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ORIGINAL ARTICLE

Inactivation of *Dekkera bruxellensis* yeasts in wine storage in brand new oak barrels using low electric current technology

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Abstract *Dekkera bruxellensis* is one of the species of yeast, which is most damaging to wine quality, and the tools available to control its growth are limited. In previous studies, non-*Saccharomyces* yeasts and *Dekkera bruxellensis* have been significantly restricted during wine-making processes using an innovative approach based on low electric current treatment (LEC). In the present study, LEC techniques were assessed for their capacity to inhibit wine spoilage by *D. bruxellensis* and to prevent formation of undesirable flavours during storage in oak barrels. Although the effect of SO_2 treatment on *D. bruxellensis* viability and ATP content was more immediate, from the 30th day onward no significant variations between LEC and SO_2 treatments were observed. At the end of the trial, LEC treatment had had a comparable effect to that of SO_2 addition. Acetic acid content was significantly lower after LEC and SO_2 treatments than in untreated wines and volatile phenols were also found to be significantly lower in the LEC treated wine. Moreover, the results from the panel test clearly indicate that no significant differences were found between the LEC and the SO_2 treated wines. These results clearly indicate that LEC technology could represent a viable tool to limit yeast spoilage caused by *D. bruxellensis*. The present work represents, to our knowledge, the first attempt to control *D. bruxellensis* during red wine storage in oak barrels using LEC. The potential industrial applications of LEC technology include the real future possibility of producing a new, marketable range of healthier wines to satisfy the requirements of modern wine consumers.

Keywords Inactivation · *Dekkera bruxellensis* · LEC technology · Wine spoilage yeast · New oak barrels

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Introduction

Brettanomyces yeasts, in particular *Dekkera bruxellensis*, are regarded as spoilage microorganisms with the potential to drastically alter the final outcome of red wine production (Silva et al. 2004; Renouf et al. 2007; Zuehlke et al. 2013;) and to cause relevant financial loss and damage to the reputations of wine producers (Santos et al. 2009; Dai et al. 2010; González-Arenzana et al. 2013).

This yeast is most commonly detected in barrel-aged wines with high pH and low sulphur dioxide content. It may proliferate from only a very few cells in contaminated barrels and can produce sensory consequences such as the generation of malodorous compounds, particularly 4-ethylphenol (Dias et al. 2003; Snowdon et al. 2006). During barrel-ageing and storage of wine, *Brettanomyces*/*Dekkera* yeasts are known to survive in barrels in areas where they are protected from sanitizing treatments such as SO_2 addition. These include the yeast lees, around bung holes, and in the oak structure (Guzzon et al. 2011). The penetrative capacity of the wine serves as a vector, carrying these yeasts up to 8 mm deep into the cracks and crevices of staves, giving the yeast cells a significant degree of protection from SO_2 treatments and allowing colonies to become established (Malfeito-Ferreira et al. 2004).

The survival of these established microbial populations becomes a greater sanitary issue with used barrels as the pores become impregnated or blocked by microbial cells, colour pigments, and other colloidal materials (Lonvaud-Funel and

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Renouf 2005). Barrels are at times impossible to sterilise (Hale et al. 1999; Oelofse et al. 2008).

As far as the effect of SO_2 on *D. bruxellensis* yeast is concerned, studies have yielded inconsistent results: this species is regarded as being either "sensitive" or "resistant" (Garde-Cerdán and Ancin-Azpilicueta 2006). Some authors found this yeast to be sensitive to free SO_2 concentrations exceeding 30 mg/L (Gerbaux et al. 2002), explaining why it may frequently be isolated from wines with low levels of SO_2 (Serpaggi et al. 2012). Others have observed yeast growth even with concentrations of free SO_2 above 30 mg/L, reflecting the resistance of certain *D. bruxellensis* strains (Agnolucci et al. 2010). The heart of this controversy, however, does not lie in the free form of SO_2, but rather in the actual effectiveness of its molecular form (Ribereau-Gayon et al. 2006; Agnolucci et al. 2014), which is dependent on many variations in wine composition, including pH, ethanol content, temperature, anthocyanin levels, and nutrient content (Blomqvist et al. 2012). The effectiveness of molecular SO_2 on a strain of *B. bruxellensis* has also been linked with oxygen availability (du Toi et al. 2005). The authors reported that 0.25 mg/L of molecular SO_2 drastically affected the ability of the strain to form a culture; however, the strain remained viable and increased in numbers after exposure to oxygen. This is of importance especially during racking and transfers throughout barrel ageing.

New alternative strategies to chemical preservation have been proposed such as ultraviolet radiation and the application of pulsed electric fields (Barbosa-Cánovas et al. 2001; Fredericks et al. 2011). More and more research studies are being directed towards achieving inactivation of microorganisms in the food system, using electrical current treatments (Knorr and Heinz 2001; Heinz et al. 2003; Toepfl et al. 2007; Puertolas et al. 2009). Inactivation of bacteria and yeast cells by electrochemical means is now well documented (Ranalli et al. 2002; Valle et al. 2007; Puertolas et al. 2010a, b; Zuehlke et al. 2013). Low electric current technology (LEC) could be included as one of these new alternative strategies. Several studies have been carried out on the effect of LEC treatments on grape must fermentation with no sulfur dioxide (SO_2) addition (Lustrato et al. 2003, 2006). The same authors revealed the significant advantages connected with such treatment and raise the strong possibility of it being a viable alternative to SO_2 addition (Lustrato et al. 2010). The aim of the present work was to assess LEC technology for preventing *D. bruxellensis* spoilage of red wine during storage in brand new oak barrels and to assess the potential of this approach as an alternative to the addition of SO_2. A further aim was to evaluate the sensory characteristics of LEC-treated, barrel stored wines.

Materials and methods

Yeast strain and conditions

Dekkera bruxellensis CBS 4481 (Centraalbureau voor Shimmelcultures, Utrecht, the Netherlands) was used in this investigation. Inocula were prepared as follows: cells from YPD agar plates were inoculated and grown for 72 h in YPD liquid medium containing ethanol at 10 % (v/v) (Vigentini et al. 2008). Cells were then harvested by centrifugation at 3,500 rpm for 15 min at 4 °C, washed once in a solution of 10 g/L peptone and adjusted to a concentration of log 6.0 colony forming units (CFU)/mL in wines. The initial cell concentration in wine was then confirmed by plate counts.

LEC equipment and oak barrels

LEC technology used in this investigation has already been described in detail (Lustrato et al. 2010). Each test was carried out using a pair of MMO (Metal Mixed Oxide, Metakem GmbH, Usingen, Germany) cylindrical electrodes (length 70 cm; diameter, 1.5 cm; current intensity 200 mA with inversion of polarity every 60 s). The energy applied to the samples in each treatment was 1.44 J/Kg, corresponding to 0.3439 cal/h. The values, in amperes and volts, applied in the chamber were measured by a specific probe Testo 175 S1/S2 current/voltage data logger (Testo, Milano, Italy) immersed in the wine samples. The weight of the electrodes was checked at the end of the trial to evaluate any corrosion phenomena and the solubility of metal ions in the wine. Brand new oak barrels were used (Renzi, Modena, Italy). Barrels were 40 L in volume, 60 cm in diameter and 80 cm tall. The oak barrels were modified to permit the introduction of two MMO-coated titanium electrodes, in parallel, at a distance of 50 cm from each other. Three aligned holes were made in the barrels, 25 cm from each other, where the LEC would be applied. The third, central, hole was used for sampling during testing. The barrels were placed horizontally in the cellar and temperature and pH values were monitored (Mettler-Toledo AG Schwerzenbach, Switzerland). The LEC equipment and oak barrels are shown in Fig. 1.

Wine sample and experimental design

To test the effect of low electric current on *Dekkera bruxellensis,* a Montepulciano d'Abruzzo red wine produced in a local winery [Cooperative San Zenone, Montenero di Bisaccia (Italy)], was used with the following properties:

pH 3.48, alcohol 14.00 % (v/v), total acidity 5.40 g/L, volatile acidity 0.55 g/L; total SO_2 50 mg/L, and free sulphites 10 mg/L. The red wine samples were filter-sterilised using a 0.20 μm sheet filter press (Velo mod. Fpc 60 Treviso, Italy)

Fig. 1 LEC equipment and brand new oak barrels used in laboratory conditions

before use. Subsequently, SO_2 was removed from the wine by passing nitrogen through it at room temperature for 24 h. The levels of free sulphur dioxide and SO_2 remaining were 5 mg/L and 0.5 mg/L, respectively. The red wine sample was inoculated with a yeast suspension in order to obtain 10^6 cells/mL and was then distributed into separate 40 L oak barrels in which the different treatments were carried out. Treatments were performed as follows: a control (wine+cells); wine+cells subjected to 200 mA applied current; and wine+cells with supplemented SO_2. In the last case, a 21.7 g/L potassium metabisulfite solution sterilised using a 0.20 μm sterile filter was supplemented in order to obtain 2.1 mg/L mSO_2 [method described by the International Organization of Vine and Wine (O.I.V 2011)]. Furthermore, an oak barrel was used as a 'witness-only' red wine sample, providing a second control, with no microorganisms, no LEC, and no SO_2 addition. The tests were performed for 90 days and took place at cellar temperature (12-15 °C). Three replicates per treatment were carried out, and for each test there was no stirring during the wine experiment. The low electric current treatment was interrupted at 90 days and the effects were monitored until 120 days.

Culturability and activity assay

At 0, 3, 10 days and then every 10 days until the 120th day, 10 mL wine samples were collected, centrifuged, and the pellets were resuspended in peptone water.

Cellular culturability was assessed by plate-counting on a YPD agar medium in Petri dishes. Yeast growth was determined after incubating the plates at 25 °C for five days. Colony counts were expressed as log CFU/mL of wine. All dilutions were performed in triplicate. Cellular activity was evaluated by ATP determination. A portable bioluminometer Biocounter model P 1.500 (Lumac B.V., Landgraaf, The Netherlands) equipped with a photomultiplier tube (PTM) set at 7,200 RLU (Relative Luminose Unit) with 200 pg ATP in 100 μL, was adopted. A Microbial Biomass Test Kit and Standard ATP assay (Celsis-Lumac B.V., Landgraaf, The Netherlands) were used to measure ATP content. Triplicate bioluminescent assays were performed in a Tris-HCl buffer solution (0.025 M; pH 7.75) by adding an adequate dilution of standard ATP to the sample as internal standard (Ranalli et al. 2003).

Microscope observations

Microbial growth and the dynamics of cell survival during the electrochemical treatment of the samples, as well as for the relative controls (no current) were observed by scanning electron microscopy (SEM) (Ranalli et al. 2002). The samples for SEM observation were left to stand overnight in a solution of 2 % glutaraldehyde (0.01 mol/L phosphate buffer), and then immersed in 1 % osmium tetroxide. A microscope operating at 10 kV was used (Zeiss DSM 940A; LEO Elektronenmikroskopie GmbH, Oberkochen, Germany).

Sensory analysis

After four months in barrels, the sensory characteristics of the wines were assessed by a panel made up of 18 judges (7 men and 11 women, from 21 to 45 years of age) who were all members of the Food Science and Technology department of the University of Molise. The panellists were well-trained judges and had previously participated in similar studies. Wines were evaluated using a completely randomised design. Each wine sample was named with a random three-digit code. In order to reduce carry-over effects, the judges could rinse their mouth with mineral water and eat unsalted bread. Four wine samples were tested in each session. The objective of this analysis was to quantitatively describe the general sensory attributes of the wines. Colour intensity, astringency, bouquet, olfactory persistence, off-flavours, and anomalous smells were evaluated, as well as a global evaluation of the taste balance/structure. The intensity of each attribute was rated on a scale of zero to nine. A score of zero indicated that the descriptor was not perceived, while a score of nine indicated high intensity. This exercise was carried out in accordance with International Standards (ISO 8586-1 1993).

Phenolic compound analysis

After four months in barrels, the wines were evaluated for the presence of undesirable off-flavours.

All organic solvents were of analytical, or superior, grades and were purchased from Carlo Erba (Rodano, MI, Italy), unless otherwise indicated. Pure phenolic compounds (4-ethylguaiacol, 4-ethylphenol, ferulic and p-coumaric acids) were purchased from Sigma–Aldrich Co. (St.Louis, MO, USA).

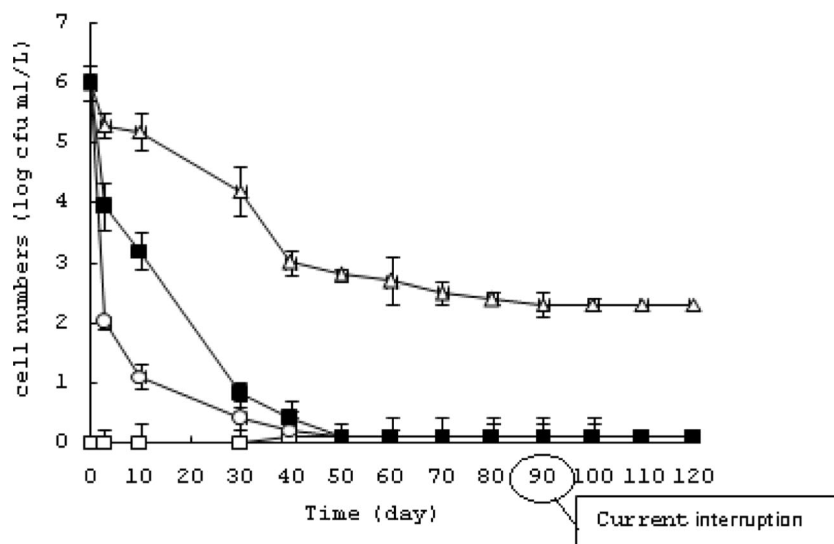

Fig. 2 *Dekkera bruxellensis* CBS 4481 population in Montepulciano d'Abruzzo red wine in oak barrels at different times and for different treatments: (□) witness (only wine); (Δ) control (wine+cells); (○) wine+cells+SO_2 (2.1 mg/L mSO_2); and (■) wine+cells+LEC at 200 mA

Before HPLC analysis, each sample was diluted with absolute methanol (1:3, v/v) and filtered through a 0.45 μm PVDF syringe filter.

All HPLC determinations were performed by a Varian, Mulgrave, HPLC system Model ProStar 230 AUS equipped with a column purchased from Kinetex 2.6 u PFP 100× 4.6 mm Phenomenex (USA), and supplied with a UV–VIS detector set up at a wavelength of 315 nm. The isocratic mobile phase was eluted at a 1 mL/min flow rate and was composed of methanol and 1 % acetic acid 55:45 (v/v). The detection limit for volatile phenol analysis was estimated to be 0.05 mg/L.

Statistical analysis

All data are expressed by means of three replications and standard deviation (± SD), submitted for statistical analyses (ANOVA); significance was defined as $P<0.05$. The SAS statistical software package (1997) and CoStat-Statistics Software version 6.3 program (www.cohort.com/costat.html) were adopted.

Results and discussion

Dekkera bruxellensis *population and ATP content*

Figure 2 shows the inactivation over time of *D. bruxellensis* in wine storage in oak barrels undergoing different treatments: control (wine+cells); wine+cells+LEC at 200 mA; wine+cells+SO_2 (2.1, mg/L mSO_2); and 'witness' (only wine).

Results showed that at the end of the trial there were no significant variations ($P<0.05$) between the two treatments (LEC treatment and the addition of SO_2) in the viable cell count of

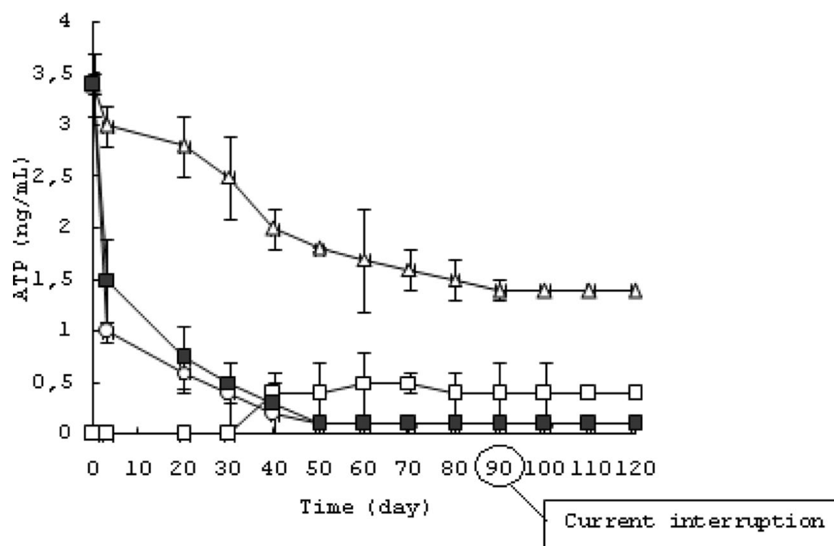

Fig. 3 Mean ATP content in Montepulciano d'Abruzzo red wine in new oak barrels at different times and for different treatments: (□) witness (only wine); (Δ) control test (wine+cells); (○) wine+cells+(2.1 mg/L mSO_2);(■) wine+cells+LEC at 200 mA

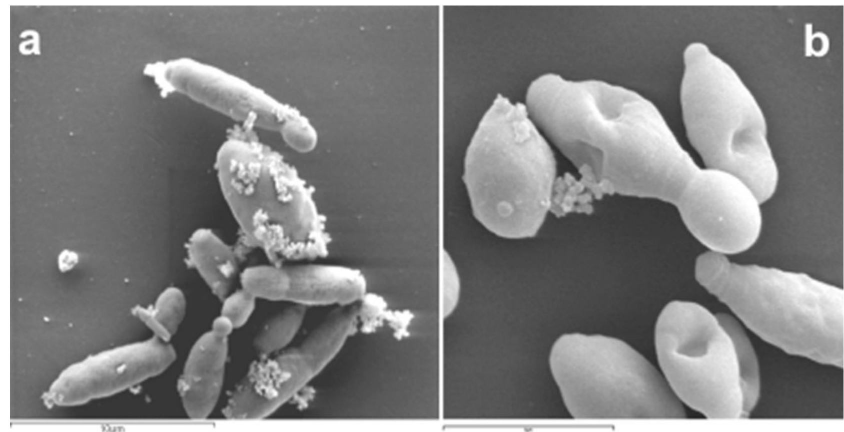

Fig. 4 Scanning electron microscope observation of *Brettanomyces bruxellensis* CBS 4481. Morphological changes are visible and are probably due to the 200 mA electric current application using MMO electrodes. (**a**) Untreated (wine+cells); (**b**) Low electric current, LEC-treated (120 days)

D. bruxellensis CBS 4481, as both LEC treatment and SO_2 addition caused comparable growth inactivation of *D. bruxellensis*.

However, from the first three days until the 20th day, SO_2 addition caused a significantly ($P<0.05$) higher inactivation of *D. bruxellensis* than the LEC treatment. Rapid antimicrobial activity of sulfur dioxide on *D. bruxellensis* has been described by du Toi et al. (2005). These authors reported decreased SO_2 antimicrobial activity of up to 30 % in a synthetic wine medium supplemented with free anthocyanins. Under the conditions present in red wine, more than 70 % of the SO_2 is combined with free anthocyanins, thus decreasing the mSO_2 level (Usseglio-Tomasset 1992).

From the 30th day of the study onward, the two treatments appear to have had a comparable effect on yeast viability, and differences in *D. bruxellensis* populations in the two test samples were not statistically significant ($P<0.05$). This remained the case even after the LEC treatment had been interrupted at the 90th day. From the 60th day onwards the level of the total viable cells in both treatment test samples (SO_2 supplemented and LEC) resulted in a 3 log of magnitude less than that found in the relative control test (wine+cells) (Fig. 2).

The total ATP content of *D. bruxellensis* CBS 4481 (Fig. 3) shows trends similar to that of the population counts. At the end of the trial, there were not significant differences ($P<0.05$) between the LEC treatment and the SO_2 addition in terms of ATP content, even after the LEC treatment had been concluded (at the 90th day). LEC treatment demonstrated inhibitory activity comparable to that of SO_2 addition. Results obtained in this investigation confirm previous observations. On a laboratory scale, 200 mA LEC treatments have been demonstrated to reduce ATP content and the activity of *D. bruxellensis* cultures (Lustrato et al. 2010).

At the end of the experiment, after stopping LEC treatment at the 90th day, SEM observations clearly showed (Fig. 4) alterations to *D. bruxellensis* CBS 4481 morphology and integrity that were probably due to LEC treatment that may have caused the rupturing of membranes with a consequent loss of cell organization. However, further investigation is required to verify this hypothesis.

Many other studies have also reported that treatment with low intensity current causes an irreversible loss of membrane function, such as in the semipermeable barrier between the

Table 1 Oenological parameters of Montepulciano d'Abruzzo wines under different treatments, after 120 days in oak barrels

Wine parameter	a) Control (wine+cells)	b) Wine+cells + SO_2 (2.1 mg/L mSO_2)	c) Wine+cells + LEC (200 mA)	d) Witness (only wine)
Ethanol (% v/v)	14.0±0.2 a	14.0±0.2 a	14.0±0.2 a	14.0±0.2 a
pH	3.2±0.04 a	3.2±0.4 a	3.2±0.4 a	3.2±0.4 a
Titratable acidity (g/L)*	6.2±0.03 b	5.4±0.3 a	5.4±0.3 a	5.6±0.3 a
Volatile acidity (g/L)†	0.6±0.01 c	0.1±0.2 a	0.2±0.2 a	0.45±0.01 b
Reducing sugar (g/L)	1.55±0.1 a	1.55±0.1 a	1.55±0.1 a	1.55±0.1 a

All parameters are given with their standard deviation ($n=3$)

* Expressed as tartaric acid.

† Expressed as acetic acid.

Values in same row followed by the same letter are not statistically different (P >0.05).

Table 2 *Dekkera bruxellensis* CBS 4481 production of volatile phenols in wine after 120 days in new oak barrels under differing experimental conditions: a) control (wine+cells); b) wine+cells with supplemented SO_2 (2.1 mg/L mSO_2); c) wine+cells+LEC 200 mA; and d) witness (only wine)

Oak barrel treatment	Concentration (mg/L)			
	p-Coumaric acid	Ferulic acid	4-Ethyl-phenol	4-Ethyl-guaiacol
a) Control (wine+cells)	0.35±0.018 a	0.12±0.006 a	0.18±0.009 b	0.15±0.008 b
b) Wine+cells+SO_2 (2.1 mg/L mSO_2)	0.57±0.029 c	0.18±0.009 b	0.16±0.008 a	0.11±0.006 a
c) Wine+cells+LEC (200 mA)	0.45±0.035 b	0.22±0.020 c	0.00±0.00	0.00±0.00
d) Witness (only wine)	1.90±0.098 d	0.42±0.021 d	0.00±0.00	0.00±0.00

All parameters are given with their standard deviation (n=3).

Values in same column followed by different letter are statistically different (P<0.05).

yeast cell and its environment (Valle et al. 2007; Puertolas et al. 2009, 2010a, b).

The temperature profile during and at the end of all the experiments was monitored. No variations were recorded in the time interval between the control set and the electrical treatment; the temperature of the wine during LEC treatment was 18 °C. In order to verify electrode stability and suitability for applications in the food industry, the weight of the electrodes was measured at the beginning and at the end of the trial, and no weight differences were found.

Chemical characteristics of the wines and volatile phenol production

Table 1 shows the main chemical characteristics of the wines subjected to the different treatments. After 120 days, both SO_2 addition and LEC treatment trials did not show significant variations (P<0.05) of wine pH (3.2) and acetic acid content(0.1±0.2 and 0.2±0.2 g/L, respectively). However the SO_2 and the LEC treatments showed a significantly (P<0.05) lower amount of acetic acid than the witness (only wine) and the control (wine+cells) did (0.6±0.2 and 1.2± 0.01 g/L, respectively). This was probably due to the inhibitory effect of the LEC treatment on *D. bruxellensis*, confirming the results of the microbiological monitoring. The volatile acidity of the witness (only wine) was significantly higher than the wines treated with SO_2 and LEC, but significantly lower than the control test (wine+cells); this phenomenon may be related to the fact that wine samples stored in oak barrels, at room temperature, can undergo spontaneous oxidation and uncontrolled alterations. Wine storage in barrels is characterized by the slow, continuous penetration of oxygen through the wood, causing the oxidation of the wine constituents, particularly polyphenols, and subsequent changes in astringency, color, and taste (González-Rompinelli et al. 2013). The increase of the volatile acidity of the witness wine, with the consequent oxidation, is due to microbial alteration caused by acetic acid bacteria as spontaneous post alcoholic fermentation. This aspect, although is not the focus of the work, was evident and supported by the ATP responses where the increased content was recorded between the 40th day to the 120th day, in the witness wine. Moreover, the average ATP content of one viable bacterial cell, as acetic acid bacteria, is 100-200 times less in the ATP content of one yeast viable cell (Ranalli et al. 2003).

Hydroxycinnamic acids (*p*-coumaric acid and ferulic acid) and volatile phenols (4-vinyl-phenol, 4-vinyl-guaiacol, 4-ethyl-phenol and 4-ethyl-guaiacol) were measured through HPLC analyses. The kinetics of off-flavour production, under various conditions, until 120 days, is shown in Table 2.

At the beginning of the experiment, the wine contained 1.96±0.10 mg/L of *p*-coumaric acid and 0.41±0.02 mg/L of ferulic acid.

In our conditions, 4-vinyl derivates were not detected, while quantifiable amounts of 4-ethyl-phenol and 4-ethyl-guaiacol accumulated in the wine over a period of 120 days.

As a consequence, 4-ethyl-derivates were produced in moderate quantities throughout tests (a) and (b), whereas they were not found in (c) and (d). Therefore, LEC treatment was useful to avoid 'off-flavour' production.

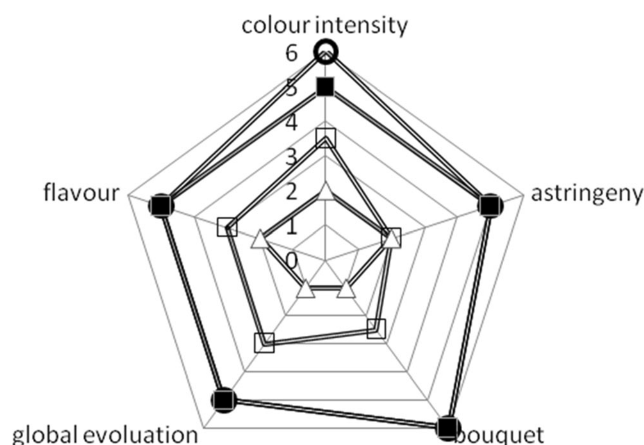

Fig. 5 Spider diagram of the sensory scores of Montepulciano d'Abruzzo wines stored in oak barrels after 120 days under different treatments.(□) witness (only wine); (Δ) control (wine+cells); (○) wine+cells+SO_2 (2.1 mg/L mSO_2); and (■) wine+cells+LEC at 200 mA.

Several papers have reported the ability of *D. bruxellensis* to produce 4-ethylphenol (Chatonnet et al. 1995; Suárez et al. 2007b). Our results have confirmed those observations. The present study suggests the importance of applying appropriate LEC treatment that limits wine deterioration in terms of off-flavour synthesis. Moreover, the determination of volatile phenol accumulation confirms that, under the conditions tested, the use of an electric field is sufficient to inhibit yeast spoilage (Suárez et al. 2007a; Puertolas et al. 2010a, b).

Sensorial characteristics of the wines

At the end of the experiment, SO_2-supplemented wine samples were compared with LEC-treated samples to ascertain whether or not any differences were found in sensory profiles after four months in the new oak barrels. Results of this analysis are reported in the spider diagram in Fig. 5.

One visual attribute (colour intensity), three mouth-feel attributes (astringency, bouquet and olfactory persistence, off-flavours, and anomalous smells) and a global evaluation like taste balance/structure of the wine were considered. No significant differences were observed between the mean values of the parameters considered for the characterisation of sensory profiles. However LEC-treated wine showed a higher mean score for taste and astringency than could be explained by the higher concentration of total phenols and tannins in this wine compared to the control. The mean score of the panel test for colour intensity was almost identical for both wines. Probably no differences in perception of colour intensity were found because the human eye is not able to detect very minor differences among samples. In summary, the results of the panel test indicated that the judges did not detect any strange tastes or off-flavours that could be caused by the LEC treatment and that the LEC-treated wine was similar to the control wine from a sensory perspective.

Conclusions

In the present work the validity of the use of LEC technology to effectively control yeast-spoilage during wine storage in brand new oak barrels has been confirmed; nevertheless, it represents, to our knowledge, the first attempt to control *D. bruxellensis* during red wine storage in new oak barrels. This may help to prevent the appearance of organoleptic anomalies in wines at the winery level. The potential industrial applications of LEC technology include a future opportunity to produce healthier, innovative wines satisfying the requirements of the modern wine consumer. From a practical perspective, further research is required into the occurrence and variability of *D. bruxellensis* in oak barrels and wineries and into the effects of LEC treatment on the sensorial properties of different wines. This would permit the technology to be expanded from the pilot scheme and to be used in production on the winery scale.

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