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Revisiting the succession of microbial populations throughout composting: A matter of thermotolerance

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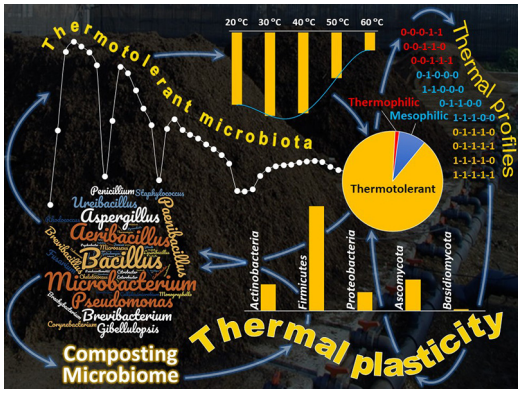
HIGHLIGHTS

- The temperature-driven succession of the composting microbial populations was revisited.
- Thermotolerance was demonstrated for a vast majority of the composting microbiota.
- Thermotolerant strains were repeatedly identified in most of the composting stages.
- *Firmicutes* and *Ascomycota* accounted for the best represented thermotolerant *phyla.*
- Thermal plasticity is a microbial reply to the ever changing composting conditions.

GRAPHICAL ABSTRACT



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ABSTRACT

Composting has been traditionally considered a process in which a succession of mesophilic and thermophilic microbial populations occurs due to temperature changes. In order to deepen in this model, 1380 bacterial and fungal strains (the entire culturable microbiota isolated from a composting process) were investigated for their ability to grow across a wide range of temperatures (20 to 60 °C). First, qualitative tests were performed to establish a thermal profile for each strain. Then, quantitative tests allowed ascertaining the extent of growth for each strain at each of the tested temperatures. The identity of the isolates enabled to position them taxonomically and permitted tracking the strains throughout the process. Results showed that 90% of the isolates were classified as thermotolerant (they grew at all tested temperatures). Only 9% and 1% of the studied strains showed to be strictly mesophilic or thermophilic, respectively. *Firmicutes* exhibited the greatest thermal plasticity, followed by *Actinobacteria* and *Ascomycota.* Most of the *Proteobacteria* and all *Basidiomycota* strains were also able to grow at all the assayed temperatures. Thermotolerance was clearly demonstrated among the composting microbiota, suggesting that the idea of the succession of mesophilic and thermophilic populations throughout the process might need a reassessment.

1. Introduction

Known for a long time, composting has become in the last 25 years a conceptually and operationally well-documented process that has positioned it in the foreground to other alternatives for organic waste

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treatment (Schaub and Leonard, 1996; Neher et al., 2013; Jurado et al., 2020). Essentially, composting is due to microbial activities. As a result, the process evolves through different stages characterized mainly by the temperature reached in the materials being treated. Four well-differentiated stages can be detected throughout a composting process: mesophilic, thermophilic, cooling and maturation (Fogarty and Tuovinen, 1991). Heat is a consequence of the excess of energy generated in exergonic reactions typically included in the aerobic metabolism of organic carbon compounds. Composting microbiota is primarily influenced by the composition of organic wastes to be composted. In addition, process evolution depends on the microbial capacity to act upon such compounds (Jurado et al., 2015). Other physicochemical factors (moisture, pH, particle size, etc.) and operational alternatives (forced aeration or static systems, outdoors or closed bioreactors, etc.) likewise influence microbial activities and hence, they are also important for the process to be successfully completed (Gea et al., 2005). In sum, given the appropriate environmental conditions, the greater the intensity of the biodegradation, the greater the quantity of energy generated and hence, the higher the temperature reached inside the composting piles.

Temperature changes throughout composting is a key factor commonly used to confirm the process is running properly. Temperature can be considered as a paradoxical factor influencing (or influenced by) most of the biological events that govern the process. Thus, microbial populations associated with composting are responsible for the temperature increases and decreases. At the same time, temperature determines qualitative and quantitatively the structure and dynamics of microbial populations throughout composting. The universally accepted approach to explain how microorganisms face these thermal changes inside the composting piles, proposes the succession of microbial groups as the process evolves. In other words, there would be an alternation between the mesophilic (growth at 10–40 °C) and thermophilic (growth at >40 °C) microbiota as thermal variations occur (Fogarty and Tuovinen, 1991; Zhao et al., 2017; Liu et al., 2018).

On the other hand, according to recent studies on composting microbiology, after an exhaustive sampling of composting piles, isolation and identification of strains, López-González et al. (2015a, 2015b) observed the existence of two distinct groups of microorganisms. One of them was composed of those widely distributed in large quantities in most of samplings during the entire process, both at the mesophilic and thermophilic stages (resident microbiota). Some other strains could only be isolated in 1–2 samplings in low numbers (transient microbiota). The composition of the resident microbiota proved to be very constant throughout the process, regardless of the predominant thermal or nutritional status. This fact poses a biological plasticity that is worth investigating. In this sense, phenotypic plasticity, that is the ability of an organism to produce different phenotypes in response to changes in the environment, has been known for a long time (Schmalhausen, 1949). Environments with rapid and, especially unpredictable fluctuations, could select reversible phenotypes, with high plasticity and diversified survival (Arnoldini et al., 2012). The adaptability of the composting microbiota, especially to temperature variations (thermotolerance), opposes the traditionally accepted model of the succession of microbial groups during the process. Therefore, performing studies to clarify this controversy is fully justified.

This study was based on a large collection of bacteria, actinobacteria and fungi (1380 strains) previously isolated through intensive sampling of a composting process and identified by molecular methods (see details in López-González et al., 2015a, 2015b). The starting hypothesis raised in this work was that high thermotolerance could be found in the microbiota associated with the composting process. In order to confirm this hypothesis, the following specific objectives were proposed: a) to establish the thermal range (from 20 to 60 °C) at which each single strain isolated from composting was able to grow, b) to quantify the actual growth of each strain at different temperatures, establishing the optimal and inhibitory temperature for growth, c) to relate the thermotolerance with the taxonomic position of the strains studied, and d) to propose a succession pattern (if any) of microbial populations associated with composting based on thermal plasticity.

2. Materials and methods

As stated above, this work derives from an exhaustive previous study of three composting piles composed of lignocellulosic waste. Results from this previous study have been published (López-González et al., 2013, 2015a, 2015b), so all the operational and methodological aspects can be easily accessed. Even so, a brief description of these aspects is provided next.

2.1. The process

Three identical piles of 500 kg were built (3.0 m L × 1.5 m W × 1.0 m H). Raw materials were a mixture (50:50 *w*/w) of ground (<30 mm) post-harvest tomato plants (lacking fruits) and pine woodchips. This starting mixture had a C/N ratio around 25. Forced aeration was supplied from the bottom of the pile to prevent oxygen concentration inside the piles to be lower than 10–12%. Moisture was kept around 50% by periodic watering. Piles were turned according to temperature values, which were monitored on-site with thermometer probes PT100 MPT2 (Lexitron-Gemisa, Madrid, Spain). The process was considered finished after 189 days. A total of 19 composite samples were collected from each pile at different times during critical points, including raw material (RM), mesophilic (MES), thermophilic (THER), cooling (COOL), and maturation (MAT) stages, as well as final product (FP). Typical physicochemical analyses (pH, moisture, bulk density, organic matter, C/N and soluble organic carbon) were performed to follow composting development. An exhaustive microbiological study was carried out. At each sampling time, total bacteria, actinobacteria and fungi, were counted (distinguishing mesophilic and thermophilic counts in each case by incubation at 30 or 55 °C, respectively). In addition, every single colony was isolated and identified by molecular methods. More than 5200 morphotypes were studied in depth. Finally, 1380 strains were considered unique strains. These strains constitute the microbial collection used in the present study.

2.2. Analysis of growth temperatures range

In order to ascertain the range of temperatures at which strains were able to grow, a liquid culture was prepared for each strain using Nutrient Broth (NB) (Panreac, Barcelona Spain) for bacteria and actinobacteria, and Potato Dextrose Broth (PDB) (Scharlab, Barcelona, Spain) for molds and yeasts. These cultures were incubated in agitation (reciprocal shaker at 200 rpm) at 30 °C for 24–72 h, depending on the moment at which an apparent turbidity could be seen in each case. These liquid cultures were used as inocula for the thermal study. Plates of Nutrient Agar (AN) or Potato Dextrose Agar (PDA) (Panreac, Barcelona Spain) were inoculated in mass by streaking sterile swabs previously soaked in inoculum liquid cultures and then incubated at 20, 30, 40, 50 and 60 °C. In general, plates were incubated for 48–72 h (bacteria), 96 h (fungi) and 96–120 h (actinobacteria), though in some cases, incubation was extended up to 7 days to prevent discarding slow growth strains at given temperatures. Tests were considered positive when visible microbial growth was evident. Three replicates were used for each combination strain/temperature.

Finally, each strain was classified according to a simple binary code assigning 1 for growth and 0 for the absence of growth for each of the five different temperatures assayed. Thus, for example, the strain code 0-1-1-0-0 stands for growth only at 30 and 40 °C.

2.3. Thermotolerance quantification: optimal temperature for growth

To quantify microbial growth at the selected temperatures, a simple microtiter assay based on Resazurin reduction was employed. The

technique described next is based on previous studies (Vega et al., 2012; Chadha and Kale, 2015) with slight modifications. Resazurin is a tetrazolium-based, non-toxic, redox dye that is reduced intracellularly in the fluorescent compound Resorufin, by the action of enzymes that act in the last region of the electronic transport chain (Vega et al., 2012). Measurement of this reduction is an indirect way of quantifying microbial growth and can be accomplished fluorometrically or colorimetrically. The assay was performed in sterile 96-well microtiter plates (Thermo Fisher Scientific, Waltham, MA, USA).

For bacteria, 150 μL of bacterial culture grown in NB for 24 h were added to six wells (6 repetitions were used in this assay). For fungi and filamentous actinobacteria strains, each of the six wells was added of 150 μL of PDB for fungi or NB for actinobacteria and then inoculated with a plug (4 mm) obtained from fungal or actinobacterial cultures grown on PDA or AN plate respectively, for 96 h. Another 6 wells added of non-inoculated culture media in each case, were used as negative control.

Microtiter plates were incubated at 20, 30, 40, 50 or 60 °C for 24 h. Afterwards, 50 μL of 0.01% (*w*/*v*) solution of Resazurin in water (Sigma-Aldrich, Darmstadt, Germany) were added to every well (included control wells) and incubated for 2 h at the specific temperature of the test (20, 30, 40, 50 or 60 °C). Subsequently, microtiter plates were read at 600 nm in a spectrophotometer (Eon, Biotek Instruments, Winooski, VT, USA) to detect the typical blue color of Resazurin. Since Resazurin is incorporated into the cells and then reduced, the lighter the blue color inside the well, the greater the microbial growth could be inferred. Obviously, control wells showed the darkest blue color. In the case of bacteria, reads were obtained directly, while for fungi and filamentous actinobacteria, 100 μL of each well were previously transferred to new microplates to avoid interference of the mycelial growth in the spectrophotometer readings.

Calculations were made subtracting the absorbance in wells containing microbial cultures from the absorbance in the corresponding control wells. Thus, the highest difference corresponded to maximum growth, and the temperature at which maximum growth was observed, was considered the optimal growth temperature in each case. For a given strain, five mean values ($Abs_{Control} - Abs_{Test}$) were obtained, one for each of the five assayed temperatures. Maximum growth (corresponding to the highest difference between $Abs_{Control} - Abs_{Test}$) was considered 100% and the rest of values (obviously lower) obtained at other temperatures were calculated as a percentage of this maximum value, so indicating the extent of growth decrease or inhibition at non-optimal temperatures.

2.4. Data processing

As it can be easily inferred, a huge amount of data had to be managed in this study, so an intensive data processing was needed. Microsoft Excel (Office 365 A1 Plus for faculty, 2019) was used to build a database including previously available information for each strain and all results obtained throughout this work. In addition, some statistical analyses were performed. One-way ANOVA was used to verify that differences in absorbance ($Abs_{Control} - Abs_{Test}$) obtained by growing each strain at temperatures selected were significantly different ($p < 0.05$). Cluster analysis was used to group data (Nearest Neighbor method, Squared Euclidean metric distance) and conglomerates were obtained for variables (from sample correlation matrix) instead of for observations. Temperatures selected (20, 30, 40, 50 and 60 °C) were grouped on the basis of the quantified growth at optimal and suboptimal temperatures. On the other hand, thermal codes (see the end of Section 2.2) were also grouped using Cluster analysis based on the quantified growth at each thermal profile. All data analyses were performed using Statgraphics Centurion 18 (StatPoint Technologies Inc., Virginia, USA).

3. Results and discussion

3.1. Composting development

According to results previously reported (see Fig. 1 in López-González et al., 2015a), composting parameters indicating process development were as typically expected. Temperature reached values above 70 °C in the thermophilic stages and decreased after each turning operation. Adequate particle size (<30 mm) and porosity, jointly with forced aeration and turning operations ensured oxygen availability. pH tended to slightly alkaline values. Organic matter and C/N ratio decreased as a result of microbial activity. Thus, in general, the process profile evolved within the standards typically found in composting of lignocellulosic materials subjected to forced aeration (Sánchez et al., 2017).

3.2. Thermal plasticity of microbial populations associated with composting

In the specialized literature, from the early review of Fogarty and Tuovinen (1991) to other works more recently reported (Hultman et al., 2010; Tian et al., 2013), the phenomenon of microbial communities succession is described throughout the different phases of the composting process. Thus, the mesophilic populations are replaced by the thermophilic ones and vice versa, several times in the course of a composting process, especially if turning operations are implemented. In order to determine the validity of such statement, for this study, pure cultures of the entire microbiota associated with composting were analyzed. Obviously, information about the composting stage and the exact values of temperature inside the composting piles at which each strain was isolated were available, and probably this information would have been enough to classify strains in the mesophilic or thermophilic range of growth. However, the fact that most of the strains could be isolated many times (at different samplings) throughout the process was indicative, at least a priori, of certain thermal plasticity. To go further on this presumption, a simple experiment was carried out. Each of the 1380 strains was plate-cultured (under laboratory conditions) at 20, 30, 40, 50 and 60 °C and growth was recorded. No attempts to quantify growth were made at that moment, only presence or absence of growth was annotated. Results were codified as thermal profiles (Table 1).

Eleven thermal profiles were found and, according to the ranges of temperatures at which growth was detected, they were included in three well-defined categories: mesophilic, thermotolerant and thermophilic. Data processing revealed that most of the strains were included in the category 'thermotolerant'. Table 2 shows very detailed information about this, including the number of strains belonging to each of the identified genera and the thermal profile and category in which each strain was included.

A graphical way of visualizing this information can be attained in Fig. 1. In brief, 1240 (89.9%) out of the 1380 studied strains showed to be thermotolerant, 122 (8.8%) were strictly mesophilic and only 18 (1.3%) were strictly thermophilic (Fig. 1a).

The results obtained clearly explain why most of the strains could be isolated in a wide range of samplings throughout the process, no matter the current temperatures inside the piles were. The changes of each category of thermal profiles in relation to composting stages is shown in Fig. 1b. As depicted in that figure, the thermotolerant populations were present at every composting sampling in huge numbers (far greater than those for mesophilic or thermophilic microbiota). They appeared profusely during most of time that the process lasted. Only after the cooling phase, thermotolerants started to decrease, though that was the general trend for all microbial groups as nutrients began to be very scarce and microbial activities could no longer be supported. At different extents, mesophilic and thermophilic microbiota followed a similar pattern, showing greater values of growth during the bio-oxidative stage of composting. It results noteworthy that high temperatures characteristic

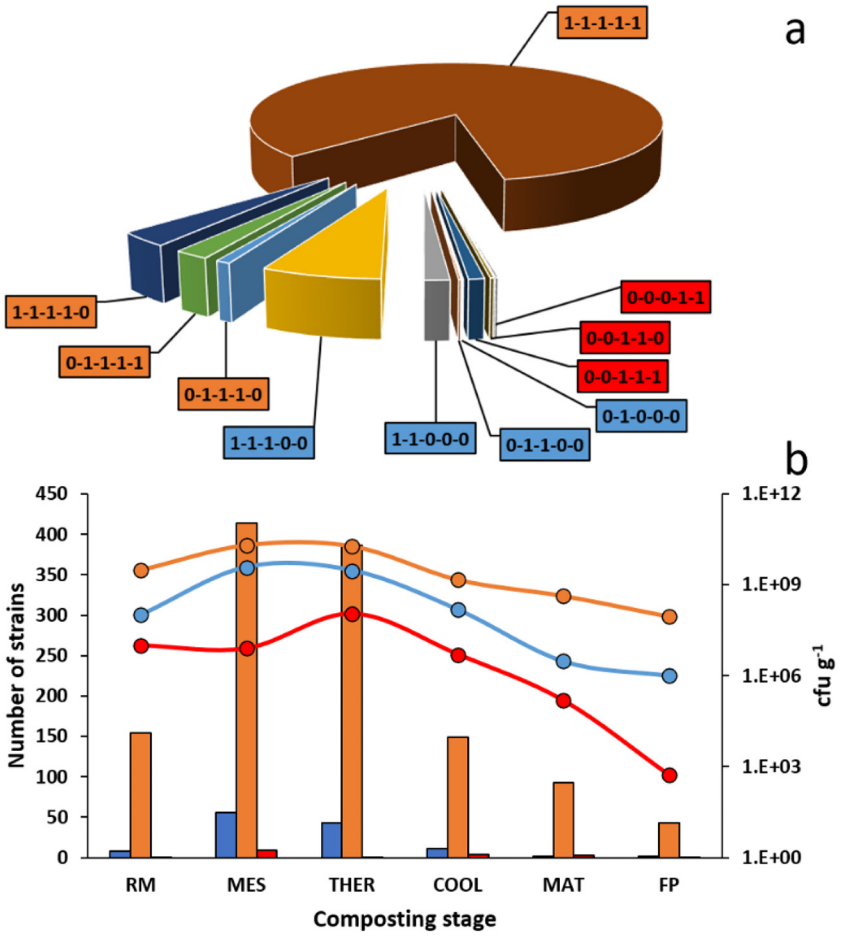

Fig. 1. Distribution (%) of the studied strains among the established thermal profiles (see Table 1) (a) and occurrence of mesophilic (●), thermotolerant (●) and thermophilic (●) strains throughout the different composting stages (b). RM (raw materials), MES (mesophilic), THER (thermophilic), COOL (cooling), MAT (maturation) and FP (final product).

of thermophilic stages seemed not to exaggeratedly affect mesophilic microorganisms, probably because many of them were spore formers or were located at peripheral positions in the piles where temperature values were lower. As said above, subsequent microbial growth declined in parallel to nutrients depletion, dropping to minimum values after the cooling phase (López-González et al., 2013). This typical pattern has been previously reported in other studies (Bathia et al., 2012). Heat generation must be considered a residual consequence of microbial metabolism (Bathia et al., 2012) which in turn depends on nutrients availability, and that is why these two phenomena should always be viewed as inevitably linked.

Table 1
Thermal profile codes for the classification of the microbial isolates according to the temperatures at which growth was detected (+) and thermal categories in which the profiles were included.

Growth at tested temperatures					Thermal profile code	Thermal category
20 °C	30 °C	40 °C	50 °C	60 °C		
−	+	−	−	−	0-1-0-0-0	Mesophilic
−	+	+	−	−	0-1-1-0-0	
+	+	−	−	−	1-1-0-0-0	
+	+	+	−	−	1-1-1-0-0	
−	+	+	+	−	0-1-1-1-0	Thermotolerant
−	+	+	+	+	0-1-1-1-1	
+	+	+	+	−	1-1-1-1-0	
+	+	+	+	+	1-1-1-1-1	
−	−	−	+	+	0-0-0-1-1	Thermophilic
−	−	+	+	−	0-0-1-1-0	
−	−	+	+	+	0-0-1-1-1	

3.3. Optimal temperature of composting microbiota

Optimal temperature of growth for each microbial strain was next determined (Fig. 2). Data presented in this figure can easily be interpreted since the darker the color, the greater the microbial growth in each row of the pictogram, thus showing optimal temperature of growth for each strain. An exact correspondence with qualitative results (thermal profiles) presented in Table 2 was obtained. Data processing showed that 30 and 40 °C were optimal temperatures for most of the strains. Specifically, the order (by number of strains) of assayed temperatures that showed to be optimal were: 40 °C (490 strains), 30 °C (472 strains), 20 °C (320 strains), 50 °C (96 strains) and 60 °C (2 strains). A simple ANOVA was performed for each strain in order to determine whether the growth values at the assayed temperatures were significantly ($p < 0.05$) different or not. It was observed that, in most of the cases, differences between growth at 30 and 40 °C were not significant ($p > 0.05$). Performing this experiment also allowed to determine the percentage of growth decrease obtained at non-optimal temperatures for each strain (in relation to growth at optimal temperature). The information relative to each of the 1380 strains analyzed is very large to be individually presented; however, in an attempt to ascertain the extent of growth inhibition at non-optimal temperatures, strains were grouped by optimal temperature and thermal categories, and the mean of

Table 2
Total number of strains belonging to the genera specified in the first column and number of strains with the indicated thermal profiles (for profile codes, see Table 1). a) Prokaryotic *phyla* b) Eukaryotic *phyla*.

	Genus	Sn[a]	Mesophilic				Thermotolerant				Thermophilic		
			01000	01100	11000	11100	01110	01111	11110	11111	00011	00110	00111
a)													
Actinobacteria	*Agromyces*	1	–	–	–	–	–	–	–	1	–	–	–
	Arthrobacter	8	–	–	–	1	–	–	–	7	–	–	–
	Brachybacterium	14	–	–	1	4	–	–	–	9	–	–	–
	Brevibacterium	37	–	–	–	10	–	–	4	23	–	–	–
	Cellulosimicrobium	12	–	–	–	2	–	–	–	10	–	–	–
	Citricoccus	4	–	–	–	–	–	–	–	4	–	–	–
	Corynebacterium	17	–	–	–	1	–	–	–	16	–	–	–
	Gordonia	2	–	–	–	–	–	–	–	2	–	–	–
	Haloglycomyces	2	–	–	–	–	–	–	–	2	–	–	–
	Isoptericola	1	–	–	–	–	–	–	–	1	–	–	–
	Jonesia	8	–	–	–	1	–	–	–	7	–	–	–
	Leucobacter	2	–	–	–	–	–	–	–	2	–	–	–
	Microbacterium	90	–	–	3	18	–	–	2	67	–	–	–
	Micrococcus	1	–	–	–	–	–	–	–	1	–	–	–
	Nocardiopsis	4	–	–	–	1	–	–	–	3	–	–	–
	Rhodococcus	16	–	–	–	1	–	–	–	15	–	–	–
	Salinibacterium	2	–	–	–	1	–	–	–	1	–	–	–
	Streptomyces	3	–	–	–	1	–	–	1	1	–	–	–
	Tsukamurella	1	–	–	–	–	–	–	–	1	–	–	–
	Total	225	0	0	4	41	0	0	7	173	0	0	0
Firmicutes	*Aeribacillus*	75	–	–	–	2	–	5	1	58	1	–	8
	Aerococcus	1	–	–	–	–	–	–	–	1	–	–	–
	Bacillus	520	1	–	–	7	1	9	8	487	1	2	4
	Brevibacillus	26	–	–	–	–	–	–	2	24	–	–	–
	Chryseomicrobium	1	–	–	–	1	–	–	–	–	–	–	–
	Geobacillus	2	–	–	–	–	–	–	–	2	–	–	–
	Jeotgalicoccus	2	–	–	–	–	–	–	–	2	–	–	–
	Lactococcus	1	–	–	–	–	–	–	–	1	–	–	–
	Lysinibacillus	11	–	–	–	–	–	–	–	11	–	–	–
	Paenibacillus	43	–	–	–	2	6	9	3	23	–	–	–
	Psychrobacillus	2	–	–	–	–	–	–	–	2	–	–	–
	Sporosarcina	1	–	–	–	–	–	–	–	1	–	–	–
	Staphylococcus	19	–	–	–	1	–	–	–	18	–	–	–
	Terribacillus	4	–	–	–	2	–	–	1	1	–	–	–
	Ureibacillus	36	–	–	–	2	1	2	–	31	–	–	–
	Total	744	1	0	0	17	8	25	15	662	2	2	12
Proteobacteria	*Acinetobacter*	2	–	–	–	–	–	–	–	2	–	–	–
	Alcaligenes	2	–	–	–	–	–	–	–	2	–	–	–
	Bordetella	4	–	–	–	–	–	–	–	4	–	–	–
	Brevundimonas	4	–	–	–	1	–	–	–	3	–	–	–
	Castellaniella	2	–	–	–	–	–	–	1	1	–	–	–
	Chelatococcus	12	–	–	–	–	3	–	–	8	–	1	–
	Citrobacter	11	–	–	–	1	–	–	1	9	–	–	–
	Cronobacter	1	–	–	–	–	–	–	–	1	–	–	–
	Curtobacterium	1	–	–	–	–	–	–	–	1	–	–	–
	Enterobacter	10	–	–	–	1	–	–	–	9	–	–	–
	Erwinia	2	–	–	–		–	–	–	2	–	–	–
	Klebsiella	3	–	–	–	1	–	–	–	2	–	–	–
	Lelliottia	1	–	–	–	–	–	–	–	1	–	–	–
	Ochrobactrum	2	–	–	–	–	–	–	1	1	–	–	–
	Pantoea	3	–	–	–	–	–	–	–	3	–	–	–
	Paracoccus	2	–	–	–	1	–	–	–	1	–	–	–
	Pigmentiphaga	1	–	–	–	–	–	–	–	1	–	–	–
	Prolinoborus	1	–	–	–	–	–	–	–	1	–	–	–
	Providencia	1	–	–	–	–	–	–	–	1	–	–	–
	Pseudomonas	72	–	2	–	19	–	–	8	43	–	–	–
	Pseudoxanthomonas	7	–	–	–	1	–	–	3	3	–	–	–
	Psychrobacter	7	–	–	–	1	–	–	–	6	–	–	–
	Pusillimonas	1	–	–	–	–	–	–	–	1	–	–	–
	Serpens	1	–	–	–	–	–	–	–	1	–	–	–
	Stenotrophomonas	1	–	–	–	–	–	–	–	1	–	–	–
	Thermovum	1	–	–	–	–	–	–	–	1	–	–	–
	TOTAL	155	0	2	0	26	3	0	14	109	0	1	0
b)													
Ascomycota	*Acremonium*	3	–	–	–	2	–	–	–	1	–	–	–
	Alternaria	5	–	–	2	–	–	–	–	3	–	–	–
	Aspergillus	44	–	–	–	–	–	–	3	40	–	–	1
	Candida	5	–	–	–	–	–	–	–	5	–	–	–
	Cephaliophora	1	–	–		1	–	–	–	–	–	–	–
	Cladosporium	3	–	–	1	–	–	–	–	2	–	–	–

(continued on next page)

Table 2 (*continued*)

	Genus	Sn[a]	Mesophilic				Thermotolerant				Thermophilic		
			01000	01100	11000	11100	01110	01111	11110	11111	00011	00110	00111
	Cyberlindnera	1	–	–	–	–	–	–	–	1	–	–	–
	Davidiella	3	–	–	2	–	–	–	–	1	–	–	–
	Emericella	2	–	–	–	1	–	–	–	1	–	–	–
	Fusarium	34	–	–	1	3	–	–	1	29	–	–	–
	Galactomyces	11	–	–	–	–	–	–	–	11	–	–	–
	Gibellulopsis	32	–	–	2	–	–	–	1	29	–	–	–
	Gloeotinia	1	–	–	1	–	–	–	–	–	–	–	–
	Graphium	1	–	–	–	1	–	–	–	–	–	–	–
	Hypocrea	1	–	–	–	–	–	–	1	–	–	–	–
	Kuraishia	1	–	–	–	–	–	–	–	1	–	–	–
	Microascus	1	–	–	–	–	–	–	–	1	–	–	–
	Monographella	12	–	–	1	–	–	–	–	11	–	–	–
	Nakazawaea	7	–	–	–	–	–	–	–	7	–	–	–
	Ochrocladosporium	1	–	–	–	–	–	–	–	1	–	–	–
	Penicillium	26	–	–	–	2	–	–	1	23	–	–	–
	Pichia	3	–	–	–	–	–	–	–	3	–	–	–
	Preussia	1	–	–	1	–	–	–	–	–	–	–	–
	Pyrenochaeta	6	–	–	3	–	–	–	1	2	–	–	–
	Scedosporium	3	–	–	–	3	–	–	–	–	–	–	–
	Scopulariopsis	15	–	–	1	1	–	–	1	12	–	–	–
	Stemphylium	1	–	–	1	–	–	–	–	–	–	–	–
	Talaromyces	1	–	–	–	–	1	–	–	–	–	–	–
	Thermomyces	7	–	–	–	–	–	4	–	3	–	–	–
	Trichoderma	4	–	–	1	–	–	–	–	3	–	–	–
	Verticillium	2	–	–	–	–	–	–	–	2	–	–	–
	Yamadazyma	6	–	–	–	–	–	–	–	6	–	–	–
	Total	244	0	0	17	14	1	4	9	198	0	0	1
Basidiomycota	*Cryptococcus*	3	–	–	–	–	–	–	–	3	–	–	–
	Kwoniella	1	–	–	–	–	–	–	–	1	–	–	–
	Rhodosporidium	1	–	–	–	–	–	–	–	1	–	–	–
	Rhodotorula	4	–	–	–	–	–	–	–	4	–	–	–
	Sporidiobolus	1	–	–	–	–	–	–	–	1	–	–	–
	Trichosporon	1	–	–	–	–	–	–	–	1	–	–	–
	Total	11	0	0	0	0	0	0	0	11	0	0	0

[a] Total number of strains belonging to the specified genus.

growth (for all the strains) at 20, 30, 40, 50 and 60 °C is shown in Fig. 3. Results presented in this figure were tremendously revealing. Mesophilic strains growing optimally at 20, 30 or 40 °C lost 100% viability at 50 and 60 °C, and reduced growth between 12 and 50% when they grew at temperatures other than optimal. Thermophilic strains only grew optimally at 40 and 50 °C. No thermophilic strain was able to grow at 60 °C. These strains lost 100% viability at 20 and 30 °C and their growth were decreased by 18 to 59% when cultured at suboptimal temperatures. Finally, thermotolerant strains were able to grow optimally at all the assayed temperatures (even at 60 °C). Inhibition of growth at suboptimal temperatures was really variable ranging between 8 and 95%; however, regardless of the value for optimal temperature of growth, there were always thermotolerant strains growing at the rest of assayed temperatures. Results presented in Fig. 3 were indicative of the high thermal plasticity characterizing composting microbiota.

Thermotolerance is surely the reason why there is such an enormous variability in reports about composting microbiota, from those that collect a huge amount of results produced by others (Ryckeboer et al., 2003a) to those including specific data from processes related to that here reported (Chandna et al., 2013).

Though under laboratory conditions, 'composting' trials carried out by Xiao et al. (2011a) at different temperatures, including uninterrupted or continuous thermophilic composting (CTC), showed a pattern for bacteria (as a group) indicative of a high level of thermotolerance; however, the authors classified the microbiota only as mesophilic or thermophilic, since no attempt was made to identify microorganisms. The selection of those species well adapted to restrictive thermal conditions is evident in that report and serves to highlight the thermal plasticity of the indigenous microbiota present in organic wastes, which coincides with the results reported here. The vast microbial biodiversity present in composting and the variability between facilities using different operative strategies suggest a cautious interpretation of results produced in studies relative to composting microbiota (Hultman et al., 2010; Partanen et al., 2010); however, even trying not to be assertive, the real fact is the results here presented clearly show a very specific pattern of selection of the thermotolerant microbiota that definitively will be in charge of producing the final compost.

A concluding analysis was carried out to verify if the results obtained from quantitative growth tests at different temperatures allowed the correct sorting and the interrelation between the thermal profiles of the studied strains, as an additional proof of the suitability of the results to support the conclusions reached. Fig. 4a shows a cluster analysis in which temperatures employed were hierarchically ordered using the growth levels of all strains to build the dendrogram. As can be seen, strictly mesophilic temperatures (20 and 30 °C) grouped together, as did the strictly thermophilic ones (50 and 60 °C), leaving 40 °C in an intermediate place as it is the transition temperature between thermal categories.

On the other hand, Fig. 4b shows an identical cluster analysis in which thermal profiles of all strains were grouped according to microbial growth levels reached at each of the assayed temperatures. Essentially, thermal profiles belonging to 'pure' thermal categories (mesophilic or thermophilic) grouped together respectively, while two thermotolerant profiles (1-1-1-1-0 and 1-1-1-1-1) grouped close to mesophilic and the other two thermotolerant profiles (0-1-1-1-0 and 0-1-1-1-1) grouped jointly with the thermophilic ones. Particularly this cluster analysis shows how the results obtained support the belonging of the studied strains to each of the established thermal profiles and how these profiles are related to each other following a given order according to the thermal categories in which they are included. These dendrograms can be considered very illustrative indeed.

3.4. Microbiological insight into thermotolerance throughout composting

Since the identity of all strains was known, it was possible to ascribe thermal profiles and categories to the different taxonomic groups in which each strain was included. The relative abundance of thermal profiles and categories ordered by *Phylum* is shown in Fig. 5a. Five *phyla* included all the strains here studied: *Actinobacteria* (225 strains), *Firmicutes* (744 strains) and *Proteobacteria* (155 strains) among prokaryotes, and *Ascomycota* (244 strains) and *Basidiomycota* (11 strains) among eukaryotes. A sole strain belonging to the *Phylum Bacteroidetes* was discarded and not included in the graphs as it was considered not representative enough. As shown in Fig. 5a, and following the plotline of this study, thermotolerant microbiota constituted the vast majority of strains included in every *phylum*. *Actinobacteria* have usually been considered a widely represented group in composting processes and a good biological indicator of the correct biotransformation

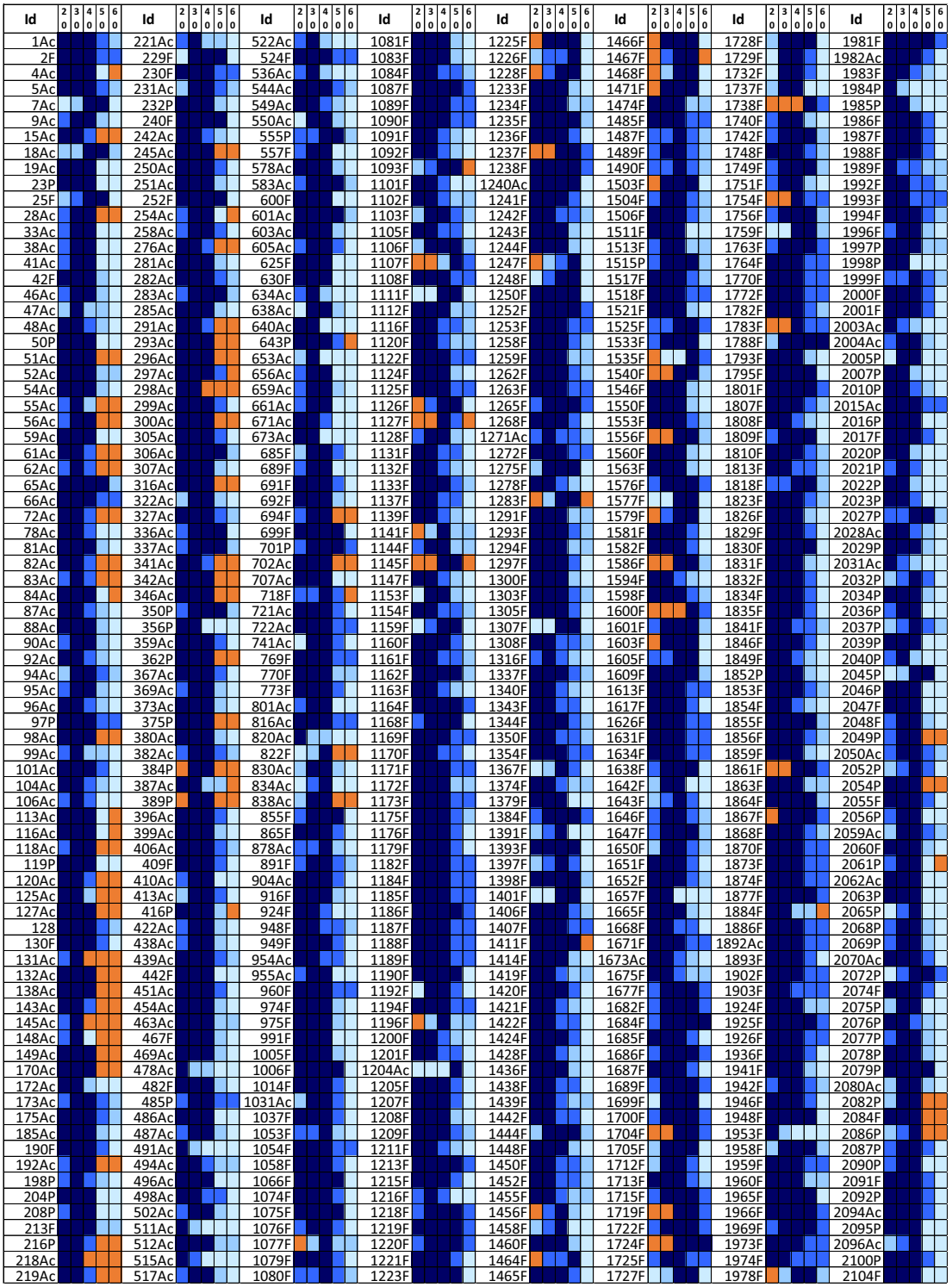

Fig. 2. Pictogram representing growth (as per the quantity of reduced resazurin) of the strains at the tested temperatures. 0–25% (■), 25–50% (■), 50–75% (■), 75–100% (■) and no growth (■). Percentages are referred to growth at optimal temperature (100%) in each row. Id numbers are followed by a letter indicating the *phyla* to which the strain belongs (Ac: *Actinobacteria*, F: *Firmicutes*, P: *Proteobacteria*, As: *Ascomycota*, B: *Basidiomycota*).

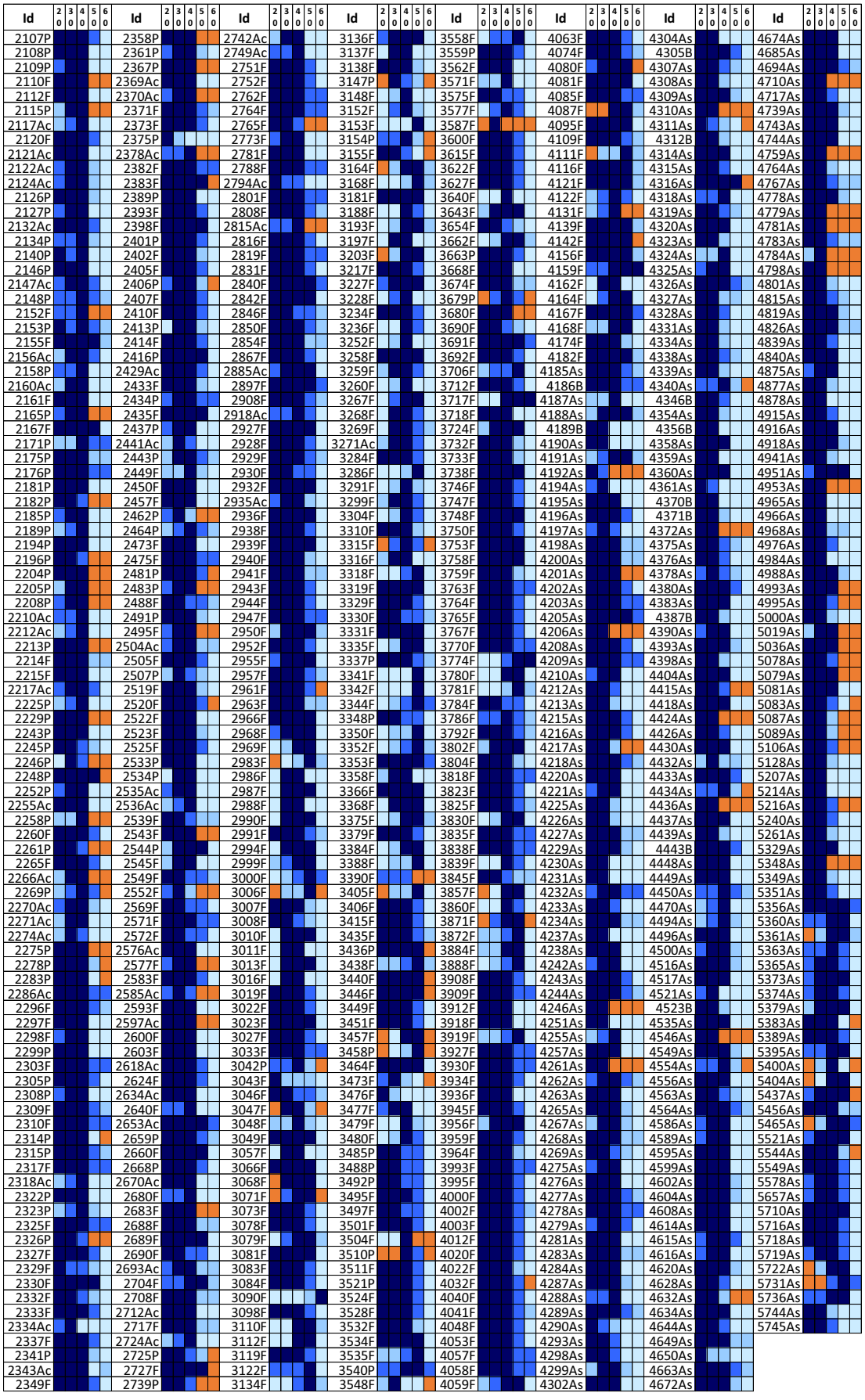

Fig. 2 (*continued*).

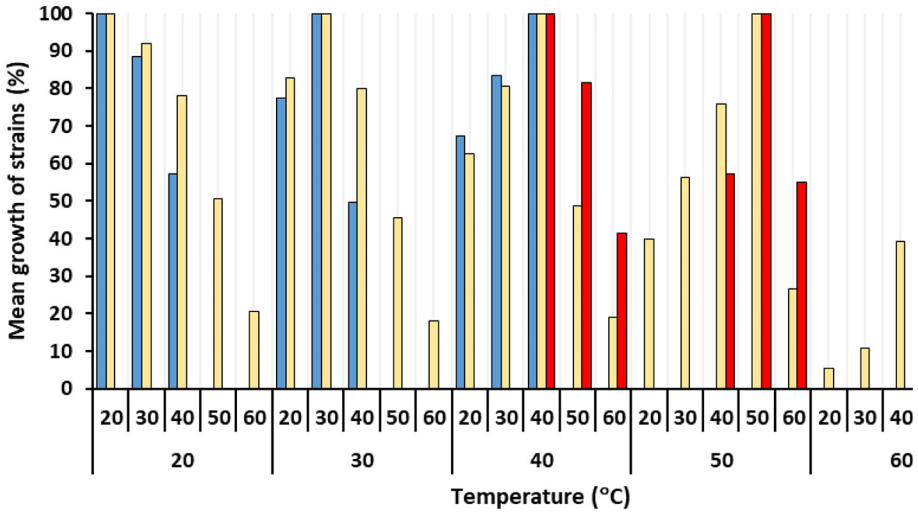

Fig. 3. Mean growth of all the strains at the tested temperatures grouped by optimal temperature of growth and thermal categories. Mesophilic (■), thermotolerant (■) and thermophilic (■) strains.

of organic matter (Xiao et al., 2011b). Traditionally, this group has been associated with the final stage of the composting process, due to its low growth rate and its ability to degrade more recalcitrant substrates (Franke-Whittle et al., 2009). Other studies, however, have demonstrated that *Actinobacteria* can be isolated throughout the whole process (López-González et al., 2015a). Results obtained in this study showed that *Actinobacteria* were represented by the highest number of species (68) and showed to be mostly thermotolerant (180 out of 225 strains). Other strains (45) were classified as mesophilic (Table 2, Fig. 5a). It is important to remark that most of the thermotolerant *Actinobacteria* were able to grow at all assayed temperatures, including 60 °C (173 strains). Actinobacterial genera *Microbacterium*, *Brevibacterium* and *Rhodococcus* accounted for the highest number of fully thermotolerant strains (1-1-1-1-1; Table 2).

Non-filamentous Gram-positive bacteria (*Phylum Firmicutes*) are usually considered the group best represented in composting processes (Ryckeboer et al., 2003b; Karadag et al., 2013). Typical characteristics related to their persistence in hostile habitats, the inclusion of spore-forming species and their relatively high growth rate make this group find composting environment a suitable place to colonize, proof of which is the fact that more than half of the strains included in the study collection belonged to this *phylum*. As shown in Fig. 5a, *Firmicutes* exhibited 9 out of the 11 thermal profiles and accounted for a sizeable majority in almost all of them. It was also the group containing the largest number (744) of strains, distributed among 59 species, mostly belonging to the genus *Bacillus* and related genera (*Paenibacillus*, *Terribacillus*, and *Ureibacillus*) (Table 2).

Thermotolerance was a fact in *Firmicutes*. It should be noted that, out of 744 strains, 676 were able to grow at 60 °C, and 662 showed to be fully thermotolerant () (Table 2). A special mention deserves the genus *Bacillus* with 505 thermotolerant strains (Table 2), and particularly remarkable was the species *Bacillus licheniformis* with 220 strains

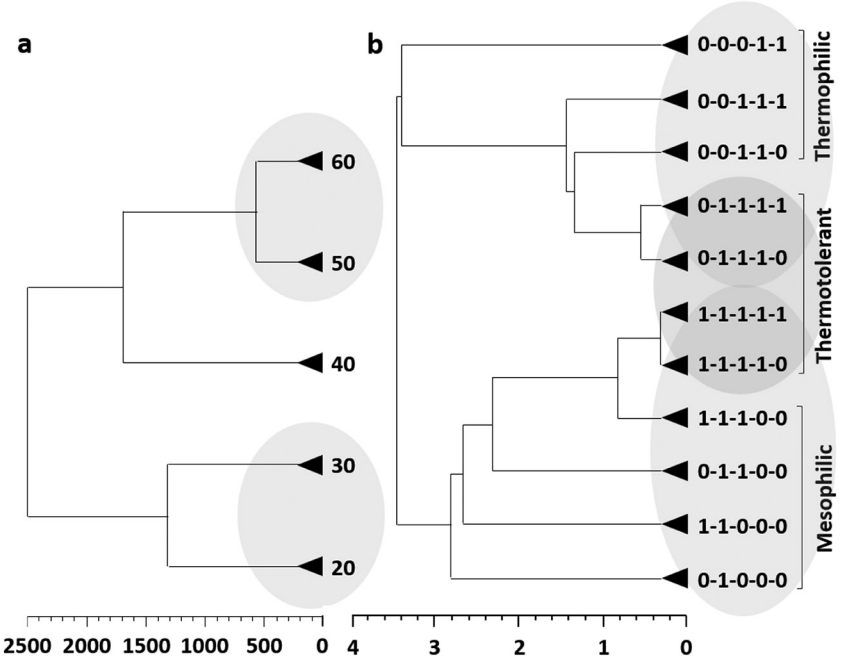

Fig. 4. Hierarchical representation of the tested temperatures (a) and thermal profiles (b) obtained from analysis of conglomerates based on growth levels of the entire strain collection. Nearest Neighbor method and Squared Euclidean metric distance were used for clustering.

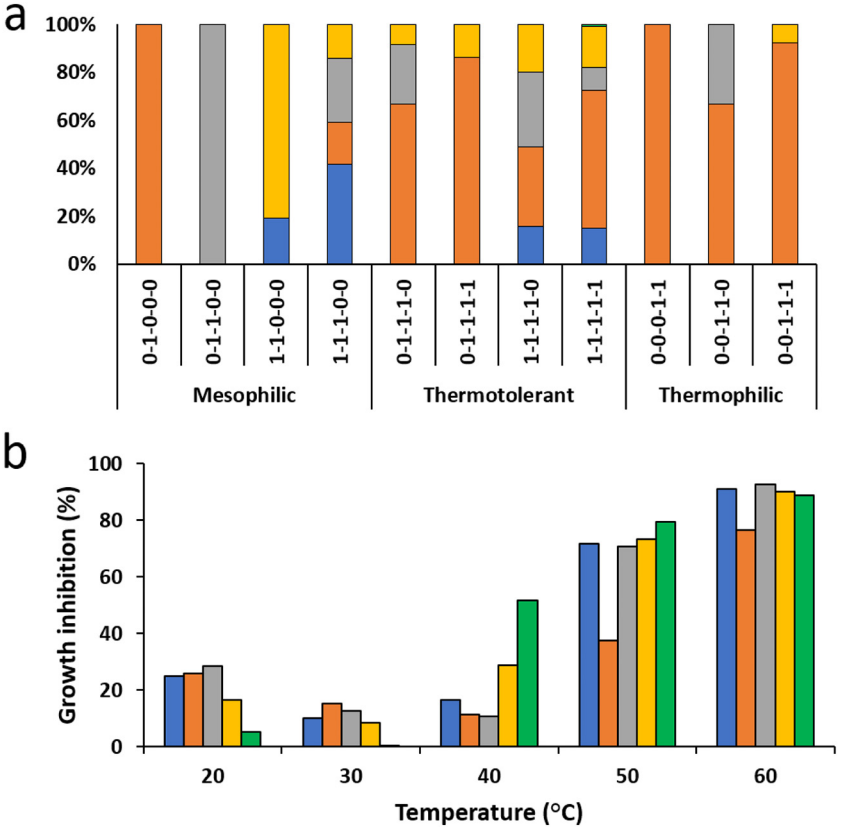

Fig. 5. Distribution of *phyla* grouped by thermal profile (a) and growth inhibition at non-optimal temperatures (b) in prokaryotic and eukaryotic strains. Graph columns are for *Actinobacteria* (■), *Firmicutes* (■), *Proteobacteria* (■), *Ascomycota* (n) and *Basidiomycota* (■).

classified in the thermal profile 1-1-1-1-1. Since *Bacillus* and related genera are spore formers and synthesize a broad assortment of biodegradative enzymes, their persistence throughout the different thermal stages of composting should not be considered surprising (Partanen et al., 2010; Jurado et al., 2014).

In regard to *Phylum Proteobacteria*, although its members participate in an ample variety of metabolic strategies and are a group closely related to the composting environment, they have often been associated with the initial stages of the process, and as soon as composting progresses, they lose preeminence (de Gannes et al., 2013; Tian et al., 2013). *Proteobacteria* was the smaller group of prokaryotes in the study collection (155 strains). Though mostly thermotolerant (126 strains), members of this *phylum* were found in the three thermal categories (Fig. 5a). Proportionally they were less abundant than other prokaryotic *phyla*, but even so, 106 strains showed to be fully thermotolerant and hence they were able to grow even at 60 °C. It is undeniable that Gram-negative bacteria are less resistant than Gram-positive to environmental factors (Russell, 2003). That is surely the reason why *Proteobacteria* were present in the composting piles to a lesser extent than *Firmicutes* or *Actinobacteria*; however, the members of this *phylum* inhabiting composting piles must have adapted efficiently to thermal variations and high competition for nutrients (Cai et al., 2018; Saarinen et al., 2018). Two genera of *Proteobacteria* deserves a special mention, *Chelatococcus* and *Pseudomonas*, as they were the best represented in composting piles (Table 2). In addition, a strain of *Chelatococcus daeguensis* proved to be strictly thermophilic with optimal growth at 50 °C. Among *Proteobacteria*, thermophilia has rarely been reported. One of the strains included in this study and identified as *Thermovum composti* (Table 2) has previously been described as thermophilic (Yabe et al., 2012). Thermal tolerance has been attributed to other Gram-negative bacteria such as *Klebsiella* spp. and *Pseudomonas* spp. (Caplenas and Kanarek, 1984; Manaia and Moore, 2002). Results related to the thermal behavior of *Proteobacteria* were surprising because this group has traditionally been considered strictly mesophilic. Perhaps the work carried out by Gram-negative bacteria in the composting process is not restricted to that of simple bystanders with little or no potential activity. Therefore, a reassessment of the role that Gram-negative bacteria plays in the composting process could be necessary.

Eukaryotic *phyla* were represented by *Ascomycota* and *Basidiomycota*, being the former more abundant (244 strains) than the latter (11 strains). According to results shown in Table 2, most strains of *Ascomycota* were thermotolerant (212) and 198 exhibited the thermal profile 1-1-1-1-1. On the other hand, all the 11 strains of *Basidiomycota* were fully thermotolerant. *Ascomycota* strains were distributed in all thermal categories, including strictly thermophilic (Fig. 5a). There was even a strain of *Aspergillus fumigatus* that grew optimally at 50 °C (Table 2). *Basidiomycota* proved to be thermotolerant (Fig. 5a), and although the 11 strains could survive at 50 and 60 °C, it was at the cost of a significant decrease in growth (Fig. 2). *Aspergillus*, *Penicillium* (*Ascomycota*) and *Rhodotorula* (*Basidiomycota*) were the most abundant genera among fungi. The presence of fungi in the composting piles is, in general, less copious than that of bacteria in terms of colony counts ($cfu \cdot g^{-1}$), though fungi play an important role in allowing the process to be successfully completed (López-González et al., 2015b). Results obtained for eukaryotes were even more remarkable than those for prokaryotes, since a higher thermal sensitivity in composting processes has traditionally been ascribed to fungi (Hassen et al., 2001).

To better understand the extent to which sub-optimal temperatures affect the growth of the different taxonomic groups constituting composting microbiota, the graph in Fig. 5b shows levels of growth inhibition at the tested temperatures sorted by *phylum*. As previously indicated, bacterial *phyla* showed to grow optimally at 40 °C though not significant differences could be found with growth at 30 °C. In contrast,

as expected, fungi showed an optimum growth temperature of 30 °C. Thermophilic temperatures (50 and 60 °C) were those that more sharply compromised microbial growth. Of special incidence was the case of the highest temperature of the study (60 °C), which reduced microbial growth by 75–90% in all the studied isolates. Analyzed by *Phylum*, the temperature of 20 °C showed a very similar influence between the three large groups of bacteria detected in the process (*Firmicutes*, *Proteobacteria* and *Actinobacteria*), reducing growth by around 25%. *Firmicutes* was the group in which the best values of thermal plasticity were obtained, since it was less affected by the thermophilic temperatures (37.57% of growth reduction at 50 °C and 76.7% at 60 °C). These values fit with the thermotolerant behavior associated with this *phylum* (Kuok et al., 2012) and provide reasons to classify this group as resident microbiota of the process. In *Actinobacteria*, the growth profiles were similar to those obtained for *Proteobacteria*. In this sense, and unlike *Proteobacteria*, there are numerous references on the importance of this bacterial group in composting processes (Mokni-Tlili et al., 2011; Wei et al., 2019) as a proof of its adaptability to thermal and nutrients changes. The analysis of thermal plasticity in fungi revealed that *Ascomycota* was the most resistant to temperatures above 40 °C, although both *Ascomycota* and *Basidiomycota* severely restricted their growth above 50 °C. In fact, what really surprised was the ability of this group to survive (and grow) at such a wide range of temperatures, thus ensuring its persistence during composting.

3.5. Succession pattern of composting microbiota based on thermotolerance

In the end, and for the purpose of this work, what really matters is the transition sequence of microbial populations through composting and there is no doubt that thermotolerance has a preeminent role in this regard. Through the study here performed, the complete microbiome of a composting process has been investigated in relation to the temperature range at which its components can grow. Other aspects have also been considered, such as the temporal presence and prevalence of each strain throughout the process and the taxonomic position of all strains.

What traditionally has been taught is that there exists a sequence of microbial successions governed by the temperature inside the composting piles, in such a way that a microbial group substitutes to another and so on, as a function of the mesophilic or thermophilic stages. This knowledge is deeply rooted in the scientific community. Examples of this are the contents of important textbooks (Paul, 2007; Insam and de Bertoldi, 2007) and many reviews or specific articles (McKinley and Vestal, 1984; Fogarty and Tuovinen, 1991; Sánchez et al., 2017; Liu et al., 2018).

The term succession, at least in this context, implies that each time the temperature experiments a change, the microbial populations existing inside the pile disappear (or radically diminish) and are replaced by others better adapted to grow at the new thermal conditions. Raw materials, at the beginning, and the surrounding environment later, feed the piles with the suitable microbes at each moment. In addition, as some of the present microorganisms are spore-formers, they can remain inactive in the material under adverse conditions and recolonize it when favorable growth conditions are restored.

As previously mentioned, temperature is a paradoxical factor that acts as cause and effect. It is the consequence of microbial metabolism and, at the same time, is a factor that exerts a tremendous pressure on the microbial viability. In response, composting microbiota has developed a unique and highly valuable tool: thermotolerance.

Although tangentially, some reports have addressed the thermotolerant microbiota of composting in the last 30 years. McKinley and Vestal (1984) highlighted the important role of what they called facultative thermophilic microorganisms in the composting process. Nakasaki et al. (1985) reported the existence of three strains of spore-forming bacteria whose vegetative forms were able to form colonies at 60 °C, although they showed no respiratory activity. Zhao et al. (2017) revealed the use of four actinobacterial strains as cellulolytic inoculants that grew in both the mesophilic and thermophilic ranges. These are just a few examples, but no reports were found in which the phenomenon of thermotolerance in the entire microbiota associated with composting was comprehensively investigated, as was done in this work.

Knowing that most of the composting microbiota is thermotolerant has an added-value. Apart from the conceptual implications related to the way in which composting proceeds and the role played by the responsible microorganisms, other consequences can be considered. In this sense, an enormous biotechnological potential could be exploited if the composting microbiota is considered as an enzymatic factory capable of producing large quantities of different enzymes functional over a wide range of temperatures and the consequent industrial and economic benefit that would derive from this (Antunes et al., 2016).

Finally, the existence of alternating thermal phases throughout composting is undeniable, however, from results here presented, it would be reasonable to think that most microorganisms involved in composting are eurythermal, mostly thermotolerant. It is possible that the universally accepted paradigm that ascribes general microbial groups to the composting stages established as a function of temperature (i.e. mesophilic, thermophilic) needs to be modified. This modification would not affect the designation of the composting stages, but rather the composition of the associated microbial populations whose separation by growth temperature would not be so evident.

4. Conclusions

Composting evolves through different thermal stages that alternate between mesophilic and thermophilic temperatures. Contrary to what has traditionally been accepted, this study demonstrates that composting microbiota has a thermal plasticity that allows it to persist and grow throughout the process. The microbial aerobic use of carbon sources and heat generation are firmly linked events. Thus, it should not be surprising that the microorganisms associated with composting are well adapted to the temperature variations that occur as a result of their own biological activities. This study shows that 90% of the composting microbiota grows at an ample range of temperatures (20 to 60 °C) irrespective of their taxonomic position. In this sense, especially noteworthy was the unusual thermotolerance observed in many gram-negative bacteria and fungi. Thermal plasticity also contributes to consider microorganisms associated to composting as a true microbiome perfectly adapted to the ever-changing conditions imposed by the process.

Data availability

Dataset related to this article can be found at http://dx.doi.org/10.17632/2bk2p87rv5.2, an open-source online data repository hosted at Mendeley Data (Moreno et al., 2020).

CRediT authorship contribution statement

J. Moreno: Project administration, Conceptualization, Investigation, Writing – review & editing. **J.A. López-González:** Methodology, Conceptualization, Investigation, Writing – original draft. **M.A. Arcos-Nievas:** Investigation. **F. Suárez-Estrella:** Conceptualization, Investigation. **M.M. Jurado:** Investigation. **M.J. Estrella-González:** Investigation. **M.J. López:** Conceptualization, Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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