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Short communication

Profiling white wine seed vinegar bacterial diversity through viable counting, metagenomic sequencing and PCR-DGGE

Vesna Milanović[a], Andrea Osimani[a,*], Cristiana Garofalo[a], Francesca De Filippis[b,c], Danilo Ercolini[b,c], Federica Cardinali[a], Manuela Taccari[a], Lucia Aquilanti[a], Francesca Clementi[a]

[a] Dipartimento di Scienze Agrarie, Alimentari ed Ambientali, Università Politecnica delle Marche, via Brecce Bianche, 60131 Ancona, Italy
[b] Department of Agricultural Sciences, Division of Microbiology, University of Naples "Federico II", Portici, Italy
[c] Task Force on Microbiome Studies, University of Naples "Federico II", Naples, Italy

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ABSTRACT

The production of traditional vinegar is usually carried out using the so-called “seed vinegar” or “mother of vinegar” that is composed of an undefined and complex pool of microorganisms deriving from a previous vinegar production. To date, there have been relatively few studies on the microbiota of seed vinegars. The present study was carried out to discover the bacterial biota of seed vinegar samples used in the homemade production of local vinegars obtained from the acetic fermentation of white wine. The seed vinegar samples were subjected to viable counting and advanced molecular analyses, namely, Illumina sequencing and PCR-DGGE. The adopted polyphasic approach allowed the bacterial diversity of the analyzed samples to be profiled, thus revealing the presence of acetic acid bacteria ascribed to the genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Komagataeibacter*. Moreover, other microbial genera as *Pseudomonas*, *Bacillus* and *Clostridium* were abundantly found in almost all the samples, together with other minority genera. The results of viable counting confirmed the well-acknowledged limitations inherent with acetic acid bacteria recovery on plate growth media. The overall results confirmed that seed vinegars have a complex and heterogeneous biodiversity, thus encouraging their exploitation for the isolation and future technological characterization of cultures to be selected for the manufacture of mixed starter cultures.

1. Introduction

Vinegar has been an appreciated product since the Ancient Roman Empire. Indeed, Pliny the Elder (n.d.) (born Gaius Plinius Secundus, 23–79 CE) argued about the beneficial properties of such a food in his “Natural History” (in Latin: *Naturalis historia*), Book 23.27, titled: “Vinegar: twenty-eight remedies”. Among the presumed medicinal effects, the healing of leprous sores, scorbutic eruptions, running ulcers, wounds inflicted by dogs, scorpions, and scolopendrae, and the bite of the shrew-mouse, was reported. According to recent studies (Budak et al., 2014; Petsiou et al., 2014), vinegars possess antimicrobial and anticancer properties. Moreover, their consumption is recommended for the regulation of lipid metabolism, control of blood glucose levels and weight loss. Today, vinegar is widely consumed as a food seasoning that in its noblest form takes the connotations of the renowned “traditional balsamic vinegar of Modena” and “traditional balsamic vinegar of Reggio Emilia”, both produced in Italy (Giudici et al., 2009).

In accordance with the Codex Alimentarius Commission, vinegar originates from the double fermentation (alcoholic and acetic) of substrates obtained from various sources (mostly cereals or fruit) that are rich in sugars (Mas et al., 2016). Moreover, Commission Regulation (EU) 2016/263 amending Annex II to regulation (EC) No 1333/2008 of the European Parliament and of the Council specifies that in some Member States, only vinegars obtained from the fermentation of agricultural products are allowed to be named “vinegars”.

The distinctive sensory trait of vinegar is imparted by acetic acid. Moreover, aldehydes, carotenoids, esters, ketones, organic acids, phenolic compounds, phytosterols and vitamins C and E can also be present (Ho et al., 2017).

Several types of vinegars are produced worldwide in accordance with industrial or traditional processes. Among these, rice-based vinegar, balsamic vinegar and white wine-based vinegar are the most popular (Ho et al., 2017).

As reported by Gullo and Giudici (2008) the production of traditional vinegar is usually carried out using the so-called “seed-vinegar” or “mother of vinegar” that is composed of an undefined pool of

* Corresponding author.
E-mail address: a.osimani@univpm.it (A. Osimani).

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microorganisms derived from a previous vinegar production.

The microbiota that lead to the production of vinegar are complex and mainly include bacterial genera belonging to the acetic acid bacteria group such as *Acetobacter*, *Acidomonas*, *Ameyamaea*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Komagataeibacter*, *Kozakia*, *Neoasaia*, *Saccharibacter*, *Swaminathania*, and *Tanticharoenia*. Among the abovementioned genera, *Acetobacter*, *Gluconacetobacter* and *Komagataeibacter* are those mainly involved in the oxidation of ethanol into acetic acid (Saichana et al., 2015; Sengun and Karabiyikli, 2011; Trček and Barja, 2015; Trček et al., 2016; Yetiman and Kesmen, 2015).

The acetic acid bacteria group comprises peritrichously or polarly flagellated bacteria that are gram-negative or gram-variable, aerobic and non-spore forming. They can grow between pH 3 and 4 with an optimum pH of 5–6.5 (Sengun and Karabiyikli, 2011). Moreover, yeasts and lactic acid bacteria can also play a key role in the definition of the sensory traits of vinegar (Wu et al., 2012).

A number of previous studies have been focused on investigating the microbiota of vinegars (Gullo et al., 2006; Haruta et al., 2006; Ilabaca et al., 2008; Nie et al., 2013, 2015; Ozturk et al., 2015; Trček et al., 2016), whereas to date, relatively few studies were focused on the microbiota of seed vinegars (mother of vinegars) (Yetiman and Kesmen, 2015; Valera et al., 2015). Indeed, the cultivation and isolation of acetic acid bacteria from these matrices are particularly challenging since they are difficult to recover on culture media that are already available. Moreover, such a bacterial group can be present in the "viable but non culturable" (VBNC) state, thus sometimes affecting the results of culture-dependent analyses (Vegas et al., 2010, 2013).

To obtain a clearer picture of the microbial communities involved in food fermentations, many molecular tools based on the study of the DNA or RNA have regularly been implemented. Among these, high-throughput sequencing techniques and Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis (PCR-DGGE) are shown to be able to detect major and minor bacterial components, as well as identify a single bacterial species, especially when they are used in combination (De Filippis et al., 2017; Ferrocino and Cocolin, 2017; Osimani et al., 2018; Trček and Barja, 2015).

The comprehension of the composition of the microbiota involved in the production of vinegars represents a pivotal activity for the improvement of process efficiency and for the subsequent exploitation of the detected microbial species in other biotechnological processes (Mamlouk et al., 2011). Hence, the present study aims to discover the bacterial biota of seed vinegars used for the homemade production of local vinegars obtained from the acetic fermentation of white wine. To this aim, six samples (collected in duplicate) of seed vinegars provided by homemade vinegar producers were subjected to viable counting and advanced molecular analyses, namely, Illumina sequencing and PCR-DGGE.

2. Materials and methods

2.1. Sampling

Seed vinegar (SV) samples were obtained from six homemade vinegar producers located in the Macerata province (Marche region, Central Italy). All homemade vinegars were produced from the oxidation of locally produced white wines obtained from *Malvasia* vine (approximately 10–11% v/v ethanol). Samples were collected during summer from 10-liters glass jars used for surface fermentation. The samples were obtained from 5-year-lasting traditionally (homemade) produced white wine vinegar under uncontrolled conditions. Indeed, all the fermenting glass jars were kept in the cellars of producers' farms, thus being influenced by seasonal temperature variations. Sample collection was carried out at the end of oxidation (approximately 2 months), just before half of the vinegar was collected for consumption and an equal amount of fresh wine was to be added to the jars to start a new fermentation.

For each producer, two seed vinegar samples, each collected from the surface of different active jars, were analyzed. Seed vinegar samples SV1, SV2, SV3, SV4, and SV6 were collected in liquid form, whereas samples collected from producer 5 (SV5) were characterized by relatively thick pellicles floating on the surface of the vinegars.

2.2. pH measurement

The pH potentiometric measurements of the homemade vinegars were carried out with a model 300 pH meter equipped with an HI2031 solid electrode (Hanna Instruments, Padova, Italy). For each sample, three independent measurements were performed.

2.3. Viable counts and bulk cell formation

An aliquot of each sample was ten-fold serially diluted and subjected to viable counts of acetic acid bacteria on the following selective media: Glucose Yeast Extract $CaCO_3$ (GYC) (10% glucose, 1.0% yeast extract, 2.0% calcium carbonate, 1.5% agar), AE (0.5% glucose, 0.3% yeast extract, 0.4% peptone, 0.9% agar, 3 mL absolute ethanol, 3 mL glacial acetic acid) and MYA (1.5% malt extract, 0.5%, yeast extract 1,5% agar 60 mL ethanol) (Gullo and Giudici, 2008). All growth media were incubated at 30 °C for 2–3 days (Wu et al., 2012).

Viable counts were expressed as log colony forming units (log cfu) per mL of sample ± standard deviation.

For bulk cell formation, all the colonies present on the surface of the countable plates were resuspended in 2 mL of sterile saline solution added with glycerol, harvested with a sterile pipette and stored at −20 °C prior to DNA extraction (Garofalo et al., 2015).

2.4. Extraction of DNA and 16S rRNA gene amplification

The extraction of microbial DNA from seed vinegar samples was carried out with the PowerFood Microbial DNA Isolation Kit (MO BIO Laboratories, Carlsbad, USA) (Cardinali et al., 2017), while the DNA from collected bulk cells was extracted following the procedure previously described by Osimani et al. (2015). The DNA quantity and purity were assessed via optical readings at 260, 280 and 234 nm using a UV–Vis Shimadzu UV-1800 spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

DNA extracts (seed vinegar matrices or bulk cells) obtained from each of the two samples collected from each producer were pooled, and 100 ng of each pooled DNA extract was amplified by PCR in a 50-μL reaction volume using universal primers for eubacteria P27f and P1495r (Weisburg et al., 1991). The PCR products were checked by electrophoresis in a 1.5% (w/v) agarose gel at 85 V for 1 h together with a 100 bp ladder (HyperLadder, Bioline, London, UK) used as a molecular weight standard. Digital images were captured using the Complete Photo XT101 system (Explera). The purification of 16S rRNA gene amplicons was carried out using the GFX™ PCR DNA and Gel Band Purification Kit (EuroClone, Pero, Italy), in accordance with the manufacturer's instructions. The purified amplicons were used for both metagenomic sequencing and PCR-DGGE analysis.

2.5. Metagenomic sequencing, bioinformatics and data analysis

Primers and amplification conditions of the V3-V4 region of the 16S rRNA gene were those already described by Berni Canani et al. (2017). The Illumina 16S Metagenomic Sequencing Library Preparation protocol was used for library multiplexing, pooling and sequencing using the MiSeq Reagent kit v2, leading to 2 × 250 bp paired-end reads.

Raw reads were joined using FLASH (Magoc and Salzberg, 2011). Joined reads were quality trimmed (Phred score < 20), and short reads (< 250 bp) were discarded using Prinseq (Schmieder and Edwards, 2011). Subsequent analyses were carried out in QIIME 1.9.1 (Caporaso et al., 2010), with a pipeline recently described (Berni