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The occurrence of fungi, yeasts and bacteria in the air of a Spanish winery during vintage

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ABSTRACT

This research studies the presence of microorganisms of enological interest (yeasts, bacteria and molds) and their evolution in the air of a wine cellar. The samples were taken throughout the winemaking campaign (September–December) in a winery of the D.O.Ca. Rioja, Spain. They were collected using an airIDEAL atmosphere sampler from Biomerieux. For the isolation, specific selective media were used for each group of microorganisms. The results obtained indicate that the presence in the winery air of the various different microorganisms studied is directly related to the winemaking processes that are taking place in the winery. Thus, the number of molds present decreases once grapes have ceased to be brought into the winery. The maximum number of yeasts in the air is found when all the vats in the cellar are fermenting, while the lactic bacteria are not detected until the first malolactic fermentation begins. The species of yeasts and molds identified are also related to the winemaking processes. The coincidence of strains of *Saccharomyces cerevisiae* among those present in the vats during alcoholic fermentation and those isolated from the air, confirms the role of the latter as a transmitter of microorganisms.

1. Introduction

Winemaking is a process in which a series of complex microbiological transformations take place, involving interactions between yeasts, bacteria and filamentous fungi. The conversion of must into wine occurs through alcoholic fermentation (AF), a process performed by yeasts. This initial transformation is followed by what is known as malolactic fermentation (MLF), caused by lactic bacteria. Both microbiological processes make an essential contribution to the final quality of the product. The origin of these microorganisms and the way in which they get into the must is a question which has been debated for some time (see for example the studies carried out by Peynaud and Domercq on microorganisms in the winery environment by Peynaud and Domercq, 1956, 1961) but nowadays it is accepted that the yeasts and bacteria which take part in the fermentation processes have two possible sources: the grapes and the material in the winery environment (Fleet and Heard, 1993; Mortimer and Polsinelli, 1999). The grapes which come into the winery have an important population of microbes adhered to the bloom, made up of yeasts, bacteria and molds. But the winery equipment (stemmer, crusher, pipework, vats, etc.) also contribute microorganisms and thus, as the must comes into contact with the different winery equipment, the yeasts, bacteria and molds which have been in contact with these surfaces pass into the must and, if the environmental conditions allow, begin to multiply and ferment.

Molds are ubiquitous, with various genera commonly found on grapes (*Aspergillus*, *Botrytis* and *Penicillium*), and to a lesser extent *Phythophthora*, *Moniliella*, *Alternaria* and *Cladosporium* (Rosa et al., 2002). Molds are also ubiquitous inside wineries and are present on surfaces and in the air (Donnelly, 1977). The yeasts associated with vinification can be grouped into two categories: *Saccharomyces* and non-*Saccharomyces*. The first of these groups includes yeasts belonging to the *Saccharomyces* genus and these are the main agents responsible for fermentation (Beltrán et al., 2002). The yeasts get onto the grapes through the effects of wind dispersal and via insects (Carrascosa et al., 2005). The microbial load of the grapes entering the winery is almost exclusively composed of non-*Saccharomyces* yeasts, so the inoculation of musts with *Saccharomyces* mainly occurs through the winery equipment (Pretorius, 2000). Lactic bacteria have been isolated from vine leaves, grapes, winery equipment, barrels, etc (Bae et al., 2006). This means that they are present during all stages of vinification and, like the yeasts, enter with the grapes and remain in the winery equipment after MLF (Fugelsang and Edwards, 2007).

For many years, winemakers have found that the first spontaneous fermentations (alcoholic and malolactic) carried out on each harvest at the wineries take longer than subsequent ones (Peynaud, 1989). The reason would be due to the increase in microorganisms which occurs in the first vat, and in their dispersal around the cellar and arrival in large quantities in other vats in which the subsequent fermentations take place, and which would start up more quickly. Recently, Granchi,

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Augruso, Ganucci, and Vicenzini (2007), have suggested that *Saccharomyces cerevisiae* which dominate the fermentation of the first vat of each harvest invade the winery environment and produce a natural inoculation in the subsequent fermentations. The dispersal of the microorganisms in the winery occurs through the machinery, tools, insects and probably the air.

The aim of the present work is to study the presence and evolution of various types of winemaking microorganisms (yeasts, bacteria and molds) in the air of a winery during the period of vinification for the 2006 campaign. In the recent literature consulted, only Connell et al. (2002), have used the analysis of air in a winery for a study of the presence of microorganisms and they found *Brettanomyces* yeasts in air samples taken from different parts of a commercial wine cellar. Previously, Donnelly (1977) used this approach in a winery bottling room. However, the analysis of the air is common in other food industries (Salustiano et al., 2003), primarily in the aseptic packaging of food, where the presence of microorganisms in the air is a source of contamination and spoilage of foodstuffs. The need to control the microbiological quality of the air to ensure that food products remain safe and wholesome throughout their shelf life is well established in food factories, because air carries many microorganisms. Concentrations of 100–10,000 microorganisms per cubic meter are quite normal (Curiel et al., 2000).

2. Materials and methods

2.1. Sample collection

The research was carried out during the months of September–December 2006 in a winery situated in the village of Tudelilla (La Rioja, Spain). Only red wines are made in this winery and all the fermentation processes, both alcoholic and malolactic are always spontaneous. Sampling of the winery air began on 27 September, 5 days before the first grapes were brought into the cellar (which happened on 2 October), and ended on 19 December, 1 month after the last malolactic fermentation was complete. In total, 13 air samples were taken in the winery (27 September; 2, 5, 9, 11, 16, 20, 24, 31 October; 8, 14, 28 November and 19 December). As can be seen, most of the samples were taken during the month of October coinciding with grape reception into the winery and alcoholic and malolactic fermentation.

Air samples were taken using the airIDEAL 3P air sampler from BioMérieux, an impaction aerobiocollector used to detect the presence of viable microorganisms in the environment to be tested by precise sampling of a given volume of air. Air is taken up with a turbine through a grid surface. The acceleration of airflow results in the impaction of airborne microorganisms on the agar. Passage of the air through the grid filters out particles, thereby facilitating the enumeration of CFU (colony forming units) after incubation of the medium.

An analysis was performed for the presence in the air of three types of winemaking microorganisms: yeasts, lactic bacteria and molds. All the samples were taken from the same point in the winery, located in the middle of the winemaking area. The sampler was placed on a platform 1 m above the ground. The study of each of the microorganisms was made using petri dishes with specific selective media for each one: Cloramphenicol Glucose Agar for yeasts (Biokar Diagnostics, France), Agar Czapek (Scharlau Microbiology, Barcelona, Spain) for molds and modified MRS-Agar (Scharlau Microbiology, Barcelona, Spain) for lactic bacteria. The volume of air analyzed varied between 20 and 100 l depending on the microorganism under study and of the stage in the vinification process. In each sampling, two different volumes of air were analyzed in duplicate for each microorganism.

Simultaneously with the analysis of yeasts in the air, the indigenous yeasts which produced three of the eight spontaneous alcoholic fermentations carried out in the winery during the whole of the campaign were analyzed. The aim of the sampling was to compare the microorganisms present at the same time in both media. The wine was fermented in 25,000-l stainless steel vats at a controlled temperature of 28 °C. The fermentation processes analyzed were: the first fermentation of the campaign, vatted on 2 October, another midway through the period, vatted on 9 October and the final fermentation of the year, vatted on 16 October. The three spontaneous fermentations were sampled at three different stages: 24 h after vatting, vigorous or tumultuous fermentation and end of fermentation. The collection of colonies was considered to be representative of the total yeast population (Santamaría et al., 2007), and it was in line with previous studies (Santamaría et al., 2005). All samples were collected in sterile bottles and taken to the laboratory for processing.

2.2. Microbial analyses

The plates obtained from the air sampling were transferred to the laboratory and incubated in the appropriate conditions for the growth of each microorganism (incubator at 25 °C for 48 h for yeasts, cool store at 20 °C for 7 days for molds, and anaerobiosis at 30 °C for 10 days in the case of lactic bacteria). Some diphenyl crystals (approximately 100 mg per plate)(Panreac Química SA, Barcelona, Spain), were added to the yeast and bacteria plates in order to impede the development of mold. After the incubation period, the number of each type of microorganism was counted. From the two volumes of air samples at any given moment, the one chosen for the count was that in which the plates contained less than 100 CFU (according to manufacturer's instructions). The result of this count was expressed as the average of the number obtained in the two repeat samples analyzed (CFU). Subsequently, this value was converted into the most probable number of microorganisms collected per plate (MPN). The MPN value is calculated from the CFU count, using Feller's law: $MPN = N \cdot (1/N + 1/N-1 + 1/N-2 + \ldots\ldots + 1/N - CFU + 1)$. A reading and statistical correction table is used to convert the number of CFU to MPN (included in the equipment's instruction manual). This statistical correction corresponds to the random passing of microorganisms through the orifices of the grid. In order to determine the most probable number of microorganisms collected per cubic meter of air (MPN/m^3), the most probable number of microorganisms collected per plate (MPN collected) was multiplied by 1000 and divided by the volume of air sampled in liters. In every plate with yeasts, ten colonies were randomly selected and stored in tubes with malt agar for later analysis.

Samples collected in sterile bottles during spontaneous alcoholic fermentations for isolation of yeasts, were processed as follows: serial dilutions were carried out and the samples were seeded onto plates containing a chloramphenicol glucose agar medium. The plates were incubated at 25 °C for 48 h. Plates containing between 30 and 300 yeast colonies were examined; ten colonies from each sample were randomly selected (10 from the start of fermentation, 10 from vigorous fermentation and 10 from the end of fermentation), making a total of 30 colonies for each tank. The colonies chosen were stored in tubes with malt agar for later analysis.

All the colonies of yeasts isolated both in the air and in fermentations were later seeded onto Petri plates containing Lysine Agar Medium (Scharlau Microbiology, Barcelona, Spain). The growth in these dishes allowed us to distinguish between *Saccharomyces* and non-*Saccharomyces* yeasts, since only the latter are capable of growing in such a medium.

Individual isolates of the *Saccharomyces* genus, isolated both in the air and in spontaneous fermentations, were identified by mithochondrial DNA (mtDNA) restriction analysis. The Restriction Fragment Length Polymorphism (RFLP) of mithochondrial DNA was used for distinguishing between strains of *S. cerevisiae* (Pulvirenti and Giudici, 2003). Yeast cells were grown overnight in a culture of 5 mL YEPD (1% yeast extract, 2% peptone, 2% glucose) (Cultimed, Panreac Química