Evaluating the influence of operational parameters of pulsed light on wine related yeasts: focus on inter- and intra-specific variability sensitivity.

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Highlights

- Pulsed light treatment was applied to 14 wine yeast species (198 strains)
- PL efficiency was driven by two main factors: Fluence per flash and total fluence
- The sensitivity to PL treatment varied depending on the species and the strains
- *Brettanomyces bruxellensis* and other wine spoilers were highly sensitive to PL
- Pulsed light treatment is promising for wine microbial stabilization

Abstract

In oenology, there is a growing demand by consumers for wines produced with less inputs (such as sulphite, frequently used for microbial control). Emerging control methods for managing microorganisms in wine are widely studied. In this study, the efficiency of pulsed light (PL) treatment was investigated. A drop-platted system was used to evaluate the impact of three PL operational parameters: the fluence per flash, the total fluence and the flash frequency. Fluence per flash appeared to be a key parameter prior to total fluence, thus demonstrating the importance of the effect of peak voltage during PL treatments. The efficiency of PL treatment was assessed on 198 strains distributed amongst fourteen yeast species related to wine environment, and an important variability in PL response was observed. *Brettanomyces bruxellensis* strains were strongly sensitive to PL, with intraspecific variation. PL was then applied to red wines inoculated with 9 strains of *B. bruxellensis*, *Saccharomyces cerevisiae* and *Lachancea thermotolerans*. Results confirmed interspecific response variability and a higher sensitivity of *B. bruxellensis* species to PL. Wine treatments with a total fluence of 22.8 $J.cm^{-2}$ resulted in more than 6 log reduction for different *B. bruxellensis* strains. These results highlight the potential of PL for wine microbial stabilization.

Keywords: Pulsed light, yeast, microbial stabilization, *Brettanomyces bruxellensis*, red wine, plate screening

I. Introduction

Wide varieties of food preservation methods are available including heating, freezing, filtration and addition of preservatives. However, these methods are cost effective and can impair food quality and therefore major efforts have been made to develop alternative technologies with a lower impact on food quality. Pulsed light (PL) is a non-thermal technology used to decontaminate surfaces or liquid by inactivating microorganisms using short-time pulses of an intense broad-spectrum light (John & Ramaswamy, 2018; Kramer, Wunderlich, & Muranyi, 2017b; Pirozzi, Pataro, Donsì, & Ferrari, 2021). PL relies on a wide wavelength range of 200–1100 nm, which includes ultraviolet (UV, 200–400 nm), visible light (VIS, 400–700 nm), and near-infrared region (IR, 700–1100 nm). PL treatment is based on the accumulation of high discharge voltage in a capacitor where the stored electrical energy is delivered as intermittent short pulses through a light source filled with xenon gas. This xenon-light source emits a broad-spectrum light flash (typically 1 to 10 pulses per second) with approximately 25% in the UV range (Keener & Krishnamurthy, 2014). Microbial inactivation is commonly attributed to photochemical damage of DNA caused by UV wavelengths, enhanced by photothermal effect due to local overheating and photophysical effect on proteins and membranes (Elmnasser et al., 2007; Ikehata & Ono, 2011). Photophysical effect is responsible for membrane disruption and vacuole extension observed in yeast (*Saccharomyces cerevisiae*) and bacterial cells (*Bacillus subtillis*) (Ferrario & Guerrero, 2017; Nicorescu et al., 2013; Takeshita et al., 2003). The concomitant action of these three mechanisms (*e.g.* photochemical, photothermal and photophysical effects), which may act synergistically, as well as the high peak power involved, can explain the generally reported higher decontamination effectiveness of PL in comparison with continuous wave UV light treatment (Clair, Esbelin, Malléa, Bornard, & Carlin, 2020). The main process parameters governing PL efficiency are the fluence ($J.cm^{-2}$) over exposure time (s), the frequency (Hz), the number of pulses applied (n), the pulse width (τ) and the peak power (W) (Barbosa-Canovas, Schaffner, Pierson, & Zhang, 2000; Pataro, De Maria, & Lyng, 2016).

Since the first works on disinfection with flash lamps performed in the late 1970s in Japan, PL has been widely explored. Various applications were developed, ranging from the decontamination of surfaces (packaging, processing equipment, medical devices, and solid

foods) to the microbial stabilization of liquid (disinfection of air, water, food liquids) (Aguirre, Hierro, Fernández, & García De Fernando, 2014; Ferrario & Guerrero, 2017; Hwang, Cheigh, & Chung, 2015; Levy, Aubert, Lacour, & Carlin, 2012; Pataro et al., 2011; Proulx et al., 2017). In liquid application, PL efficiency may be restricted by its low penetrance. This difficulty is usually overcome via the design of specific reactors maximising microorganism exposure to light (Junqua, Vinsonneau, & Ghidossi, 2020).

This innovative non-thermal control method was started to be evaluated only recently in oenology. For example, PL treatment was shown to be effective in a limited extend, and reduced the microbial density (*e.g.* yeast and bacteria) on red grape surfaces, by about 1.2 $\log_{10}$ CFU.mL^{-1} with 10.7 J.cm^{-2} (Escott et al., 2021, 2017). A reduction of 4.89 $\log_{10}$ CFU.mL^{-1} of *Escherichia coli* was found upon the application of 80 pulses (0.66 J.cm^{-2}.pulse^{-1}) to red grape juice (Xu et al., 2019). During winemaking, yeasts management is essential to ensure efficient fermentations and to limit the risk of organoleptic changes. Indeed, while *Saccharomyces* yeasts are essential for must fermentation, some species are considered as spoilers (*e.g. Brettanomyces bruxellensis*, *B. anomalus*, *Trigonopsis cantarellii*, *Zygosaccharomyces rouxii*, *Z. bailii*, *Schizosaccharomyces pombe*, etc.). Those species are able to affect the fermentation kinetics and/or to produce off-flavours and/or to modify wine's physical properties, lowering the quality of the final product (Loureiro & Malfeito-Ferreira, 2003; Padilla, Gil, & Manzanares, 2016). Among them, *Brettanomyces bruxellensis* is certainly the most feared spoiler, damaging up to 25 % of the world red wine production (Alston, Arvik, Hart, & Lapsley, 2021; Oro, Canonico, Marinelli, Ciani, & Comitini, 2019; Romano, Perello, Revel, & Lonvaud-Funel, 2008). *B. bruxellensis* yeasts are able to produce volatile phenols such as 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol, whose aromatic notes are unpleasant and described as animal, leather, horse, stable or pharmaceutical (Chatonnet, Dubourdie, Boidron, & Pons, 1992; Oelofse, Pretorius, & Toit, 2008; Schopp, Lee, Osborne, Chescheir, & Edwards, 2013). Several recent studies have shown both high genetic and phenotypic diversity of *B. bruxellensis* in different wine regions (Avramova, Cibrario, et al., 2018; Cibrario et al., 2020; Eberlein, Abou Saada, Friedrich, Albertin, & Schacherer, 2021; Gounot et al., 2020). This important diversity is reflected in the relative sensitivity of different strains to existing microbial stabilization methods used during winemaking. For instance, genetic groups resistant to sulphite addition or expressing lower sensitivity to chitosan or to UV-C treatment have been identified (Avramova, Vallet-Courbin,

Maupeu, Masneuf-Pomarède, & Albertin, 2018; Paulin et al., 2020; Pilard et al., 2021). Preliminary results on wine microbial stabilization with PL were obtained in white wine with a maximal reduction of 2.10 log of *B. bruxellensis* (Pérez-López et al., 2020). PL could thus be interesting in the wine industry to control the risk associated with *Brettanomyces* yeasts contamination.

The aim of this research was to investigate the PL sensitivity of fourteen yeast species associated with winemaking, with a focus on *Saccharomyces cerevisiae* and *Brettanomyces bruxellensis* to identify inter- and intra-specific variability. Specifically, this study explored the dose-effect relationship between PL total fluence, fluence per flash, frequency, and inactivation of microorganisms naturally present in wine. A plate-based screening approach was applied to a large collection of yeast species and strains (14 species and 198 strains). Different PL treatment's modalities (with varying fluence per flash, frequency, and total fluence) were considered and the variability within *B. bruxellensis* and *S. cerevisiae* was compared. In a second step, six *B. bruxellensis*, two *S. cerevisiae* and one *L. thermotolerans* strains were selected to evaluate PL potential to inactivate those yeasts in a highly absorbent red wine. In this part, the inoculated red wine was treated with several PL intensities using a lab-scale continuous flow PL apparatus.

II. Materials and methods

II.1. Yeast strains

The 198 strains from 14 species used in this study were collected from different laboratories or collections (Table 1, Supplemental Table 1). Strains were grown and maintained in Yeast extract peptone dextrose (YPD) plates at 24°C (10 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ glucose, 20 $g.L^{-1}$ agar, Fisher Scientific, Hampton, New Hampshire, USA). These species were selected for their relevance in the winemaking process.

Table 1: List of the yeast strains used in this work.

Species	Strains
B. anomalus	3 strains : BR 23-4 ; CLIB 304 ; NRRL Y-17522 T
B. bruxellensis	Wine 2N - 34 strains: 1961_MX_M1_E2 ; CBS 2499 ; ISA2150 ; CRBO L0469 ; CRBO L0614 ; CRBO L14163 ; CRBO L1703 ; CRBO L17111 ; CRBO L1714 ; CRBO L1715 ; CRBO L1727 ; CRBO L1751 ; YJS5302 ; YJS5319 ; YJS5320 ; YJS5345; YJS5347 ; YJS5357 ; YJS5373 ; YJS5385 ; YJS5392 ; YJS5416 ; YJS5422 ; YJS5426 ; YJS5440 ; YJS5447 ; YJS5449 ; YJS5453 ; YJS5456 ; YJS5458 ; YJS5461 ; YJS5463 ; YJS5479 ; YJS5485 Wine/Kombucha 2N - 19 strains: 15_1 ; ISA1601 ; YJS5301 ; YJS5310 ; YJS5334 ; YJS5340 ; YJS5344 ; YJS5349 ; YJS5363 ; YJS5368 ; YJS5384 ; YJS5398 ; YJS5402 ; YJS5406 ; YJS5407 ; YJS5413 ; YJS5417 ; YJS5420 ; YJS5431 Wine/Beer 3N - 25 strains: 2OT13_05 ; 2OT13_07 ; 2OT14_01 ; 2OT14_03 ; AWRI1608 ; CDR222 ; GB62 ; ISA2397 ; CRBO L0417 ; CRBO L14155 ; CRBO L14194 ; CRBO L17112 ; CRBO L1741 ; CRBO L1749 ; CRBO L1771 ; LB15107g

	; LB15110g ; MLC_296_2014_9 ; NL045 ; NL059 ; VP1519 ; YJS5396 ; YJS5397 ; YJS5400 ; YJS5454 1st Wine 3N - 19 strains: 12_LT_VGC3_c_10 ; 2OT13_02 ; 33_2 ; AWRI1499 ; CDR217 ; GB12 ; GSP1509 ; CRBO L14174 ; CRBO L14175 ; NL050 ; YJS5408 ; YJS5434 ; YJS5445 ; YJS5459 ; YJS5469 ; YJS5473 ; YJS5476 ; YJS5478 ; YJS5487 Tequila/bioethanol 3N - 6 strains: CBS 5512 ; CBS 6055 ; CRBO L14169 ; CRBO L17108 ; SJ12_4 ; UWOPS_92__298_4 2nd Wine 3N - 8 strains: ISA2211 ; CRBO L0308 ; CRBO L14190 ; CRBO L1733 ; CRBO L1782 ; VP1539 ; VP1544 ; YJS5382
H. uvarum	3 strains : CRBO L1433 ; NZ15 ; Y-1614
L. thermotolerans	3 strains : 18 ; AEB ; CLIB292
M. pulcherrima	3 strains : CRBO L0675 ; NZ268 ; Y-7111
S. cerevisiae	Beer - 10 strains: 227 ; 382 ; 650 ; Notthingham ; premium gold ; S-04 ; US-56 ; Windsor ; Y7327 ; Y7328 Bread - 10 strains: 215 ; 319 ; 324 ; 646 ; Hirondelle ; SBA ; SBB ; SBC ; SBD ; SBE Dist - 5 strains : A24 ; alcotec 48 ; Y-963 ; YB-427 ; YB-428 Food - 2 strains : Y-767 ; YB-360 Fruit - 5 strains : Y-6678 ; Y-7568 ; Y-965 ; YB-210 ; YB-4081 Fruit juice - 4 strains : Y-129 ; Y-2230 ; Y-6275 ; Y-6278 Nature - 2 strains : Y-35 ; Y-7567 Wine - 13 strains : 154 ; 157 ; 328 ; 479 ; Fx 10 ; GN ; CRBO L0431 ; CRBO L0432 ; CRBO L0433 ; CRBO L0437 ; SB ; X5 ; Y-1301
S. uvarum	2 strains : U1 ; U3
***Sc x Su (S. cerevisiae x S. uvarum* synthetic hybrids)**	2 strains : DU23 ; EU23
Schizo. pombe	3 strains : CRBO L0442 ; Y-11791 ; Y-12796
Starm. bacillaris	3 strains : 10_372 ; CRBO L0473 ; NZ12
T. delbrueckii	3 strains : B172 ; CLIB 230 ; CRBO L0705
Tri. cantarellii	3 strains : CRBO L0412 ; CRBO L0416 ; CRBO L0419
Zygo. bailii	2 strains : CLIB 213 ; CRBO L0536
Zygo. rouxii	2 strains : CLIB 233 ; CRBO L0314

II.2. PL treatment of yeast on YPD solid medium

Strains were grown in liquid YPD media (10 $g.L^{-1}$ yeast extract, 10 $g.L^{-1}$ peptone, 20 $g.L^{-1}$ glucose) for 24 hours and the population was estimated by optical density (FLUOstar Omega, MNGLabtech, France). Droplets of 2 µL of serial dilutions (0.5, 0.05, 0.005 and 0.0005 Optical Density at 600nm, corresponding to concentrations around 10^6, 10^5 10^4 and 10^3 $cells.mL^{-1}$) were spotted onto solid medium (YPD with 20 $g.L^{-1}$ agar, Ø4mm-diameter drops), aiming at obtaining 4 different densities (around 1000, 100, 10, 1 $CFU.drop^{-1}$). Drops were produced in triplicate for each condition on square Petri dishes (12*12cm). Spotted plates were then placed in a PL.Box (Sanodev, France), at 30cm distance from the flash lamp and treated with different modalities (Table 2). First, modalities 1, 5, 6, 8, 10, 11 and 12 were designed to study the impact of an increasing fluence with the same flash frequency (2 Hz) and fluence per flash (42 $mJ.cm^{-2}$). Then, modalities 2, 3, 4, 7, 8, 9, 13, 14 and 15 were designed to evaluate the impact of both the fluence per flash (22 $mJ.cm^{-2}$, 42 $mJ.cm^{-2}$ and 87 $mJ.cm^{-2}$,

according to the manufacturer) and the flash frequency (1 Hz, 2 Hz and 5 Hz) at equivalent total fluencies. Total fluence for the different treatment modalities was calculated according to the number of applied flashes and the fluence per flash ($mJ.cm^{-2}$). According to the manufacturer, the fluence was homogeneous on the 15cm*15cm surface 30 cm under the flash lamp and no statistical impact of the drop positions were evidenced (data not shown).

Table 2: List of the 15 modalities of PL used for the plate screening.

Modality	Lamp Voltage (V)	Fluence/Flash ($mJ.cm^{-2}$)	Flash Number	Treatment Time (s)	Frequency (Hz)	Total Fluence ($mJ.cm^{-2}$)
1	0	0	0	0	0	0
2	1500	22	20	4	5	440
3	1500	22	20	10	2	440
4	1500	22	20	20	1	440
5	2100	42	2	1	2	84
6	2100	42	5	2,5	2	210
7	2100	42	10	2	5	420
8	2100	42	10	5	2	420
9	2100	42	10	10	1	420
10	2100	42	15	7.5	2	630
11	2100	42	20	10	2	840
12	2100	42	25	12.5	2	1050
13	3000	87	5	1	5	435
14	3000	87	5	2.5	2	435
15	3000	87	5	5	1	435

II.3. Yeasts growth monitoring after PL treatments on YPD solid medium

II.4. After PL treatment, plates were incubated in the dark at 24 °C, a temperature chosen to support the growth of all yeast species tested. The growth was monitored every day for 10 days: plates were imaged from an illuminated desk to avoid light gleam (model DMC-FS7, Panasonic Corporation, Japan). Growth data were analysed with custom-made scripts in R (R Development Core Team, 2013) as previously described (Pilard et al., 2021): plate images were imported on R using the *OpenImageR* package. The images of the plates were cropped for superimposition and the position of the drops was determined by manual clicking using the *grid* package and the *grid.locator* function. The area of each drop (in mm^2) was calculated using automatic background subtraction. All statistical analyses were performed using R

home-made scripts. In particular, growth dynamics were analysed using K-means clustering (*cutRepeatedKmeans* function; *ClassDiscovery* R package). From 2 to 15 clusters were tested, and the optimal number of clusters (4) was determined visually.

PL treatment of yeasts inoculated in red wine

For both yeasts adaptation and PL treatments of yeasts inoculated in red wine, 18 L of Bordeaux red wine (Merlot and Cabernet Sauvignon blend of the 2019 vintage) were used. This wine was chosen for its high UV absorbance (α_{254nm}= 49 cm^{-1}) and its low free SO_2 content (< 15 $mg.L^{-1}$). To facilitate yeast strains adaptation and growth into the wine, few drops of hydrogen peroxide were added to the wine until free SO_2 content reached zero. Then, to ensure complete sterilization, wine was pasteurized (80 °C for 40 minutes) and then kept at 4 °C. *B. bruxellensis* (CRBO L1735, CRBO L1737, CRBO L1746, AWRI1499, AWRI 1608, CBS2499), *S. cerevisiae* (FX10, CRBO L0437), *L. thermotolerans* (CLIB292) strains were selected for these experiments. They were firstly grown on YPD plates, then inoculated in YPD liquid medium (24 °C) and finally in sterile red grape juice. To lower the lethality due to the inoculation in the wine during PL treatments, the proportion of pasteurized wine in the culture medium was gradually increased until it reached 90 %. Populations were counted using Malassez cell with addition of methylene blue (Ribereau-Gayon et al., 2006). Just before PL treatments, each strain was inoculated at a final concentration of 10^5- 10^6 $CFU.mL^{-1}$ in three (triplicates) 500 mL batches of pasteurized red wine. The set-up used in this study was a lamp with 8mm of inner diameter and 10mm of outside diameter filled with 600mbar of xenon. The distance between each electrode was 200mm. Wine treatments were realized in continuous flow at 9 $L.h^{-1}$ in a home-made reactor, which consists of a 2 mm inner diameter FEP tubing (16.5 m length) coiled around PVC tubing (8 cm inner diameter, 25 cm length). The reactor was placed horizontally, at 15 cm distance from the lamp in the PL treatment apparatus (LP.Box, Sanodev, France) (Figure 1). This type of reactor was chosen to limit the thickness of the liquid treated and to homogenize the residence time of the wine in the device.

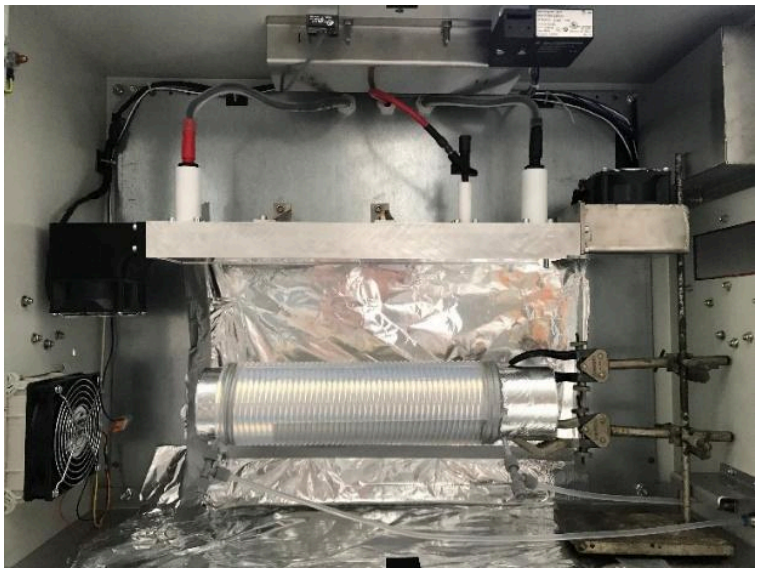

Figure 1 : Experimental setup for PL treatment of wine

To estimate the theoretical fluence applied to the wine during one pass in the reactor at 9 L.h^{-1}, the following calculation was made:

$$F_{Total} = n_{flash} * F_{flash}$$

$$n_{flash} = Freq * RTD$$

$$RTD = \frac{Q}{V_{tube}}$$

$$V_{tube} = L_{tube} * 2 * \pi * R^2$$

F_{Total} : Total fluence (mJ.cm^{-2})
n_{flash} : Number of flashes received
F_{flash} : Fluence per flash (mJ.cm^{-2})
$Freq$: Flash frequency (Hz)
RTD : Residence time (s)
Q : Flow rate (m^3.s^{-1})
V_{tube} : Tube volume (m^3)
L_{tube} : Tube length (m)
R : Tube inner radius (m)

Considering the reactor's geometry, wine was exposed to PL only half of its residence time in the reactor. Postulating this, the following approximation was made:

$$F_{Total} = \frac{n_{flash} * F_{flash}}{2}$$

Flash frequency was set at 4.5 Hz, the reactor was placed 15 at cm distance from the lamp and the input voltage was set at 4000 V, which corresponded to a fluence per flash of 169 mJ.cm^{-2} at the surface of the reactor. The measured RTD was 20 s, so the wine theoretically received up to 45 flashes resulting in a total fluence of 7.6 J.cm^{-2} per pass in the reactor. Each 500 mL of inoculated wine batches was then treated with the following modalities: 0 J.cm^{-2} (OFF: circulated in the reactor without PL treatment), 7.6 J.cm^{-2} (T1: circulated once with PL), 15.2 J.cm^{-2} (T2: circulated twice with PL) and finally 22.8 J.cm^{-2} (T3: circulated thrice

with PL). Samples (5 mL) were collected under sterile conditions right after inoculation (T0) and after each treatment (OFF, T1, T2 and T3).

II.5. Yeasts enumeration before and after red wine PL treatments

Microbial counts were determined in triplicates by plating serial 10-fold dilutions of the samples and 10 or 100 µL were plated in 9 cm diameter petri dishes. Yeasts were enumerated on YPD plates after 7 days of incubation at 25 °C. The number of colonies detected was expressed in $CFU.mL^{-1}$ and the limit of detection was 10 $CFU.mL^{-1}$. The OFF modality was used as reference for $\log_{10}$ reduction determination. Analyses of variance (ANOVA) were performed followed by post-hoc Tukey tests (*HSD.test* function from *agricolae* package, R software).

III. Results

III.1. Sensitivity to PL treatment: large screening on Petri dishes

In this first part, PL sensitivity was assessed for 198 yeast strains belonging to 14 different species (Table 1). These species are known to be associated with grape and/or wine. Between 2 and 3 strains per species were chosen to represent the genetic diversity of the species. In addition, a special attention was given to two species, namely *B. bruxellensis* and *S. cerevisiae*, for their negative and positive importance in winemaking respectively, using different strains from various origins.

III.1.1. Monitoring the impact of pulsed light treatment on yeast growth

Different modalities were studied with variation of fluence per flash, total fluence and frequencies, resulting in 15 combinations (Table 2), in order to estimate their impact on yeast growth. The growth was monitored daily, up to 10 days. For each strain, each initial density (4 densities) and each modality, the growth area was measured automatically using home-made R scripts. Triplicates were performed so that more than 350.000 data points were included in the dataset.

First, the total fluence impact was assessed (Figure 2). Modalities 1, 5, 6, 8, 10, 11 and 12 were applied at 2 Hz with a fluence per flash of 42 $mJ.cm^{-2}$, the only difference being the number of flashes applied (respectively 0, 2, 5, 10, 15, 20 or 25) which corresponded to different total fluencies (respectively 0, 84, 210, 420, 630, 840 and 1050 $mJ.cm^{-2}$). Figure 2

shows three distinct behaviours associated with total fluence increase, as well as the sensitivity to PL treatments for all the tested strains.

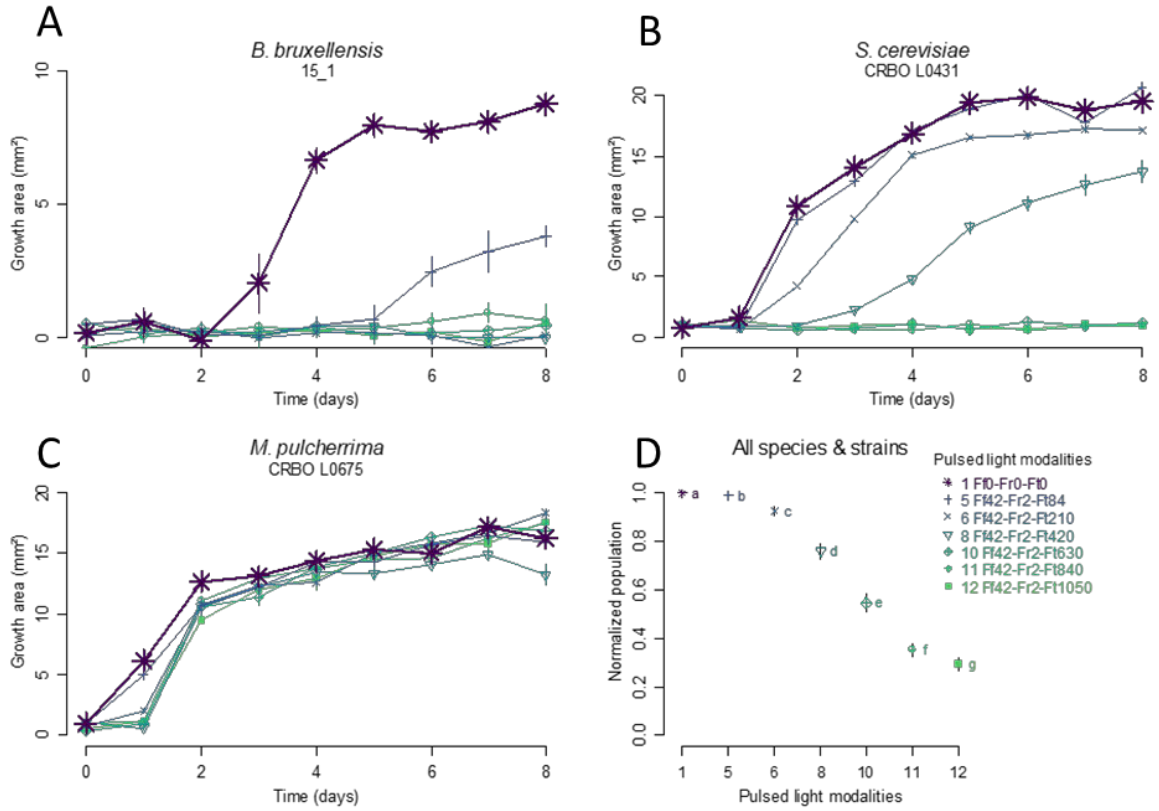

Figure 2 : Growth kinetics of strains depending on the total fluence applied during the pulsed light treatment (0, 84, 210, 420, 630, 840 and 1050 mJ.cm^{-2}). (A) B. bruxellensis 15_1, (B) S. cerevisiae CRBO L0431, (C) M. pulcherrima CRBO L0675, (D) Mean of the Normalized population (Area Under the Curve AUC normalized using the control -not-treated- modality 1) for all the strains used in this study (198).

Seven modalities were used (1, 5, 6, 8, 10, 11 and 12). Ff stand for fluence per flash (mJ.cm^{-2}), Fr for frequency (Hz) and Ft for total fluence (mJ.cm^{-2}). The density of 100 cells/drop was used for comparison. Growth area was measured in mm². For D, the letters correspond to significant differences between each modality (Kruskal-Wallis test).

B. bruxellensis strains showed a highly sensitive behaviour to PL. For example, for strain 15_1 (Figure 2A), 84 mJ.cm^{-2} (modality 5: 2 flashes at 42 mJ.cm^{-2}, 2 Hz) were sufficient to halve its growth and all other treatments were sufficient to prevent its growth. For strain *S. cerevisiae* CRBO L0431, PL treatment with 84 mJ.cm^{-2} (modality 5) did not impact its growth, while treatments with 210 and 420 mJ.cm^{-2} (modalities 6 and 8) reduced yeast growth by 20 % and 35 % respectively. Only total fluencies higher than 630 mJ.cm^{-2} (modalities 10, 11 and 12) fully prevented CRBO L0431 growth (Figure 2B). PL poorly affected *M. pulcherrima* CRBO L0675 strain growth, with none of the applied treatments being sufficient to reduce significantly the final cell growth (Figure 2C). However, treatments with 210 mJ.cm^{-2} and higher total fluencies increased the lag-phase (about 24 hours more than the control).

Considering all the strains, all modalities were significantly different from each other, indicating that the higher the fluence, the greater the reduction in growth, thus confirming that the total fluence is the main process parameter affecting yeast growth (Figure 2D).

In Figure 3, the PL modalities 1, 3, 8 and 14 were compared to study the influence of the fluence per flash on the yeast growth profiles. Four fluencies per flash were applied (0, 22, 42 and 87 mJ.cm^{-2}) with proximate total fluencies (about 430 mJ.cm^{-2}) and frequency (2 Hz).

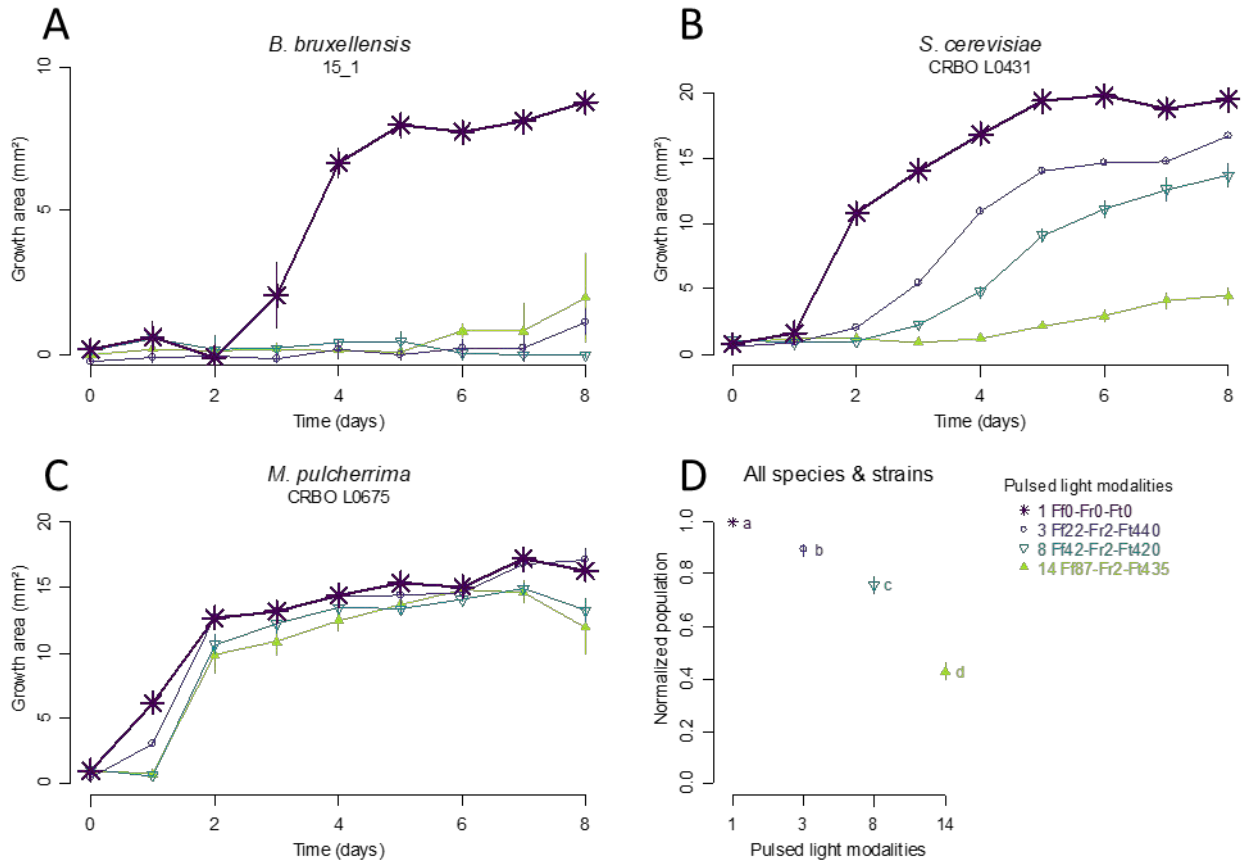

Figure 3 : Growth kinetics depending on four different fluence per flash applied during the pulsed light treatment (0, 22, 42 and 87 mJ.cm^{-2}). (A) B. bruxellensis 15_1, (B) S. cerevisiae CRBO L0431, (C) M. pulcherrima CRBO L0675, (D) Mean of the Normalized population (Area Under the Curve AUC normalized with the control modality) for all the strains used in this study (198).

Four modalities were used (1, 3, 8 and 14). Ff stand for fluence per flash (mJ.cm^{-2}), Fr for frequency (Hz) and Ft for total fluence (mJ.cm^{-2}). The density of 100 cells/drop was used for comparison. Growth area was measured in mm². D: the letters correspond to significant differences between each modality (Kruskal-Wallis test).

Results showed that the impact of fluence per flash varied depending on the yeast considered (

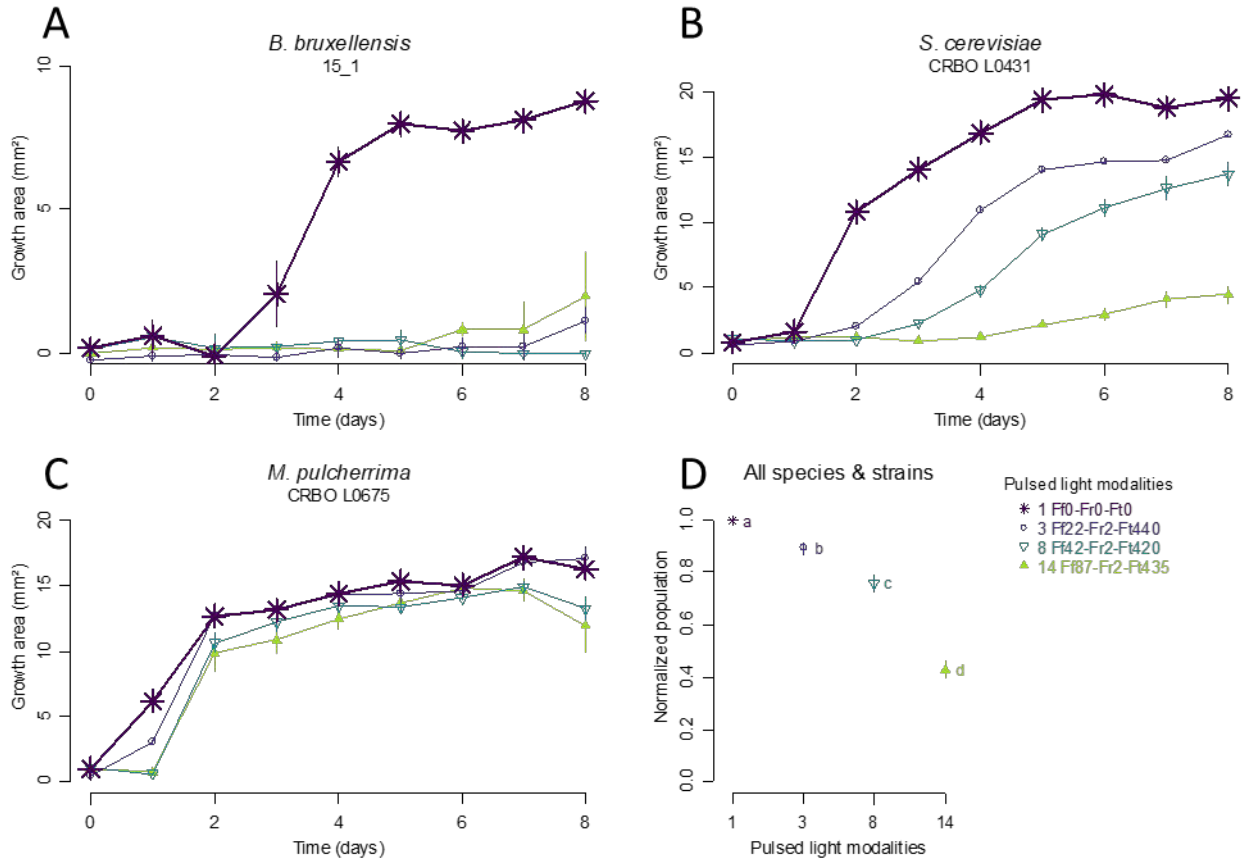

Figure 3). For example, *B. bruxellensis* strain 15_1 was highly sensitive to PL for the three fluencies per flash applied (Figure 3A). On the contrary, *M. pulcherrima* strain CRBO L0675 appeared poorly sensitive to all fluencies per flash treatments tested, only a growth delay was observed again (Figure 3C). *S. cerevisiae* CRBO L0431 showed an intermediate trend with a wide variation of response: the greater the fluence per flash, the greater the growth was impacted, 80 %, 60 % and 25 % of the maximal growth corresponding to 22, 42 and 87 $mJ.cm^2$ respectively (Figure 3B). The lag phase was also impacted. In general, yeast species were more affected by higher fluence per flash (Figure 3D). The fluence per flash of 22 $mJ.cm^{-2}$ corresponded to 90% of the maximal growth, 42 $mJ.cm^{-2}$ to 75 % and the 87 $mJ.cm^{-2}$ to 40 %. Thus, for similar total fluence, the impact of fluence per flash had an important impact on yeast growth.

Three groups of three modalities (2, 3 and 4, then 7, 8 and 9, and finally 13, 14 and 15) were used to study the impact of the frequency of flashes (at 1, 2 and 5 Hz) on yeast growth

(Figure 4). Within the three-modality groups, the effects of the fluence per flash and the total fluence were uniform.

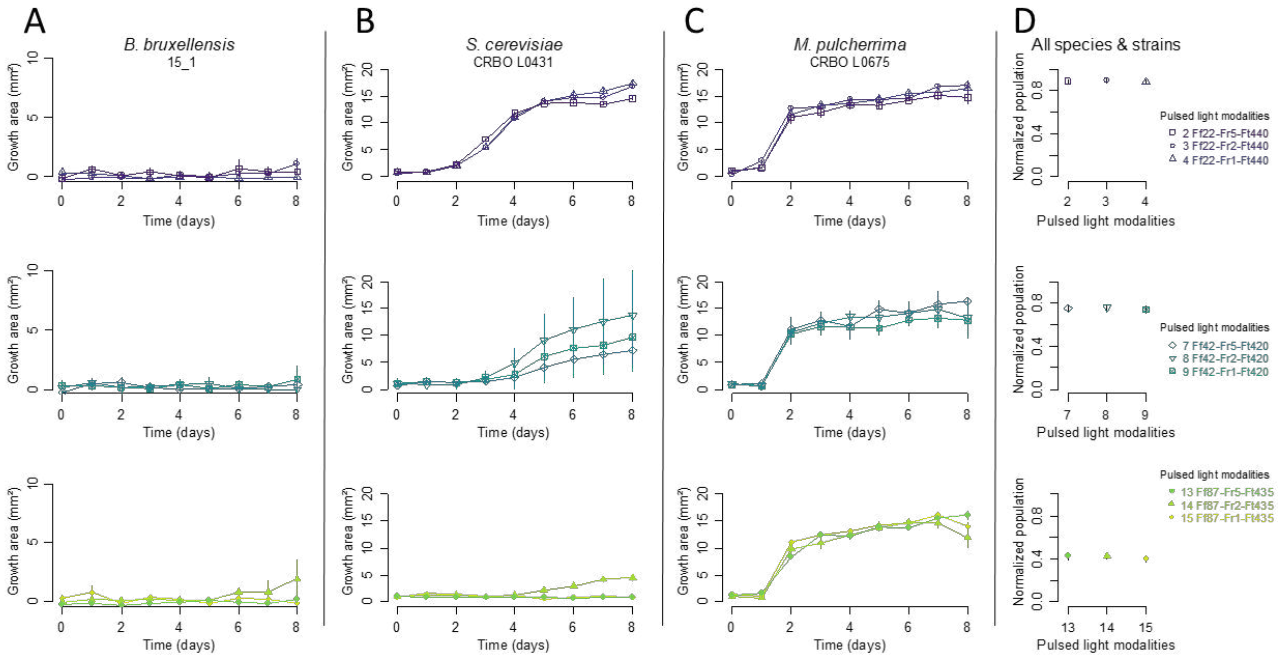

Figure 4 : Growth kinetics depending on the frequency applied on the pulsed light treatment (1, 2 and 5 Hz). (A) B. bruxellensis 15_1, (B) S. cerevisiae CRBO L0431, (C) M. pulcherrima CRBO L0675, (D) Mean of the Normalized population (Area Under the Curve AUC normalized with the control modality) for all the strains used in this study (198).

Nine modalities were used (2, 3, 4, 7, 8, 9, 13, 14 and 15). Ff stand for fluence per flash (mJ.cm^{-2}), Fr for frequency (Hz) and Ft for total fluence (mJ.cm^{-2}). The density of 100 cells/drop was used for comparison. Growth area was measured in mm².

The growth of the three strains was very similar. The three yeasts strains were not sensitive to the different frequencies used, whatever were the total fluence or the fluence per flash. No significant differences were observed for the three groups of modalities for all strains (198) (Figure 4D). These results indicate that the frequency does not affect the efficiency of the PL treatment in the tested conditions, when considering same fluence per flash and same total fluence.

III.1.2. Impact of PL treatment parameters

To precise the most important factors (fluence per flash, total fluence, frequency as well as their potential interactions) affecting the maximal population size, an ANOVA was performed for each strain. The percent of variation explained by each factor was represented on the bar plot and summarized by species or subpopulations (Figure 5 A). The

fluence per flash was the most impacting factor, explaining around 17% of the total variation of population size for all species, with important variation depending on the strain/species (ranging from 0 % to 37 %). The total fluence also had strong implication and variation regarding the strain/species, with 16.8 % of the total variation (ranging from 1.6 % to 27 %). In accordance with the results above (Figure 4), the frequency impact was small (0.6 %, range of 0 to 3 %). Moreover, interaction between fluence per flash and total fluence was low (3.2% range of 0 to 8%). The interaction between both factors can be seen on the isocurve response (Figure 5 B): at 22 mJ.cm^{-2}, a poor effect of pulsed light treatments on yeast growth was globally achieved, no matter the total fluence, compared to 87mJ.cm^{2}, where the impact of the total fluence was stronger. Indeed, the total fluence is an important factor, but only when the fluence per flash is already sufficient to impact yeast growth.

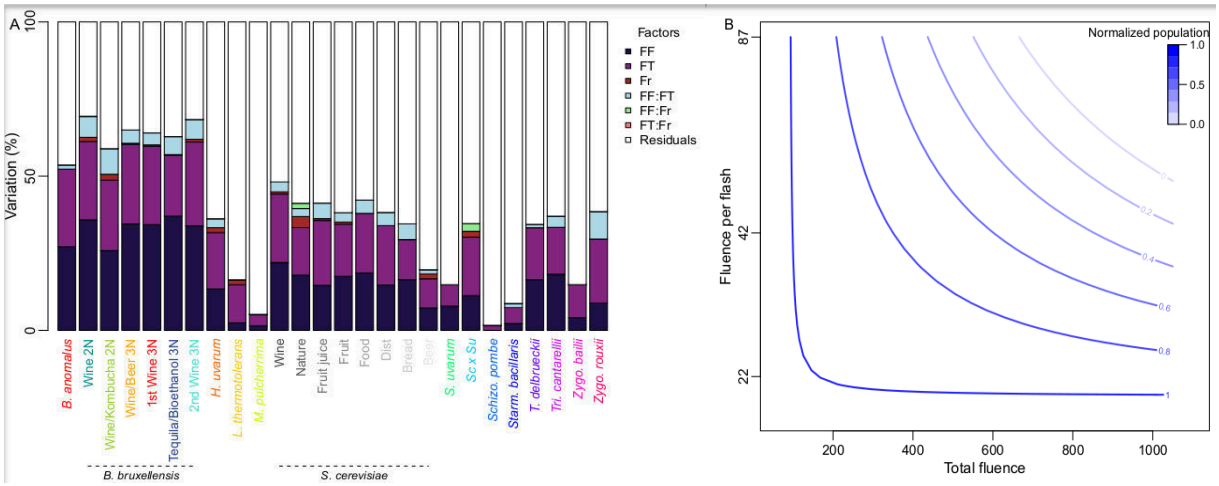

Figure 5 : Factors impacting pulsed light treatment efficiency. (A) Percentage of variation explained by the different factors (fluence per flash, total fluence and frequency) and their interactions for all tested species and strains aggregated by species or subgroups. (B) Isocurve representation of the normalized population size depending on the two main factors, fluence per flash and total fluence.

Overall, these analyses suggested that the fluence per flash is the most important factor that conditioned the efficiency of pulsed light treatment. The total fluence is the other factor that affected PL efficiency on yeast growth, but its effectiveness can be detected if a threshold value of the fluence per flash is exceeded.

These analyses also showed that the impact of these factors varied greatly depending on the species and strain. Both *Brettanomyces* species (*B. anomalus*, 3 strains and *B. bruxellensis*, 111 strains) were significantly impacted by fluence per flash and total fluence, while the three tested strains of *Metschnikowia pulcherrima* or *Starmerella bacillaris* were poorly or not impacted. *S. cerevisiae* and many other species displayed an intermediate behaviour.

III.1.3. Interspecific and intraspecific variability according to PL sensitivity

To analyse the impact of pulsed light treatments on the investigated yeast species, a K-means clustering analysis was performed. The maximal population (area in mm^2) was normalized using the maximal population recorded in absence of PL treatment (modality 1). Four growth trends were identified (Figure 6), group 1 encompassing very sensitive strains (*i.e.* sensitive at low fluence per flash/low total fluence), group 2 and 3 containing strains sensitive to higher values of fluence per flash and/or total fluence, and group 4 being sensitive only to the most efficient modalities. Interestingly, a strong variation depending on the species was observed: all tested strains of *L. thermotolerans, M. pulcherrima, Starm. bacillaris, Zygo. bailii*, *T. delbrueckii* clustered in the less sensitive group (G4). Conversely, strains of *B. anomalus* and *B. bruxellensis* were exclusively distributed in the most sensitive groups (G1-G3).

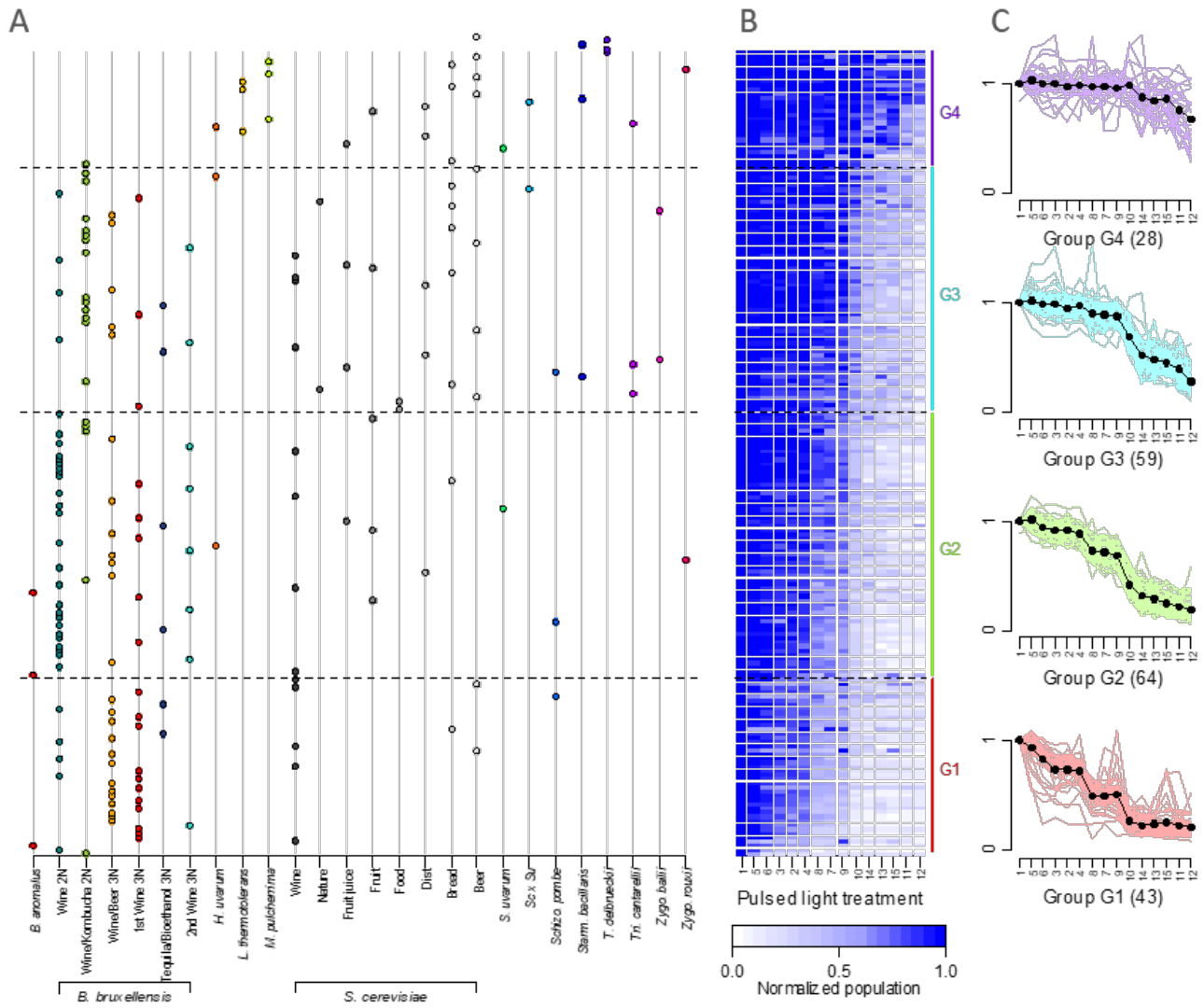

Figure 6 : Clustering of 198 yeast strains depending on their sensitivity to pulsed light treatment.

K-means clustering identified an optimum of 4 groups (G1-G4), G1 containing the more sensitive strains and G4 the less sensitive ones. Sc x Su stands for S. cerevisiae x S. uvarum. (A) Distribution of the strains in the different sensitivity groups per species and/or substrates. (B) Heatplot showing the normalized population size (one line per strain) depending on the 15 modalities of pulsed light treatment. Modalities were ordered from the less to the most impacting on yeast growth: 1, 5, 6, 3, 2, 4, 6, 7, 9, 10, 14, 13, 15, 11 and 12. (C) Bidimensional plot showing the normalized population depending on the 15 modalities of pulsed light treatment for each group.

A focus was made on *S. cerevisiae* and *B. bruxellensis* strains to explore the intra-specific diversity to PL response. To compare the different strains, the maximal growth means of all applied treatments was used as proxy and presented in Figure 7.

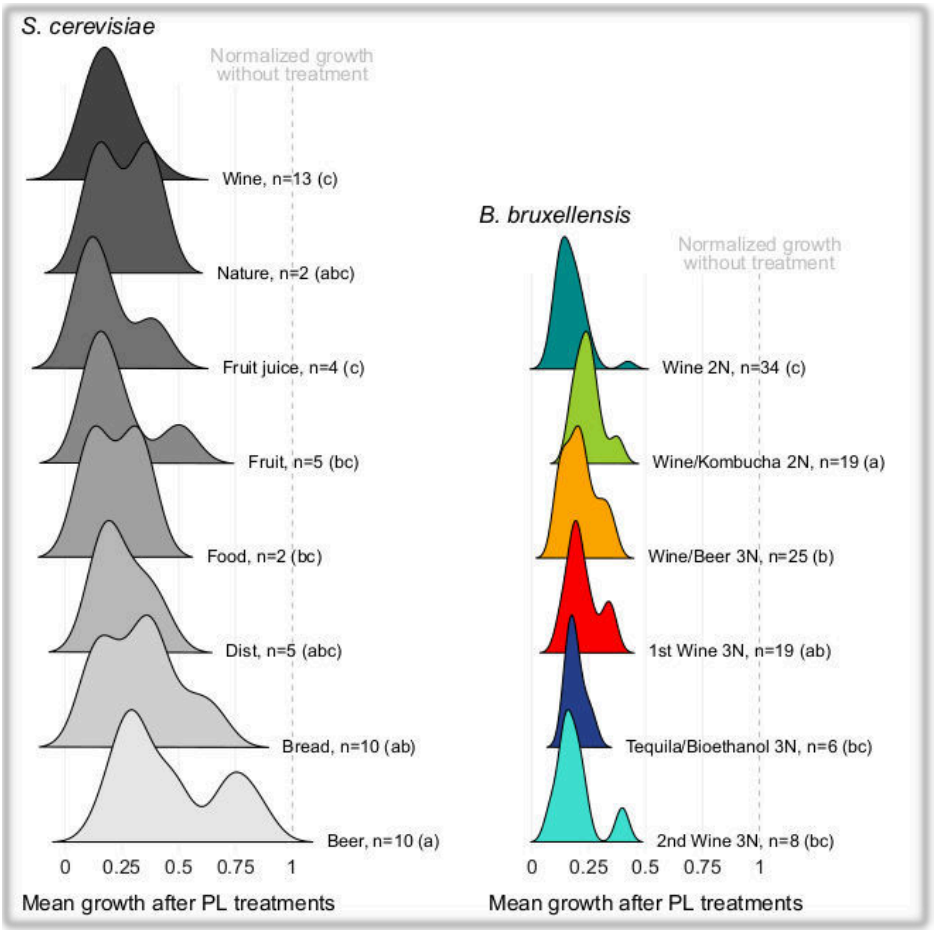

Figure 7 : Influence of pulsed light treatments on the growth of S. cerevisiae and B. bruxellensis subgroups.

AUC means 'Area Under the Curve' and represents the normalized mean growth for the PL modalities. For each subgroup, a Kruskal-Wallis test was performed on AUC to identify significant differences between groups. Different letters (in brackets) denote different significance groups. 2N and 3N stand for diploid and triploid respectively.

For *S. cerevisiae*, strains associated with brewery, bakery and distillery processes were significantly less sensitive to PL treatments compared to wine and fruit juice strains. Wild isolates and strains associated with food spoilage (Nature, Food, Fruit, Fruit juice groups) showed intermediate behaviour. By contrast, all six subpopulations of *B. bruxellensis* showed lower maximal growth than *S. cerevisiae*, confirming that *B. bruxellensis* was more sensitive to PL treatments. In the case of *B. bruxellensis*, PL impact varied depending also on the genetic group, with Wine/Kombucha 2N group less sensitive to PL treatment. Wine 2N, Tequila/Bioethanol 3N and 2nd Wine 3N were the most sensitive. Wine/Beer 3N and 1st Wine 3N had an intermediate behaviour. Altogether, these results showed that the sensitivity to PL treatments varied depending on the yeast species, but also depending on the origin of the strains.

III.2. Sensitivity to PL treatment of various yeasts species and strains in red wine

In the second part of this study, the PL potential was evaluated for the microbial stabilization of various inoculated yeasts species and strains in red wine (Figure 1). We selected six strains from *B. bruxellensis* belonging to the three main wine-related genetic groups (Avramova, Cibrario, et al., 2018) already used in another related study (Pilard et al., 2021). To compare the relative sensitivity of these species, two *S. cerevisiae* and one *L. thermotolerans* strains were also selected. Thus, nine yeast strains were submitted to four different PL treatments (Figure 8). The $\log_{10}$ reduction from the T0 population was used for the different statistical analyses, to consider variations in initial populations. ANOVA analysis was made to determine the impact of the PL and the strain groups on yeast growth (used in Figure 8). The PL treatments explained 81 % of the variation, while 7 % were explained by the strain groups.

Concerning the impact of each modality, no significant differences in viability were observed between the T0 and OFF modalities, evidencing that pumping through the apparatus had no impact on cell viability. The 3 PL modalities, T1 (7.6 $J.cm^{-2}$), T2 (15.2 $J.cm^{-2}$), T3 (22.8 $J.cm^{-2}$) were significantly different from T0 and OFF modalities and from each other, the higher the fluence, the higher the logarithmic reduction detected.

Regarding the sensitivity to PL for the three species, *B. bruxellensis* strains were all highly sensitive compared to other species, resulting approximately in 1 $\log_{10}$ $CFU.mL^{-1}$ reduction with T1 treatment, 3 to 5 $\log_{10}$ $CFU.mL^{-1}$ reduction for T2 treatment, and finally more than 6 $\log_{10}$ $CFU.mL^{-1}$ reduction (below our detection threshold for each strain) with T3 treatment (Figure 8). *S. cerevisiae* strains expressed the lowest sensitivity, with 0.5, 1 and 2 $\log_{10}$ $CFU.mL^{-1}$ reduction respectively for treatments T1, T2 and T3. *L. thermotolerans* strain showed an intermediate response to PL compared to the other two species, with 0.5, 2 and 5 $\log_{10}$ $CFU.mL^{-1}$ reduction respectively for treatments T1, T2 and T3.

Considering the variation within *B. bruxellensis* genetic groups, the Wine 2N and the 1st Wine 3N strains were significantly more sensitive than the Wine/Beer 3N strains. *L. thermotolerans* CLIB292 expressed an intermediate sensitivity, between *B. bruxellensis*

and *S. cerevisiae* strains. The two *S. cerevisiae* strains had similar response of growth reduction following PL treatments.

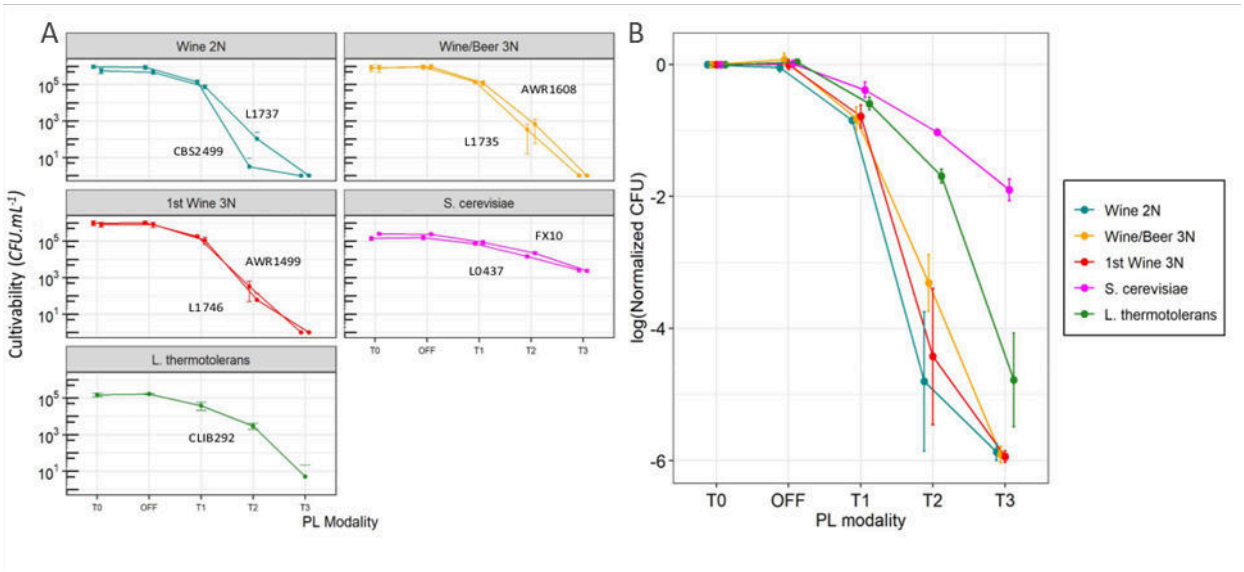

Figure 8 : Cultivability of 9 strains after different PL treatments. (A) Cultivability is expressed in CFU.mL^{-1}. (B) The log reduction was normalized using the T0 cell cultivability.

Normalized survival curves are grouped by genetic groups or by species, normalized using the mean at T0. The means of triplicates +/- standard errors were represented for each modality.

IV. Discussion

The present study aimed to explore the PL sensitivity of yeasts related to winemaking and vineyard. A large collection of yeasts was used, encompassing 14 species and 198 strains, and a rapid plate screening method using 15 PL treatments was applied. Furthermore, experiments on nine strains from 3 species were carried out in a continuous flow PL treatments chamber to evaluate the necessary dose for microbial inactivation and validate the species variability observed on plate screening.

IV.1. Total fluence and fluence per flash governing the PL efficiency on solid media

The impact of three different treatment parameters governing the PL treatment (*e.g.,* fluence per flash, total fluence and frequency) was investigated. Fluence per flash and total fluence strongly impacted the growth reduction (17 % and 16.8% respectively). An increasing dose for both parameters was correlated with a higher growth reduction. Although the two parameters were dependent and interlinked for the efficiency of the treatment, similar total fluencies did not induce the same germicidal effect depending on the way the energy was

distributed. Indeed, the minimum fluence per flash tested (22 µJ.cm^{-2}) weakly impacted cells growth whatever the total fluence applied (Figure 5 B). Two non-mutually exclusive hypotheses can be made: (i) the content of wavelengths emitted changed depending on the lamp voltage, (ii) a minimal fluence per flash threshold is needed, indicating a possible "peak effect" (Gómez-López, Ragaert, Debevere, & Devlieghere, 2007). Concerning the variation of wavelength content depending on the lamp voltage, it has been showed that UV content could decrease when lamp voltage decreased. Considering that PL efficiency relies (at least partially) on UV-C content, it could explain the lack of low fluence per flash germicidal effect observed here. In addition, some studies point up a possible additional "peak effect" with high energy treatments which could contribute to the germicidal effect of pulsed light (Levy et al., 2012). The existence of this fluence threshold is in contradiction with Bunsen-Roscoe law, which enounces that only the total fluence governs the PL efficiency (Gómez-López & Bolton, 2016; Kramer et al., 2017b). Violation of this law has already been observed on bacteria and fungi in surface treatments with PL, on *Aspergillus niger*, as well as in continuous flow treatments of *Listeria innocua* (Artíguez, Lasagabaster, & Marañón, 2011; Kramer, Wunderlich, & Muranyi, 2017a; Levy et al., 2012; Luksiene, Gudelis, Buchovec, & Raudeliuniene, 2007). Demonstrating a specific effect of high fluence per flash treatments (not due to the variations of UV content in the emitted spectrum) remains an issue, unresolved by our study whose aim was to determine the impact of three PL parameters (fluence per flash, total fluence and flash frequency) on 14 wine yeast species. Here, the frequency modulation (between 1 and 5 Hz) had almost no effect (0.06 %) on PL treatment efficiency. This indicates that for PL applications, the flash frequency can be increased (or reduced) maintaining the same total fluence without affecting the germicidal efficiency, which can be interesting to reduce the treatment time needed at the industrial scale. However, subsequent work will have to measure the possible impact of flash frequency on the wine temperature (not measured here), and its subsequent impact on wine organoleptic quality.

IV.2. Cellar resident yeasts expressed higher sensitivity to PL than vineyard yeasts

Concerning the sensitivity between the different yeast species, a great variability was observed (Figure 6). *Brettanomyces* species were highly sensitive to PL, while on the contrary

L. thermotolerans, M. pulcherrima, Starm. bacillaris, Zygo. bailii, *T. delbrueckii* were scarcely impacted. A third group, composed of *H. uvarum*, *Saccharomyces* sp., *Schizo. pombe*, *Tri. cantarelli*, *Zygo. Rouxii*, showed an intermediate behaviour. This ranking is globally in accordance with a previous plate screening study led on continuous UV-C treatments, where *Brettanomyces* species were highly impacted when other species such as *M. pulcherrima* or *Starm. bacillaris* were less affected (Pilard et al., 2021). These results are in adequation with the well-described overriding role of UV-C into PL efficiency (Keener & Krishnamurthy, 2014). Secondary metabolites (photoprotective pigments, mycosporine-like amino acids) or coat proteins (Bisquert, Muñiz-Calvo, & Guillamón, 2018; Clair et al., 2020; Gao & Garcia-Pichel, 2011; Singaravelan et al., 2008), DNA repair mechanisms (photoreactivation and dark repairs mechanisms) (Friedberg, Walker, Siede, & Wood, 2005; Zhang, Wang, & Zhong, 2017), wall thickness and composition (Beauchamp & Lacroix, 2012) and, possibly, clustering ability (shielding effect) are factors involved in the protection of microorganisms from light damages, whose amount or efficiency may vary from one species to another. The less sensitive species to PL are particularly associated with vineyards and grape berries, therefore frequently exposed to sun light (Sipiczki, 2016; Varela & Borneman, 2017; Zott, Miot-Sertier, Claisse, Lonvaud-Funel, & Masneuf-Pomarede, 2008). On the contrary, *B. bruxellensis* or *B. anomalus* are barely isolated from grape berries but mainly from anthropized environments (cellar, equipment, barrels), thus less exposed to UV radiations (Loureiro & Malfeito-Ferreira, 2003; Wang, García-Fernández, Mas, & Esteve-Zarzoso, 2015). UV radiations were already shown to influence and modulate yeast community at ecological scale (Libkind, Moliné, Sampaio, & Van Broock, 2009; Longan, Knutsen, Shinkle, & Chosed, 2017). *T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima*, which were less affected by PL treatments, can be used by the winemakers as technological auxiliaries for acidification, biocontrol agent or to improve aromas in grape musts (Roudil et al., 2019; Windholtz et al., 2021). Thus, PL treatments might be used to eliminate the unwanted yeasts, without impairing the establishment of positive ones.

Regarding the results on red wine PL treatments, the sensitivity of *B. bruxellensis* strains was also highly marked, with 6 $\log_{10}$ CFU.mL^{-1} reduction (below the detection limit) compared to *S. cerevisiae* strains (2 $\log_{10}$ CFU.mL^{-1} reduction) for a total fluence of 22.8 J.cm^{-2} (Figure 8). This result is in agreement with the plate screening results. The viability reduction of

B. bruxellensis was strong, which underlines the interest of using PL to prevent wine spoilage caused by this yeast. Surprisingly, *L. thermotolerans* strain, which was among the less sensitive strain in plate screening, was more impacted than *S. cerevisiae* in the wine. This could be explained by the physiological state of this species in red dry wine: indeed, the adaptation of this species to wine was long and tedious compared to the others (data not shown).

IV.3. Intraspecific sensitivity within *B. bruxellensis* and *S. cerevisiae*

A focus on *B. bruxellensis* (111 strains) and *S. cerevisiae* (49 strains) yeast species was made, for their relevance in beverages (Hirst & Richter, 2016; Tubia et al., 2018). Although *S. cerevisiae* species was less sensitive than *B. bruxellensis*, important variability was observed between groups in both species (Figure 7). Within *B. bruxellensis* genetic subpopulations as recently defined by Eberlein (Eberlein et al., 2021), the Wine 2N isolates were more affected than the 1st Wine 3N and Wine/Beer 3N isolates on plate screening. This trend was also observed in wine treatment, with a small subset of strains. Surprisingly, the Wine 2N group was found to be much affected by PL than the 1st Wine 3N and Wine/Beer 3N groups, while this group expressed lower sensitivity to continuous UV-C treatments (Pilard et al., 2021). This might be attributable to the additional effects of photothermal and photophysical mechanisms due to PL high intensity and large emission spectrum. Regarding *S. cerevisiae*, different behaviours were observed within the subpopulations described, but the Wine and Fruit juice isolates showed an increased sensitivity compared to isolates from Beer and Bread environments. Although being responsible of the necessary fermentation of sugar into ethanol, *S. cerevisiae* can act as spoiling microorganism causing an unwanted 'refermentation' of sweat wine due to its ethanol and SO_2 resistance (Divol, Miot-Sertier, & Lonvaud-Funel, 2006; Malfeito-Ferreira, 2019). Hence, PL utilization for *S. cerevisiae* managing in winemaking might be a promising track.

V. Conclusion

This study brings information on the impact of three factors governing PL treatments on yeast sensitivity (fluence per flash, total fluence, and frequency). In accordance with previous studies, total fluence and fluence per flash affected the PL efficiency. In addition, our results suggest that the fluence per flash must be set at or above a threshold value to

guarantee the effectiveness of PL treatment (due to UV decrease and/or peak effect). Once this threshold value is reached, higher fluencies per flash and higher total fluencies increased the treatment efficiency. By contrast, the frequency does not affect the treatment efficiency, although its impact on wine temperature remains to be assessed. Thus, a possible reduction in processing times can be envisioned and optimization for the continuous processing of liquids can be developed using efficient criteria. In addition, a strong diversity regarding the PL sensitivity was observed at inter specific level. By chance, spoilage yeasts showed higher sensitivity to PL treatments than "beneficial" yeasts. These results were confirmed in continuous flow PL treatments of red wine, *B. bruxellensis* strains being highly sensitive (6 $\log_{10}$ CFU.mL^{-1} reduction, below the detection limit, for a total fluence of 22.8 J.cm^{-2}). However, considering PL application for winemaking, the impact of the treatment on wine's organoleptic properties should be evaluated, which has not been done in this study and will be the subject of further work. The right combination of fluence per flash and total fluence should be used to efficiently stabilize wines with no or minimal impact of PL on wine's properties. Chemical and sensorial analyses of PL treated wines must be carried out to address these questions.

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VIII. Declaration of interest

The co-author Philippe Marullo is affiliated with BIOLAFFORT. This does not alter the authors' adherence to all the journal's policies on sharing data and materials.

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