Industry allocated project number	PHI allocated project number

SATI	CFPA	SAAPPA/SASPA	DFTS	Winetech
tarryn@satgi.co.za Tel: 021 863-0366	inmaak@mweb.co.za Tel: 021 872-1501	theresa@hortgro.co.za Tel: 021 882-8470	dappies@dtd.co.za Tel: 021 870 2900	andraga@winetech.co.za Tel: 021 276 0499
				X

Indicate (X) client(s) to whom this concept project proposal is submitted. Replace any of these with other relevant clients if required.

NB: The instructions in red, throughout the template, should be omitted from the final document.

FINAL REPORT
(2015)

1. PROGRAMME AND PROJECT LEADER INFORMATION

	Research Organisation Programme leader	Research Team Manager	Project leader
Title, initials, surname	Prof M du Toit and Prof FF Bauer	Dr Benoit Divol	
Present position	Professors	Senior lecturer	
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	Co-worker	**Student**	
Title, initials, surname			
Present position			
Address			
Tel. / Cell no.			
Fax			
E-mail			

2. PROJECT INFORMATION

Research Organisation Project number	IWBT Y 11-02
Project title	Evaluating the impact of yeast co-inoculation on individual yeast metabolism and on wine composition
Short title	

Fruit kind(s)			
Start date (mm/yyyy)	01/2012	**End date** (mm/yyyy)	12/2014

Key words	Non-*Saccharomyces* yeasts, *Saccharomyces cerevisiae*, mixed cultures, fermentation, wine composition

Approved by Research Organisation Programme leader (tick box) ☐

THIS REPORT MUST INCLUDE INFORMATION FROM THE **ENTIRE** PROJECT

3. EXECUTIVE SUMMARY

*This must report on the **ENTIRE** project. Address the objectives and milestones of the project as well as the impact of the study on the industry.*

The use of non-*Saccharomyces* yeasts is becoming increasingly popular in an attempt to increase wine style diversity and restore a certain terroir character. Indeed, although they are quickly outcompeted by *Saccharomyces cerevisiae*, non-*Saccharomyces* yeasts are dominant in grape juice. For a long time, they were regarded as insignificant or even as responsible for spoilage, but in the past two decades, research has shown that certain species may actually produce interesting aroma compounds, reduce the production of off-aroma compounds, and release varietal aroma from non-fragrant precursors. In this study, five species were selected based on these characteristics and investigated further. Pure and mixed culture fermentations were conducted and confirmed that these species are of oenological relevance through their production of specific aroma compounds as well as polyols (including glycerol and others), reduction of volatile acidity. However, a more in-depth study of the interactions occurring between *L. thermotolerans* and *S. cerevisiae* revealed that physical and metabolic interactions occur, limiting the survival (and therefore the impact) of the non-*Saccharomyces* yeast. Furthermore, the study highlighted the role that oxygen plays in the early decline of *L. thermotolerans*. Overall, this study started to reveal that, through the adjustment of certain parameters (in particular oxygen, nutrients), the winemakers could influence the survival of the yeasts, thereby enhancing their impact on the final wine composition. The study paves the way to future investigations that will identify the actual influence of the key factors identified here on yeast interactions, survival, metabolic activity and ultimately on wine composition.

4. PROBLEM IDENTIFICATION AND OBJECTIVES

State the problem being addressed and the ultimate aim of the project.

Certain non-*Saccharomyces* yeasts possess oenologically relevant properties, including the production of specific metabolites which differ in nature and/or concentration from those produced by *Saccharomyces* spp., and the secretion of extracellular hydrolytic enzymes. The latter aspect was investigated in the Winetech project IWBT Y11-01. As for the former, the positive characters that these species can confer to wine include reduced production of acetic acid, increased production of glycerol and organic acids and an overall ability to positively alter the wine organoleptic profile. However, the nature of these properties remains vague and incompletely characterised.
The aim of our study was therefore to systematically evaluate the actual potential of these yeasts with regards to the production of wine-active metabolites when interacting with *Saccharomyces cerevisiae*. Indeed, the fermentation capacity of these yeasts is limited and co-inoculation or sequential inoculation with *S. cerevisiae* is mandatory to ensure complete fermentation. Complex and only partially understood interactions then occur between the two species and the outcome of fermentation is not only the result of the individual yeasts' metabolism but also that of their interactions which include competition for nutrients and antagonistic behaviour.

Specific objectives:

Milestone 1: Selecting non-*Saccharomyces* yeast species/strains for their positive contribution to wine quality

Milestone 2: Testing the membrane bioreactor

Milestone 3: Evaluating the impact of the presence of the selected non-*Saccharomyces* yeasts on the metabolic activity of *S. cerevisiae* (using synthetic grape juice)

Milestone 4: Evaluating the impact of the presence of the selected non-*Saccharomyces* yeasts on the metabolic activity of *S. cerevisiae* (using real grape juice)

5. WORKPLAN (MATERIALS AND METHODS)

List trial sites, treatments, experimental layout and statistical detail, sampling detail, cold storage conditions and examination stages and parameters. Add additional rows if required.

MILESTONE 1: Selecting non-*Saccharomyces* yeast species/strains for their positive contribution to wine quality

Task 1: various strains of non-*Saccharomyces* yeasts from the IWBT and ARC culture collections will be tested for killer activity against *S. cerevisiae*. The "spot-on-the-lawn" method will be used to evaluate killer activity. The assay will be standardised in order for the assay to be conducted at the IWBT and at the ARC-Nietvoorbij. The IWBT and the ARC-Nietvoorbij have signed a broad MTA (Material Transfer Agreement) in order to ease the transfer of microbial strains between the 2 institutions.

Task 2: The strains exhibiting no killer activity against *S. cerevisiae* will be tested in co-inoculation with *S. cerevisiae* in grape juice in laboratory scale (different cultivars will be tested; grape juice will be provided by the ARC-Nietvoorbij). Population dynamics will be followed throughout alcoholic fermentation (the ability of non-*Saccharomyces* yeasts to grow in the presence of cycloheximide will be used to discriminate between non-*Saccharomyces* yeasts and *S. cerevisiae*). The non-*Saccharomyces* yeasts will be inoculated either at a concentration close to that present under real winemaking conditions or at a much higher cell density in order to mimic that of a mixed starter culture. Moreover, increased oxygen concentration will be tested as a potential tool to retard the death of non-*Saccharomyces* yeasts. Other factors, such as nitrogen concentration, will also be tested.

The equipment of the experimental cellar at the ARC-Nietvoorbij could also be used to perform the fermentations in larger volumes in order to be closer to normal winemaking conditions.

Task 3: At different time points during alcoholic fermentation (at least 3: beginning, middle and end), the concentrations of primary and secondary metabolites will be determined by GC-FID as well as the concentrations of monoterpenes (also by GC-FID). Inoculation with the pure *S cerevisiae* strain will be used as a control.

Task 4: Results from tasks 1, 2 and 3 will be combined and the best strains selected.

MILESTONE 2: Testing the membrane bioreactor

Milestone 1 will be repeated using the membrane bioreactor (the non-*Saccharomyces* strains selected in Milestone 1 will be coinoculated with *S. cerevisiae* but the two species will be inoculated in separate compartments). This will allow verifying indirectly the existence of cell-to-cell interactions between *S. cerevisiae* and non-*Saccharomyces* yeasts.

MILESTONE 3: Evaluating the impact of the presence of the selected non-*Saccharomyces* yeasts on the metabolic activity of *S. cerevisiae* - and vice-versa - (using synthetic grape juice)

Task 1: Using the membrane bioreactor, fermentations will be carried out and mRNA from the *S. cerevisiae* strain and the non-*Saccharomyces* strain will be separately extracted at different time points (same time points as in Milestone 1). Chemical composition of the wines will be determined as previously mentioned. The control will consist of the *S. cerevisiae* strain being inoculated in both chambers.

Task 2: The mRNA extracted from *S. cerevisiae* will be reverse-transcribed and the resulting cDNA will be used for microarray analysis or high-throughput sequencing in order to assess the

impact of the presence of non-*Saccharomyces* yeasts on the transcriptome of *S. cerevisiae*. The analysis of the results generated, will allow identifying the genes whose transcription is most affected.
Task 3: Quantitative real-time PCR will be used to verify the results obtained with the microarrays or high-throughput sequencing, with a specific target on the genes identified in Task 2 as being strongly influenced by the non-*Saccharomyces* yeasts.
Task 4: same as Tasks 2 and 3 for the mRNA extracted from the non-*Saccharomyces* yeasts. However, the lack of information regarding their genome sequence will oblige to use high-throughput sequencing for the analysis of the transcriptome. Moreover, the analysis of the results might be rendered difficult for the same reason.

MILESTONE 4: Evaluating the impact of the presence of the selected non-*Saccharomyces* yeasts on the metabolic activity of *S. cerevisiae* (using real grape juice)
Milestone 3 will be repeated in real grape juice. After evaluation at laboratory scale, fermentations will be conducted at a larger scale at the ARC-Nietvoorbij. The results will be compared.

6. RESULTS AND DISCUSSION

State results obtained and list any industry benefits. If applicable, include a short discussion covering ALL accumulated results from the start of the project. Limit to essential information only

Fermentation kinetics

The yeasts *Lachancea thermotolerans*, *Torulaspora delbrueckii*, *Starmerella bacillaris*, *Pichia kluyveri* and *Metschnikowia pulcherrima* were selected based on their good oenological aptitudes and the fact that, except for *S. bacillaris*, they are commercially available.
Each of these yeasts was sequentially inoculated in a synthetic grape must with *S. cerevisiae*, the latter being added 48h after the different non-*Saccharomyces* yeasts. The fermentations were noted Sc for pure *S. cerevisiae* which was used as a reference and LtSc, TdSc, SbSc, PkSc and MpSc for the mixed cultures, respectively. *S. cerevisiae* was inoculated 24 h after the non-*Saccharomyces* yeasts. The typical fermentation kinetics profiles are shown in Figure 1. The fermentation carried out by *S. cerevisiae* in pure culture was the fastest and that by the mixed culture with *P. kluyveri* the slowest. It seems that the presence of the non-*Saccharomyces* yeast induces a delay in the fermentation with the sugar concentration decreasing more slowly at the beginning. Nevertheless, all yeasts combinations fermented to dryness, within a few days after the *S. cerevisiae* pure cultures. A number of reasons could explain the delay observed in the mixed culture fermentations, including the partial depletion of nutrients used by the non-*Saccharomyces* yeasts and the presence of inhibitory compounds produced by the latter yeasts.
After about one third of the sugars had been consumed, the yeasts *L. thermotolerans*, *T. delbrueckii*, *S. bacillaris* and *P. kluyveri* started to decline (data not shown). The presence of *L. thermotolerans* could no longer be detected at half fermentation. However, *M. pulcherrima* died off very rapidly after *S. cerevisiae* inoculation (data not shown). This early decline of non-*Saccharomyces* yeasts has been attributed to a number of factors including their lower tolerance to ethanol compared to *S. cerevisiae* and the rapidity at which *S. cerevisiae* assimilates nutrients and produces biomass. Our studies show that other factors, such as the lack of oxygen could also play a role. In this regard, it should also be considered that *M. pulcherrima* is a Crabtree-negative yeast and this could also account for its very early disappearance.

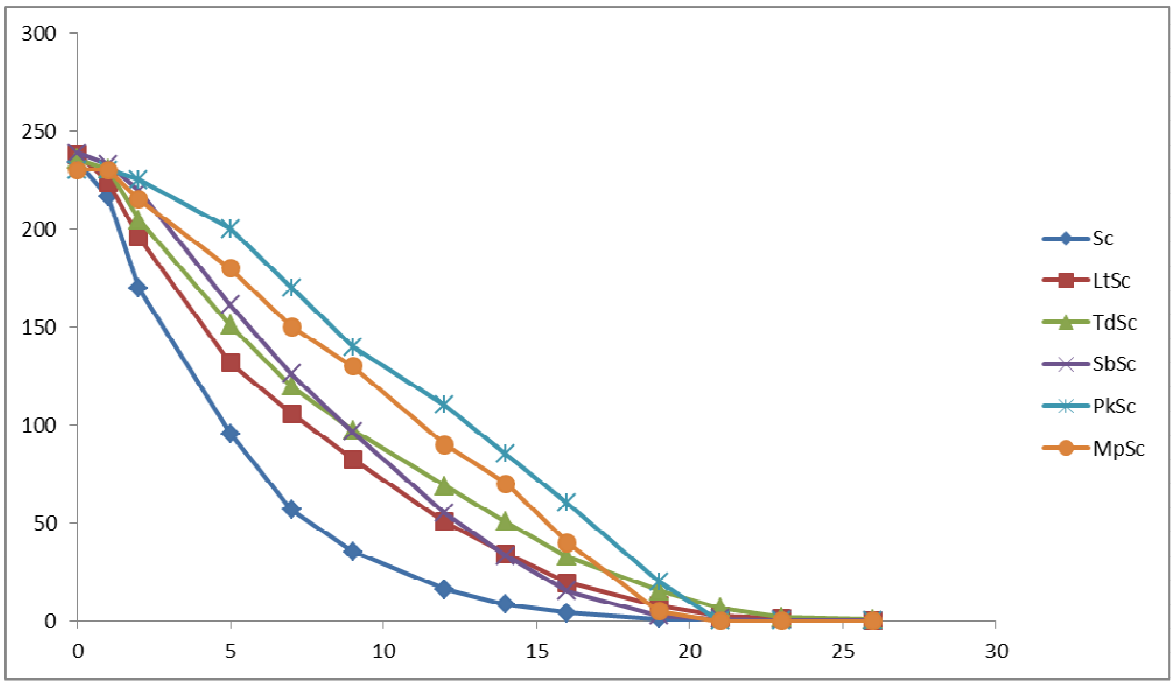

Figure 1: Typical fermentation kinetics profiles obtained when using mixed starter cultures. Mixed cultures were sequentially inoculated (*S. cerevisiae* was added 24 h after the non-*Saccharomyces* yeasts).

Alteration of wine aromatic profile

The production of a variety of compounds potentially impacting the wine organoleptic profile was evaluated using different analytical techniques (enzymatic assays, HPLC and GC-FID). Generally, the presence of the non-*Saccharomyces* yeasts greatly influenced the final wine composition, with the exception of *M. pulcherrima* which only impacted the level of medium chain fatty acids produced (Table 1).

The presence of most non-*Saccharomyces* yeasts led to a reduced concentration of acetic acid in the final wine, *L. thermotolerans* yielding the most severe reduction. However, the presence of *P. kluyveri* led to an increase. In parallel, the amount of glycerol produced was greater, especially in the case of *S. bacillaris*. It was also observed that *T. delbrueckii* and *L. thermotolerans* produced much more of the other polyols than *S. cerevisiae*. All these results suggest that the non-*Saccharomyces* yeasts respond to osmotic stress and redox balance related issues differently than *S. cerevisiae*. Further investigations are nevertheless required before final conclusions can be drawn. In future studies, the impact of the polyols produced on the wine's mouthfeel should also be evaluated.

The non-*Saccharomyces* yeasts studied also impacted the production of major volatile compounds. An increase in the concentrations of higher alcohols, acetate esters and fatty acids other than medium chain fatty acids was observed (except for *M. pulcherrima*). Of particular interest, *L. thermotolerans* produced high amounts of butanol and propanol and *T. delbrueckii* high amounts of isobutyric acid. The amount of higher alcohols produced by *L. thermotolerans* nevertheless remained under the detection threshold of these compounds and is unlikely to have a strong impact on the wine aromatic profile. The level of isobutyric acid produced by *T. delbrueckii* however, is above the detection threshold and this could impact on the overall wine aroma. Biologically speaking, these enhanced productions of higher alcohols and fatty acids also reveal different requirements of these different species for redox balancing.

In the case of *M. pulcherrima*, different strains were tested and, from our data, it is clear that strong strain variance occurs. Other studies in our environment show that, overall, this species does positively influence the aromatic profile of wine, but that this influence might be more associated with some extracellular enzyme activities, especially glycosidases activity, than with intracellular metabolism. The substrates of the reactions catalysed by these latter enzymes were however not supplied to the synthetic grape juice medium used in this study. Moreover,

certain strains of this species display extracellular acid protease activity which could also impact on wine composition. Further work is currently on-going in our environment on this topic. Finally, similar comments can be made for *P. kluyveri* which has been selected on the basis of its high potential to release volatile thiols. However, the precursors of these compounds were not supplied in our medium and the full potential of *P. kluyveri* was therefore not assessed.

Table 1: A summary of the significant alterations observed in various chemical compounds when using mixed starter cultures compared to pure culture of *S. cerevisiae* (-: decrease; 0: no change; +: increase; a double sign indicates a strong variation) – Td: *Torulaspora delbrueckii*, Lt: *Lachancea thermotolerans*, Mp: *Metschnikowia pulcherrima*, Sb: *Starmerella bacillaris*; Pk: *P. kluyveri*; nd: not determined

Compounds	**Td**	**Lt**	**Mp**	**Sb**	**Pk**
Primary metabolites					
Acetic acid	-	--	-/0/+*	-	++
Succinic acid	+	+	nd	0	
Glycerol	+	+	nd	++	
Other polyols	++	++	nd	0	
Aroma compounds					
Higher alcohols	+	++	0/++*	+	++
Acetate esters	+	+	0/+*	+	-
Medium chain fatty acids	-	-	-/+*	-	-
Other fatty acids	++	+	-/0*	+	+
Ethyl esters	0	0	0/+*	0	+

*strain dependent

Investigating yeast-yeast interactions using a membrane bioreactor

This part of the study was conducted in collaboration with Prof Patricia Taillandier, INP-ENSIACET, Toulouse, France.

The yeasts selected for this study, were *L. thermotolerans* and *S. cerevisiae*. Following mixed culture fermentations in a membrane bioreactor, it was observed that the presence of *S. cerevisiae* led to a significant decline in viability in *L. thermotolerans.* This decline was significantly less prominent in mixed cultures where the cells were in indirect contact. Together, the data provided evidence for both cell-cell and metabolic interactions whereby *S. cerevisiae* had a strong negative influence on the growth of *L. thermotolerans*. However, it was also observed that *L. thermotolerans* had some negative impact on the growth of *S. cerevisiae*, leading to a reduction in biomass (when in indirect contact) and a reduced maximum CFU/mL compared to pure cultures. The data also suggest that direct physical contact may increase the production of glycerol and propanol, but this needs further investigation.

During the study, it was also noticed that the concentration of dissolved oxygen seemed to play a crucial role on the survival and therefore on the overall metabolic activity of the yeasts, especially the non-*Saccharomyces* yeasts *L. thermotolerans*. By decreasing the frequency at which oxygen pulses were provided, a reduction in biomass and increase in fermentation duration was observed for all fermentations. However, this effect was somewhat reduced in mixed cultures. Here, no impact on fermentation duration was observed and the decrease in biomass was less compared to pure cultures. The impact of these oxygen pulses was also greater on *L. thermotolerans*. In the latter yeast's pure culture a slight increase in glycerol was observed when less oxygen was provided and in general there appeared to be no impact on acetic acid production. Furthermore, there was little or no impact on volatile production, however, more repeats might reveal different results and therefore more research is needed to confirm these results.

7. COMPLETE THE FOLLOWING TABLE

Milestone	Target Date	Extension	Date	Achievement

		Date	completed	
1. Selecting non-*Saccharomyces* yeast species/strains for their positive contribution to wine quality	2012	n/a	2013	Specific yeast species/strains were selected for further studies based on their positive contributions: production of specific aroma compounds, reduction of off-aromas (e.g. acetic acid) and production of polyols (glycerol and others).
2. Testing the membrane bioreactor	2012	n/a	2014	Yeast-yeast interactions (both physical and metabolic) occur during mixed culture fermentations. The impact of oxygen on yeast survival (and therefore impact on wine composition) was also evidenced.
3. Evaluating the impact of the presence of the selected non-*Saccharomyces* yeasts on the metabolic activity of *S. cerevisiae* - and vice-versa - (using synthetic grape juice)	2013	n/a	2014	The impact of the yeasts selected in Milestone 1 was investigated thoroughly using pure and mixed culture fermentations in synthetic wines. Features specific to the different non-*Saccharomyces* yeasts used were identified.
4 Evaluating the impact of the presence of the selected non-*Saccharomyces* yeasts on the metabolic activity of *S. cerevisiae* (using real grape juice)	2014	n/a	Not completed	As the previous milestones took longer than expected to complete, this milestone was not achieved. However, it will be completed in follow-up projects.
5. Journal publication(s) – final milestone				One popular article was submitted to Wineland for publication. Five oral presentations were delivered at conferences. Scientific articles will be submitted for publications after some results have been confirmed.

8. CONCLUSIONS

The inoculation of selected non-*Saccharomyces* yeasts in wine together with *S. cerevisiae* clearly provides a tool to winemakers to modulate the organoleptic profile of wines or to address

recurring problems such as high acetic acid. The fermentations presented above were performed several times and distinct patterns emerged. Nevertheless, the data indicate that for every species of yeast, large strain-specific variations may occur, as was clearly observed in the case of *M. pulcherrima*. It is therefore likely that current studies do not fully reflect the true oenological potential of certain species. A more systematic approach, investigating several strains of these species in real grape must is therefore being undertaken, especially on strains isolated from South African grape must as part of other Winetech-funded projects. Their full characterisation and future use could bring in unique organoleptic profiles, reinforcing the South African identity of the wines. The secretion of extracellular hydrolytic enzymes is another relevant characteristic of non-*Saccharomyces* yeasts. This aspect has also been investigated and will be reported in a follow-up article.

The results of this study also clearly show that interactions (both physical and metabolic) between yeasts do occur, especially when two yeast species are co-inoculated. Moreover, the results show that the environmental conditions play a significant role on the survival of non-*Saccharomyces* yeasts. It is extensively reported in literature that non-*Saccharomyces* yeasts do not survive more than a few days during wine fermentation because of their poor tolerance to ethanol. This statement is now being challenged by new evidence suggesting that other factors, such as oxygen availability, play a stronger role than the increasing concentration of ethanol. Unravelling the nature of the interactions occurring between yeasts and identifying the factors that play a role in the survival of yeasts during fermentation should be the subject of future projects to provide tools to winemakers to modulate the impact of yeasts on the final wine composition through *de novo* production of aroma compounds and release of aroma precursors found in the grape must.

9. ACCUMULATED OUTPUTS

List ALL the outputs from the start of the project. The year of each output must also be indicated.

a) TECHNOLOGY DEVELOPED, PRODUCTS AND PATENTS

Indicate the commercial potential of this project, e.g. Intellectual property rights or commercial product(s).

b) SUGGESTIONS FOR TECHNOLOGY TRANSFER

Provide steps taken to ensure the transfer of the gained/new information/knowledge to ultimately benefit the South African fresh fruit industry.

c) HUMAN RESOURCES DEVELOPMENT/TRAINING

Complete the following table, adding more lines if necessary.

Student Name and Surname	Student Nationality	Degree (e.g. MSc Agric, MComm)	Level of studies in final year of project	Graduation date	Total cost to industry throughout the project
Honours students					
Marli de Kock	South African		Honours	Dec 2012	
Masters Students					
Marli de Kock	South African		MSc	Dec 2014	

Natasha Luyt	South African		MSc	Dec 2014	
Arlene Mains	South African		MSc	March 2014	
PhD students					
Postdocs					
Support Personnel					

d) PUBLICATIONS (POPULAR, PRESS RELEASES, SEMI-SCIENTIFIC, SCIENTIFIC)
Please list using the format illustrated in the example below. ***ATTACH PDF COPIES OF ANY PAPERS ALREADY PUBLISHED***

Divol B, Bauer FF (2015) Altering the wine aromatic profile using multispecies yeast starter cultures. Submitted for publication in *Wineland*.

e) PRESENTATIONS/PAPERS DELIVERED
Please list using the format illustrated in the example below.

Divol B, Mains AO, Mostert TT, Bauer F.F. (2013) Specific wine yeast interactions revealed by metabolomic and proteomic approaches. Oral presentation at the 18th Biennial Conference of the South African Society of Microbiology (SASM 2013) at Bela-Bela, November 2013.

Divol B, Mains AO Bauer FF (2013) Multispecies yeast starter cultures and their impact on fermentation kinetics and outcomes. Oral presentation at the 35th conference of the South African Society of Enology and Viticulture at Somerset West, November 2013.

De Kock MC, Bauer FF Divol B (2014) Response to osmotic stress in mixed culture of *Saccharomyces* and non-*Saccharomyces* yeasts under oenological conditions. Oral presentation at the 36th conference of the South African Society of Enology and Viticulture at Somerset West, November 2014.

Divol B, Luyt N, Beaufort S, Taillandier P, Bauer FF (2015) The impact of oxygen on the behaviour and interaction of *S. cerevisiae* and *L. thermotolerans*. Poster presented at the 10th symposium of Oenology at Bordeaux, June-July 2015.

Luyt N, Bauer FF, Beaufort S, Fernandez-Lopez CL, Brandam C, Divol B, Taillandier P (2015) Comparison of direct and indirect contact during interactions between *Saccharomyces* and non-*Saccharomyces* yeasts. Oral presentation at the 10th symposium of Oenology at Bordeaux, June-July 2015.