



## How sterile is filling?



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### 1. Introduction

Wine is a product of the microbiological activity of bacteria, yeast, and other fungi. The two essential processes in the production of wine, alcoholic fermentation, and malolactic fermentation are the result of microbial metabolic activity. The microorganisms are added to the must either as a starter culture or they are found on the skin of the fruit and the surfaces of winery equipment or are introduced into the must during the processing of the grapes. Initially, all acid-tolerant microbes can multiply in the must, i.e. both yeast and bacteria. After the start of fermentation and the formation of ethanol and carbon dioxide, only a few organisms are still capable of survival and growth. After completion of the alcoholic and, if applicable, the malolactic fermentation, the yeast and bacteria cells still present in the wine are separated with the aid of the first and second draw-off and

filtration of wine. From this point on, an attempt is made to protect the wine from further microbial action by keeping the cask full up to the bung and using sulfurous acid. Before the bottles are filled, the wine is additionally sterile filtered to remove the last remnants of microorganisms that could alter the end product in the bottle.

The yeasts present in the must and the wine may be of the *Saccharomyces* or non-*Saccharomyces* genus. *Saccharomyces* are also known as "wine yeast," since they promote vigorous fermentation and are dominant in reproducing (107 – 108 CFU/ml) at the beginning of the main fermentation process. Alcohol, the product of their metabolic activity, is the most important component of the wine. For this reason, in practice, the must is further inoculated with *Saccharomyces cerevisiae*, a dry yeast preparation, to insure the complete conversion of the sugar to alcohol. The

various sub-species of *Saccharomyces cerevisiae* are best adapted to the conditions in the wine and drive out other microorganisms in the course of the fermentation. Various species of yeast belonging to the non-*Saccharomyces* genus have a limited fermentation capacity in common and dominate the yeast population (106 – 107 CFU/ml) in the must and at the start of the fermentation. However, with the increase in the alcohol content under anaerobic conditions, and after sulfurization of the must, most species of non-*Saccharomyces* are no longer capable of survival. Among the most common species occurring are *Hanseniaspora uvarum*, species of *Candida*, *Pichia* and *Rhodotorula*, as well as *Brettanomyces*. They may be responsible for the formation of off-aroma, acetic acid, and acetaldehyde, and in some cases also for the formation of film and slime in wine. Cells of *Hanseniaspora uvarum* and species of the genus *Candida* are present in large numbers

in the must and usually initiate fermentation. *Candida* and *Pichia* are among the film-forming yeasts that, under aerobic conditions, multiply on the surface of the wine and form a layer of film. Among the species most frequently occurring in wine are *Zygosaccharomyces rouxii*, *Candida vini*, *C. famata* and *C. intermedia*, as well as *Pichia membranaefaciens*, *P. anomala* and *P. fermentans*. The yeast *Rhodotorula*, also known as "slime yeast" has a low alcohol tolerance and occurs at the start of fermentation in the must or in wines that are stuck in the fermentation state. Alcohol contents of 10% (v/v) and higher and the absence of oxygen inhibit the metabolism of *Rhodotorula* significantly, so that it is of lesser importance in wine production. However, its appearance offers clues as to the wine cellar's hygiene, as it indicates a lack of adequate cleaning and disinfection measures. *Brettanomyces* spec. (*Dekkera* spp.) yeast, in contrast to the other non-*Saccharomyces*, is relatively alcohol-tolerant (approx. 15 (v/v)) and occurs primarily when wine is stored in barrels and bottles. Due to its slow growth, the appearance of *Brettanomyces* spec. is often detected only belatedly. It then becomes noticeable through intense formation of acetic acid (up to 7.2 g/l) and ethylphenol, the typical *Brettanomyces* tone.

In addition to yeast, a multitude of bacteria also enters the must, but in healthy grapes the population of these is smaller than that of the yeasts (< 10<sup>3</sup> – 10<sup>4</sup> CFU/ml). They are lactic acid bacteria of the genera *Oenococcus*, *Lactobacillus*, and *Pediococcus*, as well as acetic acid bacteria of the genera *Acetobacter* spec and *Gluconobacter* spec. Because lactic acid bacteria and acetic acid bacteria themselves are acidifiers, the low pH of the

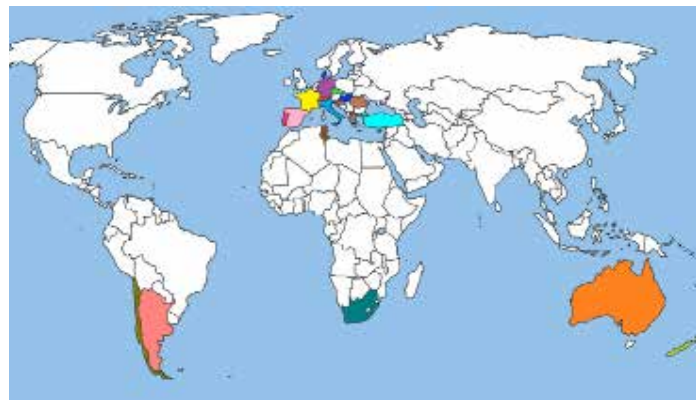
wine does not represent an obstacle to the growth of these bacteria. They are suppressed only at the beginning of fermentation and with the increasing dominance of the yeasts. If, on the other hand, the growth of the yeasts is delayed, the bacteria can multiply and become dominant, and that may inhibit the further development of the yeasts. This can lead to additional problems in fermentation. In healthy grapes, *Oenococcus oeni* predominates in terms of numbers, as it is best adapted to the conditions in the must and later in the wine. This microorganism is also added sometimes to the wine as a starter culture. *Lactobacillus* and *Pediococcus* have only a low tolerance to high concentrations of alcohol and a low pH level. They rarely occur in wine.

Acetic acid bacteria occur mainly in severely damaged grapes and in wine under extremely aerobic conditions and at a temperature of at least 10°C. The number of yeasts and bacteria in the must depends on the one hand on the climatic conditions of the winegrowing area, and on the other hand on the health and processing of the grapes. The range of species and the cell concentration of the yeasts and bacteria changes in the course of fermentation due to the selective advantages of certain species. Since the number of cells in the wine depends on the individual course of the fermentation, the only indication used is the microbial count of the must.

In general, there is no concern about pathogenic microbes in the wine. Harmful microorganisms scarcely find any breeding ground in order to multiply. Thus in this respect, the product is not at risk. There is, however, a risk in the occurrence of product spoilers. The aforementioned

microorganisms can be divided into two groups, useful microbes and beverage contaminants. Harmful microorganisms include those that adversely affect the taste and aroma of the wine caused by the formation of metabolic products or bring about a visual change through the formation of haze and sediment. The classification of a microbe as harmful depends not only on its metabolic properties and metabolites, but also on the point at which it appears and on the microbial count. The yeast *Saccharomyces cerevisiae*, for example, is essential to winemaking. If it is present in the residual sweetness of the wine at a later point, however, it can damage the product and may endanger the consumer due to the buildup of pressure in the bottle. The same applies to the *Candida* and *Pichia* yeasts found in the must at the beginning of fermentation but without causing great harm at this point. Their appearance in insufficiently filled storage containers in the

subsequent course of the aging, however, is usually associated with the wine being spoiled. Under aerobic conditions, ethanol and acids oxidize on the surface of the wine forming acetaldehyde, acetic acids, and various esters that negatively affect the aroma of the wine. Yeasts, bacteria, and fungi are present everywhere in wine cellars. They are able to multiply at a humidity level of at least 8% and wherever they can find usable sources of nutrients. Wine also contains microbe-inhibiting substances such as ethanol, sulfurous acid, and phenols, but the rule of thumb still holds: "That which has been produced biologically can also be broken down biologically". It is therefore important to control and, if necessary, to be able to eliminate the population of microorganisms. In addition to the sulfurization of the wine, the separation of microorganisms through filtration plays an important role in this case.



Source: <http://commons.wikimedia.org/wiki/File%3AVulpes-bengalensis-map.png> with color adaption by Sabrina Beerbalk

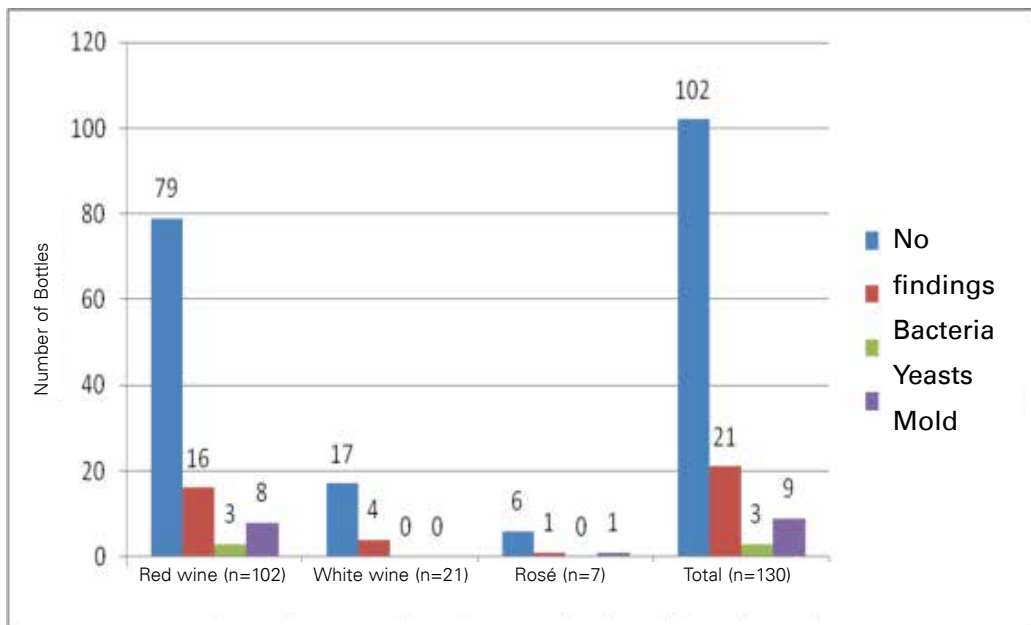
**Fig. 1** Geographic distribution of the wines used:

n = Argentina, n = Australia, n = Chile, n = Denmark,  
n = Germany, n = France, n = Greece, n = Italy, n = Croatia,  
n = Macedonia, n = New Zealand, n = Austria, n = Portugal,  
n = Romania, n = Switzerland, n = Slovak Republic,  
n = Slovenia, n = Spain, n = South Africa, n = Czech Republic,  
n = Turkey, n = Tunisia, n = Hungary

## 2. Materials and methods

For this study, 130 randomly chosen wines (102 red wines, seven rosé wines and 21 white wines) from Europe, Australia, South America, and Africa were used (see Fig. 1).

All the wines were still wines and fortified wines marketed in Germany of various varieties and quality grades from the 2002 to 2011 vintages, priced from €0.99 to €120.00 per bottle. The wines used come from the 2011 MUNDUS VINI tasting, food retailers and local vintners. In order to cultivate, isolate and identify the microorganisms present and developing in the wines, depending on the bottle contents, 250–450 ml of each of the bottles was filtered through a 0.45 µm membrane filter according to the membrane filter method (TROOST G. (1988) and MADIGAN M. and MARTINKO J. (2009)). The filters were then split in half and one half was placed on wort agar and the other half on tomato juice agar. The wort agar plates were incubated aerobically for seven days and the tomato juice agar plates for ten days at approx. 27.5°C. After the incubation period, the microbes that had grown on the membranes were examined under a microscope and pure cultures were prepared. The bacteria grown were then identified by means of 16S rRNA sequencing. The cultured yeasts were identified at the University of Applied Sciences, Geisenheim using Fourier transform infrared spectroscopy (FTIR) with the kind support of Dr. Christian von Wallbrunn.



**Fig. 2.** Results of sterile filtration of the 130 wines for bacteria, yeasts, and mold. Results are shown for red wine, white wine and rosé wine, as well as the values for all types together.

## 3. Results

A total of 78.5% of the wines, i.e. 102 of the bottles examined, were free of microorganisms. Of the red wines, 77.5% were negative, of the white wines 81.0% and of the rosé wines, 85.7% were negative.

**Bacteria** were found in a total of 21 of the 130 wines tested (see Fig. 2). This translated to a bacterial load for the wines of 16.2%. Looking at the various types of wine, bacteria were found in 15.7% of the red wines, in 19.0% of the white wines, and in 14.3% of the rosé wines. The species of bacteria that were isolated and identified were *Acetobacter pomorum*, *Bacillus coagulans*, *Bacillus bataviensis*, *Bacillus ginsengihumi*, *Bacillus subtilis*, *Bacillus thuringiensis*, *Blautia hydrogenotrophica*, *Clostridium algidixylanolyticum*, *Clostridium celerecrescens*, *Clostridium roseum*,

*Clostridium saccharolyticum*, *Oenococcus oeni*, *Paenibacillus odorifer*, *Paenibacillus stellifer*, *Pediococcus parvulus*, and *Sporolactobacillus inulinus*.

**Yeasts** were found in a total of 3 of the 130 wines tested (see Fig. 2). This translated to a yeast load of 2.9% for the wines. All the yeasts were isolated exclusively from the red wines. Of the three wines, one each was from Italy, Spain, and France. The yeasts found were *Zygosaccharomyces rouxii* or *Zygosaccharomyces bisporus*, *Candida spandovensis* and *Dekkera anomala*.

**Mold** was found in a total of 9 of the 130 wines tested (see Fig. 2). This equals a 6.9% burden of mold in the wines. Viable mold spores were isolated from 7.8% of the red wines and 14.3% of the rosé wines tested. No mold spores were observed in the white wines tested. The quantities of mold were determined but not identified.

## 4. Discussion

The study shows that 15.7% of red wines, 19.0% of white wines and 14.3% of rosé wines are bacterially contaminated. Evidence of *Clostridium* spp, *Bacillus* spp, *Paenibacillus* microorganisms was detected in the wine. They are, however, inactive. The microorganisms will grow only if an optimum breeding ground is available. The source of infection for these microorganisms is unknown. It is suspected that the infection occurred after sterile filtration. Infectious organisms such as *Pediococcus*, *Oenococcus*, and *Acetobacter* are indicated in many studies and are thus known in filled bottles.

The test results pertaining to the yeast load showed a load rate of 2.9%. The wild yeasts *Zygosaccharomyces* spp, *Candida* spp. and *Dekkera* spp. (*Brettanomyces* spp. identified were found in earlier studies.

Although older literature very often referred to non-sterile filling and to the harmful yeast organism as the cause of subsequent fermentation in filled bottles, there is a tendency to bacterial contamination in the wines offered on the market today. Even if the bacteria identified did not cause subsequent hazing in the bottles, the finding remains that a multitude of various bacteria are present in bottled wines. This also means, however, that regardless if the wine is white or red, the filtrations are either not configured optimally or reinfection occurs during filling. How sterile is filling? According to the presented study findings, although the majority of wines are bottled sterile, it is indicated that many of the samples examined were

contaminated by bacteria that are not known as wine-damaging microorganisms. The term "sterile" in the bottling of wine must be reconsidered based on these findings. This question becomes all the more significant when considered in conjunction with food hygiene.

Wine as a product has lost its special status and is being classified increasingly in the food category. Wine products must thus comply with food hygiene standards. The primary consideration here is human health. The minimum requirements for hygienic practice in commercial production, processing, shipment, storage, sale, and marketing of foods are defined in the European Union Directive 93/43/EEC on food hygiene, dated June 14, 1993. With the increasing

responsibility of the food manufacturer itself, the performance of measures and controls in its own operations is prescribed to ensure the production of safe food. The implementation of the European directive in German law was ordered by the Ordinance on Food Hygiene and on Changes in Food Transport containers" ("LMHV") dated August 5, 1997. This provision was declared to be applicable to wine production with the amendment to the Wine Law (Sec. 16 Par. 3) and the Wine Ordinance (Sec. 14) dated January 31, 1998. Since August 1, 1998 wineries are thus required to comply with the general hygiene requirements. Since February 1, 1999 companies engaging in wine production must ensure through their own measures

and controls that in their production processes, risks to health of a biological, chemical, or physical nature are identified and that appropriate safety measures are determined, carried out and monitored.

### 5 Literature

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