

DYNAMICS OF METHANE CONTENT IN BIOGAS DURING ANAEROBIC DIGESTION FOLLOWING A GLYCEROL PULSE ADDITION

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This research study investigated the feasibility of adding 1% (v/v) glycerol from the biodiesel industry to a substrate mixture of sewage sludge, manure, and paper waste that was used in the anaerobic digestion process. The highest methane (CH₄) percentage (31.6%) was identified on the second day after the introduction of glycerol, followed by a rapid decrease due to the persistent production of volatile fatty acids. In the short term, a slow improvement in CH₄ content was recorded after the simultaneous addition of a NaHCO₃ solution and biochar. The methanogenic activity of microorganisms was completely inhibited on day 10. These results indicate that the addition of glycerol at the specified dose can lead to a decrease in CH₄ content in biogas composition. To prevent inhibition, careful glycerol dosage adjustment and improved process stabilization techniques must be implemented.

Keywords: glycerol; sewage sludge; pig manure; paper waste; anaerobic digestion; methane

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1. Introduction

Biodiesel is a biofuel that, unlike non-renewable fuels, is produced from sustainable sources like vegetable oils or animal fats. To produce biodiesel on an industrial scale, a transesterification reaction is used [1-3]. In the transesterification process, triglycerides from animal fats or waste oils react with alcohol, typically methanol, to produce biodiesel and glycerol, in the presence of catalysts such as sodium methoxide, potassium methoxide, hydrochloric acid, or sodium hydroxide. The main advantage of biodiesel consists of reducing greenhouse gas emissions into

the atmosphere [4-6]. In 2023, global biodiesel production was estimated at 50 billion liters, with Indonesia (14 billion liters from palm oil) ranked first, followed by the European Union (13 billion liters from rapeseed and used cooking oil) and Brazil (8 billion liters from soybeans) [7]. Furthermore, in 2024, the European Union continued to stimulate biodiesel production as a sustainable option for replacing fossil fuels, with Germany being the country with the highest recorded percentage (24% of European Union production), followed by the Netherlands (13%), Spain (11%), France (10%), and Italy (8%) [8]. In general, the biodiesel production process generates approximately 10% (w/w) glycerol [9]. Moreover, numerous studies [10,11] have reported that producing 10 kg of biodiesel yields 1 kg of glycerol containing impurities.

Glycerol is considered harmless and can be used in biomedical applications because it can enhance both the physical and biological properties of polymeric materials by reducing moisture loss. At the same time, glycerol is available as a feedstock for animal feed and the production of citric acid, bioethanol, and biogas [12-16]. However, glycerol can contain high concentrations of salts, methanol, water, soap, and free fatty acids, which can disrupt biological wastewater treatment processes and inhibit microorganisms during anaerobic digestion (AD) [17,18]. Thus, several purification methods have been developed, including membrane separation, acidification, and ion exchange [19,20].

Currently, AD is an efficient method for valorizing both glycerol and other substrates, such as food waste, animal manure, and sewage sludge (SS), while simultaneously supporting the transition to a circular economy [21,22]. F. Goembira et al. [23] investigated the effect of glycerol on biogas production. It was introduced into 3 reactors at different concentrations (5, 10, and 15% v/v), along with a cow dung-distilled water mixture prepared at a ratio of 1:1. At the same time, a reference reactor without glycerol was set up. According to the results obtained, the maximum biogas production (380 mL/g VS) was observed for the mixture with 10% glycerol (v/v), while the highest methane (CH₄) percentage (60.2%) was detected for the mixture without glycerol. T. Srimachai et al. [24] tested the biochemical methane potential of glycerol mixed with canned sardine wastewater by anaerobic co-digestion. The process was carried out both in a single stage under thermophilic and mesophilic conditions and in two stages under mesophilic conditions. The highest CH₄ yield (634.11 mL) was observed in the two-stage mesophilic process at 4% glycerol. Additionally, the maximum CH₄ percentage in biogas was 78.48%. The aim of this research paper was to evaluate the evolution of biogas composition after adding 1% (v/v) glycerol to a mixture of SS, pig manure (PM), and paper waste (PW). The experimental methodology was adapted from our previously published work [25]. The novel elements of this research include the use of a mixture of different raw materials, the determination of the alkalinity value (TA) and the concentration of volatile fatty acids (VFAs) for the sludge sample, the

monitoring of VFAs during the process, and the theoretical evaluation of the lower calorific value (LHV) and the higher calorific value (HHV) of biogas.

2. Materials and Methods

SS was collected from a wastewater treatment plant (WWTP) near Bucharest, Romania, and acted as both inoculum and substrate due to its high content of active anaerobic microorganisms and its residual organic matter contributing to biodegradation. The PM and PW (paper towels) were obtained from a household in Arges County, Romania. The glycerol was purchased from a private company in Bucharest, Romania, and exhibited an acidic pH (5.8).

2.1. Substrate characterization and preparation

The PW was thermally pretreated at 80°C for 2 hours using a thermostatically controlled magnetic stirrer to facilitate its decomposition during the AD process [26]. Subsequently, the excess water was removed by gravity filtration. The determination of the total solids (TS) and volatile solids (VS) content for SS, PW, and PM was carried out in accordance with the *DIN EN 12880:2001* and *DIN EN 12879:2001* standards [27,28]. The substrates were placed in an oven at 105°C until a constant mass was obtained. Subsequently, the remaining residues were calcined first at 220°C for 30 minutes, then at 550°C for 2 hours. The analyses were performed in duplicate (n=2).

The pH of the substrates was also measured using a SevenExcellence S400 laboratory pH meter. To prevent their biological degradation, the samples were stored in a freezer at 4°C before starting the experiment. On the other hand, glycerol was stored in a place protected from light to avoid triggering oxidation reactions that lead to the appearance of impurities.

In addition, the WWTP determined TA value and the concentration of VFAs for the SS sample according to *ISO 9963-2:1994, Water quality—Determination of alkalinity—Part 2: Determination of carbonate alkalinity standard* [29]. Thus, the SS sample was homogenized and centrifuged for 10 minutes at 3600 rpm to separate the supernatant from the solid particles. Next, the supernatant was titrated with an automatic burette equipped with a pH electrode and a magnetic stirrer using an H₂SO₄ solution until a pH of 4.5 was reached. Following this, to determine the concentration of VFAs, the sample at pH 4.5 was boiled on a hot plate under the fume hood for 3 minutes, then cooled and titrated with a 0.025 N NaOH solution until the pH reached 7.

2.2. Experimental design and procedure

The experimental research was carried out in the Laboratory for Energy and Sustainable Environment at the Research Institute Campus of Politehnica Bucharest. The experimental setup consisted of the EDF 5.4_1 bioreactor, equipped

with a 5.4 L autoclavable vessel with a working volume of 3.41 L and a process biocontroller, as shown in Figure 1. The AD process was operated in batch mode, with glycerol, sodium bicarbonate, and biochar added periodically. The temperature was maintained in mesophilic conditions ($37 \pm 3^\circ\text{C}$) by connecting the installation to the water supply system. On the other hand, the experiment was performed without mechanical stirring, as the experimental setup was designed for liquid-phase applications.

The mixture subjected to AD was prepared with 2000 g SS, 700 g PM, and 150 g PW. The quantities were set so that the C/N ratio of the mixture was within the range necessary for the optimal development of the AD process (20-30). To ensure a humidity of 92%, 564 mL of water was added. The inoculum was used to provide the microorganisms necessary to initiate the biological activity of the system. Glycerol was introduced at 1% (v/v) on the first day of the experiment. The experimental research was conducted in a single experimental run.

To monitor the daily composition of CH_4 and other gases (carbon dioxide, hydrogen, and hydrogen sulfide) in the biogas, they were continuously transferred from the 1.99 L reactor headspace through a gas line to the TGas analyzer located behind the bioreactor. The gas analyzer was also connected to the process biocontroller, and the gas percentages could be visualized on its display. However, biogas volume measurement was not performed because the experimental facility lacked a biogas measurement system.



Figure 1. The EDF 5.4_1 bioreactor used in the AD process.

2.3. Monitoring of VFAS and pH during AD process

To identify VFAs formed during the process, samples were taken from the bioreactor for 9 days using an electronic pipette and transferred into centrifuge tubes. The samples were then sent to an accredited laboratory under refrigerated conditions to prevent their biological degradation. The samples were solubilized with purified water and then filtered using filter paper. Thus, the result was associated with the volume of liquid recovered and filtered from the samples. The

internal standard used was 2-ethylbutyric acid. The volumes analyzed, for both the standard solutions and the samples, were 0.2 mL, to which 20 μ L of 42.5% phosphoric acid solution was added. For certain components with high concentrations of VFAs in the samples, a diluted sample was also injected at a 1:10 ratio. In Eppendorf tubes, 1 mL of tert-butyl methyl ether (TBME) was added to the standard and sample volumes. The heterogeneous mixture of sample and solvent was vortexed for 5 minutes and then centrifuged at 10 °C for 10 minutes to separate the aqueous and organic layers. Next, approximately 0.8 mL of the upper layer was transferred directly into the autosampler vial. To determine the concentrations of VFAs from the samples, gas chromatography coupled with mass spectrometry (GS-MS) using a Thermo Scientific TRACE 1310-TSQ 8000 Evo system with a TriPlus RSH injector was performed. Helium, used as the mobile phase, passes through the purification devices at a constant flow rate of 1.5 mL/min and enters the TG-WaxMS polar capillary column, which separates VFAs, after their transformation into ester derivatives, from other gaseous components. Next, these derivatives were converted into ions and detected by MS.

The pH of the mixture was measured daily using a Hamilton glass pH electrode, and a NaHCO₃ solution was used to adjust it to the range necessary for the growth of methanogenic microorganisms (6.5-7.5). Simultaneously, at 24-hour intervals, biochar was introduced in different amounts to ameliorate the severe acidity, as shown in Table 1. According to J. Hu et al. [30], a 25% biochar dose, based on the VS of the substrates, stabilized the process and increased CH₄ yield. The biochar was obtained from the Explocom GK company and has a pH of 8.04.

Table 1. Biochar added in different amounts.

Biochar: 0.25 g/g VS (53.34 g total)	
Day 3	15 g
Day 4	10 g
Day 5	10 g
Day 6	7.31 g
Day 7	6.15 g
Day 8	4.88 g

2.4. Theoretical evaluation of the HHV and LHV of biogas

The HHV and LHV of biogas were calculated based on the daily measured CH₄ content, according to equations (1) and (2) [31, 32].

$$HHV_{biogas} = \sum_{i=1}^N x_i * HHV_i \quad (1)$$

where HHV_{biogas} is the higher heating value of biogas [MJ/Nm³], x_i is the daily measured CH₄ volume fraction in biogas, and HHV_i is the higher heating value of pure CH₄ [39.8 MJ/Nm³].

$$LHV_{biogas} = \sum_{i=1}^N x_i * LHV_i \quad (2)$$

where LHV_{biogas} is the lower heating value of biogas [MJ/Nm^3], x_i is the daily measured CH_4 volume fraction in biogas, and LHV_i is the lower heating value of pure CH_4 [$35.8 \text{ MJ}/\text{Nm}^3$].

Carbon dioxide (CO_2) was not considered because it is a non-flammable gas that does not support combustion, and hydrogen sulfide (H_2S) was excluded because it can affect human health and corrode various equipment, engines, and pipelines [33,34,35]. On the other hand, hydrogen (H_2) was not included because its contribution to biogas's energy content is low.

3. Results and Discussion

3.1. Substrate characterization

The VS/TS ratio, calculated after determining the TS and VS contents of the substrates, indicated that most of the components in the analyzed substrates are organic and can be biodegraded, enabling the AD process to be carried out under optimal conditions. At the same time, their pH is within the required range (6.5-7.5) for the development of methanogenic microorganisms, thereby increasing CH_4 content in biogas. The results achieved are illustrated in Table 2.

Table 2. Characterization of substrates used in AD process.

Substrates	TS, % (w/w)	VS, % (TS)	VS/TS, %	pH
SS	4.26 ± 0.01	3.20 ± 0.17	75.11	7.09
PM	22.82 ± 0.01	18.02 ± 0.13	78.96	7.32
PW	18.77 ± 0.38	15.48 ± 0.85	82.47	6.83

Table 3 summarizes the TA value and VFAs concentration for the SS. Based on these values, the VFAs/TA ratio was calculated. The value obtained (0.06) indicates that the SS has an optimal buffering capacity and can tolerate moderate variations in organic load. According to similar studies [36,37], the optimal ratio associated with stable AD processes is between 0.23 and 0.3, while a value > 0.3 indicates increased acidification of the system.

Table 3. TA value and VFAs concentration determined for SS.

Indicators	SS
TA ($\text{mg}/\text{L CaCO}_3$)	2890
VFAs ($\text{mg}/\text{L CH}_3\text{COOH}$)	168
VFAs/TA	0.06

3.2. Biogas composition

The percentages of gases in the biogas composition were measured daily and are presented in Figure 2. Based on the CH_4 content of the biogas, the results obtained showed that methanogenic bacteria activity was adversely affected under the tested conditions. The duration of the experiment was 10 days.

Glycerol was added at a concentration of 1% (v/v) on the first day of the experiment, contributing to an early increase in CH_4 from 13.7% to 31.6% on the second day due to its rapid biodegradation and substrate availability. However, from day 3 onwards, a rapid decrease in CH_4 was observed through day 6 (4.3%), followed by a temporary increase in the CH_4 percentage on days 7 and 8 due to the synergistic effect of NaHCO_3 and biochar. From day 9 onwards, the activity of methanogenic bacteria was completely inhibited, and this inhibition was attributed to the persistent formation of VFAs. The observed fluctuations in CH_4 content are like those reported in previous studies. In research conducted by Z. Wang et al. [38], it was found that CH_4 yield was 1.18% and 1.13% higher, respectively, after waste activated sludge was co-digested with 5% glycerol (v/v) and 8% glycerol (v/v). In contrast, M. Ikram et al. [39] demonstrated that co-digestion of dairy wastewater with 0.2% glycerol (v/v) led to a high CH_4 yield (59.19 mL), but when the glycerol concentration was increased to 1% (v/v), the CH_4 production started to decrease to 0.958 mL. Furthermore, X. Li and N. Shimizu [40] evaluated 2 strategies to remedy the instability of the anaerobic co-digestion process of food waste and crude glycerol. The samples were mixed in a ratio of 9:1.

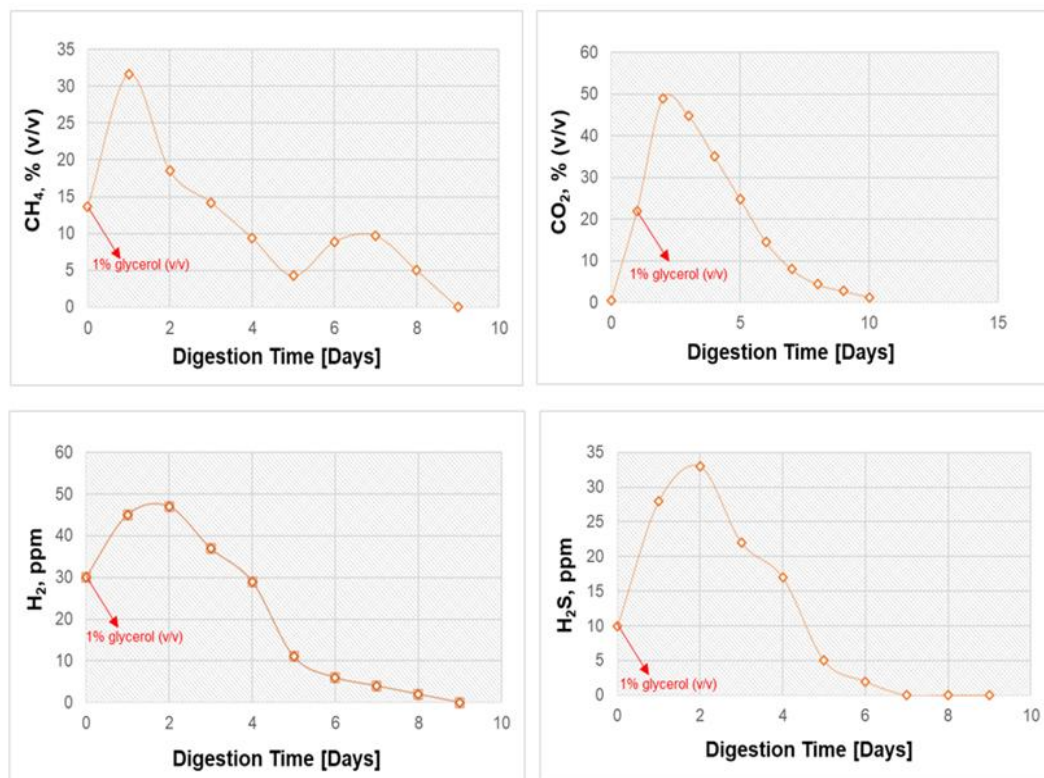


Figure 2. Variation of gases in the biogas composition after adding 1% glycerol (v/v).

The experiments were performed in different reactors under the same conditions. In the first study, the active inoculum was re-inoculated to restore the microbial consortium's activity. In the second study, biochar was introduced to facilitate the conversion of VFAs and to ensure a sustainable improvement of the process. The results showed that re-inoculation partially reactivated the methanogenic activity, while biochar significantly improved process stability and facilitated higher CH_4 yields. In contrast to the reference study, the present research did not highlight similar effects. Thus, it can be concluded that these differences can be associated with the organic loading rate and the dynamics of the formation of volatile fatty acids.

The variation of the other gases' content was directly proportional to the changes in CH_4 content. This relationship occurred because the accelerated triggering of the hydrolysis and acidogenesis stages simultaneously generated all the intermediate compounds necessary for the methanogenesis stage.

3.3. Monitoring of VFAs and pH during AD process

Figures 3 and 4 show the concentrations of VFAs formed and the variation of pH throughout the AD process. In all 9 days in which the concentrations of VFAs were analyzed, the acids identified were isobutyric acid, butanoic acid, acetic acid, isocaproic acid, hexanoic acid, propanoic acid, pentanoic acid, and heptanoic acid.

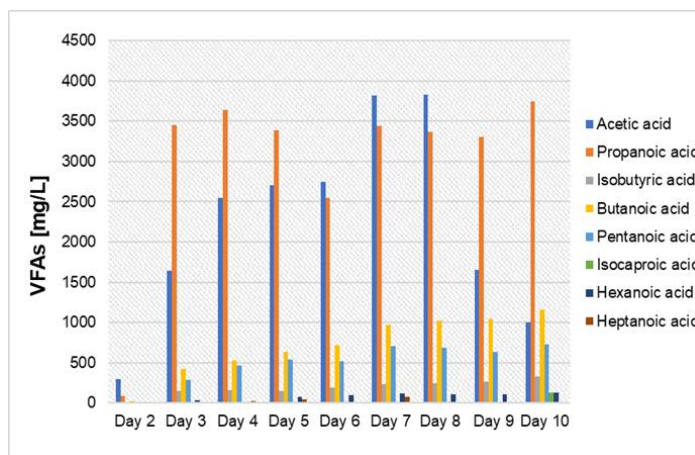


Figure 3. The concentrations of VFAs formed throughout the entire duration of the AD process.

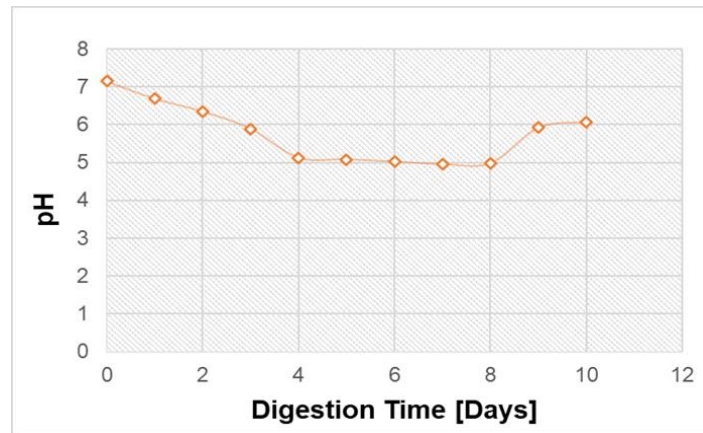


Figure 4. pH variation throughout the entire duration of the AD process.

The predominant acids formed were propanoic acid and acetic acid. The highest concentration of acetic acid was observed on day 8 (3830 mg/L) and corresponded to a pH of 4.98. On the other hand, the highest concentration of propanoic acid was indicated on day 10 (3750 mg/L), corresponding to a pH of 6.08. Both the concentrations of identified VFAs and the progressive decrease in pH from 7.19 to 4.95, followed by a slow increase to 6.08, demonstrated that the number of VFAs that formed overwhelmed the buffering capacity of the mixture and helped maintain the pH in a zone of high acidity.

Thus, in this situation, the activity of the bacteria responsible for CH_4 generation in biogas composition was inhibited. These results are aligned with the values reported in the literature. Similar studies [41,42] showed that the predominant acids formed during the AD process were acetic acid and propanoic acid. The proportion of each acid obtained varied depending on the pH.

3.4. Theoretical evaluation of the HHV and LHV of biogas

As shown in Figure 5, the highest HHV (12.58 MJ/Nm³) and LHV (11.31 MJ/Nm³) values were observed on the second day of the experiment. However, these values are below the limit reported in the literature [43], indicating that biogas use in energy applications is limited. The difference between HHV and LHV is attributed to water vapor condensation. For HHV, energy recovery is possible after condensation of water vapor, while for LHV, the heat of vaporization of water in the flue gas is excluded [44].

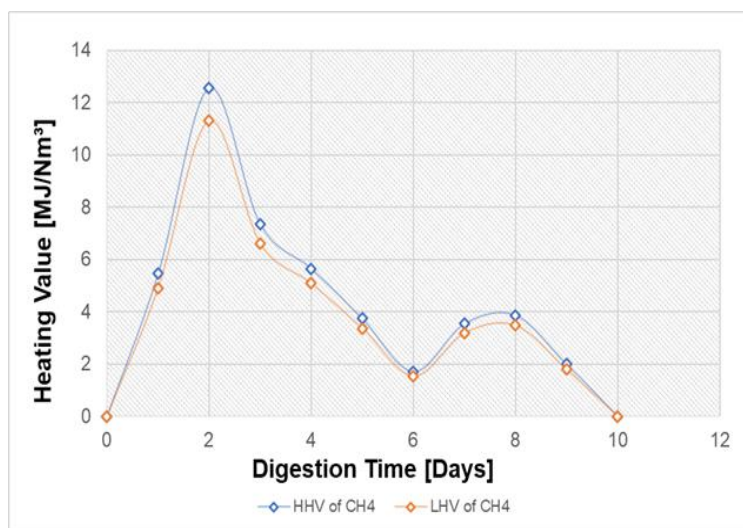


Figure 5. The evaluation of HHV and LHV of CH₄.

4. Conclusions

This research evaluated the evolution of CH₄ and the other gases during the AD process. On the first day of the experiment, 1% (v/v) glycerol was added to the mixture formed by SS, PM, and PW. Initially, a progressive increase in the CH₄ percentage was recorded, followed by a significant decrease due to the excess formation of VFAs, which contributed to the acidification of the reaction medium. On days 7 and 8 of the AD process, a slow increase in CH₄ concentration was recorded due to the combined effects of the NaHCO₃ solution and biochar, which partially increased the buffering capacity of the system. However, on day 10, the methanogenic activity of bacteria was completely inhibited due to the persistent accumulation of VFAs, in particular propanoic acid and acetic acid. Thus, future research activities focus on testing new methods to stabilize AD processes. In this case, the use of metallic oxides could be a promising strategy because they can provide micronutrients and facilitate direct electron transfer between microorganisms to avoid the inhibition [45,46]. On the other hand, due to the complexity of AD experiments, replicate runs were not performed within the scope of this study. Therefore, the results should be interpreted as indicative trends.

Author Contributions

Conceptualization, A. -M.L.; methodology, A. -M.L. and F.N.; validation, D.M.C.; formal analysis, A. -M.L. and F.N.; investigation, A. -M.L.; writing—original draft preparation, A. -M.L.; writing—review and editing, D.M.C.; supervision, D.M.C. All authors have read and agreed to the published version of the manuscript.

Data Availability Statement

Data supporting the findings of this study are available upon reasonable request from the corresponding author.

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Conflicts of Interest

The authors declare no conflict of interest.

Artificial Intelligence Disclosure

Artificial intelligence-based tools were used to improve language clarity, grammar, and readability during manuscript preparation. The tool used was Grammarly. No AI tools were used for study design, data analysis, interpretation of results, or generation of scientific content. The authors take full responsibility for the manuscript.

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