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Enhanced turnover of organic matter fractions by microbial stimulation during lignocellulosic waste composting

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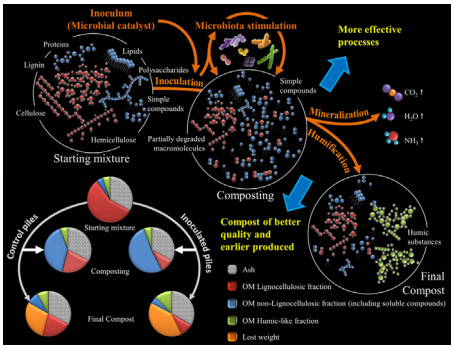
HIGHLIGHTS

- Intense turnover of organic matter was induced by inoculation of composting piles.
- More simple compounds were released from polymeric fractions in inoculated piles.
- Humification was more intense and earlier achieved in inoculated piles.
- Inoculation clearly stimulated growth and activity of the composting microbiota.
- Inoculation affected microbiota structure but its functionality remained unaltered.

GRAPHICAL ABSTRACT



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ABSTRACT

Enhanced organic matter turnover was detected in lignocellulosic composting piles inoculated with microorganisms specifically capable of decomposing polymeric compounds. In comparison to uninoculated piles, the following results were obtained in the inoculated piles: degradation of hemicellulose, cellulose and lignin were 28%, 21% and 25% respectively higher. Total organic matter, total sugars and phenolic compounds also decreased more intensely. Greater amounts of soluble organic carbon, reducing sugars and soluble proteins were available to the composting microbiota. Recycling of organic to inorganic nitrogen was improved and humification was more intense and earlier attained. Microbial community structure was also affected by inoculation. It was initially thought that these effects were due to enzymatic capabilities of inoculants, however, microbial counts, especially those corresponding to functional groups, revealed that inoculation induced a true stimulation of microbial growth and activity in the entire composting microbiota which was actually responsible for all the beneficial effects reported here.

1. Introduction

Composting is the biological decomposition of organic matter under controlled, aerobic conditions into a humus-like, stable product called compost that can be used as an organic amendment. Composting is due to the combined activity of a wide variety of microbial populations, and several physical and chemical factors related to one another govern the succession of the different environmental conditions appearing throughout the process (Moreno et al., 2013). Microbes play the key role in composting (Insam and de Bertoldi, 2007), though their importance has been often underestimated.

Organic matter is composed of readily available compounds that will be immediately used by the composting microbiota, and polymeric organic compounds that need to be enzymatically processed before they can be used as carbon and nitrogen sources by microorganisms. Among the polymers commonly found in

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raw materials for composting, lignocellulosic fractions are the most difficult to degrade and the number of microbial species capable of doing so is certainly low (Insam and de Bertoldi, 2007). The biodegradation of such polymeric materials provides microorganisms with simple, soluble compounds readily metabolizable. Besides, biodegradation of polymeric carbon, especially lignocellulose, is of critical importance for humification and stabilization of final products (Stevenson, 1994). Though indigenous microbiota usually carries out composting successfully, the completion time may become too long, as the rate at which the process proceeds is directly related to the proportion of microorganisms capable of acting upon polymeric materials. Therefore, inoculation with microorganisms capable of making available easily metabolizable compounds could be a useful strategy for enhancing the properties of final products and shortening the time needed to achieve stability (Bolta et al., 2003), though it has to be mentioned that some contradictory results have been reported on the benefits of inoculation (Golueke et al., 1954; Zeng et al., 2010). Inocula for composting are generally composed of one or a few strains with selected enzymatic capabilities (Vargas-García et al., 2005; Sarkar et al., 2010; Zeng et al., 2010), although it stands to reason that microorganisms naturally occurring in compost piles would be the best candidates for this purpose.

In this work, a consortium of enzymatically multi-functional microorganisms isolated from composting piles with the same composition and identically operated as those described here, was employed as inoculant for lignocellulosic waste composting. Differences between control and inoculated piles throughout the process were evaluated. The specific objectives considered were: (i) to examine the influence of inoculation on the structure and functionality of microbial groups during composting, (ii) to analyze the evolution of chemical parameters related to simple carbon and nitrogen fractions throughout the process, in order to identify key time periods at which microbial activity is more demanding and triggers higher organic matter decomposition rates, (iii) to investigate the evolution of polymeric materials, focusing on lignocellulosic fraction degradation and its turnover into simpler compounds readily available for fuelling microbial activities and humic substances formation, and finally (iv) to assess humification in order to establish the extent to and the moment at which maturity and stability are achieved in final products.

2. Methods

2.1. Composting process and sampling strategy

Raw materials used for composting process here described were post-harvest tomato plants (lacking fruits) and pruning pine chips (50:50 w/w). The starting mixture had a C/N ratio ≈ 25. Six identical piles were built, being three of them used as uninoculated control. The second set of three piles was inoculated as specified in Section 2.2. Pile dimensions were 3.0 m length × 1.5 m width × 1.0 m height. Raw materials were ground to a particle size below 30 mm. Piles were aerated at a rate of 7.5–9.0 L kg^{-1} every 4 h in order to maintain oxygen concentration inside the piles above 10–12%. Piles were turned over according to temperature evolution (see arrows in Fig. 1a). The process was considered finished after 20 weeks.

Samples were collected at 6 critical time points driven by temperature changes: day 0 (RM: raw materials mixture), 8th day (IMES: mesophilic stage when temperature was rising), 12th day (THER: thermophilic stage, peak temperature), 14th day (DMES: mesophilic stage when temperature was decreasing), 56th day (COOL: cooling stage) and 136th day (FPR: final product). Composite sampling was employed. Sub-samples were taken from nine different pile locations at each sampling time, thoroughly mixed, weight reduced via the quartile method and split in two fractions, one of which was air-dried at 40 °C for analysis of some parameters. The other fraction was immediately employed for analyses that required fresh samples (see below).

2.2. Microbial consortium, inocula preparation and microbial enumeration

Microorganisms used in this study were isolated during a previous composting process carried out with the same raw materials and under the same conditions described in this work and they were selected because of their enzymatic capabilities (Table 1). The consortium consisted of 4 bacteria and 2 fungi. Inoculation was carried out when the composting piles were turned at 8, 15 and 24 days after starting the process (Fig. 1a). A detailed description of microorganisms employed as inoculants in this work, including the way they were isolated, their enzymatic capabilities and the molecular methods used to identify them, has been recently published (Jurado et al., 2014).

Bacteria were grown in Nutrient Agar plates (Oxoid, UK) for 48 h, while fungal cultures were grown in Rose-Bengal Chloramphenicol Agar plates (Oxoid, UK) for 96 h. Plates were incubated at 30 °C. After incubation, microbial biomass of each strain was collected and suspended in sterile distilled water. The inoculum was prepared by mixing appropriate amounts of each individual suspension. After inoculation, final concentration of each strain in the piles was $\approx 10^6$ cfu (colony forming units or fungal propagules) g^{-1} dw. The suspension mixture (total volume 5–7 L) was spread over and injected at different pile locations to ensure the best possible distribution of the inoculum. The control piles received the same amount of distilled water.

Suitable culture media and incubation temperatures were employed for the isolation and enumeration of major microbial groups in control and inoculated piles throughout composting. Fresh samples (10 g) were suspended in 90 mL sterile saline solution (0.9% NaCl in distilled water) and shaken (150 rpm) at room temperature for 30 min. Then, ten-fold serial dilutions in sterile saline solution were performed and 100 μL volumes from dilutions were spread out over Petri plates with the required culture media. Mesophilic and thermophilic actinobacteria, bacteria and fungi were cultured in Actinomycete Isolation Agar Glycerol (Difco, USA), Nutrient Agar (Cultimed, Spain) and Rose Bengal Chloramphenicol Agar (Cultimed, Spain) respectively. Media were incubated at 30 °C (mesophilic microbiota) or 55 °C (thermophilic microbiota) for 3–4 days (actinobacteria), 2–3 days (bacteria) and 4–7 days (fungi). Microbial functional groups were also enumerated. Amylolytic, pectinolytic, lipolytic, proteolytic, xylanolytic, cellulolytic, ligninolytic, ammonifying and phosphate solubilizing activities were analyzed. Briefly, microbial strains were spread over or inoculated in differential (solid or liquid) culture media in which the enzymatic activity could be evidenced. The exact analytical procedures employed have been recently reported (Jurado et al., 2014).

2.3. Chemical parameters

Several soluble fractions were analyzed in extracts obtained from fresh samples. Soluble organic carbon (SOC) was analyzed using a TOC-VCSN analyzer (Shimadzu, Japan) in an extract 1:4 (w:v) of fresh sample in 0.5 M K_2SO_4 shaken at 200 rpm for 30 min and filtered through filter paper. Reducing sugars (RS) and soluble proteins (SP) were spectrophotometrically analyzed (UV-1800 spectrophotometer, Shimadzu, Japan) in the same extract above indicated, according to the methods described by Somogy (1952) and Herbert et al. (1971) respectively. Phenolic

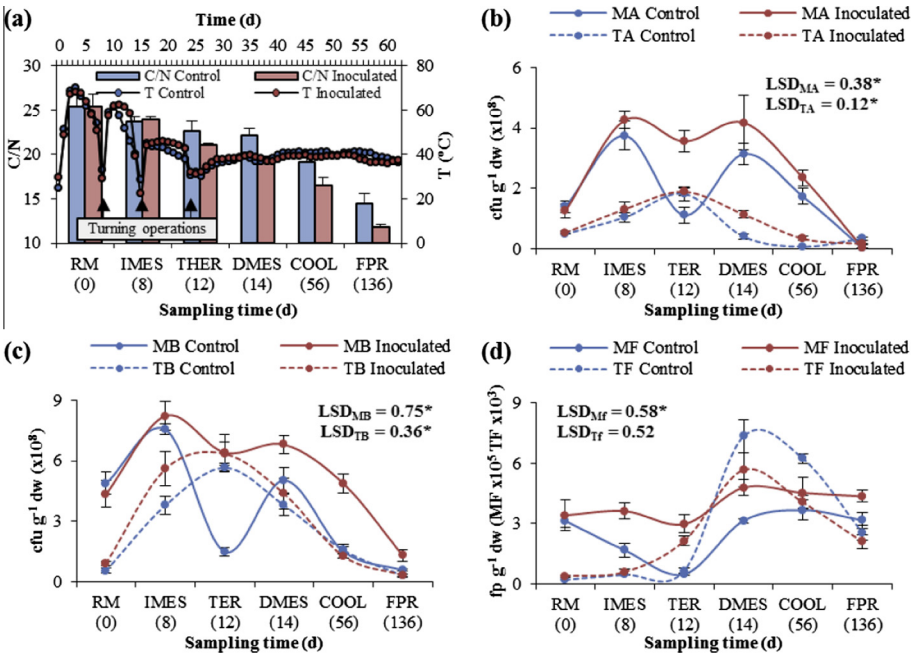


Fig. 1. Evolution of C/N ratio, temperature and major microbial groups: mesophilic actinobacteria (MA), thermophilic actinobacteria (TA), mesophilic bacteria (MB), thermophilic bacteria (TB), mesophilic fungi (MF) and thermophilic fungi (TF), throughout composting in control and inoculated piles. Results are means ($n = 3$) ± SD (vertical bars). LSD* (Fisher's Least Significant Difference, $p < 0.05$).

Table 1
Identity and enzymatic capabilities of the strains used as inoculum.

Strain	Accession number[a]	Enzymatic activities[b]								
		Amy	Pec	Hem	Cel	Lig	Lip	Amm	Pro	Pho
Bacillus altitudinis BM2	KC441789.1	+	+	+	–	–	+	+	+	+
Alternaria tenuissima FM1385	KC329619.1	+	+	+	–	–	+	+	+	–
Gibellulopsis nigrescens FM1397	HE972037.1	+	+	+	+	–	+	+	+	–
Streptomyces albus BM292	JN400097.1	+	–	+	–	+	+	+	–	+
Bacillus licheniformis BT575	HE993550.1	+	+	+	–	–	–	+	+	–
Bacillus smithii AT907	EU652724.1	+	–	–	–	+	–	+	–	–

[a] BLAST (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi).
[b] Amylase (Amy), Pectinase (Pec), Hemicellulase (Hem), Cellulase (Cel), Ligninase (Lig), Lipase (Lip), Protease (Pro), Ammonifying (Amm) and Phosphatase (Pho).

compounds (PhC) were quantified in sodium pyrophosphate extracts from solid samples according to the method described by Marambe and Ando (1992). Ammonium-nitrogen (NH_4^+-N) and nitric-nitrogen (NO_3^--N) were determined in aqueous extracts (5 g fresh sample in 100 mL distilled water after shaking at 200 rpm for 30 min) by Kjeldahl distillation in an Auto-Titration Kjeldahl Distiller Pro-Nitro A (Selecta, Spain). The nitrification index (NI) was calculated as NH_4^+-N/NO_3^--N.

Organic matter (OM) content was assessed by determination of loss on ignition at 550 °C to a constant weight. Total carbon (C) and nitrogen (N) were analyzed by dry combustion at 950 °C using a LecoTruSpec C–N Elemental Analyzer (Leco, USA). For total carbohydrate analysis, dried samples (25 mg) were hydrolyzed with 0.1 mL of 12 M H_2SO_4 for 16 h at room temperature, followed by addition of 2.4 mL of distilled water and heating in boiling water for 8 h. Total sugars (TS) in the hydrolyzate were quantified by the procedure described by Dubois et al. (1956). Cellulose (CEL), hemicellulose (HC) and lignin (LIG) were analyzed in a fiber analyzer Ankom (Ankom Technology, USA). Losses of different OM fractions were calculated from the initial (A_1) and final (A_2) ash contents and the initial (P_1) and final (P_2) fraction concentrations according to the equation of Paredes et al. (2000):

$$\%\text{Loss} = 100 - [100 \times (A_1 \times P_2)/(A_2 \times P_1)]$$

Biodegradation ratios of HC, CEL and LIG were also calculated (Wang et al., 2011):

$$R_n(\%) = [(m_0 - m_n)/m_0] \times 100$$

where R_n is the degrading ratio (%) for the n^{th} sampling, m_0 and m_n are the initial and the n^{th} sampling contents (mg g^{-1}) of lignocellulose (HC, CEL or LIG). In addition, Lignin/Holocellulose ratio (LIG/HOL) was calculated, considering Holocellulose as HC+CEL.

Humic fractions were extracted and analyzed according to Ciavatta et al. (1990). Total extractable carbon (TEC), non-humified carbon (C_{NH}), humic-like carbon (C_{HA}) and fulvic-like carbon (C_{FA}) were assessed by analysis of TOC in the corresponding fraction (TOC-VCSN, Shimadzu, Japan). In addition, some humification indices were calculated: humic polymerization degree (C_{HA}/C_{FA}), percent humic acids (P_{HA}) = (C_{HA}/TEC) × 100 (Iglesias Jiménez and Pérez García, 1992), degree of humification (DH) = (C_{HA} + C_{FA}/TEC) × 100 and humification index (HI) = C_{NH}/C_{HA} + C_{FA} (Ciavatta et al., 1990). Phytotoxicity tests using radish (*Raphanus sativus* L.) seed germination and root elongation were performed in final composts according to Tiquia et al. (1996).

2.4. Statistical analyses

Data obtained were subjected to statistical analysis using Statgraphics Centurion XVI.I (StatPoint Technologies Inc., Virginia). Student-*t* tests, analyses of variance and multiple comparison tests (Fisher's Least Significant Difference, LSD) were performed. Principal Component Analysis (PCA) was used for data reduction and to produce ordination plots. PCA data matrix was based on correlations (eigenvalue = 1).

3. Results and discussion

3.1. Influence of inoculation on microbiota evolution

Characteristic parameters indicating composting evolution followed known general trends. C/N ratio decreased as expected throughout the process (Fig. 1a). At the end of composting, C/N ratio was 53% lower in the inoculated piles in relation to its initial value. This decrease was less intense (43%) in the control piles. In general, temperature inside the piles (Fig. 1a) followed the typical evolution of composting processes subjected to turning operations. The highest temperature (65–70 °C) was recorded at around 24–48 h after the beginning of the process and as expected, thermal reactivation was promoted after turning operations. Higher thermal values were reached and maintained longer in the inoculated piles during the bio-oxidative phase of composting. Since the heat generated inside the piles is due to microbial aerobic metabolism (Insam and de Bertoldi, 2007), it could be expected that these results were a consequence of a higher microbial activity in the inoculated piles. This was confirmed by the evolution of major microbial groups throughout the process (Fig. 1b–d). Microbial growth ran in parallel in both sets of piles at the beginning and the end of the process, however, at the central stages of composting (after inoculation), growth was always significantly greater in the inoculated piles. Only thermophilic fungi counts were not different in control and inoculated piles (Fig. 1d). More specifically, bacterial and actinobacterial counts were greater than those of fungi in both control and inoculated piles all over the process (Fig. 1b–d). Similarly, mesophilic microbiota reached higher counts than thermophilic microorganisms and mesophilic bacteria accounted for the highest counts in both sets of piles. Only thermophilic bacteria and actinobacteria reached the highest counts at the thermophilic stage. Compared to prokaryotic microbiota, mesophilic and thermophilic fungi evolved according to a different trend. In general, fungi were more numerous at the final stages of composting and, in contrast to thermophilic bacteria and actinobacteria, maximum counts for thermophilic fungi were found after the thermophilic stage. Regarding to sampling times, more microorganisms were detected at the bio-oxidative phase, before and after the thermophilic stages. Fluctuations were clearly driven by temperature changes. It has been widely reported that microbial population declines to a large extent during the thermophilic stages of composting (Ryckeboer et al., 2003; Chroni et al., 2009), and so was observed in this work; however, the extent to which microbial counts decreased in the inoculated piles at the thermophilic stages, was much lower. In general, microbial evolution throughout composting was not different to what has been reported elsewhere: more actively growing microorganisms at the beginning, a decrease in counts around the thermophilic phases and a substantial lower number of fungi in comparison to prokaryotic microbiota. However, great differences arise when microbial evolution is compared between control and inoculated piles. Even though trends were very similar, much higher counts of microorganisms were found in the inoculated piles. Once first inoculation was performed, composting microbiota in the inoculated piles started to grow faster. These results suggest a growth stimulation of the composting microbiota due to inoculation and could explain the maintenance of high thermal values for longer time periods in the inoculated piles (Fig. 1a). In fact, due to enzymatic activities of inoculants (Table 1), more easily degradable compounds could have been initially released and made available to composting microbiota, which in turn would be able to reach higher counts and produce longer thermophilic phases. Even so, this interpretation needed to be confirmed by the dynamics of organic matter fractions (see below).

3.2. Inoculum microbial capabilities and turnover of organic matter fractions

Throughout composting, organic matter undergoes several transformations mainly due to microbial activities. Simple (soluble) compounds already present and those released after enzymatic attack of polymeric materials serve as nutrients for microbial growth and activity. Partially decomposed or undegraded polymeric compounds, after chemical and biologically-mediated reactions, end up contributing to the formation of humic-like substances. Microbial enzymatic capabilities of strains selected as inoculants in this work (Table 1) are related to degradation of different kind of macromolecules, all of them part of organic matter of vegetable origin. These extracellular enzymatic systems will act upon polymeric materials, causing at least, their partial decomposition, so an increase of soluble compounds readily available for microbial growth and activity could be expected. The influence of inoculation on the evolution of the most representative organic matter fractions during composting is next described.

3.2.1. Evolution of readily available organic matter fractions

According to Hofman and Dusek (2003) organic matter extracted with 0.5 M K_2SO_4 can be used as a measure of the fraction of total organic matter which is directly available to the composting microbiota. In this work, potassium sulfate extract was used to analyze soluble organic carbon (SOC), reducing sugars (RS) and soluble proteins (SP) along with microbial biomass C (C_{BIO}). Ammonium-N and nitrate-N were also analyzed in order to determine the nitrogen mobilization pattern from solid matrices (mainly proteins). The results are presented as carbon and nitrogen soluble fractions (Fig. 2).

Soluble organic carbon (SOC) and reducing sugars (RS) fractions constitute the primary source of carbon and energy for microbial growth and activity and therefore, they are expected to decrease throughout the process, as previously reported (Zmora-Nahum et al., 2005). In fact, both parameters decreased during composting in control and inoculated piles (Fig. 2a), though this diminution was much more obvious in the inoculated piles. On the other hand, according to the initial hypothesis, after inoculation, it could be expected in the inoculated piles an increased concentration of the soluble fraction resulting from the degrading activities of the inoculants, which would have liberated higher amounts of soluble compounds from the polymeric fractions. However, no increase of soluble compounds could be measured throughout the process. Even so, the results obtained should be cautiously interpreted since they also could highlight the intense microbial depolymerizing activity and the even faster utilization of solubilized carbon compounds accomplished in the inoculated piles during the bio-oxidative phase of composting. Therefore, a correct interpretation of these results will only be made once the evolution of the polymeric fractions is analyzed (Fig. 3). As for C_{BIO}, this parameter provides a direct evidence of the extent to which microbial biomass evolves during the process. Data shown in Fig. 2b served to confirm results previously presented in Fig. 1b–d where most of microbial groups are shown to reach the highest counts at the bio-oxidative stage. In

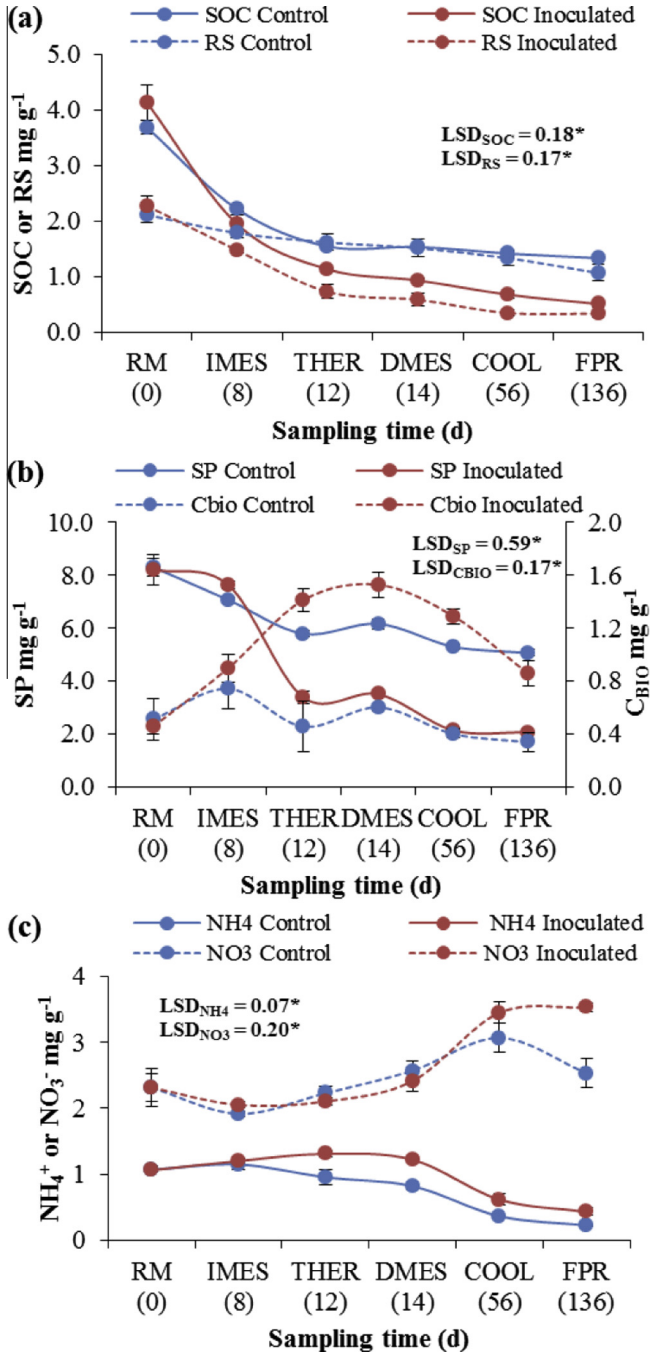


Fig. 2. Evolution of simple soluble compounds and carbon from biomass throughout composting in control and inoculated piles. (a) Soluble organic carbon (SOC) and reducing sugars (RS), (b) soluble proteins (SP) and biomass carbon (C_{BIO}), (c) ammonium nitrogen and nitrate nitrogen. Results are means ($n = 3$) ± SD (vertical bars). LSD* (Fisher's Least Significant Difference, $p < 0.05$).

fact, C_{BIO} increased until the end of the bio-oxidative phase of composting and reached markedly higher values in the inoculated piles.

Nitrogenous fraction comprises insoluble compounds (primarily proteins) and soluble nitrogenous substances coming from proteolysis and ammonification. The resulting ammonium can be directly assimilated into microbial cells (immobilization), though it can also be further oxidized to nitrite and nitrate (nitrification). Ultimately, the soluble nitrogen fraction contains ammonium, nitrite, nitrate, amino acids and small peptides. Proteolytic and ammonifying activities are mainly detected during the active phase of composting whereas nitrification has preferentially been associated to maturation (Guardia et al., 2010). This was the general profile found for the soluble nitrogen fraction during the composting processes developed in this work (Fig. 2b–c). Though the trends were very similar, significant differences were found between control and inoculated piles. According to results shown in Fig. 2c, the amounts of NH_4^+-N in the inoculated piles were initially increased (in comparison to control piles), as a consequence of the ammonifying activity. Soluble proteins concentration in the inoculated piles significantly decreased at the same time (Fig. 2b). Then, some NH_4^+-N could be immobilized, volatilized as NH_3 (during thermophilic phases) or later employed as substrate for nitrification. Regardless of these possibilities, the ammonifying activity seemed to last until final composting stages. In the control piles, ammonifying activity could not counteract immobilization, volatilization and nitrification, so NH_4^+-N concentration rapidly decreased after the thermophilic phase. This trend has been typically observed (Rashad et al., 2010; Lü et al., 2013). Organic nitrogen followed a profile quite similar to SOC and RS (Fig. 2a). Thus, soluble proteins concentration continuously decreased during composting, and this reduction was much more intense in the inoculated piles (Fig. 2b). These results are clearly related to the enhanced microbial community detected in the inoculated piles (Fig. 1b–d; Fig. 2b). Nitrification occurred during composting in both sets of piles (Fig. 2c), although nitrate formation could only be detected after thermophilic stages, when organic matter had significantly decreased and temperatures were lower. Both factors are known to have a negative effect on the first step (nitritation) of

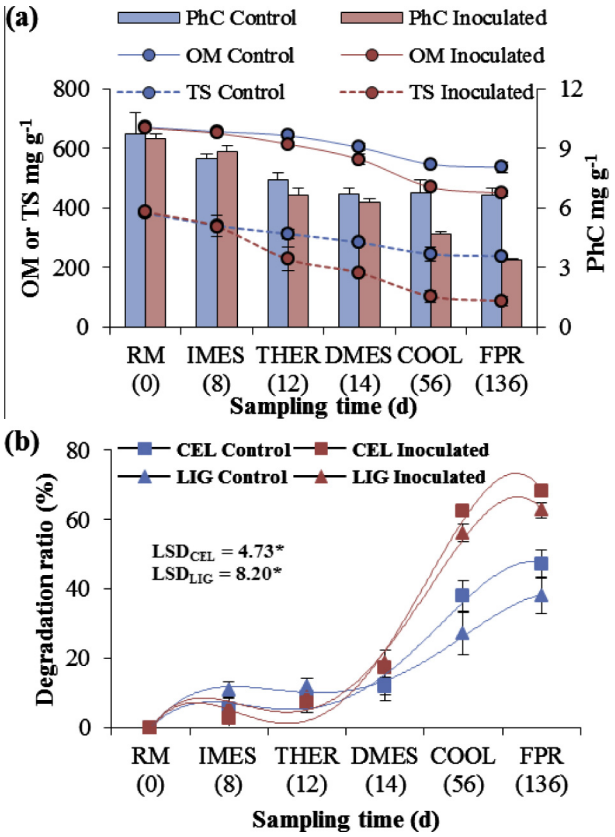


Fig. 3. Evolution of polymeric organic fractions throughout composting in control and inoculated piles. (a) Organic matter (OM), total sugars (TS) and phenolic compounds (PhC), (b) cellulose (CEL) and lignin (LIG) degradation ratio. Results are means ($n = 3$) ± SD (vertical bars). LSD* (Fisher's Least Significant Difference, $p < 0.05$).

ammonium oxidation (Zeng et al., 2013). At the maturation stage, significantly greater amounts of nitrate were measured in the inoculated piles. All these facts support the idea that recycling of organic to inorganic nitrogen was achieved to a greater extent in the inoculated piles.

3.2.2. Evolution of polymeric organic matter fractions

Among the different polymers commonly found in raw materials from vegetal origin, lignocellulose fraction is by far the most difficult to decompose. Following the changes undergone by this fraction throughout composting, can help to understand the extent to which polymeric organic matter is decomposed, providing readily available compounds for microbial growth and activity. Fig. 3 shows the evolution of lignocellulose fractions degradation, along with other related carbon fractions (organic matter, total sugars and phenolic compounds) during composting in control and inoculated piles. Organic matter decreased during composting as expected (Fig. 3a). Most of OM degradation was observed during the bio-oxidative phase in both control and inoculated piles. OM decomposition on an ash-free basis (calculated according to Paredes et al., 2000) was greater than 62% in the inoculated piles, while the corresponding value in the control piles hardly reached 45%. Total sugars (TS) represent a significant fraction of OM including not only soluble mono-, oligo- and polysaccharides but also insoluble polysaccharides such as cellulose (Šafařík and Šantrůčková, 1992). Regarding to phenolic compounds (PhC), they are mainly derived from the degradation of lignin and are used during the latter stages of composting in the formation of humic substances (Stevenson, 1994). The evolution of TS and PhC (Fig. 3a) during composting showed a decreasing trend in both sets of piles, although at the end of the process, the reduction achieved for these parameters in the inoculated piles was more than double what it was obtained in control piles. TS fraction was expected to decrease throughout the process as a consequence of microbial degradation of simple sugars and polymeric carbohydrates. On the contrary, the evolution of phenolic compounds released from the lignin matrix was expected to show an increasing profile at least at the end of the process; however, PhC also showed a decreasing tendency. Probably, as these compounds were released, they were quickly incorporated into the processes of formation of humic substances (Stevenson, 1994) thus preventing their detection to the extent that they were actually produced. In any case, results for OM, TS and PhC evolution (Fig. 3a) were clearly indicative of a more intense microbial activity in the inoculated piles.

Lignocellulose exhibits a great inertia to microbial degradation. Lignin acts as a protective factor for the cellulosic and hemicellulosic fractions and the biodegradation of lignin usually occurs tardily and at a very low rate (Malherbe and Cloete, 2002). The evolution of lignocellulosic fractions throughout composting in control and inoculated piles is illustrated in Fig. 3b. Biodegradation of HC, CEL and LIG achieved in the inoculated piles was always significantly greater than that in the control piles. HC started to be degraded since the beginning of the process in both control and inoculated piles, although degradation was 28% higher in the inoculated piles at the end of composting. HC is the easiest to degrade lignocellulose fraction and it is usually decomposed to a higher extent in comparison to CEL and LIG (Serramiá et al., 2010) which were mainly degraded once the bio-oxidative phase had finished (Fig. 3b). CEL and LIG degradation became more significant only when a substantial amount of HC had been degraded, probably due to the fact that the complex LIG-HC between cellulose fibers decreases the available surface area and prevents the ready access to CEL and LIG by microorganisms and enzymes (Komilis and Ham, 2003). These polymeric fractions are mainly decomposed by thermophilic actinobacteria and fungi (Insam and de Bertoldi, 2007), whose counts (Fig. 1b and d) were the highest coinciding with the maximum degradation ratio of CEL and LIG (Fig. 3b). According to results presented in Fig. 3b, similar profiles were observed, though important quantitative differences for CEL and LIG could be found between control and inoculated piles. In fact, the biodegradation ratios of CEL and LIG in the inoculated piles at the end of the process were respectively 21% and 25% greater than those observed in the control piles. In short, results corresponding to polymeric organic matter degradation showed a more intense depolymerizing activity in the inoculated piles, which supports an active turnover of polymeric carbon into simple soluble compounds available for microbiota.

3.3. Evolution of functionality of microbial communities

According to data presented so far, organic matter in inoculated piles experienced a greater rate of decomposition, which could be proven by high levels of polymeric material that was made available to the microbiota (Figs. 2 and 3). In order to verify a relationship between changes found in the different fractions of organic matter and the microbiota functional composition, the evolution of microbial groups with diverse enzymatic activities was analyzed in control and inoculated piles (Fig. 4). Enzymatic activities exhibited by the strains used as inoculants (Table 1) were investigated.

With the exception of cellulolytic and ligninolytic activities, all functional groups were highly detected all over the process (Fig. 4a–d). Even considering that microbial counts were somewhat lower during thermophilic stages, counts during composting ranged 0.9×10^8–2.25×10^9 cfu g^{-1}. The most abundant group was that of ammonifying microbiota, followed by proteolytic, xylanolytic, amylolytic, lipolytic, pectinolytic and phosphate solubilizing microbial groups. All these groups were more prominent during the bio-oxidative phase of composting. Cellulolytic and ligninolytic microbiota were much less represented. Their counts were found several orders of magnitude below those of other functional groups (counts ranged 10^5–10^6 cfu g^{-1}) and they were mainly detected at final composting stages as it has been previously reported (Wei et al., 2012). In general, microbial evolution profiles were quite similar in both control and inoculated piles, however, significant differences for all microbial groups were found and counts were always higher in the inoculated piles, especially after the first inoculation had been performed (IMES). Besides, total counts of each group throughout the process were significantly greater in the inoculated. A detailed study on the evolution of enzymatic activities during lignocellulosic waste composting has been previously published (López-González et al., 2014). Enzymatic activities of microorganisms used as inoculants are clearly associated to decomposition of polymeric compounds of diverse nature (proteins, lipids and carbohydrates). Thus, counting of microbial functional groups exhibiting these enzymatic activities was performed in order to relate their evolution with the dynamics of organic matter fractions during composting, an objective that was visibly achieved, since a clear relationship could be established between the expression of enzymatic capabilities (Fig. 4) and the resulting diminution of major polymeric organic matter fractions (Fig. 3). Besides, an evident increase of the whole composting microbiota (Fig. 1b–d) was demonstrated throughout the process in the inoculated piles in comparison to control piles. Thus, inoculation gave rise to greater microbial counts and a clear increase in enzymatic activities that produced the decomposition polymeric organic fractions to a higher extent and hence, a higher availability to the microbiota of simple soluble compounds.

According to results shown in Fig. 4, functional groups were maintained throughout composting in both control and inoculated piles; however, microbial counts for each functional group were greater in the inoculated piles. In other words, inoculation did

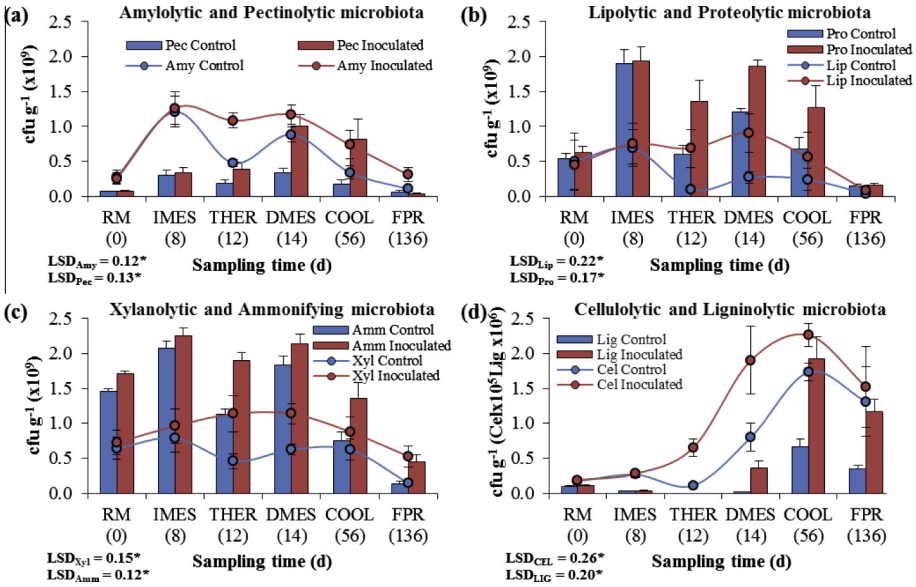


Fig. 4. Evolution of counts (colony forming units, cfu g^{-1}) of different functional microbial groups throughout composting in control and inoculated piles. Results are means ($n = 3$) ± SD (vertical bars). LSD* (Fisher's Least Significant Difference, $p < 0.05$).

not affect the composting microbiota functionality although it clearly influenced the microbial community structure.

On the other hand, differences in counts obtained in control and inoculated piles cannot be explained by the amount of inoculated microorganisms, as counts measured in inoculated piles largely exceeded these quantities and those potentially reached as a result of microbial multiplication. In fact, the effect of the inoculation was not simply the result of adding more microorganisms to the pile, not even assuming that the inoculants could grow at the same rate as the indigenous microbiota; actually, inoculation triggered a true stimulation of the entire composting microbiota. In this sense, the inoculum acted as a microbial catalyst.

3.4. Evolution of humification: achievement of stability and maturity

In addition to humification parameters, other indices are frequently used to assess compost stability and maturity. The ratio between the inorganic forms of nitrogen (NH_4^+/NO_3^-) has traditionally been used to measure the achievement of stability and maturity during the composting process. A NH_4^+/NO_3^- ratio in favor of the oxidized form (value <1) is considered desirable for mature compost whereas a high concentration of NH_4^+-N in final compost indicates instability (Rashad et al., 2010). According to results shown in Fig. 5a the nitrification index was below 1 all over the process in both sets of piles, due to high concentrations of NO_3^--N in raw materials (Fig. 2c). Organic materials, other than horticultural waste, usually contain very low (if any) NO_3^--N concentrations and as the process proceeds, these values increase due to nitrification (Kalemelawa et al., 2012). Nitrogen fertilization is heavily applied in intensive agriculture, so high amounts of nitrate can be measured in this kind of plant waste (Roorda, 1984). Thus, though in this case this index cannot be used to assess maturity, as it always took values below 1, its evolution in both control and inoculated piles showed a decreasing trend according to which final values were 80% lower than those at the beginning of the process. Significant differences were found for this index between both treatments, though similar values were reached at the end of the process.

The achievement of maturity and stability can also be assessed by the evolution of lignocellulosic fractions during the process. LIG/HOL is a very useful stability index which informs about the resistance to biodegradation of organic materials. According to Francou et al. (2008), the greater this ratio is, the more resistant to biodegradation (more stable) is the organic matter. Fig. 5a shows the evolution of LIG/HOL index during composting in control and inoculated piles. In both cases, the index increased throughout the process, indicating the progressive achievement of stability, although a significantly higher LIG/HOL index, and hence, a higher stability degree, was obtained in the inoculated piles. These results complement those shown in Fig. 3b–d.

Humic-like substances have been successfully used to assess compost maturity and stability (Iglesias Jiménez and Pérez García, 1992). The proportion of humic substances is expected to increase in relation to total organic matter throughout composting. Fig. 5b–d shows the evolution of humic substances and some humification indices during composting in control and inoculated piles. Total extractable carbon (TEC) mainly increased after the bio-oxidative stage (Fig. 5b) and its concentration showed to be 25% greater in the inoculated piles at the end of the process (in comparison to control piles). On the contrary, non-humic fraction of TEC (C_{NHS}) significantly decreased, especially in piles subjected to inoculation, where final values were 90% lower than those initially recorded (Fig. 5b). This decrease proved a very intense humification during the process.

Humification indices showed to be adequate in both final products (Fig. 5c–d); however, indices obtained from inoculated compost were numerically closer to those in typically humified and stabilized products. C_{HA}/C_{FA} reflects the degree of polymerization in humic-like materials. According to Iglesias Jiménez and Pérez García, 1992 C_{HA}/C_{FA} values over 1 indicate humification is taking place. The higher the index, the more stable the product is. P_{HA} and DH represent the proportion of C_{HA} or $C_{HA}+C_{FA}$ respectively, in relation to TEC. According to Iglesias Jiménez and Pérez García (1992), P_{HA} values greater than 62% are considered as indicative of an optimal maturation. In regards to DH, Ciavatta et al. (1990) suggested a value of 60% for humified materials such as soils and organic amendments. Finally, the humification index (HI) is based on the assumption that during humification, non-phenolic compounds are decomposed to produce polyphenolic substances (Stevenson, 1994). This index represents the proportion between

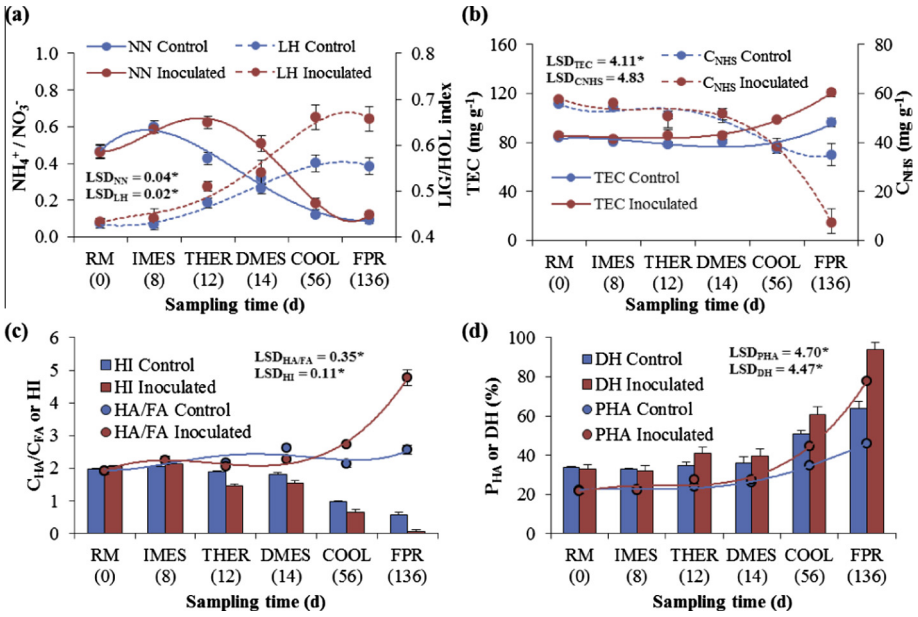


Fig. 5. Evolution of stability and maturation indices throughout composting in control and inoculated piles. (a) Nitrification index and LIG/HOL index, (b) total extractable carbon (TEC) and non-humic carbon C_{NHS}, (c) humic polymerization degree and humification index, (d) percent humic acids and humification degree. Results are means ($n = 3$) ± SD (vertical bars). LSD* (Fisher's Least Significant Difference, $p < 0.05$).

non-humified and humified organic carbon. According to Ciavatta et al. (1990), HI decreases continuously during humification up to values lower than 1 in stabilized products. Control compost showed to be correctly stabilized (only P_{HA} index was below the target value). In regard to inoculated compost, two facts can be highlighted: (a) humification was significantly greater and (b) the maximum degree of humification reached in control piles was achieved much earlier in the inoculated piles (i.e. humification indices attained at 6th sampling time in the control compost were similar to those reached at 5th sampling time in the inoculated compost), so humification was not only more intense in the inoculated piles, but it was also earlier achieved.

To exclude potential toxicity in the obtained products, phytotoxicity tests (germination indices, GI) were determined as a reliable indirect quantification of compost maturity. Both composts were found as phytotoxin-free, (GI values 84.8% and 92.12% for control and inoculated composts respectively), and hence, safe for soil application.

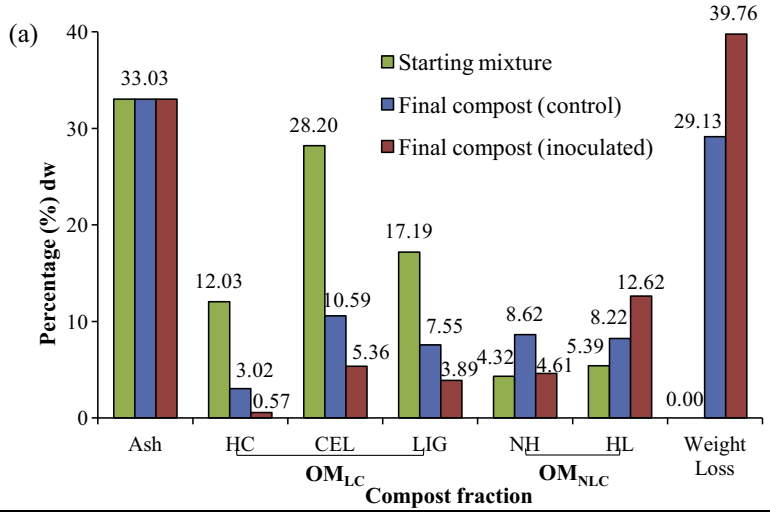
3.5. Overall analysis of results

The results previously presented show that the inoculation pattern employed in this work induced a significant increase in the biochemical capabilities of composting microbiota (Fig. 4), which directly resulted in greater microbial counts (Fig. 1b–d) as a consequence of the availability of readily metabolizable substrates (Fig. 2) released from polymeric organic matter (Fig. 3). Humification was more intense and earlier achieved in inoculated final composts, which also exhibited a higher degree of stability and maturation (Fig. 5), and demonstrated to be safe for agronomical purposes.

In addition to this, the specific effect due to this enhanced microbial activity on different organic matter fractions, could be quantitatively evaluated (Fig. 6a). This figure shows the values of different organic fractions in raw materials and final products from control and inoculated piles, as well as the percentage of change reached in relation to initial values in both final products. Lignocellulosic organic matter (HC, CEL and LIG) decreased throughout the process, being this reduction significantly greater in the inoculated piles. Non-lignocellulosic organic matter includes humic-like and non-humic-like substances. Humification was clearly higher in the inoculated piles. Non-humic materials increased during composting in the control piles whereas in the inoculated piles they maintained similar values to those in raw materials. This fraction would include degraded materials that have not been used by the microbiota nor have been converted into humic substances yet. Their accumulation in final products is certainly indicative of a less active microbiota. As a result of organic matter biodegradation, a substantial weight loss could be measured in both control and inoculated piles. In this sense, weight losses of 29.13% and 39.76% in control and inoculated piles were respectively recorded.

Finally, a principal component analysis was performed in order to identify those factors that allow distinguishing between samples from control and inoculated piles. Fig. 6b shows a biplot in which factors and cases (sampling times in control and inoculated piles) are included. The two first components accounted for 89.60% of the variability in the original data. C_{Bio} and NH_4/NO_3 were the most prominent factors though CEL, LIG, HC, SOC, RS, TS and SP were also very influential. On the other hand, sampling times were grouped according to principal components in three clearly distinguished clusters. Group I included samplings 1 and 2 for both control and inoculated piles. Groups II and III clustered together samplings 3, 4, 5 and 6 for control and inoculated piles respectively. These results show again that after inoculation, samples become clearly different in control and inoculated piles.

Inoculation of compost piles has been controversial since the very first investigations conducted by Golueke et al. (1954), who claimed that the external addition of microorganisms could be superfluous because the prevailing environmental conditions (especially nutrients availability) of compost piles determine the rate of microbial growth and hence, the amount and activity of microbes will be conditioned by the environment inside the piles. Prior statements are rigorously true; however, when the introduction of microorganisms in compost piles involves substantial changes in the prevailing environmental conditions, the effect may not be superfluous at all. In the present work, microorganisms introduced into the piles may actually be considered as bio-catalysts. Their activity initially makes available to the



Change (%) respect to initial values (ash-free basis)							
	Ash	OM_{LC}			OM_{NLC}		Weight
		HC	CEL	LIG	NH	HL	
Control	0.00	-74.87	-62.46	-56.07	99.50	52.35	-29.13
Inoculated	0.00	-95.34	-80.80	-77.54	15.90	134.02	-39.76

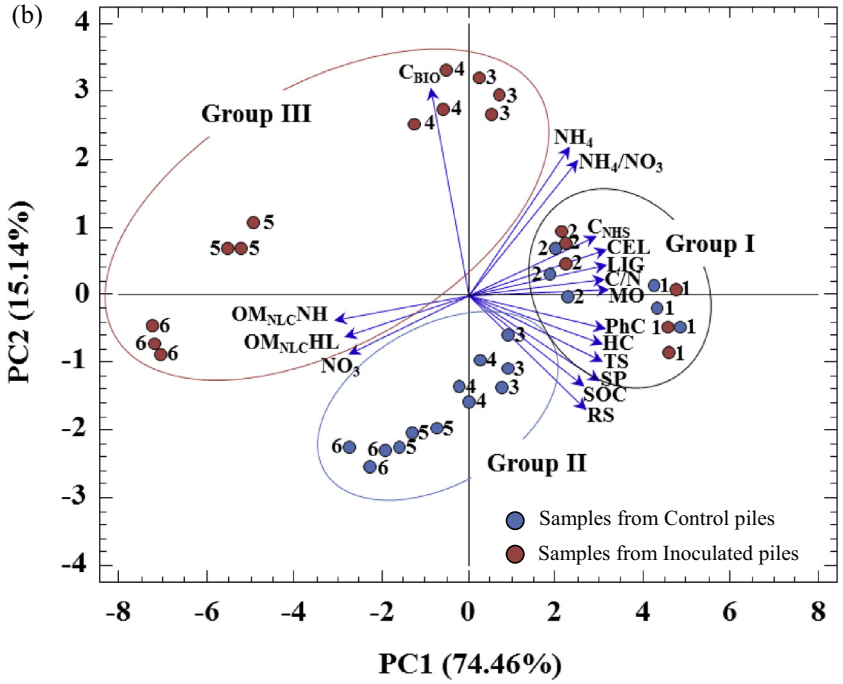


Fig. 6. (a) Effect of inoculation on different fractions of organic matter after composting and percent of change respect to initial values (ash-free basis). OM_{LC}: Lignocellulosic organic matter. OM_{NLC}: Non-lignocellulosic organic matter (NH: non-humic; HL: Humic-like). (b) Principal component analysis of data showing a biplot of variable loadings and sample distribution according to the first two components.

microbiota greater amounts of easily metabolizable nutrients, triggering an increase in the amount and biological activity of the whole composting microbiota, which effectively is conditioned by an enhanced nutritional environment radically different from that in which the initial involvement of inoculants is avoided. Furthermore, the selection of inoculants from those microorganisms naturally found among the composting microbiota seemed to be also very suitable. These microorganisms are fully capable of acting upon certain complex nutrients and they are perfectly adapted to the specific conditions of the process.

The practical application of the inoculation protocol presented here should not pose methodological difficulties as it is not operationally different from other similar protocols widely used today. Furthermore, as inoculation produces a true stimulation of the amount and activity of the composting microbiota itself, once provided the adequate environmental conditions, the expected effects

may only result in the generation of better quality products in shorter periods of time.

4. Conclusions

As a result of inoculating composting piles with a consortium of microorganisms exhibiting enzymatic capabilities related to decomposition of polymeric carbon, turnover of organic matter fractions experienced a very clear dynamic effect. Higher amounts of readily available substrates for microbial growth and activity were provided from an accelerated decomposition of polymeric fractions. Faster rates of humification and greater stability in final compost were also achieved. Microbiological analyses showed that inoculation prompted a prominent stimulation of the composting microbiota, which actually was responsible for all the above effects. Finally, inoculation clearly influenced the microbial community structure though its functionality remained unchanged.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2015.03.059.

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