Interaction between wine yeast and malolactic bacteria and the impact on wine aroma and flavour

by

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Declaration

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Summary

Wine is a product of the fermentation of grape juice. Alcoholic fermentation is mainly conducted by the yeast *Saccharomyces cerevisiae* which metabolises grape sugars to mainly ethanol, CO_2 and glycerol. Aside from these primary fermentation compounds, the yeast also produces many secondary metabolic by-products that are important to wine quality and style. Malolactic fermentation (MLF) is a secondary fermentation that normally occurs after alcoholic fermentation. Lactic acid bacteria (LAB) are responsible for the conversion of malic acid to lactic acid and CO_2 during MLF, which is important for wine deacidification and also contributes to microbial stability. Malolactic fermentation and LAB strains can also influence the aroma profile of wines. The main genera associated with this process are *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc*. *Oenococcus oeni* is the main species associated with MLF because it is able to survive the harsh physiochemical environment of winemaking. Recently *L. plantarum* has also been introduced as a commercial MLF starter culture. Research has started to focus on the potential of wine yeast and LAB interactions or combinations to alter the wine aroma profile via the production and/or degradation of aroma compounds.

The overriding goal of this study is to unravel the interactions between wine yeast and different LAB strains and their impact on wine aroma and flavour. The first aim was to assess LAB growth during co- and sequential inoculation strategies, the ability to complete MLF and the impact on the production of aroma compounds in combination with two different yeast strains in a medium containing full complement of nitrogen supplementation. Malolactic fermentation was successful in the different inoculation strategies and the bacterial combination (*L. plantarum* and *O. oeni*) completed MLF in the shortest time. The impact of the bacterial strains on the modification of aroma compounds was bigger in co- than sequential inoculation. A general increase in total esters (contributing to the fruity character of wines) especially ethyl lactate and ethyl acetate was observed. The production of esters, volatile fatty acids and higher alcohols proved to be dependent on either the yeast strain used and/or the LAB strains used.

The second aim of the research was to assess the effect of NH_4Cl (ammonium) and amino acids supplementation on yeast and LAB strains (both in co- and sequential inoculation strategies) and the impact on the aroma profile of the fermented must. Fermentations supplemented with ammonia as sole nitrogen source showed the highest total bacterial growth in terms of cell numbers. Malolactic fermentation was completed in the shortest time with *O. oeni* and the bacterial combination inoculums. The co-inoculated strategies in combination with amino acids supplementation showed the biggest impact on the aroma compound profiles of the different fermentation strategies and bacterial treatments. A general increase in total esters was observed for NH_4Cl additions with ethyl lactate and ethyl acetate showing the highest concentrations. The concentration of esters, volatile fatty acids and higher alcohols were strongly influenced by the yeast and the single LAB strains used. The results generated from this study showed that the chemical composition of the fermentation medium and the selection of yeast and LAB strains are important because these factors have an influence on the aroma and flavour profiles of wines.

Opsomming

Wyn is die produk van gefermenteerde druiwe. Die gis, *Saccharomyces cerevisiae* is verantwoordelik vir alkoholiese fermentasies waar druiwe suikers na hoofsaaklik etanol, CO_2 en gliserol gemetaboliseer word. Die gis produseer ook sekondêre metaboliete wat 'n belangrike bydrae lewer tot wynstyl en kwaliteit. Appelmelksuurgisting (AMG) is 'n sekondêre fermentasie wat gewoonlik na alkoholiese fermentasie plaasvind. Melksuurbakterieë (MSB) speel 'n sleutel rol in die omskakeling van appelsuur na melksuur en CO_2 gedurende AMG. Hierdie fermentasie lei tot 'n afname in die suurheidsgraad en verbeter die mikrobiese stabiliteit van die wyn. Appelmelksuurgisting en MSB rasse kan die aroma- en geurprofiel van wyne beïnvloed. Die belangrikste genera wat met AMG geassosieer word is *Oenococcus*, *Lactobacillus*, *Pediococcus* en *Leuconostoc*. *Oenococcus oeni* is die mees algemene ras wat vir AMG gebruik word omdat dit in uiterste wyn toestande kan oorleef. Mees onlangs is *Lactobacillus plantarum* as kommersiële aanvangskultuur vir AMG geïdentifiseer. Navorsing het onlangs meer begin fokus op gis en MSB interaksie of kombinasies as 'n strategie om die aroma profiele van wyne te verander.

Die hoofdoel van die studie is om die interaksie tussen wyngiste en verskillende MSB rasse en die effek op die aroma profile van wyne te bestudeer. Die eerste doelwit was om die impak van die twee giste op die groei en AMG vermoeë van MSB gedurende ko- en sekwensiële inokulasie praktyke en die impak op die produksie van aroma komponente, in 'n medium wat die volledige stikstof aanvullings bevat, te bestudeer. Appelmelksuurgisting was suksesvol in die verskillende inokulasie praktyke en die bakteriese kombinasie (*L. plantarum* en *O. oeni*) het AMG in die kortste tyd voltooi. Die impak van die bakteriese rasse op die modifikasie van die aroma komponente was groter met ko- as sekwensiële inokulasies. Daar was 'n toename in die totale esterkonsentrasies veral in etiellaktaat en etielasetaat. Die produksie van esters, vlugtige vetsure en hoër alkohole word beïnvloed deur die gisras en MSB rasse wat gebruik word.

Die tweede doelwit was om die impak van NH_4Cl (ammonium) en aminosure aanvullings op die gis- en MSB rasse gedurende ko- en sekwensiële inokulasie strategieë te bepaal. Melksuurbakterieë se groei was beter met die ammonium aanvulling. Appelmelksuurgisting was in die kortste tyd voltooi met *O. oeni* en die bakteriese kombinasie. Die ko-inokulasie praktyke in kombinasie met die kompleks aminosure aanvulling het die grootste impak op die produksie van aroma komponente gehad. Daar was weereens 'n toename in die totale esterkonsentrasies vir die NH_4Cl aanvulling, veral in etiellaktaat en etielasetaat. Die gis en MSB rasse speel 'n rol by die produksie en konsentrasies van esters, vlugtige vetsure en hoër alkohole. Die resultate van hierdie studie bewys dat die chemiese samestelling van die fermentasie medium, die seleksie van gis- en MSB rasse is belangrik omdat hierdie faktore die aroma en geur profiele van wyne beïnvloed.

This thesis is dedicated to my parents, Christopher and Pauline, and sister, Margaux
Hierdie tesis word opgedra aan my ouers, Christopher en Pauline, en suster, Margaux

Biographical sketch

Brenton Christopher Maarman was born on 4 January 1989 in Bredasdorp, Western Cape and matriculated at Oudtshoorn High School in 2007. Brenton obtained the BScAgric-degree (Viticulture & Oenology) at Stellenbosch University in 2011. In 2012 he enrolled at the same University for the MScAgric-degree in Oenology.

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Preface

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and is written according to the style of the journal South African Journal of Enology and Viticulture.

Chapter 1	**General Introduction and project aims**
Chapter 2	**Literature review** Understanding the complexity of interactions between different microbial populations in wine
Chapter 3	**Research results** Interaction between wine yeast and malolactic bacteria and the impact on wine aroma and flavour
Chapter 4	**General discussion and conclusions**

Contents

Chapter 1

Introduction and project aims

1. Introduction and project aims

1.1 Introduction

The chemical composition of wine is derived from the grapes as well as from the metabolic activity of the inoculated wine yeast during alcoholic fermentation (AF), the inoculated lactic acid bacteria (LAB) during malolactic fermentation (MLF) as well as the indigenous microflora associated with the vineyard or winery. Alcoholic fermentation is mainly conducted by *Saccharomyces cerevisiae* and results in the production of many metabolic by-products that are important to wine quality and style. The aroma compounds produced and the levels at which they are produced are highly strain dependent (Swiegers *et al*., 2005; Styger *et al*., 2011). MLF is mainly conducted by *Oenococcus oeni* and it is mainly initiated after alcoholic fermentation has finished although simultaneous inoculation of LAB for MLF is also a practice used today (Kunkee, 1991). Recently, *Lactobacillus plantarum* was also evaluated for its potential to conduct MLF and this species was found to perform just as favourably as *O. oeni* under wine conditions (Du Toit *et al*., 2011). MLF is characterised by a decarboxylation process in which the LAB in the wine converts the L-malic acid (a dicarboxylic acid) to L-lactic acid (a monocarboxylic acid) with the liberation of carbon dioxide (CO_2). The duration of MLF depends on physicochemical conditions and the amount of malic acid present in the wine (Muñoz *et al*., 2011). There are many LAB that occur naturally on the grape surfaces, however most of them are not able to survive in the harsh wine environment. Those that can survive are mostly of the genera *Pediococcus*, *Leuconostoc*, *Oenococcus* and *Lactobacillus*. LAB growth is favoured by the following: relatively high pH values (>3.5), sulphur dioxide (SO_2) concentrations of ≤ 50 ppm, temperatures between 25°C and 30°C and ethanol levels less than 13% v/v (Lerm *et al*., 2010).

MLF has an effect on the organoleptic properties of wine and it is known to remove vegetative and herbaceous aromas, increase the fruity and floral attributes of the wine, improve the mouthfeel and extend the aftertaste (Swiegers *et al*., 2005). Three mechanisms by which LAB are able to modify wine aroma and flavour are proposed by Bartowsky and Henschke (1995). Firstly, the bacteria are able to produce volatile compounds by metabolising grape constituents e.g. sugars and nitrogen containing compounds such as amino acids. Secondly, the bacteria can modify grape or yeast derived secondary metabolites, and lastly the bacteria can modify the must or wine by the adsorption to the cell wall of flavour compounds. Yeast autolysis results in the release of vitamins, amino acids, proteins and polysaccharides and can thus be the reason why bacterial growth and metabolic activity is stimulated after alcoholic fermentation when MLF is carried out on the yeast lees (Henick-Kling, 1993).

There are differences in the production/modification of aroma compounds by LAB strains. The study of Pozo-Bayón *et al*. (2005) showed that *L. plantarum* produced higher esters concentration than *O. oeni* while Lee *et al*. (2009) proved that *L. plantarum* produced higher levels of isobutanol, isoamyl acetate, 1-butanol, ethyl hexanoate and ethyl octanoate when compared to *O. oeni*. The study of Malherbe *et al*. (2012) showed that variations in the esters, higher alcohols and fatty acids concentrations in red wine after MLF are not vintage driven but are due to the different bacterial strains used. Sequential inoculation reduces the risk of acetic acid increases due to the low level of residual sugar and the absence of adverse interactions between yeast and bacteria (Costello, 2006; Lerm, 2010). The study of Antalick *et al*. (2012)

showed an increase in ethyl lactate and isobutyric acid in red wine after sequential inoculation with *O. oeni* when compared to uninoculated controls. The risks of sequential inoculation include a loss in viability because of the presence of high ethanol concentrations, high SO_2 concentrations, low pH, nutrient depletion and other antimicrobial compounds produced by the yeast (Larsen *et al*., 2003). Co-inoculation is a strategy that allows for an early dominance of the selected strain in the absence of inhibitory chemical parameters in the must as outlined above. Thus the outcome of MLF is more predictable but there are possible risks of undesirable/antagonistic interactions between yeast and/or bacteria, the production of possible off-odours and stuck AF (Henick-Kling and Park, 1994; Alexandre *et al*., 2004; Massera *et al*., 2009). Previous projects that were co-inoculated with *O. oeni* strains resulted in wines with increased concentrations of ethyl lactate and decreased acetate ester levels compared to sequential inoculation (Abrahamse and Bartowsky, 2012; Rossouw *et al*., 2012). Co-inoculation as a means to by-pass the problems associated with sequential MLF is gaining popularity in warmer climate regions where alcohol levels are a major concern, as well as nutrient depletion (Lerm *et al*., 2010; Du Toit *et al*., 2011). Therefore it is important to generate data on the interactions between yeast and MLF bacteria to determine the impact of co-inoculation on the chemical composition of the final product.

Previous studies have shown that *S. cerevisiae* has an impact on MLF and therefore the selection of wine yeast is crucial for a successful MLF. The interaction with the yeast can be detrimental to the bacteria through the production of ethanol, SO_2, medium-chain fatty acids, antibacterial proteins or beneficial through yeast autolysis and absorption of medium-chain fatty acids by mannoproteins (Alexandre *et al*., 2004; Arnink and Henick-Kling, 2005; Comitini *et al*., 2005; Osborne and Edwards 2006; Nehme *et al*., 2008, 2010). Interactions are very specific to a particular yeast-bacterial pairing. Different studies have shown highly strain-specific impacts on aroma compounds when different combinations of yeasts and bacteria used to carry out AF and MLF respectively. (López *et al*., 2011; Cañas *et al*., 2012; Costello *et al*., 2012). Yeast and bacteria interactions are also influenced by the must composition, the cultivar used, the vintage etc. (Lerm, 2010; Malherbe *et al*., 2012).

Wine flavour is the combination of volatile and non-volatile compounds. The grape must constituents not only provide the raw materials for the production of flavour metabolites but also serve as a nutrient source for *S. cerevisiae* to successfully carry out alcoholic fermentation (Bisson, 1999). The wine yeast *S. cerevisiae* uses ammonium ions, free amino acids and occasionally low molecular weight peptides as nitrogen sources (Henschke and Jiranek, 1993). Generally, DAP is added to the juice to serve as a nitrogen source for the wine yeast but the resulting wines are often less complex in terms of ethyl esters compared to amino acids as a nitrogen source (Smit, 2013). After alcoholic fermentation is completed the wine may lack nutrients such as essential amino acids which may negatively influence LAB growth and MLF (Remize *et al*., 2006). LAB cannot utilise ammonia from DAP and must therefore rely on commercial bacterial nutrient additives that consist of yeast extracts or yeast hulls/ghosts that contain amino acids, fatty acids, nucleic acids, vitamins and minerals (Henick-Kling *et al*., 2004). The recent sequencing of the genomes of several strains of *O. oeni* (Borneman *et al*., 2010) showed large variations between strains in terms of their ability to utilise different amino acids as nitrogen sources. These differences mean that different strains of *O. oeni* will respond differently to the various commercial nutrient additives. The different amino acid utilisation profiles of LAB strains will directly impact on the aroma compounds produced by the bacteria in

grape musts of different chemical composition. Our current understanding of the impact of differences in must composition, particularly with regard to amino acids, on the interaction between yeasts and bacteria and the aroma profile of the final wine, is far from comprehensive. A study showed that 10 compounds were essential for the growth of all wine LAB (*Oenococcus* and *Lactobacillus*) tested (Terrade and De Orduña, 2009). Nutrient requirements are strain dependent and *O. oeni* required 16 amino acids and 2 vitamins while *Lactobacillus hilgardii* required 8 amino acids and 3 vitamins. These compounds include a carbon and phosphate source, manganese, several amino acids (proline, arginine and the branched amino acids valine, leucine and isoleucine) and vitamins (nicotinic acid and pantothenic acid).

Winemakers today are challenged with new inoculation practices such as bacterial co-inoculation versus sequential inoculation, the use of different strains of *O. oeni*, and the use of other species of LAB such as *L. plantarum* (either as a pure or mixed culture) for MLF. A fair amount of research has been done on the interaction between wine yeast and LAB and the impact this has on the aroma of wine. These studies are however mostly based on pure, single strain bacterial inoculations, thus the impact of mixed inoculums containing more than one species of LAB still needs to be further investigated. Furthermore, the effect of differences in amino acid concentrations on the aroma impacts of co-inoculated fermentations has not been established.

1.2 Project aims

The aim of this study was to evaluate the metabolic impact of three different LAB treatments (*L. plantarum, O. oeni*, and a combination of *O. oeni* and *L. plantarum*) in combination with two different *S. cerevisiae* strains (Cross Evolution® and Lalvin EC1118®) in three different synthetic media (with different amino acid compositions) in both co-inoculation and sequential inoculation strategies.

The specific aims of the study were as follows:

i. to assess the impact of the yeast on the growth kinetics of LAB species during co- and sequential inoculation strategies;

ii. to evaluate the malic acid degradation of the single and combination of the LAB species in co- and sequential inoculation strategies using different yeasts;

iii. to assess the impact of these strategies on the volatile aroma profile of wine;

iv. to assess the effect of NH_4Cl and amino acids on the yeast and LAB species in co-inoculation and sequential inoculation and the impact these strategies have of the aroma profile of wine; and

v. to do multivariate statistical data analysis on all generated data sets.

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Chapter 2

Literature review

Understanding the complexity of interactions between different microbial populations in wine

2. Literature review

Understanding the complexity of interactions between different microbial populations in wine

2.1 Introduction

The interactions between different microbial populations have interested the wine microbiology community with the final aim of understanding the impact on the quality of wine. The microbial interaction in wine mainly occurs between *Saccharomyces* spp., non-*Saccharomyces* spp., lactic acid bacteria (LAB) and acetic acid bacteria (Barata *et al*., 2012). Various factors influence the composition of vineyard microbial communities namely climatic factors such as temperature, UV exposure, rainfall, sunlight and winds are some of the most important impact factors (Boddy and Wimpenny, 1992). Other elements include vineyard treatments, biotic factors (e.g. insects, birds, phytopathogenic and saprophytic moulds) geographic location and vineyard factors such as age, size, grape variety, the condition of the grapes and the vintage (Pretorius *et al*., 1999). A recent study of Barata *et al.* (2012) concluded that grape health status is the main factor affecting the microbial ecology of grapes, increasing both microbial numbers (bacterial population less than 10^6 CFU/g) and species diversity while the study of Setati *et al.* (2012) found that farming systems have a significant impact on fungal diversity and that vineyards with lower treatment levels (of pesticides, herbicides etc.) displayed higher yeast species richness. The presence of microorganisms can also influence the quality of the grapes before the harvest, during the fermentation and during the ageing and/or preservation of the wine. Therefore the quality of a wine is mainly conditioned by viticultural practices, wine making techniques and the yeast and bacterial strains used (Ciani *et al*., 2010).

The genus *Saccharomyces* belong to the Ascomycetous group. See **Table 2.1** for the four different *Saccharomyces* spp. The most important *Saccharomyces* species in the wine industry is *S. cerevisiae*. This yeast plays a fundamental role during the transformation of grape sugars into ethanol, carbon dioxide and hundreds of other secondary metabolites (Barre and Vezinhet, 1984). Selected starter cultures of *S. cerevisiae* are used in order to ensure better control over alcoholic fermentation and to supress the growth of non-*Saccharomyces* yeasts present in the must which lead to stuck fermentations and possible wine spoilage in some cases (Castelli, 1954; Amerine and Cruess, 1960). *Saccharomyces cerevisiae's* growth and metabolic activity are influenced by the amount and viability of the inoculum and the technology used in winemaking e.g. clarification procedures, temperature of fermentation, SO_2 and nutrient additions (Benda, 1982; Reed and Nagodawitha, 1988).

Non-*Saccharomyces* yeasts are normally present on grapes, must and wine making equipment (Ciani *et al*., 2010). The most important genera include *Hanseniaspora*/*Kloeckera*, *Candida*, *Pichia*, *Zygosaccharomyces*, *Schizosaccharomyces*, *Torulaspora*, *Lachancea* (*Kluyveromyces*) and *Metschnikowia* (Fleet *et al*., 1984; Heard and Fleet, 1985, 1986; Pardo *et al*., 1989, Kurtzman, 2003). See **Table 2.1** for a list of species. Several factors influence the presence and persistance of these non-*Saccharomyces* yeasts throughout the fermentation. For instance, the genera of *Hanseniaspora*/*Kloeckera* and *Candida* generally have a weak ethanol tolerance while other studies have shown that *Kloekera apiculata* and *Candida stellata* have increased ethanol tolerance at lower temperatures of 10°C to 15°C (Gao and Fleet, 1998; Erten, 2002).

The oxygen concentration also plays an important role in the growth and survival of several non-*Saccharomyces* yeasts, such as *Torulaspora delbrueckii* and *Lachancea thermotolerans* (Hansen *et al*., 2001).

Pérez-Nevado *et al*. (2006) hypothesised that the production of toxic compounds by *S. cerevisiae* can be the cause of the early death of *Hanseniaspora guilliermondii* during fermentation. Other compounds produced during fermentation such as acetic acid, medium-chain fatty acids, acetaldehyde and the synergistic action of their combinations can also inhibit certain yeast species (Ludovico *et al*., 2001; Fleet, 2003). Non-*Saccharomyces* were previously often considered as spoilage yeasts because they were often isolated from stuck or sluggish fermentation (Castelli, 1954; Ribéreau and Peynaud, 1960). Previous studies have investigated the impact of non-*Saccharomyces* yeasts on the composition, sensory properties and final flavour of wines (Lema *et al*., 1996; Egli *et al*., 1998; Henick-Kling *et al*., 1998). For these reasons, over the last decade, the role of non-*Saccharomyces* yeast in winemaking has been re-evaluated, with a view to improve the quality and enhancing the aroma complexity of wines (Rojas *et al.,* 2001; Jolly *et al.,* 2003, 2006; Swiegers *et al*., 2005; Domizio *et al*., 2007; Renouf *et al*., 2007; Anfang *et al*., 2009; Sadoudi *et al*., 2012).

Besides the inoculated yeast and the natural yeast species present in the must, different genera of bacteria are also present in, and contribute metabolically to the fermenting must. The most important of these from a winemaking perspective are the lactic acid bacteria (LAB). LAB are Gram-positive, non-sporing, non-respiring bacteria that can be described as coccoid to elongated cocci or rod-shaped bacilli (Lerm *et al*., 2010). Lactic acid is the major product formed by these bacteria during the fermentation of carbohydrates. The LAB genera associated with wine include *Oenococcus*, *Lactobacillus*, *Leuconostoc* and *Pediococcus* as these genera are most often responsible for changes to the wine matrix during malolactic fermentation (MLF) (Wibow *et al*., 1985). Certain species from the genera *Pediococcus* and *Lactobacillus* can contribute to wine spoilage in high or low pH wines. These species such as *Lactobacillus hilgardii, Lactobacillus buchneri, Lactobacillus brevis* and *Pediococcus parvulus* are also responsible for the formation of biogenic amines which can be a health risk due to their potential toxicological effects in sensitive persons (Moreno-Arribas and Polo, 2008).

The LAB species that is the best adapted to the wine matrix is *Oenococcus oeni* (Lonvaud-Funel, 1999). This species is known to improve wine quality through deacidification, production of desirable flavours and aromas and enhancement of microbial stability (Bartowsky *et al*., 2002). Recently, different studies showed that strains of *Lactobacillus plantarum* also have the ability to conduct MLF just as efficiently as *O. oeni* and possess many enzymes encoding genes important for desirable aroma production (Mtshali *et al.,* 2012; Du Toit *et al.,* 2011). Various factors negatively influences LAB growth such as pH below 3.2 (Britz and Tracey, 1990; Vaillant *et al*., 1995; Rosi *et al*., 2003), an ethanol content of 14% and above (Vaillant *et al*., 1995; Zapparoli et al., 2009) and temperature below 20°C (Britz and Tracey, 1990), in addition to the presence of some yeast inhibitory metabolites such as SO_2 concentration above 50 ppm (Henick-Kling and Park, 1994; Carreté *et al*., 2002; Osborne and Edwards, 2006; Nehme *et al*., 2008) and medium-chain fatty acids (Carreté *et al*., 2002; Alexandre *et al*., 2004; Mendoza *et al*., 2010). In literature it has been found that lysozyme and phenolic compounds can also inhibit LAB (Gao *et al*., 2002; Campos *et al*., 2009).

The focus of this literature review will be to summarise the interactions between *Saccharomyces*, non-*Saccharomyces* yeasts and LAB and the impact these interactions have on aroma compounds in wine.

2.2 The different types of interactions between *Saccharomyces* and non-*Saccharomyces* yeast

Different inoculation strategies of the grape must influence the modes and types of interactions between different yeasts and LAB. Grape juice can be inoculated with active dry yeast or it can be simultaneously/co-inoculated with non-*Saccharomyces* yeast (Bisson and Kunkee, 1993; Rodríquez *et al*., 2010; Comitini *et al*., 2010). Preliminary evidence has shown that when some yeasts develop together under fermentation conditions, they do not passively co-exist but they interact and produce unpredictable compounds and/or different levels of fermentation products which can affect the chemical and aromatic composition of wines (Howell *et al*., 2006; Anfang *et al*., 2009). See **Table 2.2** for the main interactions in mixed fermentations Several types of interactions have been identified: commensalisms, synergism, and antagonism. The main consideration in oenology is frequently the inhibition or promotion of population growth of one species by another (Fleet, 2003).

The two main types of interaction that modulate the development of different yeast populations during alcoholic fermentation are nutritional limitation, or competition, and the release of toxic compounds into the medium. Killer toxin producing strains, killer-sensitive and killer neutral strains of *S. cerevisiae* have been isolated from fermenting must (Guriérrez *et al.,* 2001). Killer strains of the genera *Candida*, *Pichia* and *Hanseniaspora* have also been isolated from wine. Some of these can assert their killer action against wine strains of *S. cerevisiae* (Fleet and Heard, 1993). Killer activity is not the only cause of antagonistic interactions between different yeast species. Competition between yeast species can also be regarded as antagonistic effects. The study of Medina *et al.* (2012) showed that the sluggish fermentation of *S. cerevisiae* and non-*Saccharomyces* yeasts (*Metschnikowia pulcherrima* and *Hanseniaspora vineae*) was due to competition for nutrients and not because yeast killer effects.

Acetic acid is one of the main yeast metabolism-derived acids present in wine. If the concentration of this acid is too high it can result in wine spoilage (Fleet, 2003), which is a problem often associated with non-*Saccharomyces* yeasts. To avoid this problem, non-*Saccharomyces* yeasts have been investigated for their wine making potential in co-culture with *S. cerevisiae (*More *et al*., 1990; Salmon *et al*., 2007*)*. The study of Bely *et al.* (2008) investigated the impact of mixed and sequential *T. delbrueckii* and *S. cerevisiae* cultures in high sugar fermentation to determine whether this would improve the quality of wines and reduce the acetic acid content. The results showed a 60% reduction in volatile acidity and acetaldehyde when the co-culture approach was used while the sequential cultures showed lower effects on the reduction of these metabolites. The studies of co-inoculated fermentations with *Lach. thermotolerans* and *S. cerevisiae* has also been showed to result in reduced amounts of volatile acidity (Renault *et al.,* 2009, Comitini *et al.,* 2011).

Other important fermentation-derived compounds which play a role in yeast-yeast interactions include the medium-chain fatty acids, such as hexanoic, octanoic and decanoic acids, which are produced during alcoholic fermentation and become inhibitory to *S. cerevisiae* above certain thresholds (Bisson, 1999). Temperature and SO_2 also influences the growth and metabolism of mixed yeast starter cultures and the study of Mendoza *et al.* (2007) investigated the influence of these factors on the growth and metabolism of *K. apiculata* and *S. cerevisiae* and showed increased viability of *K. apiculata* in mixed fermentations.

The interactions between *Saccharomyces* and non-*Saccharomyces* wine yeasts have effects not only on the persistence of the non-*Saccharomyces* yeasts but also on the behaviour of the *S. cerevisiae* strains. This can be seen by variations in the degree of flocculation in mixed cultures of *K. apiculata* and *S. cerevisiae*. The study of Sosa *et al.* (2008) showed that in mixed fermentations the flocculent strain of *K. apiculata* interacted with a non-flocculent strain of *S. cerevisiae*, inducing co-flocculation of these two yeasts. Another interaction described in mixed fermentations in wine is cell-to-cell contact. Investigations carried out in mixed cultures to evaluate cell-to-cell contact showed that *T. delbrueckii* and *Lach. thermotolerans* had a lesser ability to compete for space in comparison to *S. cerevisiae* (Nissen and Arneborg, 2003; Nissen *et al*., 2003). The causes of this behaviour are still not clear but the study of Renault *et al*. (2013) hypothesised that cell-to-cell contact may involve direct physical contact through receptor/ligand-like interaction. This type of mechanism is typically unidirectional and the mechanism may involve soluble molecules, lethal at high concentration released into the medium by *S. cerevisiae*.

In *Saccharomyces*/non-*Saccharomyces* mixed cultures, interactions should be more numerous due to the wide ranging genetic and metabolic diversities. The use of co-inoculated fermentations using different *S. cerevisiae* starter cultures have shown differences in chemical and sensory profiles from pure fermentations (King *et al*., 2008). In the case of the interaction between *S. cerevisiae* and *Starmerella bombicola* results showed a complementary consumption of glucose and fructose (Ciani & Ferraro, 1998). The exchanges of acetaldehyde between these two yeasts were highlighted when sequential, continuous fermentation and immobilised yeasts were used. The excess acetaldehyde produced by *St. bombicola* (due to the low activity of its alcohol dehydrogenase enzyme/s) was rapidly metabolised by *S. cerevisiae* (Ciani *et al*., 2000; Ciani and Ferraro, 1998). The study of Cheraiti *et al.* (2005) investigated the acetaldehyde movement between *S. cerevisiae* and *Saccharomyces bayanus*. It showed that *S. cerevisiae* also rapidly metabolised acetaldehyde produced by *S. bayanus*. A reduction in acetaldehyde was also detected in mixed fermentation using *S. cerevisiae* and *T. delbrueckii* and *S. cerevisiae* and *Lach. thermotolerans* (Ciani *et al*., 2006; Bely *et al*., 2008). The study of Gobbi *et al*. (2013) confirmed these findings. Acetoin is another compound that is similarly affected by interactions between different yeast species in mixed fermentations. This compound is largely produced by *St. bombicola* in a pure culture and completely metabolised by *S. cerevisiae* in mixed fermentation (Ciani and Ferraro, 1998).

In the quest for understanding the behaviour of yeasts under winemaking conditions, several studies have been conducted to investigate the biochemical, physiological and molecular responses of yeasts during alcoholic fermentation. Some studies investigated the physiological properties of natural and commercial *S. cerevisiae* strains, linking these to their gene expression patterns and genome sequences (Cavalieri *et al*., 2000; Hauser *et al*., 2001; Rossignol *et al*.,

2003; Varela *et al*., 2005; Wu *et al*., 2006; Borneman *et al*., 2008). Some studies showed that different yeasts investigated at different time points reveal difference in gene expression that are related to strain identity while other studies showed that over expression of genes can have a statistical impact on fermentation kinetics. The adaptation of yeast cells to wine fermentation conditions have also been investigated at the mRNA and protein level (Zuzuarregui *et al*., 2006; Rossignol *et al*., 2009, Maturano *et al*., 2011; Rossouw *et al*., 2010). The different studies showed that during alcoholic fermentations, substantial changes in protein occur.

TABLE 2.1

Dissemination and technological significance of microbial species isolated from the vineyard and winery environments (Barata *et al*., 2012)

Group	Metabolism	Genus	Relevant species	Technological significance	Main source
Yeasts					
Basidiomycetous	Oxidative	*Filobasidium*, *Cryptococcus*, *Rhodotorula* (pink yeast)	*A. pullulans* (yeast-like fungi, black yeast	Absent/unknown	Soil, bark, leaf, grape
		Aureobasidium		Absent/unknown	Soil, bark, leaf, grape
Ascomycetous	Oxidative or weakly	*Hanseniaspora*/*Kloeckera* (apiculate yeast)	*H. uvarum*/*K. apiculata*	Contamination/spoilage	Grape, grape juice, fermentation
		Candida (film-forming yeast)		Contamination	Grape, grape juice, fermentation, wine
			C. stellata or *C. zemplinina*	Contamination	Grape, grape juice, fermentation, wine
			Zygoascus hellenicus/*C. steatolytica*	Contamination	Grape, grape juices Fermentation
		Metschnikowia	*M. pulcherrima*	Contamination	Grape, fermentation,
		Pichia (film-forming yeast)	*P. anomala*	Contamination/spoilage	Grape, fermentation,
			P. membranifaciens	Contamination/spoilage	Grape, fermentation,
			P. guilliermondii	Contamination/spoilage	Grape, fermentation
		Debaryomyces	*D. hansenii*	Contamination	Grape, fermentation
		Lachancea (ex *Kluyveromyces*)	*Lach. thermotolerans*	Contamination	Grape, fermentation
			Lach. fermentati (ex *Z. fermentati*)		Grape, fermentation
	Fermentative	*Torulaspora*	*T. delbrueckii*	Spoilage	Wine, concentrated grape juices
		Zygosaccharomyces	*Z. bailii*	Spoilage	wine
			Z. bisporus	Spoilage	wine
			Z. rouxii	Spoilage	Concentrated grape juices
		Dekkera/*Brettanomyces*	*D. bruxellensis*	Spoilage	Wine
		Saccharomyces	*S. cerevisiae*	Fermenting/spoilage	Fermentation, wine
			S. bayanus	Fermenting/spoilage	Fermentation, wine
			S. paradoxus		Fermentation, wine
			S. pastorianus		Fermentation, wine
		Schizosaccharomyces	*Sc. pombe*	Spoilage	Wine
		Saccharomycodes	*Sch. ludwigii*	Spoilage	Wine
Bacteria					
Acetic acid bacteria	Aerobic	*Gluconobacter* spp., *Acetobacter* spp., *Gluconoacetobacter* spp.,		Wine spoilage, vinegar production	Grape, wine
Lactic acid bacteria	Anaerobic, semi-anaerobic	*Oenoccocus*, *Lactobacillus* spp., *Pediococcus* spp., *Weissella* spp.		Malolactic fermentation or wine spoilage	Grape, wine
Several bacterial species		*Acinetobacter* spp., *Curtobacterium* spp., *Pseudomonas* spp., *Serratia* spp., *Enterobacter* spp., *Enterococcus* spp., *Bacillus* spp., *Staphylococcus* spp.		Innocuous contaminants	Grapes

The proteins are not directly associated with changes at transcript level and this suggests that mRNA is selectively processed and/or translated in stationary phase. The interactions between *Saccharomyces* and non-*Saccharomyces* mixed cultures result in different chemical and sensorial properties due to a wide genetic and metabolic diversity of the different yeasts. The studies mentioned above need to be expanded to include more holistic studies of mixed yeast fermentations in order to understand the underlying mechanisms responsible for the different modes of interactions discussed in this review.

TABLE 2.2

Main interactions described in mixed fermentation of wines (Ciani *et al*., 2009)

Species used	Compound or behaviour	Interactions	References
S. cerevisiae *H. uvarum*	Growth and viability	Persistence of non-*Saccharomyces*	Ciani *et al.* (2006); Mendoza *et al.* (2007)
S. cerevisiae *T. delbrueckii*	Cell-to-cell contact	Increase in death rate of non-*Saccharomyces*	Nissen & Arneborg (2003); Nissen *et al.(2003)*
S. cerevisiae *C. stellata*	Acetaldehyde, acetoin, glucose and fructose	Complementary consumption	Ciani & Ferraro (1998)
S. cerevisiae *H. uvarum/guillermondii*	Ethyl acetate Esters	Reduction Increase	Moreira *et al.* (2008)
S. cerevisiae *P. anomala*	Isoamyl acetate (EAHase)*	Increase in production by *S. cerevisiae*	Kurita (2008)
S. cerevisiae *P. kluyveri*	3-Mercaprohexyl acetate	Increase in thiols	Anfang et al. (2009)
Mixed 'wild' yeasts	Volatile compounds	Increased and more complex aroma	Garde-Cerdán & Ancín-Azpilicueta (2006); Varela *et al.* (2009)

*EAHase, ethyl acetate-hydrolysing esterase

2.3 The effect of interactions between *Saccharomyces* and non-*Saccharomyces* yeast on aroma compounds

Positive interactions in terms of volatile compounds have been observed between 'wild' non-*Saccharomyces* and *S. cerevisiae* starter cultures (Garde-Cerdán and Ancin-Azpilicueta, 2006).**Table 2.3** shows the mixed fermentation processes proposed in winemaking using *S. cerevisiae* and non-*Saccharomyces* yeasts and the influence on aroma. Some studies have demonstrated increased ester concentrations in mixed fermentations compared to pure fermentations due specifically to increases in ethyl-2-methylpropanoate and ethyl caprate production (Garde-Cerdán and Ancin-Azpilicueta, 2006; Varela *et al*., 2009). The study of Moreira *et al.* (2008) confirmed the improved ester production and the reduction in ethyl acetate in mixed fermentations. In contrast, the study of Gobbi *et al*. (2013) found a significant increase in ethyl acetate concentrations where *Lach. thermotolerans* and *S. cerevisiae* were used as co-culture inoculants. Another study by Kurita (2008) investigated the effect of co-fermentations of *Pichia anomala* petite mutants (with low levels of ethyl acetate) and *S. cerevisiae*. The study concluded that desired amounts of isoamyl acetate accumulated in the mixed cultures without an excess of ethyl acetate. Comitini *et al*. (2011) also investigated the analytical profiles of wines fermented by mixed cultures, reporting significant increases in 2-phenylethanol concentrations in mixed fermentations of *T. delbrueckii* and *Lach. thermotolerans*. A study was done to compare co-inoculation of *S. cerevisiae* and *T. delbrueckii* to monoculture. The study showed that the monoculture of *T. delbrueckii* released lower amounts of ethyl acetate, propanol and isobutanol when compared to co-inoculated treatments (Barrajón *et al*., 2011). This increase in 2-phenylethanol levels contributes to a desirable floral (rose) aroma in wine (Swiegers *et al*., 2005). The study of Medina *et al*. (2013) compared spontaneous fermentation

and co-fermentation of *S. cerevisiae* and *H. vinea* in Chardonnay. The spontaneous fermentation of *H. vinea* showed increased concentrations of acetate esters, some ethyl esters and decreased concentrations of 2-phenylalcohol, 1,3 propanol, 3-methyl-1-propanol. In the co-inoculation experiments increased concentrations of ethyl acetate, ethyl caprate and ethyl succinate were reported while increases in higher alcohols were not significant. Roderiquez *et al*. (2010) compared co-inoculation of *S. cerevisiae* and *Candida pulcherima* to sequential inoculation. This study showed that sequential inoculation resulted in increases in ethyl acetate, ethyl hexanoate and hexyl acetate while the co-inoculated fermentations showed lower concentrations of isobutyl alcohol, isoamyl alcohol and 2-phenylalcohol. Positive interactions in mixed fermentations have also been shown for thiol production in Sauvignon blanc wines. (Anfang *et al*., 2009). Mixed fermentations of *P. kluyveri* and *S. cerevisiae* resulted in increases in 3-mercaptohexyl acetate (3MHA) in comparison to pure cultures. The study of Sadoudi *et al*. (2012) also showed an increase in 3MHA when *M. pulcherrima* and *S. cerevisiae* co-cultures were used to ferment Sauvignon blanc juice.

These studies show that the use of *Saccharomyces* and non-*Saccharomyces* as co-cultures to conduct fermentation have a positive impact on wine organoleptic characters and result in more complex wines in comparison with pure cultures.

TABLE 2.3

Mixed fermentation processes that have been proposed in winemaking, using *Saccharomyces cerevisiae* and non-*Saccharomyces* (Ciani *et al*., 2010)

Species used	Aim	Process	References
S. cerevisiae *T. delbrueckii*	Reduction of acetic acid production	Sequential cultures	Castelli (1969); Herraiz *et al*. (1990); Ciani *et al*. (2006); Salmon *et al*. (2007); Bely *et al*. (2008)
S. cerevisiae *Sc. pombe*	Malic acid degradation	Sequential cultures Immobilized cells (batch process) Immobilized cells (continuous process)	Snow & Gallender (1979); Magyar & Panyik (1989); Yokotsuka *et al*. (1993); Ciani (1995)
S. cerevisiae *C. stellata*	Enhancement of glycerol content	Immobilised cells (pretreatment or sequential cultures)	Ciani & Ferraro (1996); Ciani & Ferraro (1998); Ferraro *et al*. (2000)
S. cerevisiae *C. cantarellii*	Enhancement of glycerol content	Mixed or sequential cultures	Toro & Vazquez (2002)
S. cerevisiae *C. stellata*	Improve of wine aroma profile	Mixed or sequential cultures	Soden *et al*. (2000)
S. cerevisiae *H. uvarum (K. apiculata)*	Stimulation of natural fermentation (improvement of aroma complexity)	Mixed or sequential cultures	Herraiz *et al*. (1990); Zironi *et al*. (1993); Moreira (2005); Ciani *et al*. (2006); Moreira *et al*. (2008); Mendoza *et al*. (2007)
S. cerevisiae *Kluy. thermotolerans*	Reduction of acetic acid production Enhancement of titratable acidity	Sequential cultures	Mora *et al*. (1990); Ciani *et al*. (2006); Kapsopoulou *et al*. (2007)
S. cerevisiae *I. orientalis*	Reduction of malic acid content	Mixed fermentation	Kim *et al*. (2008)
S. cerevisiae *P. fermentans*	Increased and more complex aroma	Sequential cultures	Clemente-Jimenez *et al*. (2005)
S. cerevisiae *P. kluyveri*	Increased varietal thiol	Mixed fermentation	Anfang *et al*. (2009)
S.cerevisiae *Candida pulcherrima*	Improve wine aroma profile	Mixed fermentation	Zohre & Erten (2002); Jolly *et al*. (2003)
S. cerevisiae *Debaryomyces vanriji*	Increase in geraniol concentration	Mixed fermentation	Garcia *et al*. (2002)
S. cerevisiae *Schizosaccharomyces* spp. *Saccharomycodes* spp. *Pichia* spp.	Influence on sensorial and physico-chemical properties of wines	Ageing over the lees during wine maturation	Palomero et al. (2009)

2.4 Interactions between *Saccharomyces* yeast and LAB

2.4.1 The combination of the particular yeast and bacterial strain

In the literature, there are three main types of interaction; the most common kind of interaction is bacterial inhibition followed by stimulation and neutralism (Patynowski *et al*., 2002; Comitini *et al.,* 2005). Alexandre *et al*. (2004) proposed that the degree and complexity of these interactions are due to three factors. It is the yeast and bacterial combination, the uptake and release of nutrients by yeast and the third factor is the production of extracellular compounds by yeast that can have an effect on the growth of LAB. Firstly, the specific combination of the particular yeast and bacteria strains plays a key role in determining the mode of interactions and the success of MLF. A study by Nehme *et al.* (2008) made use of two *O. oeni* strains and four different yeast strains. The results showed that the same yeast strain was able to inhibit and stimulate two different bacterial strains in conditions similar to winemaking conditions. One of the yeast strains was most inhibiting to one of the bacterial strains but it showed a neutral effect to the other. The study also showed that the rate of malic acid consumption was not always correlated to the growth rate or total bacterial biomass formed. These results were confirmed by Mendoza *et al*. (2010).

Costello *et al*. (2003) proposed a method for testing the compatibility between yeast and bacteria. The aim of the study was to investigate the interaction between populations of these two microorganisms without the effect of extrinsic grape derived or processing factors like SO_2 additions, adjusted pH, sugar concentration and the presence of pesticide residues or nutrients. The study made use of four *S. cerevisiae* wine yeasts which have been characterised in terms of the growth response of a selected *O. oeni* strain. A chemically defined medium was used to successfully characterise the metabolic interactions between the yeasts and bacteria in this study. Three yeasts gave compatible interactions, while the fourth yeast inhibited bacterial growth and produced a high concentration of sulphite. A test for inhibition due to nutrient depletion was also performed but results showed that this effect was strain dependent while the treatment that removed yeast-derived inhibitory factors partly alleviated inhibition caused by the sulphite-producing yeast strain. The replacement of the synthetic media with Chardonnay juice produced similar results. This could be an effective tool for screening yeast/LAB combinations in advance to ensure compatibility and lack of antagonistic or inhibitory effects.

Another method that is also used to predict compatibility between *S. cerevisiae* and *O. oeni* is the plating method (Arnink & Henick-Kling, 2005). Results showed that the differences between vintages and grape varieties were more influential on LAB and MLF than the effect of a particular yeast/bacteria strain combination. A study done by Nehme *et al.* (2010) used different malolactic inoculation strategies to investigate the impact on MLF and reasons for antagonism exerted by yeast. The results of the sequential culture showed that the inhibition exerted by *S. cerevisiae* in terms of decreasing the rate of malic acid consumption was mainly due to ethanol (75%) and to a peptidic fraction (25%) having an MW between 5 and 10 kDa. The sequential culture consumed 0.4 g/L of L-malic acid while the co-culture consumed 3.7 g/L. There was no evidence of increased volatile acidity during the co-culture fermentations. The study concluded that the co-culture strategy was optimal for MLF with the yeast/bacteria pair studied.

2.4.2 Nutrient availability for LAB utilisation

Another important factor to consider is the uptake and release of nutrients by the yeast, which will in turn affect the nutrients available for the LAB (Lerm *et al*., 2010). At the start of AF, *O. oeni* is inhibited by *S. cerevisiae* due to the rapid uptake of certain grape metabolites from the must by the yeast. These compounds include sterols, amino acids and vitamins which result in a nutrient diminished environment for the bacteria (Larsen *et al*., 2003). Commercial yeast preparations consume more amino acids than wild *S. cerevisiae* especially during the exponential growth stage (Barrajón-Simancas *et al*., 2011). The amino acids and vitamins that are essential for bacterial proliferation are depleted during AF by yeast metabolism to such an extent, that the commencement of bacterial growth is delayed until yeast cells lyse (Nygaard and Prahl, 1997; Alexandre *et al*., 2004; Arnink and Henick-Kling, 2005).

The process of yeast autolysis plays a vital role in the release of essential nutrients for LAB proliferation and survival (Alexandre *et al*., 2004). Yeast autolytic activity can release amino acids, peptides, proteins, glucans and mannoproteins. The release of these macromolecules is yeast strain dependant (Alexandre *et al*., 2001, 2004). The release of mannoproteins can stimulate bacterial growth by adsorbing medium-chain fatty acids and thus detoxifying the wine medium. Mannoproteins can also be enzymatically hydrolysed by bacterial enzyme activity, which will enhance the nutritional content of the wine and in turn stimulate bacterial growth (Guilloux-Benatier and Chassagne, 2003; Alexandre *et al*., 2004; Diez *et al*., 2010).

Yeast metabolism has a direct effect on the nitrogen concentration available for LAB consumption. This was confirmed by Guilloux-Benatier *et al*. (2006), who found that proteolytic activity by yeast can positively affect the nitrogen composition of wine after AF, which will in turn affect the ability of *O. oeni* to grow and complete MLF. A recent study by Bach *et al.* (2011) showed that nitrogen addition during AF increased the fermentation rate but limited bacterial growth. Information on the specific nitrogen compounds that are yeast derived and that are actually of importance to LAB metabolism, besides amino acids, are limited (Alexandre *et al*., 2001). It is therefore necessary to identify the essential nutrients for which both LAB and yeast compete and to quantify these compounds to ensure the viability and growth of LAB (Arnink and Henick-Kling, 2005). One such study evaluated the effect of arginine and citrulline on the growth of two commercial *O. oeni* strains in wine in comparison to *L. buchneri* and concluded that neither arginine nor citrulline increased the growth of either of the *O. oeni* strains in comparison to the *L. buchneri* strain (Terrade and Mira de Orduña, 2009).

2.4.3 The production of extracellular compounds by the yeast

The study of Comitini *et al*. (2005) showed that the inhibitory effect of *S. cerevisiae* can be due to the production of extracellular compounds via the metabolic activity of the yeast. Therefore, the third factor to consider is the ability of the yeast to produce metabolites that can either have a stimulatory or inhibitory/toxic effect on LAB (Lerm *et al*., 2010). There are a number of yeast-derived inhibitory compounds, including ethanol, SO_2, medium chain fatty acids and proteins (Alexandre *et al*., 2004). Osborne and Edwards (2006) found that a peptide produced by *S. cerevisiae* inhibited *O. oeni* and that the degree of inhibition is influenced by the presence of SO_2. This study was performed in synthetic medium and the proposed mechanism was the possible disruption of the bacterial cell membrane. Similarly, Comitini *et al*. (2005) also reported

a LAB inhibitory compound produced by yeast which was proposed to be of a proteinaceous nature due to its sensitivity to heat and protease activity. The study of Osborne and Edwards (2007) also showed that inhibition of *O. oeni* by *S. cerevisiae* was diminished when wine was treated with proteases. In a similar study, Nehme *et al*. (2010) reported the inhibition of an *O. oeni* strain by *S. cerevisiae* that resulted in a decrease in the malic acid consumption by the LAB strain. This inhibition could be attributed, in part, to a peptidic fraction produced by the yeast.

2.4.4 The different inoculation strategies

Different inoculation strategies also play an important role in MLF and yeast and bacteria interactions. There are three possible inoculation strategies for MLF namely inoculation during and after completion of alcoholic fermentation as well as co-inoculation (Henick-Kling and Park, 1994; Rosi *et al*., 2003; Alexandre *et al*., 2004; Knoll *et al*., 2012).

The study of Rosi *et al.* (2003) found that inoculation of bacteria at the end of AF favoured the growth and malolactic activity of LAB. This could be attributed to yeast autolysis resulting in the release of vitamins, amino acids, proteins and polysaccharides which stimulate bacterial metabolism (Henick-Kling, 1993). The advantages of sequential inoculation include the lack of adverse interactions between yeast and bacteria as well as a reduced risk of acetic acid production due to lower residual sugar concentrations (Costello, 2006; Lerm, 2010). There are still risks associated with sequential inoculation and a loss in viability may be possible due to the presence of high ethanol concentrations, low pH, SO_2, other antimicrobial compounds produced by the yeast as well as nutrient depletion (Larsen *et al*., 2003).

Inoculation during AF is not a common practice because of the strong antagonism between the then dominant yeast population and bacteria (Rosi *et al*., 2003). The bacterial population shows drastic decreases in cell numbers for this type of inoculation due to various factors including the removal of nutrients by the yeast, accumulation of SO_2, ethanol production, toxic metabolite production by the yeast and acid production by the yeast resulting in a decreased pH.

Simultaneous or co-inoculation is a strategy where the grape must is inoculated with yeast and a suitable bacterial starter culture. The possible risks of simultaneous inoculation are the undesirable/antagonistic interactions between yeast and/or bacteria, stuck AF and the production of possible off-flavours (Henick-Kling and Park, 1994; Alexandre *et al*., 2004). However, studies in white grape juice have demonstrated the successful completion of MLF using co-inoculation with no adverse increases in acetic acid (Henick-Kling and Park, 1994; Scudamore-Smith *et al*., 1990; Semon *et al*., 2001; Rosi *et al*., 2003; Jussier *et al*., 2006; Zapparoli *et al*., 2006). The study of Nehme *et al*. (2010) compared co-inoculation to sequential inoculation in terms of malic acid degradation. Results indicated a decrease in the rate of malic acid consumption in the sequential inoculation because of the high ethanol concentration of the must after completion of AF.

Little is known about interactions between yeast and bacteria during co-inoculation and how such interactions may impact on the fermenting organisms, both in terms of their fermentative properties and metabolite production. A study done by Rossouw *et al*. (2012) investigated the impact of co-inoculation with *O. oeni* on the transcriptome of *S. cerevisiae*. Gene expression

analysis of the yeast suggests a strong competition between the yeast and bacteria. Further RNA sequencing analyses of both yeast and LAB in co-culture will potentially provide in depth insights the molecular regulation of microbial interactions and help to elucidate the metabolic functioning of these organisms during winemaking.

2.5 The effect of interactions between *Saccharomyces* yeast and LAB on aroma compounds

The process of MLF by LAB influences the aroma and flavour of wines via the production of volatile metabolites and the modification of grape- and yeast derived aroma compounds (Swiegers *et al*., 2005; Boido *et al*., 2009; MichImayr *et al*., 2012). The study of Costello *et al*. (2012) investigated the ability of commercial LAB to synthesise potentially flavour active fatty acid ethyl esters to augment the ethyl ester content of wine. Results indicated that LAB strains can synthesize varying concentrations of ethyl hexanoate from hexanoic acid and ethyl octanoate from octanoic acid. In this study the *O. oeni* strains exhibited greater activity than *L. plantarum* in the production of ethyl hexanoate from hexanoic acid. It has also been shown that wines that have undergone simultaneous AF/MLF tend to be less buttery, retain more fruitiness and are therefore more complex and better structured with marginally higher but sensorial insignificant levels of acetic acid (Henick-Kling, 1993; Bartowsky *et al*., 2002b; Jussier *et al*., 2006; Krieger, 2006). The studies of Semon *et al.* (2001) and Jussier *et al*. (2006) compared co-inoculation with sequential inoculation in Chardonnay wines. Jussier *et al.* (2006) found no negative impact of simultaneous AF/MLF on the fermentation success or final wine parameters while the study of Semon *et al*. (2001) showed that malic acid degradation was faster in co-inoculation than the sequential inoculation strategy and produced wines that were not significantly different from a sensory perspective. Although slightly higher levels of acetic acid were produced in the co-inoculation treatments in both studies, the differences were not statistically relevant and within the range of concentrations normally found in wine.

A study done by Abrahamse and Bartowsky (2012) showed that co-inoculation in Shiraz grape must resulted in shorter total vinification time and produced sound wines. Co-inoculation in synthetic wine resulted in higher ethyl lactate and octanoic acid concentrations in comparison to pure cultures and also reduced undesirable aroma compounds (Rossouw *et al*., 2012). No negative impact of simultaneous alcoholic and malolactic fermentation on fermentation success and on the final wine volatile aroma composition was observed. Compared to sequential inoculation, wines that were co-inoculated tended to have higher concentrations of ethyl and acetate esters, including acetic acid, 2-phenyl ethyl acetate ester, isoamyl acetate, ethyl lactate and diethyl succinate (Knoll *et al*., 2011).

A study was carried out to determine the effect of the inoculation time of the LAB on the kinetics of vinification and on chemical and sensory characteristics of Tempranillo and Merlot wines (Cañas *et al*., 2012). Important differences in volatile compound contents were observed, although there was little impact on the sensorial profile of wines. The co-inoculated treatments contained higher concentrations of ethyl acetate, ethyl butyrate, ethyl lactate and diethyl succinate compared to the sequential treatments. In contrast to this, a study in Pinotage and Shiraz by Malherbe *et al*. (2012) observed a greater increase in ethyl esters than acetate esters in sequentially inoculated MLF. The study of Lee *et al*. (2009) compared sequential inoculated

strains of *L. plantarum* and *O. oeni* in wine. The study showed that *L. plantarum* produced higher levels of propyl acetate isobutanol, isoamel acetate, 1-butanol, ethyl hexanoate, ethyl octanoate and lower levels of lactic acid than *O. oeni*.

It is evident that co-inoculation of certain LAB strains have a positive influence on the aroma and flavour of wine when compared to sequential inoculations.

2.7 Conclusions

The interactions between *Saccharomyces* and non-*Saccharomyces* wine yeast are complex and have been studied for a number of years. These relationships commence in the vineyards and continue throughout the fermentation and storage process. A variety of studies investigated the biochemical, physiological and molecular bases of yeast interactions under winemaking conditions. One of the most promising interactions is the cell-to-cell contact between *Saccharomyces* and non-*Saccharomyces* yeast. Little is known about this interaction but future research could shed light on the interaction mechanisms between yeast populations in order to optimise the aromatic impact of non-*Saccharomyces* yeast while ensuring complete alcoholic fermentation. A limitation of this study is that it is only conducted in synthetic media. Literature has shown that the interactions between *Saccharomyces* and non-*Saccharomyces* yeast have an influence on the chemical, sensory properties and final flavour of wines, resulting in more complex wines. These effects are highly strain dependent and are influenced by the specific interactions between other inoculated yeasts and also the chemical composition of the must used. More combinations of *Saccharomyces* and non-*Saccharomyces* yeasts can be assessed to investigate the impact on final flavour of wine.

The interactions between yeast and LAB have been investigated by screening particular yeast and bacterial strain combinations. Some of these studies investigated the production of extracellular compounds by the yeast and the different MLF inoculation strategies. Co-inoculation of yeast and bacteria was one of the inoculation strategies which provided the most interesting results. This strategy resulted in shorter vinification time, and more aromatic and complex wines. These studies were mostly done on *O. oeni* and more research can be done on *L. plantarum* and the combination of these strains. At this moment in time little is known about the interaction mechanisms during co-inoculation and the impact on fermenting organisms both in terms of fermentative properties and metabolite production.

Discovering and understanding the complex world of microbial interactions between different microbial populations are important because this has an influence on the quality and style of wine.

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Chapter 3

Research results

Interaction between wine yeast and malolactic bacteria and the impact on wine aroma and flavour

3. Research results

Interaction between wine yeast and malolactic bacteria and the impact on wine aroma and flavour

The aim of this study was to evaluate the metabolic impact of three different lactic acid bacteria (LAB) treatments (*L. plantarum, O. oeni*, and a combination of *O. oeni* and *L. plantarum*) in combination with two different *S. cerevisiae* strains (Cross Evolution® and EC1118®) on wine aroma and flavour. The study was performed in three different synthetic media (with different amino acid compositions) in both co-inoculation and sequential inoculation strategies. Overall co-inoculation strategies resulted in a greater impact on the final aroma compound composition. For the standard fermentations a general increase in total esters, especially ethyl lactate and ethyl acetate, was observed. The production of volatile fatty acids and higher alcohols proved to be dependent on the yeast and LAB strains used. For the nutrient addition fermentations, the amino acids supplemented fermentations resulted in higher concentrations of esters, higher alcohols and fatty acids but the concentrations were yeast-strain and inoculation strategy dependent. The results suggest that the chemical composition of the fermentation medium, and the selection of yeast and LAB strains can exert a significant influence on the aroma and flavour profile of the final wine.

3.1 Introduction

Wine is a complex end-product of alcoholic and malolactic fermentations. The chemical profile of wine is derived from the grape berry, the fermentation microflora (in particular the yeast *Saccharomyces cerevisiae*), secondary microbial fermentations that may occur such as malolactic fermentation (MLF) and the ageing and storage conditions of the wine (Styger *et al*., 2011). Yeast plays an important role in wine aroma where it can transform grape-derived metabolites via primary and secondary metabolic pathways to influence and generate various wine aroma compounds. According to Fleet (2003), yeast influences wine aroma by the following mechanisms: (1) apiculate yeast species protect the grapes against moulds by competing for nutrients, (2) the alcoholic fermentation of the grape sugars into alcohol, (3) the *de novo* biosynthesis of flavour and aroma compounds during alcoholic fermentation, (4) the metabolism of flavour-neutral grape compounds into active aroma and flavour compounds, (5) post-fermentation impact on wine via autolysis, and (6) influencing the growth of malolactic and spoilage bacteria.

The *de novo* biosynthesis of flavour and aroma compounds is probably the most important since fermentation-derived volatiles quantitatively make up the largest percentage of total aroma compounds in wine. Another important fermentation that has an effect on the organoleptic properties of wine is MLF. This secondary fermentation is known to remove vegetative and herbaceous aromas, increase the fruity and floral attributes, improve the mouthfeel and extend the aftertaste of wine (Swiegers *et al*., 2005). Three mechanisms by which lactic acid bacteria (LAB) are able to modify wine aroma and flavour are proposed by Bartowsky and Henschke (1995): Firstly, the bacteria are able to produce volatile compounds by metabolising grape constituents e.g. sugars and nitrogen-containing compounds such as amino acids. Secondly, the bacteria can modify existing grape or yeast derived secondary metabolites and, lastly, flavour-active compounds can be adsorbed by the bacterial cell wall.

While spontaneous, or uninoculated MLF is still a widespread practice in industry, many wineries are moving towards controlled inoculation of bacterial starter cultures to ensure successful and predictable MLF (Davis *et al*., 1985; Henick-Kling, 1995; Krieger-Weber, 2009). There are three inoculation strategies for MLF: co-inoculation at the start of fermentation, inoculation during alcoholic fermentation, and sequential inoculation after alcoholic fermentation (Lerm *et al*., 2010). *Oenococcus oeni* is the most preferred species selected by winemakers to conduct MLF. Strains of this species can grow in low pH musts (below 3.5) and in the presence of high levels of ethanol above 14%v/v (Muñoz *et al*., 2011). Recently, the use of *Lactobacillus plantarum* to conduct MLF showed promising outcomes (Du Toit *et al*., 2011). This species can survive high ethanol levels of up to 13%v/v, SO_2 concentration of 50 ppm and a pH ≥ 3.5. Both *O. oeni* and *L. plantarum* possess many enzymes important for desirable aroma production such as glycosidase, protease esterase, phenolic acid decarboxylase and citrate lyase (Lerm *et al*., 2010; Du Toit *et al*., 2011). A study done by Mtshali *et al*. (2010) showed that *L. plantarum* presented a more diverse enzyme profile compared to *O. oeni*, particularly for the aroma-modifying enzymes β-glucosidase and phenolic acid decarboxylase.

Diacetyl (2,3-butanedione) is considered to be one of the most important aroma compounds produced by LAB during MLF (Bartowsky and Henschke, 1995; Lonvaud-Funel, 1999). This compound is responsible for the buttery, butterscotch and nutty descriptors usually associated with wines that have undergone MLF (Bartowsky and Henschke, 1995; 2004; Martineau *et al*., 1995; Bartowsky *et al*., 2002). Diacetyl is formed as an end-product of the citric acid metabolic pathway of LAB (Bartowsky *et al*., 2002; Bartowsky and Henschke, 2004). The production of diacetyl by LAB isolates could be beneficial in adding complexity to the sensorial profiles of wines during MLF, provided that the level of diacetyl production does not exceed 5-7 mg/L.

Esters are important compounds which contribute to wine aroma and impart fruity aromas (i.e. diethyl succinate) and can also contribute to the mouthfeel and the palate of wine due to the presence of esters such as ethyl lactate (Maicas *et al*., 1999; Herjavec *et al*., 2001; Ugliano and Moio, 2005). Esterase enzymes originating from wine LAB are responsible for both the biosynthesis and hydrolysis of esters (Matthews *et al*., 2004). *Oenococcus oeni* as well as certain species of *Lactobacillus* and *Pediococcus* are able to hydrolyse esters (Davis *et al*., 1988). Some studies found that significant esterase activity levels remained under wine-like conditions (Matthews *et al*. 2006; Pérez-Martín *et al.,* 2013). Other studies showed that *Lactobacillus* spp. possesses genes coding for wine-related enzymes that could hydrolyse wine precursors to positively influence wine aroma (Mtshali *et al*., 2010, 2013). This implies that esterases originating from LAB could potentially contribute to the wine aroma profile.

LAB are also able to metabolise phenolic acids in wine, specifically *p*-coumaric acid and ferulic acid via hydroxycinnamic- or phenolic acid decarboxylases, to their vinyl derivatives. These vinyl derivatives can be reduced to produce the volatile phenols 4-ethylphenol and 4-ethylguaiacol.These compounds impart negative sensorial qualities to wine, including animal and medicinal aromas, horse sweat, horse stable, barnyard and elastoplast aromas (Cavin *et al*., 1993; Lonvaud-Funel, 1999; Swiegers *et al*., 2005). Even if LAB strains possess the potential to produce volatile phenols, it is not clear if they are able to produce significant levels of these compounds above their sensory thresholds (Gámbara *et al*., 2001; Swiegers *et al*., 2005).

Another factor which is known to impact heavily on aroma production by yeast is the nature of the nitrogen sources and amino acid composition of the grape must (Fairbairn, 2012; Smit, 2013). While numerous studies have been performed on the link between nitrogen and amino acid additions on aroma production by yeast (Bell and Henschke, 2005; Hernández-Orte *et al*., 2006), less is known regarding the impact that such treatments have on aroma production, or modification, by LAB. Nitrogen and nutrient additions are standard practice in winemaking since nitrogen is often one of the factors limiting sugar consumption and yeast growth (Ugliano *et al*., 2007). Bely *et al*. (1990) showed a reduction in fermentation time if nitrogen additions were constant and corrected before the halfway point in fermentation. The ideal nitrogen concentration for must is 200 mgN/L for successful completion of alcoholic fermentation while concentrations below 150 mgN/L can lead to stuck fermentations and concentrations above 400mgN/L can lead to wine spoilage (Ugliano *et al*., 2007). Several studies have been performed on the effect of nitrogen additions on the final aroma composition of wine (Ough *et al*., 1988; Garde-Cerdán and Ancín-Azpilicueta, 2008; Bach *et al*., 2011). The adjustment of ammonium or the amino acids content of a must has an impact on the volatile composition of the resulting wine (Hernandez-Orte *et al*., 2002, 2005).

LAB require vitamins, sugars, peptides, organic acids (pyruvate, malate, citrate), fatty acids, nucleic acids, minerals and trace elements (Na, K, Mn, Mg) for optimal growth and metabolism. Amino acids are important nutrients for LAB metabolism and survival (Nehme *et al*., 2008). Terrade and De Orduña, (2009) showed that strains of *O. oeni* and *L. plantarum* needed a carbon source and phosphate source, manganese, amino acids (proline, arginine and the branched amino acids valine, leucine and isoleucine) and vitamins such as nicotinic acid and pantothenic acid for optimal growth.

Little is known regarding the interactions between yeast and bacteria (and different LAB strains in mixed cultures) and the effect that these have on aroma and flavour production. Moreover, the impact which nitrogen and amino acid treatments have on these interactions and the resulting aroma profiles is also a largely unexplored field of research. Previous studies have shown that *S. cerevisiae* has an impact on MLF and the interaction can be detrimental to the bacteria through the production of ethanol, SO_2, medium-chain fatty acids, antibacterial proteins or beneficial through yeast autolysis and absorption of medium-chain fatty acids by mannoproteins (Alexandre *et al*., 2004, Arnink and Henick-Kling, 2005; Comitini *et al*., 2005; Nehme *et al*., 2008; 2010; Osborne and Edwards, 2006). Previous projects that used different *O. oeni* strains with the same yeast resulted in wines with different aromas (López *et al*., 2011; Malherbe *et al*. 2012; Antalick *et al*., 2013). Another study showed that co-inoculation of Shiraz must influenced the chemical composition differently compared to sequential inoculation (Abrahamse and Bartowsky, 2012). This might be due to differences in the genetic potential with regards to enzyme encoding genes of the strains resulting in different metabolic pathways being active (Mtshali *et al*. 2010). Therefore it is important to generate data on the interactions between the yeast and the LAB being used to determine the impact on the chemical composition of the final product. Furthermore, additional complexity arises as nutrient and nitrogen additions also need to be taken into consideration in terms of the impact this has on the yeast and LAB in the different inoculation scenarios.

This study used two different commercial wine yeast strains (Cross Evolution® and Lalvin EC1118®) in conjunction with *L. plantarum*, *O. oeni*, and a 50:50 combination of these two bacterial strains in both co- and sequential MLF inoculation strategies. Different nutrient addition

strategies were also included in the experimental design to assess the impact of differences in must composition on the aroma and flavour impacts of the aforementioned treatments. This approach will give insight into the interactions between selected yeast and bacteria and the impact of these combinations on flavour and aroma compounds produced in a synthetic medium. These findings can provide winemakers with appropriate knowledge to select the most appropriate yeast/bacterial combination for a desired fermentation outcome.

3.2 Material and methods

3.2.1 Bacterial isolates, media and culture conditions

This study consisted of three sets of fermentations using the synthetic medium developed by the Australian Wine Research Institute (AWRI) 1) using the medium with all nitrogen compounds added 2) using the medium supplemented with NH_4Cl as the sole nitrogen source and 3) using the medium supplemented with a complex mixture of amino acids. The synthetic medium is adapted from Henschke and Jiranek (1993). The glucose and fructose concentrations were increased from 100 g/L to 115 g/L each. The amino acids as described by Bely *et al*. (1990) were used in the synthetic medium. The composition of the synthetic medium is shown in **Table 3.1.**

TABLE 3.1

Synthetic Medium based on Henschke and Jiranek (1993) and Bely et al. (1990).

Composition		per Liter
Carbon Sources	Glucose	115 g
	Fructose	115 g
Acids	KH Tartrate	2.5 g
	L-Malic acid	3 g
	Citric acid	0.2 g
Salts	K_2HPO4	1.14 g
	$MgSO_4.7H_20$	1.23 g
	$CaCl_2.2H_2O$	0.44 g
Trace Elements	$MnCl_2.4H_2O$	200 µg
	$ZnCl_2$	135 µg
	$FeCl_2$	30 µg
	$CuCl_2$	15 µg
	H_3BO_3	5 µg
	$Co(NO3)_2.6H_2O$	30 µg
	$NaMoO_4.2H_2O$	25 µg
	KIO_3	10 µg
Vitamins	Myo-Inositol	100 mg
	Pyridoxine.HCl	2 mg
	Nicotinic acid	2 mg
	Ca Pantothenate	1 mg
	Thiamin.HCl	0.5 mg
	PABA.K	0.2 mg
	Riboflavin	0.2 mg
	Biotin	0.125 mg
	Folic Acid	0.2 mg
Lipids	Ergosterol	10 mg
	Tween 80	0.5 mL
Nitrogen source		
	NH_4Cl	0.46 g
Amino acids*	tyrosine	1.4 g
	tryptophane	13.7 g
	isoleucine	2.5 g
	aspartic acid	3.4 g
	glutamic acid	9.2 g
	arginine	28.6 g
	leucine	3.7 g
	threonine	5.8 g
	glycine	1.4 g
	glutamine	38.6 g
	alanine	11.1 g
	valine	3.4 g
	methionine	2.4 g
	phenylalanine	2.9 g
	serine	6 g
	histidine	2.5 g
	lysine	1.3 g
	cystein	1 g
	proline	46.8 g
pH adjust to 3.5 with KOH		

*Amino acids suggested by Bely *et al*., 1990

The yeast strains used in this study **(Table 3.2)** were cultivated on Yeast Peptone Dextrose (YPD) (Biolab, Merck) plates for three days. A single colony of each yeast was inoculated in YPD broth for 12 hours at 30°C and placed under aerobic conditions on an orbital shaker prior to inoculation in synthetic medium. The two strains were maintained as culture stocks in 20% (v/v) glycerol (Saarchem, Merck) at -80°C.

TABLE 3.2

Yeast and bacterial strains used in this study.

Strain	Species	Source
Cross Evolution®	*S. cerevisiae*	Lallemand
EC1118®	*S. cerevisiae*	Lallemand
S5	*O. oeni*	IWBT*
56	*L. plantarum*	IWBT

*IWBT:Institute for Wine Biotechnology, Stellenbosch University, South Africa

The *L. plantarum* strain **(Table 3.2)** was cultivated on De Man, Rogosa and Sharpe (MRS) plates containing 50 g/L MRS broth (Biolab, Merck) and 15 g/L Bacteriological agar (Biolab, Merck). The *O. oeni* strains were cultivated on MRST plates containing 50 g/L MRS and 20 g/L bacteriological agar supplemented with 10% preservative free tomato juice (All Gold, South Africa) with the pH adjusted to 5.0 with hydrochloric acid (HCl). All LAB were anaerobically cultivated using Microbiology Anaerocult sheets in anaerobic jars (Merck, Darmstadt, Germany). Agar plates for the enumeration of *L. plantarum* and *O. oeni* strains were incubated at 30°C for 4 and 7 days, respectively. For inoculation into the synthetic wine medium *L. plantarum* strains were grown at 30°C for 2 days in MRS broth and the *O. oeni* in MRS broth containing 20% preservative free apple juice (Ceres, South Africa) . The pH was adjusted to 5.2 with HCl in both the MRS and MRSA broth. All strains were maintained as culture stocks in 40% (v/v) glycerol (Saarchem, Merck) at -80°C.

3.2.2 Fermentations

Fermentations were conducted in 250 mL Erlenmeyer flasks fitted with rubber stoppers and a CO_2 gas outlet, containing 200 mL of the medium. The fermentations were conducted at 25°C. Precultures of the yeast were grown for 12 hours in YPD broth at 30°C. Cells were harvested by centrifugation at 5000 rpm and resuspended in distilled water. Yeasts were inoculated at approximately 5×10^6 cfu/mL in the synthetic medium. All fermentations were conducted in triplicate. Control fermentations were carried out for each of the yeast strains without any inoculated bacteria. The experimental design in terms of bacterial and inoculation treatments are shown in **Figure 3.1**.

The precultures of *O. oeni*, *L. plantarum* and the combination in a ratio of 50:50 were harvested by centrifugation at 2000 rpm and resuspended in sterile water. *O. oeni*, *L. plantarum* and the combination in a ratio of 50:50 were inoculated at approximately 1×10^7 cfu/mL at the start of fermentation for the co-inoculated fermentations and also at 1×10^7 cfu/mL after the completion of AF for the sequential inoculation fermentations. Control fermentations were also carried out (in triplicate) with only the different bacterial treatments inoculated into the synthetic medium.

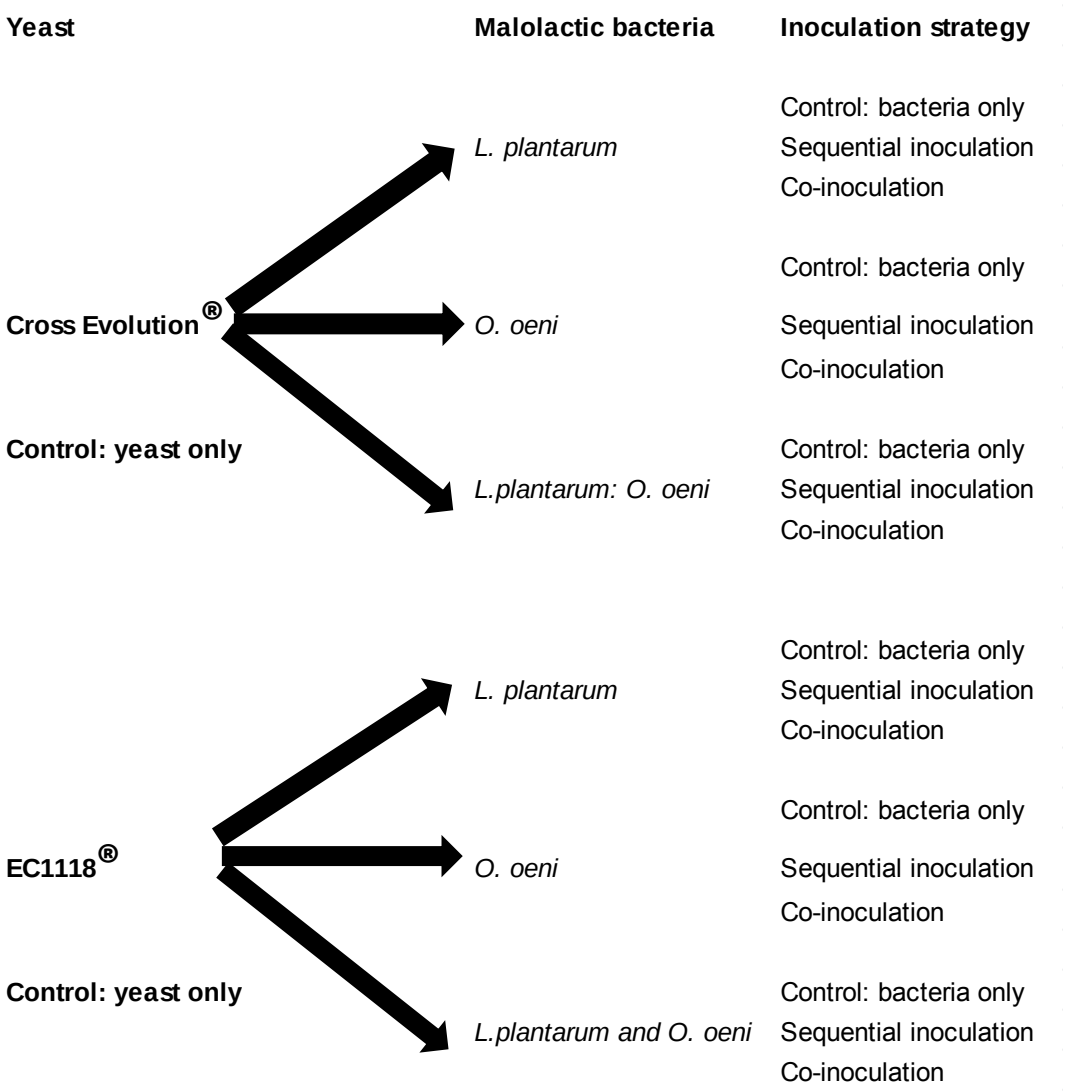

FIGURE 3.1

A summary of all the different fermentations using the yeast starter cultures, inoculation strategies and bacteria. All fermentations were evaluated in triplicate.

3.2.3 Nutrient addition fermentations

The synthetic medium described in **Table 3.1** was altered to evaluate the impact of nitrogen sources. The first set used NH_4Cl as sole nitrogen adjusted to 300 mg N/L and the second set used aromatic (tryptophan and phenylalanine) and branched (leucine, isoleucine and valine) amino acids adjusted to 300 mg N/L. The same inoculation strategies were followed as described for fermentations using the full complement of amino acids.

3.2.4 Microbial analysis

Homogenous 1 mL samples of the synthetic medium were drawn for microbial enumeration during alcoholic fermentation for the first four days and after that on a weekly basis until alcoholic fermentation was complete. For co- and sequential inoculations, samples of the synthetic medium were taken daily from the different fermentations until MLF was completed. The synthetic medium's microbiological status was monitored by plate counts of colonies formed from serial dilutions of the medium (colony forming units per millilitre, cfu/mL) on various solid media. For yeast enumeration YPD plates contained 30 mg/L Chloramphenicol (Roche Diagnostics GmbH, Mannheim, Germany) (dissolved in 1 mL of 96% ethanol) to suppress the growth of LAB. Plates were incubated aerobically at 30°C for two days before counting to determine the cfu/mL. For bacterial enumeration samples from serial dilutions were plated out on MRS or MRST plates that contained 50 mg/L Delvocid Instant (DSM Food Specialties, The Netherlands) to prevent the growth of yeasts. Plates were incubated at 30°C for four to seven days and the cfu/mL determined.

3.2.5 Standard chemical analyses

An Arena 20XT (Thermo Electron Oy, Finland) automated enzymatic kit robot was used to quantify key cellular metabolites: D-glucose, D-fructose, acetic acid, glycerol, L-malic acid, L-lactic acid, citric acid and ammonia (Thermo Fisher Scientific).

Ethanol was analysed by high performance liquid chromatography (HPLC) on an AMINEX HPX-87H ion exchange column using 5 mM H_2SO_4 as the mobile phase using the method described by Rossouw *et al*. (2012). Agilent RID and UV detectors were used in tandem for peak detection and quantification. HPChemstation software package was used to analyse the results.

3.2.5.1 Determination of volatile aroma compounds

The determination of the concentration of volatile aroma compounds was done in the finished wines. The major volatile aroma compounds were determined using gas chromatography with flame ionisation detection (GC-FID).

The extractions of major volatiles were done as previously described by Louw *et al*. (2009) and had the following exception: centrifugation of the wine/ether mixture at 4000 g for only three minutes, after which Na_2SO_4 was added to the mixture and the centrifugation step, was repeated. A Hewlett Packard 6890 Plus Gas Chromatograph (Agilent, Little Falls, Wilmington, USA) was used to analyse the major volatile aroma compounds **(Table 3.3)**. The GC was equipped with a split/splitless injector, set to a split flow rate of 98.7 mL/min, split ratio of 15:1 and a temperature of 200°C. The J & W DB-FFAP capillary GC column (Agilent, Little Falls, Wilmington, USA) was used for the separation of the compounds with dimensions of 60 m in length x 0.32 mm internal diameter with a 0.5 µm coating film thickness. as The carrier gas, hydrogen was used, at a flow rate of 6.6 mL/min. The injection volume was set to 3 µL of the extracted sample. The oven temperature program was as follows: 33°C, held for 8 minutes, increased by 21°C/min to 130°C, held for 1.3 min, then increased by 21°C/min to 170°C. An oven temperature of 170°C was held for 1 min and finally increased by 21°C/min to 240°C and held for 2.5 min. The FID was operated at 250°C with a hydrogen flow of 30 mL/min, oxygen at 350 mL/min and make-up gas flow of nitrogen at 30 mL/min. The internal standard method and

authentic standards (Merck, Cape Town) were used for the calibration of each compounds. HP Chemstation software was used for manual data collection and peak integration was performed using the (Rev. B01.03 [204])

TABLE 3.3
The 28 volatile aroma compounds quantified by GC-FID in this study

Higher alcohols	Fatty acids	Esters
Methanol	Acetic acid	Ethyl acetate
Propanol	Propionic acid	Ethyl butyrate
Isobutanol	Iso-butyric acid	Isoamyl acetate
Butanol	Butyric acid	Ethyl hexanoate
Isoamyl alcohol	Iso-valeric acid	Hexyl acetate
Hexanol	Valeric acid	Ethyl lactate
2-Phenylethanol	Hexanoic acid	Ethyl caprylate
	Octanoic acid	Ethyl-3-hydroxybutanoate
	Decanoic acid	Ethyl caprate
		Diethyl succinate
		Ethyl penylacetate
		2-Phenylacetate

3.2.5.2 Data analyses

The student's t- test was used to determine statistically significant differences between values. Principal component analysis (PCA, The Unscrambler version X10.2, Camo ASA, Norway) was used for multivariate statistical analysis.

Cytoscape software (version 2.8.2, http://www.cytoscape.org) was used to determine significant differences between treatments and compounds and to visualise the data, presented from a compound-centric viewpoint. The figures (referred to as bubble graphs) contain blue nodes (ellipses) that represents a significant lower level of the compound when treatments are compared and red nodes representing a significantly higher level. An increase in the colour intensity indicates the magnitude of the fold change observed. A significance level of 5% ($p<0.05$) was used. The figures only show significant differences; non-significant data are omitted from the figures.

3.3 Results

3.3.1 Standard fermentations

Fermentations were carried out in a synthetic medium (**Table 3.1**) to determine the impact of different combinations of yeast and bacteria and different inoculation strategies, co-inoculation and sequential, on the growth rate, the fermentation kinetics and aroma compounds produced.

Reducing sugars

The Cross Evolution control fermented glucose to less than 5 g/L within 6 days (**Addendum Table 1 A1**). The Cross Evolution® and EC1118® co-inoculated with *L. plantarum* fermented glucose to less than 5 g/L within 9 days while the EC1118® control fermentation took 11 days (**Figure 3.2**). Cross Evolution® co-inoculated with *O. oeni* consumed glucose in 12 days while EC1118® in the co-inoculated strategy consumed all glucose in 19 days (**Addendum Table 2 B1**). Cross Evolution® and EC1118® co-inoculated with the bacterial combination utilized glucose to less than 5 g/L within 12 days (**Addendum Table 3 C1**). The Cross Evolution® control fermentation utilized fructose to less than 5 g/L within 11 days (**Figure 3.3**). Cross Evolution® co-inoculated with *L. plantarum* fermented fructose to less than 5 g/L in 18 days and EC1118® in the co-inoculated strategy in 11 days (**Figure 3.3**). The EC1118® control fermented fructose to less than 5 g/L in 18 days (**Addendum Table 1A2**). The Cross Evolution® and EC1118® co-inoculated with *O. oeni* fermented fructose to less than 5 g/L within 19 days (**Addendum Table 2B2**). Fructose consumption for the Cross Evolution® and EC1118® co-inoculated with the bacterial combination was less than 5 g/L within 12 days (**Addendum Table 3C2**).

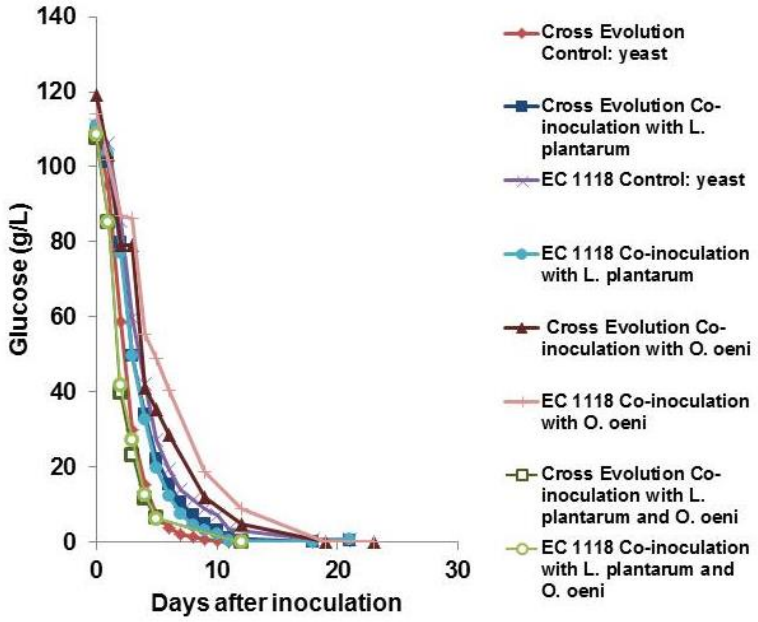

FIGURE 3.2

Glucose concentration during standard fermentation with different yeasts in combination with *L. plantarum*, *O. oeni* and the combination of *L. plantarum* and *O. oeni*. Values are the average of 3 biological repeats.

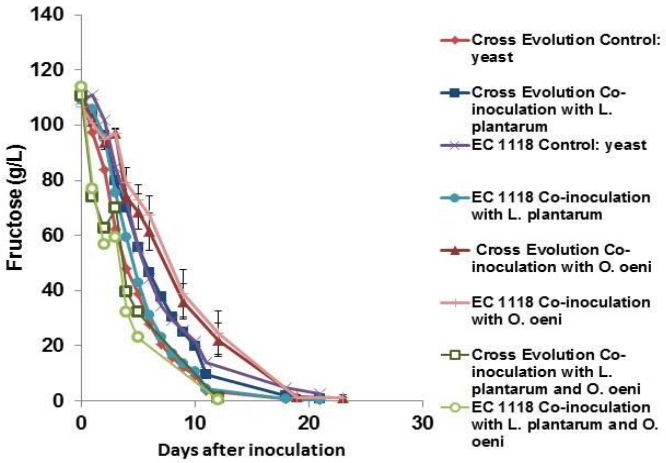

FIGURE 3.3

Fructose concentration during standard fermentation with different yeasts in combination with *L. plantarum*, *O. oeni* and the combination of *L. plantarum* and *O. oeni*. Values are the average of 3 biological repeats.

Ethanol

The ethanol concentrations at the end of the fermentations inoculated with *L. plantarum* were between 11% and 12.5% for all the treatments except the bacterial control where no yeast was added. (**Table 3.4**). At the end of the fermentations that were inoculated with *O. oeni* the ethanol concentrations for the different treatments were between 10.4% and 12.5% except for the bacterial control where no yeast was added (the concentration was 0.17%). The alcohol concentrations at the end of the fermentations that were inoculated with the combination of *L. plantarum* and *O. oeni*, for the different inoculation strategies were between 11% and 12%, except for the bacteria-only controls.

TABLE 3.4

Ethanol concentration (% v/v) of different inoculation strategies at the end of standard fermentations. Values are the average of 3 biological repeats ± standard deviation.

	Cross Evolution			EC 1118			Control
	Control: yeast-only	Sequential inoculation	Co-inoculation	Control: yeast-only	Sequential inoculation	Co-inoculation	Malolactic bacteria
L. plantarum treatments	11.93 ± 1.07	12.41 ± 1.10	11.85 ± 0.57	10.90 ± 1.99	12.18 ± 0.42	12.05 ± 0.12	0.24 ± 0.14
O. oeni treatments	11.12 ± 0.26	12.53 ± 0.29	12.15 ± 0.49	11.72 ± 0.47	10.62 ± 1.24	10.38 ± 0.88	0.17 ± 0.01
L. plantarum : *O. oeni* treatments	11.19 ± 0.71	12.06 ± 0.07	11.40 ± 0.59	11.32 ± 0.43	12.28 ± 0.19	11.32 ± 0.26	0.21 ± 0.01

Bacterial populations

The *L. plantarum* strain in co-inoculation with both Cross Evolution® and EC1118® increased from 1×10^7 cfu/mL to 1×10^8 cfu/mL before finally decreasing to between 1×10^2 cfu/mL and 1×10^3 cfu/mL after 7 days (**Figure 3.4A**). The *L. plantarum* control decreased from 1×10^7 cfu/mL to 1×10^6 cfu/mL after three days where malic acid was completely degraded

(**Addendum Table 5 A1**). *O. oeni* cell numbers in the controls increased from 1×10^7 cfu/mL to 1×10^9 cfu/mL and decreased to 1×10^6 cfu/mL after 5 days (**Figure 3.4C**). For the co-inoculatated strategies bacterial populations of *O. oeni* increased to 1×10^8 cfu/mL by day 3 and decreased to 1×10^5 cfu/mL by day 5 (**Addendum Table 5 B1**). All the bacterial combination co-inoculated strategies started at 1×10^8 cfu/mL (**Figure 3.4E**). The *L. plantarum* of the Cross Evolution® co-inoculated strategy increased to 1×10^9 cfu/mL and decreased to 1×10^7 cfu/mL at day 7(**Addendum Table 5 C1**). The *O. oeni* of the Cross Evolution® co-inoculated strategy decreased to 1×10^7 cfu/mL and increased to 1×10^8 cfu/mL at day 7(**Addendum Table 5 C1**). The *L. plantarum* and *O. oeni* populations of the EC1118® co-inoculated strategy showed similar trends to the Cross Evolution® co-inoculation strategy (**Addendum Table 5 C1**). For Cross Evolution® and EC1118® sequentially inoculated with *L. plantarum* cell numbers decreased from 1×10^7 cfu/mL to 1×10^5 cfu/mL after 19 days (**Figure 3.4B**). For the corresponding fermentations inoculated with *O. oeni* bacterial cell numbers remained constant at 1×10^7 cfu/mL to day 4 of fermentation (**Figure 3.4D**). All the sequential strategies inoculated with the bacterial combinations started at concentrations of 1×10^8 cfu/mL (**Figure 3.4F**). The *L. plantarum* population in co-inoculation with of the Cross Evolution® decreased to 1×10^7 cfu/mL after four days while *O. oeni* increased to 1×10^9 cfu/mL and decreased to 1×10^7 cfu/mL after four days. Similar trends were observed for the EC1118® sequential strategies.

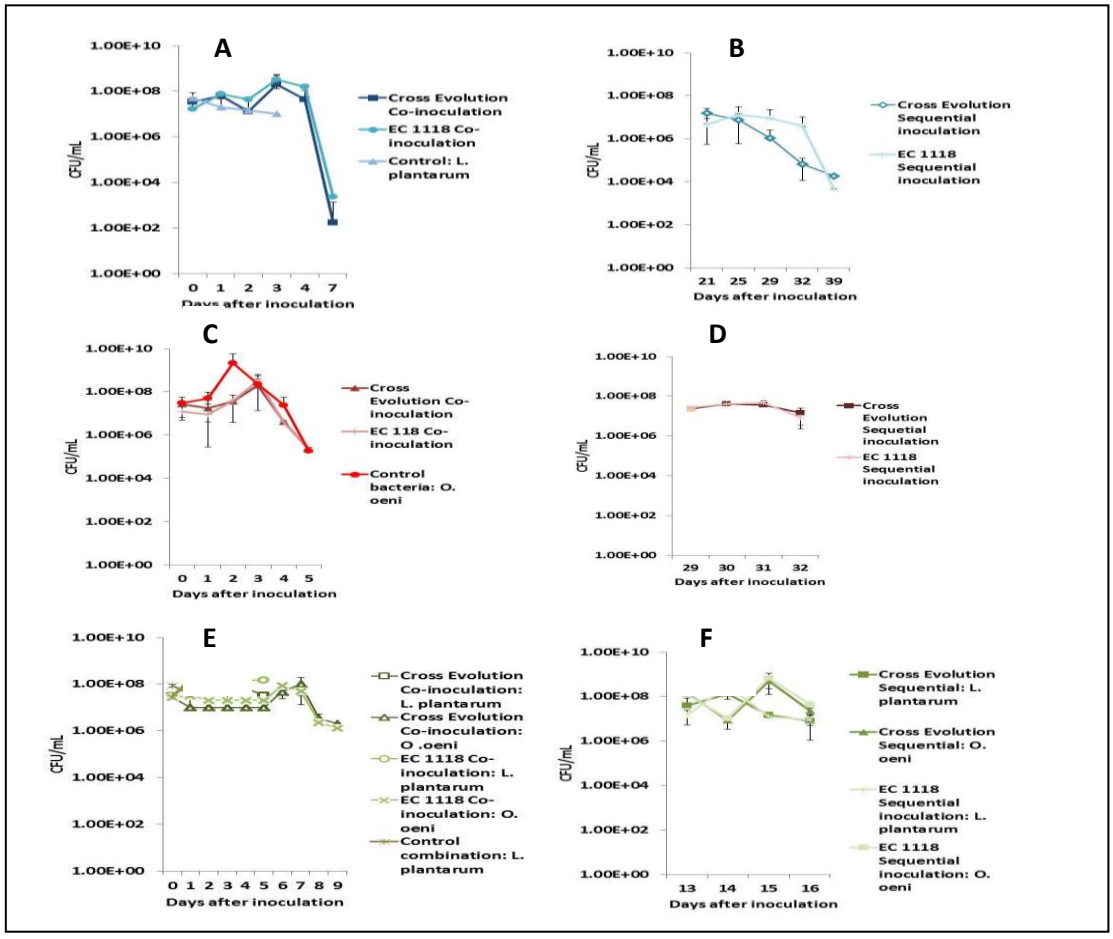

FIGURE 3.4

Bacterial populations of *L. plantarum* during co-inoculation **(A)**, the bacterial control and sequential inoculation **(B)**. Co-inoculation of *O. oeni* **(C)**, the bacterial control and sequential inoculation **(D)**. Co-inoculation of the combination of *L. plantarum* and *O. oeni* (**E**), the bacterial control and sequential inoculation **(F)**. Values are the average of 3 biological repeats ± standard deviation.

Malic acid degradation

Malic acid degradation for the fermentations co-inoculated with *L. plantarum* and *O. oeni* were completed for both Cross Evolution® and EC1118® within three days while malic acid degradation in the sequential inoculations took 12 days to complete (**Table 3.5**). The *L. plantarum* control finished within three days (the final concentration of malic acid was 0.19 g/L). Malic acid degradation for the co-inoculated strategies inoculated with *O. oeni* was completed for both yeasts within three days. See **Addendum Table 6** for the complete set of malic acid concentrations at the different time points.

TABLE 3.5

Malic and lactic acid concentration at the end standard MLF for co- and sequential inoculation of *L. plantarum*, *O. oeni* and the combination of *L. plantarum* and *O. oeni* in conjunction with Cross Evolution and EC1118. Values are the average of 3 biological repeats ± standard deviation.

Inoculation strategies	Day of MLF completion	Malic acid (g/L)	Lactic acid (g/L)
Cross Evolution			
Co-inoculation			
L. plantarum	3	0.17 ± 0.06	2.43 ± 0.07
O. oeni	3	0.07 ± 0.33	2.72 ± 0.34
L. plantarum and *O. oeni*	2	0.05 ± 0.001	2.15 ± 0.01
Sequential inoculation			
L. plantarum	12	0.03 ± 0.32	2.26 ± 0.29
O. oeni	3	0.03 ± 0	2.12 ± 0.05
L. plantarum and *O. oeni*	2	0.02 ± 0.001	2.31 ± 0.01
EC 1118			
Co-inoculation			
L. plantarum	3	0.16 ± 0.008	2.29 ± 0.12
O. oeni	3	0.06 ± 0.35	2.69 ± 0.02
L. plantarum and *O. oeni*	2	0.05 ± 0.01	2.25 ± 0.02
Sequential inoculation			
L. plantarum	12	0.03 ± 0.33	1.98 ± 0.82
O. oeni	3	0.03 ± 0.001	1.98 ± 0.02
L. plantarum and *O. oeni*	2	0.02 ± 0	2.32 ± 0.04
Control: bacteria			
L. plantarum	3	0.19 ± 3.4E-17	2.28 ± 0.08
O. oeni	3	0.18 ± 0.05	2.61 ± 0.01
L. plantarum and *O. oeni*	2	0.02 ± 0.30	1.98 ± 0.05

Citric acid

Citric acid degradation is completed when the concentrations are below 0.1 g/L. The concentrations of all the co- inoculation strategies were below 0.05 g/L at the middle of AF while the sequential inoculations took four days.

Acetic acid

For fermentations that were inoculated with *L. plantarum*, the acetic acid concentration was the highest at the end of AF for the Cross Evolution® co-inoculation (1205 mg/L) while the *L. plantarum* control fermentation showed the lowest concentration at 195 mg/L (**Table 3.6**). For the other inoculation strategies concentrations were similar ± 1000 mg/L by the end of AF. For fermentations inoculated with *O. oeni* the co-inoculated strategies with EC1118® produced the highest acetic acid concentration of 3825 mg/L. *O. oeni* in co-inoculation with Cross Evolution® resulted in acetic acid concentrations of 2783 mg/L. For the other inoculation strategies concentrations were in the area of 1000 mg/L. The concentrations of fermentations co-inoculated with the bacterial combination were higher compared to the corresponding sequentially inoculated fermentations.

TABLE 3.6

Acetic acid concentration at the end of standard fermentations with Cross Evolution and EC 1118 using *L. plantarum*, *O. oeni* and combination of *L. plantarum and O. oeni* with bacterial treatments and different inoculation strategies. Values are the average of 3 biological repeats ± standard deviation.

Inoculation strategies	Day of completion of alcoholic fermentation	Acetic acid (mg/L)
Cross Evolution		
Control yeast without inoculated *L. plantarum*	21	982.26 ± 2.34
Control yeast without inoculated *O. oeni*	23	972.20 ± 3.09
Control yeast without inoculated *L. plantarum* and *O. oeni*	12	893.06 ± 1.71
Co-inoculation		
L. plantarum	21	1205.44 ± 1.45
O. oeni	23	2783.94 ± 0.68
L. plantarum and *O. oeni*	12	1356.79 ± 0.78
Sequential inoculation		
L. plantarum	21	1144.78 ± 1.54
O. oeni	23	1128.93 ± 1.33
L. plantarum and *O. oeni*	12	958.59 ± 1.24
EC 1118		
Control yeast without inoculated *L. plantarum*	21	982.94 ± 0.63
Control yeast without inoculated *O. oeni*	23	1085.52 ± 3.79
Control yeast without inoculated *L. plantarum* and *O. oeni*	12	955.77 ± 2.35
Co-inoculation		
L. plantarum	21	1028.13 ± 6.49
O. oeni	23	3825.94 ± 1.63
L. plantarum and *O. oeni*	12	1338.38 ± 1.27
Sequential inoculation		
L. plantarum	21	1091.27 ± 0.41
O. oeni	23	1044.75 ± 1.91
L. plantarum and *O. oeni*	12	1028.12 ± 0.35
Control: bacteria		
L. plantarum	21	195.35 ± 0.75
O. oeni	23	251.23 ± 1.24
L. plantarum and *O. oeni*	12	212.58 ± 1.09

3.3.2 NH_4Cl-supplemented fermentations

The second focus area of our work relates to the impact of different nutrients and nitrogen sources on the interactions between different combinations of yeast and bacteria. In this section of the work, the synthetic medium was supplemented either with NH_4Cl as the sole nitrogen source (representing the most simple nitrogen source) or with an equimolar mix of aromatic and branched chain amino acids as the nitrogen source (the total nitrogen was the same for both treatments)

Reducing sugars

The Cross Evolution® and EC1118® control fermentations consumed glucose to less than 5 g/L by day 44 of fermentation (**Addendum Table 8 A1**). Cross Evolution® and EC1118® co-inoculated with *L. plantarum* and *O. oeni* consumed glucose to less than 5 g/L by days 28 and 29 respectively (**Figure 3.5**). For the bacterial combination glucose was similarly consumed by day 29 (**Addendum Table 10 C1**). Fructose was utilised by day 44 for all treatments (**Figure 3.6**). See **Addendum Table 8 A2**, **Table 9 A2 and Table 10 C2** for fructose consumption during the AF.

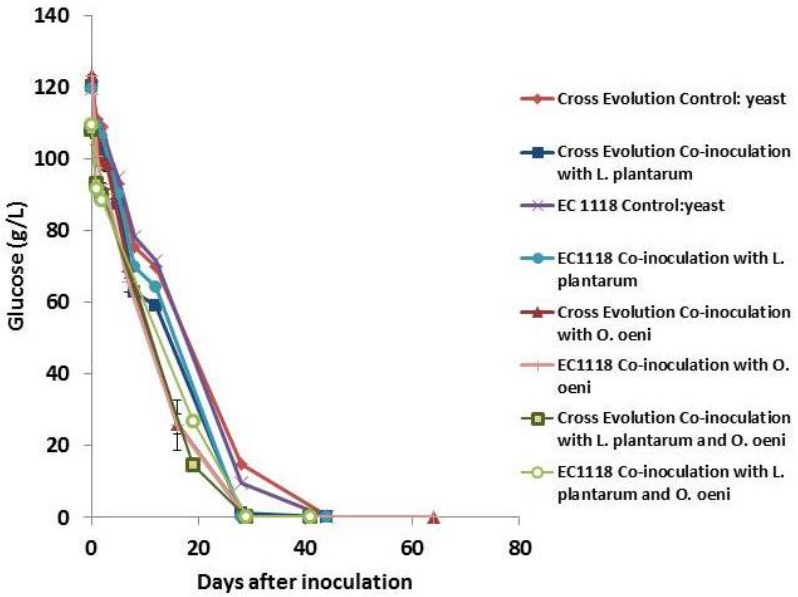

FIGURE 3.5

Glucose concentration during NH_4Cl supplemented fermentations with different yeasts in combination with *L. plantarum*, *O. oeni* and the combination of *L. plantarum* and *O. oeni*. Values are the average of 3 biological repeats.

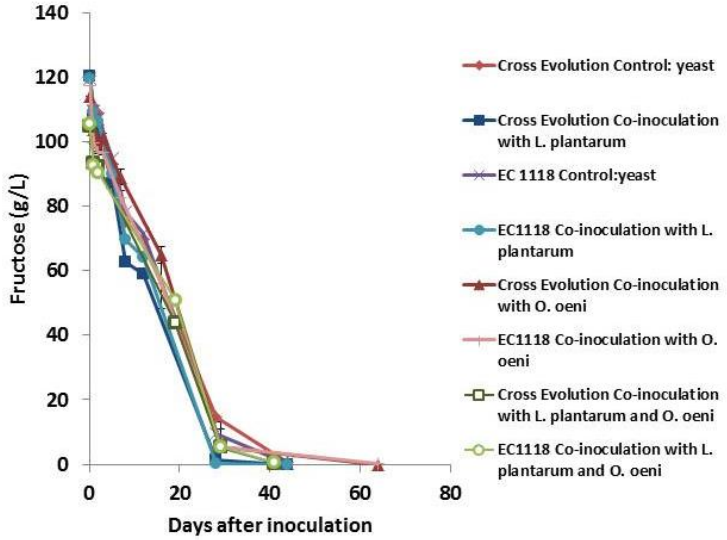

FIGURE 3.6

Fructose concentration during NH_4Cl supplemented fermentations with different yeasts in combination with *L. plantarum*, *O. oeni* and the combination of *L. plantarum* and *O. oeni*. Values are the average of 3 biological repeats.

Ethanol

The ethanol concentration of most of the fermentations that were inoculated with *L. plantarum* was between 10% and 12% (**Table 3.7**). The ethanol concentration of the fermentations that were inoculated with *O. oeni* ranged between 11% and 12% and the ethanol concentrations of the bacterial combination inoculation strategies were between 10% and 12%.

Table 3.7: Ethanol concentrations (% v/v) of NH_4Cl supplemented fermentations at the end of alcoholic fermentation. Values are the average of 3 biological repeats ± standard deviation.

	Cross Evolution			EC 1118			Control
	Control: yeast-only	Sequential inoculation	Co-inoculation	Control: yeast-only	Sequential inoculation	Co-inoculation	Malolactic bacteria
L. plantarum treatments	10.94 ± 0.25	11.51 ± 0.24	11.99 ± 0.23	11.74 ± 0.28	11.72 ± 0.07	11.35 ± 1.04	0.18 ± 0.01
O. oeni treatments	11.19 ± 0.57	11.53 ± 0.58	11.37 ± 0.61	11.40 ± 0.46	11.42 ± 0.57	11.08 ± 0.91	0.16 ± 0.04
L. plantarum : *O. oeni* treatments	11.72 ± 0.43	11.43 ± 0.49	11.03 ± 0.06	11.56 ± 0.57	12.49 ± 0.29	10.29 ± 0.15	0.16 ± 0.02

Bacterial populations

Cross Evolution® and EC1118® co-inoculated with *L. plantarum* increased from 1×10^7 cfu/mL to 1×10^8 cfu/mL after 8 days. (**Figure 3.7A**). Cross Evolution® and EC1118® co-inoculated with *O. oeni* had intial concentrations of 1×10^9 cfu/mL and decreased after 3 days to 1×10^8 cfu/mL (**Figure 3.7C**). The Cross Evolution® co-inoculated with the bacterial combination were inoculated at 1×10^8 cfu/mL and increased to approximately 1×10^9 cfu/mL (**Figure 3.7E**). Cross Evolution® co-inoculated with the bacterial combination increased from 1×10^7 cfu/mL to approximately 1×10^9 cfu/mL (**Addendum Table 12 C1**). Fermentations conducted with both Cross Evolution® and EC1118® and sequentially inoculated with *L. plantarum* had initial *L.*

plantarum levels of 1×10^8 cfu/mL which decreased to 1×10^5 cfu/mL after 9 days (**Figure 3.7B**). The Cross Evolution® and EC 1118® sequential strategies inoculated with *O. oeni* increased to a cell concentration of 1×10^8 cfu/mL after 3 days before rapidly decreasing to 1×10^7 cfu/mL by day 4 (**Figure 3.7D**). Both the Cross Evolution® and EC1118® sequential strategy inoculated with the bacterial combination were initiated at 1×10^7 cfu/mL but in the case of Cross Evolution® the cell count remained at 1×10^7 cfu/mL after 5 days while for EC1118® bacterial cell counts decreased to 1×10^6 cfu/mL after 5 days (**Figure 3.7F**). See **Addendum Table 12 A1-C2** for the bacterial population values.

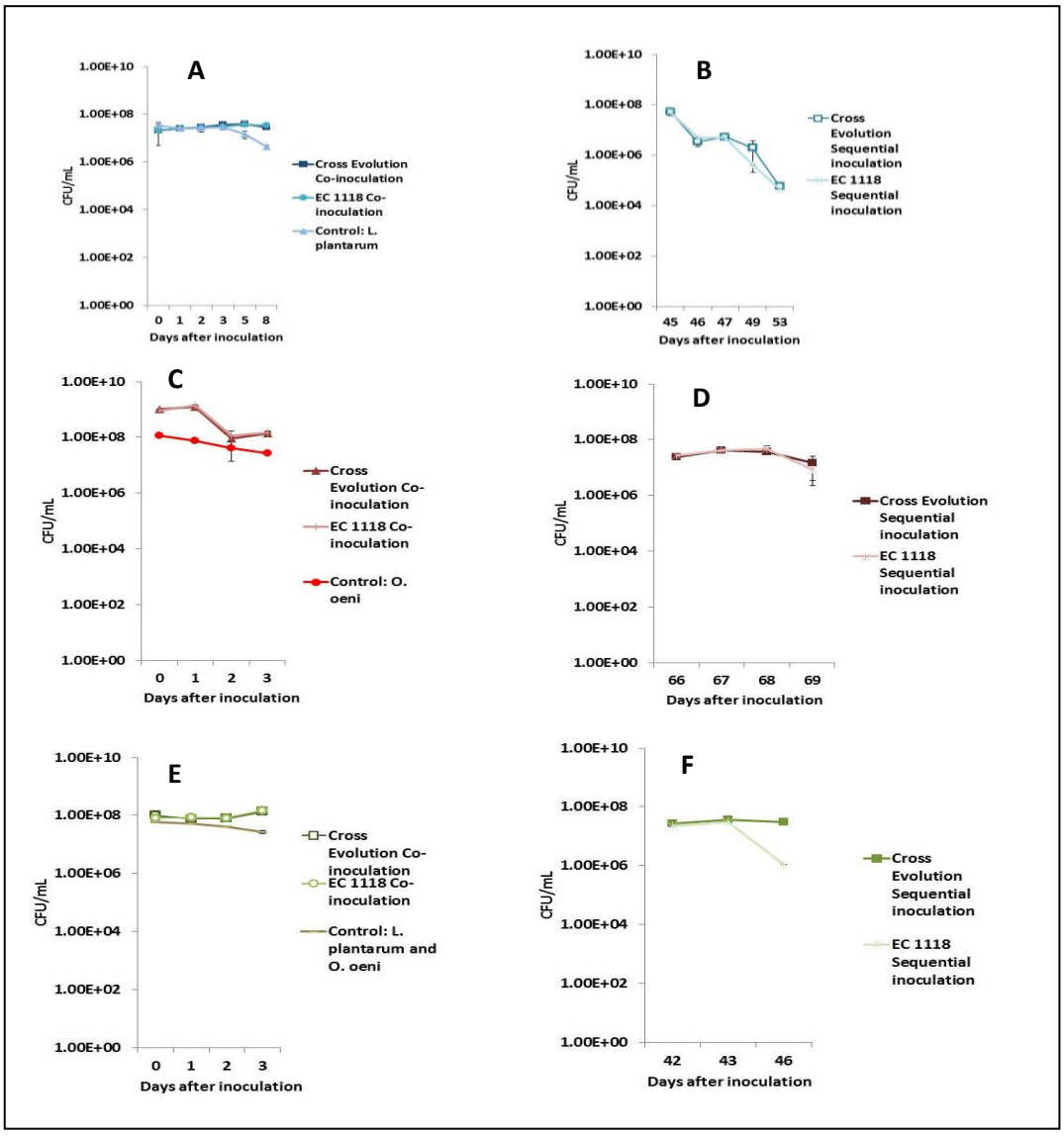

FIGURE 3.7

Bacterial populations of NH_4Cl supplemented fermentations in combination with *L. plantarum* **(A)** during co-inoculation, bacterial control and sequential inoculation **(B)**. Co-inoculation of *O. oeni* **(C)**, bacterial control and sequential inoculation **(D)**. Co-inoculation of combination of *L. plantarum* and *O. oeni* **(E)**, bacterial control and sequential inoculation **(F)**. Values are the average of 3 biological repeats ± standard deviation.

Malic acid

Malic acid degradation of fermentations co-inoculated with *L. plantarum* finished within 8 days for both yeast strains while malic acid degradation in the sequential inoculations took 9 days to complete (**Table 3.8**). Malic acid degradation of the *O. oeni* co-inoculated fermentations reached completion after 2 days for both yeasts while in the corresponding sequential fermentations MLF was finished within one day only. Fermentations of EC1118® and Cross Evolution® co-inoculated with the bacterial combination took 3 days to complete. The sequential inoculation of both yeast that were inoculated with the bacterial combination finished within 5 days. The corresponding lactic acid concentrations ranged between 1.31 g/L and 2.85 g/L. See **Addendum Table 13** for the complete malic acid and lactic acid values.

TABLE 3.8

Malic and lactic acid concentrations at the end of NH_4Cl supplemented MLF for co- and sequential inoculation of *L. plantarum*, *O. oeni* and the combination of *L. plantarum* and *O. oeni*. Values are the average of 3 biological repeats ± standard deviation.

Inoculation strategies	Day of MLF completion	Malic acid (g/L)	Lactic acid (g/L)
Cross Evolution			
Co-inoculation			
L. plantarum	8	0.22 ± 0.02	2.81 ± 0.06
O. oeni	2	0.08 ± 0.01	1.86 ± 0.01
L. plantarum and *O. oeni*	3	0.07 ± 0.002	1.92 ± 0.03
Sequential inoculation			
L. plantarum	9	0.07 ± 0.09	1.31 ± 0.17
O. oeni	1	0.08 ± 0.001	1.4 ± 0.01
L. plantarum and *O. oeni*	5	0.05 ± 0.001	1.34 ± 0.05
EC 1118			
Co-inoculation			
L. plantarum	8	0.18± 0.02	2.85 ± 0.05
O. oeni	2	0.07 ± 0.004	1.79 ± 0.01
L. plantarum and *O. oeni*	3	0.07 ± 0	1.86 ± 0.01
Sequential inoculation			
L. plantarum	9	0.03 ± 0.23	1.20 ± 0.38
O. oeni	1	0.08 ± 0.004	1.37 ± 0.03
L. plantarum and *O. oeni*	5	0.07 ± 0.004	1.32 ± 0.02
Control: bacteria			
L. plantarum	8	0.26 ± 0.4	2.05 ± 0.03
O. oeni	2	0.28 ± 0.01	1.74 ± 0.01
L. plantarum and *O. oeni*	3	0.08 ± 0.01	1.97 ± 0.02

Citric acid

Citric acid degradation was complete for all fermentations between 12 and 19 days after inoculation. No differences were observed for any of the bacterial co-inoculated treatments compared to the yeast–only controls. The concentrations were below 0.04 g/L at the middle of AF. The sequential inoculations took five days to complete.

Acetic acid

Cross Evolution® control fermentations had the highest acetic acid concentration of 1011.84 mg/L (**Table 3.9**). For most treatments the final acetic acid concentration was below 1000 mg/L. The bacterial control treatments had the lowest acetic acid concentration and the highest concentrations of acetic acid were found for the *O. oeni* and Cross Evolution® co-inoculated treatment (1015 mg/L). (**Table 3.9**). Generally no consistent trends were evident in terms of higher or lower acetic acid concentrations for either co-inoculation or sequential inoculation for a particular yeast-bacteria pair. Also no consistent trends were seen for any of the bacterial treatments in terms of generally higher or generally lower acetic acid levels across all treatments.

TABLE 3.9

Acetic acid concentration at the end of NH_4Cl supplemented fermentations. Cross Evolution and EC 1118 were inoculated with *L. plantarum*, *O. oeni* and the combination of *L. plantarum and O. oeni*. Values are the average of 3 biological repeats ± standard deviation.

Treatment	Day of completion of alcoholic fermentation	Acetic acid (mg/L)
Cross Evolution		
Control yeast without inoculated *L. plantarum*	44	1011.84 ± 0.23
Control yeast without inoculated *O. oeni*	64	938.32 ± 2.10
Control yeast without inoculated *L. plantarum* and *O. oeni*	41	802.64 ± 1.69
Co-inoculation		
L. plantarum	44	733.68 ± 2.24
O. oeni	64	1015.41 ± 0.19
L. plantarum and *O. oeni*	41	898.11 ± 0.97
Sequential inoculation		
L. plantarum	53	1003.41 ± 0.36
O. oeni	69	857.58 ± 0.66
L. plantarum and *O. oeni*	46	816.0 ± 1.08
EC 1118		
Control yeast without inoculated *L. plantarum*	44	735.76 ± 0.24
Control yeast without inoculated *O. oeni*	64	839.0 ± 1.16
Control yeast without inoculated *L. plantarum* and *O. oeni*	41	986.06 ± 0.82
Co-inoculation		
L. plantarum	44	944.63 ± 1.88
O. oeni	64	903.99 ± 1.22
L. plantarum and *O. oeni*	41	972.99 ± 0.56
Sequential inoculation		
L. plantarum	53	939.84 ± 7.58
O. oeni	69	727.03 ± 0.49
L. plantarum and *O. oeni*	46	924.64 ± 1.16
Control: bacteria		
L. plantarum	44	79.81 ± 0.58
O. oeni	64	100.38 ± 0.22
L. plantarum and *O. oeni*	41	89.76 ± 1.01

3.3.3 Amino acids-supplemented fermentations

In this section of the work, the synthetic medium was supplemented with an equimolar mix of the branched chain (leucine, isoleucine, valine) and aromatic (tryptophan and phenylalanine) amino acids. These amino acids were selected as they are known precursors for the higher alcohols and esters produced during AF (Dickinson *et al*., 2000, 2003).

Sugars

The Cross Evolution® and EC1118® control fermentations and co-inoculated strategies with *L. plantarum* consumed glucose to less than 5 g/L within 24 days (**Figure 3.8**). The Cross Evolution® and EC1118® strategies co-inoculated with *O. oeni* consumed glucose and fructose to less than 5 g/L within 69 days while the co-inoculation strategies with the bacterial combination consumed glucose and fructose within 48 days (**Figure 3.9**). See **Addendum Tables 15-17** for complete glucose and fructose values

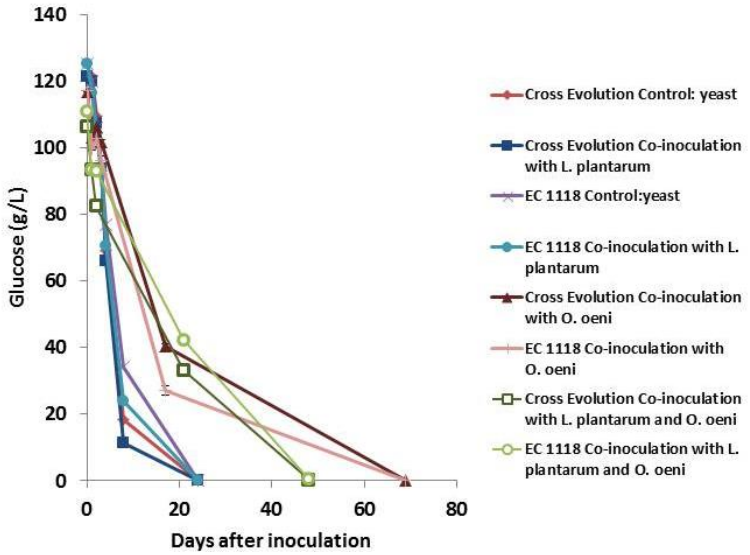

FIGURE 3.8

Glucose concentration during amino acids supplemented fermentations with different yeasts in combination with *L. plantarum*, *O. oeni* and the combination of *L. plantarum* and *O. oeni*. Values are the average of 3 biological repeats.

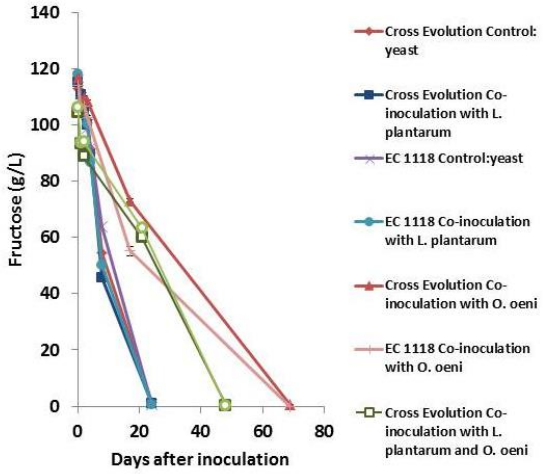

FIGURE 3.9

Fructose concentration during amino acids supplemented fermentation with different yeasts in combination with *L. plantarum*, *O. oeni* and the combination of *L. plantarum* and *O. oeni*. Values are the average of 3 biological repeats.

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Ethanol

The ethanol concentration of all the different strategies that were inoculated with *L. plantarum* ranged between 11% and 12% (**Table 3.10**). The alcohol concentration of most fermentations that were inoculated with *O. oeni* was between 10% and 12%. The ethanol concentrations of fermentations that were inoculated with the bacterial combination were between 10% and 12% (**Table 3.10**).

TABLE 3.10

Ethanol concentrations (% v/v) of amino acids supplemented fermentations at the end of alcoholic fermentation. Values are the average of 3 biological repeats ± standard deviation.

	Cross Evolution			EC 1118			Control
	Control: yeast-only	Sequential inoculation	Co-inoculation	Control: yeast-only	Sequential inoculation	Co-inoculation	Malolactic bacteria
L. plantarum treatments	11.06 ± 0.97	11.34 ± 1.04	11.79 ± 0.27	11.49 ± 0.62	11.18 ± 0.14	11.39 ± 0.11	0.18 ± 0.01
O. oeni treatments	10.09 ± 0.95	10.40 ± 0.29	11.15 ± 0.06	11.67 ± 0.17	11.53 ± 0.30	11.01 ± 0.15	0.28 ± 0.11
L. plantarum : *O. oeni* treatments	10.94 ± 0.20	10.63 ± 1.37	10.32 ± 0.58	11.89 ± 0.39	10.26 ± 0.56	10.44 ± 0.22	0.30 ± 0.27

Bacterial populations

Cross Evolution® and EC1118® fermentations were co-inoculated with *L. plantarum,* at 1×10^7 cfu/mL and remained at this concentration for 8 days (**Figure 3.10A**). The same was true for the *L. plantarum* control. Both co-inoculated strategies using *O. oeni* had an initial concentration of 1×10^8 cfu/mL and remained at this cell count for 3 days (**Figure 3.10C**). The *O. oeni* control and

co-inoculated strategies with the bacterial combination had concentrations of 1×10^8 cfu/mL and decreased after 3 days to 1×10^7 cfu/mL (**Figure 3.10E**).The Cross Evolution® sequential strategies inoculated with *L. plantarum* had an initial concentration of 1×10^7 cfu/mL and remained at this cell count for 12 days (**Figure 3.10B**). For both the sequential strategies inoculated with *O. oeni* initial bacterial concentrations were 1×10^8 cfu/mL (**Figure 3.10D**). In the case of the EC1118® sequential strategy cell numbers remained at 1×10^8 cfu/mL while for the Cross Evolution® sequential strategy viable cell numbers decreased to 1×10^5 cfu/mL after 6 days. Both sequential strategies were inoculated with the bacterial combination at 1×10^7 cfu/mL and cell numbers remained at this value for 5 days (**Figure 3.10F**). See **Addendum Table 19 A1-C2** for the complete set of bacterial population numbers for the various fermentations.

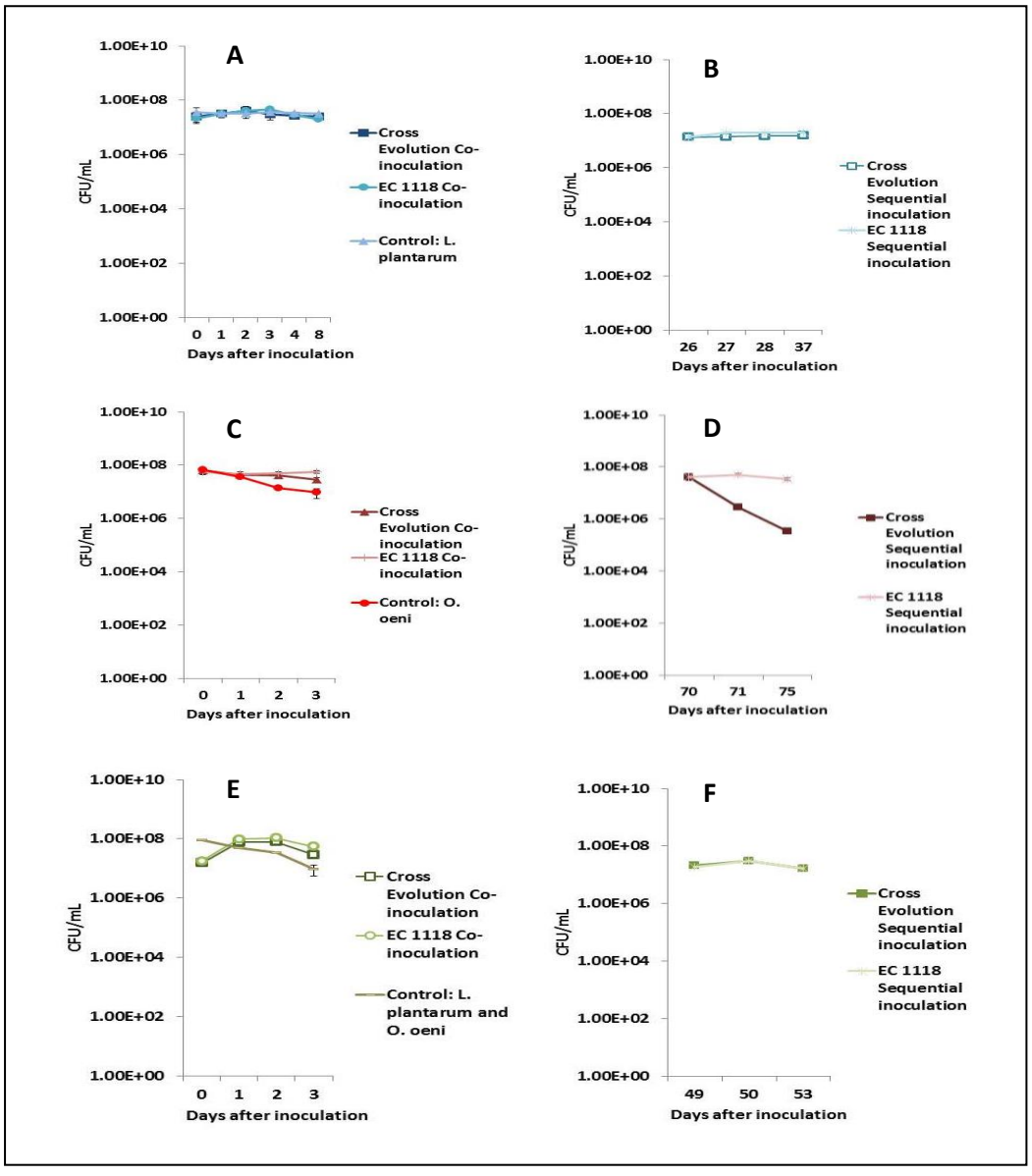

FIGURE 3.10

Bacterial populations of fermentations (with amino acids as nitrogen source) inoculated with *L. plantarum* (**A**) during co-inoculation, bacterial control and sequential inoculation (**B**). Co-inoculation of *O. oeni* (**C**), bacterial control and sequential inoculation (**D**). Co-inoculation of combination of *L. plantarum* and *O. oeni* (**E**), bacterial control and sequential inoculation (**F**). Values are the average of 3 biological repeats ± standard deviation.

Malic acid

Malic degradation of the fermentations with both yeast strains co-inoculated with *L. plantarum* took 6 days to complete. The sequential inoculation took 12 days to complete MLF (**Table 3.11**). The malic acid degradation of the strategies that were co-inoculated with *O. oeni* and the bacterial combination took 3 days to complete MLF for both yeast strains. The sequential inoculation of EC1118® and Cross Evolution® with *O. oeni* and the bacterial combination took 5 days to complete. The decrease in malic concentration resulted in a corresponding increase in lactic acid concentrations (between 1.33 g/L and 2.83 g/L). See **Addendum Table 20 A1-C2** for malic acid and lactic acid concentration during the course of MLF.

TABLE 3.11

Malic and lactic acid concentrations at end of fermentation in media supplemented with a nitrogen source consisting of branched chain and aromatic amino acids using co- and sequential inoculation of *L. plantarum*, *O. oeni* and combination of *L. plantarum* and *O. oeni* in conjunction with *Cross* Evolution and EC1118. Values are the average of 3 biological repeats ± standard deviation.

Inoculation strategies	Day of MLF completion	Malic acid (g/L)	Lactic acid (g/L)
Cross Evolution			
Co-inoculation			
L. plantarum	6	0.04 ± 0.06	2.64 ± 0.06
O. oeni	3	0.09 ± 0.01	1.88 ± 0.03
L. plantarum and *O. oeni*	3	0.10 ± 0.02	1.88 ± 0.03
Sequential inoculation			
L. plantarum	12	0.06 ± 0.05	1.89 ± 0.06
O. oeni	5	0.04 ± 0.25	2.04 ± 0.02
L. plantarum and *O. oeni*	5	0.05 ± 0.001	1.33 ± 0.001
EC 1118			
Co-inoculation			
L. plantarum	6	0.18 ± 0.07	2.83 ± 0.04
O. oeni	3	0.07 ± 0.04	1.83 ± 0.01
L. plantarum and *O. oeni*	3	0.07 ± 0.004	1.83 ± 0.01
Sequential inoculation			
L. plantarum	12	0.04 ± 0.23	1.71 ± 0.38
O. oeni	5	0.06 ± 0.09	1.01 ± 0.06
L. plantarum and *O. oeni*	5	0.04 ± 0.02	1.32 ± 0.01
Control: bacteria			
L. plantarum	6	0.05 ± 0.02	2.41 ± 0.02
O. oeni	3	0.06 ± 0.07	1.56 ± 0.06
L. plantarum and *O. oeni*	3	0.06 ± 0.07	1.56 ± 0.06

Citric acid

All of the strategies consumed citric acid within 8 days to 21 days. The concentrations were below 0.03 g/L. Four of the strategies did not degrade all the citric acid: The Cross Evolution®

control yeast (0.12g/L), Cross Evolution® sequential inoculation with the bacterial combination (0.21 g/L), the *O. oeni* control (0.14 g/L) and the bacterial combination controls (0.14 g/L).

Acetic acid

The acetic acid concentration of the EC1118® sequential inoculation strategy with *L. plantarum* was the highest (1160 mg/L) while EC1118® co-inoculated with *O. oeni* showed slightly lower concentrations of 1002.45 mg/L on average (**Table 3.12**). For the other fermentations the concentrations were below 1000 mg/L. There were no consistent trends for co-inoculation or sequential inoculation with a particular yeast-bacteria pair in terms of achieving a higher/lower final acetic acid concentration.

TABLE 3.12

Acetic acid concentrations at the end of AF in media supplemented with aromatic and branched chain amino acids as the sole nitrogen source. Values are the average of 3 biological repeats ± standard deviation.

Treatment	Day of completion of alcoholic fermentation	Acetic acid (mg/L)
Cross Evolution		
Control yeast without inoculated *L. plantarum*	24	736.12 ± 0.54
Control yeast without inoculated *O. oeni*	69	577.29 ± 0.70
Control yeast without inoculated *L. plantarum* and *O. oeni*	48	512.79 ± 0.36
Co-inoculation		
L. plantarum	24	807.93 ± 1.64
O. oeni	69	714.57 ± 1.50
L. plantarum and *O. oeni*	48	746.49 ± 1.82
Sequential inoculation		
L. plantarum	37	779.81 ± 0.22
O. oeni	74	671.24 ± 0.47
L. plantarum and *O. oeni*	53	475.07 ± 0.22
EC 1118		
Control yeast without inoculated *L. plantarum*	24	963.41 ± 0.64
Control yeast without inoculated *O. oeni*	69	644.99 ± 0.90
Control yeast without inoculated *L. plantarum* and *O. oeni*	48	836.43 ± 0.36
Co-inoculation		
L. plantarum	24	693.04 ± 0.97
O. oeni	69	1002.45 ± 1.54
L. plantarum and *O. oeni*	48	931.28 ± 0.36
Sequential inoculation		
L. plantarum	37	1156.20 ± 0.15
O. oeni	74	584.46 ± 1.22
L. plantarum and *O. oeni*	53	928.94 ± 1.22
Control: bacteria		
L. plantarum	24	91.19 ± 0.56
O. oeni	69	110.54 ± 0.63
L. plantarum and *O. oeni*	48	90.86 ± 0.57

3.3.4 Production of volatile aroma compounds

3.3.4.1 Standard fermentations

Esters

There was an increase in total ester concentrations in co-inoculated strategies compared to the yeast–only control fermentations and sequentially inoculated fermentation (**Table 3.13**). Fermentations conducted with Cross Evolution® co-inoculated with the bacterial combination showed the highest total ester concentration of 401.74 mg/L while Cross Evolution® sequential inoculated with bacterial control showed the lowest concentration of 65.31 mg/L.

Two esters that are strongly influenced by MLF are ethyl lactate and ethyl acetate. Ethyl lactate was the most dominant ester produced after MLF. The co-inoculation strategies showed higher concentrations compared to sequential inoculations (**Addendum Figure 1 A1**). For the Cross Evolution® treatments, the co-inoculation with the bacterial combination resulted in the highest concentration of ethyl lactate (362.73 mg/L) while for fermentations conducted using EC1118® the co-inoculation with the bacterial combination showed the highest concentration on average (382.23 mg/L).

Ethyl acetate was the ester produced at the second highest concentration overall. The Cross Evolution® co-inoculated strategies with different LAB species showed the highest concentrations (120.74 mg/L) compared to the sequentially inoculated strategies (109.38 mg/L) (**Table 3.13**). For the EC1118® treatments, the co-inoculated strategies with the different LAB species all showed higher concentrations (150.05 mg/L) than sequentially inoculated fermentations (129.23 mg/L). The bubble graph also indicates that fermentations with single LAB strains resulted in significantly higher concentrations than the bacterial combination (**Figure 3.11 A**).

Ethyl butyrate was not detected in all the different treatments. The other esters were detected at low quantities. The bubble graphs of ethyl caprylate (**Figure 3.11B**) and ethyl caprate (**Figure 3.11C**) showed that single LAB strains resulted in significant higher concentrations of these esters than the bacterial combination which was especially true for *L. plantarum*.

Higher alcohols

There was a decrease in total higher alcohols when co-inoculated strategies where compared to yeast control fermentations (**Table 3.14**). This was not the case for the sequentially inoculated fermentations where total higher alcohols were higher in certain cases compared to the yeast-only fermentations. For both co-inoculated and sequentially inoculated fermentations the *O. oeni* treatments showed the lowest levels of higher alcohols in combination with both yeast strains compared to the other LAB strains. The different inoculation strategies delivered no detectable 3-methyl-1-pentanol. Pentanol was detected at low quantities in co-inoculation strategies and sequential inoculations. Butanol concentrations were higher in sequential strategies than co-inoculation strategies (**Addendum Figure 1 A2**). Sequential strategies showed higher 3-ethoxy-1-propanol concentrations than co-inoculated strategies. Hexanol concentrations were higher in co-inoculation than sequential inoculation strategies. The bubble graph for 2-phenylethanol shows that the levels of this compound were significantly lower in co-inoculation strategies with single LAB strains (**Figure 3.11D**).

Isoamyl alcohol levels showed clear variations between the different inoculation treatments. The isoamyl alcohol concentrations of the co-inoculation treatments with Cross Evolution®, EC1118® and *L. plantarum* increased from 80 mg/L at the middle of AF to 100 mg/L at the end of AF (**Figure 3.12**). The isoamyl alcohol levels in the sequential inoculations with Cross Evolution® remained at 80 mg/L while EC1118® increased from 80 mg/L at the end of AF to 120 mg/L at the end of MLF. The isoamyl alcohol concentration of the co-inoculation treatments with Cross Evolution® and *O. oeni* increased from 55 mg/L at the middle of AF to 60 mg/L at the end of AF while EC1118® and *O. oeni* increased from 65 mg/L at the middle of AF to 75 mg/L at the end of AF (**Figure 3.12E**). The isoamyl alcohol concentrations in the sequential inoculations with Cross Evolution® remained at 60 mg/L while EC1118® remained at 80 mg/L. Similar trends were observed for the fermentations that were inoculated with the combination of malolactic bacteria.

Fatty acids

There was no increase in the total fatty acid concentrations when co-inoculated strategies were compared to yeast control fermentations as well as sequential inoculation strategies (**Table 3.15)**. The EC1118® co-inoculated with *L. plantarum* showed the highest concentration of 11.39 mg/L while Cross Evolution® co-inoculated with the bacterial combination resulted in the lowest concentration of 7.79 mg/L when the different co-inoculation strategies were compared. For the EC1118® treatment, sequential inoculation with *L. plantarum* resulted in the highest concentration of 11.48 mg/L and EC1118® with the bacterial combination showed the lowest concentration of 9.58 mg/L. Sequential inoculation strategies generally showed higher concentrations of propionic acid, isovaleric acid and isobutyric acid compared to co-inoculation strategies. Co-inoculation strategies generally showed higher concentrations of butyric acid, valeric acid and octanoic acid. The bubble graph of butyric acid clearly shows that fermentations with single LAB strains resulted in significant higher concentrations of this acid (**Figure 3.11E**).

Hexanoic acid was a dominant fatty acid after MLF. Sequential inoculation strategies resulted in the highest concentrations. Fermentations inoculated with *L. plantarum* showed the highest average value of 2.83 mg/L. The bubble graphs also support these findings (**Addendum Figure 1 A3**).

TABLE 3.13

Ester concentrations (mg/L) at the end of standard fermentations with the different yeast and bacterial treatments in both co- and sequential inoculation. The concentrations given for the sequential inoculations are end point values after MLF. Values are the average of 3 biological repeats ± standard deviation.

	Ethyl acetate	Ethyl butyrate	Isoamyl acetate	Ethyl hexanoate	Hexyl acetate	Ethyl lactate	Ethyl caprylate	Ethyl-3-hydroxybutanoate	Ethyl caprate	Diethyl succinate	Ethyl phenylacetate	2-Phenylethyl Acetate	Totals
Cross Evolution													
Control yeast without inoculated *L. plantarum*	52.18 ± 3.01	0.41 ± 0.01	0.79 ± 0.10	0.59 ± 0.05	0.05 ± 0	13.70 ± 0.32	1.32 ± 0.61	1.19 ± 0.02	8.95 ± 4.44	0.70 ± 0.27	0.55 ± 0.02	0.62 ± 0.04	**81.05**
Control yeast without inoculated *O. oeni*	35.02 ± 2.87	0.35 ± 0.01	0.39 ± 0.02	0.51 ± 0	0.06 ± 0	12.40 ± 0.32	0.81 ± 0.05	1.16 ± 0.03	6.32 ± 0.32	0.89 ± 0.17	0.51 ± 0	0.56 ± 0.01	**58.98**
Control yeast without inoculated *L. plantarum* and *O. oeni*	26.10 ± 2.48	0.34 ± 0	0.42 ± 0.05	0.41 ± 0.01	0 ± 0	11.59 ± 0.25	0.21 ± 0.02	0 ± 0	0.88 ± 0.31	0.67 ± 0.09	0.58 ± 0.02	0.57 ± 0.03	**41.77**
Co-inoculation													
L. plantarum	47.47 ± 5.03	0.37 ± 00.02	0.51 ± 0.0.14	0.50 ± 0.04	0.03 ± 0.001	67.45 ± 8.03	0.78 ± 0.10	1.20 ± 0.003	4.66 ± 0.82	0.60 ± 0.02	0.18 ± 0.10	0.53 ± 0.04	**124.28**
O. oeni	37.91 ± 2.37	0.35 ± 0.02	0.39 ± 0.05	0.54 ± 0.02	0.06 ± 0.0004	286.56 ± 0.48	1.08 ± 0.05	0 ± 0	7.30 ± 0.10	1.21 ± 0.02	0.11 ± 0.02	0.71 ± 0.01	**336.22**
L. plantarum and *O. oeni*	35.36 ± 2.87	0.37 ± 0.002	0.47 ± 0.05	0.44 ± 0.01	0 ± 0	362.73 ± 3.28	0.27 ± 0.04	0 ± 0	0.89 ± 0.37	0.56 ± 0.02	0.07 ± 0.04	0.58 ± 0.01	**401.74**
Sequential inoculation													
L. plantarum	44.88 ± 5.20	0.37 ± 0.004	0.48 ± 0.03	0.47 ± 0.01	0.06 ± 0.003	16.69 ± 1.19	0.72 ± 0.03	1.17 ± 0.02	5.70 ± 0.23	0.68 ± 0.03	0.90 ± 0.14	0.52 ± 0.01	**72.64**
O. oeni	33.61 ± 4.69	0.37 ± 0.02	0.42 ± 0.11	0.45 ± 0.05	0.06 ± 0.001	33.19 ± 2.17	0.45 ± 0.20	1.14 ± 0.01	4.42 ± 1.17	0.62 ± 0.03	1.04 ± 0.19	0.51 ± 0.02	**76.28**
L. plantarum and *O. oeni*	30.89 ± 5.14	0.36 ± 0.01	0.53 ± 0.08	0.42 ± 0.01	0 ± 0	28.08 ± 1.35	0.26 ± 0.02	1.14 ± 0.04	1.70 ± 0.30	0.56 ± 0.01	0.77 ± 0.07	0.60 ± 0.02	**65.31**
EC 1118													
Control yeast without inoculated *L. plantarum*	58.36 ± 0.13	0.45 ± 0	0.83 ± 0.01	0.53 ± 0.13	0.05 ± 0	14.90 ± 0.20	1.04 ± 0.07	1.20 ± 0.02	1.04 ± 0.07	0.79 ± 0.02	0.50 ± 0.37	0.57 ± 0.01	**80.26**
Control yeast without inoculated *O. oeni*	51.37 ± 6.19	0.40 ± 0.03	0.47 ± 0.15	0.50 ± 0.10	0.07 ± 0.01	12.16 ± 0.37	0.45 ± 0.26	1.17 ± 0.02	2.01 ± 1.12	1.38 ± 0.29	0.53 ± 0.02	0.56 ± 0.01	**71.07**
Control yeast without inoculated *L. plantarum* and *O. oeni*	39.96 ± 2.55	0.39 ± 0.01	0.43 ± 0.04	0.48 ± 0.02	0 ± 0	11.89 ± 0.10	0.30 ± 0.03	1.15 ± 0.02	1.29 ± 0.20	0.77 ± 0.06	0.56 ± 0.01	0.56 ± 0	**57.78**
Co-inoculation													
L. plantarum	58.96 ± 3.95	0.41 ± 0 02	0.57 ± 0.11	0.55 ± 0.08	0.10 ± 0.001	75.07 ± 12.02	0.78 ± 0.39	1.21 ± 0.07	5.46 ± 3.18	0.57 ± 0.01	0.09 ± 0.02	0.53 ± 0.02	**144.3**
O. oeni	50.04 ± 3.19	0.38 ± 00.02	0.40 ± 0.07	0.47 ± 0.06	0.06 ± 0.002	332.66 ± 2.33	0.49 ± 0.14	1.15 ± 0.03	2.21 ± 0.76	0.75 ± 0.07	0.04 ± 0.02	0.52 ± 0.02	**389.17**
L. plantarum and *O. oeni*	41.05 ± 0.59	0.38 ± 0.01	0.42 ± 0.04	0.47 ± 0.01	0 ± 0	382.23 ± 1.42	0.30 ± 0.01	1.15 ± 0.04	1.18 ± 0.05	0 ± 0	0.57 ± 0.01	0.55 ± 0	**428.3**
Sequential inoculation													
L. plantarum	52.67 ± 9.35	0.39 ± 0.02	0.51 ± 0.08	0.49 ± 0.03	0.004 ± 0.002	17.57 ± 0.89	0.62 ± 0.07	1.21 ± 0.01	5.41 ± 0.74	0.61 ± 0.04	0.78 ± 0.21	0.72 ± 0.27	**80.984**
O. oeni	45.78 ± 10.13	0.40 ± 0.02	0.47 ± 0.03	0.44 ± 0.02	0.06 ± 0.001	27.49 ± 1.85	0.41 ± 0.08	1.19 ± 0.02	2.72 ± 0.41	0.59 ± 0.02	1.49 ± 0.22	0.53 ± 0.002	**81.57**
L. plantarum and *O. oeni*	30.78 ± 3.81	0.37 ± 0.003	0.38 ± 0.05	0.43 ± 0.04	0 ± 0	32.03 ± 1.78	0.25 ± 0.13	1.15 ± 0.03	1.17 ± 0.88	0.55 ± 0.01	0.82 ± 0.19	0.54 ± 0.01	**68.47**
Control: bacteria													
L. plantarum	22.02 ± 6.25	0 ± 0	0 ± 0	0 ± 0	0 ± 0	10.70 ± 0.33	0.04 ± 0.01	0.62 ± 0.11	0 ± 0	0 ± 0	0 ± 0	0.35 ± 0.01	**33.73**
O. oeni	12.44 ± 0.03	0 ± 0	0 ± 0	0 ± 0	0 ± 0	11.79 ± 0.28	0.05 ± 0.01	0 ± 0	0.28 ± 0.03	0 ± 0	0 ± 0	0.34 ± 0.001	**24.9**
L. plantarum and *O. oeni*	12.71 ± 0.24	0 ± 0	0 ± 0	0 ± 0	0 ± 0	15.85 ± 4.75	0.05 ± 0.004	0.60 ± 0.07	0.24 ± 0.02	0 ± 0	0 ± 0	0.35 ± 0.02	**29.8**

TABLE 3.14

Higher alcohol concentrations (mg/L) at the end of standard fermentations with the different yeast and bacterial treatments in both co- and sequential inoculation. The concentrations given for the sequential inoculations are end point values after MLF. Values are the average of 3 biological repeats ± standard deviation.

	Propanol	Isobutanol	Butanol	Isoamyl alcohol	Pentanol	3-Methyl-1-pentanol	3-Ethoxy-1-propanol	Hexanol	2-Phenylethanol	Totals
Cross Evolution										
Control yeast without inoculated *L. plantarum*	53.10 ± 7.03	15.46 ± 2.10	0.88 ± 0.03	99.20 ± 0.25	0.44 ± 0.002	0 ± 0	2.92 ± 0.53	0.46 ± 0.01	19.45 ± 1.55	**191.91**
Control yeast without inoculated *O. oeni*	24.90 ± 4.12	8.97 ± 0.09	0.89 ± 0.01	65.86 ± 0.49	0.43 ± 0	0 ± 0	2.61 ± 0.0004	0.47 ± 0.003	15.01 ± 0.12	**119.14**
Control yeast without inoculated *L. plantarum* and *O. oeni*	30.90 ± 0	10.08± 0.08	0.84 ± 0.01	69.71 ± 1.61	0.44 ± 0	0 ± 0	3.75 ± 3.66E-05	0.47 ± 0.03	19.26 ± 0.46	**135.45**
Co-inoculation										
L. plantarum	39.25 ± 9.32	11.79 ± 2.09	1.00 ± 0.10	90.88 ± 2.29	0.44 ± 0.01	0 ± 0	3.28± 0.56	0.47 ± 0.001	16.84 ± 3.22	**163.95**
O. oeni	29.01 ± 5.56	10.06 ± 0.79	0.77 ± 0.05	61.92 ± 5.61	0.44 ± 0	0 ± 0	3.01 ± 0.23	0.48 ± 0.01	15.09 ± 0.63	**120.78**
L. plantarum and *O. oeni*	33.90 ± 7.56	10.68 ± 0.69	0.97 ± 0.06	77.69 ± 1.11	0.43 ± 0	0 ± 0	3.73 ± 0.38	0.48 ± 0.01	19.73 ± 1.64	**147.61**
Sequential inoculation										
L. plantarum	46.00 ± 5.18	12.62 ± 0.63	0.87 ± 0.03	81.45 ± 0.25	0.44 ± 0.002	0 ± 0	3.33 ± 0.28	0.46 ± 0.001	17.12 ± 1.21	**162.29**
O. oeni	29.65 ± 9.28	10.70 ± 1.43	0.99 ± 0.08	65.90 ± 4.81	0.44 ± 0.003	0 ± 0	2.86 ± 0.46	0.48 ± 0.004	16.54 ± 2.25	**127.56**
L. plantarum and *O. oeni*	40.60 ± 5.84	10.86 ± 0.49	0.91 ± 0.01	90.71 ± 2.05	0.44 ± 0.001	0 ± 0	4.09 ± 0.34	0.47 ± 0.003	21.38 ± 0.97	**169.46**
EC 1118										
Control yeast without inoculated *L. plantarum*	76.11 ± 5.30	20.36 ± 0.56	0.88± 0.01	113.31 ± 1.21	0.43 ± 0.002	0 ± 0	9.82 ± 0.14	0.46 ± 0.01	18.79 ± 0.37	**240.16**
Control yeast without inoculated *O. oeni*	37.47 ± 0	13.11 ± 0.01	0.89 ± 0.01	72.94 ± 1.53	0.44 ± 0	0 ± 0	13.29 ± 0.0004	0.47 ± 0.36	17.57 ± 0.43	**156.18**
Control yeast without inoculated *L. plantarum* and *O. oeni*	33.90 ± 0	10.68 ± 0.70	0.97 ± 0.02	77.69 ± 5.38	0.43 ± 0	0 ± 0	3.73 ± 0.0004	0.48 ± 1.13	19.73 ± 1.39	**147.61**
Co-inoculation										
L. plantarum	62.48 ± 12.52	17.06 ± 2.45	0.91 ± 0.08	97.90 ± 3.39	0.44 ± 0.002	0 ± 0	9.97 ± 1.29	0.47 ± 0.001	17.97 ± 1.32	**207.2**
O. oeni	30.54 ± 12.97	11.53 ± 2.10	0.87 ± 0.09	76.60 ± 1.64	0.44 ± 0.004	0 ± 0	12.32 ± 2.49	0.49 ± 0.01	13.41 ± 2.83	**146.2**
L. plantarum and *O. oeni*	49.17 ± 14.62	11.46 ± 0.18	0.81 ± 0.08	69.74 ± 4.32	0.44 ± 0.002	0 ± 0	14.28 ± 1.43	0.47 ± 0.002	20.92 ± 2.80	**167.29**
Sequential inoculation										
L. plantarum	60.67 ± 13.02	17.62 ± 1.64	0.91 ± 0.03	114.78 ± 0.49	0.44 ± 0.004	0 ± 0	10.34 ± 0.48	0.46 ± 0.002	19.08 ± 0.01	**224.3**
O. oeni	41.01 ± 5.87	14. 00 ± 0.63	0.98 ± 0.02	82.62 ± 3.50	0.44 ± 0.002	0 ± 0	13.80 ± 1.09	0.47 ± 0.001	18.68 ± 1.31	**172**
L. plantarum and *O. oeni*	35.76 ± 11.91	11.08 ± 1.95	0.83 ± 0.09	83.77 ± 3.52	0.45 ± 0.02	0 ± 0	14.07 ± 3.11	0.48 ± 0.01	19.18 ± 2.84	**165.62**
Control: bacteria										
L. plantarum	0 ± 0	0 ± 0	0 ± 0	8.51 ± 0.04	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.41 ± 0.55	**10.59**
O. oeni	0 ± 0	0 ± 0	0 ± 0	8.48 ± 0.004	0 ± 0	0 ± 0	0± 0	0 ± 0	0.85 ± 0.003	**9.33**
L. plantarum and *O. oeni*	0 ± 0	0 ± 0	0 ± 0	8.49 ± 0.01	0 ± 0	0 ± 0	0 ± 0	0 ± 0	1.39 ± 0.95	**9.88**

TABLE 3.15

Fatty acids concentrations (mg/L) at the end of standard fermentations with the different yeast and bacterial treatments in both co- and sequential inoculation. The concentrations given for the sequential inoculations are end point values after MLF. Values are the average of 3 biological repeats ± standard deviation.

	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Hexanoic acid	Octanoic acid	Decanoic acid	Totals
Cross Evolution									
Control yeast without inoculated L. plantarum	1.39 ± 0.14	0.85 ± 0.16	0.10 ± 0.03	0.88 ± 0.11	0.55 ± 0.01	3.24 ± 0.89	2.63 ± 0.40	1.14 ± 0.38	**10.78**
Control yeast without inoculated *O. oeni*	1.14 ± 0.12	0.77 ± 0.04	0.08 ± 0	0.76 ± 0.04	0.51 ± 0.03	2.73 ± 0.06	1.87 ± 0.09	0.83 ± 0.07	**8.69**
Control yeast without inoculated *L. plantarum* and *O. oeni*	1.08 ± 0.07	0.64 ± 0.02	0.05 ± 0	0.72 ± 0.01	0.56 ± 0.06	1.56 ± 0.03	1.83 ± 0.06	0.69 ± 0.06	**7.13**
Co-inoculation									
L. plantarum	1.38 ± 0.19	0.74 ± 0.06	0.09 ± 0.02	0.79 ± 0.04	0.56 ± 0.06	2.54 ± 0.13	2.06 ± 0.17	0.64 ± 0.09	**8.8**
O. oeni	1.12 ± 0.05	0.78 ± 0.02	0.09 ± 0.02	0.76 ± 0.01	0.55 ± 0.01	2.99 ± 0.12	1.93 ± 0.07	0.71 ± 0.02	**8.93**
L. plantarum and *O. oeni*	1.16 ± 0.19	0.64 ± 0.03	0.07 ± 0.02	0.74 ± 0.02	0.60 ± 0.05	1.74 ± 0.04	2.30 ± 0.10	0.54 ± 0.06	**7.79**
Sequential inoculation									
L. plantarum	1.44 ± 0.14	0.75 ± 0.03	0.10 ± 0.01	0.79 ± 0.01	0.53 ± 0.03	2.53 ± 0.04	1.99 ± 0.05	0.83 ± 0.06	**8.96**
O. oeni	1.18 ± 0.18	0.79 ± 0.05	0.09 ± 0.02	0.77 ± 0.04	0.53 ± 0.04	2.46 ± 0.29	1.97 ± 0.17	0.90 ± 0.12	**8.69**
L. plantarum and *O. oeni*	1.27 ± 0.04	0.65 ± 0.03	0.06 ± 0.01	0.74 ± 0.02	0.62 ± 0.03	1.75 ± 0.14	2.36 ± 0.23	0.88 ± 0.13	**8.33**
EC 1118									
Control yeast without inoculated *L. plantarum*	1.71 ± 0.08	1.11 ± 0.01	0.11 ± 0.01	1.00 ± 0.02	0.68 ± 0.07	3.77 ± 0.14	3.14 ± 0.08	1.37 ± 0.04	**12.89**
Control yeast without inoculated *O. oeni*	1.62 ± 0.05	0.84 ± 0.06	0.10 ± 0.01	0.88 ± 0.06	0.56 ± 0.07	2.29 ± 0.45	2.54 ± 0.34	0.93 ± 0.14	**9.76**
Control yeast without inoculated *L. plantarum* and *O. oeni*	1.31 ± 0.13	0.74 ± 0.02	0.08 ± 0.01	0.78 ± 0.03	0.61 ± 0.07	2.21 ± 0.14	2.76 ± 0.23	1.07 ± 0.14	**9.56**
Co-inoculation									
L. plantarum	1.72 ± 0.40	0.99 ± 0.11	0.13 ± 0.03	0.92 ± 0.06	0.71 ± 0.09	2.93 ± 0.85	2.97 ± 0.47	1.02 ± 0.47	**11.39**
O. oeni	1.48 ± 0.20	0.85 ± 0.04	0.09 ± 0.02	0.85 ± 0.07	0.55 ± 0.05	2.46 ± 0.52	2.76 ± 0.64	0.94 ± 0.30	**9.98**
L. plantarum and *O. oeni*	1.35 ± 0.08	0.74 ± 0.02	0.08 ± 0.01	0.81 ± 0.05	0.71 ± 0.05	2.42 ± 0.15	3.38 ± 0.19	1.06 ± 0.08	**10.55**
Sequential inoculation									
L. plantarum	1.48 ± 0.28	0.92 ± 0.12	0.10 ± 0.02	0.88 ± 0.05	0.66 ± 0.08	3.13 ± 0.51	2.99 ± 0.56	1.32 ± 0.37	**11.48**
O. oeni	1.53 ± 0.14	0.89 ± 0.05	0.10 ± 0.01	0.92 ± 0.02	0.54 ± 0.05	2.46 ± 0.19	2.70 ± 0.07	1.06 ± 0.02	**10.2**
L. plantarum and *O. oeni*	1.24 ± 0.30	0.75 ± 0.07	0.10 ± 0.03	0.76 ± 0.03	0.61 ± 0.05	2.09 ± 0.32	2.96 ± 0.49	1.07 ± 0.34	**9.58**
Control: bacteria									
L. plantarum	0.60 ± 0.01	0 ± 0	0 ± 0	0.59 ± 0	0 ± 0	0.52 ± 0.04	0.66 ± 0	0 ± 0	**2.17**
O. oeni	0 ± 0	0.47 ± 0.02	0.01 ± 0	0.60 ± 0	0 ± 0	0.57 ± 0.01	0.67 ± 0.01	0 ± 0	**2.3**
L. plantarum and *O. oeni*	1.25 ± 0.21	0 ± 0	0 ± 0	0.80 ± 0.10	0 ± 0	0.51 ± 0.02	0.65 ± 0.01	0 ± 0	**2.67**

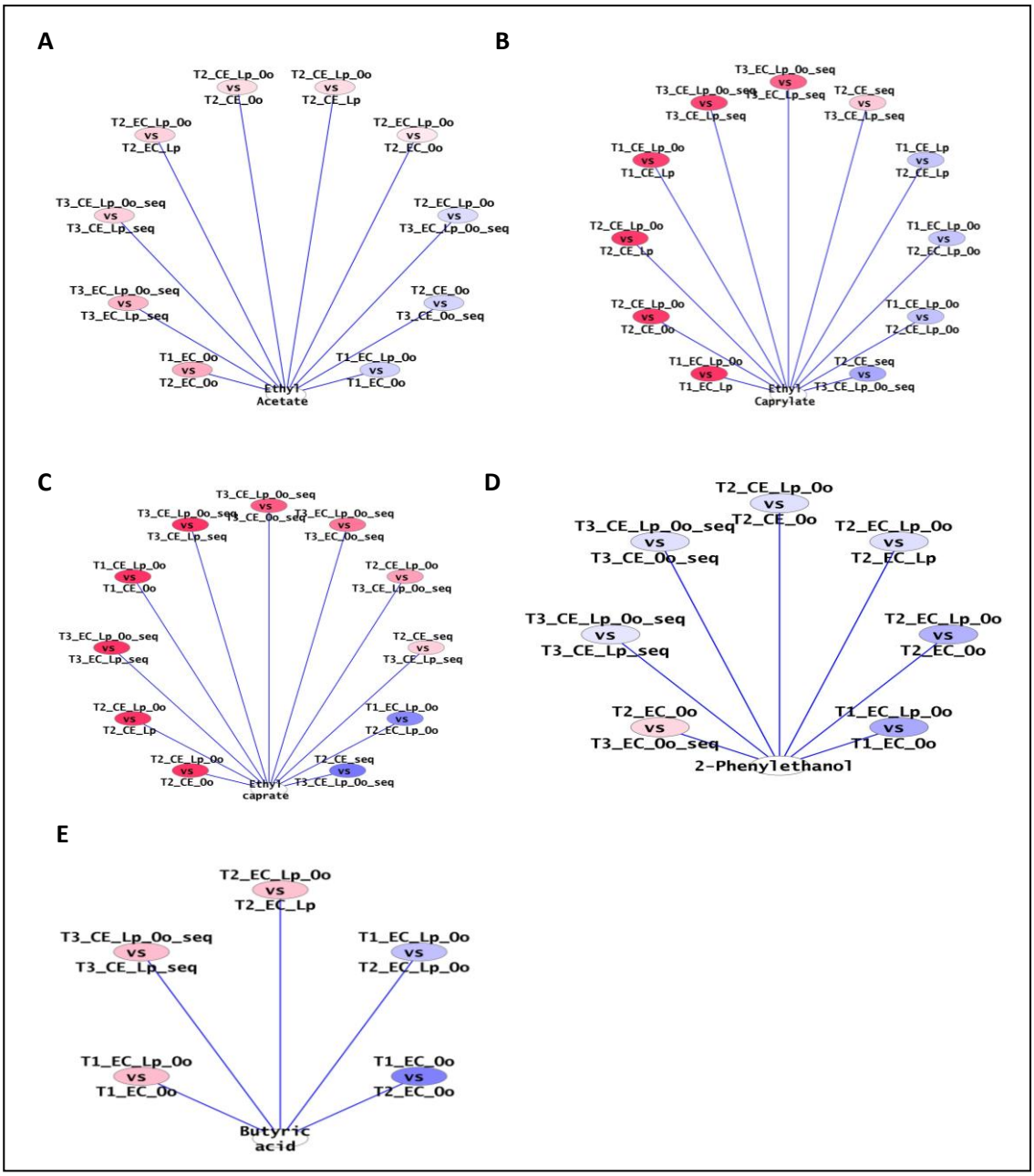

FIGURE 3.11

The aroma compounds of different treatments: ethyl acetate **(A)**, diethyl succinate **(B)**, ethyl caprylate **(C)** ethyl caprate **(D)** 2-phenylethanol and **(E)** butyric acid concentrations at the end of alcoholic and MLF. A blue node (ellipse) indicates a reduction and a red node an increase in the compounds. An increase in the colour intensity indicates the magnitude of the fold change observed. **CE_Lp**: Cross Evolution co-inoculated with *L. plantarum*; **CE_Oo:** Cross Evolution co-inoculated with *O. oeni*; **CE_Lp_Oo:** Cross Evolution co-inoculated with bacterial combination of *L. plantarum* and *O. oeni*; **EC_Lp:** EC1118 co-inoculated with *L. plantarum*; **EC_Oo:** EC1118 co-inoculated with *O. oeni*; **EC_Lp_Oo:** EC1118 co-inoculated with bacterial combination of *L. plantarum* and *O. oeni*; **CE_Lp_seq:** Cross Evolution sequential inoculation with *L. plantarum*; **CE_Oo_seq:** Cross Evolution sequential inoculation with *O. oeni*; **CE_Lp_Oo_seq:** Cross Evolution sequential inoculation with the bacterial combination of *L. plantarum* and *O. oeni*; **EC_Lp_seq:** EC1118 sequential inoculation with *L. plantarum*; **EC_Oo_seq:** EC1118 sequential inoculation with *O. oeni*; **EC_Lp_Oo_seq:** EC1118 sequential inoculation with the bacterial combination of *L. plantarum* and *O. oeni*; **T1:** half point of alcoholic fermentation; **T2:** end point of alcoholic fermentation; **T3:** end point of MLF.

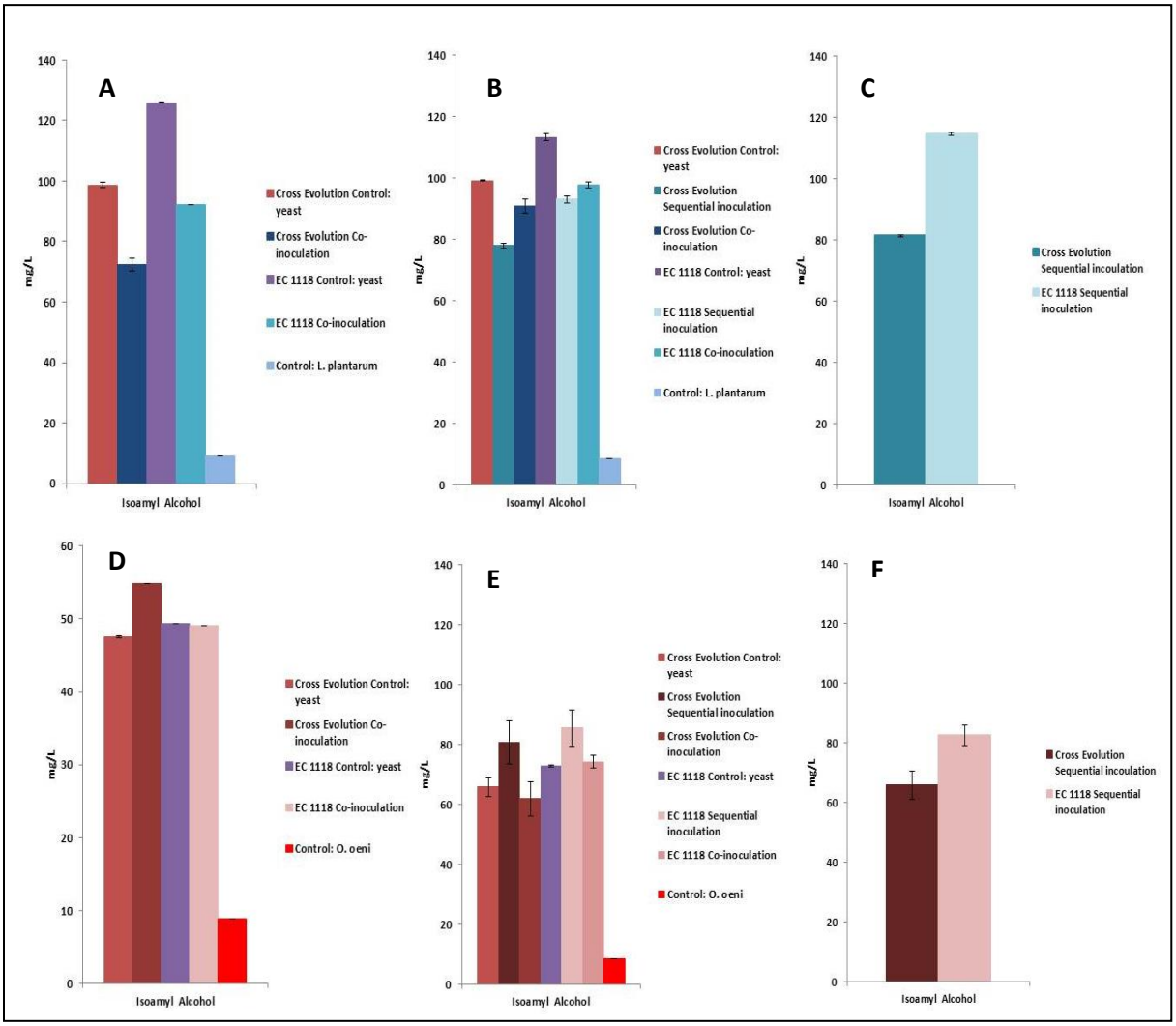

FIGURE 3.12

Isoamyl alcohol concentrations at middle AF (A), end AF (B), and the end of sequential inoculation (C) in fermentations inoculated with *L. plantarum* and at middle AF (D), end AF (E) and at the of end of sequential inoculation (F) in fermentations inoculated with *O. oeni*. Values are the average of 3 biological repeats ± standard deviation.

Multivariate statistical analysis

The principal component analysis (PCA) was conducted on the GC-FID generated data of the control fermentations to investigate correlations between different treatment samples, co-inoculated with *L. plantarum*, *O. oeni* and the combination of these bacteria **(Figure 3.13)**. Samples that are in close proximity to one another are most similar in terms of the total metabolic fingerprint (concentrations of higher alcohols and esters) of these samples. The PCA thus shows a largely separate grouping for samples inoculated with *L. plantarum*, while the *O. oeni* and bacterial combination–inoculated samples fall within the same broad clusters. This suggests that the metabolic activity of *O. oeni* in the mixed inoculum is the main driver responsible for the MLF–dependent aroma profile modification seen in these fermentations.

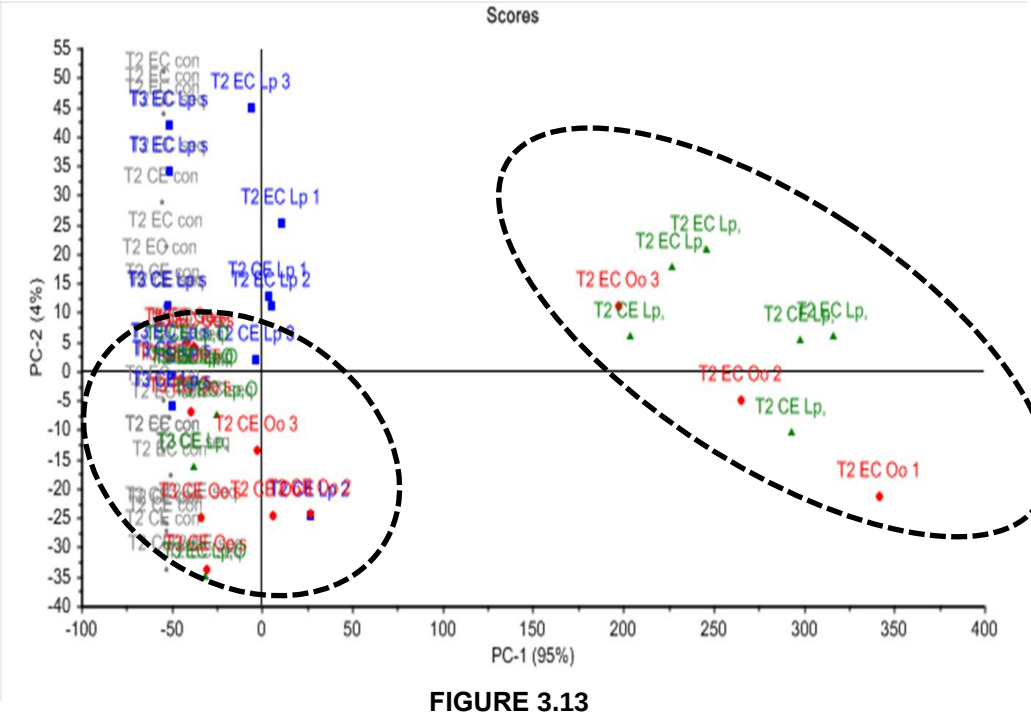

FIGURE 3.13

PCA bi-plot of scores (in blue, red and green) of the GC-FID generated data for the control fermentations after completion of MLF inoculated with *L. plantarum* (blue), *O. oeni* (red) and the bacterial combination (green). Control fermentations (yeast only) are indicated in grey.

3.3.4.2 NH_4Cl-supplemented fermentations

Esters

There was an increase in the total ester concentrations when co-inoculation and sequential inoculation strategies were compared (**Table 3.16**). Cross Evolution® co-inoculated with *O. oeni* resulted in fermentations with the highest concentration (120.74 mg/L) of total esters and EC1118® inoculated with *L. plantarum* resulted in fermentations with the lowest concentration of total esters (56.35 mg/L). The Cross Evolution® sequential inoculation strategy with the bacterial combination showed the highest ester concentration (97.49 mg/L) and EC1118® fermentations inoculated with *L. plantarum* showed the lowest concentration (50.85 mg/L).

The most dominant ester after MLF was ethyl lactate. The co-inoculation treatments showed higher concentrations than sequential inoculation strategies at the end points. Fermentations inoculated with *O. oeni* resulted in the greatest ethyl lactate production with an average value of 86.47 mg/L. According to the bubble graph the concentration of ethyl lactate was significantly higher for the NH_4Cl supplemented fermentations than fermentations supplemented with the amino acids as nitrogen source (**Figure 3.14A**).

Ethyl acetate was the second highest ester detected after MLF. Co-inoculation strategies in combination with Cross Evolution® showed the highest concentrations, especially *L. plantarum* while for sequential inoculation strategies in combination with EC1118®, the *O. oeni* treatment presented the highest concentration (**Addendum Figure 2 A1**).

Isoamyl acetate concentrations were higher in the sequential than corresponding co-inoculated fermentations. The inoculation strategies involving *L. plantarum* showed the highest final

concentrations of isoamyl acetate. The bubble graph shows that single LAB strains of amino acids fermentations resulted in higher concentrations compared to NH_4Cl fermentations (**Figure 3.14B**). The sequential inoculation strategies resulted in the highest hexyl acetate and ethyl-3-hydroxybutanoate concentrations compared to the corresponding co-inoculated fermentations. Ethyl caprate concentrations were highest for the sequential inoculations in combination with Cross Evolution® but highest for co-inoculation strategies with EC1118®. Co-inoculation of *L. plantarum* and *O. oeni* in combination with Cross Evolution® resulted in the highest ethyl phenyl acetate concentrations, however no ethyl phenyl acetate was detected in the case of the bacterial combination. For the EC1118® treatments the sequential inoculation strategy showed highest ethyl phenyl acetate concentrations compared to the co-inoculated treatments.

Higher alcohols

The sequential strategies showed higher concentrations of total higher alcohols than yeast control fermentations (**Table 3.17**). The co-inoculated strategies showed lower total higher alcohols at the end of AF compared to the yeast–only control fermentations. Of the co-inoculated treatments, Cross Evolution® co-inoculated with *O. oeni* showed the highest concentration (268.84 mg/L) and EC1118® co-inoculated with *L. plantarum* the lowest concentration (173.89 mg/L) of total higher alcohols. For the sequentially inoculated fermentations the combination of Cross Evolution® and *O. oeni* led to the highest concentration of 229.95 mg/L higher alcohols while the Cross Evolution® and bacterial combination treatment led to the lowest concentrations of 176.24 mg/L on average.

Propanol and butanol concentrations were the highest in the co-inoculated fermentations while the sequential inoculation strategies resulted in higher concentrations of isobutanol. 2-Phenylethanol production was lower in fermentations conducted with the ammonium containing medium versus the amino acids supplemented medium (**Figure 3.14C**). The co-inoculation strategies resulted in the highest 3-ethoxy-1-propanol and hexanol concentrations.

Isoamyl alcohol was produced throughout fermentation at the highest level compared to the other higher alcohols. (**Addendum Figure 2 A2**). Fermentations conducted with EC1118® co-inoculated with *L. plantarum* showed the highest average final value of 109.38 mg/L.

Isobutanol was the next most important higher alcohol (in quantitative terms) at the end of AF and MLF. The Cross Evolution® co-inoculation with the *O.oeni* showed the highest concentration (88.37 mg/L) and for EC1118® sequential inoculation, the bacterial combination showed the highest concentration (81.57 mg/L). According to the bubble graph LAB strains produced less isobutanol concentration when compared to amino acids fermentation (**Figure 3.14D**).

Fatty acids

The yeast control fermentations had the highest total fatty acid concentrations (**Table 3.18**). The co-inoculation strategies resulted in lower fatty acid concentrations compared to the yeast control fermentations except for both yeast strains in combination with *L. plantarum*. Cross Evolution® co-inoculated with *L. plantarum* showed the highest concentration of 9.76 mg/L total fatty acids and Cross Evolution® co-inoculated with the bacterial combination showed the lowest concentration of 5.29 mg/L. For the sequentially inoculated fermentations Cross Evolution® in combination with *L. plantarum* led to the highest concentration (7.34 mg/L) fatty

acids and Cross Evolution® with the bacterial combination had the lowest concentration (4.91 mg/L).

The sequential inoculation strategies resulted in higher concentrations of isobutyric acid compared to the co-inoculated fermentations. For Cross Evolution® treatments the co-inoculation treatments resulted in the highest butyric acid concentrations and for EC1118® the sequential treatments led to higher butyric acid levels at the end of AF and MLF. Conversely, the sequential inoculation fermentations with Cross Evolution® led to the highest isovaleric acid concentration while for EC1118® the co-inoculated fermentation presented higher isovaleric acid concentrations compared to the sequential fermentation using this yeast strain. This clearly points to the yeast strain-specific impact of co- versus sequential inoculation. For valeric acid concentrations, production was higher for the sequentially inoculated fermentations compared to the corresponding co-inoculated fermentations. Octanoic acid concentrations were higher for the co-inoculated than the sequential inoculated strategies. The bubble graph showed NH_4Cl fermentations production of octanoic acid was lower compared to amino acids fermentations (**Figure 3.14E**).

Isobutyric acid was the fatty acid with the highest concentrations after MLF. The sequential inoculations showed higher concentrations than co-inoculated fermentation. The Cross Evolution® co-inoculation with *L. plantarum* had the highest concentration (3.55 mg/L). For the EC1118® sequential inoculation strategy, the bacterial combination showed the highest concentration (1.61 mg/L). The bubble graph shows that NH_4Cl fermentations produced significant lower concentrations when it is compared to the amino acids fermentations (**Figure 3.14F**).

Propionic acid was quantitatively the second highest fatty acid after AF and MLF. Sequential inoculation strategies led to higher concentrations than co-inoculation. Single LAB strains resulted in higher concentrations (**Addendum Figure2 A3**). For the Cross Evolution® co-inoculation, *L. plantarum* showed the highest concentration (1.56 mg/L) while the bacterial combination showed the highest concentration for EC1118® treatment (1.27 mg/L).

TABLE 3.16

Ester concentrations (mg/L) at the end of NH_4Cl supplemented fermentations of the different inoculation strategies. Values given for the sequential inoculations are the final concentrations at the end of MLF. Values are the average of 3 biological repeats ± standard deviation.

	Ethyl acetate	Ethyl butyrate	Isoamyl acetate	Ethyl hexanoate	Hexyl acetate	Ethyl lactate	Ethyl caprylate	Ethyl-3-hydroxybutanoate	Ethyl caprate	Diethyl succinate	Ethyl phenylacetate	2-Phenylethyl Acetate	Totals
Cross Evolution													
Control yeast without inoculated *L. plantarum*	31.36 ± 1.06	0 ± 0	0.53 ± 0.19	0.39 ± 0.01	0.07 ± 0	16.97 ± 0.66	0.10 ± 0.01	0.92 ± 0.03	0.12 ± 0.01	0.47 ± 0	1.67 ± 0.19	0.48 ± 0.03	**53.08**
Control yeast without inoculated *O. oeni*	35.98 ± 2.42	0 ± 0	0.15 ± 0.02	0.21 ± 0.01	0 ± 0	17.51 ± 2.85	0.05 ± 0	1.03 ± 0.03	0.06 ± 0	0.32 ± 0.01	1.57 ± 0.01	0.25 ± 0.01	**57.13**
Control yeast without inoculated *L. plantarum* and *O. oeni*	37.36 ± 0.55	0 ± 0	0.16 ± 0.01	0.22 ± 0	0.02 ± 0	16.08 ± 0.18	0.08 ± 0	1.04 ± 0.04	0.06 ± 0	0.27 ± 0	1.63 ± 0.02	0.26 ± 0	**57.18**
Co-inoculation													
L. plantarum	35.98 ± 0.18	0 ± 0	0.34 ± 0.01	0.37± 0.01	0 ± 0	41.92 ± 0.90	0.12 ± 0.002	0.86 ± 0.01	0.12 ± 0.003	0.48 ± 0.004	0.52 ± 0.02	0.41 ± 0.01	**81.12**
O. oeni	32.71 ± 1.58	0 ± 0	0.25 ± 0.005	0.22 ± 0.01	0.03 ± 0.002	85.49 ± 1.06	0.05 ± 0.01	1.06 ± 0.0003	0.05 ± 0.01	0.28 ± 0.01	0.25 ± 0.01	0.35 ± 0.01	**120.74**
L. plantarum and *O. oeni*	26.61 ± 0.98	0 ± 0	0.11 ± 0.002	0.20 ± 0.003	0.02 ± 0.003	60.86 ± 0.35	0.05 ± 0.01	0 ± 0	0.05 ± 0.2	0 ± 0	0 ± 0	0.27 ± 0.01	**88.17**
Sequential inoculation													
L. plantarum	30.01 ± 2.69	0 ± 0	0.66 ± 0.01	0.42 ± 0.01	0.07 ± 0.004	18.35 ± 0.23	0.11 ± 0.004	0.93 ± 1.36E-16	0.13 ± 0.01	0.50 ± 0.04	0.47 ± 0.002	1.18 ± 0.03	**52.83**
O. oeni	42.22 ± 2.71	0 ± 0	0.22 ± 0.02	0.23 ± 0.01	0 ± 0	28.83 ±0.57	0.06 ± 0.002	1.01 ± 0.01	0.07 ± 0.004	0.43 ± 0.02	0.29 ± 0.01	1.22 ± 0.02	**74.58**
L. plantarum and *O. oeni*	21.93 ± 1.77	0 ± 0	0.11 ± 0.01	0 ± 0	0.02 ± 0.003	73.99 ± 0.86	0.04 ± 0.002	1.00 ± 0.01	0.05 ± 0.004	0.35 ± 0.24	0 ± 0	0 ± 0	**97.49**
EC 1118													
Control yeast without inoculated *L. plantarum*	31.52 ± 0	0 ± 0	0.46 ± 0.03	0.39 ± 0.003	0.07 ± 0.001	14.62 ± 0.46	0.11 ± 0.002	0.98 ± 0.004	0.14 ± 0.003	0.45 ± 0.004	0.88 ± 0.06	0.45 ± 0.01	**50.07**
Control yeast without inoculated *O. oeni*	38.78 ± 1.27	0 ± 0	0.11 ± 0.01	0.20 ± 0.004	0.09 ± 0.002	19.95 ± 0.71	0.05 ± 0.003	1.07 ± 0.05	0.07 ± 0.003	0.29 ± 0.01	0.77 ± 0.04	0.33 ± 0.01	**61.71**
Control yeast without inoculated *L. plantarum* and *O. oeni*	30.71 ± 0.64	0 ± 0	0.09 ± 0	0 ± 0	0 ± 0	12.50 ± 0.02	0.05 ± 0.002	1.06 ± 0.01	0.04 ± 0.002	0.49 ± 0.01	0 ± 0	0.24 ± 0.004	**45.18**
Co-inoculation													
L. plantarum	18.55 ± 0.51	0 ± 0	0.33 ± 0.02	0.34 ± 0.03	0.08 ± 0.003	35.87 ± 0.76	0.01 ± 0.002	0 ± 0	0.22 ± 0.04	0.44 ± 0.03	0.06 ± 0.002	0.45 ± 0.03	**56.35**
O. oeni	29.90 ± 0.32	0 ± 0	0.13 ± 0.01	0.21 ± 0.01	0.03 ± 0.003	87.45 ± 1.66	0.05 ± 0.002	1.04 ± 0.04	0.34 ± 0.44	0.27 ± 0.003	0 ± 0	0.26 ± 0.02	**119.68**
L. plantarum and *O. oeni*	27.38 ± 1.27	0 ± 0	0.10 ± 0.002	0.22 ± 0.03	0 ± 0	55.71 ± 0.50	0.09 ± 0.002	0 ± 0	0.08 ± 0.001	0 ± 0	0 ± 0	0.29 ± 0.02	**83.87**
Sequential inoculation													
L. plantarum	29.29 ± 0.34	0 ± 0	0.35 ± 0.03	0.38 ± 0.01	0.08 ± 0.004	18.16 ± 0.10	0.10 ± 0.001	0.93 ± 0.02	0.11 ± 0.01	0.42 ± 0.02	0.46 ± 0.01	0.57 ± 0.02	**50.85**
O. oeni	31.40 ± 1.25	0 ± 0	0.15 ± 0.04	0.23 ± 0.01	0.06 ± 0.004	29.51 ± 1.23	0.05 ± 0.002	0 ± 0	0.06 ± 0.004	0.33 ± 0.03	0.28 ± 0.004	0.38 ± 0.50	**62.45**
L. plantarum and *O. oeni*	25.76 ± 0.22	0 ± 0	0.10 ± 0.002	0.20 ± 0.0004	0 ± 0	67.74 ± 0.47	0.07 ± 0.002	1.06 ± 0.01	0.05 ± 0.002	0.24 ± 0.03	0.50 ± 0.01	0 ± 0	**95.72**
Control: bacteria													
L. plantarum	0 ± 0	0 ± 0	0.36 ± 0.04	0.25 ± 0.21	0 ± 0	12.06 ± 0.27	0.06 ± 0.002	0 ± 0	0.10 ± 0.002	0 ± 0	0.42 ± 0.01	0.67 ± 0.11	**13.92**
O. oeni	3.38 ± 0.08	0 ± 0	0 ± 0	0 ± 0	0 ± 0	8.64 ± 0.21	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	**12.02**
L. plantarum and *O. oeni*	3.17 ± 0.02	0 ± 0	0 ± 0	0 ± 0	0 ± 0	8.37 ± 0.03	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	**11.54**

TABLE 3.17:

Higher alcohol concentrations (mg/L) at the end of NH_4Cl supplemented fermentations of the different inoculation strategies. Values given for the sequential inoculations are the final concentrations at the end of MLF. Values are the average of 3 biological repeats ± standard deviation.

	Propanol	Isobutanol	Butanol	Isoamyl alcohol	Pentanol	3-Methyl-1-pentanol	3-ethoxy-1-propanol	Hexanol	2-Phenylethanol	Totals
Cross Evolution										
Control yeast without inoculated *L. plantarum*	40.16 ± 0.71	52.63 ± 1.07	3.30 ± 0.02	168.42 ± 0.22	0 ± 0	0 ± 0	0.37 ± 0.002	0 ± 0	23.31 ± 0.11	**288.43**
Control yeast without inoculated *O. oeni*	33.12 ± 0	63.15 ± 0	1.16 ± 0.01	100.76 ± 0.17	0 ± 0	0 ± 0	0.26 ± 0.004	0 ± 0	12.83 ± 0.04	**211.28**
Control yeast without inoculated *L. plantarum* and *O. oeni*	29.15 ± 2.08	86.57 ± 0.29	0.73± 0.005	101.80 ± 0.90	0 ± 0	0 ± 0	0.25 ± 0	0 ± 0	12.45± 0.07	**230.95**
Co-inoculation										
L. plantarum	39.31 ± 1.11	42.04 ± 0.77	2.79 ± 0.03	94.07 ± 0.61	0 ± 0	0 ± 0	0.38 ± 0.01	1.40 ± 0	11.07 ± 0.49	**191.32**
O. oeni	36.99 ± 0.91	88.37 ± 0.40	1.07 ± 0.02	113.31 ± 1.53	0 ± 0	0 ± 0	0.26 ± 0	1.51 ± 0.06	27.33 ± 1.37	**268.84**
L. plantarum and *O. oeni*	33.76 ± 1.32	83.72 ± 1.40	0.97 ± 0.03	77.61 ± 1.10	0 ± 0	0 ± 0	0.25 ± 0	0 ± 0	9.50 ± 0.03	**205.81**
Sequential inoculation										
L. plantarum	29.51 ± 2.48	33.26 ± 1.70	2.32 ± 6.33	117.92 ± 6.44	0 ± 0	0 ± 0	0.36 ± 0.75	1.73 ± 0.95	19.01 ± 0.22	**177.11**
O. oeni	28.81 ± 2.17	81.33 ± 1.45	1.06 ± 0.04	105.49 ± 1.59	0 ± 0	0 ± 0	0.26 ± 0.01	0 ± 0	13.00 ± 0.42	**229.95**
L. plantarum and *O. oeni*	26.04 ± 1.21	71.43 ± 0.66	0.94 ± 0.11	69.03 ± 0.89	0 ± 0	0 ± 0	0 ± 0	0.25 ± 0.002	8.55 ± 0.10	**176.24**
EC 1118										
Control yeast without inoculated *L. plantarum*	0 ± 0	0 ± 0	1.17 ± 0.01	108.25 ± 0.02	0 ± 0	0 ± 0	0.36 ± 0.001	1.89 ± 0	21.39 ± 0.03	**133.06**
Control yeast without inoculated *O. oeni*	27.39 ± 0	63.15 ± 0	1.16 ± 0.03	100.76 ± 6.86	0 ± 0	0 ± 0	0.26 ± 0.001	0 ± 0.	12.83 ± 2.07	**205.55**
Control yeast without inoculated *L. plantarum* and *O. oeni*	15.85 ± 0.62	23.86 ± 0.42	0.54 ± 0.03	28.32 ± 0.43	0 ± 0	0 ± 0	0 ± 0	0.25 ± 0.18	6.58 ± 0.002	**75.4**
Co-inoculation										
L. plantarum	30.12 ± 0	0 ± 0	0.96 ± 0.11	121.12 ± 2.71	0 ± 0	0 ± 0	0.36 ± 0	1.67 ± 0.06	19.66 ± 0.64	**173.89**
O. oeni	25.32 ± 0.55	45.85 ± 3.33	1.05 ± 0.02	105.78 ± 1.35	0 ± 0	0 ± 0	0.26 ± 0.01	3.99 ± 0.87	13.94 ± 0.88	**196.19**
L. plantarum and *O. oeni*	31.05 ± 0.74	59.99 ± 0.12	0.79 ± 0.01	76.13 ± 1.60	0 ± 0	0 ± 0	0.25 ± 0	2.63 ± 0.02	12.24 ± 0.38	**183.08**
Sequential inoculation										
L. plantarum	31.87 ± 1.70	42.75 ± 0.58	1.13 ± 0.10	104.41 ± 0.29	0 ± 0	0 ± 0	0.36 ± 0.002	0 ± 0	25.90 ± 0.42	**206.42**
O. oeni	29.89 ± 0.84	65.84 ± 1.44	0.83 ± 0.02	94.77 ± 0.15	0 ± 0	0 ± 0	0.25 ± 0.004	1.87 ± 0.03	21.14 ± 0.96	**214.5**
L. plantarum and *O. oeni*	21.92 ± 1.53	81.57 ± 0.96	1.07 ± 0.004	71.46 ± 0.35	0 ± 0	0 ± 0	0 ± 0	0.25 ± 0.002	14.98 ± 0.06	**191.25**
Control: bacteria										
L. plantarum	0 ± 0	0 ± 0	0.71 ± 0.08	7.56 ± 3.05	0 ± 0	0 ± 0	0.36 ± 0	1.68 ± 0.05	6.33 ± 0.28	**16.64**
O. oeni	0 ± 0	0 ± 0	0 ± 0	5.86 ± 0.08	0 ± 0	0 ± 0	0.25 ± 0	0 ± 0	0.59 ± 0.02	**6.7**
L. plantarum and *O. oeni*	0 ± 0	0 ± 0	0 ± 0	6.71 ± 0.02	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.79 ± 0.02	**7.5**

TABLE 3.18

Fatty acid concentrations (mg/L) at the end of NH_4Cl supplemented fermentations of the different inoculation strategies. Values given for the sequential inoculations are the final concentrations at the end of MLF. Values are the average of 3 biological repeats ± standard deviation.

	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Hexanoic acid	Octanoic acid	Decanoic acid	Totals
Cross Evolution									
Control yeast without inoculated *L. plantarum*	1.67 ± 0.09	1.92 ± 0.28	0.94 ± 0.02	0.66 ± 0.02	0.37 ± 0	0.80 ± 0.03	1.08 ± 0.03	1.01 ± 0.08	**8.45**
Control yeast without inoculated *O. oeni*	1.20 ± 0	2.77 ± 0.01	0.54 ± 0.01	0.66 ± 0.04	0.43 ± 0.01	0.43 ± 0.02	0.62 ± 0.02	0.53 ± 0.01	**7.18**
Control yeast without inoculated *L. plantarum* and *O. oeni*	1.02 ± 0.01	2.99 ± 0.04	0.50 ± 0.01	0.84 ± 0.01	0.34 ± 0.01	0.51 ± 0.01	0.66 ± 0.02	0.47 ± 0.01	**7.33**
Co-inoculation									
L. plantarum	1.56 ± 0.03	3.55 ± 0.04	1.00 ± 0.01	0.68 ± 0.03	0.36 ± 0	0.84 ± 0.05	0.96 ± 0.01	0.81 ± 0.01	**9.76**
O. oeni	1.17 ± 0.02	1.44 ± 0.04	0.48 ± 0.01	0.66 ± 0.03	0.31 ± 0.01	0.55 ± 0.03	0.74 ± 0.04	0.43 ± 0.02	**5.78**
L. plantarum and *O. oeni*	0.91 ± 0.01	1.54 ± 0.05	0.47 ± 0.01	0.60 ± 0.01	0.26 ± 0	0.50 ± 0.01	0.63 ± 0.02	0.38 ± 0.01	**5.29**
Sequential inoculation									
L. plantarum	1.21 ± 0.01	1.31 ± 0.02	0.82 ± 0.02	0.80 ± 0.04	0.37 ± 0.001	1.05 ± 0.02	0.95 ± 0.02	0.83 ± 0.03	**7.34**
O. oeni	1.03 ± 0.06	2.42 ± 0.01	0.51 ± 0.03	0.86 ± 0.05	0.38 ± 0.01	0.53 ± 0.02	0.86 ± 0.01	0.46 ± 0.03	**7.05**
L. plantarum and *O. oeni*	0.85 ± 0.01	1.36 ± 0.03	0.45 ± 0.04	0.61 ± 0.06	0.26 ± 0.01	0.46 ± 0.02	0.57 ± 0.03	0.35 ± 0.03	**4.91**
EC 1118									
Control yeast without inoculated *L. plantarum*	1.16 ± 0.01	1.22 ± 0.04	0.74 ± 0.02	0.78 ± 0.03	0.39 ± 0.01	0.84 ± 0.07	1.17 ± 0.02	0.89 ± 0.03	**7.19**
Control yeast without inoculated *O. oeni*	1.55 ± 0.04	1.92 ± 0.01	0.60 ± 0.04	0.61 ± 0.04	0.55 ± 0.03	0.61 ± 0.02	1.25 ± 0.03	0.55 ± 0.02	**7.64**
Control yeast without inoculated *L. plantarum* and *O. oeni*	1.25 ± 0.01	1.67 ± 0.03	0.50 ± 0.01	0.52 ± 0.02	0.25 ± 0	0.44 ± 0.01	0.71 ± 0.01	0.66 ± 0.01	**6**
Co-inoculation									
L. plantarum	1.24 ± 0.02	1.58 ± 0.15	0.77 ± 0.02	0.84 ± 0.04	0.39 ± 0.01	0.87 ± 0.01	1.10 ± 0.01	0.84 ± 0.02	**7.63**
O. oeni	1.04 ± 0.03	0.70 ± 0.01	0.52 ± 0.02	0.50 ± 0.01	0.28 ± 0.02	0.81 ± 0.03	1.16 ± 0.01	0.69 ± 0.08	**5.7**
L. plantarum and *O. oeni*	0.90 ± 0.03	1.17 ± 0.02	0.50 ± 0	0.59 ± 0.01	0.26 ± 0.01	0.61 ± 0.02	0.83 ± 0.03	0.59 ± 0.01	**5.45**
Sequential inoculation									
L. plantarum	1.20 ± 0.04	1.21 ± 0.14	0.74 ± 0.01	0.73 ± 0.02	0.38 ± 0.003	0.75 ± 0.03	1.05 ± 0.02	0.77 ± 0.02	**6.83**
O. oeni	0.91 ± 0.02	0.80 ± 0.01	0.45 ± 0.04	0.55 ± 0.04	0.36 ± 0.04	0.45 ± 0.03	1.14 ± 0.01	0.59 ± 0.02	**5.25**
L. plantarum and *O. oeni*	1.27 ± 0.02	1.61 ± 0.01	0.56 ± 0.02	0.53 ± 0.03	0.25 ± 0.01	0.46 ± 0.002	0.77 ± 0.003	0.63 ± 0.01	**6.08**
Control: bacteria									
L. plantarum	0.90 ± 0.07	0.77 ± 0.10	1.04 ± 0.04	0.64 ± 0.03	0.37 ± 0	0.67 ± 0.11	1.06 ± 0.03	1.07 ± 0.03	**6.52**
O. oeni	0 ± 0	0 ± 0	0 ± 0	0.63 ± 0.01	0 ± 0	0 ± 0	0.25 ± 0.003	0 ± 0	**0.88**
L. plantarum and *O. oeni*	0.65 ± 0.03	0.38 ± 0	0 ± 0	0.71 ± 0	0 ± 0	0.24 ± 0	0.41 ± 0.01	0.35 ± 0.01	**2.74**

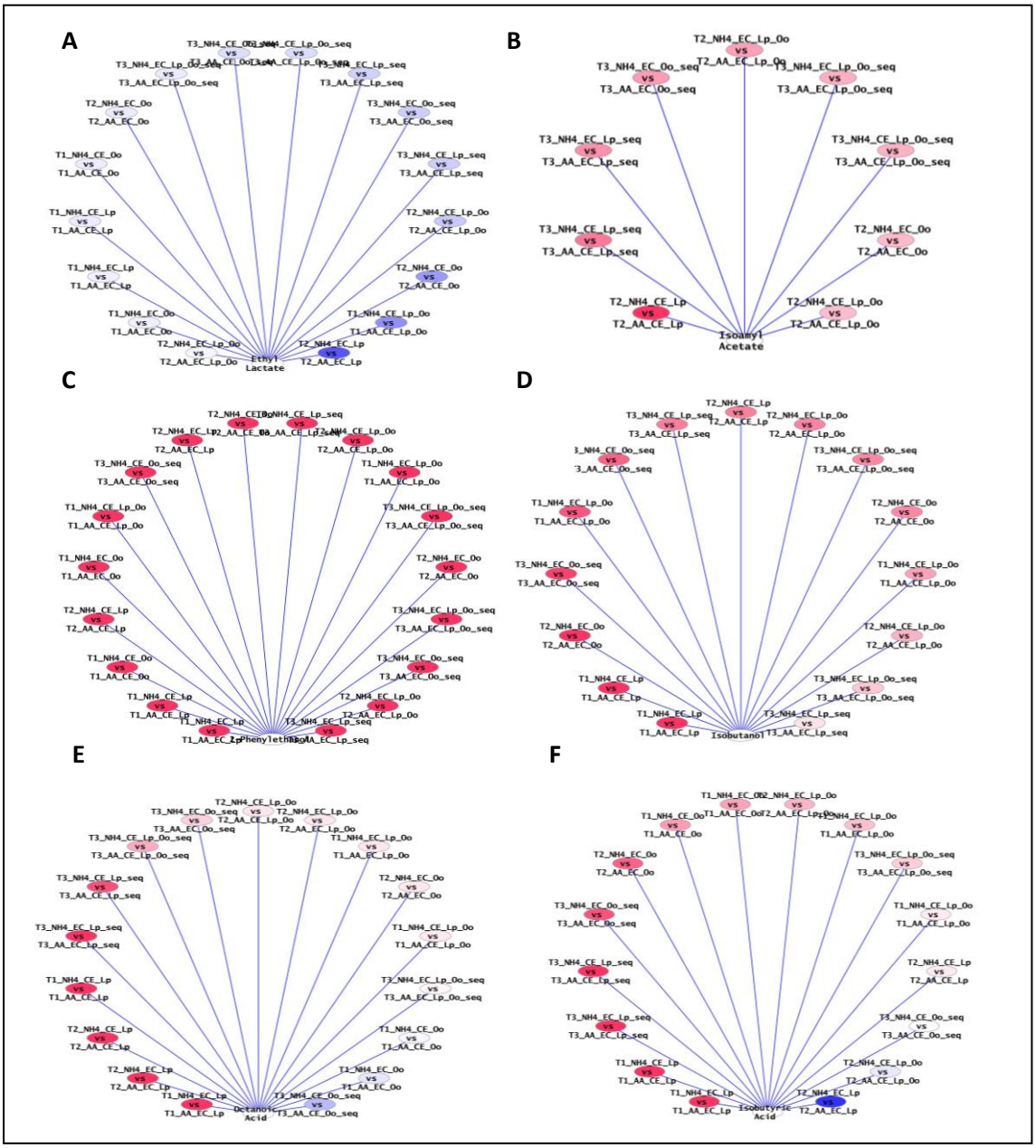

FIGURE 3.14

The aroma compounds of different nitrogen additions are compared at the end of alcoholic and MLF: ethyl lactate **(A)**, isoamyl acetate **(B)**, 2-phenylethanol **(C)** isobutanol **(D),** octanoic acid **(E)** and isobutyric acid **(F)**. A blue node (ellipse) indicates a reduction and a red node an increase in the compounds. An increase in the colour intensity indicates the magnitude of the fold change observed. **CE_Lp**: Cross Evolution co-inoculated with *L. plantarum*; **CE_Oo:** Cross Evolution co-inoculated with *O. oeni*; **CE_Lp_Oo:** Cross Evolution co-inoculated with bacterial combination of *L. plantarum* and *O. oeni*; **EC_Lp:** EC1118 co-inoculated with *L. plantarum*; **EC_Oo:** EC1118 co-inoculated with *O. oeni*; **EC_Lp_Oo:** EC1118 co-inoculated with bacterial combination of *L. plantarum* and *O. oeni*; **CE_Lp_seq:** Cross Evolution sequential inoculation with *L. plantarum*; **CE_Oo_seq:** Cross Evolution sequential inoculation with *O. oeni*; **CE_Lp_Oo_seq:** Cross Evolution sequential inoculation with bacterial combination of *L. plantarum* and *O. oeni*; **EC_Lp_seq:** EC1118 sequential inoculation with *L. plantarum*; **EC_Oo_seq:** EC1118 sequential inoculation with *O. oeni*; **EC_Lp_Oo_seq:** EC1118 sequential inoculation with bacterial combination of *L. plantarum* and *O. oeni*; **AA:** Amino acids supplementation; **NH_4Cl:** Ammonium supplementation; **T1:** half point of alcoholic fermentation; **T2:** end point of alcoholic fermentation; **T3:** end point of MLF.

3.3.4.3 Amino acids-supplemented fermentations

Esters

Overall, total ester concentrations increased in co-inoculation and sequential inoculation strategies compared to their respective control fermentations (**Table 3.19**). The EC 1118® fermentations co-inoculated with *O. oeni* resulted in the highest concentration of total esters (113.18 mg/L) while the combination of Cross Evolution® and *L. plantarum* resulted in fermentations with the lowest concentration of (19.03 mg/L). The EC1118® fermentations sequentially inoculated with the bacterial combination showed the highest ester concentration (85.77 mg/L) and Cross Evolution® sequentially inoculated with *L. plantarum* led to fermented medium with the lowest concentration of 55.87 mg/L esters.

Ethyl lactate was the dominant ester after MLF and co-inoculation strategies showed the highest concentrations. For the Cross Evolution® treatment, the co-inoculation strategy with the bacterial combination showed the highest concentration (45.94 mg/L) while for the EC1118® treatment, the co-inoculation strategy with *O. oeni* had the highest concentration (73.73 mg/L). Fermentations conducted with the amino acids supplemented media resulted in generally lower concentrations of esters compared to the fermentations supplemented with NH_4Cl as the sole nitrogen source (**Figure 3.14A**)**.**

Ethyl acetate showed the second highest concentration after MLF. The Cross Evolution® co-inoculation strategies resulted in fermentations with higher concentrations than the sequential inoculation. The EC1118® sequential inoculation had higher concentrations than the co-inoculation strategies, with the *L. plantarum* treatment resulting in the highest concentration (51.43 mg/L) (**Addendum Figure2 A1**).

The Cross Evolution® co-inoculation strategies showed the highest hexyl acetate concentrations and no concentrations were observed in the case of *O. oeni*, the bacterial combination and the sequentially inoculated bacterial combination. The ester, hexyl acetate, was not observed for the EC1118® co-inoculation strategy with *L. plantarum*, the bacterial combination as well as the sequential inoculation strategy in conjunction with the bacterial combination. For the Cross Evolution® treatment, the co-inoculation strategies resulted in fermentations with the highest ethyl caprylate concentration while in the case of the EC1118® strategies, the sequential inoculation fermentations resulted in the highest concentrations. This shows the impact of yeast strain specific effects on co- and sequential inoculation. The ethyl-3-hydroxybutanoate for the Cross Evolution®, the co- and sequential inoculation showed similar concentrations. For the EC1118® strategies the co-inoculation showed higher concentrations of this compound than the sequential inoculation strategies. The co-inoculation strategies resulted in higher concentrations of 2-phenyl acetate. For ethyl phenyl acetate the sequential inoculation strategy had higher concentrations. For isoamyl acetate, the Cross Evolution® co-inoculation strategy showed the highest concentrations and for EC1118®, the sequential inoculation strategies showed the highest concentrations. The bubble graph of isoamyl acetate shows that the amino acids fermentations with single LAB strains resulted in significantly higher concentrations than the NH_4Cl fermentations (**Figure 3.14B**).

Higher alcohols

The total higher alcohol concentrations were lower in co-inoculated and sequential inoculated strategies when compared to control fermentations (**Table 3.20**). Cross Evolution® co-inoculated with *L. plantarum* showed the highest concentration (1240.71 mg/L) and EC1118® with the bacterial combination showed the lowest concentration of 548.53 mg/L. The Cross Evolution® sequential inoculated strategy with *L. plantarum* showed the highest concentration (1063.07 mg/L) and Cross Evolution® with the bacterial combination showed the lowest concentration (630.12 mg/L).

Isoamyl alcohol was the dominant higher alcohol after MLF. The co-inoculations fermentations resulted in higher concentrations than sequential inoculation strategies. The strategies inoculated with *L. plantarum* showed the highest average value of 502.43 mg/L (**Addendum Figure 2 A2**).

2-Phenylethanol showed the second highest concentration after MLF. No inoculation trends with regards to highest total concentrations are observed. For Cross Evolution®, co-inoculation with *L. plantarum* showed the highest concentration of 484.83 mg/L and EC1118® co-inoculated with *L. plantarum* resulted in final 2-phenylethanol concentrations of 487.37 mg/L. Amino acids supplemented fermentations with single LAB strains resulted in significantly higher concentrations of this compound compared to fermentations with NH_4Cl as nitrogen source (**Figure 3.14C**).

Overall propanol and isobutanol were higher for the sequential inoculation strategies. Amino acids supplemented fermentations with single LAB strains resulted in higher isobutanol concentrations (**Figure 3.14D**). Sequential inoculation strategies resulted in fermentations with the highest pentanol and 3-ethoxy-1-propanol levels, while co-inoculation strategies showed the highest hexanol concentrations.

Fatty acids

Cross Evolution® co-inoculated with *L. plantarum* showed the highest total fatty acid concentration (20.31 mg/L) and the bacterial combination the lowest concentration (5.3 mg/L) (**Table 3.21**). The EC1118® sequential inoculation strategy with *L. plantarum* had the highest concentration of 18.67 mg/L and EC1118® with the bacterial combination had the lowest concentration of 5.72 mg/L.

The concentrations of octanoic acid were the second highest after AF and MLF. It showed similar trends as isobutyric acid. According to the bubble the amino acid fermentations with single LAB strains, especially co-inoculated strategies with *L. plantarum* resulted in significant higher concentrations than NH_4Cl fermentations (**Figure 3.14E**). Isobutyric acid showed the highest concentration after MLF. The sequential inoculations showed higher concentrations than co-inoculation strategies. EC1118® co-inoculated with *L. plantarum* strategy had the highest concentration (5.11 mg/L). For the EC1118®, the co-inoculated strategy showed higher concentrations than sequential inoculation strategy. The bubble graph shows a significant increase of the isobutyric acid concentration with single LAB strains compared to NH_4Cl fermentations (**Figure 3.14F**).

Propionic acid, valeric acid, isovaleric acid and octanoic acid were present at higher concentrations for the co-inoculated fermentations, while the sequentially inoculated fermentations showed the

highest isobutyric acid concentrations. The butyric acid concentrations for the Cross Evolution® sequential inoculations were higher than the co-inoculated fermentations. The butyric acid concentrations for the EC1118® co-inoculated fermentations were higher than the sequentially inoculated fermentations. This highlights again the impact of specific yeast strains on the outcomes of co- and sequential inoculations of a particular bacterial strain/s.

TABLE 3.19

Esters concentrations (mg/L) at the end of amino acids supplemented fermentations of different inoculation strategies. Values given for the sequential inoculations are the final concentrations at the end of MLF. Values are the average of 3 biological repeats ± standard deviation.

	Ethyl acetate	Ethyl butyrate	Isoamyl acetate	Ethyl hexanoate	Hexyl acetate	Ethyl lactate	Ethyl caprylate	Ethyl-3-hydroxybutanoate	Ethyl caprate	Diethyl succinate	Ethyl phenylacetate	2-Phenylethyl Acetate	Totals
Cross Evolution													
Control yeast without inoculated *L. plantarum*	34.90 ± 0.70	0 ± 0	2.86 ± 0.02	0.67 ± 0.03	0.06 ± 0	11.43 ± 0.15	0.33 ± 0.01	0.83 ± 0.03	0.38 ± 0.01	0.45 ± 0.01	1.46 ± 0.04	5.80 ± 0.05	**59.69**
Control yeast without inoculated *O. oeni*	31.53 ± 0.43	0 ± 0	0.21 ± 0.01	0.20 ± 0	0 ± 0	19.06 ± 0.30	0.04 ± 0	1.40 ± 0.02	0.04 ± 0	0.29 ± 0.01	0.35 ± 0.04	0.47 ± 0.01	**53.59**
Control yeast without inoculated *L. plantarum* and *O. oeni*	20.87 ± 0.55	0 ± 0	0.19 ± 0	0 ± 0	0.02 ± 0	14.11 ± 0.17	0.04 ± 0	1.10 ± 0.02	0.04 ± 0	0.28 ± 0.01	0.49 ± 0.02	0.30 ± 0	**37.44**
Co-inoculation													
L. plantarum	31.36 ± 1.33	0 ± 0	9.40 ± 0.02	0.61 ± 0.001	0.26 ± 00.01	14.83 ± 0.18	0.54 ± 0.004	0.93 ± 0.02	0.87 ± 0.02	0.54 ± 0.02	0.84 ± 0.01	6.13 ± 0.01	**66.84**
O. oeni	30.92 ± 0.84	0 ± 0	0.23 ± 0.01	0.20 ± 0.003	0 ± 0	41.76 ± 1.45	0.08 ± 0.002	1.34 ± 0.02	0.07 ± 0.001	0 ± 0	0.32 ± 0.01	0.49 ± 0.01	**75.41**
L. plantarum and *O. oeni*	20.85 ± 0.46	0 ± 0	0.18 ± 0.01	0.20 ± 0.002	0 ± 0	45.94 ± 1.81	0.07 ± 0.001	1.32 ± 0.02	0.06 ± 0.003	0 ± 0	0.12 ± 0.02	0.38 ± 0.01	**69.12**
Sequential inoculation													
L. plantarum	33.74 ± 0.45	0 ± 0	1.53 ± 0.02	0.53 ± 0.01	0.07 ± 0.001	12.45 ± 0.08	0.35 ± 0.01	0.88 ± 0.01	0.47 ± 0.02	0.46 ± 0.01	1.56 ± 0.02	3.83 ± 0.01	**55.87**
O. oeni	32.15 ± 2.42	0 ± 0	0.18 ± 0.003	0.20 ± 0.001	0.06 ± 0.01	21.77 ± 0.87	0.04 ± 0.001	1.56 ± 0.04	0.04 ± 0.002	0.29 ± 0.004	0.27 ± 0.02	0.45 ± 0.01	**57.01**
L. plantarum and *O. oeni*	16.26 ± 0.23	0 ± 0	0.19 ± 0.01	0 ± 0	0 ± 0	55.39 ± 1.08	0.06 ± 0.003	1.15 ± 0.01	0.04 ± 0.002	0.28 ± 0.005	0.15 ± 0.01	0.47 ± 0.02	**73.99**
EC 1118													
Control yeast without inoculated *L. plantarum*	51.08 ± 0.23	0 ± 0	0.93 ± 0	0.66 ± 0.02	0.06 ± 0	11.59 ± 0.26	0.46 ± 0.04	0.96 ± 0.04	0.45 ± 0.02	0.44 ± 0.01	1.53 ± 0.02	3.39 ± 0.28	**72.06**
Control yeast without inoculated *O. oeni*	36.23 ± 1.52	0 ± 0	0.26 ± 0.01	0.22 ± 0.01	0.02 ± 0	12.69 ± 1.12	0.10 ± 0.01	1.02 ± 0.01	0.11 ± 0.01	0.28 ± 0.01	0.66 ± 0.49	0.63 ± 0.05	**52.22**
Control yeast without inoculated *L. plantarum* and *O. oeni*	32.10 ± 1.10	0 ± 0	0.22 ± 0.01	0 ± 0	0 ± 0	13.74 ± 0.35	0 ± 0	1.09 ± 0	0 ± 0	0.49 ± 0.01	0 ± 0	0.52 ± 0.01	**48.16**
Co-inoculation													
L. plantarum	0 ± 0	0 ± 0	0 ± 0	0.54 ± 0.04	0 ± 0	13.52 ± 0.39	0.15 ± 0.003	0.93 ± 0.02	0.14 ± 0.0004	0.45 ± 0.0004	0.33 ± 0.001	2.97± 0.02	**19.03**
O. oeni	36.57 ± 1.21	0 ± 0	0.23 ± 0.01	0.21 ± 0.01	0.02 ± 0.0002	73.73 ± 2.05	0.10 ± 0.005	1.33 ± 0.05	0.12 ± 0.01	0.31 ± 0.01	0 ± 0	0.56 ± 0.03	**113.18**
L. plantarum and *O. oeni*	30.12 ± 0.71	0 ± 0	0.2 ± 0.006	0.23 ± 0.002	0 ± 0	50.97 ± 0.10	0.09 ± 0.001	1.56 ± 0.04	0.07 ± 0.002	0 ± 0	0.02 ± 0.001	0.48 ± 0.01	**83.83**
Sequential inoculation													
L. plantarum	51.43 ± 0.37	0 ± 0	0.73 ± 0.04	0.53 ± 0.02	0.07 ± 0.0002	12.63 ± 0.39	0.33 ± 0.04	1.04 ± 0.04	0.28 ± 0.01	0.45 ± 0.02	1.33 ± 0.03	2.27 ± 0.05	**71.09**
O. oeni	38.52 ± 0.96	0 ± 0	0.29 ± 0.01	0.22 ± 0.01	0.05 ± 0.003	20.04 ± 0.71	0.11 ± 0.02	1.06 ± 0.02	0.21 ± 0.01	0.28 ± 0.005	0.86 ± 0.02	0.58 ± 0.03	**62.22**
L. plantarum and *O. oeni*	26.33 ± 0.65	0 ± 0	0.18 ± 0.002	0.20 ± 0.002	0 ± 0	56.79 ± 1.01	0.07 ± 0.002	1.15 ± 0.01	0.05 ± 0.01	0.43 ± 0.01	0 ± 0	0.57 ± 0.02	**85.77**
Control: bacteria													
L. plantarum	6.92 ± 0.01	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.06 ± 0	0.86 ± 0	0.37 ± 0	0 ± 0	0 ± 0	0.44 ± 0.05	**8.65**
O. oeni	4.16 ± 0.03	0 ± 0	0 ± 0	0 ± 0	0 ± 0	9.30 ± 0.50	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.17 ± 0.13	**13.63**
L. plantarum and *O. oeni*	3.31 ± 0.01	0 ± 0	0 ± 0	0 ± 0	0 ± 0	8.68 ± 0.03	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.24 ± 0	**12.23**

TABLE 3.20

Higher alcohol concentrations (mg/L) at the end of amino acids supplemented fermentations of different inoculation strategies. Values given for the sequential inoculations are the final concentrations at the end of MLF. Values are the average of 3 biological repeats ± standard deviation.

	Propanol	Isobutanol	Butanol	Isoamyl alcohol	Pentanol	3-Methyl-1-pentanol	3-ethoxy-1-propanol	Hexanol	2-Phenylethanol	Totals
Cross Evolution										
Control yeast without inoculated *L. plantarum*	34.72 ± 2.25	83.95 ± 0.95	0.65 ± 0.01	554.78 ± 14.07	0.26 ± 0.01	0 ± 0	0.36 ± 0.02	4.54 ± 0.13	515.04 ± 21.60	**1194.3**
Control yeast without inoculated *O. oeni*	19.55 ± 0	197.64 ± 0	0.61 ± 0	265.14 ± 3.19	0.28 ± 0.0002	0 ± 0	0.27 ± 0.001	1.92 ± 0.002	296.88 ± 5.65	**782.29**
Control yeast without inoculated *L. plantarum* and *O. oeni*	15.78 ± 0.92	181.45 ± 3.67	0.56 ± 0.0002	324.99 ± 4.23	0.28 ± 0.004	0 ± 0	0.25 ± 3.82E-05	0 ± 0	295.50 ± 5.84	**818.81**
Co-inoculation										
L. plantarum	49.67 ± 0.32	93.98 ± 0.22	0.71 ± 0	605.41 ± 1.06	0.26 ± 0.01	0 ± 0	0.36 ± 0	5.49 ± 0.13	484.83 ± 1.57	**1240.71**
O. oeni	18.12 ± 0.48	194.26 ± 1.54	0.63 ± 0.02	373.28 ± 1.41	0.28 ± 0.01	0 ± 0	0.28 ± 0	9.28 ± 0.54	337.40 ± 1.54	**933.53**
L. plantarum and *O. oeni*	21.62 ± 1.01	143.12 ± 3.24	0.71 ± 0.03	227.43 ± 2.98	0.28 ± 0	0 ± 0	0.26 ± 0	1.65 ± 0.02	220.68 ± 1.24	**615.75**
Sequential inoculation										
L. plantarum	31.59 ± 0.13	75.74 ± 0.66	0.64 ± 0.01	492.84 ± 0.80	0.26 ± 0.002	0 ± 0	0.36 ± 0.003	3.43 ± 0.01	458.21 ± 0.41	**1063.07**
O. oeni	17. 96 ± 0.80	205.81 ± 1.48	0.64 ± 0.05	295.84 ± 1.09	0.28 ± 0.002	0 ± 0	1.80 ± 0.005	0.27 ± 0.001	335.64 ± 1.68	**858.24**
L. plantarum and *O. oeni*	13.46 ± 0.35	154.56 ± 3.21	0.58 ± 0.01	295.05 ± 0.63	0.28 ± 0.002	0 ± 0	0.26 ± 0.004	1.88 ± 0.04	164.05 ± 2.99	**630.12**
EC 1118										
Control yeast without inoculated *L. plantarum*	47.89 ± 0.59	68.16 ± 0.93	0.67 ± 0.01	464.57 ± 0.29	0.26 ± 0.004	0 ± 0	0.36 ± 0.001	54.63 ± 1.80	511.91 ± 0.55	**1148.45**
Control yeast without inoculated *O. oeni*	18.12 ± 0.03	194.26 ± 0.002	0.63 ± 0.01	373.28 ± 0.14	0.28 ± 0.004	0 ± 0	0.28 ± 0.001	9.28 ± 0.09	337.40 ± 3.90	**933.53**
Control yeast without inoculated *L. plantarum* and *O. oeni*	13.70 ± 0.001	132.72 ± 0	0.90 ± 0	223.95 ± 0.02	0.28 ± 0.005	0 ± 0	0.25 ± 0	0 ± 0	293.04 ± 0	**664.84**
Co-inoculation										
L. plantarum	0 ± 0	0 ± 0	0.64 ± 0	556.29 ± 0.58	0.25 ± 0	0 ± 0	0.36 ± 0	36.17 ± 0.23	487.37 ± 1.20	**580.08**
O. oeni	16.16 ± 1.08	158.94 ± 1.43	0.49 ± 0.02	257.44 ± 2.47	0.27 ± 0	0 ± 0	0.27 ± 0	11.71 ± 0.36	253.08 ± 2.55	**698.36**
L. plantarum and *O. oeni*	17.83 ± 0.62	132.88 ± 0.89	0.50 ± 0.01	196.39 ± 0.75	0.27 ± 0	0 ± 0	0.25 ± 0	6.39 ± 0.09	194.02 ± 1.30	**548.53**
Sequential inoculation										
L. plantarum	37.58 ± 0.19	53.82 ± 0.30	0.67 ± 0.01	355.18 ± 0.42	0.26 ± 0.01	0 ± 0	0.36 ± 0.002	37.11 ± 0.05	384.78 ± 3.52	**869.76**
O. oeni	17.82 ± 0.87	188.53 ± 3.06	0.55 ± 0.04	305.27 ± 1.80	0.28 ± 0.003	0 ± 0	7.70 ± 0.51	0.27 ± 0.002	372.53 ± 1.90	**892.95**
L. plantarum and *O. oeni*	14.01 ± 0.86	124.99 ± 2.39	0.92 ± 0.02	241.98 ± 1.59	0.28 ± 0.004	0 ± 0	0.25 ± 0.001	1.75 ± 0.05	271.64 ± 1.09	**655.82**
Control: bacteria										
L. plantarum	0 ± 0	0 ± 0	0 ± 0	8.16 ± 0.01	0.26 ± 0.01	0 ± 0	0.36 ± 0.01	0 ± 0	0.97 ± 0.02	**9.75**
O. oeni	0 ± 0	1.66 ± 0	0 ± 0	5.90 ± 0.05	0 ± 0	0 ± 0	0.25 ± 0	0 ± 0	0.64 ± 0.01	**8.45**
L. plantarum and *O. oeni*	0 ± 0	5.36 ± 0.02	0 ± 0	15.46 ± 0.58	0 ± 0	0 ± 0	0 ± 0	0 ± 0	27.73 ± 0.43	**48.55**

TABLE 3.21

Fatty acids concentrations (mg/L) at the end of amino acids supplemented fermentations of different inoculation strategies. Values given for the sequential inoculations are the final concentrations at the end of MLF. Values are the average of 3 biological repeats ± standard deviation.

	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Hexanoic acid	Octanoic acid	Decanoic acid	Totals
Cross Evolution									
Control yeast without inoculated *L. plantarum*	1.83 ± 0.03	4.35 ± 0.29	1.26 ± 0.02	2.87 ± 0.02	0.44 ± 0	2.78 ± 0.07	3.36 ± 0.05	1.95 ± 0.04	**18.84**
Control yeast without inoculated *O. oeni*	0.98 ± 0.01	2.36 ± 0.04	0.44 ± 0.01	1.08 ± 0.02	0.32 ± 0.01	0.38 ± 0.03	0.72 ± 0.02	0.56 ± 0.03	**6.84**
Control yeast without inoculated *L. plantarum* and *O. oeni*	0.65 ± 0.03	1.89 ± 0.02	0.45 ± 0	0.92 ± 0.02	0.26 ± 0	0.35 ± 0.01	0.51 ± 0.01	0.48 ± 0	**5.51**
Co-inoculation									
L. plantarum	2.08 ± 0.11	4.22 ± 0.05	1.38 ± 0.01	2.89 ± 0.05	0.72 ± 0	3.22 ± 0.04	3.53 ± 0.45	2.27 ± 0.04	**20.31**
O. oeni	1.57 ± 0.04	1.82 ± 0.08	0.43 ± 0.01	1.17 ± 0.04	0.29 ± 0.01	0.35 ± 0.02	0.64 ± 0.04	0.51 ± 0.02	**6.78**
L. plantarum and *O. oeni*	0.65 ± 0.02	1.27 ± 0.01	0.46 ± 0.01	0.83 ± 0.01	0.26 ± 0.01	0.51 ± 0.03	0.77 ± 0.03	0.55 ± 0.02	**5.3**
Sequential inoculation									
L. plantarum	1.62 ± 0.01	4.07 ± 0.03	1.16 ± 0.01	2.74 ± 0.03	0.56 ± 0.01	2.60 ± 0.05	2.60 ± 0.06	1.06 ± 0.02	**16.41**
O. oeni	0.76 ± 0.01	2.34 ± 0.04	0.47 ± 0.02	1.15 ± 0.02	0.32 ± 0.02	0.40 ± 0.01	0.51 ± 0.02	0.54 ± 0.04	**6.49**
L. plantarum and *O. oeni*	0.67 ± 0.001	1.36 ± 0.03	0.44 ± 0.004	0.96 ± 0.02	0.25 ± 0.002	0.38 ± 0.02	1.03 ± 0.01	0.63 ± 0.01	**5.72**
EC 1118									
Control yeast without inoculated *L. plantarum*	2.07 ± 0.04	7.44 ± 0.02	1.40 ± 0	3.41 ± 0.17	0.91 ± 0.01	2.85 ± 0.08	3.88 ± 0.02	2.52 ± 0.02	**24.48**
Control yeast without inoculated *O. oeni*	1.38 ± 0.01	2.04 ± 0.02	0.59 ± 0.03	1.53 ± 0.03	0.34 ± 0.02	0.78 ± 0.01	1.33 ± 0.02	1.13 ± 0.02	**9.12**
Control yeast without inoculated *L. plantarum* and *O. oeni*	0.82 ± 0.03	2.78 ± 0.04	0.51 ± 0	1.60 ± 0.02	0.26 ± 0.01	0.38 ± 0.01	0.55 ± 0.02	0.60 ± 0.01	**7.5**
Co-inoculation									
L. plantarum	2.01 ± 0.01	0.48 ± 0.01	0.66 ± 0.04	3.62 ± 0.01	1.47 ± 0.02	0.45 ± 0.01	4.64 ± 0.03	1.74 ± 0.05	**15.07**
O. oeni	1.06 ± 0.01	1.77 ± 0.01	0.54 ± 0.02	1.09 ± 0.01	0.33 ± 0	1.01 ± 0.09	1.42 ± 0.02	0.81 ± 0.02	**8.03**
L. plantarum and *O. oeni*	0.70 ± 0.02	2.03 ± 0.02	0.49 ± 0	1.01 ± 0.02	0.32 ± 0	0.71 ± 0.01	1.02 ± 0.01	0.75 ± 0.01	**7.03**
Sequential inoculation									
L. plantarum	1.53 ± 0.003	5.11 ± 0.01	1.21 ± 0.01	2.65 ± 0.03	0.67 ± 0.01	2.82 ± 0.01	3.23 ± 0.03	1.45 ± 0.004	**18.67**
O. oeni	0.75 ± 0.02	2.14 ± 0.02	0.61 ± 0.02	1.27 ± 0.02	0.35 ± 0.02	1.02 ± 0.03	1.64 ± 0.04	0.99 ± 0.02	**8.77**
L. plantarum and *O. oeni*	1.44 ± 0.04	0.84 ± 0.01	0.43 ± 0.01	0.76 ± 0.04	0.27 ± 0.03	0.68 ± 0.03	1.17 ± 0.03	0.78 ± 0.02	**6.37**
Control: bacteria									
L. plantarum	0.94 ± 0.02	0.44 ± 0.02	1.03 ± 0.03	0.57 ± 0.01	0.37 ± 0.01	0.44 ± 0.02	0.56 ± 0.05	0.65 ± 0.03	**5**
O. oeni	0 ± 0	0.42 ± 0.01	0 ± 0	0.71 ± 0	0 ± 0	0.21 ± 0	0.27 ± 0	0 ± 0	**1.61**
L. plantarum and *O. oeni*	0.64 ± 0.01	0.46 ± 0.02	0 ± 0	0.46 ± 0.01	0 ± 0	0.21 ± 0	0.28 ± 0.01	0.31 ± 0	**2.36**

Multivariate statistical analysis

The principal component analysis (PCA) was conducted on the GC-FID generated data of the NH_4Cl and amino acids fermentations to investigate correlations between different treatment samples, co-inoculated with *L. plantarum*, *O. oeni* and the combination of these bacteria (**Figure 3.15**). The samples that are close to each are most similar in terms of the total metabolic fingerprint of these samples. The PCA clearly shows distinctly separate groupings of samples fermented with either the ammonium or amino acids as nitrogen source. Separate groupings of samples inoculated with *L. plantarum*, *O. oeni* and the bacterial combination are observed for fermentations supplemented with the amino acids. However in the case of fermentations where ammonium was supplied as the sole nitrogen source all the different bacterial treatments group closely together in the same cluster. This shows that the impact of different bacterial treatments on the aroma profile produced is the greatest when amino acids are included in the fermentation medium.

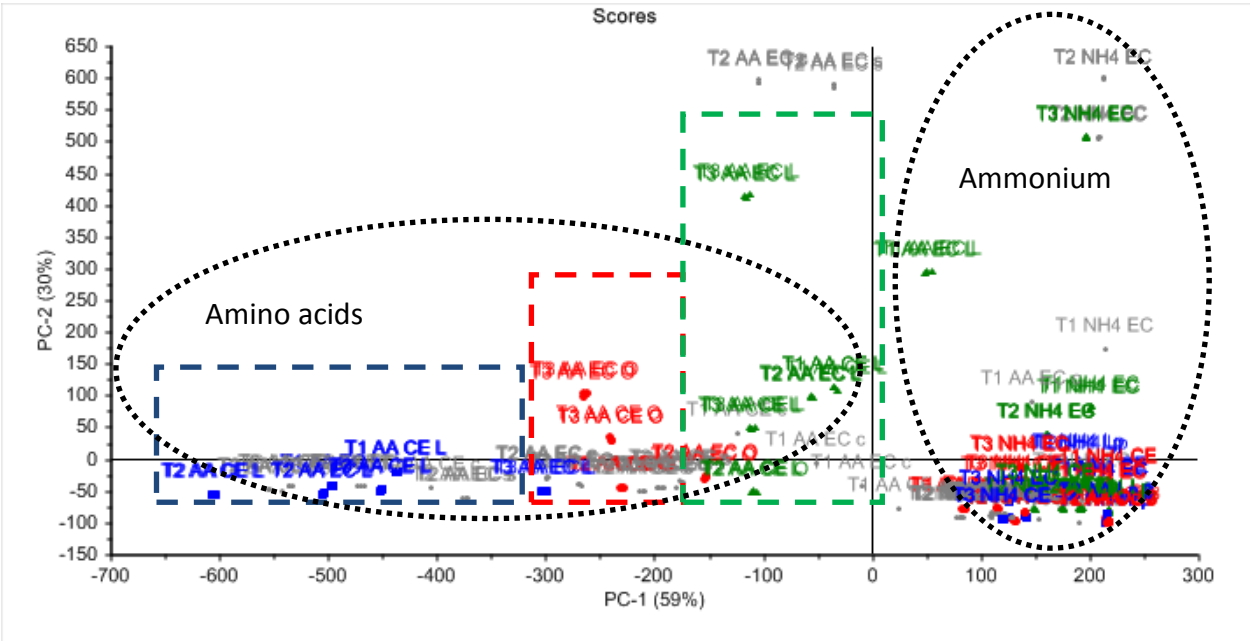

FIGURE 3.15

PCA bi-plot of scores generated from the total set of GC-FID quantified aroma compound concentrations for the NH_4CL and amino acids fermentations. These data points represent the end points of MLF and AF for both co-inoculated (prefixed by T2) and sequentially inoculated (prefixed by T3) samples. Fermentations inoculated with *L. plantarum* are shown in blue, *O. oeni* in red and the bacterial combination in green, while samples of the yeast–only control fermentations are indicated in grey.

Discussion

3.3.5.1 Standard fermentation

Fermentation kinetics

This study showed that glucose was consumed at a faster rate by Cross Evolution® yeast while EC1118® consumed fructose at a faster rate than Cross Evolution (**Addendum Tables 1-3**). The co-inoculation treatments resulted in a slightly slower rate of glucose and fructose utilization

compared to the controls. This can probably be attributed to the fact that yeast and bacteria compete for nutrients (Fleet, 2003; Alexander *et al*., 2004).

For *L. plantarum* in combination with both yeast strains the average growth rate during active MLF was 8.1 times higher compared to the bacteria-only control. This indicates that this strain of *L. plantarum* is stimulated by the two yeast strains used in our study (**Addendum Table 5**). In contrast, the maximum cell counts achieved by *O. oeni* in the bacterial control fermentations were 11.5 times higher than those of *O. oeni* in combination with either of the yeast strains. This shows that the growth of this strain of *O. oeni* is inhibited by the yeast strains used in our experiments. The *O. oeni* growth of the bacterial combination presented similar results: For fermentations inoculated with the bacterial combination, *L. plantarum* growth was higher and *O. oeni* lower than the pure culture controls. The study by González-Arenzana *et al*. (2013) showed that *O. oeni* was present at lower bacterial numbers in the early stages of fermentation whereas *L. plantarum* dominated the first few days of alcoholic fermentation due to higher bacterial population numbers. Another reason for the dominance in growth by the *L. plantarum* can be due to yeast inhibition of *O. oeni*. The study of Costello *et al*. (2003) showed that yeast strains can inhibit certain LAB strains and stimulate others. The rate of MLF for all fermentations was faster than would be expected, which could be due to homogenization associated with small fermentation volumes (**Addendum Table 6**). Vrščaj Vodošek *et al.* (2009) showed a faster rate of MLF in small fermentation volumes compared to bigger fermentation volumes.

Volatile aroma compounds

Esters

The formation of ethyl lactate occurs via the esterification of lactic acid and ethanol, thus the production of ethyl lactate is linked to lactic acid production from MLF. Ethyl lactate is favourable for wine aroma due to its fruity, buttery and creamy aromas and its contribution to the mouthfeel of wine (Lerm *et al*., 2010). An increase in the ethyl lactate concentration of the co-inoculated strategies is observed (**Addendum Figure 1 A1**). Similar studies of Lerm (2010), Abrahamse and Bartowsky (2012) and Cañas *et al*. (2012) also showed that co-inoculation of LAB for MLF in wine results in higher ethyl lactate concentrations than sequential inoculation. The co-inoculated treatments with the bacterial combination resulted in the highest ethyl lactate concentration (**Table 3.13**). The PCA plots indicate that *O. oeni* has the biggest impact on the aroma profile modification by MLF of the different treatments due to its higher esterase activities (**Figure 3.13**). A recent study done by Rossouw *et al*. (2012) confirmed that co-inoculated fermentations with *O. oeni* resulted in higher ethyl lactate concentrations.

One of the main groups of fermentation derived esters is the acetate esters. The condensation of higher alcohols with acetyl-CoA results in the formation of acetate esters (Matthews *et al*., 2004; Ugliano and Henschke, 2008). Ethyl acetate has a fruity character with an odour threshold of 12.26 mg/L (Ferreira *et al*., 2001). Co-inoculation of *L. plantarum* with EC1118® resulted in the highest final concentrations of ethyl acetate. The fermentations with single LAB strains showed significantly higher concentrations than the bacterial combination and the bubble graph supports the findings (**Figure 3.11A**). This is in contrast with the study of Sumby *et al*. (2010) which showed that *O. oeni* displayed a greater amount of esterase activity than *L. plantarum*.

Ethyl caprylate is a compound which imparts a fruity charcter (Francis and Newton, 2005). The single LAB strains resulted in higher concentrations than the bacterial combination. (**Figure**

3.11B). The study of Costello *et al*. (2012) showed that *O. oeni* produced significantly higher levels of ethyl caprylate than *L. plantarum* due to its better ester-synthesizing capabilities. Ethyl caprate contributes to the floral character of wine (Francis and Newton, 2005). Lower concentrations are observed for the bacterial combination when compared to single LAB strains. A study done by Biodo *et al*. (2009) showed that *O. oeni* produced significant levels of ethyl caprate.

Higher alcohols

Higher alcohols are formed by the decarboxylation and subsequent reduction of α-keto acids (Lerm e*t al*., 2010). At lower concentrations (less than 300 mg/L), higher alcohols can contribute to the complexity and fruity aromas in wine, whereas higher concentrations (above 400 mg/L) could be detrimental to wine aroma and quality due to harsh chemical-like aromas (Swiegers *et al*., 2005). There is an increase in higher alcohol concentrations at the end of alcoholic fermentation. The yeast control had the highest total higher alcohol concentrations, followed by the sequential and co-inoculation strategies. This can also be seen in the bubble plots figures **(Figure 3.11D**, **Addendum Figure 1 A2**).

Isoamyl alcohol was quantitatively the predominant higher alcohol after MLF (**Table 3.14**). The studies of Antalick *et al*. (2012) and Schöltz (2013) also showed increases in higher alcohol concentrations at the end of MLF although Herjavec *et al*. (2001) reported no change in the isoamyl alcohol concentrations O. oeni strains, at the end of MLF. The co-inoculation strategies that were inoculated with *L. plantarum* presented higher concentrations than the different sequential inoculated treatments. The study of Lerm (2010) found no differences in isoamyl concentrations between co-inoculations and sequential inoculations.

2-Phenylethanol has a honey, spice aroma (Francis and Newton, 2005). The bacterial combinations produced higher concentrations of this compound than the single LAB strains (**Table 3.14**). This is in contrast with a study which showed that *O. oeni* produced significant levels of this compound (Pietra Torres *et al*., 2011). Another study showed that there were no significant differences in the concentrations of this compound produced by *L. plantarum* and *O. oeni* (Lee *et al*., 2009). However, due to the genetic diversity and intraspecies variation in LAB genera, specifically *O. oeni*, general conclusions regarding the metabolic activities of this species cannot be derived from studies dealing with only one, or a few strains (Borneman *et al*., 2013).

Fatty acids

Acetic acid is the most important volatile acid produced during vinification. At concentrations of 0.2 to 0.6g/L acetic acid can contribute to the complexity of wines (Lerm *et al*., 2010). However when threshold values exceed 0.7 g/L, acetic acid leads to vinegary, pungent aromas in wine (Francis and Newton, 2005). The different strategies produced high amounts of acetic acid. A higher rate of MLF can result in higher acetic acid production (Lonvaud-Funel, 1999). The MLF rate in this medium was high and can be the cause of the high acetic acid concentrations. The acetic acid concentration of the co-inoculated treatments that used *O. oeni* was the highest while the co-inoculated treatments that used *L. plantarum* and the combination of the bacteria were slightly lower (**Table 3.6**). These trends are in agreement with previous studies which showed that co-inoculation does not result in significantly increased production of acetic acid (Abrahamse and Bartowsky, 2012; Knoll *et al*., 2012).

Hexanoic acid has a sweaty, rancid cheese, fatty aroma (Peinado *et al*., 2004). Lower production of these compounds is desired rather than higher concentrations because these compounds have low detection thresholds and undesired pungent aromas (Francis and Newton, 2005).The sequential inoculation strategies with *L. plantarum* showed the highest concentrations of hexanoic acid (**Table 3.15**). This is in agreement with previous studies which showed that *L. plantarum* led to fermentations with higher concentrations of hexanoic acid than is the case for *O. oeni* (Pozo-Bayón *et al*., 2005; Lee *et al*., 2009). This is in contrast with a study done by Maicas *et al*. (1999) which reported no significant increase in isobutyric or hexanoic acids after the completion of MLF for both *O. oeni* and *L. plantarum*.

Butyric acid is known for its cheese oudour (Escudero *et al*., 2007). This is not a desirable compound in wine. The single LAB strains produced higher concentrations of this compound than the bacterial combinations (**Figure 3.11E**). The study of Lee *et al*. (2009) showed that *O. oeni* produced less butyric acid than *L. plantarum*. This can be a reason for the bacterial combination producing less of this compound than single strains.

3.3.5.2 Nitrogen addition fermentations

Fermentation kinetics

For fermentations that were supplemented with NH_4Cl, the EC 1118® yeast fermented better than Cross Evolution® in terms of glucose and fructose consumption in the NH_4Cl supplemented media (**Addendum Table 8-10**). In contrast, the fermentations that were supplemented with amino acids favoured a faster rate of glucose and fructose utilization by Cross Evolution compared to EC1118® (**Addendum Table 15-17**).

There was an increase in the bacterial population during MLF in the synthetic media that were supplemented with NH_4Cl. For both the Cross Evolution® and EC1118® co-inoculation strategies, *L. plantarum* growth was 1.3 times lower than the bacterial control (**Addendum Table 12A1**). The *O. oeni* and bacterial combination co-inoculation strategies showed higher bacterial growth than the bacterial controls (**Addendum Table 12 B1 and C1**). The bacterial populations of the sequential inoculation treatment decreased during MLF (**Addendum Table 12 A2, B2, and C2**). A reason for this can be the yeast that produces inhibiting secondary metabolites (Lerm *et al*., 2010). During Cross Evolution® fermentations with *O. oeni*, the growth of the bacteria was better but for EC1118® fermentations, the bacterial growth decreased due to inhibition by yeast. The fermentations that were supplemented with amino acids showed that the bacterial population growth rates of *L. plantarum*, *O. oeni* and bacterial combination were higher than their respective bacterial controls (**Addendum Table 19 A1-C2**). The results showed that yeast strains used in this study stimulate the growth of bacteria in the amino acid supplemented fermentations. For all the sequential treatments bacterial numbers remained at a constant concentration except for the Cross Evolution® treatment sequentially inoculated with *O. oeni* where the bacterial population declined rapidly.

All of the NH_4Cl treatments resulted in fermentations where citric acid was completely utilized while three of the amino acids treatments that used the bacterial combination as well as the *O. oeni* control treatment did not lead to complete utilization of all the citric acid. This is in contrast with the study of Lonvaud-Funel (1999) which showed that *Leuconostoc*, *Oenococcus* and *Lactobacillus* can degrade all citric acid present in a standard grape must.

Volatile aroma compounds

Esters

There was an increase in ethyl lactate concentrations after MLF in both nitrogen addition fermentations (**Table 3.16 and 3.19**). Co-inoculation treatment concentrations were higher than sequential inoculation treatments. In all the different fermentations, *O. oeni* and the bacterial combination were the largest contributors towards the formation of this compound but a similar study done by Pozo-Bayón *et al*. (2005) showed that *L. plantarum* produced the highest concentrations of ethyl lactate. The PCA plot also indicates that *O. oeni* has the largest effect on the modification of aroma compounds (**Figure 3.15**). Another study done by Pietra Torres *et al*., 2011 showed that sequential inoculation of *O. oeni* in wine resulted in higher ethyl lactate concentrations. Recently, the study of Knoll *et al.* (2012) showed that *O. oeni* favoured the production of ethyl lactate. The co-inoculation treatments of the amino acids inoculated with the bacterial combination were lower than the NH_4Cl treatments.

The ethyl acetate concentrations were higher in the NH_4Cl sequential inoculation strategies especially when *O. oeni* was used as the inoculated culture (**Table 3.16**). For the amino acids, co-inoculation strategies with *L. plantarum* resulted in the highest concentration (**Table 3.19**). For the amino acids supplemented fermentations concentrations were significantly lower than the corresponding NH_4Cl fermentations (**Addendum Figure 2 A2**). The study of Ugliano and Moio (2005) showed no significant difference in the ethyl acetate concentrations when no MLF was compared to sequential inoculation with *O. oeni*. Lee *et al*. (2009) showed that ethyl acetate concentrations were higher in sequentially inoculated fermentations with *O. oeni* compared to *L. plantarum*.

Higher alcohols

The yeast controls produced more isoamyl alcohol than both the co-inoculation and sequential inoculations strategies (**Table 3.17 and 3.20**). In general higher concentrations were observed for inoculation strategies with *L. plantarum*. The study of Lee *et al*. (2009) showed that *L. plantarum* resulted in higher concentrations than *O. oeni* for sequential inoculations. Similar results were obtained with the amino acids supplemented fermentations and the concentration of this compound was four times higher than the NH_4Cl treatments. (**Addendem Figure 2 A2**). This can be attributed to the fact that the amino acids that were selected favours higher alcohol production (Dickinson *et al*., 2003). Isobutanol was the second highest higher alcohol. This compound at a high concentration imparts a solvent aroma (Guth, 1997). Overall the sequential inoculation strategies with *O. oeni* showed the highest concentrations of this compound. Concentrations were two times higher for most of the amino acids supplemented fermentations compared to the NH_4Cl fermentations (**Table 3.17 and 3.20**).

Fatty acids

The Cross Evolution® control of the NH_4Cl fermentations resulted in the highest acetic acid concentrations at the end of fermentation (**Table 3.9**). The co-inoculation treatments that used *O. oeni* also resulted in a high concentration while the treatments that used *L. plantarum* and the combination of these bacteria were below 1g/L. A reason for these results could be that *L. plantarum* produces less acetic acid than *O. oeni* (Du Toit *et al*., 2011). For the amino acids

supplemented fermentations the control and the sequential treatments resulted in the highest acetic acid concentrations while those of the co-inoculated treatments were lower than the control treatments (**Table 3.12**). This is in agreement with studies which showed that co-inoculation lowered acetic acid concentrations (Nehme *et al*., 2010; Knoll *et al*., 2012).

Isobutyric acid was present at the highest concentration of the fatty acids. This acid imparts a rancid, butter aroma character (Francis and Newton, 2005). For both nitrogen additions, co-inoculations with *L. plantarum* resulted in the highest concentrations(**Table 3.18 and 3.21**) but the studies of Siebert *et al*. (2005), Bartowsky (2005) showed that *O. oeni* has extensive suite metabolic pathways and enzymes that can generate volatile fatty acids at concentrations above their odour detection thresholds. It is evident that yeast plays the main role in the formation of the volatile fatty acids because the bacterial control produces less fatty acids. Studies done by Schöltz (2013) and Maicas *et al*. (1999) showed no significant increases in isobutyric acids.

Octanoic acid imparts a sweat, cheese aroma (Francis and Newton, 2005). The co-inoculation strategies with *L. plantarum* and *O. oeni* resulted in higher concentrations than the bacterial combinations (**Figure 3.14E**). Cãnas *et al*. (2012) showed that there were no significant differences in octanoic acid concentrations between co-inoculation and sequential inoculations. No significant increases in isobutyric acid for the sequential inoculation of *O. oeni* were found but significant increases were observed for octanoic acid (Lopéz *et al*., 2011). The study of Lee *et al*. (2009) showed that *O. oeni* produced more isobutyric and octanoic acid than *L. plantarum* for sequential inoculations.

3.4 Conclusions

Co-inoculation treatments with different malolactic bacteria such as *L. plantarum*, *O. oeni* and the combination of these bacteria, in combination with two different yeast strains, was compared to the corresponding sequentially inoculated fermentations to determine the impact of different inoculation strategies on the production of aroma compounds. Overall co-inoculation strategies resulted in a greater impact on the aroma profile of the wine in terms of increased concentrations of certain groups of compound (such as esters) and decreased concentrations of others (certain higher alcohol). The results also showed that the differences exist between yeast strains in terms of their impact on co- versus sequential inoculation. The malolactic bacteria, *O. oeni* and *L. plantarum* showed higher concentrations for aroma compounds compared to bacterial combination although *O. oeni* seems to be metabolically dominant in the combination. Some of the general trends showed that the bacterial combination resulted in higher ethyl lactate and lower hexanoic acid concentrations. For 2-phenylethanol and isoamyl alcohol concentrations co-inoculation with *O. oeni* resulted in the lower concentrations compared to *L. plantarum* and the bacterial combination. Interestingly, *L. plantarum* and the bacterial combination produced less acetic acid. Different nutrient additions, different inoculation strategies as well as different malolactic bacteria were compared to investigate which addition, inoculation strategy and malolactic bacteria had the greatest impact on the aroma compounds. Overall co-inoculations resulted in higher concentrations of volatile aroma compounds. Similar to the standard fermentations, the specific yeast strains also impacted on co- versus sequential inoculation. This resulted in differences in the production of volatile aroma compounds between yeast and MLF inoculation strategies. Malolactic bacteria, *O. oeni* and *L. plantarum* played an important role in the formation/modification of the aroma compounds and the PCA plots and the growth of bacterial populations confirms this. Lower concentrations of isoamyl alcohol, 2-

phenylethanol, octanoic acid and decanoic acid were observed for co- and sequential inoculation with the bacterial combination. Acetic acid concentrations were lower with *L. plantarum* and the bacterial combination. The results indicated that amino acids supplemented fermentations resulted in higher concentrations of aroma compounds than NH_4Cl fermentations. This study provides winemakers with information on what MLF practices and LAB strains to use to enhance or decrease certain aroma compounds in order to produce a certain style of wine. It also highlights the importance of fermentation medium/must and shows that amino acids present in the medium can influence the aroma profile of the wine significantly during both AF and MLF.

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Chapter 4

General discussion and conclusions

4. General discussion and conclusions

4.1 Concluding remarks and future work

Malolactic fermentation (MLF) is an important secondary fermentation that normally occurs after alcoholic fermentation in most red and some white wines (Pan *et al*., 2011). During this fermentation, lactic acid bacteria (LAB) convert malic acid to lactic acid. This process is beneficial because it decreases the acidity and increases the pH of wine, contributes to the microbial stability and changes in the aroma and mouthfeel properties of wines (Lerm *et al*., 2010). The LAB species generally associated with this practice originate from the genera *Oenococcus*, *Lactobacillus*, *Pediococcus* and *Leuconostoc*. *Oenococcus oeni* is the most preferred species because it can survive the harsh wine environment and is used in almost all commercially available starter cultures (Lonvaud-Funel, 1999; Ugliano *et al*., 2003). Winemakers are becoming aware of the advantages associated with inoculated MLF compared to un-inoculated, spontaneous MLF. This is the main reason why malolactic starter cultures are being used to initiate MLF in many red and selected white wine cultures. Recently, the use of *Lactobacillus plantarum* as commercial starter culture was introduced (Du Toit *et al*., 2011). *Lactobacillus plantarum* is tolerant to harsh wine conditions such as high ethanol and SO_2 concentrations, pH higher than 3.5 and temperatures of ± 20°C. It can conduct MLF just as efficiently as *O. oeni* under certain conditions and it possesses many enzyme encoding genes important for desirable aroma production (Mtshali *et al*., 2010; Du Toit *et al*., 2011).

The overall objective of this study was to investigate the impact of different combinations of inoculated yeast and malolactic bacteria on aroma production in different MLF inoculation strategies. Little is known about the interactions between yeast and bacteria as well as between LAB strains in mixed started cultures and the effect of these interactions on aroma and flavor production. Therefore, the first aim of this study was to assess the impact of yeast on the growth of LAB strains in co- and sequential inoculations. Bacterial growth for all three treatments was stimulated in conjunction with the yeast strain EC1118® compared to Cross Evolution®. The co-inoculation treatments showed the highest bacterial populations and this correlates well with the formation of volatile aroma compounds during this treatment. The next step was to investigate the impact of these strategies on the aroma profile of wine. An increase in ethyl lactate concentrations was observed in the co-inoculated treatments compared to the control and sequentially inoculated fermentations, which is in agreement with previous studies (Malherbe, 2010; Knoll *et al*., 2011 Schöltz, 2013). The co-inoculated treatments showed the highest concentrations, particularly treatments that were inoculated with *O. oeni*. The ethyl acetate concentrations were also increased for co-inoculated fermentations compared to their respective controls, with *L. plantarum* co-inoculated fermentations showing the highest concentrations. Literature cannot confirm these findings for co-inoculation but only for sequential inoculation. The study of Pozo-Bayón *et al*. (2005) showed that *L. plantarum* produced more ethyl acetate than *O. oeni* and the concentrations were higher in wine than synthetic medium. This is in contrast with Lee *et al*. (2009) that showed *O. oeni* produced more than *L. plantarum* but these findings are very strain specific so results using the same species but different strains would not be expected to necessarily agree.

No increase in isoamyl alcohol and 2-phenylethanol concentrations was seen compared to the yeast-only controls. For 2-phenylethanol the sequential inoculation showed a higher concentration. The bacterial combination showed the highest concentrations. The isoamyl alcohol concentration was higher in co-inoculations. The highest concentrations were found for the fermentations inoculated with *L. plantarum*. This finding is in agreement with studies by Lerm (2010) and Lee *et al*. (2009).

The bacterial combination resulted in the lowest acetic acid concentrations in the sequentially inoculated fermentations. This interesting combination can be further investigated and tested in real grape must. The co-inoculated strategy with *L. plantarum* had the lowest concentration. A possible reason for this may be that *L. plantarum* is a facultative heterofermenter and thus produces less acetic acid than *O. oeni* which is an obligatory heterofermenter (Lerm *et al*., 2010; Du Toit *et al*., 2011).

Overall co-inoculations resulted in fermentations with higher total volatile aroma compounds than sequential inoculations. This can be due to the fact that sequential inoculation inhibits bacterial growth because of high ethanol concentrations, low pH, SO_2, nutrient depletion and antimicrobial compounds produced by yeast (Larsen *et al*., 2003). This means that the impact of bacterial secondary metabolism is less pronounced under these conditions compared to co-inoculation scenarios when bacterial metabolism can have a major impact on the levels of higher alcohols and esters.

Pure cultures of *L. plantarum* and *O. oeni* generally resulted in higher aroma compound concentrations compared to the bacterial combination. A possible reason for this is that LAB has the ability to produce antimicrobial peptides that can inhibit other LAB microflora (Du Toit *et al*., 2011). Interestingly, for the bacterial combination, *L. plantarum* dominates numerically in terms of growth for the first few days but the metabolic impact of *O. oeni* activity still appears to be dominant when taking the metabolic footprint of the volatiles into consideration.

The second aim of this study was to evaluate the impact of different nutrient additions on the aroma and flavour production in the same experimental framework of the first objective. The control treatment in this case involved a simple nitrogen source of only NH_4Cl in the synthetic medium compared to a nitrogen source rich in aromatic and branched chain amino acids which are the precursors for the higher alcohols, esters and acids produced by yeast via the Ehrlich pathway (Liu *et al*., 2008). Bacterial growth was stimulated by Cross Evolution® and EC1118® in both the NH_4Cl and amino acids treatments as these fermentations showed higher bacterial populations compared to the bacteria–only controls.

Once again ethyl lactate concentrations were higher in co-inoculated fermentations for both the NH_4Cl and amino acids treatments compared to the corresponding sequentially inoculated fermentations. The co-inoculation treatments that were inoculated with *O. oeni* and the bacterial combination showed the highest concentrations of ethyl lactate compared to *L. plantarum*. A reason for this finding is that *O. oeni* has higher esterase activity than *L. plantarum* (Bartowsky and Borneman, 2011; Pérez-Martin *et al*., 2013; Sumby *et al*., 2013). Overall the amino acids treatments resulted in lower ethyl lactate formation compared to the NH_4Cl fermentations. There was also an increase in ethyl acetate concentrations in the co-inoculated fermentations compared to the yeast-only controls. The study of Ugliano and Moio, (2005) showed no

significant differences in ethyl acetate concentrations in wines that have undergone MLF compared to controls. Recently Antalick *et al*. (2012) confirmed these findings.

Isoamyl alcohol and 2-phenylethanol concentrations were lower after MLF. This is in contrast with studies that showed increases in levels of higher alcohols after MLF (Pozo-Bayón *et al*., 2005; Pietra Torres *et al*., 2011). The concentrations of several higher alcohols were greater for the amino acids treatments compared to the NH_4Cl fermentations. Overall strategies inoculated with *L. plantarum* and *O. oeni* resulted in higher concentrations compared to the bacterial combinations. For isobutyric acid it is observed that co-inoculation strategies resulted in higher concentrations. For octanoic acid strategies inoculated with *L. plantarum* resulted in higher concentrations. The study of Lee *et al*. (2009) showed that there was no significant difference in concentrations between *L. plantarum* and *O. oeni* but this finding is strain dependent.

The nutrient addition fermentations showed similar trends compared to the standard fermentations. The co-inoculation strategies again resulted in a bigger aromatic impact compared to the sequential inoculations even when the amino acid source was used (which contains all the precursors for the formation of these compounds). Yeast assimilable nitrogen (YAN) consists of ammonia, free amino acids and low molecular weight peptides. At the beginning of fermentations YAN is rapidly consumed from the fermentation medium (Bisson, 1999). This is the reason for the almost negligent aromatic impact of the sequentially inoculated bacteria since amino acids are quickly depleted during fermentation and little to none remain by the end of AF. Another trend in agreement with the standard fermentations is the fact that *O. oeni* appears to dominate metabolically in the mixed culture inoculums as indicated by the overall metabolic footprint. The aroma profiles of wines inoculated with the *O. oeni* and mixed culture are more similar to one another compared to the *L. plantarum*.

For fermentations where NH_4Cl was supplied as the sole nitrogen source there is almost no difference between the different bacterial treatments in terms of their impact on the aromatic profile of wine. This highlights the importance of the original medium amino acid composition in terms of the effect that co-inoculation of a particular strain of LAB can have. This shows that amino acids are crucial as aromatic precursors for yeast metabolism (and bacterial metabolism to a lesser extent). The study of Abrahamse and Bartowsky (2012) showed that co-inoculation with a YAN concentration of ± 300 mg/L generally resulted in higher volatile compound production compared to sequential inoculation. Cañas *et al*. (2012) reported similar trends.

This study generates a number of future research projects. The yeast and bacteria and LAB strains in mixed cultures using different inoculation strategies can be evaluated in a winemaking environment. The most interesting combinations of yeast and bacteria or bacterial mixed cultures can be repeated in a double-compartment fermenter where the yeast and bacteria have no direct interactions (Renault *et al*., 2013). This process can provide information regarding genes which are differentially expressed in the different experimental treatments and how these genes relate to aroma production. The results of the study showed that the combination of *L. plantarum* and *O. oeni* is able to successfully complete MLF and it has a positive influence on wine aroma. More research can be done to find optimal ratios of this bacterial combination in order to produce certain wine styles. Additional combinations of different yeast and LAB strains can be tested in different synthetic media and grape juices to evaluate the impact of these yeast-bacteria pairings on the aroma compounds produced. Limited literature is available

regarding the interactions between non-*Saccharomyces* and LAB strains. More research can be done to assess these combinations in different MLF inoculation strategies and to evaluate the impact on volatile aroma production/modification in wines.

Our results confirm the aromatic benefits of co-inoculation in terms of decreased higher alcohols and increased ester formation (particularly those imparting fruity aromas). Furthermore we have shown the benefit of using mixed species bacterial starter cultures in terms of retaining the favourable aromatic impact of one species (*O. oeni*) while negating some of the negative impacts (lower acetic acid production in co-inoculation using *L. plantarum*). These novel findings will pave the way for the future evaluation and application of mixed-species LAB starter cultures containing two or more different species of LAB. To this end, future research should aim to unravel the underlying mechanisms and molecular responses which determine different yeast-bacteria, and bacteria-bacteria interactions, and the impact of these interactions on secondary metabolism and aroma production by both groups of wine organisms.

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Addendum

Table 1: Glucose (A1) and fructose (A2) consumption during standard fermentation with Cross Evolution and EC1118 and *L. plantarum*

A1

	Glucose						
Days	Cross Evolution Control:yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control: yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
0	109.56 ± 0	109.56 ± 0	109.56 ± 0	110.94 ± 0	110.94 ± 0	110.94 ± 0	114.43 ± 0
1	95.09 ± 10.30	103.15 ± 3.34	101.02 ± 1.41	106.14 ± 5.86	103.37 ± 0.33	103.48 ± 1.06	112.48 ± 1.21
2	58.84 ± 10.42	75.02 ± 2.04	79.83 ± 6.95	85.69 ± 10.82	79.94 ± 6.32	77.01 ± 3.72	116.32 ± 3.30
3	29.92 ± 8.25	44.60 ± 2.45	49.47 ± 10.89	59.63 ± 13.19	53.62 ± 12.78	49.60 ± 8.16	113.04 ± 3.47
4	15.46 ± 6.95	28.25 ± 5.82	33.95 ± 11.68	42.17 ± 13.69	36.12 ± 15.63	32.63 ± 11.44	114.41 ± 1.88
5	7.19 ± 3.84	16.38 ± 7.64	22.29 ± 12.25	27.39 ± 14.12	23.36 ± 19.32	19.78 ± 13.13	115.93 ± 2.60
6	3.69 ± 1.85	10.97 ± 6.67	15.35 ± 11.46	19.50 ± 13.93	14.57 ± 19.07	12.39 ± 10.22	115.09 ± 1.37
7	2.07 ± 1.19	7.18 ± 4.94	10.59 ± 9.23	13.92 ± 10.95	10.27 ± 15.37	7.64 ± 7.11	112.33 ± 3.57
8	1.32 ± 0.89	4.95 ± 3.38	7.17 ± 6.92	11.20 ± 9.28	8.38 ± 13.28	4.51 ± 4.25	112.19 ± 3.66
9	0.88 ± 0.71	3.83 ± 2.66	4.87 ± 5.13	8.78 ± 7.47	6.54 ± 10.38	3.16 ± 2.95	112.15 ± 3.57
10	0.56 ± 0.44	2.82 ± 1.96	3.11 ± 3.46	7.13 ± 6.51	5.34 ± 8.58	2.03 ± 1.86	112.15 ± 3.57
11	0.19 ± 0.15	1.11 ± 0.80	1.02 ± 1.37	3.51 ± 3.30	2.46 ± 4.01	0.51 ± 0.46	112.15 ± 3.57
18	0.02 ± 0.01	0.08 ± 0.06	0.06 ± 0.06	0.62 ± 0.70	0.30 ± 0.47	0.02 ± 0.01	112.15 ± 3.57
21	0.48 ± 0.01	0.49 ± 0.01	0.53 ± 0.03	0.77 ± 0.26	0.53 ± 0.16	0.60 ± 0.29	112.15 ± 3.57

A2

	Fructose						
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control: yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
0	110.66 ± 0	110.66 ± 0	110.66 ± 0	109.17 ± 0	109.17 ± 0	109.17 ± 0	112.23 ± 0
1	97.34 ± 9.39	107.50 ± 3.93	103.64 ± 2.08	110.94 ± 4.76	106.22 ± 1.19	105.87 ± 0.23	109.68 ± 0.72
2	84.02 ± 9.34	95.50 ± 0.50	96.18 ± 2.07	101.64 ± 7.13	97.15 ± 2.89	95.20 ± 3.36	112.84 ± 2.25
3	62.30 ± 11.23	79.40 ± 0.86	79.73 ± 6.55	84.62 ± 10.58	78.83 ± 9.02	75.35 ± 6.76	109.45 ± 3.39
4	47.98 ± 10.76	63.24 ± 3.31	69.82 ± 11.20	72.77 ± 13.24	64.88 ± 16.40	59.48 ± 10.44	113.64 ± 4.11
5	38.78 ± 10.58	51.03 ± 7.59	55.72 ± 11.56	55.23 ± 17.70	47.39 ± 50.55	42.93 ± 15.71	110.17 ± 2.14
6	27.69 ± 8.14	41.22 ± 8.86	46.45 ± 12.85	44.20 ± 20.20	34.66 ± 25.71	31.25 ± 17.32	108.27 ± 4.31
7	20.54 ± 6.35	33.50 ± 9.38	37.75 ± 11.46	34.28 ± 18.88	24.77 ± 25.31	23.21 ± 15.52	107.56 ± 2.16
8	15.91 ± 5.17	28.13 ± 9.09	30.36 ± 11.98	29.31 ± 18.91	20.23 ± 24.27	17.37 ± 13.01	107.83 ± 1.70
9	12.19 ± 5.13	23.87 ± 7.74	24.86 ± 11.24	25.58 ± 17.13	17.48 ± 22.68	13.60 ± 10.77	107.84 ± 1.71
10	9.31 ± 4.42	20.02 ± 7.06	19.88 ± 10.05	21.77 ± 15.63	15.07 ± 18.28	10.80 ± 8.99	107.84 ± 1.71
11	3.65 ± 2.69	11.32 ± 0.01	9.57 ± 7.96	14.03 ± 12.10	9.30 ± 12.98	4.57 ± 2.68	107.84 ± 1.70
18	0.85 ± 0.43	2.65 ± 1.10	1.98 ± 1.20	4.77 ± 4.26	2.64 ± 3.49	0.70 ± 0.29	107.84 ± 1.70
21	0.38 ± 0.18	1.20 ± 0.55	0.81 ± 0.79	2.49 ± 2.20	1.34 ± 1.58	0.32 ± 0.11	107.84 ± 1.70

Table 2: Glucose (B1) and fructose (B2) consumption during standard fermentation with Cross Evolution and EC1118 and *O.oeni*

B1	Glucose						
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control: yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control bacteria: *O. oeni*
0	119.14 ± 0	119.14 ± 0	119.14 ± 0	114.12 ± 0	114.12 ± 0	114.12 ± 0	119.56 ± 0
1	104.04 ± 2.56	105.81 ± 1.43	103.11 ± 0.44	102.93 ± 1.83	102.49 ± 1.28	101.97 ± 0.75	108.29 ± 6.43
2	82.10 ± 0.34	80.57 ± 6.09	79.13 ± 3.66	84.06 ± 3.16	81.89 ± 1.79	86.96 ± 1.93	111.22 ± 5.42
3	82.12 ± 0.16	80.96 ± 6.18	79.07 ± 4.57	81.33 ± 4.49	79.24 ± 1.37	86.27 ± 3.05	108.58 ± 4.32
4	47.94 ± 2.31	44.18 ± 9.06	41.25 ± 6.39	48.87 ± 8.98	45.81 ± 2.25	55.34 ± 6.81	108.58 ± 4.32
5	40.62 ± 1.80	37.82 ± 9.06	35.25 ± 6.53	42.15 ± 8.51	38.98 ± 3.04	48.93 ± 7.28	112.09 ± 1.88
6	32.67 ± 2.40	30.44 ± 8.32	28.75 ± 6.20	34.43 ± 7.91	31.18 ± 3.15	40.40 ± 6.57	111.07 ± 3.83
9	14.83 ± 1.89	13.09 ± 5.25	11.95 ± 3.67	15.72 ± 5.98	11.95 ± 3.40	18.84 ± 6.63	112.09 ± 1.74E-14
12	6.25 ± 1.26	4.85 ± 2.47	4.76 ± 1.96	6.33 ± 3.70	3.89 ± 1.50	9.06 ± 4.45	112.09 ± 1.74E-14
19	0.033 ± 0.02	0.02 ± 0.02	0.04 ± 0.04	0.04 ± 0.06	0.002 ± 0.001	0.12 ± 0.14	111.07 ± 0
23	0.01 ± 0.003	0.004 ± 5.77E-05	0.02 ± 0.004	0.02 ± 0.03	0.01 ± 0.002	0.06 ± 0.08	112.09 ± 0

B2	Fructose						
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control: yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control bacteria: *O. oeni*
0	110.48 ± 0	110.48 ± 0	110.48 ± 0	106.63 ± 0	106.63 ± 0	106.63 ± 0	109.30 ± 0
1	103.74 ± 2.49	103.38 ± 0.95	101.45 ± 0.72	101.94 ± 1.36	100.19 ± 0.56	100.85 ± 1.17	103.87 ± 10.25
2	95.55 ± 0.37	95.26 ± 2.85	93.77 ± 2.65	93.62 ± 3.15	93.81 ± 0.57	94.94 ± 0.60	102.14 ± 4.25
3	98.82 ± 0.28	99.87 ± 2.77	96.98 ± 1.95	95.43 ± 3.18	94.59 ± 1.63	97.06 ±1.53	103.07 ± 4.00
4	81.28 ± 1.77	77.95 ± 7.35	74.09 ± 5.16	74.57 ± 8.74	72.57 ± 2.64	79.03 ± 5.48	103.07 ± 4.00
5	74.08 ± 0.91	72.54 ± 9.72	68.56 ± 6.33	67.18 ± 8.11	65.92 ± 2.57	73.04 ± 5.36	106.47 ± 0.68
6	67.65 ± 2.48	66.01 ± 8.32	61.46 ± 6.72	62.91 ± 9.12	59.48 ± 3.11	67.40 ± 6.94	106.17 ± 0.62
9	42.80 ± 4.27	41.43 ± 10.28	36.06 ± 6.45	36.20 ± 9.29	30.56 ± 5.68	39.01 ± 8.54	106.17 ± 0
12	27.85 ± 2.96	24.96 ± 7.99	22.09 ± 6.12	21.11 ± 7.75	16.09 ± 3.65	24.62 ± 7.89	108.17 ±1.74 E-14
19	1.74 ± 0.44	1.44 ± 0.87	1.58 ± 0.83	1.13 ± 0.10	0.49 ± 0.12	1.24 ± 0.70	106.17 ± 0
23	1.09 ± 0.36	0.93 ± 0.55	1.27 ± 0.79	0.81 ± 0.67	0.40 ± 0.06	0.98 ± 0.53	110.17 ± 0

Table 3: Glucose (C1) and fructose (C2) consumption during standard fermentation with Cross Evolution and EC1118 and *L. plantarum* and *O.oeni*

C1	Glucose						
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control: yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*
0	107.58 ± 0	107.58 ± 0	107.58 ± 0	108.80 ± 0	108.80 ± 0	108.80 ± 0	104.57 ± 1.95
1	97.86 ± 6.16	95.95 ± 1.10	85.34 ± 1.88	94.71 ± 0.36	94.51 ± 1.61	85.26 ± 1.16	104.58 ± 1.94
2	47.53 ± 7.29	35.21 ± 7.69	39.91 ± 6.70	50.60 ± 1.47	53.03 ± 5.07	41.66 ± 4.28	104.58 ± 1.94
3	28.41 ± 0.38	25.57 ± 0.27	22.99 ± 1.01	30.59 ± 0.18	39.66 ± 0.19	27.18 ± 0.12	106.82 ± 0.62
4	18.07 ± 6.23	8.81 ± 4.81	11.48 ± 4.16	21.79 ± 3.77	25.17 ± 5.75	12.70 ± 3.74	107.79 ± 0.66
5	11.64 ± 5.01	4.47 ± 3.69	6.47 ± 3.38	14.63 ± 3.87	17.71 ± 5.66	6.36 ± 3.15	107.70 ± 0.74
12	0.06 ± 0.07	0.004 ± 0.004	0.002 ± 0.02	0.03 ± 0.02	0.06 ± 0.05	0.002 ± 0.001	107.63 ± 0.55

C2	Fructose						
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control: yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*
0	110.73 ± 0	110.73 ± 0	110.73 ± 0	113.94 ± 0	113.94 ± 0	113.94 ± 0	101.052 ± 1.55
1	85.29 ± 19.94	80.12 ± 1.86	74.06 ± 0.84	86.51 ± 0.15	86.73 ± 0.32	76.76 ± 1.69	100.88 ± 1.74
2	77.16 ± 7.43	67.24 ± 5.81	62.55 ± 5.65	71.93 ± 1.62	75.65 ± 4.19	56.73 ± 1.85	100.88 ± 1.74
3	61.87 ± 1.60	63.24 ± 3.31	69.82 ± 11.20	72.77 ± 13.24	64.88 ± 16.40	59.48 ± 10.44	96.95 ± 8.55
4	52.87 ± 9.12	37.85 ± 7.92	39.40 ± 7.14	46.92 ± 5.16	51.09 ± 7.51	32.12 ± 4.92	98.82 ± 7.62
5	44.13 ± 10.45	28.39 ± 8.48	32.06 ± 8.16	39.31 ± 5.64	43.82 ± 8.30	23.08 ± 5.70	98.85 ± 7.65
12	2.34 ± 1.31	0.85 ± 0.71	0.95 ± 0.73	1.34 ± 0.81	1.89 ± 0.93	0.42 ± 0.20	99.16 ± 7.04

Table 4: Yeast populations of standard fermentations with *L. plantarum* (A), *O. oeni* (B) and the bacterial combination (*L. plantarum* and *O. oeni*) (C)

A

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control: yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation
0	1.07E6 ± 8.74E05	3.67E5 ± 5.51E5	6.67E5 ± 8.33E5	9.67E5 ± 4.62E5	5.67E5 ± 6.66E5	1.23E6 ± 3.79E5
1	1.37E7 ± 2.52E6	2.07E7 ± 1.55E7	1.17E7 ± 3.06E6	2.20E7 ± 7.94E6	4.20E7 ± 4.53E7	1.17E7 ± 2.52E6
2	7.4E7 ± 2.12E7	3.33E7 ± 1.93E7	2.07E7 ± 6.66E6	2.80E7 ±2.74E7	4.00E7 ± 2.52E7	3.93E7 ± 1.89E7
3	4.93E7 ± 6.66E6	4.07E7 ± 1.50E7	1.37E7 ± 8.70E6	1.83E7 ± 2.33E7	3.50E7 ± 2.25E7	4.27E7 ± 2.43E7
4	9.8E6 ± 3.66E6	6.7E6 ± 4.18E6	7.33E6 ± 4.57E6	8.87E7 ± 6.97E7	3.02E7 ± 4.32E7	5.87E7 ± 6.00E7
5	1.15E7 ± 7.48E6	8.53E6 ± 7.06E6	5.5E6 ± 3.70E6	2.39E7 ± 2.66E7	4.26E7 ± 3.61E7	2.20E7 ± 1.52E7
6	1.41E7 ±1.38E7	3.53E6 ± 2.52E5	3.9E6 ± 3.10E6	1.70E7 ± 2.09E7	1.52E7 ± 1.72E7	9.58E6 ± 7.08E6
7	5.93E6 ± 6.91E6	4.57E6 ± 3.51E6	1.07E6 ± 1.10E6	7.60E6 ± 5.96E6	5.53E6 ± 3.44E6	4.63E6 ± 4.24E6
8	4.73E6 ± 3.19E6	2.23E6 ± 1.59E6	2.43E6 ± 1.74E6	2.60E6 ± 1.95E6	5.00E6 ± 5.39E6	4.00E6 ± 3.35E6
9	1.53E6 ± 1.39E6	3.93E5 ± 2.05E5	7.13E5 ± 1.32E5	8.80E5 ± 2.72E5	2.44E6 ± 2.32E6	9.83E5 ± 5.65E5
10	1.32E5 ± 9.39E4	2.57E5 ± 5.51E4	2.67E5 ± 3.26E5	4.70E05 ± 1.30E5	5.83E5 ± 6.90E5	1.90E5 ± 1.61E5
21	7.33E3 ± 9.24E3	1.63E4 ± 8.74E3	3.33E3 ± 1.15E3	1.58E05 ± 2.53E5	1.13E4 ± 3.51E3	1.00E4 ± 5.29E3

B

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control: yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation
0	7.77E5 ± 1.07E5	4.87E5 ± 1.65E5	4.63E5 ± 3.04E5	1.02E6 ± 4.51E5	7.97E5 ± 1.88E5	2.84E6 ± 3.17E6
1	5.53E6 ± 3.11E6	6.20E6 ± 2.45E6	3.20E6 ± 2.00E5	6.87E6 ± 1.14E6	8.00E6 ± 1.41E6	1.80E7 ± 1.74E7
2	4.23E ± 1.15E6	6.97E6 ± 5.28E6	8.87E6 ± 3.44E6	1.72E7 ± 1.21E7	1.10E7 ± 3.69E6	7.60E6 ± 6.30E6
3	2.13E7 ± 2.83E7	2.28E7 ± 2.40E7	1.24E7 ± 6.96E6	1.96E7 ± 1.46E7	1.17E7 ± 3.61E6	8.73E6 ± 6.89E6
4	8.67E5 ± 3.71E5	1.66E6 ± 2.06E6	7.57E5 ± 3.41E5	3.06E6 ± 2.38E6	4.10E6 ± 2.56E6	2.09E6 ± 1.35E6
5	6.80E5 ± 3.86E5	1.07E6 ± 7.16E5	7.13E5 ± 2.30E5	3.33E6 ± 9.02E5	1.15E7 ± 3.39E6	6.80E5 ± 6.22E5
6	2.58E5 ± 2.23E5	2.82E5 ± 1.69E5	1.36E5 ± 1.18E5	3.69E5 ± 2.21E5	1.67E5 ± 2.18E5	2.97E5 ± 1.04E5
9	3.15E5 ± 2.81E5	1.17E6 ± 3.79E5	5.93E5 ± 4.09E5	5.93E4 ± 3.93E4	2.63E5 ± 2.23E5	3.11E5 ± 2.50E5
12	1.50E5 ± 9.81E4	2.26E5 ± 1.44E5	1.31E5 ± 1.13E5	6.71E5 ± 7.41E5	9.30E5 ± 1.04E5	4.27E5 ± 1.15E5
19	8.00E3 ± 2.00E3	2.67E4 ± 8.74E3	8.67E3 ± 8.50E3	9.77E4 ± 9.50E4	6.64E5 ± 7.35E5	7.03E4 ± 4.13E4
23	1.26E4 ± 1.68E4	3.77E3 ± 2.72E3	8.67E2 ± 1.03E3	2.99E4 ± 2.66E4	2.91E4 ± 1.82E4	5.90E3 ± 4.17E3

C

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation
0	9.93E5 ± 5.51E4	8.73E5 ± 8.74E4	8.43E5 ± 1.93E5	9.23E5 ± 1.46E5	1.10E6 ± 3.01E5	7.80E5 ± 1.22E5
1	1.32E7 ± 2.35E6	2.27E7 ± 5.58E6	4.36E7 ± 4.36E7	1.73E7 ± 3.04E6	2.09E7 ± 6.70E6	1.53E7 ± 4.33E6
2	2.50E7 ± 7.94E6	3.90E7 ± 1.35E7	2.97E7 ± 1.26E7	3.77E7 ± 4.73E6	3.23E7 ± 5.13E6	3.83E7 ± 1.16E7
3	2.76E7 ± 7.07E6	4.71E7 ± 3.98E7	6.24E7 ± 4.81E7	3.79E7 ± 9.30E6	2.01E7 ± 2.28E7	2.65E7 ± 1.60E7
4	3.09E7 ± 2.04E6	2.94E7 ± 1.18E6	2.84E7 ± 1.21E6	4.04E7 ± 1.21E6	3.00E7 ± 2.00E5	3.22E7 ± 2.67E6
5	1.32E7 ± 1.56E6	2.29E7 ± 2.10E6	2.80E7 ± 2.12E7	3.08E7 ± 1.53E6	2.18E7 ± 1.63E7	3.15E7 ± 7.21E5
12	1.04E7 ± 5.29E5	2.99E7 ± 1.15E5	42.25E7 ± 4.04E5	2.60E7 ± 0	1.91E7 ± 1.15E5	1.73E7 ±2.31E5

Table 5: Bacterial populations during standard co-inoculation with *L. plantarum* (A1), *O. oeni* (B1) and the bacterial combination (*L. plantarum* and *O. oeni*) (C1) and sequential inoculation with *L. plantarum* (A2), *O. oeni* (B2) and the bacterial combination (*L. plantarum* and *O. oeni*) (C2)

A1

Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
0	3.33E7 ± 3.46E7	1.67E7 ± 9.82E5	4.99E7 ± 3.44E7
1	6.47E7 ± 7.77E6	7.77E7 ± 1.72E7	2.03E7 ± 3.52E7
2	1.26E7 ± 2.08E5	4.33E7 ± 2.30E7	1.43E7 ± 1.25E6
3	2.02E8 ± 1.88E8	3.21E8 ± 3.28E8	9.90E6 ± 0
4	4.49E7 ± 4.50E7	1.54E8 ± 1.39E8	
7	1.67E2 ± 2.08E2	2.37E3 ± 1.18E3	

A2

Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
0	1.53E7 ± 1.04E7	4.63E6 ± 4.08E6
4	7.70E6 ± 7.11E6	1.32E7 ± 1.64E7
8	1.05E6 ± 1.43E6	9.20E6 ± 1.29E7
11	6.67E4 ± 5.51E4	3.83E6 ± 6.56E6
18	1.84E4 ± 0	4.67E3 ± 0

B1

Days	Cross Evolution	EC 118 Co-inoculation	Control bacteria: *O. oeni*
0	2.67E7 ± 4.50E6	1.25E7 ± 7.47E6	3.10E7 ± 2.45E7
1	1.79E7 ± 8.19E6	8.77E6 ± 4.66E6	4.81E7 ± 4.78E7
2	3.67E7 ± 3.28E7	3.80E7 ± 1.73E6	2.07E9 ± 3.45E9
3	2.02E8 ± 1.88E8	3.21E8 ± 3.28E8	2.28E8 ± 3.28E8
4	4.14E6 ± 5.60E5	3.88E6 ± 1.70E5	2.33E7 ± 3.22E07
5	2.20E5 ± 1.96E4	2.41E5 ± 1.88E4	1.89E5 ± 4.56E3

B2

Days	Cross Evolution Sequetial inoculation	EC 1118 Sequential inoculation
0	2.41E7 ± 1.73E6	2.63E7 ± 3.88E6
1	4.20E7 ± 6.56E6	4.13E7 ± 5.51E6
2	3.70E7 ± 7.55E6	4.70E7 ± 1.35E7
3	1.47E7 ± 1.12E7	8.49E6 ± 6.23E6

C1

Days	Cross Evolution Co-inoculation: *L. plantarum*	EC 1118 Co-inoculation: *L. plantarum*	Cross Evolution Co-inoculation: *O .oeni*	EC 1118 Co-inoculation: *O. oeni*	Control combination: *L. plantarum*	Control combination: *O. oeni*
0	3.10E7 ± 3.61E6	3.30E7 ± 3.00E6	2.80E7 ± 2.65E6	2.73E7 ± 8.08E6	8.67E7 ± 1.15E7	2.97E7 ± 3.51E6
1	9.73E7 ± 1.13E8	8.20E7 ± 3.06E7	1.00E7 ± 1.73E7	3.07E7 ± 4.27E7	3.97E ± 7.09E6	1.63E8 ± 4.73E7
2	8.43E7 ± 3.27E7	2.05E8 ± 1.02E7	1.00E7 ± 0	2.00E7 ± 0	1.39E8 ± 8.81E7	8.67E7 ± 4.04E7
3	1.61E8 ± 3.20E7	1.29E8 ± 2.31E6	1.00E7 ± 0	2.00E7 ± 0	3.10E8 ± 2.36E7	3.33E7 ± 1.53E7
4	3.20E7 ± 0	1.53E8 ± 2.31E6	1.00E7 ± 0	2.00E7 ± 0		
5		1.46E8 ± 4.51E6	1.00E7 ± 0	2.00E7 ± 0		
6			4.67E7 ± 2.31E7	8.67E7 ± 2.31E7		
7			1.00E8 ± 8.72E7	5.00E7 ±1.73E7		
8			3.40E6 ± 1.78E6	2.23E6 ± 4.04E5		
9			2.00E6 ± 0	1.27E6 ± 5.77E4		

C2

Days	Cross Evolution Sequential: *L. plantarum*	Cross Evolution Sequential: *O. oeni*	EC 1118 Sequential inoculation: *L. plantarum*	EC 1118 Sequential inoculation: *O. oeni*
0	3.77E7 ± 4.53E7	1.39E8 ± 2.65E7	1.43E7 ± 9.07E6	1.24E8 ± 2.11E7
1	1.29E8 ± 5.37E7	8.33E6 ± 4.93E6	3.04 ± 1.69E8	1.00E7 ± 2.65E6
2	1.43E7 ± 3.79E6	5.07E8 ± 3.84E8	1.23E7 ± 1.15E6	6.60E8 ± 4.30E8
3	8.00E6 ± 6.93E6	2.30E7 ± 8.19E6	9.27E6 ±4.45E6	4.40E7 ± 5.20E6

Table 6: Malic acid degradation during standard co-inoculation with *L. plantarum* (A1), *O. oeni* (B1) and the bacterial combination (*L. plantarum* and *O. oeni*) (C1) and sequential inoculation with *L. plantarum* (A2), *O. oeni* (B2) and the bacterial combination (*L. plantarum* and *O. oeni*) (C2)

A1

	Malic acid			Lactic acid		
Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
0	2.91 ± 0	2.91 ± 0	2.91 ± 0	0 ± 0	0 ± 0	0 ± 0
1	0.81 ± 0.16	0.56 ± 0.19	1.73 ± 0.09	1.74 ± 0.13	1.77 ± 0.04	1.19 ± 0.16
2	0.20 ± 0.01	0.18 ± 0.004	0.19 ± 0.01	1.33 ± 0.14	1.72 ± 0.02	2.21 ± 0.14
3	0.17 ± 0.01	0.16 ± 0.008	0.19 ± 3.4E-17	2.43 ± 0.07	2.29 ± 0.12	2.28 ± 0.08
4	0.16 ± 0.01	0.15 ± 0.01	0.19 ± 0.01	2.72 ± 0.03	2.66 ± 0.13	2.88 ± 0.02
5	0.15 ± 0.01	0.14 ± 0.01	0.19 ± 0.001	2.85 ± 0.08	2.91 ± 0.17	3.08 ± 0.01
6	0.14 ± 0.01	0.12 ± 0.02	0.18 ± 0.01	2.85 ± 0.20	2.82 ± 0.16	4.09 ± 0.06
7	0.12 ± 0.01	0.10 ± 0.01	0.17 ± 0.003			

A2

	Malic acid		Lactic acid	
Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
0	2.38 ± 0	2.09 ± 1.92E-7	0.04 ± 0.004	0.29 ± 0.38
4	1.96 ± 0.36	1.54 ± 0.62	0.48 ± 0.25	0.82 ± 0.63
8	1.31 ± 0.71	0.91 ± 0.74	1.16 ± 0.60	1.54 ± 0.81
11	0.32 ± 0.32	0.39 ± 0.33	1.26 ± 0.29	1.97 ± 1.29
12	0.03 ± 0.32	0.03 ± 0.33	2.26 ± 0.72	1.98 ± 0.82
18	0.01 ± 0.001	0.01 ± 0.001	2.45 ± 0	2.10 ± 0.31

B1

	Malic acid			Lactic acid		
Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control bacteria: *O. oeni*	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control bacteria: *O. oeni*
0	3.21 ± 0	3.09 ± 0	3.17 ± 0	0.04 ± 0	0.04 ± 0	0.04 ± 0
1	2.32 ± 0.09	2.15 ± 0.17	0.63 ± 0.44	0.56 ± 0.08	0.68 ± 0.17	2.11 ± 0.44
2	0.93 ± 0.31	0.92 ± 0.35	0.24 ± 0.10	1.84 ± 0.36	1.85 ± 0.54	2.53 ± 0.06
3	0.07 ± 0.33	0.06 ± 0.35	0.18 ± 0.05	2.72 ± 0.02	2.69 ± 0.48	2.61 ± 0.01
4	0.06 ± 0.01	0.05 ± 0.01	0.15 ± 0.01	2.75 ± 0.05	2.75 ± 0.02	2.64 ± 0.11
5	0.04 ± 0	0.04 ± 0.01	0.13 ± 0.01	2.77 ± 0.03	2.79 ± 0.02	2.64 ± 0
6	0.01 ± 0.33	0.04 ± 0	0.13 ± 0.02	2.80 ± 0.34	2.81 ± 0.02	2.66 ± 0.05

B2

	Malic acid		Lactic acid	
Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
0	2.38 ± 0.05	2.35 ± 0.04	0.03 ± 0	0.03 ± 0
1	1.41 ± 0.10	1.10 ± 0.07	1.04 ± 0.11	1.10 ± 0.04
2	0.47 ± 0.28	0.11 ± 0.04	1.73 ± 0.28	1.93 ± 0.08
3	0.03 ± 0	0.03 ± 0.001	2.12 ± 0.05	1.98 ± 0.02

C1

	Malic acid			Lactic acid		
Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Contol: *L. plantarum and O. oeni*	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Contol: *L. plantarum and O. oeni*
0	2.76 ± 0.002	2.75 ± 0	2.58 ± 0.58	0.04 ± 0	0.04 ± 0	0.64 ± 0.01
1	0.05 ± 0.001	0.06 ± 0.01	0.47 ± 0.36	1.86 ± 0.03	1.83 ± 0.06	1.99 ± 0.003
2	0.04 ± 0.002	0.05 ± 0.001	0.02 ± 0.30	2.16 ± 0.01	2.25 ± 0.02	1.98 ± 0.05
3	0.03 ± 0.02	0.03 ± 0.01	0.01 ± 0.001	2.58 ± 0.29	2.69 ± 0.02	2.13 ± 0.02
4	0.03 ± 0.003	0.03 ± 0.002		2.66 ± 0.07	2.82 ± 0.10	
5	0.03 ± 0.002	0.03 ± 0.01		3.03 ± 0.08	3.20 ± 0.14	

C2

	Malic acid		Lactic acid	
Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
0	2.34 ± 0.002	2.32 ± 0.02	0.02 ± 0.003	0.02 ± 0.002
1	0.02 ± 0.001	0.02 ± 0	2.31 ± 0.01	2.32 ± 0.04
2	0.02 ± 0.001	0.02 ± 0	2.34 ± 0.01	2.32 ± 0.04

Table 7: Glycerol concentrations of standard fermentations with *L. plantarum* (A), *O. oeni* (B) and the bacterial combination (*L. plantarum* and *O. oeni*) (C)

A

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5	5.62 ± 0	5.10 ± 0	5.54 ± 0.53	6.22 ± 0.001	6.01± 0.01	5.53 ± 0.41	0.01 ± 0.001
21	4.23 ± 0.25	4.33 ± 0.41	3.94 ± 0.46	4.81 ± 0.13	4.14 ± 0.19	4.57 ± 0.52	0.02 ± 0.001

B

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *O. oeni*
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
6	3.28 ± 0.01	3.20 ± 0.02	3.55 ± 0.71	4.09 ± 0.001	4.00 ± 0.03	3.50 ± 0.40	0.01 ± 0.01
23	3.97 ± 0.18	4.16 ± 0.21	4.01 ± 0.18	5.40 ± 0.14	5.34 ± 0.22	5.21 ± 0.44	0.03 ± 0.01

C

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
3	5.72 ± 0.002	5.50 ± 0.04	5.03 ± 0.34	3.88 ± 0.003	3.75 ± 0.05	6.17 ± 0.50	0.01 ± 0.001
12	5.49 ± 0.15	5.15 ± 0.46	5.29 ± 0.06	4.97 ± 0.12	5.14 ± 0.06	5.14 ± 0.23	0.01 ± 0.001

Table 8: Glucose (A1) and fructose (A2) consumption of NH_4Cl-supplemented fermentations with Cross Evolution and EC1118 and *L. plantarum*

A1

Glucose							
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
0	120.35 ± 0	120.35 ± 0	120.35 ± 0	119.63 ± 0	119.63 ± 0	119.63 ± 0	120.48 ± 0
1	111.14 ± 1.97	110.04 ± 2.23	106.90 ± 0.45	109.44 ± 2.13	110.49 ± 1.05	109.14 ± 0.50	118.9 ± 0.88
2	108.93 ± 1.74	105.97 ± 2.64	102.88 ± 0.91	106.87 ± 1.51	110.08 ± 0.28	106.33 ± 1.30	120.38 ± 0.69
5	92.85 ± 0.43	91.77 ± 0.13	87.16 ± 0.27	94.72 ± 0.68	94.47 ± 2.49	90.06 ± 0.99	120.50 ± 0.36
8	75.37 ± 1.35	73.99 ± 0.61	62.67 ± 2.06	78.30 ± 2.38	76.32 ± 1.59	69.70 ± 2.48	119.84 ± 0.62
12	69.68 ± 1.07	68.51 ± 0.53	58.93 ± 2.31	71.61 ± 2.75	70.87 ± 1.40	64.11 ± 3.26	119.84 ± 0.62
28	14.77 ± 0.89	13.11 ± 1.73	1.27 ± 0.63	9.54 ± 5.19	5.18 ± 2.39	0.09 ± 0.10	119.84 ± 0.62
44	0.47 ± 0.34	0.36 ± 0.26	0.01 ± 0	0.07 ± 0.06	0.01 ± 0	0.01 ± 0	119.75 ± 0.69

A2

Fructose							
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
0	108.82 ± 0	108.82 ± 0	108.82 ± 0	108.42 ± 0	108.42 ± 0	108.42 ± 0	109.65 ± 0
1	104.52 ± 0.26	102.57 ± 3.11	102.92 ± 0.56	103.66 ± 0.71	103.55 ± 0.68	103.35 ± 0.48	107.50 ± 0.52
2	103.61 ± 0.32	102.85 ± 0.89	102.34 ± 0.77	103.93 ± 1.87	105.04 ± 0.77	102.91 ± 0.64	108.22 ± 0.84
5	99.34 ± 0.57	98.34 ± 0.80	96.43 ± 0.24	99.08 ± 0.85	99.61 ± 1.61	96.77 ± 0.89	109.06 ± 0.54
8	93.49 ± 0.5	90.87 ± 1.53	86.08 ± 1.91	89.88 ± 1.35	91.13 ± 0.94	86.57 ± 1.28	110.37 ± 0.13
12	88.73 ± 1.61	88.02 ± 0.86	82.02 ± 2.15	86.86 ± 2.53	87.06 ± 1.49	84.20 ± 1.47	110.37 ± 0.13
28	56.46 ± 1.43	53.52 ± 1.99	25.05 ± 3.80	36.30 ± 8.63	28.76 ± 5.61	5.62 ± 5.02	110.37 ± 0.13
44	1.85 ± 0.39	1.20 ± 0.07	0.03 ± 0.03	0.54 ± 0.41	0.58 ± 0.82	0.01 ± 0	110.44 ± 0.35

Table 9: Glucose (A1) and fructose (A2) consumption of NH_4Cl-supplemented fermentations with Cross Evolution and EC1118 and *O.oeni*

B1

Glucose							
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *O. oeni*
0	123.43 ± 0	123.44 ± 0	123.44 ± 0	123.21 ± 0	123.21 ± 0	123.21 ± 0	122.17 ± 0
1	118.70 ± 1.33	119.00 ± 1.46	105.53 ± 1.57	110.62 ± 0.63	113.68 ± 3.07	100.75 ± 0.63	110.21 ± 0.29
2	110.90 ± 0.76	113.66 ± 2.70	99.42 ± 1.15	102.15 ± 1.10	104.17 ± 0.73	92.80 ± 0.54	107.68 ± 1.02
3	109.13 ± 1.06	105.63 ± 1.51	98.53 ± 1.96	98.76 ± 1.90	100.06 ± 0.46	90.11 ± 1.37	111.29 ± 0.20
7	75.85 ± 13.62	77.67 ± 2.37	70.03 ± 3.33	67.82 ± 9.76	73.75 ± 4.00	65.52 ± 2.68	111.16 ± 0.53
16	35.90 ± 22.13	37.47 ± 4.85	26.08 ± 2.71	23.20 ± 14.47	30.88 ± 6.51	25.60 ± 6.98	111.16 ± 0.53
29	7.16 ± 8.29	4.06 ± 2.50	0.01 ± 0.15	1.55 ± 2.61	1.97 ± 2.76	0.19 ± 0.30	111.18 ± 0.52
64	0.07 ± 0.11	0.02 ± 0	0.01 ± 0	0.51 ± 0.86	0.63 ± 0.87	0.01 ± 0.01	111.51 ± 0.61

B2

Fructose							
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *O. oeni*
0	114.07 ± 0	114.07 ± 0	114.07 ± 0	117.13 ± 0	117.13 ± 0	117.13 ± 0	118.53 ± 0
1	110.01 ± 1.66	110.85 ± 1.17	103.79 ± 1.06	107.49 ± 0.46	109.07 ± 0.35	99.52 ± 0.48	104.26 ± 0.34
2	108.72 ± 0.62	108.62 ± 1.03	99.46 ± 0.57	103.56 ± 1.82	103.22 ± 1.78	96.61 ± 0.39	103.40 ± 0.97
3	109.12 ± 0.28	109.41 ± 0.98	100.91 ± 1.53	104.90 ± 0.30	106.23 ± 0.09	96.63 ± 0.84	106.94 ± 0.79
7	97.65 ± 7.51	97.27 ± 2.27	89.00 ± 2.27	87.02 ± 6.05	91.41 ± 2.01	82.76 ± 2.18	105.72 ± 0.67
16	75.24 ± 20.49	79.24 ± 3.84	64.78 ± 2.62	53.62 ± 19.04	64.78 ± 6.80	56.09 ± 7.78	105.72 ± 0.67
29	32.25 ± 28.48	34.32 ± 7.78	5.31 ± 5.78	10.53 ± 15.01	15.34 ± 14.38	5.50 ± 7.57	105.72 ± 0.67
64	1.08 ± 0.98	0.38 ± 0.47	0.01 ± 0.01	0.59 ± 0.94	0.90 ± 0.89	0.05 ± 0.07	107.05 ± 1.64

Table 10: Glucose (A1) and fructose (A2) consumption of NH_4Cl supplemented fermentation with Cross Evolution and EC1118 and *L. plantarum* and *O. oeni*

C1

	Glucose						
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum and O. oeni*
0	108.16 ± 0	108.16 ± 0	108.16 ± 0	109.39 ± 0	109.39 ± 0	109.39 ± 0	109.44 ± 0
1	101.60 ± 1.10	100.89 ± 1.64	92.76 ± 0.8	101.82 ± 0.66	102.21 ± 0.37	91.44 ± 0.52	96.71 ± 1.65
2	96.89 ± 0.75	94.90 ± 4.73	89.81 ± 0.6	100.43 ± 0.42	100.79 ± 1.01	88.25 ± 1.47	95.60 ± 0.87
19	20.84 ± 3.40	22.43 ± 1.95	14.59 ± 7.09	64.53 ± 2.75	64.30 ± 3.79	26.77 ± 13.44	110.77 ± 0.58
29	7.16 ± 8.29	4.06 ± 2.50	0.10 ± 0.15	1.55 ± 2.61	1.97 ± 2.76	0.19 ± 0.30	111.18 ± 0.52
41	0.39 ± 0.37	0.25 ± 0.40	0.24 ± 0.39	0.28 ± 0.03	0.26 ± 0.06	0.24 ± 0.40	111.07 ± 0.33

C2

	Fructose						
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L .plantarum and O. oeni*
0	104.65 ± 0	104.65 ± 0	104.65 ± 0	105.65 ± 0	105.65 ± 0	105.65 ± 0	104.92 ± 0
1	102.33 ± 1.34	100.99 ± 1.00	93.40 ± 1.05	100.58 ± 0.89	100.06 ± 0.67	92.41 ± 0.8	95.47 ± 2.26
2	99.33 ± 0.46	99.44 ± 1.59	92.16 ± 0.65	99.31 ± 0.34	99.57 ± 0.47	90.20 ± 1.42	95.40 ± 1.78
19	57.10 ± 3.20	57.93 ± 1.66	43.84 ± 8.64	78.64 ± 1.59	78.99 ± 1.71	50.99 ± 14.76	106.26 ± 0.58
29	32.25 ± 28.48	34.32 ± 7.78	5.31 ± 5.78	10.53 ± 15.01	15.34 ± 14.38	5.50 ± 7.57	105.72 ± 0.67
41	0.16 ± 0.02	0.26 ± 0.13	0.26 ± 0.14	0.12 ± 0.01	0.41 ± 0.50	0.56 ± 0.48	106.54 ± 0.76

Table 11: Yeast populations of NH_4Cl-supplemented fermentations with *L. plantarum* (A), *O. oeni* (B) and the bacterial combination (*L. plantarum* and *O. oeni*) (C)

A

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation
0	3.33E6 ± 5.77E4	3.03E6 ± 5.77E4	2.45E6 ± 1.82E6	3.23E6 ± 5.77E4	3.30E6 ± 1.00E5	4.13E6 ± 5.77E4
1	3.47E6 ± 4.51E5	3.53E6 ± 5.51E5	3.80E6 ± 1.00E5	3.57E6 ±5.69E5	3.03E6 ± 7.23E5	2.67E6 ± 3.51E5
2	8.00E6 ± 2.65E6	6.67E6 ± 1.53E6	7.67E6 ± 5.77E5	6.67E6 ± 1.53E6	8.33E6 ± 2.31E6	1.80E7 ± 1.91E7
5	2.03E7 ± 1.04E4	2.00E7 ± 0	1.33E7 ± 3.51E6	7.63E7 ± 1.07E8	1.47E7 ± 3.79E6	1.67E7 ± 3.51E6
8	2.10E7 ± 1.73E6	2.33E7 ± 5.13E6	2.93E7 ± 1.11E7	9.47E7 ± 1.43E8	1.63E7 ± 4.62E6	1.77E7 ± 5.69E6
12	3.07E8 ± 7.23E7	3.13E7 ± 7.77E6	4.40E7 ± 5.72E7	6.67E7 ± 2.31E7	1.80E7 ± 1.00E6	1.83E7 ± 4.93E6
44	3.23E6 ± 5.77E6	2.97E6 ± 5.77E4	2.91E6 ± 3.61E4	7.77E5 ± 3.21E4	8.03E5 ± 5.77E03	9.53E5 ± 2.52E4

B

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation
0	2.37E6 ± 5.13E5	2.53E6 ± 4.16E5	2.80E6 ± 5.29E5	6.67E6 ± 2.31E5	5.83E6 ± 1.22E6	5.37E6 ± 9.02E5
1	4.07E6 ± 1.15E5	4.23E6 ± 5.77E4	3.47E6 ± 1.15E5	7.33E6 ± 2.89E5	7.27E6 ± 2.80E5	7.90E6 ± 1.73E5
2	4.33E6 ± 1.15E5	4.07E6 ± 5.77E4	4.33E6 ± 1.15E6	1.50E7 ± 1.73E6	1.30E7 ± 3.00E6	1.40E7 ± 2.65E6
3	1.03E7 ± 5.77E5	1.50E7 ± 3.61E6	1.00E7 ± 0	1.27E7 ± 4.62E6	1.17E7 ± 2.08E6	1.23E7 ± 2.52E6
16	8.67E6 ± 2.89E6	1.03E07 ± 5.13E6	5.67E6 ± 1.15E6	1.50E7 ± 0	1.33E7 ± 2.08E6	1.37E7 ± 3.21E6
64	3.90E5 ± 1.00E4	4.00E5 ± 0	5.00E5 ± 0	3.03E5 ± 5.77E3	3.07E5 ± 5.77E3	2.97E5 ± 5.77E3

C

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation
0	5.07E6 ± 1.53E5	4.70E6 ± 0	5.13E6 ± 5.77E4	1.17E6 ± 1.53E5	1.23E6 ± 2.52E5	3.13E6 ±1.15E5
1	7.13E6 ± 1.53E5	6.83E6 ± 2.08E5	7.83E6 ± 2.08E5	1.73E6 ±2.52E5	2.23E6 ± 2.08E5	6.00E6 ±0
2	1.01E7 ± 3.61E5	7.00E6 ± 2.00E5	8.07E6 ± 1.53E5	2.90E6 ± 2.65E5	3.30E6 ± 2.65E5	7.27E6 ± 2.08E5
19	2.21E7 ± 5.77E4	8.77E6 ± 5.77E4	1.28E7 ± 0	4.03E6 ± 5.77E4	5.93E6 ± 5.77E4	1.05E7 ± 5.77E4
41	2.83E6 ± 7.64E5	3.03E6 ± 5.77E4	3.87E6 ± 3.21E5	1.29E6 ±2.31E4	1.80E6 ±5.77E3	1.91E6 ± 5.77E3

Table 12: Bacterial populations during NH_4Cl-supplemented co-inoculation with *L. plantarum* (A1), *O. oeni* (B1) and the bacterial combination (*L. plantarum* and *O. oeni*) (C1) and sequential inoculation with *L. plantarum* (A2), *O. oeni* (B2) and the bacterial combination (*L. plantarum* and *O. oeni*) (C2)

A1

Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
0	2.09E7 ± 4.30E6	2.15E7 ± 1.65E7	3.50E7 ±1.04E7
1	2.52E7 ± 2.41E6	2.52E7 ± 4.21E6	2.60E7 ± 6.00E6
2	2.80E7 ± 1.04E7	2.67E7 ±4.73E6	2.63E7 ± 7.09E6
3	3.60E7 ± 6.24E6	3.10E7 ± 8.19E6	2.83E7 ± 5.77E6
5	3.80E7 ± 2.65E6	3.57E7 ± 2.52E6	1.50E7 ± 5.20E6
8	2.90E7 ± 3.46E6	3.50E7 ± 6.56E6	4.33E6 ± 5.77E5

A2

Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
0	5.27E7 ± 6.81E6	5.07E7 ± 1.37
1	3.33E6 ± 1.15E6	5.00E6 ± 0
2	5.33E6 ± 5.77E5	5.00E6 ± 0
4	1.97E6 ± 1.76E6	4.00E5 ± 0
8	5.73E4 ± 2.52E3	4.20E4 ± 0

B1

Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *O. oeni*
0	9.99E8 ± 1.36E7	9.33E8 ± 8.33E6	1.14E8 ± 8.62E6
1	1.26E9 ± 5.05E7	1.40E9 ± 5.51E7	7.47E7 ± 1.29E7
2	9.18E7 ± 7.82E7	1.14E8 ± 1.23E7	4.17E7 ± 2.52E6
3	1.35E8 ± 1.99E7	1.52E8 ± 1.04E7	2.70E7 ± 3.61E6

B2

Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
0	2.41E7 ± 1.73E6	2.63E7 ± 3.88E6
1	4.20E7 ± 6.56E6	4.13E7 ± 5.51E6
1	3.70E7 ± 7.55E6	4.70E7 ± 1.35E7
3	1.47E7 ± 1.12E7	8.49E6 ± 6.23E6

C1

Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum and O. oeni*
0	1.00E8 ± 2.65E6	8.20E7 ± 1.73E6	5.97E7 ± 5.77E5
1	7.63E7 ± 5.77E5	8.40E7 ± 4.58E6	5.10E7 ± 1.00E6
2	7.93E7 ± 5.77E5	8.23E7 ± 1.15E6	4.13E7 ± 3.21E6
3	1.35E8 ± 1.99E7	1.52E8 ± 1.04E7	2.70E7 ± 3.61E6

C2

Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
0	2.76E7 ± 4.21E6	2.27E7 ± 6.08E5
1	3.53E7 ± 1.53E6	3.03E7 ± 5.77E5
4	3.00E7 ± 0	1.07E6 ± 1.73E4

Table 13: Malic acid degradation during NH_4Cl-supplemented co-inoculation with *L. plantarum* (A1), *O. oeni* (B1) and the bacterial combination (*L. plantarum* and *O. oeni*) (C1) and sequential inoculation with *L. plantarum* (A2), *O. oeni* (B2) and the bacterial combination (*L. plantarum* and *O. oeni*) (C2)

		Malic acid			Lactic acid		
A1	Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
	0	2.90 ± 0	2.91 ± 0	2.92 ± 0	0.02 ± 0	0.03 ± 0	0.02 ± 0
	1	2.28 ± 0.01	2.26 ± 0.01	2.43 ± 0.02	0.63 ± 0.01	0.61 ± 0	0.55 ± 0
	2	1.90 ± 0.01	1.86 ± 0.05	2.19 ± 0.02	1.09 ± 0.01	1.08 ± 0.01	0.90 ± 0.01
	3	1.56 ± 0.01	1.53 ± 0.02	1.90 ± 0.01	1.40 ± 0.01	1.41 ± 0.01	1.14 ± 0
	5	0.86 ± 0.01	0.83 ± 0.01	1.50 ± 0.03	2.02 ± 0.02	2.11 ± 0.03	1.59 ± 0.01
	8	0.22 ± 0.02	0.18 ± 0.02	0.26 ± 0.4	2.81 ± 0.06	2.85 ± 0.05	2.05 ± 0.03

		Malic acid		Lactic acid	
A2	Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
	0	2.41 ± 0.14	2.43 ± 0.03	0.01 ± 0.002	0.01 ± 0.001
	1	2.01 ± 0.01	2.04 ± 0.04	0.17 ± 0.01	0.19 ± 0.04
	2	1.79 ± 0.05	1.78 ± 0.09	0.28 ± 0.02	0.33 ± 0.07
	9	0.07 ± 0.09	0.03 ± 0.23	1.31 ± 0.17	1.20 ± 0.38

		Malic acid			Lactic acid		
B1	Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *O. oeni*	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *O. oeni*
	0	3.05 ± 0	2.98 ± 0	3.02 ± 0	0.02 ± 0	0.02 ± 0	0.02 ± 0
	1	0.93 ± 0.08	0.89 ± 0.06	1.17 ± 0.01	1.30 ± 0.04	1.26 ± 0.03	1.17 ± 0.01
	2	0.08 ± 0.01	0.07 ± 0	0.28 ± 0.01	1.86 ± 0.01	1.79 ± 0.01	1.74 ± 0.01
	3	0.07 ± 0	0.07 ± 0	0.08 ± 0.01	1.92 ± 0.03	1.86 ± 0.01	1.97 ± 0.02

		Malic acid		Lactic acid	
B2	Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
	0	2.57 ± 0.07	2.50 ± 0.03	0.07 ± 0	0.08 ± 0
	1	0.08 ± 0	0.08 ± 0	1.40 ± 0.01	1.37 ± 0.03
	2	0.08 ± 0	0.08 ± 0	1.40 ± 0.02	1.38 ± 0.02
	3	0.08 ± 0	0.08 ± 0	1.40 ± 0	1.38 ± 0.02

		Malic acid			Lactic acid		
C1	Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*
	0	2.78 ± 0	2.84 ± 0	2.86 ± 0	0.02 ± 0	0.02 ± 0	0.02 ± 0
	1	1.30 ± 0.04	1.29 ± 0.07	1.66 ± 0.03	0.95 ± 0.01	0.90 ± 0.04	0.75 ± 0.02
	2	0.47 ± 0.03	0.39 ± 0.13	1.02 ± 0.05	1.50 ± 0.03	1.45 ± 0.05	1.15 ± 0.04
	3	0.07 ± 0	0.07 ± 0	0.08 ± 0.01	1.92 ± 0.03	1.86 ± 0.01	1.97 ± 0.02

		Malic acid		Lactic acid	
C2	Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
	0	2.39 ± 0.04	2.49 ± 0	0.06 ± 0	0.07 ± 0
	1	1.16 ± 0.25	1.29 ± 0	0.29 ± 0.40	0.10 ± 0
	5	0.05 ± 0.001	0.07 ± 0.004	1.34 ± 0.05	1.32 ± 0.02

Table 14: Glycerol concentrations of NH_4Cl-supplemented fermentations with *L. plantarum* (A), *O. oeni* (B) and the bacterial combination (*L. plantarum* and *O. oeni*) (C)

A

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
12	3.59 ± 0.04	3.52 ± 0.43	4.02 ± 0.28	3.38 ± 0.19	3.52 ± 0.23	3.90 ± 0.41	0.01 ± 0
44	5.49 ± 0.15	5.15 ± 0.46	5.29 ± 0.06	4.97 ± 0.12	5.14 ± 0.06	5.14 ± 0.23	0.01 ± 0

B

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *O. oeni*
0	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.01 ± 0
16	5.01 ± 1.98	5.24 ± 0.90	4.71 ± 0.3	5.18 ± 0.62	4.87 ± 0.30	4.34 ± 0.54	0.01 ± 0
64	5.24 ± 1.00	5.21 ± 0.11	4.97 ± 0.20	4.83 ± 0.17	4.92 ± 0.28	3.74 ± 0.88	0.01 ± 0

C

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
19	3.35 ± 0.40	3.36 ± 0.51	3.49 ± 0.58	3.55 ± 0.33	3.82 ± 0.57	3.38 ± 0.72	0.01 ± 0
41	4.41 ± 0.24	4.23 ± 0.50	4.26 ± 0.26	7.33 ± 0.11	7.47 ± 0.49	4.52 ± 0.19	0.02 ± 0

Table 15: Glucose (A1) and fructose (A2) consumption during amino acids-supplemented fermentations with Cross Evolution and EC1118 and *L. plantarum*

A1					Glucose			
	Days	**Cross Evolution Control: yeast**	**Cross Evolution Sequential inoculation**	**Cross Evolution Co-inoculation**	**EC 1118 Control:yeast**	**EC 1118 Sequential inoculation**	**EC 1118 Co-inoculation**	**Control: *L. plantarum***
	0	121.26 ± 0	121.26 ± 0	121.26 ± 0	125.25 ± 0	125.25 ± 0	125.25 ± 0	124.85 ± 0
	1	121.42 ± 0.75	123.41 ± 1.19	119.89 ± 0.18	119.81 ± 0.68	117.24 ± 2.50	116.37 ± 1.57	120.37 ± 0.95
	2	109.41 ± 2.38	106.04 ± 5.21	107.46 ± 2.80	105.98 ± 2.65	105.60 ± 2.32	103.72 ± 1.20	117.80 ± 1.52
	3	92.94 ± 1.05	93.32 ± 6.65	93.50 ± 4.19	93.57 ± 1.57	96.62 ± 4.29	93.09 ± 6.50	120.18 ± 0.83
	4	69.28 ± 7.05	72.38 ± 14.68	66.09 ± 6.97	76.92 ± 2.66	76.83 ± 10.62	70.39 ± 14.27	114.09 ± 4.53
	8	18.42 ± 7.91	28.13 ± 22.89	11.24 ± 7.58	34.13 ± 9.75	32.64 ± 20.56	24.04 ± 22.47	121.23 ± 0.21
	24	0.10 ± 0.16	0.08 ± 0.11	0.02 ± 0.03	0 ± 0	0.01 ± 0.01	0.01 ± 0.01	116.36 ± 0.81

A2					Fructose			
	Days	**Cross Evolution Control: yeast**	**Cross Evolution Sequential inoculation**	**Cross Evolution Co-inoculation**	**EC 1118 Control:yeast**	**EC 1118 Sequential inoculation**	**EC 1118 Co-inoculation**	**Control: *L. plantarum***
	0	112.98 ± 0	113.98 ± 0	114.98 ± 0	115.98 ± 0	116.98 ± 0	117.98 ± 0	118.98 ± 0
	1	111.31 ± 3.0	111.91 ± 1.4	110.79 ± 1.7	107.94 ± 1.10	107.97 ± 2.44	106.23 ± 0.83	112.58 ± 0.70
	2	105.29 ± 0.8	105.93 ± 1.8	105.80 ± 0.8	105.42 ± 1.68	105.52 ± 1.96	103.74 ± 1.54	110.80 ± 0.59
	3	101.85 ± 1.2	100.48 ± 5.2	100.12 ± 2.1	98.68 ± 0.98	101.23 ± 3.44	100.31 ± 3.34	112.08 ± 0.30
	4	91.54 ± 3.4	93.29 ± 6.8	89.28 ± 2.9	91.87 ± 0.39	93.17 ± 3.89	86.83 ± 7.74	107.76 ± 1.19
	8	54.34 ± 11.1	62.99 ± 21.5	45.81 ± 11.3	63.92 ± 9.60	60.40 ± 22.29	50.09 ± 25.13	108.84 ± 0.46
	24	1.19 ± 0.6	0.23 ± 0	0.85 ± 0.40	0.76 ± 0.60	0.51 ± 0.63	0.75 ± 0.35	106.27 ± 0.24

Table 16: Glucose (B1) and fructose (B2) consumption during amino acids-supplemented fermentations with Cross Evolution and EC1118 and *O.oeni*

B1					Glucose			
	Days	**Cross Evolution Control: yeast**	**Cross Evolution Sequential inoculation**	**Cross Evolution Co-inoculation**	**EC 1118 Control:yeast**	**EC 1118 Sequential inoculation**	**EC 1118 Co-inoculation**	**Control: *O. oeni***
	0	116.81 ± 0	116.81 ± 0	116.81 ± 0	116.94 ± 0	116.94 ± 0	116.94 ± 0	118.66 ± 0
	1	115.71 ± 1.14	115.54 ± 0.79	103.17 ± 1.19	110.13 ± 1.12	110.20 ± 0.13	99.69 ± 0.53	106.38 ± 0.92
	2	119.99 ± 1.15	119.51 ± 0.68	106.25 ± 0.09	113.94 ± 0.66	113.80 ± 2.25	102.90 ± 0.21	112.84 ± 1.14
	3	110.13 ± 2.34	112.83 ± 0.80	101.57 ± 0.71	105.59 ± 0.67	106.81 ± 0.36	96.12 ± 1.25	108.97 ± 2.24
	17	54.60 ± 2.15	55.77 ± 3.20	40.37 ± 0.74	33.33 ± 11.23	30.11 ± 2.52	27.00 ± 1.39	104.25 ± 1.94
	69	0.24 ± 0.07	0.56 ± 0.28	0.01 ± 0	0.04 ± 0.05	0.02 ± 0	0.02 ± 0	106.21 ± 0.63

B2					Fructose			
	Days	**Cross Evolution Control: yeast**	**Cross Evolution Sequential inoculation**	**Cross Evolution Co-inoculation**	**EC 1118 Control:yeast**	**EC 1118 Sequential inoculation**	**EC 1118 Co-inoculation**	**Control: *O. oeni***
	0	116.87 ± 0	116.87 ± 0	116.87 ± 0	114.13 ± 0	114.13 ± 0	114.13 ± 0	114.76 ± 0
	1	114.19 ± 0.14	114.18 ± 1.95	105.85 ± 0.21	111.05 ± 0.56	111.44 ± 1.26	103.60 ± 0.93	107.34 ± 2.25
	2	117.99 ± 0.76	117.22 ± 0.55	109.45 ± 0.78	114.37 ± 1.21	114.36 ± 0.06	106.51 ± 0.84	111.91 ± 0.86
	3	116.66 ± 0.60	116.34 ± 0.89	108.29 ± 0.64	111.31 ± 1.24	111.63 ± 0.75	102.49 ± 0.76	112.47 ± 1.35
	17	86.13 ± 1.33	88.04 ± 0.57	73.06 ± 0.49	65.11 ± 9.27	61.86 ± 2.32	55.13 ± 1.66	101.63 ± 2.91
	69	0.30 ± 0.02	0.31 ± 0.07	0.43 ± 0.33	0.49 ± 0.28	0.32 ± 0.53	0.01 ± 0	101.99 ± 0.76

Table 17: Glucose (C1) and fructose (C2) consumption during amino acids-supplemented fermentations with Cross Evolution and EC1118 and *L. plantarum* and *O. oeni*

C1

Glucose							
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*
0	106.13 ± 0	106.13 ± 0	106.13 ± 0	110.62 ± 0	110.62 ± 0	110.62 ± 0	108.69 ± 0
1	104.52 ± 0.45	104.56 ± 1.25	93.34 ± 0.64	100.36 ± 0.37	102.77 ± 3.69	93.23 ± 0.44	97.76 ± 0.50
2	102.78 ± 0.54	102.74 ± 1.18	82.41 ± 14.92	101.17 ± 0.41	101.84 ± 3.01	93.09 ± 0.90	96.57 ± 1.07
21	51.31 ± 3.42	56.78 ± 2.23	33.20 ± 5.48	71.25 ± 1.71	75.42 ± 5.93	42.31 ± 3.79	108.35 ± 0.58
48	0.46 ± 0.28	0.28 ± 0.14	0.23 ± 0.20	0.30 ± 0.05	0.30 ± 0.05	0.32 ± 0.48	107.58 ± 0.25

C2

Fructose							
Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*
0	104.26 ± 0	104.26 ± 0	104.26 ± 0	106.37 ± 0	106.37 ± 0	106.37 ± 0	105.58 ± 0
1	101.52 ± 0.31	103.15 ± 1.29	93.32 ± 0.34	98.92 ± 0.74	100.99 ± 3.12	93.84 ± 0.88	97.24 ± 0.49
2	100.62 ± 0.40	102.36 ± 0.96	88.76 ± 6.61	99.85 ± 1.02	100.12 ± 2.37	93.87 ± 0.89	96.15 ± 1.41
3	80.14 ± 3.61	81.47 ± 0.37	59.97 ± 3.92	84.31 ± 0.72	86.99 ± 4.25	63.19 ± 3.08	105.25 ± 0.58
17	0.46 ± 0.28	0.28 ± 0.14	0.23 ± 0.20	0.30 ± 0.05	0.12 ± 0	0.16 ± 0.06	105.25 ± 0.91

Table 18: Yeast populations of amino acids-supplemented fermentations with *L. plantarum* (A), *O. oeni* (B) and the bacterial combination (*L. plantarum* and *O. oeni*) (C)

A

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation
0	2.50E6 ± 2.12E6	3.00E6 ± 3.46E6	4.00E6 ± 1.73E6	2.70E6 ± 2.04E6	3.77E6 ± 4.04E5	5.33E6 ± 1.53E6
1	4.17E6 ± 9.87E5	5.70E6 ± 9.85E5	4.43E6 ± 5.03E5	6.03E6 ± 1.12E6	4.85E6 ± 7.07E4	8.10E6 ± 2.93E6
2	1.70E7 ± 4.36E6	1.43E7 ± 2.31E6	1.27E7 ± 4.16E6	1.63E7 ± 6.66E6	1.50E7 ± 4.58E6	1.03E7 ± 3.06E6
3	2.33E7 ± 5.03E6	2.67E7 ± 6.03E6	2.07E7 ± 7.09E6	2.10E7 ± 7.21E6	2.03E7 ± 4.04E6	1.73E7 ± 1.01E7
4	2.53E7 ± 1.53E6	1.80E7 ± 8.19E6	2.03E7 ± 4.51E6	2.50E7 ± 1.31E7	3.97E7 ± 7.57E6	3.23E7 ± 7.77E6
8	5.33E7 ± 2.63E7	4.90E7 ± 2.43E7	4.27E7 ± 1.85E7	4.13E7 ± 7.57E6	4.23E7 ± 1.03E7	5.07E7 ± 2.75E7
24	3.61E7 ± 1.75E7	2.11E7 ± 6.58E6	3.48E7 ± 9.81E6	2.46E7 ± 8.01E6	2.40E7 ± 8.03E6	3.44E7 ± 9.89E6

B

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation
0	4.20E6 ± 2.65E5	4.77E6 ± 2.08E5	4.37E6 ± 3.21E5	3.37E6 ± 1.15E5	3.17E6 ± 1.53E5	4.03E6 ± 5.77E4
1	4.80E6 ± 1.06E6	4.63E6 ± 3.21E5	5.57E6 ± 2.89E5	3.43E6 ± 1.15E5	4.20E6 ± 5.57E5	5.13E6 ± 1.79E6
2	7.00E6 ± 4.36E5	6.63E6 ± 2.08E5	6.03E6 ± 1.26E6	5.50E6 ± 5.20E5	5.93E6 ± 7.09E5	5.87E6 ± 1.62E6
3	6.67E6 ± 1.15E6	6.80E6 ± 0	7.33E6 ± 1.15E6	8.00E6 ± 0	7.67E6 ± 5.77E5	1.40E7 ± 0
17	7.83E6 ± 1.03E6	6.80E6 ± 2.65E5	7.23E6 ± 1.04E6	3.07E7 ± 4.02E7	1.01E7 ± 1.91E6	9.57E6 ± 1.21E6
69	5.03E5 ± 5.77E3	4.53E5 ± 5.77E3	1.60E6 ± 1.00E4	3.67E5 ± 5.77E3	5.10E5 ± 1.00E4	1.00E4 ± 0

C

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation
0	3.37E6 ± 1.53E5	4.67E6 ± 1.53E5	3.43E6 ± 5.77E4	1.37E6 ± 3.21E5	1.37E6 ± 3.06E5	1.67E6 ± 2.89E5
1	3.23E6 ± 2.52E5	4.43E6 ± 4.04E5	3.43E6 ± 3.06E5	3.17E6 ± 1.53E5	1.44E7 ± 1.87E7	9.50E6 ± 2.00E5
2	3.93E6 ± 2.08E5	3.93E6 ± 2.08E5	1.57E7 ± 2.10E7	3.97E6 ± 2.08E5	3.67E6 ± 5.77E4	9.13E6 ± 1.53E5
19	2.73E6 ± 2.52E5	3.73E6 ± 5.77E4	9.60E6 ± 1.00E5	6.23E6 ± 5.77E4	3.07E6 ± 1.15E5	1.10E7 ± 1.15E5
48	7.43E5 ± 4.51E4	5.50E5 ± 5.00E4	5.43E5 ± 5.77E3	3.57E6 ± 5.13E5	3.17E6 ± 1.53E5	6.07E6 ± 3.79E5

Table 19: Bacterial populations during amino acids-supplemented co-inoculation with *L. plantarum* (A1), *O. oeni* (B1) and the bacterial combination (*L. plantarum* and *O. oeni*) (C1) and sequential inoculation with *L. plantarum* (A2), *O. oeni* (B2) and the bacterial combination (*L. plantarum* and *O. oeni*) (C2)

A1

Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
0	2.55E7 ± 1.00E7	2.09E7 ± 7.48E6	3.60E7 ± 1.54E7
1	3.25E7 ± 2.12E6	3.31E7 ± 3.46E6	3.20E7 ± 9.54E6
2	4.00E7 ± 6.24E6	4.07E7 ± 1.97E7	3.27E7 ± 5.77E5
3	3.00E7 ±1.11E7	4.67E7 ± 2.08E6	3.60E7 ± 0
4	2.63E7 ± 4.04E6	2.87E7 ± 1.53E6	3.40E7 ± 0
8	2.58E7 ± 1.73E5	2.04E7 ± 4.04E5	3.23E7 ± 1.15E6
24	5.30E6 ± 3.95E6	2.07E6 ± 1.75E6	1.05E6 ± 4.73E4

A2

Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
1	1.35E7 ± 5.77E4	1.36E7 ± 2.31E5
2	1.38E7 ± 2.65E5	1.95E7 ± 4.51E5
3	1.47E7 ± 5.77E4	1.98E7 ± 3.21E5
12	1.51E7 ± 5.77E4	2.00E7 ± 1.00E5

B1

Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *O. oeni*
0	6.19E7 ± 9.88E6	5.56E7 ± 1.31E7	6.60E7 ± 1.08E7
1	4.50E7 ± 1.73E6	4.77E7 ± 6.66E6	3.53E7 ± 3.06E6
2	4.13E7 ± 7.09E6	4.90E7 ± 7.94E6	1.40E7 ± 2.65E6
3	2.90E7 ± 4.58E6	5.50E7 ± 7.00E6	9.33E6 ± 3.79E6

B2

Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
0	4.23E7 ± 6.66E6	4.00E7 ± 9.54E6
1	2.90E6 ± 3.61E5	5.07E7 ± 4.93E6
6	3.33E5 ± 5.77E4	3.50E7 ± 4.36E6

C1

Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*
0	1.52E7 ± 1.53E5	1.77E7 ± 1.53E5	9.23E7 ± 3.21E6
1	7.60E7 ± 1.00E6	9.77E7 ± 3.21E6	4.83E7 ± 1.53E6
2	7.93E7 ± 1.53E6	1.07E8 ± 3.21E6	3.40E7 ± 2.65E6
3	2.90E7 ± 4.58E6	5.50E7 ± 7.00E6	9.33E6 ± 3.79E6

C2

Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
0	2.09E7 ± 1.53E5	1.84E7 ± 1.53E5
1	3.03E7 ± 5.77E5	3.07E7 ± 1.15E6
4	1.67E7 ± 2.00E5	1.66E7 ± 5.77E4

Table 20: Malic acid degradation during amino acids-supplemented co-inoculation with *L. plantarum* (A1), *O. oeni* (B1) and the bacterial combination (*L. plantarum* and *O. oeni*) (C1) and sequential inoculation with *L. plantarum* (A2), *O. oeni* (B2) and the bacterial combination (*L. plantarum* and *O. oeni*) (C2)

		Malic acid			Lactic acid		
A1	Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*	Cross Evolution	EC 1118	Control: *L. plantarum*
	0	2.85 ± 0	2.88 ± 0	2.88 ± 0	0.05 ± 0	0.05 ± 0	0.05 ± 0
	1	2.27 ± 0.03	2.19 ± 0.03	2.30 ± 0	0.70 ± 0.01	0.73 ± 0.01	0.70 ± 0.01
	2	1.84 ± 0.02	1.69 ± 0.03	1.84 ± 0.01	1.11 ± 0.01	1.19 ± 0.02	1.14 ± 0.02
	3	1.54 ± 0.02	1.31 ± 0.02	1.53 ± 0.02	1.48 ± 0	1.61 ± 0.03	1.56 ± 0.02
	4	1.11 ± 0.03	0.83 ± 0.03	1.13 ± 0.02	1.88 ± 0.03	2.08 ± 0.06	1.97 ± 0.02
	5	0.78 ± 0.04	0.47 ± 0.07	0.81 ± 0.01	2.24 ± 0.16	2.55 ± 0.02	2.18 ± 0.01
	6	0.04 ± 0.06	0.18 ± 0.07	0.05 ± 0.02	2.64 ± 0.06	2.83 ± 0.04	2.41 ± 0.02

		Malic acid		Lactic acid	
A2	Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
	0	2.42 ± 0.05	2.34 ± 0.06	0.02 ± 0.01	0.02 ± 0
	1	2.26 ± 0.01	2.15 ± 0.07	0.22 ± 0.01	0.26 ± 0.01
	2	2.00 ± 0.07	1.90 ± 0.03	0.39 ± 0.02	0.58 ± 0.05
	3	1.84 ± 0.50	1.86 ± 0.22	0.51 ± 0.03	0.77 ± 0.08
	6	1.79 ± 0.03	1.17 ± 0.16	0.71 ± 0.05	1.20 ± 0.18
	8	1.70 ± 0.08	0.94 ± 0.22	0.81 ± 0.06	1.48 ± 0.27
	12	0.06 ± 0.05	0.04 ± 0.23	1.89 ± 0.06	1.71 ± 0.38

		Malic acid			Lactic acid		
B1	Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *O. oeni*	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *O. oeni*
	0	2.98 ± 0	3.00 ± 0	2.95 ± 0	0.01 ± 0	0.01 ± 0	0.01 ± 0
	1	1.31 ± 0.05	1.27 ± 0.01	1.57 ± 0.05	1.02 ± 0.02	1.00 ± 0.04	0.84 ± 0.03
	2	0.59 ± 0.04	0.52 ± 0.01	1.11 ± 0.07	1.57 ± 0.04	1.56 ± 0.01	1.24 ± 0.04
	3	0.09 ± 0.01	0.07 ± 0	0.06 ± 0.07	1.88 ± 0.03	1.83 ± 0.01	1.56 ± 0.06

		Malic acid		Lactic acid	
B2	Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
	0	2.70 ± 0.05	2.42 ± 0.05	0.07 ± 0	0.08 ± 0
	1	2.26 ± 0.14	1.37 ± 0.03	0.06 ± 0.05	0.59 ± 0.04
	5	0.04 ± 0.001	0.06 ± 0.09	2.04 ± 0.001	1.01 ± 0.06

		Malic acid			Lactic acid		
C1	Days	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*	Cross Evolution Co-inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*
	0	2.83 ± 0	2.88 ± 0	2.83 ± 0	0.01 ± 0	0.01 ± 0	0.02 ± 0
	1	1.03 ± 0.04	0.93 ± 0.07	1.30 ± 0.03	1.07 ± 0.01	1.12 ± 0.06	0.96 ± 0.01
	2	0.30 ± 0.03	0.22 ± 0.08	0.58 ± 0.03	1.56 ± 0.02	1.61 ± 0.06	1.44 ± 0.01
	3	0.10 ± 0.02	0.07 ± 0	0.06 ± 0.07	1.88 ± 0.03	1.83 ± 0.01	1.56 ± 0.06

		Malic acid		Lactic acid	
C2	Days	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation	Cross Evolution Sequential inoculation	EC 1118 Sequential inoculation
	0	2.53 ± 0.03	2.55 ± 0.01	0.08 ± 0	0.07 ± 0
	1	0.38 ± 0	0.46 ± 0.01	1.17 ± 0.01	1.09 ± 0
	5	0.05 ± 0	0.04 ± 0.02	1.33 ± 0	1.32± 0.01

Table 21: Glycerol concentrations of amino acids-supplemented fermentations with *L. plantarum* (A), *O. oeni* (B) and the bacterial combination (*L. plantarum* and *O. oeni*) (C)

A

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum*
0	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.01 ± 0	0.01 ± 0
8	5.49 ± 0.32	5.05 ± 1.02	5.84 ± 0.41	5.20 ± 0.39	5.30 ± 0.81	5.71 ± 1.08	0.01 ± 0
24	6.30 ± 0.44	5.76 ± 0.44	6.01 ± 0.12	6.08 ± 0.11	6.13 ± 0.17	6.12 ± 0.29	0.02 ± 0.02

B

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *O. oeni*
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
17	3.75 ± 0.42	3.79 ± 0.60	3.39 ± 0.17	4.10 ± 0.94	4.40 ± 0.28	3.65 ± 0.25	0.01 ± 0.01
69	5.89 ± 0.15	5.68 ± 0.38	5.24 ± 0.05	5.27 ± 0.17	5.18 ± 0.24	4.87 ± 0.07	0.01 ± 0

C

Days	Cross Evolution Control: yeast	Cross Evolution Sequential inoculation	Cross Evolution Co-inoculation	EC 1118 Control:yeast	EC 1118 Sequential inoculation	EC 1118 Co-inoculation	Control: *L. plantarum* and *O. oeni*
0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
21	2.77 ± 0.47	2.78 ± 0.27	2.97 ± 0.26	3.40 ± 0.22	3.22 ± 0.27	2.90 ± 0.18	0.01 ± 0.01
48	4.72 ± 0.46	4.45 ± 0.49	4.16 ± 0.09	7.74 ± 0.39	7.63 ± 0.19	4.92 ± 0.12	0.01 ± 0

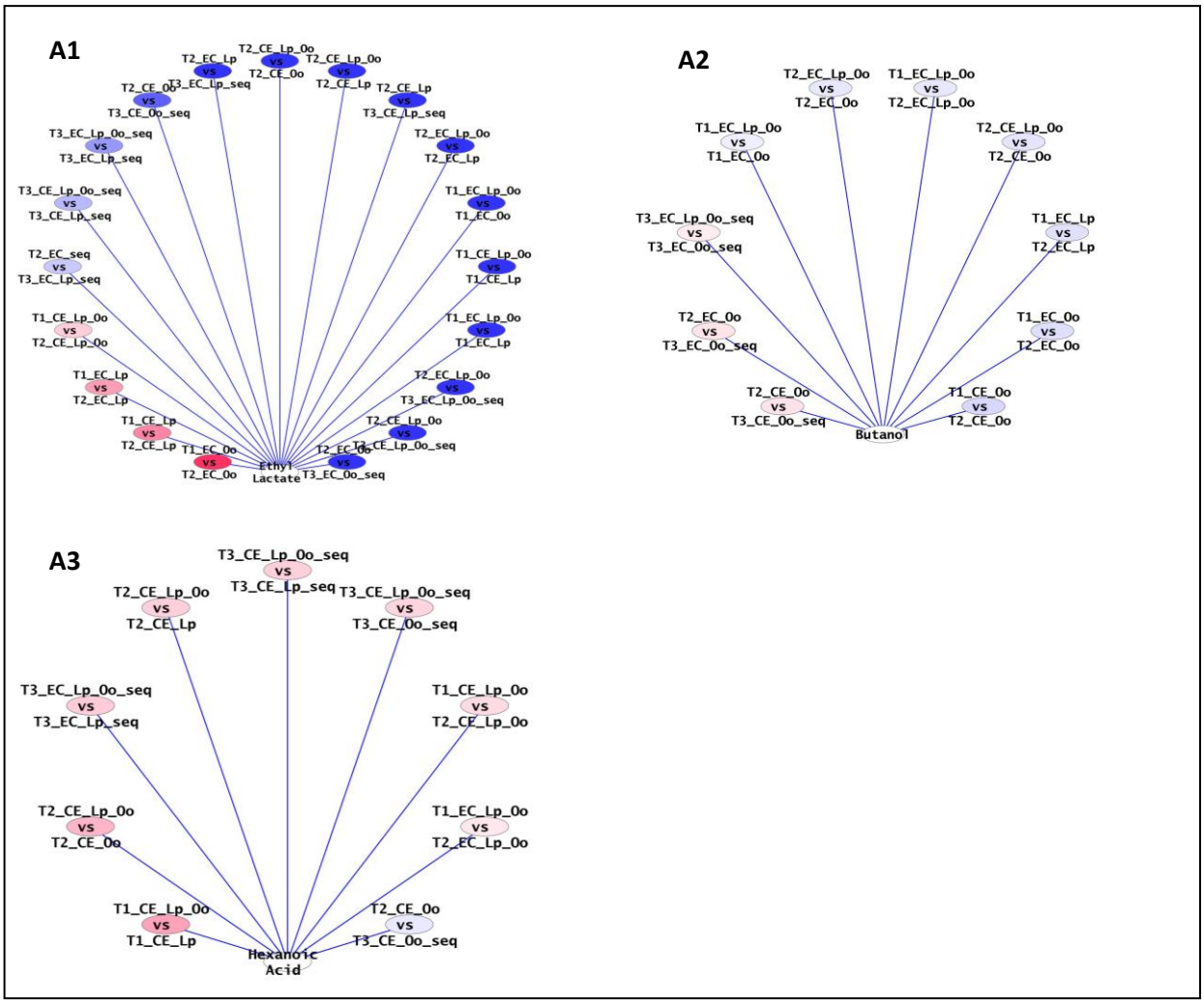

Figure1: The aroma compounds of different standard fermentation treatments that were significant different: ethyl lactate **(A1)**, butanol **(A2)** and hexanoic acid **(A3)** at different time points. A blue node (ellipse) indicates a reduction and a red node an increase in the compounds. An increase in the colour intensity indicates the magnitude of the fold change observed. **CE_Lp**: Cross Evolution co-inoculated with *L. plantarum*; **CE_Oo:** Cross Evolution co-inoculated with *O. oeni*; **CE_Lp_Oo:** Cross Evolution co-inoculated with bacterial combination of *L. plantarum* and *O. oeni*; **EC_Lp:** EC1118 co-inoculated with *L. plantarum*; **EC_Oo:** EC1118 co-inoculated with *O. oeni*; **EC_Lp_Oo:** EC1118 co-inoculated with bacterial combination of *L. plantarum* and *O. oeni*; **CE_Lp_seq:** Cross Evolution sequential inoculation with *L. plantarum*; **CE_Oo_seq:** Cross Evolution sequential inoculation with *O. oeni*; **CE_Lp_Oo_seq:** Cross Evolution sequential inoculation with bacterial combination of *L. plantarum* and *O. oeni*; **EC_Lp_seq:** EC1118 sequential inoculation with *L. plantarum*; **EC_Oo_seq:** EC1118 sequential inoculation with *O. oeni*; **EC_Lp_Oo_seq:** EC1118 sequential inoculation with bacterial combination of *L. plantarum* and *O. oeni*; **T1:** half point of alcoholic fermentation; **T2:** end point of alcoholic fermentation; **T3:** end point of MLF.

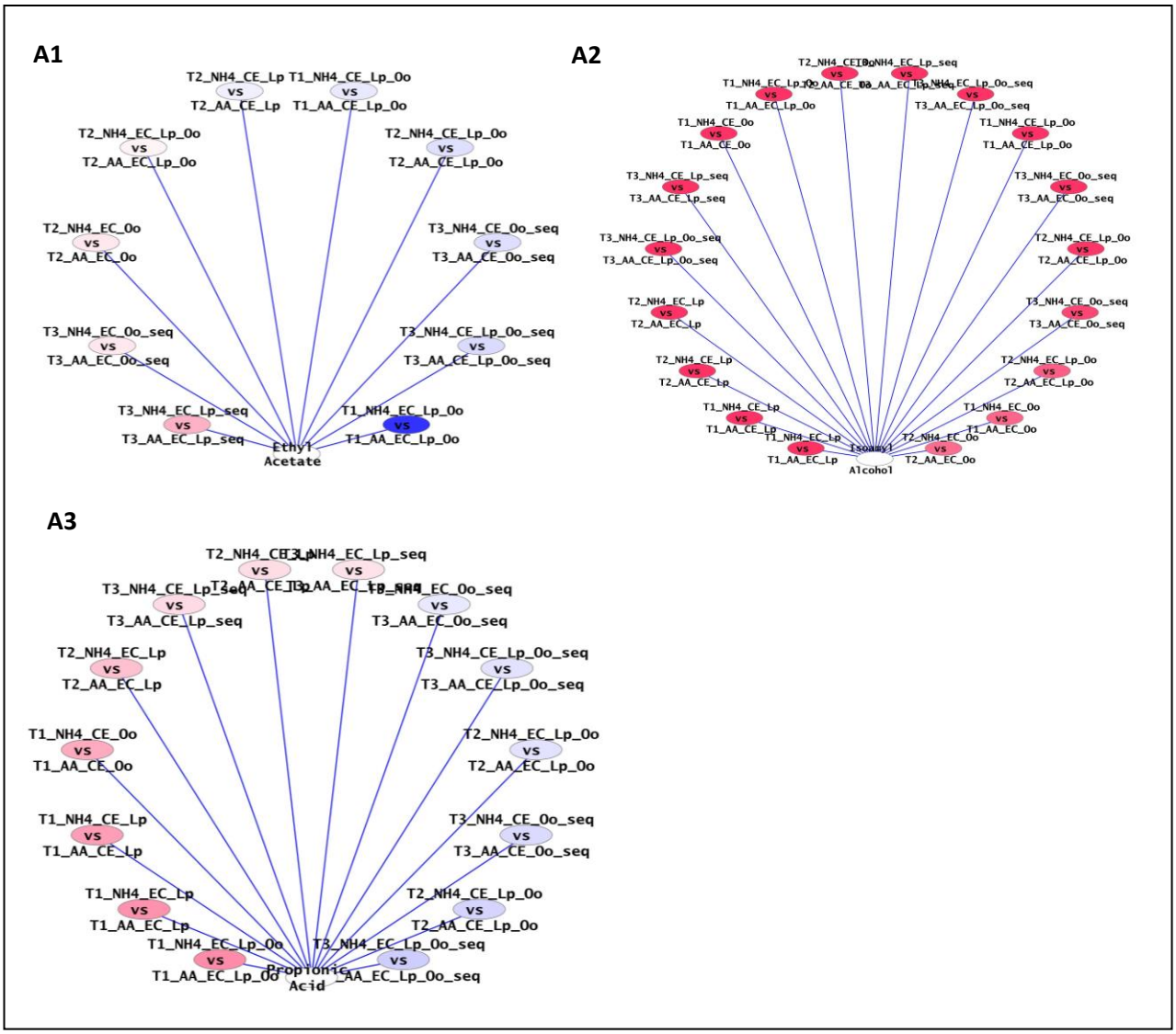

Figure 2: The aroma compounds of different nitrogen additions are compared at different time points: ethyl actate **(A1)**, isoamyl alcohol **(A2)** and propionic acid **(A3)**. A blue node (ellipse) indicates a reduction and a red node an increase in the compounds. An increase in the colour intensity indicates the magnitude of the fold change observed. **CE_Lp**: Cross Evolution co-inoculated with *L. plantarum*; **CE_Oo:** Cross Evolution co-inoculated with *O. oeni*; **CE_Lp_Oo:** Cross Evolution co-inoculated with bacterial combination of *L. plantarum* and *O. oeni*; **EC_Lp:** EC1118 co-inoculated with *L. plantarum*; **EC_Oo:** EC1118 co-inoculated with *O. oeni*; **EC_Lp_Oo:** EC1118 co-inoculated with bacterial combination of *L. plantarum* and *O. oeni*; **CE_Lp_seq:** Cross Evolution sequential inoculation with *L. plantarum*; **CE_Oo_seq:** Cross Evolution sequential inoculation with *O. oeni*; **CE_Lp_Oo_seq:** Cross Evolution sequential inoculation with bacterial combination of *L. plantarum* and *O. oeni*; **EC_Lp_seq:** EC1118 sequential inoculation with *L. plantarum*; **EC_Oo_seq:** EC1118 sequential inoculation with *O. oeni*; **EC_Lp_Oo_seq:** EC1118 sequential inoculation with bacterial combination of *L. plantarum* and *O. oeni*; **AA:** Amino acids supplementation; **NH_4CL:** Ammonium supplementation; **T1:** half point of alcoholic fermentation; **T2:** end point of alcoholic fermentation; **T3:** end point of MLF.