

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

5

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14

15    **Abstract**

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

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

28

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

31

## 32 **1. Introduction**

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

69 and at farm level (with limited process control facilities, thus representing the worst  
70 case scenario for the disposal and upgrade of wet husk through composting). The  
71 variability and the diversity of the microbial community were estimated by UPGMA  
72 analysis of the PCR-DGGE profiles and by the community diversity indices analysis.  
73

## 74 **2. Materials and Methods**

### 75 *2.1. Composting procedure, sampling and physical-chemical analyses*

76 The microbial starters used throughout this study were *Bacillus amyloliquefaciens*  
77 subsp. *plantarum* M51/II [formerly *B. subtilis*, reclassified according to Chen et al.  
78 (2009) and Borriss et al. (2011)], *Pseudomonas synxanta* 3/2, *Pseudomonas fluorescens*  
79 19/5, *Serratia marcescens* B2 (bacteria), *Streptomyces* sp. ATB 42, *Streptomyces* sp.  
80 AC 3, *Streptomyces* sp. AC 20, *Streptomyces* sp. AB 11 (actinobacteria), *Candida*  
81 *butyri* 8(4), *Rhodotorula mucillaginosa* 4(1), *Sporopachydermia lactativora* 2(3)  
82 (yeasts), and *Arthrotrrys oligospora* DSMZ 2023, *Chaetomium globosum* Ch 10,  
83 *Phanerochaete chrysosporium* ATCC 42538, *Trichoderma atroviride* T14 (microfungi).  
84 The microbial strains used in this study were isolated from wet husks (originated from  
85 the oil extraction process of olives cv, Leccino, Moraiolo, and Frantoio), except for *A.*  
86 *oligospora* DSMZ 2023, obtained from German Collection of Microorganisms and Cell  
87 Cultures, and *P. chrysosporium* ATCC 42538, obtained from American Type Culture  
88 Collection.

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

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

96 At farm level the composting process was carried out by Cooperativa Arnasco in  
97 Arnasco (Savona, Italy) in an olive oil mill that serves a 25–30 year-old 40 ha olive  
98 grove (cultivars Taggiasca and Pignola). Twenty tons of wet husk from a two-phase  
99 decanter extraction process were mixed with the starters, added with 1 ton of olive  
100 wood chips to maintain aereation and placed in a windrow pile. The mechanical turning  
101 of the pile was done occasionally, depending on the external temperature. Sampling was  
102 carried out in compliance with the methods of the Italian Ministry of Agriculture  
103 (Legislative Decree 75/2010) at time 0, 15, 60 and 90 days for composting at industrial  
104 pilot, and at time 0, 35, 100, 145 and 200 days for composting at farm level. The  
105 temperature profile of the piles and the physical-chemical traits of the samples from the  
106 industrial pilot composting process were reported in a previous paper (Echeverria et al.,  
107 2012). The methods to determine the physical-chemical traits of samples from the farm  
108 scale composting process, to extract the humic substances and to express the  
109 humification index (HI) are those described by Echeverria et al. (2011).

110

## 111 *2.2. Microbial cultures, DNA extraction and PCR amplification*

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

116 Genomic DNA was extracted from bacteria, actinobacteria and yeasts liquid cultures  
117 grown overnight at 25 ° C using “MasterPure™ Yeast DNA Purification” (Epicentre)  
118 according to the manufacturer’s protocols. For the DNA extraction from fungi, the  
119 mycelium grown on plates was resuspended with 10 mL deionized water. 2 mL of  
120 suspension were inoculated in 40 mL of Malt Broth for an overnight incubation on a  
121 rotatory shaker at 25 °C. After harvest by centrifugation at 10,000 rpm and 3 washes  
122 with MgCl<sub>2</sub> 0,1M, 400 mg of mycelium (f.w.) were transferred into a 2 mL Eppendorf  
123 tube and crushed. The other steps were the same as for bacteria and yeasts.  
124 DNA extraction from compost samples was carried out using the “PowerSoil™ DNA  
125 Isolation Kit” (Mo-Bio Laboratories San Diego, CA) according to manufacturer’s  
126 instructions. For the analysis of the bacterial community, the amplification of the  
127 variable region V3-V5 of 16S rDNA was carried out using the primers 341F  
128 (CCTACGGGAGGCAGCAG) and 907R (CCGTCAATTCCTTTRAGTTT) (Yu and  
129 Morrison, 2004). The primer 341F had at its 5’ end an additional 40-nucleotide GC-rich  
130 tail (5'CGCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCCCGCCCG-3').  
131 Amplification reaction was prepared in a final volume of 50 µL, using 1 µL of extracted  
132 DNA diluted 1:100, 10 µL of 5 x Phusion™ HF Buffer (Finnzymes), 1U of Phusion™  
133 High-Fidelity DNA polymerase (Finnzymes), 0.2 mM of each dNTPS (GeneAmp dNTP  
134 Mix, Applied Biosystem), 0.5 µM of each primers (Primm) and 3% DMSO  
135 (Finnzymes). The fragment obtained is 560 bp long. The reaction was carried out using  
136 an iCycler-iQ Multicolor Real-Time PCR Detection System (Biorad) with the following  
137 denaturation, amplification and extension procedure: 98 °C 30 sec; 98 °C 10 sec, 52 °C  
138 10 sec, 72 °C 15 sec for 35 cycles; 72 °C 10 min. The presence of amplicons was  
139 confirmed by electrophoresis in 1.5% (w/v) Agarose I (Euroclone) in TBE 1 x buffer

140 (Euroclone) gels stained with ethidium bromide  $0.5 \mu\text{g mL}^{-1}$ . All gels were visualized  
141 and captured as TIFF format file by Liscap program for Image Master VDS system  
142 (Pharmacia Biotech). For the analysis of fungal community, the amplification of the  
143 variable region D1-D2 of 26S rDNA was carried out using the primers NL1  
144 (GCATATCAATAAGCGGAGGAAAAG) and LS2  
145 (ATTCCCAAACAACACTCGACTC) (Cocolin et al., 2000). The primer NL1 had at its 5'  
146 end an additional 40-nucleotide GC-rich tail. Amplification reaction was prepared in a  
147 final volume of  $50 \mu\text{L}$ , using  $2 \mu\text{L}$  of extracted DNA diluted 1:10,  $10 \mu\text{L}$  of 5 x  
148 Phusion™ HF Buffer (Finnzymes), 1U of Phusion™ High-Fidelity DNA polymerase  
149 (Finnzymes), 0.2 mM of each dNTPS (GeneAmp dNTP Mix, Applied Biosystem), 0.5  
150  $\mu\text{M}$  of each primers (Primm) and 3% DMSO (Finnzymes). The fragment obtained is  
151 250 bp long. The reaction was carried out using an iCycler-iQ Multicolor Real-Time  
152 PCR Detection System (Biorad) with the following denaturation, amplification and  
153 extension procedure:  $98 \text{ }^\circ\text{C}$  30 sec;  $98 \text{ }^\circ\text{C}$  10 sec,  $48 \text{ }^\circ\text{C}$  10 sec,  $72 \text{ }^\circ\text{C}$  15 sec for 35  
154 cycles;  $72 \text{ }^\circ\text{C}$  10 min. The presence of amplicons was confirmed with the same  
155 procedure as for bacterial communities.

156

### 157 *2.3 DGGE analysis of bacterial and fungal community*

158 The amplicons were analyzed using the DCode™ Universal Mutation Detection System  
159 BIORAD.  $20 \mu\text{L}$  of the PCR products plus  $20 \mu\text{L}$  of buffer 2 x made with 70% glycerol,  
160 0.05% xylene cyanol and 0.05% bromophenol bleu were loaded on a 8%  
161 polyacrylamide-bisacrilamide (37.5:1) gel with a urea-formamide denaturing gradient  
162 ranging 30% to 65% (prokaryotes) and 20% to 75% (eukaryotes). A composite mix of

163 bacterial 16S rRNA gene fragments from *Pseudomonas synxanta* 2/3, *Bacillus*  
164 *amyloliquefaciens* subsp. *plantarum* M51/II, *Serratia marcescens* B2, *Streptomyces* sp.  
165 AB11, *Streptomyces* sp. AC20 and a composite mix of fungal 26S rRNA gene  
166 fragments from *Sporopachydermia lactativora* 2(3), *Candida butyri* 8(4),  
167 *Phanerochaete chrysosporium* ATCC42538, *Trichoderma atroviride* T14, *Chaetomium*  
168 *globosum* ChT3 were added on each side and in the center of DGGE gels as reference  
169 DGGE patterns. Gels were run at 90 V and 60 °C for 16 hours and stained for 30' in  
170 500 mL of TAE 1 x buffer containing 50 µL of Sybr Gold Nucleic Acid Gel Stain  
171 (Molecular Probes, Invitrogen). The profiles were visualized under UV illumination and  
172 captured as TIFF format file by Liscap program for Image Master VDS System  
173 (Pharmacia Biotech). Band patterns in different DGGE lanes were compared with the  
174 ImageMaster 1D Elite v3.00 software (Pharmacia Biotech). The lanes were normalized  
175 to contain the same amount of total signal after background subtraction and the gel  
176 images were straightened and aligned to give a densitometric curve. Bands were  
177 assigned and matched automatically and then checked manually. Band positions were  
178 converted into Rf values between 0 and 1, and profile similarity was calculated by  
179 determining Dice's similarity coefficients for the total number of lane patterns from the  
180 DGGE gel. The similarity coefficients calculated were then used to generate the  
181 dendrograms utilizing the clustering method UPGMA (Unweighted Pair Group Method  
182 Using Arithmetic Average). DGGE banding data were used to estimate four different  
183 indices treating each band as an individual operational taxonomic unit (OTU). Richness  
184 (*S*) indicates the number of OTUs present in a sample and is determined by the number  
185 of fragments. The overall diversity index of Shannon-Weaver (*H*) and the dominance  
186 index of Simpson (*D*) were calculated using the equations  $H = -\sum(P_i \times \ln P_i)$  and  $D =$



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

191

## 192 *2.4 Statistical analysis*

193 The data represent the means of three replicates (n=3). The results of physical-chemical  
194 traits were subjected to one-way analysis of variance (ANOVA) with time as a  
195 variability factor. A two-way ANOVA test was applied to diversity indices in which  
196 treatment and time are the source of variability. The means were compared by using  
197 Least Significant Difference test ( $P < 0.05$ ). Analyses were carried out with the CoStat  
198 6.4 program (CoHort software).

199

## 200 **3. Results and Discussion**

### 201 3.1 Composting at industrial pilot level

#### 202 3.1.1 *Community dynamics and microbial diversity of wet husk composted with and* 203 *without starters*

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

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

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

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

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

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

278

279 3.2 Composting process at farm level

280

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

291

### 292 *3.2.1 Physical-chemical changes during the composting process at farm level*

293 The data on the physical-chemical traits of olive husk during composting are reported in  
294 Tab. 3. The pH increased progressively shifting to values within the optimal range for  
295 composting. The values of pH were modified as a consequence of the biodegradation of  
296 acids, such as those with carboxylic and phenolic groups, as well as the mineralization  
297 of organic compounds (proteins, amino acids and peptides) into inorganic compounds  
298 (Gil et al., 2008). At the end of composting, the pH value of the end-product was 8.1,  
299 within the range allowed by the Italian law for agricultural compost application  
300 (Legislative Decree 75/2010), which is 6.0-8.5. These results are consistent with those  
301 obtained by other similar experimental composting processes that included this matrix  
302 in the starting material (Alfano et al., 2008; Cayuela et al., 2010). The electric  
303 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<sup>-1</sup>. 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<sub>2</sub> (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

328 very low value of phenols content at the end of composting indicates an efficient phenol  
329 metabolism, due to the use of the starters that enhanced the decomposition and/or  
330 polymerization of phenols, thus contributing to the formation of humic acids.  
331 According to the Italian Law, humification parameters, namely humification index (HI),  
332 humification degree (HD) and humification ratio (HR), must be used to evaluate the  
333 qualitative character of the organic matter contained in organic materials. In our work,  
334 the HI and HD appear to be the most sensitive ways to follow the humification process,  
335 while the values of the mineralization rate (HR) do not follow a well-defined trend  
336 during composting. The increasing trend of HD (from 79.6 to 93.8 mg g<sup>-1</sup>) may be  
337 explained by the formation of complex molecules as a result of polymerization of  
338 simple molecules, while the decreasing trends of HI (from 0.25 to 0.07) may be due to  
339 the biodegradation of non-humic components of the FA fraction NHC, showing a  
340 decrease of 85%, followed by the formation of more polycondensed humic structures.  
341 The end-values obtained for HI, HD and HR suggest a high degree of polymerization of  
342 the humic compounds and a high organic matter stability, according to the results found  
343 by Mondini et al. (2006), Albuquerque et al. (2009) and Echeverria et al. (2011). The  
344 results relative to pH, EC and the organic matter dynamics indicate the occurrence of  
345 two distinct phases during the composting process, i.e. an activation phase during the  
346 first 100 days (mesophilic and thermophilic phases) and then a stabilization phase due  
347 to a deceleration of all activities up to 145 days. The latter corresponds to the  
348 completion of the maturing phase.

349

350 *3.2.2 Microbial diversity and community dynamics of husk composted at farm level*

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

354 This process is generally characterized by a high diversity and a gradual turnover of  
355 bacterial populations. Based on the increase of richness and of the Shannon-Weaver  
356 index of general diversity ( $S$  and  $H$  values from 23 to 37 and from 3.108 to 3.601) along  
357 with the decrease of the Simpson index of dominance ( $D$  values from 0.045 to 0.027),  
358 the bacterial diversity increased after day 35 reaching the highest value at the day 100.  
359 Subsequently, the bacterial biodiversity decreased ( $S$  and  $H$  values from 37 to 29 and  
360 from 3.601 to 3.344). During this maturation phase the inverted trend of the values of  
361 diversity indices indicates a gradual stabilization of the bacterial populations, in  
362 agreement with the trends and the final values of the humification parameters (Tab. 3).

363 It is worthy of note that the community evenness remained almost constant during the  
364 composting process and was characterized by high  $E$  values that ranged from 0.996 to  
365 0.999. As in the industrial pilot composting process, the highest bacterial biodiversity  
366 was found in correspondence to the thermophilic phase.

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

373 The low similarity found at days 100, 145 and 200 is due to the differences in the  
374 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 is a 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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405

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504 **Table 1**

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

510

		Time (days)				511
		0	15	60	90	512
<i>S</i>	St-	24.33 bc	24.00 bc	27.67 ab	26.67 ab	513
	St+	22.00 c	30.00 a	30.33 a	30.67 a	
<i>H</i>	St-	3.179 bc	3.165 bc	3.271 ab	3.237 abc	514
	St+	3.073 c	3.385 a	3.388 a	3.400 a	
<i>D</i>	St-	0.042 ab	0.042 ab	0.038 bc	0.039 bc	515
	St+	0.047 a	0.034 c	0.034 c	0.034 c	516
<i>E</i>	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	517

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523 **Table 2**

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

529

		Time (days)				
		0	15	60	90	531
<i>S</i>	St-	17.33 ab	18.00 ab	15.66 bc	11.33 c	532
	St+	21.00 a	17.00 ab	14.00 bc	16.66 ab	
<i>H</i>	St-	2.838 ab	2.871 ab	2.733 abc	2.420 c	533
	St+	3.025 a	2.822 ab	2.582 bc	2.787 ab	
<i>D</i>	St-	0.052 c	0.057 bc	0.066 abc	0.089 a	534
	St+	0.049 c	0.060 bc	0.080 ab	0.063 bc	535
<i>E</i>	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	536

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541



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·m <sup>-1</sup> )	1080 a	812 b	690 c	565 d	568 d
Organic C (mg·g <sup>-1</sup> )	472 a	409 b	310 c	244 d	239 d
Total N (mg·g <sup>-1</sup> )	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, µg·g <sup>-1</sup> )	918 a	533 b	252 c	186 d	194 d
Total Extractable C (mg·g <sup>-1</sup> )	98.2 a	97.5 a	53.0 b	45.8 c	45.3 c
Humic acids (mg C·g <sup>-1</sup> )	50.2 a	49.8 a	30.4 b	26.2 c	24.0 c
Fulvic acids (mg C·g <sup>-1</sup> )	28.0 a	27.8 a	16.4 b	16.6 b	11.5 c
Nonhumified C content (mg·g <sup>-1</sup> )	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

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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

<b>(a)</b>	Time (days)				
	0	35	100	145	200
<i>S</i>	24	23	37	29	27
<i>H</i>	3.135	3.108	3.601	3.344	3.274
<i>D</i>	0.045	0.045	0.027	0.035	0.038
<i>E</i>	0.996	0.998	0.997	0.998	0.999

<b>(b)</b>	Time (days)				
	0	35	100	145	200
<i>S</i>	15	11	30	22	22
<i>H</i>	2.348	2.167	3.152	2.939	2.852
<i>D</i>	0.140	0.042	0.051	0.061	0.072
<i>E</i>	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.

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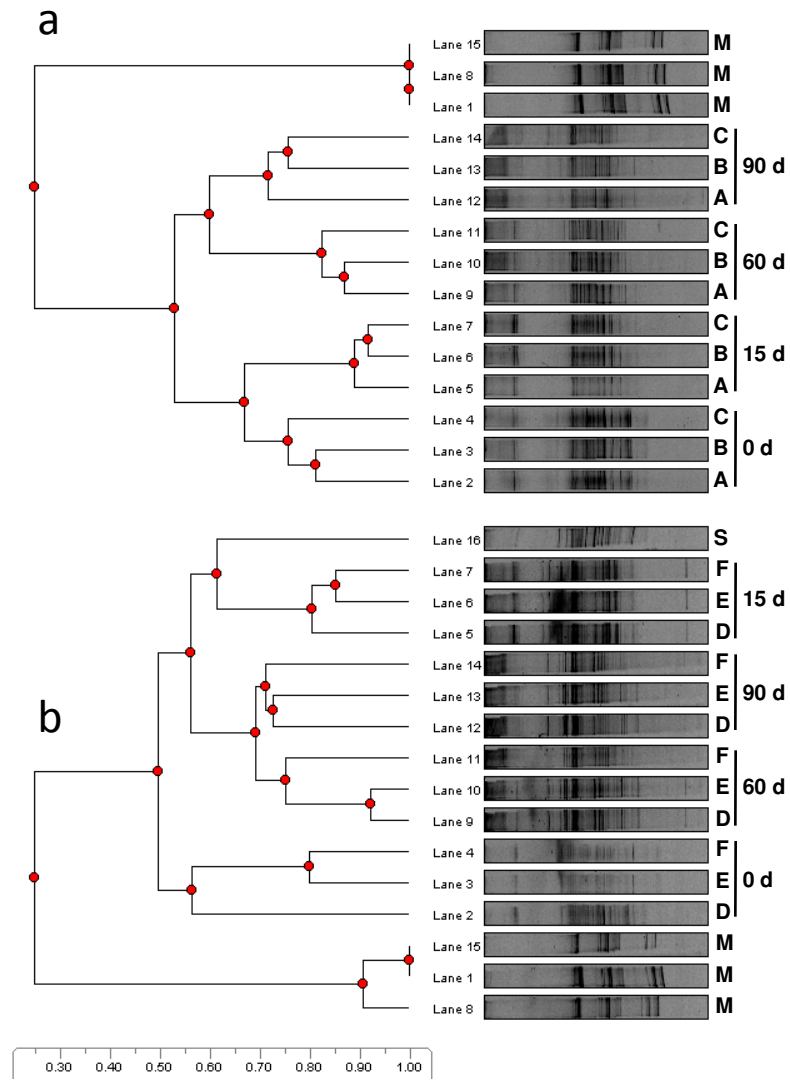


Fig. 1

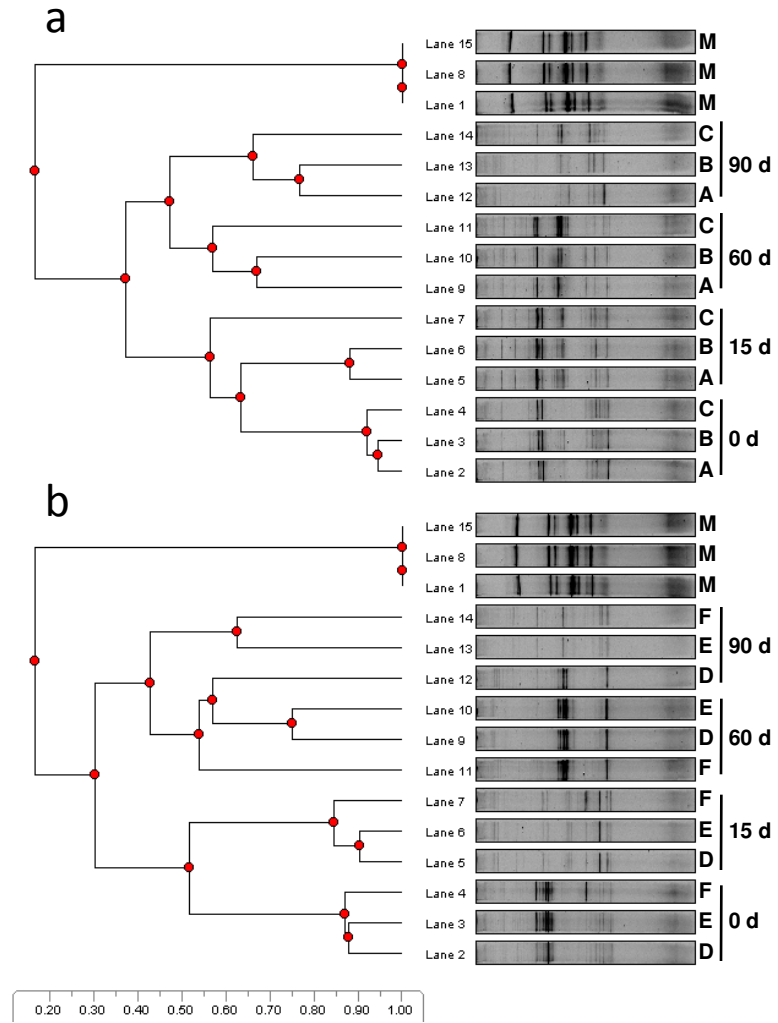


Fig. 2

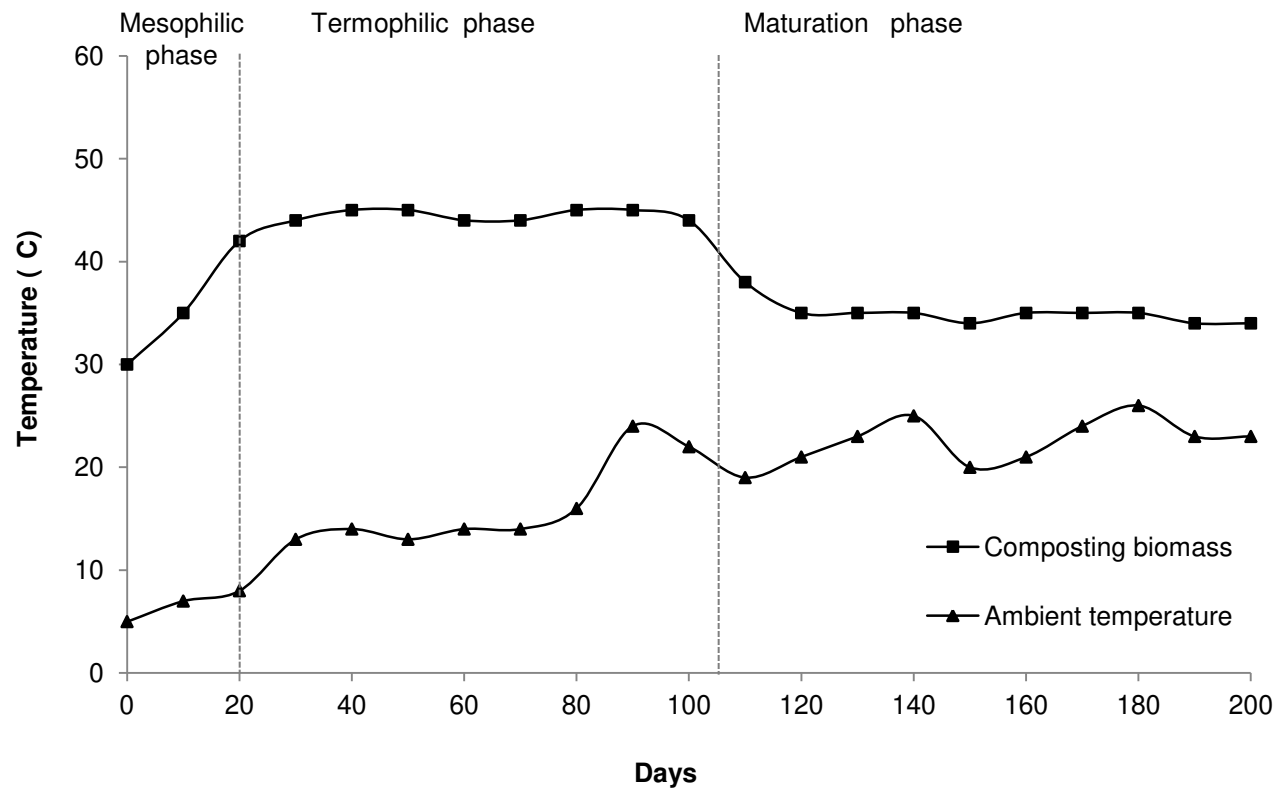


Fig. 3

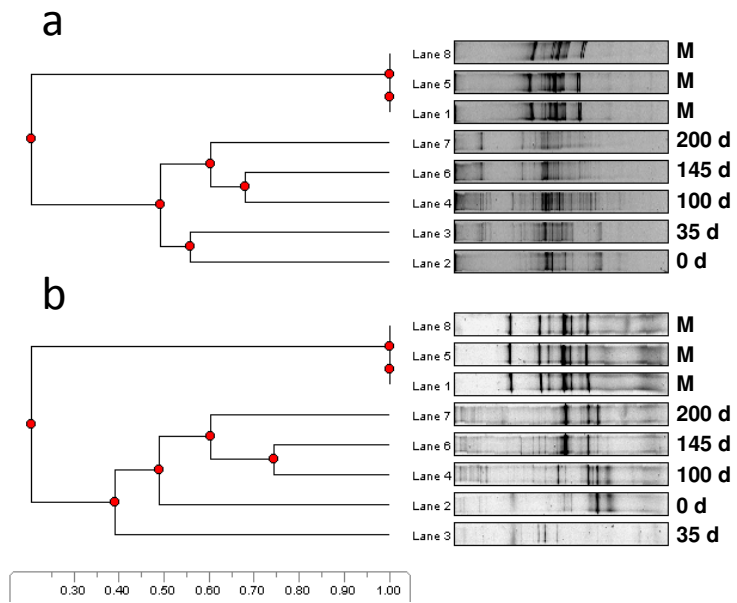


Fig. 4