Food Control 34 (2013) 261–267

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Screening of yeast mycoflora in winery air samples and their risk of wine contamination

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ARTICLE INFO

Article history:
Received 25 October 2012
Received in revised form
25 April 2013
Accepted 30 April 2013

Keywords:
Yeasts
Winery air
Non-*Saccharomyces*
Brettanomyces

ABSTRACT

The airborne yeast flora associated with four different areas in 3 Spanish wineries was analyzed over the course of a year (winter, spring, summer and fall) in order to distinguish the yeast species present in this ecosystem and to evaluate the risk of air as a means of wine contamination by spoilage yeasts. Sampling was conducted in the vinification, bottling, cask aging and bottle aging areas. 367 yeasts were identified from two different culture media: 258 in a generic medium for yeast species and 109 in a more selective medium for the *Brettanomyces/Dekkera* genus.

Low levels of yeasts were found in the air with different values depending on the activity being performed at the time: the population in the bottling area increases when bottling is being carried out, and it also increases in the air present in the winemaking premises during vinification. Most of the isolated yeasts were members of the non-*Saccharomyces* group. Yeasts from the *Saccharomyces cerevisiae* species were only detected in the air present in the vinification area during the vinification period. *Aureobasidium* and *Cryptococcus* were the dominant genera in the air and they were found permanently in the 3 wineries in every area analyzed. The highest level of yeast cells was found in the bottling line, which makes it the area in the winery that requires the greatest vigilance. However, the types of yeast detected are not a real risk to the quality of the wine being bottled. Spoilage yeast *Brettanomyces/Dekkera* was detected in the air present in the cask area of one winery when wine containing these yeast species was being handled. This suggests that air can be a means for these yeast cells to spread in the wineries and that yeast dissemination is associated with the handling of the wine containing them.

No significant differences were found between the wineries, neither in the count of yeasts in the air nor in the main genera present. Therefore, this data establishes an initial description of the yeast levels in the air, the genera and species usually present and the risk to the stability of the wine and its spoilage.

1. Introduction

An awareness of the quantitative and qualitative diversity of the microorganisms present in wineries is important, as are the mechanisms by which they transfer to the wines. The winery is an environment associated with grape fermentation, wine storage, aging and bottling, and microorganisms could be a source of contamination during the storage, aging and bottling of wines. The dissemination of microorganisms in a winery occurs through tools, equipment, workers, insects and air. The air itself does not facilitate the development of microbes and it only acts as a support medium or carrier until the microorganisms are deposited on the surrounding surfaces.

With regard to enological microorganisms, yeasts play an important role both in winemaking and in wine spoilage. When yeasts are present in the air, this air automatically becomes a dissemination medium that can lead to wine contamination. Although some authors (Stratford, 2006) have indicated that air can actually be a source of yeast infection, it seems to be less frequent than expected, since yeast cells are larger and denser than bacteria. Air can be a cause of yeast contamination from genera such as the *Rhodotorula* and *Sporobolomyces* spp., described as hygiene yeasts (Davenport, 1996). Garijo et al. (2008, 2009) suggested that air is a source of microbial dissemination and contamination in wineries during alcoholic and malolactic fermentation, particularly during the handling of wine. Previously, Donnelly (1977) had determined the extent and types of airborne microorganisms in a winery bottling room, and other authors (Mandl, Clemenz, Sterfliner, Kneifel, & Schattauer, 2008; Ocón et al., 2011) had analyzed mold content in the air of wineries.

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0956-7135/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodcont.2013.04.044

Wine is the result of the activity of various microorganisms on grape juice. However, microorganisms do not always yield positive effects and some can damage the quality of wine. Particularly, *Brettanomyces bruxellensis* is considered the main cause of wine spoilage, and it has become a major worldwide enological concern in recent years. The yeasts belonging to the species *Dekkera bruxellensis*, or its anamorph *B. bruxellensis*, have the ability of spoiling wines by producing ethylphenols (Loureiro and Malfeito-Ferreira, 2006), which are compounds responsible for the off-flavors described as animal odors, farmyards, horse sweat, medicine and animal leather (Chatonnet, Dubourdieu, Boidron, & Pons, 1992). *Brettanomyces* has been studied extensively in the past years with the aim of gaining knowledge about its ecology and establishing a strategy for adequate control measures (Suárez, Suárez-Lepe, Morata, & Calderón, 2007). *Brettanomyces* is thought to spread through a winery by importation of infected wine; poor sanitation of hoses, tanks or other equipment; the common fruit fly; and air. Beech (1993), while researching cider, found a relationship between the exposure of the liquid to air and contamination by *Brettanomyces* yeast, while Connell, Stender, & Edwards (2002) found *Brettanomyces* yeast in air samples from different areas of a commercial winemaking enterprise.

Recently, in a previous study carried out by our research team (Ocón et al., 2013), the quantitative and qualitative composition of yeasts in the air was analyzed from four areas of a Spanish winery over 12 consecutive months. Most of the isolated yeasts were members of the non-*Saccharomyces* groups, being *Sporidiobolus* and *Cryptococcus* the dominant genera in the air from every area analyzed. The *Brettanomyces* genus was not found in the air at any moment or in any sampling point. The purpose of this paper is to extend the study to other wineries with different characteristics in order to corroborate the preliminary data and establish the variations in the microbial composition of the air based on different factors, as well as the risks of contamination via this route. An extensive knowledge of these ecosystems is essential to establish the source of spoilage yeasts and their contamination routes. This information could also be useful for developing strategies aimed at the biocontrol of infections in wineries.

2. Materials and methods

2.1. Sample collection

Sampling was conducted in 3 wineries located in La Rioja (Spain) which produce and commercialize wine under the Rioja Designation of Origin. This sample collection was conducted in the vinification, bottling, cask aging and bottle aging areas. Air samples were collected in four different periods of the year: winter, spring, summer and fall. Samples were collected in duplicate from a single point in each area from each winery. During the fall, the process of vinification is the main activity carried out in the winery, whereas wine racking and bottling activities, as well as cask cleaning, are performed during the rest of the year (Table 1).

The three wineries sampled presented different characteristics and designs. Winery 1 is 10 years old, produces 500,000 L of wine per year and is designed in such a way that all the work areas are interconnected. Winery 2 is 30 years old, produces 3 million liters per year and is built in such a way that each work area is located in an independent building. Finally, Winery 3 is 40 years old, produces 7 million liters per year and is built following a traditional design in which the 4 work areas are distributed into two floors, where the lower floor is below ground level.

Air samples were collected using the airIDEAL 3P air sampler from BioMérieux, an impaction aerobiocollector used for detecting the presence of viable microorganisms in the environment to be tested by precise sampling of a given volume of air. Air is taken up by means of a turbine through a grid surface (rate flow was 100 L min^{-1}). The acceleration of airflow results in the impact of airborne microorganisms on the agar. The passage of the air through the grid filters out particles, thereby facilitating the enumeration of CFU (colony forming units) after incubation of the medium. The sampler was placed on a platform 1 m above the ground.

Table 1
Activities performed in the wineries during sampling.

Wineries	Season			
	Winter	Spring	Summer	Fall
Winery 1	Bottling **NO** Racking	Bottling **NO** Racking	**NO** Bottling **NO** Racking	**NO** Bottling **NO** Racking Winemaking
Winery 2	**NO** Bottling Racking	**NO** Bottling **NO** Racking	Bottling Racking	**NO** Bottling **NO** Racking Winemaking
Winery 3	Bottling Racking	Bottling Racking	Bottling Racking	Bottling Racking Winemaking

In each sampling, petri dishes with a diameter of 90 mm with two culture media were used in duplicate: (A) Chloramphenicol Glucose Agar (CGA) (5 g/l yeast extract, 20 g/l glucose, 0.2 g/l chloramphenicol, 15 g/l agar), which is a specific selective media for yeasts. The volume of air analyzed was 500 L; and (B) Agar MR (3 g/l TSB, 2 g/l yeasts extract, 12 g/l agar), which was used as a resuscitation media. A sterile filter with a pore diameter of 0.45 μm was placed over the agar surface. The volume of air sampled was 1500 L. Yeasts present in the air can enter into viable but non-culturable (VBNC) state in response to stresses, such as osmotic pressure, temperature and oxygen concentration. Therefore, they may not be able to grow in selective agar due to the additional stress caused by the selective agents. In addition, the number of viable yeasts present in the air may be underreported. For these reasons, a resuscitation medium was employed to recover cells from the *Brettanomyces/Dekkera* genus because cells can recover a culturable state when favorable environmental conditions are restored. The existence of a VBNC-like phenomenon in *B. bruxellensis* has been suggested by Millet and Lonvaud-Funel (2000) and by du Toit, Pretorius, & Lonvaud-Funel (2005).

2.2. Microbiological analysis

The CGA plates obtained from the air sampling were transferred to the laboratory and incubated at 25 °C for 72 h, whereas MR plates with filters were incubated at the same temperature during 24 h (resuscitation period). After that, the filters were removed from the MR plates and transferred to DBDM plates. Subsequently, these plates were incubated at 25 °C for 21 days. DBDM (0.7 g/l Yeast Nitrogen Base; 0.7 g/l Ethanol; 0,001 g/l cycloheximide; 0.01 g/l ac. p-cumaric; 0.002 g/l bromocresol green, 0.02 g/l chloramphenicol, 20 g/l agar, pH 5,4 adjusted with sorbic acid) is a specific medium for detecting the presence of *Brettanomyces/Dekkera* yeasts (Rodrígues, Gonçalves, Pereira-da-Silva, Malfeito-Ferreira, & Loureiro, 2001).

Some diphenyl crystals, approximately 100 mg per plate (Panreac Química SA, Barcelona, Spain), were added to the plates in order to impede the development of molds. After the incubation period, the number of yeasts was counted and the result was expressed as the average result obtained in the two repeat samples (CFU). Subsequently, this value was converted into the most probable number of microorganisms collected per plate (MPN) using a