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Research Article

Diversity and Functional Role of Elephant Grass Epiphytic Microbiota in the Efficiency of Anaerobic Digestion: Hydrolysis and Fermentation

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Abstract

The characterization of the epiphytic microbiota of lignocellulosic substrates used in anaerobic digestion is crucial for understanding the initial metabolic pathways of biomass degradation and their direct relationship with fermentative yield. This study integratively evaluated the microbial diversity present in elephant grass (Pennisetum purpureum) and its correlation with the biochemical performance of anaerobic reactors subjected to different operational conditions (stirring, supplementation with sucrose and acetic acid, control). The communities were analyzed through culturing in selective and differential media (MCCK, PSA, SM, TSA, VRBGA and SDA). The results showed that the epiphytic microbiota acts as a decisive modulator of the acidogenic stage, influencing the formation of organic acids, the competition between fermentative and opportunistic groups, and the balance with methanogenic archaea. The findings reinforce the need for integration between microbiological analysis and operational management to optimize biogas production.

Keywords: *Microbiological Analysis; Elephant Grass; Anaerobic Fermentation; Selective Media; Epiphytic Microbiota.*

1. Introduction

Anaerobic digestion of lignocellulosic substrates strongly depends on the initial activity of the epiphytic microbiota, responsible for the primary hydrolysis of biomass and the induction of subsequent fermentation routes. Plant biomass, including grasses, agricultural residues and various lignocellulosic materials, are renewable sources of energy (NETO *et al*., 2010; CORTEZ; LORA; GÓMEZ, 2008; MARTINS; CARRINO; LUIZ, 2023; SILVA, 2023). Its main structural components - cellulose, hemicellulose, and lignin - condition the release of monosaccharides by hydrolysis, a fundamental step for subsequent fermentations, such as ethanol production (KASCHUK, 2016; SILVÉRIO *et al*., 2021).

In elephant grass (*Pennisetum purpureum*), the leaf surface harbors an endogenous set of microorganisms - coliforms, lactobacilli, filamentous fungi, yeasts, fermentative bacteria and opportunistic species - that naturally colonize the plant epidermis. This biota establishes a metabolic microenvironment capable of initiating degradation even before the biomass enters the reactors, modulating the solubilization rate, the formation of organic intermediates and the trophic balance necessary for the performance of anaerobic digestion (NASCIMENTO, 2010).

The present study derives from an excerpt from the dissertation "INFLUENCE OF THE EPIPHYTIC MICROBIOTA OF THE PEACH PALM HEART AND ELEPHANT GRASS ON THE PROCESSES OF HYDROLYSIS, FERMENTATION AND CONVERSION TO THEIR BIOPRODUCTS", as a partial requirement to obtain the title of Master in Chemical Engineering, presented to the Graduate Course in Chemical Engineering, Technology Sector of the Universidade Federal do Paraná, under the guidance of Prof. Dr. Arion Zandoná Filho, focusing on the experimental stage aimed at the characterization of the epiphytic microbiota of elephant grass.

Although significant advances have been achieved in the design and operation of anaerobic reactors, the functional role of the epiphytic microbiota remains underrepresented as a critical variable for hydrolysis, acidogenesis, and intermediate metabolite formation. The lack of this understanding limits biological pretreatment strategies, hinders decisions about supplementation, agitation, and sanitary control, and reduces the ability to predict fermentative responses to operational disturbances. Thus, the characterization of this microbiota becomes fundamental to understand the variability of the processes and improve the management of lignocellulosic substrates. How does the composition and functional behavior of the elephant grass epiphytic microbiota influence the efficiency of hydrolysis and fermentation steps during anaerobic digestion?

Elephant grass has high productivity and lignocellulosic composition rich in carbohydrates, attributes that make it a strategic substrate for anaerobic digestion (GUIMARÃES, 2022; ASSIS, 2024; JOHANNES, 2024). It is assumed that its epiphytic microbiota - composed of fermenters, coliforms, fungi and opportunists - acts directly in the initial hydrolysis and fermentation, modulating the solubilization of organic matter, the profile of volatile fatty acids (VFAs) and the acidogenic performance of the reactors. Operational factors, such as agitation and supplementation, can alter this activity, making its characterization essential to optimize the process.

Figure 1 - Elephant Grass SOURCE: Authors (2022).
LEGEND: (A) elephant grass before collection, (B) elephant grass after collection.

Elephant grass stands out as a promising raw material for biogas given its high biomass production (SILVA CARVALHO *et al.*, 2018; ROSA *et al.*, 2019; COELHO *et al.*, 2024). Its quality, however, varies with agronomic factors such as cutting age, soil fertility, climate, and planting time (NYAMBATI; WAMBUI; OUMA, 2023; CARVALHO *et al.*, 2018; LAIANE, 2022; EMBRAPA, 2025b; EMBRAPA, 2025a).

Table 1. Chemical Composition of Elephant Grass Along its growth.

Cutting age	DM				
(days)	(%)	CP	NDF	LIG	NDT
50	9,3	9,7	60,5	3,8	50,1
70	13,8	7,7	66,3	5,8	47,9
90	16,4	6,2	68,2	7,0	46,2
100	19,7	5,6	68,6	7,7	45,6

SOURCE: Adapted from Carvalho *et al*. (2018), Rosa *et al*. (2019)
LEGEND: DM: Dry matria; CP: Crude Protein; NDF: Neutral Detergent Fiber; LIG: Lignin; NDT: Total Digestible Nutrients.

These characteristics consolidate elephant grass, especially the cultivar BRS Capiaçu, as one of the main sources of biomass in Brazil, with an approximate production of 50 t DM ha^{-1} $year^{-1}$ (COELHO, 2024). From a biotechnological point of view, understanding the metabolic dynamics of the epiphytic microbiota present in this material is essential to optimize pre-fermentation and anaerobic digestion, reduce costs, and minimize dependence on external inoculation.

Anaerobic Biodigestion

Anaerobic biodigestion consists of the microbial decomposition of organic matter in the absence of oxygen, through the stages of hydrolysis, acidogenesis, acetogenesis and methanogenesis, resulting in biogas and bioinputs.

Different reactor configurations – such as Covered Lagoon Biodigester (BLC), *Continuous Stirred Tank Reactors (CSTR),* Plug-Flow Reactors *(PFR) and Upflow* Anaerobic Sludge Blanket (RAFA)") - can be used according to the substrate, scale and climatic conditions, typically operating in the mesophilic range (30-40 °C), pH between 6.5 and 7.5 and C/N ratio between 20/1 and 30/1 (CIBIOGÁS, 2020; ALMEIDA, 2020; GONÇALVES; RAMALHO, 2021; CALEFFI, 2024).

Microorganisms in Anaerobic Biodigestion

Microbial characterization - conventional or molecular - allows the identification of microorganisms essential to the stages of hydrolysis and fermentation (NASCIMENTO, 2010; SPLABOR, 2023; SILVA *et al*., 2023). The main groups evaluated in this study are presented below, with their phenotypic and metabolic properties relevant to the anaerobic process.

Salmonella sp., are Gram-negative bacteria (a group that does not retain dye in Gram staining), which do not ferment lactose, forming transparent colonies in the medium or MacConkey Agar (MCCK). They develop between 5-48 °C (ideal 35-37 °C) and neutral pH, and can consume some sugars and produce hydrogen sulfide (H_2S) - this gas, among other characteristics, such as sugars mentioned above (arabinose, maltose, mannitol, etc.), are used as a marker, that is, they confirm their laboratory identification (MICROBIOLOGY IN PICTURES, 2015; BRASIL, 2011; CARNEIRO; COSTA, 2020; USDA, s.d.).

Shigella sp., are facultative Gram-negative and anaerobic rod bacteria, capable of living with or without oxygen. In MCCK, its presence is indicated by colorless or transparent colonies, due to the inability to ferment lactose; however, they ferment other sugars without producing gas and grow between 10 and 48 °C, depending on the species (NATIONAL LIBRARY OF MEDICINE, 2013; SILVA, 2017).

Coliforms are groups of Gram-negative bacteria (contaminants) that ferment lactose with gas production during incubation, usually between 35-37 °C and broad pH. They include Escherichia coli, Enterobacter, Citrobacter, and Klebsiella, widely used as indicators of contamination and sanitary quality (GARROTE, 2023; ECOTICIAS, 2023).

Figure 2. Escherichia Coli (Oxidase Negative)
SOURCE: Adapted from Fernandes Junior (2019). (Created with BioRender.com)

In selective media such as MacConkey Agar (MCCK), coliforms that ferment lactose form pink or red colonies, while non-lactose fermenters produce colorless or transparent colonies. This difference in color helps to visually identify them during microbiological analysis.

Pseudomonas are Gram-negative, aerobic and non-fermenting bacteria, especially Pseudomonas aeruginosa. In the medium or Cetrimide Agar (PSA), its identification occurs by the production of characteristic pigments, especially pioverdine (fluorescent yellowish-green) and pyocyanin (blue-green), typical colors associated with the genus. Some strains can still generate reddish or black pigments. They grow in 1-3 days, at 30-37 °C and pH 5.5-6.0, preferring the surface of the plate due to the higher oxygen content (HIRAI, 2020; AL-FRIDAWY; ALDARAGHI; ALKHAFAJI, 2021).

Staphylococcus are facultative aerobic, spherical, gram-positive bacteria that grow between 7-48 °C (optimum: 30-37 °C). In Salt-Mannitol Agar, S. *aureus* ferments mannitol and forms yellow colonies, while species such as S. *epidermidis* remain white, allowing differentiation between coagulase-positive and negative groups (HEXIS CIENTÍFICA, 2023). This metabolic plasticity favors its adaptation to different environments.

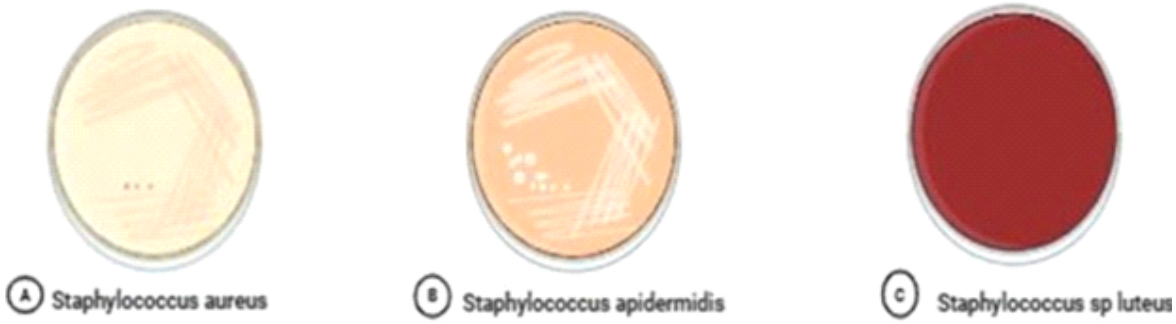

Figure 3. Staphylococcus SP. in SM Culture Medium
SOURCE: Adapted from Microbiology Note (2023). (Created with BioRender.com)

LEGEND: (A) Staphylococcus aureus, (B) Staphylococcus apidermidis, (C) Staphylococcus sp luteus.

Enterobacteria (Enterobacteriaceae) are gram-negative bacilli that include fermenting and non-fermenting lactose species, including genera such as Escherichia, Shigella, Salmonella, Citrobacter, Klebsiella, Enterobacter, Serratia, Proteus, Morganella, Providencia, Yersinia and Erwinia (USP, n.d.). In the medium or Neutral Red Violet Glucose Agar (VRBGA), after 72 h at 25 °C, fermenters form dark red/purple colonies with halo, while non-fermenters present pale or colorless colonies. Metabolically, they use aerobic oxidation of nitrogenous compounds or fermentation of carbohydrates in anaerobiosis (USP, n.d.).

Figure 4. Count of Enterobacteria in Vrbg Agar
SOURCE: Adapted from Brunetti (2023). (Created with BioRender.com)

Mesophilic aerobic microorganisms are oxygen-intensive bacteria that grow at moderate temperatures (5-50 °C, optimum 37 °C) and variable pH. They can include genera such as Escherichia, Salmonella, Shigella, Staphylococcus, Proteus, and Pseudomonas (CIDASC, 2021). They develop in various natural environments and usually form visible colonies within 48 hr.

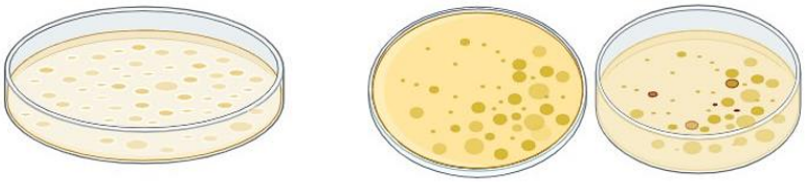

Figure 5. Mesophilic Aerobic Microorganisms
SOURCE: Authors (2025). (Created with BioRender.com)

Molds and Yeasts are fungi that differ in structure and metabolism. Molds are multicellular, formed by branched hyphae (mycelia), adaptable to different humidity and temperature conditions, growing between 25-30 °C and pH 4.5-5.0 in 48-60 h. Yeasts are unicellular, use sugars as a source of carbon and energy, performing alcoholic fermentation, with ideal growth between 25-30 °C and pH 4-4.5 in a few hours, being widely used in ethanol production and baking. In the culture medium or Sabouraud Agar (SDA), the presence of molds is indicated by filamentous and colored colonies, while yeasts form rounded, smooth, and clear colonies, allowing them to be visually differentiated and indicating their metabolic activity (BORGES, 2020).

These groups make up part of the epiphytic microbiota capable of initiating hydrolytic and fermentative routes, influencing biomass conversion and metabolite formation.

Thus, it is highlighted that the identification and characterization of microorganisms provide an essential basis to understand their diversity and functional role in the fermentation processes of elephant grass.

In view of the bioenergetic role of elephant grass and the relevance of the epiphytic microbiota in the early stages of anaerobic digestion, this study aimed to characterize this microbiota and evaluate its influence on the fermentative performance of reactors operated under different conditions. By integrating microbiological analyses and acid-fermentative responses, it is intended to clarify how native biota modulates the efficiency of lignocellulosic conversion.

Culture and Media Used in Microbiological Analyses

The selective and differential media employed - MacConey Agar (MCCK), Cetrimide Agar (PSA), Mannitol Salt Agar (SM), Neutral Red Violet Glucose Agar (VRBGA), Soy Tryptone Agar (TSA) and Sabouraud Dextrose Agar (SDA) - allow the identification of the epiphytic diversity associated with elephant grass and infer its functional potential in the hydrolysis and fermentation stages (NASCIMENTO, 2010; USP, s.d.; HIRAI, 2020; CIDASC, 2021; BORGES, 2020; BRUNETTI, 2023; SPLABOR, 2023).

Microbiological characterization makes it possible to understand the composition of the native microbiota, identify dominances, monitor contaminants, and correlate such parameters with the observed fermentative responses.

Next, the methodology is presented, describing the experimental design, the operating conditions of the reactors, the collection procedures and the microbiological analyses, ensuring rigor and reproducibility of the results.

2. Methodology

The investigation focused on the identification and quantification of microbial density, as well as the evaluation of its diversity in reactors operated under different operating conditions (agitation and supplementation). It was also sought to relate the presence of the main microbial groups to the fermentative dynamics, especially to the effects on acidogenesis and the stability of the anaerobic process.

Substrate and Preparation

Fresh elephant grass was collected at the Polytechnic Center of the Universidade Federal do Paraná (UFPR), located in region 22J (E: 0677802; N: 7184019), without any prior chemical or thermal treatment. The plants had a height between 75 and 505 cm and leaves with a maximum length of 76 cm.

Figure 6. Geolocation of the Polytechnic Center of UFPR (Curitiba)
SOURCE: UFPR/CAMPUSMAP (2025).

After collection, the biomass was manually cut with sterilized and weighed scissors (63 g), and distributed in three reactors, named Reactor 1 (A), Reactor 2 (B) and Reactor 3 (C).

In Reactors 1 and 2, the biomass was inserted into reaction vessels and completed with ultrapure water up to 500 mL, resulting in an approximate consistency of 13%. The system was conducted in the INFORS HT MULTIFORS 2 bioreactor. A solution of 50% sucrose and 4% acetic acid was used to adjust the pH to 3. Reactor 1 operated under mechanical agitation, while Reactor 2 remained static (FIGURE 7).

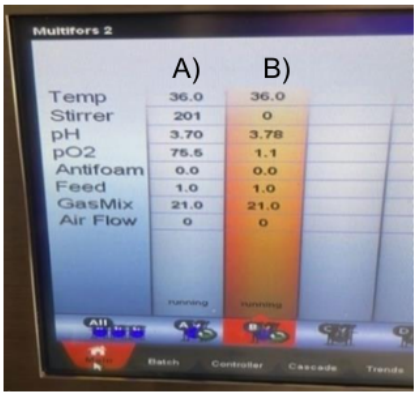

Figure 7. Physicochemical Parameters used in Reactors 1 and 2
SOURCE: Authors (2023).
LEGEND: (A) Reactor 1, (B) Reactor 2.

Reactor 3 (control) received the biomass in beaker with 500 mL of ultrapure water, without supplementation, under orbital agitation (TE-422, Tecnal®).

All reactors were operated under anaerobic conditions for 0, 10 and 20 days. The collected samples were transferred to Eppendorf-type tubes for physicochemical and microbiological analyses. The supernatant was centrifuged and treated for subsequent quantification by high-performance liquid chromatography (HPLC).

Microbiological Analyses

The characterization of the epiphytic microbiota was carried out through isolation and enumeration in selective and differential media, using a methodology standardized by the Brazilian Pharmacopoeia, 5th ed., Chapter 5.5 (ANVISA, 2017), using BIOCEN plates®.

The means used and their purposes were: MCCK: isolation of Gram-negative bacilli, differentiating lactose fermenters and non-fermenters (coliforms, enteropathogens). PSA: selection of Pseudomonas aeruginosa by formation of characteristic pigments. SM: detection of Staphylococcus spp., with differentiation by mannitol fermentation. VRBGA: enumeration of total coliforms (Enterobacteriaceae glucose fermenters). TSA: general means for assessing the total viability of the heterotrophic community and mesophilic aerobes. SDA: Isolation of molds and yeasts, fermentative groups relevant to CO_2 and ethanol production.

The analytical steps included: (i) homogenization of the biomass; (ii) preparation of microbial suspensions; (iii) aseptic inoculation in the media; (iv) incubation at 37 °C in 410/5ND ovens (Nova Ética®) e TE-394/1 (Tecnal®), according to the specific recommendations of each medium.

In the end, the results of greatest relevance to the research objectives were consolidated, emphasizing microbial behaviors associated with the operating conditions of the reactors.

3. Results and Discussion

Results and Discussion of the Physicochemical Analysis

The results and discussion of the physicochemical analysis are presented initially, since the efficiency of anaerobic digestion depends directly on the maintenance of environmental parameters appropriate to microbial development. These data provide the necessary context to consistently interpret the dynamics and performance of the epiphytic microbiota. Next, the microbiological results (identification and density) are presented. Images of the plates or culture media were not included, as the functional evaluation prioritized quantification and microbial characterization.

The efficiency of anaerobic digestion depends directly on the maintenance of physicochemical parameters appropriate to microbial development. Thus, the integrated analysis of environmental conditions and microbiological data allows a deeper understanding of the fermentation dynamics of the reactors. The results presented refer to the process conducted in the liquid phase, due to the more representative behavior of metabolic interactions.

Integrated Analysis of Physicochemical Parameters

The relationship between physicochemical factors and the microbial activity in the anaerobic reactors allowed the comparison of the performance of the optimized system - Reactor 1 (A) -, the supplemented reactor, but without agitation - Reactor 2 (B) and the control - Reactor 3 (C)

Temperature

In all reactors, the temperature remained at ~35 ± 1 °C, classic mesophilic range, ensuring operational stability and favoring the simultaneous growth of fermentative and methanogenic microorganisms. This condition is recognized for its robustness and lower sensitivity to environmental oscillations, as indicated by Angelidaki *et al*. (2018).

pH

The pH behavior directly reflected the metabolic balance of each system. In

Reactor 1 (A), values between 6.8-7.2, close to neutrality, demonstrate ideal conditions for methanogenesis. In Reactor 2 (B), a slight tendency to acidification (6.5-6.8), consistent with moderate accumulation of VFAs due to the absence of agitation. In Reactor 3 (C), values between 6.1-6.4 characterize marked acidification, incompatible with efficient methanogenic activity. These results agree with Chernicharo (2016), who emphasizes the importance of neutral pH to avoid microbiological collapses.

Carbon/Nitrogen Ratio (C/N)

The C/N ratio was determinant for the performance of the reactors. In Reactor 1 (A), ~25:1 - optimal range for biogas production, a direct result of sucrose supplementation. In Reactor 2 (B), ~22:1 - still acceptable, but with lower power availability (CAILLOT, 2017). In Reactor 3 (C), ~17:1 - excess nitrogen, indicating possible free ammonia formation and microbial inhibition (CAILLOT, 2017; SANTOS, 2022).

Volatile Fatty Acids (AGVs)

The concentration of VFAs correlated with the stability of the reactors. In Reactor 1 (A), < 2,000 mg/L, characterizing a balance between production and consumption. In Reactor 2 (B), ~3,000 mg/L, indicating partial overload. In Reactor 3 (C), > 4,000 mg/L, a clear scenario of acidification and methanogenic inhibition.

Values higher than 3,000 mg/L are widely associated with severe instability in anaerobic systems (APHA, 2017).

Volatile Solids Removed (SV)

The SV confirms the performance of each system, where in Reactor 1 (A), > 60% - excellent degradation of organic matter. In Reactor 2 (B), ~40% - intermediate performance. In Reactor 3 (C), < 25% - limited digestion, reflecting strong metabolic imbalance (LIMA; APPLEBY; LI, 2023).

Another important parameter to consider is the length of residence.

Length of residence

Shorter periods (between 0-10 days) provided maximum solubilization of useful sugars and acids. Excessive times (20 days) led to secondary degradation and formation of minor acids and ethanol. These results corroborate studies by Johannes, Silva and Costa (2024) and Sucinda *et al*. (2020), which highlight the direct effect of pretreatment intensity on sample quality.

Interpretative Synthesis

The physicochemical analysis confirms that the stability of the anaerobic process depends on the synergy between adequate temperature, neutral pH, balanced C/N ratio and low concentration of VFAs.

The optimized system - Reactor 1 (A) showed the best performance, supported by supplementation, agitation and optimal environmental parameters. The absence of agitation - Reactor 2 (B) - and the absence of supplementation - Reactor 3 (C) compromised both the microbial activity and the efficiency of organic removal and potential biogas production. The acid pretreatment directly influenced the availability of sugars and organic acids, impacting the liquid phase of the process.

Then, the results of the microbiological analysis are presented, focused on the detection of groups according to the selective character of the media.

Results and Discussion of the Microbiological Analysis

The results obtained reveal distinct patterns in the microbial colonization of elephant grass, as well as significant differences between the three reactors studied. Integrated data analysis allows understanding epiphytic microbial dynamics, their response to operating conditions, and their implications for acidogenesis and process stability.

The initial quantification of the epiphytic microbiota allowed us to compare the occurrence of coliforms and the fermentative behavior as a function of the cultivation conditions (agitation and chemical additives). The values obtained are presented in the tables, table and figure (graph).

The occurrence of epiphytic coliforms before and after fermentation is shown in CHART 1.

Chart 1. Microbial Density in MCCK Plates Between Reactors.

Reactor	Operational conditions		Microbial density	Observations
	Supplementation / Additives	Agitation		
1 (A)	+	+	High	Intense and diverse colonization
2 (B)	+	-	Moderate	Significant growth, but limited by lack of agitation
3 (C)	-	+	Low	Significant but Limited Growth

SOURCE: Authors (2025).

In CHART 1, the results indicate higher microbial density in Reactor 1 (A), associated with the combination of supplementation and agitation, favoring intense and diversified colonization. Reactor 2 (B) showed moderate growth, possibly limited by the absence of agitation, while Reactor 3 (C) exhibited low density, showing that the absence of supplementation restricted microbial development, even with agitation.

A higher incidence of coliforms was evidenced in Reactor 2 (B), suggesting that the absence of shear favors the survival of Gram-negative bacilli. The detection of total and thermotolerant coliforms, including Escherichia coli, is associated with fermentative and industrial environments, with high levels being indicative of failures in hygienic control or cross-recontamination (CAP-LAB, 2023). On the other hand, in Reactor 1 (A), a reduction in these groups was observed, possibly associated with mechanical stress and greater dispersion of microorganisms in the liquid medium.

The presence of lactose-fermenting and non-fermenting enterobacterial (*Enterobacteriaceae*) colonies was also identified, especially under conditions without agitation and without initial acidification (STATPEARLS, 2024).

Table 2. Microbial Density/Comparison in PSA Plaques Between.

Reactor	Operational conditions		Microbial density	Observations
	Supplementation / Additives	Agitation		
1 (A)	+	+	~90	Highest growth; highly favorable environment for fermentation
2 (B)	+	-	~60	Intermediate growth; limitation due to the absence of agitation
3 (C)	-	+	~25	Basal growth; minimal biomass activity

SOURCE: Authors (2025).

In TABLE 2, a higher microbial density is observed in Reactor 1 (A), as a result of the combination of supplementation and agitation, which provided highly favorable conditions for fermentation. Reactor 2 (B) showed intermediate growth, limited by the absence of agitation, while Reactor 3 (C) showed basal growth, indicating minimal biomass activity in the absence of supplementation.

Growth in PSA medium indicated the possible presence of Pseudomonas spp., especially P. aeruginosa, a gram-negative, aerobic bacterium favored by agitation conditions and nutrient availability (SOUZA *et al.*, 2021). This genus has a high adaptive capacity, with the formation of biofilms resistant to hydrodynamic stress, increasing density and structural stability under flux (JOSUE *et al.*, 2021; CHAN, 2021). Although it is not typical of strictly anaerobic environments, its occurrence in partially oxygenated systems can influence the dynamics of the fermenting microbiota and the efficiency of the reactors, by affecting mass transfer and nutrient distribution (SOUZA *et al.*, 2021; CHAN, 2021).

Chart 2. Microbial Density in SM Plates Between Reactors.

Reactor	Operational conditions		Colony density	Microbial density
	Supplementation / Additives	Agitation		
1 (A)	+	+	High	High
2 (B)	+	-	Moderate	Moderate
3 (C)	-	+	Low	Low

SOURCE: Authors (2025).

The data indicate a higher density of colonies and microbial biomass in Reactor 1 (A), associated with the simultaneous presence of supplementation and agitation. Reactor 2 (B) presented moderate values, suggesting limitation due to the absence of agitation, while Reactor 3 (C) exhibited low levels, showing that the lack of supplementation restricted microbial growth, even with agitation.

The growth observed in MS indicated the presence of staphylococci (*Staphylococcus spp.*), especially *Staphylococcus aureus*, identified by the positive fermentation of mannitol, evidenced by the yellow color of the medium. This microorganism is widely associated with both human and animal environments, as described in the literature (MBIOLOG, 2023).

Chart 3. Microbial Density in VRBGA Plates Between Reactors.

Reactor	Operational conditions		Microbial density	Observations
	Supplementation / Additives	Agitation		
1 (A)	+	+	High	Pronounced growth with well-defined colonies; strong fermentative activity
2 (B)	+	-	Moderate	Visible growth; however, lower than in Reactor 1 (A). Limited agitation influenced microbial development
3 (C)	-	+	Low	Restricted growth, indicating low microbial activity

SOURCE: Authors (2025).

The results demonstrate high microbial growth in Reactor 1 (A), reflecting the combination of supplementation and agitation, with well-defined colonies and intense fermentative activity. Reactor 2 (B) showed moderate growth, lower than that of Reactor 1, possibly limited by the absence of agitation. On the other hand, Reactor 3 (B) showed reduced growth, indicating low microbial activity in the absence of supplementation.

The growth in VRBGA and MCCK indicated the presence of enterobacteria of the Enterobacteriaceae family, such as Escherichia coli, Klebsiella, Proteus and Enterobacter, characterized by high fermentative capacity (TORTORA; FUNKE; CASO, 2017). In the production of biogas, these microorganisms act in the stages of hydrolysis and fermentation, but their predominance can cause acidification of the environment and inhibit methanogenesis, in addition to competing with more specialized microorganisms.

The TSA and SDA means confirmed the microbial viability, without allowing specific identification, in line with its non-selective character and use for broad cultivation of filamentous fungi, yeasts and, eventually, bacteria. A higher predominance of yeasts was observed in Reactor 3 (C, control), possibly associated with the absence of initial acidification and additives, favorable conditions for the development of native epiphytic fungi. The growth in TSA confirmed the presence of a viable and active microbial community, but restricted to the evaluation of global activity, since this means does not allow taxonomic differentiation, requiring association with selective means or complementary methods for specific identification (OLIVEIRA *et al*., 2021; SILVA *et al*., 2023).

CHART 4 shows the distribution of colony density and the other media, likewise, FIGURE 8 - graph (clustered bars) - shows the average density of colonies per medium and reactor, grouping all the results.

Chart 4. Overview of Microbiological Activity.

Medium / Agar	Reactor 1 (A)	Reactor 2 (B)	Reactor 3 (C)
MCCK	High (evident lactose fermentation)	Moderate (lower growth than R1)	Low (restricted growth)
PSA	Moderate (isolation of Pseudomonas)	Low (limited growth)	Low (very low growth)
SM	High (positive mannitol fermentation)	Moderate (visible growth)	Low (restricted growth)
VRBGA	High (characteristic enterobacterial colonies)	Moderate (reduced growth without agitation)	Low (discrete growth)

SOURCE: Authors (2025).

In CHART 4, the general characterization of the fermentative microbiota - characterization of the main microbial groups - is systematized. The distribution of microorganisms among the selective media revealed consistent patterns consolidating the types of microorganisms identified, their functional roles and possible interactions in the degradation of lignocellulosic biomass. Non-fermenting Gram-negative bacilli, facultative fermenting groups and organisms associated with the metabolism of organic acids stand out.

The colonies identified in MCCK and VRBGA reinforce the presence of coliforms, *Enterobacteriaceae*, especially in conditions without agitation and without initial acidification.

In PSA and SM media, typical colonies of Pseudomonas and Staphylococcus were observed, indicating metabolic diversity and potential for distinct fermentation routes. The SDA medium showed higher yeast density in Reactor 3 (C), a result consistent with the lowest interference of chemical additives and with the milder conditions of initial incubation.

The general trend observed in the three reactors suggests that agitation exerted a selective effect on mechanically sensitive microbial groups, such as coliforms, while the absence of acidification favored the establishment of epiphytic yeasts and fungi. The application of sucrose and acetic acid in Reactors 1 (A) and 2 (B) modulated the community during the first days of the process, directing fermentation to acidogenic routes, although at different intensities.

The distribution of colony density in the different selective and differential media is presented below (FIGURE 8).

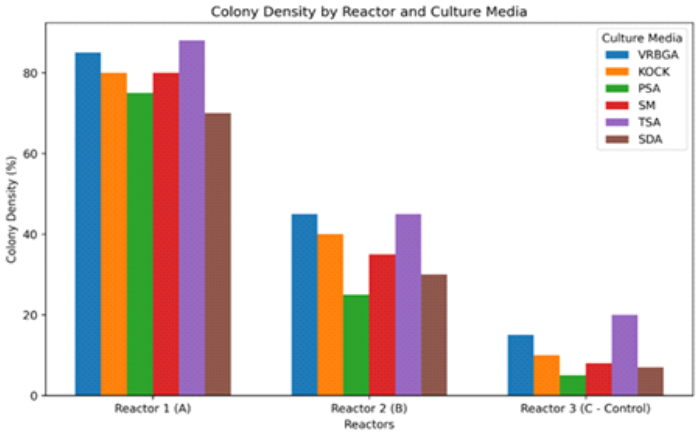

Figure 8. Average Density of Colonies by Medium and Reactor.

SOURCE: Authors (2025).

Reactor 1 (A), operated with agitation and supplementation, showed the highest colony densities in all media, indicating high microbial activity and wide functional diversity. Reactor 2 (B), without agitation, showed a significant reduction in growth, suggesting lower substrate availability and lower efficiency of biomass-microorganisms contact. Reactor 3 (C), control without additives, presented the lowest values, consistent with the absence of fermentative stimuli, however, in SDA it presented higher yeast density.

In all reactors, TSA showed the highest growth, followed by VRBGA. This uniform pattern indicates that the total heterotrophic community maintained predominance regardless of the operating conditions.

Agitation and supplementation showed a direct effect on the amplification of the epiphytic community, especially of fermenting coliforms (VRBGA) and Gram-negative bacilli (MCCK), which exhibited greater responsiveness to the most favorable conditions. The reduction observed in Reactor 2 (B) shows that the absence of agitation limits mass transfer and, consequently, the colonization of selective media. Reactor 3 (C) confirms the basal function of the natural epiphytic microbiota of elephant grass, with low metabolic expression under unstimulated conditions. These patterns reinforce the role of operational parameters in fermentative dynamics and microbial competitiveness throughout the anaerobic process (TORTORA *et al*., 2017**;** REIS *et al*., 2023).

The physicochemical results (pH, sugars and organic acids) corroborate the microbial trends observed. Although some experimental points have presented inconsistencies due to the reduced number of replicates, the set of data indicates that the dynamics of the epiphytic community is strongly conditioned to the initial experimental interventions and the operational regime of each reactor (CHERNICHARO, 2016; ANGELIDAKI *et al*., 2018).

In summary, the integrated analysis confirms that: (i) agitated conditions favor the reduction of coliforms and increase microbial dispersion; (ii) unagitated conditions tend to preserve native epiphytic groups; (iii) the initial chemical control (sucrose and acetic acid) significantly alters the microbial composition and the fermentation trajectory; (iv) Reactor 3, in turn, represents more closely the natural epiphytic ecology of elephant grass.

4. Conclusions

The present study, based on a phenotypic and functional approach to the elephant grass epiphytic community, showed that this microbiota has high metabolic diversity, composed of Gram-positive and Gram-negative bacteria, yeasts and filamentous fungi. Although the scope of the research did not include molecular, genetic or enzymatic analyses, the results obtained through selective and differential means allowed the characterization of microbial groups with distinct roles in the initial stages of anaerobic digestion.

Microbial dynamics were directly influenced by operating conditions. Agitation and supplementation modified the ecological balance of the reactors, increasing the microbial density, intensifying fermentation and redistributing the dominance between fermenters, fungi and opportunistic microorganisms. These effects demonstrate that the circulation of nutrients and the composition of the environment are determinants to modulate the activity of epiphytic populations.

The results indicate that the epiphytic microbiota exerts a significant influence on the rate of hydrolysis and acidogenesis, and may favor the release of sugars and the production of VFAs. However, this same activity, when excessive or unbalanced, can compromise subsequent methanogenic stability. Thus, understanding the composition and functional role of these microorganisms is essential to optimize anaerobic systems based on lignocellulosic biomasses.

Overall, the study confirms that the epiphytic microbiota of elephant grass is a decisive factor in the performance of anaerobic digestion, conferring potential for microbial management strategies, biological pretreatments, and targeted operational interventions.

SUGGESTIONS FOR WORK

Based on the results and methodological limitations of this study, it is recommended that future research:

Incorporate molecular characterization tools, such as PCR, 16S rRNA sequencing, and metagenomic approaches, for advanced taxonomic identification and evaluation of the structure of microbial communities.

They investigate specific biochemical mechanisms and dominant metabolic routes, allowing a more precise understanding of the contributions of epiphytic groups to hydrolysis, fermentation and formation of metabolic intermediates.

Establish correlations between detailed taxonomic profiles and reactor performance, integrating physicochemical parameters, microbial kinetics and biogas production.

Employ enzymatic, proteomic, or metabolomic analyses to elucidate specific metabolic functions, including hydrolytic, fermentative, and syntrophic interactions.

Consider kinetic modeling and mass balance, allowing to predict efficiency, optimize steps, and subsidize the scaling of processes based on lignocellulosic biomass.

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