Improving wine quality through the application of non-Saccharomyces yeast. Novel applications of lactic acid production by *Lachancea thermotolerans* (*Kluyveromyces thermotolerans*)

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Non-Saccharomyces yeast have been part of winemaking for more than 7000 years. A large number of genera can be found such as Torulaspora, Kloeckera, Lachancea, Pichia, Candida, Metchnikowia, Schizosaccharomyces, Debaryomyces, Brettanomyces, to list only a few (Jolly *et al*. 2013). During a spontaneous / natural wine fermentation, the non-Saccharomyces yeast proliferates in the first few days followed by the proliferation of Saccharomyces yeast, the latter finally dominating the fermentation. Therefore, in a natural fermentation, non-Saccharomyces yeast have a significant metabolic influence on the fermentation and subsequent final quality of the wine. However, since the advent of pure inoculums of active dried *Saccharomyces cerevisiae* wine yeast in the late 50's, the influence of non-Saccharomyces yeast on modern winemaking has diminished significantly. This is due to the fact that *S. cerevisiae* wine yeast are inoculated in a high concentration, usually 1 x 10^6 cfu / ml in the juice / grapes, thereby dominating the wine fermentation from the start and diminishing the impact that any non-Saccharomyces yeast around might have. However, since the introduction of active dried and frozen non-Saccharomyces starter cultures to the industry from 2006 through to 2013, predominantly led by the Danish biotech company Chr. Hansen, the positive influences of these yeast can today be harnessed through inoculating in a high concentration at the start of fermentation, prior to inoculating a Saccharomyces yeast, if at all.

What kind of positive impacts does non-Saccharomyces yeast have on wine fermentation?

Not all non-Saccharomyces yeast have a positive influence on wine quality. *Brettanomyces bruxellensis* is a good example of a non-Saccharomyces yeast that produces off flavours i.e. ethyl and vinylphenols, resulting in barnyard / medicinal odours and a subsequent degradation of wine quality (Oelofse *et al*. 2008). Another example is *Hanseniaspora uvarum*, which produces an excess amount of ethyl acetate that contributes to volatile acidity, giving the wine a 'nail polish' and vinegar odour (Du Toit & Pretorius 2000). However, through careful selection and many years of research, some non-Saccharomyces strains that contribute positively to wine quality have been brought to the market and these include i) *Torulaspora delbrueckii*, which produces more mannoproteins than *S. cerevisieae* yeast, resulting in an improved mouthfeel / palate weight in addition to producing a more complex ester profile *(*Comitini *et al*. 2011*);* (ii) *Pichia kluyveri*, specifically selected by Auckland University to enhance the volatile thiol concentrations in Sauvignon blanc, which has a specific metabolism to increase the release for 4-mercapto-4-methyl-pentan-2-one (4MMP), 3-mercaptohexanol (3MH) from cysteine bound precursors and then through esterification convert 3MH to the more potent 3-mercaptohexylacetate (3MHA), with these compounds boosting tropical flavours like passionfruit in very low concentrations (ng / l) (Anfang *et al*. 2009); (iii) *Lachancea thermotolerans* (previously *Kluyveromyces thermotolerans*) that has the unique ability to produce lactic acid and it will be discussed in the next section (Ribereau-Gayon et al. 1975; Comitini et al. 2011; Gobbi *et al*. 2013).

***Lachancea thermotolerans*: Tool to reduce alcohol?**

In the late 40's Brice Rankine, founding member of the Australian Wine Research institute, was distributing slants of *Saccharomyces veronae* (AWRI 173) to Australian winemakers in order to propagate and conduct wine fermentations (personal communication, Dr. Paul Henschke). The reason for this was that Australian wines were generally low in acid and high in pH, due to the hot climate, and this particular yeast could produce lactic acid from sugars, thereby bringing acidity and a lowering the pH (these were the days before tartaric acid was widely available for acid adjustments). This yeast was later re-classified as *Kluyveromyces thermotolerans* and then recently as *Lachancea thermotolerans*. Furthermore, Ribereau-Gayon already showed in 1975 that *L. thermotolerans* produced high amounts of L-lactic acid in wine combined with the low production of volatile acidity and the absence of off-flavour production. In addition, it was noted these yeast have moderate ethanol productivity, already then indicating the potential for lowering alcohol in wine (Ribereau-Gayon *et al.* 1975). The ability to moderately lower alcohol by the application of *L. thermotolerans* was later confirmed by Gobbi *et al*. in 2013 using *L. thermotolerans* strain 101 from the Yeast Culture Collection of the Dipartimento di Scienze della Vita e dell'Ambiente DiSVA of the Polytechnic University of Marche (Ancona, Italy), thereby indicating up to 1% reduction in alcohol when this strain was co-fermented with *S. cerevisiae* (EC1118). Conversion rates (gram sugar / % alcohol) varied from 17,38 to 18,15 depending on the timing of inoculation compared to *S. cerevisiae* alone that converted 16,7 gram of sugar into 1% alcohol. Benito *et al*. showed in 2015 a reduction in alcohol of up to 1% using the commercial strain of *L. thermotolerans* from Chr. Hansen (Viniflora® Concerto™).

L. thermotolerans is a common yeast found in spontaneous wine fermentations and it has been isolated in winegrowing regions of Australia, South Africa, Italy and France and a number of other wine producing countries. In a recent study, we investigated the population of yeast in Portuguese Syrah and Sauvignon blanc fermentations of vintage 2014 and though the use of metagenomics (high throughput sequencing and analysis of isolated DNA) *L. thermotolerans* represented 4% and 2%, respectively, of the genomic material in the final wines. This is a significant amount and shows that the yeast is well suited for the wine fermentation environment. Inoculation of the active dried *L. thermotolerans* resulted in 45% and 40% representation of the genomic material in the final wine (Figure 1).

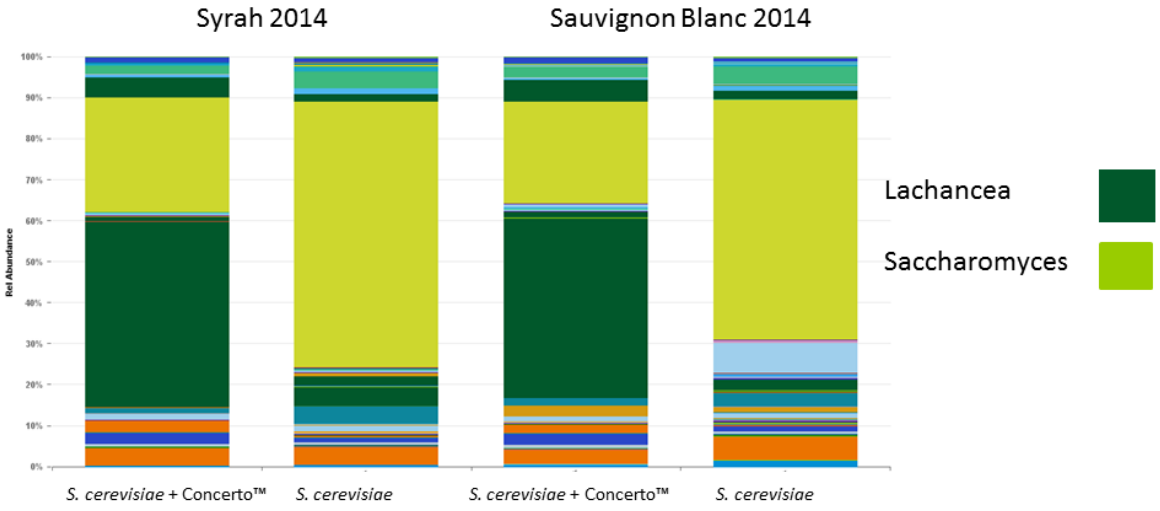

Figure 1. Relative abundance of DNA from Lachancea and Saccharomyces in Syrah and Sauvignon blanc wines inoculated with Saccharomyces wine yeast and with or without *L. thermotolerans* (Viniflora® Concerto™).

The metabolic pathway of converting sugars to lactic acid by *L. thermotolerans* is not fully known but levels of between 1-9 g/L have been reported in wine fermented with this yeast (Ribereau-Gayon *et al*. 1975; Comitini *et al*. 2011; Gobbi *et al*. 2013). The divergence of sugars to lactic acid is a logical way to reduce alcohol in wine and as described above literature reports reducing alcohol by 0.5-1%. However, this does not fully explain the reduction in alcohol and metabolites other than lactic acid could also contribute. In our own studies we show 0.3-0.5% alcohol reduction in trials conducted in Valpollicella, Italy in 2015 (Table 1). These trials were done using a relatively high dose / inoculation level (75 g/ hL) of an active dried *L. thermotolerans* (Viniflora® Concerto™) without inoculation with *S. cerevisiae* after, which gave the best results on lowering alcohol concentration (Figure 2). Fermentation rates were slightly slower in ferments with *L. thermotolerans* (Figure 2). This particular strain of *L. thermotolerans* is in fact a relatively strong fermenter and can produce up to 12% alcohol without the help of *S. cerevisiae* in sterile conditions (data not shown). In general, non-Saccharomyces yeast are weak fermenters but some do perform well, as in the case of this particular strain of *L. thermotolerans*.

	Alcohol %	Lactic acid g/L
Viniflora® Jazz™	11,26	0,12
Viniflora® Concerto™ + Jazz™	11,09	0,5
High Dose (HD) Concerto™	10,89	0,5

Table 1. Alcohol and lactic acid concentrations of Valpolicella wines fermented with *Saccharomyces cerevisiae* wine yeast (Viniflora® Jazz™) and *L. thermotolerans* (Viniflora® Concerto™). Active dried Saccharomyces yeast was inoculated at 20 g/ hl and active dried Lachancea thermotolerans at 25 g/hl in the sequential fermentation and 75 g/ hl in the Lachancea thermotolerans high dose (HD) ferment.

The reduction in alcohol by *L. thermotolerans* is significant but the question is how do we stimulate / modify it to reduce alcohol in real wine conditions even further i.e. reducing levels by 2-3%? The improved results when inoculating a high concentration is a clear indication that by increasing the presence of *L. thermotolerans* in the wine ferment, it leads to a larger reduction in alcohol percent. In addition, reducing the presence of *S. cerevisiae* by not inoculating also allows *L. thermotolerans* to have more 'time on its own' to shift grape sugars away from alcohol and thereby delay the influence of the strong fermenting *S. cerevisiae* which is very productive at producing alcohol.

Another approach would be to alter the conditions of fermentation so that the metabolism *of L. thermotolerans* shifts more to respiration than to fermentation. In fact this approach has been documented, albeit with a *Kluyveromyces lactis* strain (Quirós et.al.2014). The approach behind this work was to use Crabtree negative yeast (many non-Saccharomyces yeast are Crabtree negative) in a must which includes relatively high aeration e.g. regular pump-overs in order to shift the metabolism of the yeast from fermentation to respiration, the latter not producing alcohol. *S. cerevisiae* is Crabtree positive, meaning that when in contact with moderate amounts of sugars, it prefers fermenting, even in the presence of high concentration of oxygen. Respiration is energetically more efficient so this does not make a lot of sense but the hypothesis is that *S.*

cerevisiae chooses to produce alcohol to toxify the environment and thereby outcompete less alcohol tolerant yeast such as non-Saccharomyces yeast. We have not investigated the potential of aeration on alcohol yields with *L. thermotolerans* but this is certainly a topic for the future.

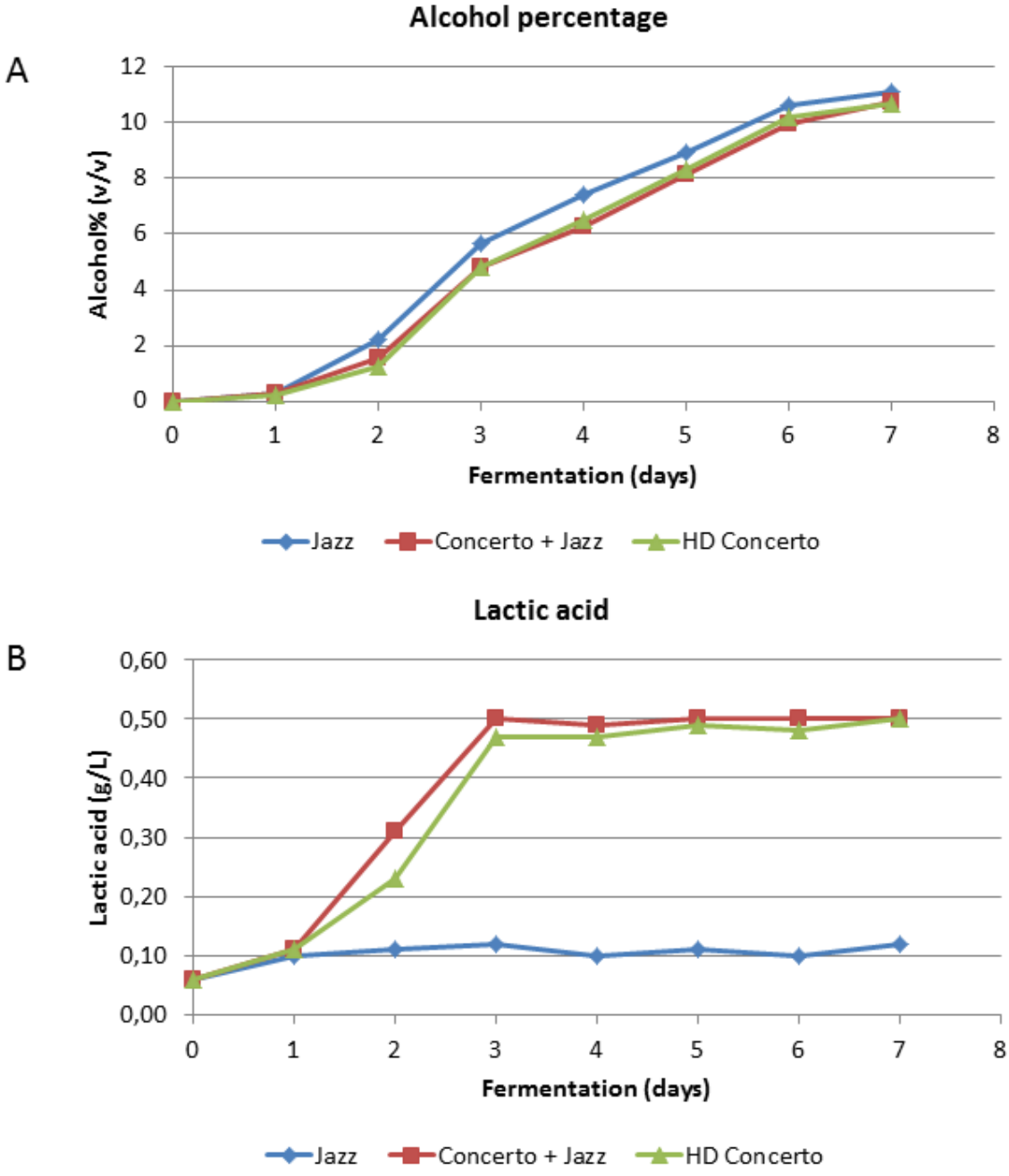

Figure 2. Alcohol and lactic acid formation rates of *S. cerevisiae* (Jazz), *L. thermotolerans* (Concerto) + S. cerevisiae and high dose (HD) *L. thermotolerans*

Another approach would be to screen a large number of *L. thermotolerans* strains and select strains with the lowest ethanol yield and or the highest lactic acid production capacity. Even with *S. cerevisiae* there is large differences in ethanol yield between strains and it is also the case for

non-Saccharomyces yeast. Large scale screening tools for yeast have been available for many years and in Chr. Hansen we are equipped with two advanced robotic screening robots that can conduct large scale screenings in relatively short time frames and this technology can be used for identifying low alcohol producing non-Saccharomyces yeasts.

Lachancea thermotolerans: Unique yeast to combat the effects global warming?

Climate change has resulted in increased temperatures in many wine growing regions in the world. Furthermore, in a worldwide stylistic drive to produce more full flavoured, fruity wines, sugar concentrations of grapes at time of harvest has steadily increased to a level where 15-16% alcohol wines are not uncommon these days. This creates an interesting dilemma for consumers, who on the one hand is asking for more fruity wines and on the other hand is demanding lower alcohol levels due to stricter drinking and driving laws and health concerns. *L. thermotolerans* offers a unique potential to counter the effect of global warming on wine grapes by producing acid during fermentation, moderately reducing alcohol levels and on top of this, producing high concentrations of the fruity / strawberry-like flavour compound ethyl lactate (using lactate as a precursor).

Another consequence of high temperatures and long hanging times for grapes are very low acid levels, resulting in wines with pH above 4.0. These wines taste flat and 'soapy' and completely undesirable for most consumers. Therefore, today it is common in warm wine growing regions to add tartaric acid and in some cases even malic acid to increase acidity and lower pH. As mentioned above, in the late 1940's, Brice Rankine had the right idea in finding a biological solution to increase acidity in the high pH Australian wines. The approach did not develop the Australian wine industry in the long term and with the advent of active dried *S. cerevisiae* yeast winemakers became less competent at growing yeast from slants. This is one of the main reasons that the use of non-Saccharomyces yeast is not more widespread today as *S. cerevisiae*, mainly due to the fact that there were no commercial products available. Non-Saccharomyces yeast are notoriously difficult to produce, partially explaining the lack of presence on the market up until recently.

Conclusions

The application of non-Saccharomyces yeast is a relatively new tool in the global wine industry. Some of these yeast have unique metabolisms and capabilities that *S. cerevisiae* does not have. Therefore, they show great potential in increasing the quality of wine and providing natural, biological solutions to technical challenges we have in the winery. *L. thermotolerans* is a yeast that we identified to be one of the clear leaders in providing solutions to the growing global problem of high levels of alcohol in wine. We are proud to be the first company in the world to market non-Saccharomces yeast, the first to produce and market *L. thermotolerans* and the first to propose this yeast as a practical way to reduce alcohol.

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