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Phospholipid fatty acid analysis to monitor the co-composting process of olive oil mill wastes and organic household refuse

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Abstract

The co-composting of olive oil mill wastes and household refuse was followed for 5 months. During the thermophilic phase of composting, the aerobic heterotrophic bacteria (AHB) count, showed a significant rise with a slight regression of fungal biomass. In the same way, phospholipid fatty acids PLFAs common in bacteria, showed a significant increase of hydroxyl and branched PLFAs. The evaluation of the ratio of octadecenoic PLFAs to stearic acid (C18:1ω/C18:0) revealed a significant reduction while a significant rise in the length of aliphatic chains evaluated by the stearic acid to palmitic acid ratio (C18:0/C16:0) was noted during the stabilization phase. The follow-up of PLFAs, indicates the degree of biodegradation that occurs during composting, it can be regarded an indicator of the stability and maturity of the end product.

Keywords: Composting; Biodegradability; Microbial biomass; PLFAs

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1. Introduction

The processing of organic waste by composting is an environmentally-aware solution, which allows organic matter recycling to approach the natural cycles of the planet. Nevertheless, its success is dependent on several factors, in particular the physicochemical parameters of the substrate and the prevailing conditions where the composting proceeds. In the same way, the composting process is the seat of microbial activity, whose nature determines the degree of organic matter transformation.

Biomass and microbial diversity can be analysed by the quantitative and qualitative study of membrane phospholipid fatty acids PLFAs [1,2]. This method is regarded as a very sensitive and reliable tool to evaluate soil microbial activity and estimate the level of organic matter decomposition [3,4]. Recently, Steger et al. [5] reported the value of fatty acid methyl esters in monitoring compost maturity. The relative content of these fatty acids can vary according to genetic characteristics, physiological conditions and the environment. The observation of bacterial cultures reveals their variation with growth phase, temperature, nutrition and the mineral composition of the culture media [6].

Although the molecular structures involved, the mechanisms of formation and the properties of these substances appear to be well established, the information obtained by following the PLFA composition at the time of microbial activity during composting is complex, probably because many factors combine to influence the final PLFA pattern, e.g. temperature, oxygenation, and mineral composition.

Normal straight chain fatty acids are common in both prokaryotes and eukaryotes but polyunsaturated fatty acids are not common in bacteria. The most common monounsaturated fatty acids in bacteria have their double bond between C9 and

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C10. Branched chain fatty acids, and fatty acids with hydroxyl and cyclopropane functions are not common in other organisms and make bacteria unique [7,8].

The physical state of phospholipid fatty acids as a function of temperature can have life-affecting consequences for the micro-organisms. It is this that conditions the quality of the fluidity to ensure the membrane functions (e.g. osmotic barrier, solute transport, and respiration). Indeed, during waste composting the high temperatures reached strongly affect the microbial growth and biodegradation kinetics [9]. The high values are directly influenced by the mode and frequency of aeration [10–12] as well as the compost's insulating properties [13].

In this study, we seek to assess the processes involved in composting olive mill wastes in a mixture with household refuse, using physicochemical parameter analysis, the quantification of aerobic heterotrophic bacteria (AHB) and total mould biomass, and also the relative abundance analysis of phospholipid fatty acids (PLFAs) common in bacteria.

2. Materials and methods

2.1. Composting tests

Two composting tests were run, they contained mixtures of solid olive waste (pile 1: 1500 kg; pile 2: 1200 kg, with a C/N ratio of 87 and 35% moisture) and liquid olive mill effluent (pile 1: 200 L, pile 2: 300 L, with a C/N ratio of 15 and 84% moisture), both recovered from the same olive oil extraction unit; 160 and 180 kg of the organic part of household refuse (C/N ratio of 12 and 67% moisture) were added to pile 1 and pile 2, respectively, giving initial C/N ratios of 32.2 and 35.2 for the two mixtures. A mineral contribution in the form of crushed rock phosphate (12 kg, with a total P_2O_5 content of 30%) was added to pile 2, to measure the effect of acidity reduction of the mixture on the biodegradation process. To aerate the mixtures, manual forking over was done every week for 5 months. The temperature measurements are taken at various points and depths in the piles using a Minitherm thermometer (Hi8751, range from: −40 to +150 °C). The overall level of humidity was kept at about 60% a proportion compatible with the elevated hydrophobicity due to the high levels of lipid present After each mixing, samples were taken at various levels in the piles, the aim being to obtain a representative and homogeneous sample. The level of humidity was checked after each mixing and when necessary, the pile was watered to compensate for losses due to evaporation.

2.2. Microbiological analysis

The micro-organism biomass which we sought to quantify was composed of heterotrophic aerobes (AHB) and total fungi. Two grams of compost were placed in sterile physiological water (9 g/l NaCl) and shaken with glass balls for 15 min. Aliquots (0.1 ml) of serial dilutions were spread on Petri dishes: 10^{-4} to 10^{-9} for AHB counts on Nutritive Gelose and 10^{-2} to 10^{-7} for fungus counts on Malt Agar. Both types of plate were incubated for 72 h at 45 °C, with a tank of sterile water inside the incubator to avoid dehydration of the culture medium.

2.3. Lipid extraction and separation

The lipid extraction was carried out on accurately weighed 15 g samples of compost. The lipids were extracted with 360 ml of a chloroform/methanol mixture (2/1, v/v) (3× 120 ml). After 24 h of maceration at 4 °C, the mixture was filtered through glass wool. The chloroform fraction containing the lipids was recovered following separation of the two miscible solvents by addition of 60 ml of 0.73% NaCl, according to the method of Folch et al. [14]; excess water was reduced by addition of anhydrous sodium sulphate. The lipid fractions were separated on silica columns. The neutral lipids, glycolipids and phospholipids were eluted by chloroform, acetone and methanol, respectively [15]. The various lipid fractions obtained were then evaporated to dryness using a Rotavapour.

The phospholipid fraction, further dried under a current of nitrogen gas, was taken up in 1 ml of heptane. The fatty acids were subjected to trans-esterification after alkaline methanolysis. The fatty acid methyl esters were analysed by gas chromatography (HP 5890 series II) coupled with mass spectrometry (HP 5971): column length 30 m, internal diameter 0.530 mm. The injection used the split mode, 1 μl sample with 1 μl hexane, carrier gas helium under 21 kPa pressure, the injector temperature was 260 °C, that of the oven: from 140 °C (4 min) to 250 °C with 4 °C/min and the detector temperature was 280 °C, the ionisation energy was 70 eV. The methyl ester peaks obtained were compared with those of external standards corresponding to bacterial fatty acid methyl esters BAMEs (26 known bacterial FAMEs, supplied by Supelco, Bellefonte, PA).

2.4. Physico-chemical analyses

The ash content was determined after heating to constant weight in an oven at 550 °C. Total nitrogen and organic carbon were determined after pyrolysis of 1 mg of matter: the two elements were oxidised to give $N_2 + CO_2$, then chromatographically separated under a stream of helium using an E.A1.04 Carbograph Auto-Analyser. Ammonium and nitrates were assayed using the Kjeldhal method with a Velp-UDK132 distillation unit. After $HNO_3/HClO_4$ digestion, phosphorus was measured by ICP-atomic emission spectrometry. Lignin was determined according to methods described by Alburquerque et al. [16]. Polyphenols were extracted with ethyl acetate and determined by the Folin-Ciacalteu method as described by Ait Baddi et al. [17].

2.5. Statistical analyses

Comparison of the averages was carried out by ANOVA test post hoc Tukey, for a confidence interval of 95%. Principal component analysis (PCA) was carried out between different PLFAs during composting.

3. Results and discussion

During composting, the initially slightly acid pH increased following organic acid degradation (Table 1); the rise was greater

Table 1
Variation of some key physico-chemical parameters during composting, expressed as a percentage of dry weight

Parameter	1 day	1 month	3 months	5 months
pH	5.49 5.77	6.11 6.76	6.06 7.34	7.08 7.62
Ash (%)	3.9 12.3	4 13.2	5.6 16.3	6.6 16.9
C/N	32.19 35.34	27.2 34.6	18.83 22.57	15.1 22
NH_4^+/NO_3^-	2.86 2.63	3.13 2.63	2.70 2.78	0.37 0.36
Total organic carbon (%)	55.4 51.6	55 50.9	52 49.2	52.6 48.9
Total nitrogen (%)	1.72 1.46	2.02 1.47	2.76 2.18	3.49 2.22
Total phosphorus (%)	0.27 4.16	0.22 4.75	0.27 5.53	0.32 6.39
Total lipids (%, TL)	29.3 22.7	22.5 15.1	6.7 9	4.2 8.2
Neutral lipids (%, TL)	83.6 68.7	73.6 83.2	34 57.8	31.3 63.9
Phospholipids (%, TL)	2.9 5.4	16.5 7.1	13.7 12.2	7.2 13.9
Total phenols (%)	0.423 1.166	0.820 0.613	0.122 0.128	0.087 0.109
Lignin (%)	59.93 51.88	53.92 49.95	42.22 43.21	40.15 42.76

The upper line corresponds to pile 1, the lower line corresponds to pile 2.

in pile 2, having received the addition of rock phosphate. The inorganic forms of nitrogen, whether oxidised as nitrate and nitrite or reduced as ammonium, showed a decrease during the last phases of composting. All nitrogen must be converted into the ammonium form before it can be assimilated by the micro-organisms. The reduction of the C/N ratio (falling to about 15 and 22 for pile 1 and pile 2, respectively) indicates the high degree of substrate decomposition. This aerobic decomposition is accompanied by a temperature variation which can be subdivided into three phases (Fig. 1), the temperature increases during phases I and II, corresponding to stabilization phase, where production of heat is greater than the losses. Then the temperature falls again in phase III representing the maturation phase, where the losses of heat are major. The ratio NH_4^+/NO_3^- fell to below 1 after 5 months of composting showing that the compost in both piles had reached maturity. Note that the changes occurring in the nitrogen forms during composting can lead to losses of the mineral forms of nitrogen. Nitrates become reduced to nitrites and even to N_2 but rarely to NH_3. The literature lists the genera of filamentous mycetes, yeasts and a certain number of bacteria and algae that possess the ability to reduce nitrates prior to its assimilation [18]. However, the level of ammonia is affected by high temperatures which can lead to losses of the gaseous form during composting [19].

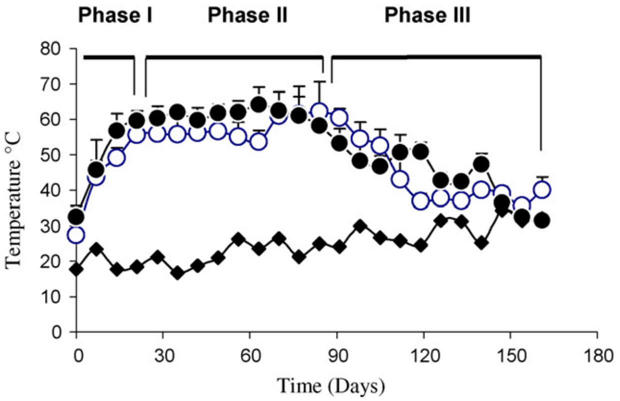

Fig. 1. Substrate temperature during composting: pile1: (○), pile2: (●) and ambient temperature (◆).

The results of AHB and mould enumeration at various temperatures during composting, presented in Fig. 2, reveal a significant increase of AHB, which peaks in the thermogenic phase. Mould counts showed an increase during phase I and then a slight regression in phase II. During the maturation phase, the AHB count fell and moulds increased. This typical evolution was reported by Ryckeboer et al. [20] quoting a low mould biomass at the beginning of composting, and relatively high values during the maturation phase.

The significantly higher decomposition rate of substrates during the stabilization phase represented in Table 2, coincides with the temperature rise (from 56 to 62 °C). This increase can be related to the micro-organism's higher metabolic activity, and heat release following progressive substrate oxidation. Although total lipids were reduced during composting, phospholipids showed a relative increase, particularly during the thermophilic phase (Table 1). This could be related to the increase in microbial biomass.

The evolution of common bacterial PLFAs (Table 3, Fig. 3) showed high values of saturated and unsaturated fatty acids during the first month (phase I); their progressive reduction was paralleled by an increase of branched and mainly hydroxyl fatty acids during the stabilization phase. The high values of saturated

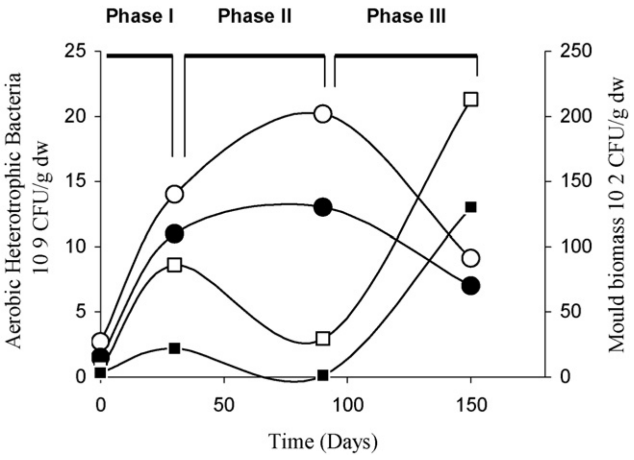

Fig. 2. Abundance of aerobic heterotrophic bacteria (AHB): pile 1: (○) pile 2: (●) and moulds, pile 1: (□), pile 2: (■) during composting.

Table 2
Level of reduction of the organic fraction, expressed as a percentage

	% Reduction		
	Phase I	Phase II	Phase III
Total lipids	16.7 37.6	52.1 50.7	31.2 11.7
Total phenols	– 33.7	69.7 54.7	30.3 11.6
Lignin*	16.3 23.4	58.5 66.3	25.2 10.4
Total organic compound*	5.5 23.6	62 63.6	32.5 12.8

Phases I, II and III, correspond to thermal stages of composting. The upper line corresponds to pile 1, the lower line to pile 2. See Fig. 1 for the definition of the three phases.

* Significant difference ($p < 0.05$) between the three phases.

Table 3
Abundance of some phospholipid fatty acid methyl esters common in bacteria, detected in the different composting phases (expressed in mg/g dry weight)

	1 day	1 month	3 months	5 months
Hydroxyl fatty acids				
2-OH C12:0	0.000 0.000	0.000 0.000	0.061 0.075	0.000 0.026
2-OH C14:0	0.000 0.000	0.059 0.159	0.378 0.245	0.035 0.133
2-OH C16:0	0.000 0.000	0.000 0.000	0.060 0.029	0.000 0.019
Branched fatty acids				
iMeC14:0	0.000 0.000	0.113 0.068	0.208 0.135	0.018 0.077
iMeC16:0	0.000 0.000	0.000 0.000	0.069 0.099	0.000 0.010
Saturated fatty acids				
C13:0	0.000 0.000	0.011 0.000	0.000 0.000	0.000 0.000
C14:0	0.000 0.000	0.063 0.052	0.075 0.059	0.000 0.028
C15:0	0.000 0.000	0.000 0.000	0.173 0.000	0.000 0.014
C16:0	0.373 0.386	1.051 0.485	0.520 0.415	0.053 0.209
C17:0	0.000 0.000	0.136 0.129	0.176 0.065	0.020 0.035
C18:0	0.058 0.090	0.236 0.141	0.207 0.181	0.030 0.092
C19:0	0.000 0.000	0.036 0.000	0.254 0.000	0.021 0.000
Unsaturated fatty acids				
C18:1ω9t, C18:1ω11c	0.038 0.000	0.184 0.075	0.093 0.000	0.012 0.075
C18:1ω9c	0.728 1.392	2.245 1.371	0.678 0.792	0.083 0.390

The unsaturations are indicated by the symbol 'ω', in position *trans* 't', position *cis* 'c'. iMe indicates a *iso*-methyl branching; OH a hydroxyl branch of fatty acid methyl esters. The upper line corresponds to pile 1, the lower line to pile 2.

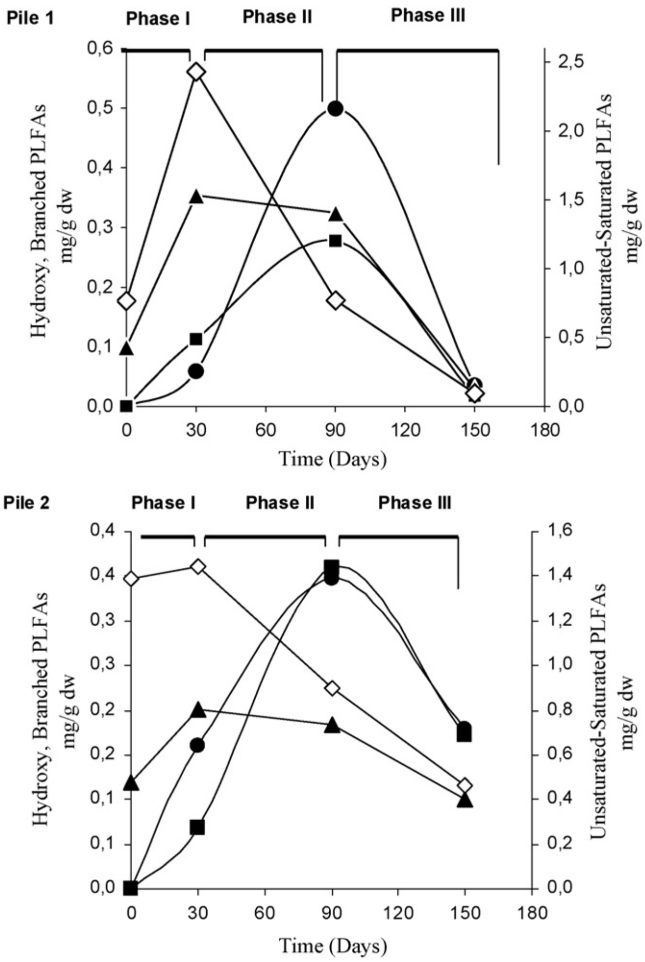

Fig. 3. Evolution of total hydroxyl (●), branched (■), saturated (▲) and unsaturated (◇) PLFAs, at different composting phases of pile 1 and pile 2.

and unsaturated fatty acids can be allotted to mesophilic AHB, but also to possible interference with other eukaryotic fatty acids from moulds, yeasts, algae and plant decomposition.

During the thermophilic phase (II), the unsaturated fatty acid content showed a significant reduction, whereas the saturated PLFA content remained relatively stable (Fig. 3). This divergent evolution is probably dependent on adaptations related to temperature rise. Indeed, the high temperatures observed are accompanied by a significant fall ($p < 0.05$) in the ratio of unsaturated octadecenoic PLFAs to stearic acid (C18:1ω/C18:0) (Fig. 4). This suggests a particular adaptation of microbial growth to high temperatures. Previous works show that the regulation of unsaturated fatty acid synthesis is influenced by growth temperatures; for example, the synthesis of *cis*-vaccenic C18:1ω by pure *Escherichia coli* cultures studied by Magnuson et al. [21] is very dependent on growth temperature. Likewise, Boulter et al. [2] reported a fall in the synthesis of monoenoic PLFAs in Gram-negative bacteria with a culture temperature increase (20–40 °C). An increase in saturated fatty acids with culture temperature in *Pseudomonas fluorescens* (5–33 °C) and *Acinetobacter calcoaceticus* (20–40 °C) cultures were abundantly reported by Cullen et al. [22] and Loffhagen et al. [23]; similar results were also published by Allen and Ingraham [24]

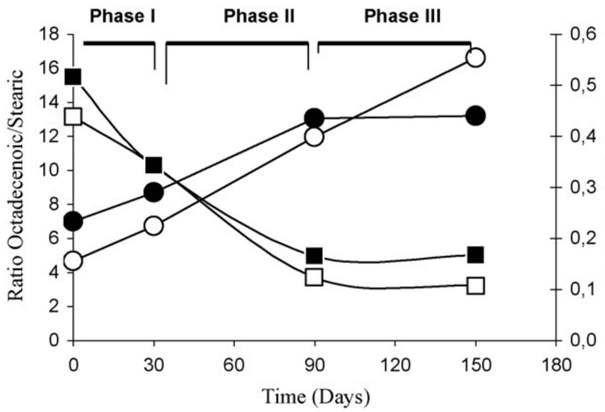

Fig. 4. Unsaturated regression represented by total octadecenoic PLFAs to stearic acid ratio (C18:1ω/C18:0), pile 1 (☐), pile 2 (■); Relative abundance of long aliphatic chains represented by stearic to palmitic acid ratio (C18:0/C16:0), pile 1 (○), pile 2 (●).

and Pugh and Emeric [25] indicating a fall in unsaturated fatty acids with temperature increase in *Escherichia coli*.

The studies in yeasts of Rattray et al. [26] show reduced levels of unsaturated fatty acids in *Candida lipolytica* and *C. tropicalis* when the growth temperature passed from 10 to 25 °C and from 28 to 38 °C, respectively. The enzymatic systems involved in the unsaturation process, reveal activation at low temperatures in *C. utilis*, and deactivation when the temperature rose from 20 to 30 °C for *Bacillus megaterium* [6].

The prevalence of saturated long-chain fatty acids is also shown to be a characteristic of microbial growth at high temperatures [27]. During a waste composting study, the ratio of stearic (melting point 69.6 °C) to palmitic (melting point 63.1 °C) (C18:0/C16:0) increased significantly ($p < 0.05$) during the stabilization phase (Fig. 4); this pattern had already been reported in other work; for example, in the psychrophilic bacterium *Micrococus cryophilus*, studied by McGibbon and Russell [28], the C18/C16 ratio of phospholipid fatty acids drops as the growth temperature drops. In the thermophilic species *Flavobacterium thermophilum*, the elongation of branched fatty acids represented by *iso*C17:0/*iso*C15:0 and *anteiso*C17:0/*anteiso*C15:0 ratios was found to occur more as growth temperatures were increased from 49 to 82 °C [29].

During composting, the beginning of the maturation phase (III) coincides with a progressive regression of branched and hydroxyl fatty acids. We also noted the stabilization of the C18:0/C16:0 ratio in pile 2 (Fig. 4), which is most probably related to the regression of thermophilic and heat-tolerant micro-organisms, following exhaustion of the nutritive elements in the medium, as reflected by a fall in substrate temperature.

Principal components analysis performed between the various PLFAs of the two piles showed grouping of unsaturated fatty acids in the top-left part, whereas branched and hydroxylated PLFAs were found in the lower right. The circle denotes a strong positive correlation between hydroxyl and branched PLFAs, but no significant correlation was found between unsaturated and hydroxyl, branched PLFAs (Fig. 5).

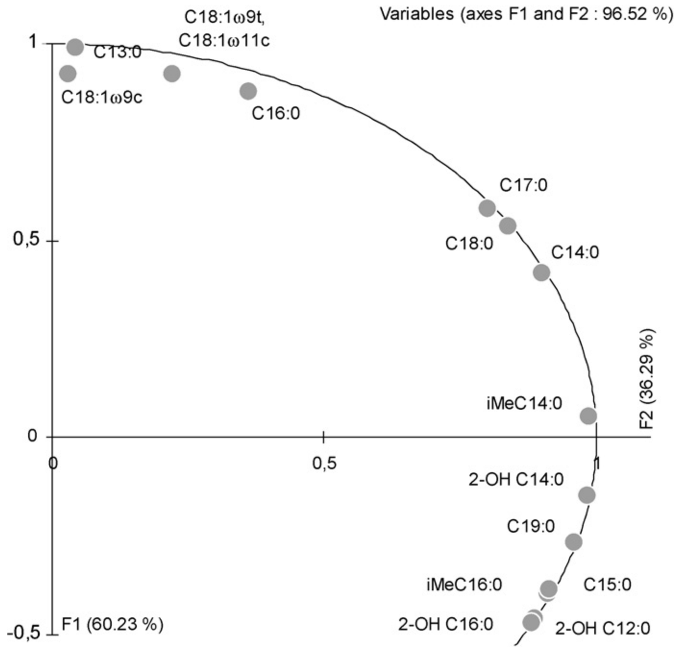

Fig. 5. Principal components analysis represented by projecting the correlation circle of PLFAs on the subspace generated by F1 and F2.

The significant increase ($p < 0.05$) of branched and hydroxyl PLFA content is an indicator of thermophilic species proliferation during phase II. Indeed, the branched and hydroxyl PLFAs are generally taken as being specific to thermophilic bacteria [3,30,31]. The preferential formation of these high-melting-point compounds probably optimises the membrane structure at high temperatures in thermophilic micro-organisms, which means their optimal growth temperatures are around 50–60 °C [32]. Boulter et al. [2] and Eiland et al. [33] noted the same pattern during composting with prevalence of branched fatty acids specific for thermophilic species in the high temperature phase. The significant synthesis of the branched fatty acids in thermophilic species of the genera *Propionibacterium* and *Bacillus* at high temperatures was reported in a paper by Kaneda [6].

The patterns of phospholipid fatty acid levels during composting, indicates the succession of mesophilic and thermophilic communities in the substrates studied. Thus, exhaustion of easily biodegradable substrate leads to reduced levels of PLFAs, related to biomass regression of thermophilic and heat-tolerant micro-organisms. The maturation phase, characterized by relatively low decomposition rates, compared to the stabilization phase, is the period during which there is a remarkable proliferation of moulds, more specialized in lignin and cellulose decomposition [3,30].

4. Conclusion

The comparison of physico-chemical and microbiological parameters during the decomposition of olive oil mill wastes mixed with household refuse, showed the strong decomposition of organic matter during the stabilization phase, characterized by a rise in aerobic heterotrophic bacteria. The reduction of mono-unsaturated fatty acids compared to stearic acid (C18:1ω/C18:0), as well the increase in the proportion of stearic compared to palmitic acids (C18:0/C16:0) and also the high levels of branched and hydroxyl fatty acids, are common features of the way bacterial PLFAs evolve. These characteristics can be used to accurately evaluate the evolution of microbial activity, making them appreciable indicators of progress of the composting process.

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