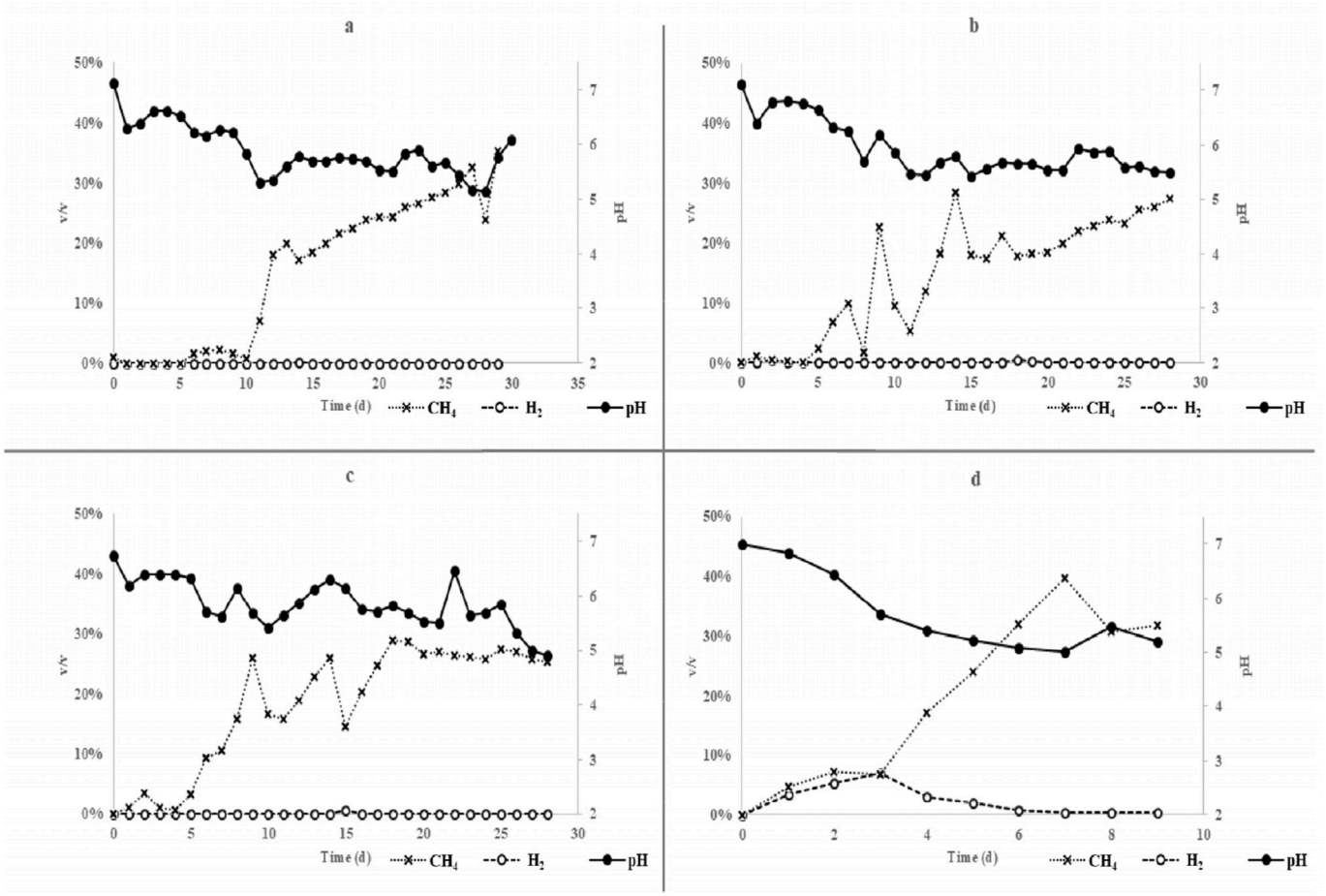

Fig. 3. Hydrogen, methane, and pH trends of CM fermentation test HRT 3 (a), HRT 3 co-digestion (d), HRT 7 (b), HRT 10 (c) days.

SR. Co-fermentation can balance the C/N ratio within the ideal range of 15–35 [20], optimizing nutrients and micronutrient availability for microorganisms while preventing inhibitory conditions.

3.2. Semi-continuous tests

Semi-continuous DF tests were conducted to evaluate biohydrogen and VFA production from the three agricultural residues. The overall results are summarized in Table 3.

3.2.1. Gas production

3.2.1.1. Results of CM trials. CM trials were conducted at HRTs of 3, 7, and 10 days. The selection of extended HRTs, uncommon in typical DF processes (which generally range from a few hours to 3 days), was justified by the CM recalcitrant nature, attributed to the high lignin and hemicellulose content. Despite different HRTs and a co-fermentation being tested, the trials failed in biohydrogen production. As illustrated in Fig. 3and in Fig. 4, the tests predominantly produced only biomethane and carbon dioxide. The following factors were identified as potential inhibitors of effective DF using CM: i) Recalcitrant Compounds: The high lignin and hemicellulose content in CM results from the fibrous cattle diet, which primarily comprises grass forage and cereals [21]. These compounds are challenging for acidogenic microorganisms to degrade, particularly those with fast metabolic kinetics [22]; ii) Low Carbon-to-Nitrogen Ratio (C/N Ratio): CM's low C/N ratio can lead to ammonia inhibition, which disrupts microbial communities and hinders biohydrogen production [23]; iii) Methanogen Presence: CM carries a significant load of methanogens from the bovine digestive tract. These microorganisms, characterized by slower growth kinetics than acidogens, can eventually dominate the microbial community and promptly shift the process to methanogenesis [2]. In order to avoid the high methanogen content, most studies found in the literature of bioH_2 production from CM employ contrivances such as working for barely a few days in batch reactors or using high temperatures, co-fermentations, or pre-treatments [22]. Hangri et al. (2024) [22] reported similar results from the fermentation of dairy manure through a pre-treated inoculum (105 °C for 1.5 h), i.e., a negligible hydrogen yield of 0.68 NmlH_2/gVS. The integration of 20% v/v of cheese whey as a co-substrate improved the process, achieving 9.29 NmlH_2/gVS. Kazimierowicz et al. (2022) [24] obtained 142.9 NmlH_2/gVS (17.6% v/v of the total gas produced) from the DF of a cattle slurry. This was achieved by i) pasteurising the slurry at 90 °C to eliminate methanogens; ii) employing a specific inoculum of *Bacteroides vulgatus*; iii) studying the process in a batch reactor, limiting the work time to 30 days. Nevertheless, these expedients were not enough to prevent the emergence of biomethane (18.7 NmlCH_4/gVS) (see Fig. 4).

To overcome these barriers, a co-fermentation strategy was adopted, combining CM with CR in a VS ratio of 20%-80% (referred to as CM* in the following discussion). This trial utilized an HRT of 3 days to mitigate the challenges posed by recalcitrant compounds and the low C/N ratio. The co-fermentation yielded improved results, with an initial peak in biohydrogen production during the first HRT. However, methanogens rapidly replaced acidogens, leading to a shift towards methanogenesis.

Yilmazel et al. [25] reports that through the co-fermentation of switchgrass added to the cattle manure, they achieved an increase in hydrogen yield from 82.5 NmL/gVS to 85.3 NmL/gVS. However, even in this study, some expedients were used to overcome the limitation of high

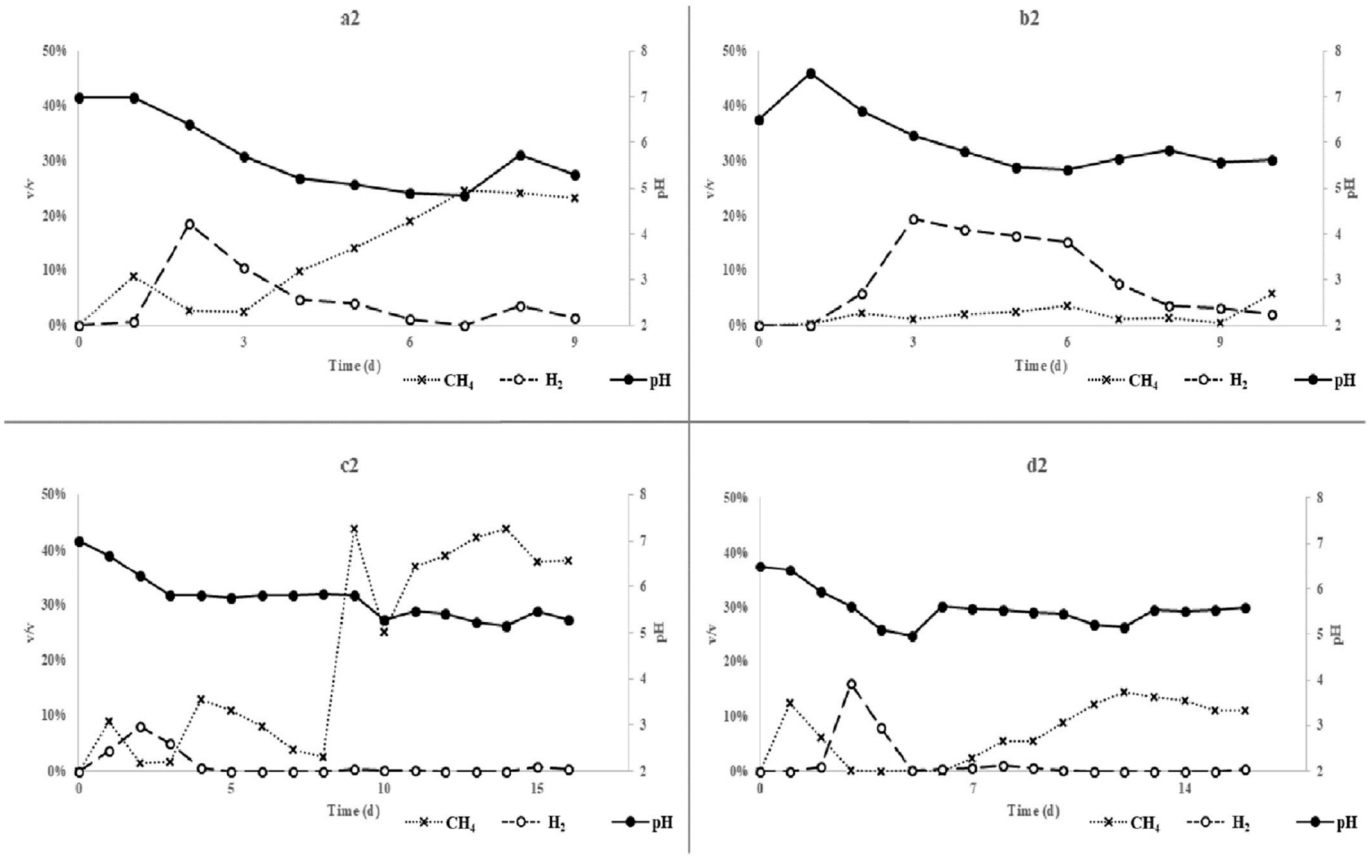

Fig. 4. CR gas and pH trends. HRT 3 d (a2), 3 d with recirculation system (b2), 5 d (c2), and 7 d (d2).

methanogens content, i.e.: i) cattle manure was sterilized at 120 °C, ii) the tests were set at thermophilic condition (78 °C), facilitating the acidogenic activity; iii) *Caldicellulosiruptor bescii* was used as specific inoculum; iv) the analysis was limited to 15 days in batch reactors. According to these evidences and the absence of methane in the biogas produced we can conclude that no methanogenic activity was taking place.

3.2.1.2. Results of CR trials. CR trials were carried out at HRTs of 3, 5, and 7 days. The initial days (approximately one HRT) of each trial exhibited biohydrogen production peaks of 18.5%, 8.1%, and 16.1%, respectively. However, biohydrogen production was no longer detected after these peaks, with the reactors transitioning to biomethane production after the first HRT.

Three primary factors were identified as contributors to the end of the biohydrogen production: i) Nutrients deficiencies: CR is deficient in nitrogen (5.6 gN/kg TS) and essential trace elements, such as Fe (99.7 mg/kg TS) and Ni (4.1 mg/kg TS) [26], which are critical for hydrogenase enzymes ([Ni-Fe] and [Fe] hydrogenases) [27]. This nutrient scarcity impairs acidogenic microbial activity and hampers biohydrogen production [28]. ii) VFA accumulation led to a drop in pH below the optimal range of 5.5, favoring alternative metabolic pathways such as lactic fermentation [22], or detoxifying processes, including solventogenic fermentation and methanogenesis [16]. The low pH also caused iii) inhibition: the acidic conditions further exacerbated the process instability, forcing microorganisms to expend energy on maintaining intracellular pH rather than biohydrogen production [29]. Even in the case of CR, the literature studies are based mainly on discontinuous reactors with methanogen-free inoculum, so this type of issue is rarely appreciated and therefore partially reported [30]. In this regard: i) Liu et al. (2023) [31] managed to produce about 23.9 $NmLH_2/g$ from the DF of corn straw, employing a pure inoculum of *Klebsiella pneumoniae* (specialized microorganism to the cellulose degradation and biohydrogen production), adding yeast powder as source of nitrogen, and ensuring hydrogenases' Fe e Ni content through $FeSO_4$ and $NiCl_2$. ii) Fu et al. (2020) [26] reached a biohydrogen yield of about 23.3 $NmLH_2/gVS$ from a 5-day DF process of corn straw. The results were achieved by inhibiting the methanogenic activity by pre-treating anaerobic sludge (inoculum) at 80 °C and working in controlled microaerophilia (oxygen loading of 5 mL/gVS). iii) 150.2 $NmLH_2/gVS$ were obtained by Zhang et al. (2017) [32] fermenting corn stalks previously pretreated with high temperature, basic reagents, and enzymes. The study was carried out by a 108 °C pretreated cow dung (as inoculum) until the biohydrogen production ended. Lastly, iv) Dong et al. (2025) [30] produced a biohydrogen yield of 33.5 $NmLH_2/gVS$ from a 2-day DF test of corn stoves. The inoculum, a sewage sludge, was previously pre-treated at 95 °C to ensure the absence of methanogenic activity.

In order to meet these challenges and also to look for solutions more feasible on the larger scale, a second reactor for methanogenesis and a digestate recirculation system were implemented. The liquid fraction of the digestate from the methanogenic phase was used as the liquid part of the fermenter feed (SRT 3 d; recirculation ratio 86%). A high recirculation ratio was intentionally set to maximize the influence of the digestate on the dark fermentation phase. In particular, this approach aimed to provide essential nutrients and to buffer the acidic pH [33]. The recirculation extended biohydrogen production to two HRTs, achieving a peak of 19.5%. Despite these improvements, the process shifted again towards methanogenesis. Ammonia inhibition was ruled out, as the TAN level was 0.66 gN/L, well below the critical threshold of 3.0–8.0 gN/L [16,23,34].

3.2.1.3. Results of SR trials. SR trials were conducted at an HRT of 3 days, selected based on the optimal results observed for CR feedstock.

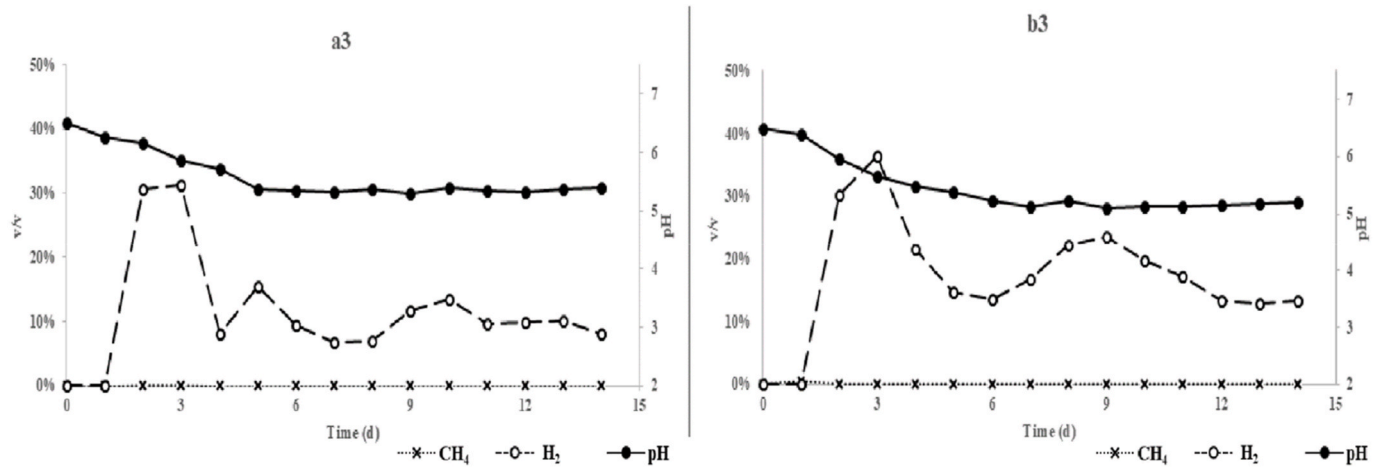

Fig. 5. Biohydrogen, biomethane, and pH trends for SR fermentation tests (HRT 3 (a3) and HRT 3 co-fermentation 50% VS with CRs (b3)).

Biohydrogen production was stable, with an average concentration of approximately 10.0% (±2.7%) and an initial peak of 31.0%. Notably, no biomethane was detected in the biogas, indicating a successful suppression of methanogenesis. The superior performance of results obtained with SR can be attributed to its high nitrogen content, which buffered pH fluctuations and supported acidogenic growth and replication [27]. SHP and SGP values were recorded at 4.7 ± 1.3 $NmLH_2/gVS$ and 47.9 ± 11.4 NmLGas/gVS, respectively.

To further enhance the process, a co-fermentation process using SR and CR as co-substrates (50% VS each) was tested at the same HRT (SR*). This strategy improved biohydrogen production, with a stable average concentration and an initial peak of 36.4%. SHP and SGP increased to 12.4 ± 7.2 $NmLH_2/g$ VS and 67.8 ± 23.4 NmLGas/gVS, respectively. The results suggest that a C/N ratio of 5.3 is optimal for acidogenic semi-continuous fermentation using these feedstocks. Gas and pH trends for SR and co-fermentation trials are depicted in Fig. 5. In conclusion, the fermentation of CR as a single substrate seems not to be so advantageous, but rather it needs a nitrogen-rich co-substrate in order to establish continuous biohydrogen production.

The biohydrogen values obtained can already be compared with literature, where, even if few, hydrogen DF experiments from soya by-products can be found. For example, Augusto et al. [35,36] research, which concerns the $bioH_2$ generation from diluted soybean molasses in Anaerobic Sequencing Batch Biofilm Reaction operating as sequential-fed batch for 3 h, OLR 26 $KgCOD/(m^3 \times d)$, and at different operative temperatures. Their first publication [35], testing the mesophilic conditions (30 °C), reports the achievement of a biohydrogen yield (about 12.42 $NmLH_2/gVS$) very similar to the SR* results. While the results of the subsequent study [36], performed at thermophilic conditions (50 °C), reached an importantly higher yield with about 57.7 $NmLH_2/gVS$. The increase in hydrogen produced was justified by the use of an unfavourable temperature for homoacetogenesis and the propionic acid pathway (biohydrogen antagonist metabolisms) but can also be attributed to the low pH (between 4.5 and 5.0). Following the limited literature and the results obtained during this experimental campaign, it can be argued that the biohydrogen continuous production without biomethane traces is given primarily by the operating parameters employed, but a decisive role is also played by the use of a nitrogen-rich substrate, contributing to stabilising the pH and balancing the nutrient content.

3.2.2. *VFAs production*

Fig. 6 reports the graphical resume of VFA concentrations observed for the different substrates tested in the experimental runs.

3.2.2.1. Results in CM trials. The CM tests conducted at HRTs of 3, 7,

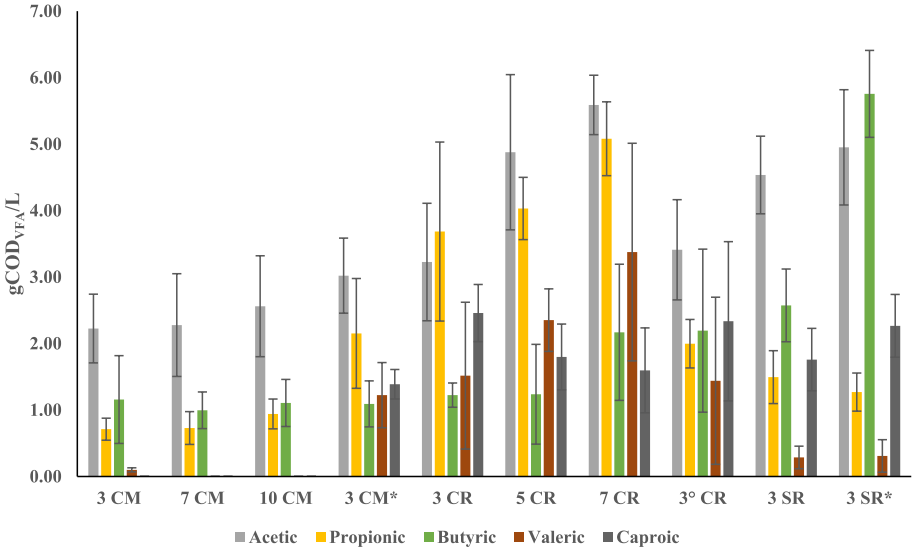

Fig. 6. Charts of average VFAs concentration of each test (* = co-fermentation; ° = recirculation system).

and 10 days produced relatively low concentrations of VFAs. The recorded concentrations were 4.56 ± 1.0 $gCOD_{VFA}/L$, 4.47 ± 1.4 $gCOD_{VFA}/L$, and 5.17 ± 1.2 $gCOD_{VFA}/L$, respectively, with corresponding yields of 0.10 ± 0.2, 0.10 ± 0.2, and 0.12 ± 0.3 $gCOD_{VFA}/gVS$. These results confirmed the challenging nature of the substrate and the difficulty in hydrolyzing the recalcitrant organic matter in CM.

The dominant VFAs produced were acetic and butyric acids, typically associated with the acidogenic phase of fermentation [37,38]. A minor presence of propionic acid was also detected, likely resulting from the degradation of proteins and lipids present in the substrate [39]. However, the limited overall VFA production correlates with the absence of detectable biohydrogen in the reactor off-gas, suggesting that the substrate composition and process conditions were not conducive to robust acidogenic activity. CM's high lignin and hemicellulose content, along with its inherently low bioavailability, necessitated extended HRTs, but even these adjustments were insufficient to significantly improve VFA production.

3.2.2.2. Results in CR trials. In contrast to CM, CR demonstrated higher VFA concentrations across all tested HRTs. Yields increased with longer HRTs, reaching 0.28 ± 0.1 $gCOD_{VFA}/gVS$ (HRT 3 d), 0.33 ± 0.1 $gCOD_{VFA}/gVS$ (HRT 5 d), and 0.40 ± 0.1 $gCOD_{VFA}/gVS$ (HRT 7 d). The higher VFA yields can be attributed to the more favorable degradation of CR's lignocellulosic components over time.

The VFA profile for CR was more diverse, including acetic, butyric, propionic, valeric, and caproic acids. Propionic acid, though present in moderate amounts, is generally considered inhibitory to biohydrogen production due to its competition for reducing co-factors such as NADH [40]. This competition may have contributed to the observed metabolic shift from biohydrogen to biomethane production. The study of Sivagurunathan et al. [41] argues that 2.36 g/L of propionic acid is sufficient to lead to a drop of biohydrogen generation, a phenomenon that could have happened also in the tests CR HRT 3 d, 5 d and 7 d (data not shown), given a similar or higher propionic acid content: 2.44 ± 0.9 g/L, 2.66 ± 0.3 g/L and 3.36 ± 0.4 g/L, respectively. Regarding valeric and caproic acids, they are indicative of chain elongation processes, wherein shorter-chain VFAs serve as substrates for specialized microbial communities capable of extending carbon chains under specific conditions [27,42]. These processes often rely on electron donors such as lactic acid, ethanol, or even biohydrogen itself, particularly in environments where pH drops below 5.5 [43]. Additionally, protein-rich substrates can drive chain elongation using amino acids as electron donors, further diversifying the VFA spectrum [27].

The implementation of a recirculation system during CR trials aimed to address nutrient deficiencies and pH instability. The recirculated digestate provided additional nutrients and buffering capacity, resulting in a more sustained VFA production. Under these conditions, the maximum VFA yield was 0.27 ± 0.1 $gCOD_{VFA}/gVS$. The recirculation setup facilitated a temporary extension of biohydrogen production, but the accumulation of VFAs and methanogens led to a decline in biohydrogen yields.

3.2.2.3. Results in SR trials. SR trials at HRT 3 d exhibited the highest VFA concentrations among all substrates tested, reaching an average of 12.4 ± 1.9 $gCOD_{VFA}/L$, with a yield of 0.27 ± 0.1 $gCOD_{VFA}/gVS$. The VFA profile for SR was dominated by acetic, butyric, caproic, and propionic acids, aligning with its higher nitrogen content and enhanced microbial activity. The stable production of biohydrogen in these trials is closely associated with the favorable balance of VFAs and the suppression of inhibitory propionic acid accumulation.

The co-fermentation of SR and CR (50% VS each) further optimized VFA production, achieving concentrations of 15.2 ± 0.8 $gCOD_{VFA}/L$ and a yield of 0.32 ± 0.2 $gCOD_{VFA}/gVS$. This improvement reflects the synergistic effects of combining substrates with complementary properties. The increased C/N ratio provided a more balanced nutrient profile, supporting sustained acidogenic activity and enhancing biohydrogen production.

The variations in VFA production across substrates and conditions highlighted the critical interplay between substrate composition, operational parameters, and microbial community dynamics. High concentrations of acetic and butyric acids are desirable for biohydrogen production, as these are the primary intermediates of acidogenic fermentation. In contrast, the accumulation of propionic acid and the onset of chain elongation pathways can divert metabolic fluxes away from biohydrogen generation.

The findings of this research emphasize the importance of optimizing substrate selection, co-fermentation strategies, and process conditions to balance VFA production and maintain an environment favorable to elevated biohydrogen production.

3.3. Metagenomic analysis

Once the fermentation trials were concluded, a metagenomic analysis was carried out for the reactor effluents at HRT 3 d for trials on CM, CR, CR°, SR*, and SR. The discussion of the metagenomic analysis is available in the Supplementary Materials.

3.4. BMP trials

BMP trials were carried out to evaluate the effect of the effluents from the different fermentation HRTs on the BMP, and compared to the fresh substrate. In particular, fresh CM, HRT 3 d CM, HRT 7 d CM, HRT 10 d CM, and fresh CR, HRT 3 d CR, HRT 5 d CR, HRT 7 d CR underwent the methanogenic trials. Similar to the previous study [18], also for these substrates, some of the fermented output reported a higher biomethane yield compared to their fresh counterpart. In detail, all the methane yields of fermented CM realized an improvement over the fresh (611.0 ± 8.1 $NmLCH_4/gVS$), i.e., HRT 3 d of 4.1% (634.0 ± 1.1 $NmLCH_4/gVS$), HRT 7 d of 11.7% (682.6 ± 93.3 $NmLCH_4/gVS$), and HRT 10 d of 18.7% (725.5 ± 80.3 $NmLCH_4/gVS$). While for the CR fermented effluents, only HRT 5 d and 7 d reported an enhancement, 4.7% (588.32 ± 29.6 $NmLCH_4/gVS$) and 34.1% (753.0 ± 22.0 $NmLCH_4/gVS$) compared to the fresh CR (561.8 ± 17.9 $NmLCH_4/gVS$). The methane yield improvement after fermentation is attributed to the optimization of hydrolysis and acidogenesis phases, making VFAs, biomethane precursors, more readily available for methanogenesis. In fact, in classic AD (mono stage) the DF commonly occurs in contemporary in the same reactor with no optimal operative parameters, and the VFA generation is not maximized. Moreover, the BMP results confirmed that the fermented effluents still contained residual biodegradable organic matter that could be converted into methane in a downstream methanogenic stage. This aspect is particularly relevant for TSAD, where the DF step should not be evaluated only in terms of biohydrogen production, but also according to its impact on the subsequent methanogenic conversion [44].

3.5. Considerations on hydrogen safety

As discussed above, the integration of biohydrogen production into TSAD systems could contribute to the partial decarbonization of gas

Table 4
Explosion properties of flammable gases.

Gas	Flammability limits	Autoignition temperature	vl
	% v/v	(°C)	(m/s)
methane	5.0-15.0	595	0.448
ethane	3.0-15.5	515	0.476
propane	2.1-9.5	470	0.464
hydrogen	4.0-77.0	560	3.25
acetylene	1.5-100.0	305	1.55
ethylene	2.7-34.0	425	0.735

networks. However, the conversion of conventional AD plants into TSAD configurations must also consider the safety aspects related to hydrogen production, handling, and storage. Compared with methane, hydrogen has a wider flammability range, lower ignition energy, and higher laminar burning velocity, as summarized in Table 4. These properties make hydrogen easier to ignite and able to burn more rapidly than most common gaseous fuels.

Therefore, the implementation of hydrogen-producing units in AD plants requires appropriate safety assessment, including the identification of potential emission sources, ventilation requirements, gas detection systems, and explosion-risk areas according to the applicable ATEX regulations.

4. Conclusions

This research emphasizes the opportunity to apply a two-stage anaerobic digestion process to manage agricultural residues, highlighting how the feedstock characteristics define and determine different results and products.

Specifically, it was emphasized that real agricultural residues cannot be considered interchangeable substrates for semi-continuous dark fermentation. Their different composition strongly affected process stability, microbial competition, and biohydrogen production. Cattle manure mainly promoted methanogenic activity, while CR showed only transient biohydrogen production due to pH instability and nutrient limitations. In contrast, soybean/CR co-fermentation improved nutrient balance and enabled stable biohydrogen production. These findings contribute to the current knowledge by showing that the practical implementation of two-stage anaerobic digestion should rely on substrate-specific strategies rather than on a generalized dark fermentation approach. The residual biomethane potential of the fermented effluents further supports the integration of dark fermentation with a downstream methanogenic stage in existing agricultural AD plants. However, some limitations should be considered: the use of real agricultural residues may introduce feedstock variability, and no specific pre-treatment was applied to selectively suppress methanogenic microorganisms. Moreover, the experiments were performed at lab scale and under a limited range of HRTs and co-fermentation/recirculation conditions. Further work should therefore focus on long-term process stability, optimization of substrate mixtures and recirculation ratios, and validation under pilot-scale conditions.

CRediT authorship contribution statement

Davide Bertasini: Data curation, Formal analysis, Investigation, Writing – original draft. **Francesco Valentino:** Funding acquisition, Supervision. **Roberto Lauri:** Supervision, Validation, Writing – original draft. **David Bolzonella:** Conceptualization, Supervision. **Federico Battista:** Conceptualization, Funding acquisition, Supervision, Validation.

Declaration of competing interests

The authors declare that have not conflict of interest including any financial, personal or other relationships with other people or organizations within three years of beginning the submitted work.

Acknowledgements

The authors declare the financial support of the project “Processi innovativi biologici e bio-elettrochimici per la produzione di idrogeno da matrici organiche di scarto” funded in the framework of the call “INAIL BRIC 2022” of the Italian government, and the European PhD scholarships funded with SEF REACT-EU resources on Green and Innovative themes, Programma Operativo Nazionale “Ricerca e Innovazione” 2014-2020 – PON.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2026.126281.

References

[1] I.Stat Istat, Il Tuo Accesso Diretto Alla Statistica Italiana, Coltivazioni, 2024. http://dati.istat.it/Index.aspx?DataSetCode=DCSP_COLTIVAZIONI.
[2] J. Köninger, E. Lugato, P. Panagos, M. Kochupillai, A. Orgiazzi, M.J.I. Briones, Manure management and soil biodiversity: towards more sustainable food systems in the EU, Agric. Syst. 194 (2021), https://doi.org/10.1016/j.agsy.2021.103251.
[3] European Union, Eu Agricultural Outlook 2023-2035, 2023, https://doi.org/10.2762/722428.
[4] Y. Davoodbeygi, A. Sabetghadam-Isfahani, S. Allami, A. Oudi, S. Eghtedari, A review on biohydrogen production technology: production methods, sources, and separation, J. Renew. Sustain. Energy 16 (2024), https://doi.org/10.1063/5.0214791.
[5] X. Zhong, R. Yuan, B. Zhang, B. Wang, Y. Chu, Z. Wang, Full fractionation of cellulose, hemicellulose, and lignin in pith-leaf containing corn stover by one-step treatment using aqueous formic acid, Ind. Crops Prod. 172 (2021) 113962, https://doi.org/10.1016/j.indcrop.2021.113962.
[6] European Biogas Association EBA, Biomethane map 2022-2023, (n.d.). https://www.europeanbiogas.eu/biomethane-map-2022-2023/.
[7] European Biogas Association EBA, Biogases Towards 2040 and Beyond. A Realistic and Resilient Path to Climate Neutrality, 2024.
[8] X. Li, Y.J. Yan, H.M. Wu, S. Ibrahim Gadow, H. Jiang, Z. Kong, Y. Hu, Enhancing mesophilic methanogenesis in oleate-rich environments through optimized micro-aeration pretreatment, Waste Manag. 193 (2025) 171–179, https://doi.org/10.1016/J.WASMAN.2024.12.005.
[9] H. Chang, Q. Yin, K. He, J. De Vrieze, G. Wu, Feeding regime selectively enriching acetoclastic methanogens to enhance energy production in anaerobic digestion systems, Biochem. Eng. J. 220 (2025) 109764, https://doi.org/10.1016/J.BEJ.2025.109764.
[10] IEA-Bioenergy, Renewable gases – hydrogen in the grid. https://www.ieabioenergy.com/blog/publications/renewable-gases-hydrogen-in-the-grid/, 2024.
[11] CPS (Center for Chemical Process Safety) New York, Guidelines for vapor cloud explosion. Pressure Vessel Burst, BLEVE, and Flash Fire Hazards, second ed., 2010.
[12] R. Oberti, A. Tenca, F. Perazzolo, E. Riva, A. Finzi, E. Naldi, G. Provolo, L. Bodria, A farm-scale pilot plant for biohydrogen and biomethane production by two-stage fermentation, J. Agric. Eng. 44 (2013) 10–13, https://doi.org/10.4081/jae.2013.s2.e115.
[13] Q. Zhang, Y. Jiao, C. He, R. Ruan, J. Hu, J. Ren, S. Toniolo, D. Jiang, C. Lu, Y. Li, Y. Man, H. Zhang, Z. Zhang, C. Xia, Y. Wang, Y. Jing, X. Zhang, R. Lin, G. Li, J. Yue, N. Tahir, Biological fermentation pilot-scale systems and evaluation for commercial viability towards sustainable biohydrogen production, Nat. Commun. 15 (2024), https://doi.org/10.1038/s41467-024-48790-4.
[14] C.N.R. Irsa, C.N.R. Irsa, Istituto Di Ricerca Sulle Acque, 2025. https://www.irsa.cnr.it/wp/?page_id=5435.
[15] I. Valdez-Vazquez, E. Ríos-Leal, A. Carmona-Martínez, K.M. Muñoz-Páez, H. M. Poggi-Varaldo, Improvement of biohydrogen production from solid wastes by intermittent venting and gas flushing of batch reactors headspace, Environ. Sci. Technol. 40 (2006) 3409–3415, https://doi.org/10.1021/es052119j.
[16] Y. Chen, Y. Yin, J. Wang, Recent advance in inhibition of dark fermentative hydrogen production, Int. J. Hydrogen Energy 46 (2021) 5053–5073, https://doi.org/10.1016/j.ijhydene.2020.11.096.
[17] C. Holliger, M. Alves, D. Andrade, I. Angelidaki, S. Astals, U. Baier, C. Bougrier, P. Buffière, M. Carballa, V. De Wilde, F. Ebertseder, B. Fernández, E. Ficara, I. Fotidis, J.C. Frigon, H.F. De Laclos, D.S.M. Ghasimi, G. Hack, M. Hartel, J. Heerenklage, I.S. Horvath, P. Jenicek, K. Koch, J. Krautwald, J. Lizasoain, J. Liu, L. Mosberger, M. Nistor, H. Oechsner, J.V. Oliveira, M. Paterson, A. Pauss, S. Pommier, I. Porqueddu, F. Raposo, T. Ribeiro, F.R. Pfund, S. Strömberg, M. Torrijos, M. Van Eekert, J. Van Lier, H. Wedwitschka, I. Wierinck, Towards a standardization of biomethane potential tests, Water Sci. Technol. 74 (2016) 2515–2522, https://doi.org/10.2166/wst.2016.336.
[18] D. Bertasini, F. Battista, R. Mancini, N. Frison, D. Bolzonella, Hydrogen and methane production through two stage anaerobic digestion of straw residues, Environ. Res. 247 (2024) 118101, https://doi.org/10.1016/j.envres.2024.118101.
[19] N. Qi, X. Zhan, J. Milmine, M. Sahar, K.H. Chang, J. Li, Isolation and characterization of a novel hydrolase-producing probiotic Bacillus licheniformis and its application in the fermentation of soybean meal, Front. Nutr. 10 (2023) 1–10, https://doi.org/10.3389/fnut.2023.1123422.
[20] D. Bertasini, F. Battista, F. Rizzioli, N. Frison, D. Bolzonella, Decarbonization of the European natural gas grid using hydrogen and methane biologically produced from organic waste : a critical overview, Renew. Energy 206 (2023) 386–396, https://doi.org/10.1016/j.renene.2023.02.029.
[21] A. Halmemies-Beauchet-Filleau, M. Rinne, M. Lamminen, C. Mapato, T. Ampapon, M. Wanapat, A. Vanhatalo, Review: alternative and novel feeds for ruminants: nutritive value, product quality and environmental aspects, Animal 12 (2018) S295–S309, https://doi.org/10.1017/S1751731118002252.
[22] S. Hangri, K. Derbal, G. Policastro, A. Panico, P. Contestabile, L. Pontoni, M. Race, M. Fabbricino, Combining pretreatments and co-fermentation as successful approach to improve biohydrogen production from dairy cow manure, Environ. Res. 246 (2024) 118118, https://doi.org/10.1016/j.envres.2024.118118.

[23] O. Yenigün, B. Demirel, Ammonia inhibition in anaerobic digestion: a review, Process Biochem. 48 (2013) 901–911, https://doi.org/10.1016/j.procbio.2013.04.012.
[24] J. Kazimierowicz, M. Dębowski, M. Zieliński, Effectiveness of hydrogen production by Bacteroides vulgatus in psychrophilic fermentation of cattle slurry, Clean Technol. 4 (2022) 806–814, https://doi.org/10.3390/cleantechnol4030049.
[25] Y.D. Yilmazel, M. Duran, Biohydrogen production from cattle manure and its mixtures with renewable feedstock by hyperthermophilic Caldicellulosiruptor bescii, J. Clean. Prod. 292 (2021) 125969, https://doi.org/10.1016/j.jclepro.2021.125969.
[26] S.F. Fu, R. Liu, W.X. Sun, R. Zhu, H. Zou, Y. Zheng, Z.Y. Wang, Enhancing energy recovery from corn straw via two-stage anaerobic digestion with stepwise microaerobic hydrogen fermentation and methanogenesis, J. Clean. Prod. 247 (2020) 119651, https://doi.org/10.1016/j.jclepro.2019.119651.
[27] O. Sarkar, U. Rova, P. Christakopoulos, L. Matsakas, Effect of metals on the regulation of acidogenic metabolism enhancing biohydrogen and carboxylic acids production from brewery spent grains: microbial dynamics and biochemical analysis, Eng. Life Sci. 22 (2022) 650–661, https://doi.org/10.1002/elsc.202200030.
[28] T. Sreethawong, T. Niyamapa, H. Neramitsuk, P. Rangsunvigit, M. Leethochawalit, S. Chavadej, Hydrogen production from glucose-containing wastewater using an anaerobic sequencing batch reactor: effects of COD loading rate, nitrogen content, and organic acid composition, Chem. Eng. J. 160 (2010) 322–332, https://doi.org/10.1016/j.cej.2010.03.037.
[29] E. Elbeshbishy, B.R. Dhar, G. Nakhla, H.S. Lee, A critical review on inhibition of dark biohydrogen fermentation, Renew. Sustain. Energy Rev. 79 (2017) 656–668, https://doi.org/10.1016/j.rser.2017.05.075.
[30] Z. Dong, S. Cao, B. Zhao, G. Wang, J. duan, A. Yuan, Enhancement of hydrogen production in dark fermentation of corn stover hydrolysates through composite electric field pretreatment: improving enzymatic efficiency and regulating microbial metabolic balance, Bioresour. Technol. 415 (2025) 131721, https://doi.org/10.1016/j.biortech.2024.131721.
[31] W. Liu, J. Pang, D. Wu, L. Zhang, D. Xing, J. Hu, Y. Li, Z. Liu, Hydrogen production by a novel Klebsiella pneumoniae strain from sheep rumen uses corn straw as substrate, Energy 282 (2023), https://doi.org/10.1016/j.energy.2023.128210.
[32] Y. Zhang, Q. Li, X. Wang, H. Yang, L. Guo, Enhanced biohydrogen production from cornstalk through a two-step fermentation: dark fermentation and photofermentation, Int. J. Energy Res. 41 (2017) 2491–2501, https://doi.org/10.1002/er.3810.
[33] D.Y. Lee, Y. Ebie, K.Q. Xu, Y.Y. Li, Y. Inamori, Continuous H2 and CH4 production from high-solid food waste in the two-stage thermophilic fermentation process with the recirculation of digester sludge, Bioresour. Technol. 101 (2010) S42–S47, https://doi.org/10.1016/j.biortech.2009.03.037.
[34] Eddy MetCalf, Wastewater engineering. Treatment and resource recovery, 5th Editio, https://doi.org/10.1002/9780470168219.ch8, 2007.
[35] I. Mehi Gaspari Augusto, C. Zampol Lazaro, R. Albanez, S. Maria Ratusznei, G. Lovato, J.A. Domingues Rodrigues, H2 production via dark fermentation of soybean molasses: elucidating the role of homoacetogenesis and endogenous substrate microorganisms by kinetic and microbial analysis, Energy 298 (2024), https://doi.org/10.1016/j.energy.2024.131301.
[36] I. Mehi, G. Augusto, C. Zampol, R. Albanez, S. Maria, G. Lovato, D. Rodrigues, Optimizing thermophilic dark fermentation of soybean molasses: the critical role of temperature in maximizing biohydrogen production, J. Environ. Chem. Eng. 13 (2025) 117804. https://www.sciencedirect.com/science/article/pii/S221334372502500X.
[37] A. Detman, D. Laubitz, A. Chojnacka, E. Wiktorowska-Sowa, J. Piotrowski, A. Salamon, W. Kaźmierczak, M.K. Błaszczyk, A. Barberan, Y. Chen, E. Łupikasza, F. Yang, A. Sikora, Dynamics and complexity of dark fermentation microbial communities producing hydrogen from sugar beet molasses in continuously operating packed bed reactors, Front. Microbiol. 11 (2021) 1–19, https://doi.org/10.3389/fmicb.2020.612344.
[38] X. Qu, H. Zeng, Y. Gao, T. Mo, Y. Li, Bio-hydrogen production by dark anaerobic fermentation of organic wastewater, Front. Chem. 10 (2022) 1–16, https://doi.org/10.3389/fchem.2022.978907.
[39] D.M. Yin, C. Uwineza, T. Sapmaz, A. Mahboubi, H. De Wever, W. Qiao, M. J. Taherzadeh, Volatile Fatty Acids (VFA) production and recovery from chicken manure using a high-solid anaerobic membrane bioreactor (AnMBR), Membranes 12 (2022), https://doi.org/10.3390/membranes12111133.
[40] O. Sarkar, U. Rova, P. Christakopoulos, L. Matsakas, Continuous biohydrogen and volatile fatty acids production from cheese whey in a tubular biofilm reactor: substrate flow rate variations and microbial dynamics, Int. J. Hydrogen Energy 59 (2024) 1305–1316, https://doi.org/10.1016/j.ijhydene.2024.02.041.
[41] P. Sivagurunathan, B. Sen, C.Y. Lin, Overcoming propionic acid inhibition of hydrogen fermentation by temperature shift strategy, Int. J. Hydrogen Energy 39 (2014) 19232–19241, https://doi.org/10.1016/j.ijhydene.2014.03.260.
[42] F. Battista, A. Zeni, M. Andreolli, E. Salvetti, F. Rizzioli, S. Lampis, D. Bolzonella, Treatment of food processing wastes for the production of medium chain fatty acids via chain elongation, Environ. Technol. Innov. 33 (2024) 103453, https://doi.org/10.1016/j.eti.2023.103453.
[43] B. Chatterjee, D. Mazumder, Role of stage-separation in the ubiquitous development of anaerobic digestion of organic fraction of municipal solid waste: a critical review, Renew. Sustain. Energy Rev. 104 (2019) 439–469, https://doi.org/10.1016/j.rser.2019.01.026.
[44] J.V. Tongco, S.H. Park, S.I. Kim, S. Hwang, Influence of fluctuating food waste concentrations on horizontal anaerobic reactor performance and biogas output, Energies 18 (2025) 5064, https://doi.org/10.3390/EN18195064, 2025, Vol. 18, Page 5064.