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Review

Oenological Impact of the *Hanseniaspora/Kloeckera* Yeast Genus on Wines—A Review

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Abstract: Apiculate yeasts of the genus *Hanseniaspora/Kloeckera* are the main species present on mature grapes and play a significant role at the beginning of fermentation, producing enzymes and aroma compounds that expand the diversity of wine color and flavor. Ten species of the genus *Hanseniaspora* have been recovered from grapes and are associated in two groups: *H. valbyensis*, *H. guilliermondii*, *H. uvarum*, *H. opuntiae*, *H. thailandica*, *H. meyeri*, and *H. clermontiae*; and *H. vineae*, *H. osmophila*, and *H. occidentalis*. This review focuses on the application of some strains belonging to this genus in co-fermentation with *Saccharomyces cerevisiae* that demonstrates their positive contribution to winemaking. Some consistent results have shown more intense flavors and complex, full-bodied wines, compared with wines produced by the use of *S. cerevisiae* alone. Recent genetic and physiologic studies have improved the knowledge of the *Hanseniaspora/Kloeckera* species. Significant increases in acetyl esters, benzenoids, and sesquiterpene flavor compounds, and relative decreases in alcohols and acids have been reported, due to different fermentation pathways compared to conventional wine yeasts.

Keywords: non-*Saccharomyces*; genome; aroma compounds; anthocyanin; mixed cultures fermentation; flavor complexity

1. Introduction

Non-*Saccharomyces* (NS) yeasts were considered unattractive in traditional winemaking, and sulphites addition was the way to prevent the risk of their growth at the beginning of the vinification process. However, today's increased knowledge about yeast diversity has demonstrated that there are many NS yeasts with beneficial properties that contribute to increasing the sensory complexity of wines [1–3]. The main NS yeasts associated with grapes are the apiculate group with bipolar budding, more precisely, the genus *Hanseniaspora* and its asexual anamorph *Kloeckera* [4,5]. In Figure 1, the plating and microscopy characteristics of two species of the genus are shown.

The *Hanseniaspora/Kloeckera* (H/K) group is currently composed of 10 recognized species associated with grapes [6–8]. One of the main characteristics of these species is the weak fermentation capacity compared to *Saccharomyces cerevisiae* (SC). However, some species, such as "*vineae*", might reach about 10% of the alcohol by volume of fermentative capacity under winemaking conditions. Furthermore, these species are important in the production of an increased diversity of volatile compounds in wine, and it was demonstrated the chemical composition of wines made with H/K in combination with SC differ from reference wines [9–12]. During these early studies about apiculate yeasts, some authors [13–15] showed that not all H/K strains formed high levels of volatile acidity and many of them produced similar levels to SC in this regard. These results indicate that although some strains of H/K can provide higher levels of ethanol than other strains, the main characteristic of

many of these known strains is the increased formation of some acetate esters. The production of other secondary metabolites—i.e., glycerol, acetaldehyde, and hydrogen sulphide—also differed between strains [16]. Thus, differences in chemical analyses of the wines were noted.

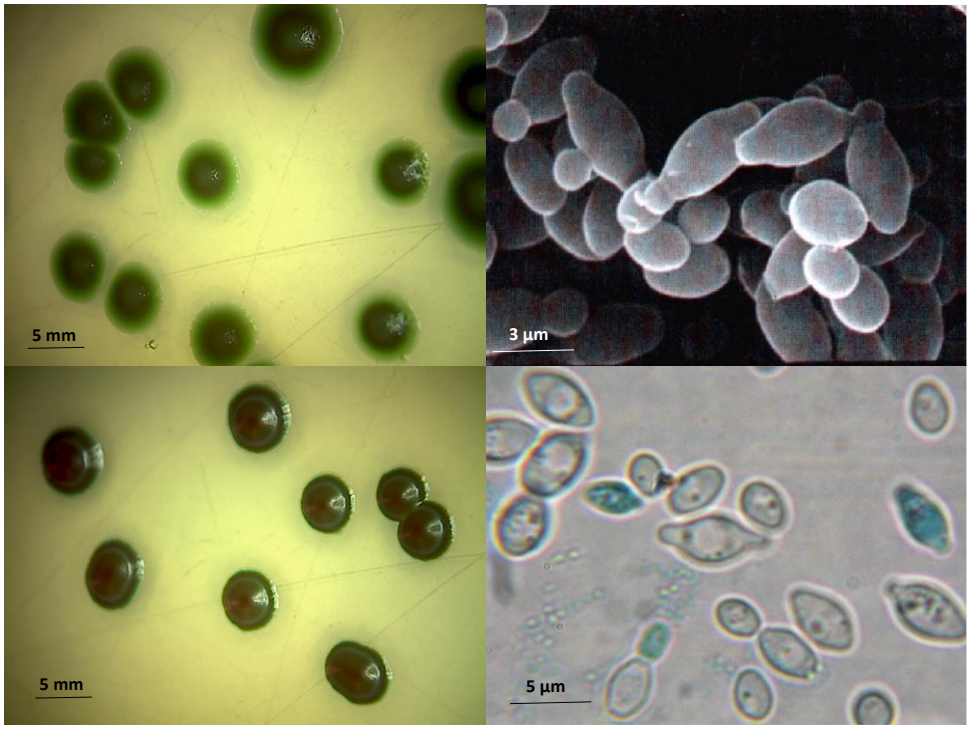

Figure 1. On the left-hand side are the typical colony color and morphology for *Hanseniaspora/Kloeckera* strains that are readily differentiated from other yeast genera in WL nutrient agar medium. However, is more difficult to distinguish between species, although some slight differences between *H. uvarum* (**upper**) colonies and *H. vineae* (**lower**) colonies can be appreciated in these photos. On the right-hand side, *H. uvarum* is visualized by electron microscopy and *H. vineae* is visualized with blue methylene stain.

The initial growth of H/K had a retarding effect on the subsequent growth of SC, as also shown for other NS species in mixed cultures [17]. Therefore, when considering the use of H/K strains at winemaking, grape must nutrient composition and competition for assimilable nitrogen by mixed cultures should be understood to prevent sluggish fermentations [18]. Some other cell interactions between H/K strains and SC that inhibit their growth were reported [19], however, H/K strains are intense removers of some vitamins, such as thiamine [20] or calcium pantothenate [21]. Medina et al. [17] found these two vitamins, in combination with ammonium salts, improved the development of SC strains to complete fermentation. Addition of yeast extract at 2 g/L was demonstrated to be more effective for *H. vineae* utilisation than ammonium salts in agave juice for tequila [22]. In white wine production, a *K. apiculata* isolate was used with SC at laboratory scale [23]. Inoculation of SC occurred 1 h after *K. apiculata,* and a dry wine of 13% by volume of ethanol was produced. A positive sensory evaluation of the Sauvignon blanc wines was obtained at 5 and 18 months after production. The production of β-phenylethyl acetate and ethyl acetate by the apiculate yeast *H. guilliermondii* has been investigated in laboratory fermentations [24]. The β-phenylethyl acetate ester contributes to 'rose', 'honey', 'fruity', and 'flowery' aroma nuances, and is formed to a greater or lesser extent by yeasts. As part of the 'fermentation bouquet', it can contribute to the overall flavor of a young wine.

The positive oenological characteristics that this H/K confers to wine have been broadly reported [18,25,26]. In addition, some studies have shown that several H/K strains have potential as

biocontrol agents against fungi, such as *Botrytis*. The competition for nutrients is the action mechanism of protection used by *H. uvarum* against fungi in grapes and apples [27,28].

2. Genetics Context of *Hanseniaspora* Species

2.1. Application of Molecular Techniques for Taxonomy and Whole Genome Analysis

Classical microbiology techniques have been extensively used in oenology to select and inoculate the best yeast strains for obtaining enhanced positive characteristics in final wines. During the last decades, the use of molecular techniques for the identification and selection of specific strains has proven invaluable for the winemaking industry. Polymerase chain reaction-based methods allow the identification of distinct species and, also, strain genotyping, resulting in a more accurate strain selection [29,30]. Based on this knowledge, culture-independent techniques have been developed to detect microorganisms present during fermentation that are not cultivable by conventional methods [31–33]. Among these culture-independent techniques, the development of next-generation sequencing permitted the description of the whole microbiota present in a specific environment, even in complex communities, such as those found throughout the wine fermentation process [34,35].

Hanseniaspora species have been widely detected in various wine-related environments, especially from soil and grapes to the early stages of vinification [36,37]. This genus is part of the apiculate group of yeast formed by *Hanseniaspora*, *Saccharomycodes*, and *Nadsonia*. The genus *Hanseniaspora* presents heterogeneous morphological, serological, and chemotaxonomic features [38]. Ten species of *Hanseniaspora* have been recovered from grapes or wines, which are taxonomically associated in two groups (Figure 2): *H. valbyensis*, *H. guilliermondii*, *H. uvarum*, *H. opuntiae*, *H. thailandica*, *H. meyeri*, and *H. clermontiae* in one cluster; and *H. vineae*, *H. osmophila* and *H. occidentalis* in the other, as revealed by partial sequence alignment of the *26S rRNA* gene. The favorable oenological characteristics that this genus confers to wine have been broadly reported [18,25,39]. However, the biotechnological potential of these species is still under evaluation at the industrial level, as compared to the traditional SC conventional strains. In this context, the development of molecular techniques and the recent identification of the whole genome sequences from *Hanseniaspora* species related to wine have created the possibility to understand and applied them from a novel, precise oenological perspective.

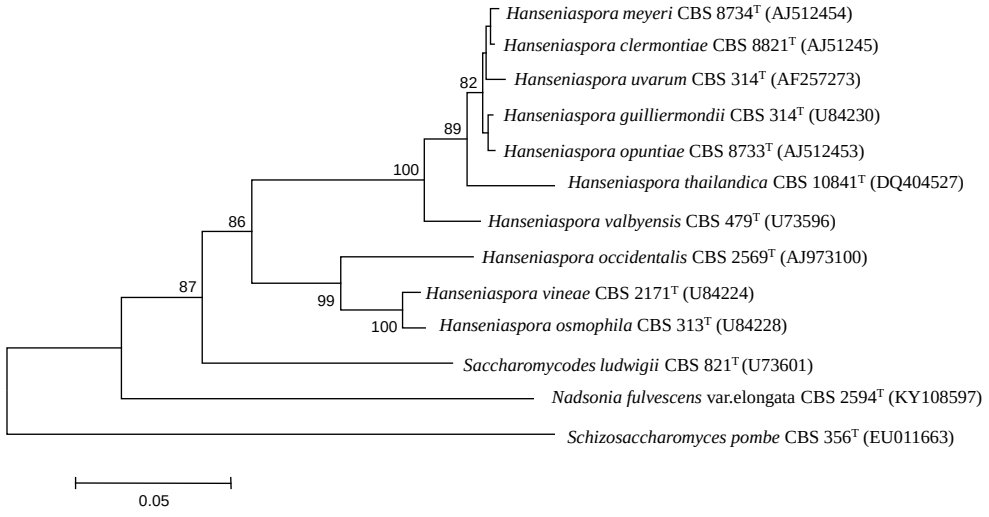

Figure 2. Phylogenetic relationships between type strains of *Hanseniaspora* species and other grape or wine-related apiculate yeasts. The dendrogram was constructed using partial *26S rRNA* gene sequences by the neighbor-joining method. The robustness of the branching is indicated by bootstrap values (%) calculated for 1000 subsets. The entries of the different genotypes include the accession numbers of the GenBank database sequences. *Schizosaccharomyces pombe* type strain was used as an outgroup.

2.2. Comparative Analysis of Hanseniaspora Genomes

Today, 10 strains belonging to six different *Hanseniaspora* species have been completely sequenced [40–43]. The data collected from these sequences (Table 1) evidence the close relation between *H. vineae* and *H. osmophila* that present a similar genome size and G + C percentage compared with the others. Moreover, the protein count is quite similar throughout the species from the genus, but the number of contigs and scaffolds reported vary widely among different species. As informed by karyotyping approaches, H/K species could present between seven and nine chromosomes [29,30]. In a recent study based on field inversion gel electrophoresis and the whole genome sequencing of type strain *H. uvarum* DSM2768, seven chromosomes were detected [44]. Notwithstanding, there are wide differences in genome size and chromosome number in karyotyping results from natural grape samples. Besides, *Hanseniaspora* genus belongs to the group of yeast that does not undergo whole-genome duplication, contrary to *Saccharomyces* [44]. These discrepancies were previously detected in the mitochondrial DNA of *H. uvarum*. It presented a reduced size compared with those from other yeasts and also a different organization of genes [45].

Table 1. *Hanseniaspora* genome assembly statistics.

Species Name (Number of Strains)	Protein Count	Number of Contigs	G + C	Scaffold Number	Assembly (Mb)	Reference
H. guillermondii (1)	4070	250	31%	208	9.04	Seixas et al., 2017 [39]
H. opuntiae (1)	4167	67	35%	18	8.53	Sternes et al., 2016 [38]
H. uvarum (4)	3552	44	32%	18	8.81	Sternes et al., 2016 [38]
H. valbyensis (1)	4772	1345	23%	647	11.46	Riley et al., 2016 [37]
H. vineae (2)	4733	277	37%	124	11.40	Giorello et al., 2014 [36]
H. osmophila (1)	4657	899	37%	17	11.37	Sternes et al., 2016 [38]

There are also some differences in the information about genes linked to interesting oenological traits. The highest number of alcohol dehydrogenases, like *ADH1*, *ADH2*, *ADH3*, *ADH4*, *ADH6*, and *ADH7*, from SC is found in the *H. vineae* genome. It presents eight genes for alcohol dehydrogenases, followed by *H. osmophila* with six. *H. uvarum*, *H. guilliermondii*, and *H. opuntiae* present just four. The highest number of copies could be related to the fermentation capacity, given the alcohol dehydrogenase activity is involved in the last step of the glycolytic pathway [46]. The fermentation ability is considered a hurdle in NS yeast relative to *Saccharomyces* strains, and thereby an improvement in fermentation performance is necessary to select a strain for wine inoculation. Limited information is available about the functional analysis of protein activities from *Hanseniaspora*. A key enzyme associated with the glycolytic pathway is pyruvate kinase. Langenberg et al. [44] recently demonstrated the correlation between pyruvate kinase activity and the enhanced fermentative ability of SC compared with *H. uvarum*. The authors explained this difference was due to a lowered specific activity rather than the structure of this enzyme. *H. vineae* and *H. osmophila* present higher sequence homology (Figure 3) in the predicted protein corresponding to the *CDC19* gene from SC than *H. uvarum* and other H/K species. Further biochemical studies will clarify the potential pyruvate kinase activity in H/K species compared to *H. uvarum* and SC.

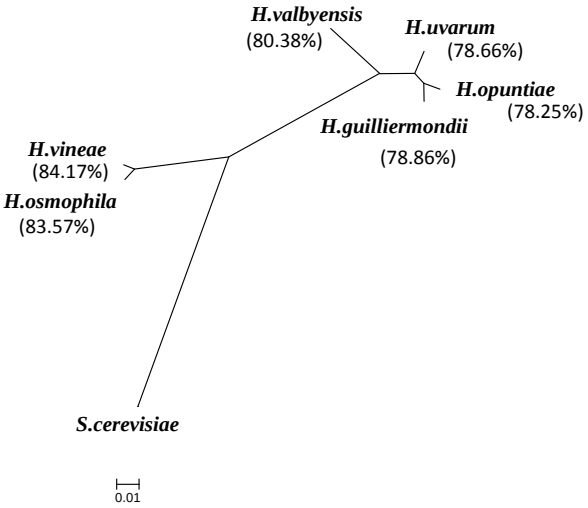

Figure 3. Relative homology of predicted protein sequences from the *CDC19* gene for pyruvate kinase activity in genome-sequenced *Hanseniaspora* strains (*H. vineae* T02/19AF; *H. osmophila* AWRI3579; *H. guilliermondii* UTAD222; *H. opuntiae* AWRI3578; *H. valbyensis* NRRL Y-1626; *H. uvarum* AWRI3580; *Saccharomyces cerevisiae* S288c). Data sequences have been collected from the NCBI protein database. Unrooted trees have been constructed using neighbor-joining analysis to calculate the percentage divergence. The percentage of identity with SC is expressed in brackets and calculated as the number of identical amino acids based on the total length.

The lack of nutrients, especially nitrogen, is a leading concern in wine fermentation that can cause stuck or sluggish fermentations [47,48]. Some genes linked to the regulation of nitrogen consumption have been identified in SC [49]. The general amino acid permease activity is attributed to *GAP1* in SC, and homologous sequences are present in a high copy number. For instance, 12 *GAP1* homologues were detected in the *H. guilliermondii* UTAD222 genome. Ammonium permeases are also involved in the regulation of nitrogen metabolism; *MEP2* homologues were found in all H/K species sequenced, and *MEP3* similar sequences were found just in *H. uvarum* and *H. osmophila*. The absence of *MEP3* in *H. vineae* might explain the inability of this species to use ammonium salts, as reported for agave juice fermentations [22].

Several enzymes that contribute to wine aroma have been extensively described in SC. One of them, *IAH1*, codifies for isoamyl acetate hydrolysing esterase, which adds to the production of desired volatile esters. The genomes of *H. osmophila*, *H. opuntiae*, *H. uvarum*, and *H. guilliermondii* present sequences that codify for a predicted protein highly similar to *IAH1*. Instead, *ATF2* and *EHT1* are both alcohol acetyltransferases. The activity of *ATF2* is affiliated with the formation of volatile esters during SC fermentation, and *EHT1* is linked to short-chain esterase activity [46]. Putative homologous alcohol acetyltransferases were predicted from DNA sequences only present in the genomes of *H. osmophila* and *H. vineae* [40].

The increase in sequences from whole genomes of *Hanseniaspora* strains available in databases is a good starting point to apply the biotechnological potential that these yeasts represent for oenology. Indeed, promising results were obtained in an attempt to genetically modify *H. guilliermondii* strains [50].

3. *Hanseniaspora*/*Kloeckera* Strains and Flavor Compounds

H/K yeasts may affect the wine fermentation directly, by producing flavors, and indirectly, by modulating the growth and metabolism of SC.

More recently, NS wine yeasts have received special attention by winemakers due to the search of different and desired oenological characteristics, compared to SC commercial strains. Diverse secondary metabolic pathways and higher enzymatic activities (esterases, β-glycosidases, lipases,

and proteases), result in sensory complexity [1,51,52] that might contribute to an increased diversity of 'flavor phenotypes'. The 'flavor phenotype' is an interesting concept for yeast selection, considering that now more than 1300 volatile compounds can be determined in wine [2,53].

In recent years, the genus H/K has been the subject of considerable study and publications, due to its positive contribution to the sensory characteristics of wines. Specifically, the yeast *H. vineae* of this genus has been of great interest because it produces several key aromatic compounds and has a good fermentation capacity. A strain of *H. vineae* isolated from Uruguayan vineyards was selected because of its positive effect on wine fermentation and contribution to the aroma profile of the final wine [18]. *H. vineae* has been demonstrated to increase fruity aromas and produce a high amount of acetate esters, such as 2-phenylethyl acetate and ethyl acetate (Figure 4), both in laboratory assays and in wines elaborated by sequential fermentation with SC [18,54].

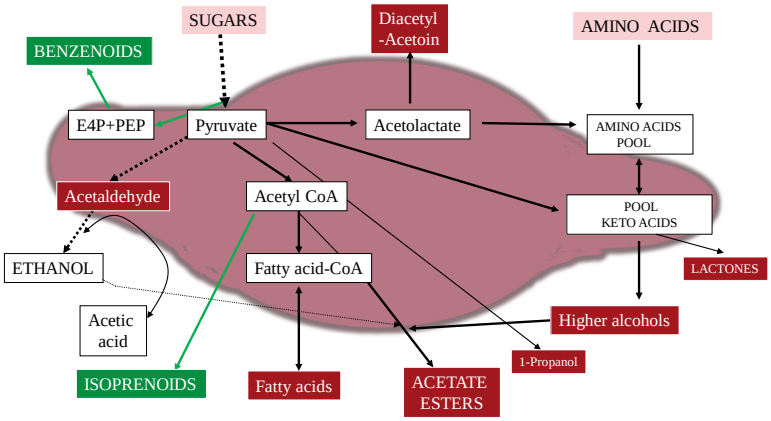

Figure 4. Fermentation flavor compounds produced by *Hanseniaspora*/*Kloeckera* yeasts during wine production (red and green boxes). Specific metabolic pathways that are highlighted in some species of this genus are shown in green (arrows and boxes). Pink boxes are the medium nutrients and doted arrows showed the main glycolysis pathway of primary fermentation.

Various groups of volatile compounds are produced during fermentation with H/K genus. For example, the use of a selected *H. uvarum* strain in mixed fermentation with commercial SC F5 increased the medium-chain fatty acid ethyl ester content in both synthetic media and grape must of Cabernet Gernischt grapes [55]. However, Medina et al. [18] did not find a significant increase in ethyl ester, using co-fermentation with *H. vineae* in Chardonnay grape must. In this work, decreases in the higher alcohols content—including 1,3-propanediol, 3-methyl 1-propanol, and tyrosol—were detected. Similar results were revealed in treatments with *H. uvarum*, finding a lower concentration of higher alcohols than the treatments inoculated with the SC isolates [56]. On the contrary, co-fermentation with *H. opuntiae* increased the amount of higher alcohols (phenylethanol and 3-methyl-butanol) and phenylacetaldehyde, in Cabernet Sauvignon grape must, intensifying the floral and sweet attributes of wine [57].

All H/K species increase the concentration of almost all the acetate esters. For example, all the acetate esters determined, except isoamyl acetate, were significantly affected by the inclusion of *H. osmophila* in a starter [58]. In this case, ethyl acetate and β-phenylethyl acetate concentrations in wine were increased when the proportion of *H. osmophila* in the culture increased. In wines fermented with the H/K:SC culture ratio of 90:10, the concentration of β-phenylethyl acetate was approximately 9-fold greater than that produced by SC pure culture [58]. In cold pre-maceration of Pinot noir grapes, inoculation with *H. uvarum* had the highest ethyl acetate level among the treatments evaluated, as well as high concentrations of the aforementioned branch-chained esters and, also, isoamyl acetate and isobutyl acetate [56]. In another report, the increased acetate ester levels were increased when *H. uvarum* was inoculated 48 h before SC, in different wine varieties, demonstrating that their enhancement could be induced by high population proportions of *H. uvarum* to SC. However, excessive *H. uvarum* yeasts in

the inoculation slowed down the fermentation rate and produced a nail polish-like odour in Cabernet Sauvignon wines, by increasing the contents of acetate esters and volatile phenols [59]. Conversely, the wines produced from Negroamaro grapes by co-fermentation with *H. uvarum* showed an increment of acetate esters (ethyl acetate, isoamyl acetate and β-phenylethyl acetate) and fatty acids esters (ethyl hexanoate, ethyl octanoate and ethyl decanoate). In particular, an increase of isoamyl alcohol and β-phenyl alcohol was shown when compared to the wines produced by the SC starter [60].

Volatile compounds produced during fermentation of Macabeo grapes inoculated with *H. vineae* and separately with SC demonstrated significant differences in the acetates and higher alcohols. The *H. vineae* vinification produced low levels of higher alcohols and 5-fold greater concentration of the acetates [26]. Interesting, in this work, three compounds, 4-ethyl guaiacol, *N*-acetyltyramine and 1H-indole-3-ethanol acetate ester, were identified in wines with *H. vineae* but not in the wine fermented with SC [26].

3.1. De Novo Synthesis of Benzenoids and Isoprenoids

Benzyl alcohol, benzaldehyde, *p*-hydroxybenzaldehyde and *p*-hydroxybenzyl alcohol, compounds typically synthesised by plants, are synthesised de novo in the absence of grape-derived precursors by *H. vineae*. Levels of benzyl alcohol produced by 11 different *H. vineae* strains were 20−200 times higher than those measured in fermentations with SC strains. The absence of *PAL* in *H. vineae* suggests that benzenoids are necessarily dependent on de novo synthesis from chorismate [61,62]. It is worth noting that the increased use of diammonium phosphate, mainly applied in winemaking for increasing ester production or avoiding hydrogen sulphide formation, will decrease the production of phenylpropanoid compounds (Figure 5), compromising the final flavor complexity of the wine [61,62].

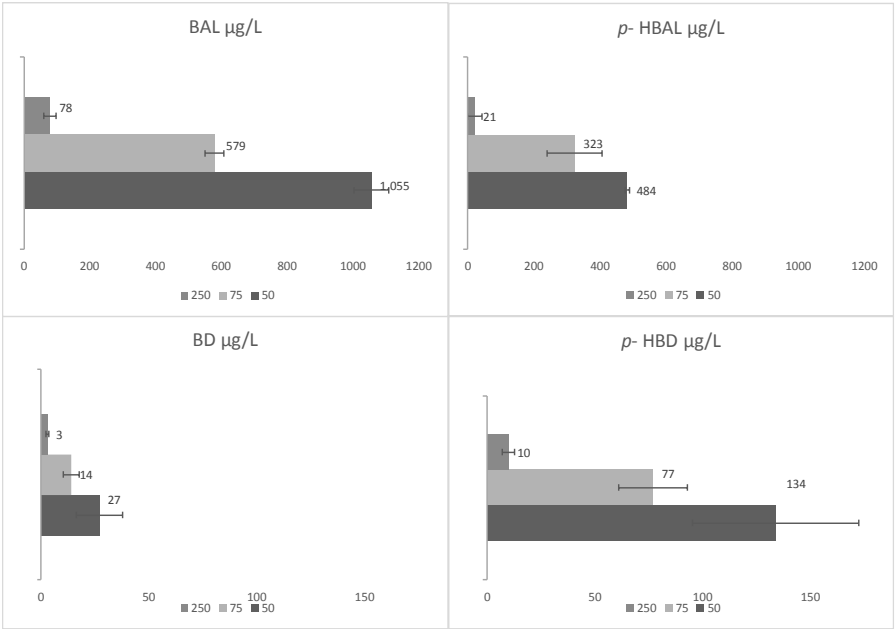

Figure 5. Formation of benzyl alcohol (BAL), benzaldehyde (BD), *p*-hydroxybenzyl alcohol (*p*-HBAL), and *p*-hydroxybenzaldehyde (*p*-HBD) by *Hanseniaspora vineae* 12/196 in the chemically-defined grape medium with three yeast assimilable nitrogen levels, where nitrogen levels of 75 and 250 mg/L were reached via the addition of diammonium phosphate. Fermentations were conducted at 20 °C; data are expressed in micrograms per liter.

Contrariwise, *H. vineae* produces high concentrations of the benzenoid and phenylpropanoid acetates. In the vinification of Macabeo grape must with *H. vineae*, 50 times more 2-phenylethyl acetate was generated than in vinifications with SC [26]. A similar trend was seen during de novo synthesis of monoterpenes by *H. uvarum*, where significant levels of citronellol were detected compared

to SC strains. More recently, studies have shown the formation of terpenes and sesquiterpenes in vinifications with different *H. vineae* strains (Figure 6) exceeded the threshold values and reached higher concentrations than sole fermentation by SC [63].

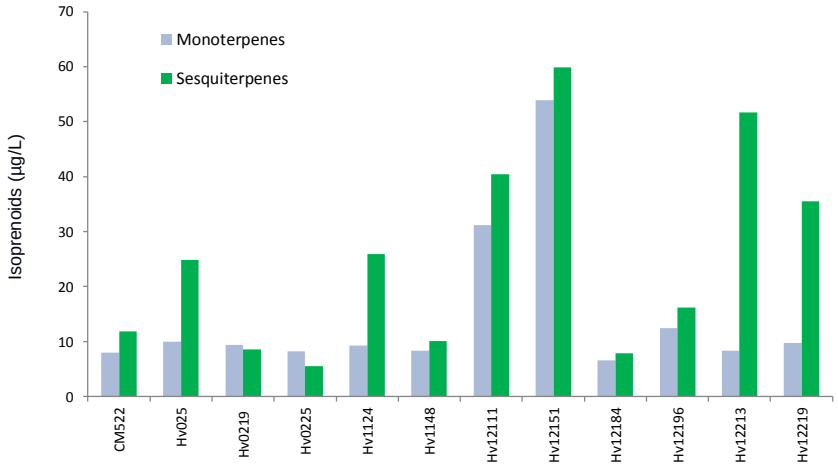

Figure 6. Production of isoprenoids (monoterpenes and sesquiterpenes) by various strains of *Hanseniaspora vineae* and the reference *Saccharomyces cerevisiae* CM522.

3.2. β-Glucosidase

Recently, Hu et al. [59] reported that β-glucosidase activity of *H. uvarum* yeast was 6.6-fold higher than that of the few naturally found SC strains. This characteristic explained why the participation of *H. uvarum* yeasts contributed to the increase of free terpene and C13-norisoprenoid contents with sensory impact [59]. However, high levels of β-glucosidase activity also increased the volatile phenol content, which might impart spicy odor traits to wines.

In a previous work, Mendes Ferreira et al. [64] studied the β-glucosidase activity using the *p*-nitrophenyl-β-D-glycoside (pNPG) as substrate in *H. uvarum* (formerly *K. apiculata*), *Pichia anomala*, and *Metschnikowia pulcherrima*, detecting the highest activity in *H. uvarum*. Furthermore, these authors demonstrated that *H. uvarum* was able to release some monoterpenols from an extract of Muscat grape juice, such as linalool, geraniol and in less quantity 3,7-dimethyl-1,7-octadien-3,6-diol and 3,7-dimethyl-1,5-octadien-3,7-diol, nerol, trans o-cimenol, α-terpineol, and citronellol [64].

The investigation of 31 H/K strains, including *H. guilliermondii*, *H. osmophila*, *H. uvarum*, and *H. vineae*, showed β-glucosidase and β-xylosidase activities (remarkable in one *H. uvarum* strain and two *H. vineae* strains) [65]. However, in this work, Muscat wine (13% *v*/*v*, initial alcohol) had only a moderate overall increase in terpene (1.1- to 1.3-fold) when treated with these strains. Specifically, these strains increase the levels of ho-trienol, β-phenylethanol, and 2,6-dimethyl-3,7-octadien-2,6-diol in the wine [65].

3.3. Effect of Hanseniaspora on the Volatile Compounds Produced during Tannat Red Grape Vinification

The vinification of Tannat grapes was conducted at three production scales: semi-pilot (20 kg), pilot (500 kg) and industrial (5000 kg) [66]. Figure 7 depicts the main flavor compound groups produced. The highest formation of acetates was detected in the vinifications with *H. vineae*, whereas, the maximum ethyl acetate concentration occurred in the vinification with *H. clermontiae*. Interestingly, the greatest concentration of norisoprenoid compounds was achieved by *H. vineae* vinification at industrial-scale compared to micro-fermentations.

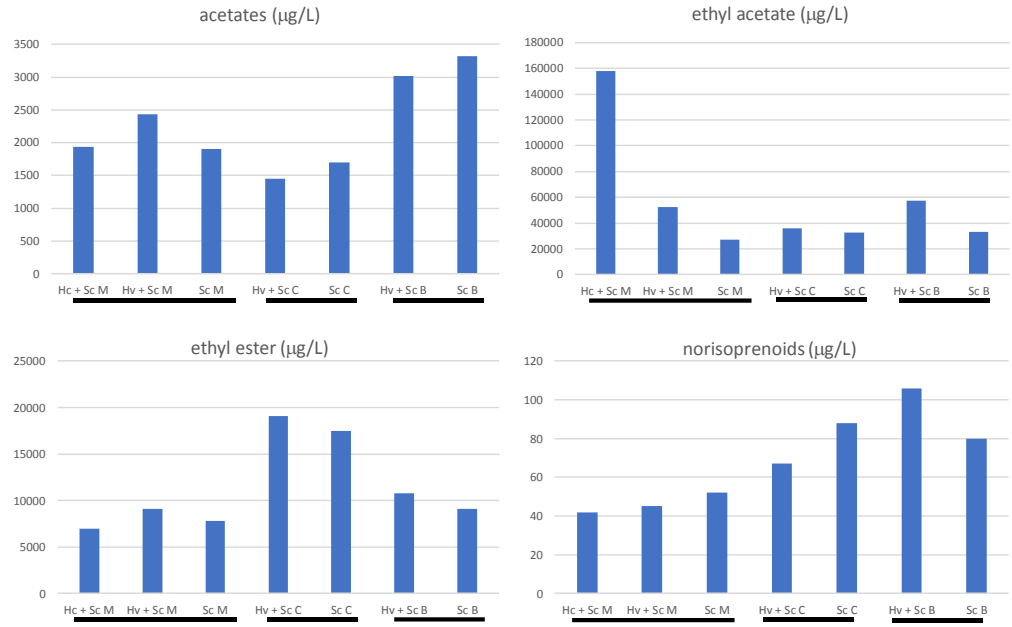

Figure 7. Concentration of principal groups of volatile compounds in vinifications at semi-pilot (M), pilot (C) and industrial (B) scale. Vinifications were inoculated with *Hanseniaspora vineae* (Hv), *Hanseniaspora clermontiae* (Hc) and *Saccharomyces cerevisiae* (Sc).

4. *Hanseniaspora/Kloeckera* Strains and Red Wine Color

The yeasts and grape maceration technology utilised during the vinification process affects pigment contents and the final red wine color [67–69]. Interactions between yeasts and anthocyanins during fermentation involve a range of mechanisms that might decrease or increase color. Yeast cell wall anthocyanin adsorption [70–72] and β-glucosidase activity, which releases the corresponding glycosylated anthocyanidin, exposing it to ready oxidation or conversion to colorless compounds [73], are well-known phenomena. Further research in the last decade has proved that some key compounds released during fermentation, such as pyruvic acid and acetaldehyde, are reactive precursors in the formation of new stable pigments. Vitisin A, vitisin B and ethyl-linked anthocyanin-flavanol pigments are examples of anthocyanin-derived compounds produced by SC strains [72,74–80]. Yeast strain selection strongly affects color intensity (CI) and the final concentrations of the anthocyanins [81,82] and other phenolic compounds [82].

Recently, studies have proven that some NS species might also be involved in wine color stabilisation [83,84]. As it was expected, some of these reactions could be attributed to the variable levels of acetaldehyde or pyruvate synthesis by different yeast species. For example, *Pichia* species generated significantly higher levels of acetaldehyde compared with *Saccharomyces* [85], and acetaldehyde increased linearly with increasing cell biomass concentration [86]. Except for a few reports on *Pichia*, *Schizosaccharomyces* and, more recently, some species of *Hanseniaspora* [83,87,88], limited information has been presented about the effect of NC strains on wine color composition. For the selection of new yeasts for the application with *Vitis vinífera* L. cv Tannat, a widely grown cultivar in Uruguay and one of the richest varieties in polyphenolic compounds [89,90], a program to select native NS yeasts and thereby increase the yeast diversity for fermentation without affecting wine color, was developed [91]. The Tannat grape juice model medium utilised allowed to screen the strains' capacity to synthesise anthocyanin-derived compounds while avoiding the interference of grape solids, such as the skin and seeds, was demonstrated previously for SC strains [72].

According to Medina [66], who evaluated 22 native H/K species for their effect on total anthocyanins (TA), CI, hue and total polyphenol index (TPI), the TPI values did not differ significantly between strains. The parameters with the greatest variation were CI with 32% (Figure 8), followed by TA with 30%, and then hue with 24%.

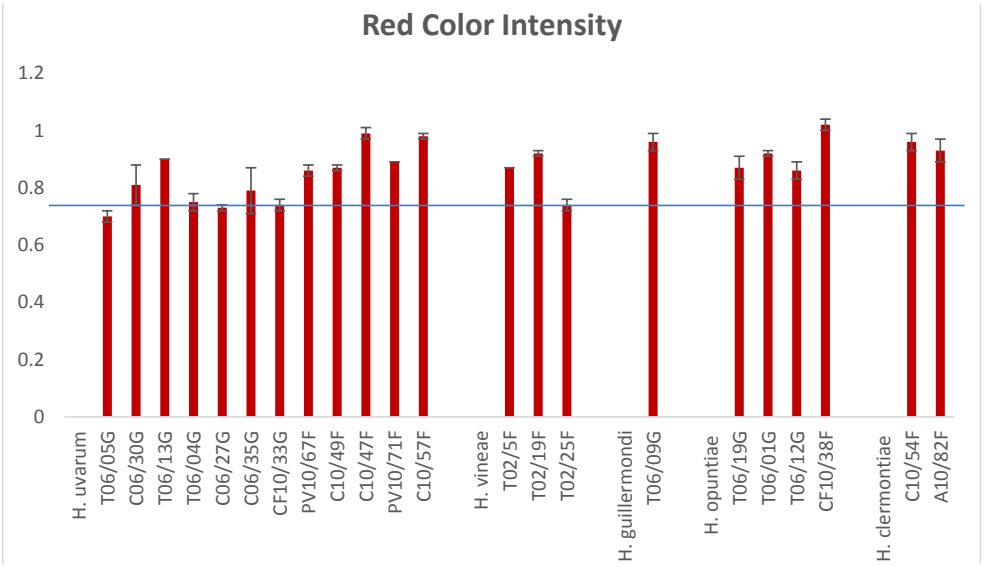

Figure 8. Mean normalised value and standard deviation of color intensity (sum of absorbance at 420, 520 and 620 nm), for 22 strains of five different *Hanseniaspora* species. The blue line indicates the average of the four lowest values obtained.

In the same study [26], consideration of the impact of CI and TA as the main color parameters in wines, the following strains were selected: *H. guilliermondii* (T06/09G), *H. opuntiae* (T06/01G), *H. vineae* (T02/5F) and *H. clermontiae* (A10/82F and C10/54F). Anthocyanin content and CI were evaluated against the best SC (882), previously selected for red grape fermentations [72]. All the previous selected H/K strains formed vitisin B, vitisin A, malvidin-3-*O*-glucoside-4-vinylphenol, malvidin-3-*O*-glucoside-4-vinylguaiacol (Figure 9, shows with letters A, B, C and D respectively).

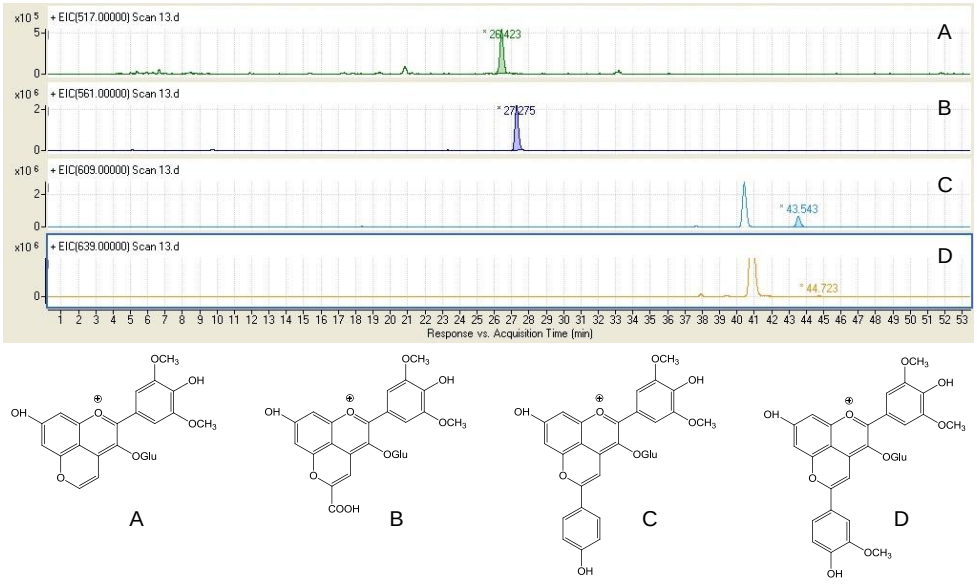

Figure 9. Identification of anthocyanin-derived pigments of Tannat grapes during fermentation by *Hanseniaspora/Kloeckera* yeasts that contribute to enhanced color stability.

Vitisin B formation has been reported previously only for SC yeasts [77,78,81,92]. Results of the anthocyanin-derived compounds formed during fermentation in the mentioned model grape medium indicated vitisin B could be linked to the increased acetaldehyde levels produced by SC when compared with NS yeasts [93]. All the NS strains selected showed vitisin B formation, despite some

relatively low concentrations recorded relative to that formed by *Saccharomyces* yeast. For vitisin A, in contrast, there was a greater formation with NS strains than SC, possibly linked to the presence of pyruvic acid in the medium [78]. In corroboration with these findings, Morata et al. [83] noticed that in comparison to SC, *Schizosaccharomyces pombe* produced more pyruvic acid. Differences in the levels of pyruvic acid production might be explained by the particular "Crabtree effect" of each yeast species [94], which is defined as a system where respiration is repressed under high concentration of sugars. SC strains display a positive Crabtree effect and, consequently, this species presents a greater ethanol fermentation efficacy than many negative-Crabtree effect NS strains [94]. According to the literature, the production of vitisin A has been reported for SC [71,77,78], *Schizosaccharomyces pombe* [83,88] and, more recently, for some species of the H/K genus (*H. guilliermondii*, *H. opuntiae*, *H. vineae*, and *H. clermontiae*) [91,93].

Conversely, another anthocyanin-derived compound (malvidin-3-*O*-glucoside-4-vinylguaiacol) was detected during alcoholic fermentation with SC 882 [72], other SC strains [79–81,95] and *Pichia guilliermondii* [87]. The first report on the formation of this derived compound for the yeast genera *Hanseniaspora* and *Metschnikowia* was relatively more recent [91]. In that work, the authors argued that the high concentration of malvidin-3-glucoside-4-vinylguaicol found for all yeast treatments might also be a consequence of the differences in the grape variety and the concentrations of the respective hydroxycinnamic acids [96,97].

Formation of pigments derived from vinylphenol and vinylguaiacol could be explained by the hydroxycinnamate decarboxylase (HCDC) activity. The HCDC activity, specifically for supplying coumaric acid, has been mentioned for strains of the genera *Pichia*, *Torulaspora*, and *Zygosaccharomyces* [98,99]. A high HCDC activity of 90% for *P. guilliermondii* was recently noticed, which significantly influenced the formation of vinyl phenolic pyranoanthocyanins [87]. The work confirmed that during mixed or sequential fermentations carried out with NS or highly fermentative SC strains, with high HCDC activity, the content of stable pigments could be increased [87].

5. Applications of *Hanseniaspora/Kloeckera* Strains in Winemaking

Mixed-culture fermentation with *Saccharomyces* wine yeast is a controlled manner to apply NS strains, where the positive effects of NS yeasts and a complete dry fermentation is obtained. Even though SC produces most of the ethanol in wine, the NS yeasts present in the grape must, play a significant role in producing aroma compounds [16,100], contributing to diverse 'flavor phenotypes'.

As mentioned above, it is currently widely accepted that the secondary metabolites formed by properly selected NS yeasts, some of them already commercially available, during alcoholic fermentation positively affect the quality of wines [1,3,100–103]. The great variety of such yeasts allows designing different selected starter cultures (in conjunction with SC). Enhanced varietal and fermentative aromas, glycerol production, or specific enzymatic activities might be obtained, based on the ability of these yeasts to ferment different wine varieties [3]. As a result, winemakers can adapt wines to consumers searching for flavor diversity [37].

Yeasts of the genus H/K are frequently isolated during the first stages of the fermentation and are also found on the surface of the grapes, as well as in the soil, cellar, harvesting machinery, and during the processing of these fruit [104,105]. Based on current knowledge, H/K is one of the NS yeast genera with a major contribution to the sensory quality of wines. The H/K tend to be the dominant yeasts in the early stages of fermentation [26,37,106–108], perhaps attributed to their high population found in grapes or high tolerance to osmotic pressure (>200 g/L).

As the fermentation process progresses, the presence of H/K decreases, as a result of their low capacity to adapt to increasing levels of ethanol [109,110], although *H. uvarum* could be found until the end of fermentation, in some situations [55]. With the aid of culture-independent molecular techniques, it was possible to verify that some *H. vineae* strains are maintained until the end of fermentation, but their proportion decreases compared to SC [26]. This behavior can be seen in Figure 10, where the

presence of *H. vineae* and other *Hanseniaspora* yeast is observed until the end of fermentation, at the semi-industrial scale of Merlot and Macabeo grapes [26].

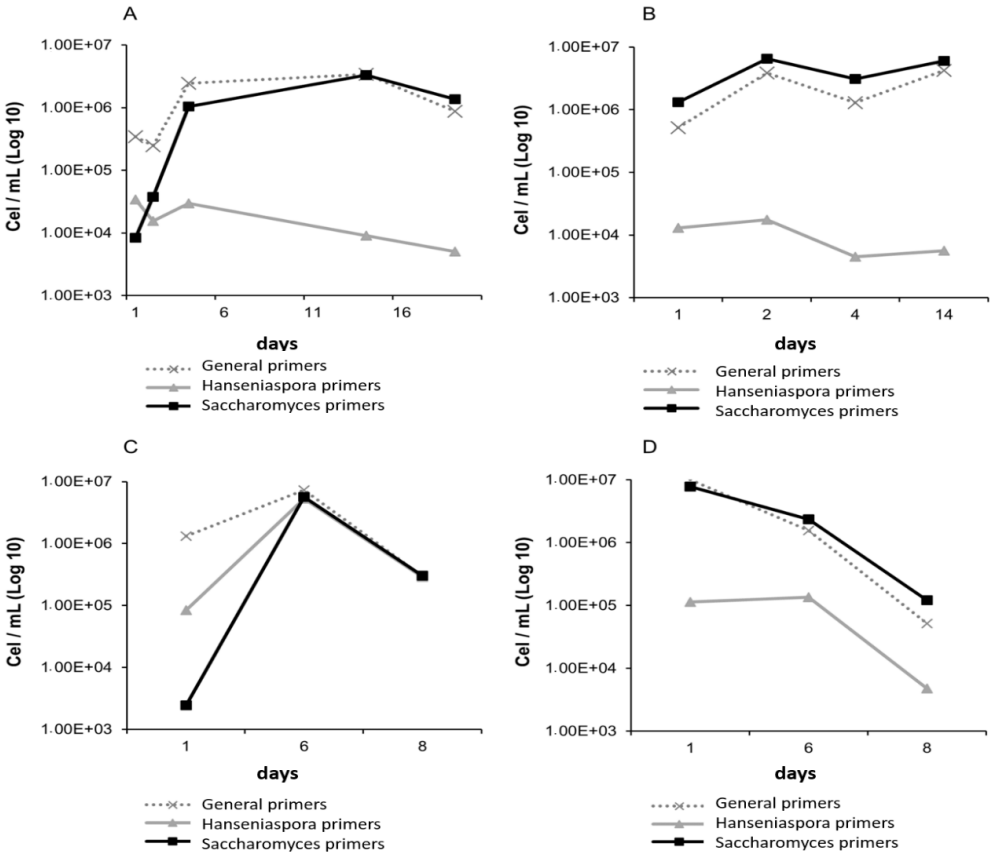

Figure 10. Monitoring of the yeast population by quantitative polymerase chain reaction, with general primers for yeasts (punctate), specific for *Hanseniaspora* spp. (grey) and specific for *Saccharomyces* (black), in Macabeo (**A**,**B**) and Merlot fermentations (**C**,**D**), in tanks inoculated with *H. vineae* (**A**,**C**) and tanks inoculated with *S. cerevisiae* (**B**,**D**).

In general, H/K shows a medium/low fermentative capacity (reaching up to 9% ethanol in some cases) [107]. Although the behavior of *Hanseniaspora* yeasts stands out from other NS yeasts [91,93] and, under certain conditions, give a better or no differences in performance against a *Saccharomyces* control [59,63], this is not a genus-dependent behavior but rather a strain-dependent characteristic. However, as seen in Figure 11, *H. vineae* is one of the main H/K fermenters, a character that corroborates the already-mentioned high homology of the pyruvate kinase gene with SC compared to the other H/K species (Figure 2). This result also justifies why is so difficult to isolate "*vineae*" species from grapes, yet readily detect them after two days of fermentation [63]. Likewise, the fermentation efficacy can be influenced by the inoculation procedures for mixed cultures. If the inoculation occurs sequentially, then the fermentation will be slowed down compared to simultaneous inoculations, due to cell retention of nutrients, and an additional nutrient addition will be necessary when the second inoculation is done [17]. Although SC has a higher capacity for fermentation than H/K strains, the lack of nutrients after 48 h will cause a sluggish fermentation process [8,59,93,111]. This tendency was also shown with different *H. clermontiae* strains [91,93]. The fermentative capacity and cell survival under mixed fermentation conditions can also be affected by the size of the inoculum. Good performances were obtained when *H. uvarum* was inoculated simultaneously and at twice the proportion of SC [60].

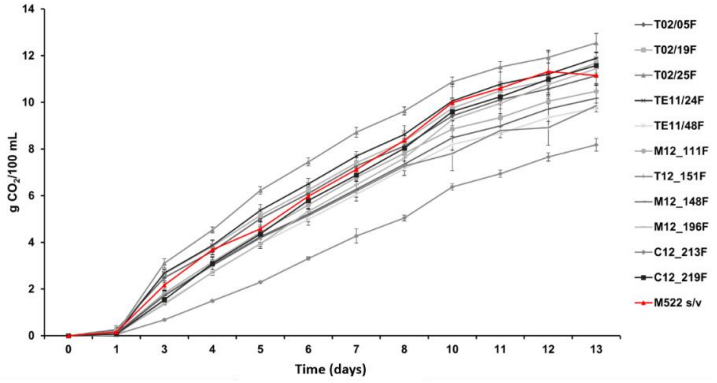

Figure 11. Fermentation kinetics of 11 strains of *Hanseniaspora vineae* at 20 °C, with a daily agitation. The yeast M522 *(Saccharomyces cerevisiae)* was used as a control (red). Data for CO_2 were obtained with cotton plug flask fermenters that include an average loss of 3 g of water vapor for every treatment.

H/K strains are considered important during vinification since they produce aromatic compounds of interest and modify the chemical composition of wines [9–11,18,112]. Two species stand out for producing high amounts of β-phenylethyl acetate, *H. guilliermondii* [113], and *H. vineae* [18,26,54,58,61,62,114]. However, this compound is not found at such high levels in the other species, as discussed above. This ester is associated with fruity, floral (rose) and honey sensory notes [100,115–117]. *H. uvarum* and *H. guilliermondii* have been reported as producing high levels of sulphur-containing aromatics [112]. At the same time, co-fermented wines with H/K strains presented more body and greater aromatic complexity in the mouth compared to SC solo fermentations [26], positively contributing to the final wine (Figure 12). Wines obtained at the semi-industrial scale from inoculums with *H. vineae* and then finished spontaneously with SC were preferred by a sensory panel than wines inoculated with a commercial SC, as a result of a higher floral descriptor, increased volume, increased structure and, ultimately, a better overall concept of the wine obtained. For body and mouth volume, no significant increase of glycerol or polysaccharides were recorded for *H. vineae* strains [63]. However, increased cell lysis was evident compared to SC commercial strains. Furthermore, the increase presence of C_{10} compounds found in wines fermented with *H. vineae*, suggest the existence of a faster autolysis rate compared to SC, as this flavor parameter was related to cell lysis by some authors for other yeast species [118,119]. The cell walls of *H. valbyensis* strains are reportedly about five times more sensitive to hydrolysis than those of SC, which is why they were used for a yeast glucan enzymatic tests [120]. Interestingly, Chardonnay barrel-fermented wines with mixed cultures of *H. vineae*/SC had significantly decreased biogenic amines and volatile acidity and increased glycerol and dry weight levels compared to pure SC fermentations [18]. The dry weight increase might also be associated with an increased cell lysis behavior of *H. vineae.* The authors of that study also showed cooperation between mixed cultures of *H. vineae*/SC with malolactic bacteria fermentation, by a significant stimulation compared to SC pure fermentation, finishing the process earlier by 45 days. More recently, these data were confirmed in red wine fermentations, also at an industrial scale [63], but further studies are needed to understand how the lactic bacteria were stimulated by this yeast.

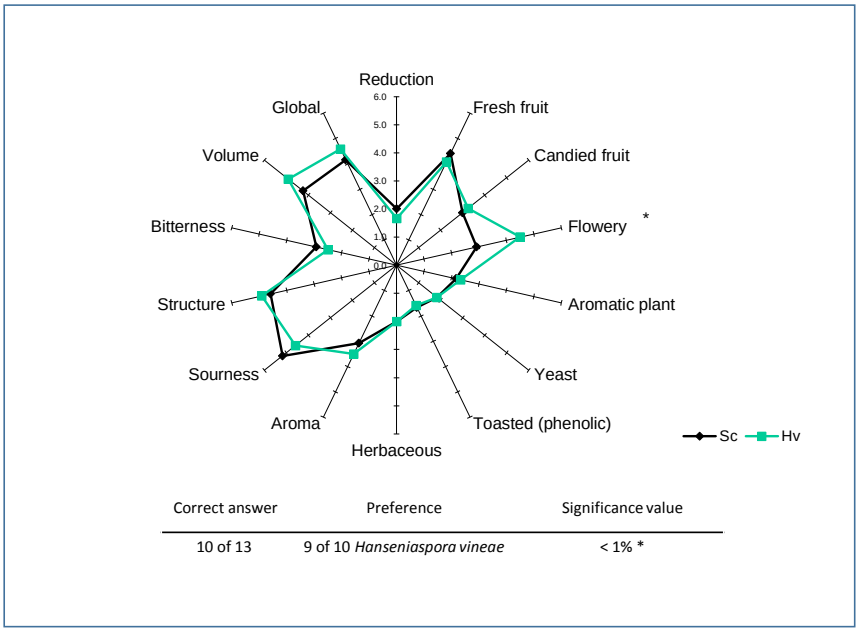

Figure 12. Results of the triangle (table) and descriptive (graphic) test of Macabeo wine fermented with *Hanseniaspora vineae* and *Saccharomyces cerevisiae*. Significant value * is indicated for flowery.

Most of these positive contributions by H/K yeasts can be explained by the presence of increased enzymatic activity compared to SC. The presence of active enzymes depends, in part, on the carbon and nitrogen sources present in the grape must. Small changes in the concentration of these nutrients can affect the nature, quantity, and diversity of the secreted enzymes [121]. The enzymes most commonly studied for their role during vinification are proteases, β-glucosidases, and pectinases since they intervene in sensory attributes, such as the color, aromas, and stability of wines [122]. Most NS yeasts have some enzymatic activity [123]. *Hanseniaspora* spp. are considered to be one of the primary producers of glycolytic and protease activities [63,105,124]. It was recently reported that within the NS yeast that contribute to the organoleptic quality of wines, *H. uvarum* had the highest enzymatic activity [1,3].

6. Conclusions

An insight into apiculate yeast biology showed that H/K is the principal genus found in mature grapes and these yeasts have interesting potential applications for wine fermentation. It is evident that selected strains of H/K yeasts might beneficially enhance the aroma and flavor attributes of wines and, more recently, this was proved for some H/K species. At the real winemaking scale, mixed cultures of *H. guilliermondii*/SC, *H. uvarum*/SC, and *H. vineae*/SC increase flavor diversity and thereby complexity. White and red grape varieties—such as Bobal, Macabeo, Chardonnay, Pinot Noir, Negroamaro, Tempranillo, Cabernet Sauvignon, Merlot, and Tannat—resulted in wines with improved sensorial profile.

Concurrently, it was confirmed that various *Hanseniaspora* species (*H. clermontiae*, *H. opuntiae*, *H. guilliermondii*, and *H. vineae*) contribute to the polyphenolic composition and color of the red wines. Thus, it was possible to demonstrate for the first time that the increased anthocyanin derived compounds generated from the mixed culture fermentation of these yeasts, enhanced the red wine color perception.

This set of mentioned characteristics (fermentative capacity, enzymatic activity, production of aromatic compounds, and ability to enhance the color of wines) makes the genus H/K a suitable stock for the selection of unconventional yeasts in commercial winemaking. Although H/K strains are still not easily available for their application, winemakers will have the opportunity to differentiate and increase regional characteristics to highlight their wines in a hugely competitive market.

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