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In situ analysis of a composting plant located in São Paulo city: fungal ecology in different composting phases

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ABSTRACT. The National Plan for Solid Waste has set out to reduce by 25% the amount of organic waste disposed of in landfills, mostly food residues from street fairs, besides determining the implementation of municipal composting plants, and it also mentions improving the capacity of plants already installed. The purpose of the study was to analyze which decomposing fungi are involved in the different composting phases, in a plant located in the city of São Paulo. Data was collected in four composting seasons from 2016 to 2017 and the analysis of 49 samples showed twelve genera belonging to the Ascomycetes and Zygomycetes phyla, but only at the mesophilic phase. In all seasons, yeasts and *Aspergillus fumigatus* were predominant with a total count of 1.0 x 10^9 cfu g^{-1} and 7.4 x 10^8 cfu g^{-1}, respectively. These fungi can be applied in future studies of biostimulation to optimize the cycle at the municipal plant.

Keywords: fungi; composting phases; mycobiota; biostimulation; biodegradation.

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Introduction

According to Veras and Povinelli (2004) one of the major challenges of contemporary society is the establishment of an effective system of solid waste management. The growth of population also increased the agroindustrial systems, and agroindustrial activity began to generate large volumes of waste, along its production chain, with a great diversity of organic solid wastes, thus constituting a social, economic and ecological problem, causing great environmental impact (Souza, Ruffato, Costa, Ruffato, & Simões, 2010). In 2010, the Federal Brazilian Law no. 12,305 (Brasil, 2010), which instituted the National Solid Waste Policy (NSWP) in Brazil, established the guidelines to solid waste management, as the following sequence: non-generation, reduction, reuse, recycling, treatment and disposal environmentally appropriate waste, which in its preliminary version pointed to a favourable target for the Southeast region of 25% reduction by 2015 of the organic portions disposed in landfills (Brasil, 2012). The Law mentions the implementation of new composting units and use of the already installed capacity of composting plants. Moreover, municipalities should implement composting systems for organic solid waste recycling, considering only those for which there is no possibility of reuse or recycling, the destination of which would be landfills.

Composting or bio-stabilization is a controlled aerobic biological process that leads to the production of the compound which results from the decomposition and humification of a mixture of organic materials waste under conditions as presence of oxygen and balance between the chemical elements of matter, by the action of microorganisms and the biodegradable waste materials are aerobically digested at a stabilized organic fraction that can be recycled for agricultural uses. It is estimated that the total process can take from 90 to 120 days (Ankidawa & Nwodo, 2012; Chiarelotto, Bottin, Spicker, Chiarelotto, & Bortoli, 2018).

According to Kiehl (1985) the composting process provides two distinct stages. The first one is the biodegradation of organic waste and the second one is maturation, cure or humification of the compound in the phase of biostabilization. Some authors characterized the composting process as having three different phases: phase 1 with an increase in temperature from ambient to above 45°C; phase 2, which has been termed the 'stabilization' phase and is characterized by the attainment of thermophilic temperatures (50°C); and phase 3, the 'maturation' phase, which is typically characterized by a reduction in temperature towards ambient (Strom, 1985; Droffner & Brinton, 1995; Tuomela, Vikman, Hatakka, & Itavaara, 2000).

Composting process becomes an alternative to the problem of waste generation and to the use of chemical fertilizer (Souza et al., 2010). According to Herbets, Coelho, Miletti, and Mendonça (2005) several factors can be easily managed for their optimization. One of them is the bio-accumulation technique, which consists in adding cultures of microorganisms with proven pollutant degrading activity to a place, ensuring that the appropriate consortium of microorganisms will be present in enough types, number and compatibility to metabolize the pollutant in a way effective. Another method is biostimulation, which is only effective when there are degrading microbial populations in the substrate. The technique is performed by stimulating the native microbiota (Yakubu, 2007).

Despite extensive research over the past twenty years into engineering aspects and the benefits of using composts, composting is still essentially considered a 'black box' process and more studies about the parameters involved in the small-scale composting process are still needed. Community structure and diversity are instrumental in manipulating compost environment to increase compost process and to improve compost quality (Agrawal, 2014). Many studies have already determined the type of bacteria and their use as microbial inoculants, with the purpose of accelerating composting and improving the final product (Zeng, Huang, Huang, & Liu, 2009; Figueiredo, Martos, Siqueira, Pereira, Silva, Rinker, & Dias, 2013). Nevertheless, further information about fungal activity in the composting system is necessary. Filamentous fungi are multicellular eukaryotes (also called mold or mould) and unicellular organisms (known as yeasts). Filamentous fungi are aerobic, but yeasts develop without the presence of oxygen (anaerobic). They prefer substrates with acid pH (yeasts), but also have activity in slightly alkaline pH (filamentous fungi). They are efficient in the thermophilic range and in the degradation of carbonaceous compounds, such as cellulose, hemicellulose and lignin (Herbets et al., 2005).

The present study aimed to elucidate which are the main genera found in all the different phases of composting, in a composting plant located in the city of São Paulo, to select native fungi as inoculants for future use in the biostimulation pathway for acceleration of the microbiological process of composting.

Material and methods

The present study was carried out at the composting plant of the Regional Office of the Municipality in São Paulo city where there is waste composting from vegetable and fruits retail, pruning and gardening of municipal services. According to Misra, Roy and Hiraoka (2003) the composting process is done by way of the static pile method with passive aeration. The format and the organic materials used permit the line to have the minimum oxygen available for aerobic decomposition without the need to be revolved (static system). The compost piles are 1.5 to 2.5m wide and 15 to 20m long and reach 1 to 1,5m high before the maturation phase at the composting cycle (Figure 1).

Figure 1. Sampling in composting piles at the São Paulo Regional Office.

Four procedures collections were made in four seasons (1- August/2016, 2- October/2016, 3- February/2017, 4- April/2017) for each different phase (1- active pile; 2- rest piles; 3- maturation; 4- compound without sifting; 5- ready composed sifted; 6- vegetable garden; 7- percolated liquid) were carried out, obtaining a total of 49 samples of the composting material. Each sample of 100 grams (g) was collected in duplicate from 3 different points of the piles, in three equidistant points from the upper strata, while low strata were collected at three depths.

Below the piles, pipes were implanted to collect the water eliminated during the process or rain water. These systems also prevent the soil from drenching and a better drainage of the tracks. The collected leachate or percolated liquid is stored in concrete boxes and a total of 8 samples of the percolated liquid

(20 mL) were also collected and transferred to a sterilized plastic flask. All material was transported in thermal bags to the microbiology laboratory.

For fungal isolation the serial dilution technique was used in surface plating, mixing 10 g of each sample in 90 mL of sterile distilled water to prepare the first dilution (10^{-1}). From this dilution, 1mL was transferred to another tube containing 9 mL of sterile distilled water, this process being repeated until dilution 10^{-6}, from which a 0.1 mL aliquot was seeded in the Petri dishes, in duplicates, with a sterile culture medium of Potato Dextrose Agar (PDA). Chloramphenicol (0.05 g L^{-1}) was added to inhibit bacteria, since chloramphenicol is a broad-spectrum antibiotic inhibitory to a wide range of gram-negative and gram-positive bacteria without affecting fungal growth (Haley, Trandel, & Coyle, 1980). The plates were incubated at 25ºC for 7 days in a standard Biochemical Oxygen Demand (BOD) incubator for growth of fungi cultures (Aquino, Lui, & Corrêa, 2017). After incubation, the Petri dishes were visually observed for fungal counts in colony forming units per gram of sample (cfu g^{-1}). For the microscopic observation of the morphology, the technique of direct mycological examination under a light microscope was used in a glass slide containing a drop of blue-cotton lactophenol. The identification of fungal genera was performed according to descriptive criteria, based on the taxonomic keys described by Pitt and Hocking (2009). In order to determine a difference between the fungal growth in the four collections, the statistical test ANOVA and Student's t-test were applied, and to determine the correlation in the biodegradation performance of filamentous fungi and yeasts, the linear correlation test Pearson and monotonic Spearman were used.

Results and discussion

The composting process time varies with the environment, type and particle size of the material to be composted (Insam & Bertoldi, 2007; Zhang & Sun, 2015). The maturity of the product at the end of composting is influenced by factors such as temperature, pH and humidity, which must be controlled throughout the process. In addition, the substrates utilized and the microbiota carrying out the process exercise a great influence on compost formation (Villar, Alves, Garrido, & Mato, 2016).

In present study, correlation between the performance of multicellular or filamentous fungi and yeasts (unicellular fungi) was determined for the biodegradation (phase 1 and 2), considering the cfu/g, the linear correlation test Pearson and monotonic Spearman (Figure 2).

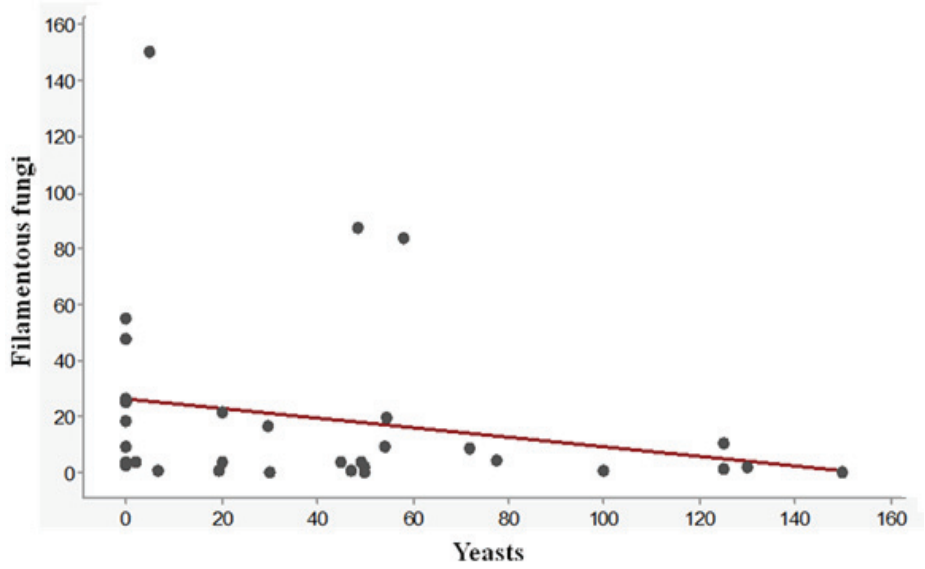

Figure 2. Correlation of filamentous fungi and yeasts at biodegradation phase.

We assumed 0 for absence of correlation, 1 for positive correlation and -1 for perfect negative correlation, and the values obtained were not very explanatory for the relationship between the two groups. From the dispersion graph it was observed that there is a negative expression in the correlation of filamentous fungi x yeasts in the composting biodegradation, since the result obtained was a linear correlation of -0.228 and the monotonic -0.372.

Most of the fungi are composed of mesophyll species, and the most common species found in the composting process as Neher, Thomas, Weicht, Bates, and Leff (2013) reported the greater abundances of *Epicoccum*, *Thermomyces*, *Eurotium*, *Arthrobotrys*, and *Myriococcum*, besides *Chytridomycota* and *Zygomycota* in composting material. Anastasis, Giovanna, and Marchisio (2005) described as fungi species in composts *Scedosporium, Penicillium, Aspergillus*. *Absidia, Cladosporium, Acremonium, Fusarium, Trichoderma, Chrysosporium, Scopulariopsis, Eurotium, Emericella, Eurotium, Epicoccum, Mucor, Chaetomium, Acremonium, Gliocladium*, etc. In the present study the genus *Aspergillus* (filamentous fungi) and yeasts were the most commonly found fungi in the four collections at the various stages of the complete composting cycle (Figure 3).

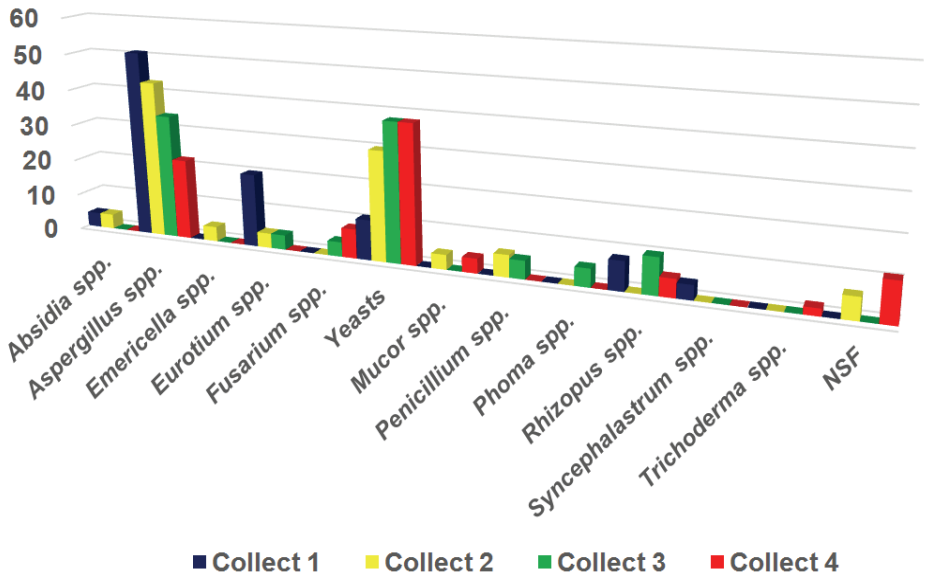

Figure 3. Fungal genera prevalence of four collections at the composting cycle.

Baeta-Hall, Sàágua, Bartolomeu, Anselmo, and Rosa (2002) found in the compost of olive pomace the count of 10^8 cfu g^{-1} of fungi, being the most common genera *Penicillium, Cladosporium, Aspergillus* and *Trichoderma*. Anastasis, Tsolakidou, Stringlis, and Pantelides (2017) showed the quantitative composition of the mycobiota of a green compost thermophilically produced from plant debris and the total fungal load was up to 8.2×10^5 cfu g^{-1} at the final compost. The results of Dehghani, Asadi, Charkhloo, Mostafaie, Saffari, Mousavi, and Pourbabaei (2012) identified fungal species during the compost process according to their frequencies as *Aspergillus* spp. (34.45%), *Microsporium* spp. (18.89%), *Trichophyton* spp. (8.89%), *Yeast* spp. (6.67%), *Mucor* spp. (5.56%), *Penicillium* spp. (4.45%), *Rhizopus* spp. (4.45%), *Fusarium* spp. (3.34%), *Cladosporium* spp. (3.34%), *Curvularia* spp. (3.34%) and other fungal species (6.62%).

According to Langarica-Fuentes, Zafar, Heyworth, Brown, Fox, and Robson (2014), fungi are known to have an important role in the composting process as degraders of recalcitrant materials such as cellulose and lignin. According to Guarro, Gené, and Stchigel (1999), Ascomycota is the largest phylum of fungi, followed by the Basidiomycota and Mucorales orders that belong to the phylum Zygomycota. The occurrence of Ascomycetes in the starting materials and at the early stages of the process is represented by yeast species, while in latter stages and in the high temperature regions of the pile, fungi from the orders Eurotiales, Sordariales, Mucorales, Agaricales and Microascales were the most prominent (Langarica-Fuentes et al., 2014).

This present study provides an improved understanding of the ecology of decomposing fungi occurring during the composting of municipal solid waste in São Paulo, and this knowledge can lead to the development of more efficient composting practices and better evaluation of the end-product quality. *Aspergillus fumigatus* and yeasts were distributed at all phases of composting process. Some fungi emerged only in a single phase (early at mesophilic stages), such as the Ascomycetes *Aspergillus candidus*, *Emericella* spp., *Fusarium solani*, *Fusarium* spp., *Phoma* spp., Non-Sporulated Fungus (NSF) and Zygomycetes such as *Absidia* spp., *Mucor* spp. and *Syncephalastrum* spp. (Figure 4).

Ascomycetes as *Trichoderma* spp. and *Eurotium* spp., besides *Rhizopus* spp. (Mucolares) are fungi associated with the active and rest pile (mesophilic phase) only, except for *Aspergillus niger* and *Penicillium* spp., ascomycetous fungi which were also found at three stages, including the maturation period (Figure 5). However, *A. niger* resurfaced in stage 7 (in percolated liquid).

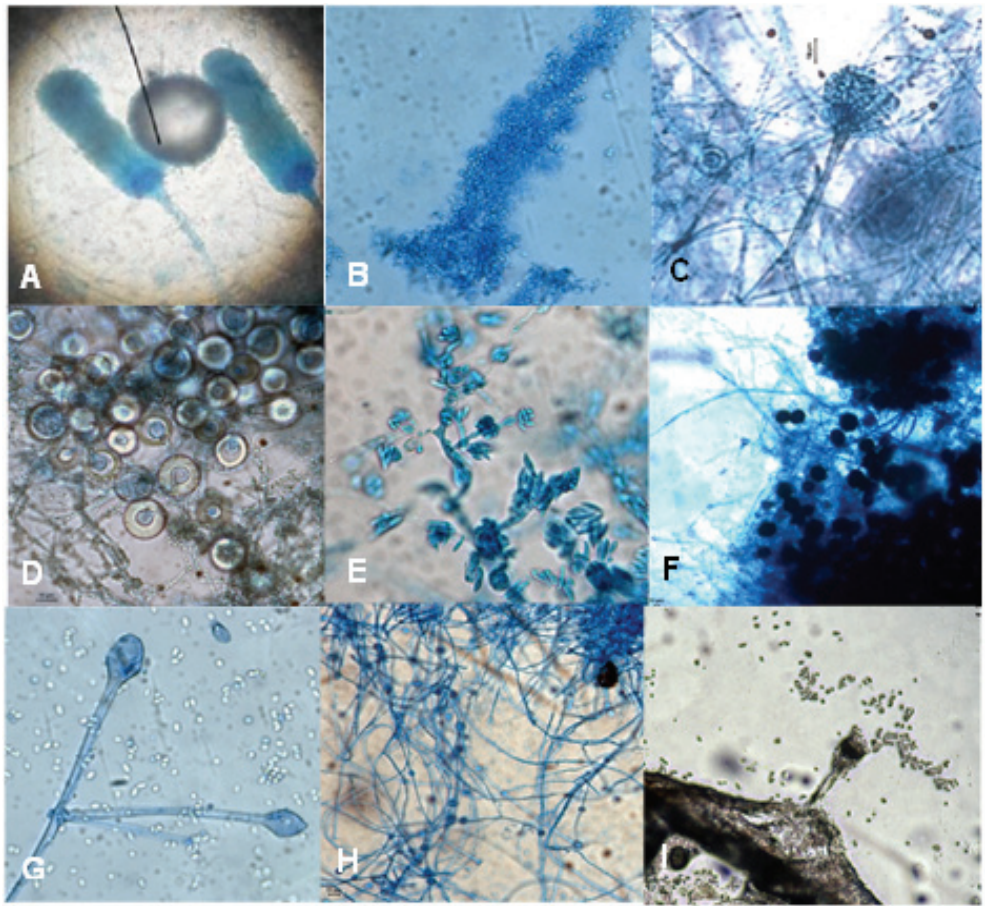

Figure 4. Examples of fungi isolated from composting material at one phase of the process. *Aspergillus fumigatus* (A), yeasts (B), *Aspergillus candidus* (C), *Emericella* spp. (D), *Fusarium solani* (E), *Phoma* spp. (F), *Absidia* spp. (G), *Mucor* spp. (H) and *Syncephalastrum* spp. (I).

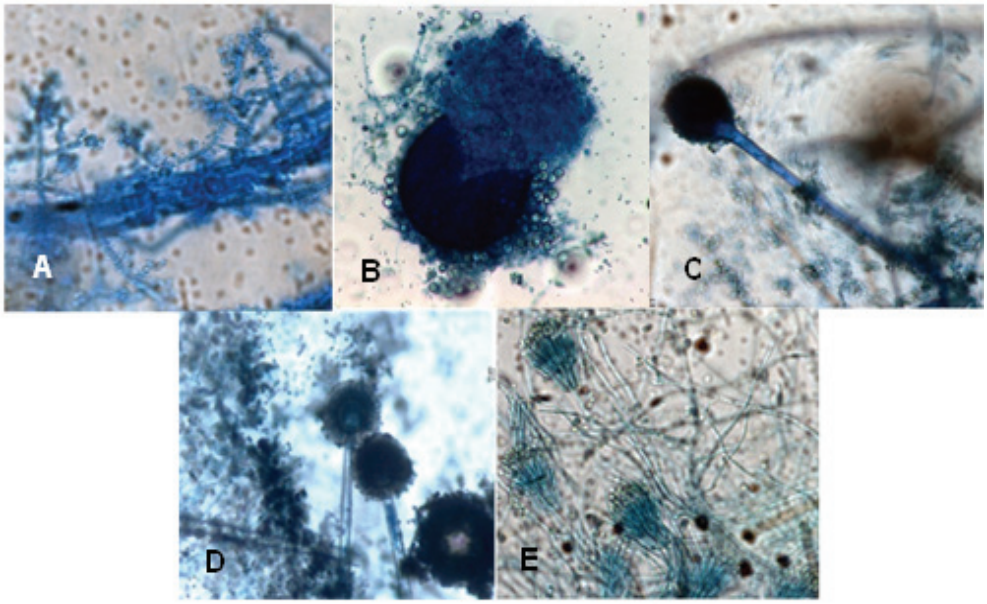

Figure 5. Examples of fungi isolated from composting material at two or three phases of mesophilic process. *Trichoderma* spp. (A), *Eurotium* spp. (B), *Rhizopus* spp. (C), *Aspergillus niger* (D) and *Penicillium* spp. (E).

The total counting of genera as demonstrated in Table 1 from the obtained data shows the cfu g^{-1} average of all stages in four seasons that ranged from 10^6 to 10^8 cfu g^{-1}.

Table 1. Genes and species of fungi isolated by phase of the composting process at the composting plant.

Fungi	Stage 1*	Stage 2*	Stage 3*	Stage 4*	Stage 5*	Stage 6*	Stage 7*
Absidia spp.	-	2×10^6	-	-	-	-	-
Aspergillus candidus	-	1×10^6	-	-	-	-	-
Aspergillus fumigatus	2×10^8	4×10^8	3×10^6	1×10^6	1×10^8	1×10^7	3×10^7
Aspergillus niger	2×10^6	2×10^7	2×10^6	-	-	-	6×10^6
Emericella spp.	3×10^6	-	-	-	-	-	-
Eurotium spp.	2×10^7	2×10^8	-	-	-	-	-
Fusarium solani	1×10^7	-	-	-	-	-	-
Fusarium spp.	-	1×10^6	-	-	-	-	-
Yeasts	1×10^8	7×10^8	5×10^6	1×10^7	1×10^8	1×10^7	1×10^8
Mucor spp.	2×10^6	-	-	-	-	-	-
Penicillium spp.	3×10^6	2×10^6	3×10^8	-	-	-	-
Phoma spp.	2×10^6	-	-	-	-	-	-
Rhizopus spp.	2×10^7	6×10^6	-	-	-	-	-
Syncephalastrum spp.	-	1×10^8	-	-	-	-	-
Trichoderma spp.	4×10^6	1×10^8	-	-	-	-	-
NSF	2×10^6	-	-	-	-	-	-
Total	$3{,}6 \times 10^8$	1.5×10^9	3.1×10^8	1×10^7	2×10^8	2×10^7	1.3×10^8

*Average counting of duplicate samples of all phases.

In the present study yeasts predominated with a total count of 1.0×10^9 cfu g^{-1} (Figure 6 A/B), in the four collections. Among the filamentous fungi, the species *Aspergillus fumigatus* had a total count of 7.4×10^8 cfu g^{-1} at all phases (Figure 6 C/D).

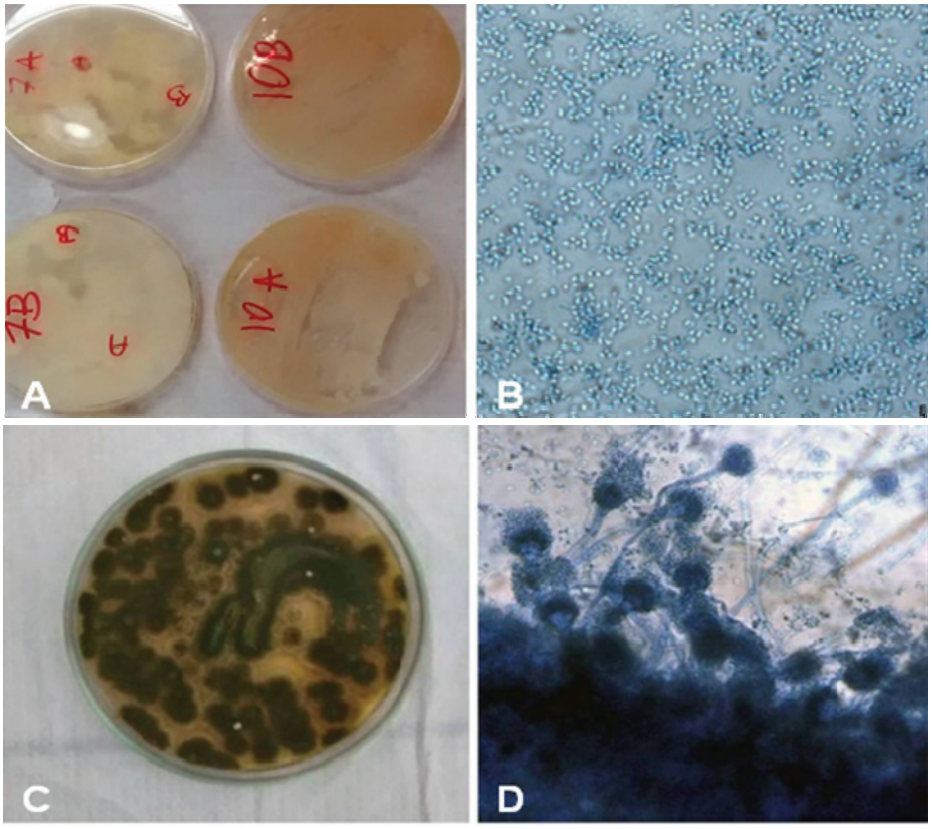

Figure 6. Yeasts (A/B) and *A. fumigatus* (C/D) isolated from composting material.

The data is in accordance to Tuomela et al. (2000) which reported that the raw material of compost contains about 10^6 microbial counts of mesophilic fungi/g of raw material and thermophilic fungi $10^3 \pm 10^6$ cfu g^{-1} and that the predominant fungi in the raw material is the thermotolerant fungus *Aspergillus fumigatus*. This was also observed by Anastasi et al. (2005) who reported that the mainly thermotolerant

filamentous fungus in the composting process is *A. fumigatus*. Lim, Chua, and Lee (2013) reported that the population of mesophilic fungi is around 10^5 to 10^7 cfu g^{-1} of compost and the microbial number of mesophilic fungi is always reestablished at the end of cooling phase and the maturity phase.

To determine if there was a difference between fungal growth in the four seasons collections, the statistical test ANOVA and Student's t-test were applied for each of the samples. The empirical distribution of the data and results obtained median and the values of the upper and lower limit of cfu g^{-1} is demonstrated in Table 2.

Table 2. ANOVA test results for the fungal total counting of four collections seasons.

Season	Mean	Standard deviation	Confidence interval 95%
Collect 1 - Aug / 2016	63.3 cfu g^{-1} x 10^7	48.3 cfu g^{-1} x 10^7	31.7- 94.9 cfu g^{-1} x 10^7
Collect 2 - Oct / 2016	34.0 cfu g^{-1} x 10^7	34.3 cfu g^{-1} x 10^7	6.3- 61.8 cfu g^{-1} x 10^7
Collect 3 - Feb/2017	58.0 cfu g^{-1} x 10^7	31.3 cfu g^{-1} x 10^7	30.3- 85.8 cfu g^{-1} x 10^7
Collect 4 - Apr/2017	94.2 cfu g^{-1} x 10^7	73.1 cfu g^{-1} x 10^7	66.5- 122.0 cfu g^{-1} x 10^7

The statistics analysis demonstrated that the p value (indicative value of variance) was 0.031 and $p > 0.05$ (not significant) were obtained between season collections 1 and 2 ($p = 0.126$); between season collections 1 and 3 ($\rho = 0.769$); season collections 1 and 4 ($p = 0.237$); between 2 and 3 ($p = 0.076$) and between 3 and 4 ($p = 0.120$). There was only significant difference in collection 2 and 4 ($p < 0.05$) with value of $\rho = 0.016$ as also demonstrated by Figure 7, that shows the empirical distribution of the data and better analysis of the results by means of the median and upper and lower limit values of cfu g^{-1} found in each sample.

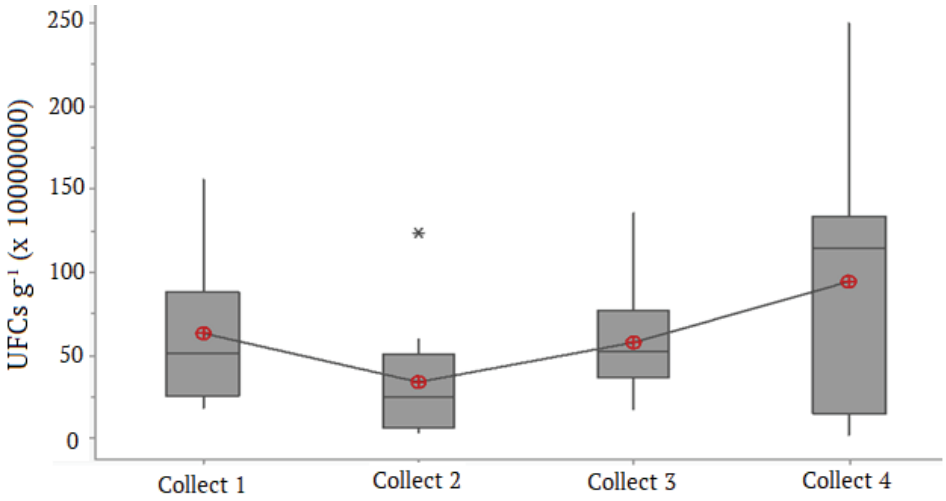

Figure 7. The median and limit values of the cfu g^{-1} obtained in each season collections.

The first three phases are also called biodegradation, when the compound passes to maturation of matter with great formation of humic substances (Inacio & Miller, 2009) and an increase of mycobiota was noticed in this present study. This observation is in accordance to Mustin (1987) that reported that at the beginning of composting biological activity settles through mesophilic microorganisms and the increase in temperatures is the result of organic matter oxidation by aerobic microbial population. This activity is important at the beginning of composting process, for it is reflected in the increase of temperature. Temperature monitoring allows an indirect measure of aerobic degradation intensity. At temperatures below 20°C, only psychrotrophic microorganisms are active. Between 20 and 40°C, mesophilic ones have their turn, but thermophilic microorganisms are active only at temperatures between 40 and 70°C (Mustin, 1987).

The collect 2 in October 2016 corresponded to stages 4 and 5, after maturation and between the formation of compound without sifting. In this period, there is usually death of microorganisms due to high temperatures, and activity of heat-sensitive microorganisms (which participate in the compost maturation) is inhibited. These temperatures become limiting for biological activity, which reduces the amount of generated heat, and the temperature stabilizes until conditions become limiting, when the piles, particularly the substrate decomposition, lead to a gradual decrease in temperature, humidity and

availability of organic matter, now entering the degradation phase of the most resistant organic substances (Makan, Assobhei, & Mountadar, 2013).

The substrates utilized and the microbiota carrying out the process exert great influence on compost formation (Villar et al., 2016). Studies have been conducted addressing the use of microbial inoculants with the purpose of accelerating composting and improving the final product (Zeng et al., 2009; Figueiredo et al., 2013). The average of fungi relative frequencies (%) obtained at each phase of the composting, during four collections, is shown by Figure 8.

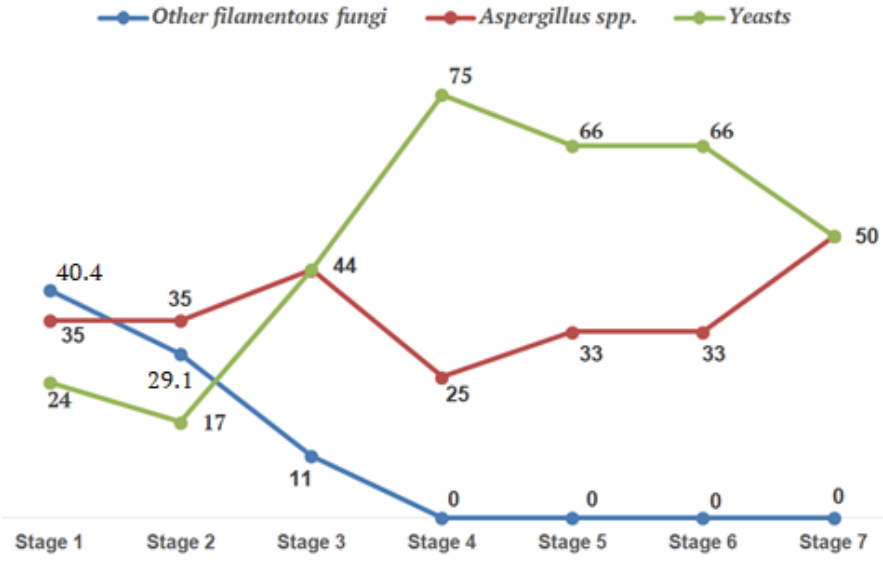

Figure 8. Relative frequency (%) of *Aspergillus* spp., yeasts and other filamentous fungi at seven stages of the composting process.

In the initial phase of composting process (phase 1 and 2) there is expansion of the mesophilic microorganism colonies and intensification of decomposition, heat release and rapid increase in temperature. Mesophilic fungi begin to predominate during this stage and are thought to be responsible for degradation and conversion of lignins (Strom, 1985; Droffner & Brinton, 1995; Tuomela et al., 2000). However, the activity of other mesophilic filamentous fungi was interrupted in step 4, due to the quick start of the thermophilic phase, characterized by temperatures above 45°C (Inacio & Miller, 2009).

Large numbers of fungal species have been found in composting materials during the mesophilic stage of the process, and it has been suggested that these species are present in the original substrate prior to the composting process (Kutzner, 2000; Huhe, Wu, & Cheng, 2017). By contrast, only a few species of thermophilic fungi have been recovered at the thermophilic stages, and these are usually well adapted to the process conditions (Kutzner, 2000). Thermophilic fungi from compost have been suggested as the main contributors to lignocellulose degradation and the optimum temperature for their growth (40-50°C) is the same optimum temperature for lignin degradation (Ryckeboer, Mergaert, Coosemans, Deprins, & Swings, 2003).

Despite the significance of fungal diversity in composting, especially in thermophilic fraction, in this study *Aspergillus* genera and yeasts have demonstrated an important role in all stages, especially in thermophilic period. *Aspergillus* genera belong to the Aspergillaceae, a family of the Eurotiales order (class Eurotiomycetes, phylum Ascomycota). Species belonging to this family have diverse physiological properties and grow in extremely low water activities due to high sugar or salt concentrations, while others can grow at low (psychrotolerant) or high temperatures (thermotolerant), low-acidity levels, and/or low oxygen levels (Houbraken, de Vries, & Samson, 2014). The *Aspergillus* spp. had a higher relative frequency (35%) comparing to yeasts in phases 1 and 2 (24 and 17%, respectively) but the activity of *Aspergillus* genera was predominant in the stage 3 (44%) and stage 7 (50%), while yeasts predominated in the stage 4 (75%) until stage 6.

The tolerance of yeast to high temperatures is a desirable characteristic in composting and industrial applications and some yeasts species ferment sugars at temperatures above 40 C (Hong, Wang, Kumagai, & Tamaki, 2007; Nonklang et al., 2008; Suryawati, Wilkins, Bellmer, Huhnke, Maness, & Banat, 2008, Hasunuma & Kondo 2012; de Souza et al., 2012; Costa et al., 2014). The equal frequency of *Aspergillus* and

yeasts occur in percolated liquid (50%) at stage 7 and these results demonstrated that these fungi resist all stages in composting process.

Studies about the complexity of microbial community dynamics and how it affected ecosystem composting process coupled to utilization of diversity indices have permitted deeper insights as to subtle changes that result in biological conditioning of compost to permit mushrooming as well as suggesting where artificial inocula could be used to hasten the composting process and associated mushroom yield. Methods to study diversity (numerical, taxonomic, and structural) are improving for both bacteria and fungi, but there is still not a clear association between diversity and function. It is generally thought that a diverse population of organisms will be more resistant to stress and more capable of adapting to environment changes in a composting system (Agrawal, 2014).

One of the factors that discourage the practice of composting is the time spent in this process, which can extend to four months or more, depending on the conditions under which it occurs. The way to accelerate the composting process is the use of microorganism-based inoculants (Wahyono & Sahwan, 1998). Ribeiro, Souza, Costa, and Dias (2017) reported that the number of studies involving microbial inoculants has been growing, and the inoculum of the composting process showed an influence on the values of temperature and on the degradation of both cellulose and hemicellulose during the thermophilic period. Wangen, Pena, Camargo, Santos, and Pires (2013) obtained good results after 90 days of avian bed compost with inoculant solution based on natural microorganisms (lactic acid bacteria and yeasts). However, it is important to know the natural or native mycobiota of each composting system for further testing with inoculant substances. It is possible to apply two predominant genera isolated in this study in all phases of composting process, yeasts and *Aspergillus fumigatus*.

Conclusion

The microbial ecology involved in the biodegradation and conversion of organic matter during the composting process is a field to be explored. The present study is an important contribution to the understanding of the fungal ecology at the composting plant in situ and to the selecting of specific fungal genera that can be applied in future biostimulation researches, using the native mycobiota itself. The results elucidated that *Aspergillus fumigatus* and yeasts are fundamental at all stages of the composting process, since they digest different materials and are thermophilic fungi. These microorganisms can be used in the laboratory as pure culture and added at all stages of the composting process to accelerate the time consumed in biodegradation, improving the capacity of municipal plant waste cycle.

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