Enhancing Sustainable and Energy-Efficient Anaerobic Digestion of Animal Manure: Effects of Intermittent Stirring and ANN-Based Methane Production Modeling

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Despite extensive research on anaerobic digestion (AD), the combined effects of intermittent stirring energy demand and substrate co-digestion on overall energy performance have remained insufficiently explored. This study evaluated the effects of intermittent stirring duration and the AD performance of cattle dung (CD), poultry droppings (PD), rabbit droppings (RD), and their mixtures on methane production and net energy recovery. Experiments were conducted in 30 L laboratory-scale mechanically stirred batch anaerobic digesters operated under mesophilic conditions and intermittent stirring durations of 1, 2, and 3 h/day. Co-digestion clearly outperformed mono-digestion, achieving higher methane yields (up to 223 L/kg VS), improved biodegradability (>90%), methane-rich biogas, and shorter digestion times (18 days). Intermittent stirring duration significantly influenced performance; moderate stirring (2 h/day) was optimal for most binary mixtures, while higher stirring (3 h/day) enhanced methane yield in balanced ternary systems. Optimized co-digestion could reach up to 7.96 MJ/kg VS despite increased mixing energy use. The statistical analyses revealed strong interrelationships among process variables, identifying stirring duration and digestion time as key drivers of methane yield and net energy production. Artificial neural network (ANN) modeling successfully predicted methane production and net energy based on C/N ratio, stirring rate, and waste composition. The optimal ANN showed high accuracy (R=0.99).

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INTRODUCTION

The continuous growth of the global population and the intensification of livestock production systems have resulted in a substantial increase in animal waste generation (Bidoglio *et al.* 2024). Improper handling of livestock manure poses serious environmental challenges, including greenhouse gas (GHG) emissions, nutrient leaching to water bodies, odor nuisance, and public health risks (Setoguchi *et al.* 2022). In recent years, anaerobic digestion (AD) has been widely recognized as a sustainable waste-management technology capable of addressing these challenges while simultaneously producing renewable energy in the form of biogas and generating nutrient-rich digestate suitable for agricultural use (Ahmed *et al.* 2025). Consequently, improving the efficiency and energy performance of AD systems treating animal waste has become a major research priority.

Biogas production from animal manure plays a critical role in climate-change mitigation by reducing methane emissions associated with conventional manure storage and treatment (Wang *et al.* 2024). Recent life cycle and carbon-footprint assessments of livestock farms have demonstrated that integrating biogas plants into manure-management systems can significantly reduce overall GHG emissions compared with traditional slurry storage, even when accounting for operational energy requirements (Setoguchi *et al.* 2022). However, despite the environmental advantages of AD, methane yield and overall energy recovery remain highly sensitive to feedstock properties and operational parameters, necessitating careful process optimization (Alrowais *et al.* 2025, 2026).

Among the many factors influencing AD performance, such as temperature, pH, hydraulic retention time, organic loading rate, solids content, and carbon-to-nitrogen (C/N) ratio, stirring is considered one of the most critical yet controversial operational parameters (Singh *et al.* 2021). In anaerobic digesters, stirring can be achieved through several methods, such as mechanical stirring, gas recirculation, or sludge recirculation, with the choice depending on digester design and substrate characteristics (Kariyama *et al.* 2018; Yang *et al.* 2019). Mechanical stirring is often favored due to its higher efficiency, including lower energy consumption, shorter mixing times, and enhanced biogas production compared with alternative techniques (Singh *et al.* 2021; Wang *et al.* 2024).

Despite these advantages, the effectiveness of stirring is highly dependent on its intensity and duration, which govern both process enhancement and potential inhibitory effects (Li *et al.* 2022). Adequate mixing enhances reactor homogeneity, improves heat and mass transfer, promotes effective contact between microorganisms and substrates, and facilitates biogas release (Wang *et al.* 2024). At the same time, excessive stirring increases power consumption and may generate shear stress that disrupts microbial aggregates, inhibits methanogenic activity, and ultimately reduces methane production (Zhang *et al.* 2023). As a result, the influence of stirring on AD performance cannot be evaluated solely based on methane yield but must also consider the associated energy demand.

Recent experimental studies have demonstrated that the effect of stirring strongly depends on substrate characteristics, solids concentration, and digester configuration (Neuner *et al.* 2024; Wang *et al.* 2024). Comprehensive reviews published in the last few years have emphasized that high-intensity or continuous mixing often leads to diminishing returns or even negative effects on methane production, particularly in digesters treating manure or high-solids substrates (Rocamora *et al.* 2020; Singh *et al.* 2021; Zhang *et al.* 2023). In contrast, intermittent or moderate stirring strategies have been shown to improve process stability and methane yield while minimizing microbial stress (Mao *et al.* 2019; Shekhar Bose *et al.* 2021; Neuner *et al.* 2024). These findings highlight that the optimal stirring regime is system-specific and cannot be generalized without experimental validation.

Other investigations have also highlighted the importance of impeller design and mixing hydrodynamics in anaerobic digesters (Kariyama *et al.* 2018; Wang *et al.* 2022, 2024); Studies comparing different impeller types have shown that mixing efficiency, dead-zone elimination, and shear distribution significantly influence microbial community structure and digestate characteristics, even when methane yields appear similar. Additionally, optimization-focused studies have increasingly treated stirring as part of a multi-variable operational framework, demonstrating that the best stirring strategy depends on trade-offs between enhanced methane productions, reduced hydraulic retention time, and increased energy consumption (Mao *et al.* 2019; Li *et al.* 2022; Neuner *et al.* 2024).

These studies underline the need for integrated assessments that explicitly consider net energy yield, rather than methane production alone.

Co-digestion of multiple wastes has been widely adopted as an effective strategy to enhance AD performance by balancing nutrient composition and improving the C/N ratio (Mao *et al*. 2019; Li *et al*. 2022; Neuner *et al*. 2024; Alrowais *et al.* 2024a, 2024b, 2026). Combining different types of animal waste can increase biodegradability and create synergistic effects among substrates (Jasińska *et al.* 2023). Recent studies have demonstrated that co-digesting of different types of animal waste such as cattle manure, poultry litter, pig manure, or rabbit manure significantly improves methane yield and process stability by mitigating ammonia inhibition and enhancing microbial diversity (Haque *et al.* 2021; Zahedi *et al.* 2022; Enokida *et al.* 2025). Moreover, integrated assessments of co-digestion systems have shown superior volatile solids (VS) reduction and higher energy recovery compared with mono-digestion, particularly under optimized substrate ratios (Bhatnagar *et al.* 2022; Li *et al.* 2022; Mohammed *et al.* 2022). However, such mixtures also introduce heterogeneity in density, particle size, and rheological behavior, which increases the importance of effective mixing (Rocamora *et al.* 2020).

Due to the complex and inherently nonlinear nature of AD processes, conventional kinetic, mechanistic, and regression-based models often exhibit limitations in accurately representing system behavior under varying operational conditions. Consequently, artificial intelligence (AI) techniques have gained increasing attention as powerful tools for modeling and optimizing biogas production systems, with artificial neural networks (ANNs) being among the most widely applied approaches (Galal *et al.* 2025). Unlike conventional modeling methods, ANNs can learn directly from experimental data without requiring predefined assumptions regarding reaction mechanisms. This capability enables them to effectively capture the complex multidimensional interactions among substrate characteristics, operational parameters, and methane production, thereby enhancing predictive accuracy and optimization performance (Abdel daiem *et al.* 2021a,b; Metwally *et al.* 2024; Abd El-wahaab *et al.* 2025).

Recent applications of ANN models in biogas research have demonstrated excellent predictive performance for methane yield, biogas composition, and energy recovery across a wide range of substrates and operating conditions (Metwally *et al.* 2024; Ahmed *et al.* 2025; Alrowais *et al.* 2025). Within this context, ANN models have gained increasing attention due to their suitability for capturing nonlinear and dynamic relationships among process variables in time-dependent and sequential datasets typical of AD systems. Unlike traditional regression models, which generally assume linearity and independence among observations, ANNs can model temporal dependencies and feedback mechanisms, thereby significantly enhancing predictive accuracy and model robustness (Abdel daiem *et al.* 2021a). This capability has been validated in several recent studies reporting the superior performance of ANN-based frameworks in predicting biogas yields and other complex environmental parameters, further supporting their applicability in advanced AD modeling and optimization, as summarized in a recent review (Galal *et al.* 2025).

Despite extensive research on either stirring strategies or data-driven modeling approaches in AD, a clear gap remains in studies that simultaneously integrate experimental evaluation of stirring–energy trade-offs with AI based optimization for co-digestion systems (Haque *et al.* 2021; Bhatnagar *et al.* 2022; Mohammed *et al.* 2022; Zahedi *et al.* 2022; Enokida *et al.* 2025). Studies have investigated either substrate co-digestion strategies or the influence of stirring conditions on AD performance (Mao *et al.* 2019; Rocamora *et al.* 2020; Shekhar Bose *et al.* 2021; Jasińska *et al.* 2023). These aspects are rarely analyzed together within an integrated energy-efficiency frame-work (Kariyama *et al.* 2018; Singh *et al.* 2021). Most previous research has focused primarily on maximizing methane yield without explicitly considering the energy required for reactor mixing or evaluating the resulting net energy balance (Kariyama *et al.* 2018; Wang *et al.* 2022). Furthermore, few studies combine experimental optimization with advanced data-driven modelling techniques capable of capturing nonlinear interactions among operational parameters and substrate characteristics (Abdel daiem *et al.* 2021a; Ahmed *et al.* 2025; Galal *et al.* 2025). As a result, the practical design of energy-efficient AD systems remains constrained by limited understanding of the trade-offs between mixing energy demand, substrate composition, and overall energy recovery. Therefore, this study addresses these gaps by integrating experimental investigation, multivariate statistical analysis, and ANN-modelling to evaluate the combined effects of stirring duration and animal-waste co-digestion on methane production and net energy recovery.

The primary aim of this study was to investigate and optimize the combined effects of stirring intervals and animal waste mixture composition on methane production and net energy yield in a laboratory-scale anaerobic digester. To achieve this objective, the co-digestion of cattle dung (CD), poultry droppings (PD), and rabbit droppings (RD) were experimentally evaluated under mesophilic conditions using different daily stirring durations at a constant stirring speed, while net energy yield is quantified by accounting for both the energy generated from methane production and the energy consumed by mechanical stirring.

The novelty of this work lies in its integrated experimental–modeling frame-work, which couples systematic laboratory experimentation with multivariate statistical analysis and ANN simulation to elucidate the relationships among operational parameters, substrate characteristics, and system performance, and to identify optimal operating conditions. Unlike conventional studies that focus primarily on maximizing methane output, this research emphasizes net energy optimization as a more realistic and practically relevant performance indicator. Furthermore, the combined use of statistical tools (*e.g.*, correlation analysis and principal component analysis (PCA)) with ANN modeling enables both interpretation of variable interdependencies and robust prediction/optimization. This allows for extrapolation beyond the tested conditions and provides valuable guidance for the design and operation of energy-efficient AD systems treating animal waste. Additionally, this integrated framework provides new insights for designing energy-efficient biogas systems capable of supporting sustainable waste management, renewable energy generation, and circular bio economy strategies.

MATERIALS AND METHODS

Experimental Setup and Reactor Configuration

The experimental study was conducted using laboratory-scale batch anaerobic digesters designed to evaluate the effect of intermittent stirring intervals and waste-mixture composition on methane production and net energy yield, as illustrated in Fig. 1. The digesters were constructed from polyvinyl chloride (PVC) with a total working volume of 30 L and operated in batch mode. Each reactor had a height of 72 cm, and an internal diameter of 35 cm. Multiple digesters were operated in parallel to ensure consistency and comparability of results across different experimental conditions. To minimize variability among treatments, substrate characteristics were determined prior to the experiments, and all feedstocks were collected from the same source and prepared following a standardized procedure to ensure consistent physicochemical properties throughout the study.

The reactors were maintained under mesophilic conditions at a constant temperature of 35 ± 1 °C throughout the digestion process. Temperature control was achieved using an external heating system, and all reactors were sealed to maintain anaerobic conditions. To minimize potential gas leakage, all reactor joints, valves, and gas collection connections were sealed using gas-tight fittings and periodically inspected throughout the experiments. Biogas production was monitored daily, and no evidence of significant gas leakage was observed. The study was conducted in batch mode using parallel laboratory-scale mechanically stirred reactors. Accordingly, sludge blanket formation was not expected under the operating conditions employed in this study. Each experimental condition was performed in triplicate to ensure reproducibility and statistical reliability, and the reported results represent the average values of the experimental runs. A schematic representation and photograph of the experimental reactor configuration are provided in Fig. 1.

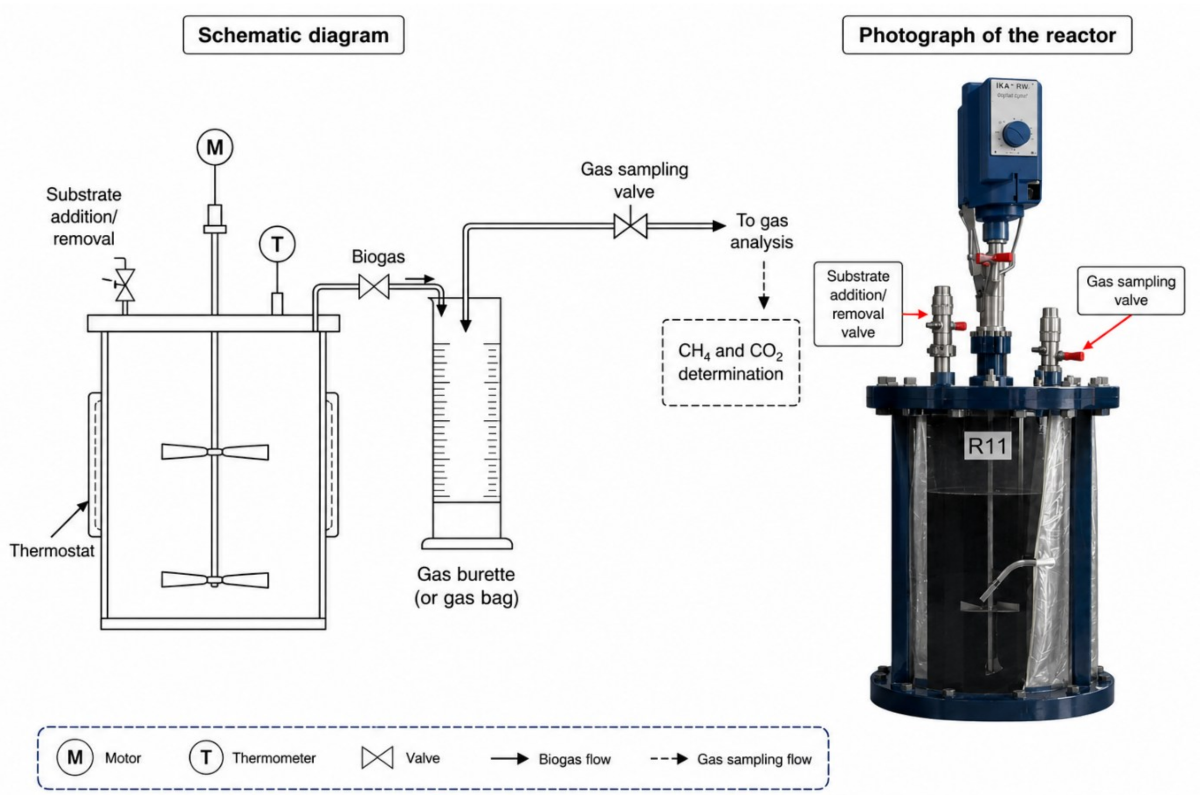


Fig. 1. Schematic diagram and photographic view of the laboratory-scale stirred anaerobic reactor used in the experimental study

Substrates and Feedstock Preparation

Three types of animal waste were used as substrates in this study: CD, PD, and RD. The substrates were collected fresh from local farms and stored at 4 °C prior to use to minimize biological degradation. Before feeding, the waste was homogenized and mixed with tap water to achieve the desired total solids (TS) concentration suitable for AD (Haque *et al.* 2021). Different mono- and co-digestion mixture ratios of CD, PD, and RD were selected based on previous investigations demonstrating their suitability for AD while avoiding excessive foaming or inhibition (Metwally 2019; Bhatnagar *et al.* 2022). The selected substrate ratios are summarized in Table 1.

Operating Conditions and Stirring Protocols

Mechanical stirring was applied using a centrally mounted six-bladed disc impeller designed to promote axial flow. The impeller had a diameter of 16.5 cm and a shaft length of 60 cm, with individual blade dimensions of 5 cm × 5 cm × 0.2 cm. The ratio of impeller diameter to reactor diameter (d/D) was approximately 0.47, which lies within the recommended optimal range for stirred-tank reactors. All digesters were operated at a constant stirring speed of 60 rpm to minimize shear stress on microbial communities while ensuring sufficient mixing. Three different daily intermittent stirring intervals were applied: 1, 2, and 3 h day^{-1}. The selection of stirring intervals and speed was based on published literature emphasizing the importance of moderate and intermittent mixing for stable AD (Singh *et al.* 2021; Neuner *et al.* 2024; Wang *et al.* 2024).

Analytical Methods

The contents of TS, VS, carbon (C), and nitrogen (N) in the substrates were determined according to standard analytical procedures described in Standard Methods for the Examination of Water and Wastewater (APHA 2005). The C/N ratio was calculated from measured carbon and nitrogen concentrations. The pH measurements were conducted using an automatic titration pH meter (ep HI 98107 pocket-sized).

Biogas volume was measured daily using the water displacement method. Methane concentration in the biogas was determined using a portable gas analyzer (IMR 1400C). Methane yields were standardized to normal temperature and pressure (273 K and 1013 mbar) to ensure consistency among measurements. Methane production was expressed as cumulative methane volume per unit of VS added (L CH_4/kg VS). The hydraulic retention time (HRT) varied between 18 and 35 days depending on the stirring interval. Each batch experiment was continued until biogas production became negligible.

Theoretical Methane Potential and Biodegradability Assessment

The theoretical methane potential (TMP) of the substrates and their mixtures studied was estimated based on the chemical composition of the organic fraction. Specifically, TMP was calculated using a stoichiometric approach that relates to methane yield to the relative proportions of carbohydrates, proteins, and fats in the feed-stock. The calculation was performed according to the method proposed in a previous study (Di Girolamo *et al.* 2013), as follows,

$$\mathrm{TMP} = 415\times(\mathrm{carbohydrates}) + 496\times(\mathrm{proteins}) + 1014\times(\mathrm{fats}) \tag{1}$$

where carbohydrates, proteins, and fats are expressed as mass percentages of VS (%VS), and the coefficients represent the theoretical methane yields (L CH_4/kg VS) associated with each biochemical component.

The biodegradability of each individual substrate and the co-digestion mixture was assessed by relating the experimentally measured actual methane yield (AMY) from the AD process to the corresponding TMP (Metwally *et al.* 2024). Biodegradability was calculated using the following equation:

$$\mathrm{Biodegradability}\ (\%) = (\mathrm{AMY}/\ \mathrm{TMP}) \times 100 \tag{2}$$

This approach provides an indicator of the extent to which the organic matter present in the feedstock was converted into methane under the applied digestion conditions.

Energy Consumption and Net Energy Calculations

In this study, only the mechanical energy consumption associated with reactor stirring was considered in the energy analysis. Thermal energy requirements for maintaining the digestion temperature were not included in the net energy calculations because of their relatively high cost and strong dependence on the heating source. Temperature control during the experiments was achieved by circulating hot water around the digesters, and it is assumed that, in pilot- and full-scale applications, this thermal demand would be supplied using renewable energy sources, specifically solar-assisted hot water systems, as discussed and recommended by a previous study (Alrowais *et al.* 2023). Moreover, heat losses resulting from radiation and convection are generally more significant in laboratory-scale digesters due to their higher surface-area-to-volume ratio. Consequently, thermal energy requirements estimated under laboratory conditions may not accurately reflect those encountered in larger-scale practical applications (Alrowais *et al.* 2023). Accordingly, heating energy was excluded to focus on the effect of stirring on the overall energy balance.

Furthermore, the primary objective of the present energy assessment was to evaluate the effect of stirring intensity on net energy recovery. Since the heating demand remained constant across all treatments, it was not expected to influence the comparative evaluation of the different operational conditions. In contrast, the energy required for mixing varied directly with the applied stirring regime and therefore represented the principal operational factor affecting net energy performance. Given that mixing is an integral component of anaerobic digestion and contributes directly to the overall electrical energy demand of the system, its inclusion in the energy assessment provided a more comprehensive evaluation of process energy performance and operational efficiency, particularly in the context of process scale-up and comparative analysis of alternative reactor configurations. The energy consumption associated with mechanical stirring was determined through a stepwise calculation procedure (Afedzi *et al.* 2023). First, the Reynolds number (*Re*) was calculated to characterize the hydrodynamic regime in the digester using Eq. 3,

$$R_e = \frac{\rho \times N \times D^2}{\mu} \tag{3}$$

where μ is the viscosity of the fermentation broth (kg/m.s), N is the impeller rotational speed (rps), ρ is the slurry density (kg/m^3), and D is the impeller diameter (m).

Following the determination of the *R*e, the corresponding power number (N_p) was obtained from standard N_p correlations based on the impeller diameter-to-tank diameter ratio d/D (0.5). The mechanical power consumption (P_c) of the impeller in W or (kg.m^2/s^3), was then calculated using Eq. 4:

$$P_c = N_P \times \rho \times N^3 \times D^5 \tag{4}$$

The energy consumption (E_c), expressed in MJ/kg VS, resulting from mechanical stirring was determined using P_c in (W), 0.0036 is a conversion factor from W to MJ/h, daily stirring duration (S_d) in h/day, digestion time (D_t) in days, and VS of substrate in kg, as given in Eq. 5:

$$E_c = 0.0036 \times P_c \times D_t \times S_d/(\mathrm{VS}) \tag{5}$$

Energy production (EP) in (MJ/kg VS) from methane production (MP) in (m^3/kg VS) was calculated using a calorific value (CV) of 36 MJ/m^3 for methane (Alrowais *et al.* 2023). Net energy yield (MJ/ kg VS) was determined as the difference between the energy produced from methane and the energy consumed by mechanical stirring.

$$E_P = M_P \times \mathrm{CV} \tag{6}$$

Artificial Neural Network (ANN) Modelling

ANN modeling was conducted to predict key performance indicators of the AD process. The experimental dataset used for ANN modelling consisted of 33 observations derived from the full experimental matrix of substrate compositions and stirring durations. Two independent ANN models were developed, each targeting a specific response variable. The first model was designed to predict cumulative methane production using the C/N ratio and stirring rate as input variables. The second model aimed to predict net energy yield based on the proportions of CD and PD in the feedstock mixture. These input parameters were selected because they represent critical operational and substrate-related factors known to influence AD performance. The C/N ratio is widely recognized as a key indicator of nutrient balance affecting microbial metabolism and methane generation, whereas stirring rate directly affects mass transfer, substrate–microorganism contact, and overall reactor hydrodynamics. Similarly, the proportions of CD and PD determine substrate composition, biodegradability, and the resulting biogas potential, which directly impacts the overall energy recovery of the system. Therefore, these variables were considered appropriate predictors for modeling methane production and net energy yield. A detailed discussion of the influence of these parameters is provided in the Results section.

All ANN simulations were implemented using the Neural Network Toolbox in MATLAB R2018b (MathWorks, Natick, MA, USA). Prior to network training, all input and output variables were normalized to a uniform range of 0 to 1 using min–max scaling to improve numerical stability and enhance training efficiency. Data normalization was performed according to the following expression,

$$x_{norm} = \frac{x - x_{min}}{x_{max} - x_{min}} \tag{7}$$

where X_{min} and X_{max} represent the minimum and maximum values of each variable, respectively, determined from the training dataset.

A feed-forward multilayer perception (MLP) architecture was adopted for both models. The optimized network configuration consisted of an input layer with 2 neurons, 2 hidden layers with 9 and 7 neurons, respectively, and an output layer with 1 neuron. The selected ANN architecture was identified through an iterative trial-and-error optimization process in which several network configurations were evaluated. The final architecture was chosen based on its superior predictive performance. Hyperbolic tangent sigmoid (tansig) activation functions were applied in the hidden layers to capture nonlinear relationships, while a linear (purelin) activation function was employed in the output layer to allow continuous-valued predictions. A schematic representation of the final ANN architecture is presented in Fig. 2.

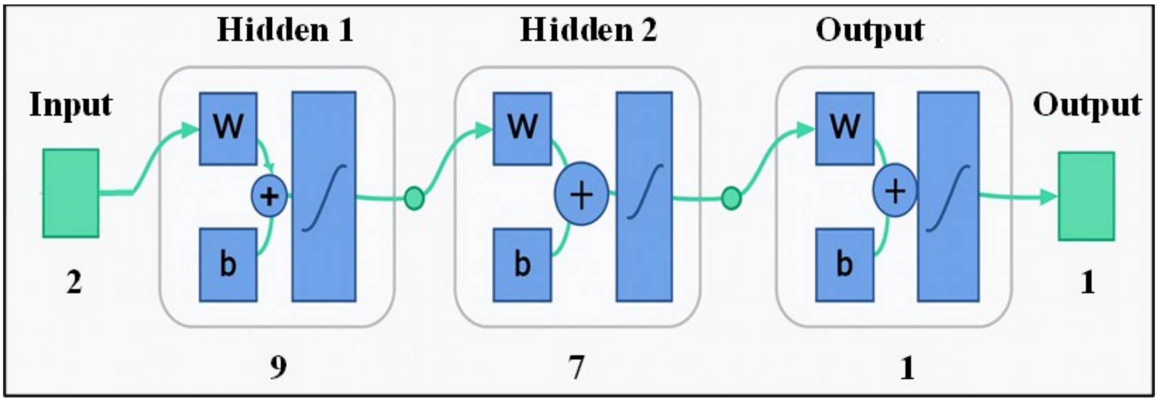


Fig. 2. Schematic diagram of the developed ANN architecture

The complete dataset was randomly divided into two subsets: 80% for model training and 20% for testing the predictive performance of the trained models on unseen data. To identify the most suitable training strategy, five backpropagation algorithms were evaluated: Bayesian Regularization (trainbr), Levenberg–Marquardt (trainlm), BFGS Quasi-Newton (trainbfg), Conjugate Gradient Backpropagation with Polak–Ribière updates (traincgp), and Resilient Backpropagation (trainrp). Comparative results for these algorithms are presented in Table 2. Based on performance evaluation, the traincgp algorithm was selected for the final ANN models due to its superior predictive accuracy.

Multivariate Statistical Analysis

Multivariate statistical analyses were conducted using Microsoft Excel and the Statistical Package for Social Sciences (SPSS, version 22.0). Pearson's correlation analysis and PCA were employed to explore and quantify relationships among the studied variables. These techniques were selected for their robustness in identifying key interactions between operational parameters and for providing a clear visualization of variable interdependencies (Ahmed *et al.* 2025; Alrowais *et al.* 2025).

RESULTS AND DISCUSSION

Physicochemical Characteristics of Substrates

The physicochemical characteristics of the individual substrates and their co-digestion mixtures are presented in Table 1. The findings showed substantial variability in parameters that are known to strongly influence AD performance. These parameters include pH, TS, VS, elemental composition, C/N ratio, and biochemical fractions. Such variability is expected due to differences in animal species, feed composition, and manure-handling practices, and it underscores the importance of substrate characterization prior to process optimization.

The pH values of the investigated substrates ranged from 7.50 to 8.10, while those of the co-digestion mixtures were largely clustered between 7.73 and 7.95. These values fall within the optimal pH range for methanogenic microorganisms under mesophilic conditions, which is generally reported as (6.5 to 8.5) (Ahmed *et al.* 2025). Similar pH ranges have been reported for cattle manure, poultry waste, and mixed animal waste systems in recent studies, indicating that the substrates used in this work are well-suited for stable methane production without requiring external pH adjustment (Bhatnagar *et al.* 2022; Zahedi *et al.* 2022; Enokida *et al.* 2025; Nleya *et al.* 2025).

The TS and VS contents varied considerably among the substrates. PD exhibited the highest TS and VS contents (29.8% and 24.5%, respectively), reflecting their relatively low moisture content and high organic loading (Mao *et al.* 2019; Sillero *et al.* 2022; Zahedi *et al.* 2022). In contrast, RD showed the lowest TS (20.0%) and VS (16.6%) values, and CD presented intermediate values (TS = 25.0%, VS = 22.2%). The co-digestion mixtures displayed TS values between 23.3% and 28.6% and VS values between 20.3% and 23.9%, generally intermediate between their constituent substrates. Such VS levels are considered favorable for AD, as they ensure sufficient biodegradable organic matter while maintaining slurry properties suitable for effective mixing and microbial contact (Haque *et al.* 2021; Zahedi *et al.* 2022; Jasińska *et al.* 2023; Enokida *et al.* 2025).

The elemental composition and corresponding C/N ratios further highlight the benefits of co-digestion. The CD exhibited the highest C/N ratio (22.4), whereas PD showed a markedly lower C/N ratio (9.30) due to its higher nitrogen content (3.83%). Low C/N ratios, such as that of PD, are commonly associated with ammonia inhibition when digested alone (Mao *et al.* 2019; Sillero *et al.* 2022). In contrast, the co-digestion mixtures achieved more balanced C/N ratios, ranging from 11.4 (1 CD:3 PD) to 19.7 (2 CD:1 RD), with most blends falling between 14 and 17.5. These values are within or close to the optimal range recommended for stable AD operation and align with the literature, which indicates that blending carbon-rich cattle manure with nitrogen-rich poultry waste effectively mitigates ammonia toxicity and enhances methane production (Haque *et al.* 2021; Jasińska *et al.* 2023; Song *et al.* 2023; Enokida *et al.* 2025).

Substantial differences were also observed in the biochemical composition of the substrates. CD was characterized by a high carbohydrate fraction (38.5% of VS) and relatively low protein (8.45%) and fat (1.35%) contents, consistent with its fibrous nature and slower biodegradation kinetics (Haque *et al.* 2021). PD, in contrast, contained a much higher proportion of proteins (28.6% of VS) and fats (7.45%), which can promote rapid biogas production (Bhatnagar *et al.* 2022). RD exhibited a more balanced profile, with carbohydrates accounting for 35.4% of VS, proteins for 15.0%, and fats for only 1.68%, suggesting moderate biodegradability and lower inhibition risk (Li *et al.* 2022).

The co-digestion mixtures displayed intermediate and more balanced biochemical compositions compared with mono-substrates. For example, the 2 CD:1 PD mixture contained 29.4% carbohydrates, 15.2% proteins, and 3.38% fats, while the 2 CD:1 RD blend maintained a high carbohydrate fraction (37.5%) with moderate protein content (10.6%). Mixtures with higher PD proportions, such as 1 CD:3 PD, showed elevated protein (23.6%) and fat (5.93%) contents, which may enhance methane. In contrast, balanced mixtures with moderate CD and PD proportions appear particularly favorable, as they combine sufficient readily degradable fractions with improved nutrient balance and reduced inhibitory potential (Bhatnagar *et al.* 2022).

Overall, the substrate characteristics summarized in Table 1 are consistent with recent literature and clearly demonstrate the advantages of co-digestion in improving feedstock quality. The balanced pH (7.7 to 8.0), optimized C/N ratios (approximately 14 to 20), and diversified biochemical fractions observed in the co-digestion mixtures are expected to enhance microbial synergy, reduce inhibition risks, and improve methane production and net energy recovery. These physicochemical results provide a strong basis for interpreting the experimental performance trends observed in this study and further support the application of co-digestion strategies in AD systems treating animal wastes.

Cumulative Methane Production

Figure 3 presents the cumulative methane production profiles for various mono- and co-digestion systems under different stirring durations, highlighting the critical role of mixing intensity on digestion kinetics and methane yield. Figure 3a shows that stirring duration strongly influenced methane production from CD. At 1.0 h/day, methane accumulated slowly and reached 120 L/kg VS after 35 days, indicating mass-transfer limitations under low mixing. Increasing stirring to 2 h/day significantly improved kinetics and yield, achieving the highest methane production (150 L/kg VS) within 20 to 22 days, reflecting improved homogeneity and microbial contact. Although 3 h/day accelerated early methane formation and shortened digestion time, the final yield decreased (135 L/kg VS), suggesting negative effects of excessive mixing, similar observations were recorded by previous study (Wang *et al.* 2022).

The observed increase in methane production with moderate stirring durations can be attributed to improved mass transfer and enhanced microbial–substrate interactions, which facilitate hydrolysis and subsequent methanogenesis. However, excessive mixing may disrupt microbial flocs, aggregates, and localized microenvironments, thereby reducing process stability and increasing energy consumption without commensurate gains in methane production. In mechanically stirred batch digesters, these effects are primarily associated with alterations in microbial interactions and mass-transfer conditions, explaining the reduced methane yield observed at prolonged stirring durations in certain substrate combinations (Kariyama *et al.* 2018; Wang *et al.* 2022, 2024). Overall, 2 h/day represents the optimal stirring duration for maximizing methane yield while reducing digestion time.

Figure 3b shows that cumulative methane production from PD is strongly affected by stirring duration, reflecting the nitrogen-rich nature of this substrate (Haque *et al.* 2021; Sillero *et al.* 2022). At 1.0 h/day, methane production was slow, reaching only ~115 L/kg VS after 35 days, indicating delayed methanogenesis likely due to ammonia inhibition (Bhatnagar *et al.* 2022). Increasing stirring to 2 h/day improved kinetics and yield, with methane reaching 127 L/kg VS within 22 days, as mixing reduced localized ammonia accumulation and enhanced microbial contact, as reported in a recent study (Enokida *et al.* 2025). The highest performance was achieved at 3 h/day, where methane production accelerated markedly; digestion time was shortened to 18 days, and the final yield increased to 143 L/kg VS. Overall, the results demonstrate that PD benefited from longer stirring, with 3 h/day providing the most favorable balance between methane recovery and digestion time, highlighting the substrate-specific nature of optimal mixing strategies. These results emphasize the importance of mixing in nitrogen-rich substrates such as PD, where localized ammonia accumulation can inhibit methanogenic activity. Increased mixing likely reduces ammonia gradients within the digester, thereby improving microbial adaptation and maintaining stable methanogenic conditions.

Figure 3c shows that cumulative methane production from RD was highly dependent on stirring duration, with RD exhibiting the strongest response to increased mixing among the tested substrates. At 1.0 h/day, methane production was slow and reached only 105 L/kg VS after 35 days, indicating limited hydrolysis and mass transfer due to the fibrous and partially lignocellulosic nature of RD (Adrover *et al.* 2020). Increasing stirring to 2 h/day markedly enhanced digestion kinetics, with methane yield rising to 184 L/kg VS within 22 days as improved mixing facilitated hydrolysis and microbial access. The highest performance was achieved at 3 h/day, where methane production accelerated sharply, digestion time shortened to 18 days, and the final yield reached 200 L/kg VS.

Table 1. Physicochemical Characteristics and Organic Composition of CD, PD, RD, and their Mixtures Used in this Study

Substrate	pH	TS (%)	VS (%)	C (%)	N (%)	C/N	Carbohydrates (% of VS%)	Proteins (% of VS%)	Fats (% of VS%)
CD	8.10	25.00	22.20	48.300	2.16	22.40	38.51	8.45	1.35
PD	7.50	29.78	24.46	35.600	3.83	9.30	11.33	28.64	7.45
RD	7.60	20.00	16.60	38.700	2.56	15.10	35.45	14.98	1.68
2 CD: 1 RD	7.93	23.33	20.33	45.100	2.29	19.68	37.49	10.63	1.46
1 CD: 1 PD	7.80	27.39	23.33	41.950	2.99	14.02	24.92	18.55	4.40
2 CD: 1 PD	7.90	26.59	22.95	44.067	2.71	16.24	29.45	15.18	3.38
1.5 CD: 1 PD	7.86	26.91	23.11	43.220	2.82	15.30	27.64	16.53	3.79
2 CD: 1 PD: 1 RD	7.83	24.95	21.37	42.725	2.68	15.97	30.95	15.13	2.96
3 CD: 2 PD :1 RD	7.82	25.76	22.02	42.467	2.78	15.27	28.94	16.27	3.44
1 CD: 3 PD	7.65	28.59	23.90	38.775	3.41	11.37	18.12	23.59	5.93
1 CD :1 PD: I RD	7.73	24.93	21.09	40.867	2.85	14.34	28.43	17.36	3.49
3 CD:1 PD	7.95	26.20	22.77	45.125	2.57	17.53	31.72	13.50	2.88

Figure 3d shows that co-digestion of CD and PD at a 3 CD: 1 PD ratio clearly improved methane production kinetics and cumulative yield compared with mono-digestion, confirming a synergistic interaction between the two substrates, as found in previous investigation (Bhatnagar *et al.* 2022; Jasińska *et al.* 2023). At a stirring duration of 1.0 h/day, methane production increased gradually, reaching about 130 L/kg VS after 35 days, but the slow kinetics indicate that limited mixing still constrained mass transfer and microbial activity. Increasing the stirring duration to 2 h/day led to a marked improvement, with methane accumulation accelerating and reaching approximately 165 L/kg VS within 20 to 22 days, reflecting improved substrate homogeneity, nutrient distribution, and buffering of inhibitory compounds. When stirring was further increased to 3 h/day, methane production occurred more rapidly in the early stages and digestion time was shortened to around 18 days; however, the final methane yield decreased to about 140 L/kg VS, suggesting that excessive mixing may have limited ultimate me-thane recovery. Overall, the results indicate that moderate stirring (2 h/day) provides the best balance between methane yield and digestion time for the 3 CD: 1 PD co-digestion system.

Figure 3e demonstrates that ternary co-digestion of CD, PD, and RD (3 CD: 2 PD: 1 RD) exhibited strong synergistic behavior, with methane production highly dependent on stirring duration. At 1.0 h/day, methane accumulated slowly, reaching about 145 L kg/VS after 35 days, indicating that limited mixing restricted full substrate interaction and hydrolysis. Increasing stirring to 2 h/day markedly enhanced digestion kinetics, with methane yield rising to approximately 180 L/kg VS within 22 days due to improved homogeneity and nutrient distribution. The highest performance was achieved at 3 h/day, where methane production accelerated sharply, digestion time was shortened to about 18 days, and the final methane yield reached around 210 L/kg VS. Overall, the results indicate that increased stirring consistently enhanced methane yield and kinetics for the 3 CD: 2 PD: 1 RD mixture.

Figure 3f shows that methane production from the 2 CD: 1 PD co-digestion system was strongly influenced by stirring duration, reflecting a balance between enhanced mass transfer and potential over-mixing effects. At 1.0 h/day, methane accumulation was slow, reaching about 115 L/kg VS after 35 days, indicating that limited mixing restricted substrate homogenization and delayed methanogenic stabilization. Increasing stirring to 2 h/day markedly improved digestion performance, with methane yield rising to approximately 170 L/kg VS within 20 to 22 days, demonstrating more efficient nutrient balance, microbial contact, and substrate utilization. Further increasing stirring to 3 h/day accelerated early methane production and shortened digestion time to around 18 days but reduced the final yield to about 145 L/kg VS, suggesting that excessive mixing favored rapid consumption of readily degradable fractions without improving conversion of more recalcitrant material. Overall, the results indicate that 2 h/day provided the optimal compromise between methane yield and digestion time for the 2 CD: 1 PD mixture.

Figure 3g shows that methane production from the 2 CD: 1 RD co-digestion system was strongly affected by stirring duration, highlighting the synergistic benefit of adding RD to CD. At 1.0 h/day, methane accumulation was slow, reaching about 120 L/kg VS after 35 days, indicating that limited mixing constrained hydrolysis and mass transfer of fibrous components. Increasing stirring to 2 h/day markedly improved digestion performance, with methane yield rising to approximately 165 L/kg VS within 20 to 22 days, reflecting enhanced substrate homogenization, improved hydrolysis, and better microbial access. Further increasing stirring to 3 h/day accelerated early methane production and shortened digestion time to around 18 days but reduced the final yield to about 145 L/kg VS, suggesting that excessive mixing favored rapid degradation of readily biodegradable fractions without improving conversion of recalcitrant material. Overall, the results indicate that 2 h/day provides the optimal balance between methane yield and digestion time for the 2 CD: 1 RD mixture.

Figure 3h shows that cumulative methane production from the ternary co-digestion mixture 2 CD: 1 PD: 1 RD was strongly dependent on stirring duration. At 1.0 h/day, methane accumulated slowly, reaching about 135 to 140 L/kg VS after 35 days, indicating that limited mixing constrained full utilization of synergistic interactions. Increasing stirring to 2 h/day significantly improved digestion performance, with methane yield rising to approximately 170 L/ kg VS within 20 to 22 days due to enhanced homogenization and mass transfer. The highest performance was achieved at 3 h/day, where methane production accelerated markedly, digestion time shortened to about 18 days, and cumulative methane yield reached nearly 200 L/kg VS. Unlike some mono- and binary systems, this ternary mixture continued to benefit from intensified stirring. This behavior suggests that substrate diversity improve system tolerance to higher mixing intensity. Overall, the results highlight the importance of substrate complexity in defining optimal stirring strategies for maximizing methane recovery.

Figure 3i shows that methane production from the 1.5 CD: 1 PD co-digestion mixture was strongly influenced by stirring duration, reflecting the balance between carbon-rich CD and nitrogen-rich PD. At 1.0 h/day, methane accumulated slowly, reaching about 120 to 125 L/kg VS after 35 days, indicating that limited mixing restricted full synergistic interaction and delayed methanogenesis. Increasing stirring to 2 h/day markedly enhanced digestion performance, with methane yield rising to approximately 175 to 178 L/kg VS within 20 to 22 days, representing the highest yield for this mixture due to improved homogenization and nutrient balance. Further increasing stirring to 3 h/day accelerated early methane production and shortened digestion time to around 18 days but reduced the final yield to about 140 to 145 L/kg VS, suggesting over-mixing effects. Overall, the results indicate that 2 h/day provided the optimal compromise between methane yield and digestion time for the 1.5 CD: 1 PD mixture.

Figure 3j shows that methane production from the 1 CD: 1 PD co-digestion mixture increased consistently with stirring duration, indicating strong synergistic behavior. At 1.0 h/day, methane accumulated slowly, reaching about 155 to 160 L/kg VS after 35 days. Increasing stirring to 2 h/day markedly improved digestion kinetics, producing approximately 180 to 185 L/kg VS within 20 to 22 days due to better homogenization and nutrient balance. The highest performance was achieved at 3 h/day, where digestion time was reduced to about 18 days and methane yield reached 220 to 225 L/kg VS. Unlike other binary mixtures, intensified stirring did not reduce the final yield. This indicates that the balanced substrate composition enhanced tolerance to higher mixing intensity and supported stable methanogenesis.

Figure 3k shows that methane production from the 1 CD: 3 PD co-digestion mixture is highly sensitive to stirring duration due to its nitrogen-rich nature. At 1.0 h/day, methane accumulated slowly, reaching about 150 L/kg VS after 35 days, indicating delayed methanogenesis likely caused by ammonia inhibition under insufficient mixing. Increasing stirring to 2 h/day markedly improved digestion performance, with methane yield rising to approximately 180 L/kg VS within 20 to 22 days as ammonia gradients were reduced and microbial acclimation improved. The best performance was achieved at 3 h/day, where methane production accelerated significantly, digestion time shortened to about 18 days, and cumulative methane yield reached 190 to 195 L/kg VS.

Figure 3l shows that methane production from the 1 CD: 1 PD: 1 RD ternary co-digestion mixture was strongly influenced by stirring duration, reflecting the balanced nature of the substrates. At 1.0 h/day, methane accumulated slowly, reaching about 145 to 150 L/kg VS after 35 days, indicating that limited mixing restricted full synergistic interaction. Increasing stirring to 2 h/day significantly enhanced digestion performance, with methane yield rising to approximately 185 to 190 L/kg VS within 20 to 22 days due to improved homogenization and microbial interactions. The highest performance was achieved at 3 h/day, where digestion time was shortened to about 18 days and cumulative methane yield reached 210 to 215 L/kg VS. Unlike some binary systems, intensified stirring did not reduce the final yield. Overall, the results confirm that increased stirring consistently improved methane yield and kinetics for this balanced ternary mixture, with 3 h/day being the most favorable condition.

In addition to the effect of stirring, the observed improvements in methane production for several co-digestion mixtures can be attributed to enhanced substrate balance. Animal manures frequently exhibit suboptimal C/N ratios when treated individually, particularly nitrogen-rich substrates such as PD, which may increase the risk of ammonia inhibition (Mao *et al.* 2019; Sillero *et al.* 2022; Bhatnagar *et al.* 2022). Co-digestion of CD, PD, and RD improved the overall C/N ratio and nutrient balance (as indicated in Table 1), thereby creating more favorable conditions for microbial activity and methane generation.

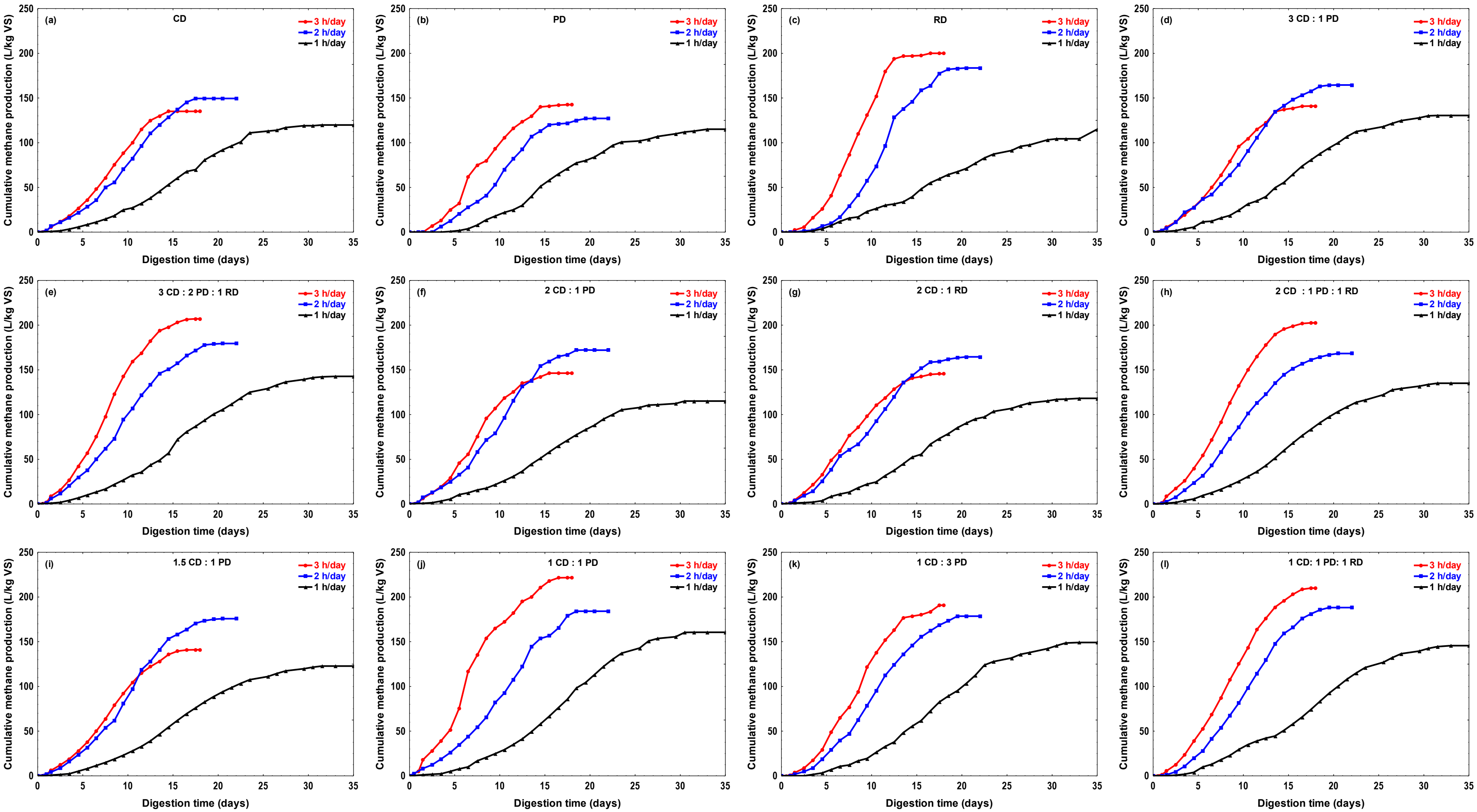


Fig. 3. Cumulative methane production under different stirring durations for mono- and co-digestion systems: (a) CD, (b) PD, (c) RD, (d) 3 CD:1 PD, (e) 3 CD:2 PD:1 RD, (f) 2 CD:1 PD, (g) 2 CD:1 RD, (h) 2 CD:1 PD:1 RD, (i) 1.5 CD:1 PD, (j) 1 CD:1 PD, (k) 1 CD:3 PD, and (l) 1 CD:1 PD:1 RD.

This finding is consistent with previous studies reporting that combining different manure types can partially overcome the limitations associated with individual substrates, enhance biodegradability, and improve AD performance (Haque *et al.* 2021; Bhatnagar *et al.* 2022; Jasińska *et al.* 2023; Song *et al.* 2023; Enokida *et al.* 2025).

Figure 3 collectively shows that intermittent stirring duration had a strong influence on methane production kinetics and yield across all digestion systems. Low intermittent stirring (1.0 h/day) resulted in slow methane accumulation and long digestion times, while moderate intermittent stirring (2 h/day) provided the best performance for most mono- and binary systems. In contrast, intensified intermittent stirring (3 h/day) was more beneficial for nitrogen-rich and multi-substrate mixtures, producing higher methane yields and shorter digestion times. Overall, the results confirm that optimal stirring intensity is substrate-dependent rather than universal. These findings demonstrate that the optimal intermittent stirring regime in AD is highly substrate-dependent and governed by the balance between enhanced mass transfer and microbial stability. Moderate mixing improves substrate accessibility and microbial contact, whereas excessive mixing may disrupt microbial aggregates and increase operational energy demand without proportional gains in methane production.

Because the present investigation was conducted using mechanically stirred batch digesters, the identified optimal stirring conditions should be interpreted within the context of this reactor configuration. Accordingly, the observed relationships between intermittent stirring duration, methane production, and net energy recovery are directly applicable to batch-operated systems and may differ from continuous-flow operating conditions. Previous studies have demonstrated that mixing performance and methane production are influenced by reactor hydrodynamics, impeller design, and digester configuration (Wang *et al.* 2022, 2024). Therefore, additional studies using continuous-flow and sludge-blanket reactor systems with different impeller configurations and positioning as well as comprehensive assessments of biogas upgrading requirements, are recommended to evaluate the transferability of these findings to larger-scale applications (Neuner *et al.* 2024). Moreover, although the observed trends are expected to remain relevant, pilot- and full-scale studies are necessary to validate the identified optimal operating conditions under practical operational environments and larger-scale process constraints.

Biodegradability and Net Energy

The performance data summarized in Table 2 provides a detailed assessment of the influence of substrate type, co-digestion strategy, and stirring duration on biogas production, methane concentration, methane yield, bio-degradability, and overall energy balance. The table shows that both feedstock composition and operational conditions strongly affected AD outcomes, particularly methane generation dynamics and net energy recovery.

Across all substrates, increasing the stirring duration from 1.0 to 2 h/day generally reduced the digestion period from 35 to 22 days and led to higher methane and biogas yields. In many cases, a further increase to 3 h/day shortened the digestion period to 18 days but did not always improve performance, indicating that excessive stirring may reduce retention time benefits without proportionally increasing methane recovery. Similar non-linear responses to mixing intensity have been reported in recent studies, which emphasize the importance of optimizing stirring duration rather than maximizing it (Zhang *et al.* 2023; Wang *et al.* 2024).

For mono-digestion systems, CD showed moderate biogas yields (192 to 233 L/kg VS) and methane contents ranging from 35.4% to 66.4% at early and late digestion stages under 1 h stirring, increasing to maximum methane values of 68.0% at 2 h stirring. PD exhibited lower methane percentages in the early digestion phase (as low as 6.00%), reflecting rapid acidogenesis and ammonia stress, but methane content increased significantly toward the end of digestion (up to 70.6%). RD displayed the most pronounced improvement with increased stirring, achieving the highest AMY among mono-substrates (200 L/kg VS) and a maximum methane content of 83.8% at 3 h stirring. These trends align well with recent reports indicating that RD is highly biodegradable but benefits strongly from enhanced mixing due to its fibrous structure and slower hydrolysis (Adrover *et al.* 2020; Jasińska *et al.* 2023).

Biogas produced during the experiments was composed primarily of CH_4 and CO_2, which together constituted the dominant fraction of the biogas. The methane concentrations measured throughout the digestion process are presented in Table 2, while CO_2 accounted for most of the remaining gas fraction. Other gaseous components, including hydrogen, were detected only at trace levels and were therefore considered negligible and excluded from further analysis.

The methane percentage ranges reported in the table explicitly reflect the minimum and maximum methane concentrations recorded from the initial to the final stages of digestion. In nearly all cases, methane content increased substantially over time, confirming a clear transition from acidogenic to methanogenic dominance. Early-stage methane values were often low, particularly for nitrogen-rich substrates such as PD and PD-rich mixtures, while final methane contents frequently exceeded 70% in optimized co-digestion systems. Such temporal methane evolution is consistent with recent dynamic biogas studies, which highlight the importance of reporting methane ranges rather than single average values (Alrowais *et al.* 2023; Zhang *et al.* 2023; Metwally *et al.* 2024).

Co-digestion systems consistently outperformed mono-digestion in terms of methane and biogas yield, biodegradability, and net energy. For example, the 1 CD:1 PD mixture achieved a biogas yield of 327 L/kg VS and an AMY of 222 L/kg VS at 3 h stirring, with methane content increasing from 56.1% in early digestion to 89.0% at the final stage. This corresponded to an exceptionally high biodegradability of 92.4%, indicating near-complete conversion of biodegradable organic matter. Similar enhancements in methane yield and methane purity through cattle–poultry co-digestion have been widely reported, primarily due to improved C/N balance and synergistic microbial activity (Bhatnagar *et al.* 2022; Jasińska *et al.* 2023; Song *et al.* 2023).

The TMP remained constant for each substrate or mixture, while biodegradability varied markedly with stirring duration and feedstock composition. Mono-digestion systems generally exhibited lower biodegradability values (43.5% to 69.4%) compared with co-digestion mixtures, many of which exceeded 75% and reached values as high as 92.4%. This confirms that co-digestion not only can increase methane yield but also it can improve the extent of organic matter conversion, a finding strongly supported by recent comparative AD studies (Bhatnagar *et al.* 2022; Sillero *et al.* 2022).

Table 2. Effect of Stirring Duration and Substrate Composition on Biogas and Methane Production, Biodegradability, and Energy Balance

Substrate	Stirring Duration (h)	Digestion Period (day)	Biogas Yield (L/kg VS)	Methane (%)	AMY (L/kg VS)	TMP (L/kg VS)	Biodegradability (%)	Energy Production (MJ/kg VS)	Energy Consumption (MJ/kg VS)	Net Energy (MJ/kg VS)
CD	**1**	35	191.66	35.43- 66.43	119.86	215.41	55.64	4.31	0.02	4.30
	2	22	233.49	39.86- 67.95	149.50		69.40	5.38	0.02	5.36
	3	18	209.87	27.24-69.01	135.05		62.69	4.86	0.02	4.84
PD	**1**	35	184.05	6.00-62.82	115.10	264.61	43.50	4.14	0.02	4.13
	2	22	198.47	23.58-67.17	127.15		48.05	4.58	0.02	4.56
	3	18	221.53	29.15-70.55	142.56		53.87	5.13	0.03	5.11
RD	**1**	35	159.30	18.68-67.20	104.37	238.46	43.77	3.76	0.02	3.74
	2	22	255.02	46.50-75.19	183.61		77.00	6.61	0.02	6.59
	3	18	294.12	21.26-83.82	200.00		83.87	7.20	0.03	7.17
2 CD: 1 RD	**1**	35	188.96	25.87-62.91	118.17	223.10	52.97	4.25	0.02	4.24
	2	22	256.92	19.34-72.76	164.75		73.76	5.92	0.02	5.90
	3	18	226.19	25.92-69.26	145.56		65.24	5.24	0.03	5.21
1 CD: 1 PD	**1**	35	230.40	26.77-71.71	160.55	240.01	66.89	5.78	0.02	5.77
	2	22	257.47	53.15-76.67	184.15		76.72	6.63	0.02	6.61
	3	18	326.89	56.10-89.00	221.65		92.35	7.98	0.02	7.96
2 CD: 1 PD	**1**	35	184.05	35.43-65.62	115.10	231.81	49.65	4.14	0.02	4.13
	2	22	268.51	41.34-66.58	172.30		74.33	6.20	0.02	6.17
	3	18	227.36	47.24-71.80	146.31		63.11	5.27	0.02	5.24
1.5 CD: 1 PD	**1**	35	196.32	27.28-62.91	122.77	235.09	52.22	4.42	0.02	4.40
	2	22	274.35	25.10-65.06	175.77		74.77	6.33	0.02	6.31
	3	18	219.65	47.24-65.97	141.36		60.00	5.08	0.02	5.05
2 CD: 1 PD: 1 RD	**1**	35	215.96	35.61-63.60	135.05	233.47	57.84	4.86	0.02	4.85
	2	22	262.67	17.07-65.36	168.29		72.08	6.06	0.03	6.04
	3	18	314.80	18.62-66.68	202.58		86.77	7.29	0.02	7.27
3 CD: 2 PD :1 RD	**1**	35	228.23	35.43-62.82	142.73	235.65	60.57	5.14	0.02	5.12
	2	22	280.19	22.67-66.30	179.51		76.17	6.46	0.02	6.44
	3	18	321.80	31.88-66.64	207.08		87.88	7.45	0.02	7.43
1 CD: 3 PD	**1**	35	228.20	15.51-68.79	149.17	252.31	59.12	5.37	0.02	5.35
	2	22	253.00	16.90-72.44	178.57		70.77	6.43	0.02	6.41

Substrate	Stirring Duration (h)	Digestion Period (day)	Biogas Yield (L/kg VS)	Methane (%)	AMY (L/kg VS)	TMP (L/kg VS)	Biodegradability (%)	Energy Production (MJ/kg VS)	Energy Consumption (MJ/kg VS)	Net Energy (MJ/kg VS)
	3	18	240.53	13.23-81.90	190.82		75.63	6.87	0.02	6.84
1 CD :1 PD: I RD	**1**	35	210.76	18.69-70.01	145.62	239.50	60.80	5.24	0.02	5.23
	2	22	263.16	10.38-74.85	188.22		78.59	6.78	0.02	6.75
	3	18	286.19	18.37-75.47	209.97		87.67	7.56	0.03	7.53
3 CD:1 PD	**1**	35	208.59	35.43-64.37	130.45	227.71	57.29	4.70	0.02	4.68
	2	22	256.84	28.06-65.13	164.60		72.26	5.92	0.02	5.90
	3	18	219.50	44.87-67.98	141.60		61.94	5.08	0.02	5.05

From an energy perspective, energy production closely followed AMY trends, with the highest energy production values observed for co-digestion mixtures under 2 to 3 h stirring. Importantly, energy consumption due to stirring remained very low and nearly constant (0.02 to 0.03 MJ/kg VS), even at higher stirring durations. Consequently, net energy was primarily governed by methane production rather than mixing energy demand. The maximum net energy value (7.96 MJ/kg VS) was achieved by the 1 CD:1 PD mixture at 3 h stirring, demonstrating the energetic superiority of optimized co-digestion strategies (Alrowais *et al.* 2023; Neuner *et al.* 2024; Wang *et al.* 2024).

Overall, the data in Table 2 clearly demonstrate that (i) methane content increased progressively from the early to late stages of digestion, (ii) co-digestion significantly enhanced methane yield, biodegradability, and methane purity, and (iii) moderate stirring durations (around 2 h/day) often provided the best balance between digestion time reduction and energy efficiency. These findings are consistent with recent studies (references) reporting that moderate intermittent mixing enhances methane production while minimizing microbial disruption and energy consumption in manure-based digestion systems. Therefore, providing strong experimental evidence supporting the integration of optimized mixing strategies with animal-waste co-digestion to maximize methane production and net energy recovery. However, the present results extend previous research by demonstrating that the optimal stirring regime is highly dependent on substrate diversity and co-digestion composition, highlighting the importance of system-specific optimization strategies.

Artificial Neural Network (ANN) Modeling

The application of ANNs to model complex AD processes requires careful selection and preparation of experimental data, as the database construction stage is considered one of the most critical steps in ANN development (Alrowais *et al.* 2025). To ensure reliable prediction performance, the selected input variables must accurately represent the underlying process dynamics. Once the relevant variables are identified, the experimental data are collected, processed, and prepared for network training (Galal *et al.* 2025).

In this study, all input and output variables were normalized to a dimensionless range between 0 and 1 prior to training. Data normalization is widely recommended in ANN modeling, as it improves numerical stability, accelerates convergence, and enhances prediction accuracy (Metwally *et al.* 2024). Since both inputs and outputs were normalized, the predicted outputs were subsequently de-normalized to enable direct comparison with the corresponding experimental measurements. Two separate ANN models were developed to analyze different aspects of system performance. The first model was designed to investigate the combined effects of stirring duration and substrate C/N ratio on cumulative methane production. The second ANN model focused on predicting net energy yield as a function of waste mixing ratios and stirring duration, aiming to identify optimal operating conditions that maximize net energy recovery.

ANN Modeling and Optimization of Methane Production

For the methane production model, the ANN inputs consisted of the C/N ratio and stirring duration, while the network output was the cumulative methane production measured at the end of the digestion period. Different digestion durations were considered depending on the applied stirring regime: 35 days for 1 h/day, 22 days for 2 h/day, and 18 days for 3 h/day. This approach ensured that the ANN predictions reflected stabilized methane yields corresponding to each operational condition.

It is well established that ANN training results can vary slightly due to random initialization of network weights, even when the same architecture and dataset are used. To account for this variability and ensure robust model selection, each ANN topology was trained and evaluated 20 times independently. The performance of each configuration was then assessed by comparing statistical indicators across all training runs to identify the most reliable architecture and training algorithm.

Table 3 presents a comprehensive comparison of different ANN architectures, defined by the number of neurons in the first and second hidden layers (L1 and L2), and combined with five backpropagation training algorithms (trainbr, trainlm, trainbfg, traincgp, and trainrp) for predicting cumulative methane production from C/N ratio and stirring rate. Model performance was assessed using total MSE and regression coefficients (R) for training, testing, and the overall dataset.

Table 3. Performance Evaluation of ANN Architectures and Training Algorithms for Predicting Cumulative Methane Production

Training Algorithm	L1	L2	MSE			Regression		
			Total	Training	Testing	Total	Training	Testing
Trainbr	5	0	0.0185	0.0151	0.0327	0.8518	0.8849	0.7164
Trainlm	5	0	0.0085	0.008	0.0103	0.9349	0.944	0.8862
Trainbfg	5	0	0.0106	0.012	0.005	0.9175	0.9169	0.9344
Traincgp	5	0	0.008	0.0086	0.0053	0.939	0.9378	0.965
Trainrp	5	0	0.0113	0.0123	0.0072	0.9125	0.9094	0.9661
Trainbr	10	0	0.0153	0.015	0.0163	0.8789	0.8946	0.6704
Trainlm	10	0	0.0260	0.0000	0.1338	0.8038	0.9998	0.4378
Trainbfg	10	0	0.0115	0.0006	0.0567	0.9319	0.9961	0.7632
Traincgp	10	0	0.0071	0.0068	0.0081	0.946	0.9487	0.9287
Trainrp	10	0	0.0098	0.0061	0.0253	0.924	0.9567	0.6742
Trainbr	3	2	0.0139	0.0156	0.007	0.8903	0.8803	0.9825
Trainlm	3	2	0.0099	0.0092	0.0129	0.9249	0.9327	0.9121
Trainbfg	3	2	0.0124	0.0132	0.0091	0.9045	0.8833	0.9839
Traincgp	3	2	0.0111	0.0112	0.0108	0.9144	0.9109	0.9168
Trainrp	3	2	0.0153	0.0168	0.0093	0.878	0.8837	0.8117
Trainbr	5	3	0.0453	0.0500	0.0261	0.6721	0.6645	0.7335
Trainlm	5	3	0.0042	0.0004	0.0196	0.9693	0.9970	0.6696
Trainbfg	5	3	0.0068	0.0068	0.0068	0.949	0.9546	0.9609
Traincgp	5	3	0.0071	0.0008	0.0334	0.9478	0.9943	0.4037
Trainrp	5	3	0.0117	0.0111	0.014	0.9087	0.9101	0.8974
Trainbr	9	7	0.0139	0.0138	0.0143	0.8926	0.8949	0.8729
Trainlm	9	7	0.0139	0.0000	0.0716	0.894	1.0000	0.1696
Trainbfg	9	7	0.0186	0.0000	0.0956	0.8935	1.0000	0.6875
Traincgp	9	7	0.0023	0.0000	0.0114	0.9856	0.9996	0.9912
Trainrp	9	7	0.0069	0.0065	0.0087	0.9470	0.9530	0.9205
Trainbr	10	10	0.0138	0.0142	0.012	0.8914	0.8891	0.82
Trainlm	10	10	0.0069	0.0000	0.0353	0.9491	1.0000	0.8289
Trainbfg	10	10	0.0191	0.0000	0.0983	0.8629	1.0000	0.4898
Traincgp	10	10	0.0108	0.0000	0.0556	0.9175	0.9999	0.3119
Trainrp	10	10	0.0041	0.0042	0.0038	0.9691	0.971	0.9773

Among all evaluated configurations, the ANN trained using the conjugate gradient algorithm with Polak–Ribiére updates (traincgp) consistently demonstrated superior predictive performance. In particular, the network architecture with two hidden layers containing 9 and 7 neurons (L1 = 9, L2 = 7) achieved the lowest total MSE (0.0023) and the highest overall R = 0.9856, with excellent agreement between predicted and experimental values for both training (R = 0.9996) and testing datasets (R = 0.9912). This indicates strong generalization capability and minimal overfitting.

Other training algorithms, such as trainlm and trainbfg, showed good performance for certain architectures but were often associated with overfitting, as evidenced by near-zero training errors coupled with significantly higher testing MSE and lower testing regression values. Similarly, architectures with fewer neurons or single hidden layers exhibited higher prediction errors and weaker regression performance, suggesting insufficient model complexity to capture the nonlinear relationships between process variables.

Overall, the results confirm that ANN performance is highly sensitive to both network architecture and training algorithm. The traincgp algorithm combined with a 9–7 hidden-layer structure provided the most reliable and accurate predictions and was therefore selected as the optimal ANN configuration for subsequent modeling, sensitivity analysis, and process optimization.

Table 4 presents the average and standard deviation (SD) of the total MSE obtained from five different ANN training algorithms; each executed over 20 independent runs. The use of both the mean and the SD provided a robust assessment of prediction accuracy and model stability. The results revealed clear differences among the examined training algorithms. The traincgp algorithm achieved the lowest average total MSE (0.0077), accompanied by the smallest SD (0.0032). This indicates that traincgp not only offered superior predictive accuracy but also exhibited high consistency across repeated training runs, which is essential for modeling complex nonlinear processes such as AD.

In comparison, the trainbr algorithm produced the highest average MSE (0.0201) and the largest SD (0.0125), reflecting relatively poorer predictive performance and lower robustness. The trainlm and trainbfg algorithms showed intermediate performance, with average MSE values of 0.0125 and 0.0132, respectively. The trainrp algorithm performed better than these methods; however, its accuracy and stability remained inferior to those of traincgp.

Based on this quantitative comparison, traincgp was selected as the optimal training algorithm for this study. Further regression analysis associated with traincgp identified the optimal neural network architecture as two hidden layers with 9 and 7 neurons, respectively. This configuration provided an effective balance between model complexity and generalization capability, minimizing prediction error without overfitting.

Table 4. Statistical Comparison of ANN Training Algorithms Based on MSE

	Average Total MSE	**SD Total MSE**
Trainbr	0.0201	0.0125
Trainlm	0.0125	0.0078
Trainbfg	0.0132	0.0048
Traincgp	0.0077	0.0032
Trainrp	0.0099	0.0039

Figure 4 illustrates the regression performance of the optimized ANN trained using the traincgp algorithm for the training dataset, testing dataset, and the complete dataset. The regression plots demonstrate a strong agreement between the ANN-predicted outputs and the corresponding experimental target values, indicating the high predictive capability of the developed model. For the training phase, an excellent correlation coefficient (R = 0.9996) was obtained, with the regression line closely matching the ideal 1:1 line. This near-perfect fit confirms that the ANN successfully learned the underlying nonlinear relationships between the input parameter, namely the C/N ratio and stirring duration, and cumulative methane production. The minimal scatter around the regression line further indicates negligible systematic error during model learning.

The testing results also showed strong predictive performance, with a high correlation coefficient (R = 0.9912). Although a slightly greater dispersion was observed compared with the training dataset, the regression line remained close to the ideal fit, demonstrating the ANN model's good generalization capability when applied to unseen data. This confirms that the model did not suffer from overfitting and was robust for predictive applications. When considering the complete dataset (training + testing), the ANN achieved an overall correlation coefficient of R = 0.9856, indicating an excellent global fit between predicted and experimental values. The consistency between training, testing, and overall regression results highlights the stability and reliability of the selected ANN architecture and training algorithm.

Overall, the strong linear relationships observed in Fig. 4, combined with the high correlation coefficients, confirm that the traincgp-based ANN model accurately captured the complex nonlinear interactions governing methane production in AD systems. These results validate the suitability of the developed ANN framework as a powerful predictive and optimization tool for assessing the combined effects of substrate characteristics and operational parameters on cumulative methane production.

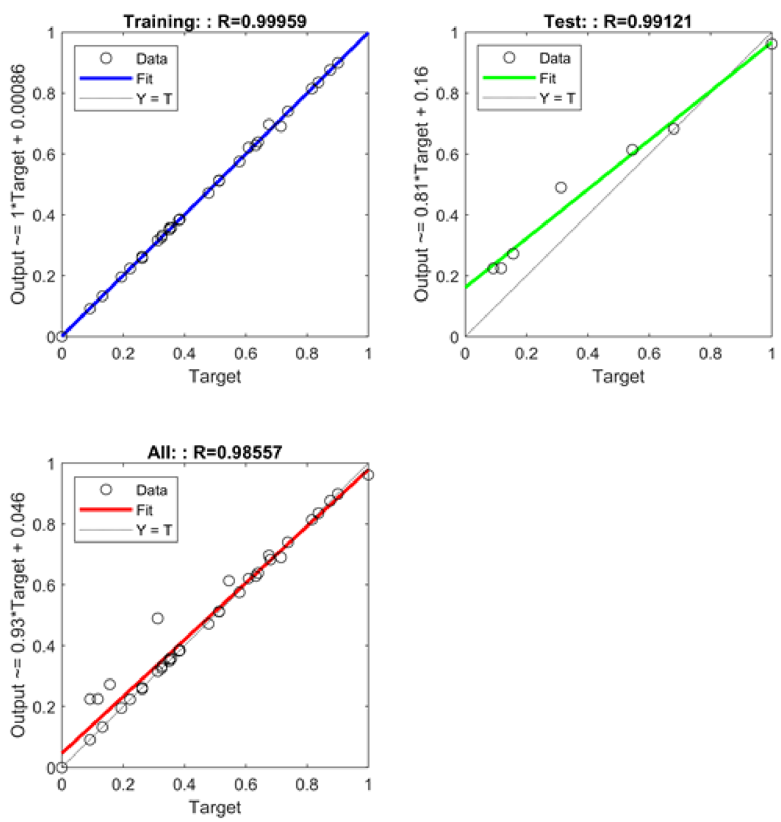


Fig. 4. Regression analysis between ANN-predicted and experimental cumulative methane production for training, testing, and overall datasets

Figure 5 presents the three-dimensional response surface generated by the optimized ANN trained using the traincgp algorithm, illustrating the combined influence of stirring rate and C/N ratio on cumulative methane production. The surface clearly demonstrates the nonlinear interaction between these two key operational parameters and highlights their critical role in governing methane yield. As evident from the response surface, methane production increased markedly with increasing stirring periods, reaching its maximum at a stirring duration of 3 h/day across a broad range of C/N ratios. This confirms that longer stirring enhances mass transfer, substrate homogeneity, and microbial–substrate contact, thereby promoting more efficient hydrolysis and methanogenesis (Neuner *et al.* 2024; Wang *et al.* 2024). The ANN predictions are consistent with the earlier experimental observations in this study, which showed that longer stirring durations led to accelerated digestion kinetics and increased methane recovery, particularly in co-digestion systems.

The effect of the C/N ratio was also clearly captured by the ANN surface. Methane production increased as the C/N ratio approached an intermediate optimal range (approximately 15 to 20), beyond which a decline in methane yield is often observed (Haque *et al.* 2021; Enokida *et al.* 2025). Low C/N ratios are associated with nitrogen-rich substrates and potential ammonia inhibition, whereas excessively high C/N ratios may indicate carbon-rich, slowly degradable material that limits microbial activity (Sillero *et al.* 2022). The response surface thus reflects the well-established requirement for balanced nutrient conditions in AD. Importantly, the highest methane production region on the surface occurs at the combination of high stirring rate (3 h/day) and moderate C/N ratio, indicating a strong synergistic interaction between mechanical mixing and substrate composition (Zhang *et al.* 2023; Wang *et al.* 2024). This interaction would be difficult to quantify using conventional linear models, underscoring the advantage of ANN-based modeling for capturing complex, nonlinear system behavior (Galal *et al.* 2025).

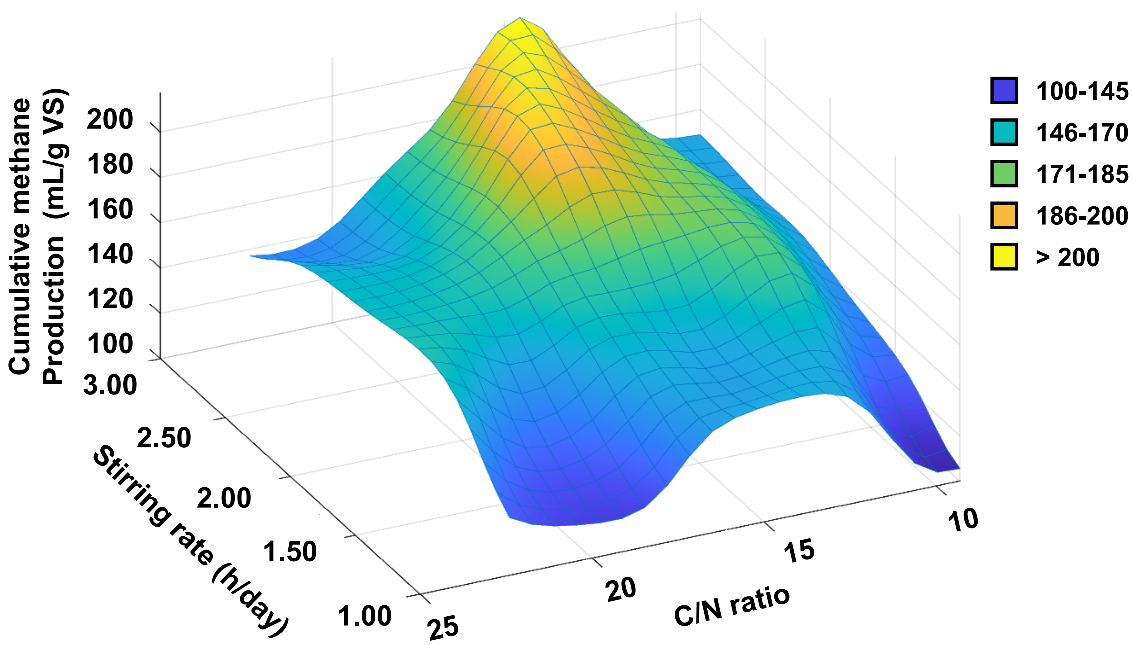


Fig. 5. ANN-predicted response surface showing the combined effect of stirring rate and C/N ratio on cumulative methane production

Overall, Fig. 5 confirms the ANN model's ability to reliably predict methane production trends and identify optimal operating conditions. The surface response provides a powerful visualization and optimization tool, demonstrating that a stirring rate of 3 h/day, combined with an appropriate C/N ratio, yields the maximum methane production. These results further validate the use of the traincgp-based ANN model for process optimization and decision support in AD systems.

ANN Analysis of Net Energy Production

This section evaluates the combined influence of stirring rate and waste composition on net energy production using ANN modeling. Three separate ANN models were developed, each corresponding to a fixed stirring duration (1, 2, and 3 h/day), to predict net energy yield as a function of waste mixing ratios. In all models, the CD ratio and PD ratio were used as input variables, while net energy was considered the output. The RD ratio was calculated implicitly from the mass balance constraint (CD + PD + RD = 1). All ANN models employed the previously identified optimal configuration, consisting of two hidden layers with 9 and 7 neurons, trained using the conjugate gradient Polak–Ribiére algorithm (traincgp). Hyperbolic tangent sigmoid (tansig) and linear (purelin) activation functions were applied in the hidden and output layers, respectively. This configuration enabled reliable prediction of net energy production across a wide range of waste compositions at each stirring rate.

Figure 6 illustrates the ANN-predicted response surfaces showing how variations in CD and PD ratios influence net energy production at stirring durations of 1, 2, and 3 h/day.

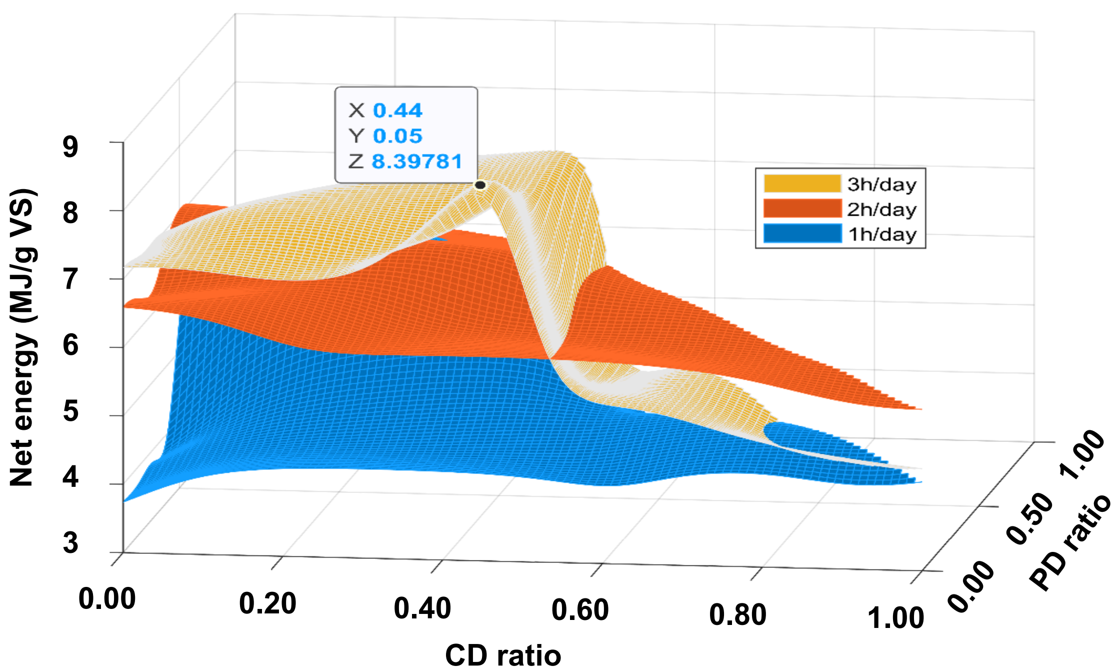


Fig. 6. ANN-Predicted effect of waste mixing ratios and stirring rate on net energy production

Overall, extending the stirring duration tended to increase net energy output; however, the magnitude of this improvement was highly dependent on substrate composition. When the CD fraction exceeded approximately 0.5, further lengthening of stirring periods did not consistently enhance net energy and may even lead to a decline.

This trend highlights the trade-off between improved methane generation, and the additional energy demand imposed by increased mixing intensity, in agreement with previous studies (Li *et al.* 2022; Neuner *et al.* 2024).

The ANN model predicted a maximum net energy yield of approximately 8.4 MJ/kg VS at a stirring rate of 3 h/day, with an optimal waste composition of CD ratio = 0.44, PD ratio = 0.05, and RD ratio = 0.51. This optimal region corresponds to a balanced combination of carbon-rich, nitrogen-rich, and fibrous substrates, which promotes high methane recovery while maintaining low relative mixing energy demand. The dominance of RD in the optimal mixture suggests that substrate diversity enhances system resilience and energy efficiency under higher stirring intensities.

Overall, the results demonstrate that maximizing net energy production requires the simultaneous optimization of stirring intensity and waste composition, rather than merely increasing stirring alone. The integration of ANN modeling with experimental data provides a robust decision-support framework, enabling accurate prediction of AD performance across a wide range of operational conditions that would otherwise necessitate extensive experimental testing. This data-driven approach not only facilitates the identification of promising operational regions and offers valuable guidance for process optimization but also enhances the understanding of underlying process dynamics and informs system design and operation. While the predicted optimal conditions require experimental validation to confirm their practical feasibility, this ANN-based modeling framework has the potential to substantially reduce the time, cost, and experimental effort associated with AD optimization. Such approaches are increasingly recognized as essential for advancing efficient waste-to-energy technologies within the emerging digital bioeconomy 3.5.

Multivariate Statistical Analysis

Correlation analysis

The Pearson correlation heatmap highlights several strong and statistically significant relationships that clarify the key drivers of AD performance and energy balance, as illustrated in Fig. 7. Among the substrate characteristics, TS and VS showed an exceptionally strong positive correlation ($r = 0.97$, $p \leq 0.01$), confirming that VS constitutes the major biodegradable fraction of TS. The C/N ratio is also strongly correlated with pH ($r = 0.90$, $p \leq 0.01$), indicating that feedstock composition directly influences buffering capacity and process stability, as reported by previous studies (Haque *et al.* 2021; Zahedi *et al.* 2022; Jasińska *et al.* 2023; Enokida *et al.* 2025).

Stirring duration emerged as a critical operational parameter. It showed a very strong negative correlation with digestion period ($r = -0.96$, $p \leq 0.01$), demonstrating that increased mixing substantially shortens the required digestion time. At the same time, stirring duration was positively and significantly correlated with biogas yield ($r = 0.58$), methane yield ($r = 0.59$), biodegradability ($r = 0.59$), energy production ($r = 0.59$), and net energy ($r = 0.59$) (all $p \leq 0.05$). These relationships confirm that enhanced mixing improves mass transfer and microbial contact, leading to higher conversion efficiency and energy recovery, as found by earlier studies (Singh *et al.* 2021; Wang *et al*. 2024).

	pH	TS	VS	C/N	Stirring duration	Digestion period	Biogas yield	Methane yield	Biodegradability	Energy production	Energy consumption	Net energy
pH	1.000	-0.170	0.060	**0.900**	0.000	0.000	-0.020	-0.140	0.080	-0.140	-0.220	-0.140
TS	-0.170	1.000	**0.970**	**-0.530**	0.000	0.000	-0.070	-0.070	-0.190	-0.070	-0.200	-0.060
VS	0.060	**0.970**	1.000	-0.330	0.000	0.000	-0.070	-0.100	-0.170	-0.100	-0.250	-0.100
C/N	**0.900**	**-0.530**	-0.330	1.000	0.000	0.000	-0.070	-0.170	0.060	-0.170	-0.080	-0.170
Stirring duration	0.000	0.000	0.000	0.000	1.000	**-0.960**	**0.580**	**0.590**	**0.590**	**0.590**	**0.390**	**0.590**
Digestion period	0.000	0.000	0.000	0.000	**-0.960**	1.000	**-0.640**	**-0.650**	**-0.650**	**-0.640**	**-0.340**	**-0.640**
Biogas yield	-0.020	-0.070	-0.070	-0.070	**0.580**	**-0.640**	1.000	**0.960**	**0.960**	**0.950**	0.200	**0.950**
Methane yield	-0.140	-0.070	-0.100	-0.170	**0.590**	**-0.650**	**0.960**	1.000	**0.970**	**1.000**	0.210	**1.000**
Biodegradability	0.080	-0.190	-0.170	0.060	**0.590**	**-0.650**	**0.960**	**0.970**	1.000	**0.970**	0.180	**0.970**
Energy production	-0.140	-0.070	-0.100	-0.170	**0.590**	**-0.640**	**0.950**	**1.000**	**0.970**	1.000	0.210	**1.000**
Energy consumption	-0.220	-0.200	-0.250	-0.080	**0.390**	**-0.340**	0.200	0.210	0.180	0.210	1.000	0.200
Net energy	-0.140	-0.060	-0.100	-0.170	**0.590**	**-0.640**	**0.950**	**1.000**	**0.970**	**1.000**	0.200	1.000

1.000
0.800
0.600
0.400
0.200
0.000
-0.200
-0.400
-0.600
-0.800
-1.000

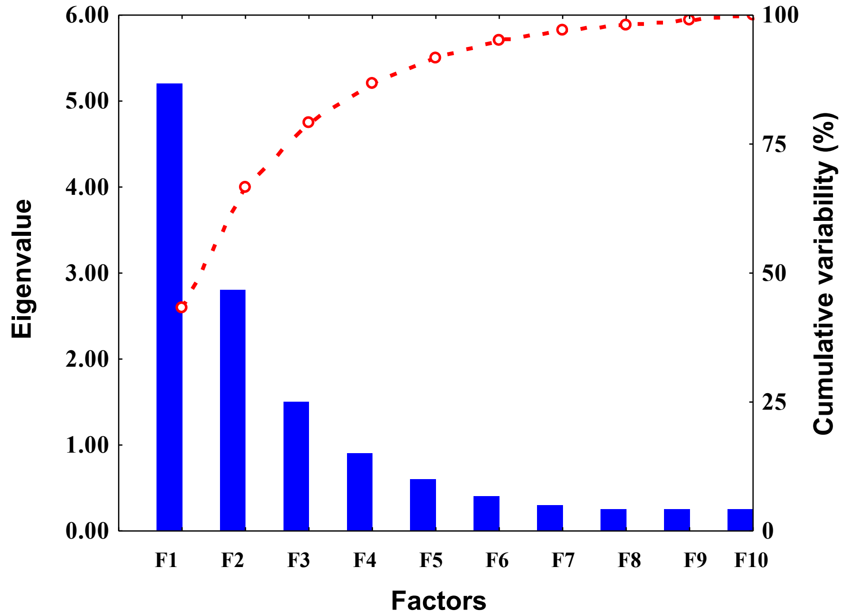
Fig. 7. Pearson correlation matrix among different parameters. Values represent Pearson correlation coefficients (r). **Bold** and **bold and underline** indicate significance at $p \leq 0.05$ and $p \leq 0.01$, respectively.

Very strong positive correlations were observed among performance indicators. Biogas yield correlated almost perfectly with methane yield ($r = 0.96$, $p \leq 0.01$), biodegradability ($r = 0.96$, $p \leq 0.01$), energy production ($r = 0.95$, $p \leq 0.01$), and net energy ($r = 0.95$, $p \leq 0.01$). Similarly, methane yield, biodegradability, energy production, and net energy exhibit near-unity correlations ($r = 0.97$–1.00, $p \leq 0.01$). These results indicate that improvements in biodegradation efficiency are directly translated into higher methane recovery and net energy gain, as reported in previous investigations (Metwally *et al.* 2024).

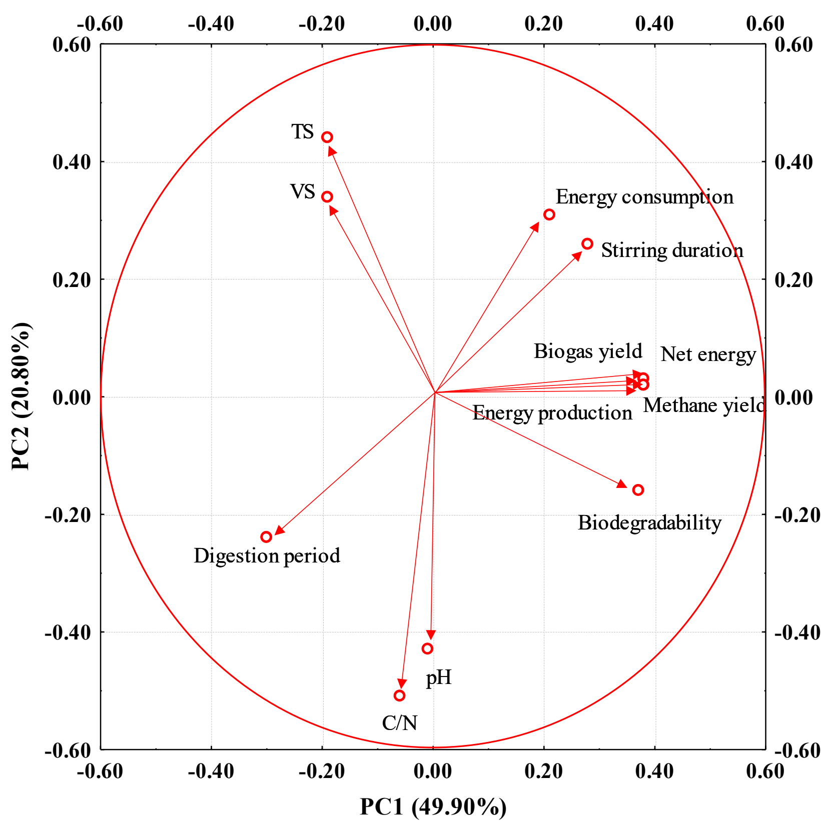
Energy consumption showed only moderate correlations with stirring duration ($r = 0.39$) and weak correlations with biogas, methane yield, and net energy ($r \approx 0.18$ to 0.21). This suggests that, within the range investigated, the additional mechanical energy required for stirring was relatively small compared with the gains in methane production and net energy output. Overall, the heatmap clearly demonstrates that stirring duration and digestion period are the dominant operational variables, while methane yield and biodegradability are the main determinants of energy production and net energy performance. Similar observations have been found in the literature (Mao *et al.* 2019; Singh *et al.* 2021; Li *et al.* 2022; Neuner *et al.* 2024; Wang *et al.* 2024).

Principal Component Analysis

Figure 8a presents a combined scree plot and cumulative variance curve, illustrating the contribution of successive factors (F1–F10) to the total variance of the dataset.



(a)



(b)

Fig. 8. PCA (a) relationships between factor number and Eigenvalues, and (b) principal component biplot chart

The bar chart shows that the first factor (F1) explains the largest share of variance, confirming that a single dominant underlying process governs much of the system's behavior. The second (F2) and third (F3) factors also contribute substantially, while the magnitude of explained variance decreases progressively for higher-order factors.

The dashed cumulative variance curve rose steeply from F1 to F3, indicating that these first few factors together accounted for a large proportion of the total variability. By the fourth factor (F4), the cumulative explained variance approached a high percentage of the total, after which the curve began to level off. This clear "elbow" behavior suggests diminishing returns in explanatory power beyond F4–F5, with subsequent factors (F6–F10) contributing only marginally.

Overall, the figure indicates that retaining approximately four factors is sufficient to capture the dominant structure of the data, in agreement with common factor-retention criteria such as the elbow method and cumulative variance thresholds. This dimensionality reduction simplifies interpretation while preserving the essential information needed to describe the relationships among physicochemical properties, operational parameters, methane production, and energy performance.

The PCA biplot (PC1 *vs*. PC2) illustrates the interrelationships among the studied variables and their relative contributions to system variability, as indicated in Fig. 8b. PC1 accounted for approximately 49.9% of the total variance, while PC2 explained about 20.8%, resulting in a cumulative explained variance of nearly 70.7%, which indicates that the first two components adequately describe the dominant trends in the dataset. Variables related to process performance, biogas yield, methane yield, biodegradability, energy production, and net energy, cluster closely and point in a similar direction along PC1, indicating a strong positive correlation among them. This grouping confirms that improvements in biodegradability directly translate into higher methane production and greater net energy recovery.

In contrast, the digestion period vector pointed in the opposite direction to these performance indicators, revealing a negative relationship; shorter digestion times were associated with higher methane and energy yields. This trend is consistent with the experimental observation that increased stirring intensity accelerated process stabilization and enhanced productivity. The vectors for stirring duration and energy consumption were oriented in a similar direction, indicating a positive association between mixing intensity and energy input. However, their relative position with respect to net energy suggests that, within the investigated range, the gains in methane and energy production outweighed the additional mixing energy demand.

The TS and VS variables were oriented differently from the main performance cluster, implying that their influence is more closely related to substrate characteristics than to direct energy outcomes. Meanwhile, pH and C/N ratio were closely aligned with PC2 and were positioned near each other, highlighting their stabilizing role in the digestive process rather than a direct control over energy yield under the tested conditions.

Overall, the biplot indicates that PC1 represents a "performance–energy" axis, dominated by methane production, biodegradability, and net energy recovery, whereas PC2 reflects operational and compositional effects, such as digestion period, stirring intensity, and substrate properties. These results emphasize that optimizing stirring intensity and substrate balance is key to maximizing methane production and net energy recovery while maintaining process stability.

Limitations and Recommendations for Future Work

Although this study demonstrated the effects of stirring duration and substrate composition on AD performance, its laboratory-scale design limits direct extrapolation to full-scale systems and excludes variable or continuous mixing regimes. In addition, the ANN models were developed using a controlled dataset, ensuring high internal validity but limiting broader applicability. Future research should validate the identified operating conditions in continuous-flow, pilot-scale, and full-scale digestion systems under realistic operating conditions. Further studies should investigate the co-digestion of animal manures with carbon-rich agricultural residues to optimize substrate balance and methane production, as well as alternative mixing approaches, including lower impeller rotational speeds and short-duration high-intensity intermittent mixing bursts, to further improve energy efficiency. Comprehensive assessments of biogas upgrading requirements should also be conducted to evaluate their impact on the overall energy balance and economic feasibility of commercial-scale biomethane production. Expanding experimental datasets will facilitate the development of more robust ANN and hybrid models, while integrating life cycle assessment, techno-economic analysis, and advanced machine-learning techniques will support real-time optimization and a more holistic evaluation of AD system sustainability.

CONCLUSIONS

1. This study evaluated anaerobic digestion (AD) of substrates cattle dung (CD), poultry droppings (PD), rabbit droppings (RD), and their mixtures under different stirring durations, supported by artificial neural network (ANN) modeling. Co-digestion clearly out-performed mono-digestion, achieving higher methane yields (up to 225 L/kg volatile solids (VS)), biodegradability above 90%, and shorter digestion times (18 days).
2. Methane content increased from low initial values to methane-rich biogas (>70%) at the end of digestion. Intermittent stirring duration strongly influenced performance, with 2 h/day optimal for most binary systems, while 3 h/day maximized methane yield in balanced ternary mixtures.
3. Net energy analysis showed that although higher stirring increased energy consumption, co-digestion systems achieved net energy up to 7.96 MJ/kg VS.
4. Multivariate statistical analysis confirmed the key factors governing methane production and net energy recovery and validated the consistency of the experimental results.
5. The ANN model trained with the traincgp algorithm (9–7 architecture) showed excellent predictive accuracy (R = 0.99, low MSE). Response surfaces identified optimal methane production at moderate C/N ratios and higher stirring rates. Overall, combining co-digestion with ANN-based optimization significantly enhanced methane recovery and energy efficiency.

This study demonstrated that optimizing AD systems requires a systems-level perspective that considers both methane generation and the energy consumed by reactor operation. By integrating experimental investigation with artificial intelligence modelling, this research provides a novel framework for evaluating the trade-offs between mixing intensity, substrate composition, and net energy recovery. The findings show that co-digestion strategies combined with optimized intermittent stirring regimes can significantly enhance methane production and overall energy efficiency, supporting the development of sustainable waste-to-energy systems.

Beyond process optimization, the results highlight the potential of anaerobic co-digestion to contribute to circular bioeconomy strategies by transforming livestock waste streams into renewable energy resources while reducing environmental pollution and greenhouse gas emissions.

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