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Fed-batch anaerobic digestion of raw and pretreated hazelnut skin over long-term operation

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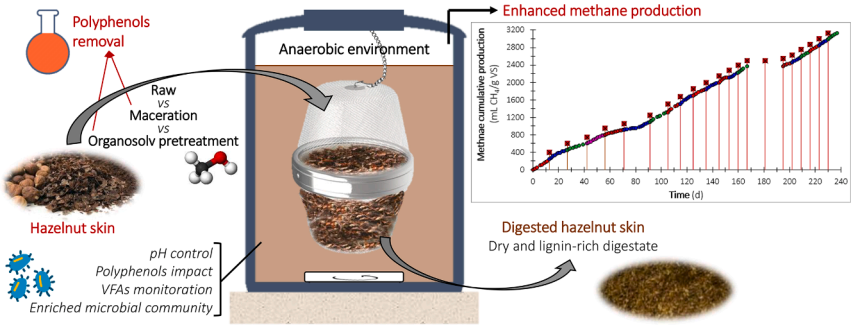
HIGHLIGHTS

- Anaerobic digestion (AD) of hazelnut skin (HS) was investigated in fed-batch mode.
- Higher CH_4 production was obtained at lower solid retention time and higher HS load.
- Maceration and organosolv pretreatment removed 82 and 97% of the HS polyphenols.
- Methanol-organosolv pretreatment increased the CH_4 potential of HS by 21%.
- Reactor configuration enabled enriching microorganisms capable of HS degradation.

GRAPHICAL ABSTRACT



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ABSTRACT

This study provided important insights on the anaerobic digestion (AD) of hazelnut skin (HS) by operating a fed-batch AD reactor over 240 days and focusing on several factors impacting the process in the long term. An efficient reactor configuration was proposed to increase the substrate load while reducing the solid retention time during the fed-batch AD of HS. Raw HS produced maximally 19.29 mL CH_4/g VS_{add}/d. Polyphenols accumulated in the reactor and the use of NaOH to adjust the pH likely inhibited AD. Maceration and methanol-organosolv pretreatments were, thus, used to remove polyphenols from HS (i.e. 82 and 97%, respectively) and improve HS biodegradation. Additionally, organosolv pretreatment removed 9% of the lignin. The organosolv-pretreated HS showed an increment in methane potential of 21%, while macerated HS produced less methane than the raw substrate, probably due to the loss of non-structural sugars during maceration.

Abbreviations: AD, anaerobic digestion; LMs, lignocellulosic materials; HS, hazelnut skin; SRT, solid retention time; VFAs, volatile fatty acids; TS, total solid; VS, volatile solid; DBM, digestate from buffalo manure; S/L, substrate to liquid; SSMC, stainless steel mesh container; R_m, methane production rate; NaOH, sodium hydroxide; Na_2CO_3, sodium carbonate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; WRC, water retention capacity; TA, total alkalinity; PA, partial alkalinity; IA, intermediate alkalinity; F-C, Folin-Ciocalteu; GAE, equivalent gallic acid; SuRT, sludge retention time.

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1. Introduction

The impact of fossil fuels on the environment and climate has led to the development of alternative strategies for energy production (Martins et al., 2019). The methane produced from renewable materials emerges as a great alternative to fossil fuels due to its high calorific value, low pollutant emission, and flexible use at different purity for various applications (Oliva et al., 2022). Methane can be produced via anaerobic digestion (AD) through different consortia of microorganisms, classified into hydrolytic, acetogenic, acidogenic, and methanogenic. Each group of microorganisms is responsible for a different phase of the AD process, i.e. hydrolysis, acetogenesis, acidogenesis, and methanogenesis. The balance between the various stages is the key for an efficient AD process (Pasalari et al., 2021).

Recently, the concept of AD evolved from a mere treatment to purify urban and industrial wastewater or sludge to an ad-hoc strategy to produce energy from undervalued waste materials (Silvestre et al., 2015). In this perspective, lignocellulosic materials (LMs) are an extraordinary opportunity for AD due to their abundance, low cost, and high organic content. Several researchers focused on the most produced LMs, such as straws (Dai et al., 2020) and grass residues (Wen et al., 2015). Nevertheless, the global interest is recently expanding to LMs coming from seasonal harvesting or specific regional production. For instance, the market of nuts is expanding year by year (International Nut and Dried Fruit Council Foundation, 2021), resulting in a huge amount of LMs that can be used for energy production without competing with the food industry. In particular, the global hazelnut production achieved 512,000 metric tons in the 2020/2021 harvesting season. Turkey (62%) and Italy (15%) were the leading producers, whereas hazelnuts are mainly exported to EU countries, China, and Canada (International Nut and Dried Fruit Council Foundation, 2021).

Most recent studies focused on optimising the AD of such residues to enhance their methane potential (Bianco et al., 2021; Şenol, 2019). The optimisation of AD for LMs often involves pretreatments to overcome the recalcitrant structure of these substrates. The main components of LMs are cellulose, hemicellulose and lignin. The presence of lignin, together with physical characteristics, such as porosity and crystallinity degree of the cellulose, hinders the hydrolytic bacteria attacking the LMs, resulting in a low hydrolysis rate and inefficient AD in terms of methane production (Xu et al., 2019).

Great progress has been made in the valorisation of LMs. However, most studies focused on small-batch applications, which require further investigation before implementation on a pilot or industrial scale. In this perspective, this article aims to close the gap between batch and fed-batch applications, providing attractive insights for the AD of hazelnut residues, i.e. hazelnut skin (HS), investigated at lab-scale (i.e. 2 L working volume) in fed-batch operation. In particular, this study proposed an alternative reactor configuration that may allow more efficient feeding and process management for LMs under fed-batch AD operation. Different operating parameters were monitored and improved to enhance the methane production from HS, such as organic load, solid retention time (SRT), and pH. Methane production, volatile fatty acids (VFAs) evolution, polyphenolic compounds accumulation, and alkalinity were monitored along with the reactor operation. The influence of pretreatments, i.e. maceration and organosolv, and the changes in the chemical composition of HS were investigated. In addition, the change in the microbial community along the variation of the operating conditions was elucidated.

2. Materials and methods

2.1. Raw substrate and inoculum

The raw HS used comes from Turkish imported hazelnuts (*Corylus avellana*). The hazelnuts were roasted, and the skin was separated from the kernel by a farming company located in the Campania region (Italy). The HS was sieved to select a range of particle sizes between 1.0 and 2.5 mm. The selected HS had a total (TS) and volatile solid (VS) content of 88.8 (±0.2) and 86.1 (±0.2)% (based on wet matter), respectively. A digestate from buffalo manure (DBM) coming from a full-scale anaerobic digester located in Eboli (Italy) was degassed to eliminate the endogenous biogas production before being used as the inoculum. The DBM showed a TS and VS content of 5.0 (±0.0) and 3.4 (±0.0)% (based on wet matter), respectively. The initial pH of the inoculum was 7.7 (±0.1), and the total alkalinity accounted for 10.7 (±0.3) g $CaCO_3$/L.

2.2. Maceration and organosolv pretreatment

The HS maceration was preliminarily tested in 50 mL falcon tubes for 1, 3, 6, 24, 48 h, 1 and 2 weeks. The falcon tubes, filled with 1 g HS and 50 mL demineralised H_2O, were kept under agitation (i.e. 130 rpm) for the desired exposure time. The maceration time allowing the higher polyphenolic compound removal was performed on a larger scale, i.e. 2 L glass bottle, keeping a substrate to liquid (S/L) ratio of 1:50.

The organosolv pretreatment was carried out following the finest condition, i.e. pretreatment for 1 h at 130 °C using a 50% (*v/v*) water-methanol solution with 0.1% (*w/v*) H_2SO_4 as a catalyst, previously proposed by the authors (Oliva et al., 2021). In the present study, the selected S/L ratio was 1:15. Maceration and organosolv pretreatment were performed individually on raw HS. Macerated and organosolv-pretreated HS was dried at 45 °C before being used as the substrate for AD.

2.3. Anaerobic digester design and methane production measurement

The AD process was performed in a 2 L (working volume) borosilicate glass reactor (Fig. 1A) designed by the authors and made by Glass Studio (Naples, Italy). The desired temperature (i.e. ∼ 37 °C) was maintained using an ED (v.2) heating bath (Julabo, Seelbach, Germany) in the service of the reactor water jacket. The pH evolution was monitored with a pH electrode (VWR, Radnor, USA). pH correction was performed using a pH/ORP 300 controller (Cole-Parmer, Vernon Hills, USA) and an EVO45 pump (Verderflex, Castleford, UK) (Fig. 1D) dosing an alkaline solution (Fig. 1C) when the pH in the reactor went below the threshold value, which was chosen depending on the desired operating condition. Reactor agitation was guaranteed by magnetic stirring. The HS was kept inside a stainless steel mesh container (SSMC) (15 × 10 × 10 cm) (Fig. 1B). The substrate container had an external grid with a mesh capable of retaining the HS, but allowing the digestate to penetrate and soak the HS. After each refeeding, the headspace of the reactor was flushed with Argon gas to ensure anaerobic conditions.

The methane production was monitored using a water displacement apparatus. The biogas produced flowed continuously through a CO_2 trap (Fig. 1E) made by 12% NaOH solution using thymolphthalein as pH indicator (Sigma-Aldrich, St. Louis, USA). The CO_2 trap allowed the methane only to displace the water in a 1.5 L glass cylinder (Fig. 1F) (Glass Studio, Naples, Italy) for a volume equal to the amount of methane produced.

The specific methane production was calculated by dividing the daily methane production by the grams of VS from HS added (VS_{add}) in the reactor. The slope of the regression line fitting the experimental data of a single feeding cycle represents the methane production rate (R_m) for that cycle. The average rate ($R_{m,av}$) was obtained by averaging the rates obtained for each cycle of the same experimental phase (Table 1).

2.4. Reactor feeding and experimental phases

The anaerobic reactor was initially filled with 697 g DBM and 1303 g H_2O, and 18.6 g HS were fed to reach an overall VS content of 20 g/L. The starting inoculum/substrate ratio was 1.5 g VS. The initial TS content was 2.6%. The operation of the reactor was divided into seven experimental phases (Table 1).

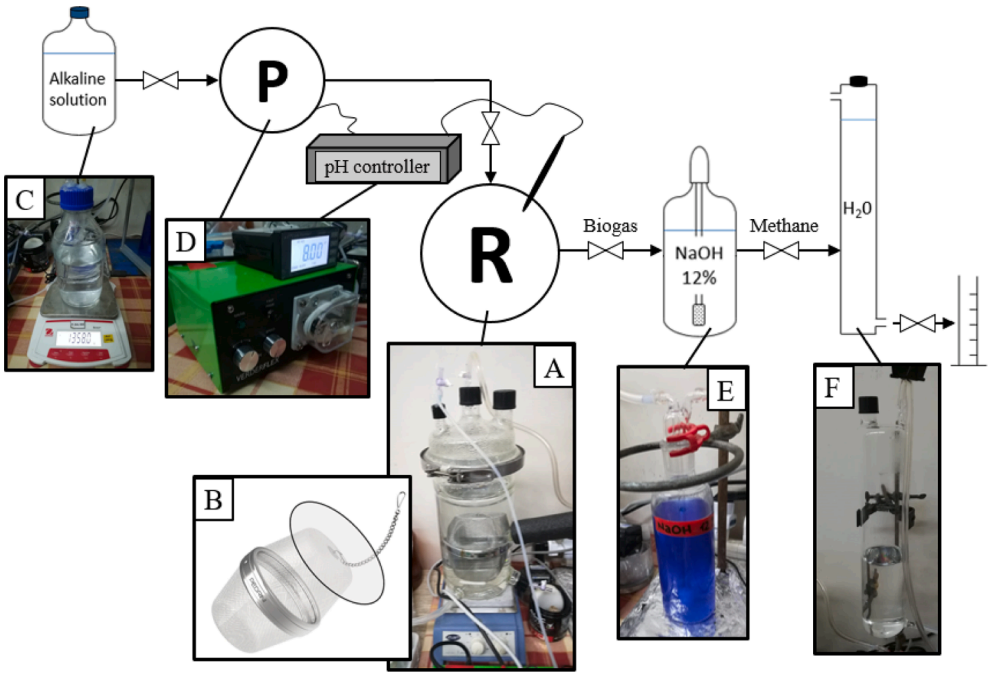

Fig. 1. Schematic representation of the experimental design including anaerobic digester (A), stainless steel mesh container for lignocellulosic refeeding (B), alkaline solution for pH control (C), pump and pH controller for alkaline solution dosing (D), carbon dioxide trap (E), and water cylinder for methane production measurement (F).

Table 1
Experimental design.

Operating parameters	Phase I	Phase II	Phase III	Phase IV	Phase V	Standby	Phase VI	Phase VII
Timeline (day)	0 – 56	56 – 105	105 – 125	125 – 145	145 – 167	167 – 195	195 – 216	216 – 237
Substrate	Raw HS	Raw HS	Raw HS	Macerated HS	Pretreated HS	Raw HS	Raw HS	Pretreated HS
HS load (g VS)	16.7 ± 0.7	26.0 ± 1.1	25.9 ± 0.7	25.2 ± 0.3	25.1 ± 1.0	24.4 ± 1.0	25.3 ± 0.8	25.0 ± 0.6
Feeding percentage (% VS)	50	50	50	50	50	50	50	50
Solid retention time (days)	28	28	20	20	14	28	14	14
No. of cycles	4	3	2	2	3	2	3	3
Length of each cycle (days)	14	14	10	10	7	14	7	7

During Phase I, HS was fed every 14 days, replacing 50% of the overall VS from HS at a time, i.e. with a SRT of 28 days, starting from HS load of 16.7 (±0.7) g VS. In Phase II, the HS load increased by roughly 55%, reaching 26.0 (±1.1) g VS, and the SRT was kept at 28 days. During Phase III, the SRT was shortened to 20 days, using the same HS load as Phase II. Phases IV and V investigated the effectiveness of pretreatments on AD of HS, keeping the same HS load and SRT selected in Phase III. In Phase IV, macerated HS was digested, while during Phase V an organosolv-pretreated HS was fed to the reactor.

During phases I to V of this study, the pH in the reactor was monitored and corrected online, as described in Section 2.2. Sodium hydroxide (NaOH) was used for pH correction until day 68. Afterwards, NaOH was replaced by sodium carbonate (Na_2CO_3). Nevertheless, due to a failure of the pumping system dosing the alkaline solution, the entire liquid phase of the reactor was contaminated on day 150, requiring the inoculation of fresh DBM and a standby phase of 28 days. During the standby phase, the reactor was fed regularly with raw HS, but methane production was not recorded.

The regular operation of the fed-batch reactor restarted with Phase VI, i.e. on day 195, by feeding raw HS with a shorter SRT of 14 days and an organic HS load of 25.3 (±0.8) g VS. Finally, in Phase VII (i.e. from day 216 to 237), organosolv-pretreated HS was used for AD, keeping the SRT at 14 days.

2.5. Microbial community analysis

Samples for DNA analysis were taken on day 0 and at the end of each experimental phase (Table 1). Additionally, the reactor was sampled before and after the extra inoculations, i.e. on days 80 and 150. DNA extraction, sequencing and bioinformatics analysis were performed by FisaBio (Valencia, Spain). Before DNA extraction, samples were homogenised by adding 1 mL of phosphate-buffered saline (PBS) to 2 mL of sample and vortexed. After two centrifugation steps at 4 °C (i.e. 2 min at 2000 rpm and 30 min at 13200 rpm), the pellet was recovered for DNA extraction.

DNA was extracted using a MAgNa Pure LC robot and a III 3,264,785,001 isolation kit (Roche, Basel, Switzerland) according to the manufacturer's protocol. External lysis was performed before DNA extraction by adding 0.23 g of lysozyme. The DNA was quantified using a Qubit dsDNA High Sensitivity kit (Qiagen, Hilden, Germany) and normalised at 5 ng/ul to start the library preparation protocol. Polymerase chain reaction (PCR) amplification, sequencing, and PCR cleanup were performed according to the Illumina protocol (Illumina Inc., 2013) that targets the V3 and V4 regions of the 16S genes with the

primers selected by Klindworth et al. (2013). The database used for the taxonomic assignation was Silva138.

2.6. Analytical methods

TS and VS of the inoculum, as well as raw, macerated and pretreated HS, were determined according to the standard methods (APHA AWWA, 2005) using a TCN115 convection oven (Argo Lab, Carpi, Italy) and a BWF 11/13 muffle furnace (Carbolite, Sheffield, UK), respectively. The HS degradation during the AD process was monitored by measuring the VS content of the digested HS before each refeeding cycle, using the same methods and equipment reported above. The water retention capacity (WRC) of raw, macerated and pretreated HS was measured as described by Sanchez et al. (2019). The chemical composition, i.e. extractives, polyphenols in the extractives, structural sugars, lignin, and ashes, of the LMs was determined by Celignis Limited (Limerick, Ireland) following the NREL procedures (Sluiter et al., 2008b, 2008a). The analysis of the structural sugars was performed using a ICS-3000 Ion Chromatography System (Dionex, Sunnyvale, USA) according to the company's protocol.

The liquid phase of the anaerobic digester was sampled regularly for alkalinity, VFAs, and soluble polyphenols analysis. Total (TA), partial (PA) and intermediate (IA) alkalinity were determined as described by Pontoni et al. (2015). PA is related to carbonate alkalinity, whereas IA refers to VFAs alkalinity. TA was obtained as the sum of PA and IA (Martín-González et al., 2013). The samples for VFAs analysis were stored and analysed according to Papirio (2020). Soluble polyphenols concentration was measured using a V-530 UV/VIS spectrophotometer (Jasco, Tokyo, Japan) following the Folin-Ciocalteu (F-C) procedure, as reported by Cubero-Cardoso et al. (2020).

3. Results and discussion

3.1. Anaerobic digestion of untreated hazelnut skin

The AD process operated under fed-batch mode showed the potential of HS for methane production. Untreated HS was fed in Phase I, II, III, and VI, showing an $R_{m,av}$ of 12.9, 10.7, 19.3, and 16.0 mL CH_4/g VS_{add}/d, respectively. The fed-batch operation here investigated allowed for fitting the experimental data with linear regression, whereas the cumulative methane production from LMs in batch experiments generally fits the first-order kinetic or a modified-Gompertz model (Mancini et al., 2018). These two models fit experimental data tending to a stationary phase after exponential growth. On the other hand, refeeding a reactor with fresh substrate more continuously than a batch system results in a more stable daily methane production, without experiencing methane production approaching zero after the biodegradation of the most available LM components (Shen et al., 2018).

During Phase I, four refeeding cycles were performed. The first cycle corresponded to the reactor start-up, with the entire amount of VS deriving from fresh HS. In the following cycles, 50% of digested HS was replaced with fresh HS up to the desired VS content of approximately 16.7 g VS. From the second cycle, a decreasing trend in methane production was observed (Fig. 2). The methane production showed a peak after refeeding the reactor, likely due to the biodegradation of easily hydrolysable unbound compounds, i.e. free carbohydrates (Tao et al., 2019). The impact of extractives on AD largely depends on their composition, which varies with the LM (Tajmirriahi et al., 2021). After the first peak, the daily methane production decreased. Another peak was observed 5 – 7 days after refeeding the reactor (Fig. 2). The second peak is likely attributed to the slower hydrolysis and subsequent

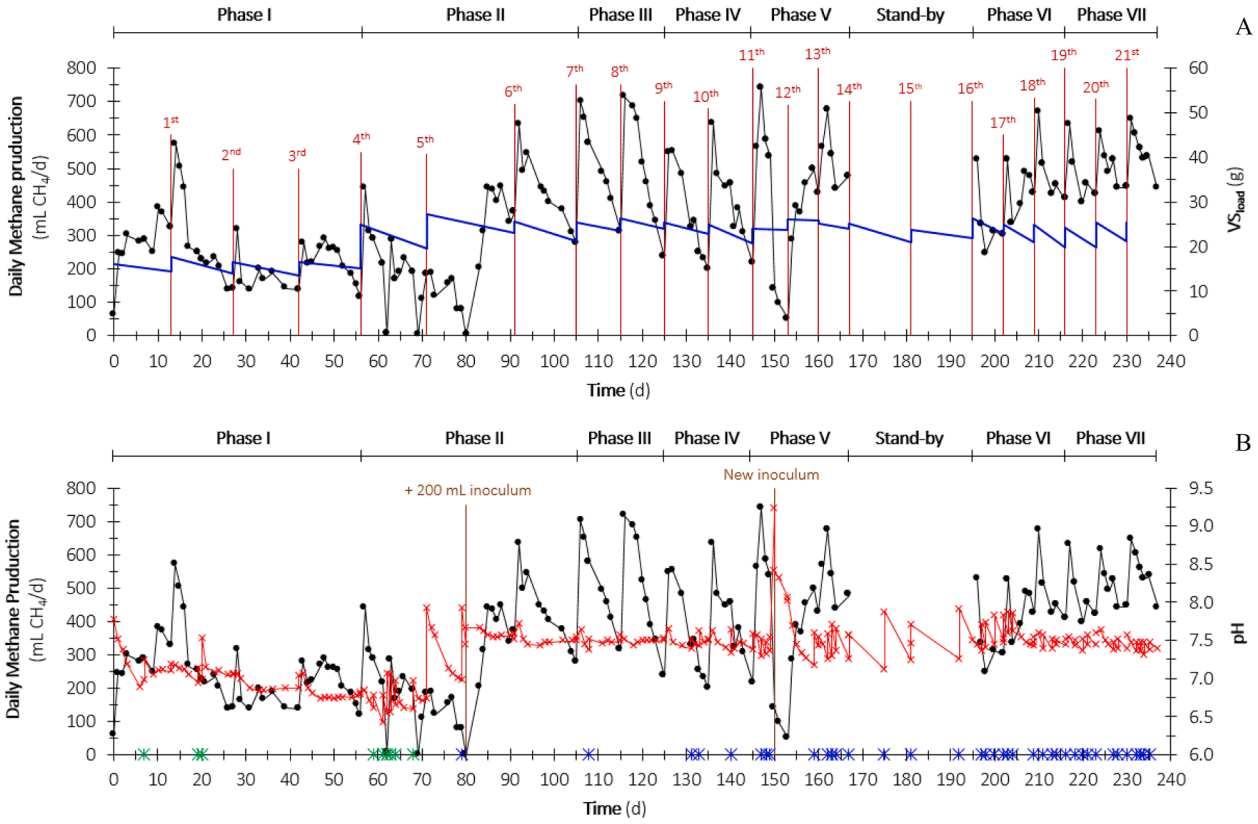

Fig. 2. Daily methane production (●) as a function of hazelnut skin refeeding (▮) (A), volatile solid content (▬) (A), pH (✖) (B), pH adjustment with NaOH (✱) or Na_2CO_3 (✱) (B), and extra inoculation (▮) (B) during the different cycles and experimental phases.

degradation of the cellulose and hemicellulose sugars (Xu et al., 2019). In the first phase, no VFAs accumulation was observed (Fig. 3A), and the total alkalinity was stable, ranging between 3.7 and 4.0 g $CaCO_3$/L (Fig. 3B), with the ratio IA/PA being below the suggested threshold value of 0.3 to digest organic wastes preserving stable reactor performance in terms of VFAs accumulation (Martín-González et al., 2013).

In Phase II, the feeding was increased to obtain an HS load of 26.0 g VS. Consequently, VFAs accumulation was observed in the reactor (Fig. 3A), and the IA/PA ratio exceeded the threshold of 0.3, reaching the maximum value of 0.8 on day 79 (Fig. 3B). Acetic acid was the only VFA detected until day 72 of operation (Fig. 3A), resulting in pH drops and a frequent pH correction with NaOH (Fig. 2B). The VFAs speciation partially switched to propionic acid in the second and third cycle of Phase II, reaching a concentration of 459 mg HAc_{eq}/L (Fig. 3A). In previous works, propionic acid exceeding 410 mg HAc_{eq} /L showed inhibitory effects on AD, which can explain the lower methane production measured in this study in the first two cycles of Phase II despite the higher HS load (Han et al., 2020).

Despite NaOH is one of the most employed pH adjusters, Na^+ ions have been reported to negatively affect the microbial activity during AD of LMs (Bianco et al., 2021; Feng et al., 2018). From day 56 to 80, the pH kept decreasing (Fig. 2B), and VFAs accumulated (Fig. 3A), suggesting that hydrolysis, acetogenesis, and acidogenesis proceeded regularly. On the other hand, methanogenic activity was inhibited albeit the increase of the HS load (Fig. 2). The inhibition of methanogens can be attributed to the VFAs buildup above 1800 mg HAc_{eq}/L and Na^+ accumulation (Rocamora et al., 2020). Hence, Na_2CO_3 was used to control the pH from day 80, and 200 mL of fresh DBM were added to restart the reactor operation. Na_2CO_3 has also the advantage of increasing the carbonate alkalinity (Jos et al., 2020). Fig. 3B shows that total alkalinity increased from day 80, and the optimal IA/PA ratio, i.e. 0.3, was established again. After day 80, the methane production restarted, allowing the reactor to work steadily with a higher organic load than that used in Phase I (Fig. 2).

The retention time of HS was reduced from 28 to 20 days in Phase III. Consequently, the reactor was fed every 10 days. During the first two phases, the daily methane production decreased over time in each feeding cycle. This trend suggests that the easily biodegradable matter was hydrolysed in the first days of the AD process (Xu et al., 2019). Therefore, shortening the SRT aimed to enhance the AD process by increasing the $R_{m,av}$ and preventing methane production from tending to zero. The peak in methane production further increased in Phase III, reaching 27.7 mL CH_4/g VS_{add} (Fig. 2). Nevertheless, a decrease in the daily methane production was observed in the subsequent days. During Phase III, the VFAs concentration was lower than in Phase II, and no significant propionic acid accumulation was observed, i.e. 46 mg HAc_{eq}/L at most (Fig. 3A).

The AD performance in Phase III can also be compared with that obtained in Phase VI, where raw HS was used as the substrate and the SRT was further reduced to 14 days. Nevertheless, this comparison is influenced by the inoculation of fresh DBM on day 150. The methane production observed in Phase VI was lower than in Phase III, suggesting that the microorganisms' adaptation during the first 150 days played a relevant role in enhancing the AD process (Pasalari et al., 2021). On the contrary, the VFAs accumulation in Phase VI was slightly higher than that observed in Phase III (Fig. 3A), and the pH correction (Fig. 2B) was more frequent than in the previous phases. Consequently, the carbonate alkalinity in the reactor increased because of the buffer capacity of Na_2CO_3 (Fig. 3A).

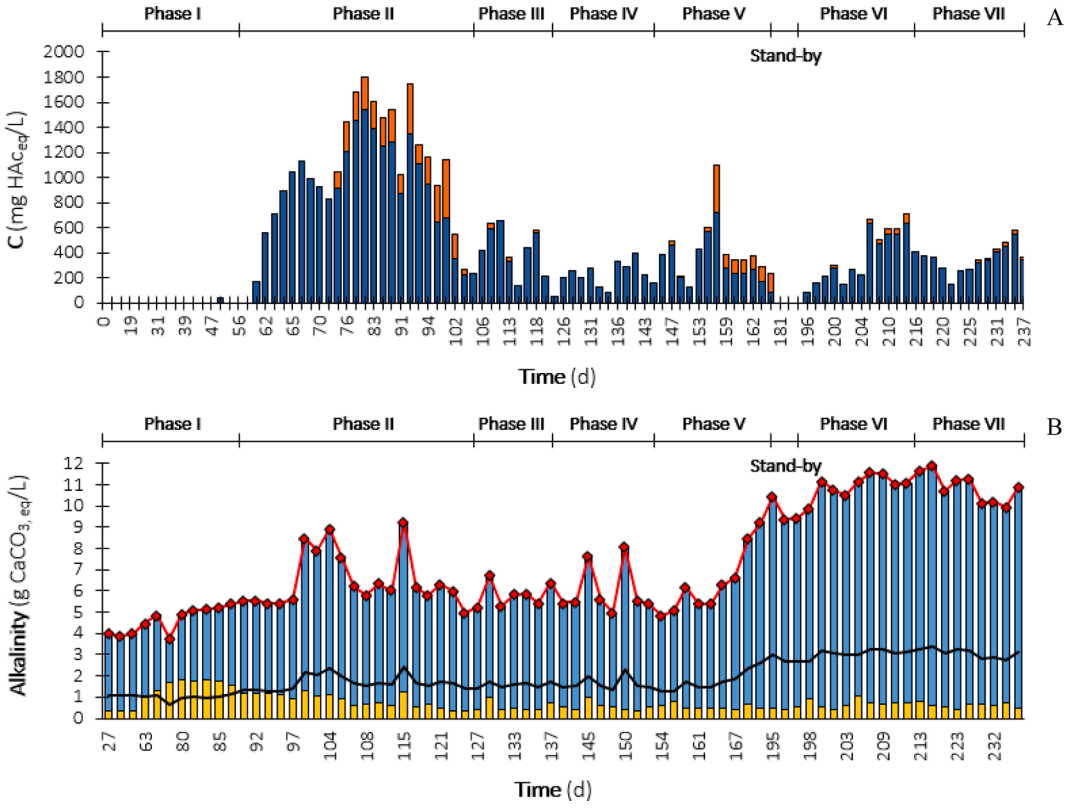

Fig. 3. Volatile fatty acids evolution (A) expressed as equivalent acetic acid: acetic acid (■) and propionic acid (■). Alkalinity evolution (B) expressed as total (◆), partial (■), and intermediate (■) alkalinity in comparison with the threshold value (▬), i.e. partial/intermediate alkalinity = 0.3, suggested in the literature (Martín-González et al., 2013).

3.2. Impact of polyphenols and pH adjustment on anaerobic digestion of hazelnut skin

The polyphenols concentration in the reactor was constantly monitored along the different operating conditions. Fig. 4A shows that a background polyphenols concentration of approximately 329.6 mg/L was observed in the reactor on day 0, i.e. prior to feeding HS. Buffalo manure is rich in acid-soluble lignin (Zeb et al., 2022). This type of lignin can be broken down into polyphenolic compounds during the AD process. Nevertheless, the complete degradation of these compounds is unlikely to occur without specific strategies, such as microaeration (Zeb et al., 2022). Therefore, the initial polyphenols concentration observed in the reactor is likely associated with the DBM used. Regarding LMs, polyphenolic compounds are generated from lignin degradation during pretreatments (Ferreira and Taherzadeh, 2020). HS is rich in Klason lignin, with the acid-soluble fraction being a minor component of the total lignin (Oliva et al., 2021). Only fungi and selected strains of bacteria can degrade lignin from LMs. In contrast, the microorganisms involved in the AD process are unlikely to degrade lignin from LMs (Oliva et al., 2022). In addition, polyphenols are also present in the extractives of HS (Ivanović et al., 2020).

Section 3.1 explained the role of Na^+ in the AD process. In addition to that, Na^+ can bind with phenols and form sodium phenolate (Li et al., 2014). Sodium phenolate inhibits microbial growth, explaining the drop in methane production observed until day 80 when NaOH was dosed in the reactor (Gellert and Stommel, 1999). The formation of sodium phenolate can also be responsible for the fluctuation of the polyphenols concentration until day 80 (Li et al., 2014). Moreover, the refeeding before day 80 corresponded to the highest polyphenols concentration observed in this study, i.e. 479.7 mg phenol/L, suggesting that the reactor acidification also inhibited the polyphenols degradation. Fig. 4A shows that polyphenols did not accumulate above the background concentration in Phase I when the organic load was 16 g VS from HS. In Phase II, the polyphenols content increased after each feeding, whereas it was lower at the end of a cycle. This trend suggests that the microbial community present in the reactor was probably capable of degrading part of the polyphenolic compounds (Zeb et al., 2022). On day 80, after inoculating 200 mL of fresh DBM, the methanogenic activity restarted along with the polyphenols degradation.

Apart from replacing NaOH with Na_2CO_3 for pH adjustment, maceration and organosolv pretreatments were tested to reduce the presence of polyphenols in the AD process. HS is indeed a polyphenol-rich substrate, the solubilisation of which can hinder the AD process by inhibiting the hydrolytic bacteria (Ivanović et al., 2020; Milledge et al., 2019). The polyphenol measurement in the liquor showed that 3 h of maceration led to the highest polyphenolic compounds extraction from HS. The 3 h maceration was repeated on a larger scale, resulting in 47.3 mg of phenols removed per gram of HS (Fig. 4B). Fig. 4A shows that the polyphenols concentration spiked up after each refeeding with macerated HS in Phase IV, despite the significant removal via maceration (Fig. 4B). The polyphenolic compounds hydrolysed from macerated HS were partially degraded during AD until reaching the background concentration mentioned previously. On the contrary, the feeding of organosolv-pretreated HS significantly reduced the polyphenols present in the reactor in Phase V (Fig. 4B). During Phase VI, the polyphenols content increased again when feeding raw HS. Finally, in Phase VII, the

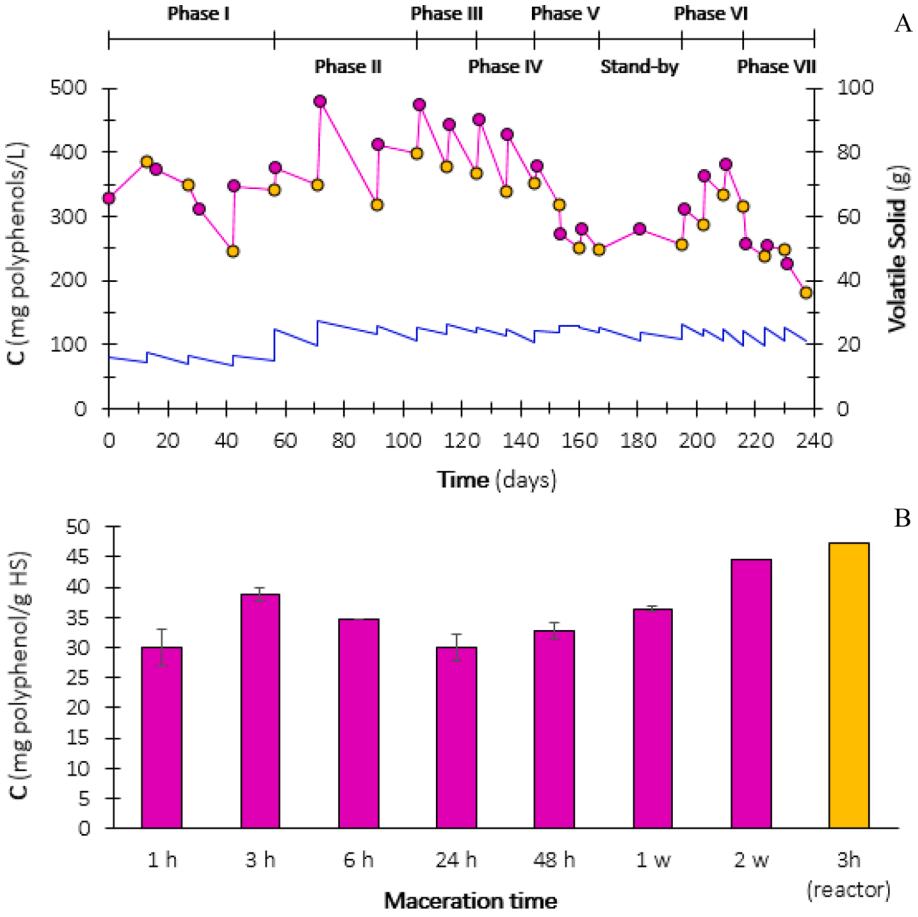

Fig. 4. Soluble polyphenols evolution during anaerobic digestion (A): polyphenols concentration at the beginning (●) and at the end (●) of each feeding cycle and evolution of the volatile solid content from hazelnut skin (▬). Polyphenols removed by maceration (B) at different exposure times on a small scale (■) and on a larger scale for the optimal condition (■).

lowest polyphenols concentration of the entire AD operation was observed, i.e. 180.1 mg phenol/L, confirming that methanol is an excellent solvent for polyphenols extraction (Vijayalaxmi et al., 2015).

3.3. Effect of maceration and organosolv pretreatment on chemical composition, porosity, and methane potential of hazelnut skin

The compositional analysis (see supplementary material) revealed that raw HS is particularly rich in lignin, i.e. 39.7 (±0.1) g/g TS. The second most abundant components were the extractives, i.e. 35.0 (±0.0) g/g TS. Extractives are usually a minor component in the LMs composition (Xu et al., 2019). Nevertheless, their abundance in HS suggests that extractives might have a crucial role during the AD process. In particular, the polyphenolic content of raw HS, expressed as equivalent gallic acid (GAE), was 106.0 (±3.9) mg/g TS. The cellulose and hemicellulose content was 10.2 (±0.1) and 3.6 (±0.0) g/g TS, respectively. The chemical composition here reported is in line with the previous study of Mancini et al. (2018). As for the porosity index, the WRC of raw HS was 1.82 (±0.08) g H_2O/g HS. The chemical composition confirmed the high recalcitrance of HS, which prompted testing various pretreatments to unlock the full potential of this substrate.

Maceration is a physical pretreatment that can enhance the biodegradation of LMs by shearing the lignocellulosic fibers and reducing the particle size of LMs with limited energy consumption and overall costs (Ariunbaatar et al., 2014). In this study, maceration of HS was performed to reduce the release of polyphenolic compounds from the hydrolysis of HS extractives in the AD process. The 3 h maceration reduced the extractives content of HS by 11%, with no relevant effect on the other components. The polyphenolic content of macerated HS was 19.3 (±0.0) mg GAE/g TS, being significantly lower than the raw substrate (see supplementary material). On the other hand, the WRC of macerated HS was 25% lower than raw HS, i.e. 1.37 (±0.12) g H_2O/g HS. Macerated HS underwent AD in Phase IV, showing a 20% lower methane production than that observed in Phase III, with an $R_{m,av}$ of 15.3 mL CH_4/g VS/d. The VFAs concentration was slightly lower than in Phase III, and the IA/PA ratio was constantly below the threshold value (Fig. 3). It is reasonable that maceration, besides polyphenols, removed other valuable water-soluble molecules, such as free sugars, entailing a lower methane potential of HS (Tao et al., 2019). Additionally, as an indicator of porosity, the lower WRC negatively influenced the biodegradation of LMs by reducing the substrate to microorganism contact (Mancini et al., 2018).

Organosolv pretreatment acts to reduce the lignin content of LMs. Additionally, depending on pretreatment parameters, hemicellulose hydrolysis and an increase in porosity can occur (Ferreira and Taherzadeh, 2020). Methanol-organosolv pretreatment greatly enhanced the AD of HS in a previous study conducted with batch bioassays (Oliva et al., 2021). In this study, the organosolv pretreatment reduced the lignin and extractives content of HS by 9 and 6%, respectively. In particular, the pretreatment removed 97% of the overall polyphenols from raw HS, resulting in 3.1 (±0.0) mg GAE/g TS in the organosolv-pretreated HS (see supplementary material). The pretreatment may have benefited from the S/L ratio of 1:15, whereas the most commonly employed ratio reported in the literature is 1:10 (Ferreira and Taherzadeh, 2020). The impact of S/L on organosolv pretreatment is still unclear and underinvestigated (Ferreira and Taherzadeh, 2020). Nevertheless, HS pretreatment may require a high S/L to completely soak HS during the pretreatment due to its high porosity. The organosolv-pretreated HS was fed to the reactor in Phase V. After 4 days of operation under these operating conditions, the $R_{m,av}$ was 25.6 mL CH_4/g VS_{add}/d. Nevertheless, technical issues with the Na_2CO_3 dosing demanded to replace the entire liquid phase of the reactor with a fresh DBM on day 150. The overdosing of Na_2CO_3 increased the pH up to 9.2 (Fig. 2B), being considerably above the optimal range (i.e. 6.5 – 7.5) for methanogenic activity (Borth et al., 2022). The performance of the reactor decreased with the new inoculation of DBM, probably due to the lack of proper microbial community adaptation to degrade HS. After two feeding cycles with the organosolv-pretreated HS, the reactor performance was restored (Fig. 2A).

After Phase V, the reactor was readapted to the LM from day 167 to 195. During this period, the reactor was fed regularly with raw HS, the pH was monitored and adjusted, but the methane production was not recorded. This standby phase represented a new baseline necessary to compare the methane potential of raw and organosolv pretreated HS. In Phase VI, the methane production from raw HS was lower than in Phase III, as described in Section 3.1 (Fig. 2). In total, 8.65 L of methane were produced in Phase VI. In Phase VII, organosolv-pretreated HS was again used, keeping the refeeding time at 7 days. The overall methane production, i.e. 10.48 L, was 21% higher than in Phase VI, confirming the effectiveness of the methanol-organosolv pretreatment on HS (Oliva et al., 2021). The $R_{m,av}$ in Phase VII was 19.8 mL CH_4/g VS/d, against 16.0 mL CH_4/g VS/d measured in Phase VI. On the other hand, no significant change in VFAs accumulation and alkalinity was observed. The increment in methane production can be attributed to the changes in chemical composition, e.g. lower lignin content, and loss of inhibitory compounds, i.e. polyphenols (Ferreira and Taherzadeh, 2020; Milledge et al., 2019). Moreover, the WRC of pretreated HS, i.e. 1.72 (±0.09) g H_2O/g HS, was comparable with the untreated substrate and is therefore not correlated with the increment in methane production.

3.4. Microorganisms involved in hazelnut skin degradation

The taxonomic classification on day 0 revealed the abundance of *Firmicutes* (i.e. 38%), *Proteobacteria* (i.e. 18%), *Synergistota* (i.e. 14%) and *Bacteroidota* (i.e. 9%) phyla (Fig. 5A). These are the most common phyla involved in hydrolysis, acetogenesis and acidogenesis phases (Pasalari et al., 2021). The DBM was a heterogeneous inoculum, with *Synergistaceae* (affiliated with the *Synergistota* phylum) being the only family above 10% of the overall microbial community (Fig. 5B). *Firmicutes* and *Proteobacteria* phyla are resilient to extreme conditions, whereas the *Bacteroidota* phylum is more sensitive to operating conditions (Pasalari et al., 2021), which suggests that the DBM was in the endogenous phase when inoculated to the reactor.

On day 80, a fresh DBM was inoculated, resulting in a step backwards in the microbial enrichment, as shown in Fig. 5A. Before adding the fresh DBM, the *Synergistaceae* (i.e. 26%) and *Bacteroidaceae* (affiliated with the *Bacteroidota* phylum) (i.e. 17%) families survived the critical operating conditions caused by the overload of HS (Fig. 5B). Around day 80, the highest VFAs accumulation was observed, i.e. 1.8 g HAc_{eq}/L (Fig. 3A). The abundance of the *Synergistaceae* family has been associated with high VFAs concentrations in the AD of corn straw (Zhu et al., 2022). In addition, enrichment (i.e. up to 6%) in the *Spirochaetota* phylum (Fig. 5A) was observed when the propionic acid concentration increased in the reactor (Fig. 3A). These bacteria can convert propionic, butyric and valeric acids into acetic acid, H_2 and CO_2, ensuring process stability (Borth et al., 2022). At the end of Phase II, once reactor stability was reached, the *Rikenellaceae* family (affiliated with the *Bacteroidota* phylum) enriched up to 24%. On the other hand, the percentage of *Synergistaceae* and *Bacteroidaceae* families dropped to 21 and 6%, respectively (Fig. 5B).

In the first three phases of this study, raw HS was fed to the reactor (Table 1). At the end of Phase III, *Bacteroidota* (i.e. 33%) and *Synergistota* (i.e. 21%) were the most abundant phyla. On the contrary, the relative abundance of *Firmicutes* and *Proteobacteria* phyla decreased to 24 and 9%, respectively (Fig. 5A). The most abundant families after Phase III were *Synergistaceae* (i.e. 21%) and *Rikenellaceae* (i.e. 15%) (Fig. 5B). Lv et al. (2019) reported the dominance of the *Synergistaceae* and *Rikenellaceae* families in the AD of a similar substrate, i.e. straw. The abundance of the *Synergistota* phylum has been recently reported in a phenol-degrading study working with concentrations comparable to this study (Li et al., 2022). The enrichment in the *Synergistota* phylum could be associated with the polyphenols released from HS. Nevertheless, further

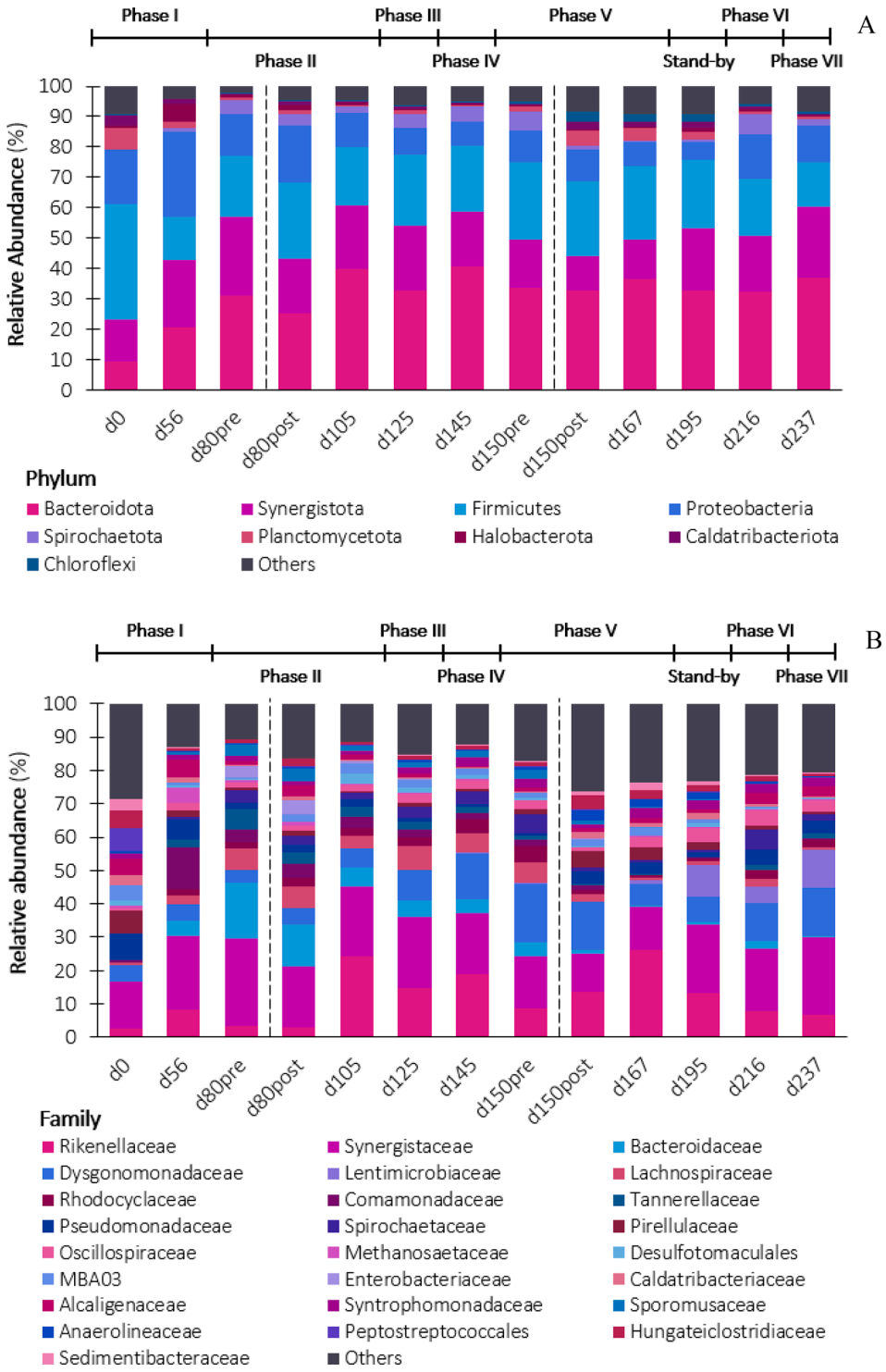

Fig. 5. Changes in the microbial community at phylum (A) and family (B) level during the anaerobic digestion of hazelnut skin under fed-batch mode operation. All the relative abundances below 3% were grouped as Others.

studies are required to confirm this hypothesis.

In Phase IV, macerated HS was fed to the reactor (Table 1). In this phase, the *Rikenellaceae* and *Dysgonomonadaceae* families (affiliated with the *Bacteroidota* phylum) enriched (Fig. 5B), resulting in a 41% relative abundance of the *Bacteroidota* phylum (Fig. 5A). In particular, the *Dysgonomonadaceae* family was enriched from Phase III and increased until day 150 of operation, reaching 18% of the relative abundance (Fig. 5B). The *Dysgonomonadaceae* family include hydrolytic genera capable of degrading recalcitrant polysaccharides, such as the carbohydrates of LMs (Owusu-Agyeman et al., 2022). As explained in Section 3.3, Phase V was affected by a new inoculation on day 150. The fresh DBM was abundant in *Rikenellaceae* (i.e. 14%), *Bacteroidaceae* (i.e. 14%), and *Synergistaceae* (i.e. 11%) families. The *Rikenellaceae* family enriched (i.e. 26%) until day 167 while feeding organosolv-pretreated HS. Nevertheless, this family gradually disappeared in the following phases to the benefit of the *Synergistaceae* family. The microbial community readapted to the substrate in the standby period. On day 195, the phyla present in the reactor were comparable with those present during Phase III

(Fig. 5A).

In Phase VI, feeding raw HS, the relative abundance of the *Proteobacteria* (i.e. 15%) and *Spirochaetota* (i.e. 6%) phyla increased. However, the most present phyla were *Bacteroidota* (i.e. 32%), *Synergistota* (i.e. 19%) and *Firmicutes* (i.e. 19%) (Fig. 5A). At the family level, *Synergistaceae* (i.e. 19%) and *Dysgonomonadaceae* (i.e. 11%) were the most abundant (Fig. 5B). The main difference with Phase III was the lower abundance of hydrolytic bacteria, which was likely the reason for the lower methane production (Pasalari et al., 2021). *Spirochaetaceae* (i.e. 6%) (Fig. 5B), affiliated with the *Spirochaetota* phylum, is a family of complex polymer-oxidising bacteria that can be associated with the increased input of polyphenols along with the feeding of raw HS (Zhu et al., 2022).

In Phase VII, organosolv-pretreated HS was fed to the reactor. *Bacteroidota* and *Synergistota* phyla reached 37 and 23%, respectively (Fig. 5A). On the contrary, the *Firmicutes*, *Proteobacteria*, and *Spirochaetota* phyla decreased (Fig. 5A). In particular, the *Spirochaetota* decrement (i.e. below 2%) seems to confirm the correlation between polyphenols and the *Spirochaetota* phylum (Zhu et al., 2022). On the contrary, *Proteobacteria* was the dominant phylum after digesting raw and pretreated rice straw for 60 days (Mirmohamadsadeghi et al., 2021). The abundance of the *Proteobacteria* phylum was previously associated with the endogenous conditions occurring when the carbon source became scarce at the end of the AD process (Pasalari et al., 2021).

In Phase VII, the most abundant families were *Synergistaceae* (i.e. 23%), *Dysgonomonadaceae* (i.e. 15%), and *Lentimicrobiaceae* (i.e. 11%). The *Lentimicrobiaceae* family (affiliated with the *Bacteroidota* phylum) is associated with cellulase and xylanase activity (Jensen et al., 2021), which can be related to the higher HS degradation and subsequent methane production observed in Phase VII (Fig. 5A).

3.5. Importance of reactor configuration for fed-batch anaerobic digestion

The organic load and retention time of the substrate are crucial parameters for efficient AD, also affecting the economy of the entire process. When digesting liquid or semisolid substrates, such as sludge or slurries, organic loading rate and hydraulic retention time are more commonly used (Pasalari et al., 2021). In contrast, for solid substrates, such as LMs, the retention time, i.e. SRT, should refer only to the solid matter.

Most of the studies investigating the AD of LMs under fed-batch operation used a stirred tank reactor and fed the reactor by replacing an aliquot of substrate and inoculum with a fresh amount of the same mixture (Lahboubi et al., 2020; Wang et al., 2021). In this study, an alternative reactor configuration was developed that decoupled the SRT of the HS from the sludge retention time (SuRT), which allows microbial growth during the SuRT while shortening the SRT (Okoye et al., 2022). A low SRT is desirable to reduce the costs of AD. However, when SRT and SuRT are coupled, shortening this time can lead to washout of microorganisms and imbalance of the microbial community at the expense of methanogens (Pasalari et al., 2021).

The core of the reactor proposed in this study is the SSMC used to retain the HS while being soaked in the reactor microbial biomass (Fig. 1B). This configuration allowed to set the desired SRT regardless of the microbial biomass, which was left enriching for the entire operating time, apart from the liquid sampling and the extra inoculation on days 80 and 150 (Fig. 2B). Microbial community acclimation is a crucial factor for AD, especially in the case of LMs, since hydrolytic bacteria need to adapt to the substrate and hydrolise it. Therefore, lowering the SuRT can result in ineffective AD (Pasalari et al., 2021; Xu et al., 2019). To the best of the authors' knowledge, the SSMC has never been used in AD to decouple SRT and SuRT.

From the perspective of upscaling this configuration, a further advantage is the easy mixing of the reactor, most of the solids being retained in the SSMC. Mixing represents one of the highest operating costs, especially in dry and semidry AD. Additionally, feeding and withdrawal of the substrate are facilitated since no pumping is required. Also, the solid content of the digested LM is higher than a digestate obtained from a stirred tank reactor since it is mainly composed of undigested lignin from the LM, which reduces the costs of drying the digestate (Peng et al., 2020).

Despite the promising perspective, this configuration also raised some concerns, such as the risk of accumulating inhibitory compounds in the liquid phase of the reactor. In addition, to ensure an optimal inoculum to substrate contact, a highly porous substrate is required, such as HS. Finally, in view of an upscaling of this configuration, further studies are required to design a SSMC that guarantees the optimal inoculum to substrate contact once the volume of the SSMC and the amount of the retained substrate increase.

4. Conclusion

The AD of HS was improved over long-term fed-batch operation by reducing the SRT and increasing the organic load. Polyphenols concentration and the pH control strategy were crucial to maintain an efficient AD. Methanol-organosolv pretreatment reduced the lignin and polyphenolic content of HS by 9 and 97%, respectively, resulting in a 21% increment of the methane potential. Maceration removed 82% of the polyphenols, but negatively affected the methane production from HS. The reactor configuration here proposed allowed the enrichment of an efficient microbial community for HS degradation, but requires further optimisation before being implemented on a larger scale.

CRediT authorship contribution statement

A. Oliva: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing – original draft, Writing – review & editing. **L.C. Tan:** Supervision, Writing – review & editing. **S. Papirio:** Supervision, Resources, Writing – review & editing, Project administration. **G. Esposito:** Supervision, Resources, Writing – review & editing. **P.N.L. Lens:** Supervision, Resources, Writing – review & editing, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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