Chapter

pH Control and Aroma Improvement Using the Non-*Saccharomyces Lachancea thermotolerans* and *Hanseniaspora* spp. Yeasts to Improve Wine Freshness in Warm Areas

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Abstract

Lachancea thermotolerans is a yeast species that works as a powerful bio tool capable of metabolizing grape sugars into lactic acid via lactate dehydrogenase enzymes. The enological impact is an increase in total acidity and a decrease in pH levels (sometimes >0.5 pH units) with a concomitant slight reduction in alcohol (0.2–0.4% vol.), which helps balance freshness in wines from warm areas. In addition, higher levels of molecular SO_2 are favored, which helps to decrease SO_2 total content and achieve better antioxidant and antimicrobial performance. The simultaneous use with some apiculate yeast species of the genus *Hanseniaspora* helps to improve the aromatic profile through the production of acetyl esters and, in some cases, terpenes, which makes the wine aroma more complex, enhancing floral and fruity scents and making more complex and fresh wines. Furthermore, many species of *Hanseniaspora* increase the structure of wines, thus improving their body and palatability. Ternary fermentations with *Lachancea thermotolerans* and *Hanseniaspora* spp. sequentially followed by *Saccharomyces cerevisiae* are a useful bio tool for producing fresher wines from neutral varieties in warm areas.

Keywords: warm areas, wine, freshness, pH control, aroma, lactic acid, 2-phenylethyl acetate, non-*Saccharomyces*, *Lachancea thermotolerans*, *Hanseniaspora* spp.

1. Introduction

Global warming is leading to increased average temperatures and irrigation difficulties in some places due to water availability affecting vineyard and wine production [1]. Wine regions affected by global warming have typical problems such as grape varieties with low acidity at harvest time, and high sugar contents that produce wines with flat taste, weak and simple aroma profile, and high alcoholic strength and pH [2]. Moreover, in red wines, the polyphenol content and especially the anthocyanins synthesis is affected, producing wines with less and more unstable colors [3]. Higher pHs make the wines less stable from a physicochemical point of view, but also more susceptible to microbial spoilage. In addition, higher pHs require strong acidity corrections, but pH is not easy to modify with tartaric acid, and wines are usually maintained at inadequate pH values. These values reduce the effectiveness of SO_2 by decreasing the molecular content that is more active as anti-microbial and antioxidant. The molecular SO_2 level of 0.6 mg/L has been proposed for maximum wine protection [4].

2. *Lachancea thermotolerans* and *Hanseniaspora* spp.

Yeast selection is a powerful tool to search for new strains with improved features that can enhance the sensory profile of wine or facilitate the technological process. Historically, vinifications have been performed with *Saccharomyces cerevisiae*, however, current enology is strongly focused on non-*Saccharomyces yeasts* [5]. Species such as: *Metschnikowia pulcherrima* [6], *Brettanomyces bruxellensis* [7], *Torulaspora delbrueckii* [8], *Aureobasidium pullulans* [9], *Hanseniaspora/Kloeckera* spp. [10], *Candida stellata* [11], *Saccharomycodes ludwigii* [12], *Starmerella bacillaris* [13], *Schizosaccharomyces pombe* [14], *Zygosaccharomyces rouxii* [15], *Wickerhamomyces anomalus* [16], *Lachancea thermotolerans* [17]. Most of them were used for their positive impact on wine aroma, flavor, mouthfeel, or color, and some of them were studied for their spoilage activity that may negatively affect wine quality.

This chapter is focused on the species *Lachancea thermotolerans* (Lt) (**Figure 1**) and the genus *Hanseniaspora* (H) spp. (**Figure 2**) because of their interesting behavior to improve the sensory profile and enhance the freshness of wines from warm areas. The main feature of Lt is the effective acidification by the formation of lactic acid from sugars [17]. Several lactate dehydrogenase sequences have been observed in the genome of Lt. Its morphology is similar to that of *Saccharomyces cerevisiae* (Sc) with ellipsoidal geometry and multipolar budding (**Figure 1**), although Lt shows a slightly smaller size. The use of Lt for wine acidification, pH control, and freshness improvement has been described

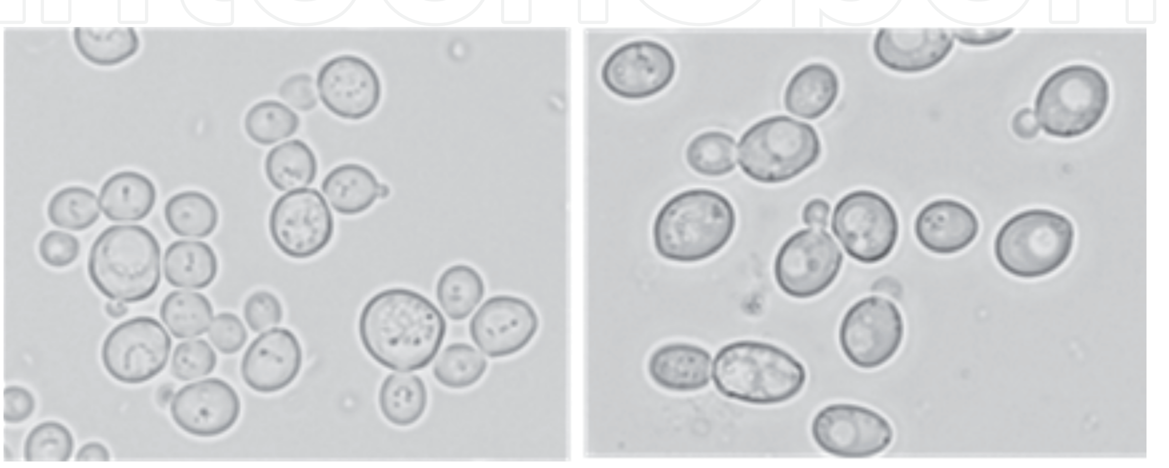

Figure 1.
Optical microscopy of Lachancea thermotolerans *(left) compared with* Saccharomyces cerevisiae *(right) both at different growth stages. Both species show an ellipsoidal shape with multipolar budding.*

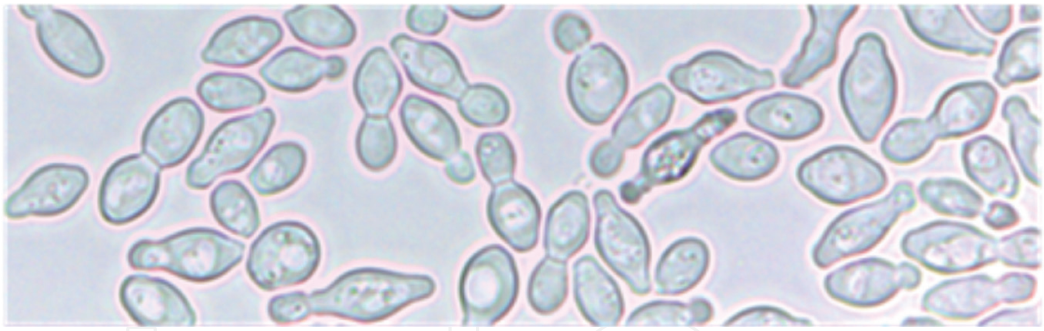

Figure 2.
Optical microscopy of Hanseniaspora vineae*, apiculate yeast with polar budding. Cells are in different stages of growth.*

in several works [18–24]. Acidification and pH control in warm areas is critical for wine quality and stability. A low pH not only produces fresher wines with a better sensory profile and improved consumer perception but also increases wine stability at the chemical and microbiological levels. So, wines with low pH are safer and more stable, and, as mentioned before, pH also favors higher molecular SO_2 content with higher antimicrobial and antioxidant performance. Therefore, biological acidification is a way to protect the wine and allows the reduction of SO_2 levels. The effect on molecular SO_2 at low pH has an impact on reducing the levels of spoilage microorganisms and, as a consequence, lowering the production of off-flavors and toxic molecules such as biogenic amines and others, thus producing safer and cleaner wines [25].

Lt shows a medium fermentative power with some strains reaching 9–10% vol. in ethanol [17]. In addition, Lt has shown other interesting features such as moderate volatile acidity [18, 22], even when used simultaneously with other species (*Metschnikowia pulcherrima*, *Hanseniaspora vineae*, *Torulaspora delbrueckii*) [23], and also reduction of volatile acidity levels in some conditions [26]. Furthermore, the positive role in the formation of thiol compounds in Sauvignon blanc has been described, releasing higher values of 3-Mercapto-1-hexanol (3MH) than the control yeast *Saccharomyces cerevisiae* (Sc) and significant contents of 4-Mercapto-4-methyl-2-pentanone (4MMP) compared to other non-*Saccharomyces* although, in this case, lower than Sc [27]. These thiol compounds are responsible for box tree (4MMP) and tropical fruit aroma (3MH) in wines that increase their complexity [28, 29]. Lt is a low producer of medium-chain fatty acids and their esters, therefore avoid heavy smells and flatness, which helps improve freshness [24].

The low pH produced by the intense biological acidification of Lt also has a positive effect on the color of white wine showing a bright and clean appearance and delaying the browning processes. This effect on browning is also evidenced by the higher levels of molecular SO_2 obtained at low pH which produces an intense antioxidant effect. Concerning red wine color, this reduction in pH favors an increase in color intensity by hyperchromic effect, but it also favors the stability of anthocyanins [30, 31].

In addition, we have observed that some Lt strains have an impact on wine structure, producing softer and full-bodied wines. However, this is not a typical feature of the Lt species, but only of some specific strains. It can be interesting to select these strains to achieve a good balance between acidity and mouthfeel.

Hanseniaspora species (*vineae*, *opuntiae*, *uvarum*, *guilliermondii*, *osmophila*, *valbyensis,* and others) are lemon-shaped apiculate yeasts with polar budding (**Figure 2**) that are typically found in grape juices at the onset of alcoholic fermentation [10], being included in the predominant indigenous yeast population of grapes. Most of them have a low fermentative power around or below 4% vol. However, some of them such as *H. vineae* can reach around 10% vol. [10].

Normally, *Hanseniaspora* spp. have been described as high producers of volatile acidity and have been removed from wine fermentation using SO_2 because of their high sensitivity to this antimicrobial agent. However, acetic acid production is quite variable among strains and some of them can reach values similar to those of Sc [32]. Some species such as *H. vineae* or *H. opuntiae* also show low values (<0.4 g/L) that can be comparable or lower than Sc [33, 34].

Several enzymatic activities have been described in *Hanseniaspora* spp., being especially interesting concerning aroma the expression of the β-D-glucosidase activity to release the free terpenes from their conjugated glucosides [35]. The latter compounds are found in higher concentrations in terpene-rich varieties, but due to their low volatility, they are odorless compounds. The use of non-*Saccharomyces* species with β-D-glucosidase activity is a way to increase wine aroma by releasing free terpenols.

Hanseniaspora vineae (Hv, anamorph sp. *Kloeckera africana*) [36] is one of the most interesting and trending species in enology, due to its medium-high fermentative power (up to 10% vol), its low volatile acidity, but especially for its high impact on wine aroma and structure. Some extra nutritional requirements have been described especially in thiamine, pantothenic acid, and YAN (yeast assimilable nitrogen) supplementation to avoid stuck or sluggish fermentations [10, 37]. The molecular proximity of Hv to Sc in phylogenetic trees is higher than that of other *Hanseniaspora* spp. (*H. opuntiae*, *H. guilliermondii*, *H., uvarum*) (**Figure 3**).

In addition to its interesting fermentative behavior with good implantation and suitable fermentation yield, Hv is useful to modulate the sensory profile of wines. The impact on the aroma is quite significant due to the formation of benzenoid compounds *de novo* by the chorismate-prephenate metabolic pathway (**Figure 4**). This pathway uses sugars as precursors and leads to the formation of floral benzenoid acetic esters such as benzyl acetate and 2-phenylethyl acetate [10, 36, 38, 39]. The production of 2-phenylethyl acetate among other fermentative compounds can

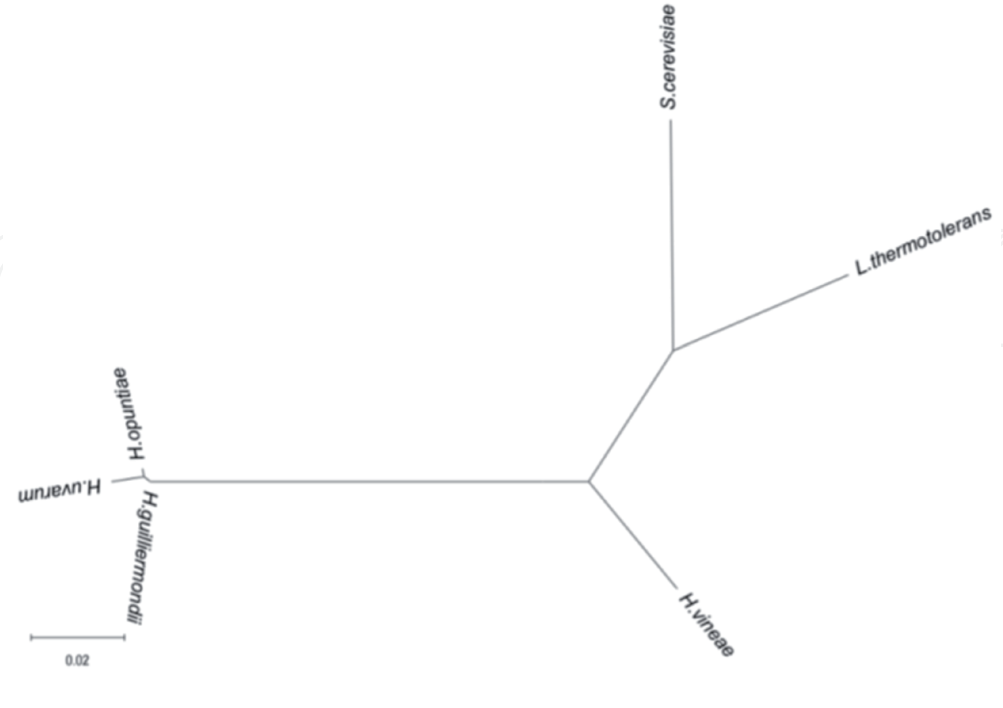

Figure 3.
Phylogenetic relationships among wine yeast species based on analysis of D1/D2 LSU rRNA gene sequences. The evolutionary history was inferred using the maximum likelihood method based on the Tamura-Nei model in MEGA7. GenBank access numbers follow strain numbers: Saccharomyces cerevisiae *NRRL Y12632/AY048154;* Lachancea thermotolerans *CBS 2803/KY108273;* Hanseniaspora uvarum *NRRL Y-1614/U84229;* Hanseniaspora opuntiae *CBS 8733/AJ512453;* Hanseniaspora vineae *NRRL Y-17529/U84224;* Hanseniaspora guilliermondii *NRRL Y1625/U84230.*

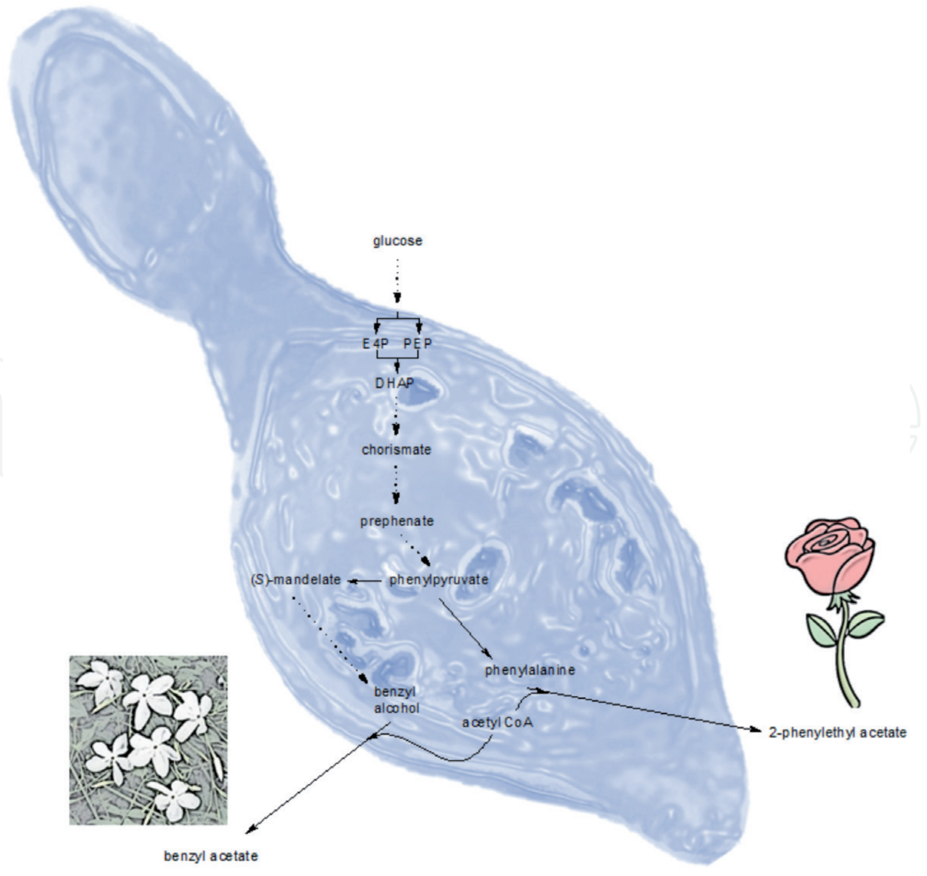

Figure 4.
De novo formation of floral esters by Hanseniaspora *spp. from sugars via the chorismate-prephenate-mandelate pathway. 2-phenylethyl acetate with rose petal aroma descriptor and benzyl acetate with jasmine aroma descriptor.*

separate, by PCA statistical analysis, the aromatic profile of Hv from Sc [34]. Benzyl alcohol concentrations in the fermentation of 11 Hv strains can reach x20-x200 the typical concentrations produced by Sc [38]. Benzyl acetate is the impact aroma of jasmine flowers and produces floral scents that help improve the sensory profile of wines produced from neutral grape varieties. Another impact compound in terms of floral aroma is 2-phenylethyl acetate, also produced by Hv. Its descriptor is rose petals and produces fresh floral perception in wines increasing complexity. This compound is also produced by other *Hanseniaspora* spp. such as *H. guilliermondii* [40], *H. uvarum* [41], *H. opuntiae* [42].

The impact of Hv on wine aroma is also related to the release or *de novo* formation of terpenes. Terpenes are aromatic compounds with a fruity and floral profile that enhance the aroma complexity and freshness of wines. Some grape varieties (Muscat, Gewürztraminer, Albariño) have terpenes produced by the plant in the form of terpenes bonded to sugars as a way to better translocate the hydrophobic free terpenes through the plant tissues. Bonded terpenes are more polar but less volatile, so less aromatic. Hv can express extracellular β-D-glucosidase releasing free terpenes during fermentation and thus improving the varietal aroma of wines [10, 35, 43]. The β-xylosidase activity has also been described in Hv [43].

De novo formation of terpenes from sugars has also been observed in fermentations with Hv. In the fermentation of the neutral variety Macabeo, the formation of a significant concentration of α-terpineol (>100 μg/L) has been observed, but below its sensory threshold [36]. Sequential fermentations with Hv followed by Sc in Albillo grapes have shown much higher concentrations of terpenes (316 μg/L) than with Sc controls (114 μg/L) [44]. Linalool, β-citronellol, and geraniol showed higher concentrations than in the Sc control (>x3, >x4, and > x2 respectively), but also above their respective sensory thresholds [44]. The balsamic terpenes terpinene-4-ol and α-terpineol were also at significantly higher concentrations but below the sensory threshold. Furthermore, several polyoxygenated terpenes showed significantly higher concentrations, but they usually have higher sensory thresholds and, therefore, less impact on the aroma.

Another interesting impact of some *Hanseniaspora* species is the effect on wine structure. Usually, wines fermented by these yeasts show a full-bodied structure and better palatability in the mouth. Fermentation of Macabeo grape must with Hv has shown a sensory profile where tasters perceived improved structure and volume [10]. When the contents of cell wall polysaccharides released by Hv were measured by size exclusion chromatography no significant differences were found with Sc. However, the absorbance at 280 nm, which can be correlated with protein, shows higher values especially at the end of fermentation with Hv [34]. When aging on lees (AOL) is extended for several months, there are no differences between Hv and Sc control. The use of size exclusion chromatography showed slightly higher molecular sizes in the polysaccharides released by Hv that may influence the more intense mouthfeel [44].

3. Use of *Lachancea thermotolerans* and *Hanseniaspora* spp. at industrial scale

The use of a new non-*Saccharomyces* strain requires a lot of experimental research in the laboratory, but also several years of pilot, semi-industrial and industrial-scale trials. **Table 1** details the fermentations, years, wineries, regions, varieties, volumes, controls, and pH effects of selected *Lachancea thermotolerans* strains L31 and A54, currently under industrial evaluation by Lallemand. The strains were tested on white and red grape varieties to see the implantation and performance of acidification on settled white must, but also on crushed red grapes with skins and seeds. Volumes ranged from 500 to 12,000 in white musts and from 1,000 kg to 15,000 kg in crushed red grapes.

In all conditions, acidification was quite effective, even in crushed grapes where the high presence of indigenous yeasts can affect the implantation by reducing the prevalence of the Lt strain. It is interesting to highlight that acidification is effective in varieties with low pHs such as Albariño (3.1) and varieties with high initial pH

Variety	Region	Scale	Year	Strain	Effect on pH	Lactic acid (g/L)
Albariño (white)	Rias Baixas	500 L	2016	L31	3.12 → 2.85	2.7
Tempranillo (red)	Ribera del Duero	1,000 kg	2017	L31	4.20 → 3.63	6.6
Tempranillo (red)	Ribera del Duero	15,000 kg	2020	L31	3.8 → 3.66	2.3
Tempranillo (red)	Mancha	8,000 kg	2020	L31	3.84 → 3.34	9.4
Airén (white)	Mancha	12,500 L	2020	A54	3.75 → 3.47	2.0

Table 1.
Performance of Lachancea thermotolerans *L31 & A54 strains on several semi-industrial trials.*

such as Airén or Tempranillo (3.75–4.20). In terms of potential alcohol, the varieties showed alcoholic strengths ranging from 11 to 12% vol. in the whites and 14–15% in the reds.

Volatile acidity was quite moderate and ranged from 0.38 to 0.46 g/L. The other fermentative volatiles were at normal values for the wines, only the ethyl lactate content was higher than the Sc controls (40–50 mg/L) due to intense lactic acid production, but below the sensory threshold for this ester (150 mg/L) [22].

It is important to note that when Lt strains are used on an industrial scale on real musts or crushed grapes it is important to keep the total SO_2 concentration below 20 mg/L. Otherwise, Lt implantation and development can be seriously affected. The typical acidification pattern shows maximum lactic acid production at the beginning of fermentation (days 3–6, **Figure 5**) depending on inoculation rate, temperature, nutrients, and must composition [22, 23, 45].

It can be observed how the high pH typical of varieties such as Tempranillo in warm areas is deleterious to wine quality, not only producing chemical and microbial instability but also making sulfites inefficient due to low molecular SO_2 levels. The natural biological acidification of Lt produces pH reductions from 4.0 to 3.5 or less resulting in molecular SO_2 levels increasing from <0.4 (dangerous) to >0.8 (safe) [25]. It should also be noted that lactic acid is a stable acid that cannot be altered or metabolized by microorganisms during wine aging. In addition, at high doses (>4 g/L) it inhibits malolactic fermentation, which can be interesting to maintain extra acidity and protect the freshness in wines from warm areas [46].

From a sensory point of view, biological acidification produces a citric freshness, which can be very crispy at high concentrations but can never be perceived as dairy acidity. This is because the milky profile of malolactic fermentation and fermented milk comes from some secondary metabolites such as acetoin or diacetyl that are found in low concentrations in Lt fermentations.

The typical sensory profile of Lt normally shows increased freshness with improved acidity (**Figure 6**) which, depending on the level of acidification, can be somewhat unbalanced and crispy. This can be controlled by the timing of Sc inoculation in sequential fermentation or, subsequently, by blending Lt wines with Sc wines. Even when Lt does not have a strong impact on the aroma, the profile is fresh, fruity, and pleasant. The body in the wines is similar to that of Sc, but, as noted above, specific strains have effects on palatability.

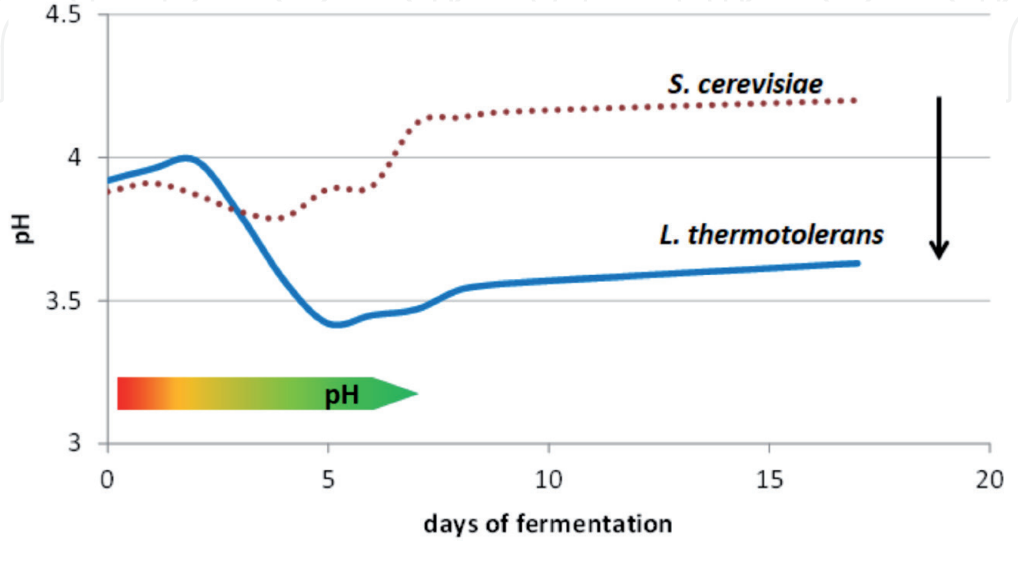

Figure 5.
Typical pH evolution in industrial fermentations driven by Lachancea thermotolerans*. The gradient color scale shows the safety of wines in terms of microbial and chemical stability as a function of pH.*

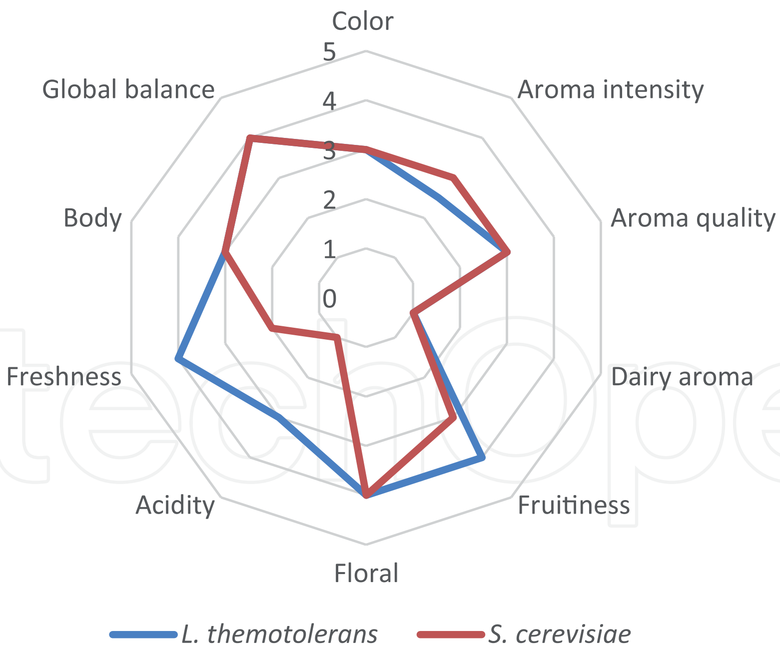

Figure 6.
Comparative sensory spider net of fermentations with Lachancea thermotolerans *and* Saccharomyces cerevisiae.

Additionally, we have compared in Airen fermentations the effect of 72 h of biological acidification with Lt (2 strains: L31 and Laktia from Lallemand) with chemical acidification using 1.5 g/L tartaric acid. Natural biological acidification produced the same effect on pH without using chemical additives [47]. Furthermore, chemical stability is higher due to the high potassium salts precipitation produced during chemical acidification with tartaric acid.

Concerning the use of *Hanseniaspora* spp. on an industrial scale, the most important species are *Hanseniaspora vineae* and *H. opuntiae*, although *H. uvarum* has also been used to some extent. We have experience fermenting Albillo (*Vitis vinifera* L.) white variety with *H. uvarum* in stainless steel and oak barrels to produce white wines aged on lees or blends of Albillo and Tempranillo (*Vitis vinifera* L.) to produce rosé wines (**Table 2**). Moreover, we have fermented must from Airen (*Vitis vinifera* L.),

Variety	Region	Scale	Year	Strain	Aroma	Mouthfeel/ Color
Albillo (white)	Ribera del Duero	150 L Stainless steel barrels	2019	Hv T02/5A	terpenes (x3) 2phenylethyl acetate (x1.33)	Improved palatability
Albillo and Tempranillo (rosé)	Ribera del Duero	150 L Stainless steel barrels	2020	Hv T02/5A	2phenylethyl acetate (x1.65)	Improved palatability Better color (red-bluish)
Albillo and Tempranillo (rosé)	Ribera del Duero	150 L Oak barrels	2020	Hv T02/5A	terpenes (x2.5)	Improved palatability Better color (red-bluish)
Airén (white)	Mancha	12,500 L	2020	Ho A56	2phenylethyl acetate	Improved palatability

Table 2.
Performance of Hanseniaspora *spp. on several semi-industrial trials.* Hanseniaspora vineae *(Hv),* Hanseniaspora opuntiae *(Ho).*

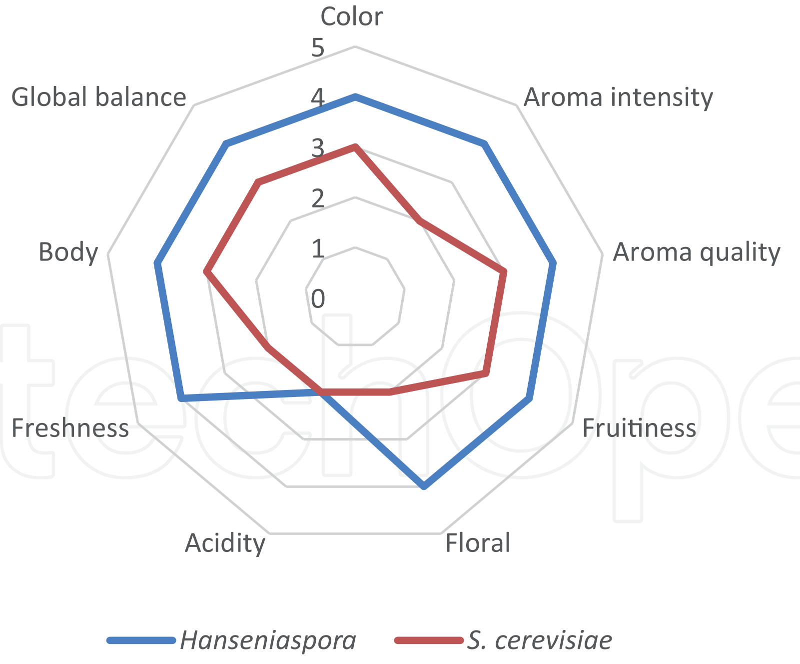

Figure 7.
Comparative sensory spider net of fermentations with Hanseniaspora vineae/opuntiae *and* Saccharomyces cerevisiae.

a neutral flat grape variety, in large stainless-steel tanks using *H. opuntiae*. This species enabled the production of wines with more body, better palatability, and floral aroma.

The formation of terpenes and floral esters by *Hanseniaspora* spp. has an interesting impact on the sensory profile, especially with neutral grape varieties such as Airén or Albillo that express fruitier and more floral wines with greater aromatic freshness. In addition, a positive effect on color can be found in rosé wines with higher anthocyanin contents in fermentations with Hv and especially some acylated derivatives [48]. **Figure 7** shows the typical sensory profile of *Hanseniaspora* spp. compared to *Saccharomyces cerevisiae*.

4. Biocompatibility

Lt and Hv/Ho can be used in mixed fermentations or independent fermentations, subsequently blending both wines in appropriate quantities. When used in mixed fermentations, biocompatibility must be taken into account due to the special sensitivity of *Hanseniaspora* to vitamins such as thiamine and pantothenate or nitrogen contents. Nutritional deficits can lead to the low formation of acetate esters and terpenes with the consequence of a low impact on the aroma. A similar situation is observed in *Lachancea thermotolerans* in which nutritional imbalances affect implantation and development of the yeast population and therefore low acidification compromising the effect on pH. Lower acidification has been observed in ternary fermentations with Lt and Hv sequentially followed by Sc under standard nutritional conditions [45]. The development of further research to carefully optimize the nutritional and physicochemical conditions (temperature, SO_2, pH) for interspecies compatibility will be a key parameter for the successful application of this biotechnology.

5. Conclusion

The combined use of *Hanseniaspora* spp. (*vineae* or *opuntiae*) with *Lachancea thermotolerans* in mixed fermentations subsequently finished sequentially by

Saccharomyces or the independent use of them and later blending their wines is interesting biotechnology to improve flat neutral varieties by increasing acidity, aroma, body, and color, and thus improving the sensory profile and freshness. Several considerations have been described to achieve successful fermentations in terms of nutritional aspects to develop and yeasts biocompatibility.

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Conflict of interest

The authors declare no conflict of interest.

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