

Article

Anaerobic Co-Digestion of Polylactic Acid (PLA) Films with Organic Fraction of Municipal Solid Waste: Biodegradation, Biogas Yields, and Metabolomic Analysis

Nicolò Montegiove ^{1,*} , Debora Puglia ² , Roberto Maria Pellegrino ³ , Franco Dominici ² , Eleonora Calzoni ³  and Daniela Pezzolla ¹ 

¹ Department of Civil and Environmental Engineering, University of Perugia, Via G. Duranti 93, 06125 Perugia, Italy; daniela.pezzolla@unipg.it

² Department of Civil and Environmental Engineering, University of Perugia, UDR INSTM, Strada di Pentima 4, 05100 Terni, Italy; debora.puglia@unipg.it (D.P.); franco.dominici@unipg.it (F.D.)

³ Department of Chemistry, Biology and Biotechnology, University of Perugia, Via del Giochetto, 06126 Perugia, Italy; roberto.pellegrino@unipg.it (R.M.P.); eleonora.calzoni@unipg.it (E.C.)

* Correspondence: nicolo.montegiove@unipg.it

Abstract

The increasing use of bioplastics in packaging applications necessitates rigorous evaluation of their fate in real waste management systems. While bioplastics are often marketed as biodegradable, their actual behavior under mesophilic anaerobic digestion (AD) is frequently insufficiently understood and often overestimated in commercial claims. Polylactic acid (PLA), one of the most widely produced bio-based polymers, has been widely characterized under these conditions, but little is known about the metabolomic changes associated with its biodegradation under mesophilic anaerobic conditions. This study investigates the mesophilic AD (37 °C for more than 3 months) of PLA films (2.5 × 2.5 cm) co-digested with the organic fraction of municipal solid waste (OFMSW). Biogas production and energy yield evaluation were assessed for AD, along with chemical parameters and metabolomic analyses. PLA biodegradation, calculated according to ISO 15985:2014, reached values close to 100% after more than 3 months, highlighting a prolonged lag phase under mesophilic AD conditions. The biogas production yielded about 380 Nm³ per t of volatile solids. Metabolomic profiling during AD revealed that the onset of PLA biodegradation, highlighted also by biogas emission, coincides with the appearance of key metabolites associated with PLA hydrolysis. These findings demonstrate that the mesophilic anaerobic co-digestion of PLA films with OFMSW did not cause any inhibition effect on biogas production. The results demonstrate the feasibility of incorporating PLA into existing organic waste treatment systems, thereby supporting both energy recovery and sustainable waste management.

Keywords: anaerobic digestion; polylactic acid (PLA); bioplastics; organic waste management; biodegradation; biogas yield; metabolomics; omics sciences



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1. Introduction

The global production capacity of biobased plastics has increased substantially in recent years, reaching 2.31 million tonnes in 2025 and projected to grow to approximately 4.69 million tonnes by 2030 [1]. Among these, polylactic acid (PLA) is one of the most commonly produced biodegradable polymers, owing to its renewable origin from starch or

sugar fermentation and its wide application in packaging, disposable items, and agricultural films [2,3]. The widespread use of PLA is due to its bio-based origin and its claimed biodegradability, particularly under industrial composting conditions [4]. However, the real fate of PLA after its use critically depends on existing waste management infrastructures, which often rely not on composting, but also on anaerobic digestion (AD) as a first treatment step for organic waste treatment. AD represents a cornerstone of circular bioeconomy strategies, converting organic substrates into methane-rich biogas and digestate suitable for agronomic use [5–8].

Bioplastics such as PLA are increasingly being found in the organic fraction of municipal solid waste (OFMSW), which feeds most full-scale AD plants [9,10]. Most bioplastics can interfere with the AD process, potentially leading to reduced biogas yields and incomplete stabilization of the digestate [11]. Anaerobic co-digestion of commercially available bioplastics with food waste has therefore gained increasing attention as a sustainable waste management strategy aimed at improving energy recovery and assessing the real biodegradation potential of these materials under operational conditions [5]. Nevertheless, most bioplastics degrade very slowly during AD, and complete breakdown is rarely achieved within the typical hydraulic retention time of full-scale biogas plants [12,13]. This incomplete degradation not only limits methane production efficiency, but also raises questions about the underlying biochemical transformations that occur during the process. Yet, most current bioplastic certification schemes, such as UNI EN 13432:2002 and ASTM D6400-21 [14,15], are designed to assess biodegradability under industrial composting conditions, while AD is generally not included in standard testing protocols. While compostability is covered by certification standards, anaerobic biodegradability is generally assessed through laboratory protocols, such as ISO 15985:2014 [16], which defines methods for determining ultimate biodegradation under AD conditions by analysis of released biogas. However, these standards are analytical rather than certifying, and AD-specific certification frameworks are still lacking. Furthermore, although PLA biodegradation under AD has been investigated in previous studies, including under mesophilic conditions typical of European biogas plants [17,18], important knowledge gaps remain regarding the metabolomic analysis associated with PLA degradation. In particular, little is known about the molecular mechanisms and metabolic changes occurring during the mesophilic anaerobic biodegradation of PLA and similar polymers, an aspect that can be elucidated through advanced omics-based approaches such as metabolomics [19]. Metabolomic approaches can provide valuable insights into the biochemical transformations associated with PLA degradation. However, studies systematically integrating metabolomic profiling with biodegradation, biogas production, and chemical analyses to characterize PLA behavior under mesophilic AD remain scarce, highlighting the need for further investigation.

The present study aims to address these knowledge gaps by providing a comprehensive evaluation of PLA biodegradation during mesophilic AD in the presence of OFMSW. Specifically, the work investigates the extent of PLA degradation over extended digestion periods, quantifies biogas production associated with PLA co-digestion, and monitors the chemical evolution of the digestate. A key component of this research is the application of metabolomic profiling to identify metabolites and biochemical changes associated with PLA degradation, offering insights into the underlying mechanisms of polymer breakdown in AD systems. Unlike biopolymer biodegradation studies, which primarily focus on biogas production and chemical parameters, the metabolomic approach adopted provides molecular-level information on the evolution of PLA during AD. Furthermore, the study assesses the compatibility of PLA-containing wastes with current AD-based waste management frameworks, contributing to a more realistic understanding of their environmental fate. This research provides experimental evidence on the anaerobic degradability of PLA,

supporting the scientific basis for evaluating bioplastics beyond composting scenarios. By integrating biogas yield, biodegradation kinetics, chemical monitoring, and metabolomic analysis, the study improves the understanding of the links between polymer degradation and energy recovery, while also highlighting potential impacts on digestate quality and process efficiency. This work provides an evaluation of PLA's fate in mesophilic AD, supporting improved end-of-life strategies and more informed environmental assessments for biodegradable plastics.

2. Materials and Methods

2.1. Tested Materials

2.1.1. Preparation of PLA Films

PLA 3051D, with a specific gravity of 1.25 g cm^{-3} , a molecular weight (M_n) of ca. $1.42 \times 10^4 \text{ g mol}^{-1}$, and a melt flow index (MFI) of $7.75 \text{ g } 10 \text{ min}^{-1}$ (210°C , 2.16 kg), was supplied by NatureWorks LLC, Plymouth, MN, USA. Neat PLA films were produced using a co-rotating twin screw micro-extruder (DSM Explorer 5&15 CC Micro Compounder, Xplore Instruments BV, Sittard, The Netherlands) equipped with a film casting system capable of producing 70 mm wide strips. The film thickness was approximately $150 \mu\text{m}$. PLA pellets were dried in a vacuum oven at 98°C for 3 h prior to extrusion. For film production, a screw speed of 150 rpm and a mixing time of 1 min were applied, with a temperature profile ranging from 180°C to a maximum of 210°C [11]. After the extrusion, the PLA film was cut into small squares ($2.5 \times 2.5 \text{ cm}$) and used for the AD as reported in a similar study on bioplastics [20]. The films were then placed in PVC mesh bags to allow for the evaluation of residual material remaining at the end of the AD process (Figure 1). The mesh bags allowed direct contact between the digestate and the PLA films while facilitating material recovery at the end of the experiment. To account for any potential effects associated with the presence of the bags, empty PVC mesh bags were also included in the control reactors.

**Mesh bag containing
bioplastic films for
anaerobic digestion**

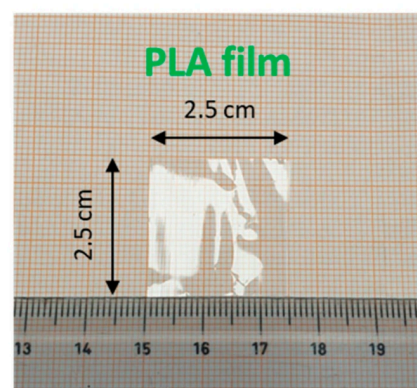


Figure 1. Preparation of biopolymer samples for anaerobic digestion: PLA films cut into $2.5 \times 2.5 \text{ cm}$ squares and enclosed in mesh bags for reactor incubation.

2.1.2. Substrate and Inoculum

The OFMSW used as the substrate for the co-digestion was prepared in the laboratory using organic residues, such as potato peels, carrot peels, lettuce waste, and other vegetable scraps. This controlled preparation ensured the absence of plastic contaminants that are often found in industrial OFMSW due to improper disposal of conventional plastics in the biowaste stream [21]. Furthermore, the laboratory-prepared mixture avoided the presence of other bioplastics that could have interfered with the assessment of PLA degradation during AD. The main physicochemical characteristics of the OFMSW are reported in Table 1.

Table 1. Main chemical characteristics of the organic fraction of municipal solid waste (OFMSW) used as substrate for the anaerobic co-digestion and of the inoculum (mean $\pm$ SEM, $n = 3$).

Parameter	OFMSW	Inoculum
Moisture(%)	88.6 $\pm$ 0.5	90.9 $\pm$ 0.2
pH	5.13 $\pm$ 0.02	8.1 $\pm$ 0.2
EC (dS m ⁻¹)	2.0 $\pm$ 0.2	27.1 $\pm$ 0.1
VS (g kg ⁻¹)	904.9 $\pm$ 4.3	742.3 $\pm$ 3.8
TOC (g kg ⁻¹)	403.4 $\pm$ 7.3	368.6 $\pm$ 7.5
TKN (g kg ⁻¹)	19.3 $\pm$ 2.2	55.7 $\pm$ 2.8
NH ₄ ⁺ -N (g kg ⁻¹)	3.9 $\pm$ 0.3	37.3 $\pm$ 0.7
Organic N (g kg ⁻¹)	15.4 $\pm$ 2.2	18.4 $\pm$ 2.9
C/N	20.9	6.6
WEOC (g kg ⁻¹)	–	53.2 $\pm$ 1.1
WEN (g kg ⁻¹)	–	40.9 $\pm$ 1.1
WEOC/WEN	–	1.3
FOS/TAC	–	0.30 $\pm$ 0.02

– indicates parameters not measured for OFMSW. EC, Electrical Conductivity; VS, Volatile Solids; TOC, Total Organic Carbon; TKN, Total Kjeldahl Nitrogen; WEOC, Water-Extractable Organic Carbon; WEN, Water-Extractable Nitrogen; FOS/TAC, ratio between volatile organic acids (Flüchtige Organische Säuren) and total inorganic carbonate (Totales Anorganisches Carbonat).

The inoculum used for AD was obtained from a full-scale biogas plant operated by Società Agricola Iraci Borgia S.S., located in Bevagna (Umbria, Italy). This facility treats agro-industrial by-products under mesophilic conditions. The main characteristics of the digestate used as inoculum are presented in Table 1.

2.2. Experimental Setup for Anaerobic Co-Digestion

Anaerobic co-digestion experiments were carried out to evaluate the biodegradation of PLA films in combination with the OFMSW under controlled mesophilic conditions. Batch tests were conducted using 1 L glass bottles, each containing 600 mL of digestate. For the treatments containing only OFMSW, 6 g of fresh substrate were added to the inoculum (inoculum+OFMSW), whereas for the co-digestion experiments, the same total substrate loading was maintained and PLA films were included at 10% on a weight basis within the substrate mixture (inoculum+OFMSW+PLA), following the dosage recommended in a previous study [22], where this proportion was considered representative of a realistic bioplastic contamination levels in OFMSW. Control bottles contained 600 mL of inoculum only. Each treatment was set up in triplicate. Prior to the start of the experiment, the bottles were flushed with nitrogen gas to remove oxygen and ensure anaerobic conditions. Digestate samples were collected periodically during the experiment for chemical analyses and process monitoring. Following each sampling event, the bottles were flushed again with nitrogen to restore anaerobic conditions within the reactors. The incubations were maintained at 37 °C, consistent with the mesophilic range typical of the source anaerobic digester and in agreement with literature recommendations for mesophilic AD studies of bioplastics [22]. The bottles were mixed daily to ensure adequate contact between

the inoculum and substrate, promoting efficient hydrolysis and microbial activity during AD. The experiment was continued until the PLA reached complete biodegradation, as estimated according to ISO 15985:2014 [16] using the cumulative gaseous carbon evolution approach. Daily biogas production was measured using a hydraulic gasometer, following the procedure described by Montegiove et al. [11], and expressed as Nm^3 per t of volatile solids (VS). Biogas composition was not determined. This setup allowed for the assessment of both the degradability of PLA in a real OFMSW matrix and its contribution to biogas yield, while providing insights into the performance of PLA under realistic mesophilic AD conditions.

2.3. Analytical Methods

The analytical characterization of the substrates and digestates was performed following standard procedures. Moisture, total organic carbon (TOC), total Kjeldahl nitrogen (TKN), and ammonium nitrogen ($\text{NH}_4^+\text{-N}$) were determined according to standard methods [23]. Moisture content was measured as the weight loss of the fresh sample after oven-drying at 105°C for 24 h using a TCN 50 Plus oven (ARGO LAB, Giorgio Bormac S.r.l., Carpi, Italy). VS were determined gravimetrically as the weight at 550°C in a FALC muffle furnace (FALC Instruments S.r.l., Treviglio, Italy). TKN was determined after microwave-assisted acid digestion using a Milestone ETHOS One microwave system (Milestone S.r.l., Sorisole, Italy), followed by distillation with a UDK 129 Kjeldahl Distillation Unit (VELP Scientifica S.r.l., Usmate, Italy) and subsequent titration. $\text{NH}_4^+\text{-N}$ was determined using the same distillation apparatus and titration procedure, but without the preliminary digestion step required for TKN analysis. TOC was measured with a Multi N/C 2100S[®] analyzer (Analytik Jena GmbH, Überlingen, Germany). The same instrument was used to determine water-extractable organic carbon (WEOC) and water-extractable nitrogen (WEN). Electrical conductivity (EC) and pH were measured on fresh samples using benchtop meters. The process stability was evaluated by means of the FOS/TAC ratio between total volatile organic acids (expressed as $\text{mg CH}_3\text{COOH L}^{-1}$) and alkalinity (expressed as $\text{mg CaCO}_3 \text{ L}^{-1}$), according to the approach proposed by Lossie and Pütz [24] and previously described in a similar study by Pezzolla et al. [25].

The potential energy yield from biogas production was estimated using the average calorific value of biogas (21.6 MJ Nm^{-3}) reported by the World Bioenergy Association [26] and previously adopted in a similar study [11]. Results were expressed as MJ per t of total solids (TS). As the biogas composition was not determined, this calculation was used to provide an estimate of the potential energy yield.

The percentage of PLA biodegradation was calculated following the procedure described in ISO 15985:2014 [16], whereby the measured biogas production was converted into gaseous carbon under standard temperature and pressure conditions. The net gaseous carbon evolved from the test material was determined after subtraction of the gaseous carbon evolved in the control, using Equation (1):

$$\% \text{Biodegradation} = \frac{m_{C,g}(\text{test}) - m_{C,g}(\text{blank})}{m_{C,i}} \times 100 \quad (1)$$

where $m_{C,g}$ represents the cumulative mass in grams of gaseous carbon evolved from the test material and blank (inoculum-only), and $m_{C,i}$ is the initial carbon content of the test material. Since the experiment involved co-digestion of PLA films with OFMSW, the contribution of the organic fraction alone was subtracted from the total biogas production to isolate the specific biodegradation attributable to PLA.

The kinetic behavior of PLA biodegradation was modeled by fitting cumulative carbon data to the modified Gompertz equation, as described by Montegiove et al. [11] (Equation (2)):

$$C = P \times \exp \left\{ - \exp \left[\frac{R \times e}{P} \times (\lambda - t) + 1 \right] \right\} \quad (2)$$

where C is the cumulative carbon in the gas phase (mg C g^{-1} bioplastic) at time t (days), P is the maximum bioplastic carbon converted to gaseous carbon (mg C g^{-1} bioplastic), R is the maximum mineralization rate (mg C g^{-1} bioplastic day^{-1}), λ is the lag phase (days), and e is Euler's number (2.71828).

2.4. Untargeted Metabolomic Analysis

Untargeted metabolomic analyses were performed on digestate samples collected at day 7 (T7) and day 65 (T65) during AD. These time points were selected based on the biodegradation kinetics observed during the experiment, with T7 representing an early digestion stage before detectable PLA biodegradation and T65 corresponding to the onset of PLA biodegradation according to ISO 15985:2014 [16]. The comparison of these two sampling times was intended to investigate metabolic changes associated with the transition from the initial digestion phase to the stage where PLA biodegradation became detectable.

2.4.1. Sample Preparation

Samples were first clarified by centrifugation at maximum speed ($\sim 16,000 \times g$) for 10 min at room temperature. An aliquot of 100 μL of the supernatant was transferred to a clean microcentrifuge tube containing 500 μL of acetonitrile/water (4:1, v/v) with 1% formic acid, vortex-mixed, and stored at 4 $^{\circ}\text{C}$ overnight to promote precipitation. The mixture was then centrifuged at $16,000 \times g$ for 15 min at 4 $^{\circ}\text{C}$. To remove residual turbidity, 100 μL of the supernatant was diluted with 900 μL methanol/acetonitrile (1:1, v/v), leading to the formation of an additional precipitate. After a second centrifugation (same conditions), the final supernatant was transferred to LC vials and analyzed. The injection volume was 2 μL .

2.4.2. LC-MS/QTOF Conditions

Chromatographic separation was performed by HILIC using a Waters XBridge BEH Amide column (2.1 $\times$ 150 mm, 2.5 μm ; cat. 186006724; Waters Corporation, Milford, MA, USA) maintained at 40 $^{\circ}\text{C}$, with a flow rate of 0.35 mL/min. Mobile phase A was water, and mobile phase B was acetonitrile (95%); both solvents contained 10 mM ammonium formate and 1.25% formic acid. The gradient program was as follows: 0–0.5 min, 100% B; 0.5–8.0 min, linear decrease to 50% B; 8.0–8.1 min, return to 100% B; 8.1–14.0 min, 100% B (column re-equilibration; stop time 14 min). Data were acquired in DDA in both ESI positive and negative modes on an Agilent 6530 QTOF mass spectrometer equipped with an Agilent JetStream source (Agilent Technologies, Inc., Santa Clara, CA, USA).

2.4.3. Data Processing

Raw data were processed using MS-DIAL (v4.9) for peak picking, alignment, and annotation, using the NIST 2020 tandem mass spectral library and matching based on MS1 and MS/MS information. Metabolite annotations were assigned at MSI level 2, based on MS1 and MS/MS spectral matching against reference databases, and were not confirmed using standards.

2.5. Statistical Analysis

Statistical analyses of chemical and process parameters were performed using GraphPad Prism version 9.0.0 (GraphPad Software, San Diego, CA, USA). All measurements

were performed in triplicate, and results are expressed as mean values $\pm$ standard error of the mean (SEM). Statistical differences among treatments were assessed through one-way and two-way analysis of variance (ANOVA), followed by Tukey's *post hoc* test for multiple comparisons. The level of statistical significance was set at $p < 0.05$. The standard error of the difference (SED) estimated from the ANOVA model was used as the measure of variability among treatment means.

Untargeted metabolomic data were analyzed using MetaboAnalyst [27]. Prior to statistical analysis, data were normalized, log10-transformed, and autoscaled to improve comparability across samples and reduce heteroscedasticity. Principal component analysis (PCA) was applied to explore global differences in the metabolic profiles of samples collected at different times during AD. Univariate analysis was performed using Student's *t*-test to identify metabolites showing significant differences ($p < 0.05$) between the time points analyzed. False discovery rate (FDR) values were calculated to account for multiple comparisons. Volcano plot analysis was conducted by combining statistical significance ($p < 0.05$) with a fold change (FC) threshold ≥ 2.0 to identify metabolites exhibiting significant changes associated with AD. A heatmap was generated using hierarchical clustering to visualize patterns in metabolite abundance across the analyzed samples. A pathway analysis was subsequently conducted using metabolites altered between T7 and T65 to provide a functional interpretation of the metabolic changes associated with the progression of AD and the onset of detectable PLA biodegradation. For pathway analysis, both FDR- and Holm-adjusted *p*-values were calculated to account for multiple comparisons.

3. Results and Discussion

3.1. Anaerobic Co-Digestion of PLA

3.1.1. Chemical Parameters

The anaerobic co-digestion of PLA with OFMSW revealed a range of chemical, process-performance, and kinetic responses that highlight the feasibility of incorporating PLA in mesophilic AD systems for treatment of the organic fraction derived from the municipal solid waste. As far as the chemical parameter evolution is concerned, the pH values remained reasonably stable across all treatments from the beginning to the end of the experiment, showing only slight variations between 8.1 and 8.3, albeit not statistically significant. Such values fit well within the functional range for both fermentative bacteria and methanogenic archaea, thereby supporting AD performance [28]. Electrical conductivity increased gradually, e.g., from 27.1 to 32.0 dS m⁻¹ ($p < 0.0001$) for digestates derived from OFMSW+PLA treatment, indicating progressive mineralisation of organic matter. Such trends are consistent with advancing AD processes, where the ongoing degradation of organic matter over time increases the concentration of soluble salts in the digestate [29].

The FOS/TAC ratio declined during the AD, reaching about 0.17–0.16 by day 112 ($p < 0.01$) in all treatments (Figure 2a). Literature indicates that values between 0.3 and 0.4 are optimal for maximizing biogas production, whereas values below 0.2 typically reflect insufficient biomass input and may require an increase in substrate loading or the addition of more readily degradable feedstocks [30]. In batch systems, such a decline is plausible, as substrate availability progressively decreases over time and the buffering capacity becomes increasingly dominant as digestion proceeds. As for VS, as expected for the batch AD test, their values decreased significantly at the end of the experiment ($p < 0.05$) for all treatments (Figure 2b), suggesting that the addition of PLA did not significantly impair organic matter removal. Moreover, in batch AD, such a progressive decline in VS is fully expected, as the absence of fresh feeding leads to the gradual consumption and depletion of degradable substrates over time [31,32]. This decline in VS aligns with the evolution of

the FOS/TAC ratio, both reflecting the progressive consumption of organic substrates as digestion proceeds.

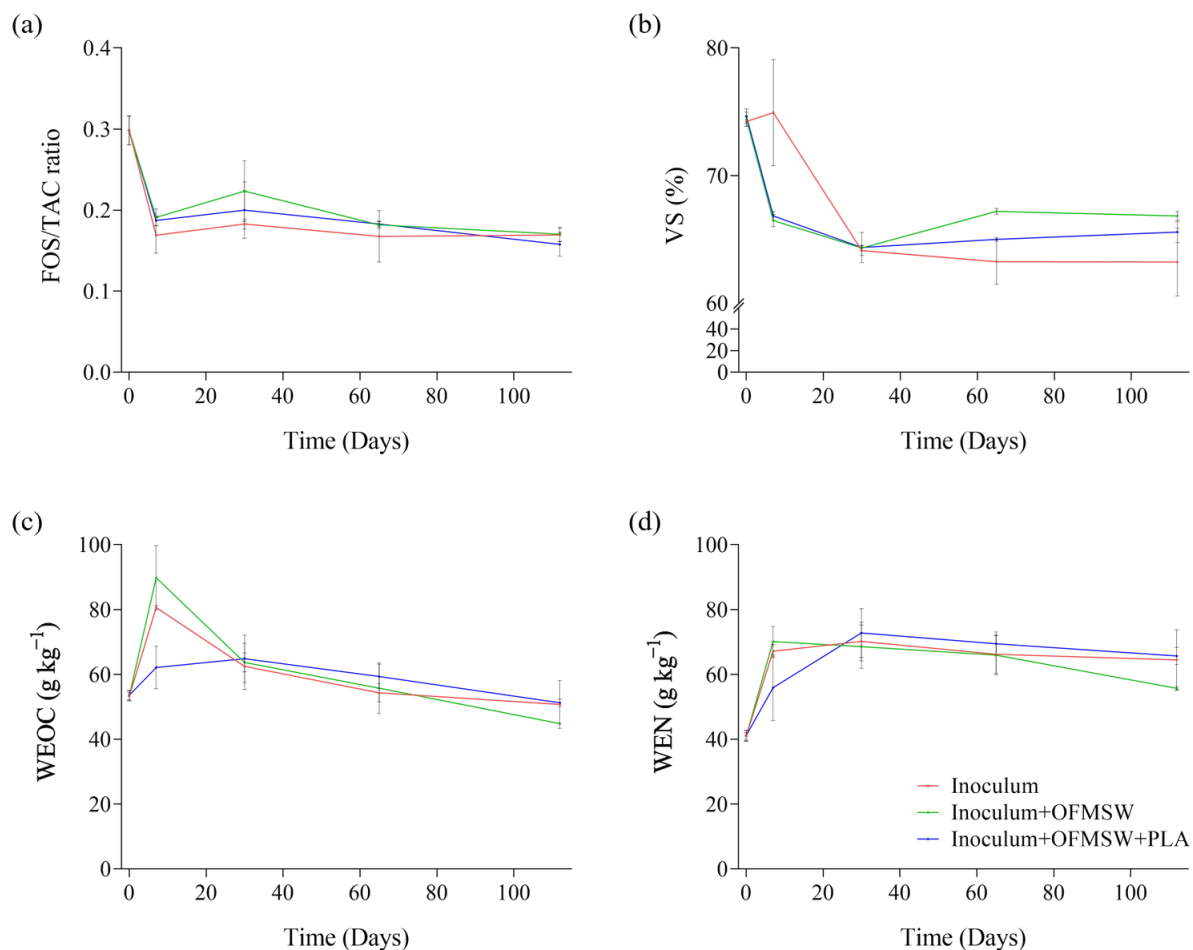


Figure 2. (a) FOS/TAC ratio (SED = 0.027), (b) Volatile solids (VS) (SED = 3.039), (c) Water-extractable organic C (WEOC) (SED = 7.316), and (d) Water-extractable N (WEN) (SED = 7.733) analysis measured over time during mesophilic anaerobic digestion of inoculum, inoculum added with organic fraction of municipal solid waste (Inoculum+OFMSW), and inoculum added with OFMSW and PLA (Inoculum+OFMSW+PLA). Data represent means of three independent experiments ($n = 3$). All values are expressed on a dry matter basis, except for the FOS/TAC ratio.

The evolution of WEOC and WEN further supports the interpretation of a progressively advancing AD process. In all treatments, WEOC exhibited an early increase, followed by a gradual decline towards the end of the AD (Figure 2c), a pattern consistent with the rapid solubilization of readily hydrolysable compounds after substrate addition and their subsequent microbial utilization as digestion progressed. Notably, the inoculum+OFMSW treatment showed the highest WEOC peak, whereas the inoculum+OFMSW+PLA reactors displayed a more moderate initial increase and a slightly slower decline. This behaviour may be consistent with the gradual hydrolysis of PLA under mesophilic anaerobic conditions, as reported in previous studies [33,34]. However, other factors, including substrate heterogeneity and microbial community dynamics, may also have contributed to the observed WEOC pattern. Additional support for PLA transformation was provided by the metabolomic analysis discussed below, which identified PLA-related hydrolysis products during the digestion process.

WEN dynamics followed a similar path, with an early rise across treatments reflecting the microbial transformation of N compounds, followed by partial stabilization in later

phases of digestion (Figure 2d). This behaviour is further supported by the evolution of $\text{NH}_4^+\text{-N}$, which increased over the course of the experiment and represented a progressively larger fraction of TKN compared to the initial inoculum composition (Table 1). At the end of digestion, ammonium N accounted for 6.1, 6.0, and 7.2% of dry weight in the inoculum, inoculum+OFMSW, and inoculum+OFMSW+PLA treatments, respectively. These results indicate an enhanced mineralisation of organic N and accumulation of ammonium during AD, regardless of substrate composition. Together, these carbon- and nitrogen-related trends corroborate the chemical and process indicators described above, showing a system in which substrate solubilization, consumption, and mineralization progressed over time without evident inhibitory effects attributable to PLA.

3.1.2. Biogas Production and Energy Yield

The cumulative biogas yields showed a rapid increase for all treatments during the first 40 days, followed by a progressive stabilization starting around day 60 towards the end of the digestion period (Figure 3). The inoculum alone reached a final yield of approximately 358 Nm^3 per t of VS, while the addition of OFMSW allowed achieving a higher performance in both inoculum+OFMSW and inoculum+OFMSW+PLA, although these differences were not statistically significant. The absence of significant differences between the treatments suggests that PLA addition did not adversely affect AD performance, biogas production, or microbial activity under the conditions tested. The detected trend confirms the good biodegradability of the organic fraction over the AD digestion process performed in mesophilic conditions, while the absence of significant differences between OFMSW and OFMSW+PLA indicates that PLA addition did not adversely affect AD performance under the tested conditions, supporting its compatibility with the co-digestion process at an application dose corresponding to 10% of OFMSW on a weight basis. This observation aligns with previous studies, albeit performed under different experimental conditions and application rates, showing that the co-digestion of PLA or PLLA with organic substrates, such as food waste or sewage sludge, generally maintains process stability, methane yields, and microbial activity, even though the polymer itself undergoes only partial biodegradation. In particular, Kang et al. [35] evaluated the co-digestion of food waste with finely ground PLA at relatively low organic loadings, i.e., 2.0 g chemical oxygen demand (COD) L^{-1} , operating batch assays for approximately 20 days, and reported synergistic effects of food waste addition on methane yield during PLA anaerobic co-digestion. Similarly, Maragkaki et al. [36] investigated PLLA co-digestion with food waste and liquid pig manure under mesophilic conditions, both in batch and pilot-scale trials. In the biochemical methane potential (BMP) tests, 0.5 g of PLLA was co-digested with 2 g of food waste in 120 mL serum bottles, with a monitoring period of approximately 125 days, whereas in the pilot reactor, 1 kg of PLLA was added to a 220 L digester treating a mixed feedstock of food waste, liquid pig manure, and inoculum, operated for 50 days. In both cases, the applied PLLA dose represented a minor fraction of the total organic load. Their results demonstrated that PLLA addition increased biogas production by 8% without affecting reactor performance at the pilot scale. Ferreri et al. [37] assessed the impact of bioplastic addition under semi-continuous mesophilic anaerobic co-digestion at bioplastic concentrations ranging from 2 to 8% (w/w OFMSW), and observed a concentration-dependent effect of bioplastics, under hydraulic residence times of 14–21 days. While low to moderate additions (2–4% w/w OFMSW) did not compromise process stability, higher bioplastic loads (8%) led to volatile fatty acid accumulation and reduced methane yields, particularly for Mater-Bi. In contrast, crystalline PLA maintained process stability but showed limited biodegradability and only modest improvements in methane production, suggesting the existence of a practical threshold beyond which further bioplastic addition becomes ineffective rather than beneficial for

AD performance. Compared with the lower or more diluted bioplastic fractions and the generally shorter digestion times reported in previous studies [35–37], the experimental setup tested in this experiment represents a prolonged assessment of PLA behaviour under anaerobic conditions. The absence of inhibitory effects and the maintenance of biogas yields comparable to OFMSW alone support the compatibility of PLA with AD even at relatively elevated inclusion rates and extended retention times.

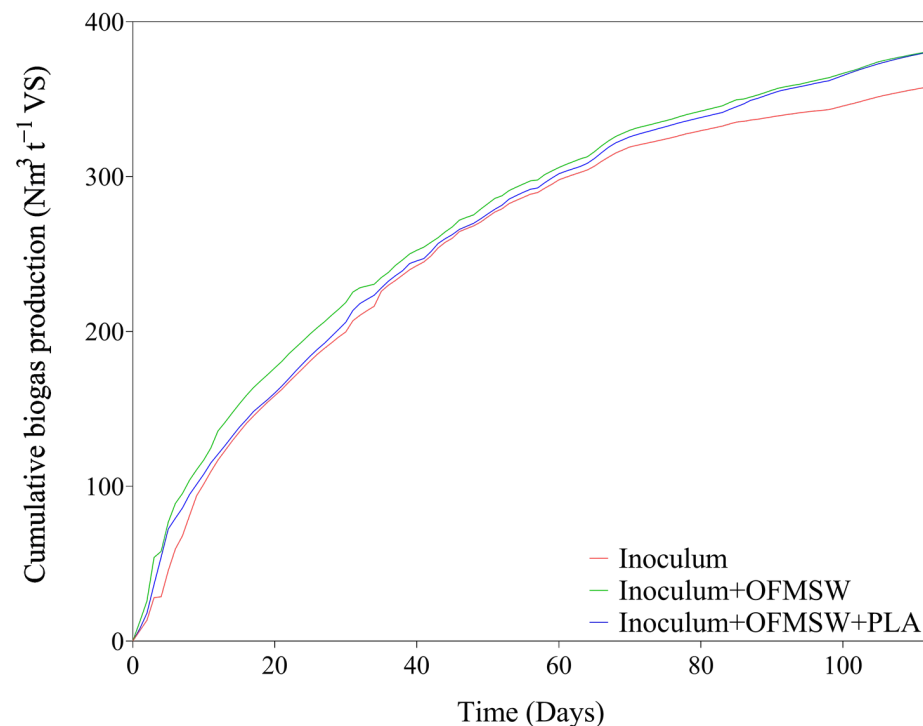


Figure 3. Cumulative biogas production during mesophilic anaerobic digestion of inoculum, inoculum added with organic fraction of municipal solid waste (Inoculum+OFMSW), and inoculum added with OFMSW and PLA (Inoculum+OFMSW+PLA), expressed as Nm^3 per t of volatile solids (VS) (SED = 30.32). Data represent means of three independent experiments ($n = 3$).

From an energy recovery perspective, these comparable biogas yields also imply that the incorporation of PLA did not compromise the overall energetic performance of the process. The energy content of biogas produced from the AD process is crucial for assessing the renewable energy potential of different substrates. Based on a calorific value of 21.6 MJ Nm^{-3} [11], the estimated energy yields at the end of the mesophilic digestion were 5738 MJ per t of TS for the inoculum, 6119 MJ per t of TS for the inoculum+OFMSW, and 6133 MJ per t of TS for the inoculum+OFMSW+PLA mixture (Table 2).

Table 2. Estimated energy production during mesophilic anaerobic digestion for the inoculum, inoculum added with organic fraction of municipal solid waste (Inoculum+OFMSW), and inoculum added with OFMSW and PLA (Inoculum+OFMSW+PLA), expressed as MJ t^{-1} of total solids (TS) (mean $\pm$ SEM, $n = 3$).

	Estimated Energy Produced (MJ t^{-1} TS)			
	Day 30	Day 60	Day 90	Day 112
Inoculum	3202 ± 318	4777 ± 332	5428 ± 332	5738 ± 333
Inoculum+OFMSW	3514 ± 409	4916 ± 413	5718 ± 414	6119 ± 414
Inoculum+OFMSW+PLA	3322 ± 299	4867 ± 301	5703 ± 302	6133 ± 303

When compared to intermediate time points, the energy yield was already considerable after 30 days, with 3202 MJ/t of TS for the inoculum, 3515 MJ/t of TS for the inoculum+OFMSW, and 3322 MJ/t of TS for the inoculum+OFMSW+PLA treatment, indicating that the highest rate of energy recovery occurred during the first month of digestion. In comparison with previous thermophilic AD studies performed at 52 °C [11], where a maximum yield for PLA was detected after about one month at 857 MJ/t of TS, the energy yields observed in the present mesophilic co-digestion experiment were higher during the first month of operation. However, the inoculum treatment also reached a higher yield of approximately 3202 MJ/t of TS after 30 days, whereas the thermophilic AD study reported approximately 1120 MJ/t of TS after 27 days for the inoculum alone [11]. Regarding the PLA treatment, the two experimental setups differed substantially in terms of substrate composition, plastic dosage, inoculum characteristics, and reactor operation. In Montegiove et al. [11], a higher dose of bioplastic was used (1 g of PLA was incubated with 67 g of wet inoculum); furthermore, the PLA was not co-digested. Therefore, the higher energy recovery observed in the present study cannot be attributed solely to temperature effects but likely reflects the combined influence of multiple factors, including the digestate used, organic loading conditions, and substrate composition. Although no direct comparison between mesophilic and thermophilic systems can be drawn from the present data, the results are consistent with previous studies reporting favourable operational performance of mesophilic AD systems. A recent study indeed reports that mesophilic AD systems generally provide higher process stability and a balanced microbial structure compared to thermophilic conditions, particularly under variable organic loading rates [38]. Although thermophilic digestion may achieve higher peak methane yields, mesophilic systems tend to maintain more stable performance and lower risk of inhibition when elevated organic load conditions occur. Mesophilic and semi-thermophilic digesters support functionally diverse microbial communities, in which acetoclastic methanogens are enriched, promoting efficient acetate conversion and stable methane production [39,40]. In addition, mesophilic and moderately elevated (semi-thermophilic) conditions (41–49 °C, typically around 45 °C) tend to reduce the accumulation of volatile fatty acids (VFAs) and ammonia compared to thermophilic systems (55 °C), thereby improving buffering capacity and ensuring greater process stability, supporting more efficient substrate conversion and sustained methane production [40].

3.1.3. Biodegradation Kinetics of PLA

The kinetic modelling using a modified Gompertz equation revealed a clear lag phase of about 60 days before measurable PLA biodegradation occurred, based on values estimated according to ISO 15985:2014 [16], followed by a slow conversion reaching values close to 100% after approximately 100 days (Figure 4). No visually detectable PLA residues were recovered from the mesh bags at the end of the digestion period. This sigmoidal biodegradation pattern is characteristic of slowly degradable polymers in AD systems, typically showing a prolonged acclimation period [34,41]. The necessity of extended retention (>100 days) for complete decomposition of PLA under mesophilic digestion underscores important operational implications for full-scale digesters treating PLA-containing organic waste streams. This may represent a practical limitation for full-scale implementation, as the hydraulic retention times commonly adopted in industrial anaerobic digesters are generally much shorter than the digestion period required to achieve near-complete PLA biodegradation in the present study [42]. Similar kinetic behaviour was reported by Yagi et al. [34], who observed negligible PLA biodegradation during the first 55 days of mesophilic anaerobic incubation (35 °C), followed by a very slow biodegradation rate of about 2.9% per week. Similarly, a study simulating full-scale mesophilic AD conditions for

OFMSW treatment reported that commercially available PLA-based materials exhibited very low biodegradation rates and remained only partially degraded even after prolonged digestion periods exceeding 250 days [43]. The delayed onset of biodegradation observed in these studies suggests that extended retention times are required to detect substantial PLA conversion under mesophilic anaerobic conditions. Differences in PLA characteristics, inoculum source, reactor operation, and substrate composition may also contribute to the variability in biodegradation extents reported in the literature. Furthermore, a previous study performed under thermophilic conditions (52 °C) further emphasizes the limited degradation of PLA during AD, where no measurable PLA degradation was observed throughout the 27-day digestion period [11]. A possible way to overcome this limit could be represented by a pretreatment aimed at increasing degradability under AD [13,44].

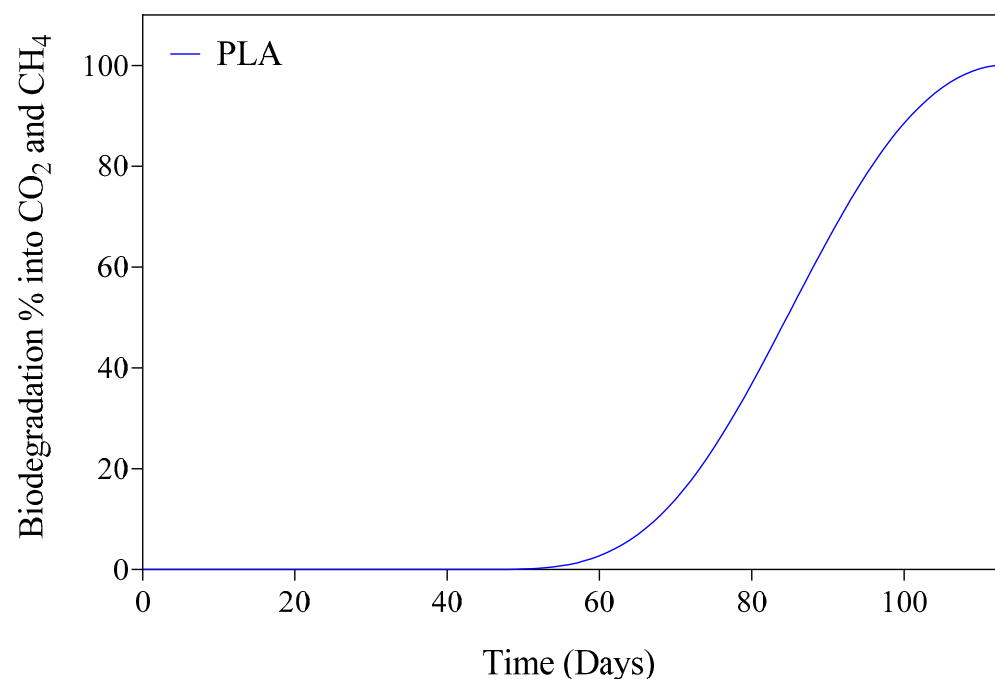


Figure 4. Biodegradation kinetics of PLA fitted to the modified Gompertz model during mesophilic anaerobic digestion, expressed as biodegradation % into CO₂ and CH₄.

The practical implications of these findings should however be interpreted within the specific waste management framework investigated in this study. AD represents an important treatment route for OFMSW in several European countries [45]; nevertheless, alternative end-of-life management strategies, including composting, recycling, or other waste treatment options, may be more prevalent in different geographical regions [46]. Therefore, the relevance and applicability of the present findings may vary according to local waste management infrastructures and regulatory frameworks.

3.2. Metabolomic Analysis of PLA Anaerobic Co-Digestion

An untargeted metabolomic analysis was also performed to complete the analytical assessment of the AD process, specifically comparing day 7 (T7) with day 65 (T65), corresponding to the stage at which PLA degradation became detectable. The metabolomic investigation was designed to complement the biodegradation and biogas production data by identifying metabolic signatures associated with PLA transformation during AD. In particular, the analysis aimed to detect potential hydrolysis products and intermediate metabolites that could provide biochemical evidence of polymer conversion. For this analysis, the Inoculum+OFMSW treatment was used as the reference control for the Inocu-

lum+OFMSW+PLA reactors. This approach allowed the metabolic changes specifically associated with PLA degradation to be distinguished from those related to the AD of the organic fraction itself. This analysis highlighted a significant reorganization of the metabolic profile involving 1290 annotated compounds (Table S1), which were characterized using MetaboAnalyst tools [27]. A multivariate statistical evaluation was performed to assess the robustness of the observed metabolic differences (F-value = 17.285; $R^2 = 0.812$; permutation $p = 0.1$), indicating a strong separation between the two digestion stages, although the permutation test did not reach the conventional significance threshold, likely due to the limited number of biological replicates. Principal Component Analysis (PCA) was first applied as an unsupervised multivariate approach to explore global differences in the metabolic profiles of samples collected at T7 and T65 during AD. The scores plot showed a clear separation between the two time points along the first principal component (PC1), which accounted for 56.2% of the total variance, while PC2 explained an additional 12.8% (Figure 5).

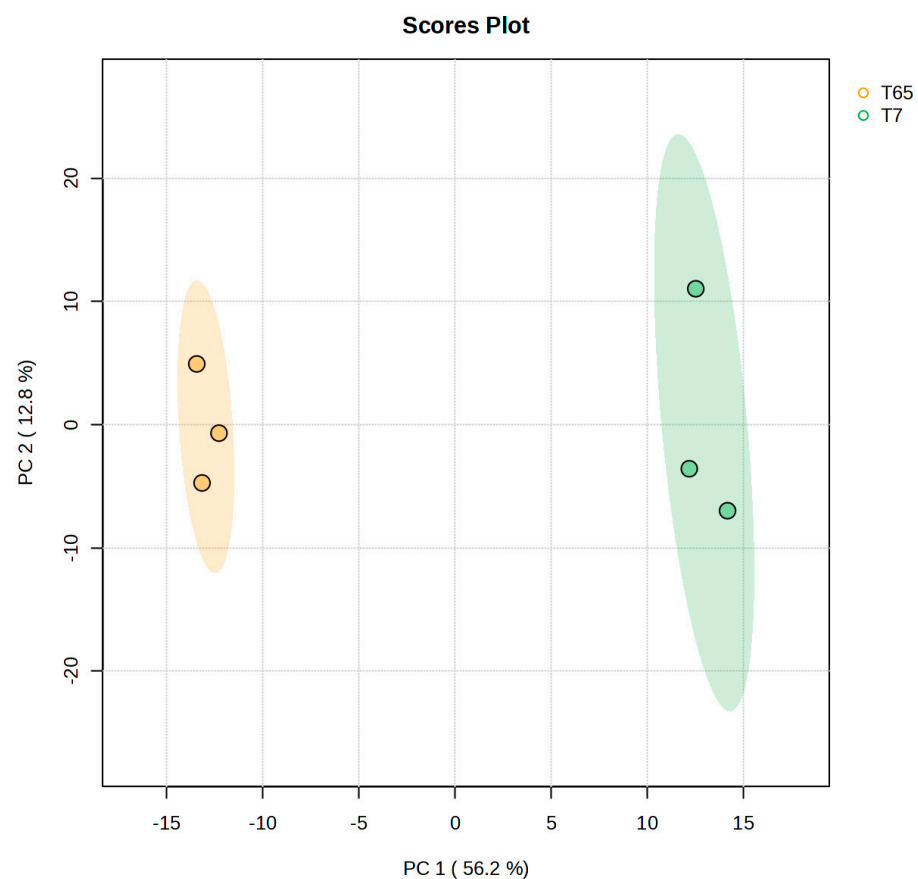


Figure 5. Principal Component Analysis (PCA) score plot of metabolomic profiles obtained from digestate samples of inoculum added with the organic fraction of municipal solid waste and PLA (Inoculum+OFMSW+PLA) collected during mesophilic anaerobic digestion at two different time points, day 7 (T7) and day 65 (T65). Each point represents an individual biological replicate ($n = 3$).

Together, the first two components described approximately 69% of the overall metabolic variability, indicating that the PCA model captured the main sources of variation in the dataset. The distinct clustering of T7 and T65 samples suggests a time-dependent shift in the metabolic profile as digestion progresses, consistent with previous observations indicating that significant PLA degradation became evident after a prolonged incubation period of approximately 2 months (Figure 4). This separation was further supported by a high F-value (17.285), reflecting a marked predominance of between-group variance over

within-group variability, and by an R^2 value of 0.812, indicating that over 80% of the total metabolic variance could be attributed to differences between the two digestion times. However, the permutation test yielded a p -value of 0.1, which does not reach the conventional threshold for statistical significance. This result may reflect the limited number of biological replicates ($n = 3$) and the inherent biological variability typical of complex AD systems. Given the exploratory nature of the metabolomic investigation and the limited number of biological replicates, the observed group separation and the metabolomic results should be interpreted as indicative of metabolic trends rather than definitive evidence of metabolite regulation. Therefore, these findings should be considered together with the biodegradation, biogas production, and chemical data obtained during the experiment. The metabolic differences observed between the different digestion times are in agreement with previous studies reporting that PLA biodegradation under anaerobic conditions typically occurs only after prolonged incubation periods, reflecting the slow hydrolysis of the polymer under mesophilic digestion conditions [34].

To further investigate the metabolites contributing to this separation, a univariate statistical analysis was performed using a Student's t -test. This analysis identified 278 metabolites (Table S2) showing statistically significant differences between T7 and T65, highlighting extensive metabolic changes occurring during the progression of AD. Among these, particular attention was given to metabolites directly associated with PLA structure and degradation pathways. Three metabolites directly related to the structure and degradation of PLA were identified: lactide (cyclic dimer), lactoyl-lactic acid (dilactate; linear dimer acid), and lactic acid (2-hydroxypropionic acid) (Figure 6).

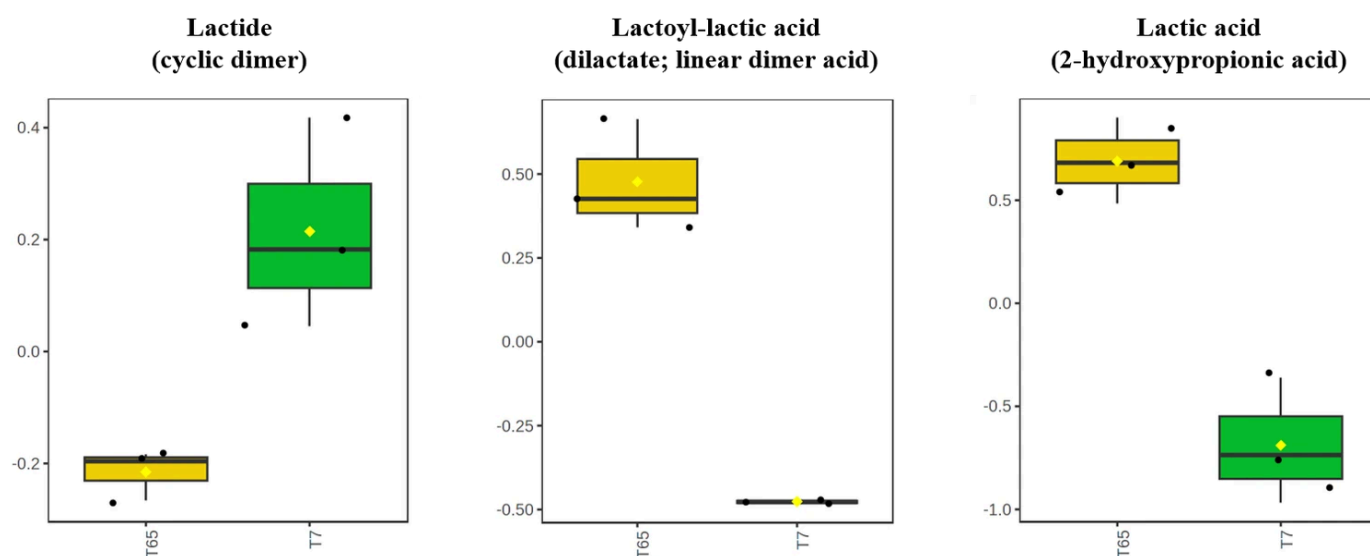


Figure 6. Box plots showing the normalized abundance of three metabolites associated with PLA degradation pathways during mesophilic anaerobic digestion of digestate samples from inoculum added with the organic fraction of municipal solid waste and PLA (Inoculum+OFMSW+PLA): lactide (cyclic dimer), lactoyl-lactic acid (dilactate; linear dimer acid), and lactic acid (2-hydroxypropionic acid). Metabolite profiles were compared between day 7 (T7) and day 65 (T65). The y -axis represents normalized metabolite abundance obtained from untargeted LC–MS/QTOF metabolomic analysis ($n = 3$).

Lactide showed a higher abundance at T7 compared to T65, suggesting that this cyclic dimer may represent a transient product associated with early PLA depolymerization. Cyclic oligomers such as lactide can be generated through intramolecular transesterification reactions occurring during PLA degradation [47]. In contrast, lactoyl-lactic acid was significantly more abundant at T65, indicating accumulation of linear dimeric species

that may arise from hydrolysis of PLA. Similarly, lactic acid exhibited a marked increase at T65 relative to T7, consistent with the release of PLA monomeric units during PLA hydrolysis [48,49]. Comparable accumulation of lactic acid has been previously observed during the anaerobic processing of PLA-containing biodegradable plastics, where lactate release was associated with hydrolytic depolymerization of the polymer matrix [50]. These metabolites therefore provide evidence of PLA hydrolysis and depolymerization processes occurring during AD, although they do not directly demonstrate complete biodegradation or mineralization of the polymer. The extent of PLA biodegradation was independently estimated according to ISO 15985:2014 [16]. The increased abundance of hydrolysis products after two months of AD provides additional evidence of PLA transformation during digestion. This observation is consistent with previous studies describing hydrolytic degradation of PLA under anaerobic conditions and its subsequent conversion during the digestion process [44,51,52].

A volcano plot was used to further visualize the results of the univariate analysis by integrating both statistical significance and magnitude of change between T7 and T65 (Figure 7). In this representation, metabolites are plotted according to their log₂ fold change (T65 vs. T7) and the corresponding $-\log_{10} p$ -value obtained from the *t*-test. Among the 278 metabolites identified as significant by the *t*-test, 233 also met the fold-change threshold applied in the volcano plot analysis ($FC \geq 2.0$).

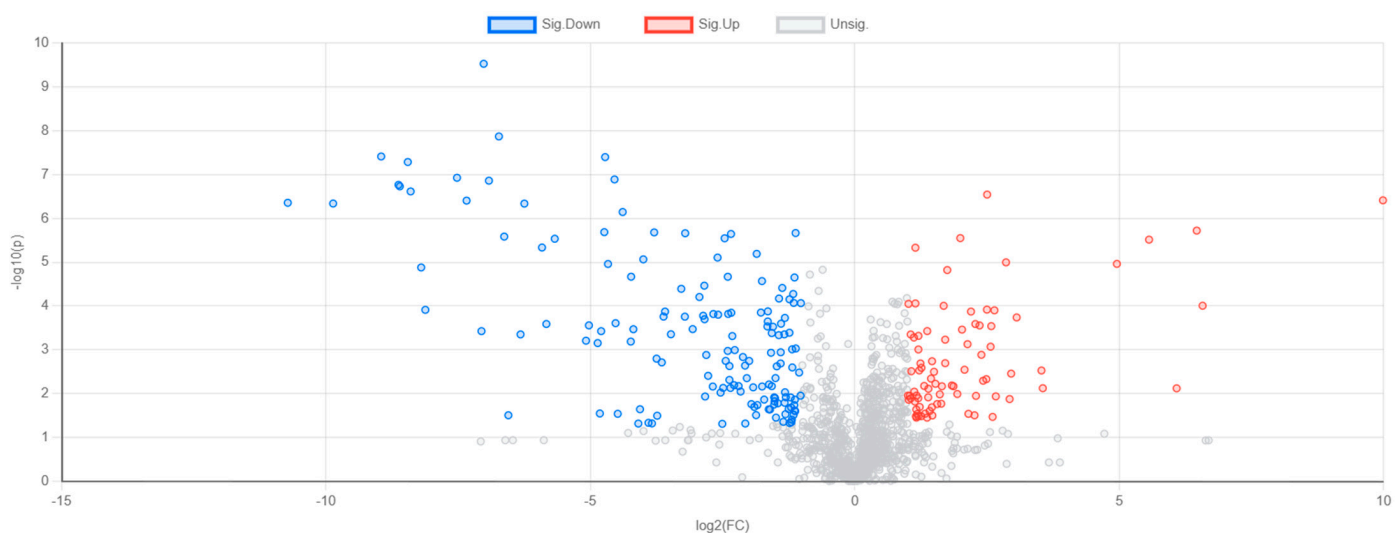


Figure 7. Volcano plot showing differential metabolite features between day 7 (T7) and day 65 (T65) during mesophilic anaerobic digestion of digestate samples from inoculum added with the organic fraction of municipal solid waste and PLA (Inoculum+OFMSW+PLA). Metabolites are plotted according to their log₂ fold change (FC) on the *x*-axis and the corresponding $-\log_{10} p$ -value derived from the *t*-test on the *y*-axis. Significantly upregulated metabolic features at T65 are shown in red, while significantly downregulated features are shown in blue; non-significant features are displayed in grey (*n* = 3).

The volcano plot revealed a substantial number of significantly altered metabolites, with 82 features significantly upregulated at T65 and 151 significantly downregulated compared to T7 (Table S3). This asymmetric distribution highlights a pronounced metabolic shift associated with digestion time, consistent with the separation observed in the PCA (Figure 5). The predominance of downregulated metabolites at T65 may reflect the depletion of readily biodegradable compounds present in the early stages of digestion, whereas the upregulated metabolites are likely associated with intermediate and end-products of anaerobic metabolism, as commonly observed in time-resolved metabolomic studies of AD [53,54].

Finally, a heatmap was generated using the metabolites identified as statistically significant by the univariate analysis to evaluate the consistency of metabolic patterns across biological replicates and to further assess group separation (Figure 8).

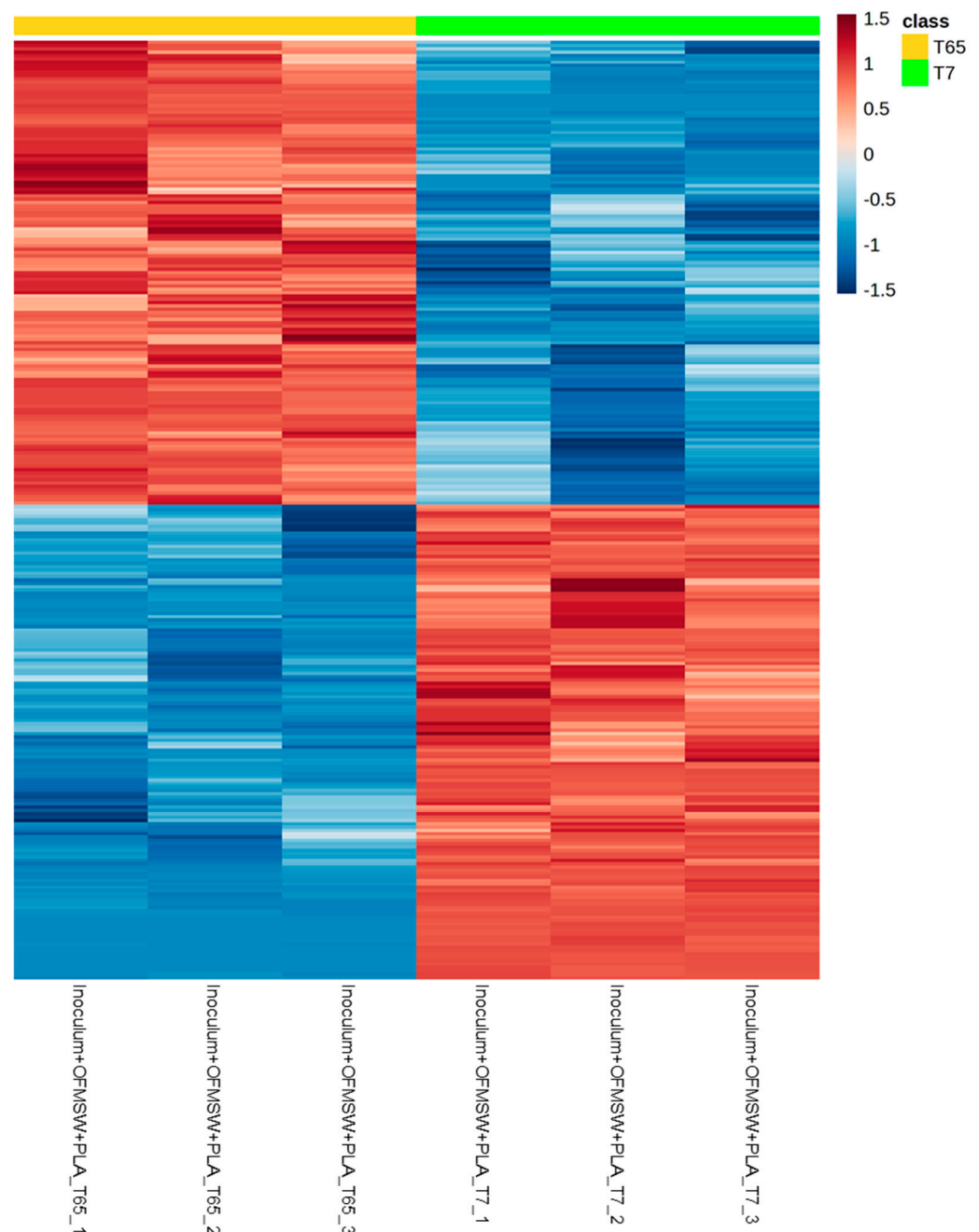


Figure 8. Heatmap showing the relative abundance of significantly altered metabolites identified by univariate analysis between day 7 (T7) and day 65 (T65) during mesophilic anaerobic digestion of digestate samples from inoculum added with the organic fraction of municipal solid waste and PLA (Inoculum+OFMSW+PLA). Metabolite intensities are represented using a scaled color gradient ranging from -1.5 (blue) to $+1.5$ (red), where higher values indicate higher relative abundance and lower values indicate lower relative abundance across samples. Data were clustered by hierarchical clustering to highlight similarities among samples and metabolites ($n = 3$).

The heatmap revealed a clear segregation of samples according to digestion time, with T65 and T7 replicates clustering separately from each other. Replicates within each time point clustered closely together, indicating limited intra-group variability. Two major

clusters of metabolites were observed: one characterized by higher relative abundance at T65 and lower abundance at T7, and a second cluster displaying the opposite trend. Metabolites showing increased abundance at T65 largely corresponded to features identified as significantly upregulated in the volcano plot, while metabolites enriched at T7 were predominantly associated with downregulated features. This reciprocal pattern further supports the presence of a time-dependent metabolic shift during AD. The marked separation suggests that digestion time was a major factor driving the observed metabolic variability. The close clustering of replicates within each sampling time further supports the presence of a consistent metabolic response associated with the progression of AD. To provide a functional interpretation of these metabolomic changes, pathway analysis was subsequently performed using the metabolites identified as altered between T7 and T65 (Figure S1). The analysis highlighted several metabolic pathways affected during the progression of AD, indicating a wider metabolic reorganization associated with the transition from the initial digestion stage to the phase in which PLA starts to be degraded. These results complement the biodegradation kinetics and biogas production data, suggesting that the onset of PLA degradation was accompanied by measurable changes in the metabolic profile of the digestate. Detailed results of the pathway analysis are reported in Table S4.

The metabolomic results are consistent with the chemical and biogas data, indicating that PLA degradation is not immediate but becomes metabolically relevant only after an extended acclimation phase. This AD pattern is typical of slowly degradable polymers and was also detected in a previous study under mesophilic conditions [34]. The emergence of PLA-derived intermediates at T65 supports the hypothesis that polymer hydrolysis represents the rate-limiting step in AD, particularly under mesophilic conditions, after which microbial communities can further metabolize hydrolysis products into central intermediates of AD, ultimately contributing to biogas production [44,51,55].

4. Conclusions

The results demonstrated that PLA can undergo biodegradation under mesophilic AD when co-digested with OFMSW, although degradation started only after a prolonged lag phase of approximately 60 days. The observed PLA degradation kinetics revealed that biodegradation approached 100% after approximately 100 days, and co-digestion with OFMSW did not negatively affect biogas production, confirming the compatibility of PLA with the AD process. Metabolomic analyses highlighted a time-dependent accumulation of metabolites associated with PLA hydrolysis, particularly lactic acid and lactoyl-lactic acid, supporting the occurrence of PLA transformation after two months of digestion.

In conclusion, this work confirms that mesophilic anaerobic co-digestion of PLA with OFMSW can effectively ensure the integration of PLA-containing bioplastics into existing organic waste treatment systems. However, the prolonged lag phase observed in this study suggests that complete degradation may require retention times longer than those commonly adopted in full-scale anaerobic digesters. A feasible way to overcome the inherent slow degradability of PLA could be represented by pretreatments designed to enhance polymer biodegradability under AD conditions.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy16141303/s1>, Table S1: Relative abundance of metabolites detected by untargeted LC–MS/QTOF analysis in digestate samples from the Inoculum+OFMSW+PLA treatment (inoculum added with the organic fraction of municipal solid waste and PLA) at day 7 (T7) and day 65 (T65). Values represent normalized metabolite intensities for individual biological replicates (n = 3 per time point); Table S2: Results of univariate statistical analysis (Student's *t*-test) performed on metabolites detected by untargeted LC–MS/QTOF analysis in digestate samples

from the Inoculum+OFMSW+PLA treatment (inoculum added with the organic fraction of municipal solid waste and PLA) at day 7 (T7) and day 65 (T65). The table reports *t*-statistics, *p*-values, $-\log_{10}(p\text{-value})$, and false discovery rate (FDR) for each metabolite. Metabolites with $p < 0.05$ were considered statistically significant ($n = 3$ per time point); Table S3: Results of volcano plot analysis performed on metabolites detected by untargeted LC–MS/QTOF analysis in digestate samples from the Inoculum+OFMSW+PLA treatment (inoculum added with the organic fraction of municipal solid waste and PLA) at day 7 (T7) and day 65 (T65). The table reports fold change (FC), $\log_2(FC)$, raw *p*-values, and $-\log_{10}(\text{raw } p\text{-value})$ for each metabolite. Metabolites meeting the thresholds of $p < 0.05$ and $FC \geq 2.0$ were considered significantly altered between the two time points ($n = 3$ per time point); Figure S1: Pathway analysis of metabolites altered between day 7 (T7) and day 65 (T65) during mesophilic anaerobic digestion of digestate samples from inoculum added with the organic fraction of municipal solid waste and PLA (Inoculum+OFMSW+PLA). Bubble size indicates pathway impact, whereas color intensity reflects pathway significance based on enrichment analysis; Table S4: Results of pathway analysis based on metabolites detected by untargeted LC–MS/QTOF analysis in digestate samples from the Inoculum+OFMSW+PLA treatment (inoculum added with the organic fraction of municipal solid waste and PLA) at day 7 (T7) and day 65 (T65). The table reports the total number of compounds associated with each pathway (Total Cmpd), the number of matched metabolites (Hits), raw *p*-values, $-\log_{10}(\text{raw } p\text{-value})$, Holm-adjusted *p*-values (Holm), false discovery rate-adjusted *p*-values (FDR), and pathway impact values (Impact).

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

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