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Quantification and characterization of microbial biofilm community attached on the surface of fermentation vessels used in green table olive processing

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ABSTRACT

The aim of the present study was the quantification of biofilm formed on the surface of plastic vessels used in Spanish-style green olive fermentation and the characterization of the biofilm community by means of molecular fingerprinting. Fermentation vessels previously used in green olive processing were subjected to sampling at three different locations, two on the side and one on the bottom of the vessel. Prior to sampling, two cleaning treatments were applied to the containers, including (a) washing with hot tap water (60 °C) and household detergent (treatment A) and (b) washing with hot tap water, household detergent and bleach (treatment B). Population (expressed as log CFU/cm^2) of total viable counts (TVC), lactic acid bacteria (LAB) and yeasts were enumerated by standard plating. Bulk cells (whole colonies) from agar plates were isolated for further characterization by PCR-DGGE. Results showed that regardless of the cleaning treatment no significant differences were observed between the different sampling locations in the vessel. The initial microbial population before cleaning ranged between 3.0–4.5 log CFU/cm^2 for LAB and 4.0–4.6 log CFU/cm^2 for yeasts. Cleaning treatments exhibited the highest effect on LAB that were recovered at 1.5 log CFU/cm^2 after treatment A and 0.2 log CFU/cm^2 after treatment B, whereas yeasts were recovered at approximately 1.9 log CFU/cm^2 even after treatment B. High diversity of yeasts was observed between the different treatments and sampling spots. The most abundant species recovered belonged to *Candida* genus, while *Wickerhamomyces anomalus*, *Debaryomyces hansenii* and *Pichia guilliermondii* were frequently detected. Among LAB, *Lactobacillus pentosus* was the most abundant species present on the abiotic surface of the vessels.

1. Introduction

Table olives are one of the most important groups of vegetables marketed and consumed as fermented. A proper fermentation procedure results in a microbiologically safe final product with enhanced sensory attributes. This is achieved primarily by the growth of LAB that metabolize fermentable sugars diffused from the olive mesocarp into the brine and produce lactic acid which decreases the pH in the brine, resulting thus in the inhibition of undesirable microorganisms and the extended preservation of the end product even at ambient temperature (Corsetti et al., 2012; Heperkan, 2013). Development of some yeast species is also desirable as they contribute in the production of aroma compounds and maintenance or even stimulation of LAB populations (Arroyo López et al., 2008, 2012a; Viljoen, 2006). The population dynamics of these diverse microbial groups throughout the fermentation process were, until recently, monitored in the cover brines. However, it has been shown that LAB and yeast communities colonize the surface of the naturally black and Spanish style table olives drupe forming mixed species aggregates characterized as biofilms (Domínguez Manzano et al., 2012; Grounta and Panagou, 2014; Nychas et al., 2002).

Biofilm formation in food processing environments has been the focus of extensive scientific research, especially in the context of food hygiene, as many outbreaks have been associated with the presence of biofilms in food industries (Srey et al., 2013). Biofilms are defined as functional consortia of microorganisms attached to a surface which are embedded in the extracellular polymeric substances (EPS) produced by the microorganisms (Monds and O'Toole, 2009). It has been realized that biofilm formation is a natural phenomenon which occurs whenever there are microorganisms and surfaces, either biotic or abiotic, surrounded by a high or a low level of nutrients (Elhariry, 2011; Giaouris et al., 2014). In these environments, the accumulation of food nutrients at the solid/liquid interface on food surfaces leads to a higher concentration of nutrients compared to the fluid phase which as a process is known as "conditioning film" (Donlan, 2002). After film conditioning, microorganisms may adhere on the food contact surface, gradually form microcolonies and finally assemble themselves in biofilms exhibiting high microbial diversity in terms of genera, species and strain levels (Borucki et al., 2003; Burmølle et al., 2006). A similar

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phenomenon takes place during table olive fermentation as the outflow of nutrients from the mesocarp into the brine medium may serve as a means of conditioning film on both the olive epidermis and the surface of the container where fermentation takes place and thus forms sites of adherence and biofilm development. The presence of biofilm on the surface of olive drupes has been extensively studied by various research groups in the last years, since the olive surface may serve as a carrier of beneficial microorganisms to the consumer, transforming thus table olives from a traditional agricultural commodity to a high-added functional food (Arroyo López et al., 2012b; Blana et al., 2014; Lavermicocca et al., 2005; Rodríguez Gómez et al., 2013). So far, the formation of biofilms on the abiotic surface of fermentation vessels has received little attention by the table olive research community. Recently, Spanish researchers (Domínguez Manzano et al., 2012) investigated the establishment of polymicrobial communities on abiotic (glass slides) surfaces that come into contact with the brine during Spanish style table olive fermentation and confirmed the ability of microorganisms to adhere and produce biofilms. The presence of this microbial community on the abiotic surfaces, composed primarily of members of the technological microbiota may have an impact on olive fermentation. Nowadays, it is well established that the natural microbiota of olives is greatly reduced during lye treatment and the effectiveness of the process depends mainly on the number of LAB introduced during the subsequent washing and brining operations (Garrido Fernández et al., 1997). However, biofilm formation is a stepwise and dynamic process consisting of several stages, among which dispersal is the last step in the biofilm cycle, allowing thus cells to revert into their planktonic form (Srey et al., 2013). In this sense, the biofilm formed on the abiotic surfaces of the fermentation vessels may contribute to the process with microorganisms of the technological microbiota, namely LAB and yeasts, through the mechanism of biofilm detachment into the brine.

The objective of the present study was to investigate the biofilm community formed on the surface of plastic containers used in the fermentation of green table olives at different sampling locations and different cleaning treatments of the vessel, as well as to characterize the microbiota assembling this community by molecular fingerprinting. To our knowledge, this is the first report on the characterization of microbial biofilms on the surface of fermentation vessels and the results of this work could provide further insights in the fermentation process of green olives.

2. Materials and methods

2.1. Sampling from the vessels and microbiological analysis

The fermentation vessels employed in this work were screw-capped plastic vessels (14 L total capacity) that were previously used in the spontaneous Spanish-style fermentation of cv. Halkidiki green olives as described elsewhere (Blana et al., 2014). At the end of fermentation olives and brine were discarded, the vessels were washed with pressurized tap water, left to dry and subsequently two sequential cleaning treatments were applied namely, (a) cleaning with hot tap water (60 °C) and household detergent (treatment A) and (b) cleaning with hot tap water, household detergent and bleach (treatment B). Before and after each cleaning treatment, the vessels were subjected to sampling at three different locations on the inside of the container, namely, (i) on the upper spot (near the opening of the vessel), (ii) on the middle spot (halfway up the side), and (iii) on the bottom of the vessel. Sampling was implemented using a fixed template of 56 cm^2 (7 cm wide × 8 cm long) for the upper and middle spots, whereas for the bottom of the vessel a fixed area of 77 cm^2 (corresponding to the half bottom area of the vessel) was sampled (see Supplementary Fig. 1). The sampling areas were thoroughly scrubbed with sterile gauze which was submerged in sterile urine collectors containing 30 mL Phosphate Buffered Saline (PBS). The gauze was left for about 20 min to allow suspension of cells in the PBS solution and microbiological analysis was carried out from this suspension. The resulting PBS suspension was serially diluted in Ringer's solution and duplicate 1 or 0.1 mL from the appropriate dilution were poured or spread on agar media. TVC were enumerated on Tryptone Soy Agar (TSA; LabM, Lancashire, UK) incubated at 25 °C for 48 h. Yeasts were enumerated on Rose Bengal Chloramphenicol agar (RBC; LabM, Lancashire, UK) incubated at 25 °C for 48–72 h. LAB were enumerated on de Man–Rogosa–Sharpe agar (MRS; LabM, Lancashire, UK) supplemented with 0.05% (w/v) cycloheximide (AppliChem, Darmstadt, Germany), overlaid with 10 mL of molten medium, incubated at 30 °C for 48–72 h. Results were expressed as log values of colony forming units per cm^2 of surface area (log CFU/cm^2). Following cleaning treatments, the vessels were left to dry and 60 days later, the vessels were subjected to the same cleaning treatments and sampling was performed in the same sampling spots to investigate any potential changes in the biofilm communities during storage of the vessels. Duplicate vessels were analyzed for each treatment denoted herewith as vessel a and b, respectively.

2.2. Preparation of bulk cells and DNA extraction

Genomic DNA was extracted from bulk cells on MRS, RBC and TSA plates for each cleaning treatment and sampling spot. The whole cultivable community from each agar plate was diluted in an appropriate volume of Ringer's solution and 1 mL of the bulk was stored in a cryoprotect vial at −80 °C for further use. DNA was extracted using the protocol described by Doulgeraki et al. (2012a). One millilitre of bulk cells was centrifuged at 9000 *g* for 5 min at 4 °C, washed with Tris–EDTA buffer (100 mM Tris, 10 mM EDTA) and finally resuspended in 0.5 mL buffer solution (1 M sorbitol, 0.1 M EDTA, pH 7.4) containing 25 mg/mL lysozyme. After incubation at 37 °C for 2 h, the samples were centrifuged at 17,000 *g* for 10 min at 4 °C and the pellet was resuspended in 0.5 mL buffer solution (50 mM Tris–HCl, 20 mM EDTA, pH 7.4). Subsequently, 50 μL of 10% SDS was added and then the samples were incubated at 65 °C for 30 min. After incubation, 0.2 mL potassium acetate 5 M was added to the samples which were left on ice for 30 min and then centrifuged at 17,000 *g* for 20 min at 4 °C. The supernatant was precipitated with 1 mL ice cold isopropanol. After precipitation, the samples were centrifuged at 17,000 *g* for 10 min at 4 °C and the resulting pellet was resuspended in 0.5 mL ice cold 70% ethanol. The samples were then centrifuged at 17,000 *g* for 10 min at 4 °C and the resulting pellet was left to dry. Finally, the dried pellet was resuspended in 50 μL sterile double distilled H_2O and stored at −80 °C until further use.

2.3. PCR-DGGE analysis

Genomic DNA from bulk cells on TSA and MRS plates was amplified by PCR targeting the variable V6–V8 region of the 16S rRNA as described by Ercolini et al. (2006). Genomic DNA from bulk cells on RBC and TSA plates was amplified by PCR targeting the 5.8S — ITS rDNA region according to Nisiotou et al. (2010a). PCR products were confirmed by gel electrophoresis in 1.0% (w/v) agarose gel followed by ethidium bromide staining. Subsequently, positive PCR products were analyzed by Denaturing Gradient Gel Electrophoresis (DGGE) using a DCode apparatus (BioRad) according to Ercolini et al. (2006) with a gradient from 22 to 55% and 20 to 60% for bacteria and yeasts, respectively. Electrophoresis was performed at 200 V for 4 h (with an initial 10 min at 50 V) at 60 °C in 1× TAE buffer. The gels were then stained with ethidium bromide for 5 min, rinsed for 20 min in distilled water and photographed using a Gel-Doc system (BioRad, Hercules, CA, USA).

The obtained DGGE profiles were compared with those of bacteria and yeast strains belonging to the Food Microbiology Culture Collection (FMCC) of the Agricultural University of Athens, previously isolated from environments associated with table olive fermentations which were used as reference strains. For bacteria characterization, *Lactobacillus pentosus* B281 and *Lactobacillus plantarum* B282 were used as reference strains (Blana et al., 2014). For yeast characterization,