Tracking down microbial communities via fatty acids analysis: analytical strategy for solid organic samples

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Analysis of phospholipid fatty acid (PLFA) composition is one of the most commonly used culture-independent tools for investigating microbial populations in ecological studies. This methodology provides qualitative and quantitative insights into the structure of the microbial community and indicates the main groups of microorganisms present and their abundance. Perhaps the biggest strength of this lipid-based approach, as compared to other microbial community assays, is that because PLFAs are rapidly synthesized during microbial growth, are not found in storage molecules and degrade rapidly during cell death, it provides an accurate census of the current living community. All cells contain fatty acids (FAs) that can be extracted and esterified to form fatty acid methyl esters (FAMEs). When the FAMEs are analyzed using gas chromatography–mass spectrometry, the resulting profile constitutes a 'fingerprint' of the microorganisms in the sample, since it contains some microbial biomarkers. Although the methods usually used for soil samples have been applied in the analysis of PLFAs in organic samples, it is important to determine the best analytical strategy for solid samples with high organic matter content. We compared different combinations of extraction and derivatization methods for determining FAs, leading to a successful analytical procedure that involves a significant reduction in the complexity and sample preparation time. The application of the optimized methodology to characterize microbial communities is shown for a variety of solid environmental samples.

Keywords Microbial communnities, fatty acids analysis, solid organic environmental samples, composting

1. Microbial Membrane Fatty Acids (PLFAs)

Basic structural components of bacterial cell are the cell wall, the plasma membrane, nuclear DNA, and ribosomes. The plasma membrane consists of a bilayer of lipids, with fatty acids attached to glycerol and proteins embedded in this double layer. Thus, the cell membranes are composed mostly of lipids and proteins. The chemical composition of the membrane of eubacteria is similar to that of an eukaryotic cell (fatty acids generally linear are linked to the glycerol by an ester-type bond: phospholipid fatty acids _PLFAs_). This is a characteristic of the domain Bacteria and Eukarya. In contrast, the membrane lipids of Archaea are composed of long and branched hydrocarbons that are joined to glycerol by an ether-type bond (phospholipid ether lipids _PLELs_). This is one of the most widely used non-genetic criteria to distinguish the three domains. In this context, the phospholipids derived from microbial cell membranes, characterized by different acyl chains, are excellent signature molecules, because such lipid structural diversity can be linked to specific microbial taxa (Figure 1).

In gram-positive bacteria (G^+) the plasma membrane is surrounded by a thick cell wall that contains peptidoglycan and teichoic acids. Gram-negative (G^-) bacteria have a much thinner wall, which in turn is surrounded by an outer cell membrane that is very similar to the plasma membrane. Cell walls of G^- bacteria contain peptidoglycan but lack teichoic acids.

The lipid-based approach to assess the general structure of the microbial community reflects the current living community, because phospholipids are found in the membranes of all living cells but not in storage products (as other lipids) and are rapidly degraded on cell death. The analysis of membrane lipids, like the nucleic acid-based molecular methods, avoids many of the disadvantages associated to culture-based methods, such as the inability to culture most of the cells of interest because optimal culture conditions have not yet been defined or due to problems associated with the dependence on other microbes for growth or with differential growth rates [1].

Most procedures of FAs analysis for the ecological studies of microbial communities have been developed for soil. However, the study of microbial communities in other solid matrixes with higher organic contents is especially interesting: e.g. the biooxidative processes for the treatment of organic wastes, as composting and vermicomposting, involve complex interactions between the organic residues and the microbial communities. Microorganisms are the main drivers of the biological mechanisms involved in these processes, thus their characterization in terms of their FAME profiles would clearly improve the understanding and optimization of such processes. Although the methods usually utilized for soil samples have been bluntly applied in the analysis of PLFAs in organic samples such as compost [2,3], it is important to determine the best combination of extraction and derivatization methods for these types of samples; because each analytical method should be tested exhaustively in the matrices in which it will be applied.

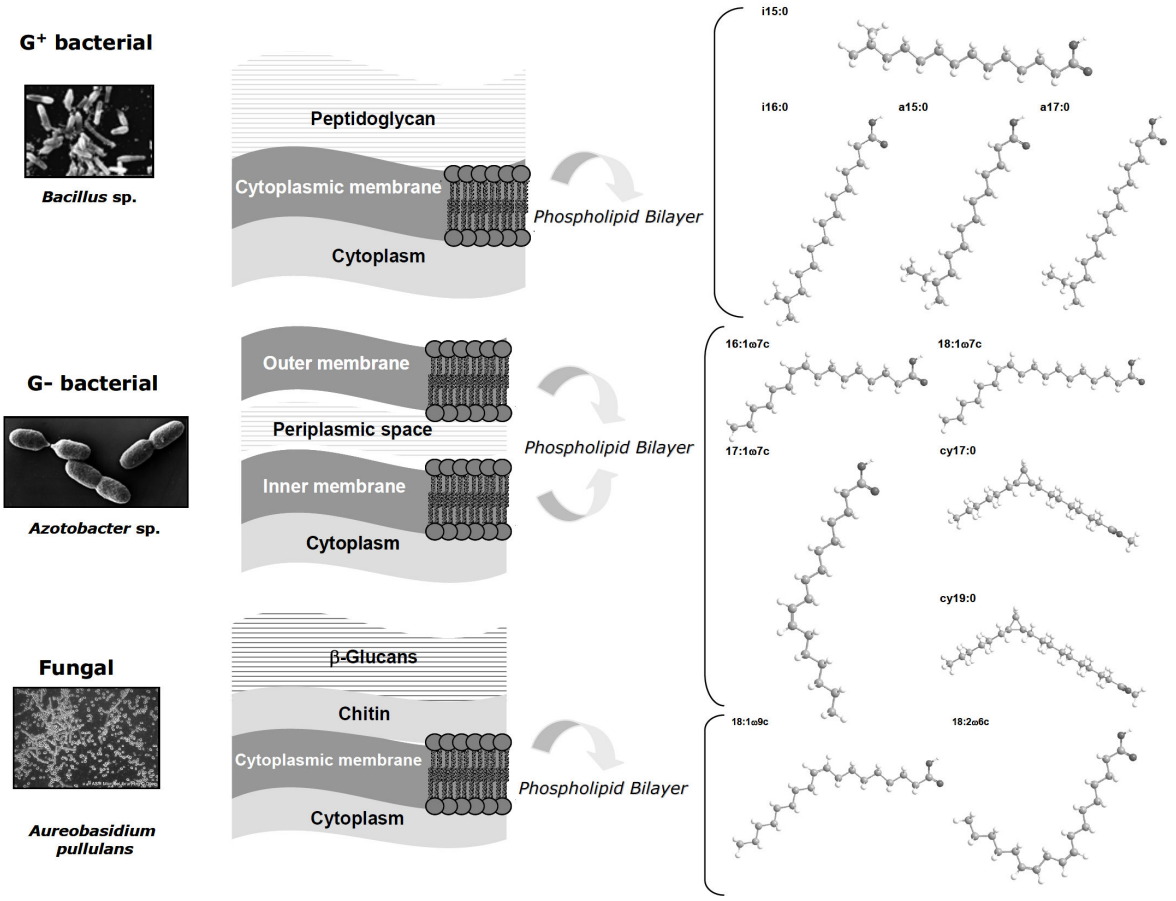

Figure 1. Marker Membrane Fatty acids in the Phospholipid Fraction of Gram$^+$ and Gram$^-$ bacteria and fungi. The prefixes ***i*** and ***a*** suggest the existence of a methyl group at the positions ***iso*** (penultimate carbon) or ***anteiso*** (antepenultimate carbon); the prefix ***cy*** symbolizes the fatty acids with an inner ring of cyclopropane.

2. Methodology Overview

Analysis of FA are divided into several stages beginning with the sample preparation and following by the extraction of the total lipids and the transformation of the fatty acids in their respective methyl esters (derivatization) and, finally, the identification and quantification of such methyl esters (FAMEs, fatty acid methyl esters). The analysis of different lipid classes requires the fractionation of the total lipid extract before the derivatization step.

Sample pretreatment is a key step in the analysis of fatty acids to achieve a better performance in the extraction stage. If the samples have high moisture content, the solubility of the target analytes in low polarity solvents may be influenced, affecting the extraction efficiency; in addition, water is a powerful electron donor and in excess may interfere with some reactions of the analytical process [4]. Drying of sample at low temperatures and under vacuum conditions or lyophilization are two of the treatments used to solve the problem. Besides, the smaller the particle size of the sample, the larger the contact surface with the solvent, and the higher the extraction yield. The homogenization and the use of ultrasonic frequency are two of the procedures used in reducing the particle size.

To extract FA, as to extract any other type of lipids, the choice of the correct procedure should consider the origin of the tissue matrix (animal, plant or microbial origin) among other general considerations. Nevertheless, fatty acids are commonly extracted by the use of mixtures of polar and non-polar organic solvents; this was originally proposed by Folch et al. [5,6], who first applied a chloroform-methanol mixture for the isolation of the total lipid content from brain lipids [5] and then a simplified version of the method to other different animal tissues besides white and gray matter, liver and muscle [6], being this paper one of the most cited scientific publications ever [7].

The Bligh and Dyer method [8] is probably the second most common extraction procedure for lipids, and it is the most likely misunderstood and abused. It was developed as an economical method of extracting the lipids from large volumes of wet tissue, from frozen fish specifically, with the minimum volume of solvent; the endogenous water in the tissue being considered as a ternary component of the extraction system [7]. Modifications of this method have typically been used for extraction of FAs from environmental samples. The most widely used modifications include addition of a buffer (rather than water) to the extraction mixture. White et al. [9] added a phosphate buffer to study microbial

communities in marine and estuarine sediments and Frostegård et al. [10] used a citrate buffer to study soil microbial communities.

Other methods of lipid extraction deal with newer instrumental methods, including pressurized or accelerated solvent extraction and microwave irradiation or ultrasonication to improve yields. Among them, microwave assisted extraction methods have been used for isolation of lipids from biological tissues [11,12] and fungal spores [13]. Recently, the authors [14] employed this technique for extraction of the total FAs from environmental matrixes with high contents of organic matter. The use of the Folch's solvents mixture in MAE has some serious limitations, so it should be replaced by solvents compatible with this extraction technique (e.g: acetone/hexane) [14].

Lipid fractionation was performed by solid phase extraction (SPE). This technique concentrates and purifies the analytes from a solution by adsorption on a solid phase, followed by elution of the analytes with an appropriate solvent for further analysis [15]. The solutions can be, as in this case, extracts from solid samples. Silica-based sorbents are the most commonly used in SPE; silanols, active sites located in the granules of silica acid, have hydroxyl groups attached directly to the silicon atom, and interact with the polar groups of the various lipid classes, while the non polar end of the lipid molecule contributes to their separation. Using three solvents of increasing polarity (chloroform<acetone <methanol) the selective elution of neutral lipids, glycolipids and phospholipids is successfully achieved.

Whatever the chosen method of extraction, the extract obtained is usually subjected to analysis by gas chromatography (GC), and thus the polar FAs are transformed into their less polar methyl ester derivatives (FAMEs) by mild alkaline methanolysis, which is a base-catalyzed reaction [16,17]. Hydroxides such as tetramethylammonium hydroxide (TMAH), trimethylsulfonium hydroxide (TMSH), and trimethylphenylamine hydroxide (TMPAH) have also been employed as derivatization agents for profiling FAs in bacteria [18], zooplankton [19], microalgae [20], soil [21], and organic matrixes [14,22].

The determination of FAMEs by GC is among the most commonplace analyses in lipid research. Quantification of FAME by GC with a flame ionization detector (FID) has been effectively performed for some time, whereas detection with mass spectrometry (MS) has been used chiefly for qualitative analysis of FAME. Nonetheless, the sensitivity and selectivity of MS methods advocate a quantitative role for GC-MS in FAME analysis, an approach that would be particularly advantageous for FAME determination in complex biological samples, where spectrometric confirmation of analytes is advisable. The capacity to combine spectrometric examination and quantitative determination advances GC-MS as a powerful alternative to GC-FID for FAME analysis [23].

3. Analytical strategy for solid organic samples

Although analysis of FAs in highly organic matrixes has already been carried out with the direct application of the methods used for FA soil analysis, the best combination of extraction and derivatization methods for these samples is still required. We studied and compared combinations of three extraction methods (modified Folch method, modified Bligh and Dyer and microwave-assisted extraction (MAE)) and two derivatization methods (alkaline methanolysis and derivatization with trimethylsulfonium hydroxide (TMSH)) for determining phospholipid fatty acids (PLFAs) in soil and solid organic samples (animal manures, compost and vermicompost) [24]. The response variables analyzed were the total amount of PLFAs (total PLFAs) expressed as the sum of the concentrations ($\mu g\ g^{-1}$) of all identified PLFAs; and the concentration ($\mu g\ g^{-1}$) of the individual PLFAs used as biomarkers of specific microbial groups, i.e., the iso/anteiso i15:0, a15:0, i16:0 and a17:0 for G^+ bacteria; the 16:1ω7c, 17:1ω7c, 18:1ω7c, cy17:0 and cy19:0, for G^- bacteria and the fungal biomarkers 18:1ω9c and 18:2ω6c [10,25].

3.1 Optimized methodology

A detailed scheme of the optimized methodology is depicted in Figure 2. Lyophilization was the chosen sample pretreatment; that is, removing of water content by freezing and subsequent sublimation at reduced pressure of the ice formed, resulting in a spongy material that can be later easily dissolved. The sample particle size has been reduced by homogenization.

The best extraction method was the *modified Folch method*; we call so because it involves the use of the same extraction mixture as described by Folch et al. [6] but without the washing step. Total FAs were extracted from 200 mg of each substrate (twelve solid environmental samples) with 60 mL chloroform–methanol, 2:1 (v/v), in 100 mL sterilized plastic jars. The jars were shaken vigorously for 30 min and the mixture was allowed to separate at room temperature for 24 h. The supernatant was filtered, collected in a glass test tube and then evaporated to dryness under a stream of oxygen-free N_2 gas. The total lipid extracts obtained were dissolved in chloroform (3 x 1 mL) and fractionated into neutral lipids, glycolipids and phospholipids, with chloroform (5 mL), acetone (10 mL) and methanol (5 mL), by means of SPE on silicic acid columns (Strata SI-1 Silica (55 mm, 70 Å), 500 mg/6 mL).

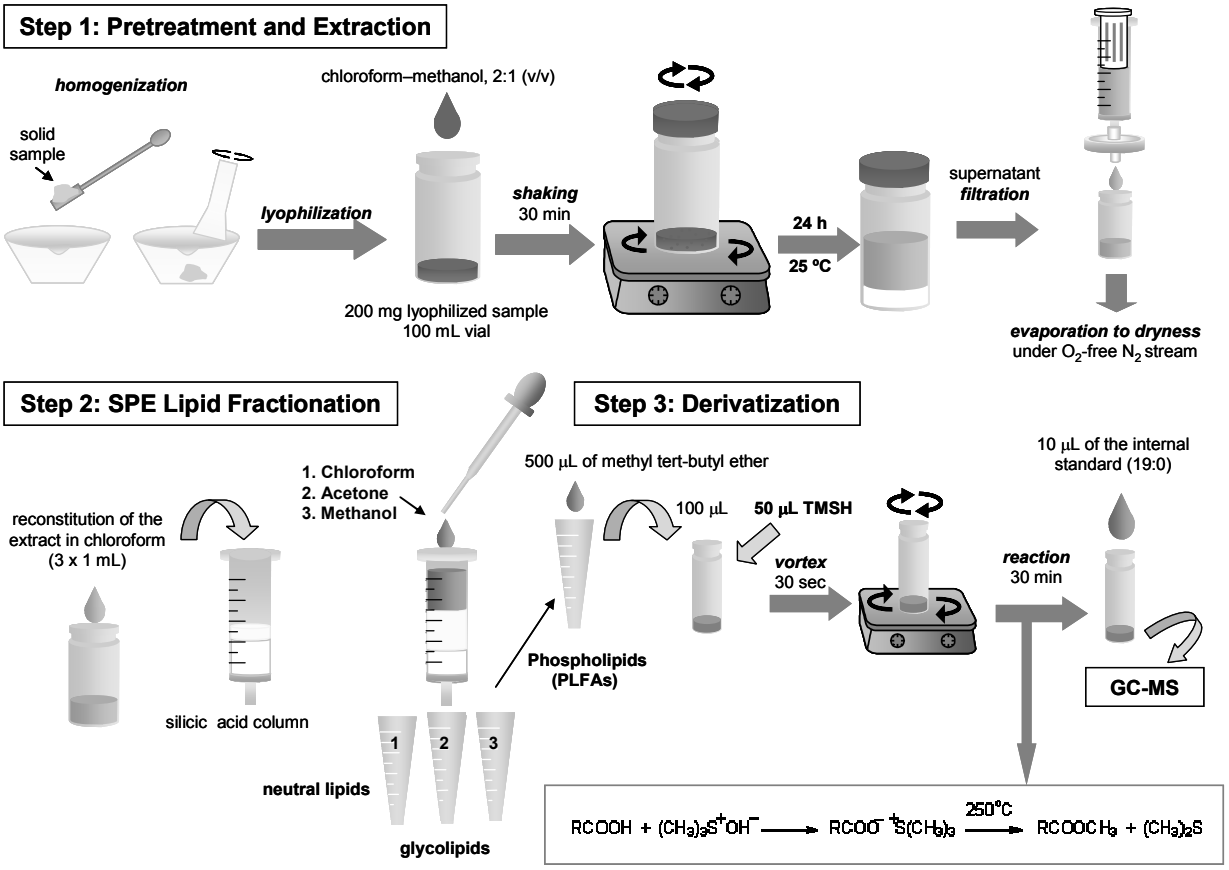

Figure 2. The optimal combination of extraction and derivatization methods proposed for the analysis of FA in solid organic environmental samples: modified Folch method and TMSH.

Phospholipid fractions were subjected to derivatization with TMSH. Briefly, phospholipid extracts were redissolved in 500 µL of methyl tert-butyl ether. This solution (100 µL) was placed in a screw-cap vial with 50 µL of the derivatization agent (TMSH), vortex-mixed for 30 s and allowed to react for 30 min; 10 µL of the internal standard methyl nonadecanoate (19:0, 230 µg mL^{-1}) was then added to the extract of FAMEs. The great advantages of TMSH derivatization are that it is a simple and a non-time-consuming procedure. Esterification with TMSH is performed at room temperature in a fast, single-step reaction, which consists of the formation of trimethylsulfonium salts by deprotonation of FAs (Figure 2). These salts are then thermally decomposed by heating, giving FAMEs and dimethyl sulfide ($(CH_3)_2S$). The byproducts of this reaction (water and dimethyl sulfide) elute with the solvent peak (during the delay period) and do not disturb the chromatographic separation of the analytes [26] which is described below.

FAMEs were separated on a CP-SIL 88 Varian Select FAME FS (50 m x 0.25 mm x 0.2 µm) capillary column in a Varian 3800 gas chromatograph equipped with a Saturn 2000 mass spectrometer (Varian Chromatography Systems, Walnut Creek, CA, USA). The GC oven temperature program was: 50 °C hold 2 min, rate 20° min^{-1} to 140 °C and then 3° min^{-1} to 250 °C. Helium (purity 99.999%) was used as the carrier gas, at a constant column flow rate of 1 mL min^{-1}. The injector was operated in splitless mode and programmed to return to the split mode 2 min after the beginning of a run. The split ratio was 1:50. The injector temperature was 280 °C. The mass spectrometer was operated in the electron ionization mode (70 eV). The mass range was scanned from 40 to 650 amu. Experimental conditions for ionization were: multiplier voltage, 1650 V; filament emission current, 10 µA; axial modulation voltage, 4 V; trap, manifold and transfer line temperatures were 170, 70, and 280 °C, respectively.

To identify the FAMEs, the retention times and the mass spectra were compared with those obtained from pure standards (Figure 3). Mass spectra were also search by means of the *National Institute of Standards and Technology* (NIST) Library. FAMEs were quantified by an internal standard calibration procedure based on peak height (see [27]). The calibration levels of the FAMEs varied in the range 0.4–250 µg mL^{-1}. The coefficients of determination (R^2) were higher than 0.99 for all calibration curves. FAMEs were described by the standard ω-nomenclature A:BωC proposed by the *International Union of Pure and Applied Chemistry- Information Unit for Conventions* [28].

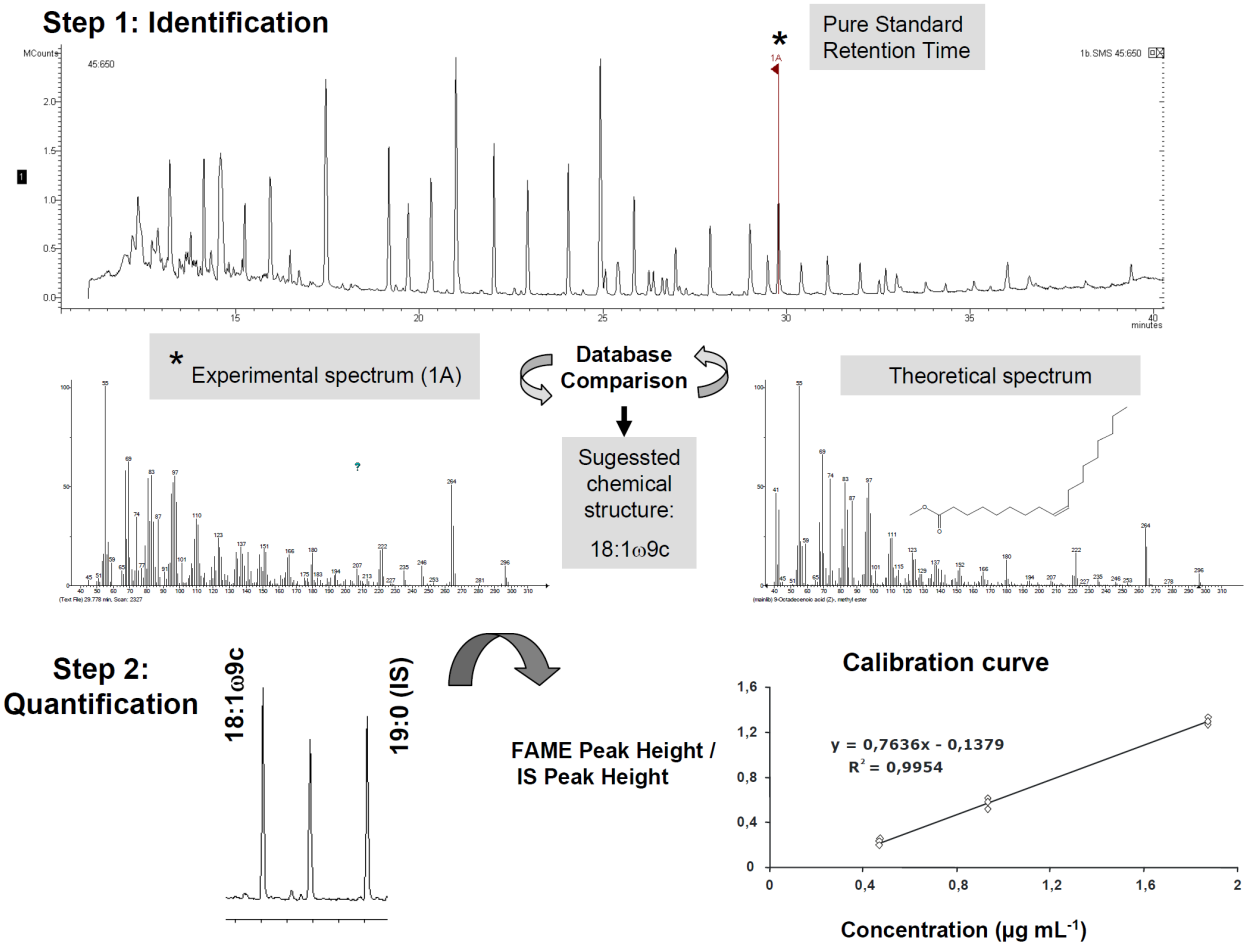

Figure 3. Identification and cuantification of FAMES by means of gas cromatography-mass spectrometry.

3.2 Application of the optimized analytical strategy to solid organic samples

Twelve solid environmental samples were analyzed in the study: soils collected from three different ecosystems (pasture, pine forest and chestnut forest, with 16-19% O.M.); three composts obtained from different organic materials (a mixture of pruning waste, litter and grass clippings; gorse (*Ulex europaeus*); and organic household waste, with 35-53% O.M.); three animal manures (cattle, horse and rabbit with 76-90% O.M.) and their corresponding vermicomposts produced in our laboratory with the earthworm species *Eisenia andrei* (54-85% O.M.).

Twenty-one PLFAs (saturated, mono- and polyunsaturated, and branched) ranging from 10 to 18 carbon atoms were identified and quantified by GC-MS in the analyzed samples. Their corresponding retention times, molecular weights and identification and quantification ions are reported elsewhere [27]. We have proved that the extraction yields of total PLFAs and bacterial and fungal PLFA biomarkers were much higher with the modified Folch method than with the modified B & D and the microwave extraction [24]. We have also indicated that the conversion of PLFAs into FAMEs was higher when TMSH was used as the derivatization agent than by alkaline methanolysis [24]. The total concentration of PLFAs and the abundance of PLFAs biomarkers of bacteria and fungi detected in the samples differed appreciably between the different types of samples, with the highest values in vermicomposts, followed by manure, compost and soil samples [24]. Figure 4 displays the distribution of the microbial groups in the analyzed samples types, showing that gram-positive bacteria are more abundant than gram-negative in all of them: soil (approximately 34 %), manure (27 %), compost (24 %) and vermicompost (45 %). Differences in fungi biomarkers are not so pronounced, varying the sum of the two PLFAs selected (18:1ω9c and 18:2ω6c) from 8.3 for soil and 22.1 µg g^{-1} for vermicompost.

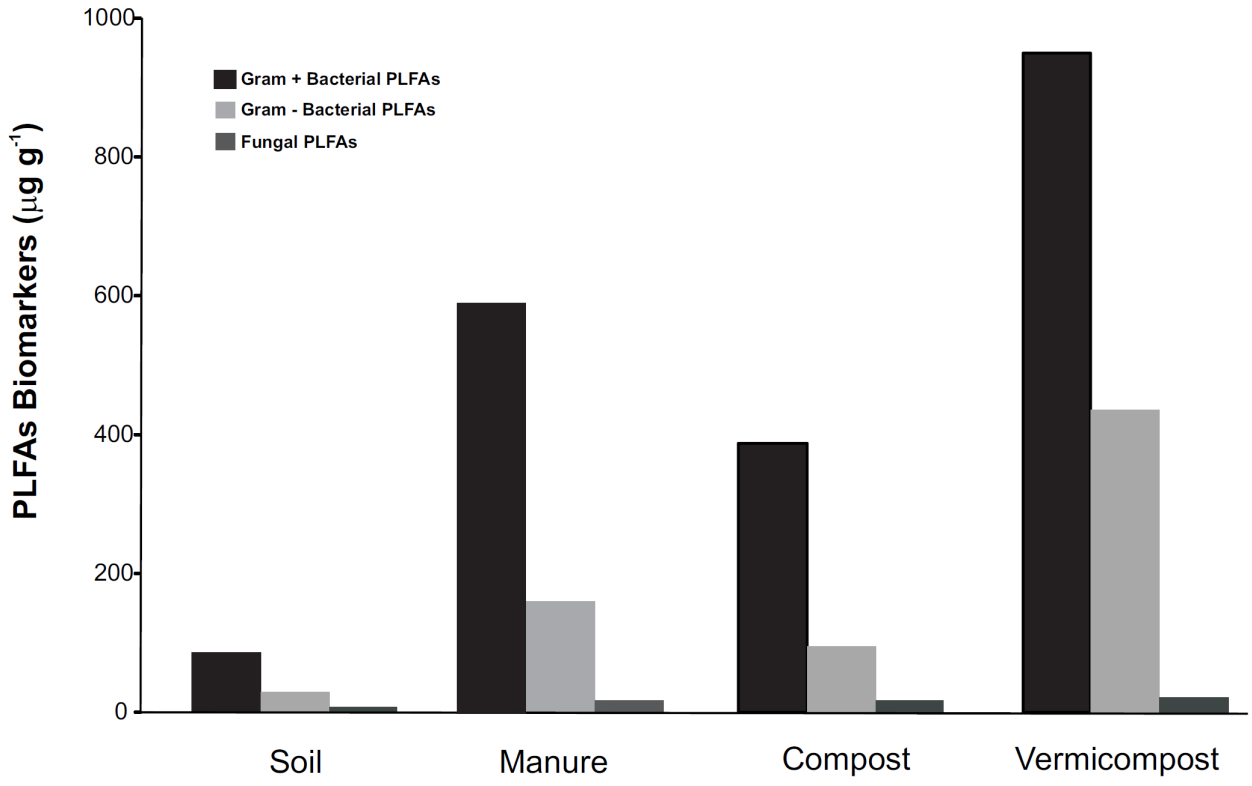

Figure 4. Abundance (μg g^{-1}) of G+ bacterial PLFAs, G^- bacterial PLFAs and fungal PLFAs in the different solid environmental samples using the best combination of extraction and derivatization methods (modified Folch method and TMSH).

4. Conclusion

In line with the results obtained, the modified Folch method and derivatization with TMSH are considered as the best options for the analysis of FAMEs in solid organic environmental samples. The modified Folch method rendered higher yields of FAMEs and the reduced manual labour of this technique results in cheaper operating costs. One great advantage of TMSH derivatization is that it is a simple and non time-consuming procedure.

This optimized analytical methodology can be used to study microbial communities by their FAME content in solid environmental matrixes with high organic matter content. This is extremely useful for comparison of microbial communities in untreated animal wastes as well as in the end products resulting from biological degradation processes (i.e. compost and vermicompost).

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