BACTERIA AND THEIR INFLUENCE ON BIOGENIC AMINES

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INTRODUCTION

Biogenic amines (BA) are organic compounds (nitrogenous bases) with low molecular weights which can be frequently found in fermented alimentary products such as wine, cheese, etc. The characteristics and biological effects of the BA are very diverse in between them, in that they can have either beneficial or harmful effects on human beings. Some amines, such as putrescine, seem to be indispensable for the cellular growth and proliferation of living organisms. Other BA, such as histamine and tyramine, on the other hand have harmful health effects, or in the case of putrescine, have effects on the organoleptic characteristics of the alimentary products. For this reason their concentration in alimentary products should be very low, or at the least not higher than certain levels (9). In the case of wines, there have been no more than 20 different BA described (20), the most abundant and dangerous of which being histamine, tyramine, phenylethylamine and putrescine, and at lower levels cadaverine and tryptamine (9).

The synthesis of the BA requires the combination of three different factors: the existence of the precursors (amino acids), the presence of microorganisms with corresponding decarboxylase activity, and the presence of adequate environmental conditions (7). The existence of amino acids is linked not only qualitatively but also quantitatively with the primary matters and the vinification technologies: grape variety, harvest timing, addition of amino acid nutrients, maceration, addition of enzymes, microbiological lysis, etc. (7). The presences of favourable conditions for microbial metabolism, such as high pH, low levels of SO2, etc. evidently encourage the synthesis of BA (15).

In the following study, we will focus our attention of the second of the three previously underlined factors, the presence of microorganisms with decarboxylase activity, more specifically bacteria, and in particular the lactic acid bacteria (LAB).

ACETIC ACID BACTERIA

Acetic acid bacteria (AAB) seem to be scarce producers of BA and in general, have not been described as a source of their synthesis in wine. In an extensive study completed by our group with numerous *Acetobacter* species (*A. aceti, A. malorum, A. pasteurianus, A. pomorum, A. tropicalis* and *Acetobacter* sp.), *Gluconacetobacter* (*G. diazotrophicus, G. europaeus, G. hansenii, G. liquefaciens, G. oboediens, G. sacchari* and *G. xylinus*), and *Gluconobacter* (*G. asaii, G. cerinus, G. frateurii* and *G. oxydans,*), it was not possible to demonstrate that any were capable of producing histamine, tyramine, phenylethylamine, putrescine, cadaverine or tryptamine, in either synthetic media or wine (9).

Concerning the AAB, during the vinification one normally tries to avoid their growth or tries to eliminate them in the fastest and most efficient manner possible. This is perhaps the reason why there are few in depth studies on the synthesis of BA by AAB. However, our results coincide with the data of other authors: it would seem that acetic acid bacteria do not represent a danger for wines for what concerns the synthesis of BA.

LACTIC ACID BACTERIA: PRODUCING SPECIES

The LAB are the main organisms responsible for the synthesis of BA in wines (9). In Table 1, it is possible to observe the correlation between the LAB species and the type of BA produced in the wine.

Especie	Amina Biógena			
	Histamina	Tiramina	Feniletilamina	Putrescina
L. brevis	-	+ (100%)	+ (100%)	?
L. buchneri	+ (30%)	-	-	+ (<1%)
L. casei	-	-	-	-
L. collinoides	-	-	-	-
L. hilgardii	+ (27%)	+ (25%)	+ (25%)	+ (<1%)
L. mali	+ (67%)	-	-	-
L. paracasei	-	-	-	-
L. plantarum	-	+ (<1%)	-	-
L. mesenteroides	+ (6%)	-	-	-
O. oeni	+ (78%)	-	-	+ (20%)
P. parvulus	+ (16%)	-	-	-
P. pentosaceus	-	-	-	-

Table 1. LAB species capable of synthesizing BA in wines.

For the case of histamine, the production of this compound depends on the strain and varies from 6% of positive strains for the *L. mesenteroides* species and up to 80% of positive strains for the *O. oeni* species (4-6, 8, 11, 13, 16, 21, 22, 26, 30, 31). It is important to underline that these percentages vary according to the method used, since less accurate systems such as the identification on plates demonstrate only the strains which produce the most elevated concentrations of BA, whereas enzymatic methods or those which use HPLC are capable of demonstrating the strains which even produce low amounts of BA (12, 13).

For tyramine, in some species such as *L. brevis* all the strains analyzed which were isolated from wines were producers, however for example only a part of the strains belonging to *L. hilgardii* are capable of synthesize this BA (1, 18). It is necessary to underline that all the strains which produce tyramine are also capable of synthesizing phenylethylamine, perhaps due to a secondary activity of the same decarboxylase enzyme (18).

The species of LAB isolated in wines that are capable of synthesizing putrescine are *L. buchneri* (31), *L. hilgardii*, *O. oeni* (8) and perhaps *L. brevis* (25) (Table 1). This characteristic seems to be more scarce in LAB with respect to their capacity to synthesize other BA. However, the number of strains capable of synthesizing putrescine seem to be continually more frequent: a few years ago LAB which synthesized putrescine were not isolated from wines, whereas now it seems that the

frequency of their isolation has increased; this could simply indicate that the methods of determination and quantification of these BA have been improved.

BIOSYTHETIC PATHWAYS OF BIOGENIC AMINES

The BA are produced starting from amino acid precursors, due to the action of corresponding decarboxylase enzymes. For example, histidine decarboxylase (HDC) synthesizes histamine from histadine, tyramine decarboxylase (TDC) syhtesizes tyramine from tyrosine and phenylethylamine from phenylalanine. The situation of putrescine is more complex (Figure 1).
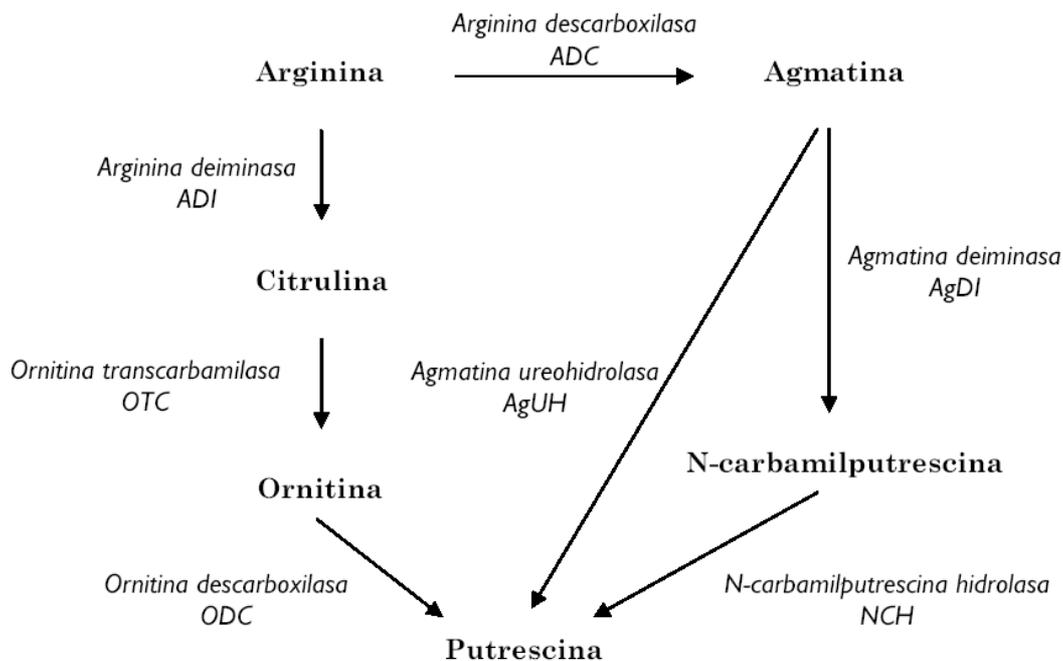

Figure 1. Possible synthesis pathways of putrescine from arginine in LAB.

In some LAB, as for *O. oeni*, arginine is subsequently transformed into citruline, ornitine and putrescine due to arginine deiminase (ADI), ornitine transcarbamylase (OTC) and ornitine decarboxylase (ODC) (8, 27-29). In other LAB, as for *L. hilgardii*, arginine is decarboxylated giving rise to agmatine via arginine decarboxylase (ADC), and in turn is transformed subsequently to N-carbamilputrescine and putrescine due to the successive action of agmatine deiminase (AgDI) and N-carbamilputrescine hydrolase (NCH) (2, 3).

IDENTIFICATION OF GENES OF STRAINS PRODUCING BIOGENIC AMINES

Presently there are specific primers for the identification of genes corresponding to the for mentioned decarboxylase enzymes. For the case of the HDC gene, an example is shown in Figure 2 (13). It is possible to see that there are strains of *O. oeni* which give false negatives with the primer pair JV16HC/JV17HC (19), whereas the strain of *L. hilgardii 5w* demonstrates an absence of reaction for the CL1mod/JV17HC primers (13).

This last pair was optimized for the identification of the HDC gene in strains of *O. oeni* (13). Due to the variability in the efficiency of gene identification according to LAB bacteria to which it is applied, it is advisable to use the primers CL1mod/JV17HC for *O. oeni* and JV16HC/JV17HC for the rest of the LAB. When the identity of the LAB is unknown, it would be important to complete a double reaction in order to avoid mistaken results.
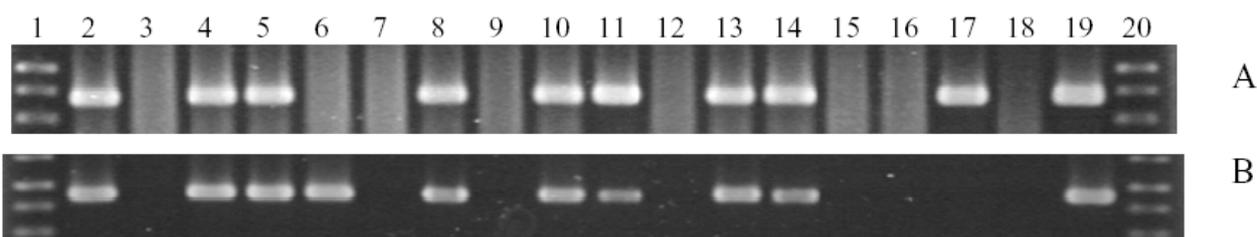.

Figure 2. Determination of the HDC gene with the primers JV16HC/JV17HC (A) and CL1mod/JV17HC (B). (lane 1 e 20) buffer ladder lane; (lane 2) positive control Lactobacillus buchneri ST2A; (lane 3) negative control Pediococcus pentosaceus 136; (lane 4) Oenococcus oeni 4042; (lane 5) O. oeni 4023; (lane 6) O. oeni 4021; (lane 7) O. oeni 4047; (lane 8) O. oeni 4010; (lane 9) O. oeni 3996; (lane 10) O. oeni 4045; (lane 11) P. parvulus 339; (lane 12) P. pentosaceus 56; (lane 13) P. parvulus 276; (lane 14) L. hilgardii 464; (lane 15) L. plantarum 98; (lane 16) L. paracasei 364; (lane 17) L. hilgardii 5w; (lane 18) Leuconostoc mesenteroides 27; (lane 19) L. mesenteroides 86.

For the TDC gene, responsible for the synthesis of tyramine decarboxylase, the use of the primer pair p0303 (23) and P1-rev (18, 24) is recommended (Figure 3).
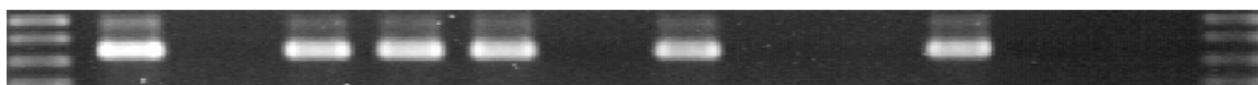

Figure 3. Determination of the TDC gene in different LAB isolated from wines

As for the case of the synthesis of putrescine, as we have seen there are two possible pathways for its synthesis: from ornitine as in the case of *O. oeni* and from agmatine as in the case of *L. hilgardii*. In the first case, normally the primers 3 and 16 for the gene ODC (6, 28, 30) are used. For the determination of genes relative to the synthesis of agmatine, we suggest the use of the primers AfuAF and AguAR for the gene aguA (AgDI), aguBF and AguBR for AguB (NCH) (10), as can be seen in Figure 4.
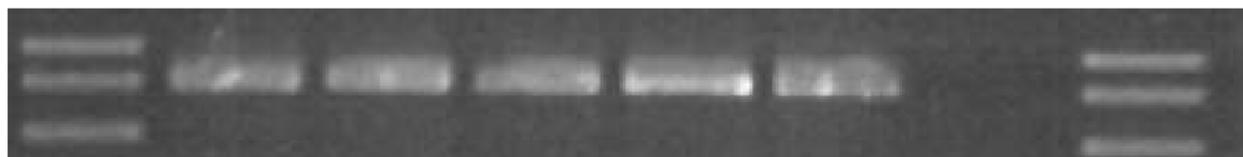

Figure 4. Determination of the aguA gene in different LAB

REGULATION OF GENES AND ENZYMES RESPONSIBLE FOR THE SYNTHESIS OF BIOGENIC AMINES

From the practical point of view, it is of interest to know not only which LAB possess certain genes, but also when, how much, how and why these genes are expressed, that which will determine the final quantity of the respective BA in the wines. It is known that a large part of O. oeni strains can produce histamine in wines, but that in the end the final quantity normally will not be very high, and that when we identify a notable quantity of histamine production, that this is due to the growth and metabolism of other LAB, especially *L. hilgardii* and *P. parvulus* (13, 14).

A determinant factor is the pH of the wine (14). As can be observed in Figure 5, with values of pH below 3.5, generally there are not high levels of histamine, which is the opposite in wines with high pH such as 3.8 or 3.9. This is due to the fact that the metabolic activities of the LAB responsible for the synthesis of BA are limited in more acidic wines, which are hence more protected. The regulation occurs at the level of the enzymatic activity and not in the expression of the genes (15, 17).
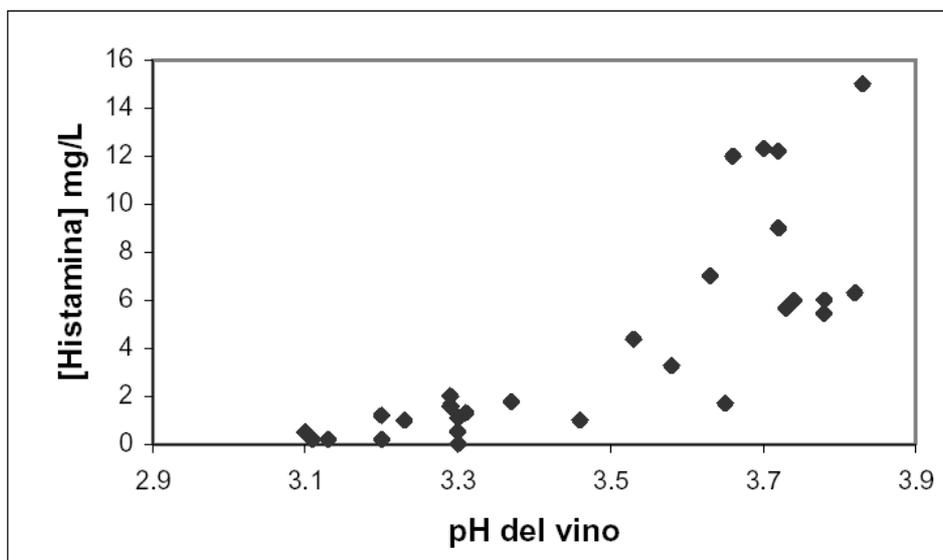
Figure 5. pH values and histamine concentration found in diverse wines once the alcoholic fermentation is complete

The activity of the enzymes involved in the synthesis of BA is at a maximum at temperatures close to 30℃, whereas at 4℃ this activity diminishes up to a 20% of the maximum (there is no effect on the expression of the genes, only on the activity of the enzymes).

Ethanol has no relevant effect on the expression of the genes coding the decarboxylases however it has a direct effect on the enzymes, in that at a concentration of around 10° of alcohol the enzymatic activity is doubled with respect to the control, as can be observed in Figure 6 (15, 17).

Glucose, fructose, malic acid and citric acid reduce the expression of the coding genes, but not the corresponding decarboxylase enzymatic activity (Figure 7). As for the lactic and tartaric acids, these do not influence either the synthesis or the activity of the enzymes implicated in the BA synthesis (15, 17).
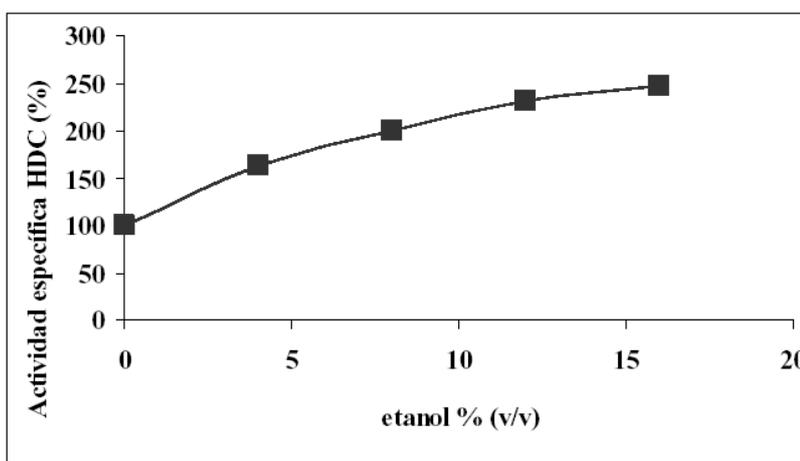

Figure 6. Effect of the ethanol concentration on the HDC enzymatic activity.

Figure 7. Effect of the malic acid concentration (from left to right 0, 0.5, 1, 2 e 4 g/L) on the expression of the HDC gene

As for SO2, this has no effect on the synthesis of or the activity of the enzymes, however it clearly has an indirect influence, since it causes a reduction in the microbiological population (15, 17).
Other factors were also studied such as histadine, histamine, the cellular growth phase or the presence of piridoxal phosphate (cofactor necessary for the decarboxylases), and their influence on the synthesis of BA was characterized (16).

TECHNOLOGICAL IMPLICATIONS OF GENE REGULATION AND ENZYMATIC ACTIVITIES

With the aim of resuming all that was covered above, it can be confirmed that glucose, fructose, malic acid, citric acid and the presence of BA in themselves reduce the expression of the genes responsible for the synthesis of BA. The precursor amino acids increase the expression of the genes. Ethanol increases the activity of the enzymes. The temperature and pH also have an influence on the enzymes but not on the genes. The growth state also influences the genetic expression. Tartaric acid, lactic acid and SO2 do not have an effect either on the synthesis or on the enzymatic activity.

From the point of view of the possible technological repercussions, all this signifies that the BA will not be synthesized as much in the must (where there is more glucose, fructose, malic acid, etc.) as in the wine (more ethanol and less energetic resources). This is logical if we imagine a system where the resources are limited as in wine, and the LAB can therefore regulate the use of metabolites (energy): to begin, they will preferentially use sugars, especially glucose and fructose. They will then follow to use organic acids as citric and malic acid (usually tartaric and lactic acid are not used and it is for this reason that they have no effect on the synthesis of amino acid decarboxylase enzymes.) Finally, they will use the amino acids to obtain energy and to maintain the intracellular pH relatively high. This is a strategy which has an aim of maintenance over a long period of time, by dosing the use of the resources in a rational manner.

In other words, the BA are not synthesized in a major way in the must but rather in the wine due to the regulation of the factors outlined above. In fact this is what is observed at the production level :the important increments of BA in wines usually arise in the aging phases more so than in the initial phases.

As for the SO2, it is necessary to consider another technologically important aspect. As was said, this compounds possesses an evident antiseptic activity, however does not influence the synthesis or the activity of the decarboxylase enzymes: we observed that the decarboxylases can continue to be active in wines even after the death of the bacteria. This signifies that even if the LAB are killed, that the enzymes can continue to synthesize BA: we studied aging wines in which the bacteria were dead, but where the content of BA was continuing to increase even after some time, due to this residual enzymatic activity (7).

USABLE TECHNOLOGICAL SOLUTIONS
From that which was just seen one can deduce that it is possible to try and avoid the appearance of BA in wines by using different strategies. Evidently the use of SO2 (or other inhibitors which can be used such as bactericides or lysozyme) can be greatly useful if used at the right time and in the adequate conditions, even if the enzymes are not eliminated. The use of selected and secure starter cultures is another possible strategy which has been demonstrated to be useful. However, unfortunately this is not always the case. In the upper part of Figure 8 the growth kinetic of LAB during the vinification in the winery is shown. This wine was treated with sulfur and was inoculated with a selected commercial starter culture. In the upper graph it can be observed that the populations of *Lactobacillus* (*L. plantarum* and *L. hilgardii*) were identified in the beginning, but not after the sulfur treatment and inoculation. The starter culture manages to grow and implant itself in the wine, leading to the malolactic fermentation (data not shown)

Along with this strain, there coexists for a certain period another strain (*O. oeni* S1), which even though it disappears after a bit of time: it is identifiable 30 days after the beginning of the fermentation but not after 250 days. Instead, another strain (*O. oeni* S2) which is not identified at the beginning and which is overtaken during the first phase by the starter culture, is capable of growing subsequently, while maintaining itself level with the inoculated strain starting from 150 after the start of the fermentation and up until the end.

When one observes what occurred during the evolution of the BA (Lower graph of Figure 8), no significant increases in tyramine or cadaverine are noted. Histamine undergoes an initial increase

coinciding with the alcoholic fermentation and with the presence of *Lactobacillus*, and successively does not present a further significant increase. However, putrescine increases in a notable manner after about 150 days, coinciding with the increased growth of the strain *O. oeni* S2. Once this strain was analyzed in the laboratory, *O. oeni* S2 was demonstrated to possess the genes and the capacity to produce putrescine in wines, whereas the *O. oeni* S1 and the starter culture strain were identified as being negative for this character. For this reason, the strain *O. oeni* S2 was responsible for the synthesis of putrescine in the advanced phases of the vinification process, and was able to grow during the aging of a wine which had undergone a successful malolactic fermentation with the use of a commercial culture.
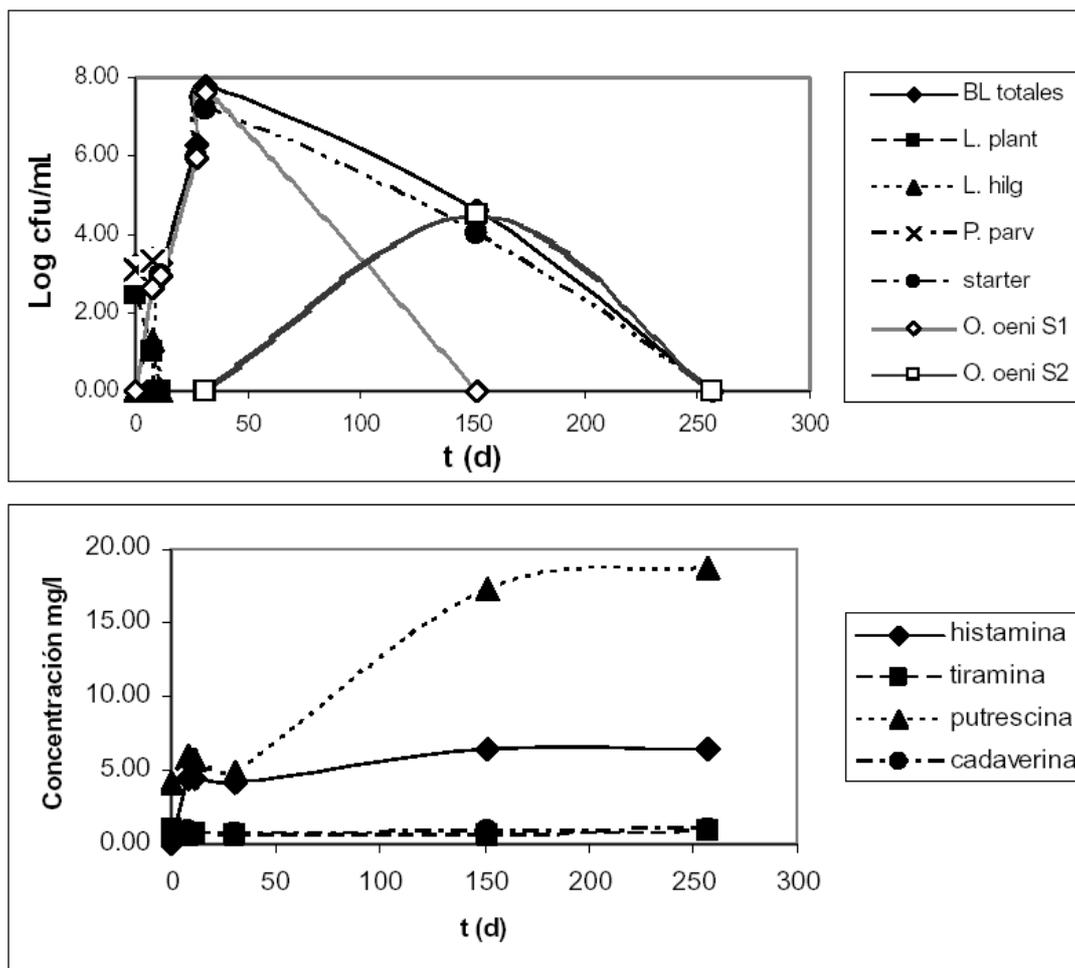

Figure 8. Evolution of the LAB population (upper panel) and AB (lower panel) during a vinification in the winery for the Tempranillo grape variety

As shown above, there is the deduction that it is necessity to stabilize the wines once the malolactic fermentation is completed, using the addition of SO2 (if possible, or another antiseptic) and to reduce the microbiological population by centrifugation or filtration, etc. The inoculation with starter cultures is also important, but it is important to remember the other practices which can allow for the control of LAB and for the avoidance of BA formation in the moments where the wine is least protected and where the metabolic conditions are ideal for the amino acid decarboxylase activity. The control of microorganisms in wines is, and continues to be a fundamental issue.

BIBLIOGRAPHY

1. Arena, M., D. Fiocco, M. Manca de Nadra, I. Pardo, and G. Spano. 2007. Characterization of a Lactobacillus plantarum Strain Able to Produce Tyramine and Partial Cloning of a Putative Tyrosine Decarboxylase Gene. Current Microbiology 55: 205-210.
2. Arena, M. E., J. M. Landete, M. C. Manca de Nadra, I. Pardo, and S. Ferrer. 2007. Factors affecting the production of putrescine by Lactobacillus hilgardii X1B isolated from wine. Journal of Applied Microbiology Accepted.
3. Arena, M. E., and M. C. Manca de Nadra. 2001. Biogenic amine production by Lactobacillus. Journal of Applied Microbiology 90: 158-162.
4. Coton, E., and M. Coton. 2005. Multiplex PCR for colony direct detection of Grampositive histamine- and tyramine-producing bacteria. J. Microbiol. Meth. 63: 296-304.
5. Coton, E., G. C. Rollan, and A. Lonvaud-Funel. 1998. Histidine carboxylase of Leuconostoc oenos 9204: purification, kinetic properties, cloning and nucleotide sequence of the hdc gene. J Appl Microbiol 84: 143-151.
6. de las Rivas, B., Á. Marcobal, and R. Muñoz. 2005. Improved multiplex-PCR method for the simultaneous detection of food bacteria producing biogenic amines. FEMS Microbiology Letters 244: 367-372.
7. Ferrer, S., and I. Pardo. 2005. Prevención de la aparición de aminas biógenas en vinos. ACE Revista de Enología 70: 6-8.
8. Guerrini, S., S. Mangani, L. Granchi, and M. Vincenzini. 2002. Biogenic amine production by Oenococcus oeni. Current Microbiology 44: 374-378.
9. Landete, J. M. 2005. Estudio y caracterización molecular de la producción de aminas biógenas por parte de bacterias lácticas de origen enológico. Tesis Doctoral. Universitat de València.
10. Landete, J. M., M. E. Arena, I. Pardo, M. d. N. M.C., and S. Ferrer. 2007. Implicated bacterial genes in putrescine production from agmatine: detection and correlation with the activity. FEMS Microbiology Letters Submitted.
11. Landete, J. M., S. Ferrer, and I. Pardo. 2007. Biogenic amine production by lactic acid bacteria, acetic bacteria and yeast isolated from wine. Food Control 18: 1569-1574.
12. Landete, J. M., S. Ferrer, and I. Pardo. 2004. Improved enzymatic method for the rapid determination of histamine in wine. Food Additives and Contaminants 21: 1149-1154.
13. Landete, J. M., S. Ferrer, and I. Pardo. 2005. Which lactic acid bacteria are responsible of histamine production in wine? Journal of Applied Microbiology 99:580-586.
14. Landete, J. M., S. Ferrer, L. Polo, and I. Pardo. 2005. Biogenic amines in wines from three Spanish regions. Journal of Agricultural and Food Chemistry 53: 1119-1124.
15. Landete, J. M., S. Ferrer, L. Polo, and I. Pardo. 2004. Influencia de factores físicoquímicos del vino sobre la producción de histamina. Tecnología del vino 19: 67-70.
16. Landete, J. M., I. Pardo, and S. Ferrer. 2006. Histamine, histidine, and growth-phase mediated regulation of the histidine decarboxylase gene in lactic acid bacteria isolated from wine. FEMS Microbiology Letters 260: 84-90.
17. Landete, J. M., I. Pardo, and S. Ferrer. 2007. Regulation of hdc expression and HDC activity by enological factors in lactic acid bacteria. Journal of Applied Microbiology Accepted.
18. Landete, J. M., I. Pardo, and S. Ferrer. 2007. Tyramine and phenylethylamine production among lactic acid bacteria isolated from wine. International Journal of Food Microbiology 115: 364-368.
19. Le Jeune, C., A. Lonvaud-Funel, B. ten Brink, H. Hofstra, and J. M. B. M. van der Vossen. 1995. Development of a detection system for histidine decarboxylating lactic acid bacteria based on DNA probes, PCR and activity test. Journal of Applied Bacteriology 78: 316-326.
20. Lehtonen, P. 1996. Determination of amines and amino acids in wine: a review. American Journal of Enology and Viticulture 47: 127-133.
21. Lonvaud-Funel, A. 2001. Biogenic amines in wines: role of lactic acid bacteria. FEMS Microbiology Letters 199: 9-13.
22. Lonvaud-Funel, A., and A. Joyeux. 1994. Histamine production by wine lactic acid bacteria: isolation of a histamine-producing strain of Leuconostoc oenos. Journal of Applied Bacteriology 77: 401-407.
23. Lucas, P., J. Landete, M. Coton, E. Coton, and A. Lonvaud-Funel. 2003. The tyrosine decarboxylase operon of Lactobacillus brevis IOEB 9809: characterization and conservation in tyramine-producing bacteria. FEMS Microbiology Letters 229: 65-71.
24. Lucas, P., and A. Lonvaud-Funel. 2002. Purification and partial gene sequence of the tyrosine decarboxylase of Lactobacillus brevis IOEB 9809. FEMS Microbiology Letters 211: 85-89.
25. Lucas, P. M., V. S. Blancato, O. Claisse, C. Magni, J. S. Lolkema, and A. Lonvaud-Funel. 2007. Agmatine deiminase pathway genes in Lactobacillus brevis are linked to the tyrosine decarboxylation operon in a putative acid resistance locus. Microbiology 153: 2221-2230.

26. Lucas, P. M., W. A. M. Wolken, O. Claisse, J. S. Lolkema, and A. Lonvaud-Funel. 2005. Histamine-Producing Pathway Encoded on an Unstable Plasmid in Lactobacillus hilgardii 0006. Appl. Environ. Microbiol. 71: 1417-1424.

27. Mangani, S., S. Guerrini, L. Granchi, and M. Vincenzini. 2005. Putrescine accumulation in wine: role of Oenococcus oeni. Current Microbiology 51: 6-10.

28. Marcobal, A., B. de las Rivas, M. V. Moreno-Arribas, and R. Muñoz. 2004. Identification of the ornithine decarboxylase gene in the putrescine-producer Oenococcus oeni BIFI-83. FEMS Microbiology Letters 239: 213-220.

29. Marcobal, A., B. las Rivas, M. V. Moreno-Arribas, and R. Munoz. 2004. Identification of the ornithine decarboxylase gene in the putrescine-producer Oenococcus oeni BIFI-83. FEMS Microbiology Letters 239: 213-220.

30. Marcobal, Á., B. d. l. Rivas, M. V. Moreno-Arribas, and R. Muñoz. 2005. Multiplex PCR Method for the Simultaneous Detection of Histamine-, Tyramine-, and Putrescine-Producing Lactic Acid Bacteria in Foods. Journal of Food Protection 68:874-878.

31. Moreno-Arribas, M. V., M. C. Polo, F. Jorganes, and R. Muñoz. 2003. Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. International Journal of Food Microbiology 84: 117-123.