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# Acetic acid bacteria from biofilm of strawberry vinegar visualized by microscopy and detected by complementing culture-dependent and culture-independent techniques

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ABSTRACT

Acetic acid bacteria (AAB) usually develop biofilm on the air–liquid interface of the vinegar elaborated by traditional method. This is the first study in which the AAB microbiota present in a biofilm of vinegar obtained by traditional method was detected by pyrosequencing. Direct genomic DNA extraction from biofilm was set up to obtain suitable quality of DNA to apply in culture-independent molecular techniques. The set of primers and TaqMan – MGB probe designed in this study to enumerate the total AAB population by Real Time – PCR detected between $8 \times 10^5$ and $1.2 \times 10^6$ cells/g in the biofilm. Pyrosequencing approach reached up to 10 AAB genera identification. The combination of culture-dependent and culture-independent molecular techniques provided a broader view of AAB microbiota from the strawberry biofilm, which was dominated by *Ameyamaea*, *Gluconacetobacter*, and *Komagataeibacter* genera. Culture-dependent techniques allowed isolating only one genotype, which was assigned into the *Ameyamaea* genus and which required more analysis for a correct species identification. Furthermore, biofilm visualization by laser confocal microscope and scanning electronic microscope showed different dispositions and cell morphologies in the strawberry vinegar biofilm compared with a grape vinegar biofilm.



## 1. Introduction

Acetic acid bacteria (AAB) are obligate aerobic microorganisms able to efficiently convert ethanol to acetic acid and, the main bacteria responsible for vinegar elaboration.

There are two main vinegar production methods: traditional and submerged. Traditional method is characterized by slow acetification carried out in wood barrels resulting in high-quality vinegar (Solieri et al., 2009). It is a static method, the so-called surface culture method or Orleans method, where AAB are placed on the air–liquid interface, developing a biofilm in direct contact with oxygen. Part of this biofilm, also called "vinegar mother," is used as starter culture to inoculate a new batch for vinegar production through a back-slopping procedure (Gullo et al., 2009).

Biofilm formation is commonly observed in several traditional fermentation processes (Domínguez-Manzano et al., 2012; Wang et al., 2012). This structure has been related with cell–cell communication via quorum-sensing signalization in some bacterial species (Davies et al., 1998), and it provides a protective growth environment that allows to tolerate the extreme conditions and to survive in a hostile medium as vinegar (Solieri et al., 2009). Biofilm produced by AAB is constituted by different exopolysaccharides (EPS), with cellulose as the main EPS (Chawla et al., 2009). The production of cellulose by *Komagataeibacter xylinus* (formerly *Gluconacetobacter xylinus*; Yamada et al., 2012) has been widely studied due to its industrial biosynthesis applicability. These studies were mainly focused on the composition and structure of these EPS pellicles (Chawla et al., 2009; Krishnamachari et al., 2011). However, other AAB genera have also been reported as cellulose producers, like *Gluconacetobacter* (Aydın and Aksoy, 2014) or *Acetobacter* (Gullo et al., 2012).

Molecular techniques have demonstrated their usefulness to determine the AAB diversity in ecological vinegar studies. Culture-dependent techniques are efficient for typing and monitoring AAB

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strains throughout vinegar elaboration process (Hidalgo et al., 2013; Wu et al., 2012), whereas culture-independent techniques are a good approach to analyze total AAB diversity present in vinegar production (Mamlouk et al., 2011; Xu et al., 2011). Moreover, some studies reported a good correlation between the results obtained by both approaches (Jara et al., 2013; Vegas et al., 2013). As far as we know, there are no studies which detect the AAB population in biofilm of traditional vinegar using culture-independent techniques. The studies from these vinegar biofilms are limited and had always been carried out after a step on culture media (Gullo et al., 2009; Nanda et al., 2001).

The culture-independent techniques based on next-generation sequencing systems, such as pyrosequencing, have been described as a more complete alternative to capture the whole complexity of the communities present in different fermented products (Ercolini, 2013; Illeghems et al., 2012; Jung et al., 2011; Roh et al., 2010) and very recently in one of famous vinegars in China obtained by solid-state fermentation technology (Nie et al., 2013). However, it has never been used to study the microbiota present in any other vinegar either in the liquid or in the biofilm matrix.

Therefore, the aim of this study was to analyze the presence of AAB in a biofilm from strawberry vinegar obtained by traditional method, using different molecular methods, including next-generation techniques. The disposition of microbial cells in the strawberry biofilm was also analyzed by laser confocal microscope (LCM) and scanning electronic microscope (SEM) and was compared with a grape vinegar biofilm also obtained by traditional method. The technological aspects of the elaboration of this strawberry vinegar and the analysis of the yeast and AAB diversity responsible for its production were previously reported (Hidalgo et al., 2010, 2013).

## 2. Materials and methods

The strawberry vinegar biofilm analyzed in this study and also the grape vinegar biofilm used for microscopy study were obtained from Mas dels Frares Experimental Cellar (Constantí, Tarragona, Spain). Both pieces of biofilms were collected from the surface of vinegar elaborated in wood barrels using the traditional method. They were samples of approximately 300 mm long × 200 mm wide and 50 g, each one. The samples were aseptically deposited in a sterile tube, transported to the laboratory, and stored at 4 °C before being analyzed. The laboratory where this research was performed meets the ISO 9001 regulations.

### 2.1. Microscopic study

#### 2.1.1. Laser confocal microscope

Three samples of 25 $mm^2$ from different parts of each vinegar biofilms (from both ends and from the middle of biofilm) were carefully and aseptically cut and disposed on a glass slide. One microliter of Syto9 dye from the Live/Dead BacLight kit (Molecular Probes, Eugene, OR, USA) was directly added to each piece of biofilm and incubated in the dark for 20 min. After that, each sample was washed with 2 μL of washing buffer (20 mM Tris–HCl, 0.9; 0.9 M NaCl and 0.1% sodium dodecyl sulfate) to eliminate the excess of dye and visualized in a LCM (Nikon, model TE2000-E).

#### 2.1.2. Scanning electronic microscope

Other three samples of 25 $mm^2$ from each biofilms were fixed in glutaraldehyde solution (6% glutaraldehyde in 0.1 M phosphate-buffered saline, pH 7.3). They were treated with 1% of osmium tetroxide for 2 h. After that, the samples were dehydrated using a graded ethanol series. Ethanol was replaced for amyl-acetate before drying to the critical point. The samples were then gold coated for their visualization in a SEM (JEOL model JSM-6400).

### 2.2. Plate growing

The strawberry vinegar biofilm was plated by triplicate streaking the loop with three samples of approximately 100 $mm^2$ onto the GY medium (5% glucose, 1% yeast extract, and 1.5% agar) supplemented with natamycin (100 mg/L) (Delvocid, DSM; Delft, The Netherlands) to suppress fungal growth. After 2 days of incubation at 28 °C, the colonies were grown on the GYC medium (10% glucose, 1% yeast extract, 2% $CaCO_3$, and 1.5% agar) to determine their acid production by the dissolution of $CaCO_3$ precipitates on plates. The colonies with a clear halo around them were subjected to the catalase test; positive results supported their putative identity as AAB and were identified by molecular methods.

### 2.3. Genomic DNA extraction

Colonies grown on plates were processed for DNA extraction using the protocol of cetyltrimethylammonium bromide (CTAB) method (Ausubel et al., 1992).

On the other hand, the remaining biofilm was analyzed by culture-independent techniques for which, DNA from biofilm was directly extracted using a protocol that consisted of a pre-treatment of the sample and the method of Ausubel et al. (1992) modified. The biofilm was aseptically cut in a total of nine portions, each one of five grams. Each biofilm portion was pre-treated to release cells from the matrix, prior to the DNA extraction, as follows: it was stirred at 200 rpm overnight in 100 mL of buffer (100 mM Tris–HCl [pH 8.0], 100 mM Na-EDTA [pH 8.0], 1.5 M NaCl, and 0.1% Tween 80), and then 1 g of glass beads was added to the samples and vortexed for 10 min. The resultant solution was vacuum filtered through a paper membrane filter grade 1 (Whatman, Maidstone, UK). Then the membrane was washed twice with 0.1 M NaCl, and the filtrated solution was centrifuged at 4500 rpm for 15 min at 4 °C. Pellets were washed twice with polyvinylpyrrolidone-EDTA (0.15 M NaCl, 0.1 M EDTA, and 2% w/v polyvinylpyrrolidone; Sigma–Aldrich, Munich, Germany) and once with sterile water for polyphenols elimination. Genomic DNA extraction from these pellets was performed by CTAB method described by Ausubel et al. (1992), with some modifications. Briefly, the pellet was resuspended with 520 μL of TE buffer (10 mM Tris–HCl, 100 mM EDTA, and 0.80 M NaCl, pH 8), 40 μL of lysozyme (20 mg/mL), and 10 μL of RNAse (1 mg/mL) and incubated at 37 °C during 1 h. A quantity of 30 μL sodium dodecyl sulfate 20% and 6 μL of proteinase K (20 mg/mL) was added and incubated at 50 °C for 30 min. The lysis concluded with CTAB, and the steps of purification with fenol–-chloroform–isoamilic alcohol (25:24:1) repeating at least twice as well as DNA precipitation were carried out, as described by Ausubel et al. (1992). DNA was finally resuspended with 50 μL of TE buffer, treated with 2.5 μL of RNAse (1 mg/mL) and stored at −20 °C until processed.

DNA concentration and purity were determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific, Whaltham, MA, USA).

### 2.4. ERIC-PCR fingerprinting

The AAB colonies recovered on plates were genotyped by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR technique as described González et al. (2004). All amplification reactions were carried out using GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA), and the amplification products