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Research Paper

Occurrence of *Brettanomyces/Dekkera* in Brazilian red wines and its correlation with ethylphenols

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Abstract

The yeast *Brettanomyces/Dekkera* can cause important spoilage in wines, with the production of ethylphenols and other off-flavor compounds. This study aimed at determining the presence of this yeast and the ethylphenols produced by them in Brazilian red wines, establishing their relationship with other chemical characteristics. Isolates of *Brettanomyces/Dekkera* were quantified by plating 126 samples of dry red wine in selective culture medium, while ethylphenols were analyzed by solid phase extraction and GC/FID. Free and total SO_2, alcohol, total dry extract, residual sugar, total and volatile acidity, and pH were also determined. *Brettanomyces/Dekkera* was present in 27% of samples. Ethylphenols were detected in most samples, with amounts higher than the threshold limit of 426 μg/L found in 46.03% of samples. The majority of wine samples showed inadequate levels of SO_2 and residual sugars, facts that might facilitate microbial spoilage. The passage in barrels and the grape varieties (Cabernet Sauvignon and Merlot), did not show any influence on the levels of contamination or ethylphenols contents. The prevalence of *Brettanomyces/Dekkera* and the concentrations of ethylphenols were high considering the sensory impact they can cause. The growth of *Brettanomyces/Dekkera* was dependent on the levels of SO_2 and alcohol of wines. Knowledge of the contamination, the presence of ethylphenols, and their relationship with the chemical characteristics of wines can entice effective measures to prevent *Brettanomyces/Dekkera* and contribute to improve the general quality of Brazilian red wines.

Key words: *Brettanomyces*, *Dekkera*, 4-ethylphenol, 4-ethylguaiacol, wine.

Introduction

Wines are subject to chemical and microbiological spoilage and yeasts of the genus *Brettanomyces* or its teleomorph *Dekkera* can be important causes of these problems, especially in red wines, with the production of ethylphenols and the formation of off-flavors, which are generally described as *phenolic*, *medicinal*, or *animal* (*horse sweat*, *stable and leather*) (Chatonnet *et al.*, 1990). Ethylphenols, 4-ethylphenol, and 4-ethylguaiacol, are formed from the transformation of *p*-coumaric and ferulic acids, naturally present in wines. These transformations occur by a chain reaction of cinnamate decarboxylase generating vinylphenols, and then ethylphenols via vinylphenol reductase (Heresztyn, 1986; Lauritsen *et al.*, 1991; Chatonnet *et al.*, 1992). Spoilage sets up after the fermentation, usually during maturation in barrels (Chatonnet *et al.*, 1990, 1992). The risk of microbial degradation of the wines is particularly high in wood barrels, due to the difficulty of cleaning and disinfection (Malfeito-Ferreira *et al.*, 2004). However, the growth of *Brettanomyces/Dekkera* and the formation of ethylphenols can also occur in concrete or stainless steel tanks (Rodrigues, *et al.*, 2001), and even in bottles (Pérez-Prieto *et al.*, 2003).

Although all wines are susceptible to contaminations, some factors will increase this risk, for instance, when using very ripe grapes, with high contents of phenolic acids and high pH, long maceration times and difficult fermenta-

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tion, which will necessarily delay the addition of sulphite (Gerbaux *et al.*, 2000; Pérez-Prieto *et al.*, 2003). Higher temperatures and pH (Dias *et al.*, 2003; Romano *et al.*, 2008), dissolved oxygen (Ciani and Ferraro, 1997), and residual sugar (Chatonnet *et al.*, 1995), as well as the lower concentration of ethanol (Gerbaux *et al.*, 2000) and sulphur dioxide (Chatonnet *et al.*, 1992, 1993; Gerbaux *et al.*, 2002; Barata *et al.*, 2008), are other factors involved with the growth of *Brettanomyces/Dekkera* and the increase of ethylphenols in wines.

Brettanomyces/Dekkera can use acetic acid (Gerós *et al.*, 2000) and ethanol (Dias *et al.*, 2003) as the sole carbon sources, but in the presence of carbon sources commonly found in wines this metabolism is blocked (Vigentini, *et al.*, 2008). Therefore, this yeast will grow in wines, taking advantage of even small amounts of residual sugars such as glucose, fructose, arabinose and trehalose (Chatonnet *et al.*, 1995), arginine (Joseph and Bisson, 2004), and organic acids (Vigentini *et al.*, 2008). Theoretically, concentrations of hexoses of 100 mg/L can support populations of *Brettanomyces/Dekkera* as large as 10^7 cells/mL (Smith, 1998). Since the production of characteristic off-flavors of *Brettanomyces/Dekkera* can start appearing in wines when its population is as low as 10^2 or 10^3 cells/mL, this becomes a major problem for the wine industry (Henick-Kling *et al.*, 2000; Fugelsang and Zoecklein, 2003). Wine spoilage caused by *Brettanomyces/Dekkera* has been reported worldwide (Heresztyn, 1986; Chatonnet *et al.*, 1992; Heimoff, 1996; Ibeas *et al.*, 1996; Fugelsang and Zoecklein, 2003). Brazil is becoming an important wine producer and exporter, with very typified products, and its industry is well known for its good technology, although climate conditions will frequently pose some challenges for grape quality. Little is known on the presence of *Brettanomyces/Dekkera* in Brazilian wines and its possible effects on wine quality. In this context, the main objective of this study was to determine the presence of these yeasts and the related ethylphenols in samples of red wines, and try to establish a relationship with other chemical characteristics of these wines, thus contributing to improve the general quality of this important product.

Materials and Methods

Chemicals

Unless otherwise stated, all chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, USA).

Wine samples

A total of 126 samples of 63 dry red wines were selected for this research. Wines were collected from 26 wineries located in the largest and most traditional wine region of southern Brazil, in the hills of the cities of Bento Gonçalves and Farroupilha (RS, latitude 29°S, longitude 51°W). Samples were arranged as 54 of Cabernet Sauvignon and 63 of Merlot, while 7 were of other red varieties, and 2 were assemblage of the mentioned varieties, distributed between 2004 and 2005 vintages. Microbiological analyses were performed immediately after the opening of the bottles; the remaining wine was frozen for later chemical analysis.

Culture medium and microbiological techniques

A selective and differential medium for *Brettanomyces/Dekkera* (DBDM) developed by Rodrigues *et al.* (2001) was used in this research, with some modifications. Its basic composition is (in g/L): Yeast Nitrogen Base, 6.7; ethanol, 60; *p*-coumaric acid, 0.1; cycloheximide, 0.01; bromocresol green, 0.022; agar, 20 (pH 5.4). The medium was modified by the addition of glucose (5 g/L) and chloramphenicol (0.1 g/L). This medium inhibits the growth of other yeast species due to the presence of cycloheximide. In addition, the medium allows the differentiation of the *Brettanomyces/Dekkera* strains by their characteristic production of ethylphenols (off-flavors) from *p*-coumaric acid, the acidification, and the long incubation time required. Addition of glucose was to improve the recovery of *Brettanomyces/Dekkera*, and chloramphenicol, to prevent growth of bacteria.

The medium was sterilized by filtration and the agar was autoclaved separately. Varying volumes of wine were plated in agar DBDM; 0.1 mL was directly spread onto plates, while volumes of 1 and 20 mL were filtered through a 0.45 μm membrane that was placed over the medium. Colonies were counted after 3 and 14 days of incubation at 28 °C.

Determination of 4-ethylphenol and 4-ethylguaiacol

Solid phase extraction (SPE) was performed using polystyrene-divinylbenzene cartridges Lichrolut-EN 200 mg (Merck, Darmstadt, Germany). The cartridges were conditioned with 4 mL of dichloromethane, 4 mL of methanol, and 4 mL of ethanol 12% (volume fraction). Subsequently, 50 mL of filtered wine containing 5 mg/L of an internal standard (3,4-dimethylphenol) were passed through the SPE cartridge at a flow rate of 2 mL/min. The sorbent was washed with 3 mL of ultra-pure water, dried-up by airflow for 3 min Finally, ethylphenols were eluted with 1.4 mL dichloromethane. The eluate was kept frozen until analysis. The recovery efficiencies of 4-ethylphenol and 4-ethylguaiacol using this methodology were always around 100%.

A gas chromatograph (CG-14B Shimadzu, Tokyo, Japan) equipped with flame ionization (FID) was used to determine ethylphenols. The analyses were performed by injecting μLof the eluate in split mode (ratio 1:10, 250 °C) into a DB-WAX column (60 m x 0.25 mm i.d. x 0.25 μm film thickness; Agilent J&W, Folsom, USA). The carrier gas was H_2 at a flow of 1 mL/min. The temperature program

was as follows: 50 °C for 1 min, first ramp to 180 °C at 10 °C/min, second ramp to 205 °C at 3 °C/min, third ramp to 215 °C at 2 °C/min, fourth ramp to 230 °C at 15 °C/min, and then kept at 230 °C for 30 min. The detector was kept at 250 °C.

General Analytical procedures

Free SO_2 in wine samples was analyzed using an Oenological Analyzer (Quick, Gibertini, Milano, Italy) equipped with SO_2 bubble, following titration with 0.02 N iodine solution. Total SO_2, was analyzed by steam distillation in an Oenochemical Distilling Unit (DEE, Gibertini, Milano, Italy) following titration with 0.02 N iodine solution. The ethanol content, density and total dry extract were determined using Alcolyzer Wine and density meter (DMA 4500, Anton Paar® GmbH, Graz, Austria). Finally, residual sugar, total and volatile acidity, and pH were determined by infrared spectroscopy using a WineScan™ FT120 (FOSS, Denmark).

Statistical analysis

Spearman correlation (r_s) analysis was used in order to correlate yeast cell numbers, concentration of ethylphenols and other chemical parameters of wines. Student's t-test was used to compare the average of results. The statistical analysis was performed using SPSS 16.0 software (SPSS Inc., USA).

Results and Discussion

Prevalence of *Brettanomyces/Dekkera* in wine samples and concentration of ethylphenols

Results for the investigation of the presence of *Brettanomyces/Dekkera* in wine are shown in Table 1. Thirty-four (27%) samples produced typical colonies of this yeast when they were plated onto the DBDM medium: colonies yellow to olive green, acidification of the medium, phenolic flavour and slow growth, reaching up to 2.25 x 10^3 CFU/mL. These values are similar to results reported by Gerbaux *et al.* (2000) and Rodrigues *et al.* (2001), in which the presence of *Brettanomyces* was found in 25 and 57% of a total of 44 and 88 samples of Pinot Noir wines from the Burgundy region and Portuguese wines, respectively, containing up to 2.5 x 10^3 CFU/mL. This high CFU counting is of great concern for the wine industry, because it is very difficult to control *Brettanomyces/Dekkera* in these environments. Concerning the samples amount of ethylphenols, they were present in high percentages, above the preference thresholds for these compounds, considering the sensory impact they may cause, but were within the range reported by others authors (Table 1). Chatonnet *et al.* (1992) have established the limit preference thresholds of 620 μg/L for 4-ethylphenol, 140 μg/L for 4-ethylguaiacol and 426 μg/L to a 10:1 mixture of 4-ethylphenol and 4-ethylguaiacol. Average and maximal values (in μg/L) found in previous studies reached up to 1,164 and 6,047 for 4-ethylphenol, and 99 and 1,561 for 4-ethylguaiacol, respectively (Chatonnet *et al.*, 1992; Gerbaux *et al.*, 2000; Pollnitz *et al.*, 2000; Rodrigues *et al.*, 2001; López *et al.*, 2002; Henschke *et al.*, 2004; Nikfardjam *et al.*, 2009; Romano *et al.*, 2009).

The yeast counts correlated ($p < 0.01$) with concentrations of 4-ethylphenol (r_s = 0.397) and 4-ethylguaiacol (r_s = 0.318) (Figure 1a). However, the prevalence of ethylphenols was higher than that of *Brettanomyces/Dekkera*, similarly observed by Rodrigues *et al.* (2001). One possibility for this unusual result might reside in the fact that ethylphenols were produced in an early stage in the wine, previously of procedures that would reduce the microbial load for bottling or, for the same reasons, the yeast cells could be in a non-cultivable state (Millet and Lonvaud-Funel, 2000). Another possibility could be the action of other yeast species such as *Pichia* and *Candida*, or bacteria such as *Lactobacillus*, which can produce ethylphenols (Edlin *et al.*, 1995; Chatonnet *et al.*, 1995, 1997; Dias *et al.*, 2003; Couto *et al.*, 2006; Rivas *et al.*, 2009).

Surprisingly, the yeast counts showed variations among different bottles of the same wine, suggesting that each bottle should be individually evaluated. Thus, it would not be strictly correct to use the average of counts of different bottles of the same wine to express the presence of *Brettanomyces/Dekkera*. These variations among bottles were also observed and reported by other authors (Di Stefano, 1985; Chatonnet *et al.*, 1993). The different environmental conditions of each fermentation tank, barrel or bottle, or different microbial loads of contaminated materials

Table 1 - Determination of *Brettanomyces/Dekkera* (CFU/mL), 4-ethylphenol (4-EP), and 4-ethylguaiacol (4-EG), in μg/L, and percentages above the preference threshold of 126 samples of Brazilian red wines.

	Brett/Dekkera	4-ethylphenol	4-ethylguaiacol	Ratio 4-EP/4-EG
Minimal	0	0	0	1.29
Maximal	2,250	3,819.65	259.67	21.98
Average ± SD	76.03 ± 314.42	593.40 ± 694.62	65.24 ± 52.69	7.77 ± 4.56
CV (%)	413.56	117.05	80.77	53.69
Above the preference threshold (%)		34.92	7.93	46.03*

CV, coefficient of variation; * refers to the mixture (10:1) of 4-EP and 4-EG.

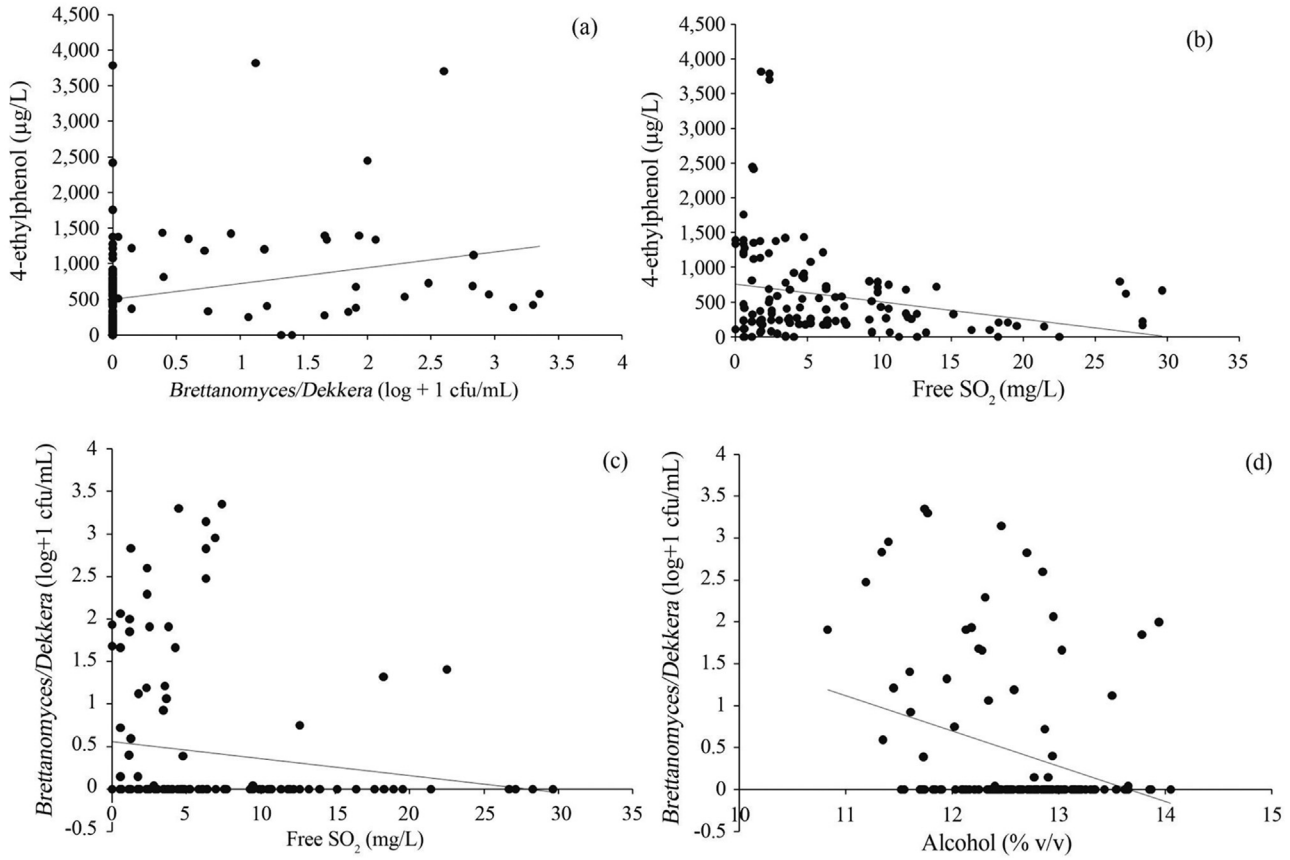

Figure 1 - Correlation between 4-ethylphenol and *Brettanomyces/Dekkera* counts (a), and free SO_2 (b); correlation between *Brettanomyces/Dekkera* counts and free SO_2 (c), and alcohol (d).

and equipment at the beginning of the bottling process may be responsible for these variations, stressing the importance of careful processing to avoid these problems.

The concentrations of 4-ethylphenol and 4-ethylguaiacol were statistically correlated (r_s = 0.860), and the ratio between them varied from 1.37 to 21.98, which is close to the range (1.6 to 38) found by Pollnitz *et al.* (2000) and Henschke *et al.* (2004). According to these authors, this ratio depends on the relative abundance of precursors, which is dependent on the geographic region and grape variety. Romano *et al.* (2008) observed the ratio of 1:1 when they used a synthetic medium containing equal concentrations of *p*-coumaric and ferulic acid, and the relationship with the precursors was confirmed.

Correlations with chemical parameters

In Table 2 are presented the chemical parameters of wine samples, while in Figure 1 are shown the possible correlations between these parameters and the presence of *Brettanomyces/Dekkera* or concentrations of ethylphenols. Among the tested chemical parameters, only free and total SO_2, alcohol, and volatile acidity correlated with counts of *Brettanomyces/Dekkera* or concentrations of ethylphenols. The average concentration of free SO_2 was low (37.3% of samples), which is indicating a permissive condition for microbial growth. Most of these samples showed high levels of ethylphenols (Figure 1b) and counts of *Brettanomyces/Dekkera* (Figure 1c), which explains the correlation between 4-ethylphenol and free and total SO_2 (r_s = -0.177 and -0.176, respectively). A free SO_2 content of 30 to 40 mg/L (Gerbaux *et al.*, 2000; Ribéreau-Gayon *et al.*, 2003) is necessary for the total elimination of viable populations of *Brettanomyces/Dekkera*, and even higher doses of SO_2 are required in wines with high values of pH (Sudraut and Chauvet, 1985). According to Henick-Kling *et al.* (2000), the wine should be maintained with at least 0.8 mg/L of molecular SO_2 in order to avoid the development of *Brettanomyces/Dekkera*. It is known that the fraction of free SO_2 in bottled wines decreases with storage time due to reactions with organic compounds and oxidation, which may explain some of the low values of free SO_2 measured in this research. Notwithstanding, the total SO_2 levels were also low (below 20 mg/L) in 16% of samples, which suggests that the addition of this preserving agent was carried out only once during production, leaving these wines unprotected against microbial deterioration.

The amount of alcohol weakly correlated (r_s = -0.282) with the counts of *Brettanomyces/Dekkera*, although most samples with high counts had up to 13% (volume fraction) of alcohol (Figure 1d), which has been suggested by some

Table 2 - Chemical parameters of 126 samples of Brazilian red wines.

	Minimal	Maximal	Average ± SD	CV (%)
Total SO_2 (mg/L)	3.07	137.43	50.36 ± 28.92	57.43
Free SO_2 (mg/L)	0	29.63	6.53 ± 6.73	103.01
pH†	3.4	4.04	3.73 ± 0.13	3.42
Alcohol (% vol.)	10.83	14.05	12.64 ± 0.66	5.25
Total dry extract (g/L)	22.52	38.78	29.51 ± 2.69	9.13
Residual sugar (g/L)	1.1	6.7	3.94 ± 1.22	30.94
Volatile acidity (g/L)	0.65	1.17	0.90 ± 0,11	12.49
Total acidity† (g/L)	4.56	7.33	5.84 ± 0.49	8.38

CV, coefficient of variation; volatile acidity as g acetic acid/L; total acidity as g tartaric acid/L. †The acidity of the wines decreased by freezing of the samples, so the pH values are approximately 0.11 above the actual values.

authors to be the minimal concentration showing inhibitory properties (Froudiére and Larue, 1988; Chatonnet *et al.*, 1993; Rodrigues *et al.*, 2001; Dias *et al.*, 2003; Silva *et al.*, 2004).

Regression plots showed that the highest counts of *Brettanomyces/Dekkera* and concentrations of ethylphenols were found in samples in the range of 5.5 to 6.7 g tartaric acid/L total acidity.

The volatile acidity of wines was high (Table 2), if the sensorial perception threshold of 0.6 to 0.9 g/L is considered (Ough and Amerine, 1988). Twenty-three samples had volatile acidity above 1 g/L, which is detrimental to the aroma of wine. In general, the production of acetic acid by *Brettanomyces/Dekkera* is related to the concentration of sugar in the medium (Ciani and Ferraro, 1997; Dias *et al.*, 2003; Abbott *et al.*, 2005; Romano *et al.*, 2008; Vigentini *et al.*, 2008). It was found a weak correlation (r_s = 0.193) between volatile acidity and *Brettanomyces/Dekkera* counts, but the production of acetic acid by this yeast can not be discharged, because of the high residual sugar of samples.

Brazilian legislation establishes that the maximal level of residual sugar in wine to be defined as "dry wine" should not exceed 5 g/L, while a more restrict definition of dry wine, obtained by the complete fermentation of sugars, should not contain more than 1 g/L of residual sugars (Ough and Amerine, 1988). Twenty-three samples (18.25%) showed levels of sugars above 5 g/L (Table 2) suggesting that these samples were susceptible to microbial contamination. Sugar levels of 300 mg/L are considered high enough to allow the development of ethylphenols in concentrations corresponding to the perception threshold (Chatonnet *et al.*, 1995). Silva *et al.* (2004) observed that residual sugar concentrations in the range of 0.1 to 12% did not show any influence on the specific growth rates of *B. bruxellensis* ISA 1791 in mineral medium containing glucose as the sole carbon and energy source. These data suggest a lack of relationship between the residual sugar and ethylphenols concentrations, which was also observed in this work.

Presence of *Brettanomyces/Dekkera* and ethylphenols correlated to the storage of wine in oak barrels

Wine samples were tested in order to investigate whether there was any effect correlated to the use of barrels during the maturation of wine on the presence of *Brettanomyces/Dekkera* and ethylphenols. Table 3 presents the results for samples that were matured in wood barrels. Surprisingly, higher counts of *Brettanomyces/Dekkera* were found for wines that were not matured, when compared with wine samples that were stored in barrels. However, the concentrations of ethylphenols were not statistically different between the two groups of samples. Wines spoilage by *Brettanomyces/Dekkera* has been associated with long times of maturation in wood barrels, which presents a number of factors that contribute to the contamination (oxygen, wood porosity, small volume, etc.) (Suárez *et al.*, 2007), but as it has been observed by some authors (Rodrigues *et al.*, 2001; Pérez-Prieto *et al.*, 2003; Coulo *et al.*, 2010), the problem associated with the presence of this yeast is much more complex. The development of *Brettanomyces/Dekkera* and production of ethylphenols is influenced by the practices of vinification (Chatonnet *et al.*, 1992, 1995) and wine storage time (Pérez-Prieto *et al.*, 2003), which may be related to the results obtained. Brazil-

Table 3 - Mean *Brettanomyces/Dekkera* counts and ethylphenols concentrations correlated to the stage of the wine in oak barrels.

	With barrel maturation	Without barrel maturation
Number of samples (*n*)	39	32
Positive for *Brettanomyces/Dekkera*	8	14
Brettanomyces/Dekkera (CFU/mL)*	9.91 ± 35.94	261.52 ± 586.24
4-ethylphenol (μg/L)	610.66 ± 761.24	495.13 ± 407.30
4-ethylguaiacol (μg/L)	65.84 ± 52.09	59.08 ± 32.06

*Different by t-test (p = 0.021).

ian wineries using oak barrels on a large scale are still very few, due to the high costs of these barrels. Thus, it appears that the use of wood barrels in a small-scale production facility and shorter maturation times have favored greater care, compared with storage in stainless steel or concrete tanks, consequently reducing contaminations.

Presence of *Brettanomyces/Dekkera* as a function of grape variety

In this research, the presence of *Brettanomyces/Dekkera* was not found to be correlated with the variety of grape used in the wine making (Table 4), even though the Cabernet Sauvignon varieties were slightly more prone to microbial spoilage than the Merlot samples due to the characteristics of their pH and total dry extract. However, there were no statistical differences for *Brettanomyces/Dekkera* counts between the two varieties, suggesting very similar winemaking practices for both varietals. The ratio of 4-ethylphenol and 4-ethylguaiacol was higher for Cabernet Sauvignon, which is linked to higher concentrations of *p*-coumaric acid (Pollnitz *et al.*, 2000), with values similar to those found by others authors: 10:1 for Cabernet Sauvignon and 8:1 for Merlot (Pollnitz *et al.*, 2000); 8:1 for Bordeaux red wines (Chatonnet *et al.*, 1992); and around 9:1 for 180 different Chilean wines (Majcenovic, 2003).

Conclusion

The prevalence of *Brettanomyces/Dekkera* and the concentrations of ethylphenols in the investigated Brazilian red wines were similar to those found for wines from other countries. The levels of contamination could be considered high, if the possible impact on sensorial aspects is taken into account. The levels of SO_2 showed to be the determinant condition for microbial spoilage, since the growth of *Brettanomyces/Dekkera* was dependent on the levels of SO_2 and alcohol, but it seemed not to be influenced by variations in sugar, acidity and total dry extract. Despite of a well-established correlation, ethylphenols were more frequent than *Brettanomyces/Dekkera* in the investigated samples, and the results showed that the stage in barrels and grape varieties did not influence the levels of contamination and concentrations of ethylphenols.

Table 4 - Presence of *Brettanomyces/Dekkera* and chemical parameters of the wines as a function of grape variety.

	Cabernet Sauvignon	Merlot
Brettanomyces/Dekkera (CFU/mL)	26.91 ± 110.97	59.49 ± 224.57
4-ethylphenol (μg/L)	628.71 ± 758.50	553.45 ± 556.87
4-ethylguaiacol (μg/L)	65.60 ± 52.77	65.88 ± 50.99
Ratio 4-EP/4-EG*	9.24 ± 4.46	7.51 ± 3.73
pH *	3.78 ± 0.11	3.68 ± 0.11
Total acidity (g tartaric acid/L)*	5.96 ± 0.41	5.76 ± 0.53
Volatile acidity (g acetic acid/L)*	0.93 ± 0.11	0.87 ± 0.10
Total dry extract (g/L)*	30.33 ± 2.16	28.76 ± 2.90
Total SO_2 (mg/L)	49.72 ± 25.19	51.67 ± 31.61
Free SO_2 (mg/L)	6.67 ± 6.56	5.99 ± 6.26
Alcohol (% v/v)	12.60 ± 0.66	12.67 ± 0.65
Residual sugar (g/L)	4.12 ± 1.32	3.95 ± 1.02

*Different by t-test ($p < 0.05$): ratio 4-EP/4-EG ($p = 0.03$); pH ($p = 5.33 \times 10^{-6}$); total acidity ($p = 0.02$); volatile acidity ($p = 0.002$); total dry extract ($p = 0.001$).

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