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ADDITION OF ADAPTED MICROORGANISMS FOR LEAF COMPOSTING

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Abstract

Techniques such as composting, a controlled process of decomposition of organic materials, have become fundamental to minimize the problems associated with the organic fraction of urban solid waste, as the potential to cause damage to public health, the environment and, consequently, to economic and administrative aspects can be related to this kind of waste. Microbial activity is also highlighted as a relevant factor since the microorganisms are fundamental in the degradation of organic matter, which can stimulate or retard the process. The aim of this study was to evaluate the microbial activation in a composting pile, through the insertion of microorganisms extracted from the degradation of leaves, from a previously completed composting process. Two composting piles were assembled, for the first one it was added a biofertilizer, i.e. final compost of a previously composting process, with characterized microorganisms. The monitored parameters were temperature, moisture content, and pH. Aeration and humidification were performed by weekly turnings. Statistical tests were applied comparing the selected monitoring parameters means and medians according to its respective normal distribution. A Pearson's correlation test between temperature values was performed. A statistically significant differences were observed when comparing the median values of temperature and pH between the thermophilic and mesophilic phases, which tends to indicate higher decomposition in the mesophilic phase. Composting showed to be an alternative for the discarding of leaves, and it is also recommended to use the biofertilizers to increase the microbial activity in the beginning of the compost process.

Keywords: biodegradation, Brazil, composting, microorganisms, organic waste

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1. Introduction

Several aspects contribute to the growing production of municipal solid waste (MSW), such as the intensification of urbanization, consumerism, the longevity of the population, planned obsolescence of products, and the increase of packages used in products. The mixture of these problems associated with the excessive generation and the final disposal of wastes represents a modern challenge (Barros, 2012; Hoornweg and Thomas, 2012; Jacobi and Besen, 2011).

According to Siqueira and Moraes (2009), if poorly managed, MSW has the potential to cause damage to public health, the environment and, consequently, to economic and administrative aspects. The MSW management should address, among other aspects, the reduction of waste generation, its reuse, and recycling, including techniques such as composting, which provides new use to the organic

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*Author to whom all correspondence should be addressed: e-mail: marcos_mol@yahoo.com.br; Phone: +55 31 33144770; Fax: +55 31 33144770 fraction of solid waste (Brasil, 2010; Kaza et al., 2018; Santos, 2007).

The remains of organic products, both of animal and plant origin, can be incorporated into the soil, where they naturally undergo a slow degradation through the activity of microorganisms. After reaching an advanced state of decomposition, the soil absorbs the nutrients present in these organics remains, which supplies physical, biological, and chemical improvements for the soil (Vital et al., 2012). However, in large urban areas, organic waste can be a major problem, due to its large production and their frequent inadequate disposal, even with their high potential for reuse (Barros, 2012; Monteiro et al., 2001; Strom and Finstein, 2000).

The usage of controlled composting, in which an accelerated decomposition process of organic materials is used, can be considered one viable solution for the waste management challenge. As a result of this process, a stable product was obtained potentially richer in mineral nutrients (Mello-Peixoto et al., 2014; Vital et al., 2012). Although the literature often classifies the composting product as humus or soil conditioner, agronomists point out that such a product could be classified as a natural fertilizer, but with a lower presence of nutrients than chemical fertilizers (Reetz Jr., 2016). According to Haug (2018) and Pereira Neto (2007), the application of this product into the soil is beneficial due to the incorporation of nutrients, among some benefits expected from the final biofertilizer characteristics.

Unfortunately, composting is still not widely applied throughout Brazil, this technique is used in less than 1% of all MSW collected in the country. This scenario is worrisome as the organic matter represents about 50% of the Brazilian MSW, which shows the high potential of this practice in the country (Brasil, 2010). This process also has a low application in North America, where less than 0.5% of MSW is destined for composting. On the other hand, Europe and Central Asia composts around 10% of all waste collected, which can be highlighted by Austria treating 31% of its MSW through composting (Kaza et al., 2018).

In order to ensure proper control of the composting process, several parameters are used, such as density, particle size, oxygen concentration, nutrient content, and carbon/nitrogen ratio. However, temperature, pH, and moisture content are the main parameters used for the control of this process and were used in this study (Abu et al., 2014; Ch'ng et al., 2013; Getahun et al., 2012; Michel Junior, 1996). Among the factors that influence the process, there are also the microorganisms present in the windrow, the main ones are bacteria (including Actinomycetes) and fungi (Barros, 2012). Moreover, periodical aeration can be emphasized as indispensable to the process, since it influences many other aspects such as temperature, moisture content, availability of oxygen, intensification of microbiological activity and, thus, accelerating the decomposition (Epstein, 2011; Pereira Neto, 2007).

According to Von Sperling (2016), microorganisms perform several fundamental functions, especially those related to the transformation of matter during biogeochemical cycles. They are responsible for the conversion reactions of organic matter, mostly heterotrophic, since they use the organic matter as a carbon source, consequently, degrading it. Also, the introduction of adapted microorganisms in new piles containing leaves and yard trimmings can stimulate the degradation process of these organic compounds, intensifying the composting process, as described by López-González et al. (2015) and Jurado et al. (2015).

The usage of green waste, such as leaves, and yard trimming, in the composting process, allows for the reduction of materials sent to landfills, also allowing a new usage to the waste that would normally be discarded. The composting process must, therefore, be encouraged in facilities that generate this type of waste, principally due to the simplicity of monitoring parameters such as moisture content, pH, and temperature. For a proper process, periodical turnings were performed to provide proper aeration, since composting, as well as any other processes of degradation of organic matter, is subject to numerous environmental and operational variables such oxygen.

Therefore, responding to the behavior of wastes with high content in lignocellulosic fractions, after being subjected to bioaugmentation strategies in composting, the aim of the present study was to evaluate the composting activation of green waste, via the insertion of microorganisms adapted from the degradation of leaves, present in the final product of the previous composting process, performed in Brazil.

2. Methodology

The methodological steps of this research contemplated the assembly of two distinct piles, in one of them it was added biofertilizer, i.e. final compost of a previously composting process, derived from a previous composting process, characterized in relation to the microorganisms present in the control pile without any additives.

2.1. Assembling and monitoring

The piles were denominated as "added biofertilizer" (AB) and "no added biofertilizer" (NAB). Only green waste (leaves and grass clippings) were used for the assembling of composting piles. After the assembly, measurements in meters of the AB and NAB lines were 2.10 x 2.30 x 1.15 (volume equal to 5.29 m^3) and 2.10 x 2.10 x 1.20 (volume equal to 5.55 m^3), corresponding to the length, width, and height, respectively. Weekly manually turnings and humidification promoted aeration, uniform distribution of waste, and maintenance of the best conditions for microorganisms. In addition to that, the parameters temperature, moisture content, and pH were monitored during the whole experiment.

2.2. Microbiological characterization of the compound

It was prepared a suspension adding 10 grams of the final product from a previous composting process and 90 mL of distilled water. The prepared suspension was added to the culture media, looking for the microorganisms' isolation. Previously, the biofertilizer was trimmed in a processor. Next, serial dilutions were made up to 10^{-6}, transferring 10 mL of the dilution to another 90 mL of distilled water. Subsequently, replicated 0.1 mL of each serial dilution in the following culture media: Nutrient agar (AN), Plate Count Agar (PCA), Tryptone Soy Agar (TSA). The selective media used were Cetrimide Agar (AC) to isolate and count *Pseudomonas aeruginosa*, *Pseudomonas* Agar (AP) and Methylene Blue Eosin Agar (EMB) for the isolation and gram-negative enteric bacilli differentiation, Simple Agar (AS), Sabouraud (SB) for identification of fungi and Xylose Lysine Agar Tergitol (XLT).

After the quantification and morphological characterization of the colonies found in the media, the microorganisms were isolated and spiked in TSB broth medium and then incubated at 37ºC for 24 hours. Some of these cultures were stored under freezing at -80°C in Tryptic Soy Broth (TSB) or Brain Heart Infusion (BHI) medium with 20% glycerol, and the working cultures were stored in tilted TSA Agar at the same time. They were, initially incubated at 37°C for 24 hours and then stored absence of direct light at temperatures of 15°C until the time of the next analyses.

Gram staining test with crystal violet solution was performed for posterior visualization in immersion objective. In case of some contamination during the microscopic analysis, the samples were passed through a purification procedure. This procedure used a bacteriological loop to isolate a colony sample from the BHI broth plate and then transferred to another BHI broth plate. The tube was taken to the incubator at 37°C for 24 hours. Soon after, the seeding and gram staining procedures were repeated, and the purity of the samples was checked in the microscope. The sample, being pure, followed the characterization process of the microorganisms.

2.3. Analytical monitoring of composting piles

The temperatures were monitored on weekdays, via the insertion of a calibrated external thermometer approximately in the center of the pile's AB and NAB. While pH and moisture content were monitored weekly in the 105 days of the experiment (June to September of 2017), starting in the day of the pile assemble. It was adopted three replicates for each pile, totaling six samples. The raw data obtained in the analytical tests were transcribed to Excel spreadsheets. The following methodologies Tedesco et al. (1995) and Manual of Methods of Soil Analysis of the Brazilian Agricultural Research Company (Embrapa, 1997), were respectively adapted for pH and moisture content.

Initially, *in natura* samples were placed in numbered porcelain capsules, of known weight, and then placed in a semi-analytical balance. Approximately 30 grams of each sample was weighed and then the capsules were transferred into an oven at 103 +/- 2°C, remaining in this condition for about 20 hours, when constant weight was identified (adapted from Embrapa, 1997).

For the pH analysis, a solution of Calcium Chloride ($CaCl_2$ 0.01 $molL^{-1}$) was used, as indicated by Tedesco et al. (1995). Triplicate tests, for each composting pile (AB and NAB), was performed, totaling six samples. In pH measurement, digital pH meter (Digimed DM-20, accuracy ± 0.01) was checked with standard solutions of pH values equal to 4.00 (4.01 at 25 ºC) and 7.00 (6.98 at 25ºC).

Approximately 30 grams of material, corresponding to each pile, were ground in a domestic multiprocessor of exclusive use, and then distributed in three 100 mL beakers. Next, 70 mL of each extraction solution was transferred to the respective beaker. Then, the samples were shaken on a mechanical stirrer (IKA Labortechnik KS-501 Digital) at 140 rpm intermittently for 30 minutes. Finally, the samples were filtered through a glass funnel with the addition of cotton, to allow only fluids to pass over the filter, and the pH of the resulting solutions was obtained using a pH meter (adapted from Tedesco et al., 1995).

2.4. Statistical analysis

Firstly, a bootstrap was used, a method of inference about a population using sample data, to obtain confidence intervals of data. Also, a normality tests was performed before data comparison, using the Shapiro-Wilk normality tests. The comparisons were performed for pH and temperature means, by the t-Student tests, for the parametric data, and Kruskal-Wallis or Mann-Whitney for the non-parametric data. In addition, the Pearson's correlation tests between the temperatures was used. The Analyses were performed using R software (version 3.5.1).

3. Results

3.1. Physical and chemical characterization the composting pile

It was possible to observe two distinct stages during composting: initially, there was an increase of temperature, maintained until the 21st day and then, with the ambient temperature dropping, there was a decrease in the temperature of AB and NAB occurred as shown in Fig. 1.

Due to the behavior observed above, the analysis was divided in two stages: The Thermophilic phase, from its start of the process until the composing temperature reached the ambient temperature; The

Mesophilic phase, from the 22nd day until the end of the experiment.

In the Thermophilic phase, the temperature of the AB presented higher values, reaching 51.3°C, whereas the NAB showed a slower temperature increase, reaching an average of 33°C in the same period. On the other hand, in the next phase, the temperatures of the piles were both closer to the ambient temperature, as highlighted in Table 1. The piles temperatures and ambient were significantly different (p-value <0.05) in the Thermophilic phase, by Student's t-test. For the final phase, the temperature of the AB did not show significant differences in relation to the ambient temperature. It was also possible to infer, during this period, a significant correlation between the temperatures of the NAB and the environment (Table 2).

Another monitored parameter was moisture, which remained in the range of 38% to 65% throughout the experiment for the two piles (Table 3).

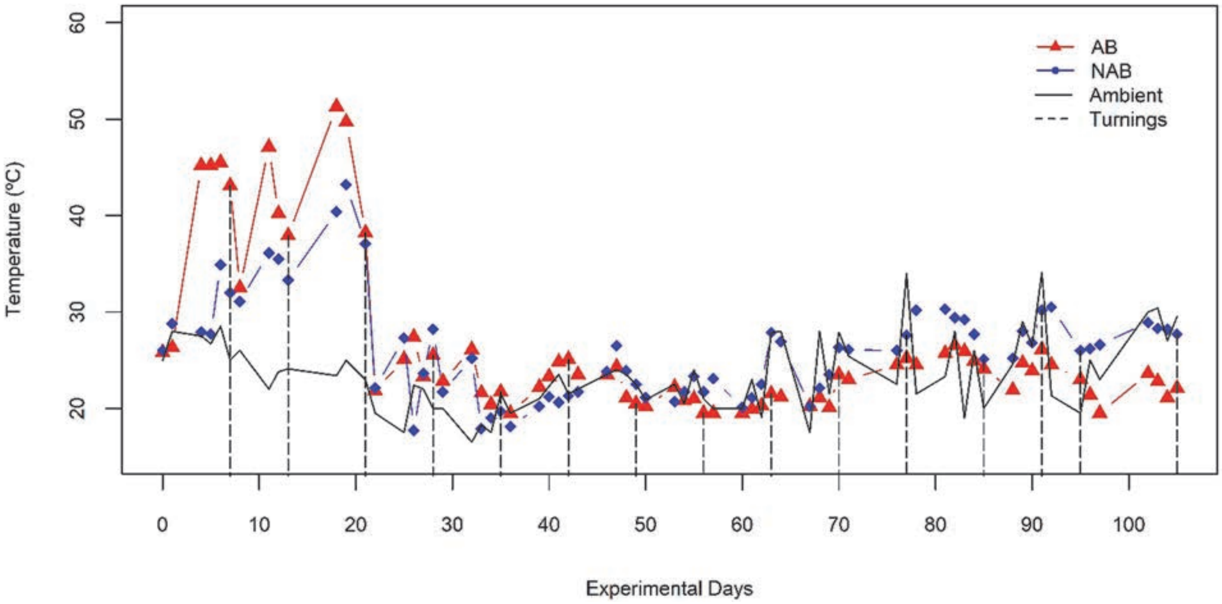

Fig. 1. Temperature variations of pile AB, NAB, and ambient temperature

Table 1. Results of the descriptive analysis in relation to the measured temperatures in the two different periods (°C)

Phase	*Source*	*n*	*Mean*	*S.D.*	*L.I.*	*Median*	*L.S.*	*p-Value*
Thermophilic	AB	12	40.82	8.57	36.31	44.15	45.43	< 0.01[a]
	NAB	12	33.08	5.25	30.42	32.65	35.96	0.01[b]
	Ambient	12	25.42	1.98	24.40	25.00	26.43	< 0.01[c]
Mesophilic	AB	57	23.00	2.96	22.29	22.80	23.77	> 0.05[a]
	NAB	57	24.66	3.93	23.73	25.10	25.63	< 0.01[b]
	Ambient	57	23.21	4.01	22.18	22.50	24.26	> 0.05[c]

Legend: NAC = no added biofertilizer; AC = added biofertilizer; n = sample number; Mean = arithmetic mean; S.T. = standard deviation; L.I. = lower limit calculated via bootstrap; L.S. = upper limit calculated via bootstrap; a = Student's t-test between AB and ambient; b = Student's t-test between NAB and AB piles; c = Wilcoxon test for Ambient and NAB

Table 2. Correlation of measured temperatures in the two different periods (°C)

Phase	*Comparison*	*Correlation*	*p-Value*
Thermophilic	AB x Ambient	-0.26	> 0.05
	NAB x Ambient	-0.48	> 0.05
Mesophilic	AB x Ambient	0.15	> 0.05
	NAB x Ambient	0.47	< 0.05*

*Legend: NAB = no added biofertilizer; AB = added biofertilizer; * statistically significant by Pearson correlation test*

Table 3. Results of the descriptive analysis in relation to the measured moisture content

Phase	*Source*	*n*	*Mean*	*S.D.*	*L.I.*	*Median*	*L.S.*	*p-Value*
Thermophilic	AB	4	0.50	0.06	0.38	0.50	0.59	0.98[1]
	NAB	4	0.51	0.06	0.42	0.49	0.65	
Mesophilic	AB	12	0.52	0.04	0.49	0.52	0.56	0.61[1]
	NAB	12	0.52	0.08	0.42	0.53	0.62	

Legend: NAB = no added biofertilizer; AB = added biofertilizer; n = sample number; Mean = arithmetic mean; S.T. = standard deviation; L.I. = lower limit calculated via bootstrap; L.S. = upper limit calculated via bootstrap;[1] = not statistically significant by t-Student test

During this same period, it is emphasized that the moisture between them did not present statistically significant differences, according to the Mann-Whitney test. When analyzing according to the phases, thermophilic or mesophilic, the ranges were different for the studies piles, as described in Table 4. Differences of pH values between AB and NAB piles was observed only to the mesophilic phase.

3.2. Microbiological characterization of the microorganisms inserted in the AB

Identification of the microorganisms inserted in the AB resulted in 75 isolated colonies of bacteria. From those, 53 samples presented growth in the characterization stage, and 23 (43%) presented similar characteristics to other samples and, therefore, were discarded. According to Symanski (2005), at the end of the thermophilic phase, characterized by elevated temperatures a recolonization by mesophilic organisms occurs in the composting pile, which could explain the low variation of possible different genera and species.

Therefore, the 30 samples that could be identified, that is, with growth and without characteristics similar to each other, were characterized in relation to the morphology and typology of Gram. About 80% of the samples had two morphologies, suggesting some kind of interaction or potential co-dependency between the microorganisms. In the same way, Nunes (2012) describes interactions between bacteria mediated by contact, through the exchange of intracellular molecules and nutrients between microorganisms of the same species and distinct species.

From the studied samples, 50% were Gram-positive bacilli, 23% Gram-negative rods, 7% Gram-positive cocci and 20% Gram-negative cocci. Counts of the isolated colonial ranged from $1.6x10^1$ to $3.3x10^3$ colony forming units per gram of sample. In a similar work, Hassen et al. (2001) also observed three large groups in the composting of organic wastes, being gram-positive cocci, gram-positive bacilli, and gram-negative rods. Another similar study by Vaz-Moreira et al. (2008) suggested a higher number of Gram-positive bacteria, indicating that they are possibly the most resistant to the elevated temperatures of the composting process when compared to Gram-negative microorganisms.

After the morphological characterization, a biochemical test was executed to identify genera or species found. The tests indicated the presence of the species *Pseudomonas aeruginosa*, *Bacillus megaterium* and the genus Bacillus spp. in the samples in which biochemical tests were performed. In a similar work, Blanc et al. (1999), Dees and Ghiorse (2001), and Hassen et al. (2001) observed that the genus *Bacillus* was dominant during the composting cycle, which may be justified by the capacity of producing bacterial endospores, which provide better resistance to high temperatures and any other unfavorable conditions present in the composting process.

Despite the fact that the present work is a composting process using only green waste, Symanski (2005), in another paper using household waste composting, observed that of all the isolated microorganisms, 52.2% belonged to the genera *Enterobacter*, *Bacillus*, *Pseudomonas*, *Escherichia*. It was also observed two of these genera in our study. In their work, Insam and Bertoldi (2007) made a survey of genera and species already found in similar works, and it was possible to identify the presence of these same genera also in their work.

The presence of fungi was also observed in the compound, and it is important to consider that they can favor the breakage of complex organic chains such as cellulose, according to Richard et al. (2002), facilitating the later decomposition of the structures by the bacteria present in the medium, when the composting began. The complementary performance between microorganisms may suggest that the best performance depends on a set of microorganisms.

4. Discussions

The temperatures data showed that the AB presented the highest temperature, reaching values above 50ºC, while the NAB temperatures did not exceed 45ºC. This may indicate higher microbial activity in the AB pile due to the insertion of the microorganisms adapted to the composting process, which possibly favored the degradation of the waste (Cerri et al. 2008; Pereira Neto 2007). On the other hand, from the 21st day of the experiment there was a decrease of the ambient temperature, with the beginning of the winter and, consequently, a reduction of the temperatures of the two lines was observed. For the mesophilic phase, the temperatures records were not higher than 39°C, possibility indicating a lower microbiological activity between the 21st and 95th day of the experiment.

Table 4. Results of the descriptive analysis in relation to the measured pH level in two different periods

Phase	*Source*	*n*	*Mean*	*S.D.*	*L.I.*	*Median*	*L.S.*	*p-Value*
Thermophilic	AB	4	7.37	0.26	7.06	7.37	7.68	0.70
	NAB	4	7.30	0.25	6.94	7.37	7.52	
Mesophilic	AB	12	7.49	0.11	7.33	7.47	7.72	< 0.01*
	NAB	12	7.24	0.18	6.91	7.23	7.49	

*Legend: NAB = no added biofertilizer; AB = added biofertilizer; n = sample number; Mean = arithmetic mean; S.T. = standard deviation; L.I. = lower limit calculated via bootstrap; L.S. = upper limit calculated via bootstrap; * statistically significant by t-Student test*

During periods of lower ambient temperatures, usually below 20°C, the microorganisms' metabolic activity is reduced, making it difficult to observe an increase of temperature inside the windrow (Tateda et al., 2002; Vandecasteele et al., 2016; Xie et al., 2017). One of the contributing factors to this reduction is the lack of favorable conditions for the growth of microorganisms where, in the case of low temperatures, it results in a slower microbiological growth phase (Madigan et al., 2016). Thus, in colder climates, elevated temperatures are not expected in a composting process (Haug, 2018; Zhao et al., 2012).

In spite of the influence of ambient temperature in the composting process, the temperature of the AB pile presented a statistically significant difference in relation to the ambient temperature in neither of the phases. Differentially, the NAB's pile temperature presented a significant correlation between its temperature and ambient temperature. This may be explained due to the presence of the microorganisms adapted to the composting process in pile AB, which raised the temperature due the microbiological activities. Corroborating with the hypothesis of this study, the upper temperature in the AB pile was due to the presence of the microorganisms adapted to the composting process, which elevated the temperature through metabolism. Similar results were presented by López-González et al. (2015) and Jurado et al. (2015) and revealed inoculation inducing a stimulation of microbial growth and activity in the entire composting microbiota, which was actually responsible for all the beneficial effects reported.

It also be noted that after day 45 the temperature was consistently higher in the NAB pile than the AB pile. This supports a finding that inoculation sped up the initial degradation of the material, since it suggests that more material remained to be degraded later without inoculation. Statistical differences were found only in the beginning of the temperature, when compared the temperature of AB and NAB. In addition, it was observed significant differences (p-values <0.05) when comparing the mesophilic and thermophilic phases between the AB and NAB piles (Tables 2, 3 and 4). These differences indicate that the insertion of microorganisms adapted to the degradation of leaves tends to accelerate the composting process.

Arias et al. (2017), in a study using pig manure and green waste, observed a similar behavior of temperature for the composting process. During an intense cold, with the ambient temperature varying between 0º and 16ºC, a reduced thermophilic phase was also reported, also a slow start was found the pile temperature did not exceed 16ºC until the 20th day of the experiment. In our study, even though the ambient temperature did not reach such extreme values, the same behavior was observed for the two composting piles, still, after the thermophilic phase temperatures were higher than 35°C.

It should be noted that several aspects of composting are inexact, and the process involves a wide variation of conditions and materials, and its duration and final substrate quality are related to the mixture of materials used at the beginning of the process (Poincelot, 1975; Rynk, 1992). Nevertheless, according to Vergnoux et al. (2009), the composting processes usually have similar characteristics, both in their operational aspects and in relation to the monitoring process, even when there is variation in the composition of the waste used. It should be noted that the implantation of composting presents technical advantages, ease implantation and simplicity in maintenance (Mello-Peixoto et al., 2014), except in relation to the microbiological characterization, which requires an analytical deepening.

Vital et al. (2012) adds that in the process of organic matter decomposition in composting the number of nutrients available in the organic remains can be reversed to be used as nutrients of plants in the gardens, as well as to maintain and beneficiate the soil fauna. However, in order to obtain an agronomical applicable biofertilizer, prior separation of the non-organic material is necessary to reduce the chances of contamination and, thus, the biofertilizer may be of better quality (Santos, 2014).

5. Conclusions

The present study suggests a higher rate of initial biodegradation with the insertion of microorganisms adapted to the degradation of leaves in the composting process and may reduce the duration period of composting process. Regarding the identification of the microorganisms, it is important to note that this study was preliminary, and only the initial identification of the microorganisms was carried out. Analysis that is more effective can be performed to identify the microorganisms, and yet, of the numerous microorganisms present in the composting process, few were cultivable.

The feasibility of using composting, is also worth noting, as an alternative for the discarding of leaves, especially in countries with a tropical climate, considering aspects such as the simplicity of assembly and monitoring, as well as the little demand for large space and time of maintenance, being accessible for situations of lesser volume of organic waste.

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