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Dynamics of bacterial microbiota during lignocellulosic waste composting: Studies upon its structure, functionality and biodiversity

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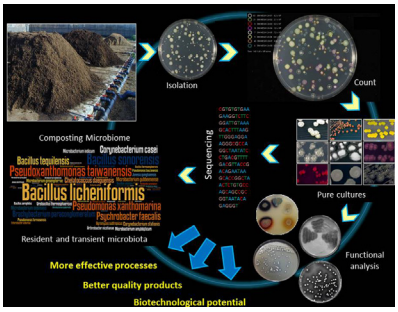
HIGHLIGHTS

- Resident and transient components of the composting microbiome were identified.
- Enzymatic decomposition of polymeric materials increased as the process progressed.
- A large fraction of composting microbiota was enzymatically multi-functional.
- Composting bacterial community proved to be thermotolerant.
- Central stages of composting shared a large proportion of bacterial species.

GRAPHICAL ABSTRACT



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ABSTRACT

An intensive isolation program carried out in three replicated composting piles allowed the identification of the resident and transient components of the composting microbiome. More than 4000 bacterial strains were isolated, enzymatically characterized and identified by partial sequencing of their 16S rRNA gene. While microorganisms isolated under mesophilic conditions were prominent throughout the process, thermophilic stages gathered the highest total counts and spore-forming bacteria prevailed at the bio-oxidative phase of composting. Enzymatic capabilities related to the degradation of polymeric materials were exhibited by most of the isolates and as a result of these activities, more soluble compounds could be made available to the entire composting microbiota. A high proportion of isolates showed to be thermotolerant as they were detected at mesophilic and thermophilic phases. Isolated strains belonged to 187 bacterial species. Biodiversity was greater at the central stages of composting and mesophilic, thermophilic and cooling phases shared 50% of species.

1. Introduction

Composting is the biological decomposition of organic matter under controlled, aerobic conditions into a humus-like, stable product called compost. From an ecological point of view, composting is a dynamic process due to the combined activity of a wide variety of microbial populations, including bacteria, actinobacteria and fungi, which are linked to consecutive environmental conditions (Moreno et al., 2013). Various physical and chemical factors related to one another determine the succession of the different environmental conditions appearing throughout the composting process (nutrients availability and composition of starting materials, temperature, oxygen concentration, moisture, pH, particle size, etc.). During composting, these parameters need to be conveniently adjusted and controlled so that microorganisms can find the best conditions to develop their biological activities. Thus, microbes play key roles in all the events related to the biotransformation of organic substrates, being bacteria those more influential due to their metabolic versatility. Therefore, an

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understanding of the bacterial community dynamics would be useful for the effective management and improvement of the efficiency of composting processes (Brown et al., 2008).

Two main protocols have been employed to date for the investigation of composting microbial communities. Culture-dependent approaches have been widely used (Ryckeboer et al., 2003b; Chandna et al., 2013) although very little is still known about community structure at different stages of the process. On the other hand, culture-independent methods based on the analysis of DNA directly extracted from environmental samples have allowed the identification of unknown sequences potentially ascribable to new taxa (Partanen et al., 2010; de Gannes et al., 2013; Franke-Whittle et al., 2014). There is no doubt that molecular methods, especially those related to metagenomics, are increasingly used, however, their application to multifaceted environmental samples such as those from composting processes might still entail some drawbacks (Bent and Forney, 2008; Chandna et al., 2013). Besides, studies aimed at establishing relationships between phylogeny and metabolic diversity, or the potential exploitation of any biological capacity require the availability of microbial cultures.

Composting microbiota has traditionally been related to temperature fluctuations inside the composting pile. Thus, a wide variety of mesophilic and thermophilic microbes have been associated to composting (Ryckeboer et al., 2003b). Lately, using metagenomic techniques, some authors (Hultman et al., 2010; Partanen et al., 2010; de Gannes et al., 2013) have analyzed samples of compost and have shown a huge variety of microorganisms, some of which had not been previously related to composting.

This study was essentially focused to microbiological aspects of lignocellulosic waste composting. Both culture-dependent methods and molecular techniques were employed.

The specific objectives considered were: (i) to analyze mesophilic and thermophilic bacteria and actinobacteria through an intensive sampling protocol in order to establish the community structure at different composting stages, (ii) to evaluate the biological capabilities of the bacterial microbiota associated to composting in relation to organic matter degradation, in order to establish functional groups throughout the process, (iii) to identify the bacterial and actinobacterial community members and finally, (iv) to asses biodiversity of bacterial microbiota throughout the process.

2. Methods

2.1. Composting process and sampling strategy

Composting process was carried out using post-harvest tomato plants (lacking fruits) and pruning pine chips (50:50 w/w). The starting mixture had an appropriate C/N ratio (around 25). Three identical piles were built. Pile dimensions were 3.0 m length × 1.5 m width × 1.0 m height. Raw materials were ground, so particle size in the starting mixture was below 30 mm. Piles were subjected to forced aeration 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%. Moisture content was kept between 45% and 55% by watering when needed. Piles were turned over according to temperature values (Fig. 1a). The process was considered finished after 189 days. Self-heating tests (TMECC, 2002) were performed to confirm maturity. Samples were collected at 19 sampling times that were selected according to temperature evolution (Fig. 1a and Supplementary Table S1). Composite sampling was employed. Sub-samples were taken from nine different pile locations at each sampling time as follows: 3 surface samples (1–10 cm depth), 3 samples at 45 cm depth and 3 samples at 90 cm depth. Sub-samples were thoroughly mixed, weight reduced via the quartile method and split in two fractions, one of which was air-dried at

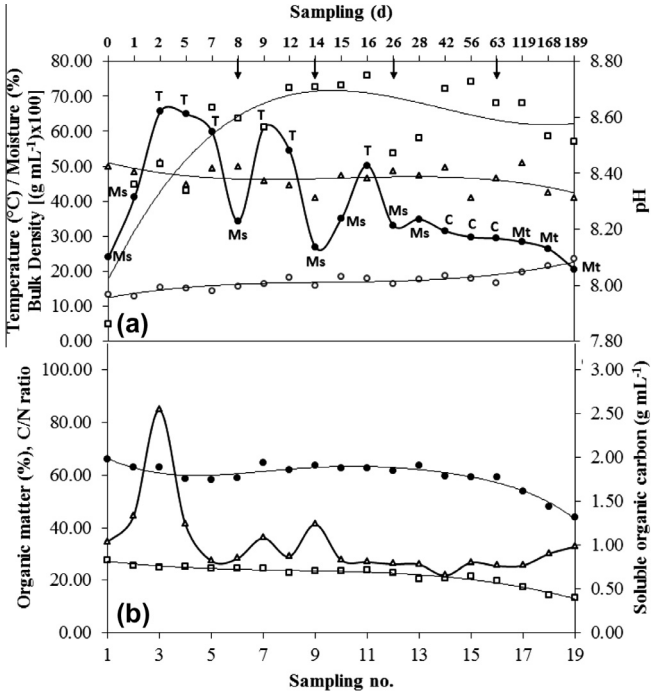


Fig. 1. Evolution of selected physical and chemical parameters throughout the composting process (adapted from López-González et al. (2013)). (a) Temperature (●), moisture (△), bulk density (○), pH (□). (b) Organic matter (●), C/N ratio (□), soluble organic carbon (△). Stages of composting: mesophilic (Ms), thermophilic (T), cooling (C) and maturation (Mt). Arrows indicate turning operations. All results are means (n = 3 piles).

40 °C for most analytical parameters, while the other one was immediately used for soluble organic carbon (see below) and microbiota analyses. Some standard physical and chemical analyses were performed in order to assess the correct evolution of the process. The process was monitored on-site for temperature with long-handled (50 cm) thermometer PT100 probes connected to a data logger. Besides, pH, moisture, bulk density, organic matter, C/N and soluble organic carbon were determined at every sampling time. Analytical procedures employed can be accessed in a detailed study on the physical and chemical aspects of the composting assays here described that has been recently published (López-González et al., 2013).

2.2. Isolation and enumeration of bacterial strains

Suitable culture media and incubation temperatures were employed for the isolation and enumeration of strains. Mesophilic (MA) and thermophilic (TA) actinobacteria were cultured in Actinomycete Isolation Agar Glycerol (Difco, Becton, Dickinson & Co., MD, USA) and incubated at 30 or 55 °C respectively for 4–5 days. Mesophilic (MB) and thermophilic (TB) bacteria were cultured in standard Nutrient Agar (Cultimed, Spain) at 30 or 55 °C, respectively for 2–3 days. 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, tenfold 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. Counts were expressed as colony forming units per gram of sample dry weight (cfu g^{-1}). After incubation, plates were photographed (Canon EOS 450D camera, lens Zoom EF-S18-55 mm f/3.5–5.6 IS). Each different colonial type (according to size, morphology, pigmentation and texture) in each plate, was separately counted and then, transferred to a new plate with fresh

medium. Plates were incubated (same times and temperatures as before indicated), checked for purity and stored at 4 °C (working pure cultures) or preserved in cryoballs Cryoinstant (Deltalab, Spain) for long-term conservation. Pure cultures were also photographed and all morphotypes were compared. In addition, microscopic observations, Gram and spore stains, catalase and oxidase tests were performed in order to eliminate repeated isolates.

2.3. Analysis of functional groups

Bacterial and actinobacterial isolates were tested for some enzymatic capabilities related to organic matter biodegradation. Amylolytic, pectinolytic, lipolytic, proteolytic, xylanolytic, cellulolytic, ligninolytic, ammonifying and phosphate solubilizing activities were analyzed. Briefly, 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). Since bacterial and actinobacterial counts had been previously determined, enzymatic activities could be quantitatively expressed as cfu of isolate/s showing a given enzymatic capacity g^{-1} sample dw.

2.4. Identification of isolates

The identities of specific isolates were determined based on partial or nearly full length 16S rRNA gene sequence analysis. For genomic DNA extraction, freshly grown colonies were suspended in 500 μL of sterile milliQ water, heated at 97 °C for 5 min and then chilled in an ice bath for 5 min. The 1/10 diluted supernatant was used as template. The amplification was carried out using MyCycler thermal cycler (Bio-Rad, CA, USA). Universal primers were used: 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Protocols for DNA amplification, cleaning and sequencing have been previously published (Jurado et al., 2014). The forward and reverse sequences were edited, assembled and aligned using the following bioinformatic tools: Sequence Scanner v1.0 (Applied Biosystem, Life Sciences, Thermo Fisher Scientific, MA, USA), Clustal X v2.0.11, MEGA v6.06, and Reverse Complement (http://www.bioinformatics.org/sms/rev_comp.html). The Ribosomal Database Project RDP's Classifier utility was used for the initial classification of strains up to the genus level (confidence threshold 80%) (Wang et al., 2007). The partial or nearly full length sequences were compared for similar nucleotide sequences with BLAST-NCBI search utility (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the Identify utility at the EzTaxon server http://www.ezbiocloud.net/eztaxon/identify.

2.5. Assessment of bacterial diversity

Several indices were calculated from the number of identified species and their counts in the three replicated piles used in this study throughout sampling times: Specific richness (S), evenness (Shannon-Wiener index, H'), equity (Pielou index, J'), dominance (Simpson index, D) and similarity (Sørensen index, I_S). *Formulae* used for these calculations were (Magurran, 1988):

Shannon–Wiener Index (H') = $-\Sigma p_i \log_2 p_i$
Pielou Index (J') = H'/H'_{max} being $H'_{max} = \log_2 S$
Simpson Index (D) = $\Sigma(p_i)^2$
Sørensen Index (I_S) = $2c/(a+b)$

where S is number of species, p_i represents n_i/N (being n_i the counts of the ith species and $N = \Sigma n$), a is the number of species appearing in sample 1, b is the number of species appearing in sample 2 and c, the number of species shared by samples 1 and 2.

2.6. Statistical analyses

Data obtained were subjected to statistical analysis using Statgraphics Centurion XVI.I (StatPoint Technologies Inc., Virginia). Analyses of variance (ANOVA) and multiple comparison tests (Tukey HSD Honest Significant Difference) were performed to compare mean values for the different factors and variables analyzed ($p < 0.05$). Principal Component Analysis (PCA) was used for data reduction and to produce ordination plots. PCA data matrix for counts of isolates (cfu g^{-1}) was standardized based on covariances. An eigenvalue = 1 was selected.

3. Results and discussion

3.1. Evolution of process monitoring parameters

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. As it was expected, a thermal reactivation was promoted after turning operations. Changes in temperature are crucial to describe the microbial activity during the entire process and achieve the organic material stability (Moreno et al., 2013). Moisture was maintained around 50% throughout the process ensuring a water activity value high enough not to restrict biological reactions and, jointly with forced aeration and porosity, allowed a correct oxygen availability. Bulk density slightly increased since starting particle size was quite small (30 mm) and pH evolved in the range 7.8–8.6. In regards to chemical parameters (Fig. 1b), they changed as expected: organic matter and C/N decreased throughout the process. Especially noticeable was the soluble organic carbon profile. A noteworthy increase of soluble organic carbon concentration could be observed nearby the thermophilic stages, followed by a quick depletion. This fact highlights the intense microbial depolymerizing activity and the even faster utilization of solubilized carbon compounds during the bio-oxidative phase of composting.

3.2. Overview of microbiological analyses

Three replicated composting piles were subjected to an intensive sampling program for the isolation and identification of bacteria and actinobacteria. Nineteen samplings throughout 6 months allowed the isolation of strains that finally constituted the collection with which the study was conducted. At each sampling time, samples were processed following four isolation programs (MA, TA, MB and TB; see Section 2.2 for detail). Once the 19 samplings had been completed, 4110 colonial morphotypes were observed. Most of them could be obtained in pure culture and used thereafter. However, a significant proportion of strains (774 ≈ 18.8%) were lost when transferred from the original isolation plate to a new one in order to obtain pure cultures, or after a second transfer. Thus, the strain collection consisted of 3336 isolates (≈81.2% out of all morphotypes originally observed). An interesting point raises from the fact that almost 800 isolates failed to grow after 1–2 transfers to fresh medium. It is obvious that those strains were not unculturable since they initially grew from a diluted suspension of the compost sample in a culture medium. According to Stewart (2012), nutrients, pH, osmotic conditions, the presence of other microorganisms, etc., are factors that can only be found in the original isolation medium. In the absence of these factors, some strains ended losing their ability to grow.

Pure cultures obtained were then compared to each other in order to pinpoint replicated strains. Simple morphological (at both macro- and microscopic levels) and biochemical tests led to the

selection of 1194 strains apparently unique from a fairly basic phenotypic point of view. These strains were called type strains being the remaining isolates (2142) considered equal to any of them. Type strains were studied for their enzymatic capabilities by growing them in differential media properly formulated and identified by molecular methods. A schematic flow diagram illustrating an overview of all microbiological analyses preformed is shown in Supplementary Fig. S1.

3.3. Bacterial community structure

The number of isolates and their counts were available for each isolation program (MA, TA, MB, TB) once all the 19 samplings had been carried out (Fig. 2). In general, more isolates were detected around the thermophilic stages and especially at the cooling-maturation phases (Fig. 2a). The number of isolates was significantly different due to sampling time and isolation program, however, no significant differences were found between the three replicated piles. Thermophilic bacteria (TB) accounted for the highest number of isolates, followed by mesophilic actinobacteria (MA), mesophilic bacteria (MB) and thermophilic actinobacteria (TA). Results presented in Fig. 2a correspond to original unprocessed data. At that time, there was not absolute certainty that microorganisms grown on MA and TA plates were actinobacteria in all cases and those grown on MB and TB plates were exclusively bacteria. Selective isolation procedures for actinobacteria are based on two main approaches: nutritional selection and antimicrobial inhibition (Hirsch and Christensen, 1983), however, up to date neither has demonstrated to be strictly selective and isolation of actinobacteria is still linked to subjective decision. Thus, some bacteria grew on culture media intended for actinobacteria isolation and *vice versa*. On the other hand, simple observation of morphotypes proved not to be efficient enough to reach a fair level of discrimination. These facts could be confirmed once the isolates were identified. Fig. 2b shows the same microbial groups rearranged in view of the true identity of isolates. A significant number

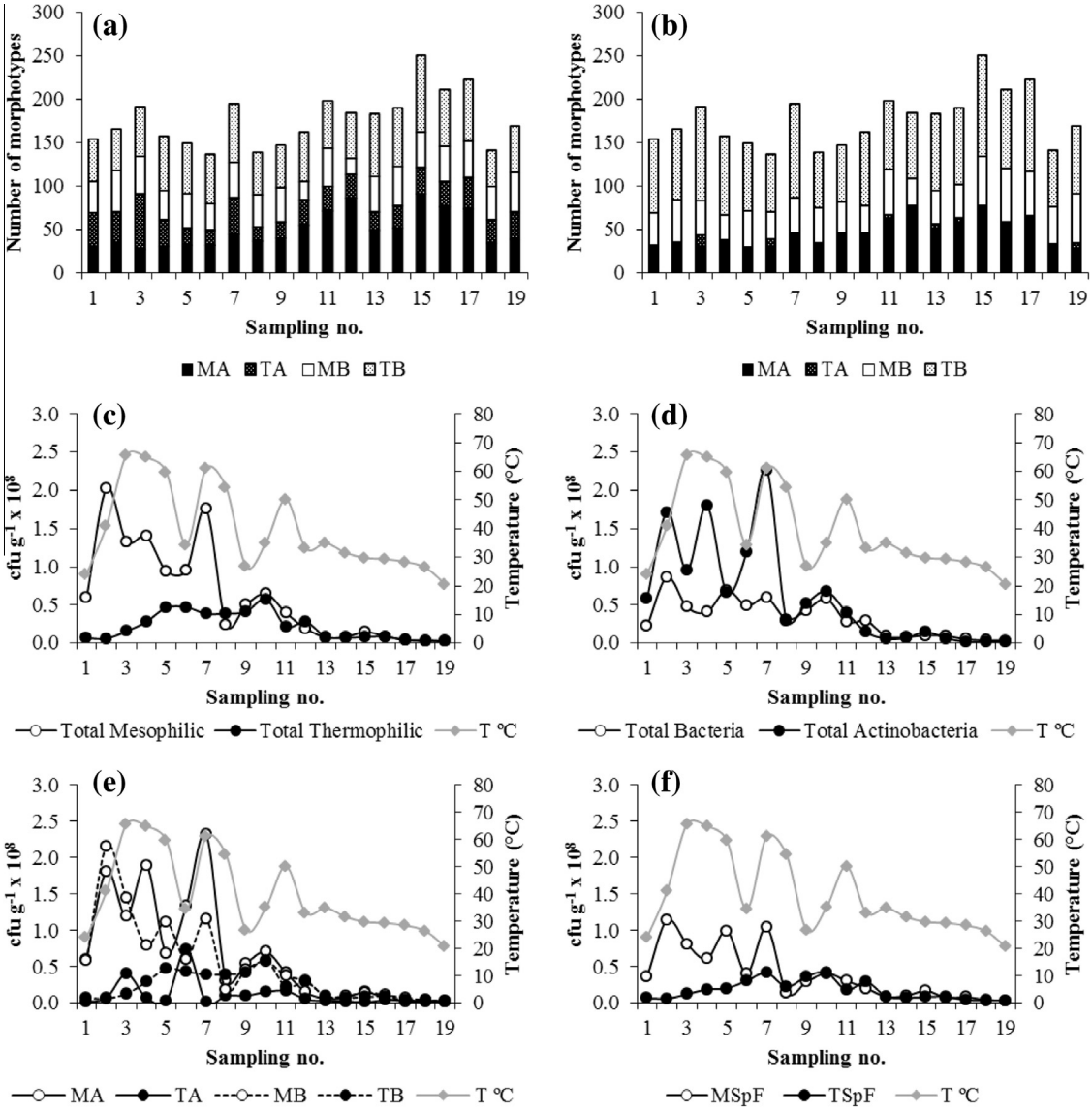


Fig. 2. Evolution of the number of morphotypes and counts (cfu g^{-1} dw) throughout composting. (a) Number of morphotypes isolated according to the isolation program: mesophilic actinobacteria (MA), thermophilic actinobacteria (TA), mesophilic bacteria (MB) and thermophilic bacteria (TB), (b) number of morphotypes once identified, that is, really belonging to each group (MA, TA, MB and TB), (c) counts of mesophilic and thermophilic bacterial microbiota, (d) counts of actinobacterial and bacterial isolates, (e) counts of isolates according to the isolation program, (f) counts of mesophilic and thermophilic spore-forming bacteria (MSpF and TSpF). All results are means (n = 3 piles).

of morphotypes initially considered actinobacteria were actually bacteria and thermophilic actinobacteria almost disappeared in favor of thermophilic bacteria.

Evolution of total counts (cfu g^{-1}) is shown in Fig. 2c–e. Results are grouped in function of different criteria to ease the observation of trends. Piles mean temperature is also depicted. Finally, Fig. 2f shows the evolution of endospore-forming (Spf) bacteria in order to correlate its presence to bacterial prevalence throughout thermophilic stages as pointed out elsewhere (Chroni et al., 2009). Significant differences between levels for sampling time and for isolation program were found in all cases. In order to summarize data presented in Fig. 2c–f, several facts could be highlighted: (i) total counts of bacteria and actinobacteria were higher at mesophilic and thermophilic stages, being mesophilic bacteria the most abundant group, (ii) thermophilic phases of composting gathered higher total counts, around 4–6 times greater than at the end of the process, (iii) bacteria and actinobacteria isolated in mesophilic isolation programs were more abundant throughout the process in comparison to those isolated on plates from thermophilic isolation programs and (iv) mesophilic SpF bacteria prevailed throughout the process, especially during the bio-oxidative phase of composting.

Even though the generated heat in a composting pile occurs in a gradient inside out and not all the pile zones are exposed to the same thermal values, it has been widely reported that microbial population declines to a large extent during the composting thermophilic stages (Ryckeboer et al., 2003a; Chroni et al., 2009). Thus, most microorganisms are supposed to disappear when high temperatures are reached inside the composting pile. Only thermophilic and endospore-forming microbiota would survive at peak temperatures. After turning operations or once the process had entered the cooling phase, recolonization by mesophilic microbiota will proceed and thermophilic microorganisms will definitely disappear from the composting pile. According to results presented here, microbial evolution throughout composting is a much more dynamic process, obviously conditioned by temperature and other nutritional and ambient factors. However, temperature did not result as deleterious as reported (Insam and de Bertoldi, 2007). Microorganisms seemed not to lose their capacity to grow. When temperature reached certain values (higher than 50 or 60 °C), actively growing microorganisms slowed down their growth rate or directly stopped growing but, most of them showed the ability to survive as thermotolerant, and as soon as temperature declined, microorganisms started to actively grow again. Therefore, it could be more appropriate to consider that a significant proportion of microorganisms growing throughout the bio-oxidative phase of composting are actually thermotolerant.

In regards to recolonization of composting piles by mesophilic microbiota at the cooling phase (Insam and de Bertoldi, 2007), data obtained throughout this study showed that was not the case. Once the bio-oxidative phase of composting has finished, high temperatures will not hamper recolonization by mesophilic biota, however, easily degradable nutrients will have been exhausted and only those microorganisms able to degrade polymeric carbon will survive under these circumstances. Therefore, an intensive mesophilic recolonization at the cooling stage will be as successful as easily degradable nutrients are available and that would prove a deficient or uncompleted bio-oxidative phase, which in turn can happen often.

3.4. Bacterial functional groups

A total of 1194 strains were investigated for their enzymatic capabilities (Fig. 3). The evolution of strains showing different

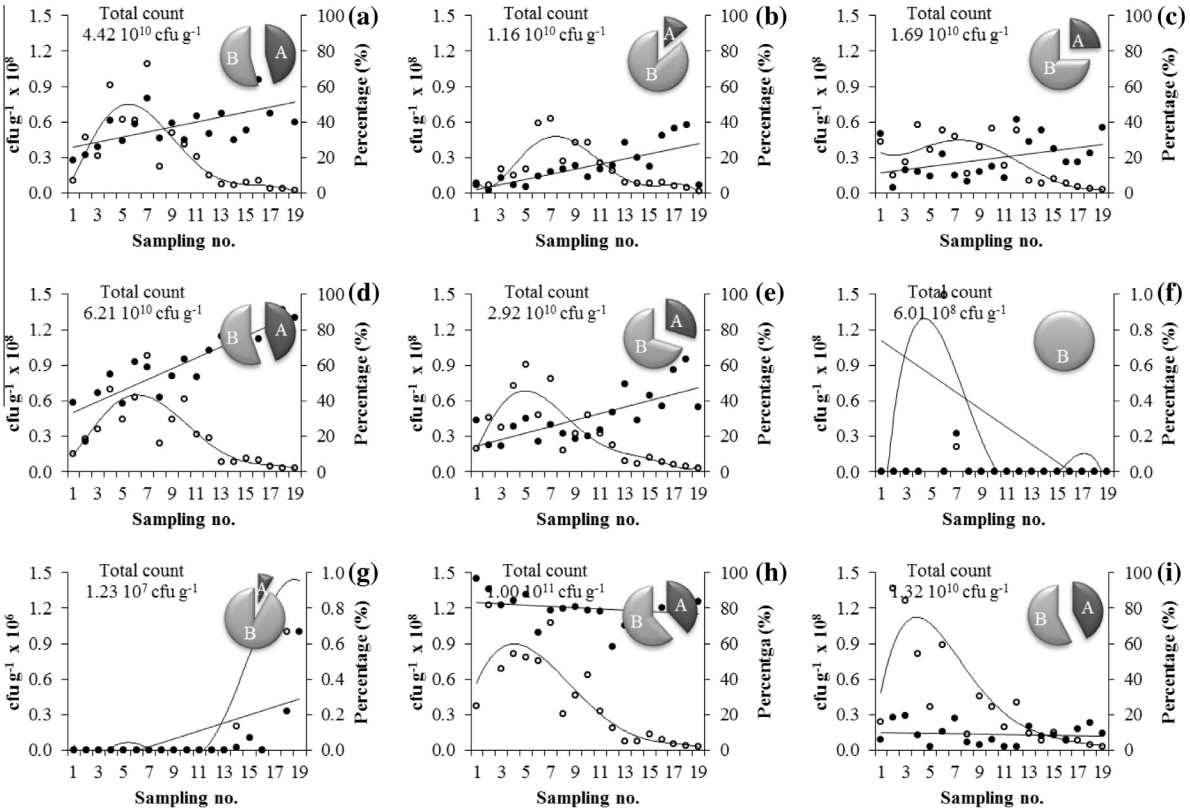


Fig. 3. Evolution of different bacterial enzymatic activities throughout composting. (a) Amylolytic, (b) pectinolytic, (c) lipolytic, (d) xylanolytic, (e) proteolytic, (f) cellulolytic, (g) ligninolytic, (h) ammonifying, (i) phosphate solubilizing activities. Counts (cfu g^{-1}) for each enzymatic activity (○), percentage of counts of isolates for each enzymatic activity in relation to total counts (●). Bacteria (B), actinobacteria (A).

enzymatic activities followed similar patterns as those for total bacteria and actinobacteria previously presented in Fig. 2. In general, enzymatic activities were better represented at the bio-oxidative phase of composting, however, most activities became more important (higher percentage of positive strains in relation to total counts) as the process progressed. Seven out of the nine activities studied were related to polymeric carbon degradation. Enzymatic activities such as cellulolysis and ligninolysis showed to be poorly distributed among the microbiota studied. These capabilities are more characteristically ascribed to fungi (Malherbe and Cloete, 2002; Mohammad et al., 2012) and although some prokaryotic isolates showed to be positive for these activities, their relative presence was very scarce. In fact, the contribution of bacteria and actinobacteria to lignocellulose degradation is restricted to secondary processes associated to the transformation of monomeric or oligomeric compounds released from primary degradation actions (Vikman et al., 2002). In addition, many lignocellulolytic microorganisms do not always express these enzymatic capabilities (Schneider et al., 2012). Cellulolytic strains (Fig. 3f) appeared around the second thermophilic phase and ligninolytic strains (Fig. 3g) were more numerous at the end of the process. Pectinolytic (Fig. 3b) and lipolytic strains (Fig. 3c) were moderately present at the bio-oxidative phase and gained a slight prominence at the final stages of composting. Most microorganisms showing those activities were bacteria. The best represented enzymatic activities were amylolysis, proteolysis and xylanolysis. High numbers of microbial cells showed those capabilities at the bio-oxidative phase and they became more prominent as process progressed. More than 46% of isolated strains were amylolytic (35.6% of total counts cfu g^{-1}). Bacteria have shown to have a major role in amylolysis during composting (Pascon et al., 2011) and so was observed in the present study (Fig. 3a). More amylolytic cells grew at thermophilic stages. This correlation to high temperatures has been reported by other authors (Simujide et al., 2013). Proteolytic activity (Fig. 3d) was the best represented among polymeric carbon degradation activities (6.21×10^{10} cfu g^{-1}) with maximum peaks at the thermophilic stages. This high proportion of proteolytic strains (67% of isolated strains accounting for 50% of total counts) might be related to the accumulation of proteins released from dead cells (Chroni et al., 2009). Proteolysis was slightly better represented among bacteria. This activity is closely related to ammonification which was shared by the highest number of microorganisms (1.00×10^{11} cfu g^{-1}) throughout composting (Fig. 3h). Up to 75% of isolated strains were able to transform organic nitrogen into ammonium (or ammonia) accounting for 80.94% of total counts. Ammonium is the preferred nitrogen source for most microorganisms (Geisseler et al., 2010), and ammonification shows the awesome role of ammonifiers in habitats containing high loads of organic nitrogen, such as compost piles. With regards to hemicellulose degraders (Fig. 3e), more than 23% of isolated strains showed this capacity, accounting for 34% of total counts and mostly appearing at the first and second thermophilic stages. Together with cellulose, pectin, proteins and lignin, hemicellulose is part of the plant cell wall (Keegstra, 2010). Contrarily to lignin, this polymer is easily degradable (Pérez et al., 2002) since plant cell wall structure offers a better accessibility to it. Thus, it constitutes an excellent carbon source for xylanolytic microorganisms. Phosphate solubilizing bacteria and actinobacteria (Fig. 3i) represented around 7% of isolated strains and a proportion of 10.6% of total counts. Their activity was more apparent at mesophilic stages and their abundance in the bacterial community slightly decreased as composting progressed. Phosphate solubilizing microorganisms have lately reached a considerable importance due to their implication in the promotion of plant growth (Vassilev et al., 2006).

Finally, it might result interesting to remark that only 82 strains showed to lack all the enzymatic activities studied. These strains theoretically depend on the availability of simple soluble carbon compounds since they do not have tools for using polymeric compounds. As every strain was tracked throughout the process, it could be corroborated that these strains only appeared at the bio-oxidative stage when a very dynamic microbial activity could release soluble compounds from which they could take advantage. On the other hand, 501 strains were positive for five or more activities. This high distribution of biochemical capacities points out the complex nature of composting and the enzymatic need to face such an intricate environment.

3.5. Bacterial identification

16S rRNA gene sequences were compared to similar nucleotide sequences with different bioinformatic tools. Isolates were placed in 4 *phyla*, 6 classes, 12 orders, 31 families, 62 genera and 187 species. Supplementary Table S2 shows the resulting identification obtained up to the genus level. There were 202 unidentified strains (6.06%). When sequences were compared to NCBI database it could be found that many strains belonging to the same species were given different accession numbers. To avoid this multiplicity of accession numbers, all the sequences were compared to EzTaxon database that only contains type strains. Only similarities above 97% were accepted. Most strains were identically classified against both databases (NCBI and EzTaxon), although there were some minor discrepancies. Only three strains were found to belong to the *Phylum Bacteroidetes* and were classified as *Sphingobacterium spritivorum* (EzTaxon Accession No. ACHA01000008). The rest of strains were placed in the *phyla Actinobacteria*, *Firmicutes* and *Proteobacteria*. Species belonging to those *phyla* are listed in Table 1. Different species were unevenly scattered throughout the process according to their counts and the number of sampling times at which they were detected. Whereas some species were found at almost every sampling time (i.e., *Bacillus licheniformis*), some others were only detected in one or two samples and with low counts (i.e., *Tsukamurella inchonensis*). Five species were isolated at all the 19 sampling times and all of them were classified as *Firmicutes* (*Aeribacillus pallidus*, *B. licheniformis*, *Bacillus sonorensis*, *Bacillus thermoamylovorans* and *Ureibacillus thermosphaericus*). On the other hand, 64 species were isolated only at one sampling time (16 *Actinobacteria*, 18 *Firmicutes* and 30 *Proteobacteria*). *Actinobacteria* were the most abundant group, followed by *Firmicutes* and *Proteobacteria*. *Bacilli* (*Firmicutes*) were the most frequently isolated group. It may result noticeable that 13 species classified as *Proteobacteria* were only detected in thermophilic samples. Only one of them had been previously described as thermophilic (*Thermovum composti*, Yabe et al., 2012). The rest of species were known mesophilic bacteria, although thermal tolerance has been attributed to some of them (*Klebsiella* spp., Caplenas and Kanarek, 1984; *Pseudomonas* spp., Manaia and Moore, 2002; *Ochrobactrum* spp., Gajdosova et al., 2011).

Several important facts could be considered in relation to the isolation and detection of cultivable microorganisms from composting piles. Taking a representative sample from a compost pile is a difficult task, even when composite sampling is used. Required quantities of material for analytical purposes are immensely small in relation to the amount of material in the pile and particle size in raw materials, especially at the early stages of the process, is often very heterogeneous. Thus, some degree of uncertainty can be expected about sample representativeness. Whether a microbial species is detected or not, is really a matter of sample homogeneity (and representativeness), and eventually depends on the detection limit of the analytical technique employed that might be above the amount in which that species is actually present in a given sample. On the other hand, composting microbiota largely depends on microorganisms present in raw materials and those coming from

Table 1
Identified species at different stages of the composting process.

1 4 7 10 13 16 19	*Actinobacteria* species (Type strain)	N[a]	Accession[b]	S[c]
	Agromyces subtropicus (IY07-56[T])	2	AB546310	97.79
	Arthrobacter arilaitensis (Re117[T])	3	FQ311875	99.75
	Arthrobacter mysorens (LMG 16219[T])	1	AJ639831	99.13
	Arthrobacter nicotianae (DSM 20123[T])	7	X80739	99.17
	Arthrobacter russicus (GTC 863[T])	25	AB071950	99.56
	Brachybacterium faecium (DSM 4810[T])	4	CP001643	98.80
	Brachybacterium paraconglomeratum (LMG 19861[T])	40	AJ415377	99.26
	Brachybacterium saurashtrense (JG 06[T])	3	EU937750	98.52
	Brevibacterium aurantiacum (NCDO 739[T])	9	X76566	98.57
	Brevibacterium celere (KMM 3637[T])	1	AY228463	97.49
	Brevibacterium epidermidis (NCDO 2286[T])	39	X76565	99.53
	Brevibacterium frigoritolerans (DSM 8801[T])	2	AM747813	99.87
	Brevibacterium halotolerans (DSM 8802[T])	53	AM747812	99.79
	Brevibacterium iodinum (NCDO 613[T])	33	X76567	99.22
	Brevibacterium linens (DSM 20425[T])	20	X77451	98.97
	Brevibacterium permense (VKM Ac-2280[T])	4	AY243343	99.28
	Cellulosimicrobium cellulans (LMG 16121[T])	6	CAOI01000359	99.56
	Cellulosimicrobium funkei (ATCC BAA-886[T])	11	AY501364	99.13
	Citricoccus nitrophenolicus (PNP1[T])	4	GU797177	100.00
	Citricoccus zhacaiensis (FS24[T])	3	EU305672	99.19
	Corynebacterium ammoniagenes (DSM 20306[T])	5	ADNS01000011	98.83
	Corynebacterium casei (LMG S-19264[T])	56	AF267152	98.63
	Corynebacterium glutamicum (ATCC 13032[T])	1	BA000036	99.15
	Corynebacterium stationis (LMG 21670[T])	15	AJ620367	99.34
	Gordonia humi (CC-12301[T])	1	FN561544	97.37
	Gordonia paraffinivorans (NBRC 108238[T])	1	BAOQ01000072	99.26
	Haloglycomyces albus (YIM 92370[T])	2	EU660053	92.65
	Isoptericola halotolerans (YIM 70177[T])	3	AY789835	99.89
	Jonesia denitrificans (DSM 20603[T])	12	CP001706	99.75
	Jonesia quinghaiensis (DSM 15701[T])	7	AJ626896	99.53
	Leucobacter kyeonggiensis (F3-P9[T])	2	JQ039895	97.39
	Microbacterium aerolatum (V-73[T])	17	AJ309929	99.26
	Microbacterium amylolyticum (N5[T])	38	HQ605925	97.80
	Microbacterium arabinogalactanolyticum (IFO 14344[T])	1	AB004715	98.89
	Microbacterium arthrosphaerae (CC-VM-Y[T])	1	FN870023	97.69
	Microbacterium aurum (IFO 15204[T])	25	AB007418	97.24
	Microbacterium barkeri (DSM 20145[T])	5	X77446	99.72
	Microbacterium esteraromaticum (DSM 8609[T])	16	Y17231	99.31
	Microbacterium foliorum (DSM 12966[T])	13	AJ249780	98.47
	Microbacterium ginsengiterrae (DCY37[T])	18	EU873314	98.87
	Microbacterium gubbeenense (DSM 15944[T])	37	AUGQ01000019	99.14
	Microbacterium halotolerans (YIM 70130[T])	10	AY376165	97.38
	Microbacterium indicum (BBH6[T])	41	AM158907	98.04
	Microbacterium invictum (DC-200[T])	6	AM949677	98.74
	Microbacterium keratanolyticum (IFO 13309[T])	4	AB004717	98.81
	Microbacterium oxydans (DSM 20578[T])	13	Y17227	97.65
	Microbacterium paraoxydans (CF36[T])	11	AJ491806	99.71
	Microbacterium phyllosphaerae (DSM 13468[T])	1	AJ277840	98.93
	Microbacterium profundi (Shh49[T])	8	EF623999	97.61
	Microbacterium resistens (DMMZ 1710[T])	2	Y14699	98.79
	Microbacterium sediminis (YLB-01[T])	18	HQ219727	97.86
	Microbacterium thalassium (IFO 16060[T])	2	AB004713	98.46
	Microbacterium yannicii (G72[T])	7	FN547412	97.56
	Micrococcus yumnanensis (YIM 65004[T])	7	FJ214355	99.67
	Nocardiopsis flavescens (SA6[T])	7	GU997639	99.13
	Nocardiopsis yanglingensis (A18[T])	1	GQ463465	98.88
	Rhodococcus artemisiae (YIM 65754[T])	1	GU367155	98.52
	Rhodococcus coprophilus (DSM 43347[T])	1	X80626	98.31
	Rhodococcus phenolicus (DSM 44812[T])	6	AM933579	98.73
	Rhodococcus pyridinivorans (PDB9[T])	6	AF173005	99.61
	Rhodococcus rhodochrous (DSM 43241[T])	22	X79288	99.26
	Rhodococcus ruber (DSM 43338[T])	1	X80625	100.00
	Rhodococcus zopfii (DSM 44108[T])	2	AF191343	98.53
	Salinibacterium xinjiangense (0543[T])	11	DQ515964	97.59
	Streptomyces albidoflavus (DSM 40455[T])	1	Z76676	99.45
	Streptomyces heliomycini (NBRC 15899[T])	2	AB184712	99.25
	Streptomyces somaliensis (NBRC 12916[T])	1	AB184243	99.55
	Tsukamurella inchonensis (DSM 44067[T])	1	X85955	99.70

(*continued on next page*)

the surrounding ambient. Later, environmental factors will be mostly responsible for microbial fluctuations, and in the end, composting microbiota depends on its own competitive effectiveness and its survival capacity. Keeping in mind these facts, and according to frequency and abundance of microorganisms, composting microbiota behaves as a true microbiome, in which a succession of microbial strains deeply dependent on each other and conditioned by both biotic and abiotic factors, evolve following general rules but maintaining a high degree of uniqueness derived from differences in raw materials, operating conditions and surrounding environment. That is the reason why such a huge heterogeneity can be found in the specialized literature in relation to microorganisms associated to composting processes. Almost each combination of materials and operating conditions leads to different microbiota composition (Ryckeboer et al., 2003a; Chandna et al., 2013; Tian et al., 2013). Thus, it is pointless confirming or refuting the identity of microorganisms isolated from different composting assays. Every study is valuable itself since a standard microbiota characterizing the composting process does not exist, and that is simply because there is not a standard composting process. However, for each composting process there are microbial groups frequently detected in moderate or high counts at all or many stages. These groups constitute the composting resident microbiota. On the other hand, those species that appear once or infrequently, generally with low counts, could be considered as the composting transient microbiota. While resident microbiota have the needed capabilities to survive and compete successfully throughout the process, transient microbiota, mainly coming from the surrounding ambient, are in competitive disadvantage and they end up disappearing after a short time. Still, though different composting processes can share certain resident and/or transient microbial groups, each compost pile has a unique microbiome. Fig. 4a shows a pictographic representation of the most abundant and frequently isolated species throughout the process here described.

In order to ascertain the relative importance of isolated strains at each composting stage, a principal component analysis (PCA)

Table 1 (*continued*)

1 4 7 10 13 16 19	*Firmicutes* species (Type strain)	N[a]	Accession[b]	S[c]
	Aeribacillus pallidus (DSM 3670^T)	116	Z26930	99.54
	Aerococcus viridans (ATCC 11563^T)	1	ADNT01000041	99.28
	Bacillus aerius (24K^T)	27	AJ831843	98.84
	Bacillus aerophilus (28K^T)	91	AJ831844	99.86
	Bacillus altitudinis (41KF2b^T)	6	AJ831842	99.93
	Bacillus amyloliquefaciens subsp. *amyloliquefaciens* (DSM 7^T)	2	FN597644	99.53
	Bacillus amyloliquefaciens subsp. *plantarum* (FZB42^T)	8	CP000560	99.53
	Bacillus borbori (DX-4^T)	1	JX274440	99.85
	Bacillus cereus (ATCC 14579^T)	5	AE016877	99.70
	Bacillus coagulans (IAM 12463^T)	3	D16267	99.31
	Bacillus endophyticus (2DT^T)	1	AF295302	99.77
	Bacillus ginsengihumi (Gsoil 114^T)	1	AB245378	99.67
	Bacillus kochii (WCC 4582^T)	2	FN995265	100.00
	Bacillus licheniformis (ATCC 14580^T)	542	AE017333	99.64
	Bacillus megaterium (IAM 13418^T)	8	D16273	99.83
	Bacillus methylotrophicus (CBMB205^T)	17	EU194897	100.00
	Bacillus mojavensis (RO-H-1^T)	8	JH600280	99.56
	Bacillus niabensis (4T19^T)	2	AY998119	99.58
	Bacillus pumilus (ATCC 7061^T)	29	ABRX01000007	98.66
	Bacillus safensis (FO-036b^T)	86	AF234854	99.78
	Bacillus siamensis (KCTC 13613^T)	7	AJVF01000043	99.37
	Bacillus smithii (NBRC 15311^T)	1	AB271749	99.93
	Bacillus sonorensis (NBRC 101234^T)	311	AYTN01000016	98.38
	Bacillus stratosphericus (41KF2aT)	21	AJ831841	99.73
	Bacillus subtilis subsp. *inaquosorum* (KCTC 13429^T)	19	AMXN01000021	99.53
	Bacillus subtilis subsp. *subtilis* (NCIB 3610^T)	54	ABQL01000001	99.87
	Bacillus tequilensis (10b^T)	113	HQ223107	99.65
	Bacillus thermoamylovorans (CNCM I-1378^T)	160	L27478	99.28
	Bacillus thermolactis (R-6488^T)	9	AY397764	99.57
	Bacillus toyonensis (BCT-7112T)	1	CP006863	100.00
	Brevibacillus borstelensis (NRRL NRS-818^T)	40	D78456	99.77
	Brevibacillus limnophilus (DSM 6472^T)	2	AB112717	99.88
	Brevibacillus panacihumi (DCY35^T)	1	EU383033	99.62
	Chryseomicrobium imtechense (MW 10^T)	1	GQ927308	99.78
	Geobacillus thermodenitrificans subsp. *thermodenitrificans* (DSM 465^T)	3	CP000557	99.85
	Jeotgalicoccus halophilus (C1-52^T)	2	FJ386517	99.89
	Lactococcus garvieae (ATCC 49156^T)	1	AP009332	99.62
	Lysinibacillus chungkukjangi (2RL3-2^T)	2	JX217747	98.25
	Lysinibacillus macroides (LMG 18474^T)	1	AJ628749	99.71
	Lysinibacillus sinduriensis (BLB-1^T)	13	FJ169465	97.47
	Paenibacillus ginsengihumi (DCY16^T)	60	EF452662	99.79
	Paenibacillus lactis (MB 1871^T)	2	AY257868	99.81
	Paenibacillus lautus (NRRL NRS-666^T)	4	D78473	99.12
	Paenibacillus pueri (b09i-3T)	1	EU391156	99.28
	Paenibacillus residui (MC-246^T)	2	FN293173	99.93
	Psychrobacillus psychrodurans (DSM 11713^T)	6	AJ277984	99.32
	Sporosarcina globispora (DSM 4^T)	4	X68415	98.42
	Staphylococcus cohnii subsp. *conhii* (ATCC 29974^T)	1	D83361	100.00
	Staphylococcus epidermidis (ATCC 14990^T)	3	L37605	99.78
	Staphylococcus equorum subsp. *equorum* (ATCC 43958^T)	18	AB009939	99.64
	Staphylococcus equorum subsp. *linens* (RP29^T)	2	AF527483	99.28
	Staphylococcus saprophyticus subsp. *saprophyticus* (ATCC 15305^T)	1	AP008934	99.93
	Staphylococcus succinus subsp. *succinus* (AMG-D1^T)	1	AF004220	99.48
	Staphylococcus vitulinus (ATCC 51145^T)	1	AB009946	100.00
	Staphylococcus warneri (ATCC 27836^T)	6	L37603	99.63
	Terribacillus halophilus (002-051^T)	11	AB243849	99.64
	Terribacillus saccharophilus (002-048^T)	1	AB243845	100.00
	Ureibacillus suwonensis (DSM 16752^T)	3	AY850379	99.41
	Ureibacillus thermosphaericus (DSM 10633^T)	94	AB101594	99.68

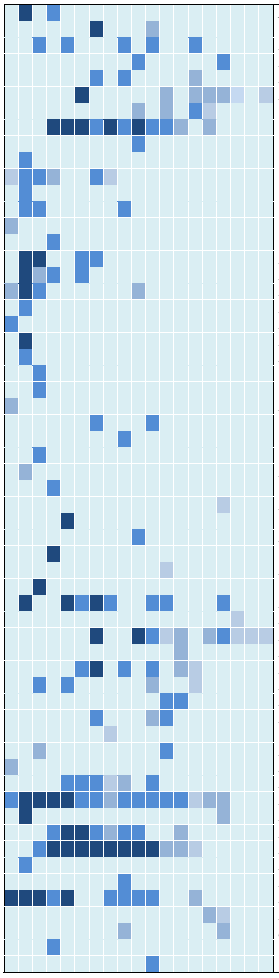
(*continued on next page*)

was performed. Fig. 4b shows an ordination graph where the first two principal components accounted for 85.23% of the variance. The plot highlights genera responsible for bacterial community differences among the main stages of composting (mesophilic, thermophilic, cooling and maturation phases). Only genera with greater coefficients are shown, being *Microbacterium* and *Bacillus* prominent among these. Other *Actinobacteria* (*Agromyces*, *Arthrobacter*, *Brevibacterium* and *Corynebacterium*), *Firmicutes* (*Paenibacillus*, *Staphylococcus* and *Ureibacillus*) and *Proteobacteria* (*Alcaligenes*, *Pseudomonas* and *Pseudoxanthomonas*) played the same role though to a lesser extent. Multivariate analysis showed that bacterial communities were different at each composting stage since samples from each phase were plotted in different areas of the ordination graph. Samples from mesophilic and thermophilic phases were grouped closer and so did samples from cooling and maturation stages. This highlights differences between microbiota from the bio-oxidative phase and that typically found at the curing stage of composting. Besides, bacterial communities became more similar in the replicate samples from the three piles studied as the process progressed.

3.6. Bacterial biodiversity

Four biodiversity indices were employed to assess biodiversity over time (composting stages) in the three composting piles studied throughout this work (Table 2). Bacterial counts in replicate piles for each isolated species were used for calculations. Analysis of variance displayed significant differences for all indices at different composting stages. In addition to sampling time, replication was also analyzed for significant differences between piles. Only richness (*S*) showed to be significantly different in the three piles; no significant differences for other indices could be attributed to replication. The diversity indices H', J' and *S* all rose markedly over the first stages of composting, what is consistent with the rapid proliferation of a range of microbial species. The diversity indices then fell, demonstrating that some of these species began to dominate the community. It has been widely reported that microbial activities are severely inhibited or reduced when temperature reaches the thermophilic range (Ryckeboer et al., 2003a; Chroni et al., 2009), though it has also been established that thermophilic or thermotolerant microbes dominate compost with strict thermogenic phases (Miyatake and Iwabuchi, 2005). In the process here described, as previously mentioned, a high proportion of microorganisms were considered thermotolerant, no matter the isolation program (mesophilic MA, MB or thermophilic TA, TB) from which they were isolated. Besides, the highest enzymatic activities were detected around the thermophilic stages (Fig. 4). Therefore, higher biodiversity indices H' and J' were measured during the bio-oxidative phase of composting (Table 2). While H' reflects the heterogeneity of a community based on the number of species present and their relative abundance (values range 0 to $\log_2 S$), J' is an evenness index that measures the proportion of actual diversity in relation

Table 1 (*continued*)

1 4 7 10 13 16 19	*Proteobacteria* species (Type strain)	N[a]	Accession[b]	S[c]
	*Acinetobacter indicus* (CIP 110367^T)	2	KI530754	99.62
	**Alcaligenes aquatilis* (LMG 22996^T)	2	JX986974	99.21
	Bordetella petrii (DSM 12804^T)	7	AM902716	98.42
	Brevundimonas bullata (IAM 13153^T)	5	D12785	99.65
	Brevundimonas olei (MJ15^T)	3	GQ250440	99.51
	Brevundimonas terrae (KSL-145^T)	7	DQ335215	99.28
	Castellaniella ginsengisoli (DCY36^T)	8	EU873313	99.82
	Chelatococcus daeguensis (K106^T)	49	EF584507	98.62
	Chelatococcus sambhunathii (HT4^T)	1	DQ322070	97.94
	Citrobacter freundii (ATCC 8090^T)	1	ANAV01000046	99.39
	Citrobacter sedlakii (CDC 4696-86^T)	10	AF025364	99.69
	Citrobacter youngae (CECT 5335^T)	1	AJ564736	99.79
	Cronobacter zurichensis (508/05^T)	3	DQ273681	98.78
	Curtobacterium plantarum (CIP 108988^T)	1	JN175348	97.29
	**Enterobacter aerogenes* (KCTC 2190^T)	1	CP002824	99.27
	Enterobacter asburiae (JCM 6051^T)	5	AB004744	98.84
	Enterobacter cancerogenus (LMG 2693^T)	4	Z96078	99.46
	Enterobacter hormaechei (ATCC 49162^T)	9	AFHR01000079	99.22
	Enterobacter soli (LF7a^T)	1	CP003026	98.97
	Erwinia toletana (A37^T)	1	AF130910	98.26
	Erwinia uzenensis (YPPS 951^T)	1	AB546198	98.21
	Klebsiella pneumoniae subsp. *ozaenae* (ATCC 11296^T)	1	Y17654	99.93
	**Klebsiella pneumoniae* subsp. *pneumoniae* (DSM 30104^T)	1	AJJI01000018	99.01
	**Klebsiella pneumoniae* subsp. *rhinoscleromatis* (ATCC 13884^T)	1	ACZD01000038	99.77
	Lelliottia amnigena (JCM 1237^T)	1	AB004749	100.00
	**Ochrobactrum intermedium* (LMG 3301^T)	2	ACQA01000003	99.92
	Ochrobactrum pseudogrignonense (CCUG 30717^T)	2	AM422371	98.64
	**Pantoea beijingensis* (LMG 27579^T)	1	KC846071	98.13
	Pantoea septica (LMG 5345^T)	1	EU216734	98.13
	**Pantoea vagans* (LMG 24199^T)	2	EF688012	98.13
	Paracoccus chinensis (KS-11^T)	1	EU660389	97.40
	**Paracoccus yeei* (G1212^T)	1	AY014173	99.29
	Pigmentiphaga daeguensis (K110^T)	1	EF100696	98.82
	**Prolinoborus fasciculus* (CIP 103579^T)	1	JN175353	100.00
	Providencia rettgeri (DSM 4542^T)	1	AM040492	99.01
	**Pseudomonas balearica* (SP1402^T)	2	U26418	99.13
	Pseudomonas bauzanensis (BZ93^T)	9	GQ161991	99.24
	Pseudomonas brenneri (CFML 97-391^T)	1	AF268968	99.39
	Pseudomonas formosensis (CC-CY503^T)	25	JF432053	99.87
	Pseudomonas guariconensis (PCAVU11^T)	1	HF674459	99.06
	Pseudomonas mendocina (LMG 1223^T)	13	Z76664	98.82
	Pseudomonas mohnii (Ipa-2^T)	5	AM293567	97.43
	Pseudomonas pachastrellae (KMM 330^T)	2	AB125366	99.71
	Pseudomonas plecoglossicida (FPC951^T)	5	AB009457	99.22
	**Pseudomonas putida* (NBRC 14164^T)	1	AP013070	99.63
	Pseudomonas rhizosphaerae (IH5^T)	2	AY152673	99.13
	Pseudomonas seleniipraecipitans (CA5^T)	2	FJ422810	99.89
	Pseudomonas stutzeri (ATCC 17588^T)	8	CP002881	99.36
	Pseudomonas xanthomarina (KMM 1447^T)	87	AB176954	98.64
	Pseudomonas xiamenensis (C10-2^T)	2	DQ088664	98.35
	Pseudoxanthomonas suwonensis (4M1^T)	20	AY927994	98.61
	Pseudoxanthomonas taiwanensis (CB-226^T)	100	AF427039	100.00
	Psychrobacter alimentarius (JG-100^T)	1	AY513645	99.16
	Psychrobacter celer (SW-238^T)	1	AY842259	99.42
	Psychrobacter faecalis (Iso-46^T)	18	AJ421528	99.37
	Pusillimonas noertemannii (BN9^T)	2	AY695828	97.55
	Serpens flexibilis (ATCC 29606^T)	3	GU269546	100.00
	**Stenotrophomonas rhizophila* (e-p10^T)	1	AJ293463	100.00
	**Thermovum composti* (Nis 3^T)	3	AB563785	99.66

[a] Number of strains identified as the species indicated in the first column

[b] Accession number (http://www.ezbiocloud.net/eztaxon/identify)

[c] Similarity percentage

* Species only isolated in thermophilic samples

Pictogram shows 19 small boxes per row corresponding to sampling times. The darker the color, the higher the count (i.e. very light blue $<10^3$ cfu g^{-1}, very dark blue $>10^8$ cfu g^{-1})

to maximum expected diversity (values range 0–1). In this sense, diversity values greater than 80% of the maximum expected biodiversity were observed at mesophilic and thermophilic stages. On the other hand, the Simpson index (D) showed the inverse trend since it measures the probability that two individuals taken at random from a sample belong to the same species. Consistently, the lowest values (maximum diversity) were found at the mesophilic and thermophilic stages.

In addition to biodiversity indices, Sorensen similarity index (I_S) was calculated for every paired combination of composting stages. This index expresses the extent to which two samples are similar based on of the species detected in them. The higher the index, the more similar the two samples (values range 0–1). According to results shown in Table 2, samples from raw materials showed to be more similar to those corresponding to late composting phases (maturation and final product). When mesophilic and thermophilic phases were compared, the highest similarity indices were displayed. High similarity was also found between mesophilic/thermophilic and cooling phases. Therefore, it could be observed that these stages shared a significant proportion of species (more than 50–60% of species were shared). In other words, bacterial community was quite similar at the central stages of composting. Samples from maturation stage and final product were also very similar, indicating that most microbes detected at maturation phase (54.2%) were also present in final compost.

Composting biodiversity is related to capabilities of resident microbiota whose prominence is due to thermal tolerance and enzymatic multi-functionality. These microbial characteristics should be ineluctably considered for a proper composting management. Multiple biochemical capabilities of microorganisms associated to composting might be exploited not only to improve the process itself, but also because they have an undeniable biotechnological potential.

3.7. Interaction between nutritional, environmental and biological factors

Organic matter is composed of readily available compounds and polymeric organic substances that need to be enzymatically processed before they can be used by microorganisms (Fig. 3). The

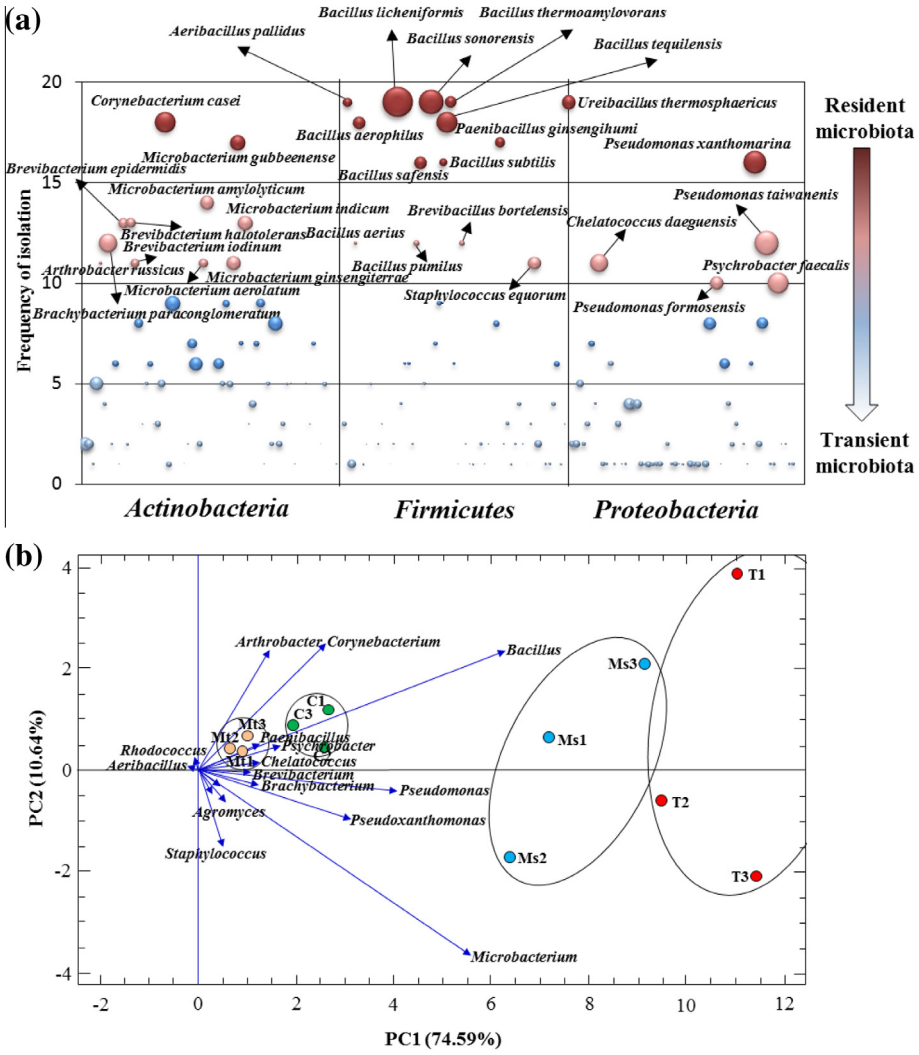


Fig. 4. Most influential bacteria and actinobacteria throughout composting. (a) Frequency of isolation (along the vertical axis) and relative abundance (bubble diameter) of identified species. (b) Principal component analysis of data (counts, cfu g^{-1}) showing loadings plots for each composting stage (Ms, mesophilic; T, thermophilic; C, cooling; Mt, maturation) in the three replicated piles with the most influential genera.

Table 2
Biodiversity and similarity indices throughout the composting process (values are mean ± standard deviation, $n = 3$ piles).

Index[a]		Raw materials	Mesophilic	Thermophilic	Cooling	Maturation	Final product
Richness (S)		22 ± 2^{A}	77 ± 2^{C}	72 ± 5^{C}	55 ± 5^{B}	34 ± 6^{A}	22 ± 3^{A}
Shannon–Wiener (H')		3.239 ± 0.102^{A}	5.118 ± 0.132^{B}	5.142 ± 0.094^{B}	4.553 ± 0.247^{B}	3.427 ± 0.325^{A}	2.766 ± 0.276^{A}
Pielou (J')		0.733 ± 0.038^{BC}	0.817 ± 0.022^{C}	0.835 ± 0.023^{C}	0.788 ± 0.025^{C}	0.678 ± 0.037^{AB}	0.624 ± 0.035^{A}
Simpson (D)		0.151 ± 0.018^{BC}	0.041 ± 0.005^{A}	0.041 ± 0.003^{A}	0.071 ± 0.018^{AB}	0.150 ± 0.040^{BC}	0.228 ± 0.041^{C}
I_S[b]	Raw materials	1.000 ± 0.000^{C}	0.323 ± 0.039^{A}	0.351 ± 0.048^{AB}	0.365 ± 0.046^{AB}	0.411 ± 0.032^{B}	0.380 ± 0.043^{AB}
	Mesophilic	0.323 ± 0.039^{A}	1.000 ± 0.000^{E}	0.597 ± 0.019^{D}	0.519 ± 0.031^{C}	0.440 ± 0.021^{B}	0.318 ± 0.030^{A}
	Thermophilic	0.351 ± 0.048^{AB}	0.597 ± 0.019^{D}	1.000 ± 0.000^{E}	0.510 ± 0.028^{C}	0.390 ± 0.021^{B}	0.306 ± 0.026^{A}
	Cooling	0.365 ± 0.046^{A}	0.519 ± 0.031^{B}	0.510 ± 0.028^{B}	1.000 ± 0.000^{C}	0.501 ± 0.023^{B}	0.414 ± 0.051^{A}
	Maturation	0.411 ± 0.032^{AB}	0.440 ± 0.021^{AB}	0.390 ± 0.021^{A}	0.501 ± 0.023^{BC}	1.000 ± 0.000^{D}	0.542 ± 0.094^{C}
	Final product	0.380 ± 0.043^{A}	0.318 ± 0.030^{A}	0.306 ± 0.026^{A}	0.414 ± 0.051^{A}	0.542 ± 0.094^{B}	1.000 ± 0.000^{C}

[a] Within a row, values labeled with the same letter are not significantly different.
[b] Sørensen similarity index (I_S) compares paired composting stages.

decomposition of such polymeric materials provides simple, soluble compounds readily metabolizable to microbial cells. As indicated above, composting microbiota behaves as a true microbiome in which every member has a key role (Tables S2 and 1 and Fig. 4). Those microorganisms with the needed enzymatic capabilities (Fig. 3) will decompose polymeric materials, being the resulting soluble compounds available to all microorganisms around. As the composting process advances, the ratio of strains exhibiting those activities increased (Fig. 3), however, the most recalcitrant polymers will accumulate and the rate at which these compounds are decomposed will be lower and lower until the energy released is not enough to maintain microbial activities. Lignocellulosic polymers are the most difficult to degrade and the number of microbial species capable of doing so is certainly low (Insam and de Bertoldi, 2007). Anyhow, once the bio-oxidative stage is over, partially decomposed or undegraded polymeric

materials will serve for the formation of humic substances that will help confer to compost the needed maturity and stability.

Enzymatic capabilities of composting microbiota are responsible for most of the facts stated above. Raw materials composition and operational conditions determine the way microbiota behaves, its structure (Fig. 2 and Tables S2 and 1) and biodiversity (Table 2, Fig. 4). There is such a complex interaction between chemical components, environmental factors and microbial activities, that it is really difficult to ascertain which of them exerts a greater influence on the others.

4. Conclusions

Once readily degradable compounds have been exhausted, microbial enzymatic activities grant the decomposition of polymeric materials, providing more soluble compounds to all the composting microbiota. In this sense, microorganisms inhabiting composting piles could be considered a true microbiome, each of whose members (resident and transient) has a key role in the process. Biodegradation of different polymeric components is likely to occur simultaneously throughout the process, due to microbiota multi-functionality. Thermal tolerance is a widespread characteristic among composting microorganisms. Maximum biodiversity is perceived at central composting stages and resident microbiota has a determining role in composting and an undisputed biotechnological potential.

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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.2014.10.123.

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