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## A peculiar cell cycle arrest at g2/m stage during the stationary phase of growth in the wine yeast *Hanseniaspora vineae*.

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## ABSTRACT

Yeasts of the genus *Hanseniaspora* gained notoriety in the last years due to their contribution to wine quality, and their loss of several genes, mainly related to DNA repair and cell cycle processes. Based on genomic data from many members of this genus, they have been classified in two well defined clades: the “faster-evolving lineage” (FEL) and the “slower-evolving lineage” (SEL). In this context, we had detected that *H. vineae* exhibited a rapid loss of cell viability in some conditions during the stationary phase compared to *H. uvarum* and *S. cerevisiae*. The present work aimed to evaluate the viability and cell cycle progression of representatives of *Hanseniaspora* species along their growth in an aerobic and discontinuous system. Cell growth, viability and DNA content were determined by turbidity, Trypan Blue staining, and flow cytometry, respectively. Results showed that *H. uvarum* and *H. opuntiae* (representing FEL group), and *H. osmophila* (SEL group) exhibited a typical G1/G0 (1C DNA) arrest during the stationary phase, as *S. cerevisiae*. Conversely, the three strains studied here of *H. vineae* (SEL group) arrested at G2/M stages of cell cycle (2C DNA), and lost viability rapidly when enter the stationary phase. These results showed that *H. vineae* have a unique cell cycle behavior that will contribute as a new eukaryotic model for future studies of genetic determinants of yeast cell cycle control and progression.

## INTRODUCTION

The cell division cycle is a fundamental biological process of living organisms that consists of a series of closely coordinated events to ensure a proper cellular proliferation, that is a series of well-orchestrated events to carry out and certify an adequate cell volume for duplication, a precise and correct DNA replication, the proper attachment and alignment of the chromosomes on the spindle, and complete chromosome segregation before cell division (Tang, 2010; Mayhew et al., 2017; Smith et al., 2002).

In the budding yeast *Saccharomyces cerevisiae*, cell division cycle begins with an initial growth phase, G1, during which cells prepare for duplication by reaching a threshold of “structures”, size or organelles needed to support partition. Transition from G1 into S phase is marked by progression through Start (the point of commitment to cell division), where genetic information is duplicated and begins the emerge of a bud. After genome duplication cells enter a G2 phase, in which cells get ready for partition and, finally, during M phase the initial cell is divided into 2

cells (Yu et al., 2006; Jiménez et al., 2015). In discontinuous growth systems, when essential nutrients are scarce, yeast cells cease mitotic division and arrest within a steady or quiescent state (G0 phase) (Zhang et al., 2019). Arrested cells in G0 acquire several metabolic characteristics that collectively define the stationary phase of growth (Herman, 2002). This series of events occurs in a similar way in all eukaryotes and because of the fundamental biological importance of cell cycle progression and its relevance in both human development and diseases such as cancer, identification of molecular determinants of specific stages of the eukaryotic cell cycle has been a subject of intense study for several decades (Yu et al., 2006, Levine and Holland, 2018).

Each step on cell cycle process is tightly regulated by external and internal signals, in order to maintain cell genomic stability through generations and to adapt cell metabolism and proliferation to environmental conditions (Brazhnik and Tyson, 2006). Numerous genes and proteins are involved in leading cells through the cell cycle phases and many cellular mechanisms control the cell cycle.

*Hanseniaspora* genus is the most common of those yeasts known as

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apiculate or bipolar budding cells and have been intensively studied to determine their effect on quality of fermented products (Martin et al., 2018; Lleixà et al., 2019; Del Fresno et al., 2020). In winemaking, *H. vineae* has been described to improve aroma quality, increasing fruity flavors, and enhancing wine complexity (Martin et al., 2018; Giorello et al., 2019).

Recently, *Hanseniaspora* yeasts have been gaining attention due to an unprecedented loss of dozens of genes involved in DNA repair and cell cycle that are broadly conserved across living organisms. Steenwyk et al. (2019) characterize two lineages within the genus *Hanseniaspora*, a

faster-evolving lineage (FEL) and a slower-evolving lineage (SEL), which differ by their acceleration in evolution rate as determined by the non-synonymous and synonymous ratio (dN/dS). Moreover, the two evolutionary groups exhibit important differences in terms of loss of genes related to DNA repair and cell cycle control, between them and when compared with *S. cerevisiae*. Other's organisms belonging to fungal phylum Ascomycota are described to also have lost genes related to cell cycle, especially related to DNA repair (Phillips et al., 2021).

The contribution of *H. vineae* in wine quality, and the discovery of a unique natural loss of genes related to cell cycle in *Hanseniaspora*

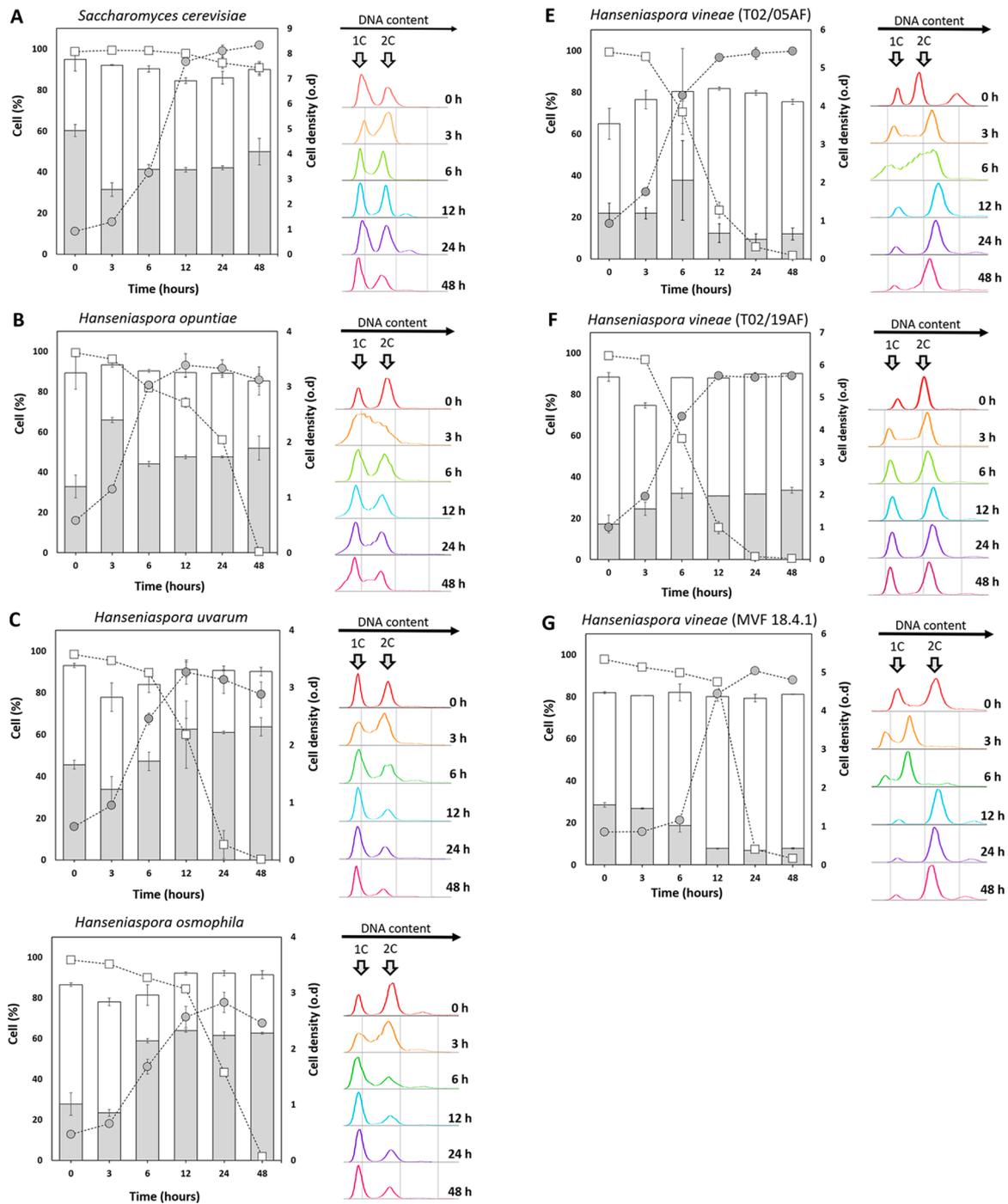


Fig. 1. Cell density (○), percentage of viable cells (□), percentage of cells with one copy of DNA content (grey bars), percentage of cells with two copies of DNA content (white bars) and the representative flow cytometry profiles of (A) *Saccharomyces cerevisiae*, (B) *H. opuntiae*, (C) *H. uvarum*, (D) *H. osmophila*, (E) *H. vineae* (T02/05AF), (F) *H. vineae* (T02/19AF) and (G) *H. vineae* (MV18.4.1) during aerobic growth. The positions of the peak of 1C and 2C DNA are indicated by arrows.

impelled us to study different species of this genus. Thus, the aim of this work was to characterize and compare the cell growth, viability, and cell cycle progression of FEL and SEL representatives from *Hanseniaspora* species along their development in an aerobic and discontinuous system.

## MATERIAL AND METHODS

Experiments were performed with three different *Hanseniaspora vineae* strains T02/05AF, T02/19F and MVF18.4.1, a *Hanseniaspora osmophila* (AWRI 3579), a *Hanseniaspora uvarum* (CZ17.1.1), a *Hanseniaspora opuntiae* (LAM 17.2.1) and a *Saccharomyces cerevisiae* (EC1118). A colony from each yeast strain was pre-grown overnight in YEPD media under constant shaking (150 rpm) at 28°C. The cells were collected by centrifugation, washed with sterile saline solution (0.9% NaCl) and inoculated into Yeast Nitrogen Base (YNB) medium (without amino acid) supplemented with 2% glucose, and incubated under constant shaking at 28°C, in order to obtain a final population of  $10^7$  cells ml<sup>-1</sup>. Cell culture aliquots were collected at 0, 3, 6, 12, 24 and 48 h of growth by centrifugation and assessed for viability, cell density and cell cycle.

The percentage of viable cell was evaluated by Trypan Blue exclusion test according to Strober (2015), which relies on the integrity of the cell membrane to distinguish between dead and viable cells. Briefly, one part of cell culture aliquots was mixed with one part of 0.4% trypan blue, incubated 1-2 min at room temperature and transferred to a Neubauer chamber, at least 200 cells/treatment were counted. To characterize exponential and stationary phase we evaluated cell density by optical density (600nm) using a spectrophotometer. Cell cycle analysis was determined by flow cytometry, according to Delobel and Tesniere (2014), using propidium iodide as fluorescent reactive dye and realized in a FACSCalibur flow cytometer (Becton-Dickinson) equipped with an argon-ion laser emitting at 488 nm.

The results were statistically analyzed by analysis of variance (one-

way ANOVA) and Tukey's test. The statistical analyses were performed using IBM-SPSS Statistics - version 22 software, and statistical significance was attributed to values of  $P \leq 0.05$ .

## RESULTS

As mentioned, the cell cycle is a complex process that involves a group of closely coordinated events that ensure the generation of new "daughter" cells, for the process to be efficient these events must have a controlled, coordinated, and sequential system monitored by internal and external signals, that ensure a proper cell cycle progress.

Fig. 1 and Table 1 show the cellular DNA content (1C and 2C), viability (%) and cell density (o.d). Cell density allowed to characterize three growth phases: lag phase, growth phase and stationary phase. All yeasts initiated the experiment with high cell viability, the highest was *H. opuntiae* with  $99.44 \pm 0.18\%$  and the lowest corresponded to *H. vineae* (MVF18.4.1) with  $97.85 \pm 0.29\%$ . As expected, *S. cerevisiae* (Fig. 1A) had a high and constant cell density increase during the growth phase (3 to 12h), after which the cell density increased slowly, indicating the stationary phase. Viability remained almost constant during the first 24h (98.69 to 93.14%) but decreased thereafter reaching 90.81% at the end of the experiment. Regarding DNA content, *S. cerevisiae* cells were inoculated after a pre-grow until stationary phase, where most of the cells had 1C of DNA content, indicating a cease in cell cycle progression and an arrest in G1/G0 phase of the mitotic cell cycle (60.25%). In the first 3h of the experiment *S. cerevisiae* population had a significant increase of cells with 2C DNA content, and after 6 hours the proportion of 1C and 2C remained similar and without significant differences, nearly 50/50 proportion.

Yeasts classified in faster-evolving lineages (FEL), *H. opuntiae* (Fig. 1. B) and *H. uvarum* (Fig. 1.C), had similar behavior regarding cell density, both reached stationary phase after 12 hours without significant

**Table 1**

Cell density, viability, one copy (1C) and two copies (2C) of cellular DNA content of *S. cerevisiae*, *H. uvarum*, *H. osmophila*, and *H. vineae* along their growth in aerobic condition.

Cell density (o.d)	T0	T3	T6	T12	T24	T48
<i>S. cerevisiae</i>	0.91 ± 0 <sup>b-D</sup>	1.29 ± 0.04 <sup>c-D</sup>	3.25 ± 0.17 <sup>b-C</sup>	7.68 ± 0.16 <sup>a-B</sup>	8.1 ± 0.23 <sup>a-AB</sup>	8.34 ± 0.09 <sup>a-A</sup>
<i>H. uvarum</i>	0.58 ± 0 <sup>d-C</sup>	0.95 ± 0.02 <sup>d-C</sup>	2.46 ± 0.1 <sup>c-B</sup>	3.27 ± 0.21 <sup>d-A</sup>	3.14 ± 0.24 <sup>c-A</sup>	2.88 ± 0.22 <sup>de-AB</sup>
<i>H. opuntiae</i>	0.58 ± 0.01 <sup>d-C</sup>	1.15 ± 0.06 <sup>c-B</sup>	3.03 ± 0.01 <sup>b-A</sup>	3.39 ± 0.21 <sup>d-A</sup>	3.33 ± 0.16 <sup>c-A</sup>	3.12 ± 0.11 <sup>d-A</sup>
<i>H. osmophila</i>	0.47 ± 0.01 <sup>e-C</sup>	0.66 ± 0.01 <sup>e-C</sup>	1.68 ± 0.13 <sup>d-B</sup>	2.57 ± 0.19 <sup>e-A</sup>	2.83 ± 0.18 <sup>c-A</sup>	2.46 ± 0 <sup>e-A</sup>
<i>H. vineae</i> (T02/05AF)	0.93 ± 0.01 <sup>b-D</sup>	1.76 ± 0.03 <sup>b-C</sup>	4.28 ± 0.01 <sup>a-B</sup>	5.27 ± 0.07 <sup>b-A</sup>	5.38 ± 0.15 <sup>b-A</sup>	5.44 ± 0.08 <sup>b-A</sup>
<i>H. vineae</i> (T02/19AF)	1 ± 0.02 <sup>a-D</sup>	1.95 ± 0.07 <sup>a-C</sup>	4.42 ± 0.04 <sup>a-B</sup>	5.67 ± 0.1 <sup>b-A</sup>	5.62 ± 0.08 <sup>b-A</sup>	5.67 ± 0.06 <sup>b-A</sup>
<i>H. vineae</i> (MVF18.4.1)	0.85 ± 0.02 <sup>c-E</sup>	0.86 ± 0 <sup>d-E</sup>	1.16 ± 0.02 <sup>e-D</sup>	4.44 ± 0.01 <sup>c-C</sup>	5.04 ± 0.07 <sup>b-A</sup>	4.8 ± 0.06 <sup>c-B</sup>
<b>Viability (%)</b>						
<i>S. cerevisiae</i>	98.69 ± 0.67 <sup>ab-A</sup>	99.28 ± 0.02 <sup>a-A</sup>	99.22 ± 0.17 <sup>a-A</sup>	97.83 ± 0.9 <sup>a-A</sup>	93.14 ± 2.65 <sup>a-AB</sup>	90.81 ± 3.05 <sup>a-B</sup>
<i>H. uvarum</i>	98.3 ± 0.35 <sup>ab-A</sup>	95.31 ± 0.3 <sup>ab-A</sup>	89.57 ± 0.19 <sup>bc-A</sup>	59.99 ± 16.18 <sup>b-B</sup>	7.4 ± 6.74 <sup>d-C</sup>	0.36 ± 0.51 <sup>b-C</sup>
<i>H. opuntiae</i>	99.44 ± 0.18 <sup>a-A</sup>	96.2 ± 1.85 <sup>ab-A</sup>	81.94 ± 1.4 <sup>c-B</sup>	74.59 ± 2.45 <sup>ab-C</sup>	56.15 ± 1.06 <sup>b-D</sup>	0.53 ± 0.75 <sup>b-E</sup>
<i>H. osmophila</i>	98.67 ± 0.29 <sup>ab-A</sup>	96.61 ± 0.75 <sup>ab-A</sup>	89.96 ± 0.91 <sup>bc-B</sup>	84.38 ± 0.49 <sup>ab-C</sup>	43.39 ± 0.35 <sup>c-D</sup>	1.89 ± 0.78 <sup>b-E</sup>
<i>H. vineae</i> (T02/05AF)	99.23 ± 0.08 <sup>a-A</sup>	97.1 ± 0.26 <sup>ab-A</sup>	70.49 ± 5.4 <sup>d-B</sup>	23.4 ± 3.8 <sup>c-C</sup>	5.76 ± 1.81 <sup>d-D</sup>	1.74 ± 0.25 <sup>b-D</sup>
<i>H. vineae</i> (T02/19AF)	98.73 ± 0.26 <sup>ab-A</sup>	96.81 ± 0.68 <sup>ab-A</sup>	58.65 ± 1.07 <sup>e-B</sup>	15.58 ± 2.98 <sup>c-C</sup>	1.45 ± 1.33 <sup>d-D</sup>	0.49 ± 0 <sup>b-D</sup>
<i>H. vineae</i> (MVF18.4.1)	97.85 ± 0.29 <sup>b-A</sup>	94.1 ± 0.95 <sup>b-B</sup>	91.46 ± 1.45 <sup>ab-B</sup>	87.01 ± 0.82 <sup>a-C</sup>	7.36 ± 1.06 <sup>d-D</sup>	2.96 ± 0.27 <sup>b-E</sup>
<b>1C (%)</b>						
<i>S. cerevisiae</i>	60.25 ± 2.9 <sup>a-A</sup>	31.55 ± 3.32 <sup>b-C</sup>	41.25 ± 2.47 <sup>ab-BC</sup>	41.1 ± 1.13 <sup>bc-BC</sup>	42.05 ± 0.92 <sup>c-BC</sup>	49.95 ± 6.43 <sup>a-AB</sup>
<i>H. uvarum</i>	45.65 ± 2.05 <sup>ab-BC</sup>	33.85 ± 6.15 <sup>b-C</sup>	47.25 ± 4.45 <sup>ab-ABC</sup>	62.65 ± 5.16 <sup>a-A</sup>	61.15 ± 0.64 <sup>a-AB</sup>	63.75 ± 4.31 <sup>a-A</sup>
<i>H. opuntiae</i>	32.9 ± 5.66 <sup>bc-C</sup>	65.9 ± 1.27 <sup>a-A</sup>	44.15 ± 1.34 <sup>ab-BC</sup>	47.6 ± 0.85 <sup>b-B</sup>	47.65 ± 0.49 <sup>b-B</sup>	52 ± 5.94 <sup>a-B</sup>
<i>H. osmophila</i>	27.8 ± 5.52 <sup>c-B</sup>	23.45 ± 1.63 <sup>b-B</sup>	58.95 ± 1.06 <sup>a-A</sup>	63.95 ± 0.78 <sup>a-A</sup>	61.65 ± 1.63 <sup>a-A</sup>	62.65 ± 0.49 <sup>a-A</sup>
<i>H. vineae</i> (T02/05AF)	21.95 ± 4.74 <sup>c-A</sup>	21.9 ± 2.69 <sup>b-A</sup>	37.7 ± 19.09 <sup>ab-A</sup>	12.33 ± 4.48 <sup>d-A</sup>	9.47 ± 2.59 <sup>e-A</sup>	11.99 ± 2.84 <sup>c-A</sup>
<i>H. vineae</i> (T02/19AF)	17.3 ± 4.24 <sup>c-B</sup>	24.65 ± 3.18 <sup>b-AB</sup>	32.1 ± 2.4 <sup>ab-A</sup>	30.9 ± 0.14 <sup>c-A</sup>	31.8 ± 0.14 <sup>d-A</sup>	33.6 ± 1.41 <sup>b-A</sup>
<i>H. vineae</i> (MVF18.4.1)	28.55 ± 1.06 <sup>c-A</sup>	26.8 ± 0.28 <sup>b-A</sup>	18.8 ± 3.11 <sup>b-B</sup>	7.77 ± 0.29 <sup>d-C</sup>	6.94 ± 0.59 <sup>e-C</sup>	7.81 ± 0.33 <sup>c-C</sup>
<b>2C (%)</b>						
<i>S. cerevisiae</i>	34.7 ± 5.66 <sup>c-C</sup>	60.6 ± 0.28 <sup>a-A</sup>	49.05 ± 1.48 <sup>ab-AB</sup>	43.45 ± 1.34 <sup>c-BC</sup>	43.95 ± 3.04 <sup>c-BC</sup>	40.05 ± 3.04 <sup>c-BC</sup>
<i>H. uvarum</i>	47.45 ± 1.06 <sup>bc-A</sup>	44.05 ± 6.86 <sup>b-AB</sup>	36.75 ± 3.75 <sup>ab-ABC</sup>	28.5 ± 3.68 <sup>d-C</sup>	29.6 ± 0.28 <sup>d-BC</sup>	26.45 ± 2.05 <sup>d-C</sup>
<i>H. opuntiae</i>	56.45 ± 8.13 <sup>ab-A</sup>	27.4 ± 1.13 <sup>c-C</sup>	46.2 ± 0.71 <sup>ab-AB</sup>	41.95 ± 2.47 <sup>c-ABC</sup>	41.5 ± 0.85 <sup>c-ABC</sup>	33.4 ± 7.07 <sup>cd-BC</sup>
<i>H. osmophila</i>	58.75 ± 0.92 <sup>ab-A</sup>	54.65 ± 1.91 <sup>ab-A</sup>	22.45 ± 5.02 <sup>b-B</sup>	28.2 ± 0.71 <sup>d-B</sup>	30.6 ± 1.27 <sup>d-B</sup>	28.8 ± 1.98 <sup>cd-B</sup>
<i>H. vineae</i> (T02/05AF)	42.95 ± 7.42 <sup>bc-A</sup>	54.6 ± 4.53 <sup>ab-A</sup>	42.65 ± 20.58 <sup>ab-A</sup>	69.45 ± 0.78 <sup>a-A</sup>	70.2 ± 1.13 <sup>a-A</sup>	63.45 ± 1.2 <sup>ab-A</sup>
<i>H. vineae</i> (T02/19AF)	71.2 ± 2.12 <sup>a-A</sup>	50 ± 1.27 <sup>ab-C</sup>	56.05 ± 0.07 <sup>a-B</sup>	57.05 ± 0.64 <sup>b-B</sup>	58.05 ± 0.78 <sup>b-B</sup>	56.55 ± 0.64 <sup>b-B</sup>
<i>H. vineae</i> (MVF18.4.1)	53.35 ± 0.49 <sup>abc-C</sup>	53.8 ± 0 <sup>ab-C</sup>	63.3 ± 4.1 <sup>a-B</sup>	72.35 ± 2.19 <sup>a-A</sup>	72.4 ± 1.84 <sup>a-A</sup>	73.3 ± 0.14 <sup>a-A</sup>

\*Small letters compare means in the columns (yeasts) and capital letters in the lines (hours).

differences in cell density thereafter. *H. opuntiae* population began to significantly lose viability just after 3 hours of growth and attaining 0.36% of viability at 48h, while *H. uvarum* started to lose viability after 6h of growth and reaching 0.36% of viability at 48h. A similar behavior through time was evidenced between *H. opuntiae* and *H. uvarum* regarding cell cycle, both strains increased cell number with 1C DNA content after 6 hours and at the beginning of the stationary phase (12h). After this time, 1C and 2C DNA content remained constant, in a 60/30 proportion to *H. uvarum* and a 50/40 proportion to *H. opuntiae*, indicating a cease in cell cycle progression and an arrest in G1/G0 phase.

Among *Hanseniaspora* species considered as slower-evolving lineages (SEL), *H. osmophila* (Fig. 1.D), reached stationary phase after 12 hours and remained with a constant cell density (from o.d 2.57 to 2.46) until the end of the experiment. Moreover, *H. osmophila* maintained a high cell viability in the first three hours (>97.5%) and fall rapidly reaching just 1.89% after 48h. Regarding cell cycle, *H. osmophila* had a significant increase in cells with 1C DNA content after 6 hours (from 27.8 to 58.95%), stabilizing thereafter in approximately 62% of the population, indicating a cease in cell cycle progression and an arrest in G1/G0 phase of the mitotic cycle. Conversely, as expected, the number of cells with 2C DNA content decreased from 58.75 to 28.8%.

The *H. vineae* (SEL group) strains T02/05AF (Fig. 1E) and T02/19F (Fig. 1F) reached stationary phase after 12 hours, while MVF18.4.1 (Fig. 1G) reached the highest cell density after 24 hours but showed a remarkable reduction of growth after 12h.

Strains T02/05AF and T02/19F showed a significant decrease in viability all over the experiment reaching 1.74 % and 0.49% of viability after 48h, respectively. However, MVF18.4.1 remained with a high viability (>90%) during the first 6h of growth and, after that time, viability decrease constantly and significantly, reaching a final viability of 2.96%.

From inoculation, through the exponential and stationary phase, and until the end of the experiment, most of the population of T02/05AF and T02/19F strains of *H. vineae* remained with a significantly higher proportion of cells with 2C DNA content. At the end of the experiment, more than 60% of T02/05AF, and 71% of T02/19F cells had a 2C DNA content, indicating that most of the cells of these strains arrested in G2/M phase. Similar behavior was evidenced regarding DNA content on MVF18.4.1, where, after reaching stationary phase (12h) and in the next evaluated times, most of the population presented a significantly higher proportion of 2C DNA content ( $72.35 \pm 2.19\%$ ). Despite the differences of growth, the three strains of *H. vineae* showed a peculiar behavior compared with *Saccharomyces* and other *Hanseniaspora* species with high proportion of cells with 2C DNA content (G2/M) at the stationary phase and quiescence.

## DISCUSSION

In the present work, we described the cell cycle in different species of the genus *Hanseniaspora* (from FEL and SEL branches) to establish the possible implications from a “natural” and unique loss of genes related to cell cycle. Therefore, to assess cell cycle progression, we analyzed asynchronous cell cultures and determined the distributions of DNA content. During the discussion, one copy (1C) or two copies (2C) of DNA content will be treated as G0/G1 and G2/M phases respectively since an accumulation of cells with either one or two copies of DNA can indicate an arrest through cell cycle progression. It is important to highlight that cell density was evaluated to identify exponential and stationary growing stages. A conventional behavior of eukaryotic cells (including yeasts) involves high levels of G2/M at the exponential phase when cell progress in their cell cycle, and a high prevalence of G0/G1 during stationary phase when cells are starved, cease mitotic division, and enter in a quiescent stage (Singh et al., 2006; Herman, 2002).

Our experimental results showed that *S. cerevisiae* populations remained with high viability throughout the experiment and increased cell density until reaching close to stationary phase (12h) of growth.

*S. cerevisiae* showed a classical behavior with high prevalence of G2/M during exponential growth, and a gradual increase of G0/G1 during stationary phase (48 h). According to Gray et al. (2004) quiescent yeast cells (G0 phase) are commonly obtained by growing *Saccharomyces* in liquid cultures to saturation in rich media, but in stationary phase, a substantial proportion of cells tend to a quiescent stage (G0). In our case, the proportion of cells in G0/G1 phase was not so evident, but as expected, at the end of experiment a larger population of quiescent cells (G0) were observed.

*H. opuntiae* and *H. uvarum* strains (FEL branch) had a proper progression in cell cycle, and as *S. cerevisiae*, they showed an increase of G0/G1 population after entering the stationary phase. Moreover, this arrest of the mitotic division events was more evident in *H. uvarum* than in *H. opuntiae*. These species start to decrease viability before they reach the stationary phase and this loss of viability could be caused by several factors. In *S. cerevisiae*, alterations of fundamental cellular pathways (such as defects in DNA replication and RNA instability), exposure to acetic acid, osmotic stress, low pH, and other stressing conditions could cause a rapid loss of viability (Falcone and Mazzoni, 2016; Carmona-Gutierrez et al., 2010). Indeed, according to Steenwyk et al. (2019), lineages belonging to FEL branch had experienced a substantial loss of genes, a greater number compared to SEL branch (661 genes uniquely lost in FEL and 23 genes uniquely lost in the SEL branch). The genes loosed in FEL branch are related to cell-cycle checkpoint genes, genome integrity, DNA damage checkpoint, DNA damage sensing, spindle checkpoint and metabolism-associated genes and, therefore, could be related to the fast loss of viability.

Regarding *H. osmophila*, belonging to SEL branch, loss of viability occurred at beginning of the stationary phase and go forward, when cells started to progress to G0/G1 phase. At the end of the experiment, most of populations ceased division and arrested on G1/G0 phase of the mitotic cell cycle. In summary, *H. osmophila*, as *S. cerevisiae*, *H. opuntiae* and *H. uvarum*, showed an expected behavior with high proportion of G2/M cells during fast-growing stages, and a gradual increase of quiescent cell (G0) during stationary phase of growth.

Strains T02/05AF and T02/19F of *H. vineae*, also belonging to SEL branch, showed a similar behavior to *H. uvarum* and *H. opuntiae*, decreasing viability prior to reach stationary phase, and strain MVF18.4.1 had an intense drop of viability at the beginning of the stationary phase, like *H. osmophila* behavior. However, the three *H. vineae* strains showed a peculiar behavior regarding cell cycle compared to the other species. As the other yeasts, *H. vineae* strains began their exponential growth with a high proportion of cells in G2/M phase, but during the last exponential phase and in stationary phase cells remain in a G2/M phase, even after cell proliferation ceased. This data indicates that *H. vineae* cells were not able to enter a proper cell cycle arrest at a G0/G1 stage. Little but consistent behavioral differences were observed among *H. vineae* strains (pronounced number of cells arrested in G2/M phase in T02/05AF and MVF18.4.1 compared to T02/19AF).

We do not have yet an explanation for the particular behavior of *H. vineae*, but there are some possible explanations. There are two key events that punctuate the cell cycle progression: DNA replication, when nuclear genome is duplicated - which defines S (synthesis) phase -, and mitosis (M phase), defined as the period in which chromosomes are condensed, sorted and then equally distributed to daughter cell (Hustedt and Durocher, 2017). During DNA replication, genotoxic stresses (such as DNA damage and incomplete replication) activate the checkpoints pathways, which prevents cell cycle progression until the damage are repair (Chao et al., 2017). These checkpoints have the role of checking if the replication have been properly accomplished, and there are no physical impediments for chromosomes to be replicated or repaired prior to nuclear division (Hartwell and Weinert, 1989; Russell, 1998). Damages incurred during the process of DNA replication is considered to be especially detrimental (Shor et al., 2020) as they can lead to cell failure to arrest in G0/G1 phase, genome instability and apoptosis (Weinberger et al., 2007). If cells with incomplete replication

or damaged DNA progress along cell cycle, they arrest at the G2/M phase (Lezaja and Altmeyer, 2018).

Fungal DNA damage responses are highly diverse (Shor et al., 2020) and, as commented before, several lineages of yeast genus *Hanseniaspora* lack homologs of dozens of genes involved in chromosome segregation, cell cycle progression, and DNA repair. Work performed by Shor et al. (2020), examined the DNA damage response of *C. glabrata* and uncovered that in the presence of DNA damage, cells did not accumulate in S phase (like *S. cerevisiae*) and proceeded to divide, giving rise to mitotic errors and significant cell death. Furthermore, cells with unrepaired DNA damage at the end of S phase also activate the G2/M checkpoint, which arrests cells in mitosis (Rhind and Russell, 1998; Lobrich and Jeggo, 2007).

Steenwyk et al. (2019) reported that 23 genes involved in cell cycle progress, biological process, cellular components with molecular function, were uniquely lost in the SEL branch (*H. osmophila* and *H. vineae*), and among those only PHO genes are known to have a direct or indirect link to cell cycle progress, were PHO5, PHO3, PHO11 and PHO12 are absent on both strains. Moreover, Spellman et al. (1998) identified more than 800 genes exhibiting cell cycle oscillation among those, and many of these genes (~300) participate in nutrient acquisition, and their transcripts show high expression in M or M/G1, among which they are repressible acid phosphatases (PHO5, PHO11, and PHO12) and constitutive acid phosphatases (PHO3) (Neef and Kladde, 2003).

Interestingly, although *H. osmophila* and *H. vineae*, both members of SEL branch, share a set of absent genes, only *H. vineae* strains were not able to enter an expected quiescent (G0/G1) cell cycle arrest. Shen et al. (2018) analyzed the genomes of 332 yeast species and found differences in genome content between those two species. The BUSCO genes analysis shows 138 genes not found in the genome and 112 genes fragmented (i.e genes with a partial sequence) in *H. vineae*, against 132 and 118 genes in *H. osmophila*, respectively. Despite these losses, the growth and development of this genus in its habitat do not seem to be disrupted, since *Hanseniaspora* yeasts have successfully diversified and are frequently isolated from grape and wine must environment (Martin et al., 2018).

It is important to highlight that cell cycle proper progression can be sensitive to many environmental conditions (e.g osmotic, oxidative, or replicative stress). These situations can slow down or even arrest cell cycle progression by different molecular mechanisms (Jiménez et al., 2015). The nutrient-induced signaling network enables yeast to profit from rich nutrient conditions by stimulating cell proliferation and to survive periods of nutrient scarceness by inducing the entry into a quiescent (G0) stage (Smets et al., 2010). Moreover, restriction of some nutrients can cause a transient G2/M phase arrest. This was reported under abrupt glucose starvation in *Schizosaccharomyces pombe* (Masuda et al., 2016), and is dependent on Wee1 tyrosine kinase that inhibit the key cell cycle regulator, CDK1/Cdc2. G2/M arrests nutrient dependent were also reported in *S. cerevisiae*. Santos et al. (2014) demonstrated that the presence of ammonium (NH<sub>4</sub><sup>+</sup>), and possible any other rich nitrogen source, inhibits a proper cell cycle arrest for cells cultured in medium with high or low amino acid concentrations. Although it is known that some genotoxic agents cause cell cycle exit in G2 phase during cancer cell treatments in human fibroblasts (Baus et al. 2003), it is unknown why this phenomenon might happen in natural cells (Gire and Dulic, 2015).

## CONCLUSION

The present work evidenced a peculiar behavior of *H. vineae* when compared with the control yeast *S. cerevisiae*, and other representatives of the FEL group and SEL group of the genus *Hanseniaspora*. While these species exhibited a typical G1/G0 (1C DNA) arrest at the end of the exponential phase and during the stationary phase, the three strains of *H. vineae* (SEL group) arrested a G2/M stages of cell cycle (2C DNA), and lost viability rapidly when enters the stationary phase. These results

have implications in the production of *H. vineae* with commercial purposes for wine fermentation, as well as in future studies of genetic determinants of yeast cell cycle control and progression. Moreover, these results open the opportunity to determine physical and nutritional conditions that may interfere with the behavior of *H. vineae*, both in aerobic and fermentative systems. Nonetheless, this work highlight the potential of this yeast species for understanding genome function and evolution in a native eukaryotic cell.

## AUTHORS' CONTRIBUTIONS

Luisa Vivian Schwarz and Sergio Echeverrigaray were involved in the planning and execution of the experimental work. All authors were involved in the MS writing and editing.

## ETHICS APPROVAL

Not applicable

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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