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Insights into Anaerobic Co-Digestion of Lignocellulosic Biomass (Sugar Beet By-Products) and Animal Manure in Long-Term Semi-Continuous Assays

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Abstract: Biogas production through anaerobic digestion has proven to be one of the most important pillars of the transition into the circular economy concept, a sustainable approach for biorefinery. This work aims to extend and improve knowledge in the anaerobic co-digestion of complementary substrates, given insights into wastes biodegradability and the influence of manure composition on the anaerobic process stability. Anaerobic co-digestion of sugar beet by-products with two kinds of animal manure (pig and cow) was investigated in semi-continuous assays, analyzing both common and non-classical parameters. Co-digestion with manure clearly mitigated the inhibitory effect of volatile fatty acids at high organic loading rates, leading to increases in methane production by 70% and 31% in comparison with individual digestion of sugar beet by-products, for co-digestion with pig and cow manure, respectively. Non-classical parameters could give more insight into the coupling/uncoupling of the anaerobic digestion phases and the involved microorganisms. Indirect parameters indicated that the process failure at the critical organic loading rates was mainly due to methanogenesis inhibition in the co-digestion with pig manure, while in co-digestion with cow manure or in individual digestion of sugar beet by-products, both hydrolysis–acidogenesis and methanogenesis phases were affected. Biomethanation degree refers to the maximum methane potential of organic wastes. Sugar beet by-products required a long digestion-time to reach high biodegradability. However, short digestion-times for co-digestion assays led to a high biomethanation degree.

Keywords: anaerobic co-digestion; sugar beet by-products; manure; semi-continuous feeding mode; methane improvement; non-classical parameters

1. Introduction

According to the European Economic and Social Committee, the success of the circular economy is based on three pillars. The first pillar is about energy consumption (i.e., the efficiency in energy sources). The second pillar is about reducing the cost of product processing (i.e., sustainable technological processes for waste-out systems designing), and the third one concerns employment.

Biogas production through anaerobic digestion (AD) has proven to provide direct benefits to the second pillar and indirect benefits to the rest of the pillars, achieving the transition into the circular economy, a sustainable approach for the biorefinery concept [1].

Currently, agriculture is one of the industrial sectors that generates the largest amount of wastes. Therefore, 1.3 billion tonnes of food are yearly discarded during production handling,

storage processing, and distribution, with an estimated carbon footprint of 3.3 billion tonnes of CO₂ equivalent of greenhouse gases (GHG) released into the atmosphere per year [2]. Agroindustrial wastes such as sugarcane bagasse, wheat straw, wheat bran, and many others are cheapest and abundantly available. Their valorization by AD has long been investigated and implemented for bioenergy production [3,4]. By-products from sugar beet plant after the sugar extraction process may be bio-converted into bioenergy through AD [5].

Similarly, a large amount of manure produced in livestock facilities is a potential candidate for clean energy production such as biogas [6]. Moreover, livestock production in the European Union is high, being Spain and Germany the largest producers of pigs, while France held the largest number of bovines [7]. It has been reported that AD of animal manure can save up to 20% of the dairy processing facility's energy demand [8].

Despite their suitability for the anaerobic treatment, the use of manure as feedstock in AD could inhibit to methanogenic archaea due to the ammonia released during the process [9,10]. Manure has been traditionally used as a fertilizer for agricultural soils due to its high content in nitrogen and phosphorus. Nevertheless, this uncontrolled practice may have environmental problems, such as groundwater contamination due to infiltration, or greenhouse gas emissions [11]. Furthermore, lignocellulosic agroindustrial wastes and by-products lack of some macro and micronutrients [12,13].

The AD of both kinds of wastes separately may be improved and optimized by co-digestion as this biomass has different/complementary characteristics [14,15]. Sugar beet by-products (SBB) are rich in carbon while manure is a potential substrate to compensate nitrogen deficiency in the anaerobic process, together with minerals and essential nutrients. Moreover, manure provides a high buffer capacity, which could neutralize volatile fatty acids (VFAs) released by agroindustrial wastes in the AD process [16,17].

Besides, the manure type plays an important role in the AD process. Hence, some manure resulted in more effective in the co-digestion process with agroindustrial wastes than others [18]. This effect returns to several factors such as the composition of each manure according to its origin (animal digestive tract), the food given to animals in the course of animal husbandry, and the synergy between the manure and agroindustrial waste used in co-digestion [19–21]. Despite the numerous benefits of co-digestion, the mixture of substrates with different characteristics could create either synergistic interactions of microorganisms or antagonist interactions, which in this case, can worsen the anaerobic process [22].

To date, several previous studies have investigated anaerobic co-digestion of agroindustrial wastes with livestock manure to enhance biogas production and in most of these studies; synergistic effect between the two kinds of substrates has been suggested to be the reason of the process improvement. However, only a few studies have thoroughly investigated the effect of manure on the neutralization of acidity from agroindustrial wastes in semi-continuously operated reactors.

Cheng and Zhong [23] have demonstrated that the use of swine manure as a co-substrate helps to neutralize the high VFAs concentration released by cotton stalk, emphasizing advantages related to the high buffer capacity provided by swine manure. Other authors have used a different strategy to minimize the acidification by VFAs in a biological process, by adding some micronutrients [12,13].

The use of the semi-continuous feeding mode in anaerobic digesters operating for a long-term period is of great interest as it gives insight into the most viable options for the organic material treatment through the AD in a view of a real scale approach. Optimization of hydraulic retention times and organic loading rates, as well as information on feedstock combination, may help in future research and decisions on agricultural and manure management and treatment for efficient and long-lasting wastes treatment plants in rural and agricultural areas.

This work aims to extend and improve knowledge in the anaerobic co-digestion of complementary substrates, given insights into wastes biodegradability and the influence of manure composition on the AD stability. Anaerobic co-digestion of sugar beet by-products with two kinds of animal manure, pig and cow, (PM and CM) was investigated in semi-continuous assays, analyzing both common

and non-classical parameters. This research addresses a deep analysis of the findings from long-term anaerobic digesters operating with agricultural and animal wastes.

2. Materials and Methods

2.1. Feedstock and Inoculum Characteristics

Sugar beet by-products were a mixture of exhausted pulp (85%) and molasses (15%). They were provided by the sugar processing company in the south of Spain (El Portal, Jerez de la Frontera, Andalusia). The used SBB have a total solids content of 80–90%, which was adjusted to 8% of TS to avoid the non-homogenization of the reactor content due to the rheological behavior of SBB [24]. For this purpose, the SBB as dried pellets were rehydrated with deionized water for 24 h previously to their use. Cow and pig manure were collected from two different livestock facilities in the same zone. The inoculum for the start-up of the single AD of SBB (Inoc1) was provided from a laboratory-scale reactor under semi-continuous operation that was fed with the organic fraction of municipal solid wastes (OFMSW), which was first adapted to the new substrate SBB for a long period before starting the herein studied assays [25]. The effluent from the reactor of single digestion of sugar beet by-products was used as inoculum for the start-up of co-digestion assays (Inoc2). In co-digestion reactors, the mixture between the two co-substrates has been established as 25% of SBB and 75% of pig manure or cow manure (v/v) [26,27].

The SBB, CM, PM, and inocula characteristics are presented in Table 1.

Table 1. Physicochemical properties of sugar beet by-products (Sugar beet by-products, cow manure, pig manure), and inocula.

Parameters (Units)	SBB	CM	PM	Inoc1	Inoc2
pH	5.7 ± 0.7	6.2 ± 1.3	6.5 ± 1.7	7.5 ± 0.7	7.6 ± 0.8
TS (g/kg)	880.3 ± 12.4	222.7 ± 11.5	225.3 ± 7.4	41.2 ± 1.3	36.1 ± 10.6
VS (%TS)	90.6 ± 1.8	77.7 ± 6.7	75.1 ± 5.2	16.3 ± 1.6	21.8 ± 3.3
CODS (gO ₂ /kg TS)	64.7 ± 2.5	77.7 ± 13.9	83.0 ± 7.9		246.5 ± 6.1
CODT (gO ₂ /kg TS)	165.8 ± 5.9	308.5 ± 6.3	220.6 ± 11.1		321.3 ± 6.9
DOC (gC/kg TS)	49.7 ± 5.1	33.7 ± 9.8	39.1 ± 6.6	150 ± 5.0	127.4 ± 19.4
TVFA (gHAc/kg TS)	2.5 ± 0.9	24.3 ± 4.9	20.4 ± 2.2	7.2 ± 2.4	47.1 ± 4.3
Alkalinity (gCaCO ₃ /kg TS)	3.3 ± 1.4	170.2 ± 0.9	169.9 ± 49.3		116.4 ± 69.3
N-NH ₄ ⁺ (gN/kg TS)	0.3 ± 0.1	15.7 ± 3.2	10.6 ± 2.6		30.5 ± 5.5
TN (gTN/kg TS)	14.5 ± 1.5	157.6 ± 6.7	145.2 ± 12.4		174.5 ± 3.6
Ratio C/N	38.9 ± 2.7	13.2 ± 1.0	13.5 ± 0.3	-	19.4 ± 2.4
Hemicellulose (%)	15.4 ± 0.3	13.9 ± 0.8	14.4 ± 0.1	-	-
Cellulose (%)	25.9 ± 0.3	23.2 ± 1.2	16.3 ± 1.2	-	-
Lignin (%)	1.6 ± 0.1	19.8 ± 1.1	16.8 ± 1.9	-	-
Mineral salts (%)	6.7 ± 0.2	26.2 ± 1.0	27.2 ± 3.1	-	-

The organic content of SBB was the highest since the total solids were almost all volatile (90%). The VS content of manure was also higher (75–77%) with a high fraction of nitrogenous material. The C/N ratio of SBB was much higher than for animal manure, which led us to expect a complementarity between the two kinds of substrates in terms of carbon and nitrogen contents. Concerning the lignocellulosic content of SBB and manure, all of them could be considered as lignocellulosic substrates with a high cellulose fraction in SBB and CM. Moreover, the mineral content of PM and CM is much higher than for SBB. Trace elements are also necessary for the AD process and are considered to stimulate the digestion of cellulosic material [26]. Therefore, manure are natural sources of trace elements and their mixture with SBB gives rise to an appropriate medium for the growth of microorganisms. Wintsche et al. [28] reported that supplementation of trace elements in anaerobic reactors leads to stable and more efficient methane production processes while deficits in these elements may cause process imbalances. Both manure have a high mineral content and important alkalinity indicating a high buffering capacity.

2.2. Semi-Continuous Digesters

Three semi-continuous stirred tank reactors (SSTR) were used for individual digestion of SBB, co-digestion of SBB with CM, and co-digestion of SBB with PM. Assays were carried out at mesophilic temperature (35 °C). Reactors are comprised of stainless steel with a useful volume of 10 L. The temperature was maintained by a recirculating bath (Ultratherm200-Selecta) through the reactor jacket. A motor installed at the top of the reactor (Heidolph-RZR-2102) with a stirring blade performed the mixing of the reactor content.

Table 2 depicts the hydraulic retention times (HRT) and organic loading rates (OLR) studied in each digester.

Table 2. The operation conditions (hydraulic retention times and organic loading rates) of the three semi-continuous stirred tank reactors (SSTR) digesters.

Reactors		Hydraulic Retention Times (Days)						
		20	18	15	12	8	6	5
SBB	Organic loading rates (gVS/Lreactor*d)	3.3	3.6					
SBB + PM		4.2	4.7	5.9	7.4	8.5	11.2	12.8
SBB + CM		3.7	4.2	4.9	6.2			

The first HRT tested was at 20 days according to literature in which the AD of lignocellulosic biomass and similar substrates to this study indicated the need for high HRT [29]. Demirer and Scherer applied HRTs with a range of 95–15 days to sugar beet silage and reported that the HRT around 25 days gave the highest biogas yield [29].

2.3. Analytical Methods

Total solids (TS), volatile solids (VS), and alkalinity were measured according to APHA Methods 2540B, 2540E, and 2320B, respectively [30]. The chemical oxygen demand (COD, total, and soluble) was determined by colorimetric techniques using a spectrophotometer instrument (HACH[®], DR/4000U), according to the method 5220C [30]. Total chemical oxygen demand (TCOD) was measured directly in the sample while for soluble chemical oxygen demand (SCOD) and the rest of the soluble parameters; samples were lixiviated and filtered through a 0.47 µm filter. Total nitrogen (TN) and ammonium (N-NH₄⁺) were analyzed by distillation (Selecta[®], Pronitro II) according to the 4500-NH₃E method [30]. Filtered samples for the volatile fatty acids (VFAs) analysis were filtered once more through a 0.22 µm Teflon[®] filter and analyzed with a gas chromatograph (Shimadzu[®], GC-2010).

The dissolved organic carbon (DOC) was analyzed in an automatized carbon analyzer (Analytic-Jena[®], multi N/C 3100), according to the combustion-infrared method 5310B [30]. The biogas was collected in a 10 L gas-bag (Tedlar[®], SKC). The biogas volume was daily measured by a high precision drum-type gas meter (Ritter[®], TG5). The composition of biogas was analyzed by a gas chromatograph (Shimadzu[®], GC-2014) [15]. For the lignocellulosic content of substrates (cellulose, hemicellulose, lignin), the Van Soest method was applied by using an automatized analyzer (Foss[®], FIBERTECTM 8000) [31].

2.4. Biomethanation Degree Calculations

The biomethanation degree (BD) of a substrate or different co-substrates refers to the potential of methane production from the selected organic wastes and by-products in specific conditions. To find out the biomethanation degree of the substrates in a semi-continuous reactor, the parameter defined in Equation (1) was used. Thus, BD was calculated as the percentage of methane yield achievable in a semi-continuous assay regarding the maximum methane yield attainable as the methane potential of organic wastes, which can be determined by a batch test in the same experimental conditions.

As previously mentioned, in an earlier study of Aboudi et al. [26], a series of batch assays were carried out to find out the best mixture ratio between the same substrates used in the present work.

Later on, the authors have studied the effect of the total solids content on the methane yield, also in a batch study with the same substrates at the best mixture ratios between them [27]. The ultimate methane potentials of SBB + CM and SBB + PM were 464.5 LCH₄/kgVS_{added} and 451.4 LCH₄/kgVS_{added}, respectively. The maximum methane potential of SBB was 308.8 LCH₄/kgVS_{added}. These ultimate methane potential productions were in agreement with other works studying the co-digestion of manure and by-products from tuber plants [32,33].

$$\text{Biomethanation degree (\%)} = \frac{100 \cdot \text{SMP}_{\text{semicontinuous}}}{\text{MBMP}_{\text{batch}}} \quad (1)$$

$\text{SMP}_{\text{semicontinuous}}$ is the specific methane production in SSTR, expressed as LCH₄/kgVS_{added} and $\text{MBMP}_{\text{batch}}$ is the maximum biomethane potential obtained in batch assays in similar conditions and the same wastes, also expressed as LCH₄/kgVS_{added}.

2.5. The Indirect Carbon-Related Parameter Calculations: Acidogenic Substrate as Carbon

Non-classical parameters measured indirectly have shown to give substantial information about the AD process, giving interesting insights into the organic material degradation and bioconversion into methane [34]. In this sense, indirect parameters such as the acidogenic substrate as carbon (ASC), which refers to the organic carbon non-converted into VFAs, could be obtained according to Equation (2), from the subtraction of two directly measured parameters: dissolved organic carbon (DOC) and volatile fatty acidity (DAC).

$$\text{ASC} = \text{DOC} - \text{DAC} \quad (2)$$

DAC is accounting the carbon contained in the different VFAs from C₂ to C₇ and it is obtained according to Equation (3).

$$\text{DAC} = \sum_{i=2}^{i=7} \frac{AiH \cdot ni \cdot 12}{MWi} \quad (3)$$

where “ AiH ” is the concentration of each volatile fatty acid; “ ni ” and “ MWi ” are the numbers of carbon atoms and the molecular weight of each acid, respectively.

These parameters have been used as a useful tool to determine the critical stage of the AD process [15,34,35]. Thus, the accumulation of the ASC in the anaerobic digester usually indicates an imbalance between the hydrolysis and the acidogenic phases, while the accumulation of the DAC has been related to an imbalance between the acidogenesis and the methanogenesis phases.

3. Results and Discussion

3.1. Methane Production Yields and Process Efficiency

Daily methane productions (DMP) in the three SSTR are depicted in Figure 1A–C.

As shown in Figure 1A, individual digestion of SBB at 20-day HRT was stable for a long period of operation with values around 840 LCH₄/m³reactor*d. The 18-day HRT showed similar productions for approximately one period of this HRT. However, production decreased by 44%, in comparison with the previous HRT. For the SBB + CM reactor Figure 1B, the decrease in HRT from 20 to 18 days and consecutively to 15 days, led to an improvement in methane production of 19% and 28%, respectively. However, operation at 12-day HRT induced a pronounced decline in methane generation by 61%. In the case of the SBB+PM reactor, daily methane productions increased by 24%, 35%, 46%, 55%, and 60% when HRT decreased to 18 days, 15 days, 12 days, 8 days, and 6 days, respectively, indicating a high system performance at lower HRTs and higher OLRs.

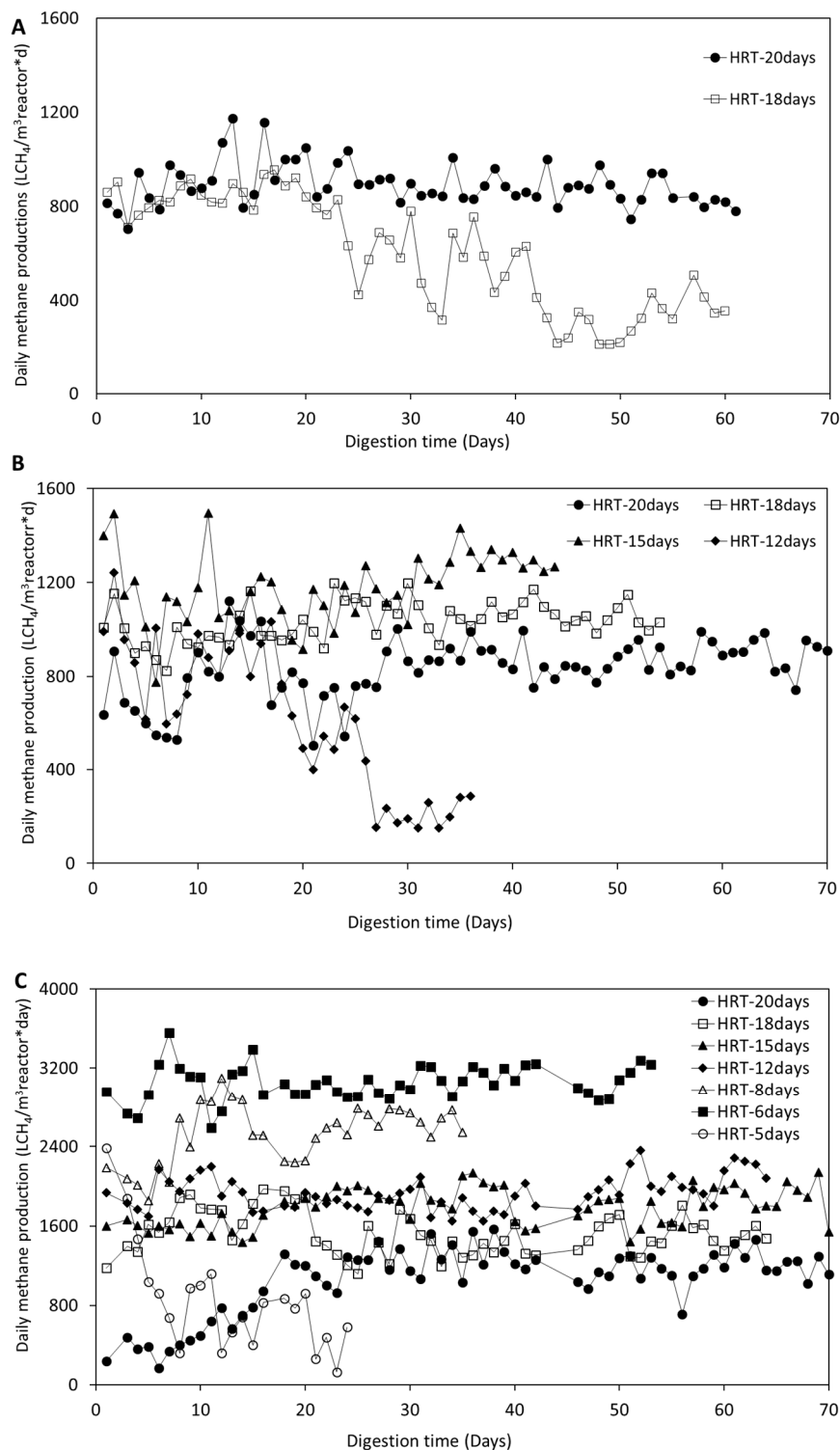


Figure 1. Daily methane productions (production rates as LCH₄/m³ reactor*day) in the three SSTR digesters at different hydraulic retention times. (A) SBB, (B) SBB + CM, and (C) SBB + PM.

Nevertheless, the operation at 5-day HRT (12.8 gVS/Lreactor*d as OLR) was critical, leading to an abrupt decrease in methane generation, obtaining only 800 LCH₄/m³ reactor*d (a drop of 28%).

In both co-digestion assays, manure addition was shown to significantly increase methane production, as well as allowing increasing the organic load supplied to the reactor. In previous studies, Li et al. [32] reported that chicken manure or pig manure co-digested with apple pulp allowed

the increase of OLR from 2.4 to 7.2 gVS/Lreactor*d, being 4.8 gVS/Lreactor*d the best OLR studied concerning the increase in methane generation. The daily methane productions increased by 56.9% and 47.4%, in comparison with individual digestion of chicken manure and pig manure, respectively. Kapajaru and Rinatal [33] studied anaerobic co-digestion of potato tuber (including stillage and peels) with pig manure in mesophilic SSTR. The authors reported that methane productions increased by 54.5% in comparison with the single digestion of PM. Similarly, Damaceno et al. [36] studied sweet potato co-digestion with sludge cake from poultry slaughtering. They found that the balanced nutrient content provided an optimum environment for the microorganism activity in co-digestion reactors, with 80% of poultry slaughtering and 20% of sweet potato. In another attempt, Panichnumcin et al. [37] reported that waste proportion in the mixture is a very important factor in co-digestion assays. They studied co-digestion of cassava pulp with PM in mesophilic SSTR at a constant OLR of 3.5 gVS/Lreactor*d and a 15-day HRT. In their study, cassava pulp up to 60% has shown the highest biogas generation. These findings are in agreement with those observed by Aboudi et al. [26] in batch studies about the optimization of the substrates mixture ratios for SBB and manure.

The methane content in the biogas produced by the SBB reactor decreased by 14.4% when decreasing the HRT to 18 days (OLR of 3.6 gVS/Lreactor*d). In co-digestion assays, the methane content in biogas from the SBB+CM reactor decreased by 46% at the HRT of 12 days (OLR of 6.2 gVS/Lreactor*d), while in reactors containing SBB+PM the methane content was almost maintained above 40% in all stages, with the highest content at the HRT of 20 and 18 days.

Figure 2 shows the obtained biomethanation degree in each assay.

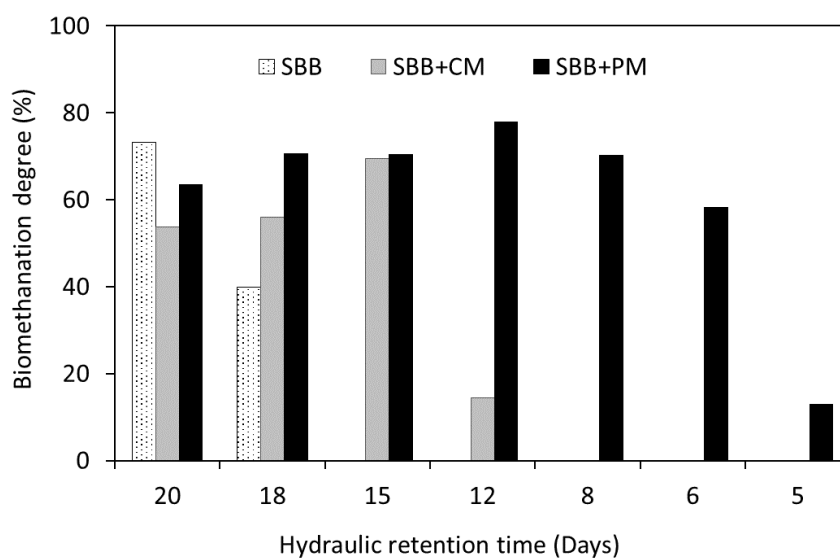


Figure 2. Biomethanation degree in the three SSTR digesters.

It can be observed that for SBB single digestion and at the optimum HRT of 20 days, a high biomethanation degree of 73% was obtained. The SBB substrate required a long time for its degradation (high HRT), and decreasing this time led to reaching low substrate biodegradability. Moreover, in both co-digestion assays, above 60% of biomethanation degree was achievable at the different HRT studied, excepting at the critical HRT tested in each experiment: 5-day HRT in SBB + PM (BD was only 13%) and 12-day HRT in SBB + CM (BD was only 14%). The highest value of the BD was around 78% in the reactor of SBB+PM at the HRT of 12 days. These results demonstrated that co-digestion played an important factor in wastes degradation likely due to the synergistic effect created in digesters, being PM more suitable to improve the biodegradability of the digester content than CM.

According to Smith and Mah [38] and from the results of their research using C^{14} tracers in an AD study of sludge, authors observed that the 73% of the total methane produced in an anaerobic process comes from the acetoclastic pathway, while the 27% usually comes from the

hydrogen-utilizing pathway. Nevertheless, recent studies found that in the anaerobic digestion of nitrogen-rich substrates, the methanogenesis shift from acetoclastic pathways to the syntrophic acetate oxidation-hydrogenotrophic (SAO-HM) route [39–41]. In a recent study by Yin et al. [41], the authors attempted to explain methanogenic pathways in anaerobic digestion of nitrogen-rich substrates. The authors studied acetate conversion by C^{13} isotope under mesophilic and thermophilic conditions.

Results showed that in both temperatures, the syntrophic acetate oxidation-hydrogenotrophic (SAO-HM) pathway was dominant and that methanogenic pathway shifting, induced by high ammonia levels, closely correlated to the process performance. In this sense, it has been deduced that the substrate characteristics condition the predominance of one pathway or another.

In the present research, the combination of SBB as a high content carbonaceous material and manure as high content nitrogenous substrates led to balance the nutrients content of the anaerobic process and hence, the inhibition by ammonia was avoided.

Aiming for a comprehensive comparison of the obtained methane productions in the present research, the criterion developed by Smith and Mah [38] has been applied to the results. Thus, specific methane productions (SMPs) for the three semi-continuous digesters are depicted in Figure 3. The highest SMP from SBB+PM (HRT from 18 to 8 days) and the highest SMP from SBB+CM (HRT of 15 days) have been selected as representatives of the optimal operation area (the blue box). The average value of the five data of SMPs was $331 \text{ LCH}_4/\text{kgVS}_{\text{added}}$. The black discontinuous line in the figure corresponded to the SMP value of 27% of the average SMP obtained ($89.5 \text{ LLCH}_4/\text{kgVS}_{\text{added}}$). The green line corresponded to the SMP value of 73% of the average SMP obtained ($241.8 \text{ LCH}_4/\text{kgVS}_{\text{added}}$).

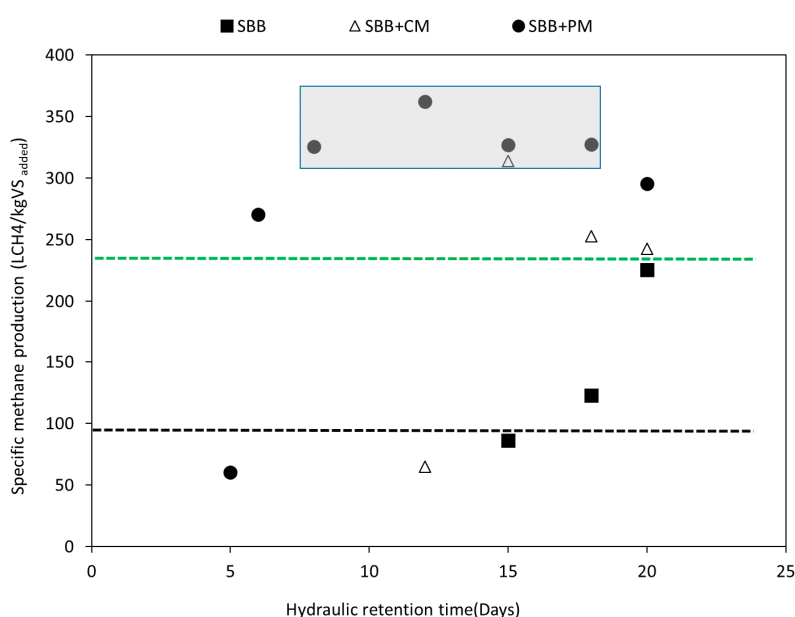


Figure 3. Specific methane productions at the different hydraulic retention time in the three SSTR digesters. Greenline: the specific methane production (SMP) value of 73% of the average SMP obtained. Blackline: the value of 27% of the average SMP obtained. Blue box: the highest SMP values in anaerobic digesters.

As can be observed, the critical operational condition in each assay (lower HRT/Higher OLR) has shown SMP values slightly below the limit value of $89.5 \text{ LCH}_4/\text{kgVS}_{\text{added}}$, indicating that acetoclastic methanogens were affected in failure periods. In critical HRTs, the accumulation of VFAs and the drop in pH to values ranged from 5.6 to 6.3 occurred indicating the inhibition of the systems. It has been reported that acetotrophic methanogens are strongly inhibited below a pH of 6.2 [41]. Figure 4 shows the average values of pH in each stage (HRTs) for the three SSTRs.

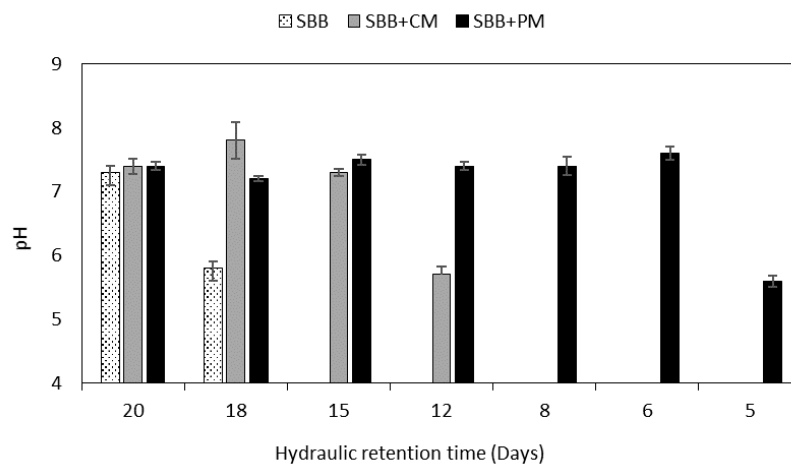


Figure 4. Average pH values in each stage in the three semi-continuous reactors.

Figure 5 shows the organic matter removal expressed as the percentage of volatile solids removed from the systems.

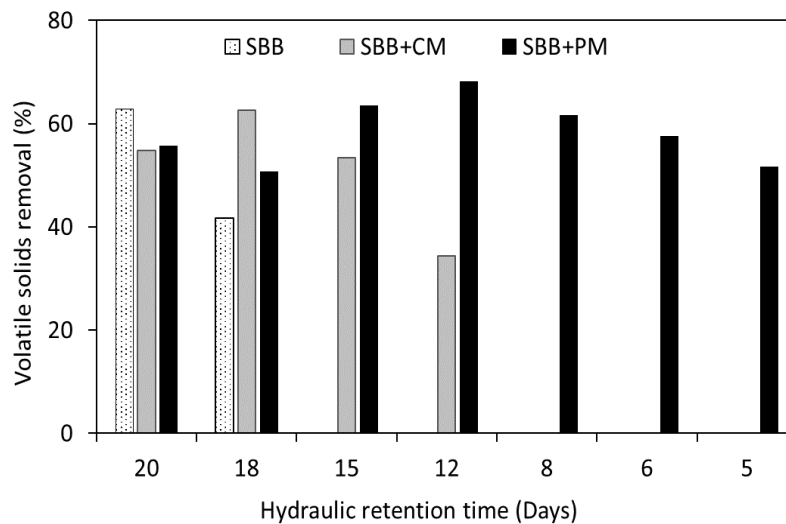


Figure 5. Volatile solids removal in the three semi-continuous reactors.

In the SBB digester, operation at a lower HRT (higher OLRs) of 18 days led to a decrease in the efficiency of the organic matter removal. In the SBB+PM reactor, the decrease in VS removal was gradual from 12-day HRT to 5-day HRT. The same behavior was observed in the SBB+CM reactor, with a gradual decrease from 18-day HRT until 12-day HRT.

3.2. Analysis of the Process Stability Based on the Indirect Carbon-Related Parameters

Fdez-Güelfo et al. [34] analyzed the indirect parameters of a semi-continuous anaerobic digester fed with the OFMSW. The authors reported that decreasing the HRT from 15 to 10 days affected the hydrolysis stage, which induced a sequential failure of the acidogenesis stage, and therefore, the methanogenesis acetoclastic pathway was disturbed. Moreover, the further decrease of HRT to 8 days has resulted in the wash-out of microbial populations involved in the anaerobic process with complete inhibition of both acetoclastic and H₂-utilizing archaea.

Figure 6 shows the evolution of the indirect parameters, together with the dissolved organic carbon (DOC) evolution in the three SSTR at the different OLRs applied.

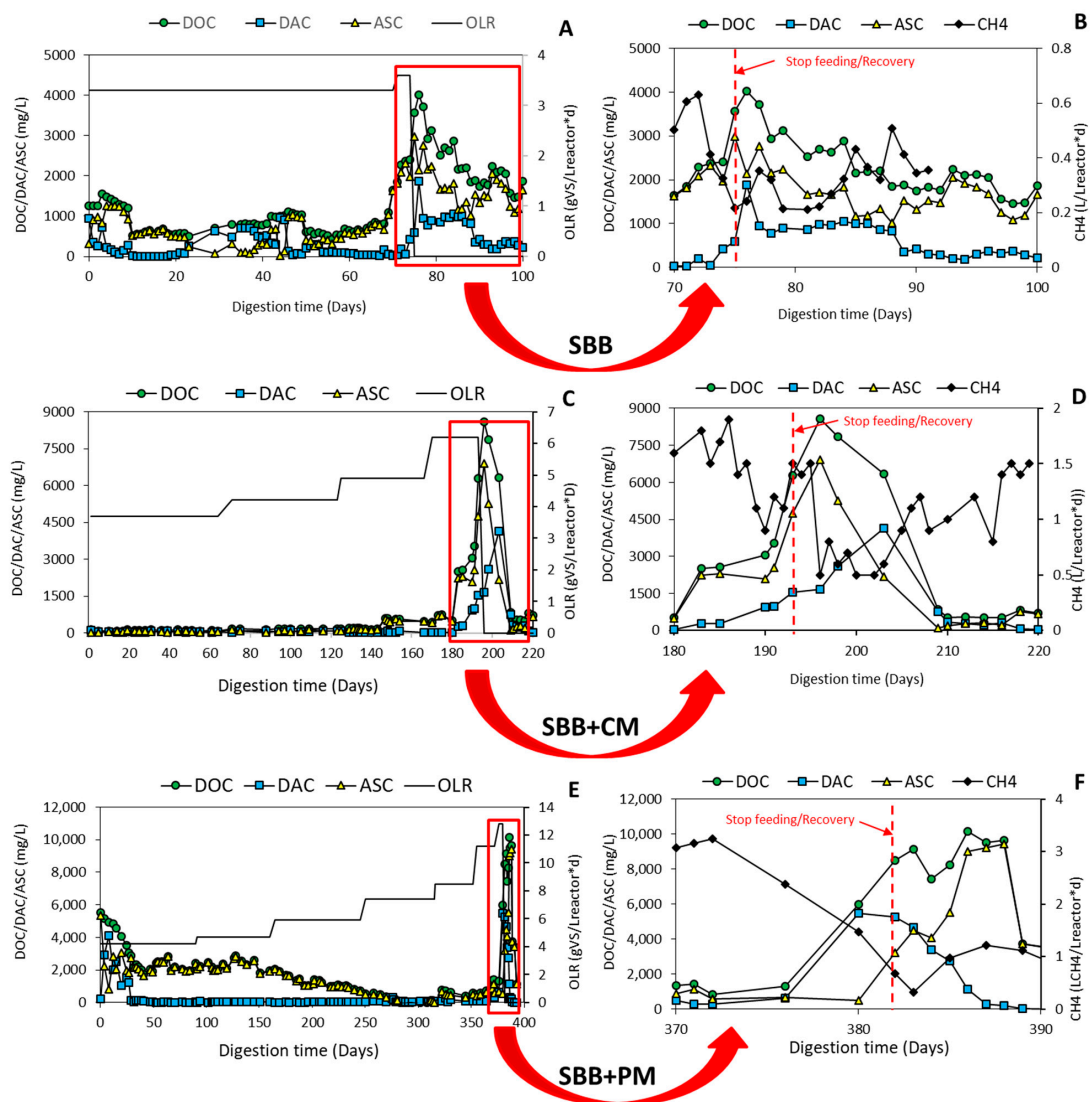


Figure 6. Evolution of the dissolved organic carbon (DOC) and indirect parameters in the three SSTR. The detailed figures of failure operation periods for each digester are illustrated on the right. (A,B) SBB digester; (C,D) SBB+CM digester; (E,F) SBB+PM digester.

At the startup period of the SBB digester and for a short period, the DOC parameter increased showing slight acidification due to the DAC increase, this behavior is usual at the inception period indicating the adaptation of microorganisms to the substrate and the operational conditions. Consecutively, the DOC and DAC values decreased gradually leading to methane generation (see Figure S1 in the supplementary material). It can be observed that in the stable operation period, the DAC values were low, being very similar to DOC values and, hence, no inhibition was observed.

The OLR increase to concentrations of 3.6 gVS/Lr*d, led to an initial increase in methane productions increased for the first few days, as a response to the organic material rise in the feedstock. The DOC and the ASC also showed a gradual increase during these few days. Nevertheless, once the DAC started increasing and despite the low proportion of the DAC with regard to the ASC, methane production dropped sharply indicating a distortion of the activity of methanogens. It has been observed that the lowest methane production (data not shown) matches the highest DAC value. The feeding was stopped to avoid further DAC accumulation and after several days, the SBB reactor has begun to recover as the DAC decreased in the medium.

In the SBB + CM reactor, operation at OLRs from 3.7 to 4.9 gVS/Lr*d showed low values of the DOC and the indirect parameters (DAC and ASC) with high methane production. It was observed

that in the mid-operation period at OLR of 4.9 gVS/Lr*d (HRT of 15 days), these parameters showed a slight increase.

The next step using a further high OLR showed an increase in the three carbon-related parameters with the predominance of the ASC. It has been observed that during the failure period, the DOC increased with the rise of both the DAC and the ASC, however, a lag period between these two parameters has been detected. Hence, the disturbance period started with fluctuations in methane productions with lower values, but the highest content of the DOC was as ASC and only when the methane decrease was pronounced, the DAC proportion in the DOC was higher. This behavior could be likely due to failure in the hydrolysis and acidogenesis firstly, followed by the inhibition of methanogenesis. In this sense, an imbalance between the AD steps occurred. In this co-digestion digester, only the increase in OLR affected the process, however, CM shows the advantage that in the startup period, no accumulation of DAC or ASC was observed, indicating that a mixture of SBB and CM as complementary substrates has benefited the process. Furthermore, CM comes from the digestive tract of cows, which are herbivores ruminants and their rumen contains microorganisms able to degrade lignocellulosic substrates [42]. Nevertheless, the characteristics of CM showed that this manure provides more cellulosic material than PM.

In the SBB+PM digester, the startup period showed high DOC and DAC concentrations, which illustrate a necessary adaption period for microorganisms in this reactor. In this case, the DAC proportion in the DOC was very low in comparison to the ASC parameter. Moreover, it can be observed that as the OLR increased, the DOC and ASC decreased gradually to reach very low values when operating at OLR between 7.4 and 11.2 gVS/Lr*d, corresponding to HRTs from 12 to 6 days. An immediate decrease in methane productions accompanied by an increase in the DAC parameter was observed at the OLR of 12.8 gVS/Lr*d (HRT of 5 days). In this case, and unlike what was observed in the two previous reactors, the ASC parameter was very low and all the DOC was in the acids form, indicating that only methanogenesis was affected when the reactor was overloaded. This behavior could likely be related to the wash-out of microbial populations involved in the anaerobic process, as previously reported by Fdz-Güelfo et al. [34]. The cease of feeding led to the DAC decrease and hence the reactor has begun to recover. In all reactors, the recovery of the process has only been possible when the DAC decreased significantly.

3.3. Analysis of the Process Stability Based on the Classical Parameters

The stability of the three SSTRs has been assessed by using the parameters alkalinity and total volatile fatty acidity, among others. Figure 7 shows the total alkalinity, the acidity to alkalinity ratio, and the propionic acid concentrations, and the propionic to acetic acids ratio (A, B, C, and D, respectively) in each operational condition.

The alkalinity of both co-digestion reactors was significantly higher than that observed in the single digestion of SBB. This fact is expected due to the characteristics of the substrates involved in each assay. The SBB is rich in carbonaceous material but is deficient in nitrogen and alkalinity. Besides, it can be observed that alkalinity in co-digestion with PM was higher than for CM. This fact is likely due to the high buffer capacity provided by pig manure, which allows neutralizing organic acids in the system, and therefore, the highest OLRs applied were reached in this reactor. Nevertheless, the critical OLR and HRT in each digester led to a decrease in alkalinity. The criteria acidity/alkalinity ratio is an indicator of systems failure due to inhibition by acidification [43–45].

Furthermore, it has been observed that when reactors failed, the propionic acid was the predominant VFAs in the medium, which justify disturbances of systems due to propionic acid, a well-known strong inhibitor in the anaerobic process [46,47]. The ratio between acetic and propionic acids (HPr/HAC) has been designed as a useful tool for AD systems failure due to acidification [46,48], being considered critical the values above 1.4. In the present study, the individual digestion of SBB at the 18-days-HRT led to a higher HPr/HAC value than the tolerated limit by the system.

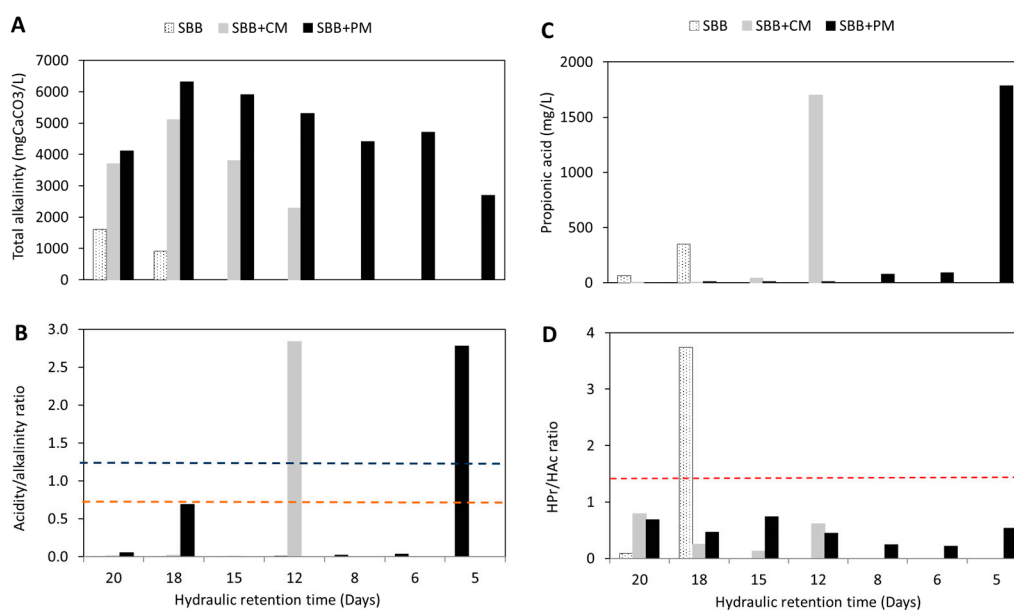


Figure 7. The classical parameters related to the process stability in the three SSTR. (A) The total alkalinity, (B) the acidity/alkalinity ratio, (C) the propionic acid concentrations, and (D) the propionic to acetic acid ratio. Values below the blue line refer to suitable values, values above the orange line indicate the critical threshold for proper operation, and values between both lines refer to alarming values that could distort the digesters. The red line refers to the threshold limit for the HPr/HAc ratio, as values above 1.4 were considered as critical for proper operation.

The characteristics of each manure in terms of alkalinity, ammonia, and the variety of microorganisms provided from the digestive tract of the animals may be the main reasons for the observed behaviors.

It has been reported that the ratio acidity to alkalinity is useful as an indicator of anaerobic reactor stability [43–45]. Thus, values above 0.8 predict the reactor instability. Values below 0.4 indicate proper operation and values with a range of 0.4–0.8 predict that the operating conditions are at the tolerance limit for microorganisms in which the destabilization of the system started. In this sense, it has been corroborated that at HRTs of 18 days, 12 days, and 5 days, respectively for SBB, SBB+CM, and SBB+PM reactors, the tolerance limit of microorganisms was altered and a lack of buffer capacity to neutralize the VFAs released occurred.

Previous studies have reported that SBB is rich carbonaceous material, suitable to be converted into biogas. However, limitations due to the high acidification resulted as a handicap for the process efficiency. Alkaya et al. [17] observed the increase of VFAs in the AD of sugar beet pulp at higher OLRs and lower HRTs. However, the use of an external source of alkalinity, decreased acidification of reactors, maintaining an adequate pH. Similarly, in a study of Demirel and Scherrer [49], the individual AD of sugar SBB (sugar beet silage) in SSTR at six different HRTs ranged from 95 to 15 days, showed better performance at the HRT of 25 days, with higher biogas production. The authors reported that SBB is a poor substrate in terms of nutrients such as nitrogen, phosphorus, and buffering capacity. In their study, authors regularly used an external source of nutrients to avoid system failure. In the present research, the use of manure as a co-substrate has a similar effect on the process, contributing to adjust the nutrients and stabilizing the system by the alkalinity supply.

Chen and Zhong [23] reported that despite the high VFAs concentration in co-digestion reactors of the cotton stalk with swine manure in comparison with single digestion of a cotton stalk, the anaerobic co-digestion assay showed higher stability with high biogas generation, indicating the role of swine manure on neutralizing VFAs in the medium. In the same way, Pagéz-Díaz et al. [50] studied semi-continuous anaerobic digestion of the solid cattle slaughterhouse (SB) and SB co-digestion with manure and various crops. They reported a failure of the individual digestion of SB. However,

stable performance, operating with higher loading rates, was observed for co-digestion with different manure sources. The authors highlighted the established synergy effect by mixing substrates with different characteristics. In a study of Li et al. [18], it has been found that AD of PM has given the highest methane yield in comparison with dairy, chicken, and rabbit manure at the same operating conditions. These findings are in agreement with the results of the present study.

In addition to macronutrients such as carbon, nitrogen, phosphorus, and sulfur, trace elements are crucial for effective biogas production due to the microbial demand for these elements [51]. In a study of Schmidt et al. [52] using continuous mesophilic digesters of slaughterhouse wastewater, the authors reported that the addition of trace elements to the digester resulted in higher biogas production and higher degradation efficiency, by improving the process stability. Moreover, higher OLR and lower HRT could be used in comparison to digesters with deficiencies in trace elements. Similarly, Demirer and Scherer [49] highlighted that micronutrients availability plays a crucial role in the process performance and stability of the AD of agricultural substrates.

Moreover, authors have pointed out that the lack of trace elements is likely the main reason for poor process efficiency. In this sense, the supply of trace elements from the animal manure studied in this research could justify the high performance of reactors in comparison with the reactor of SBB digested individually.

4. Conclusions

- Non-classical parameters could give more insight into the coupling/uncoupling of the AD phases and the involved microorganisms, revealing that process failure was mainly due to methanogenesis inhibition in co-digestion with PM, while for co-digestion with CM or individual digestion of SBB, both hydrolysis–acidogenesis and methanogenesis phases were affected.
- Co-digestion with manure contributed to reducing the inhibitory effect of volatile fatty acids at high organic loading rates (OLRs), leading to increases in methane production by 70% and 31% in comparison with AD of SBB, for co-digestion with pig and cow manure, respectively.
- Biomethanation degree (BD) refers to the maximum methane potential that can be obtained from organic wastes under specific operating conditions. SBB required a long digestion-time to achieve high biodegradability. However, short digestion-times for co-digestion assays led to high BD.

Supplementary Materials: The following is available online at <http://www.mdpi.com/2076-3417/10/15/5126/s1>, Figure S1: Evolution of the DOC, DAC, ASC, and methane productions in the three SSTR. A: SBB reactor; B: SBB+CM reactor; C: SBB+PM reactor.

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