Controlling *B. bruxellensis* with Pulsed Electric Fields: Optimization of industrial protocols and impact on the wine profile

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Abstract. Pulsed electric field application for microbial inactivation of wine spoilage microorganisms has recently become a point of interest among scientific and industry peers. *B. bruxellensis* is considered one of the most undesirable spoilage yeast in wines. Thus, this assay has the objective of contributing to better understanding the effect of PEF, not only on the inactivation of *B. bruxellensis* and impact on Total Yeast population, but also on physico-chemical and sensorial quality. 2 sets of red wine were subjected to a 15 kV/cm and 35 kJ/Kg PEF treatment at pilot-plant scale (240 L/h, 4 bar); one wine was naturally contaminated with *B. bruxellensis*, being used for microbial assessment, while the wine deemed free of contamination was subjected to physico-chemical and sensorial analysis prior and after the application of PEF. *B. bruxellensis* was effectively inactivated using a conservative PEF treatment of 15 kV/cm and 35 kJ/kg, resulting in a reduction from 2.467 x 10^3 viable cells/mL to below the detection limit of <150 viable cells/mK; Total Yeast Count decreased 80,66%. The treatment posed a ΔT = +8,5 ºC. Sensorial analysis concluded no significative differences. Small, but significant differences were found at physico-chemical level.

Being found in various food matrices, such as beer, cider and wine, the yeast of the Dekkera/Brettanomyces genus can either benefit or negatively impact the product [1], [2]. *B. bruxellensis* is mostly undesirable in the wine industry, being considered a spoilage yeast, mainly due to its ability to convert hydroxycinnamic acids (p-coumaric and ferulic) in volatile phenols (e.g., 4-ethylguaiacol and 4-ethylphenol) which attributes *off-flavour* taints described as "horsey", "barnyard", "bandaid", "pharmaceutical", or "smokey" [3], leading to a loss of fruity and varietal flavours. In addition, it is also capable of producing acetic acid, decanoid acids ("soap") and tetrahydropyridines ("mousy flavour") [4,5] Besides, it owns an extreme capacity of survival, considering that wine characteristics (presence of ethanol and compounds with antimicrobial activity such as polyphenols and low pH) present as an adverse environment for microbial growth [6].

There are two crucial stages during which *B. Bruxellensis* can thrive: a) the period between the end of Alcoholic Fermentation (AF) and the beginning of Malolatic fermentation (MLF), considering lack of competition from other microorganisms and nutrient availability [7], and b) aging, especially if the use of oak barrels or reincorporation of lees is considered [4]. Thus, *B. bruxellensis* poses as a serious economic threat to wineries worldwide.

One of the major problems of this organism is that, even at latent populations of 10^2-10^3 CFU/mL, over time it is as prejudicial as higher levels of contamination (evidence shows that it can reach up to 10^6 CFU/mL after AF) [4]. Therefore, it is essential to inactivate or reduce the population of *B.bruxellensis* once found.

Traditionally, *B. bruxellensis* control is based on physical methods, such as racking, filtration (>1µm) and heat treatments, or chemical, namely the use of additives or conservatives, e.g. fining agents and SO_2, being the latter already advised by Pasteur in 1866 [4,8]. Nonetheless, these procedures may not be entirely effective or could potentially have a negative impact on the sensory profile of the wine [9]. For instance, SO_2 presented some difficulties regarding the control of this specific yeast , requiring relatively high concentrations of Free SO_2: Barata et al., 2008 demonstrate that, for dry red wines in aging oak barrels, the development of *B. bruxellensi*s could only be prevented with a concentration of molecular SO2 of 1 mg/L (Free SO2: 40 mg/L); Licker et al., 1998, stipulated that threshold at 0.625 mg/L. [9,10]. In spite of this, it is crucial to keep in mind that, in EU, the maximum concentration of total SO2 is

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150 mg/L for red wines and 200 mg/L for white and rosé wines <5 g/L of reducible sugar, raising those values to 250-400 mg/L depending on the sugar content and specific protected designations (Comission Regulation (EC) N. 606/2009 of 10 July, 2009). For organic wines, these limits decrease to 100 and 150mg/L, for red and white wines (<2 mg/L of sugars), respectively, while all the others shall suffer a reduction of 30 mg/L (Comission Implementing Regulation (EU) N. 203/2012 of 8 March, 2012). Hence, it is imperative to maintain proper management and control of the sulphur content added over time.

More recently, the use of dimethyldicarbonate (DMDC) under the commercial name Velcorin®, as a conservative due to its antimicrobial properties capable of even stabilizing wines with high residual sugar content; however it possesses some issues, more specifically, the fact of being temporary, rapidly hydrolysing into CO_2 and methanol, which makes it necessary to do regular additions [11,12]. Mainly due to the toxicity of methanol, the application of DMDC is highly regulated; in EU the maximum permitted level is 200 mg/L, authorized only prior to bottling and to be used in wines only if they possess >5 g/L of fermentable sugars, having to guarantee that methanol content is monitored and maintained bellow DL before entering market (Commission Regulation (EC) No 643/2006 of 27 April, 2006).

Recently, consumers have demonstrated an increased awareness of sustainable and healthier product consumption, prompting producers to adopt more holistic approaches and follow organic, biodynamic, and low-intervention trends. This reflects their growing concern about additives, with sulfites use reduction being one of the most closely regarded, due to possible allergic responses in some consumers [13,14]. Thus, in later years, newer preventive and curative strategies for microbial control were proposed as an alternative, such as the use of ozone, electrolyzed water, chitosan, mycotoxins, high pressure processing (HPP), ultrasounds (US), and among them, Pulsed Electric Fields (PEF) [13-17].

PEF technology relies on a physical induced phenomena named electroporation, where the cell membrane is permeabilized by the action of the electric field, either by originating transient or permanent pores [20,21]. The pulse characteristic of the process reduces dramatically the thermal load in comparison with the traditional DC process. Depending on several factors, such as pulse parameters, electrodes & generators types, and characteristics of both cell and medium, reflect different outcomes that can vary between electrical stimulation, permeabilization of cell membranes and cell lysis [23]. This versatility contributes to the many PEF applications contemplated for the wine industry.

While studies regarding the application of PEF for mass transfer, with implication in maceration processes were on focus of the scientific community over the last decades, being the corollary for the inclusion of PEF in the *International Code of Oenological Practices* (Resolution OIV-OENO 634-2020), approving its use for the optimized extraction of valuable compounds and reduce maceration periods in wine [23,24], its capacity to inactivate spoilage microorganisms in wine only recently gained the interest of both academic and industry communities.

Several studies were performed regarding the inactivation of pathogenic and spoilage microorganisms to contribute to the preservation of fruit juices, such as apple and orange, and other liquid foods over the last decades, demonstrating its effectivity and no significant impact in the physicochemical properties of the product [26,28], contributing to the implementation of this technology for the production of commercial fruit juices. Several assays were performed to assess the viability of microbial inactivation of common spoilage yeast and bacteria in wine with the application of PEF. Puértolas et al. (2009), demonstrated the capacity to inactivate Dekkera/Brettanomyces yeasts and Lactobacillus bacteria determining an optimal protocol of 29 kV/cm and 186 kJ/kg, for which 99.9% of spoilage flora was neutralized [19]. Furthermore, he concluded that lactic bacteria displayed greater resistance to PEF treatment when compared *to D. bruxellensis*. This finding is consistent with the theory that larger cells require lower specific energy (*E*) to undergo electroporation [29]. Delsart et al. 2016 achieved total inactivation of *B. bruxellensis* with E=20 kV/cm and Ws = 320 kJ/kg [30]. Both assays were performed resorting to batch treatment chambers with linear parallel plate electrodes (P2P: Plate-to-Plate).

For the wine industry, one of the most important characteristics regarding PEF is the capacity of working in a continuous flow. As far as we could determine from our review of literature, few studies were realized regarding this. With a continuous flow of 12 L/h, González-Arenzana et al. 2019, obtained positive results with a PEF treatment of 23 kV/cm and 95 kJ/Kg, prolonging the shelf life of wine before bottling [31]. The same team also compared wines treated with PEF (33 kV/cm;158 kJ/kg), SO2 (30 mg/L), and a synergy of PEF+15 mg/l SO2 and concluded that PEF presents itself as a good alternative to SO2, given the effect on microbial population. Moreover, it did not only avoid the raise of volatile acidity, but also promoted higher colour intensity; however, when subjected to sensorial analysis, the highest scores were attributed to the combination of PEF+15 mg/L SO2[32]. Delso et al. 2023, performed a comparative study regarding several PEF protocols (10 L/h, 15 kV/cm; 39-120 kJ/kg) obtaining similar results on wine microbiota; similarly to González-Arenzana, they also demonstrated that PEF effect can be optimized by the content of Free SO_2 post treatment, due to its ability to avoid the recovery of sublethal injuries on microbial cells [33].

The above results, obtained by the application of lower electric field strengths are very promising, and in terms contribute to reduce cost and complexity of PEF commercial equipment, when thinking about the scalability for industry use.

Nevertheless, these studies were conducted at generally low flow rates, at laboratory scale; therefore, a need for assays performed with higher treatment flow rates arises. With this paper, we hope to contribute to a better understanding of the potential of the application of

this technology and optimization of the inactivation of undesired microbiota, specifically *B. bruxellensis* and other spoilage yeasts, by using energy efficient protocols capable of being applied by commercially available PEF units and easy to implement in wineries. For such, with this paper we intend to contribute not only to the optimization of a PEF protocol capable of significantly reducing *B. bruxellensis* and total yeast population, but also to assess its impact on the physico-chemical and sensorial properties of the wine subjected to the PEF treatment, at pilot-plant scale (240 L/h).

With that in mind, a PEF protocol of 15 kV/cm and 35 kJ/kg was applied for inactivation, and the same protocol was used and efficient PEF protocols to assess potential interferences of the application of this technology in wine to assess potential interferences of the application of this technology in wine.

2 Material and methods

2.1 Wine & PEF

Red wine vinified with grapes of Dão Region provenience was selected to perform this assay, being one vat naturally contaminated with *B. Bruxellensis*, while another vat was deemed "healthy". Microbial inactivation assessment was performed on wine originally from the contaminated tank, while physico-chemical and sensory analysis were conducted in the non-contaminated replica. Both vats were subjected to the same treatment conditions. This is mainly due to the fact that volatile phenols were produced by *B. Bruxellensis* and might interfere in this evaluation. The viability of the total yeast and *B. bruxellensis* population was previously assessed by flow cytometry with fluorescent *in situ* hybridization (RNA-FISH). Samples were collected in triplicate to sterile sample containers and shipped to an independent laboratory for microbial assessment.

The PEF system used is able to process up to 500L/h up to 6 bar, and is comprised of DN25 piping, pump, flowmeter, heat exchangers, and a high-voltage solid-state Marx generator with 15 kV/400A and 6 MW (EPULSUS® IBM3B-15, EnergyPulse Systems, Lisboa), able to deliver almost perfectly square monopolar and bipolar shaped pulses through a continuous co-axial treatment chamber with a distance between electrodes of d=1 cm. The main parameters to consider when applying a PEF treatment are the electrical field strength E, (kV/cm), being calculated by

$$E = \frac{U}{d} \quad (1),$$

where U is the pulse voltage applied in kV, and d is the distance between the electrodes of the co-linear chamber, and specific energy Ws (kJ/kg) being calculated by

$$W_s = \frac{W_t}{m}, \quad (2)$$

with m being the mass of material treated. In this case it's considered 1kg for every liter of wine. W_t represents the total applied energy, in J, that can be determined by the following equation:

$$W_t = UIt_{on}N\ , \quad (3)$$

where I, is the pulse current amplitude in A, t_{on} is the pulse width, in µs, and N the number of applied pulses. Temperature variation, in ºC, is also easy to assess by applying the following formula:

$$\Delta T = \frac{W_s}{C_p}, \quad (4)$$

being Cp the specific heat capacity of a material which, for wine, is regarded as ~4.3 kJ/(L.°C) [34, 35].

In our assay, the increase in temperature was previously calculated based on equation (4): ΔT= +8,5 ºC. This was posteriorly confirmed by direct measurement immediately after the treatment ($T_{initial}$: 17 ºC, T_{end}: 25.5 ºC). Furthermore, considering that Ws is directly related not only to temperature input but also to the process' energetic sustainability, it is essential to assess the optimization of an efficient PEF protocol with the lowest supply of specific energy. Delsart et al. (2016) demonstrated a relation between the increase of specific energy applied and an higher capacity of microbial inactivation [30]. Based on the results of several authors, mainly regarding the studies previously mentioned performed by Delso et al. (2023) (15 kV/cm; 39-120 kJ/kg) and González-Arenzana (23 kV/cm; 95 Kj/Kg) with successful results. However, both studies work at laboratory scale, working with low volumes (respectively 10 l/h and 12 l/h). Considering that, to the best of our knowledge, there are currently no papers published at pilot-plant or industrial scale, we hope to contribute to a better understanding of the process, while also contributing to already existing research regarding the impacts of a PEF treatment with an objective of microbial control in oenological and sensory parameters. Thus, in this paper, we contemplate one single protocol of 15 kV/cm and 35 kJ/kg (1500 V, 10 µs, 150 Hz) square wave bipolar pulses, applied at a flow rate of 240 L/h at a regulated pressure of 4 bar.

2.2 Determination *B. bruxellensis* and Total Yeast Population

Detection of viable *B. bruxellensis* was realized by an independent laboratory (Excell Ibérica, SL) with flow cytometry equipped fluorescent *in situ* hybridization (RNA-FISH), accredited by ISO 17025, with a Detection Limit (DL) of 150 viable cells/ml. Total Yeast Count was also determined in the same facilities, by flow cytometry.

2.3 Oenological Parameters Assessment

Typical oenological parameters were determined prior and post PEF treatment, to assess possible alterations caused by the treatment. Every parameter was analysed in triplicate.

pH was assessed by potentiometry, with the aid of a SesION+ pH31 benchtop pH meter (Hach, Loveland, USA). Total Acidity (TA) was by the determined protocol OIV-MA-AS313-01 stipulated in *Compendium of International Methods of Analysis*, being the results

expressed in grams of tartaric acid/L. Turbidity (T), was determined with 2100Q portable turbidimeter. (Hach, Loveland, USA), expressed by nephelometric turbidity units (NTU). Conductivity, presented in mS/cm, was measured with a portable conductivity meter (HI98304 DiST®4, Hanna Instruments, USA). Colour Intensity (CI), Tonality (TON), yellow colour compounds (%Ye), Anthocyanin content (ANT), and Total Phenols (TP) were monitored by spectrophotometry (U-2900 Spectrophotometer, Hitachi, Japan). Total and Free SO2 were determined using an automatic titrator (Titromatic KF, Crison Instruments, Allela, Spain). Total Dry Extract (TDE) and Volatile Acidity (VA) were assessed according to the procedure established by, respectively, OIV Method OIV-MA-AS2-03B and OIV (Method OIV-MA-AS313-02).

2.4 Sensory analysis

Two samples of the treated wines, being Control and PEF, underwent sensorial analysis through a wine tasting panel constituted by 8 trained individuals, allowing the detection of possible impacts and differences caused by the different PEF treatment protocols to which wine was subjected. The preference of the panellists regarding both wines was also assessed.

2.5 Statistical analysis

Data analysis, including descriptive statistics, was performed using IBM SPSS Statistics, Version 28.0.1.0 (SPSS Inc., Chicago, USA), with a statistical significance level of $\alpha = 0.05$. Microsoft Excel 2016 (Microsoft Corporation, Washington, USA) and Graphpad Prism 8.0.2 (Graphpad Software, San Diego, California) was used to create graphic representation of data.

3 Results and Discussion

3.1 *B. bruxellensis* and Total Yeast Population

As previously mentioned, regarding microbial analysis, samples were collected prior and post PEF treatment, in triplicate. The results obtained are displayed on Table 1 and Fig. 1.

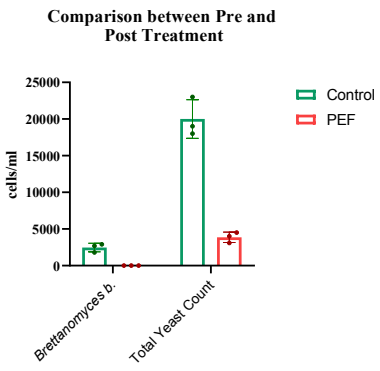

Figure 1. Total Yeast and *B. bruxellensis* inactivation results.

Table 1. Microbial Results.

	B. Bruxellensis (viable cell/ml)	**Total yeast** (viable cells/ml)
Control	2467 ± 586	20000 ± 2645
PEF	<150	3866 ± 709

Initially, *B. bruxellensis* represented 12% of the Total Yeast Count present in the wine sample, with a population of 2.467×10^3, in a universe of 2.0×10^4 viable yeast cells/ml. After treatment, *B. bruxellensis* population was undetected in the wine sample (DL = <150 viable cells/ml). A reduction of 80.66% of the viable Total Yeast Cells was also assessed. These results are in consonance with the conclusions described by Delsart et al. (2016), that observed a acceptable inactivation rate of *B. bruxellensis* and other microorganisms with a treatment of 20kV/cm and 320 kJ/Kg [30]. Delso et al. (2023), perfomed a study regarding the inactivation of another yeast species, Saccharomyces cerevisae, achieving up to 4.0 Log_{10} cycles of inactivation with a conservative PEF treatment of 15 kV/cm, 84.5-155.6 kJ/Kg [33].

3.2 Oenological parameters

The analytical results are presented in the Table 2. As observed in the table, pH did not present any differences amongst subjects. The same was concluded for TA (H=2.463, df=2, p=0.292). This result is in conformity to the ones presented by Abca & Evrendilek, 2014, which applied PEF treatments up to 31kV/cm to several wines [36]. Delsart et al. (2016) obtained similar results for pH, but reported significant differences regarding AT [30]. Ethanol content (%v/v) suffered a significant reduction in the PEF treated sample ($F(2,6)=3818$, $p<0.001$). Yet, it is important to mention that this difference falls within the margin of error for the method used. Regarding Turbidity (T), the differences between the two samples were statistically significative (H=9.00, df=2, p=0.011); however, regarding practical terms, it is believed that this difference causes little impact on the winemaking. Conductivity also displayed a subtle raise of 0.01 mS/cm. This result can be associated with the increase of the wine content in suspended particles assessed by T. Anthocyanins (ANT), Total Phenols (TP) and Colour Intensity (CI), both presented a significant increase in wine treated with PEF (p=0.043; p=0.011; p=0.043), while Tonality (TON) and %Ye remained equal amongst wines (p=0.165; p=0.165). While this increase is inconsistent with the results obtained by Abca & Evrendilek, 2014, reporting no significant changes in these parameters [36] curiously, Delsart et al, (2016) also reported a slight increase in the same parameters [30].

Total SO_2 content presented no significant alterations (p=0.296), Free SO_2 had a significant concentration reduction (p=0.043), nevertheless it was within the method's error. Total Dry Extract (TDE) and Volatile Acidity (VA) both presented small but significant differences (p=0.029; p=0.011).

Table 2. Physico-chemical parameters of Control and PEF treated wines.

	Control	PEF
pH	3.73 ± 0.00	3.73 ± 0.00
Total Acidity (g/L)	5.09 ±0.01	5.05 ±0.01
Ethanol (%v/v)	11.37±0.00	11.12±0.00
Conductivity (mS/cm)	2.61	2.62
Turbidity (NTU)	8.58 ± 0.01	8.73 ± 0.01
Anthocyanins (mg/L)	315.7±0.6	323.9±0.4
Total Phenols (u. a.)	41.2± 0.0	42.2± 0.0
Colour Intensity (u. a.)	7.872±0.165	8.021± 0.032
Tonality (u. a.)	0.615±0.002	0.614±0.003
%Yellow (%)	3.37±0.03	3.37±0.03
Free SO2 (mg/L)	17.0±0.0	14.0±0.0
Total SO2 (mg/L)	31.0±2.1	27.7±1.8
Total Dry Extract (g/L)	22.7±0.0	22.4±0.0
Volatile Ac. (g/L)	0.62±0.07	0.63±0.07

3.3 Sensory analysis

Several Parameters were selected to be evaluated, on a scale of 0-5 on this study. Visual (Clarity, Colour Intensity), aromatic (Freshness, Intensity, Metallic, Fruity, Spices) and other characteristics, such as flavour (Sweetness, Acidity, Intensity, Balance, Persistence), and textures (body and astringency). Figure 2 illustrates a comparison between the sensory results obtained.

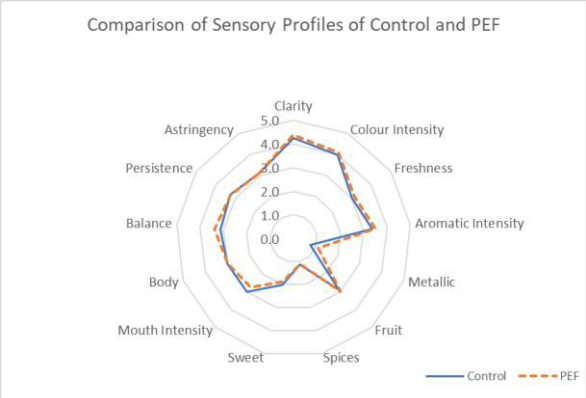

Figure 2. Sensory profiles of Control and PEF treated wine.

Statistically significant differences were not found (p<0.05) amongst the parameters understudy. Few papers published regarding this matter exist in literature; Abca & Evrendilek (2014), also perfomed an assessment of the impact of a PEF inactivation protocol on wines, presenting similar results [36]. Delso et al, (2023) performed a comparative study between the sensory profile of wines subjected to sterilizing filtration and PEF (15 kV/cm, 84.5 and 155.6 kJ/Kg) and also reached to the same conclusions. In terms of global evaluation, it was asked to the panellists to attribute a score on a scale of 0 to 20. Control scored 14.50±2.20 points, while PEF was evaluated with a score of 15.38±2,13, being the differences not statistically significative (H=1.134, df=2, p=0.567). It is also important to notice that 4 in 8 panellists attributed an higher score to PEF treated wine, in comparison to Control treatment protocol, while 3 in 8 conferred similar scores to both wines.

4 Conclusions

Our results show that a conservative PEF protocol might be applied to provide a better control of the microbiota presented in wines, while not significantly interfering on the sensorial characteristics of the final product, In physico-chemical terms, while some results present significant increase regarding control samples, such as Anthocyanin content, Turbidity, Colour Intensity and Total Phenols; however, from a practical perspective these differences might be not relevant in winemaking contextualization, even more considering the null impact on sensory quality.

Given the results obtained, it became important to assess the potential differences in terms of energetic consumption and operational costs. The operational cost of the PEF treatment considered (15 kV/cm; 35 kJ/kg), is 0.0016 €/bottle, considering a rate of 0.22 €/KWh. For example, a traditional *Brettanomyces/Dekkera* inactivation method, the application of DMDC, currently pose an estimated cost of >0.05 €/bottle [3]. This sheds a positive light on the potential of PEF to the contribution of a better environmental and economical sustainability of industrial processes. However, the surge of more studies contributing to an increase of knowledge regarding PEF application and its effects on several matrixes, allowing an holistic comprehension of this innovative technology, together with the capacity of escalating this technology is still necessary.

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