Traditional second fermentation method yeast starter culture and *prise de mousse* optimization with Inactive Dry Yeast and *Saccharomyces cerevisiae* strains IOC 18-2007 and IOC-FIZZ

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Index

Abstract

Yeast starter cultures of IOC-18-2007 and IOC-FIZZ were performed via 3 different protocols (P1: Comité Interprofessionel de Vin du Champagne, P2: 24 H and P3: 5 H) and an Institut d'Oenologie de Champagne's Inactive Dry Yeast absence and presence were followed by density kinetics and inoculated. Significant density kinetics were found in 18-2007 IDY (+), reducing the time to achieve the density goals of the CIVC protocol (P1). Contrarily, significant final pressure values after in-bottle second fermentation indicated lower pressure in the starters IDY (+), being the best protocol P1: CIVC and performance P1 18-2007 IDY (-). Total yeast sterol and fatty acid analysis at the end of the starter culture exhibited significantly better membrane adaptation in Protocol 1with a C18:1 and zymosterol composition increase, 18-2007 with higher C16:1 concentrations, and IDY (+) with higher zymosterol and C16:1 and lower C16 composition.

Key words: Champagne, Starter culture, *Pied de cuve*, IOC-18-2007, IOC-FIZZ, Inactive Dry Yeast, Second fermentation, Traditional method, *Prise de mousse*, Squalene, Zymosterol, Palmitic acid, Palmitoleic acid, Oleic acid.

Resumen

Se realizaron pies de cuba con las levaduras comerciales IOC-18-2007 y IOC-FIZZ según 3 protocolos diferentes (P1: Comité Interprofessionel de Vin du Champagne, P2: 24 H y P3: 5 H) y la presencia o ausencia de Levadura Seca Inactiva también del Institut d'Oenologie de Champagne, con seguimiento de la densidad. Se encontraron cinéticas significativamente diferentes con la combinación de 18-2007 con Levadura Seca Inactiva siendo la más efectiva para reducir el tiempo en alcanzar las densidades deseadas en cada fase del protocolo 1: CIVC. Contrariamente se encontraron diferencias significativas en las presiones finales después de la segunda fermentación en botella, donde la presencia de Levadura Seca Inactiva presentó menores valores de presión, siendo el mejor protocolo P1: CIVC y la mejor combinación P1 18-2007 sin Levadura Seca Inactiva. El análisis de esterol y ácidos grasos al final del pie de cuba mostró una adaptación de la membrana celular significativamente mejor en el protocolo 1 con un aumento de la composición de C18:1 y zymosterol, 18-2007 con una mayor concentración de C16:1 y la Levadura Seca Inactiva con composiciones mayores de zymosterol y C16:1 y menor C16.

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1. Introduction

Champagne and consequently sparkling wines as we know nowadays started in the 17th century thanks to timeless figures such as Dom Pérignon (1638 - 1715) and Frère Oudart (1654 - 1742), Jean-Rémy Moët (1758-1841) and Madame Clicquot-Ponsardin (1777-1866) in the Champagne-Ardenne region, an already wine territory since the Roman's northern Gaul campaign in the 1rst century CE, who prohibited the gale viticulture until the 3rd century to reduce the competence. Wine elaboration grew along the Rheims city specially in the 9th century and conquered the international markets already in the 13th century. By the 19th century Champagne as a sparkling wine elaboration was furtherly defined already thanks to scientific figures such as Jean-Baptiste François (1792-1838) who stablished an accurate relation between the amount of sugar added in the tirage and the pressure within the bottle, Louis Pasteur (1822-1895) who properly characterised the alcoholic fermentation's reaction in which the yeast transform sugar in alcohol and CO_2 and Armand Walfard (1873-1950) who invented the cold disgorging method. Although the phylloxera crisis starting in Europe in 1863 and the First World War (1914-1918) the region strengthened with its own controlled designation of origin (AOC) in 1935 and the Comité Interprofessionel du Vin de Champagne (CIVC) in 1941, and survived the misery of both wars which paid back in overseas success and its actual international high positioning as a luxury product (Cartwright, 2021, Charters & Spielmann, 2014).

The traditional method for worldwide sparkling wine production is based in two alcoholic fermentations. In the first one the must, originally the juice pressed out of the harvested grapes, is transformed due to yeast intervention into the base wine, a relatively low alcohol percentage wine which is then bottled with yeast again for its second fermentation (Carrascosa et al., 2011, Kemp et al., 2019, Zoecklein, 1998). Between the base wine and the final sparkling wine there is three key concepts and steps around which the project is based. On first place the *pied de cuve* (PDC) or starter, the Active Dry Wine Yeast (ADWY) preparation for the harsh conditions of the base wine. Due to the ethanol percentage, low pH and sulphites among the main perils for the new coming yeast (Porras et al., 2021), an adaptation process of the base wine is required for a complete second fermentation inside the bottle where also temperature, between 12 and 18 ºC, and pressures up to 6 or 7 bar combine for an almost impossible fermentation (Liger-Belair et al., 2017).

For in-bottle second fermentation the Comité International du Vin de Champagne stablished an acclimatation and therefore PDC standard protocol which international traditional sparkling wine producers use (**Figure S1**, Coarer & de Loire, 2008). The starter culture by the CIVC takes up to 72 hours and is mainly composed of 2 phases; firstly the rehydration where in a water medium sugar and nitrogen are added for activating the metabolism of the yeast, and afterwards the acclimatation which takes longer and adds in a wine medium more sugar and nitrogen for the yeast adaptation at similar conditions of the inoculation (Juroszek et al., 1987, Tay et al,. 2007). On second place the *tirage*, the

yeast inoculation, when the starter ends and therefore the base wine bottled are inoculated with the populated medium and a *liqueur du tirage*, a wine which contains mainly sugar for the fermentation. The third and final concept is the foaming, *prise de mousse* (PDM) (Valade, Laurent & Moncomble, 2015) the foam obtention of the second time fermented sparkling wine when opened, due to the CO2 accumulation inside the bottle (Duteurtre, 2010). Even though for champagne elaboration 12 months since inoculation until final bottling are required, the second fermentation itself takes less time approximately 4-5 weeks, when the yeast finally dies forming the desired lees which favour many scents and flavours. In this project will not be included sensory analysis nor ageing effects.

This project can be divided in two main target perspectives of the sparkling wine elaboration; an optimisation of the starter protocol itself, considering time, substrates and ADWY concentration (Benucci et al., 2016), which results in a 24 hour starter culture, the intermediate protocol (P2); a 5 hour PDC the fast protocol (P3), and of course the CIVC protocol itself of around 72 hours (P1). Previous experiments at the Université de Reims Champagne-Ardenne successfully reduced the starter's time to 18 hours. The second target of this project is the optimisation of the yeast acclimatation itself, mainly by an Institut de l'Oenologie de Champagne Inactive Dry Yeast, which has high concentrations of organic nitrogen, lipids, sterols, amino acids, vitamins, minerals, and yet unknown components. Its addition ups the total cell concentration in the PDC and guarantees better fermentative kinetics (Pozo-Bayón et al., 2009, Laurent et al, 2007, Martí-Raga et al., 2016).

Even though nitrogen plays a very important role in yeast viability, survival, biomass production, acclimatation and stuck fermentations (Beltran et al, 2005, Bisson et al., 1999, Martín-Raga et al., 2015, Sablayrolles, 2016) and at the same time can have undesired effects (Tesnière et al., 2013) nitrogen concentrations were kept constant at each phase in P2 and P3 and P1 followed the original CIVC indications. Ammoniacal and amino nitrogen concentrations of the different PDC at the beginning and ending of both phases were analysed but due to a majority of low-quality lectures, will not be included. Fatty acids and sterols analysis of the different protocols' starter cultures aimed to justify the diverse kinetics among modalities, due to their importance in yeast membrane and consequently acclimatation (Beltran et al., 2008).The main fatty acids found in the yeast membranes are the unsaturated such as C16:1 and C18:1 and the saturated C16 and C18. These acids improve its membrane structure specially the unsaturated ones; the higher the unsaturated acids percentage is the better adaptation the yeast presents in its fluidity regulation for the mentioned second fermentation conflictive conditions (Redón et al., 2008, Weber et al., 2001). The sterols are key essential lipids for the yeast membrane which improve its permeability, fluency and consequently yeast viability and adaptation to unfavourable conditions (Soubeyrand et al., 2005). Main sterols considered in this project are squalene, the initiator of the sterol biosynthesis, lanosterol and zymosterol, intermediates and ergosterol, the main sterol in yeast membranes (Daum et al., 1998, Parks, 1987).

2. Materials and Methods

2.1 Base wine and Products

Products and base wine, at **Table** 1, used and their origin.

Table 1. Overall composition of the base wine.

Champagne-Ardenne base wine composition	
pH	3.03
Total acidity (g/L H_2SO_4)	5.7
Total sugar (g/L)	0.3
Total SO_2 (mg/L)	34
Alcohol (%, v/v)	10.79
Ammonia nitrogen (mg/L)	3
α-amino nitrogen (mg/L)	31

Commercial sucrose (Carrefour, France) was used.

Diammonium phosphate (Institut Oenologique de Champagne (IOC), Épernay, France).

Methylene blue (Reactifs Ral, Paris, France)

KOH and H_2SO_4 (Panreac, Barcelona, Spain).

Heptadecanoic acid and α-cholestane (Sigma-Aldrich, Barcelona, Spain).

IOC's Inactive Dry Yeast: IDY provided by the Insitut Oenologique de Champagne.

2.2 Yeast Strains

Yeast IOC 18-2007: (IOC) is a selected strain from the Champagne region specially recommended for *prise de mousse*, difficult must, low temperature, and stuck fermentations. Specifically, it is a *Saccharomyces cerevisiae* galactose – with Killer Factor K2 active, high ethanol resistance up to 15% (v/v) and low nitrogen need. Isolated from the Champagne region is a reliable and widely used strain proven in anterior experiments at the Université de Reims Champagne-Ardenne. Related background of 18-2007 can be found in Cotea et al. 2021, Benucci et al., 2016, Berbegal et al., 2019 and Berbegal et al., 2022.

Yeast IOC FIZZ: (IOC) is a high-quality sparkling wine producer via Charmat method. It is as well a *Saccharomyces cerevisiae* galactose – with a Killer Factor K2 active and a very high ethanol resistance,

up to 18% (v/v). FIZZ is a selected high-quality sparkling wines yeast strain via the Charmat method. Related background of FIZZ can be found in Cotea et al. 2021, González-Hernández, Michiels & Perré, 2022 and Тарaн et al., 2020.

2.3 Yeast culture starter (*pied de cuve*, PDC)

PDCs were prepared according to the protocols specified in **Table** 2 in half full 250 mL Erlenmeyers closed with aluminium paper in triplicate for each protocol and its modalities. Density was the main magnitude followed with a DMA 35 (Anton Paar, Netherlands) along the starters' turbidity (data not shown) at different times according to the protocols, but mainly at the beginning and end of each phase, the rehydration and the acclimatation. Also, at the end of each phase mainly, samples were taken by centrifugation and filtration of the supernatant and sent to the IOC for total sugar and nitrogen concentration analysis. Nitrogen results were highly low quality, and therefore will not be included.

Table 2. *Pied de cuve* (PDC), ADWY[a] rehydration and acclimatation before inoculation, protocols.

Phase	Protocol 1: CIVC (**Figure S1**)	Protocol 2: 20-24 H	Protocol 3: 4-5 H
Phase 1 initial: Rehydration	• 1 h 35 ºC • 3 g/L ADWY[a] • 100 g/L Sugar • 2 g/L DAP[b] • IDY[c] (+): 10 g/L IDY[c]	• 2 h 35 ºC • 12.5 g/L ADWY[a] • 50 g/L Sugar • 0.3 g/L DAP[b] • IDY[c] (+): 10 g/L IDY[c]	• 2 h 35 ºC • 25 g/L ADWY[a] • 50 g/L Sugar • 0.15g/L DAP[b] • IDY[c](+): 10 g/L IDY[c]
Phase 1 final: End of Rehydration	• 9 h 18 ºC • Until 1,025-1,015 g/L	• 2 h 18 ºC • Density goal not defined	• 0 h 18 ºC • Density goal not defined
Phase 2 initial: Wine medium acclimatation	• 68 h 18 ºC • 1:6 wine • 75 g/L wine Sugar • 0.33 g/L wine DAP[b] • Until 995-988 g/L	• 20 h 18 ºC • 1:1 wine • 50 g/L wine Sugar • 0.9 g/L wine DAP[b] • Density goal not defined	• 3 h 18 ºC • 1:1 wine • 50 g/L wine Sugar • 0.9 g/L wine DAP[b] • Density goal not defined
Phase 2 final: Inoculation, *Tirage*.	• 750 M cells alive / bottle	• 750 M cells alive / bottle	• 750 M cells alive / bottle

[a]ADWY: Active Dry Wine Yeast [b]DAP: Diammonium phosphate. [c]IDY: Inactive Dry Yeast.

Even though there is not a following parameter for the 24 hours and 4 hours protocols, density was measured at the beginning and end of each phase. For Inactive Dry Yeast (+) starter cultures IDY was added at the beginning of the first phase, rehydration, at a concentration of 10 g/L. Protocol 1: CIVC presented timing differences between absence or presence of IDY which resulted in an invalid comparison for IDY's effect.

2.4 Inoculation and second fermentation

From the mix of 5 mL of each of the triplicates per modality, population was calculated in duplicate by yeast counting in a Thoma counting chamber 546002-T in a Labophot – 2 microscope (Nikon, Tokio, Japan), diluting in MiliQ water and Methylene Blue (Reactifs Ral, Paris, France) the dilution factor was considered prioritizing 20-30 cells per square, and 10 squares per counting.

Inoculation volumes were calculated according to each modality's population to achieve $7.5 \cdot 10^8$ alive cells / bottle and 60 mL of *liqueur de tirage* at 500 g sugar / L wine was added before closing. Inoculated bottles were kept at 18ºC in a vertical positioning. Out of the 6 bottles for each modality 3 were kept for the ending analysis which is currently ongoing.

2.5 Total yeast sterol and squalene analysis by GC-MS

Sterol determination was performed for ADWY 18-2007 and FIZZ, the IOC's IDY, and at the end of each starter of the three protocols and its 4 modalities each in triplicates, independent from the *tirage* PDCs, according to a modification of the method by Quail & Kelly (1996) from García Viñola (2021). The pellets obtained after the centrifugation (10.000 rpm for 5 min) were frozen, as in **4.6 Fatty acids analysis by GC-FID**, and resuspended in 5 mL of KOH (Panreac, Barcelona, Spain) at 12% (w/v) in methanol with 10 uL of α-cholestane (Sigma-Aldrich, Barcelona, Spain 1 µg / mL) as intern reference. The mix was placed at 70ºC for 1 hour at the dry bath, and afterwards 1 mL of MiliQ water and 0,5 mL of hexane were added. Well mixed, the sample was centrifugated at 1.000 rpm for 5 min. The organic phase was dried in the Speed Vacuum System SC110 (Savant Instrument, USA) for 30 minutes at 45ºC. After a 100 µL of hexane was added to resuspend the extract and 2 µL were injected via an automatic injector 7683B in splitless mode into GC-MS.

Gas chromatography was carried out in an Agilent System GC 6890N connected to an HP Vectra with the Chemstation Software. The column was HP – 5MS UI (Agilent Technologies, USA) 30 m x 0.25 mm x 0.25µm. The injector temperature was 160ºC the same as the starting temperature for the program, which increases up to 260ºC at a 50ºC / min rate and then at a 4ºC / min rate until 320ºC when it holds for 1 min. The sterols were identified by an inert mass selective detector MSD 5975 (Agilent

Technologies, USA). The temperatures of the MSD transfer, MS Quad and MS Source were respectively 300, 150 and 280ºC. The MSD data was obtained via electronic ionisation at 70 eV in a 60 to 700 amu range. The analysis was performed with the Chemstation Agilent MSD 1989e2005 (Agilent Technologies, USA). Each compound identification was done comparing the massive fragmentation reference of each peak with the available standard injection or described by Quail & Kelly (1996). The relative abundance was calculated according to the peak area of the identified compounds normalized with the internal standard α-cholestane. The results are presented as an individual percentage of each sterol identified.

2.6 Total yeast fatty acid analysis by GC-FID

Fatty acid determination was performed for the ADWY 18-2007 and FIZZ, the IOC's IDY, and at the end of each *pied de cuve* of the three protocols and its 4 modalities each in triplicates, independent from the *tirage* PDCs, according to a modification of the method by Borrull et al. (2015) from García Viñola (2021). Firstly 1 mL of the sample was centrifugated at 10.000 rpm for 5 minutes and once the supernatant was discarded the pellet was frozen. All pellets were resuspended in 2 mL of H_2SO_4 (Panreac, Barcelona, Spain) at 2,5% (v/v in methanol) and 10 µL of heptadecanoic acid (4 mg/mL of C17, Sigma-Aldrich, Barcelona, Spain) diluted in hexane which works as an internal standard. The mix was placed for 1 hour at 90ºC in a dry bath and afterwards 1 mL of MiliQ water and 300 µl of hexane were added. The sample was centrifugated at 3.000 rpm for 5 minutes and 2 µL of the organic phase was injected in mode splitless in the Gas Chromatograph with a Flame Ionisation Detector (FID). The extract was injected in a column HP – FFAP (Agilent Technologies, USA) of 30 m x 250 µm x 0.25 µm with an automatic injector, both from Agilent Technologies. The fatty acids determination program starts at 100ºC up to 240ºC (5 min) at a rate of 4ºC / min. Injector and detector's temperatures were 220ºC and 250ºC respectively and the carrier gas Helium at 1,2 mL / min. The relative quantification of the fatty acids was obtained for each identified fatty acid by the relation by the ratio between the area of fatty acid and the area of the C17 internal standard.

2.7 Statistical analysis

Data processing was firstly treated by the Grubbs' test for an $\alpha = 0,05$ and n according to the samples and afterwards analysed by Microsoft Office Excel 16.16.27 (Redmond, WA, USA).

XLSTAT software version 2021.5.1 (Addinsoft, Paris, France) was used to perform the ANOVAs. Data were analysed using a two-way ANOVA and with Tukey HSD (Honestly Significant Difference) tests with a 95% confidence interval at the significant level with a p value < 0.05.

3. Results & Discussion

3.1 Yeast culture starter (*pied de cuve*, PDC)

Starter cultures were carried out for each protocol (**Table 2**) and 4 different modalities among protocol, for IOC's 18-2007 and FIZZ, and with or without IOC's Inactive Dry Yeast (IDY), an addable product composed of inactive *Saccharomyces cerevisiae*. Density was the main tool for following the rehydration, first phase, and acclimatation, second phase, kinetics of the starters as in **Table 3**. According to the CIVC protocol (**Figure S1**) density must be between 1.025 and 1.015 g/L for the first phase to finish and add then the wine based medium. A timing problem evidenced when Protocol 1, CIVC, without IOC's IDY (-), the dead *S. cerevisiae*, achieved the density goal range overnight while the product including samples had a surprisingly strong downfall, which was under 1.015 g/L overnight. Protocol 1 with Inactive Dry Yeast (+) had then to be repeated, and its effect further characterised. In both cases of Protocol 2 and Protocol 3 the product impact did not interfere thus all modalities were performed simultaneously. Even though CIVC's PDC without IOC's IDY did not arrive at 998-985 g/L after 88,5 hours, due to time and space limitations, inoculation was performed at around 1.010 g/L. Values shown in **Table 3** for 18-2007's case IDY (-). P2 and P3 are as well included due to not having stablished density ranges for each phase.

Table 3. Density following-up of 18-2007's starter culture without Inactive Dry Yeast.

Protocol	Phase 1 initial density (g/L)	Phase 1 final density (g/L)	Phase 2 initial density (g/L)	Phase 2 final density (g/L)
P1: CIVC[a]	1.040 ± 0	1.025 ± 3	1.021 ± 3	1.010 ± 1
P2: 24 H	1.016 ± 0	1.011 ± 1	1.011 ± 1	1.003 ± 1
P3: 4 H	1.016 ± 0	1.017 ± 1	1.013 ± 0	1.009 ± 1

[a]CIVC: Comité Interprofessionel du Vin de Champagne.

Due to the IOC's product impact on a longer first phase of the PDC, CIVC first phase was optimised up to 7 hours of phase 1, rehydration, which allows the second phase with the wine addition to start earlier. An independent experiment regarding 18-2007 and FIZZ and Inactive Dry Yeast absence and presence was performed. 7 hours at 35ºC with agitation proved an improvement in both achieving the desired density for the second phase and in kinetics (**Figure S2**).

In the end the repetition of P1 with IOC's IDY was reduced to 2 hours at 35ºC and 5 h at 18ºC reducing the possible temperature shock, even though not studied in this project, and keeping the method closer to the initial CIVC without Inactive Dry Yeast protocol.

In **Figures 1, 2** and **3** the Δ density among the start and end of phase 1, rehydration, and phase 2, acclimatation, is represented. An ANOVA of 2 factors for each Protocol was done, and its results are indicated in the respective Figures. Due to the Protocol 1: CIVC with Inactive Dry Yeast repetition of the experiments, **Figure 1** is not considered valid for IDY effect's comparison.

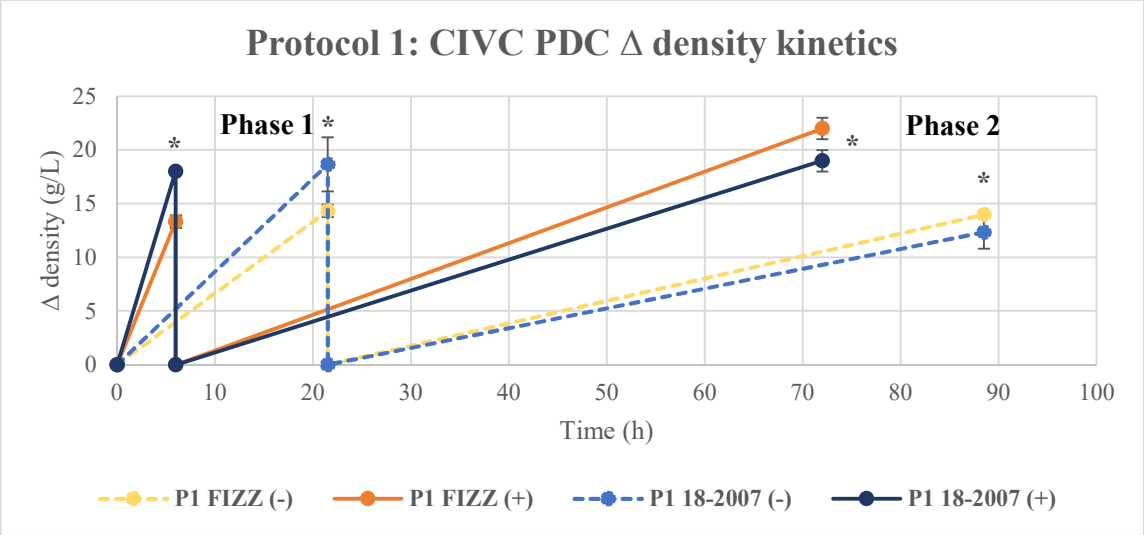

Figure 1. Protocol 1: CIVC density following up of the *pied de cuve* (starter) represented as Δ density of the two phases along the protocol's time expressed in hours. Due to repetition of the CIVC P1 Inactive Dry Yeast (+) and further optimisation of its first phase, timings and phases do not coincide and therefore will not be considered comparable among IDY (-) and (+). Significant differences (*) were found at both phase ends among yeast performance, 18-2007 and FIZZ. (-): IDY negative, (+): IDY positive.

Although Protocol 1's starter culture is not valid for IDY comparison, significant differences were found between the yeast 18-2007 and FIZZ, with a higher density downfall of 18-2007 at both ends of both phases, represented in **Figure 1**. In **Figure 2**, Protocol 2: 24 H's following of the starter presented significant differences between yeast, IDY effect and their interaction, with the significantly highest downfall of density from 18-2007 and IDY (+). In **Figure 3** significant differences were found at the end of Phase 2, among yeast, IDY effect and their interaction, with the significantly highest downfall of density from 18-2007 and IDY (+) as well. These results indicate that for *pied de cuve* following ups with density as the magnitude of reference such as the CIVC protocol for traditional method's second fermentation, 18-2007 is significantly faster at achieving the goal densities than FIZZ in combination with IDY, but FIZZ in IDY (-) conditions showed a significant increase in density's kinetics. IOC's Inactive Dry Yeast has a significant effect in increasing the density downfall independently of the yeast strain, and due to the significant interaction, 18-2007 IDY (+) is the best candidate for PDC.

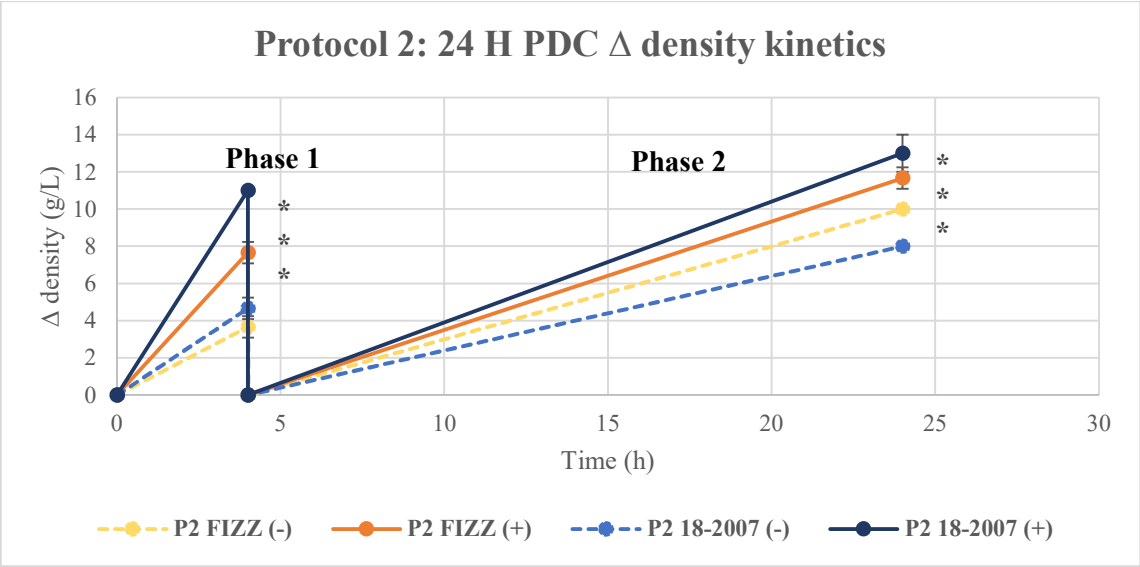

Figure 2. Protocol 2: 24 H density following up of the *pied de cuve* (starter) represented as Δ density of the two phases along the protocol's time expressed in hours. Phase 1, rehydration, lasted 4 hours. At the beginning of the second phase, adaptation, wine is added and therefore reset the initial density. Phase 2, acclimatation, lasted 20 hours since the end of the first one. Significant differences (*) were found at both ends among yeast, 18-2007 and FIZZ, Inactive Dry Yeast negative (-) and positive (+), and yeast x IDY interaction.

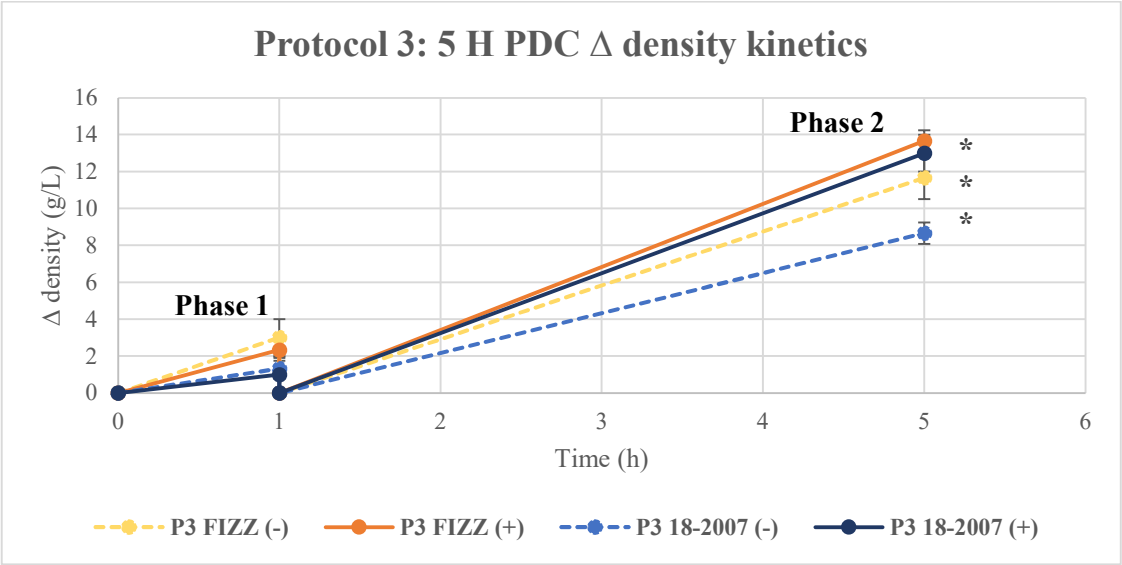

Figure 3. Protocol 1: 5 H density following up of the *pied de cuve* (starter) represented as Δ density of the two phases along the protocol's time expressed in hours. Phase 1, rehydration, lasted 1 hours. At the beginning of the second phase, adaptation, wine is added and therefore reset the initial density. Phase 2, acclimatation, lasted 4 hours since the end of the first one. Significant differences (*) were found at the end of the second phase among yeast, 18-2007 and FIZZ, Inactive Dry Yeast negative (-) and positive (+), and yeast x IDY interaction.

3.2 Inoculation and second fermentation

Inoculation was carried out according to the populations from the mix of the triplicates of each modality. Due to the IOC's Inactive Dry Yeast the total cells counting was considered as not valid because of the dead cells outnumbering the alive cells. Therefore, alive cells were the population value chosen to calculate the inoculation volumes, being $7{,}5 \cdot 10^8$ cells alive / bottle the target concentration, results in **Table 4**. As in CIVC's protocol (**Figure S1**) population is not considered, its results will not be furtherly studied.

Table 4. Tirage inoculation volumes for obtaining $7{,}5 \cdot 10^8$ alive cells / bottle for each protocol and modality. Populations were calculated from a mix of each modality's triplicate in a Thomas counting chamber in duplicate.

Protocol	Yeast 18-2007		Yeast FIZZ	
	IDY[a] (-) (mL)	IDY[a] (+) (mL)	IDY[a] (-) (mL)	IDY[a] (+) (mL)
P1: CIVC[b]	21.94 ± 9.50	12.07 ± 4.22	11.32 ± 2.00	7.77 ± 1.81
P2: 24 H	7.93 ± 1.63	3.67 ± 0.83	6.29 ± 1.26	3.01 ± 0.73
P3: 5 H	2.94 ± 0.91	1.93 ± 0.47	3.77 ± 2.19	3.49 ± 1.80

[a]IDY: Inactive Dry Yeast. [b]CIVC: Comité Interprofessionel du Vin du Champagne.

Inoculation was performed in March between the 8th and the 10th (2023), and pressure measurement was done the 5th of June (2023) at the IOC. The ending of second fermentation and mousse obtaining (PDM) is assumed. Due to Protocol 1: CIVC with Inactive Dry Yeast repetition its inoculation was performed the 24th of March, but its results will be considered as valid.

Table 5. Final bottle pressions after 2 months and a half since inoculation. Measures were performed in triplicate at the Institut d'Oenologie de Champagne, Épernay, France. A Tukey HSD test was performed (Table S1).

Protocol	Yeast 18-2007		Yeast FIZZ	
	IDY[a] (-) (bar)	IDY[a] (+) (bar)	IDY[a] (-) (bar)	IDY[a] (+) (bar)
P1: CIVC[b]	6.76 ± 0.23	6.39 ± 0.11[c]	6.40 ± 0.02	6.22 ± 0.09[d]
P2: 20 H	6.18 ± 0.02	6.12 ± 0.05	6.17 ± 0.03	6.04 ± 0.04
P3: 4 H	6.05 ± 0.03	5.98 ± 0.09	5.98 ± 0.10	5.99 ± 0.05

[a]IDY: Inactive Dry Yeast. [b]CIVC: Comité Interprofessionel du Vin du Champagne. [c]: P1 18-2007 (+) was only in duplicate due to experimental inconveniences. [d]: P1 FIZZ + had 4 samples.

Significant differences were found (**Table S1**) among the values shown in **Table 5** and represented in **Figure 4**. Firstly, between the yeast strains 18-2007 showed significantly higher pressure values than

FIZZ which is comprehensible due to each yeasts origin, being 18-2007 isolated from the Champagne region for the traditional method while FIZZ a specific high quality Charmat method producer, both from the Institut d'Oenologie de Champagne. IOC's Inactive Dry Yeast proved a significant difference as well which evidence lower final pressure values in the IDY (+) *prises de mousse*. Lastly, significant pressure differences were obtained among the different protocols, being Protocol 1: CIVC the highest, followed by Protocol 2: 24 H and Protocol 3: 5 H, all of them significant and in decreasing order. Significant interactions were found between IDY x Protocol and Yeast x Protocol. In comparison with Benucci et al., 2016, IOC's 18-2007 achieved the highest pressure (6.76 ± 0.23 bar) registered in this experiment.

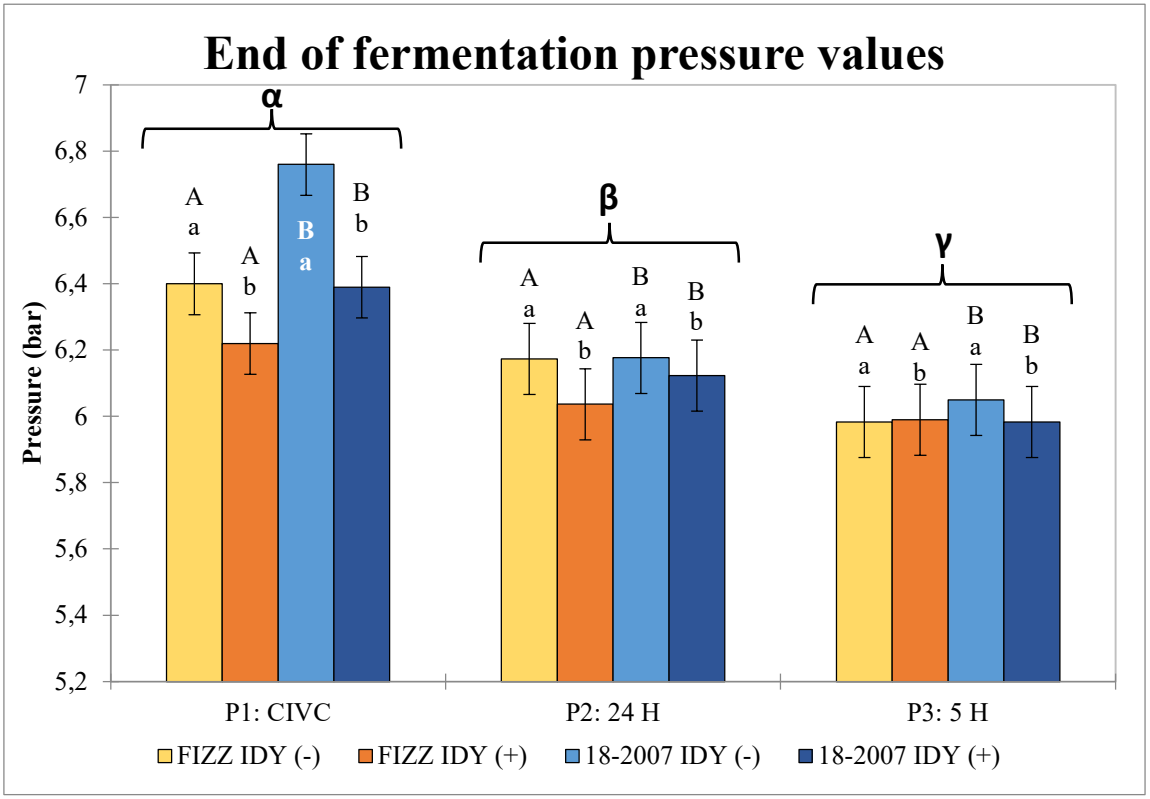

Figure 4. End of fermentation pressure values according to the different yeast strain 18-2007 or FIZZ, absence (-) or presence (+) of IOC's Inactive Dry Yeast (IDY) and the three different protocols P1: CIVC, P2: 24 H and P3: 5 H. 'α', 'β' and 'γ', 'A' and 'B', 'a' and 'b' indicate significantly different statistical group according to the HSD Tukey test performed.

Interestingly, IOC's IDY significantly improves density kinetics along the PDC but significantly impairs the *prise de mousse* (PDM), at least at the concentration applied for the three protocols, 10 g/L for the first phase of rehydration. Further analysis and different concentrations are required to properly characterise the IOC's IDY effect, due to previous bibliography as Pozo-Bayón et al., 2009, and Martí-Raga et al., 2016, proving positive effects of Inactive Dry Yeast in the pressure measures and foaming formation. Besides, significantly lower pressures were obtained in the protocols P2 and P3 than the P1: CIVC (**Figure 4**), which can be interpretated as a better adaptation and acclimatation of the yeast exposed for a longer period of time in comparison with the shortened expositions of the Protocol 2: 24 H and even shorter Protocol 3: 5 H, significantly lower than the P2. Therefore, even if actual Protocols 2 and 3 supposed an optimisation of the Active Dried Wine Yeast use due to faster density kinetics, significant consequences in pressure can be expected, further optimisation of all three protocols are yet to be pushed forward. Furthermore, a future sensorial analysis could add on the characterisation of the different protocol's effects.

3.4 Total yeast sterol and squalene analysis by GC-MS

To justify the differences in kinetics and final population between the modalities, both yeast 18-2007 or FIZZ and absence or presence of the IOC's Inactive Dry Yeast an analysis of sterol and fatty acid compositions was performed following the PDC protocols (**Table 2**) at the Universitat Rovira i Virgili.

Table 6. Effect of main factors (Yeast, Inactive Dry Yeast and Protocol) and their interaction on the total sterol and squalene composition of yeast cells. Results of 3-way ANOVA using Tuckey HSD statistical test. p-values in bold indicate a significant difference.

	Main factors			**Interaction factor**			
Sterols	**Yeast (Y)**	**IDY (S)**	**Protocol (P)**	**Y x S**	**Y x P**	**S x P**	**Y x S x P**
Squalene (Sq)	0.142	0.779	**0.001****	**0.007****	**0.028***	0.082	0.457
Zymosterol (Zym)	0.756	**0.027***	0.102	0.174	0.901	**0.039***	0.147
Ergosterol (Erg)	0.174	0.112	0.066	0.205	0.147	0.974	0.714
Lanosterol	0.185	0.323	0.234	0.878	0.394	0.159	0.646
Erg/Sq	0.278	0.349	0.319	0.225	0.266	0.306	0.340
Erg/Zym	0.881	**0.016***	0.158	0.685	0.508	0.139	0.330

*Signification codes: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

Sterol percentual composition of both yeast resuspended in stilled water, the IDY and the starter cultures of each modality at its end, the hypothetical inoculation or *tirage*, was obtained by GC-MS. Significant differences were found between the two yeast and the IDY product, presenting IDY significantly higher squalene and zymosterol compositions, and significantly lower ergosterol and therefore ergosterol / squalene and ergosterol / zymosterol in comparison with both yeasts, which did not have any differences among them (**Table S2**).

Significant differences shown at the **Table 6** were found in squalene composition in Protocol 2: 24 H as represented at **Figure 5**. Also significant interactions between the yeast strain and the absence or presence of IDY Yeast x IDY and the same Inactive Dry Yeast and the protocols Yeast x Protocol was found, with significant differences between 18-2007 IDY (+) with a higher composition than FIZZ IDY (+), but with the other two versions IDY (–) being in both statistical groups from the HSD Tukey test the interaction factor won't be considered, same case scenario for the yeast and protocol Yeast x Protocol interaction.

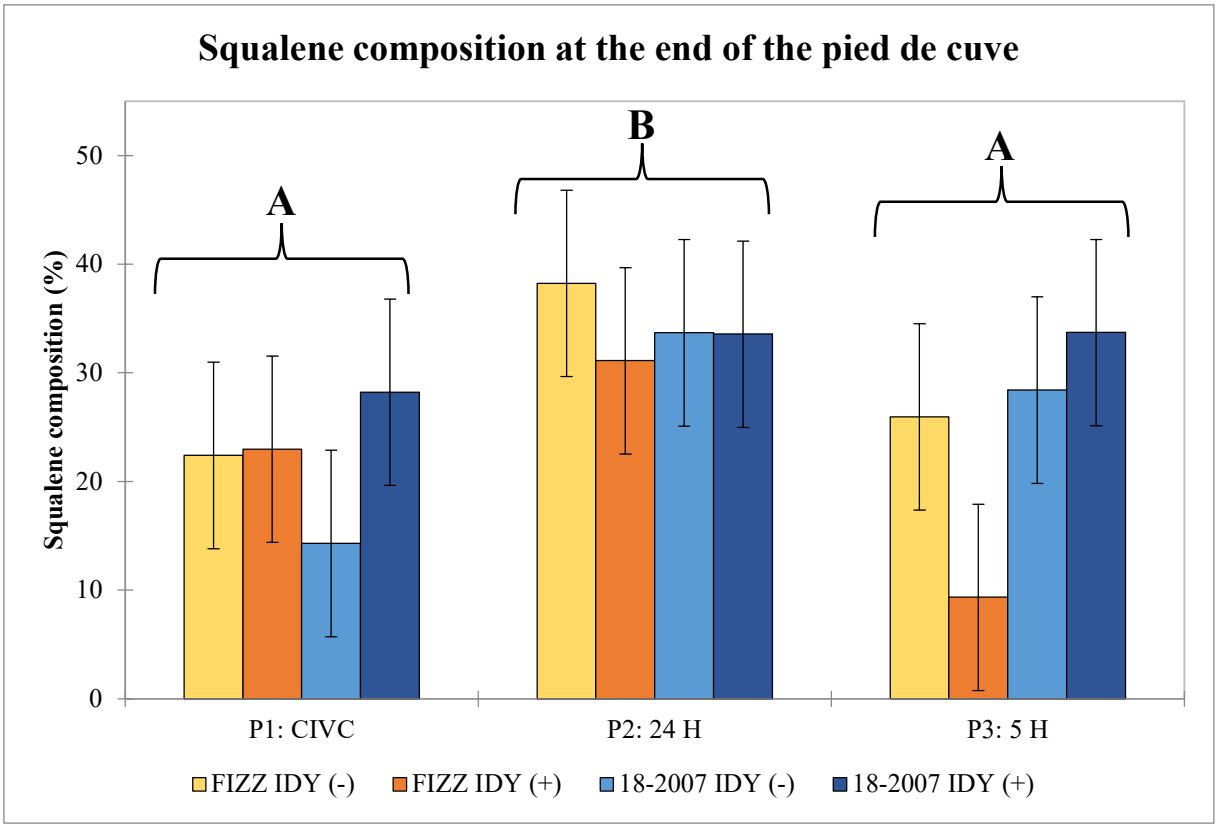

Figure 5. Representation of squalene percentual composition of the total sterol composition at the end of the PDC, all values obtained from the normalization of the GC-MS peak areas, according to the different 3 factors: yeast strain 18-2007 or FIZZ, absence (-) or presence (+) of IOC's Inactive Dry Yeast and protocol P1: CIVC, P2: 24 H or P3: 5 H. Statistical groups 'A' and 'B' belong to the performed HSD Tukey test and indicate significant difference.

Represented in **Figure 6** significant difference was found in the zymosterol composition between absence and presence of Inactive Dry Yeast with an increase in Inactive Dry Yeast (+). Once again, a significant interaction factor among sterol and protocol IDY x Protocol but this time a relevant difference between the Protocol 1: CIVC and the Protocol 2: 24 H was found, with a significatively higher zymosterol composition in P1's PDCs. The significantly higher Protocol 2: 24 H Squalene composition indicates an inhibition of the sterol biosynthesis, where squalene (**Figure 5**) is concentrated at the beginning of the path and sterols are not synthesized (**Figure S3**, Daum, Lees, Martin, & Dickson, 1998 and Parks, 1987). On the other hand, significantly higher zymosterol composition (**Figure 6**) was found in IOC's IDY presence and increased zymosterol composition in Protocol 1: CIVC significantly different than the minor Protocol 2 proves a higher activity in the sterol's biosynthesis due to zymosterol being an intermediate of the pathway. Although no other significant differences are found in ergosterol nor lanosterol, the ergosterol / zymosterol ratio is significantly lower in the PDCs with IOC's IDY (+), due to the mentioned significantly higher composition of zymosterol. As in Soubeyrand et al., 2005, IDY favours the sterol content and therefore the yeast membrane adaptation, although due to high variability most analysis were inconclusive and further conclusions apart from the significant zymosterol and squalene difference cannot be taken.

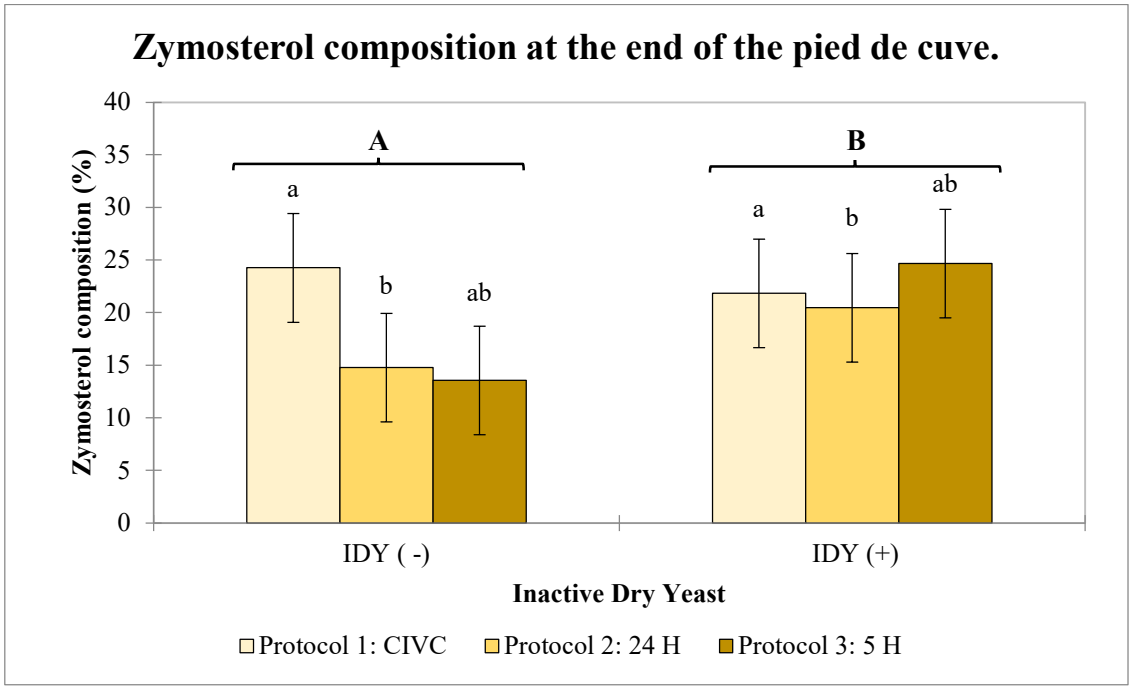

Figure 6. Representation of squalene percentual composition of the total sterol composition at the end of the PDC, all values obtained from the normalization of the GC-MS peak areas, according to the different 3 factors: yeast strain 18-2007 or FIZZ, absence (-) or presence (+) of IOC's IDY and protocol P1: CIVC, P2: 24 H or P3: 5 H. Statistical groups 'A' and 'B', and 'a', 'ab' and 'b' belong to the performed HSD Tukey test and indicate significant difference.

3.5 Total yeast fatty acid analysis by GC-FID

A 3-way ANOVA using the Tuckey HSD test was performed to analyse the differences in both percentual individual composition of C16, C16:1, C18, C18:1 and collective composition such as MCFA (Medium Chain Fatty Acids), SFA (Saturated Fatty Acids) and UFA (Unsaturated Fatty Acids).

In **Table 7**. significant differences in palmitic acid C16, **Figure 7** and palmitoleic acid C16:1 in **Figure 8** can be observed among yeast strain, 18-2007 and FIZZ, and in absence or presence of Inactive Dry Yeast. The diverse protocols show a significant difference in oleic acid C18:1 total composition, **Figure 9**. No significant differences were found among MCFA. SFA, UFA nor between any interactions among the Yeast, IDY and Protocol factors.

Table 7. Effect of main factors (Yeast, Sterol and Protocol) and their interaction on the total fatty acid composition of yeast cells. Results of 3-way ANOVA using Tuckey HSD statistical test. p-values in **bold** indicate a significant difference.

	Main factors			**Interaction factor**			
Fatty acids	**Yeast (Y)**	**IDY (S)**	**Protocol (P)**	**Y x S**	**Y x P**	**S x P**	**Y x S x P**
C16	0.051	**0.002****	0.562	0.115	0.054	0.628	0.115
C16:1	**0.020***	**0.041***	0.154	0.148	0.438	0.575	0.598
C18	0.064	0.739	0.345	0.416	0.502	0.206	0.299
C18:1	0.841	0.612	**0.005****	0.454	0.616	0.898	0.572
MCFA	0.549	0.304	0.259	0.285	0.275	0.607	0.578
SFA	0.447	0.051	0.432	0.950	0.720	0.287	0.234
UFA	0.145	0.116	0.498	0.857	0.529	0.580	0.479

*Signification codes: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

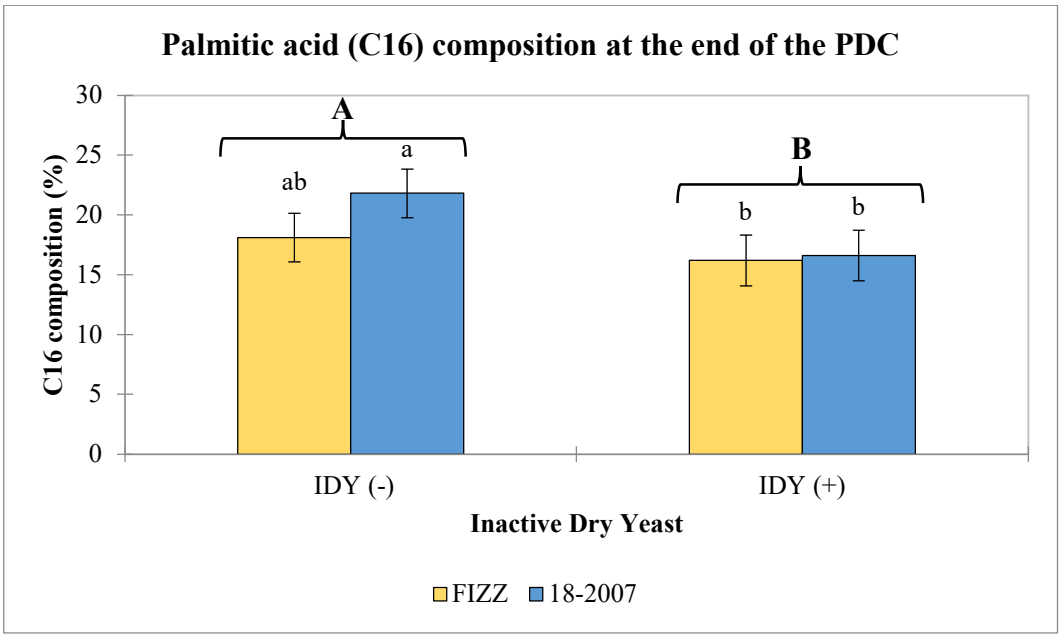

Figure 7. Representation of C16 percentual of the total fatty acid composition at the end of the *pied de cuve* (starter), all values obtained from the normalization of the GC-FID peak areas, according to 2 of the 3 different factors: yeast strain 18-2007 or FIZZ and absence (-) or presence (+) of IOC's IDY. Statistical groups 'A' and 'B', 'a', 'ab' and 'b' belong to the HSD Tukey test and indicate significant difference.

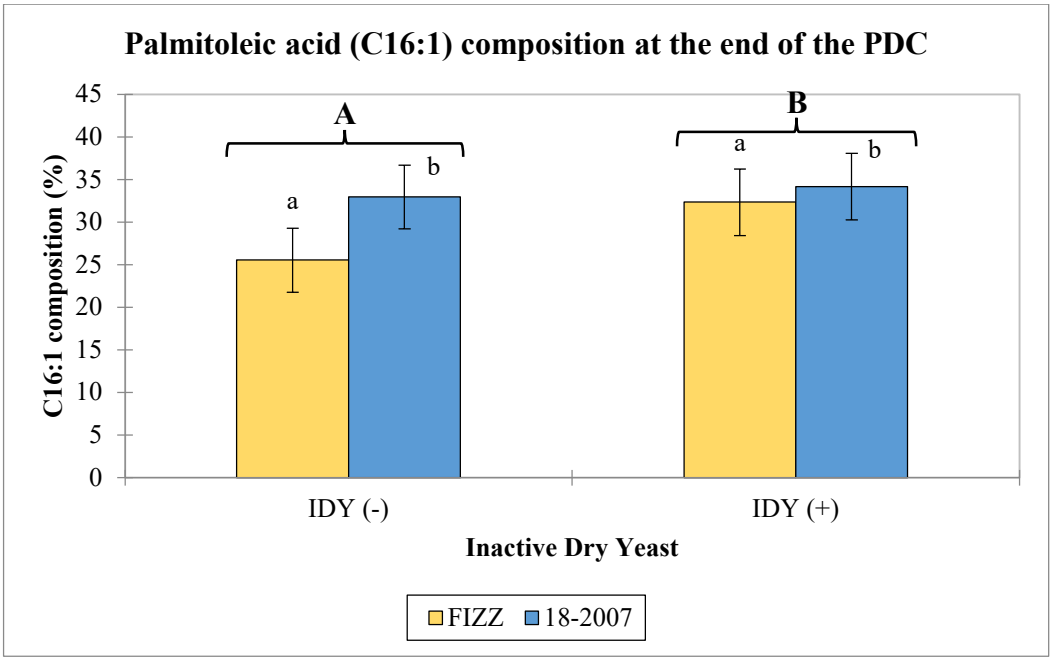

Figure 8. Representation of C16:1 percentual of the total fatty acid composition at the end of the *pied de cuve* (starter), all values obtained from the normalization of the GC-FID peak areas, according to 2 of the 3 different factors: yeast strain 18-2007 or FIZZ and absence (-) or presence (+) of IOC's IDY. Statistical groups 'A' and 'B', 'a' and 'b' belong to the HSD Tukey test and indicate significant difference.

A significant decrease in C16 composition was found among 18-2007's starter cultures with Inactive Dry Yeast in comparison with the IOC's IDY absence (**Figure 7**). Inversely, a significant increase was found in the 18-2007 and FIZZ starter cultures of C16:1 composition (**Figure 8**), and furthermore a significant difference was found among the yeast strains with a higher composition of C16:1 from 18-2007 (**Figure 9**). Finally, a significantly higher C18:1 was found in the Protocol 1: CIVC than Protocol 2: 24 H. Protocol 3: 5 H belonged to both statistical groups.

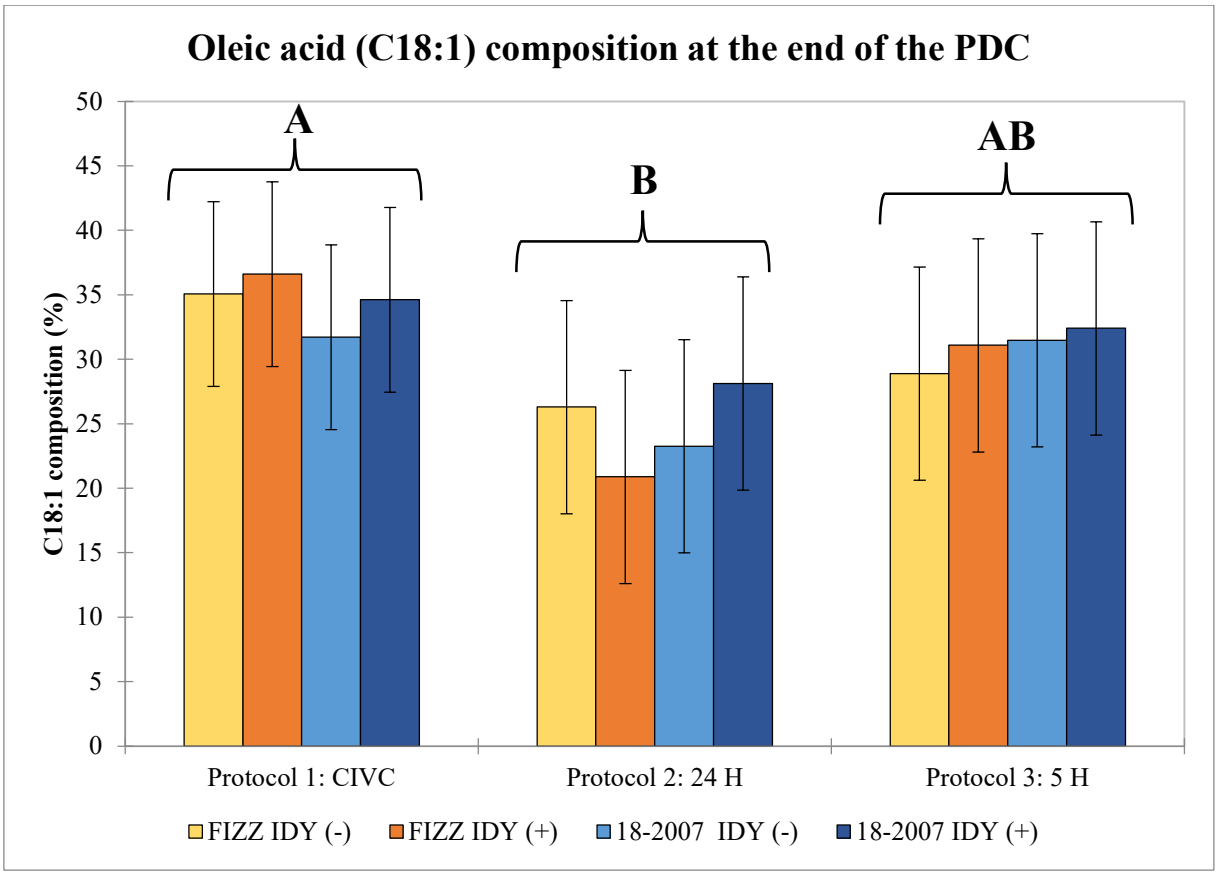

Figure 9. Representation of C18:1 percentual of the total fatty acid composition at the end of the *pied de cuve*, all values obtained from the normalization of the GC-FID peak areas, according to the different 3 factors: yeast strain 18-2007 or FIZZ, absence (-) or presence (+) of IOC's Inactive Dry Yeast and protocol P1: CIVC, P2: 24 H or P3: 5 H. Statistical groups 'A', 'AB' and 'B' belong to the performed HSD Tukey test and indicate significant difference.

Although few statistical differences among the yeast strains, the IDY absence or presence and the 3 different protocols were found, significant differences were found in the C16, C16:1 and C18:1, even though it was not the case for UFA nor SFA. IOC's IDY showed a significant decrease of C16 and a significant increase of C16:1 along the different starter cultures. The increase of palmitoleic acid, a very important membrane fatty acid, shows a better adaptation to adverse conditions such as low temperatures, as in Beltran et al., 2008, and high ethanol concentrations, resulting on higher viabilities as in Redón et al., 2008. On the same page, the yeast 18-2007 proved a significantly higher C16:1 composition than FIZZ, demonstrating a better membrane adaptation in terms of rigidity. Also, significant differences were found among the protocols P1: CIVC and P2: 24 H, with a significantly higher oleic acid composition in the PDC from the CIVC protocol, which show a positive correlation with the mentioned better adaptation and the time exposure to the adverse conditions and therefore yeast adaptation to them.

4. Conclusions

- Protocols P2: 24 H and P3: 5 H were established and performed.

- Starter culture's following by density showed significant difference among 18-2007 and FIZZ, absence or presence of Inactive Dry Yeast, and its interaction, which resulted in 18-2007 IDY (+) as the optimal combination for the fastest density kinetics.

- Final pressures after second fermentation proved significant differences between 18-2007 and FIZZ, being 18-2007 the better performer, and in absence or presence of IDY, with lower pressure values for starter cultures IDY (+). Protocol 1: CIVC had the highest pressure values, significantly followed by Protocol 2: 24 H and Protocol 3: 5 H.

- Total yeast sterol analysis showed a significantly higher Protocol 2: 24 H squalene and contrarily increased zymosterol was found in IOC's IDY (+) and in Protocol 1: CIVC; indicating a major flow downstream the sterol biosynthesis.

- Total yeast fatty acid analysis exhibited a significant C16 decrease and C16:1 increase with IDY's presence (+), indicating better adaptation to the harsh conditions. Similarly, 18-2007 and P1: CIVC proved significantly higher C16:1 and C18:1 respectively, both cases indicating a better adaptation translated to the membrane's composition.

- Further studies to characterise and optimise the different protocols are needed to fully comprehend the starter culture kinetics of the studied modalities and its justification from a complete fatty acid and sterol composition.

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6. Annex

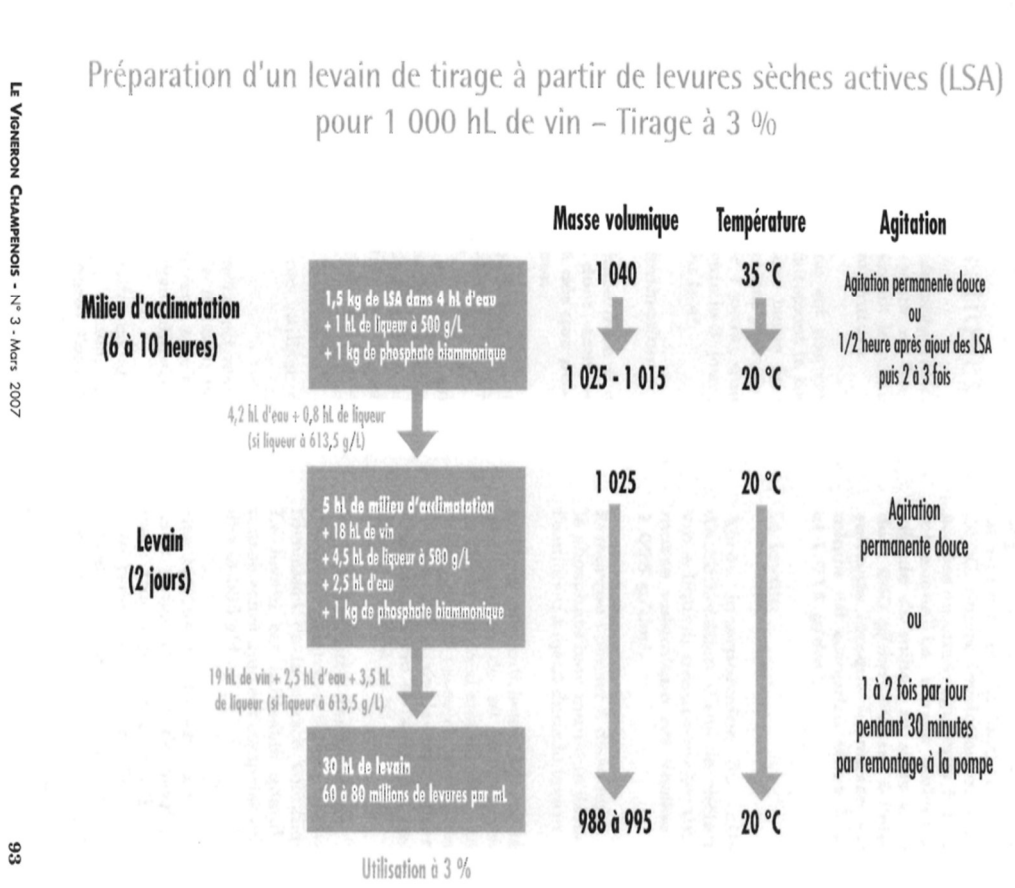

Figure S1. Comité Interprofessionel du Vin de Champagne's ADWY *pied de cuve* before second fermentation divided in *milieu d'acclimatation*, rehydration phase, and *levain*, wine-medium adaptation. *Le vigneron champenois*, Nº3 – Mars 2007.

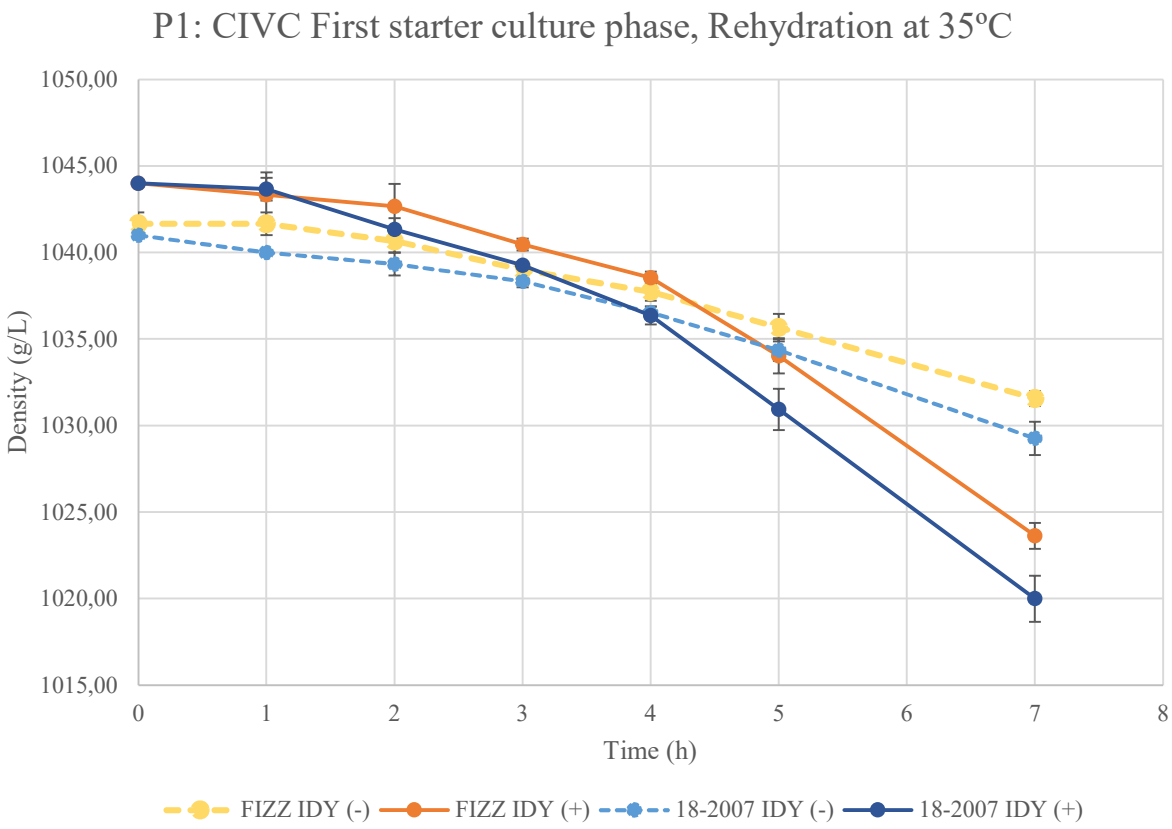

Figure S2. Density kinetics of Protocol 1: CIVC first phase, rehydration. All PDCs were kept in constant agitation at 35ºC. Density goal from the P1 for the next phase is 1.025 – 1.015 g/L.

Table S1. 3-way ANOVA via Tuckey HSD statistical test of the pressure (bar) corresponding to the end of the second fermentation, calculated at the IOC. p-values in **bold** indicate a significant difference.

Source	DF	Sum of squares	Mean squares	F	Pr > F	p-values signification codes
Yeast	1	0.113	0.113	13.980	**0.001**	**
Sterol	1	0.157	0.157	19.350	**0.000**	***
Protocol	2	1.192	0.596	73.572	**<0.0001**	***
Yeast*Sterol	1	0.008	0.008	0.980	0.332	°
Yeast*Protocol	2	0.100	0.050	6.155	**0.007**	**
Sterol*Protocol	2	0.093	0.047	5.743	**0.009**	**
Yeast*Sterol*Protocol	2	0.027	0.014	1.695	0.205	°

*Signification codes: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

Table S2. 3-way ANOVA of the ADWY and IOC'S Inactive Dry Yeast's sterol percentual composition. p-values in **bold** indicate a significant difference. Tukey HSD test showed significant differences between the group 'A' formed by 18-2007 and FIZZ and 'B', IDY, in Squalene and Zymosterol (A < B) and Ergosterol and consequently Ergosterol / Squalene and Ergosterol / Zymosterol (A > B).

	Main factors		
Sterols	**18-2007**	**FIZZ**	**Inactive Dry Yeast**
Squalene	**<0,0001*** **	**<0,0001*** **	**<0,0001*** **
Zymosterol	**<0,0001*** **	**0,003** **	**0,021***
Ergosterol	**<0,0001*** **	**0,000*** **	**0,000*** **
Lanosterol	0,463	0,181	0,600
Ergosterol/Squalene	**0,001*** **	**<0,0001*** **	**<0,0001*** **
Ergosterol/Zymosterol	**0,004** **	**0,000*** **	**0,003** **

*Signification codes: 0 < *** < 0.001 < ** < 0.01 < * < 0.05 < . < 0.1 < ° < 1*

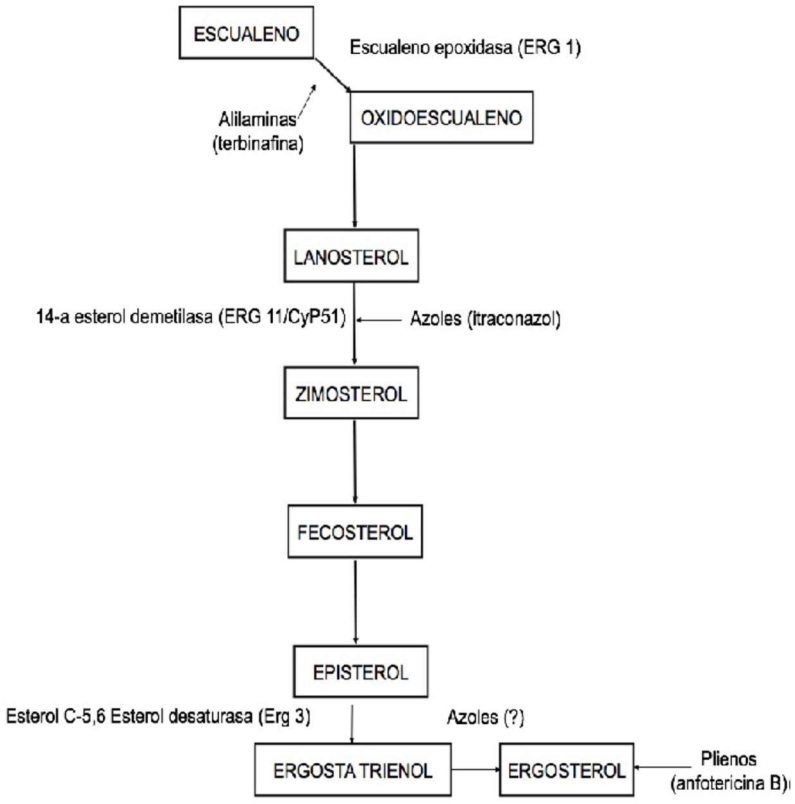

Figure S3. Sterol biosynthesis pathway, which starts at the upper part with Squalene, followed by Oxidosqualene, Lanosterol, Zymosterol, Fecosterol, Episterol, Ergosta trienol and Ergosterol. Adaptation from Daum, Lees, Martin, & Dickson (1998).