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Wine yeast species show strong inter- and intra-specific variability in their sensitivity to ultraviolet radiation

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Abstract:

While the trend in winemaking is toward reducing the inputs and especially sulphites utilization, emerging technologies for the preservation of wine is a relevant topic for the industry. Amongst yeast spoilage in wine, *Brettanomyces bruxellensis* is undoubtedly the most feared. In this study, UV-C treatment is investigated. This non-thermal technique is widely used for food preservation. A first approach was conducted using a drop-platted system to compare the sensitivity of various strains to UV-C surface treatment. 147 strains distributed amongst fourteen yeast species related to wine environment were assessed for six UV-C doses. An important variability in UV-C response was observed at the interspecific level. Interestingly, cellar resident species, which are mainly associated with wine spoilage, shows higher sensitivity to UV-C than vineyard-resident species. A focus on *B. bruxellensis* species with 104 screened strains highlighted an important effect of the UV-C, with intra-specific variation. This intra-specific variation was confirmed on 6 strains in liquid red wine by using a home-made pilot. 6624 J.L⁻¹ was enough for a reduction of 5 log₁₀ of magnitude for 5 upon 6 strains. These results highlight the potential of UV-C utilisation against wine yeast spoiler at cellar scale.

Keywords : UV-C treatment, yeast, microbial stabilization, *Brettanomyces bruxellensis*

1. Introduction

In food industry, microbial stabilization is crucial to ensure good storage and aging. In the winemaking industry, wine stabilization is historically managed by using sulphite (SO_2) that has both antioxidant and antimicrobial properties. However, there is a trend towards reducing the doses of sulphites in wines (1). This can be explained by (i) health concerns and a growing consumer preference for wines with the least possible added input; (ii) the fact that strongest sulphite doses are required for an optimal efficiency due to the increase of wine's pH (2) and (iii) because of the emergence of sulphite resistant strains amongst spoilers (3). Microfiltration and flash-pasteurization are the main technologies used in conventional winemaking even if they are both expensive, highly energy consuming and known to have potential negative impact on both colour and aromatic profile of wines. That is why other chemical and physical control methods are intensively studied (reviewed by (4–6)).

Recent studies suggested that UV-C treatment (253.7 nm) could be an alternative technology to inactivate microorganisms in grape juices and is already used for surface equipment disinfection (7–10). This technology has been widely investigated for two decades and is regarded as a promising approach for fruit juice stabilization with minimal negative impacts (11–13). Microbial inactivation caused by UV-C radiation is based on the rearrangement of the microorganism's nucleic acid and DNA-protein cross link (Cyclobutane Pyrimidine Dimers, CPDs) which directly interferes with the ability of microorganisms to reproduce (14–17). Different studies showed a wide spectrum of inactivation of wine-associated microorganisms in different types of wines and grape juices. Fredericks et al, Diesler et al, and Junqua et al demonstrated a reduction of 5 to 6 \log_{10} of magnitude in yeast and bacteria species in various wines and grape musts (7–9). Those studies showed a positive correlation between UV-C dosage and microbiological stabilisation, with the treatment efficiency depending on physical properties of the matrix (e.g. optical density at 254nm, turbidity), the considered species and the population level. For example, to obtain a reduction of 6 \log_{10} of magnitude of *S. cerevisiae*, 800 J.L^{-1} are required for rosé and white wines ($\alpha_{254\text{nm}} < 10\text{cm}^{-1}$) when 5 000 J.L^{-1} is necessary for red wine ($\alpha_{254\text{nm}} = 47\text{cm}^{-1}$) (9). To obtain a reduction of 6 \log_{10} of magnitude of *S. cerevisiae*, 600 J.L^{-1} are required for clear Riesling must (turbidity 26.7 NTU) when 800 J.L^{-1} is necessary for Müller-Thurgau must (turbidity 36 NTU) (8). Regarding population level, 600 J.L^{-1} allow the stabilization of Müller-Thurgau must

inoculated at 10^4 cell.mL⁻¹ initial cell count, when 1 400 J.L⁻¹ is not sufficient for 10E08 initial cell count (8). Considering the bacteria and yeast, 200J.L⁻¹ are sufficient to inactivate *Acetobacteria aceti* when 400J.L⁻¹ are required for *Brettanomyces bruxellensis* and 600J.L⁻¹ for *S. cerevisiae* (9). However, these studies compared only a few number of species and strains and did not take into account the intraspecific variability.

In winemaking, a plethora of yeasts are present, originating from the vineyard (e.g. present on the grape surface) or from the winery. These species can be considered as positive, neutral or negative from a winemaking viewpoint (18). Wine yeast spoilage is problematic due to contamination by *Brettanomyces bruxellensis*, *B. anomalus*, *Schizosaccharomyces pombe*, *Zygosaccharomyces rouxii*, *Z. bailii*, *Trigonopsis cantarellii*, etc. (18–20). Among them, *B. bruxellensis* is considered as the major contaminant in wine due to its ability to produce volatile phenols (4-ethylphenol, 4-ethylguaiacol and 4-vinylphenol) causing the ‘Brett’ taint described as barnyard, horse sweat or burnt plastic (21). *B. bruxellensis* can spoil up to 25-30% of red wine production (22,23). This species displays a huge genetic and phenotypic diversity (24–27). Isolates cluster in six genetic groups depending on the ploidy level, the substrate of isolation and geographical niches (24–26). *B. bruxellensis* strains isolated from wine mainly belong to three genetic groups defined as 1st Wine 3N (or AWRI1499-like), Wine 2N (or CBS2499-like) and Wine/Beer 3N (or AWRI1608-like) which respectively encompass 39%, 37% and 16% of isolates (24,28). Concerning control methods for wine production, *B. bruxellensis* is an actual challenge (4): up to 45% of wine isolates are sulphite tolerant or resistant (29). Tolerant/resistant strains mainly belong to the 1st Wine 3N group. In addition, *B. bruxellensis* isolates exhibit variable sensitivity to other treatments such as chitosan (30,31). Recently, Paulin et al showed that, in a representative collection of 53 *B. bruxellensis* isolates, 41% of strains were sensitive to chitosan, 13% of tolerant and 46% with intermediary behaviour (32). Thus, it is necessary to take into account the phenotypic variability of *B. bruxellensis* in order to properly assess its UV-C sensitivity to microbial treatment, by using a representative panel of strains belonging to the different genetic cluster of this species.

In this work, we first evaluated the efficiency of UV-C treatment using a plate screening (surface) approach. We tested 14 yeast species and 147 strains associated with winemaking, in order to appreciate the relative interspecific variability. We particularly focused on

B. bruxellensis species for which 104 representative isolates were tested. Considering the first results, we selected 6 strains of *B. bruxellensis* belonging to the three main genetic groups found in wine and harbouring different sensitivity to UV-C on solid medium in order to assess the efficiency of UV-C treatment in (liquid) red wine.

2. Materials and method

2.1. Yeast strains

The 147 strains from 14 species used in this study were collected from different origins: the CRB Oenologie collection (Centre de Ressources Biologiques Oenologie, Institut des Sciences de la Vigne et du Vin, France) or other laboratories/collections (Table 1). Strains were grown and maintained in 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).

Table 1: Strains used in this work. The reference, collection of these strains are presented in supplemental Table 1.

Species	Number and strains ID
<i>B. anomalus</i>	3 strains: BR 23-4 ; CLIB 304 ; NRRL Y-17522 T

<i>B. bruxellensis</i>	<p>1st Wine 3N - 22 strains: 2OT13_02 ; 33_2 ; AWRI1499 ; GB12 ; GSP1509 ; CRBO L0417 ; CRBO L14155 ; CRBO L14174 ; CRBO L14175 ; CRBO L14194 ; CRBO L1703 ; CRBO L17111 ; CRBO L1727 ; YJS5408 ; YJS5434 ; YJS5445 ; YJS5459 ; YJS5469 ; YJS5473 ; YJS5476 ; YJS5478 ; YJS5487</p> <p>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</p> <p>Wine 2N - 30 strains: 1961_MX_M1_E2 ; CBS 2499 ; ISA2150 ; CRBO L0469 ; CRBO L0614 ; CRBO L14163 ; CRBO L1714 ; CRBO L1751 ; YJS5302 ; YJS5319 ; YJS5320 ; YJS5345 ; YJS5347ww ; YJS5357 ; YJS5373 ; YJS5385 ; YJS5392 ; YJS5416 ; YJS5422 ; YJS5426 ; YJS5440 ; YJS5447 ; YJS5449 ; YJS5453 ; YJS5456 ; YJS5458 ; YJS5461 ; YJS5463 ; YJS5479 ; YJS5485</p> <p>Wine/Beer 3N - 21 strains: 2OT13_05 ; 2OT13_07 ; 2OT14_01 ; 2OT14_03 ; AWRI1608 ; CDR222 ; GB62 ; ISA2397 ; CRBO L17112 ; CRBO L1741 ; CRBO L1749 ; CRBO L1771 ; LB15107g ; LB15110g ; NL045 ; NL059 ; VP1519 ; YJS5396 ; YJS5397 ; YJS5400 ; YJS5454</p> <p>Tequila/bioethanol 3N - 5 strains: CRBO L14169 ; CRBO L17108 ; CRBO L1715 ; SJ12_4 ; UWOPS_92__298_4</p> <p>2nd Wine 3N - 7 strains: ISA2211 ; CRBO L0308 ; CRBO L1733 ; CRBO L1782 ; VP1539 ; VP1544 ; YJS5382</p>
<i>H. uvarum</i>	3 strains: L1433 ; NZ15 ; Y-1614
<i>L. thermotolerans</i>	3 strains: 18 ; AEB ; CLIB292
<i>M. pulcherrima</i>	3 strains: L0675 ; NZ268 ; Y-7111
<i>S. cerevisiae</i>	10 strains: A24 ; Fx 10 ; GN ; CRBO L0431 ; CRBO L0432 ; CRBO L0433 ; CRBO L0437 ; SB ; X5 ; Y7327
<i>S. uvarum</i>	2 strains: U1 ; U3
<i>S. cerevisiae</i> x <i>S. uvarum</i>	2 strains: DU23 ; EU23
<i>Schizo. pombe</i>	3 strains: CRBO L0442 ; Y-11791 ; Y-12796
<i>Starm. bacillaris</i>	3 strains: 10_372 ; CRBO L0473 ; NZ12
<i>T. delbrueckii</i>	3 strains: B172 ; CLIB 230 ; CRBO L0705
<i>Tri. cantarellii</i>	3 strains: CRBO L0412 ; CRBO L0416 ; CRBO L0419
<i>Zygo. bailii</i>	3 strains: CLIB 213 ; CRBO L0446 ; CRBO L0536
<i>Zygo. rouxii</i>	2 strains: CLIB 233 ; CRBO L0314

2.2. Surface UV-C treatment of yeast

Strains were grown in liquid media (YPD 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). Two µl of serial dilutions (0.5, 0.05 and 0.005 DO) were spotted onto solid medium (YPD), aiming at 3 different densities (around 1000, 100 and 10 CFU/drop).

Drops were performed in triplicate for each condition. Spotted plates were exposed to increasing UV-C doses using an apparatus made at the laboratory. The UV-C treatment unit consists of a box containing two 45W@UV-C low-pressure mercury lamps placed 15cm above the plates. Six increasing UV-C doses (0, 2000, 4000, 6000, 7500 and 10 000 $\mu\text{J}\cdot\text{cm}^{-2}$) were applied by varying the exposure times. UV-C doses were monitored (in $\mu\text{J}\cdot\text{cm}^{-2}$) using an UV-C sensor (HD2102.2 with LP 471 UV-C probe from DeltaOHM, Italy).

2.1.3. Growth monitoring from agar-plates

After treatment, plates were incubated at 24°C in the dark. The growth was monitored every day: plates were imaged from an illuminated desk to avoid light gleam (model DMC-FS7, Panasonic Corporation, Japan, see Figure 2A, D, G, J). Growth data was analysed with custom-made scripts in R (R Development Core Team, 2013). Briefly, 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 pixel) was calculated using automatic background subtraction. The dataset (around 90 000 measures including triplicates) was used to represent growth kinetics (Figure 2B, E, H, K).

In order to analyse and normalize the obtained dataset, the growth kinetics were expressed as AUC (Area Under Curve). This unique parameter takes into account variations in lag-phases, exponential phases and the stationary phases. For each treatment and strain, growth kinetics were fitted using a three-parameter log-logistic distribution, AUCs were calculated and then normalized against AUCs without UV-C treatment (*Normalized AUC*, see Fig 2C, F, I, L). AUC curves were subsequently analysed using K-means clustering (*cutRepeatedKmeans* function from *ClassDiscovery* package). Multi-way analyses of variance (ANOVA) were performed followed by post-hoc Tukey tests (*HSD.test* function from *agricolae* package).

2.3. UV-C treatment from liquid red wine

100 liters of SO₂ free 2018 Cabernet Sauvignon wine were used in this study for both yeasts adaptation and UV-C treatments. Wine's absorption coefficient at 254nm (α_{254}) and turbidity were equal to 31.6 cm⁻¹ and 170 NTU respectively. The wine was pasteurized and kept at

10°C until UV-C treatment. Wine sterility was controlled after pasteurization and before inoculation by plating on Total yeasts, Non-Saccharomyces yeasts, Lactic Acid bacteria and Acetic bacteria medias.

AWRI1608, L1735, L1737, CBS2499, AWRI1499 and L1746 *B. bruxellensis* strains were selected for this liquid assay. Those strains were firstly grown on YPD plates, and then inoculated in YPD liquid medium (24°C). Yeasts were adapted to wine by successive transplantings in pasteurised wine. Populations were monitored by counting on Malassez cell with addition of methylene blue. Each strain was inoculated in pasteurised red wine for test at a concentration of 10^4 CFU.mL⁻¹.

2.2.5. UV-C module treatment

The UV-C module consisted of a low-pressure mercury amalgam germicidal lamp, 78 cm long with maximum peak radiation at 254 nm, surrounded by a quartz sleeve (Suzhou Xicheng Water Treatment Equipment, China). A food grade fluorinated ethylene propylene (FEP) tube (Serto S.A.R.L, France), diameter 8/10 mm (inner/outer), chosen for its physical properties (flexibility and good UV-C transmittance), was coiled around the quartz sleeve (Figure 1).

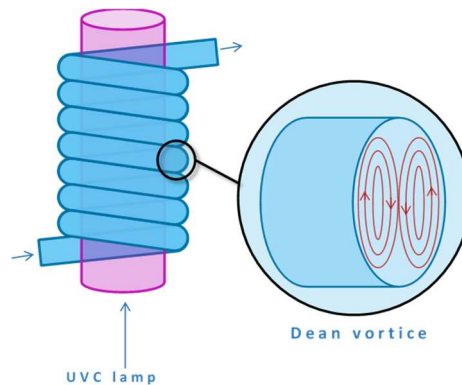


Figure 1: Schematic representation of the coiled UV-C reactor

Due to the curvature of the pipe and hydrodynamic conditions applied (9,33), Dean Vortices are generated allowing an homogenous treatment. Wine flow rate in the pipe was 200 L.h⁻¹ and the applied dose for a single cycle of treatment was 1656 J.L⁻¹. Wine samples were collected before UV-C treatment (UV0), after four cycles in the pilot with lamps turned off

(OFF), after one, two, three or four cycles (UV1 = 1656 J.L⁻¹, UV2= 3312 J.L⁻¹, UV3= 4968 J.L⁻¹, and UV4 =6624 J.L⁻¹. Between each cycles, the reactor was disinfected using 95% ethanol. Wine samples (50 mL) were collected and stored at 4°C until microbiological analyses were performed. Each trial was conducted in triplicate.

2.2.6. Microbiological analysis

Microbial counts were determined in triplicates by plating serial 10-fold dilutions of the samples and 100 µL was plated in 9cm diameter petri dishes. Yeasts were enumerated on YPD plates with chloramphenicol (0.1 mg.mL⁻¹), biphenyl (0.15 mg.mL⁻¹) and actidione (0.5 mg.mL⁻¹) after 7 days of incubation at 27 °C. All incubations were performed in the dark, as DNA repair mechanisms are known to be less effective in dark conditions (34). The number of colonies counted was expressed in CFU.mL⁻¹ and the limit of detection was 1 CFU.mL⁻¹. Multi-way analyses of variance (ANOVA) were performed followed by post-hoc Tukey tests (*HSD.test* function from *agricolae* package, R software).

3. Results

3.1. Surface UV-C treatment

3.1.1. UV-C treatment and interspecific variability

In this work, UV-C sensitivity was assessed for 147 strains from fourteen yeast species associated with grape must and wine (Table 1). Besides some *Saccharomyces* species (*S. cerevisiae*, *S. uvarum* and some of their hybrids), several non-*Saccharomyces* species considered as beneficial from an oenological viewpoint were tested: *L. thermotolerans*, *T. delbrueckii* and *M. pulcherrima*. Abundant species associated with grapes or pre-fermentative stages such as *H. uvarum* or *S. bacillaris* were also included, as well as six species mostly considered as wine spoilers: *B. bruxellensis*, *B. anomalus*, *Tri. cantarelli*, *S. pombe*, *Z. rouxii* and *Z. bailii*.

Drop-plating was used to study UV-C sensitivity of the 147 yeast strains. Six increasing UV-C doses were applied (0, 2000, 4000, 6000, 7500 and 10 000 µJ.cm⁻²) for three densities (number of initial cells per drop around 10, 100 or 1000). Petri dishes were pictured almost every day during ten days and the growth area was measured using home-made R-script.

The dataset (around 90,000 measures with triplicates) was used to represent growth kinetics with time (Figure 2).

Four different strains, representative of typical behaviours observed, are shown in figure 2 (two *B. bruxellensis*, one *S. cerevisiae*, one *M. pulcherrima*). In general, higher UV-C doses increased the lag-phase and decreased the growth rate and the maximal population (Figure 2B, E, H, K). Growth curves were then used to calculate the normalized AUC (Area Under Curve) represented in Figure 2C, F, I, L. Strain L14175 (*B. bruxellensis*) was highly sensitive to UV-C and showed a strong growth decrease for low UV-C dose (<0.25 of Normalized AUC at 2 000 $\mu\text{J}\cdot\text{cm}^{-2}$, Figure 2C). Strains YJS5456 and L0437 (*B. bruxellensis* and *S. cerevisiae* respectively, Fig2E and 2H) were sensitive to higher dose of UV-C (4000 and 7500 $\mu\text{J}\cdot\text{cm}^{-2}$). By contrast, some strains appeared poorly impacted by low UV-dose: the growth of *M. pulcherrima* strain Y-7111 (Fig1G & 1H) was poorly affected by UV-C-dose lower than 7500 $\mu\text{J}\cdot\text{cm}^{-2}$.

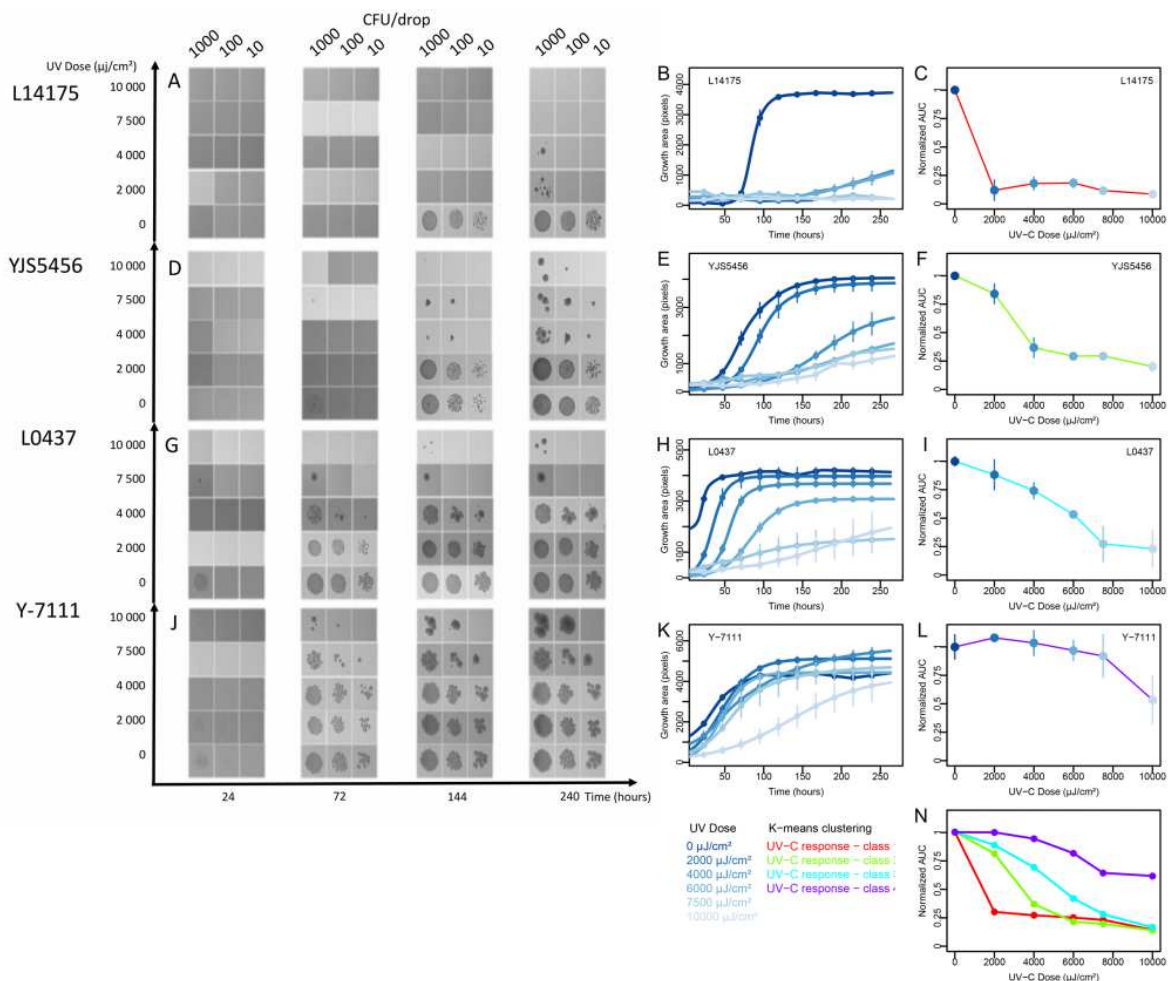


Figure 2: Impact of UV-C treatment on growth of four yeast strains. L14175 (*B. bruxellensis*, A,B,C), strain YJS 5456 (*B. bruxellensis*, D,E,F), L0437 (*S. cerevisiae*, G,H,I) and Y-7111 (*M. pulcherrima*, J,K,L). A, D, G, J are images from the three dilutions made on petri dishes with different UV-C doses. B, E, H, K show growth area (in pixels) over time for increasing UV-C treatment for the same density (1000 cells/drop). Growth kinetics were used to calculate AUC (Area Under Curve), normalized so that AUC equals 1 for UV=0 $\mu\text{J}\cdot\text{cm}^{-2}$. C, F, I, L show normalized AUC depending on UV-C treatment. Colours (red, green, turquoise and violet) represent the different AUC-UV-C dose response classes determined by K-means clustering (N). L14175 and L0437 stand for CRBO L14175 and CRBO L0423 respectively.

K-means clustering was used to sort AUC curves in different profiles, and resolved into four classes (Fig 2N). The fourteen tested species were sorted into one or two classes except for *B. bruxellensis*, which was distributed in three classes (Table 3). K-means class 1 corresponded to the most UV-C sensitive strains, and contained only *B. bruxellensis* strains. By contrast, all tested strains from *S. pombe* or *S. bacillaris* were sorted into class 4, corresponding to less UV-C sensitive behaviour.

UV-C response (K-means clustering)	Species // genetic groups	Number of strains
Class 1	<i>B. bruxellensis</i> // Wine/Kombucha 2N	3/19
	<i>B. bruxellensis</i> // 1st Wine 3N	7/22
	<i>B. bruxellensis</i> // Wine/Beer 3N	5/21
	<i>B. bruxellensis</i> // Tequila/Bioethanol 3N	2/5
	<i>B. bruxellensis</i> // Wine 2N	6/30
Class 2	<i>B. bruxellensis</i> // Wine/Kombucha 2N	10/19
	<i>B. bruxellensis</i> // 1st Wine 3N	15/22
	<i>B. bruxellensis</i> // Wine/Beer 3N	14/21
	<i>B. bruxellensis</i> // Tequila/Bioethanol 3N	2/5
	<i>B. bruxellensis</i> // Wine 2N	17/30
	<i>B. bruxellensis</i> // 2nd Wine 3N	6/7
	<i>B. anomalus</i>	3/3
	<i>Tri. cantarellii</i>	3/3
<i>Zygo. rouxii</i>	1/2	
Class 3	<i>B. bruxellensis</i> // Wine/Kombucha 2N	6/19
	<i>B. bruxellensis</i> // 1st Wine 3N	1/22
	<i>B. bruxellensis</i> // Wine/Beer 3N	2/21
	<i>B. bruxellensis</i> // Tequila/Bioethanol 3N	1/5
	<i>B. bruxellensis</i> // Wine 2N	7/30
	<i>B. bruxellensis</i> // 2nd Wine 3N	1/7
	<i>H. uvarum</i>	1/3
	<i>L. thermotolerans</i>	1/3
	<i>S. cerevisiae</i>	9/11
	<i>Saccharomyces cerevisiae</i> x <i>Saccharomyces uvarum</i>	2/2
	<i>Zygo. bailii</i>	2/3
Class 4	<i>H. uvarum</i>	2/3
	<i>L. thermotolerans</i>	2/3
	<i>M. pulcherrima</i>	3/3

	<i>S. cerevisiae</i>	2/11
	<i>S. uvarum</i>	3/3
	<i>Schizo. pombe</i>	3/3
	<i>Starm. bacillaris</i>	3/3
	<i>T. delbrueckii</i>	3/3
	<i>Zygo. bailii</i>	1/3
	<i>Zygo. rouxii</i>	1/2

Table 2: K-means classes from Normalized AUC with number of strains per species or genetic groups for the *B. bruxellensis* species.

In order to determine whether yeast growth was mostly affected by UV-C treatment, density and/or species, we performed a three-way ANOVA (Table 4, 1st ANOVA). All three factors (Species, Drop density and UV-C dose) were strongly significant, with very low p-values (<0.001). UV-C dose explained most of the percentage of variation of the data (59.99%) followed by species and density factors (8.93% and 1.71% respectively). Post-hoc Tukey tests were performed to determine significance groups (Figure 3): the different UV-C dose were significantly separated from one another, with 10 000 $\mu\text{J}\cdot\text{cm}^{-2}$ associated to the lower Normalized AUC (ie lower yeast growth) (Figure 3A). For drop density, higher initial densities were associated with higher growth (Figure 3B). Finally, Tukey tests revealed significant differences between species, with *Schizo. Pombe* and *M. pulcherrima* showing highest Normalized AUC at high UV-C doses, and *Tri. Canterellii* and *B. anomalus* showing the lowest Normalized AUC (Figure 3C) in accordance with K-means clustering. In particular, *Schizo. pombe*, *M. pulcherrima*, *Starm. bacillaris* and *L. thermotolerans* display a poor sensitivity to the two first UV-C doses used (2 000, 4 000 $\mu\text{J}\cdot\text{cm}^{-2}$, Figure 3C) with a low reduction of the Normalized AUC. For the others species, all UV-C doses affected the yeast growth.

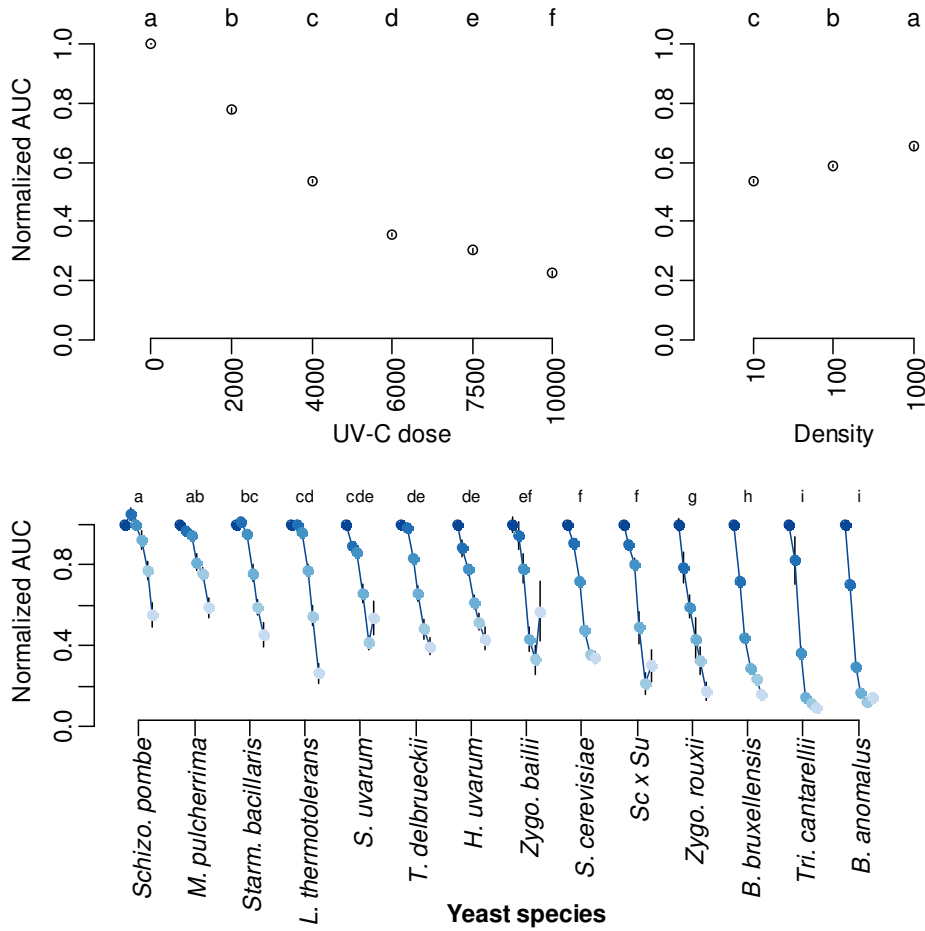


Figure 3: Impact of UV-C treatment (A), drop density (B) and species (C) on yeast growth. Yeast growth was assessed using Normalized AUC. The mean of repetitions +/- standard errors were represented for each factor. Upper letters represent significance groups (ANOVA followed by Tukey's post-hoc test, $\alpha = 5\%$). Sc x Su stands for *S. cerevisiae* x *S. uvarum* hybrids

1st ANOVA			
Factors	Degree of Freedom	Percentage of Variation	P-values
Species	13	8.93%	$<2e^{-16}$
Drop density	2	1.71%	$6.71e^{-127}$
UV-C dose	5	59.99%	$<2e^{-16}$
Residuals	10262	29.37%	

2nd ANOVA

Factors	Degree of Freedom	Percentage of Variation	P-values
Genetic group	5	0.51%	5.39e ⁻³²
Drop density	2	1.47%	1.07e ⁻⁹⁶
UV-C dose	5	74.83%	<2e ⁻¹⁶
Residuals	7197	23.18%	

Table 3 : Impact of various factors on yeast growth. Analysis of variance was performed either on the whole dataset (147 strains distributed in 14 species) or only on the *B. bruxellensis* strains (104).

3.1.2. *B. bruxellensis* sensitivity

In winemaking, the most of the spoilage treat is due to *B. bruxellensis*. Here, we included 104 strains of *B. bruxellensis* distributed in all defined genetic groups (6) to assess their sensitivity to UV-C treatment (table 1). K-means clustering sorted *B. bruxellensis* in three classes, suggesting a large intra-specific variability. To determine whether UV-C treatment, density and/or genetic group affected strain growth, we performed a three-way ANOVA followed by Tukey test (Table 4, 2st ANOVA). All three factors significantly affected *B. bruxellensis* growth (p -values<0.001), with UV-C dose, density and genetic group explaining respectively 74.83, 1.47 and 0.51% of the total variation. The higher the UV-C dose, the lower the growth (Fig 4A) while lower drop densities were associated with lowest growth (Fig 4B). When considering the effect of the genetic group, significant differences were recorded for 2000, 4000, 6000 and 7500 $\mu\text{J}\cdot\text{cm}^{-2}$ UV-C doses. At 2 000 $\mu\text{J}\cdot\text{cm}^{-2}$, three groups (2nd Wine 3N, Wine/Kombucha 2N, Wine 2N) showed higher normalized AUC (Tukey test 'a') than Wine/Beer 3N, 1st Wine 3N and Tequila/Bioethanol 3N (Tukey 'b'). For higher UV-C doses, the rank of the genetic groups changed marginally, but in general, the Wine/Kombucha 2N group was one of the less sensitive group, while the 1st Wine 3N was systematically the most impacted by UV-C.

Finally, at the higher UV-C dose tested (10000 $\mu\text{J}\cdot\text{cm}^{-2}$), the normalized AUCs were below 0.2 for all genetic groups without significant difference, indicating that UV-C treatment is efficient for all *B. bruxellensis* strains.

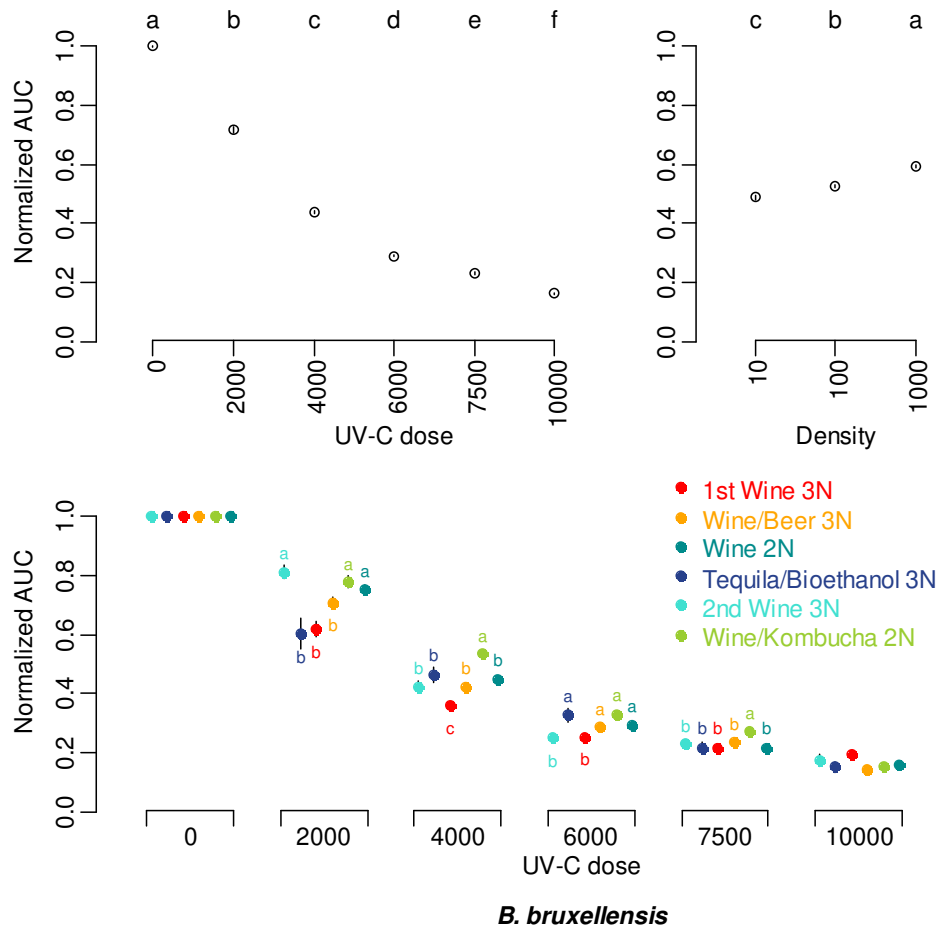


Figure 4: Impact of UV-C treatment (A), drop density (B) and species (C) on yeast growth regarding the genetic groups of 104 strains of *B. bruxellensis*.

Yeast growth was assessed using Normalized AUC. The mean of repetitions +/- standard errors were represented for each factor. Upper letters represent significance groups (ANOVA followed by Tukey's post-hoc test, $\alpha = 5\%$).

3.2. UV-C and liquid media

The impact of UV-C treatment on *B. bruxellensis* strains in liquid wine was evaluated. The UV-C pilot was designed for the treatment of large volumes of wine, and 100 liters of red wine were used to evaluate the impact of different UV-C doses on a few strains. Six strains of

B. bruxellensis were studied, belonging to the three main genetic groups associated with winemaking (Wine 2N, 1st Wine 3N and Wine/Beer 3N). Log₁₀ reduction has been used as the factor describing the strain response to UV-C treatments in wines (Figure 5). A two-way ANOVA was performed to determine the effect of UV-C dose and strain on the treatment efficiency. Both factors were strongly significant (p-value<0.001) and explained most of the percentage of data variation, with 74.4 % for UV-C dose and 3.7 % for strain.

No significant difference was recorded between UV0 and OFF modalities indicating that pumping through the UV-C reactor did not induce cell mortality. UV1 (1656 J.L⁻¹), UV2 (3312 J.L⁻¹), UV3 (4968 J.L⁻¹) and UV4 (6624 J.L⁻¹) treatments were all significantly different from both controls (UV0 and OFF) and between each other, with the greater the UV-C dose applied, the greater the logarithmic reduction achieved (Figure 5A).

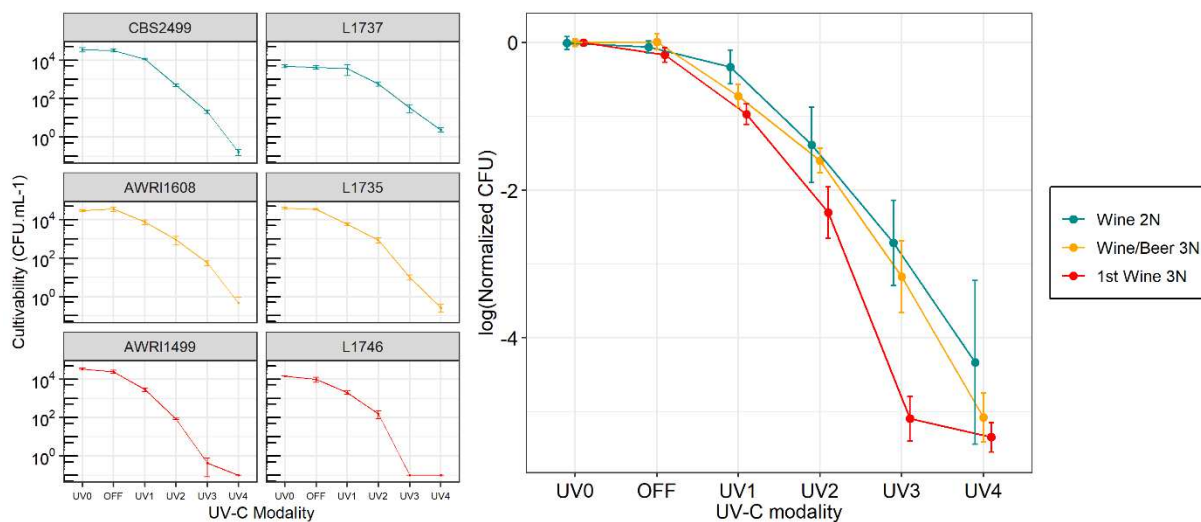


Figure 5: Survival curves of *B. bruxellensis* strains for UV-C treatment in red wine. Cultivability is expressed in CFU.mL⁻¹ (A). Normalized survival curves of *B. bruxellensis* depending on the genetic groups of the strains (B). For each measure, CFU.mL⁻¹ were normalized to the mean of the corresponding UV0 modality (Normalized CFU), the mean of triplicates +/- standard errors were represented for each modality. UV0 correspond to the wine samples collected before UV-C treatment, OFF correspond to four cycles in the pilot with lamps turned off and UV1, UV2, UV3, UV4 after one, two, three and four cycles (UV1 = 1656 J.L⁻¹, UV2 = 3312 J.L⁻¹, UV3 = 4968 J.L⁻¹, and UV4 = 6624 J.L⁻¹). The colours correspond to the genetic groups of the strains. L1737, L1735, L1746 stand for CRBO L1737, CRBO L1735 and CRBO L0423 respectively.

UV3 treatment (4968 J.L⁻¹) was enough to achieve 4.70 and 5.17 log₁₀ reduction for AWRI1499 and L1746 strains (1st Wine 3N sulphite resistant) respectively, resulting in populations lower than 1 CFU.mL⁻¹. UV4 (6624 J.L⁻¹) treatment was required to achieve the same level of population (<1CFU.mL⁻¹) for CBS2499, AWRI1608 and L1735 strains with respectively 5.33, 4.97 and 5.18 log₁₀ reduction. L1737 strain was the only one which did not achieve a cell population lower than 1 CFU.mL⁻¹ even after UV4 treatment and with the lowest initial cell population.

Considering the genetic groups of the strains (Figure 5B), the 1st Wine 3N group (L1746 and AWRI1499) was the more sensitive to UV-C treatment, the Wine 2N group (CBS2499 and L1737) was the less sensitive to UV-C while the Wine/Beer 3N group (AWRI1608 and L1735) was found to be intermediary.

4. Discussion

4.1 Surface and liquid assays are complimentary approaches to assess the impact of UV-C

The present study aimed to evaluate the sensitivity to UV-C treatment of yeast species related to winemaking with a focus on *B. bruxellensis* species. For this, a large plate screening method was developed to test 147 strains of 14 different species with a focus on *B. bruxellensis* (104 strains). We then confirmed the impact of UV-C treatment in red wine. Due to practical constrains (100 liters of wine necessary for 6 strains), only strains of *B. bruxellensis*, the main wine spoiler, were tested. Regarding plate screening method, all the tested species were significantly impacted by increasing UV-C doses. The number of cells *per* drop was also a significant factor indicating an impact of the initial cell densities. The density effect could be explained by a shadowing effect, the cells acting as absorbance particles and decreasing UV-C transmittance. This cell density impact was also observed in grape must (8). The impact of UV-C treatment was congruent with previous studies at similar UV-C doses (35–37). Surface UV-C assays revealed strong difference of sensitivity between wine species. Similar results were obtained in apple juice: *S. cerevisiae* and *Z. bailii* were less sensitives than *B. bruxellensis* and *B. anomalus* (38). Moreover, the surface plate screening revealed intraspecific variation within *B. bruxellensis*, which was confirmed by our liquid trials. Although our plate screening did not allow the determination of the log reduction after treatment, it allowed the screening of a large number of species (14 yeasts) and strains (147

distinct isolates), unachievable with liquid assays. Moreover, UV-C surface treatment could be useful for oenological equipment disinfection and such plate-screening approach is pertinent to assess its relevance. The UV4 modality (6624 J.L⁻¹) achieved the reduction of cell population lower than 1 CFU.mL⁻¹ except for L1737 strain. Those results confirmed the efficiency of UV-C treatment even for a very absorbent red wine ($\alpha_{254} = 31.6 \text{ cm}^{-1}$) contaminated by high populations (10⁴ CFU.mL⁻¹) of *B. bruxellensis* and valid our large plate screening and data analysis approach as an efficient tool for the assessment of a control method.

4.2 Wine yeast species show strong sensitivity variation to UV-C treatments

Although all yeast species showed sensitivity to UV-C, strong interspecies variations were observed within the dose range used. Numerous hypotheses can be made to explain interspecific sensitivity variation. Secondary metabolites such as photoprotective pigments (carotenoid, melanin) or mycosporine-like amino acids (MAAs) are known to protect from UV-C radiation in bacteria and fungi (35,39–43). The UV-C radiations are known to impact DNA by creating CPDs (Cyclobutane Pyrimidine Dimers) leading to the non-transcription and non-replication of DNA, ultimately inducing deformation of the DNA helix and occasioning double strand breaks (44). DNA damages caused by UV-C treatment were shown to be the primary factors affecting microorganism death (37). To repair CPDs, two DNA repair mechanisms are described, the photoreactivation performed by photolyases (45,46) and the Nucleotide Excision Repair (NER) (47). The first mechanism is highly species dependent, with some species lacking photolyase genes (48,49) indicating a low selective pressure on this function. Subsequent works will be necessary to identify the molecular mechanism(s) explaining variation in UV-C sensitivity amongst wine yeast species.

Among the considered species, *Schizo. pombe*, *M. pulcherrima* and *Starm. bacillaris* were found to be the less affected by UV-C radiations. *L. thermotolerans*, *S. uvarum*, *T. delbrueckii*, *H. uvarum* and *S. cerevisiae* exhibited an intermediary phenotype while *Z. bailii*, *Z. rouxii*, *B. bruxellensis*, *T. cantarellii* and *B. anomalus* presented the most sensitive phenotype. Our results are mostly in accordance with a previous study reporting that *M. pulcherrima* was less sensitive than *S. cerevisiae* and *H. uvarum*, and was still viable after 1.0 kJ.L⁻¹ UV-C dose

in red must (8). The same authors showed that *S. cerevisiae* and *H. uvarum* sensitivities in grape must were similar and resulted in a total loss of detectable cultivability with 600 J.L⁻¹ of UV-C dose.

Interestingly, some of the less sensitive species (*Schizo. pombe*, *Starm. bacillaris*, *M. pulcherrima*) are known to be vineyard resident, frequently isolated from grapes or other fruit surfaces (50–53). Intermediate species (*L. thermotolerans*, *S. uvarum*, *T. delbrueckii*, *H. uvarum* and *S. cerevisiae*) are ubiquitous, frequently isolated from vineyards but also from the cellar environment (fermentative grape must, wine, equipment, etc.)(50,51,53–57). By contrast, the more sensitive species (*Z. bailii*, *Z. rouxii*, *B. bruxellensis*, *T. cantarellii* and *B. anomalus*) are mainly cellar-resident, adapted to wine and/or to anthropic environments and scarcely isolated from grapes or natural environments (20,58,59). On earth, stratospheric ozone layer absorbs UV-C radiation and terrestrial UV radiations are mostly composed of UV-A and UV-B that can create the same types of damages (60). These UV radiations are known to influence and modulate yeast community composition on grapes (36,61). A weaker selective pressure on UV-C tolerance mechanisms at cellar scale could explain from an evolutionary viewpoint the differences between wine yeast species. Beside *Saccharomyces* species used to control and complete wine alcoholic fermentation, none conventional yeasts (*T. delbrueckii*, *L. thermotolerans* and *M. pulcherrima*) can be used as technological auxiliaries by winemakers for a wide range of applications as acidification, biocontrol agent or to improve aromas (62). It could be interesting to evaluate the impact of UV-C treatment in mix-inoculated wine or must on yeasts spoilers and auxiliaries in order to target specifically unwanted ones. In any case, this observation highlights the potentiality of UV-C treatment to eliminate cellar-residents species, found on oenological equipment and in wines.

4.3 *B. bruxellensis* species shows low but significant intra-specific variation to UV-C sensitivity

Regarding *B. bruxellensis*, which is highly sensitive to UV-C treatment, some intraspecific variability, low yet significant, was recorded. *B. bruxellensis* is known to be phenotypically

versatile. When focusing on the main genetic groups associated with wine, the 1st Wine 3N group is the more sensitive to UV-C, followed by Wine/Beer 3N and Wine 2N. Comparable results were obtained in our red wine trials. Some of *B. bruxellensis* strains were shown to be resistant to sulphite addition in wine (63,64). Recently, a study linked this peculiar ability to a specific genetic group, 1st Wine 3N (65). This group being the most sensitive to UV-C treatment in both surface and liquid trials, UV-C stabilization could emerge as a pertinent method to control *B. bruxellensis* in winemaking.

B. bruxellensis is a diplo-triploid species complex, resulting in diploid and triploid (comprising diploid genome and one divergent haploid genome). Polyploidy can be involved in UV-C sensitivity, by increasing the redundancy of essential genes, increasing the cell volume and organelles. In *S. cerevisiae*, it was shown that polyploids as a better survival rates compared to diploid with UV-C treatment (66,67). In our case, the triploids strains (that have probable hybrid origin) belong to the more sensitive genetic groups (1st Wine 3N, Wine/Beer 3N). We can hypothesize that interspecific hybrids have less-efficient repair systems due to the combination of divergent genomes, resulting in accrued UV-C sensitivity.

5. Conclusion

The main yeast species encountered in winemaking were compared with respect to their sensitivity to UV-C radiation first with a plate screening approach. Strong interspecific variation was observed. Interestingly, UV-C sensitivity was associated with the ecological niche of the yeast species, with the cellar resident species being more sensitive to UV-C treatment than the vineyard-resident ones. Amongst spoilers, *B. bruxellensis* was highly sensitive to UV-C compared to others species. Intraspecific variability was observed, depending on genetic groups and was confirmed in liquid trials. The strains from the 1st Wine 3N genetic group were more sensitive compared to the strains from the Wine/Beer 3N and Wine 2N groups. Indeed, 1st Wine 3N strains required 25% less energy than other strains to achieve 5 Log₁₀ reduction. Strains from this group were shown to be sulfite tolerant/resistant, thus, this support the interest of UV-C treatment in wine context. Overall, wine treatments were effective, proving that UV-C could be used to control *B. bruxellensis* even at high levels of cell population and in absorbent red wine.

This study does not address the possible wine modification induced by the process but additional work is underway to characterize the impact on organoleptic qualities at UV-C doses required for microbiological stabilization.

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