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ORIGINAL PAPER

Rapid molecular methods for enumeration and taxonomical identification of acetic acid bacteria responsible for submerged vinegar production

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Abstract The aim of the present study was to search for a rapid and reliable method to enumerate viable acetic acid bacteria (AAB) and to identify to genera and species level AAB isolates from vinegars in full acetic fermentation elaborated by the submerged method from cider, wine and spirit ethanol in industrial bioreactors. Results showed that the rapid epifluorescence staining method using the LIVE/DEAD BacLight bacterial viability kit and direct counts in Neubauer chamber rendered consistent and reliable data for viable cell counts of bacteria in all the studied vinegars. A linear correlation was shown between viable cell counts and fermentation rates. The highest fermentation rates and viable cell counts were found in cider vinegars, whereas spirit vinegars showed the lowest values for both parameters. Eighty-four AAB pure isolates were recovered from 41 different vinegar samples and were submitted to DNA extraction. PCR amplification of the 16S–23S intergenic spacer region of rDNA and subsequent sequencing were carried out to identify isolates to species level. Results showed that *Gluconacetobacter europaeus* was the predominant cultivable species, appearing in 79% of the total isolates. This was the unique species found in spirit vinegars, and this is the first time that AAB from spirit vinegars are taxonomically identified. *Ga. europaeus* was as well the predominant cultivable species in white wine vinegars. Cider vinegars presented the highest variability of species: *Ga. europaeus* (35.3% appearance among cultivable isolates), *Ga. xylinus* (35.3%), *Acetobacter pasteurianus* (17.6%) and *Ga. hansenii* (11.8%). Red wine vinegars showed cultivable isolates of the species *Ga. xylinus* (71.4%) and *Ga. europaeus* (28.6%). Summarising, both described methods for AAB enumeration and taxonomical identification proved to be fast and reliable methods, and results revealed *Ga. europaeus* as the cultivable major species in vinegars in full fermentation conducted by the submerged method, suggesting that *Ga. europaeus* strains can constitute excellent starter cultures for the elaboration of vinegars by the submerged method.

Keywords Acetic acid bacteria · ITS PCR · Epifluorescence microscopy · Vinegar elaboration submerged method · *Gluconacetobacter europaeus*

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Introduction

Acetic acid bacteria (AAB) are important microorganisms for the biotechnological industry [1] and are responsible for the production of vinegar due to their ability to oxidize ethanol to acetic acid [2]. They are gram negative, ellipsoidal to rod-shaped and can occur singly, in pairs or in chains. They have an obligated aerobic metabolism, which is their main characteristic, and oxygen availability limits their growth [3].

Taxonomically, AAB are classified in the family *Acetobacteraceae*, and their taxonomy, initially based on morphological and physiological criteria, has been submitted to continuous variations and reorientations due to the application of molecular techniques for their identification [4, 5]. Traditional identification methods were shown not to be completely reliable and, moreover, they are

time-consuming. DNA-based new methods have revealed a plethora of new genera and species, and currently in the *Acetobacteraceae* family 29 genera are recognized (available at the NCBI: http://www.ncbi.nlm.nih.gov/Taxonomy/) [6]. Nevertheless, the genera that to date have been reported in vinegar production are fewer, and are the following: *Acetobacter* (*A. aceti, A. pasteurianus, A. malorum* and *A. pomorum*) [7–10], *Gluconobacter* (*G. oxydans*) [10] and *Gluconacetobacter* (*Ga. europaeus, Ga. entanii, Ga. hansenii, Ga. intermedius, Ga. obodiens* and *Ga. xylinus*) [8–12].

From the industrial point of view, routine analysis of a high number of samples requires quick and reliable techniques for taxonomical identification of AAB. Studies reported in the last years have focused on identification of AAB from traditional balsamic vinegars [7–9, 13, 14], rice vinegar [15] and some other fermented traditional products such as cocoa beans, grapes or sugarcane [16–18]. Very few papers were published on identification of AAB from industrial vinegars produced by the submerged method [19, 20] and very few reports of the last 10 years can be found on species typing by DNA-based molecular methods of isolates from submerged method vinegars [12, 21, 22]. From the technological point of view, in the traditional process of vinegar-making, the fermentation takes place on the air–liquid interface where the AAB is placed in direct contact with atmospheric air. This type of so-called surface-culture fermentation generally is a slow process, whereas on the contrary, in industrial vinegars the AAB is submerged in the acetifying liquid and a strong aeration is continuously applied to provide the necessary supply of oxygen, resulting in very rapid processes [23]. Currently, this submerged method is the most frequently used as it reduces efficiently the production time reaching around 8–9 acetic degrees within 24–48 h [23] and allows a rigorous physical–chemical control of the whole process. Nevertheless, a deeper insight into the microbiology of the process, of the interactions between the microbiota that occur during fermentation, and in addition, the new taxonomy of AAB, requires further studies and the application of reliable and rapid DNA-based typing methods for bacteria identification. An additional issue on AAB is the lack of cultivability reported for many AAB strains [1, 9, 13]. Nevertheless, selection of the most suitable and best adapted AAB strains for vinegar production will be always restricted to those strains which are cultivable, and therefore can be grown to obtain enough biomass to be utilised as starter cultures of the acetic fermentation.

DNA-based typing methods that are currently used for AAB identification to species level are the following: DNA hybridisation, G+C content [5] and hybridisation probes [1], nevertheless, these techniques are not suitable for rapid analysis of a large number of samples. Alternative DNA-based rapid techniques that are being successfully used are repetitive bacterial DNA elements amplification by PCR (REP-PCR) [1, 16], 16S rRNA gene amplification and subsequent sequence analysis [5, 9, 14, 18], restriction fragment length polymorphism (RFLP) of PCR amplicons of the 16S rRNA gene and of the 16S–23S intergenic spacer region, and amplified fragment length polymorphism (AFLP) of the 16S rRNA gene [1, 4, 9, 18, 24–26], denaturing gradient gel electrophoresis (DGGE) of PCR amplicons obtained directly from the DNA of samples without cell culture [8, 11, 13–15], real-time PCR and nested PCR [27, 28].

The objective of the present study was to search for a rapid and reliable method to enumerate both total and viable AAB strains and to identify to genera and species level AAB cultivable isolates from vinegars elaborated by the submerged method from cider, wine and spirit ethanol.

Materials and methods

Vinegar sampling

Vinegar samples were aseptically taken from 30,000 litre bioreactors (Frings Xuzhou Bio- and Chemical Technology Co., Ltd) optimized for the submerged production of vinegar, of the company Vinagrerías Riojanas S. A., containing either wine, cider or spirit vinegars in full fermentation (fermenting rate = 0.17–0.34 acetic degrees/h) and when acetic degrees were as follows: 8–10 acetic degrees for wine vinegars, 4–5 acetic degrees for cider vinegars, and 10–11 for spirit vinegars. All samples were collected when acetic fermentations were at the stationary phase, during which the fermentation rate was maintained constant, and from different cycles of fermentation, i.e., after emptying, refilling tanks with new raw material, undergoing a new fermentation cycle and reaching again the stationary phase. A total of 41 samples were studied, 21 of white wine vinegars, 7 of red wine vinegars, 8 of cider vinegars and 5 spirit vinegars. Samples of 25 mL were collected in 50 mL sterile tubes and transported with continuous agitation, which favoured aerobic conditions. Samples were rapidly submitted (within 10 min) to microbiological analysis in the lab. Each vinegar sample was submitted to cell enumeration following the fluorescence method described below and three isolates from each sample were subcultured to purity. The obtained pure isolates were submitted to taxonomical identification by PCR amplification of the intergenic spacer region of rDNA 16S–23S as described below.

LIVE/DEAD BacLight viable and total cell enumeration

Viable and non-viable cell enumeration was performed by counting in a Neubauer chamber using the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Eugene, OR, USA) and examined under microscope (Axioscop 2 Plus Zeiss, Madrid, Spain) using Photometrics Cool Snap (Photometrics, UK) as the image holder software. The BacLight viability kit was used according to the manufacturer's instructions for bacterial cell staining. This BacLight kit is a rapid epifluorescence staining method that uses SYTO 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate viable bacterial cells. SYTO 9 stain generally labels all bacteria in a population, those with intact and damaged cytoplasm membrane. In contrast, propidium iodide penetrates only bacteria with damaged membrane, causing a reduction in the SYTO 9 stain fluorescence when both dyes are present. Following the kit usage instructions, bacteria with intact cytoplasmic membrane stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red.

One millilitre of the vinegar sample was centrifuged at 2,100×*g* (Megafuge Heraeus, Thermo Scientific, Wilmington, USA) for 10 min and the pellet was resuspended in 900 μL of sterile saline solution, mixed with 1.5 μL of each dye, and incubated in darkness for 15 min at room temperature. Five microlitre of this suspension was placed in a Neubauer chamber and examined under the fluorescence microscope at 525 nm and 620 nm for viable and non-viable cell enumeration, respectively, under magnification ×400.

Culture media, growth conditions and AAB isolation

Samples with 25 mL of wine and cider vinegars were centrifuged at 2,100×*g* (Megafuge Heraeus) for 10 min. Cell pellets of approximately 1 mL volume were collected and 100 μL of each sample were cultivated for 5 days on GY agar plates [5% glucose (Panreac Química S.A., Barcelona, Spain), 1% yeast extract (Scharlau Chemie S. A., Barcelona, Spain) and 1.5% agar (Becton–Dickinson, Madrid, Spain)] at 30 °C under aerobic conditions. Samples of spirit ethanol vinegars were centrifuged and 100 μL of cell pellets were cultivated in GY broth for 48 h at 30 °C with continuous and vigorous agitation in order to adapt AAB cells to growing in the culture medium, otherwise colonies did not grow onto the GY agar plates. These pre-enriched samples were subsequently cultivated on GY agar plates following the same procedure as described for wine and cider vinegars. After isolating procedure, colonies were submitted to gram staining and morphological analysis by optical microscopy. Three colonies with the morphology of AAB from each vinegar sample were selected at random for isolation on GY agar plates and pure isolates were stored in 20% sterile skim milk (Difco, Becton–Dickinson) at −20 °C. A total of 84 isolates were recovered. The following strains of the Spanish Collection of Type Cultures (CECT) were used as control strains: *Acetobacter aceti* CECT-298, *Gluconobacter oxydans* CECT-360, and *Acetobacter pasteurianus* CECT-474.

DNA extraction, PCR amplification and taxonomical identification

AAB were grown onto GY agar plates at 30 °C for 4 days under aerobic conditions. DNA extraction was carried out by the rapid lysis method as described López et al. [29].

The intergenic spacer of rDNA 16S–23S was used for taxonomical identification of AAB isolates. Amplifications were performed in a Biometra thermocycler (Goettingen, Germany) with the primers previously described by Ruiz et al. [30]: ITS 1 (ACCTGCGGCTGGATCACCTCC) and ITS 2 (CGGAATGCCCTTATCGCGCTC), which were synthesized by Sigma–Aldrich (Madrid, Spain). PCR amplification was carried out in a final volume of 50 μL comprising 20 μL of DNA solution (ranging from 80 to 150 ng/μL), 3 μL 10× amplification buffer (Bioline, London, UK), 0.5 μM of each primer, 0.2 μM of each of the four dNTPs, 3 mM $MgCl_2$ and 1.5U of Taq Polymerase (Bioline). PCR conditions were as described by Ruiz et al. [30]: samples were incubated for 5 min at 94 °C and then cycled 35 times at 94 °C for 30 s, 65 °C for 30 s and 72 °C for 1 min. Finally, samples were incubated at 72 °C for 5 min for the final extension and kept at 8 °C until tested. The amplification products were resolved by electrophoresis in 1% (w/v) agarose gels, separated at 80 V for 45 min, stained with ethidium bromide and photographed. The PCR products were sequenced in both strands by the company Cogenics (Meylan, France).

Computer analysis of DNA sequence

PCR product sequences of both complementary strands were aligned and the consensus sequence was compared with those included in the GenBank database using the BLAST program hosted by the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast).

Results and discussion

Viable and total cell enumeration

Cell enumeration by the standard plate counting of sample dilutions on the media recommended by the CECT (GY agar) proved to be completely unsuccessful, as AAB grew forming a continuous biofilm on the surface of the petri dish that impeded colony forming and subsequent counting. The production of exopolysaccharides by AAB has been reported [8], being dextrans, levans and cellulose the main exopolysaccharides produced by AAB glucose metabolism. This characteristic of AAB favours biofilm formation, and thus prevented colony counting on the surface of the solid growth medium. Moreover, a number of AAB strains have been reported to be non-cultivable [1, 9, 11, 25, 32], and therefore, plate counting cannot be the method of choice for quantifying viable AAB cells. The LIVE/DEAD BacLight kit proved to be the most reliable and effective method for counting total and viable cells, as previously demonstrated by Mesa et al. [31] and Baena-Ruano et al. [32]. Figure 1 shows the relationship ($r^2 = 0.90$) between viable cell counting with the LIVE/DEAD BacLight kit in 21 vinegar samples, out of the 41 total vinegar samples, which were submitted to the cell counting LIVE/DEAD method, and the fermentation rates in bioreactors. Samples taken from the four types of vinegars were included among these 21 studied samples. Table 1 shows that higher fermentation rates (0.30–0.34 acetic degrees/h) corresponded to higher cell populations (7.5–9.5 $\times$ 10^7 cell/mL), and lower fermentation rates (0.15–0.19 acetic degrees/h) corresponded to cell populations in the lower range (2.8–6.0 $\times$ 10^7 cell/mL).

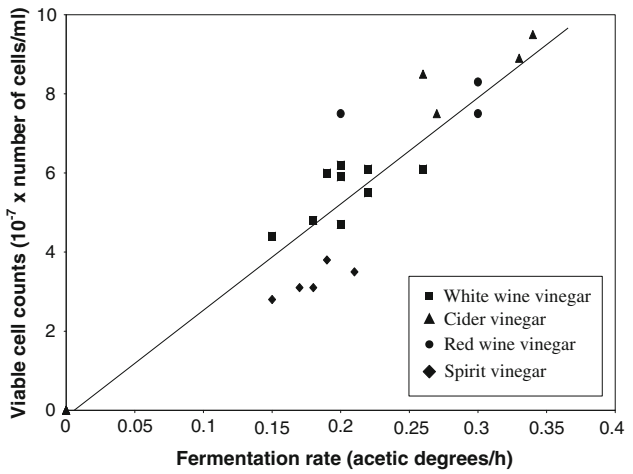

Fig. 1 Viable cell population and fermentation rates in the studied vinegar samples. *Filled square* White wine vinegar, *filled triangle* Cider vinegar, *filled circle* Red wine vinegar, *filled diamonds* Spirit vinegar

Table 1 Viable cell counts obtained for the studied vinegars by the fluorescence method

Vinegar sample	Fermentation rate (acetic degrees/h)	Viable cell counts/mL
Spirit vinegar	0.15	2.8×10^7
Spirit vinegar	0.17	3.1×10^7
Spirit vinegar	0.21	3.5×10^7
Spirit vinegar	0.18	3.1×10^7
Spirit vinegar	0.19	3.8×10^7
White wine vinegar	0.20	4.7×10^7
White wine vinegar	0.22	6.1×10^7
White wine vinegar	0.20	6.2×10^7
White wine vinegar	0.15	4.4×10^7
White wine vinegar	0.18	4.8×10^7
White wine vinegar	0.22	5.5×10^7
White wine vinegar	0.19	6.0×10^7
White wine vinegar	0.26	6.1×10^7
White wine vinegar	0.20	5.9×10^7
Red wine vinegar	0.30	8.3×10^7
Red wine vinegar	0.30	7.5×10^7
Red wine vinegar	0.20	7.5×10^7
Cider vinegar	0.33	8.9×10^7
Cider vinegar	0.26	8.5×10^7
Cider vinegar	0.27	7.5×10^7
Cider vinegar	0.34	9.5×10^7

Similarly, Baena-Ruano et al. [32] reported as well a correlation between direct AAB counts and acetic acid formation in one semi-continuous pilot bioreactor (8 litre working volume) under laboratory conditions. In our study, the highest viable cell populations were found in cider vinegars, which offered a growth medium that is rich in sugars (4%), as described by Del Campo et al. [33], and with a low content in alcohol (6%), whereas the lowest populations were shown in spirit vinegars, which presented the most stressing growth conditions for bacteria (14% ethanol and nutritionally very poor) in spite of the addition of a yeast extract supplement [34] to the acetifying liquid to facilitate its fermentation. To our knowledge, this is the first time that a correlation is reported between viable cell populations and fermentation rates in industrial bioreactors that produce a range of different vinegars by the submerged method. Each point in Fig. 1 represents a different fermentation process performed with a specific starting material (wine, cider or spirit) and under the conditions of its corresponding bioreactor. In all cases, the moment when samples were taken corresponded to the same fermentation stage: full acetic fermentation under constant fermentation rate (0.17–0.34 acetic degrees/h). It should be pointed out that a number of isolates from spirit vinegars were able to grow in liquid GY broth after a long incubation of 48 h and

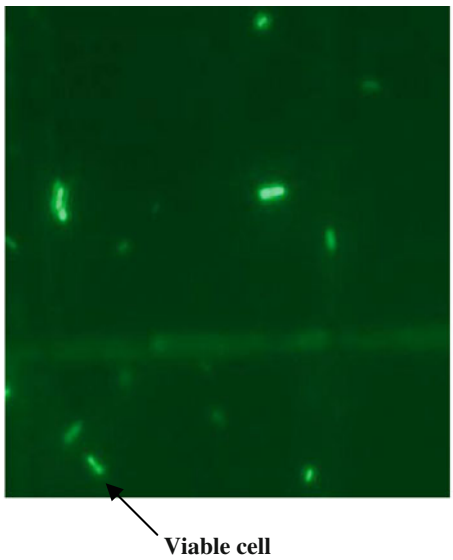

Fig. 2 Photography of the fluorescence image of viable cells (stain probe SYTO 9) of vinegar sample A (magnification ×400)

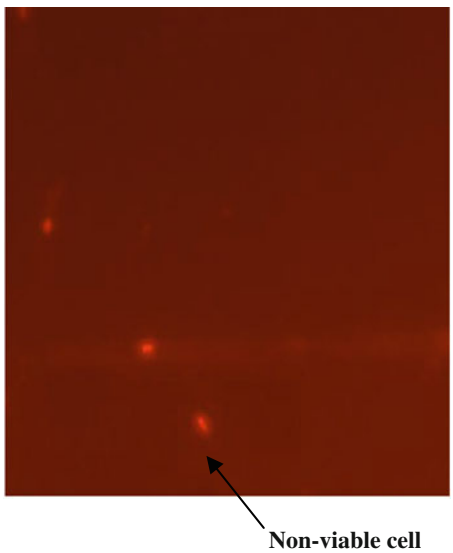

Fig. 3 Photography of the fluorescence image of non-viable cells (stained with propidium iodide probe) of vinegar sample A (magnification ×400)

under vigorous shaking. Spirit raw materials that were purchased by the vinegar company were diluted and subsequently submitted to acetic fermentation, so that the acetic acid content reached maximum values in the range of 12–13 acetic acid degrees. A low percentage of spirit vinegar samples (2% of the total vinegar samples) contained AAB that were not able to adapt to the GY broth and no isolates were recovered from those samples.

Figures 2 and 3 show the images obtained by fluorescence microscopy of viable AAB bacteria (Fig. 2) and non-viable AAB bacteria (Fig. 3). Total cell enumeration was calculated by the addition of viable and non-viable cells. AAB enumeration by the fluorescence staining method had been described by Mesa et al. [31], who applied this method to reference strains and to vinegar samples directly obtained from bioreactors. Their method included a filtering step through a black polycarbonate membrane that was observed under the epifluorescence microscope, and this step was omitted in our study as it did not improve the resolution of our analyses and increased sample manipulation. Therefore, the method reported in our study should be considered as the method of election for AAB counting in vinegar samples from industrial bioreactors fermenting vinegars from a variety of sources (wines, cider, spirit), as a replacement to plate counting that proved unsuccessful for this type of vinegars produced by the submerged method.

Species identification of AAB isolates

Eighty-four AAB isolates were obtained by cultivation from the 41 vinegar samples of our study and were identified up to species level resulting in the following four species: *Ga. europaeus, Ga. hansenii*, *Ga. xylinus* and *Acetobacter pasteurianus*. The method for species identification described in Materials and Methods section was based on that described by Ruiz et al. [30] and was modified in that it included the full sequencing in both strands of the amplicons obtained after PCR of the intergenic 16S–23S rDNA spacer region. This method offers the advantage of fast and unambiguous identification of species, rendering maximal identities (99–89%) with the sequences of AAB isolates available at GenBank database.

The identification results of the 84 isolates of this study are shown in Fig. 4. *Ga. europaeus* was demonstrated the predominant cultivable species, appearing in 79% of the total isolates and being the predominant species in both spirit and white wine vinegars. Therefore, our results showed that *Ga. europaeus* was the cultivable species that leaded fermentations of both spirit and white wine vinegars. This species was first described in vinegars elaborated by the submerged method in 1992 [35] and its high tolerance to acetic acid has been largely reported [36]. Schüller et al. [12] studied as well spirit vinegar fermentations, nevertheless they reported *Ga. entanii* in their spirit vinegars. To our knowledge, this is the first report on *Ga. europaeus* as the AAB cultivable species responsible for spirit vinegar fermentation. It should be pointed out that species identification was performed after growing the spirit vinegar samples of our study in GY broth and subsequent subculturing onto GY agar plates until obtaining pure isolates, and this procedure eliminated the possibility of identifying non-cultivable AAB strains. Nevertheless, as mentioned earlier, from the biotechnological point of view, only cultivable strains arise

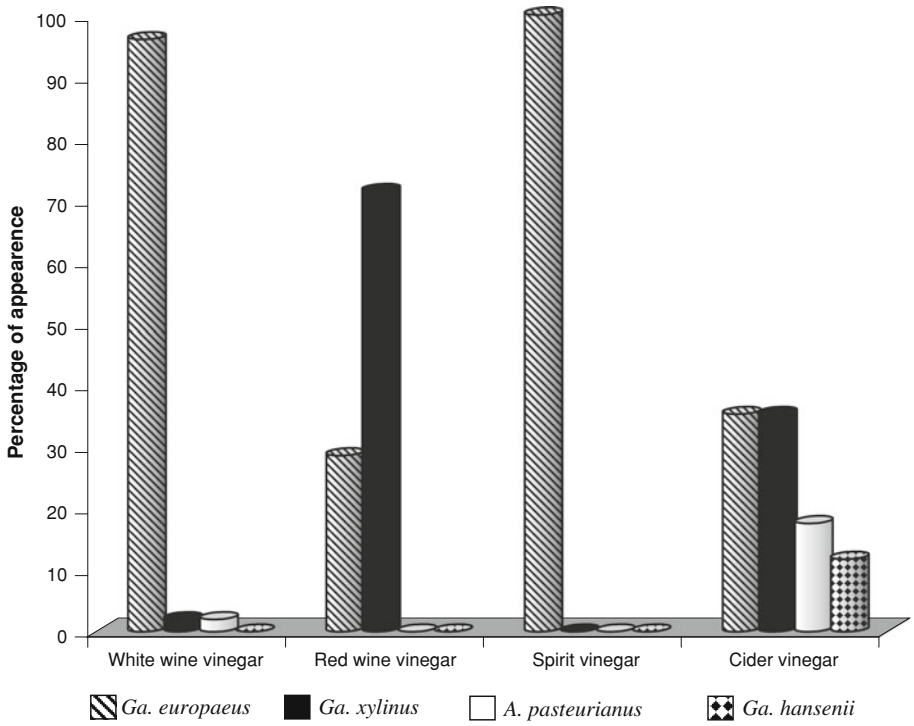

Fig. 4 Percentage of appearance of species for the four types of studied vinegars. ▧ *Ga. europaeus*, ■ *Ga. xylinus*, □ *A. pasteurianus*, ⊞ *Ga. hansenii*

interest as potential starter cultures to be selected for vinegar fermentations.

Cider vinegars in our study presented the greatest variability of species, which included *Ga. europaeus* and *Ga. xylinus,* both in 35.3% of the isolates and to our knowledge, this is the first report on these species to be responsible for cider vinegar fermentations. Other species that appeared in our cider vinegar samples were: *A. pasteurianus* (17.6%) and *Ga. hansenii* (11.8%). *A. pasteurianus* had been described in rice vinegars [15, 37] and a variety of vinegars obtained by both submerged and surface methods [38]. *Ga. hansenii* had been described in red wine [24, 39] and traditional balsamic vinegar [7].

Red wine vinegars in our study presented cultivable strains of only two species: *Ga. xylinus* (71.4% of appearance) and *Ga. europaeus* (28.6% of appearance), and this result is in accordance with reports on red wine that demonstrated the presence of these two species during alcoholic fermentation [40]. Other studies on wine vinegars and alcoholic fermentations of red wines reported the presence of *Ga. hansenii* [24, 38], *G. oxydans, Ga. liquefaciens* and *A. pasteurianus* [24], nevertheless, these species did not appear in our vinegar samples.

Other species different from the four species of our study have been described in vinegars. Thus, *A. obodiens* and *A. pomorum* were reported in red wine vinegar and cider vinegar, respectively [10] and *A. intermedius* was described in cider and spirit vinegars [41]. Nevertheless, none of our isolates revealed these species.

Currently, very few taxonomical studies can be found on AAB from industrial vinegars, and DNA-based analyses have been focused mainly on the study of traditional balsamic vinegars elaborated by the surface-culture fermentation. *Ga. xylinus, A. pasteurianus* [7, 13, 14] and *Ga. europaeus* [8] species that were identified in our vinegar samples had been also described in balsamic vinegars. Nevertheless, *A. aceti* [13] and *A. malorum* [7] species that were described in balsamic vinegars did not appear in our study. Our results show that *Ga. europaeus* is the predominant cultivable species that leaded vinegar fermentation of white wine, spirit, and a high number of cider and red wine vinegars, reaching high cell populations ($8.3–9.5 \times 10^7$ cell/mL) and high fermentation rates (0.30–0.34 acetic degrees/h). These results suggest that *Ga. europaeus* strains can constitute excellent starter cultures for the elaboration of vinegars by the submerged method. Gullo et al. [7] reported as well this species as a candidate for starter cultures for balsamic vinegars, and also suggested the possibility of its use in double selected starter culture in combination with *A. pasteurianus* [14] for the elaboration of traditional balsamic vinegars. Nevertheless, our results revealed a smaller contribution of *A. pasteurianus* than that of *Ga. europaeus* in fermentation of cider vinegars, and *Ga. europaeus* was demonstrated as

the cultivable major species for vinegar fermentations by the submerged elaboration method.

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