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Acetic acid bacteria spoilage of bottled red wine—A review

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Abstract

Acetic acid bacteria (AAB) are ubiquitous organisms that are well adapted to sugar and ethanol rich environments. This family of Gram-positive bacteria are well known for their ability to produce acetic acid, the main constituent in vinegar. The oxidation of ethanol through acetaldehyde to acetic acid is well understood and characterised. AAB form part of the complex natural microbial flora of grapes and wine, however their presence is less desirable than the lactic acid bacteria and yeast. Even though AAB were described by Pasteur in the 1850s, wine associated AAB are still difficult to cultivate on artificial laboratory media and until more recently, their taxonomy has not been well characterised. Wine is at most risk of spoilage during production and the presence of these strictly aerobic bacteria in grape must and during wine maturation can be controlled by eliminating, or at least limiting oxygen, an essential growth factor. However, a new risk, spoilage of wine by AAB after packaging, has only recently been reported. As wine is not always sterile filtered prior to bottling, especially red wine, it often has a small resident bacterial population ($<10^3$ cfu/mL), which under conducive conditions might proliferate. Bottled red wines, sealed with natural cork closures, and stored in a vertical upright position may develop spoilage by acetic acid bacteria. This spoilage is evident as a distinct deposit of bacterial biofilm in the neck of the bottle at the interface of the wine and the headspace of air, and is accompanied with vinegar, sherry, bruised apple, nutty, and solvent like off-aromas, depending on the degree of spoilage. This review focuses on the wine associated AAB species, the aroma and flavour changes in wine due to AAB metabolism, discusses the importance of oxygen ingress into the bottle and presents a hypothesis for the mechanism of spoilage of bottled red wine.

Keywords: Acetic acid bacteria; *Acetobacter*; Wine spoilage; Vinegar; Ethyl acetate; Oxygen

1. Introduction

The ubiquitous acetic acid bacteria (AAB) fall within the Acetobacteraceae family and are well adapted to various sugar and ethanol rich environments. Their ability to efficiently convert ethanol through acetaldehyde to acetic acid is utilised in culinary and medicinal vinegar production, however, in the wine industry this capability constitutes spoilage (Fleet, 1993; Sponholz, 1993; Lonvaud-Funel, 1996). Wines spoiled by AAB have characteristic volatility, a vinegar-like sourness on the palate and a range of acetic, nutty, sherry-like, solvent or bruised apple aromas and often a reduction in fruity characters (Bartowsky et al., 2003). Such wines have low commercial value but can in some cases be improved by blending or treatment by a reverse-osmosis process to lower acetic acid content.

Grapes and wine are subject to spoilage by AAB at many stages during the winemaking process (Drysdale and Fleet, 1988). Physically damaged grapes or those infected by fungi can become infected with AAB and cannot be used in wine production if the volatile acidity exceeds statutory limits (Eglinton and Henschke, 1999a). AAB growth can also occur in grape must or during stuck fermentation if exposed to the air. Most commonly wines are spoiled with AAB during maturation or storage when unintentionally exposed to air (Joyeux et al., 1984a). Bacterial spoilage has also recently been reported to occur in packaged wine such as vertically upright bottles (Bartowsky et al., 2003). This is visually evident as a distinctive ring of bacterial biomass that is deposited on the neck of the bottle at the interface between the wine and the air headspace (Fig. 1).

Prevention of AAB proliferation and wine spoilage is based on an understanding that these bacteria are aerobic in their physiology and require oxygen for growth. Such growth can be prevented by practices that include blanketing wine with an inert gas such as carbon dioxide, and ensuring that storage

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Fig. 1. Distinctive bacterial ring shaped deposit at the interface between the wine and headspace in the bottle neck (top—heavy ring, bottom—no ring). Arrow highlights the bacterial deposit formed on top bottle neck.

containers are completely filled with wine to minimize contact with the headspace of air. However, it has become evident that these bacteria may survive and even multiply, albeit slowly, under semi-anaerobic conditions, such as wine stored in tanks and barrel (Drysdale and Fleet, 1988, Joyeux et al., 1984a). Even more undesirable for the wine industry is the survival and proliferation of AAB in bottled wine, an aspect which has been, until more recently, neglected in research.

Wine spoilage by AAB has been discussed in various general reviews on wine microbiology and specific reviews on acetic acid bacteria (Vaughn 1955, Rao 1957, Amerine and Kunkee 1968, Drysdale and Fleet 1988, Sponholz 1993, Du Toit and Pretorius 2002). This review will concentrate on the spoilage of bottled wine by AAB, focussing on wine associated species, wine composition and the factors that can contribute to spoilage of bottled red wine.

2. Taxonomy of wine associated acetic acid bacteria

2.1. Taxonomy

Until recently, the taxonomy of the acetic acid bacteria had not been well characterized. Historical aspects of the wine associated AAB have been previously described (Drysdale and Fleet 1988) and this review will focus only on the recent developments.

The Gram negative AAB taxonomically belong to the family Acetobacteraceae, which falls within the *Alphaproteobacteria*, and consists of 15 genera, of which three, *Acetobacter, Gluconobacter* and *Gluconacetobacter,* are associated with grape and wine spoilage (Garrity et al., 2004). The taxonomy of AAB is complex and has been under review over recent years (see other papers in this special edition) and we will limit this discussion to the wine related AAB species. Due to differences in ethanol tolerance and their preference of ethanol as a carbon and energy source, species of *Acetobacter* are more often isolated from wine, whereas *Gluconobacter* species are isolated from grape must. *Gluconobacter oxydans* is the main species found in association with grapes and grape must. *Acetobacter hansenii* and *A. liquefasciens* have recently been reassigned as *Gluconacetobacter hansenii* and *Gluconacetobacter liquefasciens* (Yamada et al., 1998) and can be infrequently isolated in winemaking. The two species of *Acetobacter* most often isolated from wine are *A. aceti* and *A. pasteurianus*. More recently a new *Acetobacter* species, *A. oeni*, has been described which was isolated from spoiled red wine in the Dão region of Portugal (Silva et al., 2006). *Acetobacter tropicalis* has recently been isolated from spontaneously fermenting Austrian wine followed by acetic fermentation (Silhavy and Mandl 2006), whereas, previously it had only been isolated from coconut (Lisdiyanti et al., 2000).

The key biochemical differences between wine associated AAB are shown in Table 1. The major distinguishing feature between *Acetobacter* and *Gluconobacter* is the ability to oxidise acetic acid to CO_2. The *Acetobacter* species are able to oxidize ethanol to acetic acid and then to carbon dioxide and water, whereas *Gluconobacter* species do not have a complete citric acid cycle and cannot oxidize ethanol further than acetic acid. Gluconobacters are sometimes referred to as under-oxidisers and acetobacters as overoxidisers, because of their difference in oxidative potential.

2.2. Methods for isolation

AAB are fastidious microorganisms and can be difficult to isolate and cultivate on artificial medium, despite the great number of growth media proposed. Those AAB which have proliferated in bottled wine have proven at times to be more difficult to isolate and cultivate (Xia, 1997, Bartowsky et al., 2003). AAB have been ascribed to the phenomenon of viable but non-culturable state (VBNC) (Millet and Lonvaud-Funel 2000) where the population of AAB is very often under-estimated by the inability to culture the population on growth media. Epifluorescence staining techniques have been developed for the enumeration of total, viable and non-viable AAB during vinegar production (Mesa et al., 2003, Baena-Ruano et al., 2006). A real-time PCR method has been developed in order to detect these VBNC cells (González et al., 2006b).

2.3. Identification and strain differentiation

In the past, wine AAB have been identified to genus and species levels according to an array of morphological, physiological and biochemical tests as shown in Table 1. A range of PCR based methods is now available for differentiating the genera, species and strains of these bacteria. PCR based methods have been developed for the identification of *A. aceti* in wine samples using a primer pair which was based on hybridisation probes (Sokollek et al., 1998b, Bartowsky et al., 2003) and the PQQ-dependent alcohol dehydrogenase gene (Trcek 2005). The rapid identification of AAB species has also been made simpler by the recent development of PCR based techniques using ERIC-PCR and REP-PCR (Gonzales et al., 2004), RFLP of the 16S RNA and 16S–23S rDNA ITS region (Ruiz et al., 2000, Kretová and Grones, 2005, González et al., 2006a), and PCR denaturing gradient gel electrophoresis (PCR-DGGE) for AAB isolated in traditional balsamic vinegar production (De Vero et al., 2006).

Table 1
Biochemical tests to distinguish wine associated acetic acid bacteria

Distinguishing acetic acid bacteria (AAB) from lactic acid bacteria (LAB)		
	AAB	LAB
Gram stain	Negative	Positive
Catalase reaction	Positive	Negative
Motility	Motile or non-motile	Non-motile
Oxygen requirement	Obligately aerobic	Aerobic or anaerobic
Production of acetic acid from ethanol	Yes	No
Sugar metabolism	Hexose monophosphate pathway	Homo-or hetero-fermentative
G+C content (mol %)	>50	<50

Distinguishing AAB genera

	Acetobacter	*Gluconacetobacter*	*Gluconobacter*
Motility and flagellation	Peritrichous or non-motile	Peritrichous or non-motile	Polar or non-motile
Oxidation of ethanol to acetic acid	+	+	+
Oxidation of acetic acid to CO_2 and H_20	+	+	–
Oxidation of lactate to CO_2 and H_20	+	+or −	−
Growth on 0.35% acetic acid containing medium	+	+	+
Growth in the presence of 30% glucose	−	+or −	−or weak+
Ketogenesis from glycerol	+or −	+or −	+
Acid production from			
Glycerol	+or −	+	+
D-mannitol	+or −	+or −	+
Raffinose	−	−	−
Production of soluble brown pigment(s)	−	Variable	Variable
Ubiquinone type	Q-9	Q-10	Q-10

Distinguishing AAB species

	Acetobacter				*Gluconacetobacter*		*Gluconobacter*
	aceti	*oeni*	*pasteurianus*	*tropicalis*	*hansenii*	*liquefaciens*	*oxydans*
Growth on carbon sources							
Glycerol	+	+	Variable	+	+	Variable	+
Ethanol	+	−	Variable	−	−	+	+
Dulcitol	−		−		Variable	−	Variable
Sodium acetate	+		Variable		−	Variable	−
Formation from D-glucose of							
2-keto-D-gluconic acid	+	−	Variable	+	Variable	+	+
5-keto-D-gluconic acid	+	+	−	−	Variable	Variable	+
2,5-keto-D-gluconic acid	−				−	+	+
Acid production from							
D-glucose	+		Variable	+	+	+	+
D-mannose	+		−	Variable	+	+	+
D-galactose	+		Variable	+			
L-arabinose	+		Variable	−			+
D-xylose	+		Variable	+weak			
Ketogenesis from							
Glycerol	+	+	−	−	+	+	+
Sorbitol	+		−		+	+	+
Mannitol	Variable		−	−	+	+	
Nitrate reduction	−		+	+			
N_2 fixation					−	−	
G+C content (mol %)	56.2–57.2	58.1	51.8–53	55.2–56.6	58–63	62–65	56–64

Data are combined from various sources (Drysdale and Fleet 1988, Sokollek et al., 1998a, Sokollek et al., 1998b, Lisdiyanti et al., 2000, Lisdiyanti et al., 2001, Sievers and Swings 2005, Silhavy and Mandl 2006, Silva et al., 2006).
−=negative.
+=positive.
Variable=11–89% of strains positive.

The genetic differentiation of strains within a species has always been a challenge. This is exemplified with wine associated AAB, especially with the inability to easily isolate these microorganisms from wine and to maintain viable subcultures being the biggest hurdle. Most studies of wine associated AAB or those isolated from wine vinegars have been

more interested in the inter species genetic variability rather than achieving quantitative recovery or investigating the intra species genetic variability. Thus, strain differentiating techniques have not been widely used on wine associated AAB. A phenotypic technique based on whole cell protein patterns was used to differentiate AAB strains isolated from South African Cabernet Sauvignon must fermentations (Du Toit and Lambrechts 2002). The genotypic technique RAPD-PCR (randomly amplified polymorphic DNA) was successfully used to differentiate *A. pasteurianus* strains isolated from spoiled bottled red wine (Bartowsky et al., 2003). RAPD-PCR analysis of *A. pasteurianus* wine isolates and *A. pasteurianus* strains isolated from different sources, including vinegar, rice vinegar, and spoiled cider demonstrated that they formed distinct separate groups by cluster analysis (Bartowsky et al., 2003, Bartowsky and Henschke 2004). *A. pasteurianus* strains isolated from South African Cabernet Sauvignon fermentations also clustered together to form a separate group from the *A. pasteurianus* type strain (Du Toit and Lambrechts 2002). Thus, there is mounting genetic evidence that the wine associated *A. pasteurinaus* strains belong to a distinct subpopulation of this species. This may well be related to the niche environment in which wine strains reside, in that they have adapted to the high ethanol concentrations in wine (12–16%), in comparison to *A. pasteurianus* strains isolated from various other ecological habitats. Other factors may also be important, such as the ability to survive in a harsh environment for long periods with a limited availability of oxygen and during exposure to sulfur dioxide, used in wine making as an antimicrobial agent.

3. Flavour sensory changes due to spoilage by acetic acid bacteria

Wine is a complex mixture of compounds which largely define its appearance, aroma and flavour, and mouthfeel properties (Lambrechts and Pretorius, 2000; Swiegers et al., 2005). The compounds responsible for those attributes derive from three major sources, grapes, microorganisms and, when used, wood (oak). During the grape vinification process microbial metabolism results in a desirable balance of sensory compounds by modifying grape-derived molecules and by producing flavour-active metabolites. The concentration of sensorily active compounds and their interaction with other wine components play an important role in the perception of any particular compound (Francis and Newton, 2005). AAB do not contribute to the desirable microbial flora, but are rather considered spoilage organisms because their major metabolites result in disagreeable wine sensory characteristics.

Acetic acid is the major volatile acid in wine, the main constituent of wine volatile acidity (VA) and considered to be undesirable in dry wine at concentrations exceeding 0.4–0.5 g/L, depending on wine type (Davis et al., 1985, Eglinton and Henschke 1999a, Eglinton and Henschke 1999b), however, in sweet wines, such as ice wine or botrytised wine, it only becomes undesirable at 1.0–1.5 g/L (Zoecklein et al., 1999, Nurgel et al., 2004). Sensorily, acetic acid is recognised in wine as contributing a sour flavour and at high concentration a bitter, sour flavour with a vinegar-like aroma (Peynaud, 1984). Acetic acid is produced in low concentrations, usually below detection threshold, by yeast during alcoholic fermentation and by lactic acid bacteria during malolactic fermentation (Henick-Kling 1988, Eglinton and Henschke 1999b), however, concentrations can increase considerably through the metabolism of spoilage yeast, spoilage species of lactic acid bacteria and acetic acid bacteria.

Acetic acid bacteria produce acetic acid through the metabolism of ethanol to acetaldehyde to acetic acid. There are two membrane-bound enzymes catalysing the reactions: alcohol dehydrogenase (ethanol to acetaldehyde) and acetaldehyde dehydrogenase (acetaldehyde to acetic acid) (Adachi et al., 1978, Adachi et al., 1980, Ameyama et al., 1981, Adachi et al., 1987, Fukaya et al., 1989, Tayama et al., 1989). The intermediate metabolite acetaldehyde can also contribute to the sensory spoilage of wine with distinct aroma descriptors; sherry-like, bruised apple or nutty (aroma threshold 0.5 mg/L) (Francis and Newton 2005). The ethyl ester of acetic acid, ethyl acetate, has a pungent aroma, solvent-like and reminiscent of nail polish remover (aroma threshold 7.5 mg/L) (Francis and Newton 2005), and often occurs in higher concentration following the growth of AAB in wine.

Many of these aroma characteristics are immediately obvious upon opening a bottle of wine spoiled by AAB, especially the volatile vinegar-like character due to the increase in acetic acid and ethyl acetate. These wines are often considered to be oxidised and dull with a decrease in fruity aroma. In the past, wines with such a defect were allowed to 'breathe', however, consumers are much less tolerant of such wines today. In conjunction with obvious aroma defects of spoiled bottled red wine is a deposit, often noted as a visible ring of bacterial growth on the bottle neck at the junction between the surface of the wine

Table 2
Chemical and microbiological analysis of Shiraz wine in visually spoiled and unspoiled wines

Neck ring [a]		None	Fine	Medium	Heavy
Dissolved O_2	(g/L)	0.07	0.44	0.17	0.21
pH	(units)	3.5 (0.0)	3.5 (0.0)	3.5 (0.0)	3.5 (0.0)
Alcohol	(% v/v)[c]	13.3 (0.0)	13.3 (0.0)	13.3(0.0)	13.1 (0.1)
Free SO_2	(mg/L)	17 (0)	7 (1)	<5 (0)	<5 (1)
Total SO_2	(mg/L)	74 (1.1)	57 (1)	62 (6)	72 (0)
Glucose+ fructose	(g/L)	0.95 (0.01)	0.93 (0.01)	0.95 (0.02)	0.92 (0.01)
Citric acid	(g/L)	0.12 (0.00)	0.12 (0.00)	0.12 (0.00)	0.12 (0.00)
Malic acid	(g/L)	0.37 (0.02)	0.38 (0.01)	0.38 (0.03)	0.34 (0.05)
Acetic acid	(g/L)	0.5 (0.0)	0.5 (0.0)	0.6 (0.0)	3.5 (1.7)
Ethyl acetate	(mg/L)	100 (1)	103 (0)	108 (5)	730 (276)
Acetaldehyde	(mg/L)	36 (1)	45 (2)	70 (5)	160 (7)
Viable cells [b]	(cfu/mL)	6.0×10^1	1.0×10^3	2.0×10^4	9.0×10^4
Number of samples tested		12	4	4	2

Values are means and standard errors are shown in brackets.
a − Visual inspection of the neck ring formed on the bottle neck at the interface between the wine and headspace.
b − Enumeration of viable *Acetobacter pasteurianus* cells from the contents of the bottle, as determined on WL agar plates supplemented with 10% red wine (0.2 μm filtered); these figures may underestimate the true population due to the uncertainty in recovering all the bacteria.
c − v/v − volume/volume of % alcohol.

and headspace created by the bottle closure. The thickness of the neck ring is a reflection of the degree of AAB spoilage (Fig. 1).

As very few studies of AAB spoilage in bottled wines have been reported, we include some unpublished data (Bartowsky and Henschke 2004). Randomly oxidised Shiraz wine, which had been stored upright in bottles for approximately 5 months, were sorted by the presence and absence of a visible neck ring deposit and subjected to chemical and sensory analysis (Table 2 and Fig. 2). Wines lacking a visible neck ring deposit were described as being dominated by high overall fruit aroma with no vinegar-like spoilage characteristics (Fig. 2). As the degree of wine spoilage increased, as noted by the thickness of the neck ring deposit, so did the acetaldehyde, ethyl acetate and volatile acidity, with overall fruit perception being notably reduced. Interestingly, the severely spoiled wines, with a heavy neck ring deposit, had a very pronounced solvent-like ethyl acetate aroma, which concurs with the chemical data (Table 2) and most likely masked the acetaldehyde.

The chemical composition of these wines clearly changed as the *A. pasteurianus* population increased (Table 2). The cell population increased by almost four log orders of magnitude from unspoiled through to heavily spoiled red wine. The residual sugar (glucose plus fructose), pH, and citric and malic acids concentrations did not vary with spoilage, however there was a decrease in ethanol concentration and a substantial increase in acetic acid concentration. The free sulphur dioxide concentration decreased from 17 mg/L to less than detectable (5 mg/L) in the most heavily spoiled wines. There was a large increase in acetaldehyde (4 fold) and ethyl acetate (7 fold) from unspoiled to heavily spoiled wines.

Acetic acid, acetaldehyde and ethyl acetate are the main spoilage compounds produced by wine associated AAB species that have been investigated. There has been minimal research undertaken on other spoilage compounds. Mousy off flavour, as the descriptor suggests, is perceived in the mouth and is reminiscent of mice kept in a confined space (Tucknott 1977). AAB have been implicated in the production of mousy off-flavour which is caused by N-heterocycles (Vaughn 1955, Grbin et al., 1996), however, limited research has been undertaken examining their role in this wine spoilage.

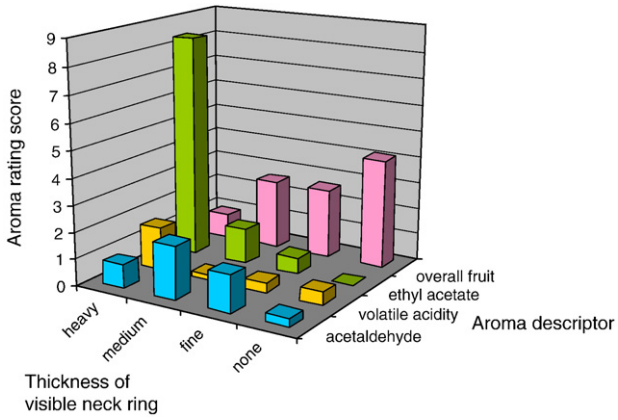

Fig. 2. Sensory analysis of Shiraz (2000 vintage) wines with varying degrees of visible spoilage. Visibly spoiled bottles were ranked as either fine, medium or heavy, as noted from the bottle neck deposit. The sensory panel was asked to rate the wines on a scale of 0 to 9 for the various attributes, where a score of 0 indicated that the attribute could not be perceived, while 9 was defined as the highest intensity observed.

4. Growth and metabolism in wine by acetic acid bacteria

4.1. Growth of acetic acid bacteria during wine production

Acetic acid bacteria have long been associated with the occurrence of volatile acidity in wines, evident as sensorially perceptible concentrations of acetic acid, and its ethyl ester, that accumulate during wine production. AAB can adversely impact on wine quality at several stages during the winemaking process, including grape maturation in the vineyard, must preparation, alcoholic fermentation, MLF, the maturation phase and packaging.

The association between volatile acidity in wine and AAB is strongest when grapes are physically damaged or infected with fungal diseases, especially *Botrytis cinerea*, which lead to colonization of the sugary substrate by a variety of secondary microorganisms, including AAB (Drysdale and Fleet 1988, Sponholz 1993). The population size of AAB is typically small (10^2–10^3 cells/g) on sound berries but can exceed 10^6 cells/g on damaged grapes (Lafon-Lafourcade and Joyeux 1981). Many factors affect colonization by AAB, including grape variety, region and season (Renouf et al., 2006b). Depending on harvest and must transport conditions, which can favour or discourage growth or survival of AAB, grapes can be an important source of AAB in winemaking. For example, unless protected from oxygen and effective doses of SO_2 are used during machine harvest and must transport, the potential for AAB growth and potential to produce acetic acid can become important.

Grape must and wine are not conducive environments for the growth of bacterial species. Even though grape must can be a rich, nutritious growth medium, high concentrations of hexoses (glucose and fructose, up to 200–250 g/L), high acidity (pH 2.9–3.7; TA 2–10 g/L as tartaric acid), and sulfites (0–100 mg/L) provide a highly selective environment for AAB that limits growth to primarily *Gluconobacter* species, especially *G. oxydans*. Furthermore, the very harsh environment of wine created by high ethanol concentration, low oxygen content and redox potential, low pH and depletion of nutrients, resulting from consumption by yeast during alcoholic fermentation, restricts growth to principally LAB and AAB genera *Acetobacter* and *Gluconacetobacter* (Joyeux et al., 1984a, Drysdale and Fleet 1985). Unless the yeast are removed from the wine immediately following completion of fermentation, various growth stimulatory nutrients are excreted into the wine, which can stimulate their growth (Alexandre et al., 2004). *G. oxydans*, metabolise the small concentrations of ethanol produced by the indigenous yeast during must and juice preparation and during the early stages of alcoholic fermentation as a carbon source, converting it to acetic acid (Joyeux et al., 1984a). In the absence of yeast, such as during must preparation, AAB populations can increase to spoil grape must by the production of acetic acid. During alcoholic fermentation, however, the population of AAB tends to decrease and can fall to below 10^2 cells/mL by the end of fermentation (Drysdale and Fleet 1988, Du Toit and Lambrechts 2002). *Acetobacter* species are better adapted to the higher ethanol

concentrations and thus tend to colonise wine rather than grape must. Evolution of AAB species during grape vinification has not been studied extensively.

4.2. Key metabolic activities of acetic acid bacteria in wine

AAB are able to alter the grape must constituents with their growth by metabolising glucose, fructose, malic and citric acids with the formation of gluconic, lactic and succinic acids, acetaldehyde and ketone compounds (Joyeux et al., 1984b). The metabolism of hexose and pentose sugars involves an initial phosphorylation and then the hexose monophosphate pathway to form acetic and lactic acids, which in some species are further oxidised via the tricarboxylic acid cycle to carbon dioxide and water, whereas, in other strains, hexose sugars may be directly oxidised to gluconate and ketogluconates (Katznelson et al., 1953, Olijve and Kok 1979, Stadler-Szöke et al., 1980). Thus, high concentrations of gluconic acid and ketogluconic acids are good markers for the presence and metabolism of AAB (Joyeux et al., 1984b, Eschenbruch and Dittrich 1986, Drysdale and Fleet 1988).

The production by *G. oxydans* of gluconic acid, its lactone derivatives, and ketogluconic acids can impact on wine quality by strongly binding SO_2, which is added during winemaking as an antioxidant as well as an antimicrobial substance. This chemical reaction decreases the antimicrobial efficacy of SO_2 and leads to an increased content of total SO_2 in wine (Joyeux et al., 1984b, Barbe et al., 2000, Barbe et al., 2002, Sponholz et al., 2004). *G. oxydans* also produces 5-oxofructose, another important SO_2 binding compound, which is of major importance in musts prepared from grapes infected with *B. cinerea* (Barbe et al., 2000). This compound further reduces the efficacy of SO_2 and leads to elevated concentrations of total SO_2.

Glycerol is an important component of wine and is formed by yeast during alcoholic fermentation. *G. oxydans* and *A. aceti* can oxidatively metabolise glycerol to dihydroxyacetone (ketogenesis) (Hauge et al., 1955, De Ley and Schell 1959) a reaction which is often used as a biochemical test for AAB identification (Table 1). Ethanol metabolism is well known and forms the basis of vinegar production during which it is oxidised to acetic acid. AAB are known to oxidise alditols to the parent sugar but, the extent to which this occurs in grape must and wine has not been determined (Fewster 1958, Ameyama et al., 1965, Drysdale and Fleet 1988).

Acetobacter species show a greater ability to metabolise organic acids than *Gluconobacter* species (Joyeux et al., 1984b), but this property has received little attention. Inorganic ammonia can serve as a sole source of nitrogen and there are no known requirements for essential amino acids (Belly and Claus 1972, Gossele et al., 1981). Other growth factors appear to depend on the available carbon source and to some extent pH. Most strains of *G. oxydans* require pantothenic acid and some require either one or more of nicotinic acid, *p*-aminobenzoic acid, niacin, and thiamin. When growing in the presence of glucose, *Acetobacter* species do not require specific growth factors (Underköfler et al., 1943, Ameyama and Kondo 1966), however when D-mannitol is the carbon source *p*-aminobenzoic acid, niacin, thiamine or pantothenic acid are required (Ameyama and Kondo 1966, Ameyama et al., 1985).

4.3. Factors affecting growth and survival of acetic acid bacteria in wines

4.3.1. Influence of other microorganisms

A variety of ethanol-tolerant microorganisms can grow in grape must and wine and their interactions can be complex (Fleet, 2003). Yeast, particularly *Saccharomyces cerevisiae*, are responsible for the alcoholic fermentation of grape must, however, the presence of other yeast and bacterial species will influence the growth and metabolism of the *S. cerevisiae*. Even though this area has received little attention, some evidence suggests that the growth of AAB species can influence the growth activities of *S. cerevisae* during alcoholic fermentation. Slow or sluggish fermentations have been attributed to the development of *Gluconobacter* and *Acetobacter* but the nature and importance the yeast-inhibitory substances has not been well established (Gilliland and Lacey 1964, Lafon-Lafourcade and Joyeux 1981, Joyeux et al., 1984b). Populations of AAB up to 10^6 cells/mL in grape musts have been shown to be sufficient to cause the death of *S. cerevisiae* (Grossmann and Becker 1984), however, the mechanism of antagonism was not elucidated. In another study, the viability of yeast was only slightly impaired by the presence of AAB during alcoholic fermentation of Muscat Gordo and Traminer musts, however the metabolism of the yeast was significantly affected (Drysdale and Fleet 1989b). The growth of AAB with *S. cerevisiae* induced classic symptoms of an incomplete fermentation with high residual sugar and lower ethanol, reduction in *iso*-amyl alcohol and glycerol, and the presence of AAB resulted in higher acetic acid and gluconic acid, and production of acetaldehyde and ethyl acetate, (Drysdale and Fleet 1989b). Comparison of an inoculated and spontaneous yeast fermentation of a Grenache grape must for AAB populations and their influence on yeast populations and alcoholic efficiency demonstrated that a rapid start of fermentation resulted in a decrease in AAB population (final AAB populations,~4×10^1 [inoculated] compared with 5×10^2 cells/mL [spontaneous]) (Guillamon et al., 2002). Factors that facilitates the growth of AAB that leads to the inhibition of alcoholic fermentation needs further study, especially with the recent trend towards spontaneous fermentation.

Grapes infected with *B. cinerea* are usually also contaminated by AAB species. Metabolic products from *Gluconobacter* species have been shown to increase the SO_2 binding power of *B. cinerea* affected grape musts by oxidising the hexoses in the grape must, glucose plus fructose, and glycerol to gluconic acid, 5-oxofructose and dihydroacetone, respectively (Barbe et al., 2001), reducing the antimicrobial activity of SO_2 and contributing to the higher total SO_2 content of wine.

4.3.2. pH

Studies in South African Cabernet Sauvignon fermentations showed that AAB numbers decreased from the beginning to the

middle of fermentation in low pH musts (pH<3.5), but this trend was lessened in higher pH musts (pH 3.7) (Du Toit and Lambrechts 2002). *G. oxydans* became undetectable by the middle stage of fermentation and was replaced by *Acetobacter* species. Similar observations were made in a Grenache study, where it was found that changes in the environment during alcoholic fermentation substantially reduced the diversity and survival of AAB (Gonzalez et al., 2005).

4.3.3. Sulfur dioxide

Sulfur dioxide (SO_2) is used as an antioxidant and antimicrobial agent during winemaking. When SO_2 is added to wine, various chemical equilibria are established with wine components, which depend on wine composition (Boulton et al., 1996). A bisulfite ion reacts with carbonyl compounds to form carbonyl bisulfite which is referred to as bound SO_2 and the free form is present as molecular SO_2, the equilibria being strongly influenced by wine pH. The molecular form of SO_2 is responsible for the antimicrobial activity and the presence of 1 mg/L of molecular SO_2 has been reported to inhibit fermentation (Sudraud and Chavet 1985). The effects of SO_2 on the survival and growth of AAB have not been well investigated with varying SO_2 inhibitory concentration being observed. The concentrations of SO_2, as used in normal winemaking practice do not appear to be sufficient to control the growth of AAB (Peynaud, 1984). *A. aceti* has been shown to grow in the presence of 25 mg/L free SO_2 and a total of at least 100 mg/L total SO_2 was required to control the growth of AAB species (Joyeux et al., 1984b, Watanabe and Iino 1984). A recent study demonstrated that low concentrations of SO_2 (0.35 mg/L molecular SO_2) were found to have minimal effect on viability and culturability of an *A. pasteurianus* strain, whereas higher concentrations were effective (1.2 mg/L molecular SO_2, equivalent to 75 mg/L free SO_2 at pH 3.6) (du Toit et al., 2005). This is in agreement with the report that 0.8 mg/L molecular SO_2 is required to prevent *A. aceti* growth in wine (Beech et al., 1979, Beech and Thomas 1985).

Cold soaking or maceration prior to alcoholic fermentation is often practiced with red wines to increase the extraction of colour from the skins. One study highlights the often ineffectiveness of normal winemaking SO_2 concentrations on controlling AAB populations in grape musts. Despite the addition of SO_2 (40 to 50 mg/kg SO_2) to commercial South African red grape musts, the AAB population increased from 10^3 to almost 10^5 cfu/mL during the 3 day cold soaking stage (15–18 °C) prior to yeast inoculation for alcoholic fermentation (Du Toit and Lambrechts 2002).

4.3.4. Storage of wine in barrel or bottle

Following alcoholic and malolactic fermentations, red wine is normally matured in tank or barrel and bottle for up to several years before sale. As indicated in Section 4.1, an important source of AAB in winemaking is grapes, especially physically damaged and fungal infected bunches. However, the maturation vessels, especially previously used wooden barrels, can also represent another important source of AAB. Although new barrels do not contain wine associated yeasts and bacteria, they quickly colonise barrels when introduced with new unsterilised wine (Renouf et al., 2006a, Renouf et al., 2006b). The porous nature of wood provides a relatively protected environment for various wine microorganisms including AAB, some of which can escape standard barrel washing procedures to infect a new batch of wine when conditions are suitable. The introduction of filtered or heat treated wine, in particular, can become contaminated by barrel microflora (Renouf et al., 2006b). Of four methods evaluated for treating barrels infected with AAB, only hot water treatment (85–88 °C for 20 min) was successful in eliminating AAB; treatment with solutions of either SO_2, chlorine or potassium carbonate proved ineffective (Wilker and Dharmadhikari 1997).

During maturation and storage of wine in tank, barrel or bottles, oxygen is either deliberately excluded or carefully controlled during storage of wine in barrel or bottle, and only when this control fails can AAB strains proliferate, though, they might remain viable for extended periods in the absence of oxygen. The mechanism of survival is not well understood but it is likely that AAB can use other wine compounds (quinones and reducible pigments) as electron acceptors (Joyeux et al., 1984a, Du Toit and Pretorius 2002). As with growth in grape must and fermenting must, the survival of the strictly aerobic AAB is very much dependent upon the presence of oxygen. The transfer of wine, containing an AAB population, from fermentation tank to storage tank or barrel may introduce sufficient oxygen through agitation and aeration to stimulate the growth of surviving AAB populations from 10^2 up to 10^5 cells/mL (Cuinier and Guerineau 1978, Joyeux et al., 1984a). Populations of AAB as high as 10^5 cells/mL, with a predominance of *A. pasteurianus*, have been observed in tank-stored Australian wines (Drysdale and Fleet 1985) but a predominance of *A. aceti* were found in Bordeaux stored wines (Joyeux et al., 1984a). It has been estimated that AAB requires all the oxygen from 1 L of air to produce an increase in volatile acidity by 0.4 g/L (as sulfuric acid) in 1 L of wine (Peynaud, 1984).

The proliferation of AAB in stored bottled wine has received little attention even though it can be a major problem for the wine industry, especially if bottled wine is stored in suboptimal conditions for an extended period of time. Bottled red wine often has a low population of microorganisms and the product is not usually sterile filtered, unlike white or sweetened wines. As red wine is generally matured for several months or years before packaging, it is usually considered to be microbially stable and a recent trend to reduce SO_2 additions has emerged. As a consequence, under conductive conditions the resident microbial population, very often AAB, might proliferate and spoil the wine. In a study of bottled Shiraz wines, the proliferation of the resident *A. pasteurianus* population was shown to result in mild to severe spoilage of the wine and was attributed to the ingress of oxygen through the bottle closure during vertical storage (Bartowsky et al., 2003).

5. Role of oxygen

Acetic acid bacteria are taxonomically classified as obligately aerobic organisms that have a respiratory metabolism which uses

oxygen as the terminal electron acceptor (Matsushita et al., 1994). Since ancient times it has been known that the surface of wine left in contact with air quickly develop acetic or vinegar-like characteristics. More recent studies have shown that momentary aeration, such as that introduced by agitation or racking of wine from one barrel into another is sufficient to encourage significant growth of resident AAB populations (Joyeux et al., 1984a, Joyeux et al., 1984b). AAB have routinely been isolated from anaerobic environments such as amongst the sediments of wine storage tanks and barrels indicating their ability to survive in the absence of oxygen for considerable periods of time (Joyeux et al., 1984a). Estimates of oxygen permeation through a barrel at a rate of approximately 30 mg/L per year have suggested that this ingress concentration is sufficient to permit the survival of low populations of AAB (Joyeux et al., 1984a).

Wine that has been fully aerated (100% saturated oxygen) can sustain the rapid growth of *A. aceti* and *A. pasteurianus* from an initial population of 10^4–10^5 to 10^8 cells/mL within a few days. At 70% saturated oxygen in wine a lower final population (10^6–10^7 cells/mL) was observed (Drysdale and Fleet 1989a). Growth for both species was greatly reduced in wine with 50% saturated oxygen. Most importantly was the observation that the bacteria could survive but not proliferate in wine containing 50% or less saturated oxygen. AAB can use compounds such as quinones and reducible dyes as electron acceptors and this is thought to contribute to their ability to survive and even grow in anaerobic or semi-aerobic environments (Joyeux et al., 1984a, Du Toit and Pretorius 2002).

In recent years the practice of micro-oxygenation of red wine with the aim to bring about desirable changes is increasing. During micro-oxygenation a controlled concentration of oxygen is introduced into the wine at a slow rate which should be slower than the rate of consumption by the wine. Desirable changes observed through micro-oxygenation in the wine include enhanced colour stability and intensity, softening of astringent tannins and decreased reductive and vegetative aromas (Parish et al., 2000). A recent study in various South African red wines showed that micro-oxygenation led to higher AAB populations (du Toit et al., 2006). Thus, all winemaking practices which could introduce oxygen into the wine need to be considered carefully for the risk of allowing the resident AAB population to proliferate and potentially spoil the wine.

Little is known about the survival of AAB in bottled red wine partially, because there is little or no perception by the research community of AAB spoilage in wine after packaging. As red wine is not usually sterile filtered and in some cases little or no SO_2 is added prior to bottling, it is not unusual to have a low population of bacteria, including AAB ($<10^3$ cells/mL) present (Bruer et al., 1999). Ingress of oxygen through the bottle closure, natural or synthetic, is known to occur but the rate of ingress varies greatly between closure type. ROTE (roll on tamper evident) closures consistently exclude the ingress of oxygen to the greatest extent, whereas, natural cork closures, the rate of oxygen ingress recently reported range from 0.1 to 122.5 μL of oxygen per day (Godden et al., 2005), and 0.5–4.4 μL oxygen per day in vertically stored bottles and 1.7–6.1 μL oxygen per day in horizontally stored bottles between the second and 12th month (Lopes et al., 2006). For AAB growth in bottled red wine, the rate of oxygen ingress is probably not as crucial as the fact that it is entering and thus providing the AAB with their vital growth limiting nutrient, oxygen.

Wine, red and white, sealed under natural cork closure has been shown to be better conserved when stored horizontally rather than vertically (Mas et al., 2002), whereas another study using two white wines found that bottle orientation had little effect on wine composition and sensory properties (Skouroumounis et al., 2005). Oxygen that enters the bottle through the closure will either enter directly, by dissolving into the wine when the bottle is stored horizontally or into the headspace between the closure and the wine in a vertically stored bottle. It is in the latter scenario that most of the spoilage of wine by AAB has been observed.

Although red wines are normally bottled with a low oxygen content, bottles that are 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 will provide the opportunity for oxygen to migrate into the headspace. The oxygen content of this entrapped gas, which is finite, and minimised by the modern bottling equipment used, does not explain the often random nature of the AAB spoilage (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 Zürn 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). This ingress of oxygen could be enhanced if the cork moisture level decreases and 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 commercially bottled wine developed spoilage characteristics for the batches of wine stored in the upright position. Nevertheless, this incidence of spoilage which was tolerated decades ago is now considered unacceptable. Notably, some of the bottled wines with the highest level of spoilage had been closed with corks that were damaged with worm holes formed by cork borers or had other physical flaws. Grading natural cork according to oxygen permeability characteristics needs to be a key criterion for the future.

6. Hypothesis for bottled wine spoilage

From our studies one of the key characteristics of bottled red wine spoilage by AAB is the random nature of its occurrence, highlighting the crucial role that oxygen ingress into the bottles, through their closure, must play. Numerous studies have shown that there is variation in natural cork oxygen permeation (Waters et al., 1996, Caloghiris et al., 1997, Godden et al., 2001, Jung and Zürn 2001). We have proposed a hypothesis based on variation of oxygen permeation in natural cork closures to explain the formation of the neck ring deposit, which contains a

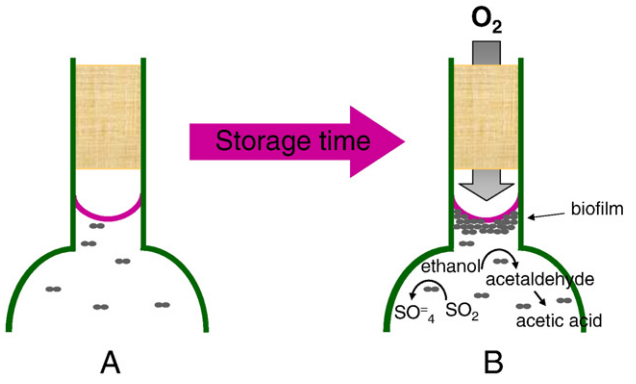

Fig. 3. Hypothetical model for formation of biofilm and bottle neck deposit to produce wine spoilage by acetic acid bacteria (AAB) growing in upright vertically-stored wine bottles. Bottle A—insufficient oxygen ingress to allow the growth of a small resident population of AAB; Bottle B—ingress of sufficient oxygen through the closure into the headspace to allow growth of AAB and to initiate the formation of a visible deposit at the wine-headspace interface.

high population of bacteria (Fig. 3) (Bartowsky et al., 2003). Initially, bacteria entrapped in a film of wine, that forms up the side of the neck of the bottle, under the action of a trace amount of 'new' oxygen provided by only those natural closures having a higher permeability to oxygen, would modify their immediate environment (contained in the thin film of wine) by a combination of chemical and metabolic oxidative reactions. This would lead to the 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 be exposed to the highest concentration of 'new' oxygen to enable metabolic oxidation processes to proceed. As the bacterial numbers increase, the formation of a neck ring will become more evident leading to wine spoilage. This bacterial wine spoilage appears to occur more frequently in vertically rather than horizontally stored bottles. Even though oxygen can enter via the closure in horizontally stored bottles, the 'new' oxygen would be consumed by reaction with wine components (Singleton 1987), particularly phenolics, before becoming available to the bacteria that are suspended in the wine immediately adjacent to the cork closure. Thus, little stimulation of bacterial growth is likely in horizontally stored bottles except when sealed with high oxygen permeable closures.

7. Conclusions

Acetic acid bacteria are part of the indigenous microflora of grapes and wine, and as such their proliferation during vinification, storage and post-bottling needs to be prevented. A variety of control measures can play a role in controlling AAB proliferation. Exclusion by sterile filtration is a possible control measure but membrane fouling, cost and time delays can limit application with red wines. The essential requirement for oxygen by AAB provides an important prevention tool in achieving this goal. The storage of bottled wine needs to be carefully considered and accordingly managed to ensure that, particularly, the bottle closure and storage position (horizontal rather than vertical) do not provide a means for oxygen ingress and thus do not provide a small resident bacterial population with a key nutrient allowing proliferation and subsequent spoilage.

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