

Evolution of Acetic Acid Bacteria During Fermentation and Storage of Wine

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Acetic acid bacteria were present at all stages of wine making, from the mature grape through vinification to conservation. A succession of *Gluconobacter oxydans*, *Acetobacter pasteurianus*, and *Acetobacter aceti* during the course of these stages was noted. Low levels of *A. aceti* remained in the wine; they exhibited rapid proliferation on short exposure of the wine to air and caused significant increases in the concentration of acetic acid. Higher temperature of wine storage and higher wine pH favored the development and metabolism of these species.

In the field of oenology, acetic acid bacteria have received little attention (3, 4, 6). This lack of interest may be explained by the fact that these bacteria are strictly aerobic, and consequently they were considered unable to grow in wines except at wine surfaces in permanent contact with air.

In recent years, wineries in the Bordeaux region have become concerned about the small increases in the levels of acetic acid sometimes encountered during the storage of wine in wooden barrels. This has provided the basis of the present investigation.

The application of new techniques has facilitated the selective isolation of acetic acid bacteria (8). In this work, the evolution of such populations is described, both in number and nature, from the grape to the wine in storage. A relationship between the bacterial growth and the formation of acetic acid in the wine at different pH and temperature conditions is established.

MATERIALS AND METHODS

Wine samples used in this study are described in the text.

Grapes were aseptically harvested from vines (7). Must and wine samples from large tanks and barrels were obtained by using large, sterilized glass pipettes. Acetic acid bacteria were selectively isolated and enumerated (8) by plating wine samples on the following medium (per liter): glucose, 10 g; Casamino Acids (Difco Laboratories), 5 g; yeast extract, 5 g; and tomato juice, 10 ml. Tomato juice (10 ml) was extracted from tomatoes by crushing and was clarified by centrifugation. The pH was adjusted to 4.5, and the medium was sterilized at 10 lb. (ca. 4.5 kg) of pressure for 20 min.

At the moment of use (7) 5.0 ml of the medium was dispensed into sterile petri dishes with 0.1 ml of 0.5% pimaricin solution to inhibit the growth of yeasts and molds and with 0.1 ml of 0.25% penicillin (250,000 IU stock solution) to inhibit the growth of lactic acid bacteria. Molten (40°C) 3% agar (5 ml) was added and then carefully mixed. Plates were incubated at 25°C for 4 to 8 days.

After purification, isolates were identified according to the tests given in Buchanan and Gibbons (5). Principal characteristics used for the differentiation of genera and species were: (i) the capacity or incapacity to degrade lactate distinguishes *Gluconobacter* and *Acetobacter* spp.; (ii) within the latter genus, the ability to oxidize glycerol or not

distinguishes *Acetobacter aceti* from *Acetobacter pasteurianus*; (iii) *Acetobacter peroxydans*, which is catalase negative like *A. pasteurianus* subsp. *paradoxus*, is distinguished by its development with ammonia as a nitrogen source.

The concentrations of acetic, lactic, and citric acids and glucose and fructose were determined by enzymatic methods (2).

RESULTS

Association of acetic acid bacteria with grapes. Studies were conducted with grapes harvested during three successive years (1978, 1979, and 1980) (A. Joyeux, Ph. D. thesis, Université de Bordeaux II, Talence, 1983) from wineries in the Bordeaux region of France. A variety of both white and red grapes were examined, as well as white grapes that had undergone "pourriture noble" by the fungus *Botrytis cinerea*. The density of acetic acid bacteria was always found to be linked to the degree of grape infection.

Freshly extracted juice from grapes at the beginning of the harvesting season contained an average of 10^2 cells per ml of acetic acid bacteria. Slightly higher levels were found on the early-picked pourriture noble grapes, but these had increased to 10^4 to 10^6 cells per ml on such grapes picked at the end of the harvesting season.

Gluconobacter oxydans was the main representative of acetic acid bacteria on sound, unspoiled red or white grapes. However, *A. aceti* and, to a lesser extent, *A. pasteurianus* became more prevalent as the grapes became spoiled. These two species accounted for 75 to 85% of the acetic acid bacteria on the *Botrytis*-infected grapes.

Development of acetic acid bacteria in white wines. The must obtained from sound white grapes contained only a very small population ($<10^2$ cells per ml) of *G. oxydans*, which quickly disappeared during fermentation. In contrast, the behavior of acetic acid bacteria was quite different during the production of Sauterne-style white wines from grapes parasitized with *B. cinerea*. An example of the development is shown in Table 1. The initial must contained around 10^6 cells per ml of acetic acid bacteria, with *G. oxydans* being the predominant species. The yeast population was about the same. During the course of the natural alcoholic fermentation, the total population of acetic acid bacteria decreased to less than 10^3 cells per ml; *A. pasteurianus* and *A. aceti* became the predominant representatives in equal proportion.

Development of acetic acid bacteria in red wines. The levels

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TABLE 1. Evolution of acetic acid bacteria during vinification of a sauterne-style white wine

Fermentation stage ^a	Acetic acid bacteria (cells/ml) ($\times 10^3$)	Microorganism (%)
Grape must	2,000	<i>G. oxydans</i> (80) <i>A. pasteurianus</i> (20)
5 days	80	<i>G. oxydans</i> (70) <i>A. pasteurianus</i> (30)
12 days	100	<i>A. pasteurianus</i> (50) <i>A. aceti</i> (50)
20 days	0.6	<i>A. pasteurianus</i> (50) <i>A. aceti</i> (50)

^a The must was obtained from Semillon grapes parasitized by *B. cinerea* and fermented at 21°C in 225-liter barrels. The initial sugar concentration was 300 g/liter, and the initial pH was 3.5.

and species of acetic acid bacteria found at different stages during the vinification of a red wine are presented in Table 2. Freshly pressed must contained about 10^4 cells per ml of *G. oxydans*. The population progressively decreased during alcoholic fermentation, and at the end of this fermentation (10 days), only 20 cells per ml were detected in the wine. The proportion of *G. oxydans* in the population decreased and that of *A. pasteurianus* increased (Table 2). On drainage of the wine from the fermenting tank, the population increased to around 3×10^4 cells per ml, and this may be explained by contamination through contact with equipment and perhaps multiplication of those cells present in the wine as a consequence of agitation and aeration. *A. pasteurianus* was the predominant species after draining.

Samples of wine taken at the commencement of the malolactic fermentation exhibited counts of ca. 10^2 to 10^3 cells per ml and consisted mainly of *A. pasteurianus* and lesser amounts of *G. oxydans* and *A. aceti* (Table 3). At the completion of malolactic fermentation (which took 3 months), cell counts remained around 10^2 cells per ml and consisted mainly of *A. aceti* and a smaller proportion of *A. pasteurianus*.

At this stage, 20 mg of SO₂ per liter was added to the wine; then the wine was mixed, filtered, and returned to the wooden barrels for storage at 10°C. Samples taken during the next 11 months of storage exhibited counts of ca. 10^2 to 10^3 cells per ml and consisted mostly of *A. aceti* and lower levels of *A. pasteurianus* (Table 3). The amount was about the same at the top and the bottom of the barrel. At the end of this period, all the acetic acid bacteria were *A. aceti* subsp. *aceti*.

During that time, wine was added to the barrels twice a week to compensate for evaporation. In a few cases, *G. oxydans* was again identified immediately after this operation (Table 3). But a week later this species had again disappeared. This suggests that its presence was due to an infection brought on either by the wine or by the equipment used in filling up the barrels.

Relationship between the growth of acetic acid bacteria and the formation of acetic acid in wines. In a preliminary laboratory study, a 15-ml sample of wine (pH 3.55; ethanol, 12%; free SO₂, 20 mg/liter) was incubated in a 20-ml test tube at 19°C. During the course of 8 days, counts, consisting of a mixed population of *A. aceti* and *A. pasteurianus*, progressively increased from an initial level of 4×10^2 cells per ml to 1.1×10^5 cells per ml. At the same time the concentration of acetic acid in the wine increased from 0.53 g/liter to 0.85 g/liter. Levels of lactic acid did not change, but the acetal-

dehyde concentration almost doubled, from 28 mg/liter to 52 mg/liter. It should be noted that in this experiment, between the second and the third days of incubation, 130 mg of acetic acid per liter was produced, although the bacteria population increased only from 8×10^2 to 12×10^2 cells per ml.

In another laboratory trial, the effects of wine pH, storage temperature, and momentary aeration on the growth of acetic acid bacteria and acetic acid production were examined. In this case *A. aceti* was the only representative of the acetic acid bacteria in the wine. The wine contained 12% ethanol and 20 mg of free SO₂ per liter. The growth of lactic acid bacteria, which were also present in the wine, was prevented by the addition of 250 mg of penicillin per liter (250,000 IU stock solution).

Wine samples were adjusted to either pH 3.4 or 3.8 and stored at either 10 or 18°C. The samples were stored in 100-ml completely filled flasks to yield an anaerobic environment. After 8 days of storage the contents of the flasks were exposed to air for 3 min by pouring them into beakers and then returning them to the flasks; under such conditions the amount of oxygen dissolved into the wine is about 7.5 mg/liter (9). Analyses were performed 8 days after this aeration.

In the samples stored in a completely anaerobic condition, for both temperatures the acetic acid bacteria decreased more rapidly at pH 3.4 than at pH 3.8; the concentrations of acetic acid stayed constant.

However, 1 week after the momentary aeration, the results (Table 4) showed little growth at 10°C, but the numbers of cells increased by 30- to 40-fold on storage at 18°C. This was accompanied by a significant increase in the level of acetic acid. Slightly higher counts of bacteria were observed at the higher pH of 3.8. The counts were still higher when no SO₂ was added.

Some practical observations. Table 5 shows some analytical data for a commercial red wine stored in new wooden barrels after completion of the malolactic fermentation. The wine samples were analyzed after 3 months of storage. Levels of acetic acid bacteria had increased by ca. 10-fold.

The levels of lactic acid bacteria increased, but this varied with the barrel. The main substrates for lactic acid bacteria are citric acid, glucose, and fructose. In barrel 1, 52 mg of citric acid per liter and 20 mg of fructose and glucose per liter were utilized, but such metabolism would not account for the production by lactic acid bacteria of 60 mg of acetic acid per liter that was also observed.

TABLE 2. Evolution of acetic acid bacteria during the vinification of red wine

Fermentation stage ^a	Acetic acid bacteria (cells/ml) ($\times 10^3$)	Microorganism (%)
Grape must	16	<i>G. oxydans</i> (100)
3 days	3	<i>G. oxydans</i> (75) <i>A. pasteurianus</i> (25)
7 days	0.1	<i>G. oxydans</i> (55) <i>A. pasteurianus</i> (45)
10 days		
Before draining	0.02	<i>G. oxydans</i> (30)
After draining	30	<i>A. pasteurianus</i> (70)

^a Must was extracted from Cabernet-Sauvignon grapes and fermented in 200-ml stainless-steel tanks. The initial sugar concentration was 200 g/liter, and the initial pH was 3.3. Samples were taken from the bottom of the tank.

TABLE 3. Evolution of acetic acid bacteria during malolactic fermentation and storage of red wine in barrels^a

Times of fermentation and storage	Acetic acid bacteria (cells/ml) ($\times 10^2$)	Microorganism (%)
Beginning of malolactic fermentation	4	<i>G. oxydans</i> (20) <i>A. pasteurianus</i> (60) <i>A. aceti</i> (20)
After malolactic fermentation (3 months)	1.2	<i>A. pasteurianus</i> (20) <i>A. aceti</i> (80)
After storage in barrels 4 months	1.6	<i>A. pasteurianus</i> (20) <i>A. aceti</i> (80)
6 months	9	<i>G. oxydans</i> (10) <i>A. pasteurianus</i> (30) <i>A. aceti</i> (60)
9 months	6	<i>A. pasteurianus</i> (20) <i>A. aceti</i> (80)
11 months	6	<i>A. aceti</i> (100)

^a For origin of the wine, see Table 2: footnote a.

Considerably more acetic acid was produced in barrels 2 and 3, where virtually no hexoses were metabolized and the same amount of citric acid as in barrel 1 was metabolized.

The data suggest that acetic acid bacteria are responsible for the greater part of acetic acid production in wines.

Barrels 2 and 3, as compared with barrel 1, had been stored for 1 year after making. It is possible that the wood of these barrels might have been dried, and being more impregnated with air, it encouraged the higher levels of acetic acid bacteria.

DISCUSSION

Overall, this study demonstrated the permanent presence of acetic acid bacteria during every stage of wine making. A change in the type of bacteria present occurs during fermentation and storage, and successively these bacteria are *G. oxydans*, *A. pasteurianus*, and *A. aceti*. Generally *G. oxydans* specifically contaminates the grapes and *A. aceti* contaminates the wine. *A. pasteurianus* contamination occurs occasionally. Nevertheless, *G. oxydans* survives during alcoholic fermentation to some extent, and its virtual disappearance is probably related to its alcohol intolerance. It may contaminate stored wine when barrels are topped up to compensate for evaporation, but it does not survive long after this event.

Although *G. oxydans* is the major representative of acetic

TABLE 4. Effect of wine pH and storage temperature on the growth of acetic acid bacteria and concentration of acetic acid in wine after aeration^a

Storage time	Storage temp	Wine pH	Acetic acid bacteria (cells/ml) ($\times 10^2$)	Acetic acid (mg/liter)
0			5	370
15 days	10°C	3.4	15	380
15 days	10°C	3.8	15	420
15 days	18°C	3.4	140	500
15 days	18°C	3.8	200	520

^a The wine from Cabernet-Sauvignon grapes contained 12% ethanol and 20 mg of free SO₂ per liter; it contained *A. aceti* only. After 8 days of storage, the contents of the flasks were exposed to air for 3 min by pouring them into beakers and then returning them to the flasks. Analyses were performed 8 days after this aeration.

TABLE 5. Production of lactic and acetic acids by residual bacterial populations during the storage in barrels for 3 months of wine^a

Sample	Bacterial population (cells/ml) ($\times 10^2$)		Acetic acid (mg/liter)	Lactic acid (mg/liter)		Citric acid (mg/liter)	Glucose (mg/liter)	Fructose (mg/liter)
	Acetic	Lactic		D(-)	L(+)			
Before storage	0.3	10	420	370	980	70	200	160
Barrel 1	2.5	800	480	430	1,080	18	180	140
Barrel 2	3.4	30	720	400	1,050	27	200	150
Barrel 3	3.4	70	650	430	1,200	18	200	150

^a Ethanol, 12.6%; pH, 3.67; free SO₂, 25 mg/liter.

acid bacteria on sound grapes, *A. aceti* becomes important on grapes infected by *B. cinerea*. Presumably, its occurrence on spoiled grapes is facilitated by the production of ethanol by yeasts which multiply where the grape skin has been ruptured. Thus, spoiled grapes represent an important source of contamination by *A. aceti*.

A. aceti can survive during the storage of wine in barrels. The quantity of oxygen which penetrates through the wood (about 30 mg/liter for 1 year) (9) prevents the complete destruction of the population, occurring when conditions are strictly anaerobic, for example, in bottles.

However, these small surviving populations (10² cells per ml) of *A. aceti* can have, during storage, unfavorable action on the quality of wines. In fact, brief exposure of the wine to air, causing the rapid dissolving of ca. 7.5 mg of oxygen per liter, can lead to a rapid multiplication of this microorganism and consequently the production of acetic acid. This phenomenon is particularly accentuated at high temperature and elevated pH.

Many observations made in different cellars show definitely that acetic acid bacteria are responsible for the small increases of acetic acid sometimes observed during wine storage in barrels, even when this is done under the best conditions. This fact is a new concept for oenology. It has always been admitted that acetic acid bacteria were strictly aerobic, so they could develop only in casks which had not been kept filled (1). In consequence it was believed (10) that anaerobic lactic acid bacteria could only develop when wine was stored in barrels, because the quantity of oxygen was insufficient to provide for the oxidation of ethanol by acetic acid bacteria.

In a normal wine-making procedure, a short period of aeration can occur, for instance, during the racking process. Then the temperature and, to a lesser extent, the pH values are the most significant factors which affect the bacterial growth and the production of acetic acid, which appears even in the subsequent absence of oxygen; free SO₂, in the concentration used as a preservative of red wines, does not sufficiently protect against the metabolism of acetic acid bacteria.

The practical consequences which arise from these results are: (i) the necessity for a low temperature (10 to 15°C) during the storage of the wine in barrels; and (ii) the necessity of avoiding any aeration at higher temperatures, for instance, during the summertime; however, in red wine making, this aeration is indispensable for the normal evolution of the phenolic compounds involved in wine color (11).

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