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Production of phytoregulators during anaerobic digestion of bovine and swine manures

Producción de fitorreguladores durante las digestiones anaerobias de estiércoles bovinos y porcinos

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Abstract

Phytoregulators intervene in plant physiological processes; currently, the production or synthesis of these compounds has different routes from pure substrates and/or isolated strains, with high costs that make it unaffordable for some sectors. The present research aims to evaluate the production of phytoregulators: indolacetic acid (IAA), gibberellic acid (GA_3), abscisic acid (ABA) and kinetin (Kn) during the anaerobic digestion of dairy cattle and swine manure and their mixture (50: 50) at different digestion times (0, 4, 8, 12, 16, 20, 24, 28 and 32 days) in a completely randomized design, using ANOVAS and Tukey (P<0. 05) with OriginLab software. The results showed that substrate and digestion time influenced the production of phytoregulators: the treatment with dairy cattle manure after 12 days of digestion registered the highest value of IAA (17.95 ± 0. 12 mg/L); to produce GA_3, the treatment with manure mixture at 8 days was the most suitable (24.55 ± 3.64 mg/L) and to produce Kn, the swine manure at 24 days of digestion was the most suitable (9.91 ± 1.98 mg/L); however, ABA production was not recorded in any of the evaluated treatments. Anaerobic digestion of dairy cattle and swine organic solid wastes can be precursors to produce phytoregulators.

Keywords: phytohormones, indolacetic acid, gibberellic acid, kinetin, sustainable development.

Resumen

Los fitorreguladores intervienen en los procesos fisiológicos de las plantas; en la actualidad, la producción de estos compuestos es inasequible para algunos sectores. La presente investigación tiene como objetivo evaluar la producción de fitorreguladores: ácido indolacético (IAA), ácido giberélico (GA_3), ácido abscísico (ABA) y kinetina (Kn) durante la digestión anaerobia de estiércoles bovino, porcino, y su mezcla (50:50) a diferentes tiempos de digestión (0, 4, 8, 12, 16, 20, 24, 28 y 32 días), bajo un diseño completamente al azar, empleando ANOVAS y Tukey (P<0.05) con el Software OriginLab. Los resultados evidenciaron que el sustrato y el tiempo de digestión tuvieron efecto en la producción de fitorreguladores: el tratamiento con estiércol bovino a 12 días de digestión registró el mayor valor de IAA (17.95 ± 0.12 mg/L); para producir GA_3 fue el tratamiento con la mezcla de estiércoles a 8 días (24.55 ± 3.64 mg/L) y, para producir Kn el estiércol porcino a 24 días de digestión fue el más adecuado (9.91 ± 1.98 mg/L); la producción de ABA no se registró en ninguno de los tratamientos evaluados. La digestión anaerobia de los residuos sólidos orgánicos de ganado vacuno y porcino pueden ser precursores para la producción de fitorreguladores.

Palabras clave: fitohormonas, ácido indolacético, ácido giberélico, kinetina, desarrollo sostenible.

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1 Introduction

Anaerobic digestion (AD) is one of the most efficient technologies for the valorization of waste to extract biogas, and other compounds of interest, such as volatile fatty acids and hydrogen (Anukam *et al*., 2019). In general, AD can be used as an organic solid waste management process that obtains economic and environmental benefits by producing clean electricity and biofertilizers, fostering a circular economy (Albalate-Ramírez *et al*., 2023). The process is based on the sequential action of specific groups of microorganisms that in the absence of oxygen are able to degrade and mineralize different organic substrates and producing biogas, comprising methane, carbon dioxide, hydrogen sulfide, ammonia, hydrogen, and water vapor through four consecutive biochemical steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Loreto-Muñoz *et al*., 2024).

Among the by-products of AD is digestate, a mixture of partially degraded organic matter, microbial biomass, and inorganic components, which has been attributed fertilizing properties because it contains organic matter, benefic microorganisms, and bioactive compounds that have the potential to promote plant growth and increase tolerance to biotic and abiotic stresses (Aguilar-Benítez *et al*., 2020). In addition, recent studies have identified digestate as a source of phytoregulators (Li *et al*., 2016).

Phytoregulators are organic molecules produced by plants to regulate their physiological processes such as germination, growth, flowering, fruit formation, response to tropisms, stress conditions, and others (Rademacher, 2015). The main groups of phytoregulators are auxins, gibberellins and cytokinins, among them indolacetic acid, gibberellic acid, and kinetin stand out (Alcántara *et al*., 2019). Commonly, they are produced in aerobic systems fed with pure substrates, resulting in high production efficiency but at a high cost (Tian-Qiong *et al*., 2017); anaerobic digestion (AD) has recently been highlighted to phytoregulators production, although there is still little information on the optimal operating conditions for its production (Castro-Ramos *et al*., 2022).

Indoleacetic acid (IAA) is an auxin that promotes stem formation and elongation, production of adventitious roots, and increases apical dominance; its organic precursor is tryptophan, and it is mainly produced aerobically in axenic cultures and pure substrates (Tian-Qiong *et al*., 2017). Gibberellic acid (GA_3) increases tissue development, promotes root elongation, young leaf elongation, flowering, nodule segment elongation, participates in flower initiation processes, induces seed germination, fruit setting and inhibition of formation of free radical which induce lipid peroxidation; its organic precursor is ent-kaurenoic acid (Mukherjee *et al*., 2022). Abscisic acid regulates and maintains seed dormancy, stimulates seed maturation, inhibits the process of plant germination, regulates cell transpiration, induces plant senescence and flowering; it has two precursors, isopentyl pyrophosphate and carotenoids (Chhaya *et al.,* 2021). While kinetin (Kn) is a cytokinin that induces root initiation and elongation, activates leaf senescence, stimulates plant photomorphogenic development and axial bud formation at the plant level, its organic precursor is adenine (Alcántara *et al*., 2019).

There is an excessive amount of manure, which in most cases is neither treated nor used, contaminating the soil and water, however it could be considered an economic and energetic waste (Salgado, 2020); in other hand there are few reports evaluating phytoregulators production on anaerobic digestion process. The objective of this research is to evaluate the effect of time (0, 4, 8, 12, 16, 20, 24, 28, and 32 days), and substrate kind (dairy cattle and swine manure, and the 50:50 mixture of both) on the production of phytoregulators (IAA, AG_3, ABA, and Kn) during the anerobic digestion process.

2 Materials and methods

2.1 *Materials and digestions*

dairy cattle manure (22.41% dry matter; pH 8.02), swine manure (29.09% dry matter; pH 6.15), and their 50:50 co-digestion mixture (25.75% dry matter; pH 6.83) were collected from a local farmer in the state of Tlaxcala, Mexico. Three anaerobic digestions were performed in triplicate in a completely randomized experimental design (Table 1), where 120 mL serological flasks were added each manure or the mixture at 5% solids, and enough water to complete 80 mL, flasks were bubbled with N, hermetically sealed, and incubated at 30°C; in total there were 81 experimental units (flasks), three flasks of each treatment (triplicate) were taken every 4 days (destructive samples) during the 32 days of digestion, the digestates were centrifuged and the supernatant was used for analysis.

Table 1. Digestions evaluated.

Substrate	Fresh dairy cattle manure (g)	Fresh swine manure (g)
dairy cattle manure (B)	17.84	0.00
swine manure (P)	0.00	13.78
Mixture B:P (50:50) (M)	8.92	6.89

2.2 Analysis of physicochemical and phytotoxic properties of digestates

At the beginning (day 0) and at the end (day 32), the chemical oxygen demand (COD), total nitrogen, phosphorus, and potassium analyses were performed using HACH water analysis kits and quantified using the HACH DR 2800 spectrophotometer; moisture, total and volatile solids were measured using a Thermolyne 1400 muffle according to standard methods for the examination of water and wastewater (APHA, 2017).

At the beginning and every 4 days were measured the pH and the phytotoxicity; the pH was measured with a HINOTEK PHS-3E pH meter. The phytotoxicity test was performed according to the methodology proposed by Camacho *et al.* (2019); thus, two concentrations of digestate were used (1 and 5 %), considering that if the germination index (GI) > 80% it is not phytotoxic, and it is said to present an inducing effect if this parameter is greater than 120 % (Castro *et al*., 2020), equation 1. For this, 10 lettuce seeds were placed in divided Petri boxes, for triplicate, with filter paper moistened with their respective percentage of digestate and then incubated for 5 days at room temperature in a dark room; for the control, the paper was moistened with distilled water.

$$\begin{aligned} RPGS &: \left(\frac{GS}{GSC}\right)(100) \\ RRG &= \left(\frac{RL}{RLC}\right)(100) \\ IG &= \left(\frac{RPGS * RRG}{100}\right) \end{aligned} \tag{1}$$

Where: RPGS is the relative percentage of germinated seeds, GS is the number of germinated seeds in the treatment, GSC is the number of germinated seeds in the control, RRG is the relative root growth, RL is the root length, RLC is the root length of the control, GI is the germination index.

2.3 Determination of biogas and methane production

Biogas production was analyzed every 4 days by water displacement in inverted test tubes, the ideal gas equation was used to obtain data under normal conditions of pressure (1 atm) and temperature (25 ºC). To quantify the methane (CH_4) production in the biogas, it was necessary to place a CO_2 trap formed by an aqueous NaOH 3 N barrier (Sosa, 2014) and for the methane content in the biogas, determined using a Perkin Elmer autosystem XL gas chromatograph, equipped with a TCD detector and a HP-PLOT Q capillary column 30 m x 0.53 mm, injection temperature of 240ºC, and detector temperature of 250ºC; using hydrogen as carrier gas. The biogas sample was obtained with a 5 μL Agilent Gold Standard Syringe and injected directly into the chromatograph. The quantification of the methane content in the biogas was carried out with the help of a calibration curve constructed with a calibration gas (methane) of the MSA brand with 99.99% purity.

2.4 Determination of phytoregulators production

The extraction of phytoregulators (indoleacetic, gibberellic, abscisic acids, and kinetin) were carried out based on the method proposed by Castro-Ramos *et al*. (2022). The digestate was centrifuged, with a Labnet Prism R Refrigerated Micro-Centrifuge, at 9000 rpm for 10 min at 20°C, 1 mL of the supernatant was adjusted to pH 2 with 1 N HCl and added 1 mL of ethyl acetate. The organic phase was evaporated at room temperature (25 ± 5 °C) and recovered in porcelain crucibles and subsequently resuspended in a mixture of methanol: water (1:1) acidified with 0.2% acetic acid.

Identification of the phytoregulators was performed on a Thermo Scientific UltiMate 3000 UHPLC/UV-VIS equipped with an EC 125/4 NUCLEODUR 100-5 C18 column at a flow rate of 0. 5 mL * min^{-1} for 8 min at two wavelengths: 280 ηm (indoleacetic acid) and 265 ηm (gibberellic acid, abscisic acid, and kinetin). An isocratic flow of 50% methanol-water with 0.2% acetic acid, 35% methanol, and 15% water was used with an injection volume of 20 μL and a temperature of 20°C. Calibration curves with specific standards (Indoleacetic acid with a purity of 98%, and abscisic acid with a purity of 98% from Sigma-Aldrich. Gibberellic acid with a purity of 90%, and kinetin with a purity of 99% from Caisson) were used to quantify the phytoregulators production.

2.5 Statistical analyses

One-way ANOVA was used for physicochemical digestate analyses, phytotoxic assay, biogas, and phytoregulators production yield; and two-way ANOVA for phytoregulators production with two factors, as follows: substrate (3 levels) and digestion time (9 levels). In both cases, the Tukey test ($p \leq 0.05$) was used to determine whether there were significant differences among treatments using Origin Lab 2021 for windows. In addition, the Pearson correlation between pH and the production of phytoregulators (IAA, AG_3 and Kn) has also been carried out in Origin Lab 2021 for windows.

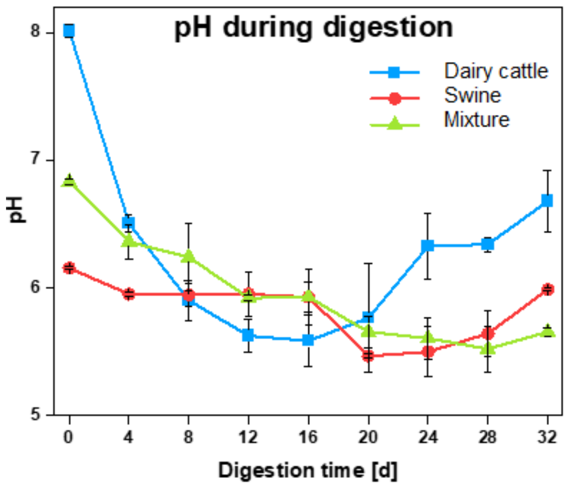

Figure 1. Dynamics of pH throughout the digestion process.

3 Results and discussion

3.1 Physicochemical properties of digestates

The pH plays an important role in anaerobic digestion, as it has an important and inconsequential relationship with each of the four anaerobic digestion steps (Figure 1).

The initial pH of each treatment was different (B: 8.02 ± 0.05, P: 6.15 ± 0.01, and M: 6.83 ± 0. 03), all digestions showed an initial decrease due to hydrolysis and acidogenesis, then the dairy cattle digestion (B) decreased even more until day 16, from day 20 the pH started to increase, due to the acetogenesis phase, because in this phase some acids are consumed; methanogenesis step improved after day 24, methanogenic microorganisms develop at a pH higher than 6.5 (Meegoda *et al.*, 2018).

For the swine digestion (P), and the mixture (M) predominate the hydrolytic and acidogenic steps. For P from day 16 to day 20 there was a high pH decrease and after day 24 an increase until 32 digestion days. The acidogenic phase is characterized for a decrease in pH, values < 6 could benefit the production of volatile fatty acids (Atasoy *et al.*, 2018), and phytoregulators such as indolacetic acid and gibberellic acid (Castro-Ramos *et al.*, 2022).

Table 2 shows the changes in physicochemical properties of the samples along the experiment. Initially, the COD of the manure was not statistically different among treatments, as well as after 32 days. However, the COD decrease in each of the digestions (Table 2), which shows that there was a consumption of organic matter because of the microbial activity during the anaerobic digestions, the highest removal of this parameter (29.96 %) was in the digestion B. Such as COD, total and volatile solids were not significantly different among the substrates used, either at the beginning or at the end of the digestion.

Total solids (TS) and volatile solids (VS) had the highest removal (33.51 and 35.33 %, respectively) with digestion P. The VS/TS ratio represents the percentage of organic matter in the total solids (Parra-Orobio *et al.*, 2015), this ratio did not change significantly: digestion B was from 75.63 % (day 0) to 75.36 % (day 32), digestion P from 69.92 % (day 0) to 67.98 % (day 32), and digestion M from 71.04 % (day 0) to 70.12% (day 32). The ratio in all treatments was about 70% even at the end of digestion. Montenegro *et al.* (2016) reported VS/TS ratios of 75% for dairy cattle and 72% for swine manure, and also mentioned that these percentages ensure that the digestion process and methane production can continue due to the availability of biodegradable matter; however, they also point out that these percentages do not guarantee methane production, which is affected by several factors as the pH.

Table 2 also shows the consumption of total nitrogen (N), total phosphorus (P), and potassium (K) during digestions, with N being the most consumed (> 90%) in all digestions. It should be noted that in each of the treatments, N and K were statistically equal at the beginning and at the end of the digestion. However, P was different to begin with, but became the same after 32 days of digesting.

The contents of N, P, and K in digestates are key indicators for evaluating the efficiency of fertilizers, they are of the essential nutrients that are required for the growth of plants. Wu & Dong (2020) mention that an N content ranges from 0.67 to 2.31 kg/m^3, P content from 0.37 to 1.11 kg/m^3 and K content from 1.12 to 1.80 kg/m^3 in digestates can be used as fertilizer, although this also depends on the crop being evaluated. According to Wu & Dong (2020), digestate B had low N and P content, digestate P has a good quantity of NPK, and digestate M had good quantities of P and K and medium of N.

According to Makian *et al.* (2024), if the N content in the anaerobic digestor is higher than 4 kg/m^3, ammonia can be produced, which, at sufficiently high concentrations (1.5 - 3 kg/m^3 ammonia), could cause direct inhibition of methanogenic archaea and indirect inhibition of acetogenic bacteria. However, Morozova *et al.* (2020) mentioned that the anaerobic digestion process can tolerate concentrations up to 11.5 kg/m^3 of N, although it can have a negative effect on the process. In this study, the N concentration in all digesters was initially higher than 7 kg/m^3 and decreased to less than 0.70 kg/m^3 after 32 days, which possibly, in conjunction with an inadequate pH, resulted in the failure to produce methane.

Table 2. Physicochemical properties of substrates (day 0) and digestates at day 32.

	Chemical Oxygen Demand (COD)		
	Day 0 (kg/m^3)	Day 32 (kg/m^3)	Remotion (%)
B	73.9 ± 19.7 Aa	53.9 ± 6.6 Aa	26.9
P	52.4 ± 13.8 Aa	45.5 ± 1.8 Aa	13.1
X M x	71.0 ± 3.3 Aa	62.1 ± 12.9 Aa	12.6
	Total Solids (TS)		
	Day 0 (kg/m^3)	Day 32 (kg/m^3)	Remotion (%)
B	13.8 ± 1.9 Aa	12.7 ± 0.7 Aa	11.8
P	11.5 ± 1.3 Aa	7.6 ± 1.8 Aa	33.5
M	13.5 ± 1.2 Aa	11.82 ± 1.7 Aa	12.4
	Volatile Solids (SV)		
	Day 0 (kg/m^3)	Day 32 (kg/m^3)	Remotion (%)
B	10.4 ± 1.9 Aa	9.17 ± 0.83 Aa	12.0
P	8.0 ± 1.2 Aa	5.18 ± 1.17 Aa	35.3
M	12.2 ± 1.4 Aa	8.29 ± 0.77 Aa	31.8
	Total nitrogen (N)		
	Day 0 (kg/m^3)	Day 32 (kg/m^3)	Remotion (%)
B	7.10 ± 0.21 Aa	0.25 ± 0.2 Ab	96.5
P	7.13 ± 0.24 Aa	0.70 ± 0.0 Ab	90.2
X M x	7.33 ± 0.13 Aa	0.55 ± 0.0 Ab	92.5
	Total Phosphorus (P)		
	Day 0 (kg/m^3)	Day 32 (kg/m^3)	Remotion (%)
B	1.1 ± 0.0 Ca	0.3 ± 0.0 Ab	74.9
P	2.5 ± 0.1 Aa	0.9 ± 0.1 Ab	66.1
M	1.5 ± 0.0 Ba	0.5 ± 0.0 Ab	67.3
	Potassium (K)		
	Day 0 (kg/m^3)	Day 32 (kg/m^3)	Remotion (%)
B	4.4 ± 0.4 Aa	3.8 ± 0.5 Aa	13.4
P	3.3 ± 1.0 Aa	2.6 ± 0.4 Aa	20.5
M	4.2 ± 0.3 Aa	3.1 ± 0.8 Aa	27.3

Means in columns (uppercase) and rows (lowercase) that do not share a letter are significantly different (SE, Tukey ($p \leq 0.05$) & One-way ANOVA).

On the other hand, there is no consensus on the optimal ratio between the organic matter to be degraded (COD) and the amount of the main macronutrients (N, P and K) in an anaerobic process; Parra-Orobio *et al*. (2020) recommend 100:1.75:0.25 COD/N/P for anaerobic digestions. In the present research, the following ratios were obtained: for digestion B was 100/9.60/1.48 initial and 100/0.46/0.51 final; for digestion P was 100/13.61/4.75 initial and 100/1.74/1.87 final; and for digestion M was 100/10.32/1.87 initial and 100/0.89/1.48 final. The above shows that the use of dairy cattle and swine manure as substrate provides a high concentration of nutrients with respect to organic matter, which may be because they have already undergone a previous digestion process (inside the animal) that has removed a large part of the organic matter.

3.2 *Phytotoxic properties of digestates*

It was observed that from day 0 and throughout the digestion process, the digestate obtained in each of the treatments was not phytotoxic, having a germination index higher than 80 % (table 3), so that it can be used as an organic fertilizer (Camacho *et al*., 2019). According to the tests carried out, digestates were not phytotoxic at any time, and in some cases the GI was higher than 120% showing and induction effect (Castro *et al*., 2020).

3.3 *Determination of biogas and methane production*

According to Figure 2, biogas production started in the first few days and continue increasing, the highest biogas rate production was since the beginning until day 16. Escorcia-Ávila *et al.* (2020),

Table 3. Analysis of digestate germination index at 1 and 5%.

	Germination Index (%) with 1 % of digestate								
Days	0	4	8	12	16	20	24	28	32
B	115.9 Af	115.6 Cf	114.4 Ag	88.1 Ch	129.4 Ad	147.1 Ab	133.9 Bc	121.1 Ae	163.9 Aa
P	106.5 Bf	116.5 Bd	95.4 Ci	105.8 Bg	109.8 Ce	137.7 Bb	124.4 Cc	103.6 Ch	140.4 Ba
X M x	102.3 Ch	130.0 Ac	100.0 Bi	114.2 Af	125.2 Bd	112.7 Cg	142.7 Aa	118.9 Be	133.1 Cb
	Germination Index (%) with 5 % of digestate								
Days	0	4	8	12	16	20	24	28	32
B	100.0 Cd	80.0 Ch	80.9 Ch	141.2 Aa	95.3 Bf	88.1 Cg	109.1 Ab	101.4 Bc	98.4 Ce
P	105.8 Be	86.9 Bh	90.9 Ag	115.2 Bc	97.7 Af	132.6 Ab	108.1 Ad	107.7 Ad	138.5 Aa
M	119.4 Ab	90.4 Ad	83.1 Bf	112.7 Cc	80.2 Cg	128.7 Ba	81.8 Bf	87.8 Ce	127.5 Ba

Means in columns (uppercase) and rows (lowercase) that do not share a letter are significantly different (SE, Tukey ($p \leq 0.05$) & One-way ANOVA).

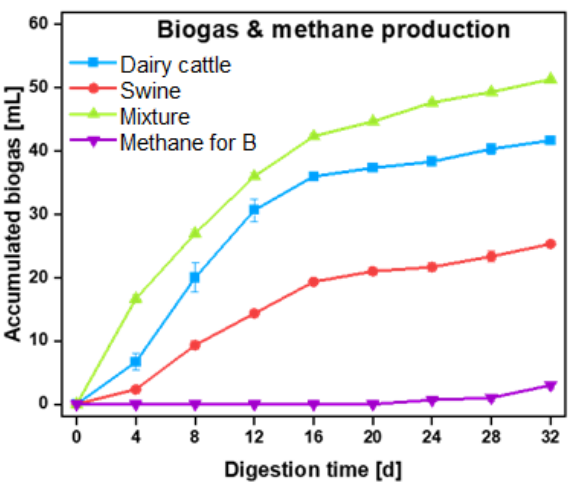

Figure 2. Cumulative biogas and methane production throughout the digestion time.

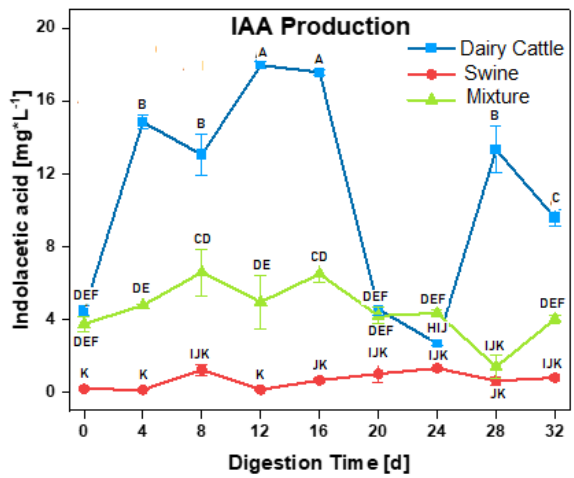

Figure 3. Indolacetic acid production throughout the digestion time. Means that do not share a letter are significantly different (SE, Tukey ($p \leq 0.05$) & Two-way ANOVA).

indicate that dairy cattle manure has a higher biogas production yield than swine manure due to its high moisture content, greater degradation and other physicochemical characteristics that make it a recommendable substrate for biogas production. Biogas production in the manure mixture was the highest, this is consistent with what was reported by Suárez-Hernández *et al.* (2018), who mentioned that the use of different substrates can significantly contribute to the improvement of the digestion process and biogas production.

According to the GC-TCD analysis, it should be noted there was no methane production in the digestions with swine manure or the mixture, only with dairy cattle manure was produced methane since day 24, and reaches its maximum on day 32, although in a very low quantity (3 mL ± 0.58), reaching only 7.2% of the total biogas composition, so it is discarded as biofuel, since it requires at least 45% of the total biogas composition (Teniza-García *et al.*, 2015).

Methane production is strongly related to the pH, comparing Figure 1 and 2, it can be seen that for B, the methane production starts between day 24 and 32 when pH increases. The main reason for the low methane yield is that a pH between 6.8 and 7.4 is the most appropriate for methane generation and below 6 methanogenic organisms cannot develop (Lozano *et al.* 2020; Meegoda *et al.*, 2018).

3.4 *Phytoregulators production*

3.4.1 *Indoleacetic acid production*

Wu and Dong (2020) reported that digestates have higher contents of indoleacetic acid than the manures this is likely microbial synthesis during the digestion process, but the bioactive compounds and their mechanism of action to carry out this biosynthesis are still not well known. Despite this, Scaglia *et al.* (2015) mention that the generation of auxins such as IAA or compounds with similar properties in digestates may result from the degradation of the lignin-cellulose complex and/or the biodegradation of aromatic amino acids such as tryptophan and phenylalanine; compounds already reported in manure digestions (Yao *et al.*, 2021).

Figure 3 shows the production of indoleacetic acid (IAA) at the different digestion times. It can be observed the presence of IAA in manure (day 0). The maximal production of IAA was in B with two relatives maximal, at 12 and 28 days; this pattern has already been documented by Castro-Ramos *et al.* (2022). For M was observed four maximal peaks, at 8, 16, 24 and 32 days. The production of this phytoregulator with P was lower than the other substrates, showing two maximal at 8 and 24 days.

According to the statistical analysis carried out, both the substrate and the digestion time have a significant effect on IAA production. The best substrate for IAA production was dairy cattle, followed by mixture and swine, and the best digestion times were on days 12 and 16. Consequently, the most recommended interaction is that of the dairy cattle manure for a period of 12 days.

The highest IAA production was achieved with dairy cattle manure on day 12 (17.37 ± 0.59 mg/L); with swine manure on day 24 (1.32 ± 0.02 mg/L); and with the mixture on day 8 (6.59 ± 1.28 mg/L). The maximum production of this phytoregulator was 3.55 times higher than that reported (4.9 mg/L) by Castro-Ramos *et al.* (2022), and very similar to that reported (17.38 ± 2.31 mg/L) by Li et al. (2016), both using dairy cattle manure within the first digestion days (4 - 12 days). It was also superior to that reported by Wu and Dong (2020) with dairy cattle manure (9.85 – 14.92 mg/L), with swine (9.95 mg/L), and poultry (11.13 – 12.03 mg/L). Therefore, it is speculated that the decrease in pH typical of the hydrolysis and acidogenic phase (Figure 1) may have a beneficial effect on the production of this phytoregulator.

Usually the IAA production is aerobic, some organisms studied for this purpose have been *Trichoderma* and *Bacillus subtilis* (González-León *et al.*, 2022). In the case of *Trichoderma*, Bader *et al.* (2020) tested different strains isolated in Argentina and obtained IAA production ranging from 13.38 to 21.1 mg/L after 7 days with tryptic soy broth. Castillo-Alfonso *et al.* (2022) achieved a IAA production of 310 mg/L with propionate and 230 mg/L with glucose in 2 days in aerobic batch reactors with *Bacillus subtilis* fed with tryptophan (a IAA precursor). Tian-Qiong *et al.* (2017) mention that this type of process with pure substrates and specific strains is expensive and unaffordable for certain social sectors, so that the use of manure in anaerobic digestion process is a viable and sustainable option, despite the enormous difference in concentrations obtained.

3.4.2 Gibberellic acid production

Figure 4 shows the amount of gibberellic acid (GA_3) produced in the different digestion times evaluated. GA_3 was present in dairy cattle and mixture manure since day 0 but not with swine manure. For dairy cattle were

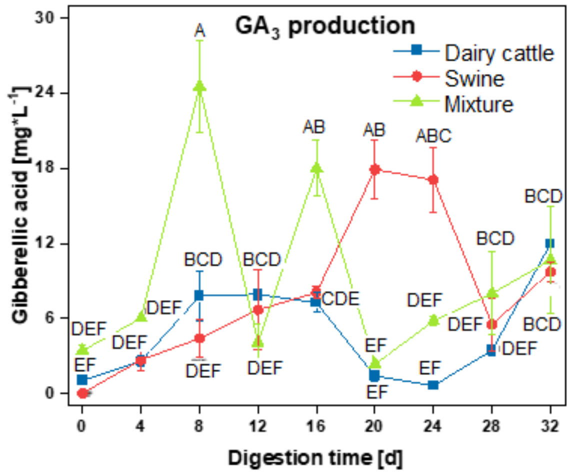

Figure 4. Gibberellic acid production throughout the digestion time. Means that do not share a letter are significantly different (SE, Tukey ($p \leq 0.05$) & Two-way ANOVA).

two maximal peaks at 12 and 32 days; for swine maximal were at 20 and 24 days, and for mixture at 8, 16, and 32 days. This could be due to the formation and degradation of ent-kaurenoic acid, a precursor of this phytohormone (Pereira *et al*., 2019). The higher production of this phytoregulator was achieve with dairy cattle manure (11.97 ± 0.06 mg/L) at day 32; with swine (17.92 ± 2.30 mg/L) at day 20; with mixture (24.55 ± 3.64 mg/L) at day 8. Results are like others, Li *et al*. (2016) reported GA_3 production with swine manure of 16.37 ± 2.16 mg/L, and dairy cattle manure of 38.53 ± 1.40. Wu and Dong (2020) evaluated the production of GA_3 from various manures: dairy cattle (11.59, 15.68 and 35.27 mg/L), swine (9.39 and 38.30 mg/L), and poultry (11.21, 15.68 and 32.29 mg/L). The phytoregulators production depend on different factor like temperature, pH, concentration, and inoculum.

According to the statistical analysis carried out, both the substrate and the digestion time have a significant effect on GA_3 production. The best digestion time was 8 days, and the best substrate was mixture and swine manures. Therefore, the most recommended interaction is that of the mixture manure for a period of 8 days.

In an aerobic process the fungus *Gibberella fujikuroi* is the main source of industrial GA_3 production, for example, Camara *et al.* (2020) using *Gibberella fujikuroi* LPB 06, a modified strain, in a 1.5 L bubble column reactor obtained 279.6 mg/L GA_3 in 7 days or Werle *et al.* (2020) studied GA_3 production using rice bran and malt residues, obtaining a maximum production of 934.41 mg in 7 days under optimal conditions with *Gibberella fujikuroi* NRRL 2278. Unfortunately, the low production yields of GA_3 lead to high process costs and restrict its use; annual production is around 100 tons, with prices ranging from 150 to 500 U.S. dollars per kilogram (Camara *et al.*, 2020). This, along with the use of modified organisms, makes it an efficient but not affordable option in a real-world environment, making it a viable option despite anaerobic digestion that requires further study to compete with the production of GA_3 by aerobic means.

3.4.3 Kinetin production

Figure 5 shows the production of kinetin (Kn), at day 0 there was no presence of this phytoregulator in any treatment. In dairy cattle manure it was not present at any time during digestion. On the other hand, swine had a maxima production peak at 24 days and mixture little peaks at 8, 24, and 32 days.

On the first 4 days, the production of this compound began for M, but after day 12 its production was minimal compared to P digestion, which had a significant increase between days 20 and 24, coinciding with a slight decrease in pH (Figure 1), so it is speculated that the decrease in pH may favor certain microbial groups that favor the increase in Kn.

The fact that this phytoregulator was not present in dairy cattle manure but was present in swine manure and in the mixture, may indicate that this type of cytokine can be developed from some compound from grains present in swine and non-dairy cattle diets.

The highest Kn production was achieved with swine manure on day 24 (9.91 ± 1.98 mg/L); and with the mixture on day 8 (2.62 ± 0.52 mg/L). According to the statistical analysis carried out, both the substrate and the digestion time have a significant effect on Kn production. The best substrate for Kn production was swine, followed by mixture, and the best digestion times were on day 24. Accordingly, the most recommended interaction is that of the swine manure for a period of 24 days.

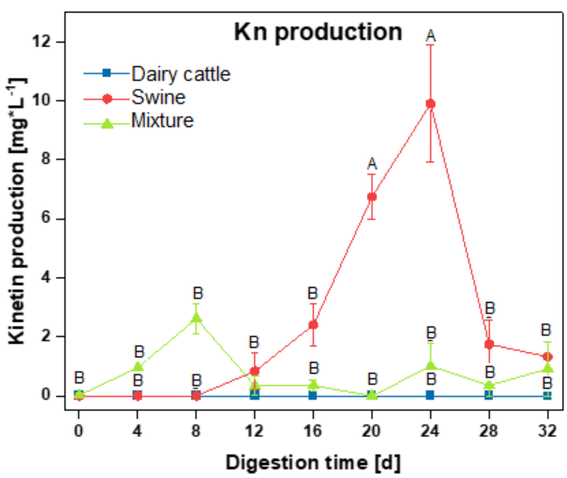

Figure 5. Kinetin production throughout the digestion time. Means that do not share a letter are significantly different (SE, Tukey ($p \leq 0.05$) & Two-way ANOVA).

3.5 Pearson correlation

A positive correlation was found between the presence of IAA and AG_3 with swine manure (0.91) and mixture manure (0.74). On the other hand, a negative correlation was found between the pH of the swine manure and the production of AG_3 (-0.83) and Kn (-0.85), which is inversely proportional to the amount of pH, in other words, when the pH decreases, AG_3 and Kn increase.

Conclusion

It is possible to produce phytoregulators using manures by anaerobic digestion, the kind of manure (substrate) and digestion time had effect on the phytoregulators production: dairy cattle manure digested for 12 days is recommended for indoleacetic acid (17.95 ± 0.12 mg/L), manure mixture for 8 days to produce gibberellic acid (24.55 ± 3.64 mg/L), and swine manure digestion for 24 days is recommended to generate kinetin (9.91 ± 1.98 mg/L).

There is a correlation between AG_3 and Kn production for swine and mixture manures. Only with swine manure, pH has an effect that is inversely proportional to AG_3 and Kn production.

The substrates at the conditions evaluated did not favor methane production. The digestates produced at any digestion time were not phytotoxic, after day 16 digestion showed an inducing effect on lettuce seeds.

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