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Wine Aroma Characterization of the Two Main Fermentation Yeast Species of the Apiculate Genus *Hanseniaspora*

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Abstract: *Hanseniaspora* species are the main yeasts isolated from grapes and grape musts. Regarding genetic and phenotypical characterization, especially fermentative behavior, they can be classified in two technological clusters: the fruit group and the fermentation group. Among the species belonging to the last group, *Hanseniaspora osmophila* and *Hanseniaspora vineae* have been previously isolated in spontaneous fermentations of grape must. In this work, the oenological aptitudes of the two species of the fermentation group were compared with *Saccharomyces cerevisiae* and the main species of the fruit group, *Hanseniaspora uvarum*. Both *H. osmophila* and *H. vineae* conferred a positive aroma to final wines and no sensory defects were detected. Wines fermented with *H. vineae* presented significantly higher concentrations of 2-phenylethyl, tryptophol and tyrosol acetates, acetoin, mevalonolactone, and benzyl alcohol compared to *H. osmophila*. Sensorial analysis showed increased intensity of fruity and flowery notes in wines vinificated with *H. vineae*. In an evolutionary context, the detoxification of alcohols through a highly acetylation capacity might explain an adaption to fermentative environments. It was concluded that, although *H. vineae* show close alcohol fermentation adaptations to *H. osmophila*, the increased activation of phenylpropanoid metabolic pathway is a particular characteristic of *H. vineae* within this important apiculate genus.

Keywords: non-*Saccharomyces* yeasts; wine aroma; fermentation clade; ester acetates; acetyl transferases; *Hanseniaspora*

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1. Introduction

The development of commercial wine yeast cultures in the last 50 years has caused most winemakers to use conventional fermentation technology based on *Saccharomyces cerevisiae* strains. However, in the last decade, some exceptional non-*Saccharomyces* strains commercially available for oenology have appeared. These strains have been developed as a reaction of the producers, searching for aroma complexity and flavor diversity as demanded by the consumers [1,2].

Among non-*Saccharomyces* yeast, *Hanseniaspora* are the main species isolated from mature grapes, as was reported in most wine regions worldwide [3,4]. However, strains of apiculate genus are not currently available for winemaking in an easy way. Traditionally, in oenological environments, the presence of apiculate yeasts such as *Hanseniaspora* genus has been considered undesirable, mainly because of the increased acetic acid production and their competition capacity for nutrients with *S. cerevisiae* which could cause stuck or sluggish fermentations [5]. Additionally, most *Hanseniaspora* species are sensitive to ethanol [6], although some strains belonging to this genus have been detected throughout the fermentation [7,8]. Due to the increased fermentation capacity of *Saccharomyces cerevisiae*

and, therefore, the production of ethanol in a few hours, this species dominates the process spontaneously up to complete sugars depletion. In addition, some flavor compounds, such as higher alcohols (2-phenylethanol and tryptophol) and medium chain fatty acids highly produced by *S. cerevisiae*, are considered inhibitors for the growth of other yeasts [9,10]. However, some *Hanseniaspora* species present positive characteristics that confer pleasant aromas and sensory complexity to the final wines [11,12], which makes them valuable for the winemaking industry and deserve to be analyzed in depth.

Hanseniaspora genus is a heterogeneous group that present several new species identified in the last decades, today including about twenty species [13]. Not all of them have been found in winemaking environments, but on other fruits, plants, and fermented foods. The categorization performed by us regarding the genetic [12] and oenological characterization of this genus [14] evidenced the existence of two separate groupings. Attending especially to their fermentative behavior, *Hanseniaspora* species can be classified in two clear technological clusters: the fruit group and the fermentation group [14]. Interestingly, an evolutionary study performed by Steenwyk et al. [15] also resulted in two clades with a similar composition among the species belonging to the *Hanseniaspora* genus, depending on characteristics related with cell cycle regulation and DNA repair.

Hanseniaspora. uvarum is the main representative of the fruit group, frequently found on grapes and early fermentation stages of wine elaboration [4]. Selected *H. uvarum* strains might enhance tropical fruity and floral aromas due to its high volatile phenols production and high glucosidase activity compared with *S. cerevisiae*, but they also produce a polish-like odor probably related with the increased production of ethyl acetate and acetic acid [16]. In fact, other *Hanseniaspora* species are able to synthesize a high concentration of acetate esters, commonly ethyl acetate [17,18].

Among *Hanseniaspora* species belonging to the fermentation group, *Hanseniaspora osmophila* and *Hanseniaspora vineae* have been previously described in spontaneous fermentations of grape, but not so frequently on fruits. These yeasts are characterized by their increased capacity to ferment and resist higher concentrations of ethanol when compared to other apiculate species [14]. However, there are some differences between these two species that make them interesting from an oenological point of view, which have not carefully been compared yet.

H. vineae is well known by its ability for improving wine flavors. Genomic, transcriptomic, and metabolomic studies in *H. vineae* have enhanced our understanding of its value within the wine industry [7,12,14,19,20]. The main characteristic of the *H. vineae* exometabolome is its increased production of acetate esters, such as 2-phenylethanol acetate, and other benzenoids compared with *S. cerevisiae,* which is a desirable trait, because esters present a lower sensorial threshold than their respective alcohols [7,12]. The reason for this difference has been argued to be due to the higher copy number of proteins with alcohol acetyltransferases (AATase) domains [20]. Therefore, *H. vineae* is characterized by intense fruity and flowery aromas and reduced levels of volatile acidity in white wines [7,8,19].

H. osmophila has been less characterized for wine production, although its capacity to enhance pleasant aromas in red wines was reported [21,22]. Higher concentrations of acetate esters, with the exception of isoamyl acetate, were found in mixed cultures with *S. cerevisiae* [21,22]. However, due to the methods performed for yeast identification at that time, *H. osmophila* was frequently confused with *H. vineae*; therefore, these findings are not so clear now. Today, we have the complete genome sequenced of 32 strains of *Hanseniaspora* available in public databases, and metabolic analysis can be correlated with genomic and transcriptomic information.

In the present work, we use genomic data of two strains of *H. vineae* [23] and one of *H. osmophila* to correlate with the vinification phenotype exhibited in the production of a Chardonnay wine. Wines elaborated with *H. uvarum* and conventional *S. cerevisiae* inoculation were used as controls. The objective was to evaluate differences in wine fermentation and flavor performance between *Hanseniaspora* strains belonging to the two

species that are classified within the fermentation group, also considering the genetic basis that could explain their different behavior during the process.

2. Materials and Methods

2.1. Yeasts and Fermentation in Natural Grape Must

Chardonnay grape must, containing 300 mg N/L of assimilable nitrogen and 200 g/L of sugars at pH 3.5, was treated with 200 mg/L dimethyldicarbonate to prevent microorganism growth. Pre-cultures of pure *H. vineae* Hv025 and *H. uvarum* AWRI1280, both isolated from Uruguayan vineyards, *H. osmophila* AWRI3579 from Australian grapes, and commercial *S. cerevisiae* ALG804 (Oenobrands, Montpellier, France) were inoculated in Chardonnay grape must and incubated at 25 °C for 12 h in a rotary shaker at 150 rpm.

Then, 125-mL Erlenmeyer flasks containing 90 mL of the grape that were closed with cotton plugs used to simulate microaerobic conditions [24] were inoculated with 10^5 cells/mL. Static batch fermentations were conducted at 20 °C to simulate winemaking conditions. Fermentation kinetics was controlled as CO_2 liberated by daily weighting. Cell growth was followed under the microscope using a Neubauer chamber and plating on WLN agar medium (Oxoid, Hampshire, UK).

After 12 days of fermentation, samples were centrifuged at 3500 rpm for 10 min. Supernatants were used for aroma characterization and sensory analysis. Residual sugars were quantified by Near Infrared Spectroscopy using a FOSS WineScan FT 120 (HilleroedDenmark).

2.2. Aroma Characterization

Aroma compounds from wine samples (50 mL) were extracted by adsorption and separate elution from an Isolute ENV+ cartridge (International Sorbent Technology Ltd., Mid Glamorgan, UK) packed with 1 g of a highly cross-linked styrene-divinylbenzene (SDVB) polymer, as described previously [25]. Samples eluted were concentrated with N_2 and Gas Chromatography coupled to Mass Spectrometry (GC-MS) analysis was performed in a Shimadzu-QP 2010 ULTRA (Tokyo, Japan) mass spectrometer equipped with a Stabilwax (30 m by 0.25 mm inside diameter [i.d.], 0.25-µm film thickness; Restek Corporation, Bellefonte, PA, USA) capillary column.

Wine aromas were identified by comparing their linear retention indices with pure standards (Sigma-Aldrich Inc., St. Louis, MO, USA). Additionally, mass spectral fragmentation patterns were compared with those stored in commercial and our own databases. Quantification by GC-MS was performed using 1-heptanol and 2-octanol 1:1 (*w*/*w*) as internal standards.

2.3. Genomic Analysis

Genomic data from the different yeast species were obtained from NCBI databases. *H. uvarum* AWRI13580; *H. osmophila* AWRI13579; *H. vineae* T02/19AF; *S. cerevisiae* S288c genome sequences were used for comparison purposes.

Protein domains predictions were carried out with Pfam protein families database [26]. Additionally, the analyzed predicted protein sequences were aligned and compared in a dendrogram that was constructed by Neighbor-Joining method using MEGA version 4 software [27].

2.4. Sensory Analysis

Sensory analysis was performed by a panel of three experts who were asked to report any defect in the wines and give an overall qualification from 0 to 10 for all the samples in triplicates, with 10 being the best and 0 the worst punctuation.

An initial approach to flavor description of tryptophol and tyrosol acetates was performed by an extended panel of six tasters. Pure standards of tyrosol acetate (Santa Cruz Biotechnology, Dallas, TX, USA) and tryptophol acetate (Angene International Ltd., Nanjing, China) were added to hydroalcoholic solutions containing 12% of ethanol to reach concentrations of 5 mg/L and 10 mg/L, respectively, considering our quantification

levels in the experimental wines. Normalized tasting glasses at 20 °C were used for all these analyses.

2.5. Statistical Analysis

All the fermentations were performed in triplicate and the statistical error for fermentation kinetics, sugar concentration, and aroma quantification were calculated as the standard deviation. To compare the concentration of volatiles quantified in each wine, variance comparison was performed by the ANOVA test carried out with STATISTICA 7.0 software. Mean rating and Tukey significant differences were calculated.

3. Results

3.1. Fermentation and Aroma Profile of Wines Elaborated with Hanseniaspora Spp.

Chardonnay musts inoculated using the four selected strains were analyzed for sugar consumption after 12 days. *S. cerevisiae* ALG804 was able to complete the fermentation and less than 2 g/L of residual sugars was detected (Table 1). However, *Hanseniaspora* strains were not able to completely deplete the sugars.

Table 1. Residual sugars in wines after 12 days of fermentation (g/L).

Strain	*S. cerevisiae* ALG804	*H. uvarum* AWRI1280	*H. osmophila* AWRI3579	*H. vineae* 025
Residual sugars (g/L)	1.6 ± 0.6	115.6 ± 11.8	86.8 ± 6.2	69.1 ± 12.1
Volatile acidity (g/L)	0.45 ± 0.05	0.91 ± 0.12	0.44 ± 0.14	0.34 ± 0.02

Among *Hanseniaspora* species, *H. vineae* was the one that showed the fastest fermentation kinetics (Figure 1), similar to *H. osmophila*. Conversely, *H. uvarum* presented the highest concentration of residual sugars after 12 days.

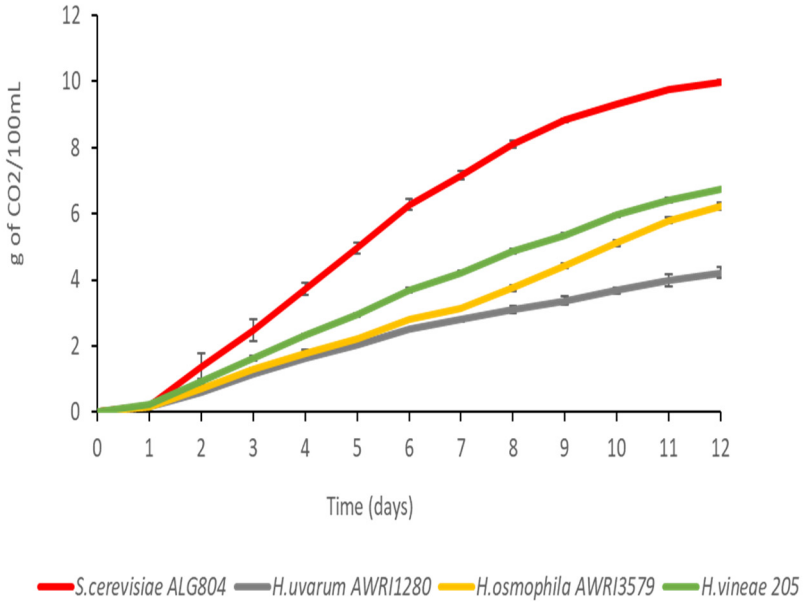
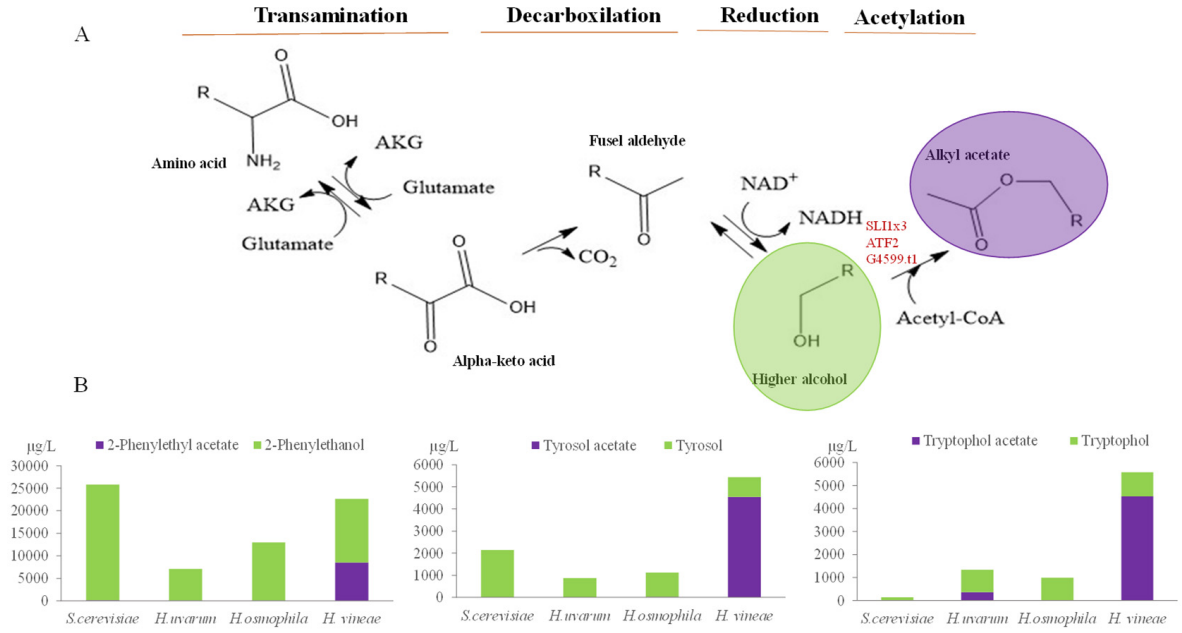


Figure 1. Fermentation kinetics of three strains of the *Hanseniaspora* genus compared with *S. cerevisiae* measured as CO_2 weight loss. Bars indicate the standard deviation for each time point.

These results are in agreement to the lower ethanol resistance presented by *H. uvarum* and *H. osmophila* compared with *H. vineae* [14], probably due to their reduced copy number of alcohol dehydrogenases genes (*ADH*) as shown in Figure 2C, where *H. uvarum* presents only four copies of *ADH* sequences, *H. osmophila* six copies, and *H. vineae* eight copies. This

fact might also explain the higher acetic acid levels of *H. uvarum* compared to *H. vineae*, an important technological difference within this yeast genus.



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	S. cerevisiae	*H. uvarum*	*H. osmophila*	*H. vineae*
Transamination	*BAT1; BAT2; ARO8; ARO9*	*BAT1; ARO8*(x2); *ARO9*	*BAT1; ARO8*(x2); *ARO9*(x4)	*BAT1; ARO8*(x3); *ARO9*(x4)
Decarboxilation	*ARO10; PDC1; PDC5; PDC6; THI3*	*ARO10; PDC1*(x2)	*ARO10*(x2); *PDC1*(x2)	*ARO10*(x2); *PDC1*(x2)
Reduction	*ADH*(x7); *AAD*(x7); *SFA1*	*ADH*(x4); *SFA1*	*ADH*(x7); *SFA1*	*ADH*(x8); *SFA1*

Figure 2. Higher alcohol and alkyl acetates biosynthesis in yeasts. (**A**) Ehrlich pathway and acetylation of higher alcohols. (**B**) Comparison of the production during the fermentation of the three alcohols and their corresponding acetates derived from aromatic amino acids phenylalanine, tyrosine, and tryptophan in the four yeast species of this study. (**C**) Genes putatively involved in the Ehrlich pathway from *S. cerevisiae*, *H. uvarum*, *H. osmophila*, and *H. vineae*. Only annotated genes in databases were considered. Gene copy numbers are detailed in brackets.

Wine aroma was analyzed by GC-MS, showing differences between the three *Hanseniaspora* species and *S. cerevisiae*. The main compounds produced with significant differences were acetate esters, such as 2-phenylethyl acetate, tyrosol acetate, and tryptophol acetate (Table 2). It is well-known that ethyl acetate is the main acetate produced in quantity by these species and with consistent concentration differences ranging between 15 and 60 mg/L [7,11,19,21]. Reports showed that *H. osmophila* synthesizes the highest concentration compared to *H. vineae* and *S. cerevisiae*, but all produce moderate levels that could impact positively the aroma and fruity character of wines (threshold aroma value 12.3 mg/L). However, ethyl acetate was not quantified in this study due to the small sample volumes utilized and the analytical methodology performed avoiding distillation.

Table 2. Aroma compounds (μg/L) detected by GC-MS in wines produced by the selected *S. cerevisiae* and *Hanseniaspora* species. Linear retention index based on a series of n-hydrocarbons reported according to their elution order on Carbowax 20 M. Average and standard deviation from triplicates were calculated.

		S. cerevisiae	*H. uvarum*	*H. osmophila*	*H. vineae*
LRI	**Alcohols**				
1221	3-Methylbutanol	113496 ^{a*} ± 35366	24130 ^{b} ± 8342	52005 ^{b} ± 10409	63075 ^{ab} ± 21210
1264	Acetoin	1764 ^{a} ± 2628	127315 ^{b} ± 4500	2351 ^{a} ± 741	20419 ^{b} ± 6535
1341	1-Hexanol	487 ± 42	294 ± 227	266 ± 134	474 ± 168
1389	3-Ethoxy-1-propanol	9868 ^{b} ± 1767	689 ^{a} ± 16	972 ^{a} ± 246	412 ^{a} ± 220
1453	2-Ethyl-1-hexanol	28 ^{a} ± 12	75 ^{a,b} ± 8	48 ^{a} ± 10	137 ^{b,c} ± 37
1526	2,3-Butanediol	1594 ^{b} ± 351	194 ^{a} ± 206	515 ^{a} ± 77	268 ^{a} ± 384
1822	Benzyl alcohol	7 ^{a} ± 1	9 ^{a} ± 2	9 ^{a} ± 1	25 ^{b} ± 5
1906	2-Phenylethanol	25710 ^{b} ± 5352	7035 ^{a} ± 1081	12959 ^{a} ± 2781	14130c^{a} ± 898
3052	Tyrosol	2149 ^{b} ± 802	879 ^{a} ± 114	1104 ^{a,b} ± 193	892 ^{a} ± 271
3514	Tryptophol	141 ^{a} ± 65	1107 ^{c} ± 13	982 ^{b,c} ± 304	1053 ^{b,c} ± 152
	Esters				
1227	Ethyl hexanoate	347 ^{b} ± 110	nd ^{a}	59 ^{a} ± 15	9 ^{a} ± 9
1341	Ethyl lactate	55 ^{a,b} ± 7	62 ^{a,b} ± 34	201 ^{c} ± 57	45 ^{a} ± 19
1439	Ethyl octanoate	1027 ^{b} ± 292	42 ^{a} ± 31	164 ^{a} ± 27	38 ^{a} ± 7
1516	Ethyl 3-hydroxybutanoate	50 ^{b} ± 17	nd^{a}	nd^{a}	15 ^{a} ± 6
1626	Ethyl decanoate	729 ^{d} ± 126	125 ^{a,b} ± 32	312 ^{c} ± 55	271 ^{b,c} ± 52
1650	1,3-Propanediol diacetate	3401 ^{b} ± 875	567 ^{a} ± 318	1257 ^{a} ± 462	1197 ^{a} ± 313
1813	2-Phenylethyl acetate	116 ^{a} ± 53	46 ^{a} ± 41	27 ^{a} ± 3	10524 ^{b} ± 3209
2130	Ethyl pentadecanoate	nd ^{a}	47 ^{a,b} ± 34	68 ^{b} ± 22	9 ^{a} ± 10
2995	Tyrosol acetate	nd ^{a}	nd^{a}	14 ^{a} ± 6	4547 ^{b} ± 3196
3405	Tryptophol acetate	nd ^{a}	108 ^{b} ± 3	nd ^{a}	6787 ^{c} ± 2007
	Acids				
1510	Propanoic acid	46 ± 25	158 ± 117	192 ± 50	59 ± 6
1588	Isobutanoic acid	138 ^{a} ± 11	394 ^{b} ± 136	410 ^{b} ± 93	834 ^{c} ± 56
1625	Butanoic acid	170 ^{b} ± 29	47 ^{a} ± 10	67 ^{a} ± 9	81 ^{a} ± 34
1650	3-Methylbutanoic acid	306 ± 160	343 ± 293	541 ± 298	313 ± 293
1843	Hexanoic acid	1365 ^{b} ± 133	234 ^{a} ± 136	290 ^{a} ± 25	211 ^{a} ± 109
2070	Octanoic acid	2143 ^{b} ± 370	182 ^{a} ± 120	382 ^{a} ± 60	128 ^{a} ± 29
2243	Decanoic acid	472 ^{c} ± 51	96 ^{a} ± 29	167 ^{a,b} ± 81	473 ^{b} ± 276
	Phenols				
2173	4-Vinylguaiacol	55 ± 19	26 ± 10	61 ± 56	33 ± 8
	Others				
1620	γ-Butyrolactone	125 ^{a} ± 28	270 ^{b} ± 76	206 ^{a,b} ± 13	147 ^{a} ± 12
1750	Valerolactone	nd ^{a}	nd ^{a}	25 ^{b} ± 8	37 ^{c} ± 4
2097	Pantolactone	nd ^{a}	nd^{a}	17 ^{b} ± 1	nd ^{a}
2007	1,4-Dimethyl piperazine	nd ^{a}	112 ^{b} ± 29	nd ^{a}	nd ^{a}
2594	Mevalonolactone	67 ^{a,b} ± 16	72 ^{a,b} ± 19	38 ^{a} ± 7	235 ^{c} ± 64

* different letters represent significant differences according to a Tukey test.

Although in the case of *H. vineae*, it is well known that this species is able to acetylate high amount of higher alcohols to esters [20] compared with *Saccharomyces*, these results showed for the first time significant differences in tyrosol and tryptophol acetates formation in Figure 2B. Other authors have described that *H. osmophila* presented high production of 2-phenylethyl acetate (up to 15 mg/L) compared with *S. cerevisiae*. In fact, *H. vineae* and *H. osmophila* present a higher copy number of *ARO8*, *ARO9*, and *ARO10* genes compared with *H. uvarum* and *S. cerevisiae* (Figure 2C), which are involved in the biosynthesis of

aromatic alcohols [18]. However, the enhanced production of these compounds was shown uniquely by *H. vineae* in our results. Further research should be done to confirm this characteristic, because in old culture collections, there were some strains of both *Hanseniaspora* species that might be confused according to the identification method that was available at that time.

Figure 2B shows that 2-phenylethyl acetate is produced in significantly higher amounts in *H. vineae* than in the other species of *Hanseniaspora* or *Saccharomyces*, presenting 10 to 20 times more concentration after 12 days of fermentation. However, a significant higher production was also found for the acetate esters derived from tyrosol and tryptophol compared to the other species. These two esters were not detected in wines fermented with *S. cerevisiae*, and neither were reported in other studies in the literature. In contrast, in this study, ethyl 2-hydroxypropanoate (ethyl lactate) was produced at significantly higher levels by *H. osmophila* (Table 2). The aroma for this compound is described as strawberry and raspberry, but its odor threshold is higher than the concentration detected in our results (60 mg/L) [28]. These acetates clearly increase when low nitrogen levels are used, and they are generally related to the composition of the must, mainly the amino acid composition and the nitrogen content. The addition of diammonium phosphate (DAP) during fermentation is known to decrease the synthesis of higher alcohols precursors [29,30]. The aroma of 2-phenylethyl acetate is well known and described as having rose, honey or tobacco notes [7]; however, to our knowledge, there are no reports about the sensory descriptions or the odor thresholds for the tyrosol and tryptophol acetates.

Acetate esters present, in general, lower odor thresholds that their corresponding alcohols. Thus, the presence of increased acetates levels is a desirable characteristic in order to improve the aroma perception of wine flavor. In a first approach, our sensory panel of experts characterized the aroma of tyrosol and tryptophol acetates, utilizing 5 mg/L and 10 mg/L, respectively, using as concentration guide the values found in this work (Table 2). Pure compounds were added to an hydroalcoholic solution of 12% by volume. Fruity and flowery primary descriptors were obtained by the six tasters, with a higher intensity impact for tyrosol acetate than tryptophol acetate. Further studies for determining the sensory threshold of these esters might be carried out, which according to our previous experience, should be higher than the value reported for 2-phenylethanol acetate (200 μg/L) but below the results obtained in this work [30].

Other distinctive characteristic is the production of benzyl alcohol, which was detected in wines fermented by *H. vineae*, but not in those obtained with the other species that were evaluated (Table 2). Its aroma was described as being floral, rose or almond [31], but the odor threshold in wine is quite high (200 mg/L) [32].

3-hydroxy-2-butanone (acetoin) was highly produced by *H. vineae* and detected in *H. uvarum*, but was not produced by *H. osmophila* fermentation (Table 2). Sensory descriptors for this compound are sour yogurt and sour milk [31]. Although its odor threshold is 30 mg/L [32], it is known that it might contribute to the palate and flavor complexity of the final wines at lower concentrations [33]. Another highly produced compound by *H. vineae* was the lactone mevalonolactone [(±)-β-hydroxy-β-methyl-δ-valerolactone, (±)-3-hydroxy-3-methyl δ-valerolactone], which is a precursor in cholesterol biosynthesis [34], being used in cosmetic applications as a skin antiaging conditioner and humectant that works at dermis and epidermis [35]. Interestingly, no data are found about the sensory potential effect of this volatile compound, but it is known that many other lactones contribute to wine aroma characteristics.

Nonetheless, the aroma profile exhibited by these yeast species was sensory analyzed by an expert panel of tasters. The overall punctuation was lower for wines inoculated with *H. uvarum* AWRI1280 that were described as possessing some defects as acetic acid and nail polish odor, results which agree to the increased volatile acidity values detected (Table 1). *H. vineae* and *H. osmophila* produced floral and fruit aromas, and no defects were detected (Figure 3).

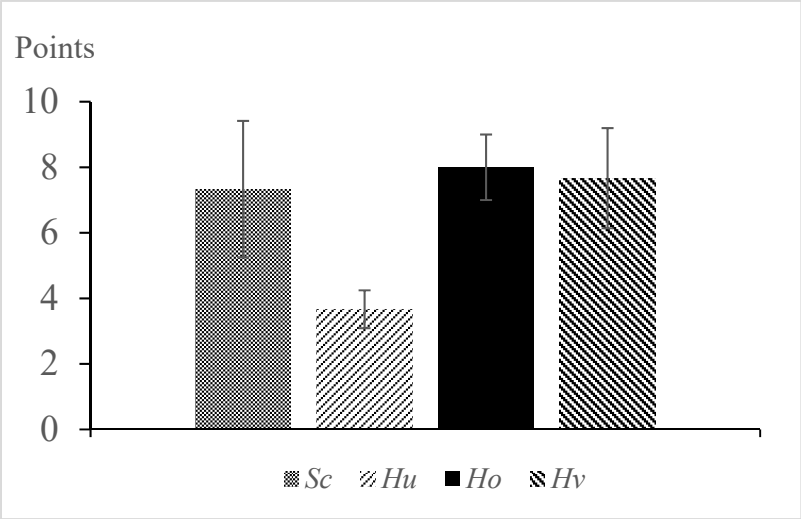

Figure 3. Sensory evaluation of the general impression of wine aroma using a scale from 0 to 10 points with an expert panel of three tasters. Bars are the standard deviation of triplicates.

3.2. Genetic Analysis of Putative Acetyltransferases in Hanseniaspora Spp.

To understand the significant increase of acetate esters produced by *H. vineae* by comparison to the other species evaluated, we made a genomic search of acetyltransferases in the four species of this work. In *S. cerevisiae*, *ATF1* and *ATF2* are genes codifying alcohol O-acetyl transferases that can mainly acetylate branched higher alcohols into their corresponding esters. Additionally, *SLI1*, which codifies in *S. cerevisiae* for N-acetyl transferase related to sphingolipid biosynthesis, presents AATase domains according to the Pfam database. Therefore, it is possible that *SLI1* homologous as those found in *H. vineae* [20] could participate in the biosynthesis of acetate esters because of its high sequence homology [36].

Regarding the results obtained from the direct comparison of sequences with AATase domains in Pfam, all the *Hanseniaspora* strains studied presented these domains throughout their genomes in different quantities. *H. vineae* presents four genes homologous to *SLI1* of *S. cerevisiae* and one of *ATF2*, as previously described by Giorello et al. (2019) [20], and in the genome of *H. osmophila*, only two were annotated as *SLI1* and one as *ATF2*, while *H. uvarum* has neither a *SLI1* nor a *ATF2* homologous sequence annotated in the databases (Table 3).

Table 3. Hypothetical proteins and annotated genes with AATase domains in three different *Hanseniaspora* species and *S. cerevisiae*.

	S. cerevisiae	***H. uvarum***	***H. osmophila***	***H. vineae***
ATF-like	*ATF1*; *ATF2*		*ATF2*	*ATF2*
SLI1-like	*SLI1*		*SLI1*x2	*SLI1*x4
Non-annotated		OEJ85955.1; OEJ85967.1	OEJ82033.1; OEJ92297.1; OEJ82035.1	g4605.1

Nevertheless, there are other two sequences with AATase domains in *H. vineae*, four in *H. osmophila*, and two in *H. uvarum*. The similarities found in the predicted amino acid sequences of these genes are shown in the dendrogram (Figure 4).

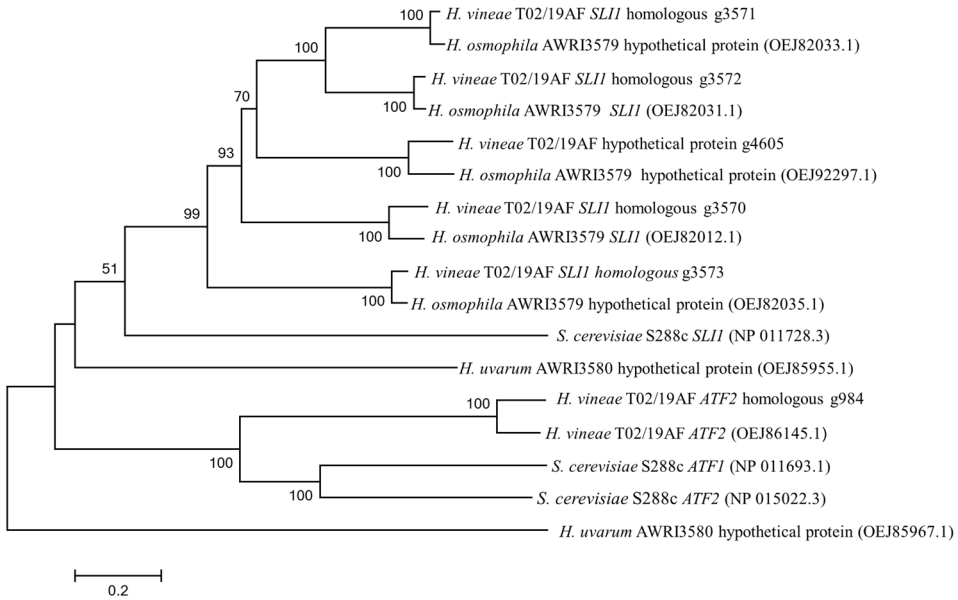

Figure 4. Dendrogram of amino acid sequences of genes from *H. uvarum*, *H. osmophila*, *H. vineae*, and *S. cerevisiae* containing AATase domains constructed using the Neighbor-Joining method. The robustness of the branching is indicated by bootstrap values (%) calculated for 1000 subsets. The entries of the different genotypes include the accession numbers of the GenBank database sequences.

SLI1 copies are localized in tandem in the *H. vineae* genome; each of them present high homology with one corresponding AATase of *H. osmophila.* Otherwise, *H. uvarum* AATases are not clustered with those from *H. osmophila* and *H. vineae*.

ATF1 and *ATF2* synthesize the production of volatile esters in *S. cerevisiae* [37], although *ATF1* is induced by high YAN level in musts [38]. In the case of *H. osmophila* and *H. vineae*, they present *ATF2* but not *ATF1*, and *H. uvarum* does not present any of them. However, there are some conserved amino acids in the sequences of *ATF2* homologous and one hypothetical protein of with AATase domains (GenBank accession number OEJ85955.1) that could reveal an acetyl transferase activity role in this species (Figure 5).

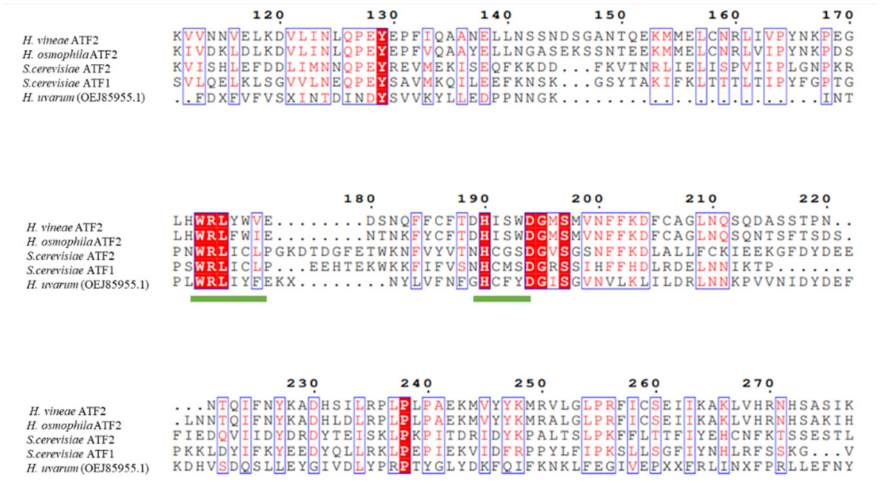

Figure 5. Sequence alignment of different *ATF* and *H. uvarum* (OEJ85955.1). The positions of the conserved region WRLICLP of *S. cerevisiae ATF1* and *ATF2* as well as conserved residues (H-X-X-X-D), putatively essential for the catalytic activity, are marked in green.

In *S. cerevisiae*, there are two essential regions for *ATF* genes. The first is the WRLICLP-motif, which is not strictly conserved throughout microorganisms [39], and the second

is the H-X-X-X-D catalytic-motif, which was found in several fruit and plant AATases. Catalytic residues His and Asp have been reported as crucial for AATase function as part of the active site in these enzymes [40,41].

Higher alcohols were reported as growth inhibitors [9], especially in non-*Saccharomyces* yeasts. One option to avoid this effect is the transformation of these metabolites into other metabolites that are more innocuous for the cell. Therefore, the detoxification role of AATases [42] could explain the better fermentative behavior of *H. vineae* and *H. osmophila*, and this could also be related to its presence throughout the fermentation process and its frequent absence in the fruit. It was recently proposed that some of these flavor compounds might explained the reason why some yeast behaves as more friendly in a fermentation niche, decreasing the inhibition effects of alcohols and acetic acid [2].

4. Conclusions

Hanseniaspora species are divided into two main technological groups according to their fermentation performance. This work showed that species from both groups are also differenced by their capacity to produce diverse flavor compounds. Within the fermentation group, the presence of different aroma profiles given in wines allowed to distinguish *H. osmophila* from *H. vineae.* Although *H. osmophila* and *H. vineae* were genetically very close, significant differences were shown by the flavor metabolites produced. Furthermore, *H. vineae* presented an increased acetylation capacity and intensely developed the phenylpropanoids metabolic pathway, also with increased copy numbers of *ARO8, ARO9*, and *ARO10* genes for the synthesis of aromatic higher alcohols. *H. vineae* might be discriminated from the other three species evaluated in this study by the quantification of benzyl alcohol, mevalonolactone, acetoin, and the three aromatic acetates, which are highly produced by this species. Interestingly, the presence of a great increased gene copy number of AATase domains in *H. vineae* might explain its characteristic to synthetize aromatic alcohols acetates in high concentrations compared to the other yeast species. *Saccharomyces* and *H. uvarum* showed a moderate capacity to acetylate these higher alcohols. Further research is being carried out by our group in order to define a specific metabolic footprinting method to evaluate the contribution of *H. vineae* to commercial wines at the winery level.

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References

1. Jolly, N.P.; Varela, C.; Pretorius, I.S. Not your ordinary yeast: Non-*Saccharomyces* yeasts in wine production uncovered. *FEMS Yeast Res.* **2014**, *14*, 215–237. [CrossRef]
2. Carrau, F.; Henschke, P.A. *Hanseniaspora vineae* and the concept of friendly yeasts to increase autochthonous wine flavor diversity. *Front. Microbiol.* **2021**, *12*, 702093. [CrossRef]
3. Romano, P.; Capece, A.; Jespersen, L. Taxonomic and ecological diversity of food and beverage yeasts. In *Yeasts in Foods and Beverages*; Querol, A., Fleet, G., Eds.; Springer: Heidelberg, Germany, 2006; pp. 13–53.
4. Albertin, W.; Setati, M.E.; Miot-Sertier, C.; Mostert, T.T.; Colonna-Ceccaldi, B.; Coulon, J.; Girard, P.; Moine, V.; Pillet, M.; Salin, F.; et al. *Hanseniaspora uvarum* from winemaking environments show spatial and temporal genetic clustering. *Front. Microbiol.* **2016**, *6*, 1569. [CrossRef] [PubMed]
5. Medina, K.; Boido, E.; Dellacassa, E.; Carrau, F. Growth of non-*Saccharomyces* yeasts affects nutrient availability for *Saccharomyces cerevisiae* during wine fermentation. *Int. J. Food Microbiol.* **2012**, *157*, 245–250. [CrossRef]
6. Pina, C.; Santos, C.; Couto, J.A.; Hogg, T. Ethanol tolerance of five non-*Saccharomyces* wine yeasts in comparison with a strain of *Saccharomyces cerevisiae*—Influence of different culture conditions. *Food Microbiol.* **2004**, *21*, 439–447. [CrossRef]

7. Medina, K.; Boido, E.; Fariña, L.; Gioia, O.; Gomez, M.E.; Barquet, M.; Gaggero, C.; Dellacassa, E.; Carrau, F. Increased flavour diversity of Chardonnay wines by spontaneous fermentation and co-fermentation with *Hanseniaspora vineae*. *Food Chem.* **2013**, *141*, 2513–2521. [CrossRef]
8. Lleixà, J.; Manzano, M.; Mas, A.; Portillo, M.d.C. Saccharomyces and non-*Saccharomyces* competition during microvinification under different sugar and nitrogen conditions. *Front. Microbiol.* **2016**, *7*, 1959. [CrossRef]
9. González, B.; Vázquez, J.; Cullen, P.J.; Mas, A.; Beltran, G.; Torija, M.J. Aromatic amino acid-derived compounds induce morphological changes and modulate the cell growth of wine yeast species. *Front. Microbiol.* **2018**, *9*, 1–16. [CrossRef] [PubMed]
10. Valera, M.J.; Morcillo-Parra, M.Á.; Zagórska, I.; Mas, A.; Beltran, G.; Torija, M.J. Effects of melatonin and tryptophol addition on fermentations carried out by *Saccharomyces cerevisiae* and non-*Saccharomyces* yeast species under different nitrogen conditions. *Int. J. Food Microbiol.* **2019**, *289*, 174–181. [CrossRef]
11. Martin, V.; Valera, M.J.; Medina, K.; Boido, E.; Carrau, F. Oenological impact of the *Hanseniaspora/Kloeckera* yeast genus on wines—A review. *Fermentation* **2018**, *4*, 76. [CrossRef]
12. Borren, E.; Tian, B. The important contribution of non-*Saccharomyces* yeasts to the aroma complexity of wine: A review. *Foods* **2021**, *10*, 13. [CrossRef]
13. Čadež, N.; Bellora, N.; Ulloa, R.; Hittinger, C.T.; Libkind, D. Genomic content of a novel yeast species *Hanseniaspora gamundiae* sp. Nov. From fungal stromata (*Cyttaria*) associated with a unique fermented beverage in Andean Patagonia, Argentina. *PLoS ONE* **2019**, *14*, e0210792. [CrossRef] [PubMed]
14. Valera, M.J.; Boido, E.; Dellacassa, E.; Carrau, F. Comparison of the glycolytic and alcoholic fermentation pathways of *Hanseniaspora vineae* with *Saccharomyces cerevisiae* wine yeasts. *Fermentation* **2020**, *6*, 78. [CrossRef]
15. Steenwyk, J.L.; Opulente, D.A.; Kominek, J.; Shen, X.X.; Zhou, X.; Labella, A.L.; Bradley, N.P.; Eichman, B.F.; Čadež, N.; Libkind, D.; et al. Extensive loss of cell-cycle and DNA repair genes in an ancient lineage of bipolar budding yeasts. *PLoS Biol.* **2019**, *17*, 1–38. [CrossRef] [PubMed]
16. Hu, K.; Jin, G.J.; Xu, Y.H.; Tao, Y.S. Wine aroma response to different participation of selected *Hanseniaspora uvarum* in mixed fermentation with *Saccharomyces cerevisiae*. *Food Res. Int.* **2018**, *108*, 119–127. [CrossRef] [PubMed]
17. Medina, K. Biodiversidad de Levaduras No-Saccharomyces: Efecto del Metabolismo Secundario en el Color y el Aroma de Vinos de Calidad. Ph.D. Thesis, Universidad de la República, Montevideo, Uruguay, 2014.
18. Rojas, V.; Gil, J.V.; Piñaga, F.; Manzanares, P. Acetate ester formation in wine by mixed cultures in laboratory fermentations. *Int. J. Food Microbiol.* **2003**, *86*, 181–188. [CrossRef]
19. Del Fresno, J.M.; Escott, C.; Loira, I.; Herbert-Pucheta, J.E.; Schneider, R.; Carrau, F.; Cuerda, R.; Morata, A. Impact of *Hanseniaspora vineae* in alcoholic fermentation and ageing on lees of high-quality white wine. *Fermentation* **2020**, *6*, 66. [CrossRef]
20. Giorello, F.; Valera, M.J.; Martin, V.; Parada, A.; Salzman, V.; Camesasca, L.; Fariña, L.; Boido, E.; Medina, K.; Dellacassa, E.; et al. Genomic and transcriptomic basis of *Hanseniaspora vineae*'s impact on flavor diversity and wine quality. *Appl. Environ. Microbiol.* **2019**, *85*, 1–20. [CrossRef]
21. Viana, F.; Gil, J.V.; Vallés, S.; Manzanares, P. Increasing the levels of 2-phenylethyl acetate in wine through the use of a mixed culture of *Hanseniaspora osmophila* and *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.* **2009**, *135*, 68–74. [CrossRef]
22. Viana, F.; Belloch, C.; Vallés, S.; Manzanares, P. Monitoring a mixed starter of *Hanseniaspora vineae-Saccharomyces cerevisiae* in natural must: Impact on 2-phenylethyl acetate production. *Int. J. Food Microbiol.* **2011**, *151*, 235–240. [CrossRef]
23. Giorello, F.M.; Berna, L.; Greif, G.; Camesasca, L.; Salzman, V.; Medina, K.; Robello, C.; Gaggero, C.; Aguilar, P.S.; Carrau, F. Genome Sequence of the native apiculate wine yeast *Hanseniaspora vineae* T02/19AF. *Genome Announc.* **2014**, *2*, e00530-14. [CrossRef]
24. Fariña, L.; Medina, K.; Urruty, M.; Boido, E.; Dellacassa, E.; Carrau, F. Redox effect on volatile compound formation in wine during fermentation by *Saccharomyces cerevisiae*. *Food Chem.* **2012**, *134*, 933–939. [CrossRef]
25. Boido, E.; Lloret, A.; Medina, K.; Farñia, L.; Carrau, F.; Versini, G.; Dellacassa, E. Aroma composition of *Vitis vinifera* cv. Tannat: The typical red wine from Uruguay. *J. Agric. Food Chem.* **2003**, *51*, 5408–5413. [CrossRef]
26. Finn, R.D.; Bateman, A.; Clements, J.; Coggill, P.; Eberhardt, R.Y.; Eddy, S.R.; Heger, A.; Hetherington, K.; Holm, L.; Mistry, J.; et al. Pfam: The protein families database. *Nucleic Acids Res.* **2014**, *42*, D222–D230. [CrossRef] [PubMed]
27. Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **2007**, *24*, 1596–1599. [CrossRef] [PubMed]
28. Lloret, A.; Boido, E.; Lorenzo, D.; Medina, K.; Carrau, F.; Dellacassa, E.; Versini, G. Aroma variation in Tannat wines: Effect of malolactic fermentation on ethyl lactate level and its enantiomeric distribution. *Ital. J. Food Sci.* **2002**, *14*, 175–180.
29. Martin, V.; Giorello, F.; Fariña, L.; Minteguiaga, M.; Salzman, V.; Boido, E.; Aguilar, P.S.; Gaggero, C.; Dellacassa, E.; Mas, A.; et al. De novo synthesis of benzenoid compounds by the yeast *Hanseniaspora vineae* increases the flavor diversity of wines. *J. Agric. Food Chem.* **2016**, *64*, 4574–4583. [CrossRef] [PubMed]
30. Carrau, F.M.; Medina, K.; Farina, L.; Boido, E.; Henschke, P.A.; Dellacassa, E. Production of fermentation aroma compounds by *Saccharomyces cerevisiae* wine yeasts: Effects of yeast assimilable nitrogen on two model strains. *FEMS Yeast Res.* **2008**, *8*, 1196–1207. [CrossRef]
31. Lopez De Lerma, N.; Bellincontro, A.; Mencarelli, F.; Moreno, J.; Peinado, R.A. Use of electronic nose, validated by GC-MS, to establish the optimum off-vine dehydration time of wine grapes. *Food Chem.* **2012**, *130*, 447–452; [CrossRef]

32. Fariña, L.; Villar, V.; Ares, G.; Carrau, F.; Dellacassa, E.; Boido, E. Volatile composition and aroma profile of Uruguayan Tannat wines. *Food Res. Int.* **2015**, *69*, 244–255. [CrossRef]
33. Kuzuyama, T.; Dairi, T.; Yamashita, H.; Shoji, Y.; Seto, H. Heterologous Mevalonate Production in Streptomyces lividans TK23. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 931–934. [CrossRef]
34. Yamashita, H. Commercial production of mevalonolactone by fermentation and the application to skin cosmetics with anti-aging effect. *Fragr. J.* **2000**, *28*, 62–65.
35. Romano, P.; Suzzi, G. Origin and production of acetoin during wine yeast fermentation. *Appl. Environ. Microbiol.* **1996**, *62*, 309–315. [CrossRef]
36. Momoi, M.; Tanoue, D.; Sun, Y.; Takematsu, H.; Suzuki, Y.; Suzuki, M.; Suzuki, A.; Fujita, T.; Kozutsumi, Y. SLI1 (YGR212W) is a major gene conferring resistance to the sphingolipid biosynthesis inhibitor ISP-1, and encodes an ISP-1 N-acetyltransferase in yeast. *Biochem. J.* **2004**, *381*, 321–328. [CrossRef]
37. Verstrepen, K.J.; Van Laere, S.D.M.; Vanderhaegen, B.M.P.; Derdelinckx, G.; Dufour, J.-P.; Pretorius, I.S.; Winderickx, J.; Thevelein, J.M.; Delvaux, F.R. Expression levels of the yeast alcohol acetyltransferase genes. *Appl. Environ. Microbiol.* **2003**, *69*, 5228–5237. [CrossRef]
38. Saerens, S.M.G.; Verbelen, P.J.; Vanbeneden, N.; Thevelein, J.M.; Delvaux, F.R. Monitoring the influence of high-gravity brewing and fermentation temperature on flavour formation by analysis of gene expression levels in brewing yeast. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 1039–1051. [CrossRef]
39. Van Laere, S.D.M.; Saerens, S.M.G.; Verstrepen, K.J.; Van Dijck, P.; Thevelein, J.M.; Delvaux, F.R. Flavour formation in fungi: Characterisation of KlAtf, the *Kluyveromyces lactis* orthologue of the *Saccharomyces cerevisiae* alcohol acetyltransferases Atf1 and Atf2. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 783–792. [CrossRef]
40. D'Auria, J.C. Acyltransferases in plants: A good time to be BAHD. *Curr. Opin. Plant. Biol.* **2006**, *9*, 331–340. [CrossRef] [PubMed]
41. Beekwilder, J.; Alvarez-Huerta, M.; Neef, E.; Verstappen, F.W.A.; Bouwmeester, H.J.; Aharoni, A. Functional characterization of enzymes forming volatile esters from strawberry and banana. *Plant. Physiol.* **2004**, *135*, 1865–1878. [CrossRef] [PubMed]
42. Mason, A.B.; Dufour, J.P. Alcohol acetyltransferases and the significance of ester synthesis in yeast. *Yeast* **2000**, *16*, 1287–1298. [CrossRef]