Spoilage of bottled red wine by acetic acid bacteria

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ABSTRACT


Aims: To determine the bacterial species associated with an outbreak of spoilage in commercially bottled red wine where the bottles had been stored in an upright vertical compared with horizontal position.

Methods and Results: Bottled wines comprising Cabernet Sauvignon, Pinot Noir, Shiraz, Merlot and blended red varieties were examined for visible spoilage. Analysis of visibly affected and non-affected wines revealed a spectrum of aroma and flavour defects, ranging from loss of fruity aroma, staleness, oxidized character to overt volatile acidity. Only acetic acid bacteria, and not yeast or lactic acid bacteria, could be isolated from both spoiled and unspoiled wines and were found to grow only on Wallerstein Nutrient (WL) medium supplemented with 10\% red wine or 1–2\% ethanol. Analysis of the 16S rRNA region and RAPD-PCR analysis showed the isolates to be a closely related group of \textit{Acetobacter pasteurianus}, but this group was differentiated from the group comprising beer, vinegar and cider strains.

Conclusions: \textit{A. pasteurianus} was the species considered responsible for the spoilage but the isolates obtained had atypical properties for this species. In particular, they failed to grow on WL nutrient medium without ethanol or wine supplementation. Storage of the bottles of wine containing \textit{A. pasteurianus} in an upright vertical position specifically induced growth and spoilage in a proportion of the bottles under conditions that were inhibitory for horizontally stored bottles. We hypothesize that the upright position created a heterogeneous environment that allowed the growth of bacteria in only those bottles sealed with cork closures that had upper limit for the natural permeability to oxygen. Such a heterogeneous environment would not exist in horizontally stored bottles as the larger volume of wine adjacent to the cork would strongly compete with the bacteria for the oxygen as it diffuses through the cork closure.

Significance and Impact of the Study: A low level of bacteria (acetic acid bacteria) in wine can proliferate and cause wine spoilage in bottles stored in an upright vertical as opposed to an horizontal position under conditions that would normally limit bacterial development.

Keywords: acetic acid bacteria, \textit{Acetobacter pasteurianus}, identification, spoilage, wine.

INTRODUCTION

Acetic acid bacteria occur within the genera \textit{Acetobacter} and \textit{Gluconobacter} and are associated with wine spoilage. Conversion of ethanol to acetic acid in the presence of a small concentration of oxygen is the main form of spoilage caused by these bacteria (Greenshields 1978; Drysdale and Fleet 1988). Acetic acid is the major volatile acid in wine and is considered to be undesirable at concentrations exceeding 0.4–1.5 g l\textsuperscript{-1}, depending on the type of wine (Davis \textit{et al.} 1985). Many countries have imposed a strict limit on the maximum concentration of volatile acids in wine (Eglinton and Henschke 1999).
Acetobacter aceti and A. pasteurianus are frequently associated with wines spoiled by high volatile acidity (Drysdale and Fleet 1988). These bacteria are generally described as aerobes, but they have been routinely isolated from wine samples taken from the bottom of tanks and barrels (Joyeux et al. 1984; Drysdale and Fleet 1985), which suggests that they are able to survive, and possibly grow, under anaerobic to semi-anaerobic conditions that occur in these environments (Drysdale and Fleet 1989). Joyeux et al. (1984) proposed that oxygen permeation through wooden barrels into wine during storage is about 30 mg l$^{-1}$ per year and is sufficient to allow the survival of a low population of these bacteria. They also suggested that even brief aeration of red wine, such as occurs during wine pumping over and transfer operations, is sufficient to encourage the growth of acetic acid bacteria and cause wine spoilage, even when sulphur dioxide had been added (Millet and Lonvaud-Funel 1999). The risk of wine spoilage by acetic acid bacteria is greatest during bulk storage of wines in the cellar prior to bottling. This risk is managed by avoiding exposure of wine to oxygen and by the addition of sulphur dioxide to wines. The spoilage of bottled wines by these bacteria is a relatively rare occurrence because of the presence of sulphur dioxide, lack of oxygen and widespread use of sterile (membrane) filtration at the time of bottling (Ough 1989; Bruer et al. 1999).

In recent years, some wine industry practitioners appear to have adopted a number of practices that increase the risk of microbial spoilage of wine after bottling (Godden 2000). In response to public health issues related to the presence of sulphur dioxide in foods and beverages, the maximum level of sulphur dioxide permitted in wine has progressively been decreased over recent decades, and some winemakers have significantly decreased the use of this preservative (Ough 1983; Stockley et al. 1993; Peterson et al. 2000). Whereas levels of approximately 300 mg l$^{-1}$ were once common in French white wines, much lower values are now found (Blouin 1993). A recent survey of more than 4000 American wines revealed the average concentration of total sulphur dioxide to be 74 mg l$^{-1}$ (Peterson et al. 2000). In Australian red wines, especially, the general increase in the highest maximum pH (from pH 3.6–3.7 to 3.9–4.5) over the past 15 years, in conjunction with low total sulphur dioxide (30–80 mg l$^{-1}$), seriously reduces the effective molecular sulphur dioxide concentration for these wines (Bruer et al. 1999). Consequently, the risk of permissive conditions for microbial growth, especially lactic acid and acetic acid bacteria, has greatly increased for some wines.

In addition to this, some winemakers believe that sterile filtration detracts from the quality of premium red wines. Consequently, there is a trend by some winemakers to omit this process (Baldwin 1996; Bruer et al. 1999). Some post-bottling factors may contribute to the risk of wine spoilage.

These include the poor performance of bottle closures that may allow the ingress of oxygen (Zurn et al. 1995; Waters et al. 1996; Caloghiris et al. 1997) and the manner in which the bottles are positioned during transport and storage (Godden et al. 2001). Generally, bottles are stored on their sides with the wine in contact with the cork closure, but there has been a recent trend to store bottles in an upright (vertical) position during transportation, leaving a headspace of gas in the neck of the bottle, between the surface of the wine and the cork closure. This practice, which is believed by some to reduce the incidence of cork taint, and to prevent wine leakage and label scuffing during transport, may under certain conditions lead to an accelerated oxidative spoilage of the wine.

This paper reports the association of acetic acid bacteria with an outbreak of spoilage in bottled wine that had been vinified with minimal use of sulphur dioxide, packaged without sterile filtration and where the wine bottles had been stored in the upright vertical position for a number of months at ambient temperature.

**MATERIALS AND METHODS**

**Wine samples**

The bottled wines in this study, comprising Cabernet Sauvignon, Pinot Noir, Shiraz, Merlot and blended red varieties, were obtained from a single winery in South Australia. The bottles had been stored upright in cases held in a warehouse at 15–20°C for up to 7 months. Prior to bottling, the majority of wines had been stored in oak barrels, contained a low concentration of residual sugar (<2 g l$^{-1}$) and had undergone malolactic fermentation (MLF) (<0.2 g l$^{-1}$ L-malic acid). The wines were subsequently filtered using diatomaceous earth and coarse grade filter pads (30S filter sheets, Cuno, Australia), and bottled with a low sulphur dioxide (SO$_2$) concentration within the range of 15–20 mg l$^{-1}$ free and 50–75 mg l$^{-1}$ total SO$_2$. Wine pH values were within the range of 3.4–3.6 (data provided by collaborating winery). The wines were bottled under conditions with consistently low, measured oxygen uptake (<1 mg l$^{-1}$). The bottles used met the Australian Standard for bore dimensions and cork closures were reference 3 grade. Some batches of wine had been sealed with roll-on tamper-evident (ROTE) aluminium screw cap closures that are relatively impermeable to air (Eric et al. 1976).

Wines with visible spoilage defects were characterized as having a circular deposit (‘ring of stain’) and/or visible biofilm at the neck of the bottle at the interface of the wine and the headspace, just below the cork closure, and/or a haze or deposit in the wine. Wines showing these defects were selected for microbiological examination and bottles of
Microbiological examination

Wine samples were diluted with 0.1% peptone (Amyl Media, Australia) and spread inoculated (0.1 ml) onto plates of various agar media for the detection of contaminating microorganisms. Lactic acid bacteria were enumerated by plating onto MRS agar (Oxoid) and MRS agar supplemented with 20% apple juice (Kelly et al. 1989). Acetic acid bacteria were examined by plating onto Glucose Yeast Extract Calcium Carbonate agar (Swings et al. 1992), Wallerstein (WL) Nutrient agar (Oxoid), and WL Nutrient agar supplemented with either 2% v/v ethanol or 10% filter sterilized red wine. Filter-sterilized cycloheximide was added to these media at 50 µg ml⁻¹ to suppress the growth of yeasts. Yeasts were detected by plating onto Malt Extract Agar (Oxoid). Inoculated plates were incubated at 28°C for 7 days under aerobic and anaerobic conditions (Oxoid Gas Generating Kit cat no. BR0038).

Isolates were identified as acetic acid bacteria according to the tests and taxonomic classification described by Swings (1992) and Swings et al. (1992). These tests included: Gram stain, catalase reaction, growth on ethanol, sodium acetate and dulcitol, over-oxidation of ethanol, keto genesis of glycerol, oxidation of lactate, and production of water-soluble brown pigments.

Molecular methods

A polymerase chain reaction (PCR) method was developed to identify *A. aceti* using a primer pair, which was based on hybridization probes used by Sokollek et al. (1998) (primers AAC1 and 97 KV) yielding a PCR product of 915 bp. To confirm that a negative result was not due to insufficient or non-amplifiable DNA in the sample, two general primers (Gen-1 and Gen-2) were used to demonstrate that the sample contained amplifiable DNA (Bartowsky and Hensche 1999) (data not shown). Primers were synthesized by Gibco BRL Life Technologies (Melbourne, Australia). A thermocycler (PTC-100, MJ Research Inc., Watertown, MA, USA; with a hot bonnet for oil-free reactions) was programmed to give an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min with a final extension at 72°C for 5 min. Reaction conditions were: 0.2 µM of primer, 0.6 µM each of deoxy nucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Pharmacia Biotech), 2.5 mM MgCl₂, 5 µl of bacterial DNA template (one single colony resuspended in 100 µl of sterile water), 0.5 units of thermostable DNA polymerase (Advanced Biotechnologies) and buffer [20 mM (NH₄)₂SO₄, 75 mM Tris–HCl (pH 8.8), 0.01% Tween 20 (w/v)] as supplied by the manufacturer, in a final volume of 20 µl.

The DNA of the isolates and reference strains was also profiled using the randomly amplified polymorphic DNA (RAPD) method. Primers, 9- or 10-mers, used for the RAPD analysis included #1 (ACGGGCCCCT), #11 (OPA7-GAAACGGGTG), #12 (OPA4-AATCGGCTG) and #16 (OPG81-GTGGCTCTCC) and were synthesized by Gibco BRL Life Technologies (Melbourne, Australia). The thermocycler was programmed to give an initial denaturation at 94°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 72°C for 2 min with a final extension at 72°C for 5 min. Reaction conditions were: 0.2 µM of primer, 0.6 µM each of deoxy nucleotide triphosphate (dATP, dCTP, dGTP, dTTP; Pharmacia Biotech), 2.5 mM MgCl₂, 5 µl of bacterial DNA template (one single colony resuspended in 100 µl of sterile water), 0.5 units of thermostable DNA polymerase and buffer [20 mM (NH₄)₂SO₄, 75 mM Tris–HCl (pH 8.8), 0.01% 20 (w/v) Tween] as supplied by the manufacturer, in a final volume of 20 µl. The DNA fragments produced were separated on a 1.4% agarose gel in TBE buffer, stained with ethidium bromide and visualized by UV illumination as described by Sambrook et al. (1989). The amplified RAPD products were scored as 1 (band present) or 0 (band absent). This information was used to compile a binary data matrix that was analysed using NTSYS-pc (Numerical Taxonomy and Multivariate Analysis System, Version 2.0, Exeter Software, New York). The dendrogram showing genetic similarities were constructed with the Unweighted Pair Group Mathematical Average (UPGMA) and the SAHN algorithm.

A representative isolate based on RAPD-PCR was used for sequencing the 16S rRNA region. Chromosomal DNA of the isolate was purified using the Prepman Kit™ (Applied Biosystems), the 16S rRNA gene was amplified using universal eubacterial primers (MicroSeq® Microbial Identification system, Applied Biosystems), the fragment purified by spin column and sequenced using the automated deoxy chain terminator method (Sanger et al. 1977) by National Collections of Industrial, Marine and Food Bacteria (NCIMB, Aberdeen, Scotland).

RESULTS

Wine spoilage characteristics

A survey of wine stocks stored in the warehouse in an upright (vertical) position revealed that the occasional bottle, at random, had visible spoilage in the form of a circular deposit/stain adjacent to the wine and headspace interface.
and/or a visible biofilm, and/or a haze or deposit in the wine (Table 1). Microscopic examination of samples of this biofilm showed small rod-shaped bacteria, mostly arranged in pairs. Tasting of the visibly spoiled wines revealed a considerable spectrum of aroma and flavour defects, ranging from loss of fruity aroma/dulled aroma, staleness, oxidized/ aldehydic character to overt volatile acidity, which was described as ethyl acetate. Analysis of 22 visibly affected bottles confirmed increased concentration of acetaldehyde, ethyl acetate and volatile acidity, and decreased free sulphur dioxide content (Table 2) \((P < 0.05)\). Relative to wines without visible spoilage, acetaldehyde content had increased by up to 2–3-fold, volatile acidity by up to 0–3 g l\(^{-1}\) and ethyl acetate had increased by up to 8–12 mg l\(^{-1}\). Furthermore, the free sulphur dioxide content of many of the spoiled wines approached zero, whereas the total sulphur dioxide content of these wines was not obviously reduced (bottled variation was marked). As no visible spoilage was observed in all bottles inspected sealed with the ROTE closure, a single bottle was subjected to chemical and microbiological analysis (Tables 1 and 2).

### Isolation and tentative identification

The wines, whether visibly spoiled or not, were characterized by the presence of bacteria that were only detected by plating of samples onto WL. Nutrient agar supplemented with 10% wine incubated under aerobic conditions. Bacterial populations from wines sampled from either spoiled or unspoiled bottles ranged between \(10^2\) and \(10^3\) CFU ml\(^{-1}\). The colonies formed on these media were pinpoint in size (0.5–1 mm diameter) and green in colour. When examined under the microscope, cells appeared as short rods that were arranged singly, in pairs (mostly) or short chains. Their cellular morphology was similar to that observed for samples of biofilm taken from the surface of the wine, at the neck of the bottle. On subculture, isolates continued to grow as small, pinpoint colonies. Incubation of plates for 5–7 days was necessary for colony development.

The weak growth of these bacteria was an obstacle to their further study. Therefore, a range of media was examined for improved growth. The isolates did not grow in Tryptone Soya Agar or Broth, Nutrient Agar or Broth, Brain Heart Infusion Agar or Broth, and MRS Agar or Broth, unsupplemented or supplemented with 1–2% ethanol. The isolates also failed to grow on Glucose Yeast Extract Calcium Carbonate medium but gave weak growth on this medium when it was supplemented with 2% ethanol, and on modified Carr’s medium, which contained yeast extract and 2% v/v ethanol (Carr 1968). Bacterial growth on WL Nutrient agar was not increased by the addition of 1–5% ethanol, 2% casamino acids (Difco), 2% yeast extract (Oxoid) or 10% red grape juice. However, growth on WL Nutrient agar was enhanced by the addition of 10–20% filter sterilized red wine. Colonies on this medium were only slightly larger than when cultivated on WL Nutrient agar without wine, but they developed full size within 5 days compared with 5–7 days. Increased growth was achieved on agar media compared to that in liquid media.

The isolates were Gram negative, catalase positive, aerobic rods with an ability to utilize ethanol and oxidize acetic acid through to carbon dioxide (i.e. ‘overtoxider’). They oxidized lactate and grew on dulcitol. However, their biochemical reactions were generally very weak or negative, and

### Table 1 Incidence of microbial deposit in vertically upright stored bottled Shiraz wine sealed with ROTE* or two different brands of cork closures†

<table>
<thead>
<tr>
<th>Closure</th>
<th>Number of bottles tested</th>
<th>Visible stain, film or deposit</th>
<th>% affected</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROTE</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cork 1</td>
<td>13</td>
<td>9</td>
<td>69.2</td>
</tr>
<tr>
<td>Cork 2</td>
<td>17</td>
<td>13</td>
<td>76.5</td>
</tr>
</tbody>
</table>

*ROTE – roll-on tamper-evident.
†Data provided by collaborating winery.

### Table 2 Association between microbial deposit and chemical composition of bottled Shiraz wine sealed with ROTE* or two different brands of cork closures†

<table>
<thead>
<tr>
<th>Closure</th>
<th>Deposit</th>
<th>Volatile acidity (g l(^{-1}))</th>
<th>Ethyl acetate (mg l(^{-1}))</th>
<th>Acetaldehyde (mg l(^{-1}))</th>
<th>Free SO(_2) (mg l(^{-1}))</th>
<th>Total SO(_2) (mg l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ROTE</td>
<td>Absent</td>
<td>0.61</td>
<td>87</td>
<td>24</td>
<td>7</td>
<td>58</td>
</tr>
<tr>
<td>Cork 1</td>
<td>Absent</td>
<td>0.65 [0.01] (0.63–0.67)</td>
<td>79 [82] (54–89)</td>
<td>21 [12] (16–26)</td>
<td>10 [1.5] (8–13)</td>
<td>53 [3.6] (46–58)</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>0.72 [0.03] (0.62–0.94)</td>
<td>91 [4.1] (81–114)</td>
<td>51 [87] (31–96)</td>
<td>1 [0.5] (0.3)</td>
<td>52 [0.4] (50–54)</td>
</tr>
<tr>
<td>Cork 2</td>
<td>Absent</td>
<td>0.65 [0.01] (0.61–0.66)</td>
<td>75 [82] (54–94)</td>
<td>18 [10] (16–20)</td>
<td>10 [0.3] (10–11)</td>
<td>52 [2.0] (48–54)</td>
</tr>
<tr>
<td></td>
<td>Present</td>
<td>0.73 [0.03] (0.61–1.07)</td>
<td>83 [0.9] (80–88)</td>
<td>37 [6.8] (11–72)</td>
<td>6 [1.8] (0.8)</td>
<td>53 [0.6] (49–56)</td>
</tr>
</tbody>
</table>

*ROTE – roll-on tamper-evident.
†Data provided by collaborating winery.
Standard errors of the mean value given in brackets.
Range of data given in parentheses.

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SPOILAGE OF BOTTLED RED WINE BY ACETIC ACID BACTERIA

311
difficult to interpret due to poor growth. They grew at pH 4.0. Based on these responses and the fact that the organisms were isolated from wine, they were tentatively identified as species of Acetobacter.

Identification by molecular methods
Isolates from the spoiled wine did not give a PCR product using the A. aceti specific primers (data not shown), thereby suggesting that they were probably A. pasteurianus. Sequencing of the 16S rRNA of one of the isolates confirmed the species as A. pasteurianus (99-751% identity, a good match). Various isolates from the spoiled wines gave similar RAPD profiles, which were found to cluster together (Figure 1), but were distinct from strains of A. pasteurianus isolated from other sources, such as beer, rice vinegar and cider.

DISCUSSION
Although acetic acid bacteria are well known for their potential to spoil wines during bulk storage and handling in wineries, they are not normally associated with the spoilage of wines after bottling (Drysdale and Fleet 1985, 1988; Bruer et al. 1999). Our study suggests that a combination of modified processing and handling conditions has provided the basis for an unusual, widespread outbreak of bottled wine spoilage by acetic acid bacteria. In particular, the wines were not filter-sterilized at the time of bottling and contained a low concentration of the preservative, sulphur dioxide, which was insufficient to eliminate viable bacteria from both spoiled and unspoiled bottles. Significantly, although the wines were bottled with low oxygen content, the bottles were stored in a vertical position, leaving a headspace of gas between the surface of the wine in the neck of the bottle and the cork closure. The oxygen content of this entrapped gas, which is finite, and minimized by the modern bottling equipment used, does not explain the random nature of the spoilage noted (Caloghiris et al. 1997). However, various studies with natural cork implicate variation in their oxygen permeation characteristics as measured by the rate of oxidation of wine constituents, such as ascorbic acid, sulphur dioxide and phenolics (Waters et al. 1996; Caloghiris et al. 1997; Godden et al. 2001; Jung and Zurn 2001), and it has been estimated that over a year, several millilitres of oxygen could enter a bottle via this route (Ribéreau-Gayon et al. 1976; Casey 1992). There is a growing body of evidence to suggest that vertical storage of bottles, where the wine is not in contact with the cork closure, may decrease the ability of the cork to exclude air (G. Skouroumounis and E.J. Waters, AWRI, personal communication). In our study, only a small proportion of bottles developed spoilage characteristics for the batches of wine stored upright. Notably, some of the bottled wines with the highest level of spoilage had been sealed with corks that were damaged with wormholes formed by cork borers or other physical flaws. None of the bottles sealed with the ROTE closure showed evidence of wine spoilage. Collectively, these observations suggest that ingress of oxygen through the corks in vertically stored bottles may have been a contributing factor to the spoilage outbreak. This type of spoilage problem has not re-occurred as the winery reverted to storage of their bottled wines in a horizontal rather than vertical position.

A possible explanation for the observation that bottles stored in the upright position exhibited an increased incidence of microbially induced oxidative spoilage could be due to either a greater permeability of the cork closure to oxygen or to the development of a ‘heterogeneous environment’ in the film of wine that forms up the side of the bottle neck, adjacent to the inner surface of the cork. At the present time, the evidence for cork closures in upright bottles to become more permeable to gaseous exchange over

Fig. 1 Dendrogram generated by cluster analysis of the similarity matrix of 23 winery isolates of presumptive Acetobacter pasteurianus and 10 A. pasteurianus isolates from other alcoholic beverages. Superscript T refers to type strain. The analysis was performed with the NTSYS-PC (Numerical Taxonomy and Multivariate Analysis System, version 2.0, Exeter Software, New York) using unweighted pair-group method arithmetic average (UPGMA) and the SAHN algorithm. Numbers refer to numerical similarities.
storage time is not strong. However, the second hypothesis only depends on oxygen being the limiting factor for bacterial growth as well as the natural variation in oxygen permeability characteristics of natural cork closures that can be inferred from recent studies (Godden et al. 2001). In this hypothesis, bacteria entrapped in a film of wine, which forms up the side of the neck of the bottle, under the action of a trace amount of ‘new’ oxygen provided by only those cork closures having a higher permeability to oxygen, would modify their immediate environment by a combination of metabolic and chemical oxidative reactions. This would lead to commencement of growth, with cells spreading from the side of the neck of the bottle to form a biofilm on the surface of the wine. In this way, the biomass would intercept and be exposed to the highest concentration of ‘new’ oxygen to enable metabolic oxidation processes to proceed. However, the bacteria, suspended in the wine immediately adjacent to the cork closure in horizontally placed bottles, due to the competing high demand for oxygen by wine (Singleton 1987), would conceivably not modify the wine environment sufficiently to allow cell growth, and obvious spoilage to result. That oxygen entering the bottle via the cork closure was the trigger for visible spoilage was shown by the fact that ROTE sealed bottles did not spoil. All studies published to date have demonstrated the reduced rate of wine oxidation in bottles closed with the ROTE closure (Godden et al. 2001).

No information was obtained to suggest that the spoiled bottles contained a genotypically different or physiologically ‘fitter’ strain of bacteria from the unspoiled ones. Furthermore, the fact that bacteria were found in spoiled and unspoiled bottles alike did not support the possibility that only some bottles received a sufficient dose of bacteria to cause the random spoilage observed, assuming that no other factors were limiting for growth. The random distribution of spoiled bottles also argues against systematic processing faults, such as an amount of contaminated wine at the commencement of bottling, contaminated bottles or corks, aeration of a quantity of wine caused by a mechanical fault during a filling shift, and spoilage-enabling conditions in a section of the warehouse.

From a microbiological perspective, there are some anomalies to explain in relation to this spoilage outbreak. Although A. pasteurianus was diagnosed to be the organism associated with this spoilage, the isolates were not typical for this species. They were very difficult to culture in various standard microbiological and biochemical test media, although the presence of ethanol as well as other factors in wine enhanced their growth. This compromised their identification by conventional procedures. They were very difficult to culture, and their poor growth in various microbiological media and biochemical tests compromised identification by conventional procedures. Nevertheless, several molecular techniques confirmed this identification. The RAPD data also provided evidence that the strains obtained from the wines in the study are different from strains of A. pasteurianus isolated from other ecosystems. The presence of ethanol as well as other factors in wine seemed to encourage their growth. A detailed, systematic study of A. pasteurianus from various ecological habitats is needed to resolve and understand the unusual observations we have made.

Neither yeasts nor lactic acid bacteria were detected in the spoiled wines. As the wines of this study were several months into storage, it is possible that the low levels of viable acetic acid bacteria (10^2–10^4 CFU ml^-1) detected in the spoiled bottles were the remnants of a population that had grown to much greater levels (e.g. 10^6–10^7 CFU ml^-1) and had already died off. However, this did not seem to be the case, as the spoiled wines, overall, were not visibly turbid and yielded only a slight sediment of bacteria on centrifugation. Moreover, all of the bottles of unspoiled wines examined were found to contain low populations (10^1–10^3 CFU ml^-1) of Acetobacter. Drysdale and Fleet (1985) have previously reported that A. pasteurianus and A. aceti occurred at 10^1–10^3 CFU ml^-1 in many wines during bulk storage in wineries, without causing spoilage. This apparent low population of recovered viable cells may, however, be underestimated due to the bacteria being present in a viable non-culturable state caused by the low oxygen content of bottled wine and other stresses imposed by the wine environment. Millet and Lonvaud-Funel (2000) have shown that acetic acid bacteria enter a viable non-culturable state when deprived of oxygen in red wine. According to Joyeux et al. (1984), such low populations can be activated to produce significant volatile acidity on exposure to air. Furthermore, Millet and Lonvaud-Funel (1999) reported that the free sulphur dioxide (15–20 mg l^-1) concentrations typically added to wine following MLF for storage in barrels are not necessarily sufficient to suppress the growth of acetic acid bacteria when exposed to small amounts of oxygen.

In summary, we have reported an unusual outbreak of bottled wine spoilage by acetic acid bacteria. The problem was most likely initiated by a combination of circumstances including bottling of wine without sterile filtration, a lower concentration of sulphur dioxide in wine prior to bottling, and storage of bottles in an upright vertical position. Such upright vertical orientation of the wine bottles could encourage the ingress of air through the cork closure, or alternatively, provide a heterogeneous environment from which a bacterial growth focus developed and migrated to the surface of the wine. A. pasteurianus was the species considered responsible for the outbreak but the isolates obtained were atypical and require further physiological and biochemical characterization.
REFERENCES


