The use of Pulsed Light to reduce native population on the pruina of grapes, and the use of *Lachancea thermotolerans* as red wine acidifier

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Abstract. Pulsed light is an emerging technology used to limit the proliferation of microorganisms in food matrices. The treatment consists on the emission of ultra-short high intensity white light pulses. The light is composed by ultraviolet, visible and near infrared spectra. Its use in enology allows the winemaker to carry on ternary (simultaneous) and sequential fermentations. The PL working conditions were determined through this investigation at the same time that the implantation feasibility of yeast and bacteria for the acidification of red wine was assed. The experimental set up evaluated different doses (number of pulses and energy density) on destemmed grapes. The grapes were placed inside a laboratory-scale cabinet inside a tray and the grapes were mixed randomly three times within the treatment. The microorganisms (both native and inoculated) were followed up with selective and differential growing media. The yeast population decreased 1.2 $\log_{10}$ UFC/mL, although the reduction is less sensitive when the initial population is already low (e.g. 1×10^2 UFC/mL). The use of PL favored the accumulation of lactic acid, produced by either yeast or bacteria, in treated musts. The concentration of lactic acid was higher when using *L. thermotolerans* against the use of *O. oenni* in coinoculation or sequential MLF.

1 Introduction

The pulsed light is an emerging technology basically used to limit the microbial growth on foodstuff in food technology. It is a superficial treatment based on the emission of white light pulses of ultra-short length [1]. The high intensity pulses comprise wavelengths from the ultraviolet range to the near infrared range including the visible light spectrum [2]. The intensity of the pulsed light may exceed several times the energy of solar radiation achieved on the earth's surface at sea level [3]. It is the UV-C fraction from the total light irradiated that has antimicrobial activity when used as treatment; the UV-C fraction corresponds to wavelengths between 200 nm and 280 nm [4].

The effects associated to the UV-C radiations comprise photo-chemical through the formation of pyrimidine dimers between contiguous bases of thymine or between thymine and cytosine; these dimers are new bonds created in the polynucleotide chains that inhibit the production of new DNA for the cellular replication [5]. Another effect would be the breakage of single or double bonds in the DNA chains. In the meantime, there are photo-physical and photo-thermal effects which contribute to inhibit the proliferation of microorganisms in foodstuff. These effects are related to a variation in the ions flux from and into the cell, changes in the polarity of the membrane, membrane depolarization, and localized heat production [1].

Regarding this last aspect, although temperature can reach 130ºC at cellular structure level [6], the global temperature registered in the foodstuff remains constant, or experiences slight variations (< 10ºC) considered non-significant to modify organoleptic properties. Thus, this technology may be considered as non-thermal as in the case of other emerging technologies currently under use or under study for cold sterilisation. Such technologies are high-hydrostatic pressure (HHP), ultra-high pressure homogenisation (UHPH), pulsed electric fields (PEF), etc. The pulsed light has been used on a variety of different foodstuff like fruits and vegetables (tomatoes, lettuce, avocado, raspberry, apple, strawberries, etc.), and meat products [7].

The experimental set up was carried out in a laboratory where the pulsed light treatment was performed on a tailor-made cabinet. The treatment was done over destemmed grapes simulating a pretreatment applied at a selection table before crushing. The essays seek to decrease the microbial populations, both yeast and bacteria, to carry on alcoholic fermentation and malolactic fermentation in comparison to untreated conditions. The strains selected are prone to producing lactic acid, metabolite of interest, to assess the implantation of such microorganisms under both conditions. An increase of lactic acid during winemaking may improve organoleptic perception of red wines.

2 Materials and methods

2.1 Pulsed light treatment

The treatment consisted in the release of two sets of 10 pulses with power of 1 MW/pulse and amplitude of 0.2 to 2 milliseconds over destemmed Tempranillo grapes. After the first set of pulses the tray with grapes was shaken to emulate the movement that grapes experienced on a conveyor belt in a selection table.

The treatment area was a tray with the following dimensions: 25 cm × 13 cm. The working distance, measured from the neon lamps to the grapes, was 7 cm. After the treatment, the grapes were pressed and the must filtered with sterile muslin. Untreated grapes were also pressed and filtered in the same way to get the control must.

2.2 Atomic Force Microscopy

Topographic measurements of the grape's skins (treated and untreated) were done with Nano-Observer AFM (Concept Scientific Instruments, Les ULIS, France) in resonance mode. A silicon rectangular cantilever 1 N/m (model Fort, AppNano, Mountain View, CA, EUA) with an 8 nm radius tip was used for the determinations. An amplitude of 4-5 volts was used to compensate topographic variations (1-4 micron).

2.3 Yeast strains and micro-fermentations

The experimental set up comprised a fermentation designed to assess the production of lactic acid biologically with the use of *Lachancea thermotolerans* in coinoculation with *Saccharomyces cerevisiae*, and *Oenococcus oenni* through malolactic fermentation in coinoculation or sequential fermentation with *Saccharomyces cerevisiae* (Fig. 1). The yeast used were: *Lachancea thermotolerans* (Lt) strain L3.1 (Lallemand Bio, Madrid, Spain); *Saccharomyces cerevisiae* (Sc) strain 7VA (ETSIAAB, UPM, Spain); and *Oenococcus oenni* (Oo) strain Enoferm Alpha™ (Lallemand Bio, Madrid, Spain).

In the fermentative essay, a control was carried out by pure culture fermentation with Sc in must from untreated grapes (F1-C). A second control was carried out also with Sc in must with treated grapes (F1-LP) to compare the performance after the PL treatment. The juice of the two musts was divided into nine amber glass flasks each, for a total of eighteen micro-fermenters. The volume essayed was 250 mL in ISO flasks filled with 220 mL for a headspace of 30 mL. The grapes had less than 10^2 CFU/mL reason why apiculate yeast (*Hanseniaspora opuntiae*) and *Saccharomyces cerevisiae* yeasts were sprayed over the grapes in both cases, regardless they underwent PL treatment or not. Microbiological analysis showed an initial population of 7.1 $\log_{10}$ CFU/mL for inoculated *Saccharomyces* and non-*Saccharomyces* yeasts in the musts. The last two fermentative scenarios were coinoculation of Sc and Oo with untreated and treated grapes, F2-C and F2-LP respectively; and lastly, a coinoculation of Lt and Sc with untreated and treated grapes, F3-C and F3-LP, respectively. The musts had 1105 specific gravity and pH 4.21-4.23. The expected alcohol was 14.5% v/v.

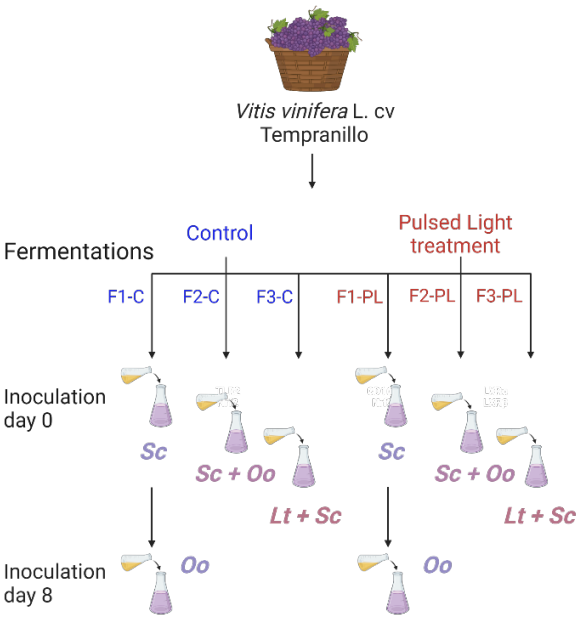

Figure 1. Experimental set up for untreated (control) and treated grapes (pulsed light treatment). Sc-*S. cerevisiae*; Oo-*Oenococcus oenni*; Lt-*Lachancea thermotolerans*.

2.4 Oenological parameters

Organic acids (g/L), total and reducing sugars (g/L), volatile acidity (expressed as g/L of acetic acid) and ethanol (% v/v) were determined with the use of FOSS (OenoFoss™, Foss Iberia, Barcelona, Spain). The samples needed previous filtration with 0.45µm PMMA filters and the release of trapped CO_2.

2.5 Volatile profile

The aromatic volatile compounds were determined with gas chromatography with a flame ionisation detector (GC-FID). The equipment is an Agilent Technologies™ 6850 (Palo Alto, CA, USA) with a column DB-624 (60 m x 250 µm × 1.4 µm). The temperature set for the injector was 250ºC and for the detector 300ºC. Finally, the temperature went from 40ºC for 5 min to 250ºC with a gradient of 10ºC/min and was maintained for 5 min. Hydrogen was the carrying gas with a flow of 2.2 L/min and split ratio set at 1:10. One hundred µL of 4-methyl-2-pentanol (500 mg/L) were used as an internal standard in accordance with an internal procedure [8].

2.6 Anthocyanins profile

Anthocyanins were identified and quantified with a series 1100 HPLC (Hewlett-Packard, Palo Alto, CA, USA) equipped with a diode array detector (DAD). Gradients of solvents A (water/formic acid, 95:5 v/v) and B (methanol/formic acid, 95:5 v/v) were used in a reverse-phase Poroshell 120 C18 column (Phenomenex,

Torrance, CA, USA) (50 × 4.6 mm; particle size 2.7 µm) as follows: 0-2 min, 15% B (working flow 0.8 mL/min); 2-10 min, 15–50% B linear; 10-12 min, 50 % B; 12-13 min, 50-15% B linear; and 13-15 min, re-equilibration. Detection was performed by scanning in the 400–600 nm range. Quantification was performed by comparison against an external standard at 525 nm and expressed as milligram per litre of malvidin-3-O-glucoside (Extrasynthese, Genay Cedex, France) (r2 = 0.9999). Anthocyanins were grouped by family into non-acylated, acylated (acetyl, coumaryl and caffeoyl derivatives), pyranoanthocyanins, and polymeric pigments. Twenty microliter sample of previously filtered (0.45 µm membrane) wines was injected into the HPLC apparatus. The detection limit was 0.1 mg/L. The method was adapted from C. Escott et al. [9].

2.7 Sensory analysis

The wine sensory analysis was assessed with a panel of ten experts. The sensory evaluation was performed at the Chemistry and Food Technology Department of the School of Agricultural, Food and Biosystems Engineering (ETSIAAB) at Universidad Politécnica de Madrid (Spain). The pour size used for this tasting was 60mL (approximately 2 ounces) in transparent wine-tasting cups so the colour could also be assessed. The panel comprised five females and five males with ages between 25 and 55 years old.

The panel evaluated 15 attributes for appearance, aroma, and mouth. The panellists rated the attributes on a five-point scale from low perception (1) to high perception (5). The descriptive attributes were: appearance (colour intensity, hue, and transparency); aroma (intensity, quality, flowers, herbs, fruitiness, reduction, and oxidation); mouth (general acidity, astringency, body, bitterness); a final general overall note was also asked. The hue was rated on a separate scale from red (1) to orange (5). The results were treated with statistical analysis, and the average values were plotted in a radar chart.

2.8 Statistical analysis

The means, the standard deviations, and the differences were examined using one-way ANOVA and the least significant difference (LSD) test. A principal component analysis (PCA) was also obtained with the use of PC Statgraphics v.XI software (Graphics Software Systems, Rockville, MD, USA). The significance was set at $p < 0.05$.

3 Results and Discussion

Right after the pulsed light treatment, skins from the trated and untreated grapes were kept at –80º C to freeze-dry them. The morphology analysis performed with a AFM showed that some damages are visible in treated grapes more extensively than in untreated berries (Fig. 2). Nonetheless, this technique does not assess the damage done on the berries as they penetrate from the outside to the inner parts of the grapes. Pulsed light, as it has been reported [10], does not penetrate deeply in the vegetal tissues, therefore, a significant increase in polyphenols and pigments extractions is not expected.

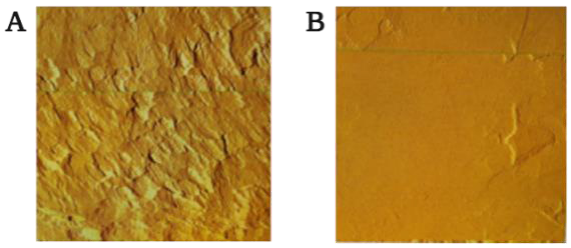

Figure 2. Detail of the surface of treated (A) and untreated (B) grape skin under the AFM.

The effect of the pulsed light is not only perceived on the topography of the berries, but also in the microbial counts, which is the main purpose why this technology is used. The population of total yeast was reduced from 7.1 $\log_{10}$ CFU/mL to 5.8 $\log_{10}$ CFU/mL in the surface of the grapes. This reduction in the population counts must have allowed a better implantation of selected yeast and lactic acid bacteria strains as it was seen in previous experiments [11], and as it can be seen in some oenological parameters shown hereafter.

In both cases, the volume of ethanol produced is slightly lower for the fermentations with Lt. The reductions accounts for approximately 0.5% v/v with respect to both fermentations with Oo (see Table 1). A reduction of ethanol is commonly observed in fermentations were the concentration of lactic acid reaches more than 2 g/L [12]. This is expected as part of the sugars are consumed by yeast to accumulate lactic acid from the enzymatic conversion from pyruvate.

Table 1. Oenological parameters for finished wines produced with untreated grapes (C), and treated grapes (LP). Different letters indicate statistical significant difference ($p < 0.05$).

	Ethanol	Glucose / fructose	pH	Total acidity
	% v/v	g/L		g/L
F1-C	13.7 ± 0.1b	1.9 ± 0.3a	4.1 ± 0.0a	4.2 ± 0.3c
F2-C	13.7 ± 0.1b	1.7 ± 0.2ab	4.1 ± 0.0a	4.1 ± 0.3c
F3-C	13.2 ± 0.3c	1.4 ± 0.3c	3.6 ± 0.1b	6.1 ± 0.6b
F1-PL	14.4 ± 0.1a	2.0 ± 0.1a	4.1 ± 0.0a	4.3 ± 0.1c
F2-PL	14.2 ± 0.2a	1.9 ± 0.1a	4.2 ± 0.0a	4.1 ± 0.2c
F3-PL	13.7 ± 0.3b	1.3 ± 0.2bc	3.5 ± 0.1b	7.4 ± 0.8a

As a consequence of the accumulation of lactic acid, there is an impact in the total acidity and the pH of the wines. The concentration of lactic acid, when in low concentration, does not modify the pH nor the total acidity as it has happened in this experimental trial. Total acidity is 2 g/L higher in the untreated trial whilst it is 3 g/L higher in the treated trial when Lt is used. This increment in acidity has reduced pH 0.5 and 0.7 from the average value obtained with MLF with Oo. The increment in acidity is not only due to the accumulation

of lactic acid, but to the fact that malic acid is not consumed when Lt is used during winemaking (Fig. 3). The difference between this mixed fermentation and the other two is that lactic acid bacteria metabolises malic acid, the concentration of malic acid decreases, and thus, there is an increment on the pH values towards the end of the fermentation [13].

Another observation in this regards is the fact that the variability in the production of lactic acid was reduced in treated must together with a sensitive increase in lactic acid production (see Fig. 3 – F3PL and F3C). A better implantation of yeast after the use of PL might have happened and therefore, a more controlled fermentation with enhanced lactate productions was achieved.

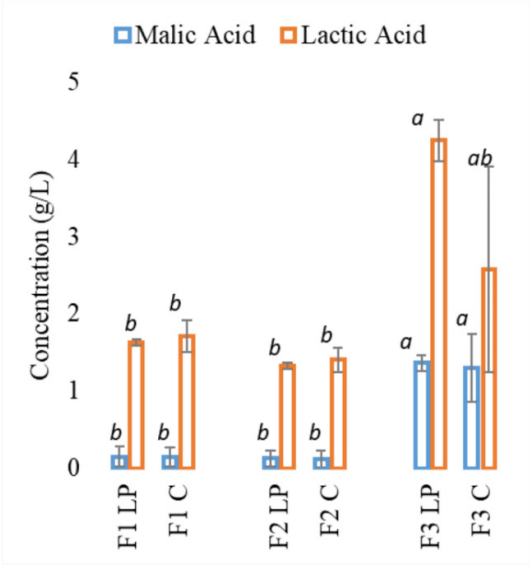

Figure 3. Concentration of malic acid and lactic acid in fermentations with untreated grapes (C) and treated grapes (LP).

At the same time that the total acidity increased with the use of Lt, there was a decrease in the accumulation of aromatic compounds in comparison to the mixed and sequential MLF with Oo (Table 2), and a slight decrease in the concentration of anthocyanins as well (Table 3). The potential influence of the PL on these results is yet to be determined.

Table 2. Aromatic volatile compounds grouped by family. Different letters indicate statistical significant difference ($p < 0.05$).

	Ethyl esters	Acetate esters	Higher alcohols	Carbonyl compounds
	mg/L	mg/L	mg/L	mg/L
F1-C	57.6±7.9b	95.6±7.0a	431.3±14.7ab	785.6±26.3c
F2-C	76.1±4.5a	96.0±5.7a	458.1±37.4a	645.5±43.8d
F3-C	42.6±2.1c	41.4±2.8c	389.6±24.4b	617.0±54.4d
F1-PL	21.5±1.1d	71.2±0.9b	431.6±16.2ab	988.8±25.5a
F2-PL	24.8±6.9d	64.2±4.8b	436.4±45.1ab	894.4±17.0ab
F3-PL	53.3±8.7b	23.6±1.4d	335.0±2.7c	862.0±89.6bc

Regarding aroma volatiles, the concentration is higher when using Oo for MLF in both fermentative scenarios, with and without treatment, but the concentration is always higher for untreated grapes when compared to its treated counterpart. One explanation to this, is the fact that the native microbial population, and by native we refer to the concentration of Ho poured before the PL treatment, have produced larger amount of esters during the first stages of the alcoholic fermentation. *Hanseniaspora* spp. Strains are known for producing larger amounts of esters before strains of Sc implant and thrive over the other species during AF [14].

Table 3. Anthocyanins grouped by family. Different letters indicate statistical significant difference ($p < 0.05$).

	Non-acylated anthocyanins	Acylated anthocyanins	Pyranoant hocyanins	Polymeric pigments
	mg/L	mg/L	mg/L	mg/L
F1-C	578.3±34.6a	90.8±2.2a	18.4±2.2c	21.9±0.2ab
F2-C	520.6±32.8ab	82.9±2.1ab	20.0±2.7c	20.2±0.4c
F3-C	458.6±56.7b	76.8±5.0b	23.9±1.9ab	20.7±0.5bc
F1-PL	579.9±49.0a	91.8±10.3a	20.4±1.0bc	22.4±1.3a
F2-PL	533.9±2.2a	87.9±4.0a	24.9±1.7a	21.6±0.5ab
F3-PL	516.7±22.9ab	90.7±3.9a	20.3±1.8bc	22.1±0.3a

The concentration of carbonyl compounds, including 2-butanediol, is higher in treated musts probably due to fact of having less Ho colonies and less enzymatic activity shown by Lt.

As for the pigments, the amount of anthocyanins detected in finished wines after fermentation does not differ as much between counterparts of the same fermentative scenario. The major loss is observed in non-acylated anthocyanins. The effect of the reduction of anthocyanins observed in wines produced with Lt might be due to molecular interactions between the pigments and cellular structures. These interactions are strain related and apparently are larger with the strain L3.1 (Lt) than with the strain 7VA (Sc). Morata et al. [15] have reported higher colour retention by lees with strains from the following species: *Saccharomyces cerevisiae* and *Lachancea thermotolerans*.

Nonetheless, and despite the anthocyanins decrease when using Lt to ferment red musts, the colour expressed due to the high acidity obtained in these wines is not perceived negatively. In this regard, the colour intensity has no significant difference (Fig. 5), while the hue was perceived red for Lt wines and yellowish or more orange in the wines with MLF with higher pH values.

Lastly, the sensory analysis, besides the assessment of the colour perceived in wines, has evaluated the aroma profile and the global perception. Figure 4 reflects the results regarding the aroma profile, whilst Figure 5 shows the results regarding visual, body, and global perception.

In general, the wines had scarce statistical differences, and these were basically related to the fruity aroma and the quality of the aroma (Fig. 4), as well as the hue, the bitterness, the acidity, and the global perception. The black lines refer to the fermentations done with Lt; the wines with untreated grapes have dotted lines, and the wines with treated grapes have a continuous line. As it can be seen, the continuous black line (treated grapes with Lt) were perceived as fruiter with better quality aroma, more acid and with the best global perception. It is important to notice that this wine had lower concentration of volatile compounds, but somehow the fruity aroma is enhanced in this matrix. A higher acidity, and a brighter red colour may also influence the freshness perceived in this wine.

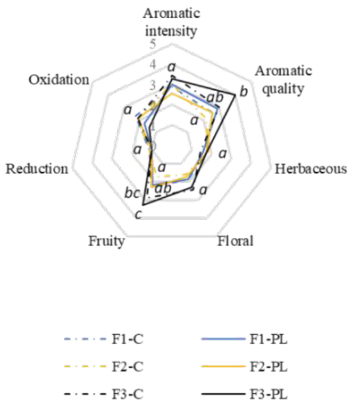

Figure 4. Two-dimensional star plot of wine-tasting aroma descriptors. Different letters indicate a significant difference between means ($p < 0.05$).

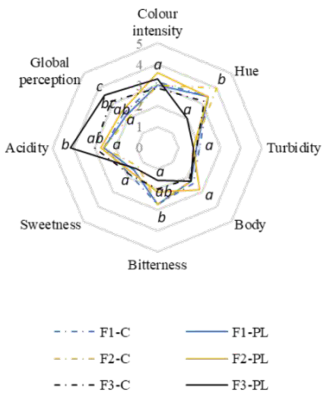

Figure 5. Two-dimensional star plot of wine-tasting visual and mouth descriptors. Different letters indicate a significant difference between means ($p < 0.05$).

The perception of red wines as fresher associated to a decrease in pH values or an increase in total acidity has been reported previously [16]. In this way, a fermentative biotechnology able to increase the accumulation of lactic acid may change the way in which consumers perceive red wines. In the same time, the high production of lactic acid reduces the concentration of ethanol which may also decrease the heat produced by high alcohol volume in mouth associated to red wines produced in war areas.

4 Conclusions

The use of pulsed light on grapes allows the reduction of native microorganisms. In this way, the use of selected yeast strains, either commercial or fresh from isolates, would allow a better control of the alcoholic fermentation of musts. In particular, the use of pulsed light has improved the accumulation of lactic acid with the use of acidifying strains of yeast and bacteria in coinoculation with *S. cerevisiae*. Under experimental conditions, both in the alcoholic fermentation and the malolactic fermentation, there is a larger accumulation of lactic acid in the musts obtained with treated grapes. The effect might be attributed to a lower competence that acidifying microorganisms encountered after the use of PL in comparison to a larger population found in untreated grapes. Nonetheless, there are drawbacks in the use of this technology as a potential reduction of anthocyanins and colour modification due to oxidative reactions. This negative effect could be reduced by having longer maceration times at lower temperatures to counteract the effect of pulsed light. Finally, it is recommended to apply this technology continuously on selection tables to reduce over-irradiation on certain areas of the berries and the release of must before the crusher.

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