

Nisin in the biopreservation of *Bordô (Ives)* and *Niágara* table wines from Santa Catarina, Brazil

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ABSTRACT

The searches for new antimicrobial compounds to control the lactic acid bacteria in winemaking are based in the increasing global concern about the chemical preservatives restrictions and the development of super-resistant strains continuously exposed to sulfites in wineries for decades. The antimicrobial activity of the nisin as a biopreservative was studied for Bordô (Ives) and Niágara grape wines from Santa Catarina, Brazil. Measurements of antimicrobial activity were made by well-diffusion assays. From the eleven previously assessed strains of nisin-susceptible lactic bacteria (nisin concentration 1000 IU ml⁻¹), four were selected for definitive assays with nisin (100 IU ml⁻¹) dissolved in the wines. Positive results for inhibition were obtained for the four strains selected. Next, the direct inhibitory action was assessed in wines artificially inoculated and then treated with nisin. After 60 days of storage, there was reduction in the total bacterial population as compared to control, especially in Bordô (Ives) wine, while the physic-chemical parameters were not influenced by the nisin treatment. The inhibitory activity of nisin was not affected when it was dissolved in wine in the antimicrobial assays, and its potential utilization as biopreservative should be able to aid on the control of autochthonous microbiota, but further studies are required to conclude more precisely the nisin effects at long term in wines.

Keywords: Antimicrobial, lactic acid bacteria, malolactic fermentation, vinification, bacteriocin.

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RESUMO

As buscas por novos compostos antimicrobianos capazes de inibir a proliferação de bactérias ácido-lácticas na vinificação são motivadas pela crescente preocupação global com as restrições do uso de conservantes químicos, e ainda pelo risco de desenvolvimento de linhagens super-resistentes continuamente expostas aos sulfitos durante décadas nas vinícolas. A atividade antimicrobiana da nisina como bioconservante foi estudada para vinhos Bordô e Niágara de Santa Catarina. Os ensaios de atividade inibitória foram realizados pela técnica de difusão em poços, inicialmente para 11 bactérias lácticas que se mostraram susceptíveis sob a concentração de 1000 UI mL⁻¹. Dentre estas, quatro foram selecionadas para os ensaios definitivos de nisina dissolvida em vinho sob a concentração de 100 UI mL⁻¹. Foram obtidos resultados positivos para as quatro linhagens. Em seguida, a ação inibitória foi diretamente avaliada em vinhos artificialmente inoculados com as mesmas quatro linhagens e depois tratados com nisina. Depois de 60 dias de estocagem, houve uma redução na população bacteriana total quando comparada ao controle, especialmente para amostra de vinho Bordô (Ives), ao mesmo tempo, os parâmetros físico-químicos do vinho não foram alterados pelo tratamento com nisina. O potencial inibitório da bacteriocina não foi perdido quando dissolvido em vinho, durante os ensaios antimicrobianos em ágar, e seu potencial de utilização como bioconservante poderia contribuir no controle da microbiota autóctone, sendo necessários outros estudos para concluir precisamente os efeitos da nisina nos vinhos em longo prazo.

Palavras-chave: Antimicrobiano, bactéria ácido-láctica, fermentação maloláctica, vinificação, bacteriocina.

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INTRODUCTION

The wine's microbiology is composed, besides of ethanogenic yeasts, for lactic acid bacteria (LAB) responsible by the secondary fermentation, which is an important step to improve the quality and guaranteed the wine stabilization. The main source from these bacteria is the surface of grapes and they remain in the must after the milling step as negligible populations during the first stage of vinification (tumultuous fermentation by yeasts). The malolactic fermentation (MLF) occurs precisely at the moment when the LAB population overcomes the adaptation phase and shows an intense proliferation, which may be facilitated by adding starter cultures (selected LAB strains), a common practice in the winemaking worldwide.

Despite the extension of the LAB group, only four genera are acknowledged to be able to develop during the MLF in such limiting conditions, characterized by an acidified pH, high ethanol concentration and the presence of sulfur dioxide, widely used as a preservative in the process. *Oenococcus oeni* is the best adapted species and it is recognized for its beneficial effects on the sensory evolution of the wine after the MLF (Fugelsang & Edwards, 2007). Due to its physiological properties, *O. oeni* has higher tolerance to the sulfites than other LAB, thereby ensuring its prevalence in the winemaking environment. Different tolerance mechanisms of *O. oeni* to sulfites are discussed by several authors (Guzzo *et al.*, 1998; Rojo-Bezarez *et al.*, 2007). Others LAB species related to winemaking are the *Lactobacillus* (Stratiotis & Dicks, 2002) and, less frequently, the *Pediococcus* strains (Rhodes *et al.*, 2003; Du Plessis *et al.*, 2004). Among lactobacilli, the heterofermentative species are prevalent, especially *L. delbrueckii* (Costantini *et al.*, 2009). *Leuconostoc mesenteroides* has been isolated in wine samples (Du Plessis *et al.*, 2004), however its low incidence is probably due the greater sensitivity to alcohol compared with other genera (Oliva-Neto & Yokoya, 2001).

During advanced stages of winemaking, some populations of the LAB that are best adapted to the medium may remain in the wine during storage and aging. Although they multiply slowly during these stages, the presence of such bacteria after the MLF is undesirable due to the risk of formation of residual compounds which are deteriorative to the sensorial quality of the wine,

generating an increase in volatile acidity, excessive rancidity, the presence of bitterness, among other well-known aromatic defects such as mannitol taint, mousiness, ropyness and geranium off-flavor (Fugelsang & Edwards, 2007; Costantini *et al.*, 2009).

Traditionally the bacterial control in winemaking is performed by the use of chemical compounds derived from sulfur, which are incorporated since the beginning process - into grape must - until the advanced stages, as salts in aqueous solution, or as sulfur dioxide gas (SO₂). Nowadays the use of sulfites is considered a compulsory treatment in vinification processes worldwide due to its antioxidant, antioxidase and antimicrobial effects, especially against native microorganisms. However it is known that excessive SO₂ concentrations can preclude completely the proliferation of LAB and generate depreciative residual compounds, as hydrosulfates and mercaptans, which have negative effects on the olfactory quality of the wine. Sulfites should be used cautiously due also to legal determinations, for instance quantity limits set for industrialized foods and beverages, as well as, in some countries, specific labeling for products containing sulfites. There is a strong global trend to reduce the concentration of chemical preservatives in winemaking (Constantini *et al.*, 2009).

Bacteriocins have been investigated as an alternative for the microbial control of undesirable LAB. In the context of vinification, two decades ago the pioneering works of Radler (1990a, 1990b) and Daeschel *et al.* (1991) already suggested that the use of sulfites should be reduced by incorporating nisin into the process. Nisin is produced by strains of *Lactococcus lactis* subsp. *lactis* and it has been known since 1928 (Rogers & Whittien, England). It has had GRAS (Generally Regarded as Safe) status since 1988 and it is used in the food processing in many countries (Arauz *et al.*, 2009; Zacharaf & Lovitt, 2012), including in Brazil through of MERCOSUL Resolutions n. 79/1994 and 82/1996.

Nowadays, it is known that nisin is stable to the conditions of vinification and does not affect yeast cell growth or the sensory profile of wine (Knoll *et al.* 2008), although authorization for industrial use in winemaking has been difficult to obtain. This undefined situation is strengthened by contradictory studies, as the results about the

nisin and phenolic compounds interactions (Daeschel & Bower, 1993-1994; Knoll *et al.*, 2008), by the immense heterogeneity of the wine profiles (physicochemical properties influenced by grape variety and the type of vinification that is carried out) that are produced in different regions and, besides these, the strong resistance of wine producers, which are one of the most traditional industrial sector. All these constraints tend to hinder to take general conclusions about new alternatives of wine preservation. Thus, there is a need for deeper and specific studies for each wine producer region. In this study, nisin was evaluated as a possible microbial control agent for the wine varieties that are typical of the state of Santa Catarina, Brazil, which are made from *Bordô (Ives)* and *Niágara* grapes.

MATERIAL AND METHODS

Microorganisms and culture conditions

The LAB strains used in this study were obtained from the culture collection of the Laboratory of Food Microbiology of the University of the West of Santa Catarina (UNOESC), Videira campus, and also isolated from the wine microbiota kindly provided by the Laboratory of Microbiology of the EPAGRI Experimental Station in Videira, Santa Catarina, Brazil. All LAB were reactivated from stock strains which were kept under freeze (-20 °C) with 10% glycerol. For reactivation they were cultivated in De Man, Rogosa & Sharpe (MRS) broth, except those of the genus *Enterococcus*, grown in Brain Heart Infusion broth (BHI). Incubation was performed in microaerophilic condition and at 35 °C, except for *Oenococcus oeni*, *Lactobacillus casei* and *L. brevis* which were incubated at 30 °C.

Preliminary screening for nisin sensitivity

Eleven strains (Table 1) among the genera *Lactobacillus*, *Oenococcus*, *Enterococcus*, *Leuconostoc* and *Pediococcus* were evaluated for the inhibitory potential of commercial nisin (Nisaplin[®], Aplin & Barrett). The evaluation was carried out by well diffusion assay (Ammor *et al.*, 2006) inoculating each indicator microorganism from an overnight-enriched culture into plates that received the MRS or BHI media (pour plate) with pH previously adjusted to 5.0. After solidification, the agars were perforated in two places - one for the addition of 50 µl of nisin

solution diluted in HCl 0.02 M and containing a final concentration of 1,000 IU ml⁻¹, and another for the addition of 50 µl of sterile distilled water (negative control). All the plates were incubated at 30 °C or 35 °C/24 hours in according each indicator microorganism. The results were determined by the presence or absence of an inhibition halo on the indicator lactic culture at the end of the assay.

Confirmation of inhibitory activity

Four strains (*Lactobacillus brevis*, *L. delbrueckii* subsp. *lactis*, *Pediococcus acidilactici* and *Oenococcus oeni*) were selected for definitive inhibition assays using nisin solution at a final concentration of 100 IU ml⁻¹, and sulfur dioxide (SO₂) dissolved in a sample of red *Bordô* wine or of white *Niágara* wine to a initial concentration of 32 mg l⁻¹ of free SO₂. In this assay, the MRS agar was supplemented with 2% sterilized *Niágara* table wine when *Lactobacillus* spp. and *Pediococcus* sp. were used as target strain. In the *Oenococcus oeni* assay, the malolactic culture medium (MLO) was used and supplemented with 3% tomato extract, as described by Rojo-Bezares *et al.* (2007), with modifications (original tomato extract concentration as 10%). Four holes were perforated on the agar plates for the addition of one of the following substances: 50 µl of nisin (100 IU ml⁻¹), 50 µl of sterile distilled water (control), 50 µl of *Bordô (Ives)* wine containing SO₂ (32 mg l⁻¹), and 50 µl of *Niágara* wine containing SO₂ (32 mg l⁻¹). The agar plates were incubated at 30°C in microaerophilic condition for 48 hours (to MRS medium) or for 4 days (to MLO medium). All evaluations were performed in triplicate and in two repetitions. At the end of each incubation period the inhibition halo diameter obtained on the indicator microorganisms previously inoculated (pour plate) on agar plates was measured in millimeters (mm).

Activity of nisin in wine

A 10 ml sample of *Niágara* wine containing 32 mg l⁻¹ of SO₂ was supplemented with 1.0 mg of commercial nisin (10⁶ IU g⁻¹ initial concentration) diluted directly into the wine to obtain a final nisin concentration of 100 IU ml⁻¹. The antimicrobial potential of this sample was evaluated through of 50 µl aliquots in well diffusion assay, according to describe above, for each one of the four indicator microorganisms in

the same test conditions. Comparatively, 50µl of the same wine containing SO₂ was used in each assay as control (without nisin addition).

Viability assessment of bacteria and physicochemical evaluation of wine with nisin

A sample of 720 ml *Bordô (Ives)* wine and *Niágara* wine received inocula of lactic acid bacteria (10⁴ CFU ml⁻¹ each) to form a mix from *Lactobacillus brevis*, *L. delbrueckii* subsp. *lactis*, *Pediococcus acidilactici* and *Oenococcus oeni*. These inocula were obtained by centrifugation (1,300 xg, 5 minutes) from an initial culture as previously described, and inoculated in each wine sample at time zero (t=0). In parallel others two samples of 720 ml (one *Bordô* wine, one *Niágara* wine) received commercial nisin (final concentration of 100 UI ml⁻¹), besides of the inocula. A third sample of each wine was kept under the same conditions without inoculum or nisin to further comparisons (original sample).

The microbial monitoring of the LAB added to the wines was made at time zero (t₀=0) and at the end of the storage period (t_f=60 days) for all 720 ml samples of wine, which were kept under environmental conditions of low humidity and the absence of light. For the quantification of LAB (CFU ml⁻¹) after the incubation period, 1 ml aliquots of each sample were plated on MRS agar (pour plate) and incubated at 30 °C under microaerophilic conditions for 7 days. All analyses were performed in duplicate.

Physicochemical analyses were performed to monitor the parameters of density (mg l⁻¹), alcoholic degree (% v/v), total acidity (meq l⁻¹) and free sulfur dioxide - SO₂ (mg l⁻¹), accord to standard methods described by Brazilian Ministry of Agriculture, Livestock and Supply (BRASIL, 2006), in triplicate for all samples in t₀ and t_f. All the data obtained from the microbial and physicochemical parameters were statistically evaluated by variance analysis (ANOVA).

Table 1. Qualitative assessment of the spectrum of action of nisin on lactic acid bacteria commonly found in wine microbiota

Target microorganism	Origin	Susceptibility
<i>Enterococcus faecium</i>	Culture collection - ATCC 6569	+
<i>Enterococcus faecalis</i>	Culture collection - ATCC 19433	+
<i>Oenococcus oeni</i>	Commercial strain (Biolact Acclimaté PB1025, AEB Group®)	+
<i>Leuconostoc mesenteroides</i> subsp. <i>cremoris</i>	Isolated from meat*	+
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	Culture collection - ATCC 7830	++
<i>Lactobacillus fermentum</i>	Culture collection - ATCC 9338	++
<i>Lactobacillus plantarum</i>	Culture collection - ATCC 8014	+
<i>Lactobacillus casei</i>	Isolated from wine**	++
<i>Lactobacillus brevis</i>	Isolated from wine**	+
<i>Pediococcus pentosaceus</i>	Culture collection - ATCC 33314	++
<i>Pediococcus acidilactici</i>	Culture collection - ATCC 8042	++

* UNOESC Collection (Videira, SC, Brazil)

** EPAGRI Experimental Station (Videira, SC, Brazil)

Key: (+) weak inhibition halo, (++) well-defined inhibition halo.

RESULTS AND DISCUSSION

Inhibitory activity assays

In the preliminary screening for the antimicrobial activity, all 11 LAB strains were observed to be sensitive to 1000 UI ml⁻¹ of the bacteriocin.

Among the species tested, *Pediococcus* spp. and *Lactobacillus* spp. (except *L. plantarum*) showed greater susceptibility when compared to *Enterococcus* spp., *Oenococcus oeni* and *Leuconostoc mesenteroides*, as shown in Table 1. The records for the inhibition halos obtained in

the second assay with nisin are shown in Figure 1 for the four lactic acid bacteria selected at this stage. The following measures of inhibition halos, excluding the diameter of the well, were obtained for each microorganism: (a) *Lactobacillus brevis* 10.6mm, (b) *Lactobacillus delbrueckii* subsp. *lactis* 8.4mm, (c) *Pediococcus acidilactici* 4.0mm, and (d) *Oenococcus oeni* 7.8mm. It can be drawn from the well-defined inhibition halos on the surface of each agar that the effectiveness of the bacteriocin remained satisfactory even at a concentration 10 times lower than that used in the first assay (Table 1). *Lactobacillus* spp. was again the most sensitive. *O. oeni* proved to be more susceptible than *P. acidilactici* in this assessment. As expected, no inhibition halo was observed on the wine samples containing SO₂. In addition, similarly to the studies of Rojo-Bezares *et al.* (2007), this study has revealed an effective antimicrobial action against wine isolates (*Lactobacillus casei* and *L. brevis*).

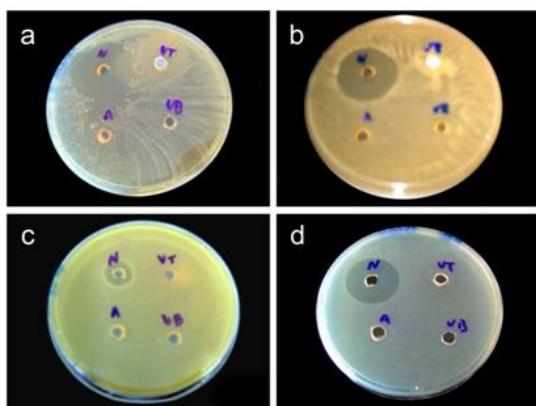


Figure 1. Antimicrobial action of nisin against: (a) *Lactobacillus brevis*, (b) *Lactobacillus delbrueckii* subsp. *lactis*, (c) *Pediococcus acidilactici*, (d) *Oenococcus oeni*.

Key: N = nisin; A = sterilized water; VT = *Bordô* wine control; VB = *Niágara* wine control.

These results are interesting because currently there is a concern over the development of super-resistant strains in wineries where cultures are routinely exposed to sulfur compounds, thus the susceptibility of LAB to other inhibitory compounds is appreciated. The survival of a viable population in the bottled product is the most worrying contamination, responsible by the known "second growth" which can use of

residual L-malate as carbon source (Fugelsang & Edwards, 2007).

An effective control of *O. oeni* by alternative antimicrobial compounds is really needed if we consider that it can survive in a concentration of 100 mg l⁻¹ of free SO₂ (Lafon-Lafourcade *et al.*, 1983). Rojo-Bezares *et al.* (2007) reported that *O. oeni* has low resistance to nisin, which was ascertained from the fact that the minimal inhibitory concentration (MIC) for the *Oenococcus* group was always much lower compared to other LAB composed of strains of *Lactobacillus* spp., *Pediococcus* spp. and *Leuconostoc mesenteroides* for the same treatments with nisin, nisin + ethanol and nisin + metabisulfite. In our assays (with nisin diluted on HCl), the halos between *L. delbrueckii* subsp. *lactis* and *O. oeni* are very close, but when nisin was dissolved directly in the wine (next assays), some differences are observed about the halos diameter in the same four target bacteria, as presented below.

Figure 2 shows the antimicrobial activity of nisin dissolved in wine, which has not lost its activity. This is evidenced by the formation of well-defined inhibition halos against the four LAB evaluated. These inhibition halos had the following measures of diameters: *Lactobacillus brevis*: 6.4 mm, *Lactobacillus delbrueckii* subsp. *lactis*: 4.7 mm, *Pediococcus acidilactici*: 4.3 mm, and *Oenococcus oeni*: 9.0 mm. The activities against *Lactobacillus* strains were partly reduced (to 61% and 54% in *L. brevis* and *L. delbrueckii*, respectively), whereas the *O. oeni* halo increased 15%, in comparison to the first inhibitory assay with 100 IU ml⁻¹ of nisin. In this case, our results are in agreement with Rojo-Bezares *et al.* (2007) about the higher susceptibility of the *O. oeni* than *Lactobacillus* spp. in nisin assays.

Bacterial viability in treated wines

It was found that the *Niágara* wine samples stored for 60 days showed a reduction in bacterial population when compared to the initial count (control), both in the absence and in the presence of nisin, with a reduction of approximately 2.1 and 2.3 log cycles respectively. However there was no effect of nisin compared to the group without nisin after 60 days. Therefore, the effect of the nisin was not significant in reducing of countable lactic acid bacteria in *Niágara* wine (Figure 3). In the *Bordô* (*Ives*) wine samples there was a reduction of 1.5 log cycles for the

treatment with inoculum only in comparison with control, and a reduction of approximately 2.4 log cycles for the treatment with inoculum and nisin after 60 days, in comparison with control (with inoculum at day zero). This suggests a possible antimicrobial effect by nisin, although no significant difference was observed considering the standard deviation. In the original samples (not shown in the graph), which did not receive neither inoculum nor nisin, LAB populations were also detected in the order of 10^3 UFC ml^{-1} in all samples (already considered to be in the control group).

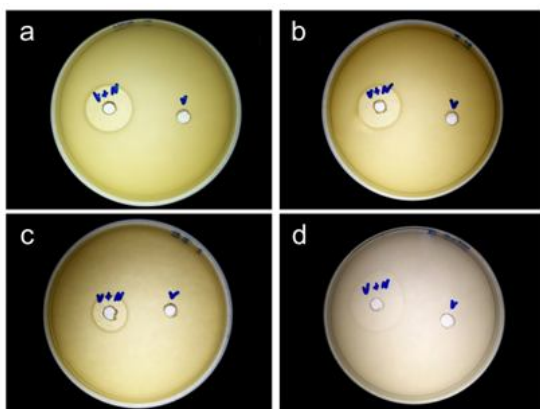


Figure 2. Antimicrobial action of nisin (100 IU ml^{-1}) diluted in Niágara wine, where: (a) *Lactobacillus brevis*, (b) *Lactobacillus delbrueckii* subsp. *lactis*, (c) *Pediococcus acidilactici*, (d) *Oenococcus oeni*.

Key: V+N = Niágara wine + nisin; V = control, only wine.

Physicochemical evaluations in treated wines

According to Table 2, the presence of LAB and/or nisin during the 60 days of (white and red) wine storage did not promote any relevant changes in the physicochemical properties tested, despite of the parameter free SO_2 which decreased with time in all samples. This is a normal tendency which always occurs in the any wine storage due to its volatility and, thus, it is not assigned to nisin presence. In respect to total acidity, slight differences were observed and the values at the end of storage time were equal or less than the initial total acidity. This is a favorable situation, because if a microbiologic contamination occurs normally the acidity is increased by organic acid formation, especially by lactic acid bacteria. Preliminarily, the nisin seems not depreciative to the wine quality, but

other evaluations at different storage times and wine profiles are required to conclude more precisely the nisin effects at long term.

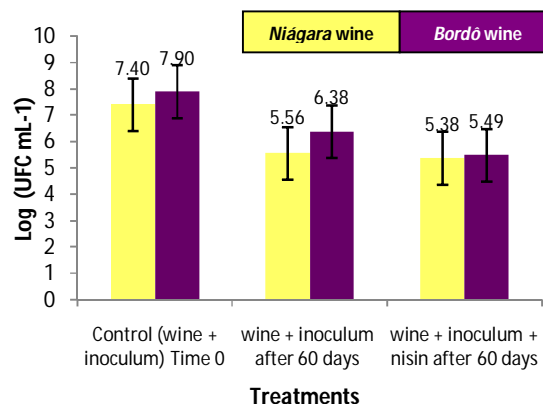


Figure 3. Cell viability of mix of lactic acid bacteria in Niágara and Bordô (*Ives*) wine in the presence or absence of nisin after 60 days of storage.

*Error bars respect the standard deviation.

CONCLUSION

The results of this study contribute to extend the well known antimicrobial action of nisin on general lactic acid bacteria against wine isolated bacteria also. The utilization of nisin as a complementary preservative in the Niágara and Bordô (*Ives*) winemaking would be able to aid on the control of autochthonous microbiota responsible for microbiological diseases, and could reduce the sulfite concentration required currently. Further studies might also examine the joint use of sulfite + nisin in industrial processes, which may certainly improve the preservative effect. Overall the experimental microbial contamination did not affect the physicochemical parameters during the study period, but studies involving longer periods should be carried out to assess the interference both of lactic acid bacteria as nisin on the analytical and sensory quality of the wine.

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Table 2. Physicochemical evaluations of *Niágara* and *Bordô* (*Ives*) wine during storage

<i>Physicochemical parameters</i>	<i>Sample</i>	<i>T = 0</i>		
		Control	With inoculum only	With inoculum + nisin
Relative Density (mg l⁻¹)	<i>Niágara</i>	0.99 ^a	0.99 ^a	0.99 ^a
	<i>Bordô</i>	0.99 ^a	0.99 ^a	0.99 ^a
Alcohol (% v/v)	<i>Niágara</i>	10.30 ^a	10.50 ^a	10.50 ^a
	<i>Bordô</i>	10.50 ^a	10.30 ^a	10.50 ^a
Free SO₂ (mg l⁻¹)	<i>Niágara</i>	32.00 ^a	22.40 ^b	25.60 ^c
	<i>Bordô</i>	32.00 ^a	19.20 ^b	19.20 ^b
Total Acidity (meq l⁻¹)	<i>Niágara</i>	100.00 ^a	96.00 ^b	98.00 ^b
	<i>Bordô</i>	103.00 ^a	99.00 ^b	103.00 ^a

*For each treatment: different letters represent significant statistical differences when p = 0.05.

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