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Evolution of bacterial diversity during two-phase olive mill waste (“alperujo”) composting by 16S rRNA gene pyrosequencing

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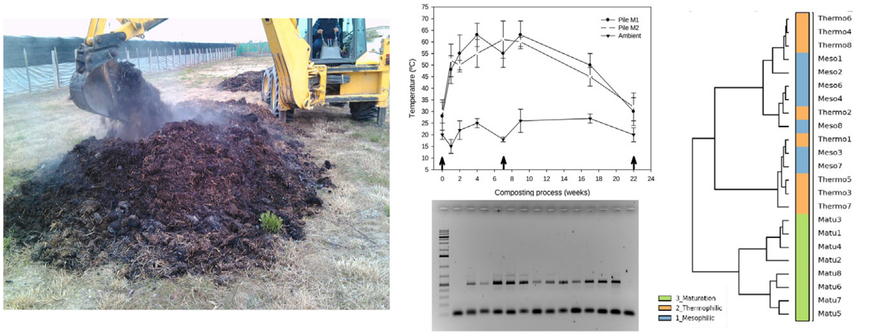
HIGHLIGHTS

- Actinobacteria, Bacteriodetes, Firmicutes and Proteobacteria were the main phyla detected.
- Mesophilic and thermophilic phases did not affect bacterial population.
- Maturation increased bacterial diversity, especially due to new bacterial population were detected.
- *Planomicrobium* and *Ohtaekwangia* are proposed as biomarkers of AL composting maturation.

GRAPHICAL ABSTRACT



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ABSTRACT

Microorganisms are the main contributing factor responsible for organic matter degradation during composting. In this research, the 454-pyrosequencing of the 16S rRNA gene was used to elucidate evolution of bacterial diversity during mesophilic, thermophilic and maturation composting stages of the two-phase olive mill waste (“alperujo”), the main by-product of the Spanish olive oil industry. Two similar piles were performance composting AL with sheep manure as bulking agent. Actinobacteria, Bacteriodetes, Firmicutes and Proteobacteria were the main phyla found in genomic libraries from each composting phase. Shannon and Chao1 biodiversity indices showed a clear difference between the mesophilic/thermophilic and maturation phases, which was mainly due to detection of new genera. PCA analysis of the relative number of sequences confirmed maturation affected bacterial population structure, and Pearson correlation coefficients between physicochemical composting parameters and relative number of genera sequences suggest that *Planomicrobium* and *Ohtaekwangia* could be considered as biomarkers for AL composting maturation.

1. Introduction

To promote sustainable development, the European Union has adopted a legislative initiative called the Circular Economy (de Man and Friedge, 2016) whose objectives focus on reducing the use of raw materials, minimizing waste production, and encouraging reuse and recycling. In Spain, the olive oil industry generates a large amount of a solid by-product called “alperujo” (AL), a highly contaminative organic waste that needs to be treated for its revalorisation (Alburquerque et al., 2004). In order to achieve that, composting can be performed due to it being a simple, inexpensive and effective method for transforming organic waste as AL into organic amendments and fertilisers (Tortosa et al., 2012; Muktadirul Bari Chowdhury et al., 2013).

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Composting is a biological process by which the organic matter from the raw materials is degraded by native microbial population due to its metabolic activity. This transformation is carried out by a complex temporal succession of a large number of microorganisms included bacteria, actinobacteria and fungi (López-González et al., 2015). Indeed, these population developments are directly affected by the evolution of several environmental factors of composting substrates like moisture, nutrient availability (especially carbon and nitrogen), oxygen rate, physical structure (particular size), pH, salinity, etc. (Insam et al., 2010). One of the main selective parameters in microbial evolution during composting is probably temperature, which defines the four phases of the process: mesophilic (25–40 °C), thermophilic (35–65 °C), cooling and maturation (Insam et al., 2010).

Bacterial diversity in composting has been investigated for decades using different approaches (culture and cultured-independent methods). Molecular methods like clone library sequencing, DNA fingerprinting, diagnostic microarrays or qPCR can be used successfully to monitor microbial community composition in detail (Hultman et al., 2010). Recent molecular tools based on DNA high-throughput sequencing technologies are considerably increasing the knowledge of microbial communities involved in composting. Until now, there are few examples of microbial ecology studies using this promising technology. Bibby et al. (2010) composted biosolids from wastewater treatment plants with sawdust, woodchips, or green waste, using a full scale windrow composting system. Partanen et al. (2010) worked in a pilot-scale compost plant, as well as a full-scale composting system using municipal biowaste mixed with wood chips. de Gannes et al. (2013) worked in a in-vessel rotary composting reactors (200L), using rice straw, sugar cane bagasse and coffee hulls which were amended with either cow or sheep manure. Neher et al. (2013) carried out the most complete study of microbial evolution during composting until now. They studied different compost recipes, composting systems (windrow, aerated piles and vermicomposting) and composting time. Tkachuk et al. (2014) used a static composting method for carcass, beef and cattle mortalities. Storey et al. (2015) worked in an industrial windrow composting plant, using shredded green waste and spent brewery grains with calcium ammonium nitrate or sludge from a wastewater plant as nitrogen amendments. Zhang et al. (2016) investigated in a 90-m^3 industrial-scale fermentor cornposting corn cobs mixed with fresh cow dung. Currently, no report using high-throughput sequencing applied to olive mill waste composting has been published. So, the information about bacterial communities evolution and dynamics during AL composting remains scarce.

In Tortosa et al. (2012), we demonstrated that composting is a feasible biotechnology for AL treatment and its revalorisation. Indeed, the physicochemical evolution of the organic matter degradation was studied in detail. In this research, we have focused on microbial aspects. We have used 454-pyrosequencing to study how microbial population and its evolution could be affected by the composting process. Also, we aimed at finding specific biomarkers for AL composting, with emphasis on the maturation process.

2. Materials and methods

2.1. Composting performance, raw material characterization and sampling procedure

Two trapezoidal piles, M1 and M2, of about 10 t each were arranged by mixing two-phase olive mill waste (“alperujo”, AL) and sheep manure (SM) (1:1, AL:SM, fresh weight) using a backhoe loader as previously indicated (Tortosa et al., 2012). The composting system was open and seven mechanical turnings were applied according to bioxidative phase and temperature evolution (Fig. 1), being more frequently during thermophilic phase (four turnings within the first two months). The substrate moisture was controlled by an aspersion system and kept above 40%.

To control and monitor the composting process, several parameters were analysed. Moisture content and temperature, pH, and electrical conductivity (EC), total nitrogen (T_N), total organic carbon (T_{OC}), total organic matter (OM), fat content, water-soluble carbon (WSC), water-soluble phenolic substances (WSPH), water-soluble carbohydrates (WSCH), lignin, cellulose and hemicellulose, and phytotoxicity by germination index (GI) tests with cress (*Lepidium sativum*) were determined as previously referred (Tortosa et al., 2012). The humification indices, humification ratio (HR), humification degree (HD) and percentage of humic acids (P_{AH}), were calculated according to Alburquerque et al. (2009).

Thirty (30) subsamples were randomly taken from the 0–25 cm layer of each pile after the mechanical turnings at the 1st, 7th and 22nd weeks, corresponding to the mesophilic, thermophilic and maturation phases, respectively (Fig. 1). Then, the samples were mixed, kept in a portable fridge, brought to the laboratory within 24 h of collection and maintained at −80 °C until DNA extraction and agrochemical characterization. For all experiments, a sample was defined as a composite of 30 subsamples collected at random depths from a given pile that were mixed to be representative of a pile. For each M1 and M2 piles, 4 analytical replicates were obtained from each composite sample corresponding to the mesophilic, thermophilic and maturation phases. A total of 24 replicates, 12 per pile, were then analysed.

2.2. DNA extraction and quantification

Frozen compost samples (~5 g) were homogenized with pestle and mortar under liquid nitrogen according to the recommendations by Neher et al. (2013). Then, DNA was extracted from 250 mg using the commercial PowerSoil® DNA isolation kit (MO-BIO) after mechanical breakage using a minibead beater cell disrupter (Mikro-Dismembrator S; Sartorius Stedim Biotech) for 30 s at 1600 rpm. Quality and size of DNAs were checked by electrophoresis on 1% agarose gel (40 min at 80 mV) stained with GelRed™ (Biotium) under UV light. DNAs were also quantified by spectrophotometry at 260 nm using a Nanodrop 1000 Spectrophotometer (Thermo Scientific).

2.3. Amplification and pyrosequencing of DNA

PCR amplification of the hypervariable V4-V5 regions of the 16S rRNA gene was performed over each individual DNA extraction using the coded-primer approach to multiplex pyrosequencing (Parameswaran et al., 2007). PCR was performed with an 8 bp key-tagged sequence joined to universal primers U519F and U926R (Baker et al., 2003). PCR reactions (50 µl) were done using a Master Taq kit 5 PRIME 1000U (http://www.5prime.com/) and contained 0.4 µM of each primer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs mix (0.1 mM each), 1× MasterTaq buffer (1 mM Mg^{2+}), 1× Taq Master PCR Enhancer, 0.75 U of Taq DNA Polymerase, molecular-biology-grade water and 1–150 ng of the DNA template. The PCR program consisted of an initial denaturation step at 94 °C for 3 min, 25 cycles with denaturation at 94 °C for 15 s, primer annealing at 55 °C for 45 s and extension at 72 °C for 1 min, and finally, a heating step at 72 °C for 8 min for final extension. For each sample, only one amplicon per PCR reaction was generated and amplicon bands were quantified directly from agarose gel using BIO-RAD Quantity One software. All bands were equilibrated to the same concentration and checked again in 1% agarose gel. After that, samples were combined in equimolar amounts in one sample to reduce

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