MICROBIOLOGICAL ANALYSIS AND CONTROL OF THE FRUIT VINEGAR PRODUCTION PROCESS

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Microbiological analysis and control of the fruit vinegar production process

DOCTORAL THESIS

Doctoral Thesis presented by
Mr **Claudio Esteban Hidalgo Albornoz**

to receive the degree of Doctor with International Mention
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CERTIFY

That the Doctoral Thesis entitled **Microbiological analysis and control of the fruit vinegar production process**, presented by Mr **Claudio Esteban Hidalgo Albornoz** to receive the degree of Doctor with International Mention by the Rovira i Virgili University, has been carried out under our supervision in the Department of Biochemistry and Biotechnology of this University. All the results presented in this thesis were obtained in experiments conducted by the above mentioned student.

Tarragona, 3th September 2012

Dr. María Jesús Torija Martínez

Dr. Estibaliz Mateo Alesanco

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JUSTIFICATION

AND

OBJETIVES

This thesis work has been carried out in the "Oenological Biotechnology" research group within the Biochemistry and Biotechnology Department of the Faculty of Enology at Rovira and Virgili University (URV) between the years 2007 and 2012. During my first year, my research was focused on the aims of the European Project entitled "WINEGAR: Wood Solutions to Excessive Acetification Length in Traditional Vinegar Production". This was a Cooperative Research Project (CRAFT) of the European Commission, Reference 1321 U07 E40 N-WINEGAR/BJ02. The results of this work were presented as the final project to obtain a Master's degree in Oenology from URV.

In the following years (2008-2012), I worked on the project "Microbiological Analysis and Control of the fruit condiment production process", which was funded by the Ministry of Education and Science (MEC), Spanish Government, Project AGL2007-66417-C02-02, Reference BES-2008-007881.

Limited data about the production of fruit vinegars and the use of selected acetic acid bacteria (AAB) to inoculate the different acetification processes was available at the beginning of this thesis. Vinegar composition and quality are the result of many different variables at multiple production steps, and one of the most important variables is the type of microorganism involved in each step. Thus, it was essential to understand and control the microorganisms that conduct both alcoholic fermentation and acetification in the production of fruit vinegars.

The working hypothesis of the present study is as follows: **it is possible to produce vinegar from any fruit because of the presence of specific microbiota that will take over the process.**

To test this hypothesis, our general aim was to study vinegars produced from different fruits, allowing us to identify and characterize the biodiversity present in each sample. Specifically, our goal was to select possible starter strains in an attempt to shorten and have a better control over these processes.

This general objective can be divided into the following specific objectives:

1. We aimed to study the native microorganisms involved in fruit vinegar production.

The first step to achieve our general aim was to determine the biodiversity present in different fruits, which may be participating in the production of vinegar. For this reason, ecological studies were conducted on different fruits during vinegar production (including strawberry, persimmon or highbush blueberries). These ecological studies allow us to assess not only the biodiversity of these samples but also the succession of these microorganisms throughout the process. This information is essential for the selection of strains to be used as starter cultures. Prior to this work, no ecological studies on fruit vinegars had been reported. Therefore, the lack of information about these processes at microbiological level made the development of these ecological studies necessary.

2. We aimed to test whether selected microorganisms isolated from the ecological studies could be used as starter cultures in the vinegar production process.

The use of starters is very common in fermentation processes, such as wine production, allowing better control of the process and producing more reproducible outcomes. However, at the beginning of this work, no inoculation studies with selected AAB had

been reported for the production of traditional vinegar. Inoculation in vinegars has traditionally involved the use of a "mother of vinegar" or back slopping, which consist of undefined cultures that do not ensure the success of the process. The use of indigenous microorganisms, which are isolated from the same matrix from which they will be used, is considered a good selection criterion because these strains are assumed to be better adapted to the medium. Therefore, in this work, yeast and AAB selected from different spontaneous fruit vinegars have been tested as individual starter cultures to initiate and perform the alcoholic fermentation and acetification of these fruits.

3. We aimed to develop a method for DNA extraction from different types of vinegars which is suitable in both purity and quantity to be used in culture-independent molecular techniques.

One of the main drawbacks to the study of AAB isolated from vinegar samples is the low culturability of the AAB in synthetic solid media. To overcome this problem and to better observe the biodiversity present during the acetification processes (without the bias of only detecting culturable bacteria), the development of culture-independent techniques is essential. The limiting step for the application of these techniques is obtaining DNA templates of adequate quantity and quality. In the Oenological Biotechnology group, a DNA extraction method has been optimized to be used in wine and wine vinegar samples. However, this method is not suitable for the extraction of the DNA from other complex media, such as traditional balsamic vinegar or fruit vinegars.

4. We aimed to compare the use of culture-independent and culture-dependent molecular techniques to identify and quantify the microorganisms responsible for the processes involved in fruit vinegar production.

As mentioned above in the previous objective, it is essential to determine if the microorganisms recovered using solid media are representative of the species present during vinegar production. Comparing the results obtained by culture-dependent and culture-independent techniques is the best way to obtain this information.

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EXPERIMENTAL DESIGN

To achieve the objectives proposed, we utilized the following experimental design, which is detailed below.

CHAPTER ONE: Effect of barrel design and the inoculation of *Acetobacter pasteurianus* selected strain in wine vinegar production

In this study, three different strategies were tested in an attempt to improve and shorten the acetification process.

- The wood of the barrel was changed. The woods used to construct the barrels were acacia, cherry and oak, which have different porosity.
- The shape of the barrel was changed. Two prototype barrels were constructed to modify the barrel shape. These prototypes had higher liquid-air interfaces compared to standard barrels.
- An *Acetobacter pasteurianus* pure culture that has been previously isolated in an ecological study conducted in similar conditions was used as a starter.

To determine the effect of these variables, the increase of the acidity and the consumption of the ethanol were monitored at the following stages of the acetification process: initial mixture (T_0); 3% (w/v) acidity (mid-acetification); and 6% (w/v) acidity (final acetification).

Furthermore, a microbiological study was performed to analyze the imposition of the inoculated *A. pasteurianus* strain throughout the acetifications in the different conditions tested. The species identification was carried out by Restriction Fragment Length Polymorphism (RFLP)-PCR of the 16S rRNA gene and 16S rRNA gene sequencing. The typing was achieved by Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR and $(GTG)_5$-PCR (Appendix 1). Moreover, the effect of

inoculation with this *A. pasteurianus* strain using the superficial and the submerged method was compared.

CHAPTER TWO: Production of fruit vinegars: Technological process for persimmon and strawberry vinegars

In this study, vinegars from persimmons and strawberries were produced by the traditional method under laboratory conditions. For each fruit, two different conditions were compared at the kinetic level. In one condition, both processes (alcoholic fermentation (AF) and acetification) occurred spontaneously. In the other condition, the AF was inoculated with the commercial *Saccharomyces cerevisiae* wine strain QA23, and the acetification was allowed to proceed spontaneously. These processes were carried out in glass containers at room temperature and repeated in triplicate. The temperature, the pH, and the concentration of free amino nitrogen (FAN), sugars, ethanol, and acetic acid were analyzed throughout the processes.

CHAPTER THREE: Identification of yeast and acetic acid bacteria isolated from the fermentation and acetification of persimmon (*Diospyros kaki*)

In this chapter, a microbiological study was conducted on persimmon vinegar, the production of which is described in Chapter 2. Sampling was conducted during the initial mixture stage, at 3% (w/v) acidity (mid-acetification stage) and at 6% (w/v) acidity (final-acetification stage).

An ecological study of the AF processes was performed by yeast identification and typing using the following techniques (Appendix 1):

- RFLP-PCR of the rRNA gene and sequencing of D1/D2 region of 26S rRNA gene were used for identification at the species level.

- RFLP analysis of mitochondrial DNA (mtDNA) was used for fingerprinting.

The ecological study of acetification was conducted by AAB identification and typing using the same techniques applied in Chapter 1.

CHAPTER FOUR: Effect of inoculation on strawberry fermentation and acetification processes using native strains of yeast and acetic acid bacteria

In this chapter, a microbiological study was conducted on the production of strawberry vinegar. The strawberry vinegar was produced as described in the Chapter 2 and by using a yeast and AAB strain isolated in this previous microbiological study. These inoculated processes were compared with the spontaneous processes as a control.

All experiments were performed in triplicate. The AF processes were carried out in glass containers, and the acetification processes were performed in the following three different materials: glass containers or wooden barrels of oak or cherry.

Sugar consumption and ethanol production were monitored during AF, and ethanol consumption and acetic acid production were monitored during acetification. Microbiological analyses of the yeast and AAB strains were conducted using the same techniques described above in Chapter 3.

CHAPTER FIVE: *Acetobacter* strains isolated during the acetification of blueberry (*Vaccinium corymbosum* L.) wine

In this study, highbush blueberry wine acetification was performed with naturally occurring microorganisms and with an inoculated *Acetobacter cerevisiae* strain. This strain was isolated from grapes from the northern Chilean valleys and was selected because of its ethanol resistance and level of acetic acid production. Acetifications were

carried out in triplicate using the Schützenbach method at a controlled temperature. Wood shavings were used as the bacterial support material.

The processes were monitored by measuring ethanol consumption and acetic acid production. AAB identification and typing was performed using the techniques described above in Chapter 1.

CHAPTER SIX: Evaluation and optimisation of bacterial genomic DNA extraction for no-culture techniques applied to vinegars

Six different DNA extraction methods were tested on 12 intermediary products of special vinegars, fruit vinegars and condiments produced from different raw materials and procedures. These DNA extraction methods were based on chemical, enzymatic or resin-mediated protocols.

The quality of gDNA extracted using the different methods was checked by PCR amplification of the region V7 to V8 of the 16S rRNA gene. The amplicons obtained were resolved by denaturing gradient gel electrophoresis (DGGE). The DGGE bands were sequenced to identify the microorganisms present in the samples.

INTRODUCTION

1. Vinegar

In human history, vinegar appears at the beginning of agriculture with the discovery of alcoholic fermentation from fruits, cereals and vegetables. The genesis of vinegar can hardly be distinguished from the origin of wine. Although vinegar has always been considered among the lowest quality products of fermented foods, it has also been widely used as a food condiment, as a preservative agent and, in some countries, as a healthy drink (Solieri and Giudici, 2009).

The definition of vinegar itself differs from country to country. The available definition from the Codex Alimentarius (1987) indicates that vinegar is ‘‘a liquid, fit for human consumption, produced from a suitable raw material of agricultural origin, containing starch, sugars, or starch and sugars, by the process of double fermentation, first alcoholic and then acetous”. This definition includes a wide variety of vinegars, such as grain vinegar, spirit vinegar, and fruit vinegar. The raw materials used in vinegar production include rice, grapes, malt, apple, honey, potatoes, whey or any other sugary food (Bamforth, 2005; Solieri and Giudici, 2009). Many different varieties of vinegar are produced all over the world. Although, most of them have a plant origin, vinegars can also be produced from animal sources (Table 1).

Vinegar is a solution of acetic acid produced by a two-step bioprocess. In the first step, fermentable sugars are transformed into ethanol by the action of yeast. In the second step, AAB oxidize the ethanol into acetic acid in an aerobic process.

Table 1. Overview of vinegars from around the world: raw materials, intermediate product, vinegar name, and geographical distribution (Solieri and Giudici, 2009).

Category	Raw material	Intermediate	Vinegar name	Geographical distribution
Vegetable [a]	Rice	Moromi	Komesu, kurosu (Japanese)	East and Southeast Asia
			Heicu (Chinese)	
	Bamboo sap	Fermented bamboo sap	Bamboo vinegar [b]	Japan, Korea
	Malt	Beer	Malt vinegar	Northern Europe, USA
	Palm sap	Palm wine (toddy, tari, tuack, tuba)	Palm vinegar, toddy vinegar	Southeast Asia, Africa
	Barley	Beer	Beer vinegar	Germany, Austria, Netherlands
	Millet	Koji	Black vinegar	China, East Asia
	Wheat	Koji	Black vinegar	China, East Asia
	Sorghum	Koji	Black vinegar	China, East Asia
	Tea and sugar	Kombucha	Kombucha vinegar	Russia, Asia (China, Japan, Indonesia)
	Onion	Onion alcohol	Onion vinegar	East and Southeast Asia
	Tomato	_	Tomato vinegar	Japan, East Asia
	Sugarcane	Fermented sugar cane juice	Cane vinegar	France, USA
		Basi	Sukang iloko	Philippines
			Kibizu	Japan
Fruit	Apple	Cider	Cider vinegar	USA, Canada
	Grape	Raisin	Raisin (grape) vinegar	Turkey and Middle East
		Red or white wine	Wine vinegar	Widespread
		Sherry wine	Sherry (jerez) vinegar	Spain
		Cooked must	Balsamic vinegar	Italy
	Coconut	Fermented coconut water	Coconut water vinegar	Philippines, Sri Lanka
	Date	Fermented date juice	Date vinegar	Middle East
	Mango	Fermented mango juice	Mango vinegar	East and Southeast Asia
	Red date	Fermented jujube juice	Jujube vinegar	China
	Raspberry	Fermented raspberry juice	Raspberry vinegar	East and Southeast Asia
	Blackcurrant	Fermented blackcurrant juice	Blackcurrant vinegar	East and Southeast Asia
	Blackberry	Fermented blackberry juice	Blackberry vinegar	East and Southeast Asia
	Mulberry	Fermented mulberry juice	Mulberry vinegar	East and Southeast Asia
	Plum	Umeboshi [c] fermented plum juice	Ume-su	Japan
	Cranberry	Fermented cranberry juice	Cranberry vinegar	East and Southeast Asia
	Kaki	Fermented persimmon juice	Persimmon vinegar	South Korea
			Kakisu	Japan
Animal	Whey	Fermented whey	Whey vinegar	Europe
	Honey	Diluted honey wine, tej	Honey vinegar	Europe, America, Africa

[a] Vegetable is not a botanical term and it used to refer to an edible plant part; some botanical fruits, such as tomatoes, are also generally considered to be vegetables.

[b] Obtained by bamboo sap fermentation.

[c] Umeboshi are pickled *ume* fruits. *Ume* is a species of fruit-bearing tree of the genus Prunus, which is often called a plum but is actually more closely related to the apricot.

1.1. Elaboration of vinegar

In vinegar production, one of the critical steps is the preparation of the raw material. This phase is required to obtain the fermentable sugar and juice solution to be acetified. The processing will differ depending on the raw material used. In general, fruits require less preparation than seeds; however, seeds are more easily stored and preserved after harvest. Fruits are highly perishable, rich in water, and need to be processed very quickly. Therefore, basic safe food handling practices, storage, and processing are essential to prevent the growth of pathogenic microorganisms. These microorganisms could alter the quality of the final product or even produce dangerous toxins, such as aflatoxin (Solieri and Giudici, 2009).

After raw material preparation, the alcoholic fermentation and acetification processes play a key role in vinegar production. Different biotransformations can take place depending on the environmental factors (temperature, pH, water activity) or the nutrients (sugar sources) and the microbial diversity present in the raw material. Microbial species involved in fermentations may range from yeast and lactic acid bacteria to molds and AAB (Nanda et al., 2001; Haruta et al., 2006; Wu et al., 2010).

Alcoholic fermentation (AF) is a fermentation step common to all vinegars. This is a biological process in which sugars, such as glucose, fructose, and sucrose, are converted into cellular energy, ethanol and carbon dioxide (CO_2). This process is mainly carried out by yeast. Among yeast, *Saccharomyces cerevisiae* is the most widespread species (Ribéreau-Gayon et al., 2006). However, non-*Saccharomyces* species have also been found during AF in vinegar production (Solieri et al., 2006). Microorganisms such as lactic acid bacteria can also play a role in obtaining ethanol from heterofermentative metabolic pathways (Obilie et al., 2003; Parrondo et al., 2003).

Much of our knowledge regarding AF in fruits has been influenced by studies of wine and beer fermentation. In these specific fermentations, the limiting parameters include the availability of different vitamins or minerals and the lack of equilibrium between fermentable sugar and available nitrogen (Ribéreau-Gayon et al., 2006). Furthermore, in the case of beer fermentation, the use of a selected yeast strain is a requirement and significantly contributes to the characteristics of the final product. However, in wine, use of a selected yeast strain is not necessary, but it is a very common practice, especially following the development of active dry wine yeast technology. Even so, some still advocate for spontaneous fermentations performed with natural wild yeasts present on the surface of grapes or the winery equipment because of the authenticity of the final products. For both beer and wine, most of the yeasts available for use as starter cultures have been selected for brewing or winemaking because they are good performers, have low nutritional requirements, start fermentations quickly, provide good fermentation rates, and produce byproducts that are appreciated by consumers. Furthermore, because a particular yeast strain can give uniform characteristics to the final product, it is a common practice to select a local wine yeast strain. However, no yeast strain is currently available for the fermentation of other fruits, and most of these fermentations are performed with wine yeast or spontaneously.

Acetification is commonly known as the oxidation of the ethanol. Once the sugar has been converted into ethanol, the second bioprocess is carried out by AAB and consists of an oxidation that is highly dependent on the availability of oxygen.

These microorganisms are part of the natural microbiota of fruits, and they can survive AF despite the adverse conditions (Du Toit and Pretorius, 2002). At the final stages of the AF, higher aeration due to wine replacement and racking may stimulate the growth of AAB, which could start acetification (Joyeux et al., 1984; Drysdale and Fleet, 1989).

Therefore, this process can be carried out spontaneously (Holzapfel, 2002). Nevertheless, in vinegar production, the use of back slopping is a very common practice to start the acetification. It consists of using part of a previously acetified batch, which is called "vinegar mother", to inoculate a new batch. This practice makes the process more reliable and faster than the spontaneous one.

The vinegar mother is an undefined starter culture that increases the initial number of AAB cells. During the acetification process, a selective pressure is exerted on the indigenous microorganisms, and those best adapted eventually dominate the process. These dominant microorganisms may be good candidates to be tested for use as starter cultures. However, although back slopping is a primitive precursor of the starter culture method (Solieri and Giudici, 2009), the use of well-defined starter cultures is lacking in vinegar production.

Technological methods used for the vinegar elaboration play an important role in the obtained product. One of the most used systems is the traditional method, also called the superficial, surface or Orleans method. It is a static method that is traditionally employed for the manufacture of high-quality vinegars. In this case, the presence of AAB is limited to the surface of the acidifying liquid. In other words, they are placed on the air–liquid interface in direct contact with air and hence with the available atmospheric oxygen to allow the conversion of alcohol into acetic acid (Laguno and Polo, 1991). In a vinegar factory, this method used to be a perpetual cycle carried out in barrel. The process consists of drawing off some volume of vinegar when the expected acidity is reached and adding new wine to the barrel. Therefore, the bacteria can grow and feed on the alcohol contained in the new wine that was added. The barrels are never emptied but are always partly filled, and they have an open hole allowing air contact with the alcoholic solution.

To start this type of acetification, a "vinegar mother" is usually used, which is a gooey film (mainly cellulose) that appears on the surface of the alcoholic product. Normally, this biofilm, which holds the highest concentration of AAB, is skimmed off from the top of the liquid, and it is added to subsequent batches of wine to speed the formation of vinegar.

Currently, this method is employed for the production of traditional and selected vinegars because of the quality of the products obtained. Nevertheless, the main drawback of this method is the long period of time required to obtain a high acetic acid concentration, resulting in increased production time and costs.

Alternative devices have been developed for industrial vinegar production to increase the speed of the AAB biological reaction (Tesfaye et al., 2002). At the moment, the most common technology used in the vinegar industry is the submerged method (De Ory et al., 1999). In this system, AAB are suspended in the acetifying liquid, and a strong aeration is applied to this liquid to provide adequate oxygen (Ormaechea, 1991). Some improvements, such as control of the stirring and heating, have allowed this acetification system to become the most widely used at industrial scale for the elaboration of most consumed vinegars (Tesfaye et al., 2002).

This process uses stainless steel fermentation tanks, working discontinuously or semicontinuously, with the following different control systems: air supply, cooling, and foam formation. The discontinuous method implies three phases: loading of the raw material and the starter (previously prepared in an appropriate medium), acetification and the complete unloading of the biotransformed product (vinegar). A semicontinuous process is similar to the discontinuous process, but in this case, only a part of the finished product is unloaded. The rest of the product is left in the vessel and used as starter to begin the next cycle (Nieto et al., 1993). Several methods and different types

of bioreactors have been designed for submerged acetification. The Frings acetator is the most widely used and commercially successful reactor (Adams, 1998).

The main advantages of the submerged method in comparison to the traditional one include a high acetification speed, which is capable of producing a high acetic acid concentration in a short time (1-2 days), the production of large volumes of vinegar, and control of the environment to create the optimal conditions for AAB acetification. However, one of the main problems with this method is loss of volatile compounds, such as ethanol, acetic acid or ethyl acetate, due to the recirculation system. This system reduces the production yield and the quality of the product, and it increases the operational costs (Romero and Cantero, 1998).

Alternative vinegar elaboration methods have been designed to reduce the time needed for the acetification but to replicate the quality of the final product that one obtains with traditional methods. The Schützenbach method increases the acetification surface contact (air contact) by using wood shavings as a bacterial support material. The acetification occurs in a container with two chambers. The upper chamber, which is filled almost to the top with wood chips or other solid materials, is separated from the lower chamber by a screen, through which air is injected. The alcoholic solution is then distributed evenly over the top of the material and allowed to percolate through it. The resulting liquid is pumped back to the top and recirculated until the acidity reaches the expected concentration. Once the process is finished, the vinegar is drawn off and fresh alcohol solution is added (Laguno and Polo, 1991).

1.2. Wine vinegar

In this section, we will only consider vinegar derived from grapes. Wine vinegar is the most common vinegar used in Mediterranean countries and Central Europe, and it is made from either red or white wine (Sellmer-Wilsberg, 2009). Differences between wine and vinegar are well established. While the maximal acetic acid content in wine is 1.2 g/L, the titratable acidity must be higher than 6% (w/v) and residual ethanol lower than 1.5% (v/v) in wine vinegar. Particularly, in Spain (Real Decreto 2070/1993, B.O.E.: 8/12/93), the residual ethanol in vinegar must be less than 0.5% (v/v).

Generally, the wines used for acetification have low ethanol content (7%−9% v/v). If wines with high alcohol content are to be used, they should be diluted appropriately to avoid the inhibition of AAB due to a high concentration of ethanol (Raspor et al., 2008). Similar to wines, there is a considerable range in vinegar quality. In fact, only a few types of vinegar, such as the Traditional Balsamic Vinegar from Modena and from Reggio Emilia (Italy) and sherry vinegar from Jerez and the Condado de Huelva vinegar from Huelva (Spain), are protected by the Denomination of Origin (PDO). These vinegars are produced under the supervision of different Regulating Councils according to the Official Production Regulation (Disciplinare di produzione, 2000; Consejería de Agricultura y Pesca, 1995; Council regulation (EC) No. 813/2000).

1.3. Fruit vinegar

Fermented juices from a wide variety of fruits (other than grapes) can also be used to produce vinegar. Although high quality products are produced from fresh and high quality juice fruit, it is technically feasible to produce them from second quality fruit and even waste fruit (Monspart-Sényi, 2006). However, the main reason that fruits are not commonly used to produce fruit vinegar is their low sugar content. Despite the

similarities between the processes and the long tradition and knowledge available regarding the elaboration of wine vinegars, this process is not fully comparable to the production of fruit vinegars. Apart from the differences in sugar concentration between fruits, there are other factors to be considered as well. These factors include the difficult extraction required to obtain the juice of some fruits, which leads to the use of commercial pectinolytic enzymes, and the high concentration of organic acids in some fruits, which can hinder the growth of some microorganisms.

It is important to note that many fruit vinegars are made by distillation of an alcoholic solution, and the further addition of fruit juice or fruit puree is provided for their aromatization. These types of "non-natural" fruit vinegar are commonly available in some Asian countries, such as China, where the market has no specific regulations for this type of product (Chang et al., 2005). Even in Europe, clear regulation of these products does not exist.

In Table 1, a summary of different vinegars obtained from fruits around the world is presented. In recent years, different studies have been conducted on these products that mainly focused on their organoleptic characteristics and their quality parameters, which has been analyzed by chemical and sensory methods. Some examples include the studies carried out with rabbiteye blueberry (Min-Sheng and Po-Jung, 2010), apple (Liu et al., 2008; Sakanaka and Ishihara, 2008), lemon, peach (Liu et al., 2008), persimmon (Sakanaka and Ishihara, 2008; Ubeda et al., 2011b), plum (Liu and He, 2009), and strawberry (Ubeda et al., 2011a; Ubeda et al., 2012) vinegars.

2. Microorganisms involved in the vinegar production

The microorganisms involved in the elaboration of vinegars are mainly yeasts and AAB. The former are the responsible for the AF, and the latter are needed for the acetification. Although both groups of microorganisms are very important, this thesis focuses on the AAB. For that reason, this introduction contains a short description of yeast and a more detailed AAB description.

2.1. Yeasts

As mentioned above, yeasts are the most important microorganisms during AF because they influence fermentation speed, wine flavor and other wine qualities (Pretorius, 2000; Fleet, 2003; Loureiro and Malfeito-Ferreira, 2003; Jolly et al., 2006).

Yeasts are defined as unicellular ascomycetous or basidiomycetous fungi, and their vegetative growth results predominantly from budding or fission. They do not form their sexual states within or upon a fruiting body (Kurtzman and Fell, 1998).

Of the 1500 yeast species listed (Kurtzman et al., 2011), at least 215 are important in foods. Furthermore, although 32 yeast genera are associated with fruits and fruit products (Worobo and Splittstoesser, 2005), only 15 are directly associated with winemaking. These genera include *Brettanomyces* and its sexual ("perfect") equivalent *Dekkera; Candida; Cryptococcus; Debaryomyces; Hanseniaspora* and its asexual counterpart *Kloeckera; Kluyveromyces; Metschnikowia; Pichia; Rhodotorula; Saccharomyces; Saccharomycodes; Schizosaccharomyces*; and *Zygosaccharomyces* (Ribéreau-Gayon et al., 2006; Fugelsang and Edwards, 2007).

The *Saccharomyces* genus is the most commonly used in beverage industry. The *Saccharomyces* genus has several unique characteristics that are not found in other genera, such as their higher capacity to ferment sugars (Fleet and Heard, 1993). This

ability allows them to colonize sugar-rich media and predominate over other yeasts, which are not as tolerant to alcohol (Fleet and Heard, 1993; Barrio et al., 2006). On the other hand, non-*Saccharomyces* yeasts, commonly known as wild yeasts, are mostly present on grapes and at the beginning of the fermentation (Fugelsang and Edwards, 2007). The imposition of *S. cerevisiae* along the AF is associated with the increasing presence of ethanol, the anaerobic conditions, the use of sulfites during harvesting and the high concentration of sugar in the must (Fleet and Heard, 1993; Fleet, 2008). Accordingly, most of the non-*Saccharomyces* wine-related species possess low fermentation activity and low SO_2 resistance (Ciani et al., 2010). However, the development of these non-*Saccharomyces* species can have an important impact on the complexity of the aroma of the final product (Gil et al., 1996; Grbin, 1999; Soden et al., 2000).

The current strategy employed to ensure the correct development of AF, especially during winemaking process, involves inoculation of the must with selected *S. cerevisiae* strains, usually added as active dried yeast. This practice results in a shorter lag phase, a rapid and complete fermentation of the must, and a more reproducible final product (Fleet and Heard, 1993; Bauer and Pretorius, 2000). The selection of the *S. cerevisiae* strain used is based on the fact that each strain presents different biotechnological properties (Ribéreau-Gayon et al., 2006). These properties can include different performance during the AF. Therefore, it is important to be able to identify and characterize the different species and strains that participate in fermentation.

For many years, the methods used for the identification of yeasts have been based on morphological and biochemical criteria (Barnett et al., 1990). These methods are laborious, time consuming and dependent upon the physiological state of the yeast, and therefore, they are not useful for precise identifications (Querol et al., 1992). More

recently, different methods based on the analysis of total cell proteins (Vacanneyt et al., 1991), isoenzyme profiles (Duarte et al., 1999), and analysis of fatty acids by gas chromatography (Moreira da Silva et al., 1994) have been used. As with the classical techniques, the reproducibility of these techniques is questionable because, in many cases, they depend on the physiological state of the yeasts (Golden et al., 1994). Finally, molecular techniques based on the direct genomic analysis have been developed and successfully applied to the identification and characterization of yeasts. These techniques have the advantage of not being dependent on the physiological state of the cell.

2.1.1. Yeast Species Identification

Different molecular techniques for the identification of yeast species have been developed. These methods could be classified depending on whether they require a previous culturing step. Among the culture-dependent techniques, the methods most commonly used are those based on the amplification of the ribosomal genes such as the sequencing of ribosomal DNA (Kurtzman and Robnett, 1998) or the restriction analysis of the ribosomal DNA (Guillamón et al., 1998; Esteve-Zarzoso et al., 1999). On the other hand, the main culture-independent techniques applied to the identification of yeast at species level are Denaturing Gradient Gel Electrophoresis (PCR-DGGE) (Cocolin et al., 2000; Di Maro et al., 2007; Stringini et al., 2009), Temperature Gradient Gel Electrophoresis (PCR-TGGE) (Hernán-Gómez et al., 2000), Fluorescence in situ hybridization (FISH) (Andorrà et al., 2011), and Real-Time Polymerase Chain Reaction (RT-PCR) (Hierro et al., 2006; 2007).

Among all these molecular techniques, the restriction analysis of ribosomal genes (PCR-RFLPs of rDNA) has been used for the identification of yeast species in this

thesis. In this technique, the region containing the 5.8S gene and the adjacent intergenic regions, ITS1 and ITS2, are amplified. This DNA is then digested with different restriction enzymes (*Hin*fI, *Cfo*I, *Hae*III) to obtain species-specific profiles. Guillamón et al. (1998) and Esteve-Zarzoso et al. (1999) used this technique for the rapid identification of yeasts present in wines and other beverages.

2.1.2. Yeast Typing

There are several techniques useful for the typing of yeasts, most of them based on the use of Polymerase Chain Reaction (PCR) to detect DNA polymorphisms. The techniques most frequently used to characterize yeast genotypes are Random Amplified Polymorphic DNA (RAPD)-PCR and microsatellite analysis (Baleiras Couto et al., 1996; Torriani et al., 1999; Richards et al., 2009). Other techniques such as the amplification of Delta sequences and intron splice sites have been developed specifically to differentiate between genotypes of the species *S. cerevisiae* in wine (Ness et al., 1993; Fernández-Espinar et al., 2001; Schüller et al., 2004). On the other hand, Amplified Fragment Length Polymorphism (AFLP)-PCR (Vos et al., 1995; De Barros Lopes et al., 1999) is a technique that combines the use of PCR and restriction enzymes, but its complex methodology has reduced its application in yeast characterization. Other techniques not based on PCR have been widely used for yeast fingerprinting, such as Pulsed-Field Gel Electrophoresis (PFGE) (Esteve-Zarzoso et al., 2001, 2003) and the restriction analysis of mitochondrial DNA (RFLP of mtDNA). The latter technique is used for the *S. cerevisiae* typing in this thesis and will thus be further explained.

This RFLP of mtDNA technique is one of the most commonly applied for the genotyping of *S. cerevisiae* strains (Fernández-Espinar et al., 2001; Torija et al., 2001; Beltran et al., 2002; Schüller et al., 2004). This technique relies on the different

restriction profiles obtained between the mitochondrial and nuclear DNA during a total DNA digestion with GCAT restriction enzymes. These differences are based on the composition of A-T and G-C base pairs in the yeast mtDNA, resulting in a small number of restriction sites in the mtDNA and a large number of sites in the nuclear DNA. Therefore, the mtDNA bands can be clearly visualized over the background shadow of the digested nuclear DNA by electrophoresis with agarose gels (Querol et al., 1992). In the case of *S. cerevisiae*, the restriction enzymes *Hin*fI and *Hae*III are the most appropriate for use in characterization.

This technique has been even applied for the genotyping of some non-*Saccharomyces* such as *Dekkera bruxellensis* (Ibeas et al., 1996), *Zygosaccharomyces* (Guillamón et al., 1997; Esteve-Zarzoso et al., 2003), *Candida stellata*, *Metsnikowia pulcherrima*, *Torulaspora delbrueckii* (Pramateftaki et al., 2000) and *Pichia guilliermondii* (Martorell et al., 2006).

2.2. Acetic Acid Bacteria

2.2.1. General characteristics

AAB are gram-negative or gram-variable bacteria, with ellipsoidal to rod-shaped morphologies. They are motile due to the presence of flagella, which can be either peritrichous or polar. Their size varies between 0.4-1 μm wide and 0.8-4.5 μm long. Under microscopy, they are observed as individual cells, in pairs or in chains. They have a strict aerobic metabolism with oxygen as the terminal electron acceptor, and they are catalase positive and oxidase negative.

Most AAB grow between pH 5.4-6.3 (Holt et al., 1994), but they also can grow at pH values lower than 4. Du Toit and Pretorius (2002) reported that AAB can also be isolated at pH values of 2.0-2.3 in media containing acetate, if they are aerated. The

optimal temperature for growth is 25-30 ºC, but they can also grow between 38-40 ºC (Saeki et al., 1997; Ndoye et al., 2006) and weakly at temperatures as low as 10 ºC (Joyeux et al., 1984). AAB can present pigmentation in solid cultures and produce different types of polysaccharides (De Ley et al., 1984).

These bacteria are usually found in substrates containing sugar and/or ethanol. These substrates include fruits, flowers, food and fermented beverages, such as fruit juices, wine, cider, beer, cocoa and vinegar (Thompson et al., 2001; Nielsen et al., 2007; Yamada and Yukphan, 2008)

2.2.2. AAB taxonomy

The AAB are classified in the *Acetobacteraceae* family, in α-class of Proteobacteria (De Ley et al., 1984; Sievers et al., 1994) in the following thirteen genera: *Acetobacter*, *Gluconobacter*, *Acidomonas*, *Gluconacetobacter*, *Asaia*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neoasaia*, *Granulibacter*, *Tanticharoenia, Ameyamaea*, and *Neokomagataea*. Until recently, the genera with the highest diversity of species were *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter*, which contain 20, 17, and 13 species, respectively. Recently, Yamada et al. (2012) have proposed to transfer 12 species of the *Gluconacetobacter* genus to a new genus, *Komagatabacter*, however, this taxonomic change has not yet been accepted (Table 2).

The history of AAB classification began in the early nineteen century. They were first observed and isolated in 1837 by F.T. Kützing, who obtained the organism from naturally fermented vinegar and called it *Ulvina aceti* (Asai, 1968). A few years later, Louis Pasteur (1868) performed the first systematic study of acetic acid fermentation. He was the first to describe the “vinegar mother” as a mass of live microorganisms, which induced acetic acid fermentation. He also determined that this process was not

possible in the absence of these microorganisms. Hansen observed in 1879 that the microbiota, which converted the alcohol into acetic acid, were not a single strain and were comprised various bacterial species, and Beijerinck proposed *Acetobacter* as the first genus of AAB in 1899.

In subsequent years, the AAB taxonomy began to rely on morphological, biochemical, and physiological criteria. Visser't Hooft first proposed an AAB classification based upon these criteria in 1925. Ten years later, Asai (1935) formulated the proposal to divide the AAB into two genera: *Acetobacter* and *Gluconobacter*. Later, Frateur (1950) proposed a new classification based essentially on five physiological criteria: catalase activity, production of gluconic acid from glucose, oxidation of acetic acid to carbon dioxide and water, oxidation of lactic acid to carbon dioxide and water, and oxidation of glycerol into dihydroxyacetone. These criteria allowed the subdivision of *Acetobacter* into four groups: *peroxydans*, *oxydans*, *mexosydans*, and *suboxydans* (reviewed by Barja et al., 2003).

Another example of the reclassification of the AAB was introduced by Yamada et al. (1997). These authors transferred the following species, formerly classified as *Acetobacter*, to the genus *Gluconacetobacter* based on differences in the ubiquinone system: *A. xylinus*, *A. liquefaciens*, *A. hansenii*, *A. diazotrophicus* and *A. europaeus*. The *Acetobacter* genus uses Q-9 as the main respiratory quinone, but the *Gluconacetobacter* genus uses Q-10.

Although morphological, biochemical, and physiological criteria have been commonly used to differentiate AAB genera, using only these phenotypic tests to classify AAB is not reliable and, therefore, is not adequate (Cleenwerck and de Vos, 2008).

The history of the taxonomic criteria applied to bacterial species has been provided in the different editions of *Bergey's Manual of Determinative Bacteriology*, which has

become in a reference for bacterial taxonomy. In recent editions of *Bergey's Manual of Systematic Bacteriology*, molecular tests, such as fatty acid composition, electrophoresis of soluble proteins, guaninecytosine (GC) content, and DNA-DNA hybridization, have been included among the taxonomic criteria (De Ley et al., 1984).

DNA-DNA hybridization is a powerful molecular technique that was suggested by McCarthy and Bolton in 1963 to discriminate between closely related species of bacteria. The use of phenotypic tests has decreased, as more people prefer the use of DNA-DNA hybridization and other molecular DNA-based methods, such as sequence analysis of 16S rDNA genes (Ruiz et al., 2000) and analysis of the internal transcribed spacer sequences (ITS) of the 16S-23S rDNA genes (González and Mas, 2011). Sequence analysis of that ITS region has proven very useful to differentiate closely related species as *Acetobacter malorum* and *Acetobacter cerevisiae* (González et al., 2006; Valera et al., 2011).

Although the taxonomy of AAB is continually being revised and reorganized due to the use of molecular techniques, difficulties in recovering, identifying and preserving AAB samples have limited our knowledge of their phylogenesis. Therefore, the reorganization of the taxonomy of the AAB has not been completely established, and rearrangements of the groupings are still in progress (De Vero and Giudici, 2008). In addition, the new molecular techniques allow the detection of new species, which have never been previously described.

Table 2. Current classification of AAB genera and species. Table is adapted from Sengun et al. (2011) and updated from Kommanee et al. (2011), Yukphan et al. (2011), Tanasupawat et al. (2011a and 2011b), and Yamada et al. (2012).

Genus	**Species**		
Acetobacter	*A. aceti*	*A. malorum*	*A. peroxydans*
(20 species)	*A. cerevisiae*	*A. nitrogenifigens*	*A. syzygii*
	A. cibinongensis	*A. oeni*	*A. fabarum*
	A. estunensis	*A. orientalis*	*A. ghanaensis*
	A. indonesiensis	*A. orleanensis*	*A. senegalensis*
	A. lovaniensis	*A. pasteurianus*	*A. farinalis*
	A. pomorum	*A. tropicalis*	
Gluconobacter	*G. albidus*	*G. oxydans*	*G. kanchanaburiensis*
(13 species)	*G. cerinus*	*G. roseus*	*G. uchimurae*
	G. frateurii	*G. sphaericus*	*G. nephelii*
	G. japonicus	*G. thailandicus*	
	G. kondonii	*G. wancherniae*	
Gluconacetobacter	*Ga. azotocaptans*	*Ga. entanii* [a]	*Ga. rhaeticus* [a]
(17 species)	*Ga. diazotrophicus*	*Ga. europaeus* [a]	*Ga. saccharivorans* [a]
	Ga. sacchari	*Ga. hansenii* [a]	*Ga. swingsii* [a]
	Ga. johannae	*Ga. sucrofermentans* [a]	*Ga. nataicola* [a]
	Ga. liquefaciens	*Ga. intermedius* [a]	*Ga. oboediens* [a]
	Ga. xylinus [a]	*Ga. kombuchae* [a]	
Asaia	*As. bogorensis*	*As. siamensis*	*As. krungthrpensis*
(5 species)	*As. lannensis*	*As. spathodeae*	
Neokomagataea	*Nk. thailandica*	*Nk. tanensis*	
(2 species)			
Acidomonas	*Ac. methanolica*		
Neoasaia	*N. chiangmaiensis*		
Swaminathania	*Sw. salitolerans*		
Kozakia	*Kz. baliensis*		
Granulibacter	*Gr. bethesdensis*		
Saccharibacter	*S. floricola*		
Tanticharoenia	*T. sakaeratensis*		
Ameyamaea	*Am. chiangmaiensis*		

[a] These twelve species have been proposed to be transferred to a new genus *Komagatabacter* (Yamada et al., 2012), however, this taxonomic change has not yet been accepted.

2.2.3. General aspects of AAB metabolism in acetic acid production

AAB are obligate aerobes, therefore their growth is highly dependent upon the availability of molecular oxygen, which acts as a terminal electron acceptor. When oxygen is limited (for instance, during alcoholic fermentation), alternative terminal electron acceptors, such as quinones, can be used.

Furthermore, AAB have a strong ability to incompletely oxidize several alcohols and sugars, which can lead to the accumulation of intermediate metabolites in the media without toxicity for the bacteria (De Ley et al., 1984). This is the case of ethanol, which is converted into acetic acid by two membrane-bound enzymes. First, ethanol is oxidized to acetaldehyde by alcohol dehydrogenase. Second, acetaldehyde is oxidized into acetate by aldehyde dehydrogenase. In both reactions, electrons are transferred and then accepted by oxygen (Adachi et al., 1978; Saeki et al., 1997; Yakushi and Matsushita, 2010). The alcohol dehydrogenase enzyme uses pyrroloquinoline as a cofactor, is independent of $NADP^+$ and has an optimal pH of 4. A cytoplasmatic $NADP^+$-dependent alcohol dehydrogenase has also been identified. However, its low specific activity and high optimal pH in comparison to the membrane-bound enzyme limits its contribution to the oxidation of ethanol (Adachi et al., 1978; Takemura et al., 1993; Matsushita et al., 1994). On the other hand, the aldehyde dehydrogenase is also a $NADP^+$-independent enzyme, and its optimal pH is between 4 and 5. However, it can catalyze the oxidation of acetaldehyde to acetic acid at lower pH values (Adachi et al., 1980). This enzyme is more sensitive to the presence of ethanol than alcohol dehydrogenase (Muraoka, 1983), and its activity decreases with low concentration of oxygen, accumulating acetaldehyde.

Acetobacter and *Gluconacetobacer* genera are able to oxidize acetic acid completely to carbon dioxide and water, whereas *Gluconobacter* is unable to perform a complete oxidation of ethanol. This oxidation occurs via the tricarboxylic acid cycle, and it is inhibited by ethanol (Saeki et al., 1997; Ribéreau-Gayon et al., 2006). Furthermore, the *Acetobacter* genus is reported to produce more acetic acid than *Gluconobacter*, which could be due to the higher stability of the *Acetobacter* alcohol dehydrogenase enzyme under acetic conditions (Matsushita et al., 1994).

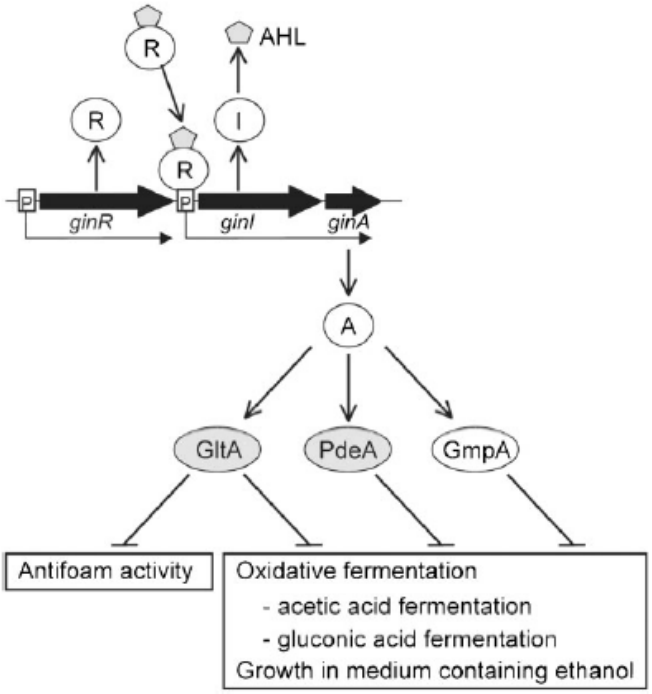
The resistance of AAB to high concentrations of acetic acid is reported to be due to the citrate synthase enzyme, which detoxifies acetic acid by its incorporation into the tricarboxylic or glyoxylate cycles only when ethanol is absent from the media (Fukaya et al., 1990). However, the high tolerance to acetic acid is strain dependent (Nanba et al., 1984), and it appears that adaptation to high acetate concentrations is a prerequisite for high acetic acid tolerance (Lasko et al., 2000). *Acetobacter* strains decrease their internal pH in response to a lower external pH (Menzel and Gottschalk, 1985).

Recently, the GinI/GinR Quorum Sensing (QS) system, homologous to the LuxI/LuxR system described in *Vibrio fischeri*, has been reported to be responsible for the repression of acetic acid oxidation in *Gluconacetobacter intermedius* (Iida et al., 2008a). QS is a cell density-dependent system, which is used to regulate diverse physiological functions, such as biofilm formation and secondary metabolite production. *N*-acylhomoserine lactones (N-AHLs) are autoinducers that are involved in many QS mechanisms that regulate gene expression in gram-negative bacteria. Three AHLs with different acyl chains have been reported to regulate these GinI/GinR proteins (Iida et al., 2008a).

GinA is a protein of 89 amino acids, the production of which is induced by the QS system that controls, via a still unknown mechanism, the GmpA protein. This GmpA

protein belongs to the OmpA protein family, and it represses oxidative processes, including acetic acid and gluconic acid production (Iida et al., 2008b). There are also four novel GinA-inducible genes: gltA, which encodes for a putative glycosyltransferase; pdeA, a putative cyclic-di-GMP phosphodiesterase; pdeB, a putative phosphodiesterase/diguanylate cyclase; and nagA, a putative N-acetylglucosamine-6-phosphate deacetylase (Iida et al., 2009). These authors reported that the genes gltA and pdeA, together with the GmpA protein, are involved in the repression of antifoam activity, growth in medium with ethanol and acetic acid and gluconic acid fermentation (Figure 1).

Figure 1: Image from Iida et al. (2009) as a possible model for the QS system in *Ga. intermedius*.



2.2.4. Isolation and growth

AAB are generally considered to be *fastidious microorganisms* because of their poor recovery on laboratory media. This trait has been observed in AAB samples isolated from environments with high levels of acetic acid (Entani et al., 1985). They do not generate endospores as a resistance form. However, it has suggested that AAB cells may undergo a transition to a survival state, the so-called viable but non-culturable (VBNC) state, when exposed to an extreme medium, such as wine or vinegar (Millet and Lonvaud-Funel, 2000). Bacteria in this state fail to grow on routine bacteriological media, on which they would normally grow and develop into colonies, but they are alive and capable to renew their metabolic activity (Oliver, 2000).

Clear growth differences have been observed between AAB species isolated from fruits, flowers and fermented foods, and they have displayed differing abilities to grow using different culture media depending on the available nutrients (Lisdiyanti et al., 2003). The poor recovery on culture media has also been associated with the lack of a suitable synthetic media, as not all synthetic media equally support the growth of AAB and could even be selective among strains (Gullo et al., 2006). However, considerable progress in AAB isolation has been made with the development of various culture media. The most widely used culture media to isolate AAB are shown in Table 3. Some of these media, such as the AE medium and its modification, RAE medium, have been designed to promote the growth of AAB adapted to high concentrations of acetic acid (Entani et al., 1985; Sokollek and Hammes, 1997; Sokollek et al., 1998).

However, among these culture media, GY and GYC media are the most widely used to recover AAB strains from grape must, wine (Du Toit and Lambrechts, 2002; Bartowsky et al., 2003; González et al., 2004) and different types of vinegars (Gullo et al., 2006; Prieto et al., 2007; Vegas et al., 2010; Valera et al., 2011). The presence of calcium

carbonate in the GYC medium is used as an acid indicator to detect if the isolates are producing acid, which is an important feature of an AAB. Furthermore, it is often recommended to supplement the media with antifungal and antibiotic agents to suppress the growth of fungus, yeast and unwanted bacteria. Natamycin, pimaricin and cycloheximide are commonly used to avoid fungal and yeast growth, and penicillin is used to avoid lactic acid bacteria. Incubation times vary from two to eight days at temperatures between 25°C and 30°C.

Table 3. Most common media used to isolate AAB.

Media	Quantity	Media	Quantity
[a]**GYC agar**		[b]**GY Medium**	
D-Glucose	5.0% (w/v)	Glucose	2.0% (w/v)
Yeast extract	1.0% (w/v)	Yeast extract	1.0% (w/v)
Calcium carbonate	0.5% (w/v)	Agar	2.0% (w/v)
Agar	2.0% (w/v)		
GYC Medium		[d]**AE-medium**	
Glucose	10.0% (w/v)	Glucose	0.5% (w/v)
Yeast extract	1.0% (w/v)	Yeast extract	0.3% (w/v)
Calcium carbonate	2.0% (w/v)	Peptone	0.4% (w/v)
Agar	1.5% (w/v)	Agar	0.9% (w/v)
		Absolute ethanol	3 ml (v/v)
		Acetic acid	3 ml (v/v)
[c]**YPM Medium**		[e]**RAE-medium**	
Yeast extract	0.5% (w/v)	Glucose	0.4% (w/v)
Peptone	0.3% (w/v)	Yeast extract	0.1% (w/v)
Mannitol	2.5% (w/v)	Peptone	0.1% (w/v)
Agar	1.2% (w/v)	Absolute ethanol	0 - 4% (v/v)
		Citric acid	0.015% (w/v)
		Na_2HPO_4	0.038% (w/v)
		Agar	0.5-1% (w/v)
V50			
Yeast extract	0.4% (w/v)		
Glycerol	0.2% (w/v)		
Tartaric acid	0.2% (w/v)		
K_2HPO_4	0.05% (w/v)		
$MgSO_4.7H_2O$	0.05% (w/v)		
Na acetate	0.1% (w/v)		
$MnSO_4$	0.02% (w/v)		
$CaCl_2$	0.01% (w/v)		
Ethanol (v/v)	6% (v/v)		
pH 5			

[a] Glucose yeast extract Calcium carbonate medium

[b] Glucose yeast extract medium

[c] Yeast extract peptone mannitol medium

[d] Acetic acid ethanol medium

[e] Reinforced-AE medium

2.2.5. Molecular techniques

The appearance of the molecular biology in the 1950s and the development of new techniques and tools revolutionized all areas of biology. The study of AAB also benefited from this advance, which provided reliable identification and characterization results in a shorter time. These new techniques have allowed both more precise AAB species identification and a clear discrimination between different strains of a species via genotyping. The results have been interesting both to taxonomy studies and from an industrial point of view.

During the next 60 years, a wide variety of molecular techniques have been developed, and many of them have been applied to AAB analysis. Depending on the degree of discrimination obtained, some of these techniques are more suitable for genera detection, species identification, and characterization of isolates or typing.

Among the molecular methods, PCR-based methods are preferred due to their rapidity, specificity, reliability and sensitivity. However, the validity and robustness of the results obtained from such molecular techniques depends on the efficient recovery of bacterial DNA. DNA extraction is usually affected by factors such as incomplete cell lysis, DNA adsorption to a particular material, coextraction of enzymatic inhibitors and degradation or damage of DNA (Miller et al., 1999). Clearly, the application of a suitable DNA extraction protocol for a specific sample is essential for correct estimation of microbial diversity. The DNA extraction method must be simple, quick and efficient. Safety, cost and DNA quality must also be considered. DNA quality is critical because the efficiency of PCR amplification can be reduced by inhibitors from the matrix. DNA extraction has therefore been highlighted as a limitation of culture-independent methods (Abriouel et al., 2006; Cankar et al., 2006).

2.2.5.1. Genera detection and species identification

The following techniques have been applied in the discrimination between species of AAB in this thesis:

- ***PCR-RFLP and sequencing of the 16S rRNA gene***:

This technique is based on the amplification of the 16S rRNA region and subsequent digestion with restriction enzymes to generate a series of fragments of variable size. This technique allows species identification on the basis of their phylogenetic relationships. It was initially used to analyze strains of species belonging or related to the genus *Brevibacterium* (Carlotti and Funke, 1994).

Ruiz et al. used this technique in 2000 for rapid AAB identification. In this study, eight endonucleases were tested, and two of them (*Rsa*I and *Taq*I) were selected for their higher power in discrimination between AAB isolates from wine samples. However, due to the description of new AAB species and several AAB rearrangements, the number of endonucleases needed for correct species identification has increased (González et al., 2006). Vegas et al. (2010) used mainly the *Taq*I and *Alu*I endonucleases for a first approximation analysis of AAB isolates from vinegar samples, and they then applied *Bcc*I, as reported in Torija et al. (2010). In that study, the technique was used to differentiate between species of the genus *Gluconacetobacter* (*Ga. hansenii*, *Ga. europaeus* and *Ga. xylinus*).

The sequencing of the 16S rRNA gene is a powerful tool to identify AAB species. Currently, it is the main technique used to confirm the identification performed by PCR-RFLP of 16S rRNA gene. However, this gene is highly conserved, and the homology between the AAB species can be up to 99.7%. This extremely high homology can make the correct AAB identification difficult (Cleenwerck and De Vos, 2008), and it can be

impossible to differentiate some AAB species, especially those as closely related as *A. cerevisiae* and *A. malorum* (Valera et al., 2011).

- ***PCR-RFLP and sequencing of the 16S-23S rRNA genes Internally Transcribed Spacer (ITS) region:***

This technique is identical to the previous one, but the amplification target is the ITS between the 16S and 23S rRNA genes present in all eubacteria. This technique had been successfully used to classify and identify AAB (Sievers et al., 1996; Ruiz et al., 2000; Trcek and Teuber, 2002; González et al., 2005; Trcek, 2005; Gullo et al., 2006). However, Ruiz et al. (2000) observed some ambiguous results in isolates from wine samples, and later studies have tested other endonucleases to obtain a more accurate AAB identification (Trcek and Teuber, 2002; González et al., 2005).

Recently, González and Mas (2011) reported that analysis of the ITS of the 16S-23S rRNA genes is a good tool for AAB species identification, as well as for taxonomic identification. They confirmed that all the AAB species tested could be differentiated by this phylogenetic analysis, excluding some problems in relation to closely related species (such as the differentiation between *A. malorum* and *A. cerevisiae*, which could not be resolved by 16S rRNA gene sequencing) (Valera et al., 2011).

- ***Denaturing gradient gel electrophoresis (DGGE):***

DGGE is a culture-independent technique that is commonly used to determine the biodiversity present in samples. It was developed to characterize microbial communities from specific environmental niches (Muyzer and Smalla, 1998).

In this technique, DNA fragments of the same length but with different sequences can be separated because of their different electrophoretic mobility in denaturing conditions. The region amplified is usually a ribosomal DNA fragment, the most common of which

are the 16S and 23S rRNA genes. Separation of the amplicons is based on the decreased electrophoretic mobility of a partially denatured double-stranded DNA molecule when run on a polyacrylamide gel with a linear denaturing gradient of urea and formamide. (Muyzer and Smalla, 1998). During the electrophoretic process, the DNA remains doubled stranded until it reaches the gel zone at which the denaturing conditions are the same as the melting temperature (Tm) of the DNA. At this point, the double-stranded DNA is partially denatured, and its motility is reduced. To avoid the complete dissociation of the two DNA strands into single strands, the 5' primer has a poly GC tale of approximately 40 bp, which acts as a high-melting domain. The advantage of this method is that it does not require the previous isolation of the microorganisms.

DGGE has been used to characterize microbial communities from environmental (Muyzer and Smalla, 1998; Muyzer, 1999) and food samples (Kesmen and Kacmaz, 2011; Minervini et al., 2012). This technique has also been applied to characterize microorganisms present in wines (Lopez et al., 2003; Andorrà et al., 2008) and vinegars (De Vero et al., 2006; Haruta et al., 2006; Gullo et al., 2009).

- ***Real Time PCR (RT-PCR):***

This culture-independent technique is a fast, sensitive and accurate tool for detecting and enumerating microorganisms. It consists of monitoring the progress of a PCR reaction in each cycle by detecting the increase in fluorescence produced by a reporter molecule as the amplification proceeds. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA, such as SYBR® Green, or sequence specific probes, such as TaqMan® or TaqMan® - MGB probes. The latter are very specific probes due to the presence of a non-fluorescence quencher and a minor groove binder (MGB) at the 3' end. This MGB group increases the melting temperature (Tm)

of the primer, allowing detection of single-base mismatches (Kutyavin et al., 2000; Torija et al., 2010).

Few studies have applied this technique in the detection and characterization of AAB strains. Some have quantified the total number of AAB cells in wine and vinegar samples using SYBR® Green as fluorescent reporter (González et al., 2006, Andorrà et al., 2008; Torija et al., 2010). Other authors have designed TaqMan® or TaqMan® MGB probes to detect and quantify different genera or species of AAB (Gammon et al., 2007; Torija et al., 2010), and others have simply applied some of these probes in vinegar samples (Jara et al., 2012).

2.2.5.2. Fingerprinting

Currently, different techniques have been tested for AAB typing to establish categories that allow the appropriate characterization of these microorganisms, which include the following:

- ***Random Amplified Polymorphic DNA-PCR (RAPD-PCR):***

This technique is based on the use of arbitrary oligonucleotides (10 nucleotides) to initiate the amplification of genomic DNA, which yields a band pattern that should be characteristic of a particular bacterial strain (Caetano-Anolles et al., 1991; Meunier and Grimont, 1993). The technique has been used to characterize strains of AAB present in spirit vinegar production (Trcek et al., 1997) and in rice vinegar (Nanda et al., 2001).

- ***Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) and Repetitive Extragenic Palindromic-PCR (REP-PCR):***

ERIC and REP elements are highly conserved palindromic sequences found in enteric bacteria (Pooler et al., 1996). However, these sequences also appear to be present in the genomes of various bacterial groups. The distribution of these sequences in the AAB genome produces a unique pattern at the strain level, due to the different size of the fragments between these elements in the different strains. These techniques have been used to characterize AAB isolates from wines (González et al., 2004) cereal vinegars (Nanda et al., 2001; Wu et al., 2010), Traditional Balsamic Vinegars (Gullo et al., 2009), wine vinegars (Vegas et al., 2010), submerged vinegars (Fernández-Pérez et al., 2010), and grapes (Valera et al., 2011).

- ***$(GTG)_5$-PCR fingerprinting technique:***

This technique is based on PCR-mediated amplification of DNA fragments located between specific interspersed repeated sequences in prokaryotic genomes. Versalovic et al. (1994) proposed the use of this rep-PCR to obtain a genomic fingerprint in individual bacterial strains. The first report in bacterial communities related to the food industry was published by Gevers et al. (2001), and they reported that the $(GTG)_5$-PCR fingerprinting technique is a promising genotypic tool for rapid and reliable speciation and typing of LAB in food-fermentation industries. Moreover, the validation of this technique for identification and classification of AAB was successfully tested at species level by De Vuyst et al. (2008) and at the strain level by Papalexandratou et al. (2009). Recently, several ecological studies have been performed using this technique, which have demonstrated its usefulness in AAB typing (Vegas et al., 2010; Valera et al., 2011).

3. Ecological studies and inoculation

Fresh fruits have characteristics such as a waterproof, wax-coated protective covering or skin, which functions as a barrier for entry of most plant pathogenic microorganisms. However, the skin of fruit harbors a natural microbiota, which is varied and includes both bacteria and fungi (Hanklin and Lacy, 1992). These microorganisms remain on the surfaces of the fruit, as long as the skins are healthy. Any cuts or bruises that appear post harvest or during other processing operations allow entry by microorganisms to the less protected internal tissue. Nevertheless, depending on the specific composition of the fruit (polysaccharides, sugars, organic acids, vitamins, minerals) and specific environment, some microorganisms present in the natural fruit microbiota could persist during the fruit processing (Kalia and Gupta, 2006). These microbes could then become dominant populations in the must and initiate the fermentation or acetification.

Since ancient times, many fermented food and beverages, including vinegars, have been spontaneously elaborated. In regards to vinegar production, each bioprocess (alcoholic fermentation and acetification) depends on the microbial composition, as not all yeast or AAB strains present in the raw material have the same ability to ferment the sugars to ethanol (Fleet, 2008) and to oxidize ethanol into acetic acid (Gullo and Giudici, 2008). Therefore, it is important to be able to discriminate between the yeast and AAB strains to determine how many strains are involved in the processes and which one is leading the biotransformation. The best way to obtain this information is to perform an ecological study. The fundamental limitation of these studies is the recovery and isolation of microorganisms on a specific solid culture medium. However, the current methodologies for typing are culture-dependent, and therefore, the culturing step cannot be bypassed. After conducting an ecological study, the availability of information about the microorganisms involved in the process will allow the selection of the strains best

adapted to the biotransformation conditions for further characterization to be used as possible starter cultures (Singleton, 2004).

The microorganisms involved in the elaboration of wine and beer have been widely studied for many years (Degre, 1993), and species of *Saccharomyces* are the primarily responsible for both processes (Ribéreau-Gayon et al., 2006). Despite the number of publications about other fruit wines (Akubor et al., 2003; Reddy and Reddy, 2005; Santos et al., 2005; Duarte et al., 2009), wine vinegars (Vegas et al., 2010) or fruit vinegars (Su et al., 2010; Ameyapoh et al., 2010), very few of these studies have focused on performing ecological studies. This is also true for the processes carried out by AAB, and little information about them is available. It is known that the *Acetobacter* and *Gluconacetobacter* genera are present in fermented products, and the *Gluconobacter, Asaia* and *Frateuria* genera are found in flowers and fruits (Lisdiyanti et al., 2003). In regards to the AAB characterization in vinegars, *Acetobacter pasteurianus* and *Gluconacetobacter* species have been reported to be the main species in vinegar. *A. pasteurianus* has been found in vinegars with a low concentration of acetic acid, and *Gluconacetobacter* (*Ga. europaeus*, *Ga. xylinus*, *Ga. oboediens* and *Ga. intermedius*) has been found in vinegars with high acetic acid concentrations (Sokollek et al., 1998; Schüller et al., 2000; Nanda et al., 2001; Haruta et al., 2006).

The use of selected starters is a common practice in fermented foods to control the process and to predict and ensure the quality and reproducibility of the final product (Hammes, 1990; Holzapfel, 1997; Ribéreau-Gayon et al., 2006). In beverages such as wine (Pretorius, 2000; Ribéreau-Gayon et al., 2006) and beer (Dufour et al., 2003; Hutkins, 2006; N'Guessan et al., 2008), yeast inoculation has been widely used. In contrast, the AAB inoculation practice in vinegar production has been limited to the use of vinegar mother or back slopping. In this case, the product obtained is the result of the

competition between the microorganisms, specifically AAB present in an undefined starter. However, this method does not ensure the control of the process or the quality of the final product. Few studies have tested the use of a selected AAB culture as starters for the production of vinegar both by the submerged method (Sokollek and Hammes, 1997; Saeki et al., 1997) and by traditional methods (Gullo et al., 2009). In the last study, a selected *A. pasteurianus* strain was tested for use as a starter culture in the production of traditional balsamic vinegar (Gullo et al., 2009). The abovementioned studies demonstrate interest in the inoculation practice in fermentation and acetification, which is necessary to avoid microbial deviations and to help to complete the bioprocess (Ribéreau-Gayon et al., 2006).

Therefore, knowledge of the indigenous yeasts and AAB present in spontaneous processes and the subsequent selection of the most suitable strains to carry out these transformations may be a good strategy for the improvement of vinegar production. In addition, this research in appropriate starter cultures may also improve the quality of the final product, maintaining the natural characteristics that are desired in a new vinegar.

4. References

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Chapter 1

Effect of barrel design and the inoculation of *Acetobacter pasteurianus* in wine vinegar production

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Abstract

The traditional production of wine vinegar is a lengthy process with little or no microbiological control. The aim of this study was to shorten the acetification process via three different strategies: changes in wood type; barrel shape; and the inoculation of an *Acetobacter pasteurianus* pure culture. The barrel shape was modified by constructing two prototypes with higher liquid-air interface. We compared the changes in Acetic Acid Bacteria (AAB) population dynamics in these barrels with those of a submerged method. The wood type had no effect on the acetification length, whereas the shape of the barrel resulted in a significant shortening of the acetification length. Although the selected AAB strain did not always take over, it reduced the biodiversity of the AAB. The inoculated strain was predominant in oak barrels, whereas in the highly aerated prototypes *Gluconacetobacter* species (*Ga. intermedius* and/or *Ga. europaeus*) displaced *A. pasteurianus*, as occurs in the submerged method.

Keywords: *Gluconacetobacter europaeus*, *Gluconacetobacter intermedius*, Oak, Cherry, Acacia, Traditional vinegar, Submerged method

1. Introduction

Traditional wine vinegar is obtained by spontaneous wine acetification conducted by acetic acid bacteria (AAB). These spontaneous acetifications typically result from the competitive activities of a variety of microorganisms (Hopzapfel, 2002). Those best adapted eventually dominate the process, and may be good candidates for starter cultures. In the case of vinegar, the process is usually initiated by the vinegar mother, which is an undefined starter culture obtained from previous vinegar, a process known as back-slopping (Hopzapfel, 2002). There are several reasons for the use of a starter culture in a fermentative process. One concerns the control of the process in order to predict and to ensure the quality and reproducibility of the final product (Hammes et al., 1990; Hopzapfel, 1997, Ribéreau-Gayon et al., 2000). The use of undefined cultures such as the vinegar mother does not ensure total control of the acetification or the quality of the product. Therefore, the control of the vinegar making process to date has been limited or non-existent. One of the reasons for this could be the absence of well defined starter cultures similar to selected microorganisms used in other food fermentation processes (wine, cheese, yogurt, sausages, etc.) (Ayad, 2009; Cocolin et al., 2006; Constanti et al., 1998; Coucheney et al., 2005; Jussier et al., 2006; Simova et al., 2008). In fact, as far as we know, only one study has tested an AAB selected culture as starter for the production of vinegar by traditional method (Gullo et al., 2009).

Selection of starter cultures is often preceded by ecological studies, where the presence and dominance of different species and strains is reported and the ones capable of leading the fermentative process are selected for further characterization of the final products. AAB ecological studies have been conducted in order to identify the main species and strains involved in wine vinegar production. The use of molecular techniques for AAB characterization has shown that *Acetobacter pasteurianus* and

Gluconacetobacter species are the main species in vinegars. *A. pasteurianus* was mostly found in vinegars with a low concentration of acetic acid whereas *Gluconacetobacter* (*Ga. europaeus*; *Ga. xylinus*; *Ga. oboediens* and *Ga. intermedius*) was present in high acetic acid concentrations (Haruta et al., 2006; Ilabaca et al., 2008; Nanda et al., 2001; Sokollek et al., 1998; Schüller et al., 2000; Vegas et al., 2010).

Traditional wine vinegar production is characterized by the use of wooden barrels and a slow acetification process that may take several months. The combination of AAB metabolism, wood contact and simultaneous ageing and acetification yield a high quality product (García-Parrilla et al., 1999; Morales et al., 2001; Natera et al., 2003; Tesfaye et al., 2002;). In this process, AAB develop a biofilm in the liquid-air interface to keep the bacteria in close contact with oxygen. Such a lengthy process introduces a spoilage risk that could be reduced with appropriate control. In contrast, industrial vinegar production takes place in stainless steel fermentors where air is applied to the liquid, producing a "submerged" culture of AAB. In such cases, AAB act as a bioreactor transforming ethanol very quickly into acetic acid with a considerable loss of aromas (Morales et al., 2002). Thus, the result is a fast process (about 24 hr) with a low quality product.

We aimed to reduce the time of fermentation by introducing two innovations: On one hand, to change the wood of the barrel and, on the other, to change the shape of the barrel. These changes were carried out together with the use of an appropriate inoculum of *Acetobacter pasteurianus*. The analysis of the resulting changes in microbiota and the effect of wood type and shape of the barrel are reported in the present study. We also analysed AAB population dynamics in submerged acetifications inoculating the same *Acetobacter pasteurianus* strain.

2. Materials and methods

2.1. Starter preparation

The AAB used was an *A. pasteurianus* strain (Ap0) isolated in an earlier ecological study conducted in La Guinelle vinegar plant (AOC Banyuls, France), (Vegas et al., 2010). This isolate had been identified by 16S rRNA gene sequencing and genotyped by both $(GTG)_5$-rep-PCR and ERIC-PCR. These techniques allowed us to monitor this strain all along the biomass production in the laboratory and proliferation in the vinegar plant. During the preparation of the inoculum the isolates always showed the same typing profile as that of the inoculated strain.

Under laboratory conditions, this strain was recovered in 25mL-Glucose broth (GY: 1% Yeast extract, 5% Glucose) and afterwards, mixed with the vinegar plant's wine and water in a proportion of 25:50:25 (laboratory inoculum: wine: water), producing the initial vinegar mother. The characteristics of the wine used for vinegar mother, propagation and acetification are shown in Table 1. To increase the mother's volume, diluted wine (50% in water) was added before the AAB exhausted the ethanol until the volume reached an amount of 7 L. In the vinegar plant, this vinegar mother was increased up to 100 L in an oak-barrel, maintaining the abovementioned proportions. The whole process was carried out using the wine usually employed in the vinegar plant. This vinegar mother was used to carry out the acetification by both methods (submerged and surface).

Table 1. Acetification length and main components of the starting wines.

Wine substrate	Acetification method	Acetification processes or wood type	Barrel Shape	Duration
Alcohol (%, v/v): 15.2 Acidity (%, w/v): 0.6 Glucose + Fructose: 28.76 g/L + 61.99 g/L pH 3.4 Variety: 100% Grenache	Submerged method	1	-	36 hours
		2	-	30 hours
		3	-	30 hours
	Surface method	Acacia	S	52 days (5%)
			P1	36 days
			P2	36 days
		Cherry	S	52 days (5.6%)
			P1	36 days
			P2	36 days
		Oak	S	52 days (5.7%)
			P2	36 days

2.2. Acetification conditions

2.2.1. Surface method

Eight surface acetifications in barrels (60 L capacity) of different woods and shapes were performed in triplicate and monitored. The 24 barrels were constructed by Boteria Torner (Barcelona, Spain) and specifically designed for this experiment. We worked with three types of barrels: Standard (S), Prototype 1 (P1) and Prototype 2 (P2). The S and P2 barrels were constructed in three different woods: acacia, cherry and oak, whereas P1 was made only of cherry and acacia. Essentially, the main differences in the design of these prototypes in relation to the standard barrels were an increase of ca. 30% air- contact surface and the fact that the S and P1 barrels presented a top hole of 400 and 375 cm^2, respectively, while the opening in the P2 was bigger, 625 cm^2. In all the cases, this top hole was covered by a cloth to keep out insects, dust, etc.

To begin the acetification process, the vinegar mother was mixed with diluted wine in a proportion of 10:65:25 (vinegar mother: wine: water). The alcohol content of this

mixture was 9.5% (v/v) and the acetic acid content was 0.9 (w/v). The barrels were filled with 40 L of the initial mixture, leaving an air chamber of 20 L.

For the microbiological study, sampling was conducted at different moments during the acetification: initial mixture (T_0); 3% (w/v) acidity (mid-acetification); and 6% (w/v) acidity (final acetification). Samples from vinegar mother and wines were also analysed. Acetifications were conducted at room temperature. The oxygen dissolved, the temperature, the titratable acidity and the concentrations of ethanol and residual sugars were analysed throughout the process (in the samples above mentioned).

Temperature and oxygen dissolved in the acetifying liquid were measured using a LDO^{TM} HQ10 Portable Dissolved Oxygen Meter (HACH Company, Colorado, USA). Titratable acidity was determined by titration with 0.1 N NaOH and phenolphthalein as the indicator (Ough and Amerine, 1987). Ethanol and residual sugars (glucose and fructose) were measured with enzymatic kits (Boehringer, Mannheim, Germany).

2.2.2. Submerged process

A laboratory scale fermentor (B. Braun Biotech, SA.) was used to produce wine vinegar by a submerged method. This fermentor was equipped with: a cylindrical concave bottom glass culture vessel of 5 L capacity with a height-to-diameter ratio of 2:1; an air supply system with air filters and inlet pipe with sparger ring; a refrigeration system (Frigomix® cooling unit, Sartorius, Goettingen, Germany) with cold water to prevent loss of volatile components; an electrical heater jacket 230 V; a stirrer with 6-bladed disc impellers; a Pt-100 pH-electrode and a *p*O2-electrode; a sensor for temperature measurement Pt-100; a micro-DCU 300 measurement and control system; a MCU-200 stirrer speed control and a dosing pump-300.

As reported elsewhere (Tesfaye et al., 2000) the optimum conditions used for the efficient elaboration of vinegar samples were air flow 150 L/h, temperature 30°C,

stirring speed 450 rpm, working volume 3.4 L and a loading proportion of 50:50 (wine: vinegar) which results in discontinuous acetification processes.

Throughout the process, the ethanol and acidity were analysed and sampling for the microbiological study was conducted at the end of the last three acetification processes.

2.3. Acetic acid bacteria isolation and genomic DNA extraction

AAB were isolated by plating samples on GY at an adequate dilution, supplemented with natamicine (100 mg/L) (Delvocid, DSM; Delft; The Netherlands). Between ten and fifteen colonies were randomly isolated in each point and plated on GYC (10% glucose, 1% yeast extract, 2% $CaCO_3$, 1.5% Agar) to confirm the acid production by the formation of a halo around the colony. Gram staining and catalase tests were conducted to all halo-forming colonies.

For AAB identification, total DNA was extracted by the CTAB method (Cetyltrimethylammonium bromide) described by Ausubel et al. (1992).

2.4. AAB species grouping by RFLPs-PCR 16S rRNA gene

The 16S rRNA gene was amplified using the method described by Ruiz et al. (2000). The primers used (16Sd: 5′-GCTGGCGGCATGCTTAACACAT-3′ and 16Sr: 5′-GGAGGTGATCCAGCCGCAGGT-3′)) were synthesized by Invitrogen-Life Technologies (Glasgow, UK). Briefly, reactions were carried out in 50 µl final volumes which contained 15 pmol of each primer, 200 µM of each of the four dNTPs, 5 µl 10 × amplification buffer (ECOGEN; ARK Scientific), 3 mM $MgCl_2$ and 2.5 U *Taq* DNA polymerase (ECOGEN; ARK Scientific). The reactions were performed on a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, USA) using the amplification conditions proposed by Ruiz et al. (2000). In all cases, amplified DNA was detected by electrophoresis on a 1.0% (w/v) agarose gel in TBE buffer. The gels were stained with ethidium bromide and photographed. The amplified products were digested with three

restriction enzymes: *Taq*I, *Alu*I and *Bcc*I (González et al., 2006; Ruiz et al., 2000; Torija et al., 2010). Restriction fragments generated by these enzymes were detected using 3% agarose electrophoresis gel. Lengths of amplification products and restriction fragments were detected by comparison against a 100 bp DNA ladder (Gibco-BRL, Eggenstein, Germany). The 16S rRNA gene amplicons of the typing profiles obtained were purified and sequenced by Macrogen Inc. (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. These sequences were deposited in the GenBank Database with the following accession numbers: HM046976, HM046977, HM046978, HM046979 and HM046980. Phylogenetic and molecular evolutionary analyses of these sequences were conducted using *MEGA* version 4 (Tamura, et al., 2007)

2.5. AAB typing

For AAB genotyping, we used the ERIC-PCR (Versalovic et al., 1991) and the $(GTG)_5$-rep-PCR fingerprinter technique (Versalovic et al., 1994; Gevers et al., 2001). The reactions were carried out using a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, USA). ERIC and $(GTG)_5$ amplification products were detected by electrophoresis gels on a 1.5% and 0.8% agarose (w/v), respectively. In both cases, pattern band lengths were determined by comparison against a 100bp DNA ladder (Gibco-BRL) for the smallest bands and by the mixture of λ phage DNA digested with *Hin*dIII-*Eco*RI and *Hin*dIII (Boehringer Mannheim) for the largest bands. The gels were stained with ethidium bromide and photographed.

Sizing by electrophoresis was compared to automated capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). The DNA 7500 LabChip® kit was used to size the amplified products on the bioanalyzer. The Bioanalyzer sizes PCR products quickly and automatically. The Bioanalyzer fluorescence detection system leads to greater detection sensitivity, and the DNA

sample size is estimated by comparison with external standards (DNA sizing ladder) and internal standards (DNA markers), thus providing accurate and reproducible sizing (Nachamkin *et al*. 2001, Panaro *et al*. 2000;).

3. Results

3.1. Acetification kinetics

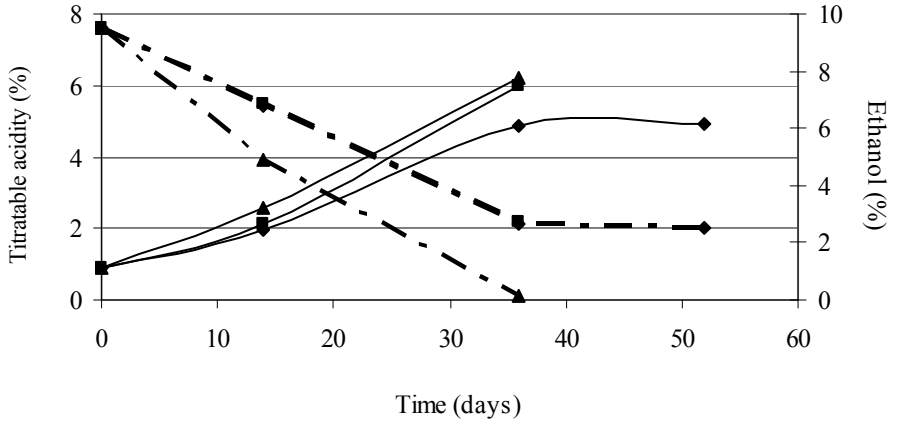
In the submerged method, we studied three acetification processes with an initial acidity >3% (w/v) to ensure the process. Each process was considered to have finished when the acidity reached at least 7% (w/v) and took an average of 33 hours (Table 1).

In the surface method, we studied the effect of wood type and barrel shape. As a general criterion we considered final acetification at 6% (w/v) of acidity. No differences were observed with regard to wood type in the duration of the process. However, the different barrel shape resulted in a marked decrease in the time required to complete the acetification. Both prototypes took less time (36 days) than the standard barrel (52 days) (Table1). Moreover, it is important to note that in S barrels the final point samples did not reach the expected 6%. Therefore, this change in the barrel shape reduced the length of the process by over one third.

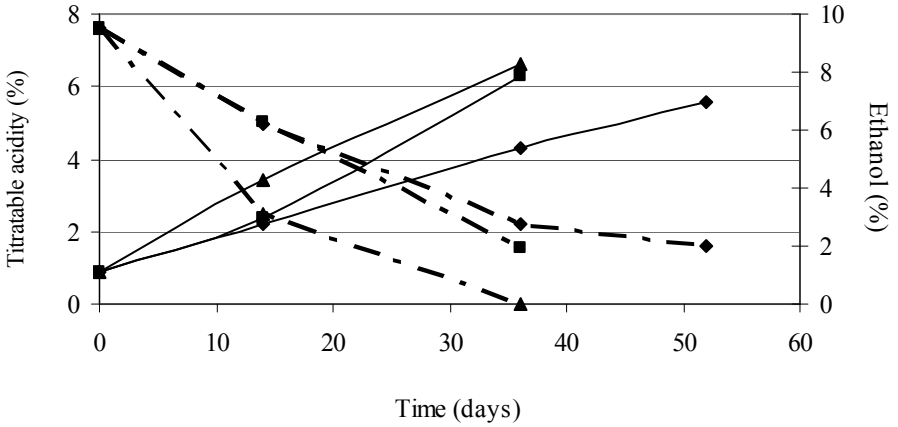
The P2 acetifications presented a faster start and acetification rate at the beginning of the process than the other casks. Nevertheless, this did not lead to a shorter process since P1 completed the acetification in the same time (Fig. 1).

Figure 1. Variation of titratable acidity (solid line) and ethanol (dotted line) in wood barrels [a) acacia; b) cherry; and c) oak] during the acetification process according to the different forms (♦S;■ P1;▲ P2).

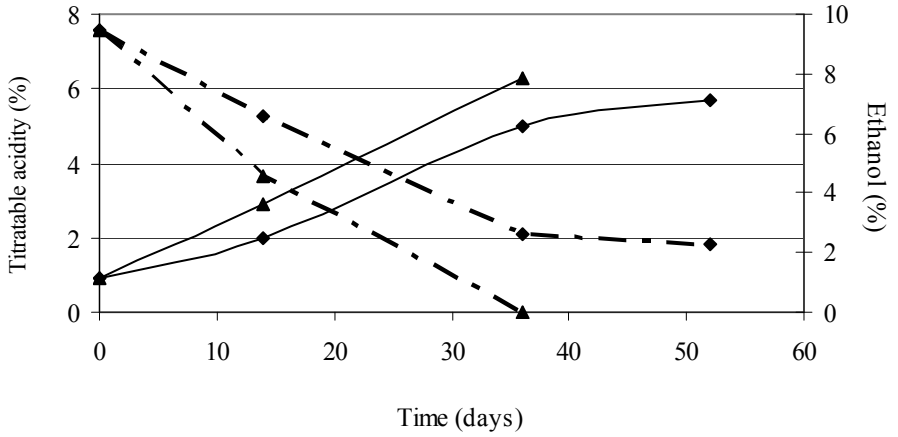
a)



b)



c)



During the acetification process in the surface method, the concentration of ethanol and dissolved oxygen were measured. No differences were observed between the different woods used. However, the barrel shape had a considerable effect in both parameters.

At the end of the process, in P2 acetifications no ethanol was detected while in the other barrels the remaining ethanol was about 2.5% (v/v). This difference in ethanol is particularly marked between both prototypes, which presented a similar acetic acid concentration at this point (Fig. 1). In the case of dissolved oxygen, throughout the process, the levels of oxygen were very low. Nevertheless, in P2 the oxygen content was higher (20 mg/mL) than in P1 and S acetifications (10 mg/mL).

The temperature measured in the acetifying liquid throughout the process was constant (25 ± 1 ºC) and no significant differences were found between barrels.

3.2. AAB identification

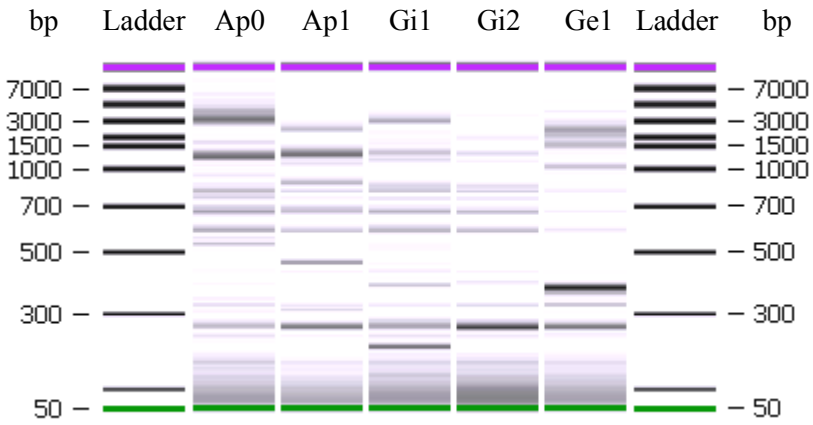
During the preparation of the vinegar mother with the pure culture of *A. pasteurianus,* Ap0 was the only AAB detected by plate recovery and typing methods.

In the submerged method, it should be noted that the acetification processes studied were carried out after three periods of preadaptation of the AAB culture in the laboratory fermentor. In the three acetification processes studied, the inoculated species was not detected and the species isolated were *Ga europaeus* and *Ga. intermedius.* At typing level, two profiles were identified by both $(GTG)_5$-rep-PCR and ERIC-PCR techniques, showing a different evolution throughout the acetification (Fig. 2). In the first acetification process, the Ge1 profile completely took over. However, this profile decreased in subsequent acetification processes due to the emergence of profile Gi2, which reached 55% in the second process and 70% in the third process (Table 2).

Table 2. AAB identification and typing in the different acetifications.

Acetification method			AAB species (GTG_5/ERIC profile)	
	Wood type	**Barrel shape**	**Middle Acetification**	**Final Acetification**
Surface method	Acacia	S	80% *A. pasteurianus* (Ap1) 20% *Ga. intermedius* (Gi2)	20% *A. pasteurianus* (Ap1) 80% *Ga. intermedius* (Gi2)
		P1	100% *A. pasteurianus* (Ap1)	100% *A. pasteurianus* (Ap0)
		P2	100% *A. pasteurianus* (Ap1)	100% *Ga. intermedius* (Gi1)
	Cherry	S	80% *A. pasteurianus* (Ap0) 20% *Ga. intermedius* (Gi1)	20% *A. pasteurianus* (Ap1) 80% *Ga. intermedius* (Gi1)
		P1	100 % *A. pasteurianus* (Ap1)	100% *A. pasteurianus* (40% Ap1; 60% Ap0)
		P2	100% *A. pasteurianus* (Ap1)	100% *Ga. intermedius* (Gi2)
	Oak	S	100% *A. pasteurianus* (Ap0)	100% *A. pasteurianus* (Ap0)
		P2	100% *A. pasteurianus* (75%Ap1; 25% Ap0)	100% *Ga. intermedius* (78.5% Gi1; 21.5% Gi2)
Submerged method	**Acetification processes**		**Final acetification processes**	
	1		100% *Ga. europaeus* (Ge1)	
	2		45% *Ga. europaeus* (Ge1); 55% *Ga. intermedius* (Gi2)	
	3		30% *Ga. europaeus* (Ge1); 70% *Ga. intermedius* (Gi2)	

Figure 2. ERIC-PCR profiles of the isolates from both submerged (Gi2 and Ge1) and surface (Ap0, Ap1, Gi1, Gi2) acetification methods.

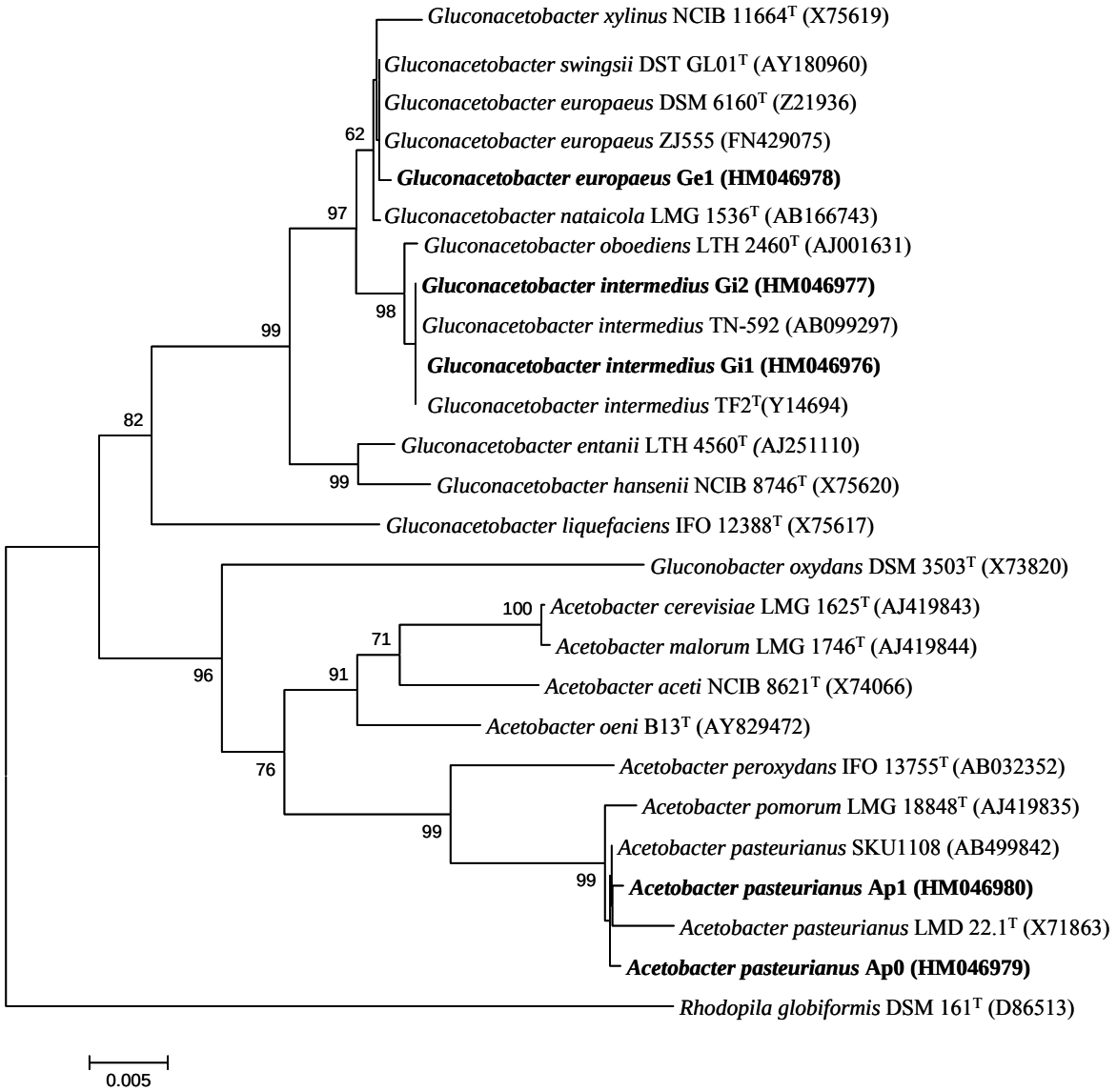


During acetification in the surface method, we detected two species: *A. pasteurianus* and *Ga. intermedius*. In the oak S and P1, only *A. pasteurianus* was isolated while in P2, a succession of species was observed (in mid-acetification *A. pasteurianus* and in final acetification *Ga. intermedius*). In contrast, in the acacia and cherry S barrels, both AAB species coexisted during the acetification process, *A. pasteurianus* and *Ga. intermedius* being the main species in the middle and at the end of the process, respectively.

At typing level, 100% the inoculated profile (Ap0) took over throughout the acetification only in the oak S barrels, although this profile was found in other casks (Table 2). Surprisingly, another profile of *A. pasteurianus* (Ap1) was identified more often than the inoculated profile. In the case of *Ga. intermedius*, the $(GTG)_5$-rep-PCR and ERIC-PCR analysis also revealed two profiles (Gi1 and Gi2) (Fig. 2). These profiles were not detected together in any barrel with the exception of oak P2. Moreover, when one of these profiles appeared in the middle of the acetification, it survived at the end of the process (Table 2).

Fig. 3 shows a phylogenetic tree based on 16S rDNA sequences reflecting the distant relationships of the isolates obtained in the surface and submerged methods.

Figure 3. Phylogenetic relationships of the isolates obtained from both submerged and surface acetification processes with some AAB species. *Rhodopila globiformis* was used as an outgroup. A phylogenetic tree based on 16S rDNA sequences was constructed using the neighbour-joining method. The robustness of the branching is indicated by bootstrap values (%) calculated for 1000 subsets.



4. Discussion

In this study, we tested the ability of a selected strain of *A. pasteurianus* to carry out an acetification process via two systems (submerged and surface). This strain had been isolated in an earlier study during a traditional wine vinegar acetification as one of the main actors (Vegas et al., 2010).

It is well known that the growth of AAB is determined by the presence of dissolved oxygen in the medium. This oxygen requirement produces AAB development on the liquid surface, usually forming biofilms (Ribéreau-Gayon et al., 2000). Therefore, to increase the availability of oxygen for AAB and, thus, achieve a faster process in the case of surface acetifications, we also studied the effect of two variables: type of wood and barrel shape. The purpose of modifying the wood type was to enhance the oxygen diffusion resulting from differences in porosity (De Rosso et al., 2009) whereas the change in the barrel shape was intended to increase the air-liquid interface and give higher exposure to oxygen.

The submerged method, for its part, involves a strong aeration to ensure the oxygen AAB demand, which results in a very quick process (24 – 36 h) (Adams, 1998). In this study, the inoculated profile was not detected in any of the acetification processes studied. Instead, we identified two profiles belonging to the *Ga. europaeus* and *Ga. intermedius* species. The presence of these species is not unusual because the production of vinegar by submerged system is associated with different species of the *Gluconacetobacter* genus and, more specifically with species such as *Ga. europaeus* (Callejon et al., 2008; Sievers et al., 1992, Trcek et al., 2000), *Ga. intermedius* (Boesch et al., 1998, Trcek et al., 2000), *Ga. entanii* (Schüller et al., 2000), *Ga. oboediens* (Sokollek et al., 1998). The absence of the *A. pasteurianus* inoculated profile during the process could be explained by a lack of adaptation of this species to the submerged

systems. In fact, as far as we are aware, this species had never previously been isolated in industrial vinegar fermentors. The occurrence of *Ga. europaeus* and *Ga. intermedius*, which had already been detected in an earlier ecological study conducted in this vinegar plant (Vegas et al., 2010), could easily be related to contamination during the final stages of starter scale-up production carried out in the vinegar plant. Therefore, in the starter culture, although the only profile identified in solid media was the inoculated one, we expected to find the coexistence of other species and strains resulting from the contamination at the vinegar plant. During the preadaptation process of the submerged system, the species/strains better adapted to these conditions (such as those belonging to *Ga. europaeus* and *Ga. intermedius)* overgrew, avoiding the detection of *A. pasteurianus*.

The influence of the studied variables in the kinetics of the surface acetification was very different. Wood effect was minimal, akin to what has been previously described by Torija et al. (2009) in a similar study. According to Joyeux et al. (1984), oxygen permeation through oak barrels into wine during storage is about 30 mg/L per year. The use of woods with higher porosity than oak (De Rosso et al., 2009) in this study did not improve the dissolved oxygen concentration since no significant differences were detected in oxygen values. However, shape variable had a marked effect on the development of the process. Both prototypes needed shorter times to complete the acetification than the standard barrels. It is noteworthy that the design of both prototypes was focused on the increase of the surface/volume ratio. In these new designs, the contact surface of the AAB with atmospheric air (oxygen) was augmented *ca.* 30% over the standard barrels. This broader surface allows larger AAB populations on the air-liquid interface. Hence, more bacteria will have enough oxygen to transform the ethanol more efficiently into acetic acid. As a consequence, the acetification length

is reduced. The shortening of the process is achieved by increasing the contact surface of the AAB with atmospheric air (oxygen) more than by the effect of the different porosity determined by the wood type.

Nevertheless, although there were no differences in the process length between the two prototypes, only in P2 ethanol was absent at the end of the acetification. This reduction was associated with a higher evaporation through the opening on the top of the barrel. P2 presented a top hole 56% bigger than those of the other two casks. This higher open surface favored the entry of oxygen, confirmed by a higher dissolved oxygen concentration in these prototypes, but this larger opening also enhanced evaporation. The absence of ethanol in the media is a huge handicap for vinegar production because the main genera responsible (*Acetobacter* and *Gluconacetobacter*) are capable of oxidizing the acetic acid to water and carbon dioxide. This oxidation occurs via the tricarboxylic acid cycle, but this reaction is inhibited by ethanol (Ribéreau-Gayon et al., 2000; Saeki et al., 1997). Consequently, the vinegar barrel's design must be a compromise between the increase of the air-contact surface and the reduction of the evaporation effect.

In traditional wine vinegar, back-slopping is the most common practice used to initiate the process for shortening the initial phase and reducing the risk of acetification failure (Holzapfel et al., 2002). Prior to this study only one AAB inoculation test had been reported using the traditional method (Gullo et al., 2009) and two using submerged methods (Saeki et al., 1997, Sokollek and Hammes, 1997). Gullo et al (2009) inoculated a selected strain of *A. pasteurianus* in traditional balsamic vinegar but did not find it at the end of the process. In our study, the profile from the selected strain took over only in the S oak barrels. In fact, this profile had previously been isolated in the same conditions (Vegas et al., 2010), suggesting that it could be well-adapted to these

acetification characteristics. In the other acetifications, the main species in mid-acetification was *A. pasteurianus* whereas at the end of the process, the presence of *A. pasteurianus* or *Ga. intermedius* species depended on the shape of the barrel. The fact that the selected strain did not take over completely throughout the acetification and the development of *Ga. intermedius* is most likely due to the different tolerance to acetic acid of these two species. In fact, at the subspecies level we detect the same profiles as in the previous ecological study in the same vinegar plant (Vegas et al, 2010). Therefore, all these profiles were indigenous to the ecological niche formed in this vinegar plant over years of production. The other *A. pasteurianus* profile (Ap1), which was mainly present in the middle of the acetification, seems to exhibit a lower acetic acid tolerance due to its minimal presence at the end of the process. In the case of P2, the presence of *Ga. intermedius* could be related to a higher concentration of dissolved oxygen observed throughout the acetification as *Ga. intermedius* is a species usually involved in high aerated processes (Boesch et al., 1998,, Trcek et al., 2000). Nonetheless, one of the clear effects of inoculation is the reduction in biodiversity, since the earlier study performed in the same vinegar plant and with the same wine yielded 27 different typing profiles (Vegas et al., 2010) whereas the present study yielded only 4.

From all these results, what stands out is that when the conditions were not favorable for *Ga. intermedius*, as in P1, the inoculated strain *A. pasteurianus* Ap0 was the AAB responsible for completing the acetification. In contrast, in other casks a species succession was observed. This succession was also recently described in a traditional balsamic vinegar study (Gullo et al., 2009). These authors proposed *A. pasteurianus* as the pioneer bacteria and *Ga. europaeus* as the subsequent species responsible for completing the process due to its high resistance and tolerance to acetic acid, which is corroborated in this study under certain conditions.

In conclusion, using an *A. pasteurianus* selected strain as a starter was not as successful as expected. This strain took over on the surface system depending on the type of barrel tested. It seems that the presence of higher oxygen and acetic acid concentration favored the development of *Gluconacetobacter* (*Ga. europaeus* and *Ga. intermedius*) species, as in the case of submerged methods or P2. We also demonstrated that the way to reduce the acetification duration in traditional wine vinegar production is to modify the barrel design. This modification must focus on the increase of the air contact surface to facilitate the development of AAB. We observed important differences between the two prototypes studied, both at technological and microbiological level, considering P1 to be the most appropriate one. This prototype was characterized by lower ethanol evaporation, shorter acetification and the development of the *A. pasteurianus* species, which has always been linked to traditional wine vinegar production. As for our results, the mixed inoculum of *A. pasteurianus* and selected *Gluconacetobacter* species are the most likely candidates for use as starting cultures, as *A. pasteurianus* is well prepared for low concentrations and *Gluconacetobacter* species are more resistant to high concentrations of acetic acid. In any event, analytical studies are required to evaluate the impact of these barrels on organoleptic quality.

Acknowledgments

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Chapter 2

Production of fruit vinegars: Technological process for persimmon and strawberry vinegars

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Abstract

Fruit surplus is common in intensive agriculture in many countries. This ecological and economic problem requires alternative uses to be found for fruit. The aim of this study was to use surplus fruit to produce vinegar by traditional methods (alcoholic fermentation and acetification) from persimmon and strawberry. The process was performed with naturally occurring microorganisms and compared with inoculated commercial wine yeast for alcoholic fermentation. The alcoholic fermentation proceeded faster when inoculated due to the length of the lag phases observed in the spontaneous fermentations. The alcoholic fermentations of the strawberry mash were faster than those of persimmon. On the other hand, acetifications were much faster in persimmon (30 days) than in strawberry (70 days), in which some acetifications stuck. From the technological point of view, to produce persimmon and strawberry wine and vinegar it is better to avoid fruit pressing and perform the process with fruit mash. For persimmon, inoculation is recommended; for strawberry it is required.

Key words: Wine, Vinegar, Fruit seasonings, Acetic acid bacteria

1. Introduction

Every year large amounts of different fruits are wasted because the surplus cannot be consumed directly by the market and because some fruit does not fulfil market requirements (second or third quality fruits). Although some alternatives to direct consumption have already been enforced (jams, fruit concentrates, fruit juices, nectars, purées, etc.) a large amount of fruit is still left in the fields to rot or be collected and later disposed as waste.[1] These practices create both an ecological and an economic problem: large amounts of organic matter have to be recycled and money must be spent on agrochemicals, labour force and machinery for both the fruit that is consumed and the fruit that is disposed but all the costs are borne by the fruit that is consumed. Thus, higher prices and environmental contamination are the result of fruit surplus.

Other alternatives have been proposed and enforced in some countries, mostly transformations by fermentation. The resulting product, fruit wine, has a variable alcohol concentration and it is often distilled, as the market for fruit wines is not large. Some of the wines reported are, for instance, mango,[2] banana,[3] acerola,[4] apricot,[5] apple,[6] gabiroba[7] and are popular in some places. However, in a global alcoholic beverage market dominated by grape wine and beer, the impact of such wines is very limited because consumers are reluctant to try them. Furthermore, new alcoholic beverages are not very well received by consumers and sometimes even have legal problems with being authorised because they can cause health concerns in both the general public and the food safety authorities.

On the other hand, the preservation of fruit components and their lack of transformation make fermentation one of the more environmentally friendly processes. Furthermore, transformation by fermentation can add some value, as some of the microorganisms produce vitamins and other compounds that can improve the healthy components of the

fruit. Thus, the option of using fermented fruits is a good one if alcohol content can be avoided. The transformation of ethanol into acetic acid fulfils some of these requirements, as it maintains most of the fruit components, produces a stable product because of the acetic acid content and the pH reduction and can be used to season food directly or while cooking. Our alternative, then, is to use some fruits to produce food seasonings by double fermentation: alcoholic and acetous (or more appropriately acetic oxidation or acetification). Most of the knowledge available about these transformations has been generated by the wine and wine vinegar sector, as it is a well known product and transformation.[8,9,10] The first transformation, alcoholic fermentation, is done by yeasts, especially *Saccharomyces* yeasts, although some non-*Saccharomyces* yeasts may also participate actively, at least in the early stages.[11,12] Once the sugar has been converted into ethanol, the second process consists of an oxidation that is highly dependent on the availability of oxygen, as the main actors, the acetic acid bacteria are clearly dependent on its abundance. The amount of oxygen available is considerably reduced during alcoholic fermentation so, once the sugar has been exhausted and the oxygen concentration increases by aeration due to racking, pumping over, etc, acetification generally proceeds.[13] Although most of the vinegar is produced from wine or alcohol, some fruit vinegars are also available.[9]

The aim of this work was to study whether fruit vinegar can be produced by two different processes (alcoholic fermentation and acetification) from persimmon and strawberry, and, if so, to optimize the procedure. The kinetics of the process has been analyzed in both spontaneous alcoholic fermentations and fermentations inoculated with commercial wine yeast. After the alcoholic fermentations, acetifications were also studied, but they were allowed to proceed with no further intervention.

2. Materials and methods

The study was carried out in 2008 with two different types of fruit: persimmon (*Diospyros kaki*, Sharoni variety) and strawberry (*Fragaria ananasa*, Camarosa variety). They were picked in Huelva, Spain, during the season for each fruit (November for persimmon and April for strawberry).

2.1. Conditions for producing persimmon and strawberry fruit vinegar

The vinegar was produced in a two-step process: first an alcoholic fermentation and then an acetification, which were carried out using crushed pulps of persimmon and of strawberry. The fruit was cleaned (by removing the green parts) and crushed using a Philips HR 2094 Liquidiser. To the crushed pulp, we added 60 mg/L sulphite and 3 g/hL of pectolitic enzymes (1.5 g/hL of Depectil Clarification and 1.5 g/hL of Depectil Extra Garde FCE) (Martin Vialatte Oenologie, France).

For each fruit, two different processes were carried out in triplicate experiments. One of the processes was a spontaneous alcoholic fermentation followed by spontaneous acetification (in both cases natural microbiota was allowed to proliferate). In the other process, the alcoholic fermentation was inoculated at the beginning with the commercial *Saccharomyces cerevisiae* wine strain QA23 (Lallemand, Inc. Canada) at a concentration of 2×10^6 cells/mL. In this case, acetification also proceeded without acetic acid bacteria inoculation.

In the case of strawberry, the process was repeated in single experiments (spontaneous and inoculated) using the liquid obtained after crushing and pressing the fruit pulp. The strawberries were crushed in the same way as above but pressed in a 10-L vertical press. The experiments were as above, with the only difference that pressed juice was used instead of mashed pulp.

The fourteen processes (six for persimmon and eight for strawberry) were conducted under laboratory conditions in 8-L glass containers with a broad top hole of 10 cm in diameter. During alcoholic fermentation and acetification, this top hole was covered by a cloth to keep out insects, dust, etc. The glass containers were filled with 6 L of the initial pulp or liquid, leaving an air chamber of 2 L. To fill the containers in triplicate, a total of 50 kg of persimmon and 65 kg of strawberry was required. When strawberry was pressed, we required 9 L of crushed strawberry pulp to obtain 6 L of liquid. As a general criterion AF was considered to have finished when the sugar had been consumed (< 2 g/L) and the acetification when the ethanol concentration had fallen below 1% (v/v). The fermentations were done at room temperature (23 ± 3 ºC).

2.2. Chemical analysis

The vinegar production processes were carried out at room temperature. The temperature, pH, and the concentration of free amino nitrogen (FAN), sugars, ethanol, and acetic acid were analysed throughout the processes. Temperature was measured using a digital thermometer (Hanna, HI 145-00) and pH using a pH meter (Crison, micro-pH 2002). FAN concentration was analyzed using the formol index method.[14] The sugar concentrations (glucose, fructose and sucrose) and the ethanol were measured with enzymatic kits (Boehringer Mannheim, Mannheim, Germany). Titratable acidity was determined by titration with 0.1 N NaOH and phenolphthalein as the indicator.[15]

2.3. Microbial analysis

The imposition of the inoculated yeast was analyzed during alcoholic fermentations. Samples of the spontaneous and inoculated processes were taken at the beginning, middle and end of the fermentation and plated on YPD (Glucose 20 g/L; Peptone 20 g/L; Yeast extract 10 g/L; Agar 20 g/L; Cultimed, Barcelona, Spain). Twenty colonies of each point were analyzed by RFLP of mtDNA.[16]

2.4 Statistical analysis

Each fermentation condition was performed in triplicate. The data were statistically treated using SPSS 17 software package. By Student T test, we determined the differences between the wild and mutant strain (the statistical level of significance was set at $P \leq 0.05$).

3. Results

We studied the production of vinegar from two fruits: persimmon and strawberry. All the processes were carried out with crushed fruit. The fresh pulp was also pressed in some of the experiments with strawberry. In order to analyse the vinegar process and to prevent side effects caused by wood, we used glass containers, which were cleaned with boiling water and bleach.

3.1. Alcoholic fermentation

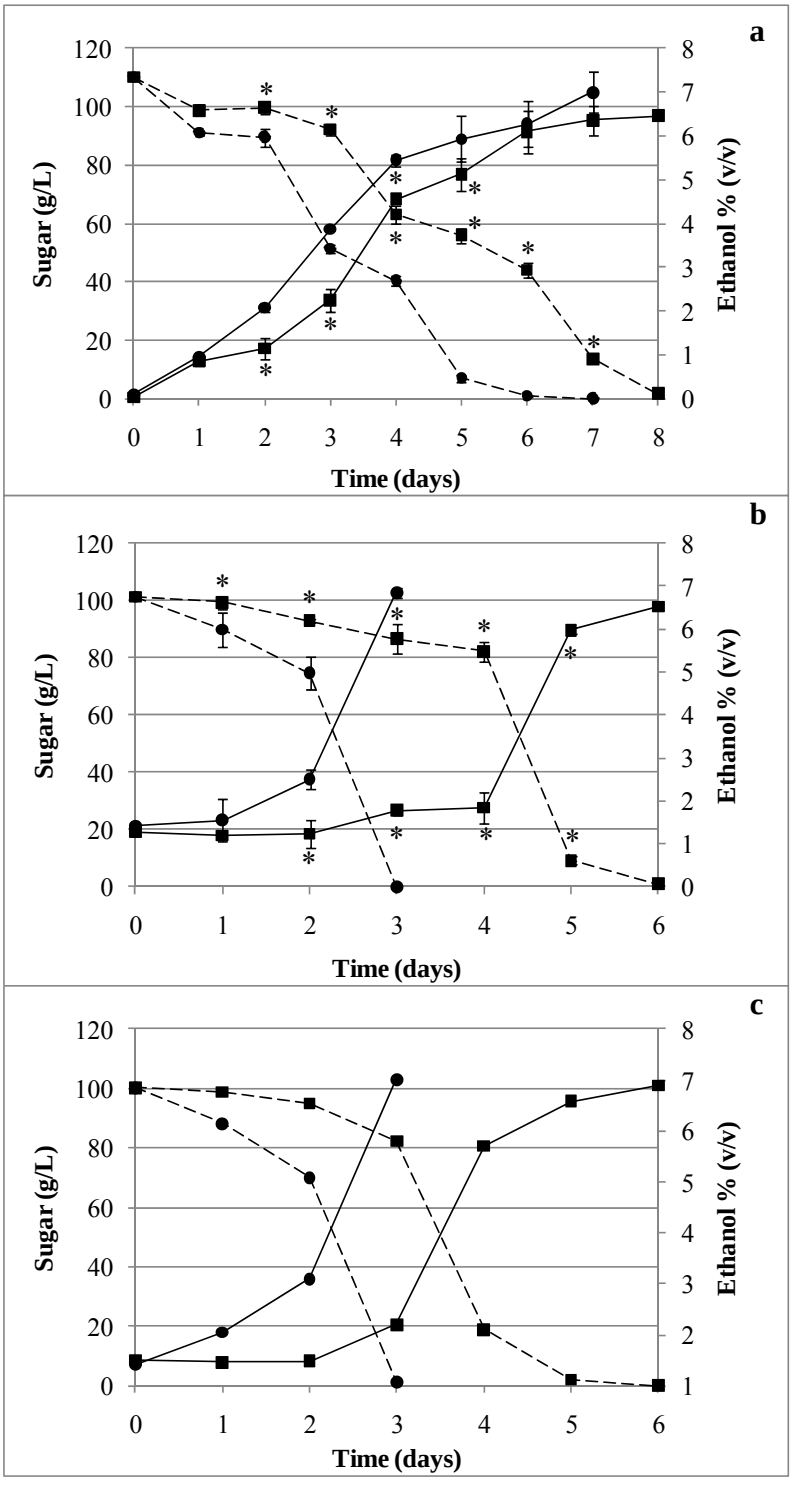
The initial sugar concentration of the persimmon fruit mash was 110.1 g/L. The amount of sucrose was rather low in comparison with fructose and glucose (Table 1). The initial pH was 5.5 and decreased sharply to 3.8 after 24 hours in both the spontaneous and inoculated alcoholic fermentations. This value remained constant throughout the alcoholic fermentation. The FAN was not a limiting factor for alcoholic fermentation, yet in all cases it was completely consumed. The inoculated alcoholic fermentation was faster than the spontaneous one (Figure 1a) because the lag phase was shorter and the fermentation rate similar. Furthermore, the alcohol concentration was 0.5 % (v/v) higher in the inoculated fermentation.

Table 1. Chemical analysis of the persimmon and strawberry pulp before vinegar production.

Parameter	Persimmon	Strawberry
Total sugars	110.1	28.4
Fructose	44.8	15.8
Glucose	57.3	8.3
Sucrose	8	4.3
FAN (mg/l)	120	224
pH	5.5	3.5
Titratable acidity (%, w/v)	0.6	0.9
Ethanol (%, v/v)	-	1.4

The strawberry fruit mash contained a very low initial sugar concentration (28.4 g/l, Table 1), which was mostly fructose. In order to proceed with the alcoholic fermentation, we added sucrose to a final sugar concentration of 100 g/L. The pH was 3.5 and remained constant throughout the alcoholic fermentation. FAN was high, yet it was also completely consumed. Overripe fruits were transported to the laboratory in good and healthy conditions, although some alcohol had already been produced, probably due to some alcoholic maceration. The alcohol content of the fruit mash was 1.4 % (v/v). Again, the inoculated fermentation was faster than the spontaneous one, in which the lag phase was very long and the fermentation rate similar (Figure 1b). The same occurred when the strawberry juice was fermented, not the crushed pulp, in a process that was exactly alike (Figure1c). The levels of alcohol were similar in this case. In all the cases, the inoculated strain took over the alcoholic fermentation with a presence over 80% of the recovered colonies in all the sampling points in the inoculated fermentations, whereas it was always absent in the spontaneous ones. At the end of the inoculated fermentations the starter was the only yeast recovered. The yeast populations achieved levels of 2×10^7 cfu/mL in all the cases, with a longer lag phase (2 days) in the spontaneous fermentations than in the inoculated ones (less than 24 hours).

Figure 1. Development of alcoholic fermentation (**a**, persimmon; **b**, strawberry; **c**, pressed strawberry). Sugar consumption (---) and ethanol production (—) during spontaneous (■) and inoculated (•) alcoholic fermentations. * Statistically significant differences $p \leq 0.05$.



3.2. Acetification

Independently of the alcoholic fermentation, the acetification process in the persimmon fruit proceeded similarly (Figure 2a). The overall acetification process finished in 30 days, and in both cases the acetic acid content was 4.5 % (w/v). In both processes, the pH decreased to 3.4.

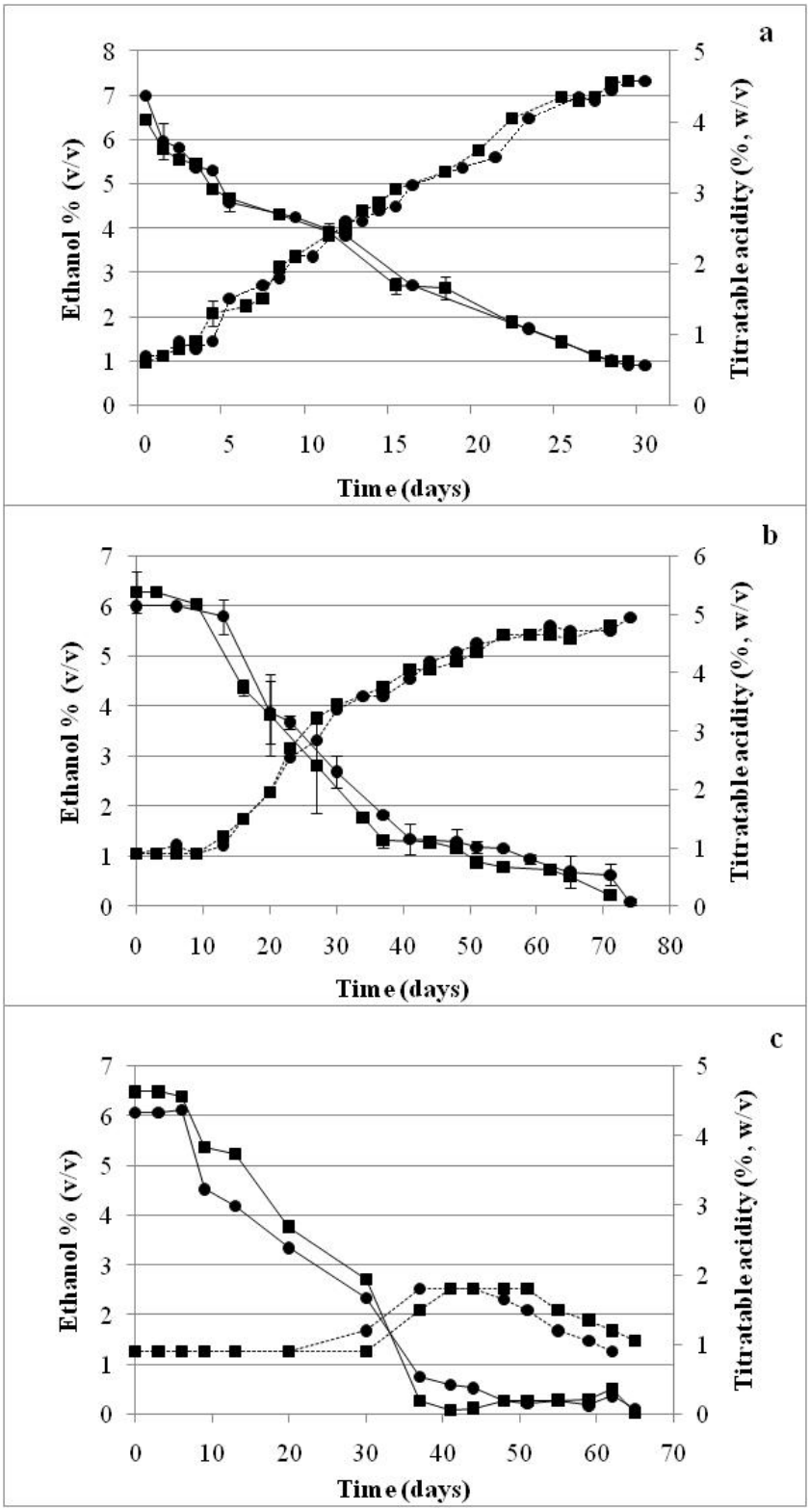
The initial titratable acidity at the end of the alcoholic fermentation of the strawberry fruits was 0.9 % (w/v) in all cases. However, only four of the six glass vessels of crushed pulp successfully completed the acetification (two in each experiment). The other two containers also consumed the ethanol, yet no increase in the titratable acidity was observed (1 % final titratable acidity, data not shown). The successful strawberry acetifications took longer than the persimmon when the crushed fruit was used (70 days in strawberry *vs* 30 days in persimmon, Figures 2a and 2b). In strawberry the yeast inoculation had a similar lack of effect as all the acetifications proceeded in parallel and similar amounts of acetic acid were recovered. The pH values decreased to 3.1 only in the successful acetifications while in the others the pH was the same as that observed at the end of alcoholic fermentation (3.5). The acetification performed with the pressed strawberries also showed poor acetic acid production (Figure 2c). The titratable acidity was only 1.8 % (w/v) after 41 days, and the acetic acid concentration remained constant for 10 days before finally decreasing to 1 % (w/v) after 14 more days. However, the ethanol concentration decreased to below 1 % (v/v) after 65 days. The pH value decreased slightly to 3.4.

3.3. The fruit vinegar yield

Fruit vinegar was only obtained when fruit pulp was used. However, the pulp was still dense and had to be pressed to remove the solid debris and obtain a clear product. Despite the obvious differences between the two processes, the vinegar yields were

similar. In terms of percentage of final product (liquid vinegar, in liters) obtained per initial amount of fruit (in kg) the results obtained with persimmon were 64.8 ± 5.4 % for the inoculated process and 63.2 ± 2.9 % for the spontaneous one. For the strawberry vinegar, the yield was only calculated for those processes in which the acetic acid was about 5 % (w/v) and the value was 66.2 ± 2.5 %. In terms of liquid recovery after pressing, these values are similar to those observed when the fresh strawberry was pressed and the strawberry juice was obtained (66.6 %).

Figure 2. Development of acetification (**a**, persimmon; **b**, strawberry; **c**, pressed strawberry). Ethanol consumption (---) and titratable acidity production (—) during spontaneous (■) and inoculated (•) acetifications.



4. Discussion

Our knowledge of the alcoholic fermentation of fruits is influenced a great deal by the fermentations of the wine and beer industry, in which the limiting parameters are mostly the lack of equilibrium between fermentable sugar and available nitrogen and the availability of different vitamins or minerals.[13] Furthermore, the use of selected yeast is a requirement and makes a significant contribution to the characteristics of the final product in beer. In wine, however, it is not so necessary, but it is a very common practice, especially after the development of the Active Dry Wine Yeast technology.[17] Even so spontaneous fermentations performed with the natural wild yeasts that are present on the surface of grapes or the winery equipment still have defenders because of the authenticity and typicity of the final products.[18] For both beer and wine, most of the yeasts available for starting cultures have been selected from brewing or winemaking because they are good performers, have low nutritional requirements, start fermentations quickly, provide good fermentation rates, and produce secondary metabolites that are appreciated by consumers.[17] Furthermore, as a particular yeast can give the final product uniform characteristics, it is also common practice to select a local wine yeast.[12,18]. However, for the fermentation of other fruit no yeast is available so most of the fermentation is performed with wine yeast or spontaneously. The present study has made a detailed analysis of both processes (inoculated and spontaneous fermentation of fruits) in order to obtain not wines but vinegars after acetification of the initial wines. We had no problems with the alcoholic fermentation, whether spontaneous or inoculated. The fruits used (strawberry and persimmon) have a large amount of available nitrogen, considering the fermentable sugar and comparing with that present in grapes. Other nutrients and vitamins are also available in both fruits.[19,20] In both cases the inoculated fermentation proceeded faster than the spontaneous one, as happens in

wine,[21] largely because of the shorter lag phase when the active yeast is seeded. In the regular transformation into wine, sugar is expected to convert into ethanol (approx. 17 g sugar produces 1 % ethanol v/v).[13] Interestingly, no differences were observed in strawberry fermentation with pressed fruit and fruit mash. It should be pointed out that overripe strawberries produced a certain amount of alcohol, as observed in the alcoholic maceration of grapes, where the alcohol content in the process easily produces over 2 % ethanol (v/v).[13,22]

Although yeast inoculation was not really needed to produce these fruit wines, specific yeast for inoculation will be highly recommended in the industrialisation of both the wine and the vinegar process. The industrialisation requires shorter production periods and a repetitive product, which could be obtained by the practice of inoculating selected strains.

While the alcoholic fermentation of grape is a well-known process, the acetification process is still only partially understood. Most of the vinegar is produced from alcohol and a mix of nutrients in industrial processes in which the seed culture is submerged in a highly aerated vat and maintained continuously throughout a batch process, with a daily refilling system. Wine vinegar can also be produced in this way, although high quality vinegars are produced with the traditional surface culture method. In this method the acetic acid bacteria lie on the liquid-air surface and produce a biofilm that uses oxygen directly from the air or from the limited amounts of air that pass through the wood pores. However, most starter cultures in both cases have very limited availability and are poorly characterised.[23] In fact, most industrially available cultures come in liquid form as mixed, non-characterised cultures and require some time to perform acetification. In contrast, traditional methods use the “vinegar mother” that is normally whether vinegar in process or the biofilm that is spread on top of a new batch. However,

it is well known that acetic acid bacteria are part of the natural microbiota of grapes and wines, and they often survive until the end of alcoholic fermentation.[24] In our case, we allowed the natural population of acetic acid bacteria to acetify the fruit wines. We had no problem with the persimmon fruit and the acetification proceeded at a reasonable rate with the natural microbiota present in the fruit, as already observed by other authors.[25] Wine acetification with traditional methods is much slower, probably due to a higher alcohol concentration (10-15 % ethanol v/v) in the starting wine.[26] In fact, most of the wine used for vinegar production is diluted with water or vinegar. In strawberry, although the starting alcohol concentration was similar, the acetification was much slower and, in some cases, did not finish or produce acetic acid. In fact, if we bear in mind that the pressed strawberry is less acetified than the fruit mash, it is easy to draw the conclusion that some nutrients in the solid particles of the mash are needed for the acetic acid bacteria to perform well. It is evident, then, that to produce strawberry vinegar, wine composition needs to be analysed and appropriate starter cultures need to be used because there is a high risk of unfinished acetifications.

The yield in terms of final product (wine or vinegar) is acceptable as it was always well over 60%. We performed the whole process at the laboratory level, with such limiting factors as the strength of the press and the recovery of fruit pulp on a small scale. Scaling up to higher volumes and with industrial equipment will produce higher yields, similar to those observed in wine. The final product obtained in both cases showed good colour (pink for strawberry, pale yellow for persimmon) and good organoleptic characteristics. Strawberry vinegar had an intense strawberry flavour, which compensated the pungent smell of the volatile acidity, and proved to be a very promising product. Further chemical characterisation of both products is under way.

From the technological point of view, most of the protocols used in wine and wine vinegar production can be used to produce persimmon and strawberry wine and vinegar. However, initial pressing of the fruit is not recommended because of the strength of the fruit (persimmon) and the lack of some characteristics required for vinegar production (strawberry). Although the alcoholic fermentation and acetification of persimmon proceeded at good rates and took a reasonable length of time, the use of selected starter cultures is recommended for to shorten the time and increase the safety of the product. In strawberry, although starter cultures are not essential for alcoholic fermentation, they are required for producing the vinegar repetitively and efficiently. In our laboratory we are now analysing the possible use of native microbiota associated to persimmon and strawberry as starter cultures for both alcoholic fermentation and acetification.

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Chapter 3

Identification of Yeast and Acetic Acid Bacteria Isolated From the Fermentation and Acetification of Persimmon (*Diospyros kaki*)

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Abstract

Persimmon (*Diospyros kaki*) is a seasonal fruit with important health benefits. In this study, persimmon use in wine and condiment production was investigated using molecular methods to identify the yeast and acetic acid bacteria (AAB) isolated from the alcoholic fermentation and acetification of the fruit. Alcoholic fermentation was allowed to occur either spontaneously, or by inoculation with a commercial *Saccharomyces cerevisiae* wine strain, while acetification was always spontaneous; all these processes were performed in triplicates. Non-*Saccharomyce*s yeast species were particularly abundant during the initial and mid-alcoholic fermentation stages, but *Saccharomyces cerevisiae* became dominant towards the end of these processes. During spontaneous fermentation, *S. cerevisiae* Sc1 was the predominant strain isolated throughout, while the commercial strain of *S. cerevisiae* was the most common strain isolated from the inoculated fermentations. The main non-*Saccharomyces* strains isolated included *Pichia guilliermondii*, *Hanseniaspora uvarum*, *Zygosaccharomyces florentinus* and *Cryptococcus* sp. A distinct succession of AAB was observed during the acetification process. *Acetobacter malorun* was abundant during the initial and mid-stages, while *Gluconacetobacter saccharivorans* was the main species during the final stages of these acetifications. Four additional AAB species, *Acetobacter pasteurianus, Acetobacter syzygii, Gluconacetobacter intermedius* and *Gluconacetobacter europaeus*, were also detected. We observed 28 different AAB genotypes, though only 6 of these were present in high numbers (between 25%-60%), resulting in a high biodiversity index.

Keywords: Fruit wine, Food condiments, Traditional production

1. Introduction

Persimmon (*Diospyros kaki*) is one of the most important fruits cultivated in Japan, Korea and China. Among the Mediterranean countries, Spain has shown the largest and fastest growth in terms of field expansion and production of persimmon, and became the largest European producer of this fruit in 2010 (Caccioni, 2010).

A wide variety of seasonal fruits are also cultivated in Spain, and some, among which persimmon is included, spoil quickly. The perishability of persimmon makes long-term storage difficult, and the fruit often needs to be consumed shorty after harvesting. Currently, the market for fruit juices and preserves is almost saturated. Therefore, the manufacture of food seasonings or vinegar represents a solution to the problem of excess secondary and tertiary quality persimmon.

Nowadays, most vinegar is produced by distillation following the fermentation process, which results in the loss of many characteristics of the fruit, including antioxidants and naturally occurring aromas. Some of these losses are supplemented by the use of artificial flavor additives. Few fruit vinegars are currently traditionally fermented and acetified; only Korea and a few other countries in southeastern Asia produce commercially available persimmon vinegar on a small scale. Traditionally, persimmon is alcoholically fermented and matured in jars to yield a final product with at least 3% (w/v) acetic acid.

Little is known about the ecology of the organisms involved in the traditional production of vinegar. However, there have been microbiological studies focused on the production of wine vinegar (Hidalgo et al., 2010b; Vegas et al., 2010; Ilabaca et al., 2008), traditional balsamic vinegar (De Vero et al., 2006; Gullo et al., 2009) and cereal vinegar (Wu et al., 2010; Haruta et al., 2006; Nanda et al., 2001). To date, however, the diversity and succession of microorganisms involved in fruit vinegar production, mainly

persimmon vinegar, remain unstudied. To our knowledge, only two studies have reported the isolation of acetic acid bacteria (AAB) from persimmon vinegar. One study used different strains to study bacterial cellulose production (Kim et al., 2006), while another examined overall acetic acid production (Kim et al., 2005).

The overall aim of our project was to use persimmon to develop new products by traditional methods that preserve its healthy properties. We focused on the production of food seasoning from persimmon using two processes, alcoholic fermentation and acetification, and we aimed to analyze the diversity and succession of microorganisms involved in both processes by molecular methods, in an attempt to gain insight and improve the biotechnological process.

2. Materials and methods

Persimmon (*Diospyros kaki* var. Sharoni) was collected in March of 2008 in Huelva, Spain. Research was completed under laboratory conditions (Tarragona, Spain) that meet 9001 ISO regulations. The details of persimmon condiment production have been reported previously by Hidalgo et al. (2010a). Briefly, 50 kg of persimmon was crushed and distributed into six 8 L glass bottles with a working volume of 6 L. Persimmon pulp was subjected to a chemical analysis prior to beginning the experiment. The total sugar concentration was determined to be 110 g/L (57.27±3.10 g/L glucose, 44.76±3.87 g/L fructose and 8.01±1.63 g/L sucrose), the initial titratable acidity was 0.6% (w/v) and the content of free amino nitrogen was 119 mg/L. Alcoholic fermentation was performed either spontaneously or by inoculation with 2 x 10^6 cells/ml of a commercial *Saccharomyces cerevisiae* wine strain, *S. cerevisiae* QA23 (Lallemand, Inc., Canada). Acetification was always allowed to occur spontaneously, following the traditional method. These experiments were repeated in triplicate, and sugar, ethanol and acetic acid concentrations were monitored.

The populations of yeast and AAB were monitored by plating at various times throughout the experiment. Samples were taken three times during alcoholic fermentation: at the initiation of the process, at a point midway through fermentation (when the sugar was half consumed) and at the point when the residual sugar concentration was below 2 g/L. To monitor the acetification process, sampling was conducted during the initial mixture stage, at 3% (w/v) acidity (mid-acetification) and when the batch reached 6% (w/v) acidity (final acetification).

2.1. Yeast isolation, identification and typing

Yeasts were isolated by plating samples on YPD agar (Yeast extract (Cultimed, Panreac, USA) 10 g/L, Bacteriological Peptone (Cultimed, Panreac, USA) 20 g/L, D-glucose (Panreac, Spain) 20 g/L, Agar (Cultimed, Panreac, USA) 20 g/L) for 48 h at 28 ºC. Between 25 and 30 colonies were randomly picked and plated on a selective Lysine medium to differentiate *Saccharomyces* and non-*Saccharomyces* yeasts. *Saccharomyces* spp. are unable to grow on selective Lysine medium (Angelo and Siebert, 1987).

To identify the yeast, cells were directly collected from a fresh colony using a tip and suspended in a PCR reaction mix, and RFLP-PCR of rDNA was performed (Esteve-Zarzoso et al., 1999). The PCR reactions were done with a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, USA), and the amplification products were digested with the restriction endonucleases *Cfo*I, *Hae*III and *Hin*fI (Boehringer Mannheim, Germany). Additionally, the endonuclease *Dde*I was used to differentiate *Hanseniaspora* species. After digestion, the PCR products and restriction fragments were analyzed on 1.4% and 3% agarose gels, respectively. Band sizes were estimated after being compared to a DNA standard (100 bp DNA ladder, Gibco-BRL, Eggenstein, Germany). Representative amplification products from the obtained restriction profiles were purified and sequenced by Macrogen, Inc. (Seoul, South Korea) using an ABI3730

XL automated DNA sequencer. DNA sequences were compared with those in the GenBank databases. In all the cases, the identity was established by 100% sequence homology with the available sequences.

Saccharomyces genotyping was accomplished using mitochondrial DNA (mtDNA) as described by Querol et al. (1992). Restriction fragments obtained after digestion with the restriction endonuclease *Hin*fI (Boehringer Mannheim, Germany) were separated by electrophoresis on a 0.8% agarose gel, and the products were evaluated against the DNA Molecular Weight Marker II and DNA Molecular Marker III (Roche, Germany).

2.2. AAB isolation, identification and typing

AAB were isolated by plating samples on GYC medium (10 % Glucose, 1 % Yeast extract, 2% $CaCO_3$ (Panreac, Spain) and 1.5 % agar) supplemented with natamicine (100 mg/L) (Delvocid, DSM, Delft, The Netherlands). We analyzed 45 colonies at each time point, and colonies with a halo around them were subjected to Gram staining and the catalase test, which verified their identity as AAB. Total DNA was extracted using the CTAB method (cetyltrimethylammonium bromide), as described by Ausubel et al. (1992).

All AAB isolations were genotyped using ERIC-PCR (González et al., 2004) and the $(GTG)_5$-rep-PCR fingerprinting technique (De Vuyst et al., 2008). Amplification was performed in the Gene Amp PCR System 2700 (Applied Biosystems, Foster City, USA), and products were detected and analyzed by electrophoresis in 1.5% (w/v) agarose gels. Amplicon size was determined by comparing the smallest products to a 100 bp DNA ladder (Gibco-BRL, Eggenstein, Germany), and a mixture of DNA Molecular Weight Marker II and DNA Molecular Marker III (Roche, Germany) was used to determine the weight of the largest fragments. The determination of fragment size was accomplished by using automated capillary electrophoresis with the Agilent

2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany). The DNA 7500 LabChip® kit was used determine the size of the amplified products by comparison with external standards (DNA sizing ladder) and internal standards (DNA markers), allowing for more accurate and reproducible size determination.

RFLP-PCR of the 16S rRNA gene (Ruiz et al. 2000) was used to differentiate bacterial species. Amplicons were analyzed by electrophoresis in 1.0% (w/v) agarose gels, and digested with three restriction enzymes: *Taq*I, *Alu*I and *Bcc*I (Roche Diagnostics GmBh, Germany) (González et al., 2006; Ruiz et al., 2000; Torija et al., 2010). Restriction fragments were detected and analyzed by electrophoresis on a 3% (w/v) agarose gel. The length of the amplification products and restriction fragments were determined by comparison with a 100 bp DNA ladder (Gibco-BRL, Eggenstein, Germany). The 16S rRNA gene amplicons representative of the different genotypes were purified and sequenced by Macrogen, Inc. (Seoul, South Korea), using an ABI3730 XL automated DNA sequencer. DNA sequences were compared with those in the GenBank databases. The identity was established by 100% sequence homology with the available sequences in all the cases, except with *Acetobacter syzygii* whose sequence homology was only 99.1% and this sequence was deposited in the GenBank database under the accession number JF951749.

2.3. Biodiversity analysis

Simpson's biodiversity index was used to calculate the biodiversity index for both yeast and AAB. Simpson's biodiversity index uses probability to determine if two randomly selected isolates are different strains. The biodiversity index was calculated using $1-\Sigma p_i^2$, where p_i is equal to the number of isolates of the same strain divided by the total number of isolates.

3. Results

3.1. Kinetics of fermentation and acetification

The inoculated alcoholic fermentations were completed two days prior to the spontaneous fermentations, and all fermentations had a final ethanol concentration between 6.5% and 7% (v/v). The acetification process was similar in all experiments, with a final acetic acid concentration around 4.5% (w/v) after 30 days. The detailed kinetic properties of both processes were presented by Hidalgo et al. (2010a).

3.2. Microbial analysis

The results of the total yeast and AAB counts performed by microscopy and plating are presented in Table 1. Naturally occurring yeast populations were found to number about 10^4 cells/mL, and most of them could be recovered by plating. Culturing from the inoculated alcoholic fermentations was more difficult (>10^6 cells/mL). In both the spontaneous and inoculated alcoholic fermentations, the yeast population reached a maximum number of >10^7 cells/mL, and 10 – 25% were culturable.

Table 1. Enumeration of yeast and bacteria by plating and microscopy.

	Samples[b]	Cell/mL	CFU/mL
SPONTANEOUS AF[a]	F_i	4.03±0.45E+04	3.62±0.34E+04
	F_m	4.38±0.53E+07	8.32±0.20E+06
	F_f	8.20±0.18E+07	2.06±0.92E+07
INOCULATED AF	F_i	1.43±0.60E+06	6.53±0.50E+05
	F_m	3.40±0.18E+07	1.58±0.18E+07
	F_f	6.57±0.83E+07	1.93±0.14E+07
ACETIFICATION FROM SPONTANEOUS AF	A_i	1.82±0.22E+08	3.75±0.96E+06
	A_m	8.79±0.81E+07	3.01±0.86E+06
	A_f	1.46±0.19E+07	6.10±0.82E+04
ACETIFICATION FROM INOCULATED AF	A_i	3.28±0.18E+08	3.97±0.80E+06
	A_m	1.69±0.89E+08	1.37±0.20E+05
	A_f	2.24±0.44E+07	4.83±0.24E+04

[a] Alcoholic Fermentation
[b] F_i: initial fermentation, F_m: mid fermentation, F_f : final fermentation; A_i: initial acetification; A_m: mid acetification; A_f: final acetification

Microscopy revealed that the bacterial population was high at the beginning of acetification, about 10^8 cells/mL, which decreased by an order of magnitude at the end of the process. Only $>10^6$ CFU/mL were recovered during the initial stages, however, and this number decreased throughout the acetification processes, until only $>10^4$ CFU/mL were recoverable at the final stage, a reduction of 99%. A considerable reduction in bacterial growth was observed during plating throughout both acetification processes. Most colonies produced a clear halo around when plated on media containing $CaCO_3$. All halo-forming colonies were Gram negative and catalase positive, which confirmed they were AAB.

3.3. Yeast isolation, identification and typing

A total of 453 yeast isolates were analyzed. Among them, 226 were isolated from the spontaneous fermentation process, of which 180 grew on L-lysine agar media. Another 227 colonies were isolated from inoculated fermentation process, including 29 that grew on L-lysine agar media.

Table 2. Identification of yeast species by RFLP-PCR of rDNA during the alcoholic fermentation process.

	Samples [b]	Number of isolates studied	Species (%)
SPONTANEOUS AF [a]	F_i	78	*Pichia guilliermondii* (58.97%) *Metschnikowia pulcherrima* (17.95%) *Hanseniaspora uvarum* (8.97%) *Zygosaccharomyces florentinus* (7.69%) *Cryptococcus sp.* (5.13%) *Dekkera anomala* (1.28%)
	F_m	74	*Hanseniaspora uvarum* (37.84%) *Pichia kluyveri* (18.92%) *Pichia guilliermondii* (14.86%) *Cryptococcus sp.* (12.16%) *Saccharomyces cerevisiae* (10.81%) *Zygosaccharomyces florentinus* (5.41%)
	F_f	74	*Saccharomyces cerevisiae* (51.35%) *Pichia guilliermondii* (18.92%) *Zygosaccharomyces florentinus* (12.16%) *Pichia kluyveri* (12.16%) *Hanseniaspora uvarum* (4.05%) *Cryptococcus sp.* (1.35%)
INOCULATED AF	F_i	68	*Saccharomyces cerevisiae* (92.65%) *Hanseniaspora uvarum* (5.88%) *Metschnikowia pulcherrima* (1.47%)
	F_m	77	*Saccharomyces cerevisiae* (68.83%) *Hanseniaspora uvarum* (23.38%) *Cryptococcus sp.* (5.17%) *Pichia guilliermondii* (1.3%) *Pichia kluyveri* (1.3%)
	F_f	82	*Saccharomyces cerevisiae* (100%)

[a] Alcoholic Fermentation
[b] F_i: initial fermentation, F_m: mid fermentation, F_f : final fermentation

3.3.1. Spontaneous alcoholic fermentation

Throughout spontaneous fermentation in these batches (three replica), 8 species of yeast, both *Saccharomyces* and non-*Saccharomyces*, were isolated and identified (Table 2). At each stage, 6 different species were identified, but only 4 of them were present throughout the process (*Pichia guilliermondii, Hanseniaspora uvarum, Zygosaccharomyces florentinus* and *Cryptococcus* sp.*)*. Non-*Saccharomyces* yeasts were dominant during the early stages of the fermentation process, comprising 100% of the community at the beginning and decreasing over the course of fermentation. *S. cerevisiae* strains were increasingly detected during the mid and final stages of the process. *P. guilliermondii, H. uvarum* and *S. cerevisiae* were the dominant species isolated at the beginning, mid and final fermentation time points, respectively.

The characterization of *S. cerevisiae* isolates is detailed in Table 3. A total of 5 different mtDNA profiles were detected, where *S. cerevisiae* Sc1 represented the dominant profile.

Table 3. Profiles of mtDNA obtained from *S. cerevisiae* isolates during the alcoholic fermentation process.

	Samples [b]	Number of *S. cerevisiae* isolates studied	Number of different mtDNA strains	mtDNA strains (%)	Biodiversity Simpson's index
SPONTANEOUS AF [a]	F_m	9	3	Sc1 (77.8%), Sc3/Sc5 (11.1% each)	0.35
	F_f	38	5	Sc1 (86.8%), Sc3 (5.4%), Sc2/Sc5/ Sc4 (2.6% each)	0.24
INOCULATED AF	F_i	63	2	QA23 (98%), Sc1 (2%)	0.031
	F_m	53	1	QA23 (100%)	0
	F_f	82	1	QA23 (100%)	0

[a] Alcoholic Fermentation
[b] F_i: initial fermentation, F_m: mid fermentation, F_f : final fermentation

3.3.2. Inoculated alcoholic fermentation

Six different yeast species were isolated during the three analyzed processes (Table 2). *S. cerevisiae* was present throughout the fermentation process. The genotypic variability analysis revealed that only 2 different mtDNA profiles were present, including that of the inoculum (QA23), which was present throughout the process, particularly at the middle and final stages of fermentation, where it was the only strain detected. At the beginning of fermentation, *S. cerevisiae* Sc1 was also present, which was the same genotype identified during the spontaneous fermentation process (Table 3).

Non-*Saccharomyces* yeasts, particularly *H. uvarum,* were detected in the early stages of fermentation, and a succession of other species was observed at very low numbers during the remainder of the experiment.

3.4. AAB isolation, identification and typing

A total of 270 AAB isolates were analyzed during these persimmon acetifications by the traditional methods (Table 4), and 7 species were identified by 16S rRNA RFLP-PCR and 16S rRNA gene sequencing. The acetifications performed after the inoculated or spontaneous alcoholic fermentations were very similar. Many of the isolates were found to be either *Acetobacter malorum* or *Gluconacetobacter saccharivorans*, and a succession process was observed between these two species. *A. malorum* was always the dominant species isolated during the first half of the acetifications, and at final stages, this species was recovered in higher numbers in acetifications from inoculated fermentations than those from spontaneous ones. Despite this, *G. saccharivorans* became dominant towards the end of the process. Other commonly detected species, regardless of the fermentation type, included *Gluconacetobacter europaeus* and *Gluconacetobacter intermedius*. Small numbers, occasionally only one colony, of

Acetobacter cerevisiae, Acetobacter syzygii and *Acetobacter pasteurianus* were also isolated.

We used two different typification methods, but they yielded the same polymorphism. The typification of all isolates yielded a total of 28 different electrophoretic profiles or genotypes. The biodiversity index was high (0.6-0.8) due to the high number of different genotypes in similar proportions. Overall, we identified 12 different genotypes present in multiple stages of acetification and 6 identical genotypes from both acetification experiments (Am1, Am14, Gs11, Gs3, Gi27, Ge5). Only one genotype was detected in all stages of the acetification that followed the inoculated fermentation (Am14), and it also appeared at the end of the acetification following spontaneous fermentation.

The same succession observed at the species level was confirmed when the genotypes were analyzed. In one of the acetifications, the main genotypes were Am1 and Am2 at the beginning and mid-point of acetification, while Gs10 appeared midway through the process, and became dominant by the end. In the other acetification experiment, Am14 was the dominant at the beginning and midway through acetification, and it was also detectable at the end of the process. One of the two main genotypes identified at the end of the acetification was also present midway through acetification (Gs11), and the other only appeared at the end of the process (Gs25).

Table 4. Isolation, identification and typing of AAB during the acetification of persimmon.

	Samples[b]	Number of isolates studied	Number of different genotypes	Species (%)	GTG_5/ERIC genotype (%)	Biodiversity Simpson's index[b]
ACETIFICATION FROM SPONTANEOUS AF[a]	A_i	45	7	*A. malorum* (63.3%) *Ga. saccharivorans* (25.0%) *Ga. europaeus* (8.3%) *A. cerevisiae* (3.3%)	Am1 (33.3%), Am2 (30.3%) Gs3 (23.3%), Gs4 (1.7%) Ge5 (5%), Ge6 (3.2%) Ac7 (3.2%)	0.74
	A_m	45	9	*A. malorum* (55%) *Ga. saccharivorans* (34.2%) *A. cerevisiae* (7.5%) *Ga. intermedius* (3.3%)	Am2 (30%), Am8 (25%), Am9 (2.1%) Gs10 (26.7%), Gs3 (4.2%), Gs11 (3.3%) Ac7 (3.3%), Ac12 (2.1%) Gi27 (3.3%)	0.76
	A_f	45	9	*A. malorum* (6.7%) *Ga. saccharivorans* (76.7%) *Ga. europaeus* (4.9%) *Ga. intermedius* (11.7%)	Am14 (6.7%) Gs10 (60%), Gs11 (6.7%), Gs4 (6.7%), Gs3 (3.3%) Ge3 (3.3%), Ge12 (1.6%) Gi27 (6.7%), Gi13 (5%)	0.62
ACETIFICATION FROM INOCULATED AF	A_i	45	5	*A. malorum* (100%)	Am14 (39.5%), Am15 (21%), Am16 (19.5%) Am17 (15%), Am1 (5%)	0.74
	A_m	45	10	*A. malorum* (66.7%) *Ga. saccharivorans* (23.3%) *Ga. europaeus* (4.5%) *A. syzygii* (3.3%) *A. pasteurianus* (2.2%)	Am14 (36.7%), Am8 (16.7%), Am18 (6.7%), Am19 (6.7%) Gs3 (17.8%), Gs11 (3.3%), Gs20 (2.2%) Ge5 (4.4%) As21 (3.3%) Ap22 (2.2%)	0.79
	A_f	45	10	*A. malorum* (20.0%) *Ga. saccharivorans* (68.3%) *Ga. europaeus* (1.7%) *Ga. intermedius* (10.0%)	Am14 (13.3%), Am23 (3.3%), Am24 (3.3%) Gs11 (26.7%), Gs25 (16.7%), Gs26 (13.3%), Gs3 (11.7%) Ge5 (1.7%) Gi27 (8.3%), Gi28 (1.7%)	0.84

[a] Alcoholic Fermentation
[b] A_i: initial acetification. A_m: mid acetification. A_f : final acetification.
[c] Biodiversity was calculated from obtained genotypes

4. Discussion

Traditional persimmon vinegar production consists of two biotransformations, fermentation and ethanol oxidation (acetification). The microbes involved in the alcoholic fermentation process were analyzed during the spontaneous process, which is performed by wild yeasts that are present on the persimmon fruit, and also after inoculation with a strain of commercial wine yeast. Yeast inoculation is a very common practice in brewing and winemaking, in order to ensure the quality and reproducibility of the final product (Degre, 1993). In this study, alcoholic fermentation proceeded quickly and efficiently, regardless of the starting method used. The initial yeast population in persimmon pulp (10^4 cells/mL) was low, though the cells were numerous enough to successfully complete the spontaneous fermentation process. In both the spontaneous and inoculated fermentations, the yeast population reached nearly 10^8 cells/mL by the end of the process. These values are similar to those obtained when studying the traditional fermentation of grape must to make wine (Parish and Carroll, 1985; Fleet and Heard, 1993; Fleet, 2003, Beltran et al., 2002), as well as to values obtained from the pulp and spontaneous fermentation of other fruits, including gabirobas (Duarte et al., 2009), strawberries (Cavaco et al., 2007), pineapples (Chanprasartsuk et al., 2010) and apples (Morrissey et al., 2004).

It is generally understood that non-*Saccharomyces* yeasts begin the process of spontaneous alcoholic fermentation, and *S. cerevisiae* eventually takes over and dominates the process. This has been described with grape wine (Fleet, 1993) and gabiroba wine (Duarte el al., 2009); although in fermentations that yield a low final alcohol content, *Saccharomyces* may not always appear (Chanprasartsuk et al., 2010). During persimmon fermentation, the dynamic changes in yeast populations were similar to those described previously. Non-*Saccharomyces* yeasts were isolated in both the

spontaneous and inoculated fermentations, but a high diversity was only present throughout in the spontaneous fermentations. These results are not surprising because inoculation with selected yeasts reduces the growth of native yeasts (Beltran et al., 2002).

The presence of various non-*Saccharomyces* yeasts is the result of differences in fruit composition; for example, the differences in sugar composition and concentration and the presence of organic acids, among others. In the spontaneous fermentation of persimmon, *P. guilliermondii, H. uvarum*, *Z. florentinus* and *Cryptococcus* sp. were repeatedly isolated throughout the process. These species have been widely described when studying different beverages (Chanprasartsuk et al., 2010; Duarte et al., 2009; Escalante et al., 2008) and food fermentation processes (Avallone et al., 2001; Obilie et al., 2003; Aponte et al., 2010; Yoshikawa et al., 2010).

The presence of *H. uvarum* as a starter yeast during wine making is well known, because it is generally the main species involved in the fermentation of grapes. However, this species seems to disappear very quickly after the initial production of alcohol (Constanti el al., 1998, Torija et al., 2001). This disappearance has been only partially confirmed by culture-independent methods, though it seems more likely that *H. uvarum* may have a limited ability to grow on plates, rather than undergoing autolysis (Andorrà et al., 2010). It is not the main species present during the initial stages of persimmon fermentation, but it becomes the prevalent non-*Saccharomyces* species as the alcohol concentration increases, though its numbers decrease by the end of the alcoholic fermentation, when *S. cerevisiae* becomes the dominant yeast species. The increasing levels of alcohol and the progressive imposition of *S. cerevisiae* during wine fermentation have been indicated as factors behind the low culturability of some non-*Saccharomyces* species (Andorrà et al., 2010). Due to the low alcohol content of these

fermentations, when compared to grape must fermentations, it is likely that a large population of non-*Saccharomyces* yeast is still present at the end of fermentation but is not culturable.

Mitochondrial DNA analysis was used to examine the succession of *S. cerevisiae* in the inoculated and spontaneous fermentations. In the spontaneous fermentations, strain Sc1 was prevalent throughout fermentation and was always the main strain recovered. Other minor strains were isolated, but only one or two colonies were recovered at each point of analysis. This low diversity is often observed during wine making in cellars where the repeated use of selected yeasts results in the contamination of the cellar, resulting in cellar-resident strains (Beltran et al., 2002). However, in non-contaminated or new cellars, *S. cerevisiae* diversity tends to be higher (Constanti et al., 1997, Torija et al., 2001), and environmental contamination of the cellar may facilitate the presence of *S. cerevisiae* strains. In our study, the dominance of a single strain could be due to the low diversity of *S. cerevisiae* on the fruit itself, or the semi-sterile conditions of the laboratory where the fermentations were performed.

As expected, the inoculation modified the indigenous microbiota because the inoculated strain was the most frequently isolated strain. The main genotype isolated during spontaneous fermentation (Sc1) was only identified during the initial stages of fermentation. Its disappearance is indicative of the presence of large numbers of the commercial inoculation strain, which is considered to be good for wine making. However, it is surprising that 30% of the isolates were of non-*Saccharomyces* species halfway through fermentation, when the commercial strain generally becomes dominant in wine fermentation (Andorrà et al., 2008).

AAB are part of the natural microbiota of fruits, and they can survive during alcoholic fermentation despite the adverse conditions (Du Toit & Pretorius, 2002). At final stages

of the alcoholic fermentation, higher aeration due to wine replacement and racking may stimulate the growth of these microorganisms, which will oxidize ethanol to acetic acid (Joyeux et al., 1984; Drysdale & Fleet, 1989). In this study, persimmon wine was acetified at a reasonable rate by the AAB present on this fruit. In this work, however, the wine was not aerated; a large air chamber above of the surface remained, which may have allowed acetification to proceed.

The AAB diversity detected throughout both processes was similar, which indicated that the inoculation with commercial wine yeast did not influence the acetification process. In both processes, the AAB recovered on plates were two or three orders of magnitude lower than what was observed by microscopy. The highly acidic conditions at the middle and late stages of acetification suggest that most of the bacteria present were AAB, despite the fact that we were unable to differentiate them by microscopy. The low recovery of culturable AAB is consistent with results obtained previously, in studies on wine elaboration (Bartowsky et al., 2003; Millet and Lonvaud-Funel, 2000) and vinegar acetification (Vegas et al., 2010; Ilabaca et al., 2008; Sokollek et al., 1998; Trcek, 2005).

A. malorun was the main AAB species identified at the initial and middle stages of both processes, and *Ga. saccharivorans* was the main species isolated during the final stages of acetification. This succession of genera was observed previously in wine vinegar production (Gullo et al., 2009; Hidalgo et al., 2010b). The *Gluconacetobacter* genus is known to have a higher tolerance to acetic acid than *Acetobacter*. *A. malorum* has been previously reported to be present during traditional balsamic vinegar preparation (De Vero et al., 2006) and in the making of pulque, a fermented beverage made from the agave plant that contains an ethanol concentration between 3% and 6% (v/v) (Escalante et al., 2008). Interestingly, *Ga. saccharivorans* has been recently identified in

Chardonnay white wines (Kato et al., 2011), which demonstrates its ability to tolerate high alcohol concentrations (more than 11.5 % (v/v)). *Acetobacter* species have always been linked with traditional wine vinegar production, while *Gluconacetobacter* species are associated with vinegar production in submerged systems, where the conditions are more extreme (Gullo et al., 2006; Sokollek et al., 1998; Trcek et al., 2000; Callejón et al., 2008). As reported in previous studies (Gullo et al., 2009; Hidalgo et al., 2010b), our results suggest that a mixed inoculum of *Acetobacter* and *Gluconacetobacter* species could be used as a starter culture in traditional persimmon vinegar production because they will secure the start and the end of the acetification process. *A. malorum* and *Ga. Saccharivorans* are highly adapted to the composition of persimmon and thus, their mixture could be highly recommendable to be used as starter culture. Large numbers of genotypes from these species were isolated, though neither became particularly dominant over the course of the acetification process. A future study focusing on these isolates (e.g., Am1, Am2, Am14, Gs3, Gs10 and Gs11) is necessary in order to evaluate their potential use as starter cultures.

Several additional AAB species were detected during both processes. These species have been previously isolated but were involved in different processes. *Ga. europaeus* and *A. pasteurianus* are commonly associated with wine or alcohol vinegar production, and they have been detected in traditional balsamic vinegar production (Gullo et al., 2009; De Vero et al., 2006), in traditional wine vinegar production (Vegas et al., 2010; Hidalgo et al., 2010b), in rice vinegar production (Nanda et al., 2001; Haruta et al., 2006) and during the submerged production of vinegar (Sievers et al., 1992, Trcek et al., 2000, Callejón et al., 2008,). *A. cerevisiae* was originally isolated from beer (White, 1970; Cleewerk et al., 2002) and later from grapes (Prieto et al., 2007). *Ga. intermedius* has been detected in wine vinegar (Hidalgo et al., 2010b), kombucha beverage, cider

and spirit vinegar (Boesch et al., 1998), and *A. syzygii* was isolated from apple juice (Lidsiyanti et al., 2002). These studies demonstrate that these particular AAB species can be found in a wide variety of ecological niches, likely far more than expected when these species were initially described.

Furthermore, it should be emphasized that we had already reported the chemical analysis of these persimmon wines and vinegars (Úbeda et al., 2011). We observed significant chemical changes in wines with a relationship between the production of several compounds and the inoculation with selected yeast. Wines produced by inoculated alcoholic fermentation had higher amounts of higher alcohols (1-propanol, 2-methyl-1-butanol and 3-methyl-butanol) and acetaldehyde than the spontaneous one. However, chemical composition differences between vinegars were not relevant.

In conclusion, the microbiota isolated from persimmon, both during fermentation and acetification, was highly diverse and capable of carrying out both processes without outside input. The use of a commercial starter culture reduced the length of the alcoholic fermentation and increased control of the process. The main yeast present naturally on persimmon, *S. cerevisiae* (Sc1), was capable of leading alcoholic fermentation, and has the potential to be used as a starter culture. During the acetification process, a clear succession of species was observed, as described previously in the production of other vinegars. It may be interesting to analyze the effects that co-inoculation with different species may have on the acetification process. *A. malorum* and *Ga. saccharivorans* would be appropriate choices in an attempt to explore this process using persimmon. To the best of our knowledge, this is the first report describing the identification and population dynamics of yeast and AAB communities during persimmon alcoholic fermentation and acetification.

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Chapter 4

Effect of inoculation on strawberry fermentation and acetification processes using native strains of yeast and acetic acid bacteria

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Abstract

The aim of this work was to analyze the microbiota involved in the traditional vinegar elaboration of strawberry fruit during a spontaneous and inoculated process. In the spontaneous processes, low biodiversity was detected in both alcoholic fermentation (AF) and acetification. Nevertheless, a strain of *Saccharomyces cerevisiae* and of *Acetobacter malorum* were selected and tested as starter cultures in the inoculation study. The inoculated processes with these strains were compared with another spontaneous process, yielding a significant reduction in time for AF with a total imposition of the *S. cerevisiae* strain. The resulting strawberry wine was acetified in different containers (glass and wood) yielding an initial imposition of the *A. malorum* inoculated strain, although displacement by *Gluconacetobacter* species was observed in the wood barrels.

Key words: Fruit condiments, Traditional production, *Saccharomyces cerevisiae*, *Acetobacter malorum*

1. Introduction

Strawberry is one of the most popular berries and is mainly consumed as fresh fruit. The relevant nutritional value represented by micronutrients and phenolic substances and the potential health benefits of strawberry fruits have been widely studied (Hannum, 2004; Giampieri et al., 2012).

Strawberry is an easily perishable fruit, which makes it impossible to store it long term; thus, it should be used very quickly after harvest. For these reasons, many products such as juice, jelly, nectar, puree, concentrate or jams have been developed (Barret et al, 2005; Hui, 2006). Despite the demand for these products, there remains an excess of strawberry crops. A possible alternative is to generate new foods that retain a maximum amount of the fruit's original characteristics. Among food storage systems, biotransformations, such as wine or vinegar production, could be a good solution because they allow products to be maintained in alcohol or acetic acid.

Although the number of studies about fruit wines (Joshi et al., 1990; Joshi et al., 1991; Akubor et al., 2003; Reddy and Reddy 2005; Santos et al., 2005; Duarte et al., 2009) and fruit vinegars (Hidalgo et al 2010a; Su et al., 2010; Ameyapoh et al., 2010; Hidalgo et al, 2012) has recently increased, no study has focused on the production of strawberry vinegar. Microbiological studies in strawberry have been focused on the natural endophytic (Dias et al., 2009; De Melo Pereira et al., 2012) and epiphytic bacterial communities of plants (Krimm et al., 2005) as well as on the role of yeast proliferation in the degradation of strawberry quality during storage (Ragaert et al., 2006a; Ragaert et al., 2006b).

The use of selected starters is a common practice in fermented foods to predict and ensure the quality and reproducibility of the final product (Hammes, 1990; Holzapfel, 1997; Ribéreau-Gayon et al., 2006), and they play an important role in controlling the

fermentative process (Constantí et al., 1998; Jussier et al., 2006; Ayad, 2009). Yeast inoculation in different beverages such as wine (Pretorius, 2000; Ribéreau-Gayon et al, 2006; Fleet, 2008) and beer (Dufour et al, 2003; Hutkins, 2006; N'Guessan et al., 2008) have been widely used to obtain a product with a predictable quality and according to scheduled times (Degre, 1993; Hutkins, 2006). In contrast, the inoculation practice in vinegar production has traditionally been limited to the use of vinegar mother or back slopping. In this case, the product obtained is the result of the competition between the microorganisms, specifically acetic acid bacteria (AAB) present in an undefined starter, which does not ensure the control of the process or the quality of the final product. Recently, the use of AAB pure cultures to carry out vinegar acetification has been tested (Gullo et al., 2009; Hidalgo et al., 2010b). Although these AAB inoculation results were not totally successful, these inoculation processes were favored, and the possibility of using mixed culture inoculations was proposed.

In the present study, we analyze samples from a strawberry vinegar production that proceeded spontaneously to determine the degree of biodiversity present in this fruit (Hidalgo et al., 2010a). Then, from the isolates we tested their capacity as possible starter cultures to carry out both processes involved in vinegar production, alcoholic fermentation (AF) and acetification in a more controlled conditions. Once the yeast and AAB strains were selected, they were tested in an inoculation assay to determine their ability, as pure cultures, to produce strawberry vinegar. Additionally, different containers (glass, oak and cherry barrels) were used during acetification to evaluate their effect on the process.

2. Materials and methods

Strawberry (*Fragaria ananasa*, Camarosa variety) was collected in Huelva (Southern Spain), and the research was conducted in Tarragona (Northeastern Spain) under laboratory conditions that meet 9001 ISO regulations. The fruits were collected in the 2008 and 2009 harvests. In 2008, a spontaneous production was performed to analyze the native microbiota associated with the strawberries (ecological study). In 2009, we inoculated the selected strains of yeast and AAB from 2008, and we performed a spontaneous process as a reference (inoculation study).

The details of strawberry vinegar production have been previously reported by Hidalgo et al. (2010a). Briefly, 100 kg of strawberry was cleaned and crushed using a Philips HR 2094 liquidizer. Sulfite (60 mg/L), and pectolytic enzymes (3 g/hL) (ROHAPECT®, AB Enzymes, Germany) were added to the crushed pulp. The processes were conducted in 8-L glass containers or in 10-L wood barrels (oak or cherry), which were constructed by Boteria Torner (Barcelona, Spain). The containers were previously cleaned and sterilized by boiling water. Glass containers were filled with 6 L of crushed fruit pulp in the case of alcoholic fermentation or with 6 L of strawberry wine in acetification processes, whereas the wood containers used for acetification were filled with 7 L of strawberry wine. In all cases, an air chamber was left open covered with a cheese cloth. All experiments were performed at room temperature (23±3 °C).

The production of strawberry vinegar was tested using spontaneous and inoculated conditions. For the inoculation study, yeast and AAB strains used as starter cultures were selected from the ecological study (Hidalgo et al., 2010a). The microbiota from this ecological study was isolated and analyzed by molecular methods as described below.

2.1. Yeast isolation, identification and typing

Yeasts were isolated by plating samples onto YPD agar (1% yeast extract (Cultimed, Panreac, USA), 2% bacteriological peptone (Cultimed, Panreac, USA), 2% D-glucose (Panreac, Spain), and 2% agar (Cultimed, Panreac, USA)) for 48 h at 28 ºC. The colonies studied were chosen randomly and plated onto a selective lysine medium to differentiate *Saccharomyces* and non-*Saccharomyces* yeasts (Angelo and Siebert, 1987). To identify yeast species, colonies were directly resuspended in a PCR reaction mix and analyzed by Restriction Fragment Length Polymorphism (RFLP) analysis of the ribosomal Internal Transcribed Spacer (ITS) region (Esteve-Zarzoso et al., 1999). The PCR reactions were performed with a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, USA) and analyzed on a 1.4% agarose gel. Next, the amplification products were digested with the restriction endonucleases *Cfo*I, *Hae*III and *Hinf*I (Boehringer Mannheim, Germany), and the restriction fragments were analyzed on a 3% agarose gel. Band sizes were estimated by comparison with a DNA standard (100 bp DNA ladder, Gibco-BRL, Eggenstein, Germany). Representative amplification products from the different restriction profiles obtained were purified and sequenced by Macrogen, Inc. (Seoul, South Korea) using an ABI3730 XL automated DNA sequencer to confirm the species identification. The sequences obtained were compared with those in the GenBank databases.

Saccharomyces genotyping was accomplished using the restriction analysis of mitochondrial DNA (RFLP mtDNA) as described by Querol et al. (1992). Restriction fragments obtained after digestion with the restriction endonuclease *Hinf*I (Boehringer Mannheim, Germany) were separated by electrophoresis on a 0.8% agarose gel, and the products were evaluated against the DNA Molecular Weight Marker II and DNA Molecular Marker III (Roche, Germany).

2.2. AAB isolation, identification and typing

AAB were isolated by plating samples on GY (10% glucose and 1% yeast extract (Panreac, Spain) and 1.5 % agar) at an adequate dilution supplemented with natamycin (100 mg/L) (Delvocid, DSM; Delft; The Netherlands) to suppress yeast growth. Then, the colonies were randomly selected and plated on GYC (10% glucose, 1% yeast extract, 2% CaCO3, 1.5% agar) to confirm acid production by the formation of a halo around the colony. These colonies were tested by the catalase test, and those with positive results were considered putative AAB colonies and analyzed using molecular techniques.

Total DNA was extracted using the modified CTAB method (cetyltrimethylammonium bromide), as described by Ausubel et al. (1992).

All AAB isolations were genotyped using Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) (González et al., 2004) and the $(GTG)_5$-rep-PCR fingerprinting technique (De Vuyst et al., 2008). Amplification was performed with the Gene Amp PCR System 2700 (Applied Biosystems, Foster City, USA), and products were detected on a 1.5% (w/v) agarose gel. Firstly, band weight was determined by comparing the smallest products to a 100 bp DNA ladder (Gibco-BRL, Eggenstein, Germany) and the largest ones to a mixture of DNA Molecular Weight Marker II and DNA Molecular Marker III (Roche, Germany). Next, the determination of a more precise band size was accomplished by using automated capillary electrophoresis with the Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany).

Different genotypes were identified at the species level by RFLP of amplified 16S rDNA (RFLP-PCR) and of the amplified 16S-23S rRNA gene ITS region (Ruiz et al. 2000). Amplicons were analyzed by electrophoresis on a 1.0% (w/v) agarose gel and digested with the following restriction enzymes: *Taq*I, *Alu*I, *Bcc*I and *Cfo*I (Roche

Diagnostics GmBh, Germany) (González et al., 2006; Ruiz et al., 2000; Torija et al., 2010). Restriction fragments were detected and analyzed by electrophoresis on a 3% (w/v) agarose gel. The length of the amplified products and restriction fragments were determined by comparison with a 100 bp DNA ladder (Gibco-BRL, Eggenstein, Germany). The 16S rRNA gene and the 16S-23S rRNA gene ITS region amplicons representative of the different genotypes were purified and sequenced by Macrogen, Inc. (Seoul, South Korea), using an ABI3730 XL automated DNA sequencer.

2.3. Preparation of starters

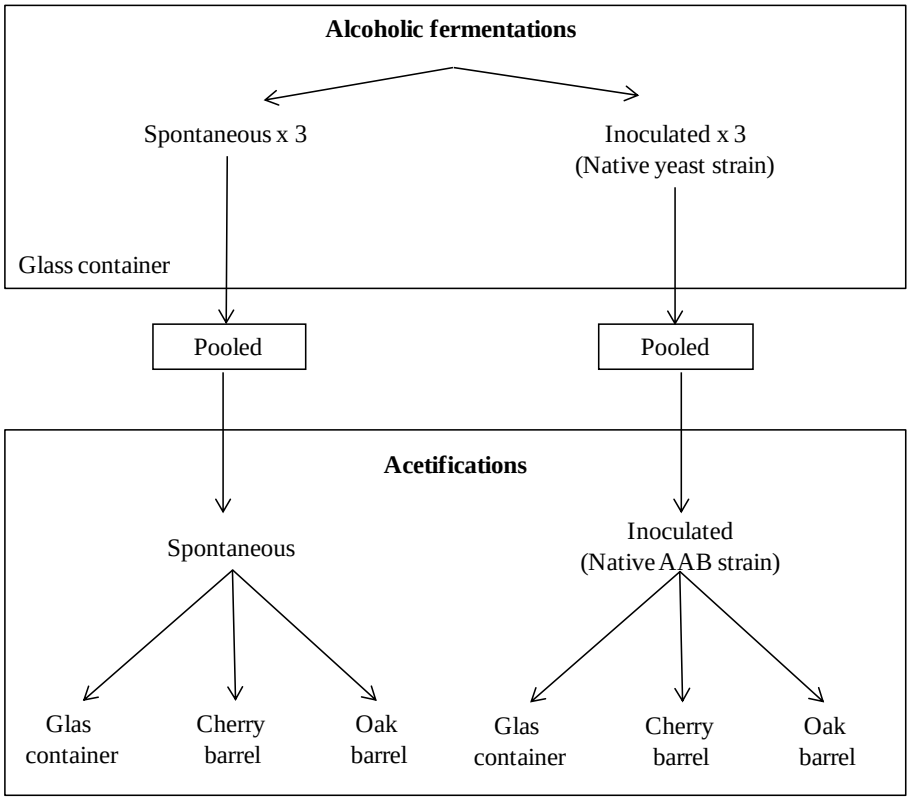
The yeast starter was grown in YPD broth (1% yeast extract (Cultimed, Panreac, USA), 2% bacteriological peptone (Cultimed, Panreac, USA), 2% D-glucose (Panreac, Spain)) for 24 h at 28°C. Then, cells were recovered by centrifugation (5 min, 8000 rpm) and added to crushed strawberry pulp at a concentration of 2×10^6 cells/mL. In the case of AAB, the starter strain was first grown for 48 h in GY broth (10% Glucose and 1% Yeast extract), and after that, cells recovered by centrifugation (5 min, 8000 rpm) were inoculated in 200 mL of strawberry wine to obtain the "vinegar mother." This process was performed in different steps, increasing the volume by the addition of strawberry wine when the titratable acidity reached 3% (w/v) to a final volume of 3 L. Afterwards, this vinegar mother was mixed with strawberry wine at a ratio of 10:90 to carry out the acetification process by the traditional method. The maintenance of the pure strain throughout the process was tested by the molecular methods previously described.

2.4. Experimental design of inoculation study

Figure 1 shows the experimental design for the inoculation study. A total of six alcoholic fermentations (AF) were conducted; three were under spontaneous conditions, and three were inoculated with the selected yeast strain. Once finished, the AF of each type of wine (spontaneous and inoculated) was pooled and distributed in three different

types of containers: glass or wooden barrels of oak or cherry. Wine was allowed to acetify spontaneously, whereas the vinegar mother from the AAB starter was added to the inoculated wine to perform the acetification.

Figure 1. Experimental design of fermentations and acetification processes



2.5. Chemical and microbiological analysis

AF was monitored by sugar consumption (glucose and fructose) and ethanol production. Acetifications were monitored by measuring ethanol consumption and total titratable acidity increase (which was mostly due to acetic acid). The concentration of residual sugars and ethanol was measured by enzymatic kits (Roche, R-Biopharm AG, Germany), and total titratable acidity was determined by titration with 0.5 N NaOH and phenolphthalein as the indicator (Ough and Amerine, 1987).

For the microbiological study, samples were taken at three different stages of AF and acetification: beginning, mid-point (when the sugar and ethanol were half consumed in AF and acetification, respectively) and the end of the process. The AF was considered finished when the residual sugar concentration was below 2 g/L and the acetification when the ethanol concentration was below 1% (v/v).

Yeast and AAB populations were monitored by plating and microscope counting (Olympus Latin America Inc.) using a Neubauer improved counting chamber (0.0025 mm^2 and 0.02 mm deep). Microbial identification and typing were carried out as described in the above sections.

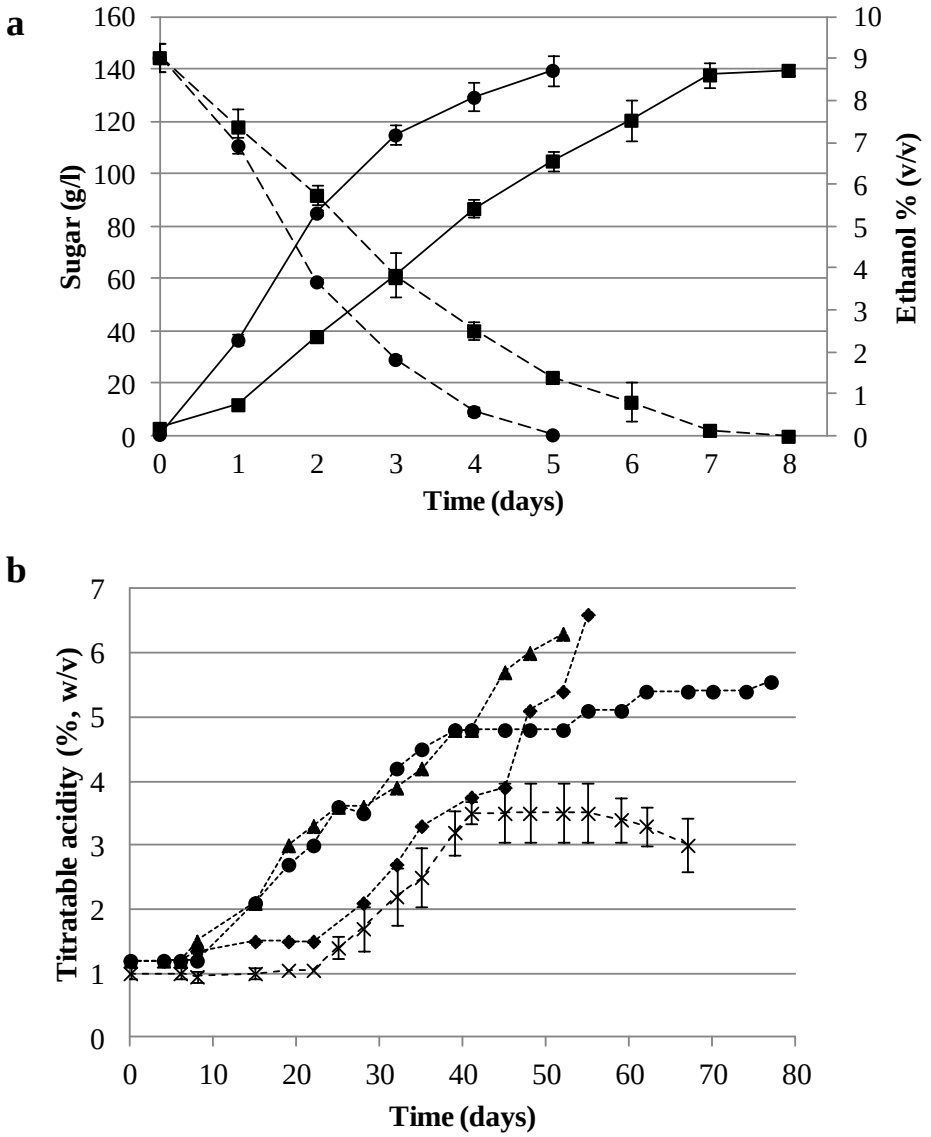
3. Results

3.1. Kinetics of the process

The strawberry fruit mash contained a low initial sugar concentration (70 g/l); therefore, to proceed with AF, we added sucrose to a final sugar concentration of 140 g/l to reach the appropriate quantity of ethanol to produce the desired final concentration of acetic acid (5.5-6 % (w/v)).

The inoculated AF was faster than the spontaneous one, which took three days longer; the total times were 5 and 8 days, respectively. In both cases, the ethanol concentration reached was 8.7% (v/v) (Figure 2a). Regarding the acetification processes (Figure 2b), the inoculated acetifications performed in wood barrels were approximately 25 days faster than those carried out in glass containers. However, in the oak barrel, a delay in the beginning of the acetification was observed compared to that of the cherry barrel.

Figure 2. Kinetics of the alcoholic fermentation (a); spontaneous alcoholic fermentations (■) and inoculated alcoholic fermentation (•), where the line (—) is the ethanol production, and (---) is the sugars consumption. Kinetics of acetification (b); spontaneous acetification average (x), cherry-barrel inoculated acetification (▲), oak-barrel inoculated acetification (♦), glass-container inoculated acetification (•).



The acetic acid concentration reached was 6.6% (w/v) in acetifications performed in wood barrels and 5.5 % (w/v) in glass containers. On the other hand, the acetification processes performed spontaneously did not reach more than 3.5 % (w/v) of acetic acid, and after 40 days, the concentration of acetic acid started to decrease.

3.2. Microbial enumeration

The results of total yeast and AAB population counted by microscopy and plating are shown in Table 1. In spontaneous AF, yeast populations were 10^6 cells/mL and 10^5 cells/mL in the initial stage of the ecological and inoculation study, respectively. Throughout the process, a population increase was observed, achieving populations of 10^7 cells/mL at the end of the process in both studies (ecological and inoculation studies).

Table 1. Enumeration of yeast and bacteria by plating and microscopy.

Process	Process	Samples [b]	Cell/mL	CFU/mL
Ecological study	**SPONTANEOUS AF**[a]	F_i [d]	5.50±0.50E+06	2.67±0.23E+05
		F_m	2.02±0.12E+07	1.27±0.02E+06
		F_f	5.38±0.55E+07	1.47±0.06E+07
	SPONTANEOUS ACETIFICATION FROM SPONTANEOUS AF	A_i	3.10±0.22E+05	5.43±0.95E+03
		A_m	2.49±1.25E+07	8.45±0.16E+04
		A_f	4.75±0.11E+07	4.10±013E+04
Inoculation study	**SPONTANEOUS AF**	F_i [d]	5.09±0.18E+05	4.50±0.16E+05
		F_m	2.27±0.14E+06	2.08±0.13E+06
		F_f	4.87±0.40E+07	2.14±0.34E+07
	INOCULATED AF	F_i	3.02±0.74E+06	9.92±0.96E+05
		F_m	8.25±0.46E+06	3.78±0.43E+06
		F_f	5.23±0.27E+07	1.94±0.90E+07
	IFIA[c] Glass	A_i	1.20±0.62E+05	1.16±0.09E+04
		A_m	3.08±0.17E+06	1.56±0.32E+05
		A_f	6.00±0.31E+06	6.94±0.13E+04
	IFIA Oak	A_i	5.56±0.34E+05	5.16±0.21E+04
		A_m	2.29±0.15E+06	1.77±0.81E+04
		A_f	1.83±0.14E+07	6.86±0.71E+04
	IFIA Cherry	A_i	5.76±0.30E+05	5.16±0.21E+04
		A_m	4.80±0.38E+06	1.54±0.11E+05
		A_f	1.62±0.82E+07	6.86±0.71E+04

[a] Alcoholic Fermentation.
[b] F_i: initial fermentation, F_m: mid fermentation, F_f : final fermentation; A_i: initial acetification; A_m: mid acetification; A_f: final acetification
[c] IFIA Inoculated acetification from inoculated fermentation
[d] Natural population

In all acetifications, the initial population observed by microscopy was approximately 10^5 cells/mL in the ecological study and approximately 10^6 cells/mL in the inoculation study. Finally, the bacterial population increased to 10^7 cells/mL by the end of the process. However, the results of the plate recovery were one to three orders of magnitude lower than the microscopy counts. On the other hand, no cells were observed by microscopy or recovered by plating in the spontaneous acetifications of the inoculation study.

3.3. Microbial identification and typing of the ecological study

We analyzed a total of 151 yeast isolates (Table 2). Only two species of yeast (*Saccharomyces cerevisiae* and *Issatchenkia terricola*) were identified. Both species were present throughout the process, although their relative percentages changed. Surprisingly, *S. cerevisiae* was clearly dominant (86%) at the beginning of the fermentation, but its percentage decreased over the course of the process. On the other hand, *I. terricola*, despite never being the dominant species, increased its presence in the middle and final fermentation points. The typing of *S. cerevisiae* isolates showed only one mtDNA profile. This strain was deposited in the CECT collection (CECT 13057) and used as starter culture in the inoculation study.

Regarding the acetification process, a total of 60 AAB isolates were analyzed (Table 3). This study revealed the presence of only one genotyping profile, belonging to the *Acetobacter malorum* species, which was identified using the 16S-23S rRNA gene ITS region. This strain was also deposited in the CECT collection (CECT 7749) and used as starter culture in the inoculation study.

Table 2. Identification and typing of yeast during the alcoholic fermentation of strawberry pulp.

Process	Alcoholic Fermentation points: Process / Container / (No. of isolates studied)	Species and mtDNA profile	Percentage of presence: Initial	Mid	Final
Ecological study	Spontaneous / Glass / (151 isolates)	*Issatchenkia terricola*	14%	44%	43%
	Spontaneous / Glass / (151 isolates)	*Saccharomyces cerevisiae* (CECT 13057)	86%	56%	57%
Inoculation study	Spontaneous / Glass / (120 isolates)	*Saccharomyces cerevisiae* (CECT 13057)	0%	22.5%	100%
		Hanseniaspora uvarum	100%	77.5%	0%
Inoculation study	Inoculated (CECT 13057) / Glass / (120 isolates)	*Saccharomyces cerevisiae* (CECT 13057)	100%	100%	100%

3.4. Microbial identification and typing of the inoculation study

A total of 240 yeast isolates were analyzed (Table 2). In the inoculated AF, the unique mtDNA profile was that of the inoculated strain of *S. cerevisiae*, indicating its imposition. During the spontaneous AF of this study, two species were detected: *Hanseniaspora uvarum* and *S. cerevisiae*. The first species was the unique species detected at the initial stage and the predominant one at the middle stage. However, it was not detected at the final stage. On the other hand, *S. cerevisiae* was the only species detected at the end of the process. Remarkably, only the profile of the inoculated strain (CECT 13057) was isolated during this spontaneous AF.

In the acetifications, a total of 180 AAB isolates were analyzed (Table 3). During the spontaneous acetifications, no AAB colonies were recovered on plates from any container tested. In the inoculated processes, the identification results varied depending on the type of container used. The inoculated strain was the unique profile detected throughout the process in the glass container and in the initial and middle stages in wood barrels. However, at the final stages of acetifications in wood barrels, AAB profiles that were different from the inoculated one were identified. In cherry barrels, only one profile (Gx2) belonging to *Gluconacetobacter xylinus* was detected, whereas in oak barrels, two different genotypes (Gs1 and Gx1) were identified belonging to *Gluconacetobacter saccharivorans* and *Ga. xylinus*, respectively, the latter being the dominant species.

Table 3. AAB identification and typing during the acetification of strawberry wine.

Process	Acetification points				
	Process / Container / (No. of isolates studied)	Species and GTG_5/ERIC genotypes	Percentage of presence		
			Initial	Mid	Final
Ecological study	Spontaneous / Glass / (60 isolates)	*Acetobacter malorum* (CECT 7749)	100%	100%	100%
Inoculation study	Spontaneous / Glass, Oak standard barrel, Cherry standard barrel	Nothing was recovered			
	Inoculated (CECT 7749) / Glass / (60 isolates)	*Acetobacter malorum* (CECT 7749)	100%	100%	100%
	Inoculated (CECT 7749) / Oak standard barrel / (60 isolates)	*Acetobacter malorum* (CECT 7749)	100%	100%	0%
		Gluconacetobacter saccharivorans (Ga1)	0%	0%	80%
		Gluconacetobacter xylinus (Gx1)	0%	0%	20%
	Inoculated (CECT 7749) / Cherry standard barrel / (60 isolates)	*Acetobacter malorum* (CECT 7749)	100%	100%	0%
		Gluconacetobacter xylinus (Gx2)	0%	0%	100%

4. Discussion

In this study, we tested the possibility of using native yeast and AAB strains as starter cultures to carry out the production of strawberry vinegar. These starters were selected from an ecological study performed on strawberry spontaneous processes.

Development of knowledge about the yeast and AAB community of local ecosystems is essential for producing strawberry vinegars with consistent attributes and for improving the vinegar-making practice. Ecological studies are an essential step towards the preservation and exploitation of indigenous microorganism wealth.

Regarding the dynamics of the microbial groups studied in the spontaneous processes, the yeasts present in strawberry pulp were sufficient to complete the spontaneous AF. Similar initial population sizes have been reported in pineapple fruit (Chanprasartsuk et al, 2010) and grapes under different conditions (Fleet and Heard, 1993). Moreover, in the strawberry AF, a low yeast biodiversity was detected. As observed in other fermented fruits, *S. cerevisiae* was the main species present throughout the process. It is well known that this species is considered to be the best adapted to AF conditions, such as high alcohol and sugars concentrations. However, in strawberry, the sugar content is low, and other non-*Saccharomyces* yeast could also perform the AF. Thus, *S. cerevisiae* was not the only species detected at the end of the process; the presence of these other species was most likely related to a low final ethanol concentration compared to other fermentative processes, such as wine fermentations, where the initial sugar concentration is much higher. Regardless, this result is not uncommon because in fermentations with low final alcohol content, *Saccharomyces* may even not appear (Chanprasartsuk et al., 2010).

Two non-*Saccharomyces* species, *H. uvarum* and *I. terricola*, were detected. The presence of *H. uvarum* in fruit crushed pulp and in the initial stages of an AF is very

common, especially in wine fermentations (Dequin et al., 2003; Hierro et al., 2005; Fugelsang and Edwards; 2007; Clavijo et al., 2010). *H. uvarum* has also been detected in the persimmon fermentation processes (Hidalgo et al., 2012). This species is known to have a low tolerance to ethanol, and its growth stops when the ethanol level reaches approximately 4% (v/v) (Rainieri and Zambonelli, 2009). Furthermore, it has also been associated with the spoilage of damaged strawberry (Fleet et al., 2003).

I.a terricola was detected along all the spontaneous fermentation. This species has been previously reported in grape must in Spain (Mora and Mulet, 1991; Mora et al., 1988). The study of this species could be interesting as this yeast has high β-glucosidase activity that promotes aromas (González-Pombo et al., 2011), which could be easily expressed under AF condition used for strawberry (low sugar concentration and low pH).

Only one strain of *S. cerevisiae* (CECT 13057) was identified throughout all the AF. The use of this strain as a starter culture in the inoculation study was completely successful, with a total imposition of this strain. In addition, the inoculated process was faster than the spontaneous one, confirming the adaptation of this strain to the strawberry conditions. However, there are two possible alternatives to explain the presence of the inoculated strain in the spontaneous processes. The yeast used as a starter belongs to the natural microbiota, which is very well adapted to the strawberry growing area. Thus, the strain could survive to every season and could come from picked strawberry. The other possible alternative is that despite having worked under controlled laboratory conditions, the spontaneous processes suffered cross-contamination during fermentation.

Regarding acetification, the traditional acetification process is known to be a good alternative for preserving fruit characteristics and for obtaining a product with

interesting organoleptic qualities. In recent years, the study and knowledge of AAB has increased considerably. These studies are not only related to the production of wine vinegar (Vegas et al., 2010; Hidalgo et al., 2010b) but also to the improvement of vinegars made from other raw materials, for example, hawthorn (Zheng et al., 2010), mango (Ameyapoh et al., 2010), strawberry (Hidalgo et al., 2010a) or persimmon (Hidalgo et al., 2010a; 2012).

The natural AAB population was not always able to complete the acetification. Strawberry wine conditions seem to be less favorable for the development of AAB than other fruit studied because the initial population counted in the spontaneous acetifications was three orders lower than those quantified, for example, in persimmon wines, which were produced under similar conditions (Hidalgo et al., 2012).

During the ecological study, we expected to find an important AAB biodiversity. However, in contrast to most ecological studies performed on different processes of vinegar production (Gullo et al., 2009; Hidalgo et al., 2010b; Wu et al., 2012; Hidalgo et al., 2012), only one profile belonging to *A. malorum* species was recovered from strawberry wine. This low AAB recovery and diversity under strawberry conditions could be responsible for the lack of acetification in some spontaneous processes. In fact, during the inoculation study, no spontaneous acetifications were able to reach the expected acidity. The species of *A. malorum* identified in this study had previously been isolated from many different niches such as rotting apple (Cleenwerck et al., 2002), fermented beverage of agave plant, pulque (Escalante et al., 2008) and grapes (Valera et al., 2011).

Instead, all inoculated containers reached the end of the acetification, but when the AAB identification and typing was performed, the recovered profile was not always the same as that of the starter culture. In fact, in the acetification carried out in the glass

container, the inoculated strain was the only genotype identified. By contrast, when the culture starter was inoculated in wood containers, genotypes of different species from *A. malorum* were observed at the final stage of the acetifications; *Ga. saccharivorans* and *Ga. xylinus* were recovered from oak barrels (one genotype in each case), and another different genotype belonging to *Ga. xylinus* was identified in cherry barrels. It is important to note that the wood barrels had been previously used for wine vinegar production. Therefore, although the barrels were properly cleaned (Wilker and Dharmadhikarf, 1997), it is also well known that there is not a "definitive" treatment to rid the AAB contamination from a barrel when the bacteria have penetrated deeply into the wood (Schahinger and Rankine, 2002); by contrast, glass containers are easier to sterilize. Therefore, these AAB species could have been present in the barrels and developed when the conditions were appropriate.

Although the inoculated strain of *A. malorum* was not able to finish the acetification in wood barrels, it is evident that the inoculation of a starter improved the process. In contrast to spontaneous processes, the use of starter cultures induced a fast beginning/start of the acetification and provided the appropriate conditions for the correct development of the process, avoiding stuck acetifications. The succession of two genera (*Acetobacter* and *Gluconacetobacter*) during the acetification had been previously observed in wine vinegar production (Gullo et al., 2009; Hidalgo et al., 2010b; Hidalgo et al., 2012). *Gluconacetobacter* species may appear at the final stages most likely due to the ability of some species of this genus to grow under high acidity conditions (Schüller et al., 2000). The lower ability of *A. malorum* to withstand high acidities could be the cause of the *Gluconacetobacter* species imposition at the end of the strawberry vinegar elaboration.

Finally, the use of wood barrels instead of glass containers improved the kinetics of the process, which was significantly faster in wood barrels. As previously reported, the size and shape of the container and even its material have important effects on the development of the process (Torija et al., 2009; Hidalgo et al., 2010b). It has to be considered that the oxygen permeability through staves of the barrels also most likely supports the survival of AAB (Du Toit et al., 2006).

The determination of major volatile compounds by static headspace gas chromatography–mass spectrometry method was carried out during this strawberry vinegar production. The results of this analysis demonstrated that inoculated acetification carried out in wood barrels yielded vinegars with a better aroma profile, as these contained higher levels of most compounds except acetaldehyde (Ubeda et al, 2011).

To our knowledge, this report is the first on the isolation and utilization of selected yeast and AAB for strawberry vinegar production. The use of native microorganisms as starter cultures ensured strawberry vinegar production. In this study, a strain of *S. cerevisiae* and another one of *A. malorum* were successfully tested as possible starter cultures for the alcoholic fermentation and acetification, respectively. However, the AAB starter was displaced by other AAB species at the end of acetification. Thus, we are now working on testing mixed cultures for AAB to ensure correct and fast development of the acetification.

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Chapter 5

Acetobacter strains isolated during the acetification of blueberry (*Vaccinium corymbosum L.*) wine

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Abstract

Highbush blueberries (*Vaccinium corymbosum* L.) are known to have positive health benefits. The production of blueberry vinegar is one method to preserve this seasonal fruit and allow extended consumption. In this study, blueberry wine acetification was performed with naturally occurring microorganisms and with an inoculated *Acetobacter cerevisiae* strain. Acetifications were carried out in triplicate using the Schützenbach method. The successful spontaneous processes took up to 66% more time than the processes involving inoculation. The isolation of acetic acid bacteria (AAB) and the analysis of these AAB using molecular methods allowed the strains involved in the processes to be typed and identified. Although the *A. cerevisiae* strain was the predominant strain isolated from the inoculated process samples, *Acetobacter pasteurianus* was isolated from samples for both processes and was the only species present in the spontaneous acetification samples. To the best of our knowledge, this is the first report describing the identification and variability of AAB isolated during blueberry acetification. The isolated *A. pasteurianus* strains can be used for large-scale blueberry vinegar production or as a starter culture in studies of other vinegar production methods.

Keywords: Vinegar, Schützenbach method, Starter culture.

1. Introduction

Acetic acid bacteria (AAB) are important microorganisms in the food and biotechnological industries because of their ability to oxidize many types of sugar and alcohols (De Ley et al., 1984). The production of vinegar is one of the most important industrial processes in which these bacteria are involved.

Vinegars derived from different raw materials, such as grapes, cereals, onions, persimmons, berries and whey, have been produced and studied (Solieri and Giudici, 2009). In some of these studies, molecular techniques have been used to identify the AAB species and genotypes present in these niches. In the single study done on blueberry vinegar, AAB were isolated and identified using biochemical tests. Several different AAB genera were identified (*Acetobacter*, *Gluconobacter*, *Asaia*, *Gluconacetobacter* and *Swaminathania)* from different varieties of blueberry (Gerard et al., 2010).

Highbush blueberries (*Vaccinium corymbosum* L.) are a rich source of dietary antioxidants (Borges et al., 2010; Seeram et al., 2006; Gu et al., 2004) that have multiple beneficial biological effects. This fruit is native to North America, and the largest blueberry industries in the world are in the United States and Chile. Currently, Chile has a dominant position in the Southern Hemisphere, and blueberry cultivation is an important economic activity in this country (Brazelton, 2011).

As with any activity related to the production of fresh fruit, there are substandard fruit, seasonal surpluses and fruit waste generated during the cultivation, and these materials could be used to produce fruit vinegar. In the case of blueberries, the production of vinegar could be a good option to preserve the healthy properties of this fruit.

There are two well-defined methods used to produce vinegar: traditional or surface processes and submerged methods. The primary differences between these two methods

are related to the time needed to complete the acetification process and to the quality of the final product. In the traditional method, the time required to obtain the expected level of acetic acid is longer than in the submerged method due to the strong aeration used in the latter method to meet the oxygen demand of the AAB. However, as consequence of the production system, the quality of the final product is significantly higher in the traditional method.

Alternative systems for the production of vinegar have been designed to achieve faster rates of production than those for traditional methods while preserving the quality of the final product to the greatest extent possible. These alternative methods, among which is the Schützenbach method, are focused on the use of inert materials, such as bacterial supports, to mimic the air-liquid interface created in the traditional method to allow direct contact with atmospheric air. The Schützenbach method uses wood shavings as a bacterial support material, and to increase the oxygen accessibility, the acetifying liquid is pumped through the wood shavings, achieving relatively high acetification rates (Laguno and Polo, 2001).

The use of well-defined starter cultures in vinegar elaboration to date has been limited or non-existent. Few studies have tested the use of a selected AAB culture as a starter for the production of vinegar by the traditional method (Gullo et al, 2009; Hidalgo et al, 2010) or the submerged method (Sokollek and Hammes, 1997; Saeki et al., 1997; Hidalgo et al, 2010), and no studies have tested such starters with the Schützenbach method. Therefore, the aim of this study was to produce blueberry vinegar by the Schützenbach method using spontaneous acetification and using inoculation with an *Acetobacter cerevisiae* strain that was previously isolated from grapes (Prieto et al, 1997).

2. Materials and methods

2.1. Acetification conditions

Spontaneous and inoculated highbush blueberry wine acetifications were performed in triplicate and monitored daily for acetic acid production. The strain used as a starter was isolated from grapes from the northern Chilean valleys (Prieto 2007) and was selected because of its ethanol resistance and level of acetic acid production. This strain belongs to the *Acetobacter cerevisiae* species (Ac0) and was inoculated at a concentration of $1x10^8$ cell/mL. The inoculum was prepared by growing the pure culture of this strain on glucose medium (GY) (10% glucose, 1% yeast extract, 1.5% agar). Cells were recovered by centrifugation (5 min, 10.000 rpm) and used to inoculate blueberry wine.

The six acetification processes were carried out using the Schützenbach method under laboratory conditions. French oak shavings (1 g/L), serving as a bacterial support material, and 5 L of blueberry wine (7 % (v/v) ethanol and 0.6% (w/v) acetic acid) were used to carry out the acetification processes. A system of PVC pipes of 4 cm in diameter with a submersible 300 L/h pump was designed to move the acetifying liquid through the bed of oak shavings arranged in two chambers at different heights. The acetification temperature was controlled at 23ºC.

The following samples were taken during acetification for microbiological analysis: initial mixture (T0); mid-acetification (when the ethanol was half consumed); and final acetification (when the ethanol concentration had fallen below 1% (v/v)). Samples of the vinegar mother and wines were also analyzed.

The titratable acidity was determined by titration with 0.1 N NaOH and phenolphthalein as the indicator (Ough and Amerine, 1987). The levels of ethanol and residual sugars (glucose and fructose) were measured with enzymatic kits (Boehringer, Mannheim, Germany).

2.2. AAB isolation and molecular analysis

Bacteria were counted by light microscopy using an improved Neubauer counting chamber (0.0025 mm^2 and 0.02 mm deep) and plated at an adequate dilution on GY agar (GY medium with 1.5% agar) supplemented with natamycin (100 mg/L) (Delvocid, DSM; Delft; The Netherlands) to suppress fungal growth. After incubation at 28ºC for 3-5 days, between ten and fifteen colonies were randomly isolated at each point and plated on GYC (GY agar medium supplemented with 2% $CaCO_3$). Each bacterial colony that produced a clear halo on GYC was subjected to a catalase test, and the positive colonies were considered putative AAB isolates and analyzed by molecular methods.

Total DNA was extracted by the modified CTAB (cetyltrimethylammonium bromide) method described by Ausubel et al. (1992).

AAB genotyping was carried out using ERIC-PCR (González et al., 2004) and $(GTG)_5$-PCR (DeVuyst et al., 2008). In both cases, the molecular profiles were determined by both electrophoresis on 1.5% (w/v) agarose and analysis using an Agilent 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany) with the 7500 Labchip and 12000 Labchip kits (Panaro et al., 2000).

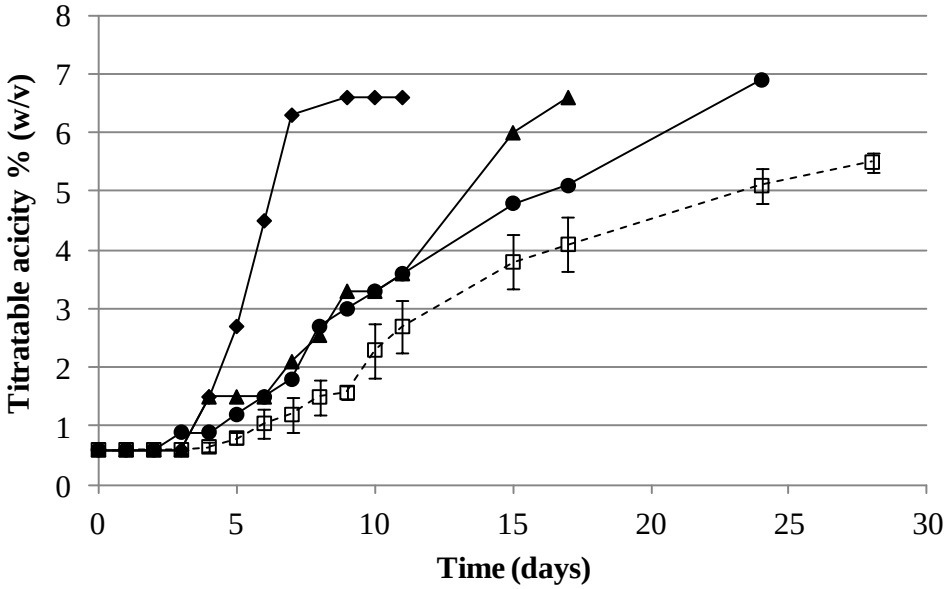
AAB identification was performed by amplifying and sequencing the 16S rRNA gene (Ruiz et al. 2000). The PCR products were purified and sequenced by Macrogen Inc. (Seoul, South Korea). The DNA sequences were compared with those in the GenBank database. Identity was established by 100% sequence homology with available sequences in all the cases.

3. Results and discussion

3.1. Acetification kinetics

The kinetics of the spontaneous and inoculated processes were followed by measuring the increase in acidity over time (Figure 1). The blueberry wine had an initial acetic acid concentration of 0.6% (w/v) and a pH of 3.15. During vinegar production, no variation in the pH was observed. The three spontaneous acetifications had similar kinetics, reaching maximum acidity (5.5 % (w/v)) after 28 days. However, the three samples inoculated with *A. cerevisiae* reached higher acidity values (6.6 to 6.9% (w/v)), and the highest acidity levels were reached sooner than the highest levels for the spontaneous process, but the exact time needed to reach the highest level of acidity varied among the three samples (9, 17 and 24 days).

Figure 1. Kinetics of acetification represented by the titratable acidity. Average of spontaneous acetifications (--□--) and individual inoculated acetification (replicates 1, •; 2, ▲; and 3, ♦).



In this study, the Schützenbach method was used successfully to produce blueberry vinegar. This system was designed to achieve acetification rates that are faster than those obtained with traditional methods (Laguno and Polo, 2001). The elaboration of vinegar from grape wine or from other fruit wines using traditional methods requires more than 30 days for the complete process (Vegas et al., 2010; Hidalgo et al., 2010pro; Hidalgo et al., 2012). Therefore, the Schützenbach method can be used to produce fruit vinegars, such as blueberry vinegar, in a shorter time than that required for the traditional method.

3.2. AAB enumeration

The bacteria were counted by microscopy and plating (Table 1). The initial population size determined by microscopy showed that the cell population in spontaneous acetification samples was approximately 10^6 cells/mL and increased by one order of magnitude by the end of the process. In contrast, the number of bacteria in the inoculated acetification samples remained constant at 10^8 cells/mL throughout the entire process. The level of plate recovery, however, decreased to the order of 10^4 CFU/mL for both acetification conditions. Poor AAB recovery on plates relative to the enumeration by microscopy during vinegar elaboration has been previously reported (Entani et al., 1985; Ilabaca et al., 2008; Sievers et al., 1992; Sokollek et al., 1998; Trcek, 2005). In this study, this effect was observed, especially at the end of processes when the acetic acid concentration was higher. Different explanations have been proposed for the limited AAB recovery on plates: (i) the high acetic acid concentration of vinegar, resulting in a medium that is considered extreme (Sievers et al., 1992; Sokollek et al., 1998; Trcek, 2005); (ii) the formation of bacterial aggregates that allows the bacteria to survive in this medium, which could complicate bacterial growth on culture plates (Ilabaca et al., 2008) and (iii) the possible entrance of AAB into a viable

but non-culturable state due to the adverse conditions (Millet & Lonvaud-Funel, 2000; Mesa et al., 2003; Baena-Ruano et al., 2006). Despite all these drawbacks, the completion of ecological studies requires methods based on culture-dependent techniques to characterize the genetic variation of AAB; therefore, the culture step cannot be bypassed at this time. In addition, having pure cultures of different AAB strains is essential to perform an in-depth study to determine their possible technological potential.

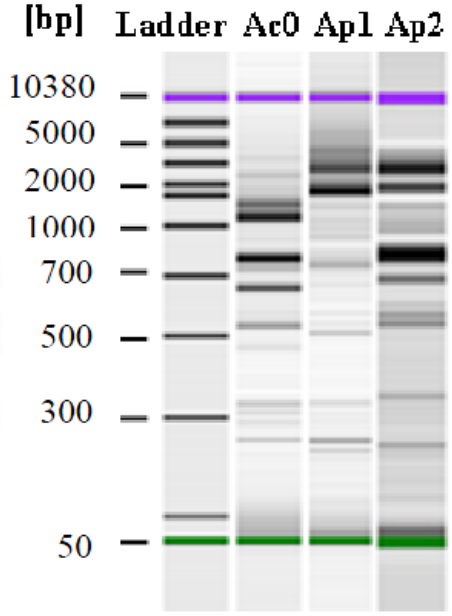
Table 1. Enumeration of AAB by plating and microscopy.

Type of process	Samples	Cell/mL	CFU/mL
Spontaneous acetification	Initial	8,00E+06	2,50E+06
	Mid	1,42E+07	4,57E+06
	Final	7,64E+07	1,83E+04
Inoculated acetification	Initial	3,68E+08	1,30E+08
	Mid	3,39E+08	3,60E+06
	Final	4,89E+08	8,70E+04

3.3. AAB typing and species identification

Among AAB typing methods, the analysis of highly conserved repetitive DNA elements, such as $(GTG)_5$ or Enterobacterial Repetitive Intergenic Consensus (ERIC) sequences, has been described as appropriate to study the genetic variation of AAB (Nanda De Vuyst). In this study, both methods (ERIC-PCR and $(GTG)_5$-PCR) were used, and the results obtained were identical. Three different genotypes were detected (Figure 2), one of which was the inoculated genotype (Ac0).

Figure 2. Different profiles isolated from highbush blueberries vinegar process.



Several isolates of each genotype were analyzed to identify the bacteria at species level by RFLP-PCR based on the 16S rRNA gene and by sequencing. The genotype Ac0 was correctly identified as the expected species, *A. cerevisiae*, and the other two genotypes (Ap1 and Ap2) were identified as *A. pasteurianus*. Because these species are closely related to *Acetobacter malorum* and *Acetobacter pomorum*, respectively, further identification by sequencing the 16S-23S ITS rRNA gene region was performed.

The presence of these genotypes is detailed in Table 2. In the spontaneous processes, the only species detected was *A. pasteurianus*. The profile Ap1 was the sole genotype identified at the beginning of the acetification processes and was replaced by Ap2, which became the predominant genotype in the middle and end of the process. The three inoculated acetifications did not have the same microbial population. The fastest acetification (9 days) was carried out by the inoculated *A. cerevisiae* strain (Ac0), which accounted for 100% of the identified bacteria. However, in the other two acetifications, although the inoculated genotype was also the primary genotype detected, the two *A. pasteurianus* genotypes (Ap1 and Ap2) found in the spontaneous process samples were

also isolated. The persistence/appearance of these *A. pasteurianus* genotypes in the inoculated samples suggests that these strains are better adapted to the composition of blueberry wine. The inoculated strain (Ac0) belonged to *A. cerevisiae*, which was the only species isolated in the original ecological study on grapes cultivated north of parallel 35 (latitude S) (Prieto et al., 2007). Among all of the strains isolated, Ac0 was selected for its good performance during the ethanol resistance test and the acetic acid production test (data not shown). Nevertheless, the two genotypes of *A. pasteurianus*, which were present in most of the processes and took over the spontaneous acetification samples, could also be good candidates for starter cultures.

Table 2. Isolation, identification and typing of AAB during acetification.

	Samples	Replicate	Number of profiles	Species (%)	GTG_5/ ERIC profiles (%)
Spontaneous Acetification	Initial	1	1	*A. pasteurianus* (100%)	Ap1 (100%)
	Mid	Triplicate	1	*A. pasteurianus* (100%)	Ap2 (100%)
			1		
			1		
	Final	1	2		Ap2 (60%), Ap1 (40%)
		2	1	*A. pasteurianus* (100%)	Ap2 (100%)
		3	2		Ap2 (82%), Ap1 (18%)
Inoculated Acetification	Initial	1	1	*A. cerevisiae* (100%)	Ac0 (100%)
	Mid	1	1	*A. cerevisiae* (100%),	Ac0 (100%),
		2	2	*A. cerevisiae* (80%), *A. pasteurianus* (20%)	Ac0 (80%), Ap1 (20%),
		3	3	*A. cerevisiae* (82%), *A. pasteurianus* (18%)	Ac0 (82%), Ap1 (9%), Ap2 (9%)
	Final	1	1	*A. cerevisiae* (100%),	Ac0 (100 %),
		2	3	*A. cerevisiae* (25%), *A. pasteurianus* (75%)	Ac0 (25%), Ap1 (42%), Ap2 (33%)
		3	2	*A. cerevisiae* (58%), *A. pasteurianus* (42%)	Ac0 (58%), Ap2 (42%)

Furthermore, *A. pasteurianus*, which has always been linked to traditional wine vinegar production, could be suitable for use in alternative vinegar production methods such as the Schützenbach method.

The presence of strains other than the inoculated strain and the eventual dominance of these strains at the end of the acetifications was recently reported in two studies of AAB inoculation carried out using traditional methods to produce Traditional Balsamic Vinegar (Gullo et al., 2009) and wine vinegar (Hidalgo et al., 2010). In these studies, *A. pasteurianus* was used as the starter, and although this species was not the dominant one at the end of the process, inoculation with this species clearly improved the vinegar production process.

For the first time, blueberry vinegar produced by the Schützenbach method based on spontaneous acetification and inoculation of an *A. cerevisiae* strain was studied. Although the spontaneous acetifications finished within an acceptable length of time, the use of the AAB starter culture resulted in a reduction in the time required of up to 66%. The identities and genetic variability of the AAB strains involved in the acetification processes were determined, and two genotypes of *A. pasteurianus* were isolated from both types of acetification. These results suggest that these *A. pasteurianus* genotypes can be used as starter cultures in the production of blueberry vinegar or in studies of other vinegar production methods.

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Chapter 6

Evaluation and optimisation of bacterial genomic DNA extraction for no-culture techniques applied to vinegars

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Abstract

Direct genomic DNA extraction from vinegars was set up and suitability for PCR assays performed by PCR/DGGE and sequencing of 16S rRNA gene. The method was tested on 12 intermediary products of special vinegars, fruit vinegars and condiments produced from different raw materials and procedures. DNAs extraction was performed on pellets by chemical, enzymatic, resin mediated methods and their modifications. Suitable yield and DNA purity were obtained by modification of a method based on the use of PVP/CTAB to remove polyphenolic components and esopolysaccharides. By sequencing of bands from DGGE gel, *Ga. europaeus*, *A. malorum/cerevisiae* and *A. orleanensis* were detected as main species in samples having more than 4% of acetic acid content. From samples having no acetic acid content, sequences retrieved from excised bands revealed high similarity with *prokaryotes* with no function on vinegar fermentation: *Burkholderia spp, Cupriavidus spp*., *Lactococcus lactis* and *Leuconostoc mesenteroides*. The method was suitable to be applied for no-culture study of vinegars containing polyphenols and esopolysaccharides allowing a more complete assessment of vinegar bacteria.

Keywords: gDNA extraction, Acetic acid bacteria, Vinegar, CTAB/PVP, PCR/DGGE

1. Introduction

Vinegars are products obtained by the biological process of double biotransformation, alcoholic and acetous, from liquids or other substances of agricultural origin. Common raw materials to make it are cider, wine, cereals and fruits (fresh and dried). Closed to vinegar a large category includes condiments (sauces, powders, seasonings and other) generally at lower acidity respect to vinegars. Main features of all these products are the occurrence of polyphenols components, low pH, the presence of metabolites deriving from biological processes such as alcoholic fermentation and acetification and in some cases compounds deriving from cooking and ageing processes. In recent years interest on the microbiological aspects and function of acetic acid bacteria (AAB) responsible for acetification processes has arisen. A number of studies dealing with species diversity by culture and no-culture methods as well as others on the functionality of AAB and their mechanisms of resistance to vinegar environment have been published (Gullo et al., 2006; De Vero et al., 2006; Ilabaca et al., 2008; Gullo and Giudici, 2008, Wu et al., 2010; Torija et al., 2010; Hidalgo et al., 2010; Kanchanarach et al., 2010). Moreover, particularly attention has been devoted to the understanding of viable but non-culturable state of AAB. Basic requirement to apply no-culture methods is the availability of a standardised and efficient genomic DNA (gDNA) extraction method. So-called matrix effects, food processing and reagent/approaches used to extract gDNA are documented as sources of the following biases: low yield, no suitable purity, degradation, altered information on diversity richness by lost of representative member and/or preferential amplification. In vinegars pitfalls arise mainly from esopolysaccharides produced by AAB and polyphenols components. What is more, special vinegar and condiments contain high percentages of carbohydrates that undergo different modifications during cooking and ageing other than enzymatic transformations leading the formation of

sugar-derived condensation products that can co-precipitate with DNA and interfering with enzymatic reactions performed for DNA analysis (Di Bernardo et al., 2005; Gryson, 2010).

The aim of this work was to set up and evaluated gDNA extraction from special vinegars, condiments and fruit vinegars suitable for no-culture-PCR-based applications.

2. Materials and methods

2.1. Bacterial reference strains

The following type and reference strains were used: *Acetobacter (A.) pasteurianus* DSM 3509^T; *A. malorum* DSM 14337^T; *Gluconobacter (G.) oxydans* DSM 2003; *Gluconacetobacter (Ga.) xylinus* DSM 6513^T; *Ga. hansenii* DSM 5602^T; *Ga. liquefaciens* DSM 5603^T. To have fresh cultures, aliquots of -80°C glycerol stock cultures were inoculated on GY broth (glucose 1%, yeast extract 1%) and incubated aerobically at 28 °C for 3-5 days.

2.2. Samples

Samples were intermediary products of special vinegars and condiments collected from an Italian factory and fruit vinegars collected from lab-scale processes in Spain (Table 1). Each sample was divided into aliquots such that DNA could be isolated from replicates, stored at 4°C and then processed. Direct observation to optical microscopy (100X) was done using C. Zeiss microscope apparatus (Axiolab). The following chemical physical parameters were determined: pH (CRISON, MicropH, 2002); acetic acid % (wt/wt; neutralizing samples at pH 7.2 with 0.1 N NaOH; it was assumed that all media acidity was due to acetic acid); ethanol expressed as % (v/v) by densimetry measure using a hydrostatic balance after distillation.

Table1. Samples used for gDNA extraction.

Intermediary product of	Description	Code	pH	Ethanol % (v/v)	Acetic acid % (wt/wt)
Special vinegar	Acetifying grape cooked must for *TBV	TBV1	2.69	0.21	6.65
		TBV2	2.92	1.48	5.28
Condiment	Acetifying grape cooked must for condiments	BV1	2.98	1.14	4.68
		BV2	3.04	1.56	5.09
		BV3	3.11	2.04	4.70
		BV4	2.99	0.87	5.85
Fruit vinegar	Strawberry fruit	SF	3.04	0.00	0.60
	Fermented strawberry juice	SW	3.21	6.52	0.90
	Acetifying strawberry juice	SV	3.10	0.22	4.95
	Persimmon fruit	PF	5.50	0.00	0.60
	Fermented persimmon juice	PW	3.88	6.50	0.60
	Acetifying persimmon juice	PV	3.46	0.90	4.55

*Traditional balsamic vinegar of Reggio Emilia (Regulation (EC) N. 813/2000)

2.3. Genomic DNA extraction

Genomic DNA from AAB reference strains was extracted by sodium dodecylsulfate (SDS) proteinase-cethyltrimethyl ammonium bromide (CTAB) treatment as previously reported (Gullo et al., 2006). To extract gDNA from samples, aliquots (0.5 and 1 ml for fruit vinegar and from 6 to 15 ml for special vinegar and condiments) were washed and cells were harvested by centrifugation (2500 *xg*, 20 min, 4°C). The extractions were performed on pellets by chemical, enzymatic, resin mediated methods and their modifications as reported in Table 2. Protocol of method 6 (method 5 modified) is full reported as Supplementary material (S1).

DNA was visualised by electrophoresis on agarose gel (1%) by ethidium–bromide staining under UV light and quantified by spectrophotometric measure (NanoDrop ND-1000). 260/280 nm absorption ratio between 1.7 and 2.0 was considered to assess purity of DNA.

2.4. 16S rRNA PCR/DGGE (Denaturing Gradient Gel Electrophoresis)

The quality of gDNA extracted using the different methods was checked by PCR amplification of the region V7 to V8 of the 16S rRNA gene. For this assay, aliquots (1 µl) of diluted (1:10 and 1:100) and no diluted gDNAs were amplified using the primers WBAC1 (5'-GTCGTCAGCTCGTGTCGTGAGA-3'; nt 1069–1090) with GC-clamp and WBAC2 (5'-CCCGGGAACGTATTCACCGCG-3'; nt 1374-1394) according to PCR conditions as previously described (Lopez et al., 2003; De Vero et al., 2006). DGGE of PCR products was performed on an 8% (w/v) polyacrylamide gel with urea and formamide as denaturants. The denaturing gradient was between 40% and 60%. Electrophoresis was performed in 1X Tris-acetate EDTA (TAE) buffer at 60 °C at constant voltage of 200 V for 4 h. Subsequently, gel was stained with ethidium bromide (50 µg/ml) in 250 ml of 1X TAE buffer for 15 minutes. After, the gel was destained with 250 ml of 1X TAE buffer for 20 minutes and photographed by BioDocAnalyze (BDA; Germany).

2.5. 16S rRNA gene sequencing

Individual PCR/DGGE bands were cut out from gel and incubated overnight at 4 °C in 30 µl dH_2O. An aliquot (1 µl) was used in a PCR with the same primer set used for DGGE but without the GC-clamp attached to the WBAC1 primer. Amplified products were purified using the kit DNA Clean & Concentrator™-5 (ZymoResearch) and automated sequenced (Eurofins MWG Operon service, Germany). Sequences contigs were assembled using CHROMASPro (Version 1.41), and similarities searched using BLAST program (Zhang et al., 2000). The nucleotide sequences matching significant similarity with rDNA of AAB were deposited into EMBL databases under the accession numbers reported in Table 4.

Table 2. Extraction methods and suitability of gDNA for PCR assay.

Method	Approach/Principle	Sample	ng/µl	$OD_{260/280}$	16S rRNA gene amplification
1. (SDS/Triton)	Lysis by chemical and enzymes (Lysozime, proteinase K and SDS/Triton) Alcohol isoamilic:cloroform extraction DNA precipitation with 2 vol of absolute ethanol	TBV1	175± 27	1.02 ± 0.01	-
		SV	1816±49	1.55±0.29	-
2. (Phenol-chloroform)	Lysis by chemical and enzymes (SDS, lysozime, proteinase K) Phenol:chloroform extraction DNA precipitation with 2 vol of absolute ethanol with 0.1 volume of 3 M sodium acetate	TBV1	1793 ±888	1.36 ± 0.27	-
		SV	161±17	1.32±0.08	-
3. (Chelex, Sigma Corp.)	Lysis by heating at 95ºC for 20 min Use of chelating resin	TBV1	321±248	1.29±0.07	-
		SV	ND	ND	-
4. (PVP-CTAB)	Lysis by chemicals and enzymes (SDS, lysozime and proteinase K) CTAB to remove polysaccharides; Phenols absorption (*PVP) Alcohol isoamilic:cloroform extraction DNA precipitation with 2 vol of absolute ethanol	TBV1	25±13	1.09±0.06	-
		SV	59±6	0.76±0.06	-
5. (CTAB-Poresbki et al., 1997)	Lysis by chemicals and heating (ß-mercaptoethanol and 60ºC for 60 min); High salt concentration to remove polysaccharides; PVP to remove polyphenols Alcohol isoamilic:cloroform extraction DNA precipitation with 2 vol of absolute ethanol	TBV1	89±37	1.53±0.15	+
		SV	716±118	1.70±0.01	+
6. (CTAB-Poresbki et al., 1997 modified)	Modifications respect to method 5: An additional washing step with saline EDTA/PVP for special vinegar. Reduced incubation times	TBV1	157±40	1.77±0.09	+
		SV	113±30	1.76±0.06	+

ND, not detected.
*PVP: Polyvinyl-pyrrolidone

3. Results

3.1. Samples: collection and analysis

For special vinegars and condiments, collection of samples was done from the superficial part of barrels and was constituted of liquid and esopolysaccharides fraction; and for fruit vinegars, homogenised samples of mashed fruit, fermented and acetifying juice were collected from the glass containers. Optical microscopy observation of fresh samples showed high number of free cells as well as aggregates of cells within the matrix, making cell counting questionable and not informative. Intermediary products of special vinegar and condiments had acetic acid content ranging from 4.68 and 6.65%, pH within 2.69 and 3.11 and ethanol ranging from 0.21 to 2.04%. Fruits and fermented juices showed no significant acetic acid content and pH values in the range of 3.04 and 5.50. Ethanol was no detected in strawberry and persimmon fruits, whereas in fermented juices it was around 6.50%. Both acetifying juices showed similar acetic acid content (4.55-4.95%) and pH (3.10-3.46) (Table 1).

3.2. Genomic DNA recovery: sample preparation and extraction

The six different methods were tested on duplicates of TBV1 and SV samples. Using methods 1, 2, 3 and 4, gDNA was recovered with yields ranging from 25±13 to 1793±888 ng/µl and $OD_{260/280}$ between 1.02±0.01 and 1.36±0.27 for sample TBV1, whereas concentration between 59±6 and 1816±49 ng/µl and $OD_{260/280}$ in the range of 0.76±0.06 and 1.55±0.29 were obtained for SV. However no amplicons of 16S rRNA gene where produced when diluted and undiluted gDNAs were used as template. By method 5, yields of 89±37 and 716±118 ng/µl were obtained and $OD_{260/280}$ of 1.53±0.15 and 1.70±0.01 for TBV1 and SV, respectively. Moreover undiluted gDNA was suitable for 16S rRNA gene amplification producing amplicons of ca. 300 bp.

Method 6 was set up from the following modifications of method 5: after adding cold ethanol, precipitation was done at -20°C for 10 minutes instead of overnight; drying step at 37°C was reduced to 30 minutes instead of 1h, then pellet was resuspended in TE buffer and RNAse added without overnight incubation (See Supplementary material S1). Method 6 tested on samples TBV1 and SV produced yields of 157±40 and 113±30 ng/µl respectively and $ODs_{260/280}$, higher than those obtained using all the other methods. Furthermore, undiluted gDNAs obtained by this method was amplified with primers WBAC1/WBAC2 allowing to obtain a single PCR product with the expected size (about 300 bp) (Table 2). Therefore, it was used to recover gDNA from the remaining samples. Genomic DNA was obtained from all the samples with yields and $OD_{260/280}$ values suitable for 16SrRNA amplification (Table 3).

Table 3. Genomic DNA recovered from intermediary products of special vinegars, condiments and fruit vinegars using method 6.

Sample	**ng/µl**	**$OD_{260/280}$**
TBV2	43±35	1.75±0.15
BV1	162±39	1.81±0.02
BV2	89±10	1.91±0.05
BV3	481±15	1.93±0.01
BV4	30±20	1.77±0.00
SF	89±48	1.82±0.09
SW	122±97	1.86±0.16
PF	87±16	1.70±0.01
PW	84±39	1.78±0.08
PV	105±58	1.74±0.05

Table 4. Sequences of 16S rRNA gene fragments obtained from PCR/DGGE bands with WBAC primer-pairs.

Sample	DGGE Band	Size (bp)	*16S rRNA sequence accession number	Closest hit (Species name accession number)	Percentage (%) similarity
TBV1	W	295	FR832712	*A. malorum* (NR025513.1) *A. cerevisiae* (NR 025512.1)	100
SV	C	332	FR832719	*A. orleanensis* (NR_028614)	100
TBV1	T	272	FR832713	*A. malorum* (NR_025513.1) *A cerevisiae* NR_025512.1	100
TBV2	K	330	FR832714	*A. malorum* (NR025513.1) *A. cerevisiae* (NR 025512.1)	99
BV1	A	330	FR832715	*Ga. europaeus* (NR_026513.1)	100
BV2	D	329	FR832716	*Ga. europaeus* (NR_026513.1)	100
BV3	F	331	FR832717	*Ga. europaeus* (NR_026513.1)	100
BV4	H	332	FR832718	*Ga. europaeus* (NR_026513.1)	100

*Sequences matching AAB species (this study).

3.3. 16S rRNA PCR/DGGE and analysis

PCR/DGGE was performed on gDNA of reference AAB strains and gDNA from samples extracted with method 6. Single migration pattern was obtain for each amplified product of reference AAB strains (Fig. 1; lanes 1 to 6) and complex pattern for the mix of references strains amplicons loaded together for which each band comigrated with the bands of the respective single amplicon (Fig. 1; Lane 7). All condiments analysed showed a similar migration pattern with a comigrant intense band (Fig. 1; lanes 13 to 16, bands A, D, F, H). Analysis of bands sequences showed that PCR fragments have 100% of similarity with *Ga. europaeus* species (Accession number NR_026513.1) (Table 4). Special vinegars showed a similar profile but different from those of condiments. In particular bands K, W, T (Fig. 1 lanes 9 to 11, respectively) comigrated. Sequences analysis revealed high percentage of similarity (99 and 100%) with the phylogenetically very closely related species *A. malorum/A. cerevisiae* (accession number NR_025513.1 and NR_025512.1). PCR/DGGE of gDNA from fruit vinegars revealed complex patterns both for strawberry and persimmon samples. Fermented and acetifying strawberry samples showed similar profiles but different from those of persimmon samples. Band C (Fig. 2; Lane 2) from acetifying strawberry juice had 100% of similarity with *A. orleanensis* (NR_028614).

Other than bands whose sequences matched AAB species and that were deposited on EMBL database (Table 4), other sequences retrieved from excised bands revealed high similarity with other prokaryotic species that have no function on vinegar fermentation such as *Burkholderia* and *Cupriavidus* spp. In particular, for special vinegars, band Z (Fig. 1; Lane 9) and band O (Fig. 1; Lane 12) showed high similarity (99%) with *Cupriavidus* (C.) *spp*, (*C. taiwanensis* NR 028800.1; *C. oxalaticus* NR 025018.1; *C. pauculus* NR 024944.1), and band N (Fig. 1; lane 12) 98% of similarity with

Burkholderia (*B.*) *spp.* (*B. fungorum* NR 025058.1; *B. phenazinium* NR 029212.1; *B. caledonica* NR 025057.1). From gDNA of condiments no detectable bands for sequences belonging to bacteria different from AAB were found. In intermediary products of fruit vinegars, *Burkholderia* and *Cupriavidus spp.* were also detected (Fig. 2; Lane 2 bands A and B). Moreover in fermented persimmon juice (PW) no AAB were detected but only sequences matching with lactic acid bacteria sequences; sequence of band E had 100% of similarity with *Lactococcus lactis* (NC_013656.1) (Fig. 2; Lane 5) and of band D (Fig. 2; Lane 5) 99% with *Leuconostoc mesenteroides* (HM443957.1).

Figure 1. PCR/DGGE showing 16SrRNA amplified gene from gDNA of special vinegars and condiments. 1: DSM 5603 *Ga. liquefaciens;* 2: DSM 14337 ^{T} *A. malorum;* 3: DSM 5602 ^{T} *Ga. hansenii;* 4: DSM 6513^{T} *Ga. xylinus;* 5: DSM 2003 *G. oxydans*; 6: DSM 3509 ^{T} *A. pasteurianus;* 7: Mix of type and reference strains; 8: *TBV2; 9: *TBV2; 10: * TBV1; 11: *TBV1; 12: *TBV1; 13: BV4; 14: BV3; 15: BV2; 16: BV1.

* Replicates.

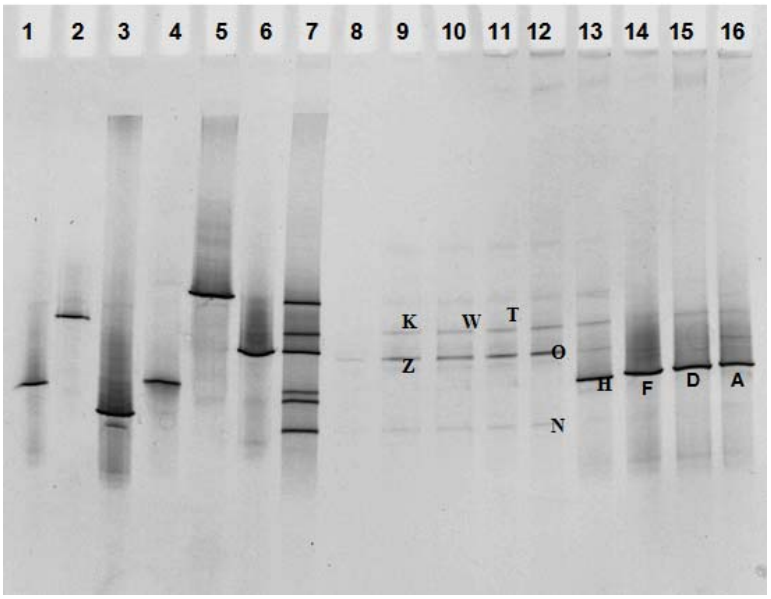
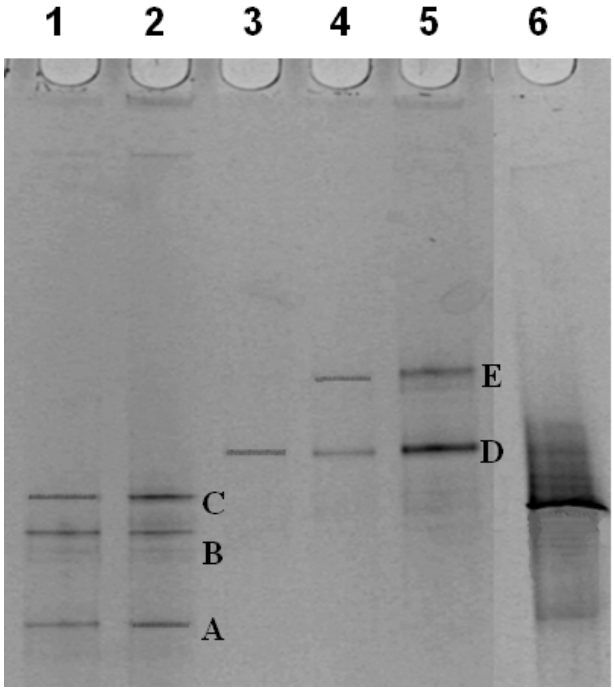


Figure 2. PCR/DGGE showing 16SrRNA amplified gene from gDNA of fruit vinegars. 1: Fermented strawberry juice (SW)*;* 2: acetifying strawberry juice (SV)*;* 3: persimmon fruit (PF)*;* 4: fermented persimmon juice (PW)*;* 5: fermented persimmon juice (PW); 6: DSM 3509 ^{T} *A. pasteurianus.*



4. Discussion

Understanding the microbial composition of vinegars is one of the most important goals for the improvement of processes management and for the evaluation of potential microbiota with impact in other biotechnological applications. No-culture-based methods can be used to obtain information on the taxonomic composition and relative abundance of vinegar's bacteria. The most critical step through out these methods is recovering representative and high quality gDNA. The extraction method can affect the quantity and quality of DNA, as well as the detectable diversity and, therefore, choosing the most suitable method is crucial. So far, gDNA extraction procedures for no culture-based methods applied to fermented and acidic beverages have been proposed for wine

(Millet and Lonvaud Funel, 2000), balsamic vinegars (De Vero et al., 2006), wine and wine vinegar (Jara et al., 2008). By these studies AAB and lactic acid bacteria were only documented.

In order to recover representative gDNA, cells need to be accessible for efficient lyses and accordingly protocols need to be adapted. One of the main drawbacks in vinegar is due to the presence of polyphenols from raw material and the unpredictable amount of esopolysaccharides deriving from AAB metabolism. Both factors affect the standardisation of classical extraction protocols and the applicability of the majority of kits based methods. Actually, Power Soil (MoBio), Wizard (Promega), Nucleospin (Clontech) and generation capture column kit (Qiagen) isolation kits did not allow to obtain suitable gDNA when tested for fruits, balsamic vinegars and condiments (data not shown). In our study, microscopic observation of samples revealed free and entrapped cells within the matrix. Temperature, cooking, pH, fermentation, drying, enzymatic degradation are recognised as factors interfering with DNA efficiency and PCR biases. In this respect, other than for vinegars (Jara et al., 2008), recovery of suitable gDNA from fermented products like miso, sufu, tofu, soybeans and cocoa beans has been reported to be affected by the length of fermentation time (Gryson, 2010; Garcia-Armisen et al., 2010). Samples of this study were acidic products for which the duration of the fermentation steps in wood barrels and glass containers could be very long (from few to several months), and some of them contained relevant amount of cooked grape juice. Although, the acetic fermentation of special vinegar and condiment occurs after the cooking step, releasing of gDNA entrapped within sugar-derived condensation products leading to main biases (Di Bernardo et al., 2005). Therefore, methods combining physical and chemical approaches were tested. Large quantitative differences in the extraction efficiency, up to one order of magnitude, were

observed. By methods 1 and 2 the highest gDNA yield was obtained for fruit and special vinegar, respectively. However, the gDNA was not suitable for PCR assays. Method 3, based on chelating resin, was not successful in term of yield and quality of the obtained gDNA. The PVP/CTAB method (Method 4) is usually applied for the extraction of gDNA from polysaccharides matrices, but surprisingly gave the lowest recoveries. Recently, a similar method has been successfully used to extract gDNA from AAB strains inoculated in wine and vinegar (Jara et al., 2008). The inconsistency between the two studies could be due to the different composition and structure of a true fermenting substrate and one on which bacteria are artificially added. In the latter case, the influence of the matrix is not persistent comparing to a long fermented product on which bacteria grow strictly within the matrix. Hence, methods from 1 to 4 were not suitable for the extraction of gDNA from special and fruit vinegars, both for the low recovery and the occurrence of some major components such as polyphenols and polysaccharides (and its derivatives), that co extracted with gDNA and acted as inhibitors of PCR reactions.

Method 5, by which suitable gDNA was obtained, originally, was set up to extract DNA from strawberry leafs, which contained large quantities of polyphenols, tannins and polysaccharides (Porebski et al., 1997). Key steps of this method are the use of high concentration of salts to remove polysaccharides and of PVP to remove polyphenols. In addition, an extended RNase treatment and a phenol-chloroform extraction to improve the gDNA purification. The lysis buffer containing β-mercaptoethanol, CTAB and PVP seemed to be more efficient than the lysis procedures of the other methods. The β-mercaptoethanol acts as a strong reductant by breaking intramolecular disulphide bonds in proteins and prevents oxidation of polyphenols and oxidative damages of nucleic acids (Herzer, 2001; Sreelakshmi et al., 2010), while CTAB, a cationic surfactant, is

added to solubilize the membranes, remove capsular polysaccharides and form a complex with DNA (Spitzer and Spitzer, 1992; Zidani et al., 2005). Furthermore, the additional purification steps after precipitation improved the gDNA quality recovered. However main drawbacks applying method 5 were the high number of manipulation steps that increase the risk of contamination, degradation and lost of representative richness as well as the long work time required to obtain gDNA. Since an extraction method should be as much as simple, quick and efficient, we modified method 5 as reported in supplementary material S1. The optimised method allowed the combination of high purity and quality of the gDNA and the reduction of extraction time. To find out if gDNA recovered by our method was suitable for no-culture methods, the 16S rRNA gene region was examined by PCR/DGGE, a technique commonly used in microbial ecology to determine the genetic diversity of complex microbial populations (Muyzer et al., 1993). Bands from 272 to 332 bp showing identity of 99 and 100% with vinegar related AAB were recovered from the acetifying products showing acetic acid content more than 4.0%. *Ga. europaeus* was found in intermediary products for condiments, whereas *A. malorum/A. cerevisiae* in special vinegars and *A. orleanensis* in acetifying strawberry juice. These species are associated to vinegar fermentations; *Ga. europaeus* is the main AAB species of industrial vinegars (Sievers et al., 1992) and its strains have been detected as indigenous bacteria of superficial vinegar productions too (Vegas et al., 2010; Gullo et al., 2009). Peculiar phenotypic characteristic of this species is the requirement for acetic acid, the low cultivability as well as the greater ability and stability of ADH (alcohol dehydrogenase) in acetate media (Trcek et al., 2007). *A. malorum* species has been previously detected in fruits and at the end of fermentation of pulque, whereas *A. cerevisiae* in beer (Cleenwerck et al., 2002; Escalante et al., 2008).

Strains of *A. orleanensis* species occur in the biofilm of rice vinegar, palm wine, nata de coco and beer (Lisdiyanti et al., 2001; Cleenwerck et al., 2002).

By PCR/DGGE we also detected groups of bacteria with no obvious functionality in vinegars. They included the plant pathogens and soil bacteria *Burkholderia* and *Cupriavidus spp* (Vandamme and Coenye, 2004). *Burkholderia spp*. are reported as occuring in water, foods, stream bacterioplankton and clinical speciamen (Palleroni, 2005). Moreover, species of both genera have been detected also in fermented beverages produced from rice (Thanh et al., 2008). *Leuconostoc mesenteroides* is a lactic acid bacterium associated to fermentation processes e.g. sauerkraut and kimchi (Plengvidhya et al., 2007; Jung et al., 2011). *Lactococcus lactis* which important habitats are found in the various niches of the dairy industry environment have also commonly been detected in plant material, including corn, beans, cabbage and fruits (Teuber and Geis, 2006). We presume that these bacteria were introduced along vinegar processes from raw materials (grapes, persimmon and strawberry) and from the wood of barrels on which the processes were performed.

PCR/DGGE as applied in our study, using primers set WBAC1GC-WBAC2, targeting the region V7 to V8 of the 16S rDNA, allowed for the first time to detect bacteria different from AAB in vinegars. These primers set were originally designed to amplify wine bacteria such as lactic and acetic acid bacteria (Lopez et al., 2003). However, when tested in silico (data not show) they show specificity with a great number of targets sequences within the domain *Bacteria* including *Burkholderia* and *Cupriavidus* spp.

In this study a method to extract gDNA from vinegars was evaluated and optimised. The use of PCR/DGGE to test the suitability of gDNA for no-culture applications was successful for the detection of AAB species as well as other bacteria member occurring by contamination of the raw material or as consequences of fermentation processes.

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GENERAL DISCUSSION

The current market trends point to a desire for new products with high added value, with special emphasis on an efficient production and food quality. Vinegar production is not exempt from this current situation, and this product has gone from being considered a byproduct to acquiring some status, which largely due to the wide acceptance of some of vinegars such as sherry vinegar or Traditional Balsamic Vinegar.

The most widely known methods of producing vinegar are the superficial and the submerged methods. As been described in this thesis, superficial methods are those in which the wine acetification is performed statically in wood barrels. Its main advantage is the high quality of the obtained vinegars, and its main disadvantage is the long time of production (Adams, 1998). In contrast, the submerged methods present a high industrialization. Over the years, the common characteristic of this method is the design of different types of acetators to provide a continuous aeration. Although industrial implementation requires a significant initial investment, their main advantage is the high production speed, resulting in a rapid vinegar production. However, the low quality of these vinegars does not allow them to compete with those produced traditionally. In addition to the development of new technologies used in vinegar production, a better knowledge of the microorganisms involved in these bioprocesses and the potential use of some of them as starter cultures to ensure the success of the process could be a good alternative to improve the acetification process.

On the other hand, the study of the use of different raw materials to produce vinegar could expand the market for vinegar. Some of these materials are highly perishable fruits that cannot be stored long term; thus, they should be used very quickly once they have been harvested. This is the case of the fruits used in this work: grape, persimmon, strawberry and highbush blueberry.

Many products, such as juice, jelly, nectar, puree, concentrate, and jams, have been developed to take advantage of these fruits (Barrett et al., 2005; Hui, 2006). However, despite the demand for these products, there remains an excess of fruit crops. A possible alternative is to generate other products, such as vinegars. It is well known that vinegar production consists of two biotransformations; the alcoholic fermentation (AF) and the acetification. Both bioprocesses could be a good choice for food storage systems because of the preserving properties of alcohol or acetic acid.

The hypothesis of this thesis is that it is possible to produce vinegar from any fruit because of the presence of specific microbiota that will take over the process. The best way to study the microbiote that take over this process, is to conduct an ecological study that will reveal the species and strains present during the vinegar production and allow us to select starter cultures to control the process. Therefore, this work was focused on the microbiological analysis and control of the vinegar production process from grapes, persimmons, strawberries and highbush blueberries.

In this study, when persimmon and strawberry were used for the production of vinegar, the process started in the fruit, so the AF must also be performed. Traditionally, our knowledge of the AF is based on the wine (Ribéreau-Gayon et al., 2006) and beer (Hutkins, 2006) industry. However, in those processes the limiting parameters are the lack of equilibrium between the fermentable sugars and the availability of essential compounds (e.g., nitrogen, vitamins or minerals) (Ribéreau-Gayon et al., 2006). In this study, practices used in wine, such as the addition of SO_2, the addition of pectolytic enzymes, and the inoculation of commercial strain yeast, were also applied. However, some limitations were observed due to the characteristics of the raw material. Some of them, such as the sugar concentration or the viscosity, may even block the production of vinegar. The concentration of fermentable sugars is a limiting parameter because it will

determine the level of ethanol obtained and therefore the level of acetic acid. In fact, the level of acetic acid will depend on this parameter as well as how the vinegar is produced and the microorganisms involved in the process (Adams, 1998).

The main chemical parameters related to vinegar production are the sugar and acetic acid content. The minimum level of acetic acid, which is needed to maintain the stability of the product over time, depends on each type of vinegar, in compliance with local standards (Adams, 1998). On the other hand, local standards are related to the way to produce the vinegar. For example, in Spain, the traditional wine vinegar is produced using diluted wine with water (Consejería de Agricultura y Pesca, 1995; Council regulation (EC) No. 813/2000). Conversely, Traditional Balsamic Vinegar is produced by concentrating the sugars by heating, following the "protected denomination of origin" protocol (Council regulation (EC) No. 813/2000). In this work, the sugar concentration of persimmon was much higher than that of strawberry (110 g/l and 28.4 g/l, respectively). However, with the sugar naturally present in persimmon, the product obtained did not achieve 5% (w/v) acidity after the acetification, so the product was considered a fruit condiment and not vinegar (Real Decreto 2070/1993, B.O.E.: 8/12/93).

Regarding the strawberry, the content of sugar was very low, and an increase in sugar concentration was mandatory to be able to produce vinegar. There are three ways to concentrate the juice: evaporation of water, cryoconcentration, and reverse osmosis (Horváth-Kerkai, 2006). In this work, the concentration of strawberry puree was tested in the laboratory by heating and the AF process was performed without problems. However, "stuck" acetifications were observed (data not shown in the thesis). This concentration method involves, together with water loss, the concentration of other compounds. These other compounds can include organic acids, which will result in a

significant decrease in pH that likely produces an extremely acid medium; this could be restrictive for microorganisms. These restrictive conditions delayed the acetification (data not shown in this thesis). The use of commercial strawberry concentrated puree was also assayed. In this case, the high initial acidity was corrected by $CaCO_3$ addition, which allowed the bioprocesses to occur without setbacks. Without this practice (increasing pH by $CaCO_3$ addition), acetifications did not initiate at all, or they acetified a low amount of ethanol (data not shown in this thesis). The easier way to increase the sugar concentration and to avoid the above problems mentioned is the direct addition of sucrose to the fruit pulp. This practice was applied successfully to achieve the expected acidity in all the cases, even in the commercial concentrated puree. Therefore, the only drawback of this practice is that this sugar comes from an exogenous source, and it is not naturally present in the fruit.

As mentioned above, the high viscosity of this raw material could be a problem for the production process. Therefore, the use of pectolytic enzymes is essential to increase the extraction of juice and the processing efficiency (pressing and solid settling) to render an attractive and clear final product (Höhn et al., 2005). The pectolytic enzymes commonly utilized in the wine and fruit industry were applied in this study, and a massive quantity of water and insoluble plant particles were observed throughout the process. This pulp turbidity should be considered because it affects the viscosity and therefore the mobility of the microorganisms into the solution as well as the O_2 available for acetification. These particles should be partially or totally eliminated to avoid the turbidity and precipitation and to improve sensory attributes (smell, taste, and color) (Horváth-Kerkai, 2006). Therefore, the use of pectolytic enzymes results in an increase in the fluidity of the medium, which may favor homogenization and the mobility of microorganisms. Another alternative used in this study to avoid the problems of

viscosity and turbidity of the raw material was to ferment the clear juice obtained after strawberry pressing instead of the mashed pulp. In this case, no disadvantages were observed in the AF process, but the acetification showed poor acetic acid production. A possible explanation is that the microbiota present will vary depending on the fruit processing steps, such as pressing, clarification, and fining (Lozano, 2006). It has been reported that crushing and pressing techniques lead to different yeast populations (Sturm et al., 2006). Regarding our results, the pressing practice seems to have reduced the microbial population (especially the bacterial population) or have removed some essential compounds present in the mash pulp, which could affect the growth of the bacterial community. Therefore, to produce strawberry vinegar, the wine composition needs to be analyzed and appropriate starter cultures need to be used because there is a high risk of unfinished acetifications. Therefore, the use of commercial pectolytic enzymes in the elaboration of fruit juices should be considered to obtain a clearer juice. In addition, testing different doses of these enzymes prior to their use is mandatory.

The fruit surface harbors a diverse range of microorganisms (Kalia and Gupta, 2006), which could be associated both with the fruit and with the area where those fruit were cultivated (Jay et al., 2005). Both yeast and AAB live in different niches and under different conditions. The starters are related to the niches and the better adaptation of the microorganisms to the bioprocess involved (in our case, AF and acetification). The use of selected starters in fermented foods is common to predict and to ensure the quality and reproducibility of the final product (Hammes, 1990; Holzapfel, 1997; Ribéreau-Gayon et al., 2006). The use of selected yeast to carry out the AF process is a very common practice in different food industries, especially after the development of the active dry yeast technology (Degre, 1993). In this study, a commercial *S. cerevisiae* wine strain was used to study the effect of inoculation on strawberry and persimmon

fruit. In both cases, the inoculated AFs proceeded faster than the spontaneous ones. Although we had no problems with the spontaneous AF processes to produce fruit wines, industrialization requires shorter production periods and a repetitive product, which could be obtained by inoculating with selected strains (Fleet and Heard, 1993). Currently there is a clear preference for using starter cultures that have been selected from the environment where they are going to be used because the wild microbiota is supposed to be well adapted to the specific conditions. On the other hand, the study of indigenous fermentation under different conditions has been widely researched in the wine industry (Jolly et al., 2006; Torija et al., 2001; Jemec et al., 2001). Today, there is a renewed interest in this practice among the wine industry because stylistic distinction tempts winemakers to accept the risks involved in these natural fermentations (Fugelsang and Edwards, 2007). In contrast, few studies have been published on other fermented fruits. Some examples include those performed with gabirobas (Duarte et al., 2009), strawberry tree fruit (Cavaco et al., 2007), pineapples (Chanprasartsuk et al., 2010) and apples (Morrissey et al., 2004). However, these studies were mainly focused on the possibility of producing wine from the fruits and some on ecological studies, but none focused on yeast selection.

The ecological studies carried out during spontaneous AF of persimmon showed great microbiological diversity, but strawberry spontaneous AF showed very low diversity. As explained before, the presence of one species or another may be determined by the better adaptation of these microorganisms to the specific conditions, which are related to different parameters, such as pH, nutrient content, and antimicrobial constituents (Jay et al., 2005). In the case of the persimmon, high diversity could be explained by the high pH found in the pulp (pH 5.5). Yeasts and molds are known to grow well when the pH is higher than 4.4 because low pH affects the functioning of respiring microbial

enzymes and the transport of nutrients into the cell (Kalia and Gudpa, 2006). In contrast, the low diversity observed in strawberries could be explained by the presence of compounds with antifungal and antibacterial defensive activities, which have been described for this fruit (Amil-Ruiz et al., 2011). Other external factors, such as fungicides and pesticides used in the growing plants, have to be considered and will have a negative impact on the populations of microorganisms. However, some pesticides have also been reported to stimulate certain yeasts, such as *K. apiculata*, which was tested in laboratory fermentations (Cabras et al., 1999).

Despite the different yeast species identified in both fruits, the AFs were similar. Non-*Saccharomyces* species were present at the beginning of the process, and a clear imposition of *S. cerevisiae* was observed at the end. This behavior has also been described in grape (Fleet and Heard, 1993) and gabiroba (Duarte et al., 2009) wines. However, in fermentations with low final alcohol content, *Saccharomyces* may not even appear (Chanprasartsuk et al., 2010). In both our persimmon and our strawberry studies, one strain of *S. cerevisiae* was predominat at the end of the spontaneous AF processes (Sc1 in persimmon and CECT 13057 in strawberry). However, a total imposition of these strains was not observed because non-*Saccharomyces* species were present until the end of the process, most likely due to the low final ethanol concentration.

Within these non-*Saccharomyces* species, *H. uvarum* is the only species that appeared both in strawberries and persimmons. This species is closely related with wine fermentation, but it also seems to be well adapted to other fruits (Morrissey et al., 2004). In winemaking, *H. uvarum* usually appears at the beginning of AF and disappears very quickly after the initial production of alcohol (Constantí et al., 1998; Torija et al., 2001). However, this disappearance has been only partially confirmed by culture-independent

methods, and the problem with this species appears to be its limited ability to grow on plates (Andorrà et al., 2010).

The yeast inoculation assays were carried out only for the AF of strawberry fruits. Total imposition of the indigenous strain (CECT 13057) isolated from strawberry spontaneous AF was observed, and the process was faster than the spontaneous one. Therefore, this strain could be considered a good starter culture for the strawberry conditions.

Once the persimmon and strawberry wines were obtained, the acetification was carried out by the superficial method. Most of the available information regarding the production of vinegars at microbiological level is related to grape and cereal vinegars (Solieri and Giudici, 2009; Llaguno y Polo, 1991; Haruta et al., 2006; De Vero et al., 2006; Ilabaca et al., 2008; Vegas et al., 2010; Wu et al., 2012). In this work, the acetifications were initially carried out with naturally occurring microorganisms. It allowed us to obtain a more complete understanding of the vinegar production from these fruits.

AAB diversity was higher in persimmon than in strawberry process. As mentioned above, the presence of antimicrobial substances in the strawberry fruit could be the reason for its low diversity (Jay et al., 2005; Terry et al., 2004). It has been reported that grapes infected by *Botrytis cinerea* may exhibit a sour rot that results in the buildup of AAB (over 10^6 cells/ml) (Drysdale and Fleet, 1988). In the strawberry, the antifungal activity over *B. cinerea* has been attributed to the antifungal compounds present in this fruit (Terry et al., 2004). Therefore, the synergy observed among molds and AAB in grapes may not occur in strawberries because of the absence of gray mold, which may also prevent the buildup of AAB. Additionally, the presence of the antibacterial compounds in strawberry fruit could also reduce the AAB population (Amil-Ruiz et al.,

2011). Another important aspect to be considered is the physicochemical composition of the fruit. This factor plays an important role in biodiversity because the physicochemical composition of the fruit restricts the biodiversity of microorganisms. Therefore, this work highlights the importance of the complex ecology associated with the fruit.

In persimmon acetifications, *A.malorum* was the main AAB species identified at the initial and middle stages, and *Ga. saccharivorans* was the main AAB species identified at the end of the process. At strain level, more than twenty-five strains were detected, and no clear candidate was indicated as possible starter to undertake this process. Therefore, selection studies with the different strains isolated need to be performed. In contrast, only one strain of *A.malorum* (CECT 7749) was isolated throughout the strawberry acetification process, which was subsequently tested as starter culture.

The inoculation practice in vinegar production has been traditionally limited to the use of a vinegar mother or back slopping (Solieri and Giudici, 2009). In both cases, vinegar is the result of the competition between the microorganisms (mainly AAB) present in an undefined starter, which does not ensure the control of the process or the quality of the final product. Nevertheless, the advantage of these inoculation systems is that the AAB are physiologically actives and are adapted to the adverse conditions present in the medium (high alcohol level or high acetic acid level).

In this work, we have evaluated AAB inoculation for the production of vinegar from different fruits: grape, strawberry and highbush blueberry. Although this work mainly focused on the superficial method, alternative elaboration methods, including the submerged and the Schützenbach method, were also tested.

The first study of AAB inoculation was carried out in wine vinegar by the superficial method. The inoculated *A. pasteurianus* strain was not totally imposed in the different conditions tested, but the inoculation clearly improved the process, considerably shortening the vinegar production time. A similar result was obtained when the inoculation was tested in strawberry using a selected strain of *A. malorum*. In both fruits, when there was no imposition of the inoculated strain, a succession of *Acetobacter* and *Gluconacetobacter* species was observed, similar to that observed in spontaneous acetification of persimmon fruit. This succession of genera has already been reported in the production of Traditional Balsamic Vinegar (Gullo et al., 2009), and it could be explained by the higher acetic acid tolerance of the *Gluconacetobacte*r genus compared to the *Acetobacter* (Gullo et al., 2006).

The detection of other AAB different from the inoculated strain could be due to multiple factors. (i) These AAB could come from the fruit, survive the AF process and grow and participate in acetification when the conditions were appropriate. (ii) In those acetifications carried out under vinegar plant conditions (in our case, the wine vinegar experiment), a contamination during the final stages of the starter scale-up production, which was performed in the vinegar plant, is possible. In our case, the other strains detected had been already identified in a previous ecological study conducted in the same vinegar plant for the selection of the starter culture (Vegas et al., 2010). (iii) The inoculation into barrels previously used for vinegar production could provide another source of AAB. Although the barrels were properly cleaned prior to the inoculation study, it is known that there is not a "definitive" treatment to eliminate AAB contamination from a barrel when the bacteria have penetrated deeply into the wood (Schahinger and Rankine, 2002). Therefore, these AAB could have been present in the barrels and developed when the conditions were appropriate.

Regarding the barrels used, different designs were also developed and tested to reduce the acetification time. These designs were focused on increasing the air-contact surface to facilitate the development of AAB, resulting in important differences at the technological and microbiological levels. Among the three types of barrels tested, the prototype P1 was the most appropriate because of the lower ethanol evaporation, shorter acetification and the imposition of the *A. pasteurianus* species along all the process, which is related to traditional wine vinegar production.

In the case of the grape wine acetified by the submerged method, the *A. pasteurianus* inoculated strain was replaced by genotypes belonging to *Gluconacetobacter* species (*Ga. europaeus* and *Ga. intermedius*). These strains are most likely due to a contamination from the vinegar plant (either of the wine or the starter production), and their growth and development during the submerged acetification in relation to A. *pasteurianus* strain reveals a better adaptation to these conditions. The presence of *Gluconacetobacter* genus is common in this method. *Gluconacetobacter* species, including *Ga. europaeus* (Callejón et al., 2008; Sievers et al., 1992; Trcek et al., 2000), *Ga. intermedius* (Boesch et al., 1998; Trcek et al., 2000), *Ga. entanii* (Schüller et al., 2000), and *Ga. oboediens* (Sokollek et al., 1998), have been described as better adapted to the strong aeration of the submerged method. This finding could explain why the inoculated *A. pasteurianus* strain rapidly disappeared in the vinegar production by this method.

A. pasteurianus species was also detected in the spontaneous process carried out with highbush blueberry by Schützenbach method. The diversity isolated from the blueberry samples was very low, similar to that of the strawberry samples. Only two genotypes of this species were identified throughout the blueberry acetifications. The more restrictive conditions of berry fruits may be a tool to select microorganisms because the more

stringent bacterial growth results in a greater selective pressure exerted on the indigenous microorganisms (Solieri and Giudici, 2009). Furthermore, the detection of these *A. pasteurianus* genotypes in the inoculated processes reveals adaptation of these microorganisms to the blueberry conditions. Despite the presence of these *A. pasteurianus* strains in the inoculated processes, there was a clear imposition of the *A. cerevisiae* inoculated strain throughout the acetification, resulting in a considerable reduction of the production time. In these processes, a high AAB population (10^8 cells/ml) was directly inoculated, unlike other processes where the inoculated AAB population was lower (approximately 10^6 cells/ml) or not controlled. This is similar to the use of a vinegar mother obtained from a pure culture strain, where a defined volume of the vinegar mother is added (usually between 10 and 25% of the total volume) as a starter into the wine. Although this inoculation (10^8 cells/ml) was successful, it is not easy to implement this technique in plant vinegar, as it is difficult to obtain the volume necessary with this high population to inoculate industrial acetators. Furthermore, the use of microscopy and centrifuge to count and recover the cells required for this inoculation strategy would be too costly to transfer to vinegar plant. Therefore, the best alternative seems to be continuing to work on the production of a vinegar mother with an adequate amount of pure culture from one strain or a mixed culture of *Acetobacter* and *Gluconacetobacter* strains to carry out the different processes. This vinegar mother should be added at the beginning of the process, and an increasing volume of wine should be added when the titratable acidity reaches 3% (w/v) until a predetermined final volume. In this manner, it is possible to increase the volume of wine in the acetator without too much change to the bacterial population, the ethanol concentration, or the acidity. However, an in-depth study must be carried out to optimize this important part of the vinegar elaboration process.

Culture-independent molecular methods should be used to complement the information obtained by these ecological studies (culture-dependent methods) to obtain more complete information about the composition of the microbial community and their relative abundance in the studied vinegars. The most critical step for the application of these culture-independent methods is the recovery of representative and high-quality genomic DNA (gDNA). Some gDNA extraction procedures for culture-independent methods applied to fermented and acidic beverages have been proposed for wines (Millet and Lonvaud Funel, 2000), balsamic vinegars (De Vero et al., 2006) and wine vinegars (Jara et al., 2008). However, when these methods were used to obtain gDNA from Traditional Balsamic Vinegar, persimmon vinegar or strawberry vinegar, adequate results were not obtained. Therefore, a direct gDNA extraction from these types of vinegars was tested, and the suitability for PCR assays was queried by PCR-DGGE and sequencing of the 16S rRNA gene. Suitable yield and DNA purity were obtained by modification of a method based on the use of PVP/CTAB to remove polyphenolic components and esopolysaccharides. *Ga. europaeus*, *A. malorum/cerevisiae* and *A. orleanensis* were detected as the main species in samples having more than 4% (w/v) acetic acid content. These results are similar to those observed in culture-dependent molecular methods. In the case of strawberry vinegars, *Acetobacter orleanensis* was the only strain detected by DGGE, and *Acetobacter malorum*, which is closely related to *A. orleanensis*, was the only strain recovered by the culturing method. On the other hand, the lack of AAB information from the persimmon samples by DGGE could be explained by the high diversity observed on the culturing plate, and this suggests that populations of many species were present that were below the detection limit of the DGGE technique. Therefore, the extraction method was suitable to be directly applied

for culture-independent study of vinegars containing a high content of polyphenols and esopolysaccharides.

Finally, as additional information regarding the vinegar quality, several major volatile components were measured during the production of these strawberry and persimmon vinegars. The antioxidant activity and total phenols index (TPI) of persimmon vinegars were higher than the values obtained for commercial white- and red-wine vinegars, indicating that persimmon vinegar may be a competitive product in the market (Ubeda et al., 2011a). On the other hand, the antioxidant activity, total phenols and monomeric anthocyanins parameters in the strawberry vinegars increased when sulfur dioxide and pectolytic enzymes were added to substrates and when a semi-pilot scale was used (Ubeda et al., 2012). In addition, the use of wood barrels improved the volatile profile of strawberry vinegars, particularly the use of the cherry wood barrels (Ubeda et al., 2011b).

These results support the hypothesis that the microbiota isolated from fruit, both during fermentation and acetification, was highly capable of carrying out both processes without additional input. Although starter cultures are not essential for alcoholic fermentation, they are required for producing vinegar repetitively and efficiently. We recommend the use of a starter cultures to produce fruit vinegars. On the other hand, fruits in general, and strawberries in particular, have a high potential to be used for the production of vinegar by traditional methods, yielding a promising product with a high added value.

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GENERAL CONCLUSIONS

1. Microbiota present in fruits appear able to carry out vinegar production. In fact, genuine vinegars from strawberry, highbush blueberry and persimmon were produced. However, a suitable preparation of raw material (i.e., addition of pectolytic enzymes or sugar correction) is a critical step for this elaboration.

2. Yeast inoculation improves the kinetics of the alcoholic fermentation of fruit juices. The yeast strain (*S. cerevisiae* CECT 13057) isolated from strawberry samples has proven to be a good starter culture for strawberry alcoholic fermentation.

3. The AAB strains used as culture starters improved the kinetics, shortening the time in the vinegar production. However, in general, there was no imposition of these strains at the final stages of these acetifications.

4. The use of wood barrels with a high air-contact surface facilitated the development of AAB, which is necessary to reduce the acetification time in traditional vinegar production.

5. Both culture-independent and culture-dependent techniques are required to obtain a global overview of the microorganisms involved in the vinegar elaboration process.

APPENDIXES

APPENDIXES INDEX

APPENDIX 1: Material & Methods

APPENDIX 2: Complementary articles

APPENDIX 1

1. Physicochemical analysis

1.1. Titratable acidity (Ough and Amerine, 1987)

During the acetification, the variation in the total acidity is due to the production of acetic acid. Therefore, in this case, the volatile acidity can be determined as the total acidity.

Total acidity is analyzed by acid-base titration with phenolphthalein as an indicator and is expressed in grams of acetic acid per 100 mL of vinegar or percentage (% w/v).

Apparatus and Materials:

• 50 mL burette

• 10 ml graduated pipette

• 250 mL Erlenmeyer flask

Reagents:

• 0.5 N sodium hydroxide

• Phenolphthalein Indicator (Dissolve 1 g of phenolphthalein in water and add ethyl alcohol (95 to 96 % v/v) until a volume of 100 mL is reached)

Procedure:

One milliliter of vinegar, 100 mL of distilled water and 2-3 drops of phenolphthalein are titrated with 0.5 N NaOH until a faint pink color persists for 30 sec. At the equivalence point, the indicator changes from colorless to pink.

Calculations:

Titratable acidity (%) =

$$\text{mL NaOH} \times \frac{0.5 \text{ eq NaOH}}{1{,}000 \text{ mL}} \times \frac{1 \text{ eq } CH_3COOH}{1 \text{ eq NaOH}} \times \frac{60 \text{ g } CH_3COOH}{1 \text{ eq } CH_3COOH} \times 100 =$$

= (mL NaOH × 3) g of CH_3COOH per 100 mL

1.2. Concentration of sugars

The residual amount of glucose and fructose is determined using an enzymatic kit (Boeringher Mannheim).

Determination of D-glucose:

At pH 7.6, the enzyme hexokinase (HK) catalyzes the phosphorylation of D–glucose by adenosine-5'-triphosphate (ATP), with the simultaneous formation of adenosine-5'-diphosphate (ADP). In the presence of glucose-6-phosphate dehydrogenase (G6P-DH), the D–glucose-6-phosphate (G-6-P) formed is specifically oxidized by nicotinamide-adenine dinucleotide phosphate (NADP) to D-gluconate-6-phosphate, with the formation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The NADPH formed in this reaction is stoichiometric to the amount of D–glucose and is measured by means of its light absorbance at 334, 340 or 365 nm.

Determination of D-fructose:

Hexokinase also catalyzes the phosphorylation of D-fructose to D-fructose-6-phosphate (F-6-P) with the aid of ATP. F-6-P is converted by phosphoglucose isomerase (PGI) to G-6-P. G-6-P reacts again with NADP with the formation of D-gluconate-6-phosphate and NADPH (2 molecules). The amount of NADPH formed now is stoichiometric to the amount of D-fructose.

1.3. Ethanol concentration

The ethanol is measured using the enzymatic kit from Boehringer Mannheim. This method is based on the oxidation of ethanol to acetaldehyde by nicotinamide-adenine dinucleotide (NAD) in the presence of the enzyme alcohol dehydrogenase (ADH). The equilibrium of this reaction lies on the side of ethanol and NAD. It can be completely displaced to the right side at alkaline conditions and by trapping of the acetaldehyde

formed. Acetaldehyde is quantitatively oxidized to acetic acid in the presence of aldehyde dehydrogenase. NADH is determined by means of its light absorbance at 334, 340 or 365 nm.

1.4. Oxygen dissolved content

Temperature and oxygen dissolved in the acetifying liquid are measured using a LDO™ HQ10 Portable Dissolved Oxygen Meter (HACH Company, Colorado, USA).

2. Culture media

2.1. YPD (Yeast Extract Peptone Dextrose)

Yeast Extract Peptone Dextrose is a general medium to grow yeast.

Glucose 20 g/L in distilled water

Peptone 20 g/L

Yeast Extract 10 g/L

This medium can be made as a liquid or as a solid by adding 20 g/L of agar. This medium is autoclaved at 121ºC for 15 min.

2.2. LYS (Lysine) (Angelo and Siebert, 1987)

This medium supports the growth of non-*Saccharomyces* yeast. *Saccharomyces* yeast cannot grow in a medium with lysine as the unique source of Nitrogen. This medium is used to distinguish between *Saccharomyces* and non-*Saccharomyces* yeast.

Lysine medium: 66 g/L distilled water

Lactate potassium solution: 4 ml/L (composition: 18 ml lactic acid 85%; 14 g KOH)

This medium is heated for the complete dissolution of the ingredients at constant agitation to avoid any overheating. When the medium is approximately 50ºC, 1 ml of

10% lactic acid is added to adjust the pH at 5. The medium is distributed in plates, with approximately 20 ml per plate.

2.3. GYC (Glucose Yeast Calcium carbonate)

GYC is a general medium to grow AAB.

Liquid medium:

Glucose 100 g/L in distilled water

Yeast Extract 10 g/L

Solid medium:

Glucose 100 g/L in distilled water

Yeast Extract 10 g/L

$CaCO_3$ 20 g/L

Agar 15 g/L

Calcium carbonate is used to detect acid production. When acid is produced, a halo is formed around the colony.

The medium is autoclaved at 121ºC for 15 min. Once the medium is warm, natamicine (100 mg/L) can be added to avoid yeast growth.

3. DNA extraction

3.1. Methodology for Culture-dependent methods

3.1.1. Yeast DNA extraction

	Work-step	Volume	Stock-concentration
1	Overnight growth of yeast cells in a tube with YPD medium at 26ºC	5 mL	
2	Transfer to other tubes culture medium with cells grown	1.5 ml	
3	Centrifugation (10,000 rpm/2 min) and removing the supernatant		
4	Washing cells with sterile distilled water	1.5 mL	
5	Centrifugation (10,000 rpm/2 min) and removing the supernatant		
6	Buffer 1 addition	500 µl	(T1:Sorbitol 0,9M, EDTA 0,1 M pH7,5)
7	Zymolyase addition	30 µl	(1,5 mg in 1300 µl of T1)
8	Incubation in a water bath (37°C/20 min)		
9	Centrifugation (10,000 rpm/2 min) and removing the supernatant		
10	Buffer 2 addition	500 µl	(T2: Tris 50MM pH7,4, EDTA 20mM).
11	SDS addition	13 µl	10%
12	Incubation in a water bath (65°C/10 min)		
13	Potassium acetate addition and mixing	200 µl	5M
14	Incubation in ice 10-15 min		
15	Centrifugation 10 min/12,000 rpm (+4°C)		
16	Transfer supernatant to other tubes (2 ml)		
17	In the new tube, isopropanol addition and incubation at room temperature for 5 min	700 µl	
18	Centrifugation 10 min/12,000 rpm (+4°C) and removing the supernatant		
19	Ethanol addition	500 µl	70%
20	Drying pellet (vacuum centrifuge) 10-15 min		
21	TE buffer addition	15µl	(Tris 10 mM pH 7,4, EDTA 1mM pH 8,0).

3.1.2. DNA extraction for AAB

Total DNA is extracted using the modified CTAB method (cetyltrimethylammonium bromide), as described by Ausubel et al. (1992).

	Work-step	Volume	Stock-concentration
1	Cell pellet resuspension in TE Buffer	520 µl	10mM Tris-HCL; 1mM EDTA, pH 8
2	SDS addition	30 µl	20%
3	Proteinase K addition	6 µl	20mg/ml
4	Incubation in a water bath (37°C/60 min)		
5	NaCl addition	150 µl	5M
6	CTAB addition	140 µl	
7	Incubation in a water bath (65°C/10 min)		
	Incubation in ice 10-15 min		
8	1 volume Chloroform/ Isoamilic alcohol addition	± 850 µl	(24:1)
9	Mix by inversion until a homogeneous emulsion is obtained		
10	Centrifugation 10 min/10000 g (+4°C)		
11	Transfer the supernatant to a new 2 ml tube		Repeat the Chloroform/ Isoamilic alcohol step until interface is not observed.
12	Isopropanol addition	380 µl	
13	Mix by inversion		
14	Incubation -20ºC/ 5 min		
15	Centrifugation 10 min/10000 g (+4°C) and removing the supernatant		
17	Ethanol addition	150 µl	70%
18	Centrifugation 5 min/10000 g (+4°C)		
19	Removing ethanol (with pipette)		
	Drying pellet (vacuum centrifuge) 10-15 min		
20	TE buffer addition	50µl	

3.2. Methodology for culture-independent methods

3.2.1. DNA extraction from bacteria.

This is a modification of the CTAB-Porebski et al., 1997 method (previously described as method 6 from Chapter 6)

A different initial washing step is performed depending on the sample:

Special vinegars and condiments: Fifteen milliliters of the sample is centrifuged (2,500x*g* (Hermle Z 383K)/20 min/4°C) to collect a pellet. The pellet is resuspended in 5 ml of saline EDTA-PVP solution (0.15 M NaCl + 0.1 M EDTA pH 8 with NaOH, 2 % PVP) and then recentrifuged.

Fruit vinegars: One gram of the sample is used. The sample is washed with distilled water and then centrifuged (2,500x*g*/20 min/4°C).

Step	Description	Amount	Stock-concentration
1	Extraction buffer Mix addition	5 ml heated at 60 ºC	Note: from 0.5 to 1.0 g of pellet. 100 mM Tris base; 1.4 M NaCl; 20 mM EDTA, pH8; 2% CTAB; 0.3% ß-mercaptoethanol
2	PVP addition	50 mg/0.5 g of pellet	PM 40000 mol/g
3	Tubes inversion (5 min)		
4	Incubation (60 ºC/60 min); Shaking each 20 min		
5	Cooling at room temperature (5 min)		
6	Chloroform/Isoamyl alcohol addition and mixing by inversion to form an emulsion	6 ml	24:1
7	Centrifugation (1,000x*g*/20 min)		
8	Transfer of top aqueous solution to new 15ml centrifuge tubes		
9	Return step 2 until removing cloudiness (PVP) in aqueous phase		
10	NaCl addition to the final solution recovered	½ Volume (V)	5 M NaCl
11	Inversion of tubes (10 times)		
12	Ethanol addition (-20 ºC)	2 V	Ethanol 95%
13	Mix by inversion. Cooling (-20 ºC/10 min)		
14	Centrifugation (1,000 x*g*/6 min)		
15	Removing of upper fase, washing pellet with ethanol (-20ºC)	2 V	70% v/v
16	Transfer to other tubes (2 ml)		
17	Centrifugation [13,000 x*g* Microfuge 22R/ 5 min] [2]		
18	Drying pellet (37ºC/30 min)		
19	Dissolution in TE	100 µl	TE buffer: Tris HCl 1 mM, EDTA 1 mM pH 8.4
20	RNase A addition	3 µl	(10 mg/ml)
21	Incubation (37 ºC/60 min)		
22	Proteinase K addition	3 µl	(1mg/ml)
23	Incubation (37 ºC/30 min)		
24	Phenol: chloroform: isoamylic alcohol addition	300 µl	25:24:1 pH 6.6/8.0
25	Vortex briefly and centrifugation (17,000 x*g*/15 min)		
26	Collection of upper layer in new 1.5 ml tube		
27	TE addition to phenol phase	50 µl	
28	Vortex, spin (17,000 x*g*/15 min), upper layer removing and addition to sample of step 26		
29	Na acetate addition	1/10 V	2M
30	Ethanol addition	2 V	Absolute
31	Tubes inversion (10 times)		
32	Overnight incubation (-80 ºC)		
33	Centrifugation (17,000 x*g* /20 min)		
34	Pellet washing with ethanol	500 µl	70% v/v
35	Centrifugation (17,000 x*g* /15 min)		
36	Removing ethanol and drying of DNA pellet (30-60 min/37ºC)		
37	TE addition	25-100	

4. Molecular techniques

4.1. Identification of microorganisms

4.1.1. Yeast

4.1.1.1. Restriction analysis of ribosomal genes (PCR-RFLP rDNA) (Guillamón et al., 1998)

The first stage of this technique is the amplification via PCR. The primers used are as follows:

ITS1 5'- TCCGTACGTGAACCTGCGG - 3'

ITS4 5'- TCCTCCGCTTATTGATATGC - 3'

The amplification mix contains a final volume of 50 µl:

Primer ITS1 (10 µM)	1 µl
Primer ITS4 (10 µM)	1 µl
dNTPs (32 µM)	4 µl
$MgCl_2$ (2.5 mM) (Ecotaq)	3 µl
Buffer Taq 10x without Mg. (Ecotaq)	5 µl
Taq DNA polymerasa (ARK Scientific) (0.2 U)	0.5 µl
H_2O milli-Q	33 µl
DNA	2.5 µl

PCR conditions:

The samples are incubated for 5 min at 95ºC and then cycled 35 times at 95ºC for 30 seconds, 52ºC for 1 min and 72ºC for 1 min. The samples are incubated for 7 min at 72ºC for final extension and kept at 4ºC until tested.

The amplicons are analyzed by electrophoresis in 1.0% (w/v) agarose gels. The gel is prepared in 1X TBE buffer (Tris 0.9 M; boric acid 0.9 M; EDTA 20 mM; pH 8) and 1 µL of ethidium bromide (Fluka Biochemika) per each 25 ml of TBE solution is added.

Sample preparation:

Five microliters of the amplified DNA are mixed with 2 µL of bromophenol blue (10 mL of a stock solution is prepared with 10 mg of bromophenol blue, 5 mL of glycerol, 1 mL of TBE, 4 mL of Milli-Q water, pH 8.3). The visualization of the amplified band is performed using a UV transilluminator. The length of the amplification products is determined by comparison with a 100 bp DNA ladder (Gibco-BRL, Eggenstein, Germany)

Once the amplified fragment is obtained, the digestion is performed. The following restriction enzymes are used: *Cfo*I, *Hae*III, *Hin*fI, *Dde*I (Roche Diagnostics).

The digestion mix contains a final volume of 20 µl:

Enzyme	1.5 µl
Specific buffer for each enzyme	2.0 µl
H_2O milli-Q	8.5 µl
DNA amplified	8.0 µl

This reaction is incubated overnight at 37ºC. The digested DNA is mixed with 4 µL of bromophenol blue. Restriction fragments are detected and analyzed by electrophoresis on a 2% (w/v) agarose gel. The length of the restriction fragments is determined by comparison with a 100 bp DNA ladder. The restriction patterns are compared with the ones reported by Esteve-Zarzoso et al. (1999).

4.1.1.2. Analysis of the region D1/D2 from rDNA (Kurtzman and Robnett, 1998)

The amplification is performed with the following primers:

NL-1 5'- GCATATCAATAAGCGGAGGAAAAG - 3'

NL-4 5'- GGTCCGTGTTTCAAGACGG - 3'

The amplification mix contains a final volume of 50 µl:

Primer NL-1 (50 µM)	1 µl
Primer NL-4 (50 µM)	1 µl
dNTPs (32 µM)	1 µl
$MgCl_2$ (2.5 mM) (Ecotaq)	2.5 µl
Buffer Taq 10x, without Mg. (Ecotaq)	5 µl
Taq DNA polymerasa (ARK Scientific) (0.2 U)	0.5 µl
H_2O milli-Q	37 µl
DNA	2 µl

PCR conditions:

The samples are incubated for 3 min at 95ºC and then cycled 36 times at 95ºC for 1 min, 52ºC for 2 min and 72ºC for 2 min. The samples are incubated for 5 min at 72ºC for final extension and kept at 4ºC until tested.

The 16S rRNA amplicons are purified and sequenced by Macrogen, Inc. (Seoul, South Korea). The sequences obtained are compared with the sequences in the GenBank database using the BLAST alignment tool.

4.1.2. Acetic Acid Bacteria

4.1.2.1. Restriction analysis of the amplified 16S rDNA (PCR-RFLP 16S rRNA) (Ruiz et al., 2000)

The amplification mix contains a final volume of 50 µl:

Primer Aceti I (10 ρM)	1.5 µl
Primer Aceti IV (10 ρM)	1.5 µl
dNTPs (each dNTP 10 mM) (Boehringer Mannheim)	1 µl
$MgCl_2$ (100 mM) (Ecotaq)	3 µl
DMSO (Dimethyl sulfoxide)	5 µl
BSA (Bovine serum albumin) (20 mg/mL)	0.4 µl
Buffer Taq 10x, without Mg. (Ecotaq)	5 µl
Taq DNA polymerasa (Ecotaq)	0.4 µl
H_2O milli-Q	29.2 µl
DNA	3 µl

PCR conditions:

The samples are incubated for 1 min at 94ºC and then cycled 35 times at 94ºC for 1 min, 60ºC for 45 seconds, and 72ºC for 2 min. The samples are incubated for 10 min at 72ºC for final extension and kept at 4ºC until tested.

Five microliters of the amplified DNA are mixed with 2 µL of bromophenol blue and detected by electrophoresis on a 1% (w/v) agarose gel (Boehringer Mannheim).

Once the amplified fragment is obtained, the digestion is performed. The following restriction endonucleases used are: *Taq*I, *Alu*I (Roche diagnostics), and *Bcc*I (Biolabs).

Enzyme	1 µl
Specific buffer for each enzyme	2 µl
H_2O milli-Q (or 6.8 µl for *Bcc*I)	7 µl
BSA (Bovine serum albumin) (only for *Bcc*I)	0.2 µl
DNA amplified	10 µl

Samples are incubated for 3 hours at 37ºC (for *Alu*I and *Bcc*I) or 65ºC (for *Taq*I).

The digested DNA is mixed with 4 µL of bromophenol blue and detected by electrophoresis on a 3% (w/v) agarose gel. The length of the restriction fragment is determined by comparison with a 100 bp DNA ladder (Gibco-BRL, Eggenstein, Germany).

4.1.2.2. Restriction analysis of the amplified 16S-23S rRNA gene ITS region (PCR-RFLP ITS 16-23S rRNA) (Ruiz et al., 2000).

Primers used to amplify the ITS 16S-23S rDNA are:

Its1, 5'-ACCTGCGGCTGGATCACCTCC-3'

Its2, 5'-CCGAATGCCCTTATCGCGCTC-3'.

The amplification mix contains a final volume of 50 µl:

Primer ITS1 (10 ρM)	1.5 µl
Primer ITS2 (10 ρM)	1.5 µl
dNTPs (each dNTP 10 mM) (Boehringer Mannheim)	1 µl
$MgCl_2$ (100 mM) (Ecotaq)	3 µl
Buffer Taq 10x, without Mg. (Ecotaq)	1 µl
Taq DNA polymerasa (Ecotaq)	0.5 µl
H_2O milli-Q	40.5 µl
DNA	1 µl

PCR conditions:

The samples are 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. The samples are then incubated for 7 min at 72°C for a final extension and kept at 4°C until tested.

Five microliters of the amplified DNA are mixed with 2 µL of bromophenol blue and detected by electrophoresis gel on a 1% (w/v) agarose gel (Boehringer Mannheim). The length of the amplification product is determined by comparison with a 100 bp DNA ladder (Gibco-BRL, Eggenstein, Germany)

Once the amplified fragment is obtained, the digestion is performed.

The restriction endonuclease used is *Cfo*I (Roche diagnostics).

Enzyme Specific buffer for each enzyme	1 µl
H_2O milli-Q	2 µl
DNA amplified	7 µl
	10 µl

The digested DNA is mixed with 4 µL of bromophenol blue and detected by electrophoresis on a 3% (w/v) agarose gel. The visualization of the restriction fragments is performed using a UV transilluminator and compared with the 100 bp DNA ladder (Gibco- BRL, Eggenstein, Germany).

4.1.2.3. PCR-DGGE (Denaturing Gradient Gel Electrophoresis) (Lopez et al., 2003; De Vero et al., 2006)

The PCR amplification of the region V7 to V8 of the 16S rRNA gene is performed using the following primers: WBAC1 (5'-GTCGTCAGCTCGTGTCGTGAGA-3'; nt 1069–1090) with GC-clamp and WBAC2 (5'-CCCGGGAACGTATTCACCGCG-3'; nt 1374-1394).

PCR conditions:

The samples are incubated for 5 min at 95°C and then cycled 30 times at 95°C for 1 min, 67°C for 30 s and 72°C for 1 min. The samples are then incubated for 5 min at 72°C for a final extension and kept at 4°C until tested.

Once the amplicons are obtained, the next step is to prepare the denaturing gradient gel. Solutions for DGGE:

TAE 50 X	Trizma base	242 g
	Acetic acid glacial	57.1 g
	EDTA 0.5 M (pH8)	100 ml
	dH20	up to 1000 ml
	Autoclave 121ºC during 15 min	
EDTA 0.5 M pH 8	EDTA	186.12 g
	Adjust the pH at 8 with NaOH	
	dH20	up to 1000 ml
	Autoclave 121ºC during 15 min	
0% denaturing solution	40% bisAcrilamida	10 ml
	50X TAE	1 ml
	dH20	to 50 ml
100% denaturing solution	urea	21 g
	formamide	20 ml
	40% bisAcrilamide	10 ml
	50X TAE	1 ml
	dH20	to 50 ml

10% Ammonium persulphate (APS)
0.1 g ammonium persulphate in 1 ml dH_20
TEMED (N,N,N,N'-tetra-methyl-ethylenediamine)

DGGE of PCR products is performed on an 8% (w/v) polyacrylamide gel with urea and formamide as denaturants.

To build the gel assembly, the glass plates are cleaned with ethanol and distilled water. The gel sandwich is assembled by placing the small glass plate on top of the large plate, correctly placing a 1 mm spacer along each edge of the plate assembly. The plate clamps are attached (tight enough to hold everything together), and the entire assembly is placed into the rear slot of the pouring stand. The clamps are loosened slightly and the spacing card is used to assure the proper spacer alignment. The plate clamps are tightened (to prevent leakage), and the plate assembly is removed from the pouring stand. The plate assembly is inspected to ensure that the two glass plates and the spacers form a flush surface across the bottom of the assembly. A foam gasket is placed into one of the two front slots of pouring stand, and the plate assembly is inserted and clamped into place. The well comb is placed firmly in between the plates.

Once the gel assembly is ready, the acrylamide gel can be prepared. It is prepared with a mixer gradient pump. Both 0% and 100% denaturing solutions are needed, and approximately 20 ml of each solution is kept on ice while the gel is built. Into each 20 ml solution, 20 µl TEMED and 200 µl APS are added. Fifty microliters of colorant can be added to the 100% denaturing solution to see the denaturing gradient when it is created. The denaturing gradient is prepared with an interval between 40% and 60% (v/v). The gel is left for 1 hour to polymerize.

Once the gel is ready to be used, the PCR products are loaded into the wells of the gel. Electrophoresis is performed in 1X Tris-acetate EDTA (TAE) buffer at 60°C at a constant voltage of 200 V for 4 h. Subsequently, the gel is stained with ethidium bromide (50 µg/ml) in 250 ml of 1X TAE buffer for 15 minutes. The gel is then

destained with 250 ml of 1X TAE buffer for 20 minutes and photographed by BioDocAnalyze (BDA; Germany).

Individual PCR-DGGE bands are cut out from gel and incubated overnight at 4°C in 30µl dH_2O. An aliquot (1 µl) is used in a PCR reaction with the same primer set used for DGGE but without the GC-clamp attached to the WBAC1 primer. The PCR products are purified and sequenced by Macrogen Inc. facilities (Seoul, South Korea) using an ABI3730 XL automatic DNA sequencer. The sequences obtained are compared with the sequences in the GenBank database using the BLAST alignment tool.

4.2. Typing

4.2.1. Restriction analysis of mtDNA for *Saccharomyces* yeasts

The DNA obtained using the protocol described in section 3.1.1. is digested at 37ºC for 6-7 hours with the restriction enzyme *Hin*fI.

Enzyme *Hin*fI	1 µl
Specific buffer for *Hin*fI	2 µl
H_2O milli-Q	8 µl
Rnase (10 mg/ml)	1 µl
DNA	8 µl

Twenty microliters of the digested DNA is mixed with 2 µL of bromophenol blue. The fragments obtained are detected by electrophoresis on a 0.8% (w/v) agarose gel (Boehringer Mannheim).

Amplicon size is determined by comparing the smallest products to a 100 bp DNA ladder (Gibco- BRL, Eggenstein, Germany). A mixture of DNA Molecular Weight Marker II and DNA Molecular Marker III (Roche, Germany) is used to determine the weight of the largest fragments.

4.2.2. Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) for AAB (González et al., 2004)

The amplification mix contains a final volume of 25 µl:

Primer Eric I (10 ρM)	1 µl
Primer Eric II (10 ρM)	1 µl
dNTPs (each dNTP 10 mM) (Boehringer Mannheim)	1.25 µl
5XGB (1M $(NH_4)_2SO_4$; 1M Tris-HCl; 1M $MgCl_2$; 0.5M EDTA (pH 8.8); β-mercaptoethanol 14.4M)	5 µl
BSA (Bovine serum albumin) (20 mg/mL)	0.2 µl
DMSO (Dimethyl sulfoxide)	2.5 µl
H_2O milli-Q	10.65 µl
Taq DNA polymerasa (Ecotaq)	0.4 µl
DNA	3 µl

PCR conditions:

The samples are incubated for 5 min at 94°C and then cycled 30 times at 94°C for 30 s, 57°C for 30 s and 65°C for 4 min. The samples are then incubated for 8 min at 65°C for a final extension and kept at 4°C until tested.

Eight microliters of the amplified DNA is mixed with 2 µL of bromophenol blue and detected by electrophoresis on a 1.5% (w/v) agarose gel (Boehringer Mannheim).

Amplicon size is determined by comparing the smallest products to a 100 bp DNA ladder (Gibco- BRL, Eggenstein, Germany). A mixture of DNA Molecular Weight Marker II and DNA Molecular Marker III (Roche, Germany) is used to determine the weight of the largest fragments.

4.2.3. $(GTG)_5$-PCR for AAB (De Vuyst et al., 2008)

The amplification mix contains a final volume of 25 µl:

Primer $(GTG)_5$ (10 ρM)	1 µl
5XGB (1M $(NH_4)_2SO_4$; 1M Tris-HCl; 1M $MgCl_2$; 0.5M EDTA (pH 8.8); β-mercaptoethanol 14.4M)	5 µl
BSA (Bovine serum albumin) (20 mg/mL)	0.4 µl
dNTPs (each dNTP 10 mM) (Boehringer Mannheim)	1.25 µl
DMSO (Dimethyl sulfoxide)	2.5 µl
H_2O milli-Q	13.45 µl
Taq DNA polymerasa (Ecotaq)	0.4 µl
DNA	1 µl

PCR conditions:

The samples are incubated for 5 min at 94°C and then cycled 30 times at 94°C for 1 min, 40°C for 1 min and 65°C for 8 min. The samples are then incubated for 16 min at 65°C for a final extension and kept at 4°C until tested.

Eight microliters of the amplified DNA is mixed with 2 µL of bromophenol blue and detected by electrophoresis on a 0.8% (w/v) agarose gel (Boehringer Mannheim).

Amplicon size is determined by comparing the smallest products to a 100 bp DNA ladder (Gibco- BRL, Eggenstein, Germany). A mixture of DNA Molecular Weight Marker II and DNA Molecular Marker III (Roche, Germany) is used to determine the weight of the largest fragments.

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APPENDIX 2

These scientific manuscripts were developed in collaboration with the University of Sevilla. These articles have been presented in the thesis work of Cristina Ubeda, and they are attached to this thesis as complementary work.

- Article 1

  Ubeda, C., Hidalgo, C., Torija, M. J., Mas, A., Troncoso, A. M., Morales, M. L. (2011). Evaluation of antioxidant activity and total phenols index in persimmon vinegars produced by different processes. Food Science and Technology, 44: 1591-1596.

- Article 2

  Ubeda C., Callejón R. M., Hidalgo C., Torija M. J., Mas A., Troncoso A. M., Morales M. L. (2011). Determination of major volatile compounds during the production of fruit vinegars by static headspace gas chromatography–mass spectrometry method. Food Research International, 44: 259–268.

- Article 3

  Ubeda, C., Callejón, R. M., Hidalgo, C., Torija, M. J., Troncoso, A. M., Morales, M. L. (2012). Employment of different processes for the production of strawberry vinegars: Effects on antioxidant activity, total phenols and monomeric anthocyanins LWT. Food Science and Technology. Doi:10.1016/j.lwt.2012.04.021.

Article 1

Evaluation of antioxidant activity and total phenols index in persimmon vinegars produced by different processes

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Evaluation of antioxidant activity and total phenols index in persimmon vinegars produced by different processes

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ABSTRACT

The total phenols index (TPI) and antioxidant activity of persimmon vinegars produced by different processes were evaluated. A novel extraction method was designed and optimised for this purpose with respect to the type and concentration of solvent and ultrasonication time. The best extraction conditions found were the use of 80% ethanol and 25 min of ultrasonication. Antioxidant capacity was determined by the oxygen-radical absorbance capacity of fluorescein (ORAC-FL) and 2,2′-diphenyl-1-pycrylhydrazyl (DPPH) free-radical assays. The antioxidant activities were the same in the fruit and the vinegar, except in the ORAC assay, which showed a significant decrease during the acetification process. The results showed that using the wild yeast strain native to the persimmon produced vinegars with higher antioxidant activity than that of an inoculated alcoholic fermentation. Finally, a comparison between our vinegars and other commercial examples was made. The TPI and antioxidant activity values of persimmon vinegars were always higher than those obtained from white and red-wine vinegars. The antioxidant activity and total phenols of the final product indicate that persimmon vinegar is a competitive product in the market.

1. Introduction

Currently, consumer interest in the health benefits of foods is increasingly important, motivating more research in this area in recent years. Furthermore, consumers are demanding value-added products with new characteristics; therefore, the purpose of many investigations has been to elaborate new products providing health benefits. The main raw materials used to obtain these new products are fruits and vegetables. Several studies have shown a negative correlation between the consumption of fruits and vegetables and risks for cardiovascular disease, cancer, inflammation or problems associated with ageing (Dillard & German, 2000; Garcia-Closas, Gonzalez, Agudo, & Riboli, 1999; Joseph et al., 1999; Prior & Cao, 2000; Steinmetz & Potter, 1996; Wargovich, 2000).

Each year a large fraction of every fruit harvested is discarded because their size is outside the standard range, deformations or overproduction. For this reason, we proposed a study of the utilisation surplus fruit for vinegar production. Persimmon was one of the fruits selected for this purpose; it is mainly consumed fresh and the processing industry is scarcely developed. Persimmon is widely consumed in China and traditionally used for medicinal purposes such as coughs, hypertension, dyspnoea, paralysis, burns and bleeding (Mowat, 1990). It has also been demonstrated to have an inhibitory effect on human lymphoid leukaemia cells (Achiwa, Hibasami, Katsuzaki, Imai, & Komiya, 1997), and in some persimmon varieties such as *Mopan* a positive effect on hypercholesterolemia has been reported (Gorinstein et al., 1998). It is assumed that these "nutraceutical" properties are due to the antioxidant components of this fruit, including phenolic compounds (Yokosawa & Okumura, 2007), vitamins and carotenoids.

There are many methods available for the evaluation of antioxidant activity; most are colorimetric assays, so it is necessary to have a sample or extract free of solid particles. Sometimes an extraction method is required due to sample consistency. The established techniques for the extraction of antioxidant compounds differ in some parameters such as the kind of solvent used, but the main objective of the extraction stage is always to recover as much of the bioactive fraction as possible with the highest efficiency (Spigno, Tramelli, & De Faveri, 2007a). Previous studies have reported the influence of several parameters (ultrasonication time, solvent type, temperature and percentage of extractant) in the extraction of phenolic molecules and antioxidant compounds in general (Alothman, Bhat, & Karim, 2009; Pinelo, Del Fabbro, Manzocco, Núñez, & Nicoli, 2005a; Spigno et al., 2007a).

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Table 1
Samples description.

Type of sample	Treatment/Fermentation	Sample codex
Puree	No treatment	K7Z1
Puree	Pectolytic enzymes and sulphur dioxide	K7Z2
Wine	From K7Z2 by spontaneous alcoholic fermentation	K7WE1-K7WE3
Wine	From K7Z2 by inoculated alcoholic fermentation	K7WI1-K7WI3
Vinegar	From K7WE made by spontaneous acetification	K7VE1-K7VE3
Vinegar	From K7WI made by spontaneous acetification	K7VI1-K7VI3

The aim of this work was the evaluation of the antioxidant activity and total phenols index of persimmon vinegar[1] at each production step in a double fermentation process (alcoholic and acetic); the effect of spontaneous versus inoculated alcoholic fermentation on these parameters was of special interest. For this purpose, an extraction method was designed in which the following variables were optimised: the kind of solvent, solvent-to-water ratio and ultrasonication time. Finally, the values obtained for our vinegars were compared with some commercial vinegars.

2. Materials and methods

2.1. Samples

In this work we have employed three different persimmon (*Diospyros kaki var. Sharoni*) batches. Persimmons were harvested at commercial ripeness in November, 2007. This variety belongs to the group of non astringent persimmon. Batch 1 and batch 2, were acquired in the market and employed for the extraction process optimization. The batch 3, provided by Agromedina company, was used for the vinegar production. The elaboration process was performed in the laboratories of the Department of Biochemistry and Biotechnology (Faculty of Enology, University Rovira i Virgili, Tarragona), according to the following procedure: ~50 kg of persimmon fruit was crushed with a beater to obtain 45 L of puree. 60 g/L of sulphur dioxide were added to avoid undesirable microbial growth. Additionally, two pectolytic enzymes were incorporated: Depectil extra-garde FCE® for volatiles release and Depectil clarification® to help clarify the product (Martin Vialatte Oenologie, Epernay, France), both at a concentration of 15 mg/L. This puree was then distributed into six glass vessels, with 6 L of sample in each. Three of these vessels were inoculated with the enological yeast QA23 at the concentration of 2×10^6 cells/mL and a spontaneous alcoholic fermentation was allowed to occur in the other three vessels. The resulting wines were acetified by a spontaneous process to produce the persimmon vinegars. At each fermentation stage, samples were taken (Table 1). Samples were stored in 30-mL amber glass flasks at −20 °C until analysis.

For solvent and percentage selection we used puree prepared in our laboratory from persimmon batch 1 and for the ultrasonic extraction time selection we have employed puree from persimmon batch 2.

2.2. Chemicals

The reagents acetone, methanol, Folin-Ciocalteu reagent, ethanol, anhydrous dipotassium hydrogen phosphate, sodium dihydrogen phosphate monohydrate, potassium chloride, sodium acetate and anhydrous sodium carbonate were provided by Merck (Darmstadt, Germany). Fluorescein sodium and gallic acid were supplied by Fluka (Madrid, Spain). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid ("Trolox"), 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) free-radical were purchased from Sigma–Aldrich (Steinheim, Germany).

2.3. Sample-extraction process

Due to the different consistencies of the samples studied, it was necessary to establish an extraction system for the determination of total phenols index and antioxidant activity. To design the extraction method, we modified the procedures proposed by Gorinstein et al. (1999) and Chen, Fan, Yue, Wu, and Li (2008). Optimisation of the most influential parameters in the extraction method was required; the parameters optimised were type of solvent (acetone, methanol or ethanol), percentage of solvent (50%, 80% or 100%) and ultrasonic extraction time (15, 25, 35 or 50 min). The selection of the best extraction parameters was made by taking into consideration the maximum values obtained in each assay as well as economy of time and solvent use. The extraction conditions are shown in Fig. 1.

2.4. Antioxidant-activity assays

2.4.1. Oxygen-radical absorbance-capacity assay (ORAC-FL)

ORAC-FL was performed in a black 96-well microplate (BD Falcon, BD Biosciences, UK), following the method described by Dávalos, Gómez-Cordovés, and Bartolomé (2004) with some modifications. This assay was realised with a Multidetection plate reader (Synergy HT, Vermont, USA). Previously, fluorescein (60 nM) and appropriate dilutions of the samples were prepared along with solutions of different Trolox concentrations (0.5, 2, 3.5, 5, 6.5, 8,

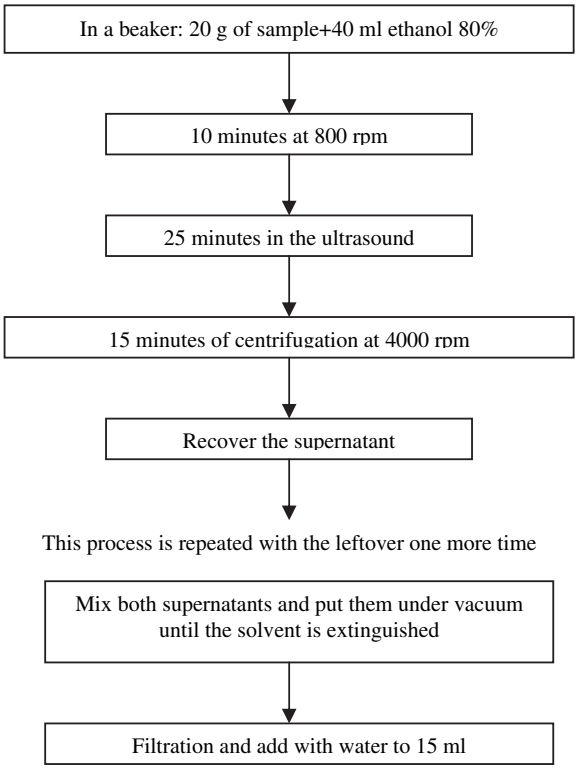

Fig. 1. Extraction process.

[1] Footnotes: Given the acidic nature of these products and the lack of a suitable alternative term, we decided to refer to these products as vinegars throughout the text, despite the fact that according to Spanish regulations some of these products are not sufficiently acidic to be classified as vinegars.

9.5 μM) used to construct the calibration curve, all in 75 mM phosphate buffer (pH 7.4). First, the wells at the edges of the microplate were filled with 200 μL of buffer to moderate the temperature throughout the assay. Then all of the wells containing 50 μL of the sample (buffer, Trolox or assay sample) at the required dilution plus 50 μL fluorescein were preincubated for 15 min at 37 °C. Afterwards, 50 μL of fresh AAPH (15 mM in phosphate buffer) was rapidly added to the reaction using a multichannel pipette. Fluorescence measures were taken at intervals of 5 min over a period of 90 min. The excitation wavelength was set at 485 nm and the emission wavelength at 528 nm. All the reaction assays were realised in triplicate. The results are expressed as the area under the curve (AUC) as calculated by the Cao and Prior (1999) equation:

$$\text{AUC} = (0.5 + f_5/f_0 + f_{10}/f_0 + f_{15}/f_0 + \ldots f_{90}/f_0) \times 5$$

where f_0 is the initial fluorescence and f_i is the fluorescence at time i (minutes).

The final AUC values were calculated by subtracting the AUC of the blank from all of the results. For each experiment, a blank was assayed and a calibration line with different Trolox concentrations was made to obtain the regression equation and calculate the ORAC-FL final values, expressed as μmol Trolox equivalents (TE)/kg of sample.

2.4.2. DPPH radical-scavenging assay

The DPPH method employed to determine the radical-scavenging capacity of each sample was based on Brand-Williams, Cuvelier, and Berset (1995). Here, 0.1 mL of appropriately diluted sample was added to 3.9 mL of DPPH solution (0.025 g/L in methanol). The absorbance of the mixtures was measured at 515 nm using a cuvette filled with methanol as a blank. Readings were taken at $t_i = 0$ (the time of sample addition) and $t_f = 60$ min (when the reaction reached steady state). A UV/vis spectrophotometer U-2800 Digilab coupled to a Peltier themostatic system (Hitachi,Tokyo, Japan) was used. Six different concentrations of Trolox (0.02, 0.06, 0.1, 0.14, 0.18 and 0.22 mM) were used in the same sample conditions to construct a calibration curve. The antiradical activity was calculated by considering the variation of the absorbance obtained, given by:

$$\text{Absorbance variation} = \text{Abs}_{t=60} - \text{Abs}_{t=0}$$

This absorbance variation was plotted versus the concentration of Trolox, the regression equation obtained and the sample values found by extrapolation. The final values were expressed as μmol Trolox equivalents (TE)/kg of sample. All the determinations were realised at least in triplicate.

2.5. Total phenols index (TPI)

This parameter was determined using the Folin-Ciocalteu method following the procedure of Waterhouse (2001). The concentrations of standards chosen to create the regression line were 50, 75, 100, 125, 150, 200, 250 and 500 mg/L of gallic acid. The absorbance of each coloured mixture was determined at 765 nm against a blank (distilled water). The assays were performed in triplicate and the results expressed as gallic acid equivalents (mg/L).

2.6. Statistical analysis

All statistical analyses were performed using the Statistica version 7.0 software package (Statsoft, Tulsa, USA).

3. Results and discussion

3.1. Optimisation of the extraction process

The criteria selected for optimisation of the extraction parameters (solvent, percentage of solvent and ultrasonication time) were the maximum values of antioxidant activity, total phenolics, and time and solvent savings.

3.1.1. Selection of the solvent

Despite being probably the most investigated parameter, solvent selection is still a complicated issue because extract yields and resulting antioxidant activities of the sample are strongly dependent on the nature of the extracting solvent. This is due to the presence of different antioxidant compounds of various chemical characteristics and polarities that may or may not be soluble in a particular solvent (Sultana, Anwar, & Ashraf, 2009). In our case, the solvents selected for the assays were acetone, methanol and ethanol. In these assays we used for all cases a mixture solvent: water at 80% and set an ultrasonication time of 15 min.

Fig. 2 shows that when we used ethanol as the solvent we got the best results in the ORAC assay (3630 μmol TE/kg) and TPI determination (330 mg gallic acid/kg). These values were significantly different from those obtained with acetone and methanol. These results may be explained based on the composition of persimmon, this kind of fruit contains different compounds (polyphenols, carotenoids, sugars, polysaccharides, vitamins, etc.) which provide antioxidant activity having different solvent affinity and response to the selected assays.

Using acetone as the extractant, we obtained the maximum antioxidant capacity in the DPPH assay (1730 μmol TE/kg). Significant differences were found between these values and those with the other solvents. However, this solvent gave the worst results in the case of the TPI determination and ORAC assay. With methanol, the extracts obtained the worst results for the DPPH assay and intermediate values for the ORAC and TPI assays.

3.1.2. Effect of solvent percentage

Some studies have suggested that the recovery of phenols is dependent on the fruit type and the kind and percentage of solvent used (Alothman et al., 2009). Because ethanol was the best solvent, we assayed aqueous solutions with the following percentages of ethanol: 50%, 80% and 100%, the ultrasonication time was set at 15 min. As shown in Fig. 3, maximum values for all of the parameters studied were obtained with the solvent:water ratio of 80:20. Our results are in agreement with those of Sultana et al. (2009), who evaluated methanol and ethanol and their mixtures with

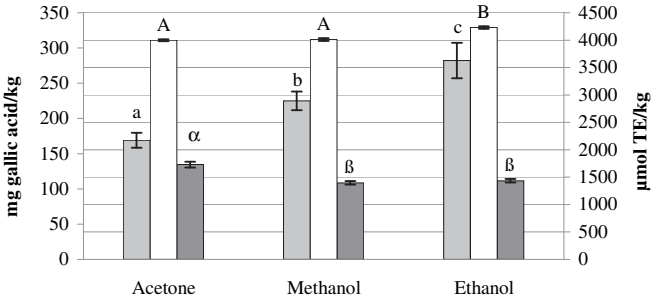

Fig. 2. ORAC, DPPH and TPI values of persimmon puree (batch 1) for the different extraction solvents tested □ TPI (mg gallic acid/kg); ■ DPPH (μmol TE/kg); □ ORAC (μmol TE/kg). The bars in the same assay with different letters show significant differences ($p < 0.05$) (a, b, c: ORAC assay; A, B, C: IPT; α, β, γ: DPPH test). ORAC and DPPH values are on the right axis and TPI values are on the left.

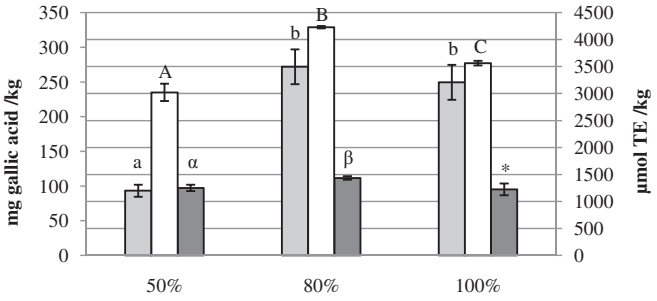

Fig. 3. ORAC, DPPH and TPI values of persimmon puree (batch 1) for the different solvent percentages tested □ TPI (mg gallic acid/kg); ■ DPPH (µmol TE/kg); ▨ ORAC (µmol TE/kg). The bars in the same assay with different letters show significant differences ($p < 0.05$); *: no significant differences with α and β (a, b, c: ORAC assay; A, B, C: IPT; α, β, γ: DPPH test). ORAC and DPPH values are on the right axis and TPI values are on the left.

water (80%) as extraction solvents for medicinal plants. Aqueous solutions exhibited better antioxidant activities and higher phenolic contents. Pinelo et al. (2005a) also concluded that mixtures of alcohols and water showed better recoveries of phenolic compounds than the corresponding monocomponent solvent systems. Accordingly, in the ethanol extraction of grape-seed powder, Yilmaz and Toledo (2005) reported an increase in extracted phenol content (as gallic acid equivalents) when they increased the amount of water in the mixture from 0% to 30%; phenol contents remained constant for 30, 40 and 50% water and decreased at higher percentages.

3.1.3. Impact of ultrasonication time

The mechanical effects and the acoustic cavitations produced in the solvent by the passage of an ultrasound wave allow for better penetration of the solvent into the sample matrix (Rostagno, Palma, & Barroso, 2003; Wang, Sun, Cao, Tian, & Li, 2008). Hence, the duration of ultrasonication is an important parameter to optimise. The best results with respect to antioxidant capacity were obtained using 25 min of ultrasonication, yielding 3595 (ORAC) and 1230 (DPPH) µmol of TE/kg, respectively (Fig. 4). With respect to TPI, the values were very similar, with no significant differences among the values at different sonication times. The TPI was 264.3 mg/kg of gallic acid at 25 min.

Extraction times longer than 25 min produced significant decreases for the parameters measured. These results agree with those of previous studies on the extraction of flavonoids from plants. Zhang, Shan, Tang, and Putheti (2009) tested different extraction times (20, 25, 30, 35, and 40 min) and the recovery of flavonoids decreased as ultrasonication time was extending beyond 25 min. On the other hand, the acoustic cavitations of ultrasound produce a progressive increase of temperature in the internal structure of the sample. Some authors (Pinelo, Rubilar, Jerez, Sineiro, & Nuñez, 2005b; Spigno & De Faveri, 2007b; Yilmaz & Toledo, 2005) have pointed out that temperature increases can denatured some phenolic compounds, so this fact could explain the loss of antioxidant activity too.

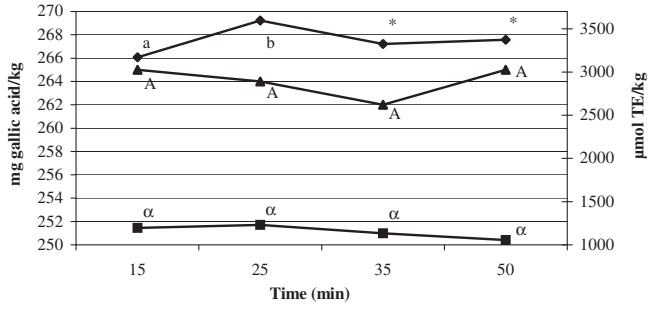

Fig. 4. ORAC, DPPH and TPI values persimmon puree (batch 2) for the different ultrasound times tested —▲— TPI (mg gallic acid/kg) —◆— DPPH (µmol TE/kg) —■— ORAC (µmol TE/kg). The markers in the same assay with different letters show significant differences ($p < 0.05$); *: no significant differences with a and b (a, b, c: ORAC assay; A, B, C: IPT; α, β, γ: DPPH test). ORAC and DPPH values are on the right axis and TPI values are on the left.

3.1.4. Evaluation of the extraction process

To test the efficiency of our extraction method we compared it with a centrifugation process. A persimmon wine sample was divided into two equal portions. One of them was subjected to a centrifugation process and the other was extracted by our method, the final volume of the obtained liquid extracts by both methods were adjusted to 15 mL. We then measured, the antioxidant activity and total phenols index of the supernatants collected from the centrifuged and the extracted samples. The ORAC, DPPH and TPI results using our extraction method was about 70%, 50% and 20% higher, respectively, than with a simple centrifugation.

3.2. Evolution of the antioxidant activity and total phenolics during the production of persimmon vinegars

After the optimisation of the extraction process, antioxidant activity and total phenols index were measured in the fruit puree, wine and vinegar samples.

3.2.1. Substrates

For the production of persimmon vinegars, the starting substrate was a puree of this fruit (batch 3). After obtaining the puree, sulphur dioxide and pectolytic enzymes were added. As can be observed in Table 2, this addition had technological benefits and a positive effect on the antioxidant character of the persimmon puree, increasing it and the phenols in solution. Phenols have been reported to be linked to cell-wall polysaccharides by hydrophobic interactions and hydrogen bonds. The release of these phenols may be improved by cell-wall degradation catalysed by enzymes (Pinelo, Arnous, & Meyer, 2006). The polysaccharides liberated from the cell-wall by the addition of pectolytic enzymes have antioxidant activity, as has been reported by several authors (Aguirre, Isaacs, Matsuhiro, Mendoza, & Zuniga, 2009; Chattopadhyay et al., 2009; Chen, Tsai, Huang, & Chen,

Table 2
Values of ORAC, DPPH and TPI for purees, wines and vinegar analyzed.

Sample	ORAC[a]	DPPH[a]	TPI[b]
Puree			
K7Z1	1891 ± 106	1289 ± 22	277 ± 22
K7Z2	2841 ± 66	1540 ± 39	424.1 ± 2.4
Wine			
K7WE1	2542 ± 215	1758 ± 75	288 ± 13
K7WE2	3192 ± 341	1838 ± 15	295.6 ± 2.2
K7WE3	3557 ± 232	1870 ± 162	320.6 ± 6.6
K7WI1	2816 ± 195	1421 ± 134	245 ± 20
K7WI2	3142 ± 282	1649 ± 88	300.3 ± 4.4
K7WI3	3637 ± 70	1699 ± 44	300.3 ± 4.4
Vinegar			
K7VE1	2111 ± 1	1731 ± 64	317.5 ± 5.1
K7VE2	1894 ± 334	1615 ± 18	268.0 ± 3.2
K7VE3	1854 ± 205	1698 ± 88	273.2 ± 1.9
K7VI1	1780 ± 12	1627 ± 88	303.6 ± 4.5
K7VI2	2022 ± 182	1457 ± 64	384.8 ± 5.5
K7VI3	1479 ± 29	1482 ± 53	397.5 ± 3.9

[a] Expressed in µmol TE/kg.
[b] Expressed as mg gallic acid/kg.

2009). Moreover, phenols and compounds with antioxidant activity confined in the vacuoles inside the cell could be released; this is the case with grapes (Pinelo et al., 2006). Conversely, sulphur dioxide may act through two different pathways: as a protector against oxidation (Delteil, Feuillat, Guilloux-Benatier, & Sapis, 2000) and as a phenol extractor (Lee & Wrolstad, 2004). One possible explanation was given by Cacace and Mazza (2002), who reported that the addition of SO_2 reduced the dielectric constant of water and consequently increased the solubility of phenols, but the mechanism remains unknown.

3.2.2. Wines

Wines were obtained by two different kinds of alcoholic fermentations: spontaneous and inoculated. The measured values of the three parameters varied among the three replicates from the same type of fermentation (Table 2). For this reason, we did not find significant differences between the inoculated and spontaneous wines with respect to ORAC and TPI, although the average antioxidant activity of inoculated wines was higher. However, for the DPPH assay, the spontaneous wines were significantly higher than the inoculated ones. The inoculation seemed to have no significant impact on persimmon purees with respect to TPI. Moreover, the contrary behaviour of the ORAC and DPPH assays did not allow us to come to a clear conclusion regarding the merits of inoculation of the substrates versus spontaneous fermentation.

3.2.3. Vinegars

Vinegars were obtained from spontaneous and inoculated wines by spontaneous acetification. With respect to the ORAC assay, values for the vinegars from spontaneous wines were higher than those from inoculated wines, but there was no significant difference between them (Table 2). DPPH values for vinegars from spontaneous wines were significantly higher than in the vinegars from the inoculated ones. Concerning the total phenols determination, vinegars from inoculated wines had significantly higher amounts of total phenols than the vinegars from spontaneous wines.

In summary, after the acetification process, vinegars from inoculated wines had higher TPI, whereas the vinegars from spontaneous wines had higher antioxidant activity. It should be noted that the two fermentations (alcoholic and acetic) took place in succession and therefore acetification was carried out in the presence of yeast lees, which might explain the variations in TPI and antioxidant activity values. In microbiological characterization of the whole process, differences were just found in the alcoholic fermentation. The results revealed that different kind of yeasts carried out the alcoholic fermentations: spontaneous and inoculated (data not shown). In spontaneous alcoholic fermentation are involved mainly strains of non-Saccharomyces yeast and the strain used in inoculated was not detected. The yeast may influence in two different ways: capturing polyphenols (Mazauric & Salmon, 2005; Razmkhab et al., 2002) and releasing antioxidant compounds, differently than the polyphenols, from inside cell and from cell-wall (Aredes-Fernández et al., 2010; Jaehrig, Rohn, Kroh, Fleischer, & Kurz, 2007). The way in which the yeast influences the antioxidant activity depends on the yeast strain. Since this was the only factor that varied between both processes this may be the reason for the different values of TPI and antioxidant activity found.

3.2.4. Overall changes

The changes in the studied parameters between the substrate and the vinegars are shown in Fig. 5. Regarding the ORAC assay, we observed an overall decrease of 34.6% from fruit (K7Z2) to vinegar. DPPH values also showed an increment during the alcoholic fermentation followed by a decrease after the acetic fermentation, resulting in an overall increase of 3.8%. With respect to the total phenols index, there was a decrease from the substrate to the wines and an increase from the wine to the final vinegar, for an overall increase of 1.6%. The final content of polyphenols was similar to that obtained by others for Hiratanenashi persimmon vinegar (Sakanaka & Ishihara, 2008).

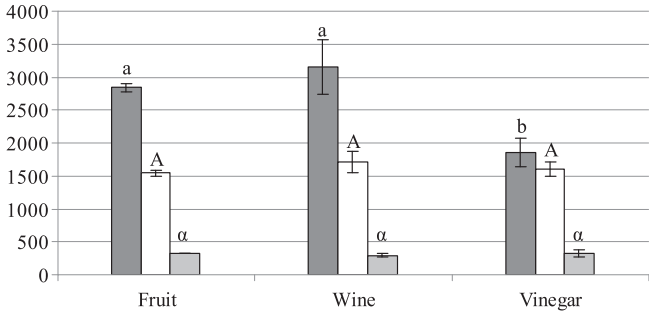

Fig. 5. Evolution of antioxidant capacity parameters (ORAC and DPPH) and TPI in the production process of persimmon vinegars (means values of K7Z2 substrates, wines set and vinegars set) ▒ TPI (mg gallic acid/kg) □ DPPH (μmol TE/kg) ▒ ORAC (μmol TE/kg). The bars in the same trial with different letters show significant differences ($p < 0.05$) (a, b, c: ORAC assay; A, B, C: IPT; α, β, γ: DPPH test). ORAC and DPPH values are on the right axis and TPI values are the left.

A likely explanation for the different behaviours of the ORAC and DPPH assays might be the different reaction mechanisms of the substances in the reaction medium. The ORAC assay is a hydrogen-atom-transfer (HAT) reaction which quantifies hydrogen-atom donor capacity, whereas the DPPH method is a single-electron-transfer (ET) reaction which measures the antioxidant reducing capacity (Huang, Ou, & Prior, 2005). The overall balance was positive because only the ORAC underwent a significant decrease while DPPH and TPI had constant values.

3.3. Comparison with commercial vinegars

Several common vinegars were selected from the market to compare them with our persimmon vinegars. In Table 3, it can observed that the average antioxidant values of our vinegars were lower than balsamic, sherry and cider vinegars but always higher than red and white wine vinegars.

The antioxidant activity and total phenols index values in our persimmon vinegars were lower than those reported by previous authors (Sakanaka & Ishihara, 2008). This may be because the persimmon varieties used in their study are astringent, so they have tannins in their composition and the persimmon used to produce our vinegars is a variety non astringent and have a lesser content of tannins. Several studies have shown that tannins are the components mainly responsible for the antioxidant activity of persimmons (Gu et al., 2008).

Table 3
Antioxidant activities and TPI values of different kinds of commercial vinegars and mean values of our persimmon vinegars.

Sample	ORAC[a]	DPPH[a]	TPI[b]
Balsamic vinegar	40049 ± 663	8842 ± 163	2539 ± 6
Apple vinegar	8986 ± 106	2036 ± 75	343 ± 10
Sherry vinegar	7879 ± 270	2066 ± 23	467 ± 6
Persimmon vinegar[c]	1857 ± 220	1601 ± 111	324 ± 55
Red wine vinegar	1462 ± 3	1229 ± 66	229 ± 16
White wine vinegar	973 ± 153	939 ± 29	137 ± 10

[a] Expressed in μmol TE/kg.
[b] Expressed as mg gallic acid/kg.
[c] Mean values of K7VE1, K7VE2, K7VE3, K7VI1, K7VI2 and K7VI3.

4. Conclusions

We determined that in the case of *Diospyros kaki var. Sharoni* the use of 80% ethanol and 25 min of ultrasonication were the best conditions among the variables assayed to obtain the greatest extraction of phenolic compounds and the highest values of antioxidant activity. The addition of sulphur dioxide and pectolytic enzymes had a positive effect on the antioxidant activity and total phenols index.

Comparing the two kinds of alcoholic fermentation, the spontaneous wines produced vinegars with higher antioxidant activity than the inoculated wines. Therefore, the isolation of the yeast strains involved in the spontaneous alcoholic fermentation and their use in this production process could be an important issue in improving the antioxidant activity of these vinegars.

The DPPH and TPI values remained constant during the processing from the fruit to the final vinegar, and the ORAC assay showed significant decrease after acetification. The antioxidant activity of the final vinegars was lower than what was reported by other authors because the variety used in this work belongs to the group of non astringent persimmons; however, it possessed higher values than other commercial vinegars like white and red-wine vinegars. These results suggest that persimmon vinegar has health-promoting qualities and could be a competitive product in the commercial market.

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Article 2

Determination of major volatile compounds during the production of fruit vinegars by static headspace gas chromatography–mass spectrometry method

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ABSTRACT

A static headspace gas chromatography coupled to mass spectrometry (SHS–GC–MS) method was validated to determine several major volatile components during the production process of fruit vinegars. The method is simple, fast, linear in the working range, suitably sensitive, repeatable and reproducible, and has a good degree of accuracy for most of the compounds studied. Different conditions were tested in the production process of vinegars by means of double fermentation. The addition of SO_2 and pectolytic enzymes produced a considerable increase in methanol and acetaldehyde, especially in strawberry purees, whereas pressing led to a loss of these volatile compounds. In the alcoholic fermentation of persimmon and strawberry purees, the *Saccharomyces cerevisiae* strain used had a great influence on the production of acetaldehyde and higher alcohols in wines. Considering the influence of these studied compounds in the final profile of the vinegars, our results showed that the *S. cerevisiae* strain isolated in this study produced the most suitable wine substrates for the production of vinegars. Moreover, semisolid fruit substrate provides better results than liquid substrate. Inoculated acetification in wood recipients yielded vinegars with a better volatile profile, as these contained higher levels of most compounds except acetaldehyde.

1. Introduction

Vinegar is one of the most widespread and common products in the world because it is available in every country in several different varieties (Mazza & Murooka, 2009). The traditional use and integration of vinegars in numerous cultures can be traced back to ancient times. Today, the most widely marketed vinegar is wine vinegar, although vinegar can be produced from a variety of very different raw materials.

In today's market, there is a growing demand for fruit vinegar sold as a health food product (Ou & Chang, 2009). This consumer trend has led to the development of new products with the aim of expanding the range of vinegars available on the market. Furthermore, the production of these vinegars provides a use for surpluses of second quality fruit.

Different quality parameters should be studied in selecting the best production procedure for new fruit vinegars. Such parameters should include volatile compounds responsible for aroma and close attention should be paid to which of these compounds might be influenced by the production process.

Aroma is certainly one of the most important determinants of food quality and acceptance. The particular aroma of vinegar is the result of high quantities of volatile compounds. These compounds may come from the raw material or may be formed during the production process. Different authors have pointed out the importance of the production process in the final aroma of vinegars and therefore in their organoleptic qualities (Morales et al., 2001; Callejón et al., 2009). Moreover, the content of several major volatile compounds found in vinegar such as methanol is restricted by Spanish legislation (<1 g/L) (Presidencia del Gobierno, 1993).

Gas chromatography coupled to a mass spectrometry detector is widely used in the study of volatile compounds. To analyse these constituents in a liquid sample, the sample is introduced into a gas chromatograph, the volatile components are evaporated, and their vapour is carried through the column by the mobile phase (Ettre, 2002). However, the non-volatile matrix remains in the injector, thereby contaminating it. Researching volatile components present in a solid sample is even more complicated. This type of sample obviously cannot be introduced into an instrument; it requires an elaborate sample preparation procedure that includes extracting the volatile components, among other steps (Ettre, 2002).

Headspace is a fast, simple, efficient and environmentally friendly sampling method used with capillary GC for the analysis of volatile fractions in many food samples. Headspace (HS) is essentially a sampling method that permits analysts to take an aliquot of the gas phase in equilibrium with a liquid or solid phase (Ettre, 2002). During static HS analysis, equilibrium between the sample and the headspace above is achieved, and a fraction of this headspace gas phase is

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withdrawn for GC analysis (Bylaite & Meyer, 2006). In equilibrium, the distribution of the analytes between the two phases depends on their partition coefficients. The composition of the original sample can therefore be established from the analytical results of this aliquot (Ettre, 2002).

Static HS-GC works well with high precision and accuracy for liquid samples since calibration can be performed easily by either external or standard addition without any serious problems (Li et al., 2009). With static headspace sampling, sample headspace volatiles are automatically brought directly to the GC, thus offering good validation as well as the possibility for a high number of samples to be processed (Sriseadka et al., 2006). The main disadvantage of static HS-GC compared to dynamic HS-GC is its relatively low sensitivity (Snow & Slack, 2002). However, sensitivity can be increased by salting-out, pH control or increasing the equilibration temperature during sample heating (B'Hymer, 2003). Static headspace GC is mostly useful for applications in the high-ppb to percent concentration ranges (Wang et al., 2008). In the headspace analysis, parameters such as temperature and equilibrium time, headspace volume and instrumental conditions must be carefully standardized (Ariseto & Toledo, 2008).

The overall goal of this work was to develop and to optimize a simple and fast method based on GC–MS to monitor the evolution of major volatile compounds in the production process of fruit vinegars. Firstly, to monitor changes in these compounds a sampling method had to be selected that was suitable for all three products studied: raw material (fruit puree), fruit wine and fruit vinegar[1], which all have very different consistencies. We decided to test headspace sampling. Next, we optimized the static headspace sampling and injection conditions. Finally, the method was successfully applied to determine the major volatile compounds in these kinds of matrices.

2. Materials and methods

2.1. Chemicals and reagents

All the chemicals used were analytical-reagent grade and provided from the following sources: acetaldehyde, methyl acetate, methanol, ethyl acetate, 1-propanol, isobutanol, isoamyl acetate, 2-methyl-1-butanol, 3-methyl-1-butanol, ethanol, acetic acid and 4-methyl-2-pentanol (IS) from Merck (Darmstadt, Germany); sodium chloride from Sigma–Aldrich (Madrid, Spain); and water from a Milli-Q purification system (Millipore, Bedford, USA).

2.2. Standards and sample preparation

6 g of sample saturated in sodium chloride (2 g) and 10 μL of internal standard (391 μg kg^{-1}) were placed into a 20 mL HS vial and sealed immediately with a white silica/PETF lined septum and aluminium crimp cap (VWR International Eurolab S.L., Barcelona, Spain) and then placed in the autosampler tray for HS sampling.

A standard mix was used to establish the best injection volume. A dearomatised fruit puree spiked with standards was used to select sample incubation temperature and time. Fruit was dearomatised as follows: 5 mL of dichloromethane were added to 20 g of fruit puree. This mixture was stirred with a stir bar over night, and then was centrifuged at 4000 rpm for 10 min and the dichloromethane was withdrawn. This procedure was repeated. To eliminate remains of dichloromethane, the puree was submitted to a nitrogen stream for 20 min. After this, 5 mL of acetone were added and the mixture was stirred for 3 h, followed by centrifugation (4000 rpm for 10 min), the solvent was withdrawn and a nitrogen stream was subsequently applied for 20 min. We spiked a commercial fruit puree and vinegar with the analytes for repeatability, intermediate precision and recovery assays.

2.3. Vinegars production and samples studied

Fruit processing and pre-treatment was performed as follows: fruit was crushed with a beater; 60 mg L^{-1} of sulphur dioxide were added to prevent the growth of undesirable micro-organisms; 15 mg L^{-1} of each of two kinds of pectolytic enzymes (Depectil extra-garde FCE® and Depectil clarification® from Martin Vialatte Oenologie, Epernay, France), were then added to the puree. 50 g L^{-1} and 75 g L^{-1} of sucrose were also added to 2008 and 2009 strawberry puree respectively to ensure an appropriate final acidity in the resulting vinegar. Samples of fruit puree were taken before and after the addition. One portion of 2008 strawberry fruit puree was pressed to study the effect of two types of starting substrates (semisolid and liquid) (Table 1).

The alcoholic fermentation of the fruit substrate was similar in persimmons and 2008 strawberries and slight modifications were made in the case of 2009 strawberries. 6 L of fruit puree was distributed into various glass recipients: six for persimmons, eight for 2008 strawberries (four of purees and two of liquid substrate) and eight for 2009 strawberries. These recipients were then divided into two groups: half of them were inoculated with the oenological yeast *Saccharomyces cerevisiae* QA23 at a concentration of 2×10^6 cells mL^{-1}, and spontaneous alcoholic fermentation was allowed to take place in the other half. The inoculated fermentation in the 2009 strawberries was performed with the yeast strain *S. cerevisiae* RP1, isolated during the spontaneous alcoholic fermentation of the 2008 strawberry puree.

Acetification was carried out in glass vessels by spontaneous processes except for strawberry wines from the 2009 harvest. These wines were acetified in three different containers: a glass vessel, and oak and cherry wood barrels. Each of them was filled with 5.5 L of wine. All the wine obtained from inoculated alcoholic fermentation was mixed and dispensed in the recipients mentioned earlier and inoculated with acetic acid bacteria. The wines from spontaneous alcoholic fermentation were processed in the same way and acetified spontaneously.

All vinegars obtained in 2007 and 2008 were pressed. Additionally two different final treatments were applied to strawberry vinegars from the 2008 harvest: some were centrifuged and others pasteurized. Strawberry vinegars from 2009 were only pasteurized. The 2007 persimmon vinegars presented an average acetic degree between 4.4 (from inoculated wines) and 4.5 (from spontaneous wines). The acetic acid contents average in 2008 strawberry vinegars were 4.8 (from spontaneous wines) and 4.9 (from inoculated wines). Finally, inoculated vinegars from 2009 harvest reached an acetic degree of 5.5 (glass vessel), 6.6 (oak barrel) and 6.3 (cherry barrel).

Furthermore, part of the puree from the 2009 strawberries was concentrated by heating to test another form of increasing the sugar content and prevent having to add it in; the resulting product was a cooked must (Table 1). One liter of this substrate was fermented by a spontaneous process and 1 L by inoculating it with RP1 strain yeast. Finally, the inoculated wines were acetified by adding the selected acetic acid bacteria and the spontaneous wines were left to acetify spontaneously.

Different samples were taken throughout these production processes and a total of 53 samples were analysed: 6 fruit purees and 1 liquid substrate, 22 wines and 24 vinegars. All the samples were stored in 30 mL amber glass flasks at −20 °C until the analysis. The codes and characteristics of the samples are shown in Table 1.

2.4. Optimization of static headspace conditions and method validation

Several headspace conditions were optimized: spit ratio, injection volume, time and temperature of incubation. Different split ratios

[1] Given the acidic nature of these products and the lack of a suitable alternative term, we have decided to refer to these products as vinegars throughout the text, despite the fact that according to Spanish regulations, some of these products are not sufficiently acidic to be classified as vinegars.

Table 1
Treatment and codex of samples.

Fruit and harvest	Treatment	Puree sample	Treatment	Substrate sample	Alcoholic fermentation	Wine sample	Acetification	Treatment o recipient	Vinegar sample
Persimmon 2007	Crushed	K7Z1	SO2 Pectolytic enzymes	K7Z2	Inoculated	K7WI1–K7WI3	Spontaneous	Pressing Centrifugation	K7VE1–K7VE3
					Spontaneous	K7WE1–K7WE3	Spontaneous	Pressing Centrifugation	K7VI1–K7VI3
Strawberry 2008	Crushed	F8P1	SO2 Pectolytic enzymes sucrose	F8P2	Inoculated	F8WI1–F8WI3	Spontaneous	Centrifugation	F8SVI1C–F8SVI2C
								Pasteurization	F8SVI1P–F8SVI2P
					Spontaneous	F8WE1–F8WE3		Centrifugation	F8SVE1C–F8SVE2C
								Pasteurization	F8SVE1P–F8SVE2P
	–	F8P2	Pressing	F8L	Inoculated	F8LWI	–	–	–
					Spontaneous	F8LWE			
Strawberry 2009	Crushed	F9P1	SO2 Pectolytic enzymes sucrose	F9P2	Inoculated	F9WI1–F9WI4	Inoculated	Glass vessel	F9SVIG
								Oak barrel	F9SVIO
								Cherry barrel	F9SVIX
					Spontaneous	F9WE1–F9WE4	Spontaneous	Glass vessel	F9SVEG
								Oak barrel	F9SVEO
								Cherry barrel	F9SVEX
		–	Heating Concentrated	F9MC	Inoculated	–	Inoculated	Glass vessel	F9MCVI1–F9MCVI2
					Spontaneous	–	Spontaneous	Glass vessel	–

(2, 5, 10, 15, 20 and 40) and injection volumes (250 and 350 μL) were tested.

We studied different incubation times (10, 20, 30 and 40 min) and temperatures (55, 65, 75 and 85 °C). A sample of commercial fruit puree was spiked with all the compounds studied for these trials. The quantities added were roughly 25 mg kg^{-1} except for ethyl acetate, which was 150 mg kg^{-1}.

The method was validated with respect to linearity, sensitivity (LOQ), precision (repeatability and intermediate precision) and accuracy.

The quantification limits were obtained injecting successive dilutions of standards and were calculated as the concentration which would result in a signal-to-noise ratio higher than or equal to 10. These values were determined for liquid and semisolid matrices.

Repeatability and intermediate precision were checked using a dearomatised commercial fruit puree and vinegar spiked with the analytes. These spiked samples were injected six times in a single day for the repeatability assay and three times a day on six different days for the intermediate precision assay. The results, expressed as relative standard deviation (%RSD).

The accuracy of the method was evaluated only in the case of vinegar since the calibration lines were built using hydroacetic solutions instead of a real matrix. A commercial vinegar was spiked with standards at three levels of concentration.

2.5. Static headspace GC–MS instrumentation and conditions

Analyses were conducted using an Agilent 6890 GC system coupled to an Agilent 5975inert quadrupole mass spectrometer and equipped with a Gerstel MP2 headspace autosampler (Müllheim an der Ruhr, Germany).

Static headspace equilibration was performed at 65 °C for 20 min, while a low shaking at 250 rpm was applied during sample heating. 350 μL of headspace gas were injected using a heated (85 °C) gastight syringe (1 mL) in split mode 10:1. The split/splitless inlet temperature was 200 °C. Syringe injection speed was 50 μL s^{-1}.

Separation was performed on a CPWax-57CB column (50 m × 0.25 mm, 0.20 μm film thickness, Varian, Middelburg, The Netherlands). The carrier gas was He at a constant flow rate of 1 mL/min. The column oven temperature was initially set at 35 °C for 5 min, and then was increased to135 °C at 4 °C min^{-1} and then at 10 °C min^{-1} to 200 °C and held for 5 min.

The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 250 °C, respectively. Electron ionization mass spectra in SIM mode were recorded at 70 eV electron energy. A solvent delay of 3.0 min was used and the following ions were monitored: 31, 43, 44, 45, 55, 57, 61 and 74. All data were recorded using an MS ChemStation. The samples were analyzed in triplicate and blank runs were done before and after each analysis.

2.6. Qualitative and quantitative analyses

Compounds were identified based on the comparison of the retention times of individual standard and computer matching with the reference mass spectra from the NIST 98 library. Acquisition was performed in selected ion monitoring mode (SIM). Initially, standard solutions and several samples were analysed in full scan mode (mass range: 29–350 amu). These data were acquired to identify the compounds and determine appropriate ions for the later acquisition in SIM mode.

The quantitative determination of volatile compounds was performed by using the relative area calculated as the ratio between the target ion of each compound and the internal standard (4-methyl-2-pentanol). Calibration curves at seven levels and three replicates per level were built by adding a standard mixture of all compounds in both matrices: a commercial dearomatised fruit puree enriched with ethanol and hydroacetic solution. This procedure was performed in keeping with that described in Mestres et al. (2002) in order to obtain a matrix that was as representative as possible and to ensure that the calibration graphs were applicable to the majority of the real sample. The range of the calibration curves was chosen to cover the possible concentrations in real samples (Tables 2 and 3).

2.7. Statistical analysis

All statistical analyses were performed using Statistica software (StatSoft, 2001). One-way ANOVA was used to evaluate significant differences (significance levels $p<0.05$).

A principal component analysis (PCA) was carried out as an unsupervised method in order to ascertain the degree of differentiation between samples and which compounds were involved. Data were auto-scaled before PCA.

3. Results and discussion

The main aim of this work was to explore the possibility of using the headspace sampling method in major volatile GC–MS analysis. Headspace gas chromatography (HS-GC) is a powerful technique for the analysis of volatile compounds in food and non-food products (Linssen et al., 1995). There are many instrumental parameters of the headspace autosampler that can affect the sensitivity, precision and

Table 2
Analytical characteristics of the method for vinegar.

Compound	Retention time (min)	m/z	Linear range (mg kg^{-1})	r^2	LOQ (μg kg^{-1})	Added (mg kg^{-1})	Recovery (%)	Mean recovery (%)	Repeatability (%RSD)	Intermediate precision (%RSD)
Acetaldehyde	3.95	44	1–200	0.998	0.30	37	65.1	68.0 ± 3.6	1.88	3.80
						50	67.0			
						62	72.0			
Methyl acetate	5.01	74	2–500	0.998	0.15	15	103.5	102.3 ± 2.0	2.64	4.10
						20	100.0			
						25	103.5			
Ethyl acetate	6.03	61	74–2002	0.9995	0.095	450	82.0	73.7 ± 7.3	3.90	1.60
						600	70.8			
						750	68.3			
Methanol	6.54	31	10–700	0.9992	4.0	150	90.0	88.5 ± 2.5	1.65	2.22
						200	90.0			
						250	85.6			
Propanol	10.8	31	1–75	0.9999	0.24	3.37	90.3	88.8 ± 3.3	2.09	3.00
						4.50	91.0			
						5.62	85.0			
Isobutanol	12.7	43	1–124	0.9998	0.21	9	96.0	102.6 ± 6.9	1.54	1.97
						12	109.7			
						15	102.0			
Isoamyl acetate	13.3	55	0.57–20.5	0.9999	0.015	0.375	84.1	83.5 ± 6.2	4.92	5.20
						0.500	89.4			
						0.625	77.0			
2-Methyl-1-butanol	16.9	57	1–75	1.000	0.11	2.62	102.7	98.1 ± 4.1	0.87	2.52
						3.50	95.0			
						4.37	96.5			
3-Methyl-1-butanol	17.0	55	1–76	0.9993	0.13	10	99.0	108.2 ± 9.0	2.54	3.97
						14	108.7			

accuracy of static headspace analysis. We therefore optimized this sampling technique by evaluating the effect of the following parameters: injection volume, temperature and equilibrium time. The addition of salt into the aqueous extract determined an increment of the ionic strength for the analytes resulting in an increase of their diffusion into the headspace and of the sensitivity (Pawliszyn, 1997). Although the effect of salting-out may play a key role in headspace sampling, taking into account our previous work (Callejón et al., 2008) in which the saturation of samples with salt gave the best results, it was not considered among parameters to optimize and we decided to use an enough amount of sodium chloride to saturate the samples. Good chromatographic data, maximum recovery, sensitivity, and time saving were selected as criteria for optimization. The method was then validated and, finally, applied to the analysis of real samples.

3.1. Optimization of static headspace conditions: the effect of injection volume, equilibrium temperature and time

Among the different split ratios tested, the lowest (2:1 or 5:1) provided poorly defined peaks and the highest resulted in small peaks. The best results were obtained with 350 μL injection volume and a 10:1 split ratio.

After the injection conditions were selected, we studied the incubation parameters. As shown in Fig. 1, we found that the higher the extraction time, the lower all relative areas of chromatographic peaks. However, no significant differences were found among relative areas obtained between 10 and 20 min of extraction. Between 10 and 30 min we found significant differences for isoamyl acetate, and between 10 and 40 min for ethyl acetate and isoamyl acetate. Therefore, we considered 20 min to be an appropriate extraction time. On the other hand, incubation temperature showed different trends depending on the compound (Fig. 2). Relative areas of 1-propanol and 2-methyl-1-butanol clearly increase as temperature rises. However, the values of relative areas for ethyl acetate, isoamyl acetate and acetaldehyde decrease as temperature increases. These decreases begin to be statistically significant for isoamyl acetate when the temperature rises from 65° to 75 °C.

An increase in temperature entailed a loss of sensitivity in some of the compounds studied; because no significant losses were observed at 65 °C, this is the incubation temperature we chose. In summary, the best incubation conditions were established at 20 min at 65 °C.

3.2. Method validation

The method was evaluated with respect to linearity, sensitivity (LOQ), precision (repeatability and intermediate precision) and

Table 3
Analytical characteristics of the method for wine and puree of fruit.

Compound	Linear range (mg kg^{-1})	r^2	LOQ (mg kg^{-1})	Repeatability (%RSD)	Intermediate precision (%RSD)
Acetaldehyde	1–200	0.9986	4.63	4.85	5.75
Methyl acetate	0.9–170	0.9982	2.77	3.12	4.19
Ethyl acetate	61–4500	0.9960	3.1	4.24	4.30
Methanol	51–3000	0.9991	38.1	4.26	5.19
Propanol	1–200	0.9989	2.40	4.96	6.00
Isobutanol	1–200	0.9991	1.54	4.70	6.88
Isoamyl acetate	0.05–10.4	0.9989	0.17	2.86	7.08
2-Methyl-1-butanol	1–200	0.9989	0.27	6.93	8.15
3-Methyl-1-butanol	1–202	0.9967	0.30	0.83	5.73

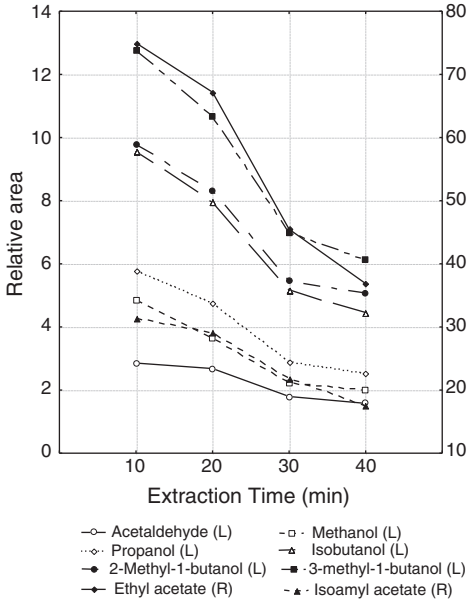

Fig. 1. Optimization of headspace conditions. Effect of incubation time on relative areas of volatiles compounds.

accuracy. The relationship between detector response measured in terms of relative area and amount of standard was linear as suggested by the correlation coefficient obtained (0.996–1.000). The linearity ranges, the equation of linear regression and the correlation coefficient are shown in Tables 2 and 3.

The quantification limits obtained were low enough to quantify the different kinds of samples of this study.

Repeatability and intermediate precision results are in agreement with the values proposed by AOAC (1993) for both kinds of matrices (fruit puree and vinegar).

The recovery percentage obtained in the accuracy assays ranged between 68.0 and 108.2. In general, a good degree of accuracy was achieved for most of the compounds, except for acetaldehyde and ethyl acetate.

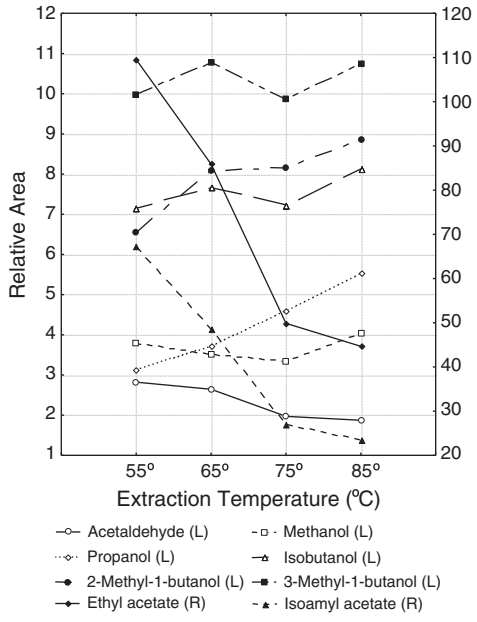

Fig. 2. Optimization of headspace conditions. Effect of incubation temperature on relative areas of volatiles compounds.

3.3. Sample analysis

The optimized method was applied to study the changes in nine major volatile compounds throughout the production process of fruit vinegars. These products were obtained through a double fermentation process (alcoholic and acetic). Different conditions were tested at each stage of production. We will discuss the results considering the effect of each stage on the concentration of these compounds. They are involved directly in the aroma of products because they either provide particular aromatic notes such as ethyl acetate or isoamyl acetate or contribute to the overall aromatic profile. Moreover, some of them are also precursors of other volatile compounds present in vinegars. For example, acetaldehyde undergoes condensation reactions to produce acetoin, a volatile compound characteristic of vinegar. On the other hand, vinegars have a considerable content of volatile acids formed from higher alcohols, especially isovaleric acid from 3-methyl-1-butanol. This alcohol is also a precursor of isoamyl acetate.

3.3.1. Pre-treatments of fruit puree

Methanol was the most abundant compound in the initial fruit puree, especially in the persimmon puree (Tables 4–6). The addition of SO_2 and pectolytic enzymes gave rise to a notable increase in this compound (about 100 mg kg^{-1}) in the strawberry samples. Added pectolytic enzymes act as hydrolysing pectins releasing methoxyl groups and producing an increase in methanol, as Ribéreau-Gayon et al. (2006) described for red wines. The second compound that underwent a considerable change in concentration was acetaldehyde. This aldehyde is a natural aroma component in almost all fruits. This compound appears as a result of fruit metabolism during ripening (Pesis, 2005). In our case, the fruit puree (persimmon and strawberry) presented values between 5.4 and 10.4 mg kg^{-1}. These amounts increased after the addition of SO_2 and pectolytic enzymes, especially in the strawberry samples. In grape must, SO_2 combines with acetaldehyde to form a stable compound (Ribéreau-Gayon et al., 2006). Therefore, the addition of this substance may cause a loss of acetaldehyde. However, we observed an increase, leading us to deduce that pectolytic enzyme may favour the release of acetaldehyde. This effect seems to be stronger than the loss caused by combination with SO_2.

The remaining compounds increased in most cases, the highest changes were found in the strawberry samples except for methyl acetate, which mainly increased in persimmon puree.

One portion of strawberry puree from the 2008 harvest was pressed to obtain a liquid substrate. The pressing process resulted in a decrease in all the compounds (Table 5), especially ethyl acetate and acetaldehyde, which diminished by up to 80%.

3.3.2. Alcoholic fermentation

Two types of alcoholic fermentations were performed. One part of the fruit puree was spontaneously fermented and the other part was inoculated with a selected strain of *S. cerevisiae* yeast.

In general, as can be seen in Tables 4–6, the higher alcohols increased in all cases as expected; in some cases, reaching concentrations close to the lowest values of the content range found in grape wine (Ribéreau-Gayon et al., 2006). During alcoholic fermentation, yeast can synthesize these compounds through two metabolic pathways, one of which is amino acid metabolism (Ribéreau-Gayon et al., 2006; Bayonove et al., 2000). Just as occurs in grape wines, the higher alcohol that reached the largest amounts was 3-methyl-1-butanol (Romano et al., 2003; Garde-Cerdán & Ancín-Azpilicueta, 2007).

If we compare the two kinds of fermentations, the inoculated alcoholic fermentation of persimmon puree produced higher alcohol contents than spontaneous fermentation, except for isobutanol, which reached a similar concentration in both types of fermentations. However, in 2008 strawberry wines produced by spontaneous fermentation

Table 4
Changes in volatile compounds during the elaboration of persimmon vinegars.

Samples	Mean concentration of compounds (mg kg^{-1}) ± SD								
	Acetaldehyde	Methyl acetate	Ethyl acetate	Methanol	1-Propanol	Isobutanol	Isoamyl acetate	2-Methyl-1-butanol	3-Methyl-1-butanol
K7Z1	10.4 ± 0.3	9.5 ± 0.9	n.q.	343 ± 9	2.27 ± 0.01	1.340 ± 0.003	0.1177 ± 0.0004	0.140 ± 0.004	n.q.
K7Z2	28.2 ± 1.9[a]	18.1 ± 1.7[a]	n.q.	376 ± 39	2.97 ± 0.08[a]	1.99 ± 0.05[a]	0.140 ± 0.004	0.31 ± 0.02[a]	n.q.
K7WE1	32.1 ± 1.9	36.1 ± 1.3[b]	1221 ± 45[b]	551 ± 17[b]	8.9 ± 0.6[b]	15.8 ± 1.3[b]	0.94 ± 0.03[b]	7.69 ± 0.25[b]	27.98 ± 1.04[b]
K7WE2	25.1 ± 3.2	34 ± 3[b]	1046 ± 107[b]	554 ± 41[b]	8.5 ± 0.5[b]	15.6 ± 1.3[b]	0.82 ± 0.06[b]	8.5 ± 0.4[b]	33 ± 3[b]
K7WE3	30.8 ± 1.9	42.2 ± 0.7[b]	1459 ± 17[b]	758 ± 18[b]	11.10 ± 0.05[b]	20.5 ± 0.5[b]	1.33 ± 0.08[b]	8 ± 1[b]	38 ± 4[b]
K7WI1	39.2 ± 0.7[b,c]	38.8 ± 0.9	1094 ± 58[b]	581 ± 40[b]	14.8 ± 1.2[b,c]	15.3 ± 0.9[b]	1.31 ± 0.15[b]	10.466 ± 0.024[b,c]	40.6 ± 0.5[b,c]
K7WI2	40.47 ± 0.14[b,c]	67 ± 5	1942 ± 90[b]	695 ± 6[b]	15.46 ± 0.15[b,c]	16.67 ± 0.03[b]	2.87 ± 0.19[b]	10.93 ± 0.03[b,c]	42.1 ± 0.3[b,c]
K7WI3	36.8 ± 1.6[b,c]	47.8 ± 0.9	1354 ± 140[b]	539 ± 74[b]	16 ± 2[b,c]	16.3 ± 1.7[b]	1.86 ± 0.07[b]	9.3 ± 0.9[b,c]	41 ± 3[b,c]
K7VE1	37 ± 3	103 ± 7[b]	1447 ± 152[b]	471 ± 42	3.07 ± 0.07[b]	7.01 ± 0.03[b]	1.25 ± 0.16	5.19 ± 0.11[b]	16.0 ± 0.6[b]
K7VE2	32.81 ± 0.19	79.89 ± 0.17[b]	1203 ± 24[b]	444 ± 31	3.42 ± 0.14[b]	7.59 ± 0.15[b]	0.89 ± 0.04	5.3 ± 0.4[b]	17.9 ± 0.3[b]
K7VE3	47 ± 3	86 ± 6[b]	1278 ± 100[b]	464 ± 12	3.37 ± 0.07[b]	8.17 ± 0.13[b]	0.90 ± 0.07	5.91 ± 0.16[b]	17.5 ± 0.3[b]
K7VI1	61 ± 4	86.10 ± 5.03[b]	1094 ± 59[d]	374 ± 19[b,d]	4.8 ± 0.1[b,d]	5.83 ± 0.04[b,d]	0.9980 ± 0.0001	4.9 ± 0.4[b]	17.55 ± 0.24[b]
K7VI2	33.4 ± 2.1	67 ± 4[b]	921 ± 70[d]	326 ± 22[b,d]	4.47 ± 0.15[b,d]	5.38 ± 0.13[b,d]	0.89 ± 0.03	5.14 ± 0.07[b]	17.1 ± 0.3[b]
K7VI3	38.1 ± 2.4	87 ± 6[b]	1024 ± 84[d]	385 ± 8[b,d]	4.169 ± 0.002[b,d]	5.11 ± 0.12[b,d]	0.95 ± 0.12	4.6 ± 0.1[b]	15.3 ± 0.3[b]

n.q.: concentration under quantification limit.
[a] Significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p<0.05$) with respect to its substrate (ANOVA).
[c] Significant differences ($p<0.05$) with respect to spontaneous process (ANOVA).
[d] Significant differences ($p<0.05$) with respect to the vinegars obtained from spontaneous wines (ANOVA).

were richer in isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol than inoculated wines, with the latter containing higher levels of 1-propanol than those produced with spontaneous fermentation. Persimmon and strawberry purees were inoculated with the same yeast strain, but the only common trend found was the production of 1-propanol in greater proportion than any other higher alcohol. This alcohol is synthesized by yeast in relation to the metabolism of amino acid sulphur (Bayonove et al., 2000). Otherwise, the observed increases in 2-methyl-1-butanol and 3-methyl-butanol in the inoculated processes were similar in both substrates. These results suggest that the production of 1-propanol could be further conditioned by the type of substrate and the production of the other two alcohols by the yeast strain. Ibarz et al. (2005), pointed out that the production of higher alcohols in grape wines depends on both factors: the yeast and must used.

Interestingly, the results of the 2009 wines showed opposite changes in higher alcohols to those observed in 2008 wines, being these changes for the inoculated 2009 wines similar to the 2008 spontaneous wines and vice versa (Tables 5 and 6). As explained in Section 2.3, the yeast strain used in the production of 2009 inoculated strawberry wines was isolated from 2008 spontaneous wines. Therefore, the strain involved in the fermentation process has a strong influence on the end levels of these compounds in wines (Torrea et al., 2003; Ribéreau-Gayon et al., 2006).

Methanol levels increased in persimmon and 2008 strawberry during alcoholic fermentation, although these differences were only statistically significant in the case of persimmon. Methanol is a non-fermentative alcohol; therefore, the only source of this compound during alcohol fermentation is the hydrolysis of pectins. In these reactions, ester bonds between galacturonic acid and methanol are

Table 5
Changes in volatile compounds during the elaboration of strawberry vinegars in harvest 2008.

Samples	Mean concentration of compounds (mg kg^{-1}) ± SD								
	Acetaldehyde	Methyl acetate	Ethyl acetate	Methanol	1-Propanol	Isobutanol	Isoamyl acetate	2-Methyl-1-butanol	3-Methyl-1-butanol
F8P1	9.9 ± 0.5	6.9 ± 0.6	96 ± 10	190 ± 14	4.46 ± 0.11	11.6 ± 0.5	0.249 ± 0.004	3.4 ± 0.1	15.5 ± 0.9
F8P2	52.9 ± 2.2[a]	9.30 ± 0.11[a]	140 ± 7[a]	292. ± 9[a]	5.97 ± 0.14[a]	16.9 ± 0.7[a]	0.360 ± 0.007[a]	5.3 ± 0.4[a]	22.5 ± 1.2[a]
F8L	7.2 ± 0.3[d]	1.93 ± 0.08[d]	53 ± 2[d]	244 ± 7[d]	3.51 ± 0.09[d]	10.3 ± 0.2[d]	0.107 ± 0.015[d]	3.7 ± 0.4[d]	12 ± 9[e]
F8LWE1	9.2 ± 0.6	2.1 ± 0.4[e]	59 ± 9[e]	489 ± 33	49.9 ± 2.1	65.0 ± 2.4[e]	0.96 ± 0.07[e]	16 ± 2[e]	80.9 ± 1.8[e]
F8LWI1	27.4 ± 2.4[c,e]	2.9 ± 0.4[e]	86 ± 6[c,e]	530 ± 14[e]	90.3 ± 0.4[c]	40.44 ± 0.08[c,e]	0.678 ± 0.024[c,e]	11.0 ± 0.3[c]	72.0 ± 1.1[c,e]
F8WE1	21.8 ± 0.9[b]	9.2 ± 0.7	170 ± 11	462 ± 9	37 ± 2[b]	83 ± 3[b]	0.95 ± 0.06[b]	34.0 ± 0.3[b]	108.3 ± 0.6[b]
F8WE2	19.0 ± 0.6[b]	7.62 ± 0.22	127 ± 5	290.1 ± 2.3	24.9 ± 0.9[b]	61.2 ± 2.3[b]	1.14 ± 0.08[b]	24.3 ± 1.3[b]	80.1 ± 1.9[b]
F8WE3	19.8 ± 1.4[b]	8.1 ± 0.4	129 ± 11	303 ± 22	26.0 ± 1.7[b]	69 ± 7[b]	0.969 ± 0.021[b]	26.2 ± 1.9[b]	92 ± 7[b]
F8WI1	51 ± 5[c]	8.47 ± 0.09	173 ± 6[b]	305 ± 3[b]	43.9 ± 0.8[b,c]	33.9 ± 0.4[b,c]	1.382 ± 0.007[b,c]	14.4 ± 0.3[b,c]	62 ± 5[b,c]
F8WI2	46 ± 4[c]	8.9 ± 0.5	184 ± 11[b]	317 ± 16[b]	44.8 ± 1.7[b,c]	35 ± 1[b,c]	1.50 ± 0.12[b,c]	12.1 ± 0.7[b,c]	64 ± 3[b,c]
F8WI3	52.5 ± 1.4[c]	9.07 ± 0.25	207 ± 17[b]	327 ± 28[b]	45 ± 4[b,c]	34.3 ± 2.4[b,c]	1.52 ± 0.15[b,c]	12.3 ± 1.6[b,c]	58.0 ± 1.9[b,c]
F8SVE1C	34.3 ± 0.4[b]	17.7 ± 0.5[b]	439 ± 31[b]	259 ± 13	4.40 ± 0.09[b]	11.93 ± 0.04[b]	0.610 ± 0.023[b]	5.74 ± 0.21[b]	13.7 ± 0.4[b]
F8SVE1P	40 ± 4[b]	19.4 ± 1.9[b]	483 ± 47[b]	246 ± 33	4.3 ± 0.3[b]	11.7 ± 0.5[b]	0.61 ± 0.08[b]	5.1 ± 0.3[b]	13.7 ± 0.3[b]
F8SVE2C	75.9 ± 3.0[b]	13.00 ± 0.11[b]	368 ± 15[b]	195 ± 12	4.16 ± 0.23[b]	11.6 ± 0.6[b]	0.46 ± 0.07[b]	5.47 ± 0.16[b]	14.2 ± 0.6[b]
F8SVE2P	79 ± 4[b]	14.2 ± 0.9[b]	374 ± 34[b]	181 ± 14	4.05 ± 0.13[b]	11.4 ± 0.3[b]	0.31 ± 0.03[b]	5.4 ± 0.3[b]	14.4 ± 0.3[b]
F8SVI1C	67.9 ± 2.3[b]	14.0 ± 0.5[b]	374 ± 11[b]	174 ± 10[b]	6.78 ± 0.24[b,c]	5.32 ± 0.22[b,c]	0.271 ± 0.003[b,c]	3.0 ± 0.4[b,c]	9.7 ± 0.4[b,c]
F8SVI1P	77.4 ± 2.3[b]	16.5 ± 0.9[b]	446 ± 24[b]	180 ± 5[b]	7.3 ± 0.4[b,c]	5.8 ± 0.3[b,c]	0.251 ± 0.013[b,c]	2.98 ± 0.19[b,c]	10.5 ± 0.8[b,c]
F8SVI2C	89.2 ± 1.6[b]	15.50 ± 0.19[b]	424.11 ± 2.23[b]	179 ± 5[b]	7.72 ± 0.25[b,c]	5.87 ± 0.19[b,c]	0.260 ± 0.016[b,c]	2.93 ± 0.03[b,c]	10.85 ± 0.15[b,c]
F8SVI2P	98 ± 4[b]	18.2 ± 0.8[b]	498 ± 34[b]	181 ± 15[b]	8.24 ± 0.13[b,c]	6.3 ± 0.3[b,c]	0.248 ± 0.014[b,c]	2.9 ± 0.3[b,c]	11.6 ± 0.3[b,c]

[a] Significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p<0.05$) with respect to its substrate (ANOVA).
[c] Significant differences ($p<0.05$) with respect to spontaneous process (ANOVA).
[d] Significant differences ($p<0.05$) with respect to F8P2 sample (ANOVA).
[e] Significant differences ($p<0.05$) with respect to semisolid wines obtained with similar alcoholic process (spontaneous or inoculated) (ANOVA).

Table 6
Changes in volatile compounds during the elaboration of strawberry vinegars in harvest 2009.

Samples	Mean concentration of compounds (mg kg^{-1}) ± SD								
	Acetaldehyde	Methyl acetate	Ethyl acetate	Methanol	1-Propanol	Isobutanol	Isoamyl acetate	2-Methyl-1-butanol	3-Methyl-1-butanol
F9P1	5.4 ± 0.1	3.21 ± 0.05	n.q.	159 ± 12	2.34 ± 0.02	1.371 ± 0.011	0.118 ± 0.001	0.211 ± 0.002	n.q.
F9P2	95 ± 8[a]	4.6 ± 0.4[a]	n.q.	293 ± 31[a]	3.47 ± 0.12[a]	2.60 ± 0.08[a]	0.133 ± 0.001	1.37 ± 0.07[a]	3.9 ± 0.3[a]
F9WE1	65.1 ± 0.7[b]	11.5 ± 0.3[b]	639 ± 11[b]	237 ± 10[b]	14.4 ± 0.5[b]	25.0 ± 0.7[b]	2.94 ± 0.04[b]	16.5 ± 0.5[b]	48.8 ± 0.9[b]
F9WE2	55.1 ± 0.6[b]	12.4 ± 0.1[b]	761 ± 8[b]	254 ± 11[b]	15.0 ± 0.5[b]	26.0 ± 0.8[b]	2.85 ± 0.04 [b]	13.4 ± 0.4[b]	48.6 ± 1.3[b]
F9WE3	44 ± 1[b]	11.3 ± 0.4[b]	667 ± 31[b]	239 ± 8[b]	14.4 ± 0.4[b]	25.0 ± 0.4[b]	2.66 ± 0.18[b]	15.19 ± 0.11[b]	47.6 ± 0.7[b]
F9WE4	49 ± 4[b]	11.1 ± 0.7[b]	633 ± 47[b]	222 ± 3[b]	13.6 ± 0.3[b]	23.9 ± 0.3[b]	2.55 ± 0.11[b]	12.69 ± 0.15[b]	46.0 ± 0.5[b]
F9WI1	23.6 ± 1.3[b,c]	4.72 ± 0.07[c]	n.q.	303 ± 4	12.81 ± 0.22[b,c]	69.7 ± 0.5[b,c]	2.64 ± 0.06[b]	52.7 ± 1.3[b,c]	171 ± 7[b,c]
F9WI2	25.1 ± 1.9[b,c]	4.45 ± 0.15[c]	n.q.	279 ± 16	12.05 ± 0.22[b,c]	67.6 ± 1.1[b,c]	2.60 ± 0.17[b]	42.4 ± 0.8[b,c]	167 ± 10[b,c]
F9WI3	23.2 ± 1.3[b,c]	4.02 ± 0.12[c]	n.q.	235 ± 5	11.1 ± 0.1[b,c]	59.4 ± 0.7[b,c]	1.98 ± 0.06[b]	39 ± 3[b,c]	152 ± 5[b,c]
F9WI4	20.0 ± 0.6[b,c]	4.52 ± 0.03[c]	n.q.	277 ± 12	11.9 ± 0.5[b,c]	67.2 ± 2.4[b,c]	2.72 ± 0.08[b]	44 ± 3[b,c]	173 ± 11[b,c]
F9SVEG	1.43 ± 0.07	7.0 ± 0.5	45 ± 5	120 ± 1	0.71 ± 0.01	1.569 ± 0.022	n.q.	2.111 ± 0.003	2.739 ± 0.004
F9SVEO	23.6 ± 0.6	16.2 ± 0.5	148 ± 5	165.6 ± 0.4	1.16 ± 0.01	3.036 ± 0.012	0.065 ± 0.007	2.914 ± 0.008	5.64 ± 0.07
F9SVEX	63.15 ± 0.11	14.22 ± 0.02	439 ± 17	198.2 ± 1.1	2.001 ± 0.003	5.176 ± 0.014	0.158 ± 0.014	4.67 ± 0.07	9.5 ± 0.3
F9SVIG	129 ± 5	3.4 ± 0.3	83 ± 5	146.7 ± 0.9	1.493 ± 0.024	11.5 ± 0.3	0.27 ± 0.04	8.81 ± 0.22	27.0 ± 0.7
F9SVIO	42 ± 3	20.4 ± 1.4	682 ± 41	276 ± 5	2.364 ± 0.012	24.7 ± 0.9	1.4 ± 0.1	21.2 ± 0.6	47.5 ± 0.06
F9SVIX	64.4 ± 1.0	17.3 ± 1.1	663 ± 5	278 ± 16	2.82 ± 0.09	26.2 ± 0.8	1.282 ± 0.023	23.3 ± 1.0	52.1 ± 0.4
F9MCVI1	719 ± 58	22.8 ± 2.1	341 ± 17	318 ± 15	11.3 ± 0.4	9.9 ± 0.6	0.57 ± 0.03	9.1 ± 0.6	43 ± 3
F9MCVI2	410 ± 17	25.4 ± 2.1	452 ± 39	370 ± 27	15.1 ± 0.8	11.3 ± 0.4	0.65 ± 0.05	11.3 ± 1.0	48.9 ± 1.9

n.q.: concentration under quantification limits.
[a] Significant differences ($p<0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p<0.05$) with respect to its substrate (ANOVA).
[c] Significant differences ($p<0.05$) with respect to spontaneous process (ANOVA).

cleaved, releasing this alcohol into the medium, which is carried out by pectin esterases (Fernandez-Gonzalez et al., 2005). Several authors have shown that some *S. cerevisiae* strains have pectin-esterase activity (Pretorius & Van der Westhuizen, 1991; Gainvors et al., 1994; Fernandez-Gonzalez et al., 2005). Thus, the increase in methanol in this fermentative stage may have come from two possible hydrolytic pathways: due to the pectin-esterase activity of the yeast and/or to the pectolytic enzymes added to the substrate that continued to act.

Acetaldehyde is a secondary product of yeast alcoholic fermentation; it is produced during the first days of fermentation (Bosso & Guaita, 2008). This aldehyde increased in persimmon case, being slightly higher in inoculated fermentations than in spontaneous fermentations, although the changes were not statistically significant. Meanwhile, in strawberry alcoholic fermentation acetaldehyde values decreased, especially in spontaneous fermentation. Strawberries are rich in anthocyanins, which are responsible for the berry's red colour. In the production of red wines, these compounds undergo condensation reactions in which different molecules are linked by acetaldehyde bridges (Bosso & Guaita, 2008). These reactions involve a loss of this aldehyde. These types of reactions could explain the diminution of acetaldehyde in strawberry wine production. Opposing trends were found in terms of the final amount of this compound in strawberry wines depending on the year of harvest. In 2008, strawberry wines from inoculated fermentation, "inoculated wines," were found to have higher values than "spontaneous wines." However in 2009 strawberry wines, the highest results for acetaldehyde were found in spontaneous wines. As mentioned earlier, the yeast strain employed for the production of 2009 inoculated wines was the same as that used for 2008 spontaneous wines. Furthermore, these 2008 spontaneous wines and 2009 inoculated wines presented similar values for this compound. The influence of the *S. cerevisiae* strain on the differing production of acetaldehyde has been reported by several authors (Antonelli et al., 1999; Regodon et al., 2006).

Among the esters studied, the most abundant in our fruit wines was ethyl acetate followed by methyl acetate and isoamyl acetate, this last related to a fruity aroma.

Ethyl acetate is the most prevalent ester in grape wines (Ribéreau-Gayon et al., 2006). In persimmon puree, the concentration of this ester was below the quantification limit; however the wines presented extremely high levels compared to the normal values in grape wines (30–110 mg kg^{-1}, Regodon et al., 2006). In 2008 strawberry, ethyl acetate underwent a slight increase only during alcoholic fermentation in the inoculated wines. Although the starting concentrations in 2009 wines were very low (below the quantification limit), the wines obtained through spontaneous fermentation presented high concentrations (633–761 mg kg^{-1}) while in those obtained through inoculated fermentation this compound was not detected. Several authors have shown that the formation of esters during alcoholic fermentation is closely related to the enzymatic activity of the yeast strain (Barre et al., 2000). In keeping with this, we observed that this compound was not produced in the 2009 inoculated process and it was only produced in one case in 2008 spontaneous wines (Tables 5 and 6). The ester isoamyl acetate increased in all cases studied.

Methyl acetate is formed by the condensation of methanol and acetic acid. We found that during the alcoholic fermentation of persimmon the amount of this ester doubled. This is consistent with the high levels of methanol found in persimmon substrate.

This compound remained practically unchanged in strawberry wine production except in the case of the 2009 spontaneous process, in which the levels of methyl acetate concentration increased. Finally, all compounds were found to have increased in the alcoholic fermentation of strawberry liquid substrate. Figuring among the most outstanding changes, we might mention a considerable increase (up to 70%) in acetaldehyde, higher alcohols and isoamyl acetate. The liquid substrate was fermented in the absence of solid colorants so the binding reaction between acetaldehyde and monomeric anthocyanins did not frequently occur. This is a likely explanation for why levels of this aldehyde were found to increase in wines from this substrate. Furthermore, the largest increase in acetaldehyde occurred in inoculated alcoholic fermentation. We observed the same behaviour for higher alcohols as in the fermentation of semisolid substrate, showing the highest contents of 1-propanol in inoculated wines and the other three higher alcohols in spontaneous wines. These results again indicate the relevance of the yeast strain in the production of higher alcohols.

Comparing the final content of the volatile compounds analysed in wines from different substrates (liquid and semisolid), it is clear that methanol and 1-propanol reached higher values in liquid wines than in wines from semisolid substrate. Wines from liquid resulted in

lower values of methyl and ethyl acetate than wines from the other type of substrate.

3.3.3. Acetic fermentation

In the acetic fermentation of persimmon wine, levels of acetaldehyde increased in most cases. In 2008 strawberry vinegar, concentrations of this compound increased in all cases. The transformation of ethanol to acetic acid takes place in two steps, with acetaldehyde being the intermediary product. These reactions can be performed by acetic acid bacteria as well as by chemical oxidation. When performed by a micro-organism, each step is catalyzed by different enzymes (alcohol deshidrogenase and aldehyde deshydrogenase, respectively). In chemical oxidation, the step from acetaldehyde to acetic acid depends on the presence of oxygen (Ribéreau-Gayon et al., 2006).

The acetification process in samples from the 2009 harvest was carried out in different containers (glass vessels, cherry and oak wood barrels). In the vinegar from glass vessels, we noticed a remarkable amount of acetaldehyde together with lower levels of ethanol and acetic acid than in vinegar produced in wood barrels. The main difference between these kinds of recipients is the better oxygen transference that occurs through wood pores. This might suggest that ethanol is being transformed into acetaldehyde while the second reaction is not taking place at a similar rate, probably due to the lower proportion of oxygen in the glass vessel. This result coincides with that reported by other authors on the accumulation of this aldehyde during acetification due to oxygen impoverishment (Polo & Sanchez-Luengo, 1991). Acetaldehyde tends to accumulate under low oxygen conditions instead of being oxidized to acetic acid (Zoecklein et al., 1995). Furthermore, we have observed increases in acetaldehyde in previous studies during glass bottle aging of red vinegars in which acetification and aging processes took place simultaneously (Callejón et al., 2010). And during accelerated aging in glass vessels with wood chips we observed an increase in acetaldehyde due to the chemical oxidation of ethanol (Tesfaye et al., 2004). Although these studies prove that the accumulation of acetaldehyde in vinegars can take place by means of the two pathways mentioned earlier (microbiological or chemical oxidation), in our case, microbiological transformation is the most likely cause of the accumulation of this compound.

The samples from cherry wood barrels had higher concentrations of acetaldehyde than those from oak barrels, regardless of the type of acetification. This compound may be released into the liquid medium from this type of wood, as this phenomenon has been observed in white wine vinegars aged in different kinds of wood (oak, cherry, chestnut and acacia) (Callejón et al., 2010).

A loss of higher alcohols occurred during the acetification stage. Callejón et al. (2009) showed that acetic acid bacteria consume other alcohols apart from ethanol, with 3-methyl-1-butanol being the most frequently consumed followed by isobutanol and 2-methyl-1-butanol, in keeping with the abundance order in the substrate. In our case, a similar behaviour was observed, and in agreement with these authors, the pattern of higher alcohols consumption varied depending on the abundance of these alcohols in the starting wines. In other words, the higher the concentration of the alcohol, the more it was consumed.

The 2009 strawberry wines were divided into two groups: one underwent spontaneous fermentation and the other was inoculated with acetic acid bacteria. In the inoculated processes the vinegars reached 6°Ac while spontaneous processes they only reached 4°Ac as a consequence of the unexpected halt of the acetification process. Therefore, in terms of the changes in higher alcohols, the consumption of these compounds was more pronounced in vinegars produced using selected acetic acid bacteria.

Although the consumption of methanol by acetic acid bacteria has not been previously reported, the acetification process implied a decrease in this alcohol. Generally, these micro-organisms have a defence mechanism that transforms alcohols into less toxic products such esters. Persimmon vinegars showed a reduction in the concentration, with about 150 mg kg^{-1}, and a similar diminution was observed for 2008 strawberry samples. In the 2009 acetification processes, spontaneous fermentation produced a larger decrease in methanol than did inoculated fermentation and this difference was more pronounced in samples produced in glass vessels. The concentration of methanol in all final products was below the legal level allowed for vinegars (Presidencia del Gobierno, 1993).

On the other hand, methanol is involved in the synthesis of methyl esters, in this case, especially of methyl acetate. We observed higher levels of methyl acetate in persimmon vinegars, and as in alcoholic fermentation, during the production process the content of this ester doubled. In 2008 strawberry samples, acetic fermentation produced significant increases in this compound. However, these condensation reactions alone are not sufficient to explain the diminution of methanol mentioned earlier.

In samples from the 2009 harvest, both strawberry vinegars produced in glass vessels experienced a similar decrease in methyl acetate. However, an increase in methyl acetate was found in the vinegar produced in wood barrels, with slightly higher levels recorded in the case of oak barrels, which may be due to concentration phenomena. Furthermore, we might point out a considerable increase in inoculated processes in barrels. In general, despite the different evolutions observed, the final concentrations of methyl acetate in vinegars were correlated with initial concentrations of methanol ($r = 0.7$).

Different trends were found in levels of ethyl acetate, a characteristic compound of vinegar, which were especially conditioned by the fruit substrate used. In persimmon, the concentrations of this ester in the resulting vinegars were similar to those in wines and no clear tendency was observed (Table 4). In 2008 strawberry vinegars, ethyl acetate reached more than twice the concentration of that in wines. From the 2009 harvest, the vinegars obtained through inoculated acetification showed values between 83 for glass vessels and 663–682 for the others. This indicates a considerable formation along with a slight concentration of this compound in wood recipients. The results of the spontaneous acetifications in the 2009 samples were the opposite because a hydrolysis of ethyl acetate was taking place. This behaviour has been observed by several authors who have shown that the active consumption of ethanol by acetic acid bacteria induces the hydrolysis of most ethyl esters (Callejón et al., 2009).

Isoamyl acetate usually increases during surface acetification processes, however, in our vinegars in most cases it was found to diminish. This might be explained again by a hydrolysis reaction due to the consumption of alcohol 3-methyl-1-butanol by acetic acid bacteria.

Comparing the two final treatments applied to the 2008 strawberry vinegars, pasteurization and centrifugation, no statistically significant differences in the volatile compounds studied between them were found (Table 5).

Special vinegars were also produced for this study which used cooked strawberry must (Table 6). Only inoculated acetifications we obtained final products. The main difference in these heated strawberry vinegars was the high levels of acetaldehyde compared to vinegars obtained from uncooked strawberry fruit puree. These high levels would adversely affect the organoleptic properties of the end product.

3.4. Principal component analysis

The compounds studied underwent a series of changes during the production of the vinegars. Several principal component analyses were performed to evaluate whether these changes were great enough to distinguish the different samples obtained throughout the production process based on substrate, production stage or production method. In the case of persimmons, the PCA allowed us to

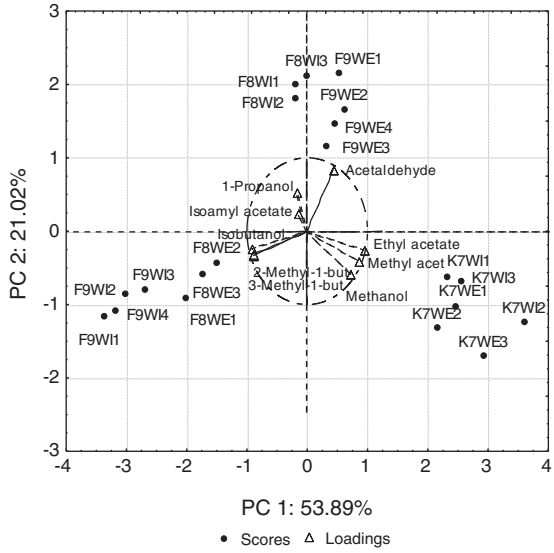

Fig. 3. Data scores and variable loadings plot on the plan made up of the first two principal components (PC1 against PC2) of wine samples.

separate the samples into three groups: the substrate, wines and vinegars, with the first three components accounting for 93.9% of the variance. Similar results were obtained when the PCA was applied to the 2008 strawberry sample data. However, in the products obtained from the 2009 harvest the separation was not so clear.

Moreover, this analysis was applied to the data of the strawberry puree substrates to study the influence of the addition of enzymes and SO_2. Each sample appears in a different quadrant in the plan of the two principal components. The PC1 is able to separate the substrates depending on the harvest and the PC2 separates the samples with and without treatment.

PCA of strawberry wines from 2008 harvest reveals that substrate pressing affects more than the inoculation. This is deducted from the samples separation into the plan of two first PC. The liquid wines inoculated and spontaneous appear in the same quadrant whist the group of wines from inoculated semisolid substrate are separated in different quadrant from the spontaneous group.

On the other hand, the result of this analysis on the data obtained from all the wine samples showed that the principal three components explained 92.6% of the variance. Data scores and variable loadings are plotted simultaneously into the plan made up of the first two principal components in Fig. 3. This figure shows that the samples are distributed into three groups. The figure shows that PC2 successfully separates the 2008 strawberry spontaneous and 2009 inoculated wines from the other strawberry wines. Thus, the wines obtained through the use of the same yeast strain appear together in the same quadrant. This reinforces the theory that the yeast strain has a strong influence on these compounds of the aromatic profile. We confirmed a high degree of association between strawberry wines inoculated with the RP1 strain and the production of higher alcohols such as 2-methyl and 3-methyl-1-butanol and isobutanol. Moreover, if we consider only the persimmon and 2008 strawberry wines, the PCA revealed that PC1 allows us differentiates between persimmon wines and strawberry wines and PC2 distinguishes between inoculated and spontaneous wines. PC1 was positively correlated with acetaldehyde, the three acetates and methanol, and PC2 was positively correlated with acetaldehyde, isoamyl acetate and propanol. In the analysis of the final vinegars, the score plot obtained by selecting the first two PCs as axes showed that the samples were distributed in three groups, one formed by persimmon vinegars, another which included 2008 strawberry vinegars and 2009 strawberry vinegars produced in a glass vessel, and a third group, very far from the previous ones, comprised of the 2009 strawberry vinegars produced in barrels. This shows the importance of the type of recipient in which the acetification is carried out on the final content of these compounds.

4. Conclusions

The headspace sampling method proposed has proved to be a valuable methodology for the determination of major volatile compounds during the production process of fruit vinegars. From a practical point of view, this method does not require any complicated sample preparation. The validation of the method was satisfactory, recovery values and limits detection are acceptable for most of the compounds studied, and the method was successfully applied to real samples.

The addition of SO_2 and pectolytic enzymes produced a considerable increase in methanol and acetaldehyde, especially in the strawberry samples. However, pressing led to a loss of these volatile compounds. In alcoholic fermentation, the *S. cerevisiae* strain used had a great influence on the production of acetaldehyde and higher alcohols in wines. Taking into account the influence of these compounds studied in the final profile of vinegar, the results show that the *S. cerevisiae* strain isolated in this study produces the most suitable wine substrates for the production of vinegars. Moreover, the use of semisolid fruit substrate provides better results than the use of a liquid substrate.

In terms of acetic fermentation, inoculated acetifications in wood recipients resulted in vinegars with better volatile profiles as these presented higher levels of most compounds except acetaldehyde.

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Article 3

Employment of different processes for the production of strawberry vinegars: Effects on antioxidant activity, total phenols and monomeric anthocyanins

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ABSTRACT

The use of strawberry surpluses for the production of added value products seems to be a good solution choice to avoid the waste of this fruit. We produced strawberry vinegars through double fermentation (alcoholic and acetous) from three different harvests of *Fragaria x ananassa var. Camarosa.* The objective was to study the evolution of antioxidant activity, total phenols and monomeric anthocyanins during the vinegar production process. These parameters increased when sulphur dioxide and pectolytic enzymes were added to substrates. Inoculation with the *Saccharomyces cerevisiae* strain RP1 produced wines with half the anthocyanins with respect to the spontaneous fermentations. The use of wood barrels, particularly cherry wood barrels, had a positive effect on all the parameters determined. All measured parameters decreased during the double fermentation process. In general, the acetification stage led to a high loss of antioxidant compounds. Moreover, the production of these vinegars at a semi-pilot scale yielded final commodities with the best values for antioxidant activity, total phenols and monomeric anthocyanins comparing with the vinegars obtained in 2008 and 2009 harvest.

1. Introduction

Strawberries are a widely researched fruit for their nutritional and health benefits as well as their organoleptic properties. This fruit is rich in vitamins, minerals, fibre and phytochemicals. In addition, strawberries contain potentially bioactive compounds and are a great source of phenolic compounds such as flavonoids and phenolic acids (Aaby, Skrede, & Wrolstad, 2005; Määttä-Riihinen, Kamal-Eldin, & Törrönen, 2004; Seeram, Lee, Scheuller, & Heber, 2006). All of these phenolic compounds have been shown to prevent oxidative processes, particularly those caused by reactive oxygen species (ROS) (Aaby, Ekeberg, & Skrede, 2007; Cerezo, Cuevas, Winterhalter, Garcia-Parrilla, & Troncoso, 2010a). These compounds make strawberries a highly antioxidant fruit (Aaby et al., 2005; Wolfe et al., 2008) with potential health benefits. Among the numerous healthy properties described in the literature are anti-proliferative effects on cancer cells (Meyers, Watkins, Pritts, & Liu, 2003; Olsson, Andersson, Oredsson, Berglund, & Gustavsson, 2006) and the antioxidant and anti-inflammatory effects that have been shown to reduce cardiovascular disease risk factors in several prospective cohort studies (Hannum, 2004).

According to the latest data from the FAO (FAOStat, FAO, 2011), Spain is the second-largest strawberry producer in the world; a large portion of this production is harvested in Huelva (Andalucía). Every year, part of the crop is discarded for various reasons, including size or deformations of the berries, or overproduction which leads to surpluses. Because vinegar is generally an inexpensive product, its production requires low-cost raw materials, such as sub-standard fruit and seasonal agricultural surpluses (Solieri & Giudici, 2009). In addition, there is a growing demand for fruit vinegars, which are sold as a health food (Shau-mei & Chang, 2009). The use of strawberries of second quality, which are still suitable for human consumption, to production healthy vinegars with special organoleptic nuances may be a good method to reduce losses due to discarding the fruit.

For this purpose, we have produced strawberry vinegars using second-quality strawberries employing two-stage fermentation and assessed different conditions and treatments. The aim of this work was to evaluate the changes in the antioxidant activity (AA), total phenols index (TPI) and total monomeric anthocyanins (TA) during the production process of strawberry vinegar. In addition, an adequate extraction method to perform these determinations was designed.

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2. Materials and methods

2.1. Chemicals

The reagents acetone, methanol, Folin–Ciocalteu reagent, ethanol, di-potassium hydrogen phosphate (anhydrous), sodium di-hydrogen phosphate 1-hydrate, potassium chloride, sodium acetate and sodium carbonate (anhydrous) were purchased from Merck (Darmstadt, Germany). Fluorescein sodium and gallic acid were supplied from Fluka (Madrid, Spain). 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma–Aldrich (Steinheim, Germany).

2.2. Samples

For the optimisation of the extraction process, we used strawberries (*Fragaria ananassa var. camarosa*) acquired at the market. The fruit was crushed in our laboratory, distributed into amber glass flasks and frozen at −20 °C.

For the production of the vinegars, we employed three different batches of strawberries (*Fragaria ananassa var. camarosa*) from the Huelva area (Spain), corresponding to three harvests: 2008, 2009 and 2010. The production processes were performed in the laboratories of the Dept of Biochemistry and Biotechnology, Faculty of Oenology, Univ Rovira i Virgili (Tarragona). In 2008 and 2009, the substrate employed were purees prepared in the laboratory using a beater. In 2010, we used a commercial puree provided by the Hudisa Company (Huelva). Sulphur dioxide (60 mg/L), sucrose and two types of pectolytic enzymes (Depectil extra-garde FCE® and Depectil clarification® from Martin Vialatte Oenologie, Epernay, France), both at a concentration of 15 mg/L, were added to the puree. After this point, the procedures were slightly different in each harvest.

2.2.1. 2008 harvest

One portion of the strawberry puree was pressed to study the effect of two types of starting substrates (semi-solid and liquid) (Table 1). Six glass containers were filled with 6 L of fruit substrate (four purees and two liquids). Half of the containers of each type of substrate were inoculated with the yeast *Saccharomyces cerevisiae* QA23 at a concentration of 2×10^6 cells/ml, and spontaneous alcoholic fermentation was allowed to occur in the other half. All wines were spontaneously acetified keeping it in the same containers. Two final treatments were tested in vinegars: pasteurization or centrifugation. The average acetic degrees in the 2008 strawberry vinegars were 4.8.

2.2.2. 2009 harvest

For the vinegar production in 2009, eight glass vessels were filled with 6 L of strawberry puree each. Half of these vessels were inoculated with the yeast strain *S. cerevisiae* RP1, isolated during the 2008 spontaneous alcoholic fermentation, and spontaneous alcoholic fermentation was allowed to occur in the other half. All of the wines obtained from the inoculated alcoholic fermentation were mixed and dispensed in three different types of containers: a glass vessel and oak or cherry wood barrels. Samples were then inoculated with a strain of acetic acid bacteria isolated from the 2008 acetification. Wines from the spontaneous alcoholic fermentation were processed in the same way and left to acetify spontaneously. The vinegars obtained were pasteurised. Inoculated vinegars from the 2009 harvest reached an acetic degree of 5.5 (glass container), 6.6 (oak barrel) and 6.3 (cherry barrel).

A portion of the puree from the 2009 strawberries was concentrated by heating in a water bath at 80 °C during 10 h, to test another method of increasing the sugar content; the resulting product was a cooked must (Table 1). The sucrose final concentration was 140 g/L. One litre of this substrate was fermented by a spontaneous process and 1 L was inoculated with the RP1 strain of yeast. The inoculated wines (IWs) were acetified with the same acetic acid bacteria isolated in 2008, and the spontaneous wines (SWs) were left to acetify spontaneously.

2.2.3. 2010 harvest

In this harvest, the pectolytic enzymes added were Rohapect® (12 mg/hL) and the pH was adjusted to 3.5 with 2 g/L $CaCO_3$. In this case, 45 L of puree were fermented in a stainless steel container on a semi-pilot scale, after inoculation with *S. cerevisiae* RP1. The acetous fermentation was performed in a cherry wood barrel. The vinegar had an acetic degree of 6.3.

All vinegars from 2009 to 2010 harvest were pasteurized as final treatment.

Forty-one samples, taken throughout these production processes, were analysed. The codes and characteristics of the samples are shown in Table 1. In addition, five commercial vinegars were also

Table 1
Samples description.

Harvest	Treatment	Puree Sample	Treatment	Sample substrate	Alcoholic fermentation (time)	Wine Sample	Acetification (time)	Treatment or Recipient	Vinegar sample
2008	Crushed	F8P1	SO_2 Pectolytic enzymes Sucrose (50 g/L)	F8P2	Inoculated (4 days)	F8WI1–F8WI4	Spontaneous (2 months)	Centrifugation	F8VIC1–F8SVIC2
								Pasteurization	F8SVIP1–F8SVIP2
					Spontaneous (5 days)	F8WE1–F8WE4		Centrifugation	F8SVEC1–F8SVEC2
								Pasteurization	F8SVEP1–F8SVEP2
	–	F8P2	Pressing	F8L	Inoculated (4 days)	F8LWI	–	–	–
					Spontaneous (5 days)	F8LWE			
2009	Crushed	F9P1	SO_2 Pectolytic enzymes Sucrose (75 g/L)	F9P2	Inoculated (5 days)	F9WI1–F9WI4	Inoculated (2 months)	glass vessel	F9SVIG
								oak barrel	F9SVIO
								cherry barrel	F9SVIX
					Spontaneous (8 days)	F9WE1–F9WE4	Spontaneous (2 months)	glass vessel	–
								oak barrel	–
								cherry barrel	–
			Heating Concentrated	F9MC	Inoculated (7 days)	F9MCWI1–F9MCWI2	Inoculated (5 months)	glass vessel	F9MCVI1–F9MCVI2
					Spontaneous (7 days)	F9MCWE1–F9MCWE2	Spontaneous (2.5 months)	glass vessel	F9MCVE1–F9MCVE2
2010	Crushed	F10P1	SO_2 Pectolytic enzymes Sucrose (65 g/L) $CaCO_3$	F10P2	Inoculated (4 days)	F10WI	Inoculated (1.5 months)	cherry barrel	F10VI

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analysed to carry out comparative studies: Aceto Balsamico, red wine and white wine vinegars, apple vinegar and sherry vinegar.

2.3. Sample-extraction procedure

The consistency of the samples (purees, wines and vinegars) made it necessary to establish an extraction system prior to analysis. The method employed was based on the extraction procedures designed and optimised previously by Ubeda, Hidalgo, et al. (2011). Twenty grams of sample were mixed in a beaker with 40 ml of extract for 10 min while shaking at 800 rpm. The sample was then subjected to ultrasonication followed by a centrifugation at 4000 rpm for 15 min. The supernatant was recovered, and the pellet was re-extracted with 40 ml of solvent following the same procedure. Both extracts were subsequently mixed, and the organic solvent was removed under vacuum. Finally, the extract was filtered, and MilliQ water was added to a final volume of 15 ml. Every extraction was performed in duplicate. We tested different condition to get the maximum values of AA, TPI and TA as well as economy of solvent used and time. Thus, the parameters studied to select the best extraction conditions were: type of solvent (acetone, methanol or ethanol), percentage of solvent (80% or 100%) and ultrasonic extraction time (15, 25, 35 or 50 min).

2.4. Assays and methods

2.4.1. ORAC-FL assay

The Oxygen Radical Absorbance Capacity assay (ORAC-FL) was performed in a Black 96-well microplate, following the procedure described in Ubeda, Hidalgo, et al. (2011). This assay was conducted in a Multi-detection plate reader (Synergy HT, Vermont, USA) located at the Centre for Research, Technology and Innovation at the University of Seville (CITIUS). All reaction assays were performed in triplicate. Results were expressed as μmol Trolox equivalents (TE)/kg of sample.

2.4.2. DPPH radical scavenging assay

To determine the radical scavenging capacity, the DPPH assay described by Brand-Williams, Cuvelier, & Berset (1995) was used. For this test, we used an UV/Vis spectrophotometer U-2800 Digilab coupled to a Peltier themostatic system (Hitachi, Tokyo, Japan). Results were expressed as μmol Trolox equivalents (TE)/kg of sample. The assays were performed in triplicate.

2.4.3. Total phenols index

This parameter was determined in triplicate, using the Folin–Ciocalteu method following the procedure described in Waterhouse (2001). Results were expressed as mg gallic acid/L.

2.4.4. Total monomeric anthocyanins

The determination of total monomeric anthocyanin content (TA) was measured following the pH-differential method described in Giusti & Wrolstad (2001). TA was expressed as pelargonidin-3-glucoside (Plg-3-glu), which is the major anthocyanin in strawberry fruit with a $\lambda_{vis\text{-}max}$ at 510 nm (Swain, 1965). Two buffers were prepared: potassium chloride buffer pH = 1 (0.025 M), and sodium acetate buffer pH = 4.5 (0.4 M). We measured the absorbance at 510 and 700 nm against a cuvette filled with distilled water as a blank.

We then calculated the absorbance of the diluted sample (A) as follows:

$$A = (A_{510} - A_{700})_{\text{pH 1.0}} - (A_{510} - A_{700})_{\text{pH 4.5}}$$

The monomeric anthocyanin concentration in the original sample was calculated using the following formula:

$$\text{TA}[\text{Plg} - 3 - \text{glu (mg/L)}] = (A \times \text{MW} \times \text{DF} \times 1000)/(\varepsilon \times 1)$$

where, A = Sample absorbance, MW = Molecular weight of Plg-3-glu (487.5), DF = Dilution factor, ε = Absorption coefficient of Plg-3-glu (17,330).

The results were expressed as mg Plg-3-glu/kg of sample.

2.5. Statistical analysis

All statistical analysis was performed using the Statistica version 7.0 software package (Statsoft, Tulsa, USA).

3. Results and discussion

3.1. Selection of the best extraction conditions

Several factors, such as solvent composition, time of extraction, temperature, pH, solid-to-liquid ratio and particle size, may significantly influence solid–liquid extractions (Azizah, Ruslawati, & Tee, 1999; Pinelo, Del Fabbro, Manzocco, Nunez, & Nicoli, 2005). In our case, the parameters that were evaluated to determine the best extraction conditions were the type of solvent, the solvent–water ratio and ultrasonication time. The criteria used to select the extraction parameters were the maximum values of antioxidant activity, total phenols, anthocyanins and time and solvent savings.

The type of solvent is one of the most influential variables in the extraction process. We tested acetone, ethanol and methanol. The extraction with methanol gave the worst results in all the assays. As shown in Fig. 1, acetone yielded the highest values for DPPH (8327 μmol Trolox equivalents (TE)/kg) and TPI (2090 gallic ac. mg/kg), with significant differences in this last parameter. However, we obtained the best results for the ORAC assay (24,329 μmol TE/kg) and for the TA determination (26.78 mg Plg-3-glu/kg) using ethanol, but no significant differences were found between these values and those with acetone (26.30 mg Plg-3-glu/kg). Henríquez, Carrasco-Pozo, Gomez, Brunser, & Speisky (2008) reported that the antioxidant activity of strawberry extracts obtained with acetone/water was higher than that with ethanol/water and aqueous extracts. Taking into account this and other studies (Garcia-Viguera, Zafrilla, & Tomás-Barberán, 1998; Pinelo et al., 2005) and our results, we selected acetone for the strawberry extractions.

The solvent–water ratios assayed were 100 and 80:20 (acetone:water) (Fig. 2). The best results for all the parameters measured were obtained using a ratio of 80:20.

Finally, the extraction potential of ultrasound technique depends on the application time, so, we assayed 15, 25, 35 and

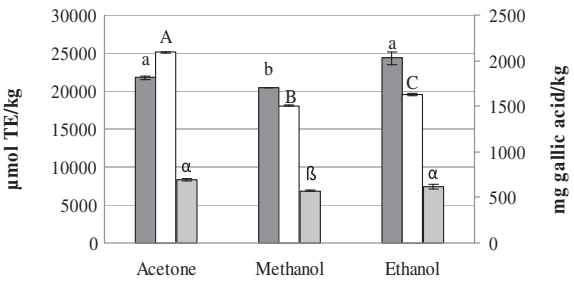

Fig. 1. ■ ORAC, □ DPPH (left axis) and □ TPI (right axis) values for the different extraction solvents tested in strawberries acquired at the market. The bars in the same assay with different letters show significant differences ($p < 0.05$) (ORAC assay: a, b, c; IPT: A, B, C; DPPH test: α, β, γ).

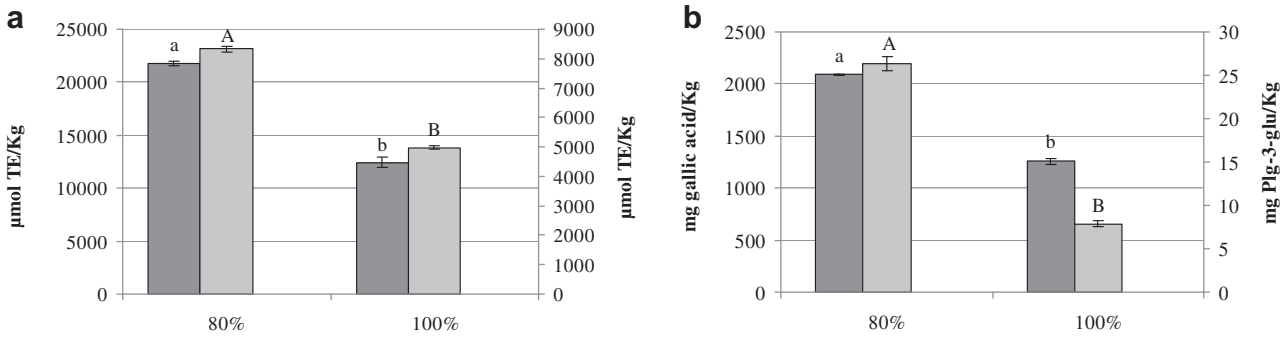

Fig. 2. Effect of solvent percentages. a) ORAC and DPPH values. b) TPI and TA values of strawberries acquired at the market. The bars in the same assay with different letters show significant differences ($p < 0.05$) (ORAC and TPI assays: a, b; DPPH and TA tests: A, B).

50 min. The ultrasonication time chosen was 25 min, since at this time ORAC, TPI and TA reached the highest values (Fig. 3).

3.2. Changes in AA, TPI and TA during the production of strawberry vinegars

3.2.1. Substrate pre-treatments

Three different strawberry purees were employed in this study. These purees presented similar values for all parameters, except the high values of TA in the substrate of the 2009 harvest. After the pre-treatments (pectolytic enzymes and SO_2 addition), we observed significant increases in almost all of the measured parameters, comparing P1 and P2 samples of each harvest (Tables 2–4). Considering the increases percentage, we observed a good correlation between the DPPH with TA ($r^2 = 0.998$) and with TPI ($r^2 = 0.971$) percentages. This could mean that these phenolic compounds are responsible for a percentage of the increases of AA.

Previous studies have shown that pectolytic enzyme treatment is very useful for the release of phenols and anthocyanins from different kinds of berries (Klopotek, Otto, & Boehm, 2005; Meyer, 2002). These enzymes were effective for the release of other phenolic compounds such as ellagic acid, which has been described as the main phenolic compound in berries from the *Fragaria* (strawberry) genus, representing 51% of the compounds analysed (Häkkinen et al., 1999). On the other hand, SO_2 protects against oxidation (Delteil, Feuillat, Guilloux-Benatier, & Sapis, 2000) and may be extracting anthocyanins and phenolic compounds. This effect was observed in blueberries (Lee & Wrolstad, 2004).

The 2008 liquid substrate had significantly lower values for all parameters when compared to the puree substrate.

The cooked must from 2009 harvest had more AA than the original substrate. Because of this result, and taking into account that the starting substrate was concentrated 2.13 times, it seems that the AA was affected by the heating as expected. In addition, anthocyanins were strongly affected by this treatment, decreasing 84%. This same effect was observed by Verbeyst, Oey, Van der Plancken, Hendrickx, & Van Loey (2010), who showed that anthocyanins are more rapidly degraded at higher temperatures on strawberry puree.

3.2.2. Alcoholic fermentation

Alcoholic fermentation was associated with a decrease in all parameters studied. The decline was statistically significant in most cases when the substrate employed was a puree, except in the case of cooked must, in which AA increased obtaining a very high antioxidant product. The decrease in anthocyanins was larger than in the rest of parameters (63–85%). This result is similar to the values obtained in other studies (decrease of 69–79%) (Klopotek et al., 2005). In general, the final values of AA and TPI in wines were similar in the three harvests.

In 2008, we found significantly differences between types of alcoholic fermentation, i.e. inoculation (IW) and spontaneous (SW) for DPPH, TPI and TA values. Total phenolic content was higher in SWs, and anthocyanin contents were higher in IWs, regardless the type of substrate used (semi-solid or liquid). In the wine from the liquid substrate, we observed that the AA and the TPI were lower than semi-solid substrate. However, the levels of anthocyanins in both types of wines were similar.

In the 2009 wines, strawberry SWs had higher significantly values of TA than inoculated wines, even in wines made from cooked must, showing a trend contrary to that observed in the wine production of 2008. It is important to note that the yeast strain (RP1) employed for the production of 2009 IWs was isolated from the 2008 spontaneous alcoholic fermentation. For this reason, we

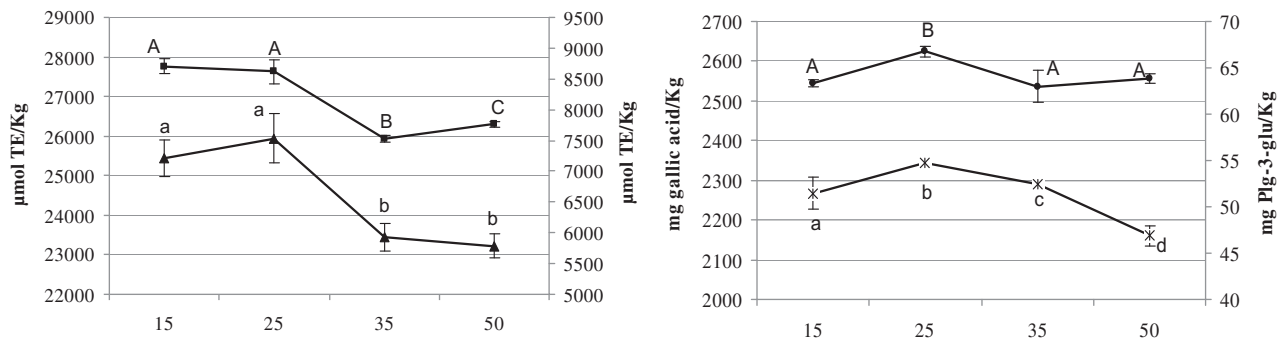

Fig. 3. Effect of different ultrasonication times a) ORAC and DPPH values. b) TPI and TA values of strawberries acquired at the market. The markers in the same assay with different letters show significant differences ($p < 0.05$) (ORAC and TPI assays: a, b, c; DPPH and TA tests: A, B, C).

Table 2
Changes in 2008 samples on ORAC, DPPH, TPI and TA during strawberry vinegar production (average ± standard deviation).

Samples		ORAC (μmol TE/kg)	DPPH (μmol TE/kg)	TPI (mg gallic acid/kg)	TA (mg plg-3-glu/kg)
Substrates	F8P1	21,792 ± 221	8327 ± 99	2090 ± 10	26.3 ± 0.8
	F8P2	26,714 ± 910[a]	10,116 ± 88[a]	2298 ± 0[a]	69 ± 0[a]
	F8L	20,642 ± 111[b]	5907 ± 516[b]	1615 ± 33[b]	43 ± 0[b]
Wines	F8LWE	12,757 ± 267[b,c]	2837 ± 59[b,c]	868 ± 29[b,c]	12.2 ± 0.2[b]
	F8LWI	13,497 ± 227[b,c]	2898 ± 129[b,c]	858 ± 13[b,c]	17.9 ± 0.2[b,d]
	F8SWE1	25,314 ± 650	8200 ± 58[b]	1907 ± 26	13.1 ± 0.7[b]
	F8SWE2	24,696 ± 70	7879 ± 70[b]	1773 ± 32	12.9 ± 0.6[b]
	F8SWE3	25,458 ± 403	7689 ± 82[b]	1757 ± 45	12.4 ± 0.7[b]
	F8SWI1	27,987 ± 1227[b]	7241 ± 35[b,d]	1670 ± 9[b,d]	16 ± 0[b,d]
	F8SWI2	25,451 ± 429[b]	8004 ± 35[b,d]	1584 ± 19[b,d]	18.0 ± 0.3[b,d]
	F8SWI3	23,745 ± 15[b]	6515 ± 67[b,d]	1548 ± 6[b,d]	17.3 ± 0.6[d]
Vinegars	F8SVE1C	9202 ± 390[b]	3256 ± 205[b]	769 ± 13[b]	0.4 ± 0.0[b]
	F8SVE1P	9849 ± 413[b]	3368 ± 352[b]	774 ± 23[b]	0.5 ± 0.1[b]
	F8SVE2C	9215 ± 338[b]	3210 ± 129[b]	781 ± 0[b]	1.1 ± 0.2[b]
	F8SVE2P	10,869 ± 190[b]	3252 ± 234[b]	683 ± 10[b]	0.6 ± 0.0[b]
	F8SVI1C	10,139 ± 341[b,e]	3227 ± 117[b]	751 ± 16[b]	1.3 ± 0.0[b]
	F8SVI1P	11,611 ± 89[b,e]	3388 ± 64[b]	744 ± 6[b]	0.9 ± 0.1[b]
	F8SVI2C	11,054 ± 40[b,e]	3260 ± 246[b]	694 ± 16[b]	0.8 ± 0.1[b]
	F8SVI2P	11,082 ± 86[b,e]	3380 ± 76[b]	712 ± 9[b]	1 ± 0[b]

Sample codes are located in Table 1.
[a] Significant differences ($p < 0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p < 0.05$) with respect to the sample from which was produced (ANOVA).
[c] Significant differences ($p < 0.05$) with respect to semi-solid wines obtained with similar alcoholic process (spontaneous or inoculated) (ANOVA).
[d] Significant differences ($p < 0.05$) with respect to spontaneous process (ANOVA).
[e] Significant differences ($p < 0.05$) with respect to the vinegars obtained from spontaneous wines (ANOVA).

believe that the diminution of TA may be related in some way to the yeast strain involved in fermentation. There are several possible explanations: the adsorption of anthocyanins to the cell walls of the used yeast strain (Morata, Gomez-Cordoves, Colomo, & Suarez, 2005) and condensation reactions with acetaldehyde (Bosso & Guaita, 2008). Perhaps the *Saccharomyces* strains involved in the 2008 spontaneous fermentations had a greater tendency to adsorb these molecules than the strain used in the inoculated processes.

The condensation reactions involve a loss of the aldehyde and the diminution of anthocyanins. We have previously reported (Ubeda, Callejón, et al., 2011) wines obtained by spontaneous alcoholic fermentations in 2008 and inoculated in 2009 contained less acetaldehyde and TA (mentioned above) than their corresponding opposite type of fermentation. In any case, the yeast strain had a greater influence in TA values than the strawberry harvest.

Finally, in the alcoholic fermentation at semi-pilot scale in a stainless steel tank (2010), the loss of AA, TPI and TA was smaller than the losses in the 2008 and 2009 harvests. Probably, the difference found may be due to the lower volume to size of contact surface with oxygen ratio in the stainless steel tank.

3.2.3. Acetous fermentation

In most cases, the acetification process was associated with a decrease in the parameters studied, being TA the most affected. Some of the loss of anthocyanins can be attributed to polymerisation or condensation reactions with other phenols, as noted in vinous substrates (Andlauer, Stumpf, & Fürst, 2000; Cerezo, Cuevas,

Table 3
Changes in 2009 samples on ORAC, DPPH, TPI and TA during strawberry vinegar production (average ± standard deviation).

Samples		ORAC (μmol TE/kg)	DPPH (μmol TE/kg)	TPI (mg gallic acid/kg)	TA (mg plg-3-glu/kg)
Substrates	F9P1	23,176 ± 868	9964 ± 193	2028 ± 82	173.0 ± 3.7
	F9P2	28,998 ± 1893[a]	10,117 ± 88	2085 ± 67	183.8 ± 3.1[a]
	F9MC	37,472 ± 1419[b]	17,897 ± 176[b]	3741 ± 21[b]	27 ± 1[b]
Wines	F9WE1	24,945 ± 276[b]	6898 ± 132[b]	1853 ± 67	52 ± 1[b]
	F9WE2	25,998 ± 795	6992 ± 299[b]	1683 ± 0[b]	55.3 ± 0.4[b]
	F9WI1	25,723 ± 564	7079 ± 53[b]	1705 ± 123[b]	26.3 ± 0.6[b,c]
	F9WI2	27,771 ± 1086	7135 ± 114	2017 ± 29	30.9 ± 1.1[b,c]
	F9MCWE1	49,755 ± 2015[b,c]	19,413 ± 141[b,c]	3380 ± 87[b,c]	24.1 ± 1.5[c]
	F9MCWE2	46,290 ± 279[b,c]	18,493 ± 105[b,c]	3001 ± 63[b,c]	23.3 ± 2.1[c]
	F9MCWI1	45,446 ± 2536[d]	17,747 ± 105[d]	3026 ± 29[d]	7.4 ± 0.6[c,d]
	F9MCWI2	43,095 ± 2576[d]	20,726 ± 271[d]	3416 ± 53[d]	6 ± 0[f,d]
Vinegars	F9VIG	15,163 ± 341[b]	6235 ± 72[b]	1099 ± 55[b]	3.07 ± 0.17[b]
	F9VIO	17,446 ± 107[b]	6902 ± 31	1844 ± 56	6.5 ± 0.9[b]
	F9VIX	19,077 ± 161[b]	7163 ± 31	1693 ± 45	4.80 ± 0.17[b]
	F9MCVE1	33,779 ± 974	14,907 ± 103	2377 ± 45	2.9 ± 0.5
	F9MCVE2	31,643 ± 1832	14,428 ± 41	2480 ± 56	4.0 ± 0.4
	F9MCVI1	30,685 ± 1377[e]	14,119 ± 305[e]	2536 ± 45[e]	1.70 ± 0.02[e]
	F9MCVI2	26,278 ± 1409[e]	14,283 ± 123[e]	2377 ± 22[e]	1.79 ± 0.15[e]

Sample codes are located in Table 1.
[a] Significant differences ($p < 0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p < 0.05$) with respect to the sample from which was produced (ANOVA).
[c] Significant differences ($p < 0.05$) with respect to spontaneous wines from F9P2 (ANOVA).
[d] Significant differences ($p < 0.05$) with respect to inoculated wines from F9P2 (ANOVA).
[e] Significant differences ($p < 0.05$) with respect to inoculated vinegars from F9WI wines (ANOVA).

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Table 4
Changes in 2010 samples on ORAC, DPPH, TPI and TA during strawberry vinegar production (average ± standard deviation).

Samples		ORAC (μmol TE/kg)	DPPH (μmol TE/kg)	TPI (mg gallic acid/kg)	TA (mg plg-3-glu/kg)
Substrates	F10P1	20,409 ± 431	10,218 ± 171	1800 ± 122	46.4 ± 1.6
	F10P2	23,783 ± 649[a]	10,592 ± 237	1886 ± 79	54.8 ± 1.4[a]
Wine	F10WI	22,910 ± 315	9652 ± 378[b]	1691 ± 36[b]	20.2 ± 0.5[b]
Vinegar	F10VI	19,784 ± 117[b]	9113 ± 331	1605 ± 95	10.6 ± 0.9[b]

Sample codes are located in Table 1.
[a] Significant differences ($p < 0.05$) with respect to the initial fruit puree (ANOVA).
[b] Significant differences ($p < 0.05$) with respect to the sample from which was produced (ANOVA).

Winterhalter, Garcia-Parrilla, & Troncoso, 2010b). Again, as occurred in alcoholic fermentation, we observed the lowest decreases in all of these parameters in the 2010 samples.

In 2008, vinegars were subjected to two different final treatments. In assessing the antioxidant activity (Table 2), we observed that the ORAC and DPPH values were slightly higher in pasteurised vinegars than in centrifuged vinegars. The centrifugation procedure removes suspension particles being able to produce losses of antioxidant compounds. Moreover, this result could also be explained by the formation of Maillard reaction products such as melanoidins that are produced by the heat of pasteurisation. Several authors who have studied vinegar melanoidins have concluded that contribute to the total antioxidant capacity of it (Xu, Tao, & Ao, 2007).

In the 2009 (Table 3), spontaneous and inoculated acetifications were performed. However, the spontaneous fermentation stopped, so we only obtained inoculated vinegars. Regarding the effect of the type of container used in the acetification, the vinegar produced in glass vessel displayed the lowest values for all the parameters studied. These results were expected due to concentration phenomena and compounds extraction in wood barrels. The vinegar from cherry barrel had the highest AA, at levels significantly different from the oak vinegar. From the oak barrel, we obtained vinegar with the highest amount of total phenols and anthocyanins, but significant differences were not found with the vinegar from cherry barrel. These results were similar to those of Cerezo et al. (2008), who reported a generally decreasing trend of TPI and TA in vinegars acetified in cherry and oak barrels, being slightly lower in oak. The lower final levels of TA in vinegar from cherry barrel may be explained by the different porosity of wood (higher in cherry wood than in oak). Oxygen permeation through the wood favours the formation of stable anthocyanin-derived compounds (Cano-López, Pardo-Minguez, López-Roca, & Gómez-Plaza, 2006), decreasing monomeric anthocyanins. According to these results, it seems that cherry wood barrel is the best to produce high antioxidant strawberry vinegars rich in phenols.

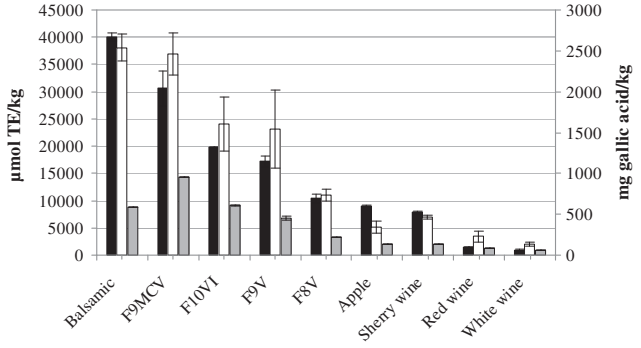

Fig. 4. Comparison of ■ ORAC, □ DPPH (left axis) and ■ TPI (right axis) values of strawberry vinegars with commercial varieties. Sample codes: F9MCV (mean value of all vinegars from cooked must), F9V (mean value of all vinegars from 2009 harvest) and F8V (mean value of all vinegars from 2008 harvest).

Vinegars from cooked must had the highest AA and TPI of all of the vinegars produced.

Otherwise, the 2010 vinegars produced on a semi-pilot scale had the highest AA and TA values of all the vinegars obtained from strawberry purees without heating. As mentioned above, the important losses of TA that occurred in the 2008 and 2009 vinegars did not occur in 2010, where losses were only around 50% from wine to vinegar. These results indicate that the production of vinegars on a semi-pilot scale allowed getting vinegars with better antioxidant properties.

Finally, we compared our vinegars with common vinegars from the market. The results are given in Fig. 4. Vinegars produced in this research project were surpassed only by the Aceto Balsamico. Cooked must vinegar had AA and TPI values close to this one.

4. Conclusions

The addition of SO_2 and pectolytic enzymes to the substrate increased AA, TPI and TA.

Although the cooked must vinegar presented the highest AA and TPI values, this substrate must be discarded for the strawberry vinegars production at an industrial scale because of their obtaining process is very slow and complex. Concerning the acetification stage, the use of wood barrels was an improvement in all of the parameters determined; specifically, cherry barrels were the best to produce high antioxidant strawberry vinegars rich in phenols. The most appropriate final treatment was the pasteurisation with reference to AA. All measured parameters decreased during the double fermentation process. In general, acetic fermentation was associated with higher decreases in AA and polyphenols than alcoholic fermentation, except in the semi-pilot scale case. Moreover, anthocyanins were severely influenced by this process. So, for substrate selection the parameter more important to take into account is the TA content. We also noted that the production of these vinegars on a semi-pilot scale resulted in final products with the best antioxidant properties and phenolic content. The antioxidant properties of these vinegars point to them as products with potential health benefits that could make them competitive commodities in the market.

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