MICROBIALLY-ENHANCED COMPOSTING OF OLIVE MILL SOLID WASTE (WET HUSK): BACTERIAL AND FUNGAL COMMUNITY DYNAMICS AT INDUSTRIAL PILOT AND FARM LEVEL

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Abstract

Bacterial and fungal community dynamics during microbially-enhanced composting of olive mill solid waste (wet husk), used as a sole raw material, were analysed in a process carried out at industrial pilot and at farm level by the PCR-DGGE profiling of the 16 and 26S rRNA genes. The use of microbial starters enhanced the biotransformation process leading to an earlier and increased level of bacterial diversity. The bacterial community showed a change within 15 days during the first phases of

composting. Without microbial starters bacterial biodiversity increased within 60 days. Moreover, the thermophilic phase was characterized by the highest bacterial biodiversity. By contrast, the biodiversity of fungal communities in the piles composted with the starters decreased during the thermophilic phase. The biodiversity of the microbial populations, along with physico-chemical traits, evolved similarly at industrial pilot and farm level, showing different maturation times.

Keywords: olive mill solid waste, wet husk, bacterial and fungal diversity, microbial dynamics, PCR-DGGE.

1. Introduction

Composting and co-composting of olive mill solid waste (wet husk) is receiving an increasing attention (Alfano et al., 2008; Hachicha et al., 2009), and the resulting compost has recently been shown to improve nutraceutical traits of horticultural crops (Ulrichs et al., 2008) and to represent a fertilizer for short-term crops (Altieri and Esposito, 2010). Detoxification of fats, organic acids and polyphenols is achieved throughout the process, resulting in an odourless product with a good germination and humification index (Echeverria et al., 2011). These results are due to the biotransformation activity of microorganisms, leading to a rapid succession of specialized bacterial populations during co-composting (Federici et al., 2011). Various attempts have been carried out to identify the microbial species and to enumerate the physio-taxonomical groups (bacteria and fungi) during the three phases of husk composting or co-composting, namely the activation, thermophilic, and maturation

45 phase, using cultivation-dependent methods. However, the results are erratic and
46 provide underestimates due to the constraints of culture media and cultivation
47 conditions (Principi et al., 2003; Bru-Adan et al., 2009), as well as to the presence of
48 microbial communities in viable but non-culturable state.
49 Culture-independent approaches are becoming prominent to study microbial
50 communities structure and dynamics, and molecular methods such as PCR-DGGE have
51 been used to analyze microbial biodiversity during the composting process of different
52 matrices (Novinscak et al., 2009; Takaku et al., 2006; Zhang et al., 2011). According to
53 such studies, the microbial communities are highly variable during the various phases of
54 the composting process and tend to get stabilized at compost maturity. This approach
55 has been recently used by Vivas et al. (2009) to analyze the bacterial community
56 structure in the final vs. in the initial matrix of a mixure of fresh olive waste and sheep
57 manure processed by co-composting or vermicomposting. They found that the bacterial
58 diversity was markedly affected by vermicomposting but not by co-composting. The
59 use of starter cultures to speed up the composting process or to obtain improved
60 compost has been controversial for long time, probably due to the complexity of the
61 physical-chemical and biological events occurring during the process (Vargas-Garcia et
62 al., 2006). Indeed, the selection of appropriate microbial strains can represent a valid
63 alternative to the traditional treatment of wet husk, as it improves both
64 biotransformation speed and the quality traits of the final product (Echeverria et al.,
65 2012).
66 This study aims at profiling the fungal and bacterial communities during a composting
67 process of olive mill solid waste (wet husk) as a sole raw material enhanced by the use
68 of microbial starters at industrial pilot (representing the best biotransformation scenario)

and at farm level (with limited process control facilities, thus representing the worst case scenario for the disposal and upgrade of wet husk through composting). The variability and the diversity of the microbial community were estimated by UPGMA analysis of the PCR-DGGE profiles and by the community diversity indices analysis.

2. Materials and Methods

2.1. Composting procedure, sampling and physical-chemical analyses

The microbial starters used throughout this study were *Bacillus amyloliquefaciens* subsp. *plantarum* M51/II [formerly *B. subtilis*, reclassified according to Chen et al. (2009) and Borriss et al. (2011)], *Pseudomonas synxanta* 3/2, *Pseudomonas fluorescens* 19/5, *Serratia marcescens* B2 (bacteria), *Streptomyces* sp. ATB 42, *Streptomyces* sp. AC 3, *Streptomyces* sp. AC 20, *Streptomyces* sp. AB 11 (actinobacteria), *Candida butyri* 8(4), *Rhodotorula mucillaginosa* 4(1), *Sporopachydermia lactativora* 2(3) (yeasts), and *Arthrobotrys oligospora* DSMZ 2023, *Chaetomium globosum* Ch 10, *Phanerochaete chrysosporium* ATCC 42538, *Trichoderma atroviride* T14 (microfungi). The microbial strains used in this study were isolated from wet husks (originated from the oil extraction process of olives cv, Leccino, Moraiolo, and Frantoio), except for *A. oligospora* DSMZ 2023, obtained from German Collection of Microorgamisms and Cell Cultures, and *P. chrysosporium* ATCC 42538, obtained from American Type Culture Collection.

The use of microbial starters for the biotransformation of wet husk was the one described by Echeverria et al. (2012) for both industrial pilot and farm level. At the industrial pilot level, the composting was run for 90 days with 1.2t of wet husk,

maintaining the humidity of the piles (200 kg each) without starters (A, B, C) and with starters (D, E, F) at 40-60%. During the composting period, the piles were mechanically turned every time the internal temperature reached or exceeded 55-60°C to allow aeration and to decrease temperature.

At farm level the composting process was carried out by Cooperativa Arnasco in Arnasco (Savona, Italy) in an olive oil mill that serves a 25–30 year-old 40 ha olive grove (cultivars Taggiasca and Pignola). Twenty tons of wet husk from a two-phase decanter extraction process were mixed with the starters, added with 1 ton of olive wood chips to maintain aereation and placed in a windrow pile. The mechanical turning of the pile was done occasionally, depending on the external temperature. Sampling was carried out in compliance with the methods of the Italian Ministry of Agriculture (Legislative Decree 75/2010) at time 0, 15, 60 and 90 days for composting at industrial pilot, and at time 0, 35, 100, 145 and 200 days for composting at farm level. The temperature profile of the piles and the physical-chemical traits of the samples from the industrial pilot composting process were reported in a previous paper (Echeverria et al., 2012). The methods to determine the physical-chemical traits of samples from the farm scale composting process, to extract the humic substances and to express the humification index (HI) are those described by Echeverria et al. (2011).

2.2. Microbial cultures, DNA extraction and PCR amplification

DGGE markers were prepared with the same microbial strains as the starters with the addition of *Streptomyces* sp. ABT42, *Sporopachydermia lactativora* 2(3), *Chaetomium globosum* ChT3. Bacteria and actinobacteria were maintained on Nutrient agar (Oxoid, Milan, Italy), yeasts and fungi on Malt agar (Oxoid, Milan, Italy).

Genomic DNA was extracted from bacteria, actinobacteria and yeasts liquid cultures grown overnight at 25 ° C using "MasterPure™ Yeast DNA Purification" (Epicentre) according to the manufacturer's protocols. For the DNA extraction from fungi, the mycelium grown on plates was resuspended with 10 mL deionized water. 2 mL of suspension were inoculated in 40 mL of Malt Broth for an overnight incubation on a rotatory shaker at 25 °C. After harvest by centrifugation at 10,000 rpm and 3 washes with $MgCl_2$ 0,1M, 400 mg of mycelium (f.w.) were transferred into a 2 mL Eppendorf tube and crushed. The other steps were the same as for bacteria and yeasts.

DNA extraction from compost samples was carried out using the "PowerSoil™ DNA Isolation Kit" (Mo-Bio Laboratories San Diego, CA) according to manufacturer's instructions. For the analysis of the bacterial community, the amplification of the variable region V3-V5 of 16S rDNA was carried out using the primers 341F (CCTACGGGAGGCAGCAG) and 907R (CCGTCAATTCCTTTRAGTTT) (Yu and Morrison, 2004). The primer 341F had at its 5' end an additional 40-nucleotide GC-rich tail (5'CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3'). Amplification reaction was prepared in a final volume of 50 µL, using 1 µL of extracted DNA diluted 1:100, 10 µL of 5 x Phusion™ HF Buffer (Finnzymes), 1U of Phusion™ High-Fidelity DNA polymerase (Finnzymes), 0.2 mM of each dNTPS (GeneAmp dNTP Mix, Applied Biosystem), 0.5 µM of each primers (Primm) and 3% DMSO (Finnzymes). The fragment obtained is 560 bp long. The reaction was carried out using an iCycler-iQ Multicolor Real-Time PCR Detection System (Biorad) with the following denaturation, amplification and extension procedure: 98 °C 30 sec; 98 °C 10 sec, 52 °C 10 sec, 72 °C 15 sec for 35 cycles; 72 °C 10 min. The presence of amplicons was confirmed by electrophoresis in 1.5% (w/v) Agarose I (Euroclone) in TBE 1 x buffer

(Euroclone) gels stained with ethidium bromide 0.5 µg mL^{-1}. All gels were visualized and captured as TIFF format file by Liscap program for Image Master VDS system (Pharmacia Biotech). For the analysis of fungal community, the amplification of the variable region D1-D2 of 26S rDNA was carried out using the primers NL1 (GCATATCAATAAGCGGAGGAAAAG) and LS2 (ATTCCCAAACAACTCGACTC) (Cocolin et al., 2000). The primer NL1 had at its 5' end an additional 40-nucleotide GC-rich tail. Amplification reaction was prepared in a final volume of 50 µL, using 2 µL of extracted DNA diluted 1:10, 10 µL of 5 x Phusion™ HF Buffer (Finnzymes), 1U of Phusion™ High-Fidelity DNA polymerase (Finnzymes), 0.2 mM of each dNTPS (GeneAmp dNTP Mix, Applied Biosystem), 0.5 µM of each primers (Primm) and 3% DMSO (Finnzymes). The fragment obtained is 250 bp long. The reaction was carried out using an iCycler-iQ Multicolor Real-Time PCR Detection System (Biorad) with the following denaturation, amplification and extension procedure: 98 °C 30 sec; 98 °C 10 sec, 48 °C 10 sec, 72 °C 15 sec for 35 cycles; 72 °C 10 min. The presence of amplicons was confirmed with the same procedure as for bacterial communities.

2.3 DGGE analysis of bacterial and fungal community

The amplicons were analyzed using the DCode™ Universal Mutation Detection System BIORAD. 20 µL of the PCR products plus 20 µL of buffer 2 x made with 70% glycerol, 0.05% xylene cyanol and 0.05% bromophenol bleu were loaded on a 8% polyacrylamide-bisacrilamide (37.5:1) gel with a urea-formamide denaturing gradient ranging 30% to 65% (prokaryotes) and 20% to 75% (eukaryotes). A composite mix of

bacterial 16S rRNA gene fragments from *Pseudomonas synxanta* 2/3, *Bacillus amyloliquefaciens* subsp. *plantarum* M51/II, *Serratia marcescens* B2, *Streptomyces* sp. AB11, *Streptomyces* sp. AC20 and a composite mix of fungal 26S rRNA gene fragments from *Sporopachydermia lactativora* 2(3), *Candida butyri* 8(4), *Phanerochaete chrysosporium* ATCC42538, *Trichoderma atroviride* T14, *Chaetomium globosum* ChT3 were added on each side and in the center of DGGE gels as reference DGGE patterns. Gels were run at 90 V and 60 °C for 16 hours and stained for 30' in 500 mL of TAE 1 x buffer containing 50 µL of Sybr Gold Nucleic Acid Gel Stain (Molecular Probes, Invitrogen). The profiles were visualized under UV illumination and captured as TIFF format file by Liscap program for Image Master VDS System (Pharmacia Biotech). Band patterns in different DGGE lanes were compared with the ImageMaster 1D Elite v3.00 software (Pharmacia Biotech). The lanes were normalized to contain the same amount of total signal after background subtraction and the gel images were straightened and aligned to give a densitometric curve. Bands were assigned and matched automatically and then checked manually. Band positions were converted into Rf values between 0 and 1, and profile similarity was calculated by determining Dice's similarity coefficients for the total number of lane patterns from the DGGE gel. The similarity coefficients calculated were then used to generate the dendrograms utilizing the clustering method UPGMA (Unweighted Pair Group Method Using Arithmetic Average). DGGE banding data were used to estimate four different indices treating each band as an individual operational taxonomic unit (OTU). Richness (*S*) indicates the number of OTUs present in a sample and is determined by the number of fragments. The overall diversity index of Shannon-Weaver (*H*) and the dominance index of Simpson (*D*) were calculated using the equations $H = -\sum(P_i \, x \, lnP_i)$ and $D =$

$\sum P_i^2$ respectively, where the relative importance of each OTU is $P_i = n_i/N$ and n_i is the peak intensity of a band and N is the sum of all peak intensities in a lane. Evenness index (E), which allows the identification of dominant OTUs, was calculated as $E = H/lnS$.

2.4 Statistical analysis

The data represent the means of three replicates (n=3). The results of physical-chemical traits were subjected to one-way analysis of variance (ANOVA) with time as a variability factor. A two-way ANOVA test was applied to diversity indices in which treatment and time are the source of variability. The means were compared by using Least Significant Difference test ($P < 0.05$). Analyses were carried out with the CoStat 6.4 program (CoHort software).

3. Results and Discussion

3.1 Composting at industrial pilot level

3.1.1 *Community dynamics and microbial diversity of wet husk composted with and without starters*

DGGE profiling of PCR- rDNA fragments was used to study the dynamics and the diversity of the bacterial and fungal communities during the composting process of wet husk with and without microbial starters.

The DGGE profiles obtained were analysed considering each fragment as a species or an individual group of species having 16S rDNA sequences with similar melting

behavior, while the band intensity indicated the relative abundance of the species. The variability and the diversity of the microbial community during the composting process were estimated by UPGMA analysis of the PCR-DGGE profiles; moreover, the community diversity indices were determined.

For the bacterial community, the diversity indices derived from the analysis of the DGGE profiles of the samples collected during the composting process of wet husk are reported in Tab. 1. The increase of Richness (*S*) and Shannon-Weaver (*H*) indices and the decrease of the dominance index of Simpson (*D*) indicated that in the piles without starters biodiversity increased slightly at the 60^{th} day, while in the piles with starters biodiversity increased significantly in the first 15 days. Similar results are evidenced by the dendrogram of Figure 1a, which shows the evolution of the bacterial community in the piles composted without starters: two main clusters can be identified, each formed by two subclusters. The first cluster spans over time 0 to 15 days and the second over 60 to 90 days. These results suggest a gradual change in the bacterial community, which explains the absence of a true thermophilic phase (temperature above 45°C) in the piles without starters.

The dendrogram reported in Fig. 1b shows the evolution of the bacterial community in the piles composted with starters: in this case the bacterial community shows a change during the first phases of composting, leading to a higher diversity at day 15. This result indicates that the use of starters affects the structure of bacterial community during the early phase of composting (*i.e.* activation), which corresponds to an increase in temperature (from 25°C to 60°C). The increased biodiversity remained high throughout the thermophilic phase (*i.e.* $\geq$ 50°C at days 14-27, including the sanitization peak at 55-60°C), and throughout the cooling and maturation phases. This finding confirms the

233 hypothesis (Federici et al., 2011) that high diversity in the thermophilic phase is a
234 typical trait of the biotransformation of wet olive husk. This could be due to the oil
235 content of this matrix, that lead to a thermal protective effect on the bacterial population
236 (Senhaji, 1977; Ababouch and Busta, 1987). Indeed, Principi et al.(2003), when
237 investigating the microbiological aspects of humid husk composting, observed that the
238 density of mesophilic microorganisms remained unchanged from the beginning to the
239 end of the process.
240 On the contrary, in the composting process of other raw materials a different trend was
241 found. Ishii et al. (2000) evaluated the microbial succession during a garbage
242 composting process by using DGGE and observed fewer bands when the temperature
243 increased. Similarly, Takaku et al. (2006) reported that the DGGE patterns drastically
244 changed during a garbage composting with rice hull as a bulking agent and found fewer
245 bands when the temperature increased.
246 As to the fungal communities, in the piles without starters the diversity indices (Tab. 2)
247 showed a higher biodiversity in the first 15 days. Subsequently, we observed a
248 decreasing biodiversity up to 90 days, as evidenced by the corresponding decrease of
249 the Richness (*S*) and Shannon-Weaver (*H*) indices and by the increase of the dominance
250 index of Simpson (*D*) (see indices at 60 and 90 days in Tab 2). In the relative
251 dendogram (Fig. 2a), two main clusters with a low similarity can be identified: the first
252 one is represented by the piles at 0 and 15 days, the second by the piles at 60 and 90
253 days. Moreover, cluster analyses reflect the trend of biodiversity indices, showing that
254 the two sub-cluster at 0 and 15 days are more similar than those at 60 and 90 days.
255 These results indicate a gradual shift of the fungal community up to 90 days, with a

decrease in biodiversity indices and in the intensity of fragments indicating the stabilization of the composted matrix in agreement with the humification index values. Considering the intensity of fragments from DGGE profiles of piles composted with starters (Fig. 2b), different groups of more active fungal communities can be identified. In the mesophilic phase at time 0, there are fragments with high intensity which are different from those of the following sampling times. In the thermophilic phase, during which thermo-sensitive fungi and yeasts succumb, Richness (*S*) and Shannon-Weaver (*H*) indices decreased, and fragments presumably corresponding to thermo-tolerant species appeared (Fig. 2b and Tab. 2). Following the temperature decrease, the species responsible for the maturation prevailed at day 60 and at day 90 the intensity of fragments decreased, indicating the stabilization of the composted matrix. According to Cahyani et al. (2004) the fungal community during composting of rice straw can be split into two groups, one predominating before and the other one after the thermic peak. In our study, the biodiversity of fungal communities in the piles composted with the starters did not increase during the thermophilic phase, differently from bacterial communities. The fungal community seems to be more sensitive to high temperature than the bacterial community, as highlighted by other Authors. Among them, Hassen et al. (2001) observed that the fungal population decreased significantly at 55-60°C during the composting of municipal solid waste. Similarly, Zhang et al. (2011), evaluating some physico-chemical parameters (*i.e.* temperature, water soluble carbon, pH and C/N ratio) during agricultural waste composting, found that pile temperature induced the most significant variation in the fungal community composition.

3.2 Composting process at farm level

Based on the results of the industrial pilot experiment, a trial of wet husk composting with starters was run also at farm level, *i.e.* in conditions of lower stringency for the process control but closer to the real situation of the composting process run by small enterprises with limited production of olive wet husk. These conditions are common to the majority of Italian olive growers. The composting process at farm level was carried out in an open air facility next to the mill and lasted for 200 days due to initial low environmental temperature (5-13 °C for the first 80 days). The activation phase lasted for about 20 days and the thermophilic phase remained at 45 °C during the following 80 days (Fig. 3). The cooling and maturation phases were monitored up to 200 days after the pile formation.

3.2.1 Physical-chemical changes during the composting process at farm level

The data on the physical-chemical traits of olive husk during composting are reported in Tab. 3. The pH increased progressively shifting to values within the optimal range for composting. The values of pH were modified as a consequence of the biodegradation of acids, such as those with carboxylic and phenolic groups, as well as the mineralization of organic compounds (proteins, amino acids and peptides) into inorganic compounds (Gil et al., 2008). At the end of composting, the pH value of the end-product was 8.1, within the range allowed by the Italian law for agricultural compost application (Legislative Decree 75/2010), which is 6.0-8.5. These results are consistent with those obtained by other similar experimental composting processes that included this matrix in the starting material (Alfano et al., 2008; Cayuela et al., 2010). The electric conductivity (EC), which reflects the degree of salinity of compost and may be related

304 with the product's toxic effects on microorganisms and plants when applied to soil,
305 decreased progressively during composting as reported by Echeverria et al. (2011). At
306 the end of the process, EC values decreased from 1080 to 568 dS m^{-1}. The volatilization
307 of ammonia and the precipitation of mineral salts might be the causes of the EC
308 decrease at the end of biodegradation (Montemurro et al., 2009).
309 The total organic C value decreased during the biodegradation process of the studied
310 mixture because of the oxidation of organic C to CO_2 (Paredes et al., 2002). According
311 to recent investigations (Gil et al., 2008), during the first stages of composting TOC
312 content decreased rapidly, from an initial value of 47.2 in the raw material to 40.9%
313 after 35 days. This behavior is usually attributed to the mineralization of labile organic
314 compounds which mainly occurred during the thermophilic phase. The TOC amount
315 continued to decrease up to 24.4% after 145 days while, from this point on, the values
316 stabilized for the rest of process.
317 Levels of total N remained steady for most of the period, then decreased in the mature
318 compost. At the end of composting, the total nitrogen content was lower by 6.9% than
319 in the starting mixture, probably due to losses through volatilization of ammonia during
320 the organic matter degradation or through volatilization of gaseous N by denitrification
321 processes. The change in the C/N ratio reflects the organic matter decomposition and
322 stabilization during composting. As a consequence of the trend of C and N, the C/N
323 ratio decreased from an initial value of 36.1 to about 19. These values may be
324 considered satisfactory for a ready-to-use compost (Legislative Decree 75/2010).
325 Phenols are important components of wet husk and are related with the compost
326 stability and degree of maturity. In accordance to previous results (Alfano et al., 2008;
327 Baeta-Hall et al., 2005), composting induced a marked decrease of phenols -79%-. The

very low value of phenols content at the end of composting indicates an efficient phenol metabolism, due to the use of the starters that enhanced the decomposition and/or polymerization of phenols, thus contributing to the formation of humic acids.

According to the Italian Law, humification parameters, namely humification index (HI), humification degree (HD) and humification ratio (HR), must be used to evaluate the qualitative character of the organic matter contained in organic materials. In our work, the HI and HD appear to be the most sensitive ways to follow the humification process, while the values of the mineralization rate (HR) do not follow a well-defined trend during composting. The increasing trend of HD (from 79.6 to 93.8 mg g^{-1}) may be explained by the formation of complex molecules as a result of polymerization of simple molecules, while the decreasing trends of HI (from 0.25 to 0.07) may be due to the biodegradation of non-humic components of the FA fraction NHC, showing a decrease of 85%, followed by the formation of more polycondensed humic structures. The end-values obtained for HI, HD and HR suggest a high degree of polymerization of the humic compounds and a high organic matter stability, according to the results found by Mondini et al. (2006), Alburquerque et al. (2009) and Echeverria et al. (2011). The results relative to pH, EC and the organic matter dynamics indicate the occurrence of two distinct phases during the composting process, i.e. an activation phase during the first 100 days (mesophilic and thermophilic phases) and then a stabilization phase due to a deceleration of all activities up to 145 days. The latter corresponds to the completion of the maturing phase.

3.2.2 *Microbial diversity and community dynamics of husk composted at farm level*

For the bacterial community, the diversity indices derived from the analysis of the DGGE profiles of the samples collected during the composting process are reported in Tab. 4a.

This process is generally characterized by a high diversity and a gradual turnover of bacterial populations. Based on the increase of richness and of the Shannon-Weaver index of general diversity (*S* and *H* values from 23 to 37 and from 3.108 to 3.601) along with the decrease of the Simpson index of dominance (*D* values from 0.045 to 0.027), the bacterial diversity increased after day 35 reaching the highest value at the day 100. Subsequently, the bacterial biodiversity decreased (*S* and *H* values from 37 to 29 and from 3.601 to 3.344). During this maturation phase the inverted trend of the values of diversity indices indicates a gradual stabilization of the bacterial populations, in agreement with the trends and the final values of the humification parameters (Tab. 3). It is worthy of note that the community evenness remained almost constant during the composting process and was characterized by high *E* values that ranged from 0.996 to 0.999. As in the industrial pilot composting process, the highest bacterial biodiversity was found in correspondence to the thermophilic phase.

The relative dendrogram reported in Fig. 4a contains two subclusters: the first one includes the samples at days 0 and 35, which are more similar (0.64) between themselves than those with a higher maturation level (0.58), where a wider diversification of microbial species has taken place due to the progressive biotransformation process; the second one includes samples at a higher maturation level (at day 100, 145 and 200) with a low similarity level of 0.67.

The low similarity found at days 100, 145 and 200 is due to the differences in the composting phase: day 100 corresponds to thermophilic phase, day 145 to the end of

375 maturation, and day 200 to the refining phase. The latter represents the biochemical
376 stabilization of the substrate and of microbial populations.
377 Considering the diversity indices (Tab. 4b) for fungi and yeasts, the fungal community
378 is highly differentiated, even though less than the bacterial community. In the activation
379 phase of wet husk composting, fungal diversity is reduced as in the case of bacteria
380 (time 0 and at day 35) and increases considerably after the day 35, as indicated by high
381 *S* and *H* values increasing from 11 to 30 and from 2.167 to 3.152 respectively. During
382 cooling and maturation (at day 145), fungal diversity decreased (*S* and *H* values from 30
383 to 22 and from 3.152 to 2.939), remaining stable at the day 200, thus suggesting a
384 gradual stabilization of the populations. The dendrogram (Fig. 4b) computed from
385 DGGE profiles shows a cluster containing samples from the different composting
386 phases (at days 100, 145 and 200), at a low similarity level (0.60), as observed for the
387 bacterial community.
388 Like the bacterial community dynamics, also the fungal one evolved in parallel with the
389 composting phases, thus confirming the trend of the diversity indices (Tab. 4b).
390
391 **4. Conclusions**
392
393 Our study shows that the starters enhanced biotransformation leading to an earlier and
394 increased level of bacterial diversity throughout the wet husk composting process.
395 Results indicate that the biodiversity of microbial populations during biotransformation,
396 along with physical-chemical traits, evolves similarly at industrial pilot and at farm
397 level, although with different maturation times, and confirm that a high diversity in the
398 thermophilic phase isa typical trait of the biotransformation of this matrix. This suggests

399 that the applicability of this type of composting process could be extended to a wider
400 range of operational situations, frequently encountered in the Mediterranean area.
401

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Table 1

Richness (*S*), Shannon-Weaver (*H*), Simpson (*D*), and Evenness (*E*) indices calculated from DGGE profiles of the bacterial community of samples from compost piles obtained without starters (St-, *i.e.* mean of piles A, B, C), and compost piles obtained with starters (St+, *i.e.* mean of piles D, E, F) at industrial pilot level. Means with different letters are significantly different ($P < 0.05$).

		Time (days)			
		0	15	60	90
S	St-	24.33 bc	24.00 bc	27.67 ab	26.67 ab
	St+	22.00 c	30.00 a	30.33 a	30.67 a
H	St-	3.179 bc	3.165 bc	3.271 ab	3.237 abc
	St+	3.073 c	3.385 a	3.388 a	3.400 a
D	St-	0.042 ab	0.042 ab	0.038 bc	0.039 bc
	St+	0.047 a	0.034 c	0.034 c	0.034 c
E	St-	0.996 ab	0.996 ab	0.985 c	0.986 c
	St+	0.997 a	0.995 ab	0.994 b	0.995 ab

Table 2

Richness (*S*), Shannon-Weaver (*H*), Simpson (*D*), and Evenness (*E*) indices calculated from DGGE profiles of the fungal community of samples from compost piles obtained without starters (St-, *i.e.* mean of piles A, B, C) and compost piles obtained with starters (St+, *i.e.* mean of piles D, E, F) at industrial pilot level. Means with different letters are significantly different ($P < 0.05$).

		Time (days)			
		0	15	60	90
S	St-	17.33 ab	18.00 ab	15.66 bc	11.33 c
	St+	21.00 a	17.00 ab	14.00 bc	16.66 ab
H	St-	2.838 ab	2.871 ab	2.733 abc	2.420 c
	St+	3.025 a	2.822 ab	2.582 bc	2.787 ab
D	St-	0.052 c	0.057 bc	0.066 abc	0.089 a
	St+	0.049 c	0.060 bc	0.080 ab	0.063 bc
E	St-	0.995 bc	0.996 b	0.993 c	0.998 a
	St+	0.994 bc	0.998 a	0.993 c	0.999 a

542 **Table 3**

543 Physical-chemical traits of the compost obtained from wet husk composted at farm

544 level. Means with different letters are significantly different ($P < 0.05$).

545

Parameters	Time (days)				
	0	35	100	145	200
pH	6.3 d	6.8 c	7.8 b	8.0 a	8.1 a
Electrical conductivity ($dS \cdot m^{-1}$)	1080 a	812 b	690 c	565 d	568 d
Organic C ($mg \cdot g^{-1}$)	472 a	409 b	310 c	244 d	239 d
Total N ($mg \cdot g^{-1}$)	13.0 a	12.9 a	13.2 a	13.1 a	12.1 b
C/N	36.3 a	31.7 b	23.5 c	18.6 d	19.7 d
Hydrosoluble phenols (p-coumaric acid, $\mu g \cdot g^{-1}$)	918 a	533 b	252 c	186 d	194 d
Total Extractable C ($mg \cdot g^{-1}$)	98.2 a	97.5 a	53.0 b	45.8 c	45.3 c
Humic acids (mg $C \cdot g^{-1}$)	50.2 a	49.8 a	30.4 b	26.2 c	24.0 c
Fulvic acids (mg $C \cdot g^{-1}$)	28.0 a	27.8 a	16.4 b	16.6 b	11.5 c
Nonhumified C content ($mg \cdot g^{-1}$)	20.0 a	19.9 a	6.2 b	3.0 c	2.8 c
Humification index	0.25 a	0.26 a	0.13 b	0.07 c	0.07 c
Humification degree	79.6 c	79.6 c	88.3 b	91.4 a	93.8 a
Humification ratio	16.6 c	18.9 a	15.1 d	17.5 b	17.8 b

546

547

548

549 **Table 4**

550 Richness (*S*), Shannon-Weaver (*H*), Simpson (*D*) and Evenness (*E*) indices calculated
551 from the bacterial community **(a)** and fungal community **(b)** DGGE profiles obtained
552 from samples of wet husk composted at farm level.

553

(a)	Time (days)				
	0	35	100	145	200
S	24	23	37	29	27
H	3.135	3.108	3.601	3.344	3.274
D	0.045	0.045	0.027	0.035	0.038
E	0.996	0.998	0.997	0.998	0.999
(b)	Time (days)				
	0	35	100	145	200
S	15	11	30	22	22
H	2.348	2.167	3.152	2.939	2.852
D	0.140	0.042	0.051	0.061	0.072
E	0.878	0.905	0.927	0.952	0.923

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559 **FIGURE LEGEND**

560

561 **Fig. 1.** Dendrogram obtained with the clustering method UPGMA (Unweighted Pair
562 Group Method Using Arithmetic Average) based on the DGGE profiles of the bacterial
563 community of samples from compost piles (A, B, C) obtained without starters **(a)** and
564 compost piles (D, E, F) obtained with starters **(b),** at different maturation time (d=days).
565 Composting is at industrial pilot level. The relationships among samples are based on
566 the similarity, evaluated by using the Dice coefficient. S: starter pile; M: molecular
567 marker.

568

569 **Fig. 2.** Dendrogram obtained with the clustering method UPGMA (Unweighted Pair
570 Group Method Using Arithmetic Average) based on the DGGE profiles of the fungal
571 community of samples from compost piles (A, B, C) obtained without starters **(a)** and
572 compost piles (D, E, F) obtained with starters **(b),** at different maturation time (d=days).
573 Composting is at industrial pilot level. The relationships among samples are based on
574 the similarity, evaluated by using the Dice coefficient. M: molecular marker.

575

576 **Fig. 3.** Temperature evolution of the compost obtained from wet husk composted at
577 farm level.

578

579 **Fig. 4.** Dendrogram obtained with the clustering method UPGMA (Unweighted Pair
580 Group Method Using Arithmetic Average) based on the DGGE profiles of the bacterial
581 community **(a)** and fungal community **(b)** of wet husk samples composted at farm level,
582 at different maturation time (d=days). The relationships among samples are based on the
583 similarity, evaluated by using the Dice coefficient. M: molecular marker.

584

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Figure

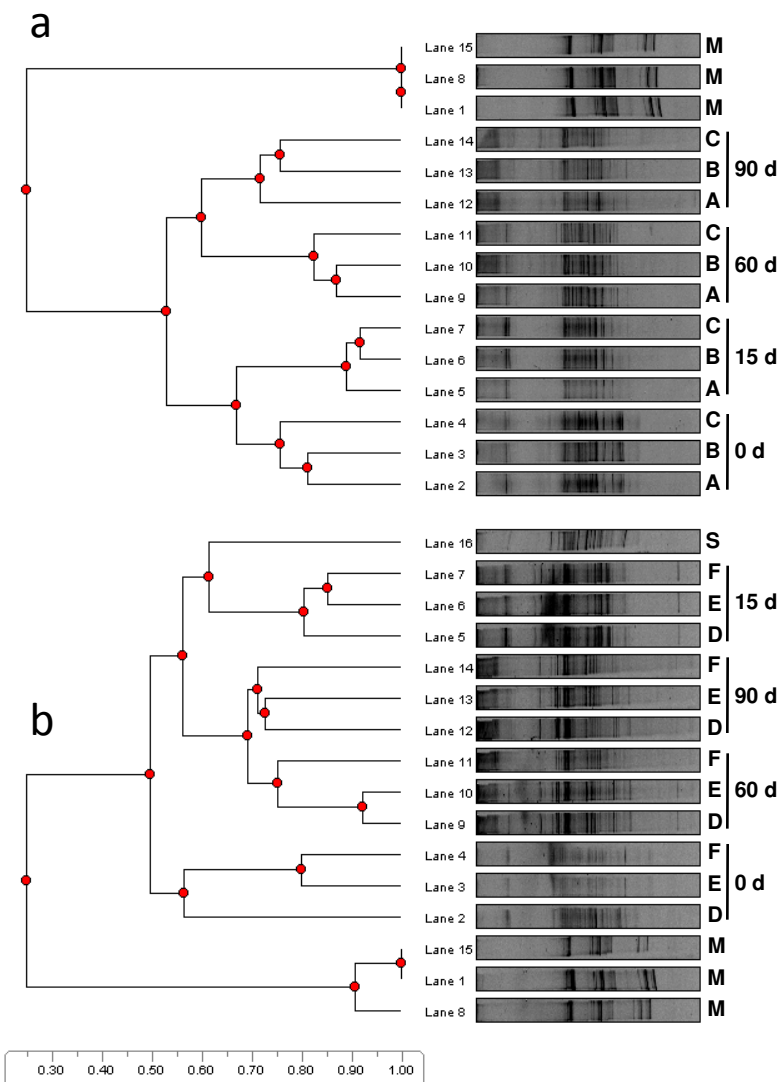
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Fig. 1

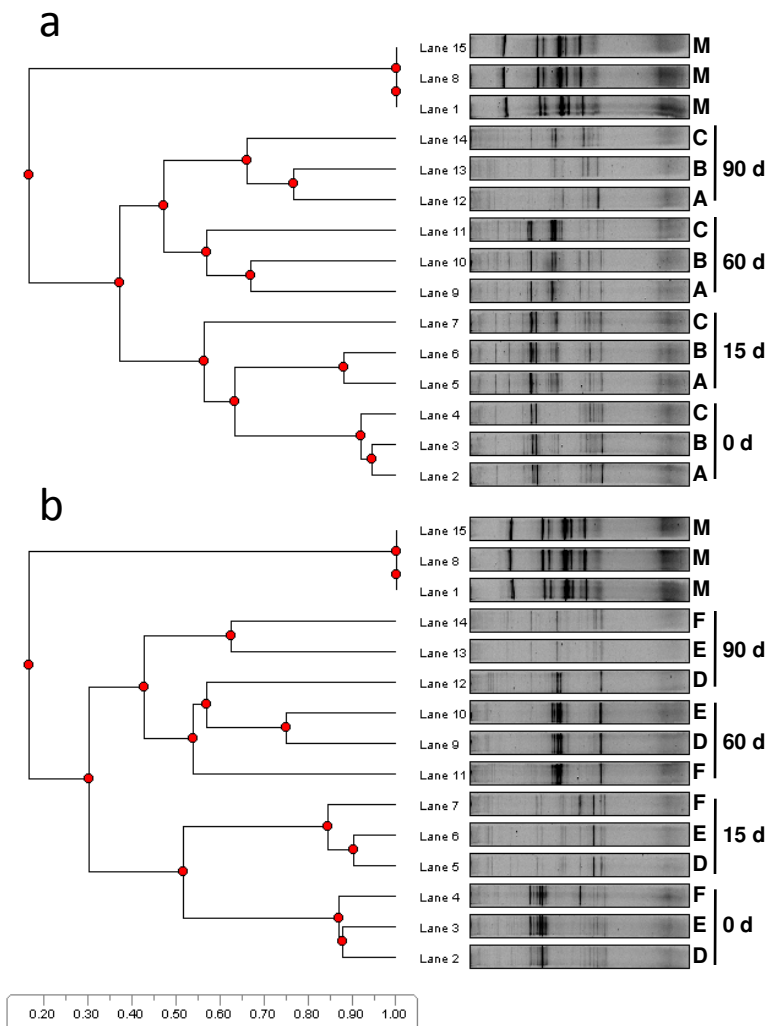
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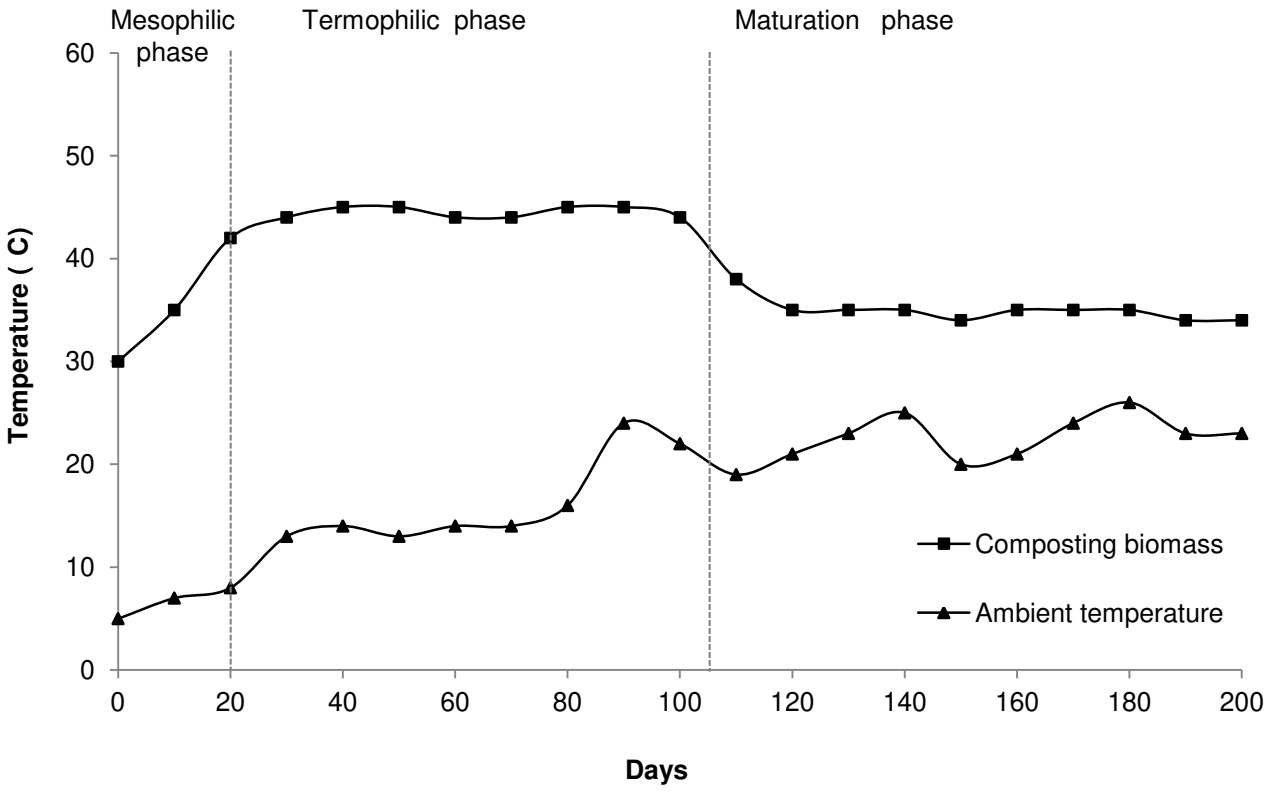
Fig. 2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Days
Composting biomass
Ambient temperature

Fig. 3

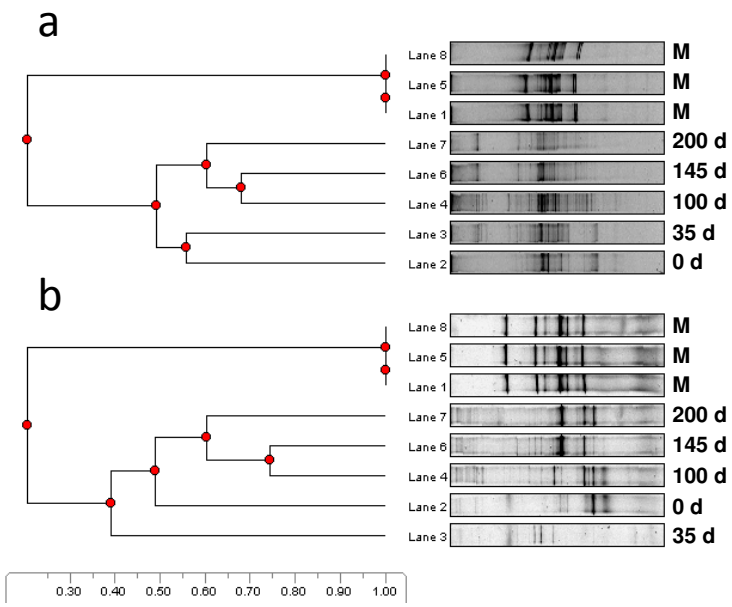
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Fig. 4