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Fungal Pretreatments on Non-Sterile Solid Digestate to Enhance Methane Yield and the Sustainability of Anaerobic Digestion

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Received: 2 September 2020; Accepted: 12 October 2020; Published: 15 October 2020

Abstract: Fungi can run feedstock pretreatment to improve the hydrolysis and utilization of recalcitrant lignocellulose-rich biomass during anaerobic digestion (AD). In this study, three fungal strains (*Coprinopsis cinerea* MUT 6385, *Cyclocybe aegerita* MUT 5639, *Cephalotrichum stemonitis* MUT 6326) were inoculated in the non-sterile solid fraction of digestate, with the aim to further (re)use it as a feedstock for AD. The application of fungal pretreatments induced changes in the plant cell wall polymers, and different profiles were observed among strains. Significant increases ($p < 0.05$) in the cumulative biogas and methane yields with respect to the untreated control were observed. The most effective pretreatment was carried out for 20 days with *C. stemonitis*, causing the highest hemicellulose, lignin, and cellulose reduction (59.3%, 9.6%, and 8.2%, respectively); the cumulative biogas and methane production showed a 182% and 214% increase, respectively, compared to the untreated control. The increase in AD yields was ascribable both to the addition of fungal biomass, which acted as an organic feedstock, and to the lignocellulose transformation due to fungal activity during pretreatments. The developed technologies have the potential to enhance the anaerobic degradability of solid digestate and untap its biogas potential for a further digestion step, thus allowing an improvement in the environmental and economic sustainability of the AD process and the better management of its by-products.

Keywords: digestate; solid fractions (SFDs); lignocellulose; pretreatment; fungi; biogas

1. Introduction

The awareness about the environmental damage caused by the massive exploitation of fossil fuels has led to a green revolution in energy production models in favor of environmentally friendly, cost-effective, and sustainable energy systems [1]. Anaerobic digestion (AD) is one of the most attractive technologies to produce renewable energy [2]. It is a multistep biological process that foresees the conversion of organic feedstocks into biogas—i.e., a gaseous mixture of methane (45–70%), carbon dioxide (25–55%), and traces of other compounds (nitrogen, oxygen, hydrogen sulfide, and water). Biogas can be burned to obtain heat or steam, but it is commonly used for cogeneration (the production of both heat and electricity) [2].

AD is a flexible technology since, theoretically, any organic substrate could be exploited as a feedstock [3]. It is thus a captivating technology for the treatment of a wide range of organic wastes [2]. However, the use of some feedstocks is often limited due to the process microbiology, plant technology,

country legislation, and biomass properties [3]. The feedstock deeply affects the yields and quality of the AD products [4]. Agricultural biogas plants (ABPs) that are more widely diffused in Europe operate in co-digestion with a mixture of zootechnical effluent, energy crops, and agriculture-related residues [2,3]. The increased use of dedicated crops (such as corn, sorghum, rye, etc.) for energy purposes has led to competition with food and feed production and for land use [5]. Nowadays, the use of energy crops is strongly discouraged at European and national regulatory levels. In Italy, the Ministerial Decree 23 June 2016 provided lower subsidies for plants that use crops in order to discourage this practice. Alternative sustainable lignocellulosic biomasses, such as agro-industrial wastes and by-products (corn stover, wheat straw, and rice straw), may contribute to improve the overall sustainability of AD [6].

In addition to biogas, AD has the potential to produce various by-products as digestate, which mainly contains water, inorganic compounds (e.g., nitrogen, N; phosphorus, P; potassium, K), and undigested organic matter [4]. Although digestate can be managed in its raw form, the liquid and the solid fractions (SFDs) are often mechanically separated to facilitate handling, storage, and transport [7,8]. The liquid fraction contains a high amount of K and ammoniacal N, whereas the SFD is rich in P and residual fibers [9]. Since plant cell wall polymers (PCWPs), such as cellulose and lignin, undergo little change during AD, they are conveyed from the lignocellulosic feedstocks mainly into the SFD [10]. The fate of digestate fractions commonly involves agricultural purposes, such as in organic fertilizer and soil amendment [11]. However, their safe management and ever-increasing production is posing some issues [4]. A huge amount of digestate is indeed continuously produced but it cannot be used immediately on farmlands due to its stabilization level, crop growth stage, soil type, and the limits posed by the European Nitrate Directive [12–14]. The required storage of digestate usually involves aboveground uncovered tanks, though undesired emission into the atmosphere can still occur due to the presence of undigested organic matter [15–17]. Moreover, the increasing number of ABPs and their confluence in a specific geographical area (e.g., northern Italy) might lead to local oversupply [14] and the need to transport the excess to areas with nutrients deficits, increasing the overall costs of the process [10]. These technical problems ultimately lead to a consistent environmental impact and the loss of energetic efficiency [17].

Innovative and alternative valorization routes for digestate are the next frontier [4]. According to the circular economy approach, the possibility of exploiting the residual undigested organic matter retained in the SFD as a feedstock for ABPs has been hypothesized [4,9,18]. This solution could also improve the economical profitability and environmental efficiency of the AD process, allowing the mitigation of greenhouse gases and ammonia emissions and the concomitant recovery of economically attractive amounts of biogas-methane [9,10,16].

Unfortunately, the SFD from agrozootechnical residues has been widely recognized as a recalcitrant biomass, and its use in biogas production challenges the microbial community involved in AD [19]. Cellulose crystallinity and lignification degree decrease its digestibility and limit the theoretical biogas yields [20]. In order to enhance the anaerobic degradability of refractory biomasses and improve the biogas recovery, pretreatment processes can be applied [21]. Among biological treatments, the use of fungi for feedstock treatment has increasingly gained importance, since they represent a valuable and environmentally sustainable alternative to physicochemical methods [20]. Basidiomycota and Ascomycota are well-known producers of non-stereoselective enzymes that oxidize a wide range of refractory compounds, including lignocellulose complex [20]. Causing mechanical and enzymatic modifications, these fungi could make structural polysaccharides more accessible for AD microorganisms, resulting in an increased biogas yield [20,22]. In the literature, only a few studies have dealt with fungal treatment to make the SFD more susceptible to a further digestion step and valorization. Nowadays, any application at a large scale is prevented by the lack of knowledge about how to control the process, the operative parameters to be monitored, the microbial resources to be exploited, etc. The present work wants to fill this gap, focusing the attention on the characteristics and residual biomethane potential of the SFD and processes that could lead to its (re)use as an AD

feedstock. This study aims to evaluate the role of pretreatments in order to transform lignocellulose and consequently enhance the methane recovery. Biological techniques based on filamentous fungi were then the tool used to achieve this goal. Three different fungal strains (*Coprinopsis cinerea* MUT 6385, *Cyclocybe aegerita* MUT 5639, and *Cephalotrichum stemonitis* MUT 6326), belonging to the Basidiomycota and Ascomycota phyla, were used. For each strain, the effects of two different pretreatment times (10 and 20 days) were investigated.

2. Materials and Methods

2.1. Biomass Sampling and Storage

The SFD was collected from a storage facility of a mesophilic full-scale ABP operating in north-west Italy (Piedmont region). The selected ABP was a stirred tank reactor with 1 MWel of installed power fed with maize silage (75%), triticale silage (13%), and other cereals (12%). The organic loading rate was 2.25 kg volatile solids (VS) m^3 digester/day. The hydraulic retention time was about 60 days. The resulting digested slurry (approximately 70 t/day) was processed through a screw-press (CRIMAN® mod. SM260) to separate the SFD (about 5 t/day) from the liquid fraction (about 65 t/day). The SFD was dried and N-stripped before storage in a static heap on an uncovered platform.

2.2. Biomass Characterization

Homogenous composite samples of untreated and fungal-pretreated SFD were prepared. The total solids (TS), VS, total nitrogen (TN), total ammoniacal nitrogen (TAN), and pH were analyzed according to the Association of Official Analytical Chemists (AOAC) [23] and Dinuccio et al. [24]. The total fiber composition was estimated as neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL) using the detergent Van Soest methods [25]. The hemicellulose and cellulose contents were calculated as the difference between the NDF and ADF, and the ADF and ADL, respectively [24]. The TS, TN, and TAN data were expressed as percentages of the raw wet biomass. On the contrary, the VS, NDF, and PCWP data were expressed as percentages of the TS content of the dry biomass, avoiding any bias due to samples with different water contents. The percentage loss of biomass components (TS, VS, NDF, and PCWP) during the fungal pretreatment was calculated according to Zhao et al. [26]. Normalization with the contribution of the fungal biomass (6.7% of TS) was not performed because the Van Soest method cannot discriminate between the fungal and plant insoluble components [27].

2.3. Fungal Pretreatments

C. cinerea MUT 6385, *C. aegerita* MUT 5639, and *C. stemonitis* MUT 6326 were selected as promising candidates to perform the whole cell pretreatment on SFD since, in a preliminary screening, they showed the ability to efficiently colonize the biomass in non-sterile conditions. The strains are preserved at the Mycotheca Universitatis Taurinensis (MUT) of the Department of Life Sciences and Systems Biology, University of Turin (Italy). The nucleotide sequences of *C. cinerea* MUT 6385 and *C. stemonitis* MUT 6326 were deposited at the GenBank NCBI database under the accession numbers MT151631 and MT151633, respectively.

Fungi were pre-grown in Malt Extract Agar (MEA: 20 g/L of malt extract, 20 g/L of glucose, 2 g/L of peptone, 18 g/L of agar) and incubated at 25 °C for 10 days. They were then grown in submerged fermentation in 500 mL Erlenmeyer flasks containing 350 mL of diluted (1:10) Malt Extract Broth with 10 g/L of maize silage. Flasks were incubated at 25 °C in agitation (120 rpm) in the dark. After 7 days, mycelial pellets were filtered and washed in order to avoid adding to the SFD any component of the exhaust medium and the fungal metabolites produced during the liquid fermentation. The fungal biomasses used as inoculum had an average TS content of 7.3%, of which 97.4% was VS. Then, 20 g dry weight (dw) of fungal biomass was inoculated in 300 g dw of non-sterilized SFD. Water was added (2:3 ratio *w/v*) to obtain a final moisture content of about 70–75%, indicated in the literature as optimal

for lignin decomposition [28]. The inoculated SFD was incubated at 25 °C for 10–20 days. According to the literature data [29,30] and preliminary trials, a few weeks of pretreatment is optimal for fungal growth. SFD without fungal inoculum was set up as a negative control. At the end of the aerobic pretreatments, the biomasses were analyzed and used as feedstocks for AD in lab-scale experiments.

2.4. Biochemical Methane Potential Tests

Biochemical Methane Potential (BMP) tests were performed through batch trials according to Dinuccio et al. [24] and VDI 4630 [31]. The inoculum was the separated liquid fraction of the digested slurry produced by selected ABP. Batch reactors (2 L) were filled with water (700 g) and a mixture of feedstock and inoculum (800 g) with a 1:2 ratio based on the VS content. Inside each batch reactor the same amount of VS was added, for a total of 29.1 g VS. Batches containing untreated SFD were used as a control. Blank batches trials (inoculum only) were also carried out to determine the productivity and correct the yields from the tested biomasses. The potential biogas production derived from the addition of VS from fungal biomass was not compensated, and it was included in the calculations of the final yields. For each trial condition, three replicates were set. Biogas was collected in a Tedlar bag (3 L capacity), connected to the glass taps of each batch reactor by means of tygon tubing. Trials were performed under mesophilic conditions (40 ± 2 °C) in a climatic chamber for 75 days. The biogas volume and composition were monitored every 4 days for the first 3 weeks and then weekly, until no more biogas production was detected. The volume of biogas produced was measured by means of a Ritter Drum-type gas volume meter (TG05/5, Ritter Apparatebau GmbH and Co. KG, Bochum, Germany). The methane concentration in the biogas was determined with a gas analyzer equipped with infrared sensors (model XAM 7000, Drägerwerk AG and Co. KgaA, Lübeck, Germany). The recorded data were normalized according to VDI 4630 [31] and the specific yields of biogas and methane were expressed as normal liters (L_N) per kg of VS. The daily rate (L_N/kg VS d) and cumulative (ΣL_N/kg VS) biogas and methane production were calculated according to the procedure described by Dinuccio et al. [24].

2.5. Statistical Analyses

Data of the TS, VS, and cumulative biogas and methane yields were statistically analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's means grouping tests using the software package RStudio Version (3.4.3).

3. Results

3.1. Fungal Inoculum Addition and Pretreatment of SFD

The chemical characterization of the untreated and fungal-pretreated SFD is presented in Table 1. All the samples showed an alkaline pH with a negligible TAN content (0.01%). Lignocellulosic fibers (NDF) were the major components of organic matter (VS), representing, on average, approximately 80% of the TS. The hemicellulose content was minimal (1.1–2.7% of TS), whereas cellulose and insoluble lignin (ADL) were the main fibrous components (around 30% and 48% of the TS, respectively).

The fungal inoculum addition and pretreatments greatly affected the SFD. Decreased values of pH and TN content were observed in comparison with the untreated SFD. Similarly, the TS concentration significantly ($p < 0.05$) decreased 23.8%, 25.4%, and 28.5% in samples pretreated with *C. aegerita*, *C. stemonitis*, and *C. cinerea*, respectively. In detail, the addition of water from the fungal inoculum caused an approximately 20.6% reduction in the TS. On the other hand, the VS content did not change ($p > 0.05$) compared to the untreated controls.

Fungal pretreatments also changed the concentration and composition of the fibers. The percentage of NDF losses ranged from 1.6% to 10.4% in samples pretreated with *C. cinerea* for 10 days and with *C. stemonitis* for 20 days, respectively. The different strains showed a similar behavior towards the PCWP, causing a higher reduction in hemicellulose (18.5–59.3%) as compared to lignin (1.0–9.6%)

and cellulose (0.2–8.2%). However, the levels of conversion of each PCWP are different among the strains tested. Noteworthy, the highest reductions in all the PCWP components were obtained with pretreatment with *C. stemonitis* for 20 days, which resulted in 59.3%, 9.6%, and 8.2% hemicellulose, lignin and cellulose reductions, respectively. Compared to *C. stemonitis*, the Basidiomycota strains gained a lower lignin reduction (1.6–3.2% and 1.0–3.9% with 10 and 20 days of pretreatment with *C. cinerea* and *C. aegerita*, respectively). The pretreatments with *C. aegerita* achieved lower reductions in cellulose (from 0.2% to 0.8% with 10 and 20 days, respectively), while those performed with *C. cinerea* reached a lower hemicellulose reduction (from 18.5% to 22.2% for 10 and 20 days, respectively).

Table 1. Characteristics and fiber compositions of the solid fraction of digestate (SFD), untreated (control) and pretreated with *Coprinopsis cinerea*, *Cyclocybe aegerita*, and *Cephalotrichum stemonitis* for 10 and 20 days (d).

	Untreated SFD (Control)	**Fungal-Pretreated SFD**					
		C. cinerea		***C. aegerita***		***C. stemonitis***	
		10d	**20d**	**10d**	**20d**	**10d**	**20d**
pH	9.4	8.2	8.5	8.8	9.0	9.0	9.1
TS [%]	31.7	22.7	22.6	24.1	24.2	23.9	23.4
Humidity [%]	68.3	77.3	77.4	75.9	75.8	76.1	76.6
VS [% TS]	88.1	87.4	87.7	88.7	88.2	87.8	89.1
TN [%]	0.8	0.6	0.6	0.7	0.6	0.7	0.6
TAN [%]	0.01	0.01	0.01	0.01	0.01	0.01	0.01
NDF [% TS]	82.4	80.6	79.9	81.1	79.7	79.6	73.8
ADF [% TS]	79.7	78.4	77.8	79.3	78.1	77.7	72.7
ADL-Lignin [% TS]	31.1	30.6	30.1	30.8	29.9	29.9	28.1
Hemicellulose [% TS]	2.7	2.2	2.1	1.8	1.6	1.9	1.1
Cellulose [% TS]	48.6	47.8	47.7	48.5	48.2	47.8	44.6

The concentrations of biomass components (TS, VS, NDF, and PCWP) generally decreased as the duration of the pretreatments increased. Most of reductions occurred during the first 10 days, while minor changes were observed when prolonging the pretreatments to 20 days. For instance, *C. cinerea* and *C. aegerita* exhibited a relatively limited variation in the TS and PCWP content between 10 and 20 days of pretreatment.

3.2. BMP Tests

Figure 1 shows the daily biogas production rates and respective methane concentrations of untreated and fungal-pretreated SFD. All the samples showed a similar profile of daily biogas yields, with a peak at day 4, followed by a progressive decrease, which dropped to zero after about 75 days. The SFD samples inoculated and pretreated with fungi did perform better than the untreated control. In particular, the samples pretreated with *C. cinerea* for 10 days produced the highest daily biogas yield (15.6 ± 0.8 L_N/kg VS d), which was approximately double with respect to the untreated SFD (8.8 ± 0.1 L_N/kg VS d). The daily methane concentration was comparable among all samples (Figure 1); the profile showed a gradual increase (from about 20–30% up to 55%) during the first 10 days of AD, when the plateau was reached and was then maintained until the end of the trials.

At the end of the anaerobic incubation, the cumulative biogas and methane yields from all the SFD samples inoculated and pretreated with fungi were significantly ($p < 0.05$) higher than those of the untreated control (Figure 2). The AD worked better with SFD treated by *C. stemonitis* for 20 days, which led to approximately three-fold higher biogas and methane yields (+182% and +214%, respectively) than the untreated SFD. Comparable ($p > 0.05$) cumulative biogas yields were achieved with pretreatments by *C. stemonitis* and *C. aegerita* for 10 days and *C. cinerea* for 20 days (Figure 2). On the other hand, the cumulative methane produced with *C. stemonitis* was significantly higher ($p < 0.05$) than that obtained with *C. aegerita* and *C. cinerea* for both 10 and 20 days.

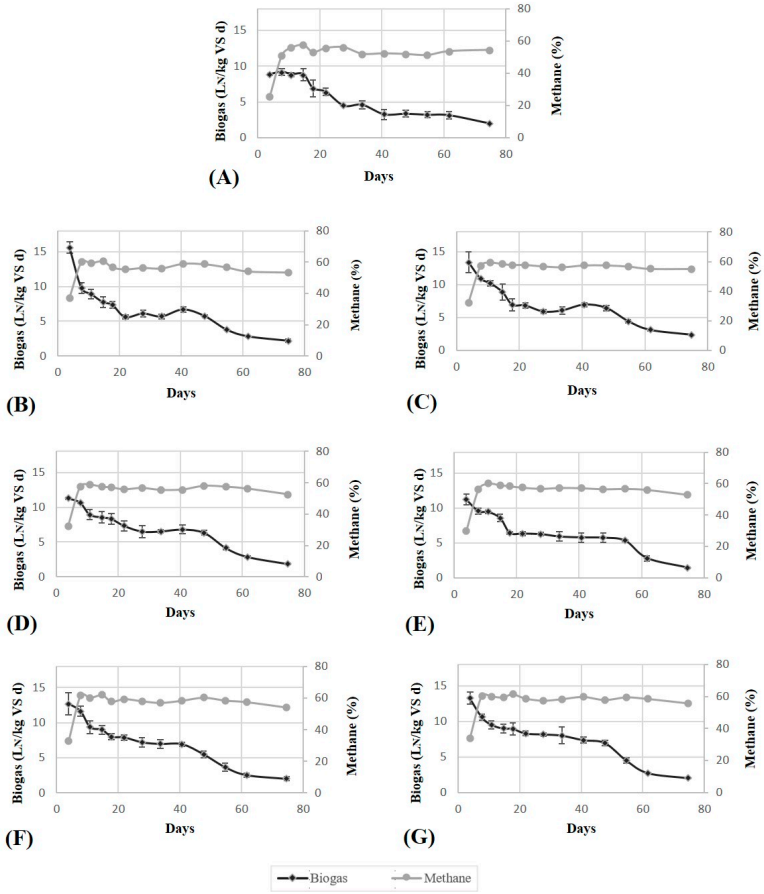

Figure 1. Daily biogas yield (L_N/kg Volatile Solids day) and methane concentration (%) of the solid fraction of digestate (SFD), untreated (**A**) and pretreated with *Coprinopsis cinerea* (**B**,**C**), *Cyclocybe aegerita* (**D**,**E**), and *Cephalotrichum stemonitis* (**F**,**G**) for 10 (**B**,**D**,**F**) and 20 (**C**,**E**,**G**) days.

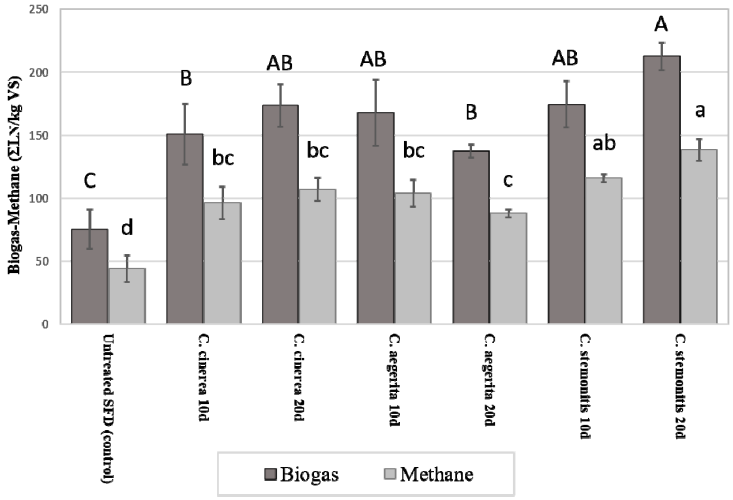

Figure 2. Cumulative biogas and methane yields (ΣL_N/kg Volatile Solids) of the solid fraction of digestate (SFD), untreated (control) and pretreated with *Coprinopsis cinerea*, *Cyclocybe aegerita*, and *Cephalotrichum stemonitis* for 10 and 20 days (d). Results are expressed as mean ± standard deviation from three replicates. Capital letters (ABC) and lowercase letters (abc) were used to show the results of statistical analyses on biogas and methane production, respectively. Different letters indicate significant differences ($p < 0.05$); means sharing a letter are not significantly different ($p > 0.05$).

Within a process carried out by the same fungal strain, no significant differences ($p > 0.05$) in the cumulative yields were observed between 10 days and 20 days of pretreatment.

4. Discussion

4.1. SFD Characteristics

The characteristics and residual biogas potentials of SFD can widely vary according to different parameters, such as the feedstock quality, organic loading rate, hydraulic retention time, storage parameters, and type of separator used on the raw co-digested slurry [4,15]. According to the literature [9,10], the currently studied SFD has an alkaline pH (>8) (Table 1). Menardo et al. [17] also reported pH values ranging from 8.6 to 9 for the SFD of three biogas plants treating mainly manure and energy crops. The low TN and TAN concentrations were related to the N-stripping and drying process performed on-site before storage [8]. Biomass alkalinity without the presence of ammonia (TAN) could be a favorable condition for the here-suggested SFD reuse, since it prevents pH and ammonia inhibition inside the digester tanks [32]. However, excessive alkalinity could represent a limiting factor for biological pretreatment, since the optimum pH for fungal growth usually ranges around acidic conditions (5 to 6.5) [33]. Moreover, alkaline conditions could be detrimental for enzymatic production and activity, with negative consequences for the PCWP degradation [6].

The observed TS and VS contents (Table 1) were comparable to those reported by Zhong et al. [19], who observed for the SFD from the co-digestion of animal manure and food wastes TS and VS contents of 30.60 ± 2.13% and 89.18 ± 0.29% on TS, respectively. However, lower VS contents were also found, as reported by Sambusiti [10] (83.8 ± 0.3% on TS) and Musatti et al. [33] (80% on TS).

In the present study, the SFD was confirmed a recalcitrant biomass, still containing a high organic matter (VS) content (approximately 88% of TS) due to the presence of not-degraded lignocellulosic fibers (around 80% of TS), mostly composed of cellulose and lignin. These findings agree with the literature data [19,33,34]. It is known indeed that lignin is not biodegradable under anaerobic conditions, while cellulose is degraded at a slower rate than hemicellulose, since cellulose degradation is negatively affected by its degree of crystallinity and interconnection with other PCWPs [4,33]. This results in an accumulation of more refractory cellulose and lignin, while hemicellulose can be largely exploited during AD [10]. Considering that the SFD fiber composition mainly depends on the original feedstock pattern [4], the obtained results were not unexpected. In fact, the AD of energy crops (maize silage) usually lead to SFD with a higher cellulose and lignin content and a lower hemicellulose content than that obtained from organic food wastes. For instance, Opatokun et al. [35] reported for the digestate from food wastes a fiber composition characterized by lignin, hemicellulose, and cellulose contents of 13.4%, 33.5% and 32.3% of TS, respectively. The SFD analyzed by Zhong et al. [19], derived from the co-digestion of animal manure and food-processing wastes, was characterized by a comparable lignin content (30.31% TS), while the cellulose content was lower (26.52% TS) and hemicellulose was higher (13.31% TS). In comparison with other agro-industrial wastes and by-products exploitable for biogas production, such as wheat straw (lignin 11.2%, hemicellulose 30%, and cellulose 40.2%) [36] and rice straw (14.11 ± 0.5% lignin, 27.9 ± 1.3% hemicellulose, and 36.3 ± 1.2% cellulose) [37], the SFD used in this work was characterized by a higher lignin and cellulose content and a lower hemicellulose content. In general, higher cellulose and hemicellulose contents are desirable, as they constitute the main sources of sugars available from lignocellulosic feedstocks for microorganisms involved in AD [34].

Interestingly, the analyzed SFD showed characteristics (Table 1) comparable and representative of those obtained in most of European mesophilic full-scale ABPs, such as those collected in the regions of northern Italy [9,10,38], which are areas characterized by a local oversupply of digestate [14]. In this context, the importance of SFD (re)use as an AD feedstock is even higher, since it could permit a better and complete exploitation of the original feedstocks into added-value product (methane), contributing to improve the economic and environmental efficiency of the AD process [4,9].

4.2. Fungal Inoculum Addition and Pretreatments: Effects on SFD

The fungal pretreatments performed in this study deeply affected the features of non-sterile SFD (Table 1). It should be considered that the applied method, as with others presented in the literature [27], is not actually capable of discriminating the contribution of fungal and plant insoluble components. Hence, the data presented in Table 1 include a marginal fraction of NDF, ADF, and ADL ascribable to the mycelium. Fungi showed alkaline tolerance, but during the pretreatment they caused a pH lowering, probably to establish an environmental niche more closely related to their physiological needs (i.e., acidic conditions), including the optimal conditions for their oxidative enzymes to operate [33].

The decreases in TS after the pretreatments were mostly caused by dilution due to the fungal inoculum water content and by PCWP degradation [26,29]. Similar results were obtained by Nuchdang et al. [39] when pretreating lignocellulosic grass with *C. cinerea* (27%). Carrere et al. [40] and Baldrian et al. [41] reported a slightly lower decrease (10–20%). It is well-known that fungal pretreatments could lead to considerable TS losses, especially in the case of easily degradable substrates. For instance, Liu et al. [42] reported a decrease of up to 55.3% in the TS content of corn stover silage following fungal pretreatment. Instead, in line with the results obtained in this study, decreases in TS for recalcitrant materials, such as SFD and albizia biomass residues, are generally lower and not so relevant for the efficiency of the AD process [40,43].

Surprisingly, only small variations (<1%; $p > 0.05$) in the VS content were observed among the untreated and fungal-pretreated SFD. Noteworthily, the data reported in the literature showed higher VS losses than those obtained in this work. For instance, Ge et al. [43] described a VS degradation of 11.2% on albizia chips pretreated with *Ceriporiopsis subvermispora*. Similar results were also reported by Kainthola et al. [44] with *Pleurotus ostreatus* and *Ganoderma lucidum* on rice straw (17.2% and 11.6% VS loss, respectively). Since an excessive VS degradation during pretreatment could negatively affect the AD process, leading to lower biogas production [44], it was fundamental for pretreatment success to find suitable microorganisms and experimental conditions that allowed the reduction in these losses [40]. Interestingly, samples pretreated with *C. cinerea* and *C. stemonitis* had a slightly ($p > 0.05$) increased VS content, probably due to fungal growth and metabolism. After 10 days, the fungi seemed to consume easily degradable compounds (i.e., hemicellulose) together with the more recalcitrant PCWP (lignin and cellulose) to sustain their growth and metabolism, lowering the TS and VS content of SFD. When the pretreatment lasted longer, the VS losses accountable to fungal degradation of PCWP may have been compensated by the grown mycelial biomass, causing the increase in the overall VS content.

All the tested strains affected the PCWP composition. This was not completely unexpected, since the selected fungal strains may produce lignocellulolytic enzymes [39,45,46] potentially useful for improving the bioconversion of lignocellulosic feedstocks [47]. As expected, distinct fungal species caused a different degradation profile and a longer treatment caused major PCWP modifications. The easily accessible hemicellulose was the main target by all strains and the reductions obtained fall within the range of those observed in the literature. For instance, Nuchdang et al. [39] reported the ability of *C. cinerea* to reach a hemicellulose degradation of 27% on lignocellulosic grass. Similar results were reported for *P. ostreatus* and *Trichoderma reesei* on rice straw, which reduced the hemicellulose content by about 23% [29]. In the work of Isikhuemhen et al. [46], *C. aegerita* appears to degrade hemicellulose components from 14% to 53.9%, depending on the combinations of solid waste from digester effluent, wheat straw, and millet used as substrate. *C. stemontis* showed the highest levels of hemicellulose degradation, even higher than those obtained in the literature with well-known white-rot fungi. For example, Zhi and Wang [36] and Liu et al. [42] obtained with *P. chrysosporium* a hemicellulose degradation rate lower than 50% on wheat straw and corn stover silage (31.2% and 32.4–48.4%, respectively). Chen et al. [48] stated that some levels of hemicellulose removal can enhance digestibility, since the process increases the porosity of lignocellulosic material. However, an excessive degradation of this heteropolymer can lead to a decrease in the biogas yields, as it is a fundamental

source of AD microorganisms [20,34]. Considering the initial poor hemicellulose content in SFD, the relatively high losses obtained in this study could be neglectable for the downstream AD process.

The more recalcitrant and abundant lignin and cellulose were also reduced by all fungi, but less efficiently compared to hemicellulose. When comparing the different strains, the SFD pretreated with *C. aegerita* and *C. cinerea* showed the lowest lignin reduction. A similar result was reported by Nuchdang et al. [39], which observed that lignin degradation of lignocellulosic grass treated with *C. cinerea* was not significantly different from that obtained by natural decay. Isikhuemhen et al. [46] reported variable levels of delignification for *C. aegerita*, obtaining values similar to those observed in this work (0.6%) and values far higher (21.8%), depending on the mix of substrates used. Although Basidiomycota species are reported as the most powerful organisms in delignification [20], the pretreatment with Ascomycota *C. stemonitis* leads to an almost triple delignification compared to *C. cinerea* and *C. aegerita* strains. However, the maximum lignin reduction obtained with *C. stemonitis* (9.6%) was relatively scarce respect to data reported in literature. For instance, Ge et al. [43] obtained a delignification rate of 24% pretreating albizia chips with *C. subvermispora.* Zhi and Wang [36] reported that *Phanerochaete chrysosporium* was able to reduce wheat straw lignin content by 36%. A higher delignification rate (35.3%) was described also by Mustafa et al. [29], pretreating rice straw with *P. ostreatus*. However, according to Muthangya et al. [49], even a small depletion of lignin may lead to a significant increase in methane production during subsequent AD. In fact, lignin degradation is reported as the main factor for a successful fungal pretreatment, as it increases the accessibility of AD microorganisms to the more easily degradable structural carbohydrates and directly contribute to the enhancement of anaerobic digestibility [26].

Notably, the cellulose reduction rates obtained in this study were lower than those generally described in the literature. For instance, Nuchdang et al. [39] reported a 16% cellulose degradation for *C. cinerea* grown on lignocellulosic grass. Higher cellulose reductions (ranging from 27.7 to 55.2%) were described also by Isikhuemhen et al. [46] using *C. aegerita*. Conversely, lower cellulose losses, comparable to those obtained in this study, were reported using specific Basidiomycota characterized by a selective delignification system. For example, Wan and Li [28] reported that *C. subvermispora* lacks a complete cellulolytic system, thus produces negligible cellulose degradation (<5%) in all the different lignocellulosic substrates tested. Low cellulose decomposition rates are generally desirable when operating pretreatment processes, as this polysaccharide constitutes one of the main sources of sugars for microorganisms involved in AD [20,34].

On the whole, the changes in SFD can be ascribable both to the addition of fungal biomass and to the fungal activity during pretreatment. As also indicated by literature data [47], the PCWP modifications could increase the digestibility of SFD during AD. However, the heterogenicity of lignocellulosic biomass and the complicated nature of AD prevent to predict the performance of the process solely based on the composition of feedstocks [50]. In this work, BMP tests in batches were carried out to assess the actual effect of fungal pretreatments on biogas and methane production.

4.3. BMP Tests

The results obtained in the BMP tests highlighted that SFD still contains residual biogas and methane potential, confirming that it could be a suitable feedstock for biogas plant feeding [17]. However, the poor biogas and methane yields obtained from the untreated samples (Figure 2) confirmed that a large fraction of SFD organic matter is not readily biodegradable as lack of easily degradable carbon source. The fungal pretreatments were capable of increasing SFD hydrolysis and BMP yields during the subsequent AD. The improved digestibility can be explained mainly to the reduced biomass recalcitrance caused by the PCWP degradation (Table 1). A higher level of PCWP degradation seems indeed correlated with higher biogas and methane production. However, the fungal biomass added to the system may have also played a role. In fact, the fungal biomass contains lipids, proteins, and other molecules that may boost the microorganisms involved in AD. As demonstrated by Hom-Diaz et al. [51], exhausted fungal biomass could be profitably used as a substrate in AD

processes, producing 281-595 L methane/kg VS. Jasko et al. [52] tested the BMP of fungal biomass of *Paxillus involutus* and *Phaeolus schweinitzii* and they obtained a biogas production of 607.3 L/kg VS and 137.9 L/kg VS, respectively. The difference between the results obtained with different fungal strains was accounted to the difference in VS composition or to the production of bioactive compounds able to inhibit or enhance the AD process [52]. In this study, considering the average methane productivity reported in literature per g of fungal VS (about 382.5 ± 169.2 mL/g VS) [51,52] and the amount of fungal VS introduced inside each batch reactor (0.71 g VS), the fungal biomass could have contributed to approximately 5–10% of the total methane productivity.

In the literature, the time of pretreatment is reported as one of the most critical factors for the efficiency of AD performance [53]. Mustafa et al. [29] treated rice straw for 10, 20, and 30 days with *P. ostreatus* and *T. reesei*; the best process performances were obtained with 20 days pretreatment, with higher biogas and methane yields. Shorter incubation times did not degrade enough PCWP, while a longer period would lead to excessive organic matter losses [29]. Different results were obtained by Phutela et al. [30] treating rice straw with *T. reesei* and *Coriolus versicolor* for 5, 10, 15, 20, and 25 days. The best treatment last 10 days, while in the other cases, the biogas and methane yields decreased (up to 50.90% on day 25). Surprisingly, in this work no significant ($p > 0.05$) differences were observed in the cumulative biogas and methane yields between 10 days and 20 days of processing. It would probably take longer to see significant differences, but longer treatment times could cause a decrease in the biogas production during AD [40,49]. Considering that one disadvantage of biological pretreatments is that they are often time consuming [22], it is a positive finding that the developed process allowed us to obtain a significant increase in the AD yields also with relatively short pretreatment times (10 days).

The use of different fungal strains and species for pretreatment turned out to be the most important factor affecting the AD performance, with *C. stemonitis* producing almost triple cumulative biogas and methane with respect to the untreated samples (Figure 2). In comparison with other authors who performed fungal pretreatments on digestate and other recalcitrant biomass, the improvement in biogas and methane yields obtained in this study were far higher and, therefore, encouraging. For instance, Lopez et al. [47] concluded that the treatment of lignocellulosic anaerobic digestate with the white-rot fungus *Phanerochaete flavido-alba* was not useful to increase the biogas production. Bremond et al. [54] tested on solid digestate the effectiveness of two different Basidiomycota strains (*P. ostreatus* and *Stropharia rugoso-annulata*), but the experimental conditions were not optimized and led to uncontrolled organic matter losses and subsequent decreases in the methane yield (up to 50%). Liu et al. [42] treated corn stover with *P. chrysosporium* and obtained an improvement of biogas production by 10.5% to 19.7% and methane yield by 11.7% to 21.2%, though a pretreatment duration that was longer (30 days). Similarly, Phutela et al. [30] reported an augmentation in biogas yields of 20.8% and 26.2% treating rice straw with *T. reesei* and *Coriolus versicolor*, respectively.

It should be noted that most of the literature work on the pretreatment of lignocellulosic feedstocks is focused on white-rot basidiomycetes inhabiting wood. The Ascomycota and fungi of other taxonomic groups or habitats have only scarcely been investigated [55]. According to our results, Ascomycota species could be equally able or even more competent in disrupting recalcitrant PCWP and enhance AD yields. Indeed, *C. stemonitis* was the most efficient in reducing hemicellulose, lignin, and cellulose and increasing the AD yields. Other authors also confirmed the effectiveness of fungal pretreatments performed with Ascomycota. For instance, the works of Mutschlechner et al. [53], Wagner et al. [56], and Deng et al. [57], reported a significant increase in the biogas yields when pretreating biowaste with *Trichoderma* species. Unfortunately, studies on Ascomycota focusing mainly on a restricted number of taxa (e.g., *Trichoderma* and *Aspergillus*), while this work demonstrated that it would be worthy to expand the research to other potentially suitable fungal species. At present, poor information is available on the potential of *C. stemonitis* to improve the AD bioconversion of lignocellulosic biomass. The findings emphasize the importance of investigating fungal biodiversity to identify new and promising species suitable for the development of effective pretreatment processes.

Previous studies [10,38] have investigated physical and chemical pretreatments to get a higher energy recovery from SFD, but surprisingly they often obtained poor results compared to those that are here reported. For example, Sambusiti et al. [10] revealed that thermal and alkaline treatments did not have a beneficial effect in enhancing the methane potentials of SFD, while enzymatic treatment resulted in an increase in the methane yield of only 13%. Menardo et al. [38] assessed in four different mechanically separated SFDs the effects of a thermal pretreatment, and they obtained no statistically significant effect on the daily yields of thermal pretreated samples, but reported a significant increase in the cumulative methane yields that ranged from 35% to 171% depending on nature of the organic matter of the considered samples. Cumulative biogas increments of 165% were reported by Mustafa et al. [58] using on rice straw a combination of physical and biological pretreatments (i.e., milling and fungal pretreatment with *P. ostreatus*). However, the beneficial income gained from the enhanced biogas yields need to be evaluated together with the higher energy requirements, operational costs, and environmental issues linked to abiotic pretreatments.

In lab-scale experiments, the sterilization of feedstocks is a routine step prior to fungal pretreatment to kill indigenous undesired microbes and assure inoculated microorganisms the best working conditions. However, sterilization is extremely expensive and requires additional energy, materials, and time, preventing its application to an industrial-scale level [26,40]. The proposed technology, which directly uses fungal inocula to pretreat unsterilized biomass, represents a fundamental advantage for future industrial application [26]. Despite the fact that this process developed in non-sterile conditions would allow an easier scale-up and a reduction in the productivity expenditure, very few works about it can be found in the literature. Moreover, pretreatments applied on unsterilized substrates are often destined to fail, since it is extremely challenging to set the optimal conditions necessary to get an efficient colonization and to keep inoculated fungi prevailing over indigenous microflora [20,26]. For instance, the pretreatment performed by Zhao et al. [26] on unsterilized yard trimmings inoculated with *C. subvermispora* was unsuccessful. Similar results were also described by Reid [59], who used *Phlebia tremellosa* to inoculate unsterilized aspen wood. The results reported by these authors and obtained in this study emphasize the key role played by the right experimental set up, working condition, and type and origin of the feedstock in pretreatment success.

5. Conclusions

Considering the low inputs and risks, the developed biological treatments appear to be a cost-effective and environmentally friendly technology to ultimately makes the SFD more susceptible to a further digestion step and increase its biogas and methane yields. Favoring the (re)use of SFD as a feedstock for AD, the fungal pretreatments contribute to the development of a next-generation by-product management strategy. According to the literature, the reuse of SFD in the anaerobic digester has the potential to improve the total methane production of the ABP by between 4% and 8%. By significantly enhancing the biogas and methane potentials, the fungal pretreatments of the SFD could permit us to obtain an even higher extra electrical production that could correspond to a significant economical income for ABP owners.

New questions have been arisen, such as the precise contribution of the fungal biomass itself to the AD process. The literature is scarce, creating a lack of knowledge that must be filled with targeted investigations. The exploitation of spent fungal biomass as AD feedstocks would bring benefits in terms of renewable energy production and sustainable waste disposal, since it is produced in large volumes by many industrial processes.

Ultimately, the integration of the fungal pretreatment of SFD and its subsequent reuse in the anaerobic digester has the potential to allow greenhouse gaseous loss abatement and concurrent energy recovery, leading to environmental and economic benefits that make the overall sustainability of AD technology even more attractive and effective.

Author Contributions: Conceptualization, A.Z., F.S., L.R., G.C.V., and E.D.; Data curation, A.Z.; Formal analysis, A.Z.; Funding acquisition, G.C.V. and E.D.; Investigation, A.Z.; Methodology, A.Z., F.S., and L.R.; Project

administration, G.C.V. and E.D.; Resources, G.C.V. and E.D.; Software, A.Z. and L.R.; Validation, F.S., L.R., G.C.V., and E.D.; Visualization, A.Z.; writing—original draft, A.Z.; writing—review and editing, F.S., G.C.V., and E.D. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Acknowledgments: The authors would like to thank the biogas plants owners for allowing the sampling of biomasses. The technical assistance of MUT staff and Giulia Blanchi is greatly acknowledged. A special thanks to the JRU MIRRI-IT for the technical and scientific support.

Conflicts of Interest: The authors declare no conflict of interest.

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