A Study of the Biogenesis of Amines in a Villard Noir Wine

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The occurrence of seven biogenic amines in red wine, as a result of bacterial metabolism, was studied. Four homogeneous lots of Villard noir must were vinified under the following conditions: 1) 22°C, pH adjusted to 3.3 and 3.8; 2) 13°C, pH adjusted to 3.3 and 3.8. After the alcoholic fermentation was completed, the wines were filter-sterilized and given various treatments: a) addition of 100 mg free SO_2/L to prevent bacterial metabolism; b) natural malolactic fermentation (MLF); c) inoculation with *Leuconostoc oenos*; d) inoculation with *Pediococcus cervisiae*; e) inoculation with both *L. oenos* and *P. cerevisiae*. Samples for analysis were removed before and after the completion of the MLF. Phenylethylamine was absent from all samples. Musts contained ethanolamine and tyramine in minute amounts (40 and 3.5 μmol/L, respectively). Cadaverine and histamine, absent from the must, were detected in highest quantities after the alcoholic fermentation but decreased significantly after completion of the MLF (5.0 *vs* 1.0 μmol/L and 10.0 *vs* 4.0 μmol/L, respectively). The tyramine content increased during both the alcoholic fermentation and the MLF (21 and 37 μmol/L, respectively). Agmatine, putrescine and ethanolamine were produced during alcoholic fermentation (3, 3 and 340 μmol/L, respectively) and their concentrations did not change appreciably during MLF. Temperature, pH and type of organism affected the content of the various amines in different ways. This study does not support the contention that amines are the products of the malolactic flora.

Isolation and identification of malolactic bacteria from wines of different wine-producing countries (except Canada) is well documented (1,22). Malolactic bacteria have been extensively studied with respect to their optimal growth conditions and formation of lactic acid, but information concerning their ability to form amines via a decarboxylase enzyme system is, however, not available. Although there is little documented evidence that malolactic bacteria are responsible for the generation of amines in wines, many authors have been prompt to incriminate these bacteria (5,6,8,20,24,27). Their reasoning is based on the observation that wines having undergone malolactic fermentation (MLF) have a higher histamine content than wines which have not.

In recent years, the generalization of this concept has been challenged by some authors. Weiller and Radler (31) observed that only one out of 28 strains of *Pediococcus cerevisiae* had the ability of forming histamine from histidine. Rice and Koehler (23) in their study of various strains of *P. cerevisiae* and *Lactobacillus plantarum* were unable to detect decarboxylase activity. Umezu (29) demonstrated the formation of tyramine from *Leuconostoc mesenteroides* var. *Saké* and from *Lactobacillus Saké*. Umezu *et al.* (30) also demonstrated amine oxidation by lactobacilli. However, these bacteria are not normally found in wines.

Lafon-Lafourcade (13) tested 59 strains of bacteria chosen from the four major groups of lactic acid bacteria found in wine. She was unable to demonstrate histamine formation by any of them under optimal growth conditions. However, under non-proliferating conditions (MLF in red wines), histamine was produced in amounts varying from 1.2 to 7.3 mg/L. She concluded that histamine production by lactic acid bacteria might be the consequence of unfavorable environmental conditions. Mayer *et al.* (14) surveyed 282 wines to assess their histamine contents. They stated that "the histamine formation was due mainly to coccoid lactic acid bacteria". The coefficients of determination ($100r^2$) between the histamine content and the presence of lactic cocci were, however, low: 29.3% for red wines and 9.8% for white wines. In the same study, 54% of the red wines and 15% of the white wines contained more than 2 mg of histamine per liter. Mayer and Pause (15), from periodical controls made on 25 wines, confirmed the formation of histamine by *P. cerevisiae* during the course of the MLF. Tyramine, putrescine, cadaverine and 2-phenylethylamine were also identified as products of bacterial metabolism. Kunsch *et al.* (12) observed the formation of histamine (40 mg/L) by *P. cerevisiae* but not by *Leuconostoc oenos*.

The role of malolactic bacteria in the formation of amines in wines is still a matter of controversy. Designed experiments to demonstrate production of amines during the course of the MLF under controlled conditions are scarce. The aim of this study was to demonstrate the production of amines, particularly histamine and tyramine, by MLF bacteria under controlled conditions of experimentation.

More specifically, the objectives were: 1) relate the disappearance of a specific amino acid with the production of the associated amine, *e.g.* histidine *vs* histamine; 2) determine whether the temperature of the vinification process (alcoholic and malolactic fermentations) has an effect on the amine content; 3) determine whether the pH of the must influences the amine content of the wine; 4) contrast the amine content of wines which have and which have not undergone MLF; 5) contrast the amine content of wines with a natural malolactic flora and wines inoculated with *Pediococcus cerevisiae* or with *Leuconostoc oenos* or with both.

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Materials and Methods

Grapes: A total of 2000 kg of Villard noir grapes was provided by T. G. Bright and Co., Ltd., Niagara Falls, Ontario, Canada. Batches of 1000 kg each were processed in one operation. Each batch was treated as a replication in the experimental design.

Yeast: Two strains of *S. cerevisiae* were used: R- 107 (courtesy Dr. R. Eschenbruch, Min. Agric. and Fisheries Res. Div., Ruakura Agricultural Station, Hamilton, New Zealand) and G-74 (courtesy Dr. H. Becker, Leiter des Inst. Rebenzüchtung, Rebenveredlung Forschungsanstalt Geisenheim, 6222 Geisenheim-Rhein, Eibinger Weg 1, West Germany).

The yeasts were maintained at 13°C on potato dextrose agar (Difco) by transfers every two weeks.

Saccharomyces cerevisiae R-107 and G-74 inocula were prepared separately by inoculating 2 L of nutrient broth from one-week-old PDA slants. The yeasts were grown at 13°C under heavy aeration and agitation using a Bellco unit. After three days of growth, the cultures were sedimented at 4°C for 12 hours.

Maréchal Foch grapes obtained on 13 September 1980, were destemmed, crushed and frozen at −29°C until needed. After thawing, the grapes were pressed; half of the juice was inoculated with the R-107 sediment and the other part with the G- 74 sediment. This represented an inoculum of 10% (2 L into 20 L). After three days of fermentation, equal amounts of R-107 and G-74 inoculated Maréchal Foch musts were used to inoculate Villard noir crushed grapes. This represented an inoculum of about 6% (10 L of R-107 Maréchal Foch + 10 L of G-74 Maréchal Foch into 340 L of must).

Bacteria: The *L. oenos* strain (OENO™) was provided by Microlife Technics (Sarasota, FL 33578). Three strains of *P. cerevisiae* (identified as 'Y', '12', and '18' were obtained from Dr. K. Mayer (Eidgenossische Forschungsanstalt, Wädenswil, Switzerland).

L. oenos and *P. cerevisiae* strains were maintained in Deman, Rogosa and Sharpe (MRS) broth to which was added 0.5 mL of a 10% aqueous solution of L-malic acid per 10 mL broth. The screw-capped test tubes (1.6 cm i.d. × 15 cm) containing 10 mL inoculated broth were loosely capped and incubated at 13°C in a BBL Gaspak jar (Canlab, Toronto, Ontario) under an atmosphere of carbon dioxide. The cultures were transferred every three weeks.

L. oenos and *P. cerevisiae* Y, 12, and 18 inocula were prepared separately by inoculating 2 L MRS broth plus malic acid with 10-day-old cultures. After incubation for nine days at 20°C under static conditions, the broth was aseptically transferred by portions into 200 mL sterile centrifuge tubes. After centrifugation (16 000 *g*, 15 min, 5°C), the supernatant was discarded and more of the same broth added to the tubes. This was repeated until all 2000 mL were centrifuged. The sediment representing the bacteria grown in 2 L was suspended aseptically into 4.5 L filter-sterilized Maréchal Foch wine. This wine had not undergone MLF and had not been sulfited. After three days of fermentation, the Maréchal Foch wine was used to inoculate the Villard noir wines which were themselves filter-sterilized.

For the treatment 'Lo' (*L. oenos*), 1000 mL of Maréchal Foch wine inoculated with *L. oenos* were added to 19 L of Villard noir wine. For the treatment 'Pc' (*P. cervisiae*), 350 mL of M. Foch wine inoculated with *P. cerevisiae* strain Y, 350 mL of M. Foch wine inoculated with *P. cerevisiae* strain 18, and 350 mL of M. Foch wine inoculated with *P. cerevisiae* strain 12 were added to 19 L of Villard noir wine. For the treatment 'L+P', 160 mL each of M. Foch wine inoculated with *P. cerevisiae* strains Y, 18, and 12, and 480 mL of M. Foch wine inoculated with *L. oenos* were added to 19 L of Villard noir wine. This represented an inoculum of about 3% (v/v).

For the treatment 'Natural MLF', Villard noir wines were not filter-sterilized. MLF was carried out by bacteria already present in the wine.

Equipment for processing the grapes: The grapes were processed using a destemmer-crusher (Diraspatrice Mod., Firenze, Italy). The juice was extracted by a hand-operated basket-type press.

The fermentation on the skins proceeded in 1000 L stainless steel containers which were filled to only half capacity in order to prevent any overflow during the fermentation. The vessels were covered with plastic sheets.

Chaptalization and deacidification were done in a stainless steel vessel equipped with an agitator and a valve at the bottom to draw out the must.

Glass jars of 19.6 L (5 US gal) were used throughout the vinification process.

The polishing filtration was carried out with a Horm Filter Press Model St-80/512/4-3 and non-asbestos, cellulosic Seitz filter medium, No. 140 with a pore size of 5 μm (Bowers Machine Co., Ltd., Montreal, Quebec) using nitrogen gas under pressure. The sterile filtration was carried out using a Millipore filtration unit, Model YY3015250 Sterilizing F/H, with Millipore Prefilter Thick, AP2512450, Prefilter AP1512450 and Filter DAWP14250 (Millipore Ltd., Mississauga, Ontario).

Chemicals: Malic dehydrogenase, E.C. 1.1.1.37 (from porcine heart), β-nicotinamide adenine dinucleotide from yeast, hydrazine sulfate, ethylene diamine tetraacetic acid, glycine, L-malic acid, and amine standards were purchased from Sigma Chemical Co. (St. Louis, MO). Unmatured alcohol (95% v/v) was obtained from Consolidated Alcohols (Toronto, Ontario). Calcium carbonate used for deacidification and sucrose used for chaptalization were of food grade. All other chemicals used for analyses and potassium metabisulfite used for wine processing were of A.C.S. quality.

Analytical methods: The methods for total acidity, pH, total soluble solids and free SO_2 are described by Amerine and Ough (2). Alcohol determination was according to the modified chemical oxidation method of Crowell and Ough (9). Residual sugars were determined using the anthrone method (11) after clean-up of the samples (2).

Paper chromatography was used for monitoring the MLF (21). The exact content of malic acid in the samples

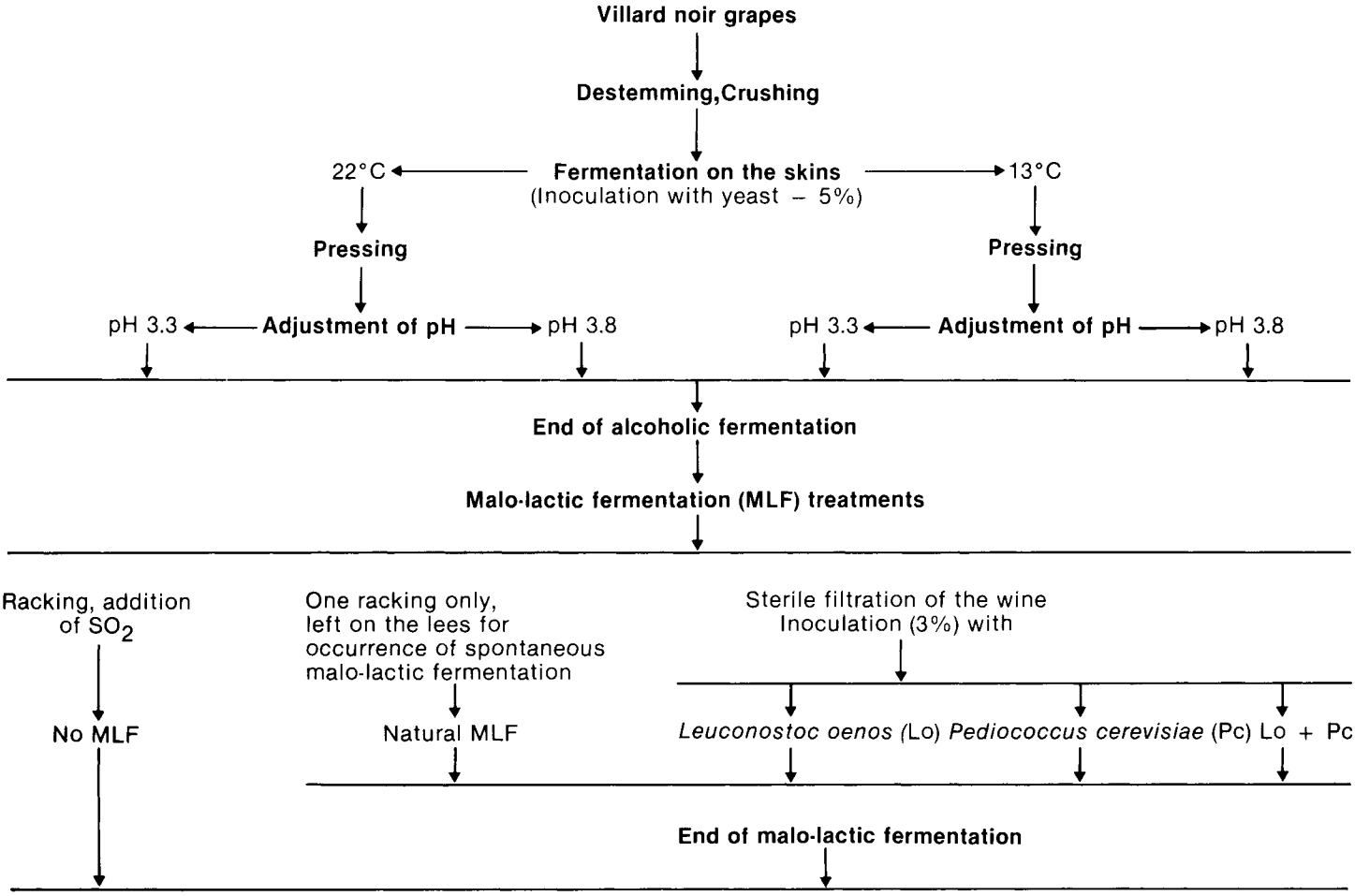


Fig. 1. Schematic diagram of operations.

was determined using the enzymic method described by Amerine and Ough (2). A standard curve obtained with malic acid standards was used for quantitation. Amino acid analysis was done on the non-protein fraction of the juice and wine samples using a Technicon Sequential Multi-Sample Analyzer (Technicon Instruments Corp., Tarrytown, NY).

Amine analysis has been described by the authors (4). The method of external standards was used for quantitation.

Method of vinification: The vinification process for the different treatments involved (temperature, pH, MLF, flora) is presented in Figure 1.

Upon arrival at the laboratory, the grapes were destemmed, crushed, and separated into two lots of 454 kg each; one lot was vinified at 22°C and the other at 13°C. At crushing time, the temperature of the berries was about 4°C. Samples were removed for determination of soluble solids and acidity. After fermentation for three days on the skins, the mash was pressed. The yield was 0.75 L/kg, representing 340 L of must per lot.

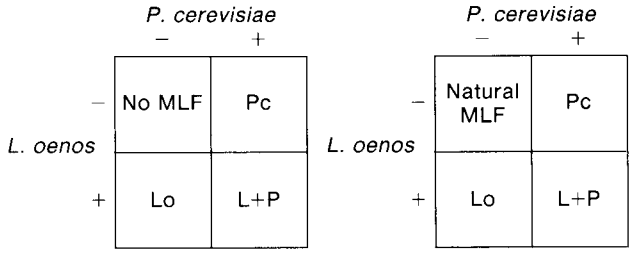
The sugar content of the must was adjusted from 18° to 22.6° Brix in order to achieve an alcohol content of 12.5% (v/v).

The experiment was designed to have a difference of 0.5 pH unit between two lots of must for each respective temperature (22° and 13°C). Part of the must was, therefore, deacidified after pressing with calcium carbonate. Once the alcoholic fermentation was completed (less than 0.2 g residual sugars/L), the wines were racked off the lees, stored in completely filled containers at 2°C and processed as follows: a) The wines for the treatment 'No MLF' received enough sulfurous acid (as $K_2S_2O_5$) to maintain the level of free SO_2 at 50 mg/L. They were then processed according to steps d and e; b) The wines for treatment 'Natural MLF' were stored at 22°C or at 13°C, respectively, until completion of the MLF without any other treatment. These were then processed according to steps d and e; c) The wines for the treatments Lo, Pc, and L+P were filter-sterilized immediately after the end of the alcoholic fermentation. This was difficult to achieve because at that stage the wines were cloudy and did not filter easily. Trials on small quantities of wines with different sizes and types of filters and clarification with egg albumin were not successful. A coarse filtration with diatomaceous earth was adopted. This filtration was then followed by a sterile filtration. The wines were kept in alcohol-sterilized jars and then inoculated. These oper-

ations of filtration and inoculation were carried out under a laminar air flow unit. At the end of the MLF, the wines were processed according to steps d and e; d) The wines were racked twice at two-week intervals and SO_2 was added to keep the level of free SO_2 at 50 mg/L; e) Tartrate stabilization followed and was carried out at −6°C for three weeks. The wines were then bottled after filter-sterilization.

Design of the experiment and statistical analysis: Pertinent null hypotheses for the five objectives listed in the introduction were tested.

The experiment consisted of a 2 × 2 × 5 × 2 factorial arrangement carried out according to a multi-split-plot design in two replications (7). The main plot treatments consisted of two temperatures of vinification (22° and 13°C), the split-plot treatments of two pH levels of the must (3.3 and 3.8), the split-split-plot treatments of five malolactic flora (No MLF, Natural MLF, Lo, Pc, L+P) and the split-split-split-plot of two times of sampling (before and after the MLF). Two 2 × 2 factorial arrangements were formed by the five flora treatments as indicated below:



The experimental unit was 20 L of wine made from one of the 20 combinations of temperature, pH, and flora. Response criteria were pH, total acidity, malic acid, amino acids, and amines.

All sets of data were processed and summarized so that all treatment comparisons could be made on the basis of mean squares appropriate to individual degrees of freedom. This included 2-, 3-, and 4-factor interactions and treatment main effects. The manner in which the analysis of variance technique was used to accomplish this is indicated below:

Analysis of Variance Plan

Source of variation	Degrees of freedom
Replicates (A)	1
Temperature (B)	1
Error (a)	1
pH (C)	1
B × C	1
Error (b)	2
Flora (D)[a]	4
No MLF vs Lo + Pc + L+P (D1)	1
Natural MLF vs Lo + Pc + L+P (D2)	1
Lo *vs* Pc (D3)	1
Lo *vs* L+P (D4)	1
Pc *vs* L+P (D5)	1
B × D	4
B x D1	1
B x D2	1
B x D3	1
B x D4	1
B x D5	1
C × D	4
C × D1	1
C × D2	1
C × D3	1
C × D4	1
C × D5	1
B × C × D	4
B × C × D1	1
B × C × D2	1
B × C × D3	1
B × C × D4	1
B × C × D5	1
Error (c)	16
Time of sampling (E)	1
B × E	1
C × E	1
D × E	4
D1 × E	1
D2 × E	1
D3 × E	1
D4 × E	1
D5 × E	1
B × C × E	1
B × D × E	4
B × D1 × E	1
B × D2 × E	1
B × D3 × E	1
B × D4 × E	1
B × D5 × E	1
C × D × E	4
C × D1 × E	1
C × D2 × E	1
C × D3 × E	1
C × D4 × E	1
C × D5 × E	1
B × C × D × E	4
B × C × D1 × E	1
B × C × D2 × E	1
B × C × D3 × E	1
B × C × D4 × E	1
B × C × D5 × E	1
Error (d)	20
Total	79

All tests of significance were performed at the probability level of $\alpha = 0.05$.

[a]MLF: malo-lactic fermentation: Inoculation with *Leuconostoc oenos* (Lo) or *Pediococcus cerevisiae* (Pc) or with both.

All analyses of variance were performed by the NWAYANOVA procedure of Gibson *et al.* (10). The sums of squares for individual degrees of freedom for the factor flora were determined by the MREG program of Smillie (25). Both programs were taken from APL libraries, Institute of Computer Science, University of Guelph, Guelph, Ontario.

Mean differences and their associated confidence limits were also determined. When the value zero is encompassed in the interval between the lower and the upper limits, the population mean difference is deemed not to be statistically significant.

The relationship between the content of certain amino acids and certain amines in wines, before and after the MLF, was tested by determining coefficients of determination for the terms of the regression equation. This was

accomplished through the use of the MREG program of Smillie (25), where the coefficients of determination were obtained from the ratio of the sums of squares for linear and for quadratic regressions to the total sum of squares for each pair. The following pairs were tested: histidine-histamine, tyrosine-tyramine, arginine-agmatine, lysine-cadaverine, ornithine-putrescine, arginine-putrescine, and serine-ethanolamine.

Results and Discussion

Malolactic fermentation: All musts were fermented to less than 0.2 g residual sugars/L. The alcohol content of all wines ranged from 12.5 to 13.5% (v/v). Delays occurred between the time of inoculation and the onset of the MLF for the treatments Lo, Pc, and L + P. The delays were extended most markedly for the wines inoculated with *L. oenos* and fermented at 13°C as indicated in the table of statistical interactions between the different treatments (Table 1). These interactions were statistically significant.

The duration of the MLF was five times longer at 13°C than at 22°C (Table 2). All other main effects were not statistically significant.

Table 1. Significant 3-factor interaction for days between the time of inoculation and the onset of the malolactic fermentation (MLF).

Flora treatments[a]	Duration before the onset of the MLF (days)			
	Temperature			
	22°C		13°C	
	pH 3.3	pH 3.8	pH 3.3	pH 3.8
Lo	26	20	178	58
Pc	15	11	15	11
L+P	15	6	15	11

[a]Inoculation with either *Leuconostoc oenos* (Lo) or *Pediococcus cerevisiae* (Pc) or with both organisms (L+P).

Table 2. Duration of the malolactic fermentation (MLF) in days - main effects contrasts.

Contrasts	Means (days)	Mean difference	95% Confidence limits[a] for mean difference	
			Lower	Upper
Temperature				
22°C	24.4	102.7*	+ 32.9	+ 172.6
13°C	127.1			
pH				
3.3	92.5	33.5	− 44.8	+ 113.8
3.8	59.0			
Flora[b]				
Natural MLF	63.9	15.8	− 18.6	+ 50.2
Lo + Pc + L+P	79.7			
Lo	75.8	7.2	− 34.9	+ 49.4
Pc	83.0			
Lo	75.8	4.6	− 37.5	+ 46.8
L+P	80.4			
Pc	83.0	2.2	− 39.5	+ 44.8
L+P	80.4			

Factors	Experimental error	
	Degrees of freedom	Mean square
Temperature	1	242
pH	2	2784
Flora	12	1497

[a]95% Confidence limits = Mean difference ± Least significant difference
[b]Inoculation with *Leuconostoc oenos* (Lo) or *Pediococcus cerevisiae* (Pc) or with both (L+P)
*: Significant at $\alpha = 0.05$

The wines assigned to the treatment No MLF did not undergo any bacterial fermentation as was observed when comparing total acidity, pH, and malic acid of these wines, before and after fermentation (Table 3). All other treatments completed MLF.

Amine content: The amines spermine, spermidine, tryptamine and phenylethylamine gave no chromatographic peaks for any of the treatments.

Table 3. Averages for total acidity, pH and malic acid values of wines, for all treatments, before and after malolactic fermentation (MLF)[a].

Temperature of vinification	pH of the must	Flora treatments[b]	Total acidity[c] (g/100 mL)		pH of the wines		Malic acid (g/100 mL)	
			Before	After	Before	After	Before	After
22°C	3.3	No MLF	1.03	0.93	3.58	3.60	0.59	0.56
		Natural MLF	1.01	0.70	3.68	3.78	0.57	0.00
		Lo	1.01	0.68	3.66	3.86	0.61	0.00
		Pc	0.97	0.70	3.52	3.78	0.58	0.00
		L+P	0.97	0.69	3.56	3.78	0.57	0.00
22°C	3.8	No MLF	0.87	0.81	3.88	3.91	0.60	0.57
		Natural MLF	0.84	0.61	3.91	4.08	0.57	0.00
		Lo	0.85	0.52	3.90	4.20	0.60	0.00
		Pc	0.85	0.53	3.88	4.08	0.58	0.00
		L+P	0.84	0.52	3.83	4.10	0.56	0.00
13°C	3.3	No MLF	1.02	0.91	3.55	3.56	0.60	0.57
		Natural MLF	1.05	0.62	3.58	3.70	0.60	0.00
		Lo	0.99	0.65	3.54	3.70	0.60	0.00
		Pc	0.97	0.69	3.53	3.68	0.58	0.00
		L+P	0.98	0.61	3.49	3.70	0.59	0.00
13°C	3.8	No MLF	0.87	0.81	3.83	3.89	0.60	0.60
		Natural MLF	0.86	0.58	3.78	4.15	0.63	0.00
		Lo	0.86	0.51	3.89	4.08	0.63	0.00
		Pc	0.84	0.51	3.78	4.08	0.62	0.00
		L+P	0.84	0.49	3.80	4.05	0.61	0.00

[a]Averages of 2 observations
[b]MLF: malolactic fermentation; Inoculation with *Leuconostoc oenos* (Lo) or *Pediococcus cerevisiae* (Pc) or with both (L+P)
[c]Expressed as tartaric acid

Table 4. Agmatine (μM) — Significant 2-factor interaction of flora treatments, Lo vs Pc, x time of sampling.

Flora treatments[a]	Time of sampling	
	Before MLF	After MLF[b]
Lo	2.69[c]	1.92
Pc	1.46	5.20

[a]Inoculation with *Leuconostoc oenos* (Lo) or *Pediococcus cerevisiae* (Pc)
[b]MLF: malolactic fermentation
[c]Means are of 8 observations

Putrescine was not found in must samples but was detected in six out of 40 wines (range of concentration: 1.5 - 7.3 μM) over the two replicates after the alcoholic fermentation and in two out of 40 wines after the MLF (concentration: 1.6, 2.1 μM). Because most wines did not have levels of putrescine exceeding the detection limits of 0.6 μM, treatment effects were non-significant. Putrescine has been reported in red and white wines in amounts exceeding 250 μM (16,32).

Agmatine was not found in must, but when detected in wines, the levels were very low. The highest concentration was 11.3 μM. The interaction between the flora treatments, Lo *vs* Pc, and the time of sampling was the only one of statistical significance. Observation of the means for this interaction (Table 4) revealed opposite trends in the agmatine content before and after the MLF depending on the organism involved in this fermentation. The wines inoculated with *P. cerevisiae* had more agmatine after the MLF than before, and the opposite occurred in the wines fermented by *L. oenos*. Main effects contrasts not involved in the interaction mentioned above were not statistically significant (Table 5). Agmatine has not been reported in musts and wines to the knowledge of the authors.

Table 6. Cadaverine (μM) — Significent 3- and 2-factor interactions involving temperature, pH, flora and time of sampling.

A-Temperature × No MLF vs Lo + Pc + L+P, × time of sampling

Temperature	Flora treatments[a]	Time of sampling	
		Before MLF	After MLF
22°C	No MLF	5.92[b]	3.30[b]
	Lo + Pc + L+P	2.91[c]	1.28[c]
13°C	No MLF	2.35	2.85
	Lo + Pc + L+P	7.33	1.92

B-pH × Lo vs Pc, × time of sampling

pH	Flora treatments	Time of sampling	
		Before MLF	After MLF
3.3	Lo	4.10[b]	1.85
	Pc	6.58	0.78
3.8	Lo	6.82	1.38
	Pc	3.30	1.52

C-Natural MLF vs Lo + Pc + L+P, × time of sampling

Flora treatments	Time of sampling	
	Before MLF	After MLF
Natural MLF	7.02[d]	0.60[d]
Lo + Pc + L+P	5.12[e]	1.60[e]

[a] MLF: malolactic fermentation; Inoculation with *Leuconostoc oenos* (Lo) or *Pedicoccus cerevisiae* (Pc) or with both (L+P)
[b] Means are of 4 observations
[c] Means are of 12 observations
[d] Means are of 8 observations
[e] Means are of 24 observations

Although cadaverine was not detected in the must samples, 32 out of 40 wines had a cadaverine content greater than the detection limit of 0.6 μM after the alcoholic fermentation. The ratio dropped to 13 out of 40 wines after the MLF. Interactions that were statistically significant are presented in Table 6. For the interaction, temperature × No MLF *vs* Lo + L+P × time of sampling, the level of cadaverine decreased after the MLF for the treatments Lo + Pc + L+P and the rate of decrease was

Table 5. Main effect contrasts for the amines, agmatine, cadaverine, ethanolamine, tyramine and histamine (2 replicates, 20 treatments).

Contrasts	Agmatine				Cadaverine				Ethanolamine				Tyramine				Histamine			
	Means (μM)	Mean diff.	95%Con.lim.[a] Lower	Upper	Means (μM)	Mean diff.	95%Con.lim.[a] Lower	Upper	Means (μM)	Mean diff.	95%Con.lim.[a] Lower	Upper	Means (μM)	Mean diff.	95%Con.lim.[a] Lower	Upper	Means (μM)	Mean diff.	95%Con.lim.[a] Lower	Upper
Temperature																				
22°C	3.51	0.70	−11.38	+12.96	—[c]				336.94	14.33	−21.91	+50.57	35.85	13.98*	+13.03	+14.93	6.72	0.09	−11.32	+11.50
13°C	2.72				—				351.27				21.87				6.63			
pH																				
3.3	2.60	1.02	− 0.84	+ 2.88	—				—				25.42	6.88	− 6.43	+20.19	7.98	2.61	− 8.82	+14.04
3.8	3.62				—				—			32.30				5.37				
Flora[b]																				
No MLF	2.33	1.04	− 0.72	+ 2.80	—				328.80	20.39	− 3.70	+44.48	—				3.50	4.11	− 0.03	+ 8.25
Lo + Pc + L+P	3.37				—				349.19				—				7.61			
Natural MLF	3.88	0.50	− 1.22	+ 2.22	—				344.20	4.99	−19.10	+29.08	33.95	4.60	− 0.89	+10.09	7.06	0.55	− 3.59	+ 4.69
Lo + Pc + L+P	3.37				—				349.19				29.34				7.61			
Lo	—				—				—				30.54	1.20	− 5.52	+ 7.92	3.78	5.07	0.00	+10.14
Pc	—				—				—				29.34				8.85			
Lo	2.30	1.42	− 1.73	+ 3.57	3.54	0.04	−2.92	+3.00	358.72	26.02	− 3.48	+55.52	30.54	2.39	− 4.33	+ 9.11	3.78	6.41*	+ 1.34	+11.48
L+P	3.72				3.50				332.70				28.15				10.19			
Pc	3.33	0.39	− 1.76	+ 2.54	3.04	0.46	−2.50	+3.42	356.14	23.54	− 6.06	+52.94	29.34	1.19	− 5.53	+ 7.91	8.85	1.34	− 3.73	+ 6.41
L+P	3.72				3.50				332.70				28.15				10.19			
Time of sampling																				
Before MLF	—				—				—				—				9.40	5.45*	+ 2.48	+ 8.42
After MLF	—				—				—				—				3.95			

Factors	Experimental error		Experimental error		Experimental error		Experimental error		Experimental error	
	df	Mean square	df	Mean square	df	Mean square	df	Mean square	df	Mean square
Temperature	1	18.34	—	—	1	162	1	0.11	1	17.96
pH	2	3.75	—	—	—	—	2	191.31	2	141.21
Flora	16	8.24	16	15.56	16	1549	16	80.40	16	45.72
Time of sampling	—	—	—	—	—	—	—	—	20	40.47

[a]95% Confidence limits = Mean difference ± Least significant difference
[b]MLF: malolactic fermentation; Inoculation with *Leuconostoc oenos* (Lo) or *Pediococcus cerevisiae* (Pc) or with both (L+P).
[c]—: No main effect contrasts presented since the interaction effects were significant.
*: Significant at $\alpha = 0.05$.

more marked at 13°C than at 22°C. The concentration in cadaverine remained stable for the treatment 'No MLF' at 13°C but decreased at 22°C. This could not be explained on the basis of contamination by malolactic bacteria in the wines 'No MLF' since the malic acid content remained the same before and after the MLF (Table 3). The authors cannot provide a satisfactory explanation at this time. For the interaction, pH × Lo *vs* Pc × time of sampling, the level of cadaverine again decreased after the MLF, and the rate of decrease depended on the pH of the must and the organism involved. The concentration in cadaverine at the end of the alcoholic fermentation was different for all the wines. *P. cerevisiae* at pH 3.3 and *L. oenos* at pH 3.8 decreased the concentration in cadaverine by 5.5 units, while *L. oenos* at pH 3.3 and *P. cerevisiae* at pH 3.8 decreased it by 2.0 and 1.5 units, respectively. The different rates of decrease of cadaverine concentrations seem, therefore, dependent on the bacteria involved in the MLF. This is further supported by a decrease of 6.4 units in cadaverine content when the MLF was carried out with a natural flora (significant interaction of Natural MLF *vs* Lo + Pc + L+P × time of sampling, Table 6). Neither of the two remaining main effects contrasts not involved in significant interactions, were statistically significant (Table 5). Cadaverine was reported in concentrations of up to 27 μM in wine by Woidich *et al.* (32), but Mayer and Pause (16) concluded that cadaverine was not present in wines.

Ethanolamine was present in musts at a concentration of 40 μM. This concentration increased markedly during the alcoholic fermentation to approximately 340 μM (average over all treatments). The content of ethanolamine in the wines was different depending on the various levels of the different factors. Two of such interactions were statistically significant: pH 3.3 *vs* pH 3.8 × time of sampling, and Lo *vs* Pc × time of sampling. The means for these interactions (Table 7) indicated that *L. oenos* reduced the ethanolamine content of the wines while *P. cerevisiae* increased it. Ethanolamine levels increased during the MLF in wines with pH values above 3.8 but remained fairly stable (slight decrease) in wines at pH values less than 3.7 (Tables 3,7). For the treatments not involved in significant interactions, none of the main effects was found to be statistically significant (Table 5).

Table 7. Ethanolamine (μM) — Significant 2-factor interactions involving pH, flora and time of sampling.

A-*Flora treatments, Lo vs Pc, × time of sampling*		
Flora treatments[a]	Time of sampling	
	Before MLF[b]	After MLF
Lo	368.86[c]	348.58
Pc	334.81	377.46
B-*pH × time of sampling*		
pH	Time of sampling	
	Before MLF	After MLF
3.3	338.90[d]	324.54
3.8	336.24	376.72

[a] Inoculation with *Leuconostoc oenos* (Lo) or *Pedicoccus cerevisiae* (Pc)
[b] MLF: malolactic fermentation
[c] Means are of 8 observations
[d] Means are of 20 observations

Table 8. Tyramine (μM) — Significant 2-factor interactions of the flora treatments, No MLF vs Lo + Pc + L+P, × time of sampling.

Flora treatments[a]	Time of sampling	
	Before MLF	After MLF
No MLF	22.34[b]	22.28[b]
Lo + Pc + L+P	20.00[c]	38.69[c]

[a] MLF: malolactic fermentation; Inoculation with *Leuconostoc oenos* (Lo) or *Pediococcus cerevisiae* (Pc) or with both (L+P)
[b] Means are of 8 observations
[c] Means are of 24 observations

Ethanolamine has been reported by a few authors to be present in red and white wines. The levels observed in this study were, however, much higher than those reported in the literature; 340 μM average of this study *vs* 4.9, 14.7, 49.1, and 130.9 μM maximum values reported by Puputi and Suomalainen (18), Spettoli (26), Zappavigna and Cerutti (33), and Mayer and Pause (16), respectively. It is suggested that the reason for this difference might reside in the analytical method since ethanolamine is volatile, and any cumbersome technique of extraction and quantitation might cause a reduction in the concentration of this amine.

Tyramine was the only amine in addition to ethanolamine that occurred in the must at a concentration of 3.5 μM. Tyramine levels increased during the alcoholic fermentation to values of 15.3 and 28.5 μM at 13°C and at 22°C, respectively. Those levels still increased after the MLF whether the fermentation was carried out by 'Natural flora', by *L. oenos*, *P. cerevisiae*, or by *L. oenos* plus *P. cerevisiae* (Table 8). Tyramine levels remained unchanged in the wines assigned the treatment 'No MLF' (Table 8). The main effect on tyramine contents of wines made at two different temperatures was statistically significant (Table 5). No other main effects proved to be of statistical significance (Table 5). Tyramine has been reported in wines in concentrations ranging from 0 to 262.4 μM (15,16,18,26,32,33). In the present study, tyramine was the amine found in highest concentrations after ethanolamine; the values obtained were, however, relatively low (average of 15-28 μM).

Data obtained for histamine ranged between less than 0.5 and 25.8 μM. None of the interactions between the different treatments was statistically significant. Temperature and pH main effect contrasts were not significant (Table 5). The factor flora, had one significant contrast, Lo *vs* L+P, and two contrasts which just failed to be statistically significant at $\alpha = 0.05$, No MLF *vs* Lo + Pc + L+P, and Lo *vs* Pc. Wines fermented with *L. oenos* contained, therefore, less histamine (3.78 μM) than wines fermented with a mixture of *L. oenos* plus *P. cerevisiae* (10.19 μM) or with *P. cerevisiae* alone (8.85 μm). The MLF resulted in a decrease in histamine content of the wines (Table 5: 3.95 μM after MLF *vs* 9.40 μM before MLF). These results were not in agreement with those reported in the literature where histamine formation was attributed to the malolactic flora.

Relation between amino acids and amines before and after the malolactic fermentation: The relationship between chosen pairs of an amino acid and its corresponding amine is given in Table 9 in terms of the coefficients of determination, R^2. None of the pairs

Table 9. Coefficients of determination for the linear and quadratic regressions for chosen pairs of amino acid-amine.

Pairs	Coefficients of determination (%) Linear r^2	Quadratic r^2	Total R^2
Histidine-histamine	0.01	0.01	0.02
Lysine-cadaverine	0.57	0.48	1.05
Ornithine-putrescine	0.71	2.05	2.76
Arginine-putrescine	0.43	3.07	3.50
Arginine-agmatine	12.16	0.62	12.77
Serine-ethanolamine	1.94	7.92	9.86
Tyrosine-tyramine	45.47	0.66	46.13

studied exhibited a high correlation. Only three out of seven of the R^2 for the linear and the quadratic terms were equal to or greater than approximately 10%, and only one R^2 approached 50% (tyrosine-tyramine). Metabolic pathways are very intricate and more than one compound could give rise to the same end-product or a single compound could be the source of many products. Therefore, an attempt to establish a direct relationship between certain amines and amino acids did not meet with much success.

Conclusions

The results of this study do not support the contention that amines in general, and histamine in particular, are the products of the malolactic flora. Agmatine, cadaverine, ethanolamine, histamine, putrescine, and tyramine were produced in highest quantities during the alcoholic fermentation. The levels of cadaverine, histamine, and putrescine decreased during MLF; the concentration of ethanolamine remained fairly stable and only the contents of agmatine and tyramine increased further during MLF. More agmatine was produced by *Pediococcus cerevisiae* than by *Leuconostoc oenos*, but the levels remained so low that they were of no practical concern. These results suggest that the yeasts, rather than the malolactic bacteria, are probably responsible for the production of amines.

The fact that red wines having undergone MLF are reported to contain amines, particularly histamine, in higher concentration than white wines or than red wines without MLF, could be explained on the basis of differences in the vinification process.

White wines are usually treated with bentonite in the later stages of their processing. Bentonite adsorbs amines (6,16,26) but also coloring matter, thus reducing its usefulness for the treatment of red wines.

Red wines intended to undergo MLF, are left on the lees (yeast cells) in order to provide essential nutrients for the malolactic flora. Yeast extracts are known to contain high concentrations of histamine and tyramine (3). Autolysis of the yeast cells results in the release of cellular amines into the wine.

Yeast and bacterial inocula used in certain winery operations could be contaminated during their preparation. These inocula are propagated in non-selective media where contaminating bacteria (*Enterobacteriaceae*) and wild yeasts could easily proliferate and produce various amines. These amines would be added to the must or wine at the time of inoculation. Although diluted, this might not always be sufficient to decrease the levels of added amines to innocuous concentrations.

Some members of the family *Enterobacteriaceae* such as *Klebsiella*, *Proteus*, *etc.*, are known to be active producers of amines and could be partly responsible for the formation of amines during the early stages of the vinification process.

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