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Yeast CA-11 fermentation in musts treated with brown and green propolis

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Worldwide laws are being created to reduce the amount of antimicrobial residues in foods and beverages. Distilled alcoholic beverages, such as cachaça should be detached, because some microorganisms infect the fermentation process and decrease the product quality, making it to require microbiological control. Thus, the objective of this research was to evaluate the green and brown propolis extract, as well as the previous physical-chemical treatment of sugarcane juice as antimicrobial agents and their effects on yeast CA-11 fermentation for cachaça production. The experiment was arranged in a completely randomized design in split splots with three replications. Main treatments constituted four antimicrobials (sodium monoensin, green propolis extract, brown propolis extract and physical-chemical treatment) and an untreated control. Secondary treatments were the five fermentation cycles. The amount of yeast cell and bud viability, yeast bud rate during the fermentation, pH, total acidity, glycerol and alcohol contents were evaluated in wines. The use of antimicrobial agents, especially the green and brown propolis extracts, improved the maintenance of the amount of live yeast cells and buds, and yeast budding rate as compared to the control treatment. Ethanol levels produced by the yeast strain 'CA-11' during fermentation were found to be around 6 to 7%, which are not statistically significant among the treatments. Results indicate great potential for the use of propolis as antimicrobial in fermentatiton process for production of distilled beverages, like cachaça.

Key words: Distillate beverages, cachaça, antimicrobial agents, *Saccharomyces cerevisiae*.

INTRODUCTION

To meet the market demand, technical and scientific advances are necessary, because they provide the basis for better production process control and consequently higher quality products. This is especially valid for fermented and distillated beverages to meet food quality and safety requirements in Brazil and the world (Venturini Filho, 2011).

Despite alcoholic beverages were classified by their properties (as a raw material and alcohol content), they were obtained by a biochemical process called ethanolic

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fermentation. The physical-chemical and microbiological characteristics of this process were directly related to chemical and sensorial characteristics of the beverage (Alcarde et al., 2012).

Cachaça is an important distilled beverage in the world. According to SEBRAE (2013), it is the second most consumed alcoholic beverage in Brazil and third distilled beverage in the world. Cachaça is defined as a typical and exclusive sugarcane rum beverage produced in Brazil, with alcohol content between 38 and 48% in volume; it is obtained from the distillation of sugarcane fermented must, containing unique sensorial characteristics; it has up to 6 g/L of sugars expressed in sucrose (Brasil, 2005).

Yeasts and substrates used in the fermentation process are important, because they may contain bacterial and fungal contaminants (Antonangelo et al., 2013). These microorganisms affect the fermentation biochemistry, altering must and wine compositions. Also, yeast viability is affected, resulting in lower alcohol yield and lower profitability to the industry (Nobre et al., 2007).

Many studies have been done on quality of raw material and care in processing (Cantão et al., 2010). Synthetic antimicrobials and antiseptics are frequently used during fermentation to prevent contamination. These products present different action mechanisms over one or more groups of microorganisms. However, international rules limit the use of synthetic agents because they are not compliant with food and beverage regulators with regards to residues both in the distilled beverage and yeasts (FSA, 2011). On the other hand, the use of natural antimicrobial in alcoholic fermentation processes allows residue-free production of beverages and by-products.

Propolis may therefore be an important alternative because of its wide biocide action, mainly against gram-positive and some gram-negative bacteria, through changes in bio-energetic status in cell membrane and motility inhibition. Propolis is one of the most heterogeneous and complex mixtures found in nature and contain more than 300 substances identified or characterized, as flavonoids, aromatic acids, terpenoids, phenylpropanoids and fatty acids (Arvey and Egea, 2012; Lustosa et al., 2008).

The aim of this study was to compare the antimicrobial activity of propolis extracts and physical-chemical treatment in the sugarcane juice, and their effects on fermentation performance and wine quality from the yeast strain CA-11.

MATERIALS AND METHODS

The experiment was set in Jaboticabal-SP, Brazil, during the 2013/2014 season and arranged in a completely randomized design in split-plots with three replications. Main treatments corresponded to four different methods of bacteria control [nonoensin-based synthetic antimicrobial, green propolis ethanol extract (PEE), brown propolis ethanol extract, previous physical- chemical juice treatment (PCT)]. Untreated juice was used as control. Secondary treatments corresponded to five fermentation cycles.

Sugarcane source

A third ratoon field of the sugarcane variety RB867515 was manually harvested without burning trash. Sugarcane stalks were obtained from organic certified production unit, in Jaboticabal-SP, Brazil, in October 2013.

Antimicrobial solutions preparation

The green and brown PEEs were prepared with raw propolis collected in Bebedouro- SP and Formiga-MG, Brazil (Mello et al., 2010), respectively. Determination of minimal inhibitory concentration (MIC) was performed in preliminary assays, and the concentrations used for green and brown PEE were 3 and 2 µg/mL, respectively. The PEEs were analyzed for pH (using a digital pHmeter), total flavonoids and oxidant activity (Woisky, 1996).

The synthetic antimicrobial sodium monoensin was prepared by direct dilution in 50% ethanol solution and used at a concentration of 3 µg/mL.

Must preparation

Juice was extracted by crushing cane and filtered in 60-mesh filter to remove coarse impurities (soil, cane bagasse). Juice was standardized to 16°Brix and heated to 28 - 32°C (Must 1).

To obtain must 2, the juice was clarified with adjustment of pH to 6.0 by adding calcium hydroxide (6°Bé) to obtain 16°Brix juice. The treated juice was heated until ebullition to facilitate the reaction between calcium and phosphorus found in solution (Albuquerque, 2011). The heated juice was put in inox decanter containing 5 mg/L of *Moringa oleifera* Lamarck leaf extract (Costa et al., 2014); it was prepared according to Ghasi et al. (2000), to facilitate impurity removal.

After 1 h, the supernatant was removed using a siphon. This originated the PCT, which was cooled at room temperature. The pH was not corrected during the preparation of both musts, and the natural pH of the must was considered. Juice and musts were characterized by pH, Total Reducing Sugars (TRS) (Lane and Eynon, 1934) and total acidity (CTC, 2005) analyses.

Inoculum preparation

The steps of cell multiplication, adaption and activation of flocculant yeast of *S. cerevisiae* CA-11 were conducted using a concentration of 30 g dry yeast per liter of must. Dry yeast was submitted for hydration process, using 600 mL of potable water. After 30 min, 3.0 L of sterile juice at 10°Brix was added in the fermenting vat. When the soluble solid level was 2°Brix, another 6.0 L of juice was added.

Fermentation process

Fermentation was done in batch procedure with yeast recovery by sedimentation in 6 L fermenting vat. Inoculum was prepared using 7.5% of active yeasts, previously diluted in 1.5 L of must at 6°Brix. The first vat loading was done after 30 minutes with 2.0 L, and the second after 1.5 h, with 2.5 L of must at 16°Brix. The end of the fermentation was established as 20 h after inoculation, or when the level of soluble solids was lower than 1°Brix. After the end of each fermentation cycle, 2/3 of vat volume corresponding to the wine was removed through a lateral siphon.

Table 1. Means of results obtained to total acidity in original must (1) and clarified must (2), used in fermentation cycles.

Cycle	Must 1 Total acidity (g/L H_2SO_4)	Must 2 Total acidity (g/L H_2SO_4)
1	0.73A	0.41A
2	0.61A	0.24B
3	0.58A	0.31AB
4	0.54A	0.30AB
5	0.58A	0.30AB
F test	0.97ns	4.58*
LSD	0.35	0.13
CV	21.40	15.81

*Significant at 5% of probability ($0.01 < p < 0.05$); ns = not significant; CV = coefficient of variation (%) ; LSD = less significant difference.

From the 2nd to the 5th cycle, material remaining at the bottom of the third cycle was washed with 700 mL of 0.75% sterile saline solution to remove toxic elements; and was kept without stirring. After 1 h, excess was removed and biocide treatments were applied. In the untreated control, saline solution was added again. After 1 h, new juice was fed, starting a new fermentation cycle.

In the 3rd and 5th cycle, the vat bottom was cleaned to remove inert material and dead yeast cells. Yeast cell and bud viability and budding rate were analyzed after 1 h biocides treatments, after 40 min of the second vat feeding, and at the end of the fermentation, using the method of Lee et al. (1981). Brix and pH analyses were also performed.

Wine and distilled analysis

Wines were centrifuged at 1650 x*g* and 25°C for five minutes (HIMAC CR 21G); and total acidity (CTC, 2005), pH (using a digital pHmeter) and glycerol (McGowan et al., 1993) were analyzed. Wine volatile fractions were separated through distillation (alcohol microdistiller TE-012 Tecnal); 20 mL of distillate per 60 mL of wine was recovered.

Samples were submited for alcohol content by a digital densimeter (Anton Paar DMA-48). Data were submitted for ANOVA and means were compared by Tukey test (5%), using the statistical program ASSISTAT version 7.7 beta.

RESULTS AND DISCUSSION

PEE characterization

Green and brown PEEs presented the following characteristics, respectively: pH 4.77 and 5.18 at 25°C; 0.48 and 0.70% of total flavonoids; all oxidant reaction was completed for 20 and 13 s. Results are based on the parameters established in a Brazilian technical regulation and propolis identity book ["Regulamento Técnico de Identidade e Qualidade da Própolis" (Brasil, 2001)], which show that the maxim oxidation activity is 22 s with 0.25% (m/m) of total flavonoids. Bispo Junior et al. (2012) describe that propolis antibacterial activity can be directly associated with their flavonoids content and anti-oxidant activity.

Sugarcane and must

Raw sugarcane used in the experiment had adequate technological quality for processing: 22.2°Brix, more than 90% of purity, 18.9% of total reducing sugars (TRS), 0.55% of reducing Sugars (RS), total acidity of about 0.8 g/L H_2SO_4 and pH 5.3.

After raw sugarcane analysis, extracted juice was standardized to 16° Brix; in the PCT calcium hydroxide was added and juice was heated. After dilution, must TRS decreased by 26% in relation to the extracted juice. However, these values were sufficient to activate the anaerobic metabolism in yeasts and fermentation process via Crabtree effect, which occurs when sugar concentrations in the substrate are above 6% (Venturini Filho et al., 2013).

Average pH values were found to be 5.2 in must 1, and 5.9 in must 2. This difference was expected because must 2 was treated with calcium hydroxide. The musts pH were not corrected to 4.5 before yeasts inoculation, as commonly used in production units (Cardoso, 2013), since yeast CA-11 metabolism produces by-products capable of reducing substrate pH. So, this strain does not require the sulphuric acid treatment normally used by beverage producers.

The average total acids values in musts 1 and 2 were 0.6 and 0.24 to 0.41 g/L H_2SO_4, respectively, during the five fermentation cycles (Table 1). The difference of acidity in musts is due to the previous physical-chemical treatment in juice, which removed some acids by adsorption or transport by calcium phosphates produced in solution (Albuquerque, 2011). Considering the fermentation cycles, it is observed that the PCT resulted in less reduction of these biomolecules only in the first cycle; however, the values were lower than that obtained in must 1. High concentration of these compounds negatively impacts yeast physiology, decreasing the amount of live yeast cells (Dorta et al., 2006). According to Maiorella et al. (1983), 4% of organic acids in fermentation are enough to reduce 80% of the yeast cell viability.

Fermentation process

Inoculum presented cell and bud viability higher than 90% and budding rate of 19%. These results corroborate with Lima et al. (2001) recommendations. At the beginning of the fermentation process (Figure 1A), a reduction in viable cells during the cycles was observed, mainly in the 4th and 5th cycles. However, in fermentations using musts treated with synthetic antimicrobial and brown PEE, the yeast percentage maintained a constant growth. These results corroborate with that of Oliveira Filho (2010), who tested brown PEE and press baker yeast and observed stable cell viability during five fermentation cycles, but lower viability in untreated control.

There was 8% reduction in viable yeast cells at the beginning of the 4th fermentation cycle when green PEE

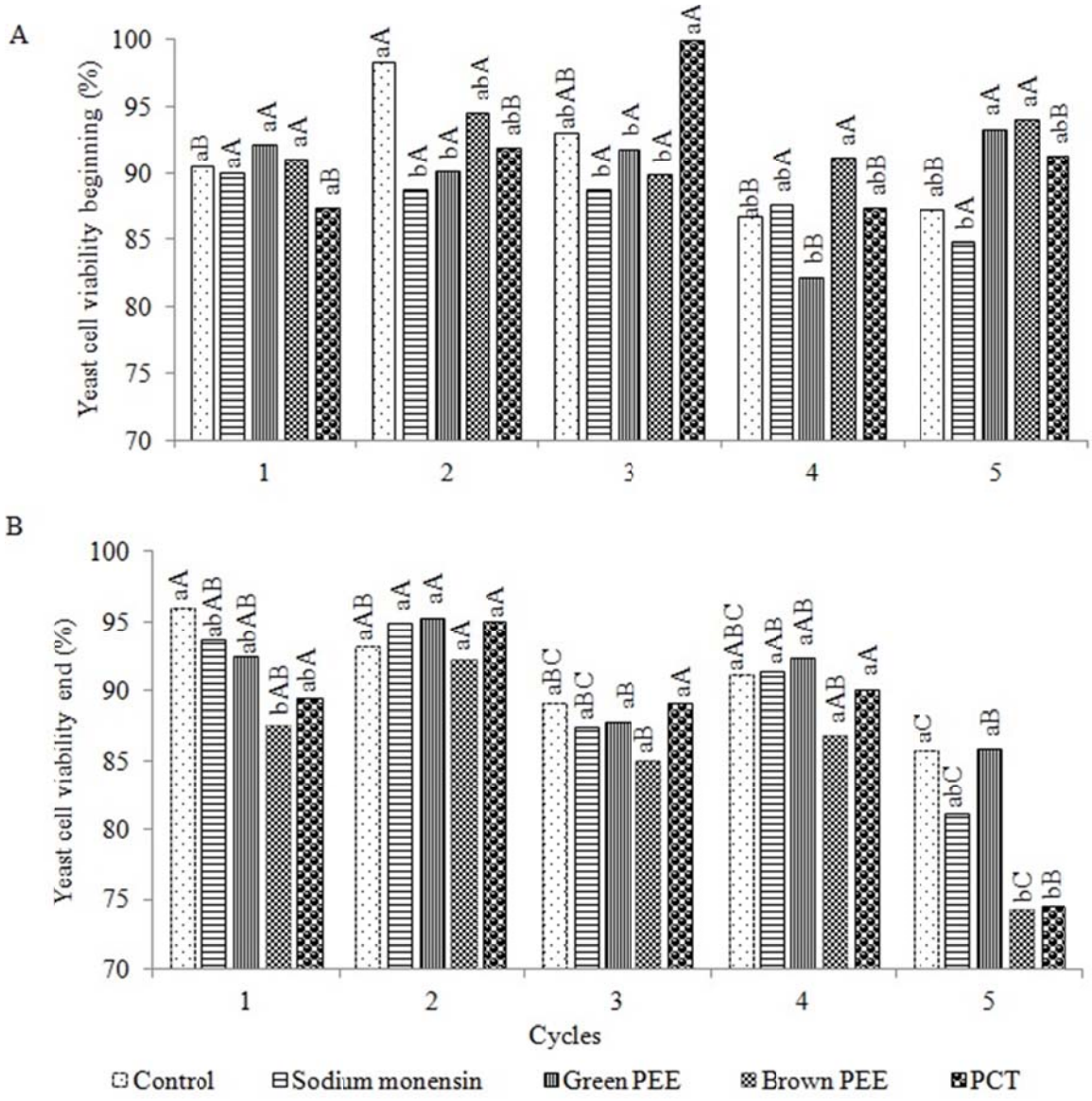

Figure 1. Interaction between must treatments and fermentation cycles for yeast CA-11 cell viability in beginning and end of fermentation process. Jaboticabal-SP-Brazil, 2013/2014season. Upper case letters compare means of the cycles. Lower case letters compare means of the must treatments in each cycle.

was used. Nevertheless, this value was about 82% and met the expectation, since an efficient fermentation requires yeast cell viability ranging from 80 to 90% (Amorim et al., 1996). In the PCT treatment, a high yeast cell viability was also observed, with values between 87 and 100%.

Also, an increase in yeast cell viability at the end of the fermentation was observed (Figure 1B) in all treatments, except in the 5th cycle, which had reductions of 84% for the check, 81% for sodium monoensin, 86% for green PEE, 74% for brown PEE and 75% for PCT. These data differ from those found by Bregagnoli et al. (2009) and Oliveira Filho (2010), who observed high cell viability only in treatments containing synthetic biocide. This divergence may occur as a result of different yeast strains or environmental conditions.

Mendes et al. (2013) related that, at the end of a fermentation cycle, the reduction in yeast cell viability is common, and frequently attributed to environmental intrinsic factors, as concentration of inhibitory metabolites such as ethanol, acids and temperature. As this was not observed in this work, it seems that the yeast strain CA-11 may have adapted to the production process environment, which favors the maintenance of its metabolic activity.

Figure 2 shows the results obtained for budding rate, at

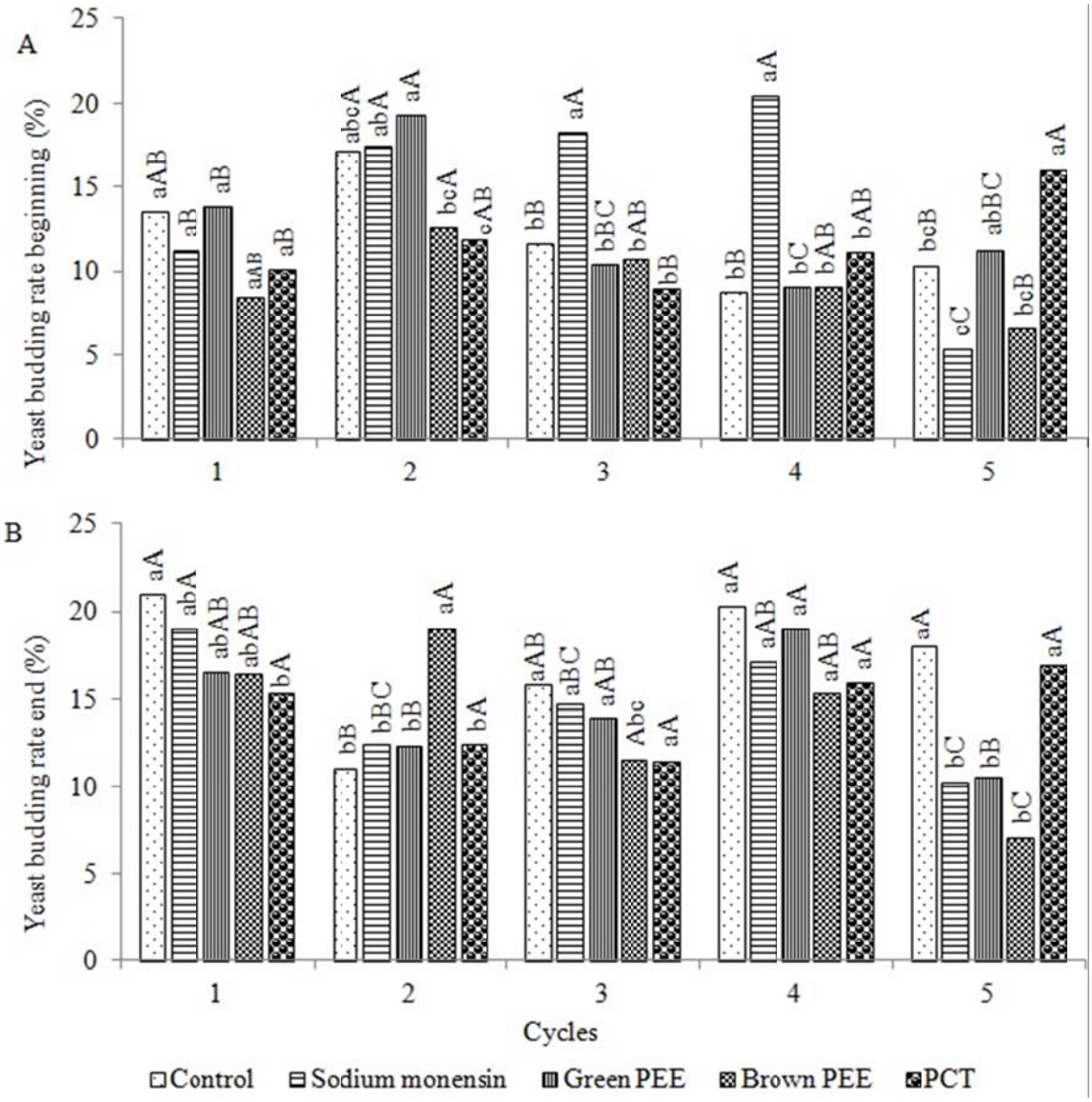

Figure 2. Interaction between must treatments and fermentation cycles for yeast CA-11 budding rate in beginning and end of process. Jaboticabal-SP-Brazil, 2013/2014 season. Upper case letters compare means of the cycles. Lower case letters compare means of the must treatments in each cycle.

the beginning (Figure 2A) and end of the fermentation (Figure 2B). During the fermentation cycles, the yeast budding rate observed ranged from 5 to 20%. These results are in accordance with Amorim et al. (1996) who verified that the adequate budding percentage in the fermentation process may vary from 5 to 15%. The use of green PEE and PCT in musts increased yeast budding rate during the fermentation cycles, but in the presence of brown PEE, the values remain stable at the end of fermentation process.

The high yeast bud rate can explain the yeast adaptability in various substrate conditions, and maintenance of cell viability was favored. However, microorganisms metabolize sugar into ethanol, glycerol or ATP and biomass production, so this high budding rate in ethanol fermentation can result in low ethanol yield and consequently the fermentation efficiency is affected (Lima et al., 2001).

Table 2 shows yeast bud viability at the beginning and end of fermentation. At the beginning of the process, the use of propolis extracts resulted in an increase of bud viability. The results of this study differ from that of Oliveira Filho (2010) and Halabi (2010), who did not find a significant difference between control, synthetic antimicrobial and propolis extract in fermentations using yeasts strains PE-2, CAT-1 and press baker yeast. In this context, a positive response of yeast CA-11 to PEE treatments was observed.

Table 2. Means of values obtained for yeast CA-11 bud viability in the fermentation cycles. Jaboticabal-SP. Season 2013/2014.

Parameter	Bud viability beginning(%)	Bud viability final (%)
Treatments (B)		
Control	88.60B	96.26A
Sodium monoensin	89.40B	89.74AB
Green PEE	93.56AB	94.62A
Brown PEE	95.97A	84.94B
PCT	91.70AB	96.55A
F test	5.80*	5.92*
LSD	5.84	9.55
CV	5.29	8.59
Cycles (C)		
1	91.86AB	91.47AB
2	88.62B	96.96A
3	92.68AB	89.78AB
4	90.07AB	95.56AB
5	96.00A	88.32B
F test	3.54*	3.67*
LSD	6.02	7.82
CV	6.28	8.11
F test (BxC)	1.67 ns	1.12ns

**Significant at 1% of proability ($p < 0.01$); *significant at 5% of probability ($0.01 < p < 0.05$); ns = no significant; CV = Coefficient of variation (%); LSD = Less significant difference.

Table 3. Means and results of ANOVA for glycerol concentration in wines.

Parameter	Glycerol (%)
Treatments (B)	
Control	0.69A
Sodium monoensin	0.59B
Green propolis	0.59AB
Brown propolis	0.48C
PCT	0.63AB
F test	12.71**
LSD	0.10
CV	14.09
Cycles (C)	
1	0.58A
2	0.57A
3	0.58A
4	0.62A
5	0,62A
F test	1.09ns
LSD	0.09
CV	15.63
F test (BxC)	1.88ns

**Significant at 1% of proability ($p < 0.01$); * significant at 5% of probability ($0.01 < p < 0.05$);, ns=no significant; CV = Coefficient of variation (%); LSD = Less significant difference.

Through fermentation cycles, there was a reduction in bud viability in the second cycle, probably associated with less cell energy production that contributes to a reduction of cell multiplication rate. However, bud viability values were higher than 88%, and considered excellent ($p < 0.05$). Yeast bud viability is important to maintain yeast population levels, because they will be reused in the next fermentation cycles, often impacting the fermentation yield (Ravaneli et al., 2006).

At the end of the fermentation, the lowest yeast bud viability was observed in brown PEE treatment. These results disagree with that of Oliveira Filho (2010), who did not observe reduction in bud viability. Although, the values are in acccordance with that of Halabi (2010), who obtained 86% of bud viability. Considering the yeast growth conditions, treatments did not show negative effects on the fermentation process.

Wine characteristics

pH and total acidity

According to Camolez and Mutton (2005), high acid levels in wine are responsible for pH reduction. This behavior was observed in treatments where the synthetic antimicrobial, green and brown PEE were applied (Figure 3A), after the second fermentation cycle. Similar results were obtained by Bregagnoli et al. (2009) and Oliveira Filho (2010), who also verified a reduction in total acidity when antimicrobial control was used.

Glycerol and Alcohol yield

Table 3 shows the glycerol concentration in wines obtained by bacterial control of the fermentation. Glycerol is synthetized by yeast to maintain the cell redox equilibrium, which is altered when organic acids and biomass production occur (Lima et al., 2001). Thus, it was observed that bacterial control resulted in less acid production and consequently the glycerol content was reduced. This performance was more evident in wines treated with brown PEE, where 0.21% of reduction was found as compared to the untreated control.

These results are higher than the observations of Ferrari (2014), who obtained results between 0.23 to 0.40% of glycerol; but lower than Balli et al. (2003), who found values of 1.10% in sucrose fermentation process at 33°C.

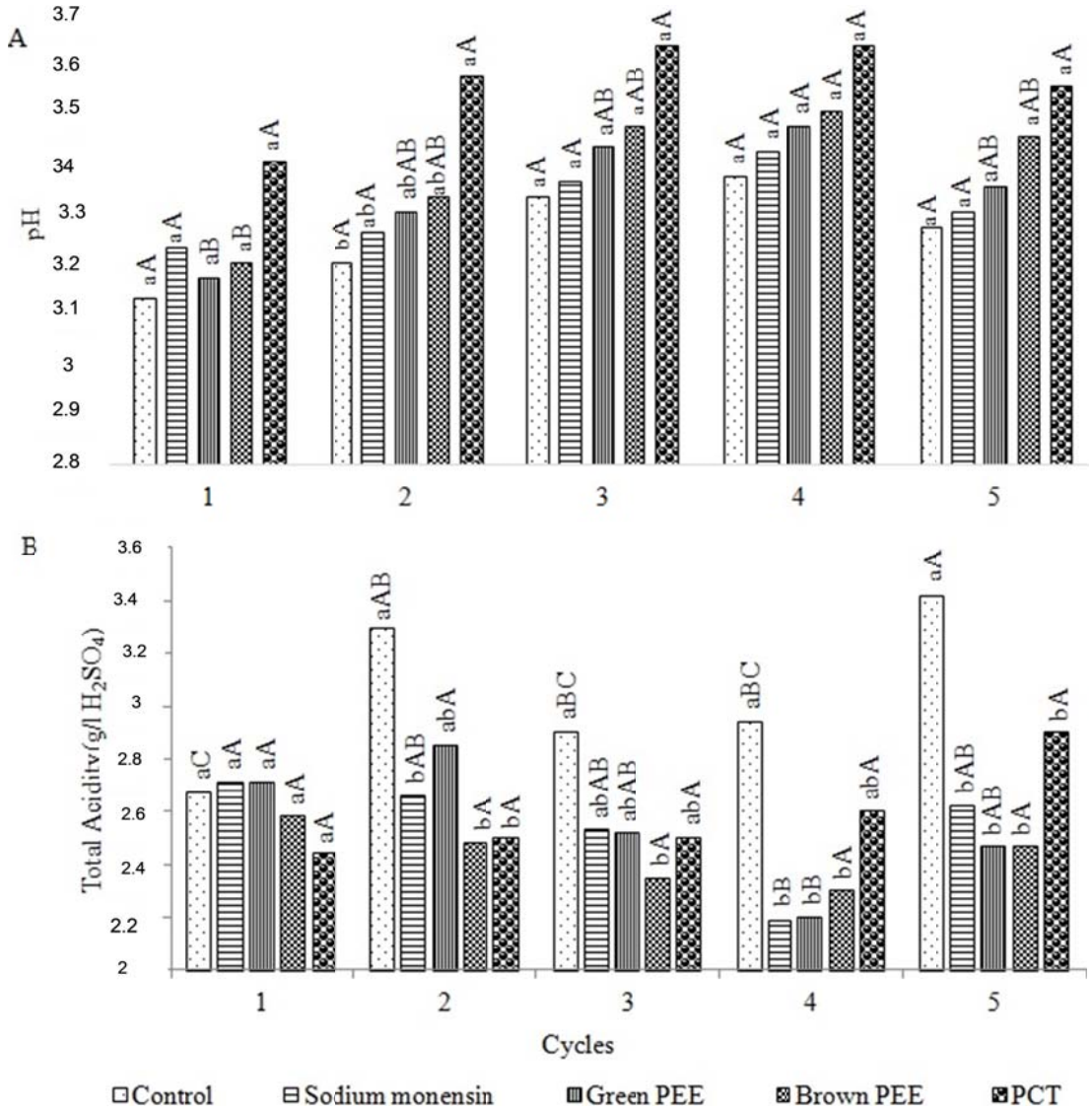

Figure 3. Interaction between must treatments and fermentation cycles for wine pH and total acidity. Jaboticabal-SP-Brazil, 2013/2014 season. Upper case letters compare means of the cycles. Lower case letters compare means of the must treatments in each cycle.

In *S. cerevisiae,* glycerol is produced in the ethanol pathway, as a by-product, competing for NADH, which is why its concentration is inversely proportional to etanol concentration. Therefore, its formation is unfavorable to fermentation process (Wang et al., 2001). Ingledew (1999) reported that up to 1% of glycerol formation is common during ethanol production process.

Ethanol rate produced by yeast CA-11 varied between 6 and 7%, and difference was not statistically significant to all treatments. Similar results were related by Bregagnoli et al. (2009) and Oliveira Filho (2010), who observed values between 6 and 7% in fermentation process using press baker yeast, and Bergamo and Uribe (2013) that used the yeast CA-11.

These results emphasize that propolis has significant antimicrobial activity to control contaminant microorganisms in alcoholic fermentations, and suggest that the use of commercial biocides can be reduced to meet the current world demand for safer food and beverage products.

Conflict of interest

The author(s) have not declared any conflict of interests.

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