

Histamine contents in Georgian qvevri wines with different wine faults

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Seventy-two qvevri wine samples from different regions of Georgia were studied for histamine and first ever results for Georgian qvevri wines were presented. Organoleptic analysis detected several wine faults that gave the possibility to search for correlation between elevated histamine contents and wine faults. Histamine content of the samples is presented according to the groups which were composed according to the tasting results and lactic/malic acid measurements. The influence of the activity of lactic acid bacteria (LAB) on the formation of histamine was clearly confirmed in the category of qvevri wines where Malolactic

Fermentation (MLF) did not take place. Wines produced according to spontaneous MLF had higher histamine contents than the wines where MLF was induced by inoculated commercial LAB species but included low concentrations as well. This underlined the unpredictability to forecast histamine production by spontaneous MLF and confirmed the benefit of using commercial *Oenococcus oeni* LAB species for lower histamine accumulation in wines. Other categories represented wines where spontaneous MLF took place and contained one or more wine faults. Wines with “tourne” and lactic smell with elevated volatile acidity (VA) had higher levels of histamine but wines with mice flavour did not so. These results suggest that the ability of bacteria to cause some of the wine faults: “tourne”, lactic smell + elevated VA, might be associated with their ability to produce higher amounts of histamine as well.

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Introduction

One of the most important Biogenic Amines (BA) in foods is histamine which was found to play a role as a mediator of allergic reactions. In food like meat, fish and dairy products, poisoning by histamine happens quite often. Thus, great attention is paid to histamine control.¹ In alcoholic drinks like beer and wine, biogenic amines were found in fewer quantities than in the above mentioned products, but still at non-desirable levels that may result in headaches, skin itching, red eyes, etc.² Based on international studies, some attempts have been made to introduce regulations on histamine control in wines. Research on histamine in wine has been carried out in different countries for more than 30 years.³ Moreover, some levels of BA in food were established as one of the indicators of food quality (Regulation 1019/2013/EU).⁴ In 2011 published ‘OIV code of good vitivincicultural practices to minimise the presence of biogenic amines in vine-based products’, the importance of the subject was highlighted for the wine sector.⁵ However, no official regulation was set regarding histamine content in wines so far.⁶ No studies have been performed on histamine contents or other BA contents in Georgian wines. Monitoring the amount of some of the most abundant BA can be an advantage also from a marketing point of view.

Histamine is one of the most important BA produced by enzymatic decarboxylation of the amino acid histidine.⁷ 2 to 10 mg/L of histamine is a recommended limit in some European countries. It may have importance in the future if this issue arouses more interest in consumers for food safety.⁸ According to Caruso *et al.*,³ histamine was detected in red and white wines from different countries with higher concentrations in red wines. The value represents the mean histamine content from the following countries: Spain – 4.76 mg/L, Argentina – nd – 5.22 mg/L, China - nd – 9.64 mg/L, Turkey – 0.03 – 2.8 mg/L, South Africa – 4.8 mg/L, Italy – 4.76 mg/L, France – 7.47 mg/L. According to Constantini *et al.*,⁹ histamine has been detected in wines from different European countries even in higher concentrations: Portugal - 23.1 mg/L, Italy - 10.8 mg/L, France - 14.05 mg/L. Spanish, German and Austrian

wines - 11.1 mg/L, 14.8 mg/L and 12.1 mg/L, respectively.

As other BA, histamine can be produced by yeast or Lactic Acid Bacteria (LAB). Its concentration depends on the abundance of amino acid precursors in the grape juice and other parameters.¹⁰ Different factors may influence the level of amino acids as precursors and, hence, histamine content in wines. One of the most important is the vinification method that may lead to longer maceration or aging on the lees that finally contributes to amino acid enrichment in the wine.¹¹ Along with amino acids, some of the LAB can also utilize peptides or proteins by proteolytic enzyme activity.¹² Higher amounts of histamine are usually found in red wines rather than in white and rose wines.^{13,14} This fact is associated with MLF when LAB start metabolic processes.¹⁵ According to some of the studies conducted in different countries, BA in wines are mainly produced at the end of MLF, although some data indicate no significant changes during the course of MLF.¹⁶

MLF is required in red and some white wines for stylistic reasons. During this process, wines are improved in taste and reduced in acidity due to the conversion of malic acid into the less acidic lactic acid. As a consequence, wines become more balanced on the palate and enriched by secondary aroma compounds contributing to aroma complexity. Traditionally, this process occurs spontaneously due to the growth of indigenous strains of LAB but in modern winemaking, the process is usually initiated by inoculation with selected commercial starter LAB strains. It is observed that the highest amount of BA is produced mainly during spontaneous MLF in comparison with the processes conducted by commercial starter cultures.^{17,18} According to different studies on BA in wines, *Oenococcus oeni* bacteria have been considered as the dominant species in the production of histamine in wines but the formation seems to be strain-dependent.^{19,20,21} It was noted that wine acidity may influence BA formation in wines. According to some of the results, high histamine concentration was observed in lower acidity wines with high pH,²² consequently, high pH wines were reported to offer good conditions for bacteria development, thus raising the probability of having more histamine producing strains.²³ In general, for generating biogenic amines, wines should meet some basic criteria, such as containing the respective amino acids acting as precursors, LAB, and conditions for their development.¹⁶

Spontaneous MLF can take place during, at the end, or long after alcoholic fermentation, but can't be always properly regulated. Sometimes the processes may occur in an undesirable way due to LAB properties resulting in wine faults.²⁴ Therefore, spontaneous MLF may be considered as unreliable because the bacteria flora may differ. In this case, uncontrolled counts of potential spoilage microorganisms, even decarboxylase-positive LAB, may dominate. In order to reduce the risk of elevated histamine levels and wine faults generated by spontaneous MLF, it is usual in modern winemaking to inoculate commercial LAB strains that have been specifically selected for their histidine decarboxylase-negative properties.^{25,26}

LAB of the main three genera *Lactobacillus*, *Pediococcus* and *Oenococcus* were shown to be responsible for different wine faults, as some of the LAB species may dramatically change the composition of wines. Degradation of tartaric acid results in "tourne", radically changing the physical and chemical parameters of wines, finally making them unsuitable for consumption. Another fault often referred to as "bitterness" may occur due to glycerol degradation, whilst wine may also increase in viscosity due to the properties of LAB species.²⁷ Today these wine faults are more common in traditional winemaking, and mainly small family wineries are facing these problems. "Mousiness" or "mice flavour" is an off-flavour reminiscent of mouse fur or mouse cage that is perceived only on the palate several minutes after swallowing the

wine.²⁸ The obligatory heterofermenters (*Oenococcus oeni*, *Lactobacillus brevis*, *Lactobacillus hilgardii*) degrade sugars into lactic acid, ethanol and acetic acid, the latter resulting in an increase of volatile acidity (VA). The LAB species: *O. oeni*, *L. plantarum*, *L. mesenteroides*, and *L. casei* can efficiently metabolise citric acid producing diacetyl, with a distinct buttery odour, turning to the so-called "lactic smell", sometimes associated with increased VA.^{29,30}

Food safety is of great concern for Georgian qvevri wines, because they are produced according to the oldest winemaking method of the world. According to archaeological investigation, excavated egg-shaped clay vessels, called qvevri in Georgia, provided chemical and archaeobotanical evidence of winemaking dating back to the Neolithic period – 6,000-5,800 BC.³¹ Traditionally grapes are crushed directly into the vessels buried till the neck in the ground of the traditional wine cellar or sometimes even outside - under the open sky or different fruit trees. During alcoholic fermentation, the wine is enriched with phenolic compounds extracted from the grape solids (skin, pipes, stalks) during several months.³² This makes the difference between the Georgian qvevri method of winemaking and modern methods used worldwide where fermentation and maturation on grape solids is excluded for white winemaking. During the process the phenolic compounds extracted from the solids are partially oxidized and may turn the white wine colour from light golden to dark amber, sometimes associated with orange colour and leading to the notion of *Orange wines*.³³ As a proof of its cultural significance "The ancient Georgian tradition of qvevri winemaking" was assigned by UNESCO on the representative list of Intangible Cultural Heritage of Humanity.³⁴ Today wine is produced almost in every Georgian family where grapes are cultivated. There are plenty of small family-run wineries producing wine in qvevri vessels without using any additives, thus increasing consumers' trust but on the other hand having less control over technological and microbiological processes, finally resulting in different wine faults and other problems that are common for Georgian qvevri winemaking.

Research on histamine has never been performed in the context of qvevri winemaking. Hence, it is reasonable to look for a relationship between LAB development and histamine production, as well as between LAB development and some other wine faults produced.

There are no studies on wine faults caused by LAB in relation to histamine accumulation. Mice flavour and "tourne" are the two wine faults caused by LAB that are rarely met in the global winemaking practice but still occur quite often among the faulty wines of Georgia.³⁵ To ensure the marketing success and sustainability of qvevri wine, reliability in terms of food safety is required with regard to the production methodology and the vessel specificity. This study aims at investigating a potential correlation between histamine content and certain wine microbial fault in Georgian qvevri wines. For this purpose, histamine contents were determined in selected samples, wine faults identified by sensory means, and correlations between histamine levels and wine faults calculated.

Materials and Methods

Sampling

A total of 72 wine samples were collected from traditional wineries of the western and eastern regions of Georgia. The wines were produced from local grape varieties (Rkatsiteli, Kakhuri Mtsvane, Khikhvi, Kisi, Chinuri, Goruli Mtsvane, Tsitska, Tsoolikouri) in tradi-

tional Georgian clay vessels – qvevries. The wineries were small family-run operations that use no additives or have scarce knowledge of winemaking methods, thus producing low-intervention wines. Eleven out of the 72 samples were collected from producers who inoculated commercial lactic acid bacteria (IOC Extraflores – *Oenococcus oeni*) for MLF, in all other wines MLF took place spontaneously or didn't occur. During the sensory evaluations, the wine samples were identified according to categories of wine faults and production methods, which include MLF with commercial strains of lactic acid bacteria or spontaneous microflora, respectively.

Determination of lactic and malic acids

Organic acids were measured according to OIV methods: OIV-MA-AS313-04 by HPLC.³⁶

For calibration, L-(+)-malic acid (purity $\geq 99\%$) and L-lactic acid (purity $\geq 99\%$) were obtained from Merck KGaA (Darmstadt, Germany). Stock solutions were prepared by dissolving the standards in distilled water to a concentration of 1 000 mg/L. The data were expressed in mg/L.

Chromatographic analysis

Malic and lactic acids were determined using an Agilent 1260 Infinity II HPLC System equipped with a UV detector set at 210 nm, an auto-sampler, and a binary pump. The separation was performed using a C18 reversed-phase column (250 mm \times 4.6 mm, 5 μ m). The mobile phase consisted of (A) 0.01% phosphoric acid in water and (B) acetonitrile. A gradient elution was applied as follows: i) 0-5 min: 95% A / 5% B; ii) 5-10 min: 90% A / 10% B; iii) 10-15 min: 80% A / 20% B; iv) 15-20 min: 70% A / 30% B; v) 20-25 min: 60% A / 40% B; vi) 25-30 min: 50% A / 50% B; vii) 30-35 min: 40% A / 60% B; viii) 35-40 min: 95% A / 5% B (re-equilibration). The flow rate was set to 0.8 mL/min, with an injection volume of 20 μ L. The column temperature was maintained at 25°C. The data were acquired and processed using Agilent OpenLab CDS software (version 2.3).

Organoleptic research - tasting panel

A group of professional wine tasters was selected. The members of that tasting commission have undergone extensive training and passed the final qualification examination at the National Wine Agency of Georgia. The tasting panel was composed of 5 members. The tasting was conducted according to the German DLG system which can be described as follows: Wine is rated on a 5-point scale

that was introduced according to ISO-Norms. ISO-Norms (<https://www.iso.org/>) regulate the vocabulary to use in sensory science³⁷ as well as about designing a sensory test³⁸ and the equipment being used for wine testing.³⁹ The wine testing according to DLG was established following German testing guidelines in order to describe German wines by trained panelists (DIN-Norm 10952).^{40,41} They have to score “blind” (unidentified) wine samples on a 5-point-scale and shortly describe the characteristics of the wines. A score of less than 1.5 points means rejection of the wine, in this case at least one fault or a general lack of minimum quality must be indicated. The following list indicates the reasons for possible rejection on the basis of faults: oxidation, volatile acidity (VA), reductive off-odours (H₂S, mercaptans), *Brettanomyces*, lactic smell, mice flavour, earthy/mouldy smell, geranium odour, excessively high free sulphur dioxide level, atypical ageing (ATA), lack of typicity.

Histamine measurement

For histamine quantification, the R1605 - RIDASCREEN® Histamine (enzymatic) test kit was used. This enzymatic determination is based on the histamine dehydrogenase which catalyzes the oxidative deamidation of histamine in the presence of an electron carrier that converts a dye to a colored product. The color intensity is directly proportional to histamine concentration and is measured at 450 nm. The electron carrier and dye are coated on the microtiter plate. The test kits and decolorants were purchased from R-Biofarm (Darmstadt, Germany). The whole methodology is described at the official web source: <https://food.r-biopharm.com/wp-content/uploads/2012/06/r1601-histamin-20-08-28.pdf>⁴²

Statistical treatment

The objective of the statistical treatment was to search for a direct relationship directly between the histamine content and some of the wine faults. From the data obtained, groups were separated and box-plots created to check homogeneity of dispersions where Leven's test was applied. Due to no equality in dispersions ($p - 0.0006$) ANOVA tests could not be applied and the data were treated by a non-parametric method for testing differences in medians. The Kruskal-Wallis Rank Test was applied to determine if there are statistically significant differences between the distributions of the independent groups (Figure 1). Standard deviation and means of each group are presented in Table 1.

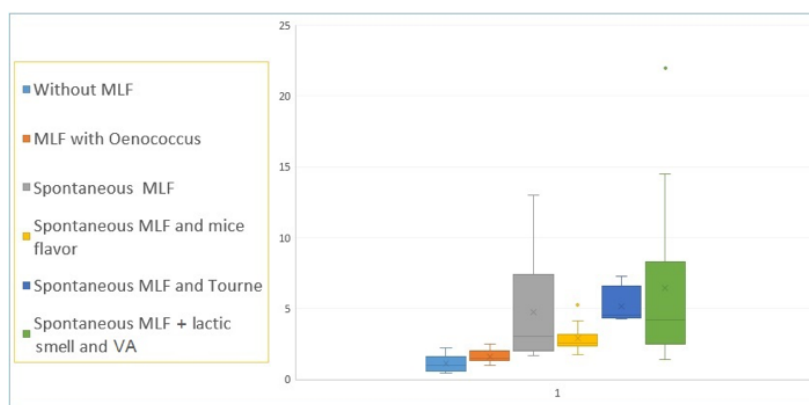


Figure 1. Results of histamine screening according to categories (mg/L).

Results

L-lactic and L-malic acids were measured in all wine samples to differentiate the wines with and without MLF. Thirteen wine samples were identified where MLF did not take place and no wine faults were detected, fifty-nine wines had MLF finished, among them 11 samples inoculated with *Oenococcus oeni* commercial strains with no sensory faults detected either. The rest 48 wines passed MLF spontaneously, twenty-eight of them identified as faulty by the tasting panel and 20 wines without any fault.

Histamin contents of the samples is presented in Table 1 with experimental wine groups formed according to the defects

revealed by the tasting evaluations. These groups are: i) wines where MLF was not carried out, without any faults detected; ii) wines with MLF performed by commercial *Oenococcus oeni* species, without faults detected; iii) wines with spontaneous MLF, without faults detected; iv) wines with spontaneous MLF, with detected fault – “Mice flavor”; v) wines with spontaneous MLF, with detected fault – “Tourne”; vi) wines with spontaneous MLF, with detected fault – “Lactic smell and VA”

The contents of malic and lactic acid in the experimental wines are presented in Figure 2 as boxplots, created according to the groups where no MLF was provided or took place naturally or by inoculated commercial LAB species.

Table 1. Histamine contents in experimental Georgian qvevri wines (mg/L).

Without MLF	MLF with <i>Oenococcus</i>	Spontaneous MLF	Spontaneous MLF and mice flavor	Spontaneous MLF and “tourne”	Spontaneous MLF + Lactic smell and VA
0.48	1.00	1.70	1.73	4.29	1.40
0.50	1.07	1.80	2.13	4.43	2.19
0.50	1.35	1.84	2.33	4.67	2.34
0.71	1.40	1.85	2.44	7.26	2.92
0.77	1.44	1.98	2.50		3.03
0.96	1.47	2.13	2.52		4.04
1.00	1.64	2.24	2.62		4.32
1.31	1.82	2.50	2.87		5.79
1.36	2.01	2.54	3.10		6.23
1.49	2.22	2.99	3.24		8.96
1.73	2.53	3.11	4.13		14.47
1.95		3.45	5.29		21.95
2.23		5.27			
		5.64			
		6.54			
		7.73			
		8.52			
		9.94			
		10.00			
		13.0			
X = 1.15	X = 1.63	X = 4.74	X = 2.91	X = 5.16	X = 6.46
S = 0.57	S = 0.47	S = 3.42	S = 0.96	S = 1.40	S = 6.07

X, means of each group; S, standard deviation of each group.

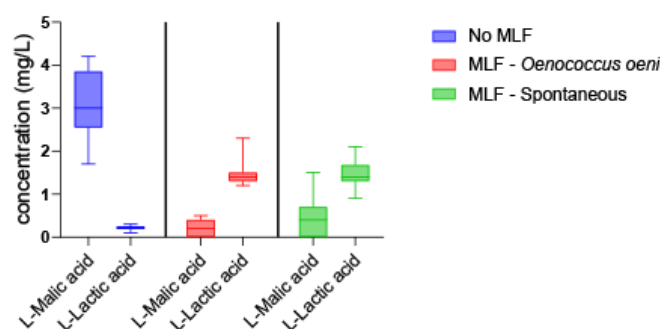


Figure 2. L-malic/L-lactic acid concentration in the experimental Georgian qvevri wines from different grape varieties.

Discussion

It becomes apparent that the presence or absence of MLF plays a key role for histamine content. The wines that did not undergo MLF showed the lowest histamine levels. This initial observation suggests that the bacteria responsible for MLF, are the main contributors to histamine formation during the winemaking process. When commercial LAB strains were used to provide MLF, the resulting wines had lower levels of histamine compared to those subjected to spontaneous fermentation. This result supports the idea that certain LAB strains may be useful in minimizing histamine-related risks, offering a practical approach to improving wine safety by limiting the content of potentially harmful biogenic amines such as histamine

However, when comparing inoculated fermentation to spontaneous MLF, a certain unpredictability emerges. Spontaneous MLF tends to produce higher histamine levels but the results are not always similar, as some spontaneous fermentations result in lower concentrations of histamine. This variability underscores the challenge winemakers face when relying on natural processes, as the unpredictability of the bacterial species involved, makes it difficult to forecast the levels of histamine that will be produced. This could have implications for winemakers who are concerned about the potential risks associated with biogenic amines, as it becomes clear that spontaneous MLF is not a reliable method for controlling histamine production.

The last three categories represent wines with faults where spontaneous MLF took place. Wines with “tourne” and lactic smell showed highly elevated levels of histamine, but wines with mice flavour did not so. This suggests a nuanced relationship between specific bacterial strains involved in spontaneous MLF and the development of wine faults as well as the production of histamine. The correlation between the production of certain fault-associated aromas and higher histamine levels implies that the bacteria responsible for these off-flavors may also be more prone to producing histamine, potentially due to their metabolic pathways or the conditions under which they develop.

Ultimately the wide range of histamine concentrations in spontaneous MLF samples could be related to the bacterial diversity in the spontaneous processes where bacterial species and their histamine-producing activities may differ.

The main question of this research - are certain wine faults related to an increased content of histamine? - can be answered affirmatively for two faults, “tourne” and “lactic smell+VA”. However, this finding does not apply to all faults, since histamine content in “mousy” wines is much lower both in average rate and group data range compared to other faulty wine groups. Conversely, wines with other faults (“tourne”, “lactic smell+VA”) show a much higher than average tendency to accumulate histamine. However, the technological connection with the formation of histamine, which is caused by the way of conducting processes of MLF - spontaneously and non-spontaneously, has been confirmed. According to the obtained results, the concentration of histamine varies in the range from 0.48 to 21.95 mg/L. This range does not differ much from the data of other winemaking countries.

Conclusions

The present study reports first data regarding the concentrations of histamine in traditional Georgian qvevri wines. MLF significantly increased histamine levels as compared to wines that have not undergone MLF. Furthermore, spontaneous MLF pro-

duced significantly higher amounts of histamine than MLF induced by inoculation with commercially selected bacteria strains, thus underlining the benefit of selected strains. Wine faults such as “tourne” and “lactic smell + VA” are associated with elevated histamine levels which suggest the involvement of spontaneous MLF bacteria in the formation of these faults.

The obtained results are a good prerequisite for the future study for determining the correlation between histamine concentration and different technological factors in the production of Georgian qvevri wines.

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