

**Evaluation of citrate metabolism in  
*Oenococcus oeni* and *Lactobacillus  
plantarum***

by

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## Summary

Citrate positive ( $\text{cit}^+$ ) *Oenococcus oeni* and *Lactobacillus plantarum* are able to degrade citrate during fermentation. These bacteria possess the citrate permease and citrate lyase enzymes which are responsible for citrate transport and degradation, respectively. Citrate negative ( $\text{cit}^-$ ) strains lack either one of these genes rendering them incapable of citrate degradation. Citrate metabolism results in the production of compounds such as D-lactate, acetate, diacetyl, acetoin and 2,3-butanediol, which can influence the aroma profile and quality of wine. Of these compounds, diacetyl is the most important compound produced via citrate metabolism that can give rise to a buttery aroma important to create certain wine styles. The aim of this study was to determine the effect of glucose, fructose and pH on citrate metabolism by a  $\text{cit}^+$  and a  $\text{cit}^-$  strain from both species of *O. oeni* and *Lb. plantarum*. The effects of these factors were determined by focussing specifically on the consumption of citrate, the relative expression (RE) of the beta subunit (*citE*) of the citrate lyase gene and the production of D-lactate, acetate, diacetyl and acetoin. Synthetic wine was used to determine the influence of different glucose and fructose concentrations (115 g/L, 50 g/L and 2.5 g/L) and different pH levels (3.0, 3.5, 4.0 and 5.0) as these represent the differences either between warm and cool climate or co-inoculation and sequential malolactic fermentation.

The results obtained for all the treatments showed that glucose had the smallest impact on citrate metabolism. In the glucose treatments, the  $\text{cit}^+$  *O. oeni* strain completely consumed citrate and the  $\text{cit}^-$  *O. oeni* did not consume citrate but both strains produced trace amounts of diacetyl and acetoin. The  $\text{cit}^-$  strain produced less D-lactate and acetate than the  $\text{cit}^+$  *O. oeni* strain in most of the glucose treatments. The lowest RE of *citE* in the  $\text{cit}^+$  *O. oeni* strain was seen in the 115 g/L glucose treatment where malate and citrate were consumed the fastest. Both the *Lb. plantarum* strains partially consumed citrate. The  $\text{cit}^+$  *Lb. plantarum* strain produced more diacetyl and acetoin than the  $\text{cit}^-$  *Lb. plantarum* strain in all the glucose treatments. In the 2.5 g/L glucose treatment, the D-lactate and acetate concentrations were lower than in the other two treatments, which might be due to the lower initial glucose concentration in this treatment. However, the  $\text{cit}^+$  *Lb. plantarum* strain produced more diacetyl and acetoin in the 2.5 g/L glucose treatment compared to the other two treatments. The *Lb. plantarum* strains produced more D-lactate, diacetyl and acetoin than the *O. oeni* strains.

In the fructose treatments, the  $\text{cit}^-$  *O. oeni* strain did not consume any citrate and the  $\text{cit}^-$  *Lb. plantarum* strain partially consumed citrate. The  $\text{cit}^+$  *Lb. plantarum* strain was able to consume more citrate in the fructose treatments and therefore produced more diacetyl and acetoin in these treatments. The highest RE in the  $\text{cit}^+$  *O. oeni* strain was seen in the 115 g/L fructose treatment where the malate and citrate were slightly delayed. The  $\text{cit}^+$  strains produced similar amounts of D-

lactate and acetate than the cit<sup>-</sup> strains. The *Lb. plantarum* strains produced more D-lactate, diacetyl and acetoin than the *O. oeni* strains, as was seen in the glucose treatments.

In all the pH conditions tested, the cit<sup>-</sup> *O. oeni* strain did not consume citrate and produced less D-lactate, diacetyl and acetoin than the cit<sup>+</sup> *O. oeni* strain. The cit<sup>+</sup> *O. oeni* strain consumed more citrate and produced more diacetyl and acetoin in the pH 3.0 and 3.5 treatments than in the pH 4.0 and 5.0 treatments. The *Lb. plantarum* strains consumed more citrate and therefore produced more D-lactate, acetate, diacetyl and acetoin in the pH 3.5, 4.0 and 5.0 treatments than in the pH 3.0 treatment. The highest RE in the cit<sup>+</sup> *O. oeni* strain was seen in the pH 3.0 treatment where the malate and citrate consumption were slightly delayed as was seen in the fructose treatments. The *Lb. plantarum* strains produced more D-lactate and acetoin than the *O. oeni* strains in most of the treatments. The cit<sup>+</sup> *Lb. plantarum* strain produced the highest diacetyl in the pH 4.0 and 5.0 treatments, but the cit<sup>+</sup> *O. oeni* strain produced the highest diacetyl in the pH 3.0 and 3.5 treatments. The measurement of 2,3-butanediol could not be performed in this study to determine whether acetoin was converted and if this is the reason why diacetyl concentrations were relatively low taking into consideration the amount of citrate consumed in some of the treatments.

In conclusion, this study showed that different sugar concentrations, pH levels and the lactic acid bacteria strains used to induce malolactic fermentation can influence citrate metabolism, which can ultimately influence the final diacetyl and acetoin concentrations and therefore the wine style. The results obtained in this study can be used by winemakers to obtain or avoid a buttery, creamy aroma in their wines.

## Opsomming

Sitraat positiewe ( $\text{sit}^+$ ) *Oenococcus oeni* en *Lactobacillus plantarum* is in staat om sitraat af te breek gedurende die fermentasie. Hierdie bakterieë besit die sitraat permease en sitraat liase ensieme wat verantwoordelik is vir die vervoer en afbraak van sitraat, onderskeidelik. Sitraat negatiewe ( $\text{sit}^-$ ) bakterieë is nie in staat om sitraat af te breek nie, omdat hul nie die gene besit wat kodeer vir hierdie ensieme nie. Verskeie metaboliete naamlik D-laktaat, asetaat, diasetiel, asetoïen en 2,3-butaandiol word geproduseer tydens sitraat metabolisme wat die aroma en kwaliteit van die wyn kan beïnvloed. Van hierdie komponente is diasetiel die belangrikste metaboliet wat geproduseer word vanaf sitraat en gee 'n botteragtige karakter wat belangrik is vir spesifieke wynstyle. Die doel van hierdie studie was om te bepaal watter effek glukose, fruktose en pH sal hê op sitraat metabolisme van  $\text{sit}^+$  en  $\text{sit}^-$  *O. oeni* en *Lb. plantarum* isolate. Die effek van hierdie faktore was bepaal deur spesifiek te fokus op die sitraat verbruik, relatiewe ekspressie (RE) van die beta subeenheid (*citE*) van sitraat liase geen en die produksie van D-laktaat, asetaat, diasetiel, asetoïen en 2,3-butaandiol. Sintetiese wyn was gebruik om die invloed van verskillende glukose en fruktose konsentrasies (115 g/L, 50 g/L en 2.5 g/L) en verskillende pH vlakke (3.0, 3.5, 4.0 en 5.0) te evalueer, aangesien hierdie faktore die verskil tussen ko- en sekwensiële appelmelksuurgisting asook tussen warm en koel klimaat verteenwoordig.

Die resultate verkry tydens hierdie studie het aangedui dat glukose die kleinste impak gehad het op sitraat metabolisme. In al die glukose behandelings het die  $\text{sit}^+$  *O. oeni* alle sitraat verbruik en die  $\text{sit}^-$  *O. oeni* geen sitraat afgebreek nie, maar beide het min diasetiel en asetoïen geproduseer. Die  $\text{sit}^-$  het ook minder D-laktaat en asetaat geproduseer as die  $\text{sit}^+$  *O. oeni* in meeste van die glukose behandelings. Die laagste RE van *citE* in die  $\text{sit}^+$  *O. oeni* was gesien in die 115 g/L glukose behandeling waar die malaat en sitraat die vinnigste afgebreek was. Beide die *Lb. plantarum* isolate het net 'n gedeelte van die sitraat verbruik. Die  $\text{sit}^+$  *Lb. plantarum* het egter meer diasetiel en asetoïen geproduseer as die  $\text{sit}^-$  *Lb. plantarum* in al die glukose behandelings. In die 2.5 g/L glukose behandeling was die D-laktaat en asetaat heelwat minder as in die ander twee behandelings as gevolg van die laer aanvanklike glukose konsentrasie. Die  $\text{sit}^-$  *Lb. plantarum* het egter meer diasetiel en asetoïen in die 2.5 g/L glukose behandeling geproduseer in vergelyking met die ander twee behandelings. Die *Lb. plantarum* isolate het ook meer D-laktaat, diasetiel en asetoïen as die *O. oeni* isolate geproduseer.

In die fruktose behandeling het die  $\text{sit}^-$  *O. oeni* weereens nie die sitraat afgebreek nie en die  $\text{sit}^-$  *Lb. plantarum* net sitraat gedeeltelik af te breek. Die  $\text{sit}^+$  *Lb. plantarum* isolaat was instaat om meer sitraat te gebruik in die fruktose behandeling en het dus meer diasetiel en asetoïen geproduseer in die hierdie behandeling. Die hoogste RE van *citE* in die  $\text{sit}^+$  *O. oeni* was gesien in die 115 g/L fruktose behandeling waar die malaat en sitraat verbruik effens vertraag was. Die  $\text{sit}^+$  isolate het egter min of meer dieselfde hoeveelheid D-laktaat en asetaat geproduseer as die  $\text{sit}^-$

isolate. Die *Lb. plantarum* isolate het meer D-laktaat, diasetiel en asetoïen geproduseer as die *O. oeni* isolate soos wat gesien was in die glukose behandeling.

In al die pH behandeling het die sit<sup>-</sup> *O. oeni* nie die sitraat afgebreek nie en dus minder D-laktaat, diasetiel en asetoïen as die sit<sup>+</sup> *O. oeni*. Die sit<sup>+</sup> *O. oeni* het meer sitraat afgebreek en dus meer diasetiel en asetoïen in die pH 3.0 en 3.5 behandeling as in die pH 4.0 en 5.0 behandeling. Die *Lb. plantarum* isolate het meer sitraat afgebreek en dus meer D-laktaat, asetaat, diasetiel en asetoïen in die pH 3.5, 4.0 en 5.0 behandeling as in die pH 3.0 behandeling. Die hoogste RE van citE in die sit<sup>+</sup> *O. oeni* was gesien in die pH 3.0 behandeling waar die malaat en sitraat verbruik effens vertraag was soos wat gesien was in die fruktose behandeling. Die *Lb. plantarum* isolate het meer D-laktaat en asetoïen geproduseer as die *O. oeni* isolate in meeste van die behandeling. Die sit<sup>+</sup> *Lb. plantarum* isolate het meer diasetiel as die ander isolate in die pH 4.0 en 5.0 behandeling geproduseer, maar die sit<sup>+</sup> *O. oeni* het meer diasetiel in die pH 3.0 en 3.5 behandeling geproduseer. Die een beperking van die studie was dat 2,3-butaandiol nie gemeet kon word nie en daarom kon daar nie vasgestel word of die asetoïen omgeskakel was nie en of dit die rede is hoekom die diasetiel konsentrasies so laag was al was sitraat heeltemal gemetaboliseer in sommige behandeling.

Ten slotte, hierdie studie het aangedui dat verskillende suiker konsentrasies, pH vlakke en tipe melksuurbakterieë wat gebruik word om appelmelksuurgisting te induseer 'n invloed het op sitraat metabolisme wat uiteindelik die finale diasetiel en asetoïen konsentrasies kan beïnvloed en dus die wyn styl. Die resultate verkry deur die studie kan moontlik gebruik word deur wynmakers om 'n botteragtige aroma te verkry of te vermy in hul wyne.

This thesis is dedicated to my family and Wian Mostert

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Hierdie tesis is opgedra aan my familie en Wian Mostert

"Sometimes our light goes out, but is blown again into instant flame  
by an encounter with another human being."

- Albert Schweitzer

## Biographical sketch

Natasha Pretorius was born on 30 March 1992 in Gauteng, South Africa. She matriculated in 2010 at Driehoek High School in Gauteng. She obtained a BSc Molecular Biology and Biotechnology *cum laude* degree in 2013 at Stellenbosch University. Thereafter, she enrolled for HonsBSc in Wine Biotechnology in 2014 at Stellenbosch University and then continued with her MSc in Wine Biotechnology in 2015 at the same institution.

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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 South African Journal of Enology and Viticulture.

**Chapter 1**      **General introduction and project aims**

**Chapter 2**      **Literature review**

Citrate metabolism in lactic acid bacteria and the impact of different factors

**Chapter 3**      **Research results**

Evaluation of citrate metabolism in *Oenococcus oeni* and *Lactobacillus plantarum* strains under different winemaking conditions

**Chapter 4**      **General discussion and conclusions**

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## List of Abbreviations Used

Acetate .....	Hace
Acyl carrier protein .....	ACP
Adenosine triphosphate .....	ATP
Analysis of variance .....	ANOVA
Citrate negative bacteria .....	cit <sup>-</sup>
Citrate positive bacteria .....	cit <sup>+</sup>
Citric acid .....	H <sub>3</sub> cit
Colony forming units .....	cfu
Comparative critical threshold .....	$\Delta\Delta C_t$
Divalent citrate .....	Hcit <sup>2-</sup>
Embden-Meyerhof-Parnas .....	EMP
Generally recognised as safe .....	GRAS
Lactic acid bacteria .....	LAB
<i>Lactobacillus</i> .....	<i>Lb</i>
<i>Lactococcus</i> .....	<i>Lc</i>
<i>Leuconostoc</i> .....	<i>Leuc</i>
Malolactic fermentation .....	MLF
Monovalent acetate .....	ace <sup>-</sup>
Monovalent citrate .....	H <sub>2</sub> cit <sup>-</sup>
Monovalent lactate .....	lac <sup>-</sup>
Monovalent pyruvate .....	pyr <sup>-</sup>
National Centre for Biotechnology Information .....	NCBI
Nicotinamide adenine dinucleotide phosphate .....	NADPH
Nicotinamide adenine dinucleotide .....	NADH
Oxaloacetate .....	oxace <sup>2-</sup>
Polymerase chain reaction .....	PCR
Proton motive force .....	PMF
Pyruvate .....	Hpyr
Quantitative real time PCR efficiency .....	<i>E</i>
Quantitative real time PCR .....	qPCR
Real-time PCR efficiency .....	<i>E</i>
Relative expression .....	RE
RNA polymerase .....	RNAP
Sulphur dioxide .....	SO <sub>2</sub>
Threshold cycle number .....	C <sub>t</sub>
Trivalent citrate .....	cit <sup>3-</sup>

# Chapter 1

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General introduction and project  
aims

# 1 General introduction and project aims

## 1.1 Introduction

Malolactic fermentation (MLF) is a secondary fermentation that takes place either simultaneously or upon the completion of alcoholic fermentation. This process is carried out by lactic acid bacteria (LAB), mainly species from the genera *Leuconostoc*, *Pediococcus*, *Lactobacillus* and *Oenococcus*. During this process the bacteria converts the dicarboxylic L-malate to L-lactate and carbon dioxide. Although, this fermentation can occur spontaneously, it is beneficial to induce the process by the addition of commercially available *Oenococcus oeni* and *Lactobacillus plantarum* starter cultures. The use of starter cultures to induce MLF is preferred to avoid the risks associated with spontaneous MLF. Malolactic fermentation is overall a desirable process which increases the microbial stability, decreases wine acidity and contributes to the organoleptic properties of wine (Bartowsky and Henschke, 2004; Pozo-Bayón *et al.*, 2005; López *et al.*, 2011; Malherbe *et al.*, 2012; Ruiz *et al.*, 2012).

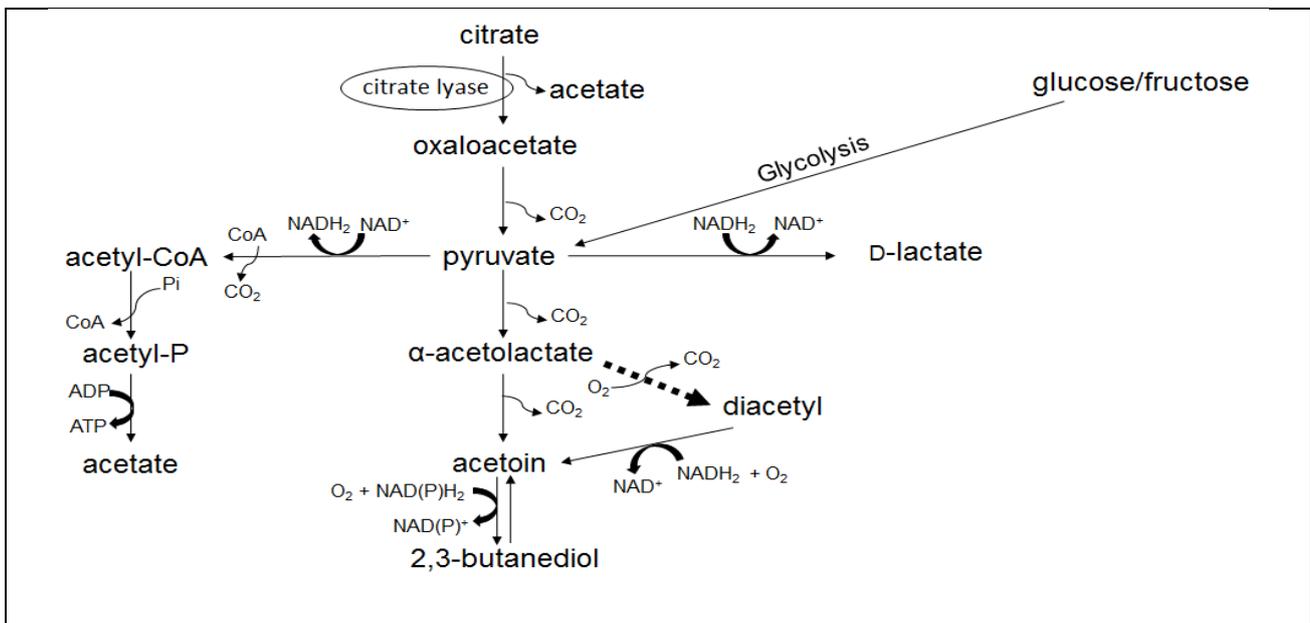
Lactic acid bacteria can positively or negatively influence wine quality and aroma by producing volatile compounds and secondary metabolites or by reducing flavour compounds (Malherbe *et al.*, 2012). The production of volatile aroma compounds such as esters and higher alcohols often improve the fruity and floral aroma of MLF wines. Some esters produced during MLF include ethyl lactate, diethyl succinate and 2-phenylethyl acetate (Pozo-Bayón *et al.*, 2005; López *et al.*, 2011; Malherbe *et al.*, 2012). However, LAB can also spoil wine by producing compounds such as acetate, D-lactate, mannitol, acrolein, ethyl carbamate and biogenic amines (Du Toit and Pretorius, 2000; Bartowsky, 2009; Ruiz *et al.*, 2012). These compounds are either considered harmful to consumers or lead to the production of off-flavours such as geranium, mousiness and bitterness (Du Toit and Pretorius, 2000; Bartowsky, 2009; Ruiz *et al.*, 2012). The modification of wine quality and aroma by LAB usually occurs through 3 different mechanisms namely: (I) the metabolism of grape constituents; (II) the modification of grape- or yeast-derived secondary metabolites; and lastly (III) by the adsorption of flavour compounds to their cell walls (Malherbe *et al.*, 2012).

Citrate is one of many grape constituents that are metabolised by some LAB which contributes to the formation of important carbonyl flavour compounds namely diacetyl, acetoin and 2,3-butanediol (**Figure 1.1**). Citrate positive ( $\text{cit}^+$ ) LAB are able to transport and degrade citrate, whereas citrate negative ( $\text{cit}^-$ ) LAB are unable to utilise citrate. Citrate is transported into the cell by citrate or malate permeases which are encoded by *citP* (Martín *et al.*, 1999; Siezen *et al.*, 2012) or *maeP* (Kawai *et al.*, 1997; Olguín *et al.*, 2009), respectively. Citrate is then cleaved by citrate lyase to acetate and oxaloacetate which is converted to pyruvate and ultimately to the carbonyl flavour compounds (**Figure 1.1**). Citrate lyase consists of three subunits namely  $\alpha$ ,  $\beta$  and  $\gamma$ , which are synthesised by the *citD*, *citE* and *citF* genes, respectively (Bekal *et al.*, 1998; Martín *et al.*, 1999).

Citrate negative LAB usually lack one or all of these genes. However, these bacteria can still produce the carbonyl flavour compounds as well as acetate and D-lactate from pyruvate formed during glycolysis, which can enter the citrate pathway (**Figure 1.1**).

The main products of citrate metabolism in LAB, diacetyl, acetoin, 2,3-butanediol, acetate and D-lactate, can have a positive or negative effect on wine quality and aroma depending on their concentrations. Diacetyl has a buttery, nutty and/or toasty aroma with the threshold varying from 0.2 mg/L to 2.8 mg/L depending on the wine type (Martineau and Henick-Kling, 1995a; Ramos *et al.*, 1995; Bartowsky and Henschke, 2004; Mink *et al.*, 2014). Diacetyl concentrations above 5 mg/L mask the fruity, floral and/or vegetative aromas and are therefore considered as spoilage, whereas diacetyl concentrations within 1 mg/L to 4 mg/L contribute to a desirable wine aroma (Bartowsky and Henschke, 2004). The diacetyl content of wine is usually within the desirable concentration and ranges from 0.05 mg/L to 4.1 mg/L (Martineau and Henick-Kling, 1995a; Ramos *et al.*, 1995; Bartowsky and Henschke, 2004; Mink *et al.*, 2014). Diacetyl is reduced to the less sensory active acetoin and 2,3-butanediol as the fermentation progresses (**Figure 1.1**). Acetoin content in wine varies from 3 mg/L to 31.8 mg/L and has a buttery, creamy aroma when above the sensory threshold of 150 mg/L (Romano and Suzzi, 1996; Du Toit and Pretorius, 2000; Malherbe *et al.*, 2012). The concentration of 2, 3-butanediol in wine varies from 0.2 mg/L to 3.0 mg/L and gives the wine a slightly bitter taste when its concentration is above the sensory threshold of 600 mg/L (Romano *et al.*, 1998; Ehsani *et al.*, 2009).

The production of these metabolites associated with citrate metabolism is influenced by various factors. These factors include the malolactic bacterial strain, the inoculation dosage, MLF inoculation time, fermentation temperature, citrate concentration, sulphur dioxide content, pH, oxygen and contact with active yeast lees (Bartowsky and Henschke, 2004). Some studies have been performed to investigate the effect of these factors. Olguín *et al.* (2009) investigated the effect of ethanol combined with a pH of 3.5 or 4.0 on the citrate metabolism in *O. oeni*. Nielsen and Richelieu (1999) investigated the effect of aeration on the diacetyl and acetoin concentrations. Martineau and Henick-Kling (1995b) and Malherbe *et al.* (2012) determined the effect of different LAB strains, mainly belonging to the species *O. oeni*, on the diacetyl and acetoin concentrations. Malherbe *et al.* (2012), Antalick *et al.* (2013) and Versari *et al.* (2015) have investigated the effect of co- and sequential inoculation on wine aroma profile in terms of being more or less fruitier due to the diacetyl content. Understanding the citrate metabolism can help winemakers make informed decisions when using malolactic starter cultures to create different wine styles.



**Figure 1.1** Citrate metabolism in lactic acid bacteria. Modified from Ramos *et al.* (1995) and Olguín *et al.* (2009).

## 1.2 Project aims

As co-inoculation is becoming a more common practice for MLF where the bacteria are exposed to juice conditions which are very different to wine, we need to gain knowledge on the impact of sugars and other factors associated with juice on the potential production of diacetyl. This knowledge will assist in managing MLF to drive a buttery style wine or not. Therefore, the overriding goal of this study was to evaluate the impact of glucose, fructose and different pH levels on citrate metabolism in four LAB strains, two *O. oeni* strains (one citrate positive and one citrate negative) and two *Lb. plantarum* strains (one citrate positive and one citrate negative). The study mainly focused on citrate degradation, the expression of *citE* that encodes the citrate lyase β subunit and the production of D-lactate, acetate, diacetyl and acetoin.

The specific aims of this study were as follows:

- i. to determine the influence of glucose on citrate degradation, the expression of *citE* as well as on the production of metabolites associated with the citrate metabolism;
- ii. to determine the influence of fructose on citrate degradation, the expression of *citE* as well as on the production of metabolites associated with the citrate metabolism; and
- iii. to determine the effect of pH on citrate degradation, the expression of *citE* as well as on the production of metabolites associated with the citrate metabolism

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# Chapter 2

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## Literature review

### Citrate metabolism in lactic acid bacteria

## 2 Literature review

### Citrate metabolism in lactic acid bacteria

#### 2.1 Introduction

Lactic acid bacteria (LAB) are Gram-positive, non-motile, non-spore forming bacteria (Todorov and Franco, 2010). These bacteria are classified into different genera mainly based on their morphology (cocci, bacilli or cocci-bacilli) and on their growth requirements such as the presence or absence of oxygen and certain nutrients (Salminen and Von Wright, 2004). The most common LAB genera include *Lactobacillus* (*Lb*), *Lactococcus* (*Lc*), *Enterococcus*, *Leuconostoc* (*Leuc*), *Pediococcus*, *Streptococcus* and *Oenococcus* (Leroy and De Vuyst, 2004; Todorov and Franco, 2010). Several LAB such as *Lb. acidophilus* (GRAS Notice No. 000357), *Lb. casei* (GRAS Notice No. 000429), *Lc. lactis* (GRAS Notice No. 000065) and *Oenococcus oeni* (GRAS Notice No. 000120) have been generally recognised as safe (GRAS) by the Food and Drug Administration and are used as starter cultures for the production of various fermented products and probiotics.

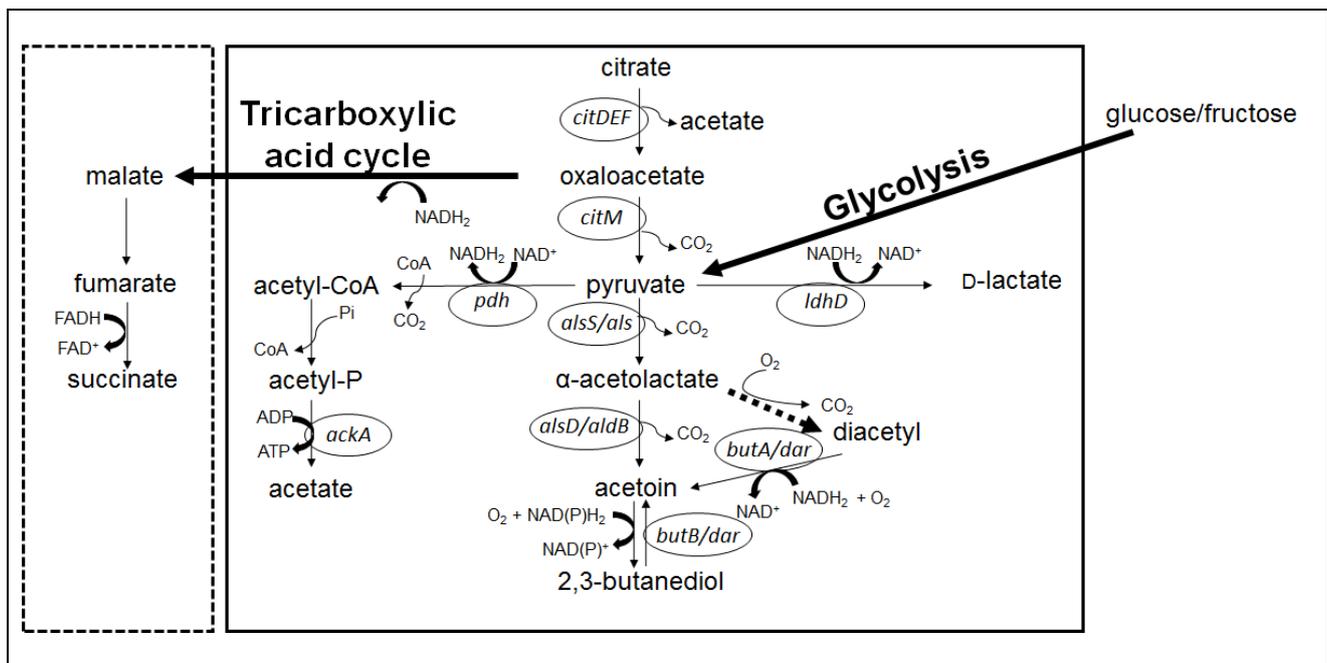
Besides their GRAS status, LAB are used in the food and beverage industry due to their ability to produce lactate from carbohydrates. The production of lactate from carbohydrates can occur through the Embden-Meyerhof-Parnas (EMP) pathway or the pentose phosphate pathway (Du Toit *et al.*, 2010; Todorov and Franco, 2010). The type of pathway depends on the fermentation characteristics of the specific LAB genera. Homofermentative LAB, such as *Lactococcus*, *Streptococcus*, *Pediococcus* and some *Lactobacillus* species, produce lactate as the only end-product from carbohydrates through the EMP pathway (Liu, 2003; Du Toit *et al.*, 2010). Obligate heterofermentative LAB produce lactate as the main end-product, while producing acetate or ethanol as by-products through the pentose phosphate pathway. These obligate heterofermentative bacteria include *Leuconostoc* species, *Oenococcus* species and some *Lactobacillus* species such as *Lb. brevis* and *Lb. hilgardii* (Liu, 2003; Du Toit *et al.*, 2010). Other LAB are categorised as facultative heterofermentative and are able to shift between the EMP pathway and the pentose phosphate pathway depending on the type of carbohydrate available and environmental conditions (Sharpe, 1979; Du Toit *et al.*, 2010). The end-products produced via these pathways by LAB play an important part in the organoleptic properties and quality of fermented products.

Lactic acid bacteria can also influence the perceived acidity of fermented products by utilising organic acids such as malate and citrate. Lactic acid bacteria capable of utilising citrate as carbon and energy source are referred to as citrate positive ( $\text{cit}^+$ ) and have the genes encoding permeases for citrate transport and citrate lyase necessary for citrate degradation (Farkye and Vedamuthu, 2002; Drider *et al.*, 2004). Citrate negative ( $\text{cit}^-$ ) LAB lack either both or only one of

these genes rendering them incapable of citrate utilisation (Farkye and Vedamuthu, 2002; Drider *et al.*, 2004).

Citrate lyase is the first intracellular enzyme involved in citrate metabolism. This enzyme cleaves citrate to oxaloacetate and acetate as indicated in **Figure 2.1**. Oxaloacetate can then either be converted to malate via the tricarboxylic acid cycle (dashed line block in **Figure 2.1**) under aerobic conditions or to pyruvate and carbon dioxide via citrate metabolism (solid line block in **Figure 2.1**) under anaerobic conditions (Torino *et al.*, 2005; Cabral *et al.*, 2007; Kang *et al.*, 2013). Malate is then converted to succinate via fumarate as an intermediate (**Figure 2.1**), while pyruvate produced either through citrate metabolism or from glycolysis is converted to acetate, D-lactate and C<sub>4</sub> flavour compounds (Ramos *et al.*, 1995; Bartowsky and Henschke, 2004). The formation of carbon dioxide and C<sub>4</sub> flavour compounds play an important role in the texture and aroma of fermented products. The C<sub>4</sub> flavour compounds include diacetyl, acetoin and 2,3-butanediol, which contribute to a buttery, nutty, creamy and/or toasty aroma (Bartowsky and Henschke, 2004; Ehsani *et al.*, 2009; Malherbe *et al.*, 2012).

The buttery aroma can be regarded as either negative or positive depending on the concentration of the C<sub>4</sub> flavour compounds as well as the fermented product. High diacetyl concentrations of 1 mg/L to 6 mg/L are required to create the buttery aroma in certain dairy products such as cheeses (Rattray *et al.*, 2003; Weimer, 2007), whereas high diacetyl concentrations of 5 mg/L in wine and beer can contribute to an undesirable aroma (Martineau and Henick-Kling, 1995a; Ramos *et al.*, 1995; Menz *et al.*, 2010; Mink *et al.*, 2014a). The different industries thus need to have a good understanding of citrate metabolism at a genetic level and knowledge of the factors influencing this metabolism to be able to achieve ideal concentrations of the C<sub>4</sub> compounds in their products.



**Figure 2.1** Citrate utilisation and production of flavour compounds by lactic acid bacteria. The utilisation of citrate via citrate metabolism under anaerobic conditions is indicated in the solid line block and via the tricarboxylic acid cycle under aerobic conditions in the dashed line block. Genes involved in citrate metabolism are indicated in circles and encode the following enzymes: *citDEF*: citrate lyase, *citM*: oxaloacetate decarboxylase, *ldhD*: D-lactate dehydrogenase, *pdh*: pyruvate dehydrogenase, *alsS/als*:  $\alpha$ -acetolactate synthase, *alsD/aldB*:  $\alpha$ -acetolactate decarboxylase and *butA/dar*: diacetyl/acetoin reductase. Modified from Jyoti *et al.*, 2004; Torino *et al.*, 2005; Olguín *et al.*, 2009 and Karakas-Sen and Akyol, 2012.

## 2.2 Genes involved in citrate metabolism

Citrate is transported into the cell by a transporter protein after which citrate is degraded by  $\text{cit}^+$  LAB to oxaloacetate and acetate. Several genes are involved in this metabolic pathway (see **Figure 2.1**) and are grouped together into the following three categories: (I) the transport of citrate; (II) the conversion of intracellular citrate to pyruvate and finally (III) the conversion of pyruvate to different flavour compounds. The function of these genes as well as the differences or similarities amongst different LAB genera will be discussed according to these three categories.

### 2.2.1 Transport of citrate

Citrate can be transported either by a citrate permease or malate permease into the cell. Most LAB such as *Lc. lactis* (Magni *et al.*, 1996; García-Quintáns *et al.*, 1998; López *et al.*, 1998), *Weissella paramesenteroides* (Martín *et al.*, 1999), *Lb. plantarum* (Siezen *et al.*, 2012) and *Leuc. mesenteroides* (Bandell and Lolkema, 2000) transport citrate through a citrate permease, while other LAB such as *O. oeni* (Mills *et al.*, 2005; Olguín *et al.*, 2009) transport citrate through a malate permease. The citrate and malate permeases are homologous proteins with a conserved C-terminus and are encoded by *citP* and *maeP* genes, respectively (Kawai *et al.*, 1997; Bandell and Lolkema, 2000). However, in *Enterococcus* and some other *Lactobacillus* species, the citrate

transporter is encoded by *citH* (Blancato *et al.*, 2008; Suárez *et al.*, 2011; Martino *et al.*, 2016) and *citT* (Illegheems *et al.*, 2015), respectively.

The expression of these genes, encoding the permeases, is regulated on a transcriptional level by polypeptides. In *Lc. lactis* the expression of *citP* is regulated by a CitQ-CitR-polypeptide encoded by *citQ* and *citR* genes. These genes are grouped together with the IS982 region in a single operon, which is controlled by three promoters named P1, P2' and P2 (García-Quintáns *et al.*, 1998). This operon is carried on a pCIT264 plasmid in *Lc. lactis* subspecies *lactis* biovar *diacetylactis* as shown in **Figure 2.2** (López *et al.*, 1998). In *Lb. plantarum* and *W. paramesenteroides* the expression of *citT* or *citP* is regulated by a single polypeptide encoded by *citR*, because of the absence of the *citQ* gene in these species (Martín *et al.*, 1999; Siezen *et al.*, 2012). The *citP* and *citR* genes form a single operon that is carried on a plasmid in *Leuc. mesenteroides* (Marty-Teyssset *et al.*, 1995; Vaughan *et al.*, 1995; Levata-Jovanovic and Sandine, 1996). In *Lb. plantarum*, the *citT* and *citR* genes are located on chromosomal DNA (Medina de Figueroa *et al.*, 2000; Illegheems *et al.* 2015). In other LAB such as *W. paramesenteroides* the *citP* and *citR* genes are clustered with several other genes, involved in citrate metabolism, in a single operon which is carried on a plasmid (**Figure 2.2**; Martín *et al.*, 1999).

### 2.2.2 Conversion of citrate to pyruvate

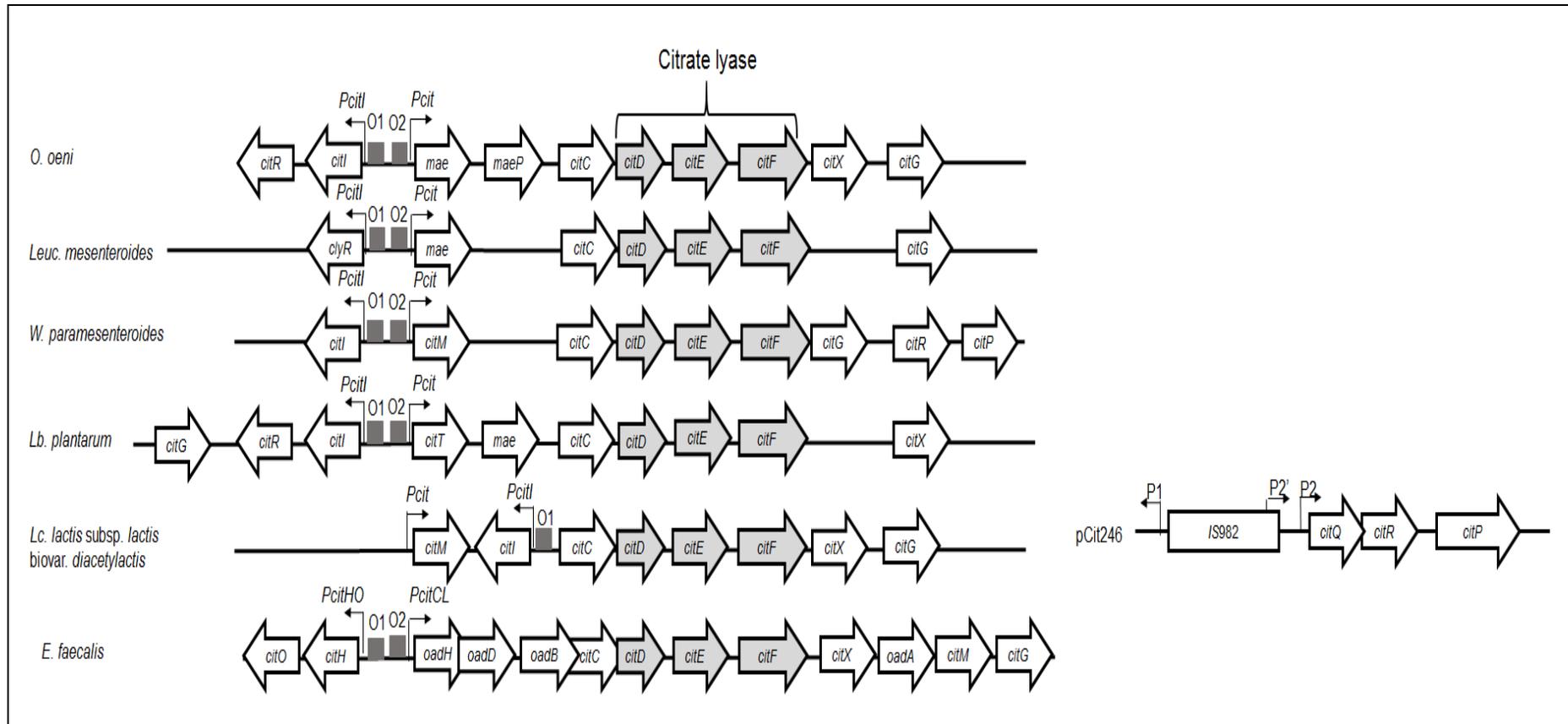
Once citrate has been transported into the cell it is cleaved to acetate and oxaloacetate by citrate lyase. This enzyme consists of three subunits namely  $\alpha$  (acetyl- acyl carrier protein [ACP]: citrate ACP-transferase),  $\beta$  (Citryl-ACP oxaloacetate lyase) and  $\gamma$  (ACP), encoded by *citD*, *citE* and *citF* genes, respectively (Bekal *et al.*, 1998a; Martín *et al.*, 1999; 2004). A study by Mtshali *et al.* (2010) observed that a large majority of wine associated *Lactobacillus* species had all three of these genes encoding citrate lyase and therefore could potentially utilise citrate. However, some of the lactobacilli strains screened lacked one or more of these genes rendering them incapable of citrate utilisation. Another study by Lerm *et al.* (2011) observed that all the *Lb. plantarum* and *O. oeni* strains screened positive for *citE* gene, but some of the strains lacked either *citD* or *citF* genes.

As soon as these *citDEF* genes are transcribed and the subunits produced to form the citrate lyase complex, this enzyme must be activated. This activation involve a adenosine triphosphate (ATP)-dependent acetylation of the prosthetic group (2-5''-phosphoribosyl-3'-dephospho coenzyme A) linked to the  $\gamma$  subunit (Martín *et al.*, 1999; 2000). This reaction is catalysed by the *citC* gene encoding an acetate: thiol-citrate lyase ligase that converts HS-ACP to acetyl-S-ACP (Martín *et al.*, 1999; 2000). This gene together with *citD*, *citE* and *citF* genes are conserved amongst LAB genera (Bekal *et al.*, 1998b; Martín *et al.*, 1999; García-Quintáns *et al.*, 2008a). The *citG* and *citX* genes also play a role in the activation of citrate lyase, which encode enzymes that are needed for the synthesis of the prosthetic group (Martín *et al.*, 2004). The *citG*

gene encodes triphosphoribosyl-dephospho-coenzyme A synthase and *citX* encodes apo-citrate lyase phosphoribosyl-dephospho-coenzyme A transferase (Martín *et al.*, 2004). However, it should be highlighted that the *citX* gene is absent in *W. paramesenteroides* and *Leuc. mesenteroides* (**Figure 2.2**).

After citrate lyase has been activated and citrate cleaved to oxaloacetate and acetate, oxaloacetate is further metabolised to pyruvate through a reaction catalysed by a putative oxaloacetate decarboxylase. Martín *et al.* (2004) indicated that the putative oxaloacetate decarboxylase displays homology to the malolactic enzyme and is encoded by the *citM* or *mae* gene, located upstream of the *citD*, *citE* and *citF* genes in most LAB (**Figure 2.2**). The oxaloacetate decarboxylase in *Enterococcus* species, however, consists of four subunits encoded by *oadH*, *oadD*, *oadB* and *oadA* genes (**Figure 2.2**).

The genes encoding citrate lyase, the activation proteins as well as oxaloacetate decarboxylase usually form a single operon controlled by the *PcitI* and *Pcit* promoters and a regulatory protein, belonging to the SorC transcriptional regulator family (Martín *et al.*, 2000; 2004; 2005). The regulatory protein in most LAB is encoded by *citI* (**Figure 2.2**), but in *Leuc. mesenteroides* (Bekal-Si Ali *et al.*, 1999) and in *Enterococcus* (Blancato *et al.*, 2008; Suárez *et al.*, 2011; Martino *et al.*, 2016) the regulatory protein is encoded by *clyR* and *citO*, respectively. The regulatory protein regulates the *cit* operon by binding two operator sites, O1 and O2, which are conserved amongst most LAB. The O1 operator (TTTTAAA-WA-TWWRAAD) is recognised with less affinity by the regulatory protein, whereas the O2 operator (TTTWAAA-WA-TTTAAAA) is the high affinity site. The *cit* operon of *Lc. lactis* subsp. *lactis* biovar *diacetylactis* is regulated by the CitI regulatory protein binding only to the O1 operator site, since these bacteria lack the O2 operator site (**Figure 2.2**).



**Figure 2.2** Genetic organization of the genes involved in the citrate metabolism of different LAB genera. Arrows indicate the transcription direction and shaded arrows indicates the three subunits of citrate lyase, which are conserved amongst LAB genera. The *Pcitl*, *Pcit*, P1, P2' and P2 promoters together with the O1 and O2 operator sites, involved in the regulation of these genes, are also indicated in this figure. Adapted from López *et al.* (1998), Martín *et al.* (2005), Mills *et al.* (2005), García-Quintáns *et al.* (2008b) and Illegghems *et al.* (2015).

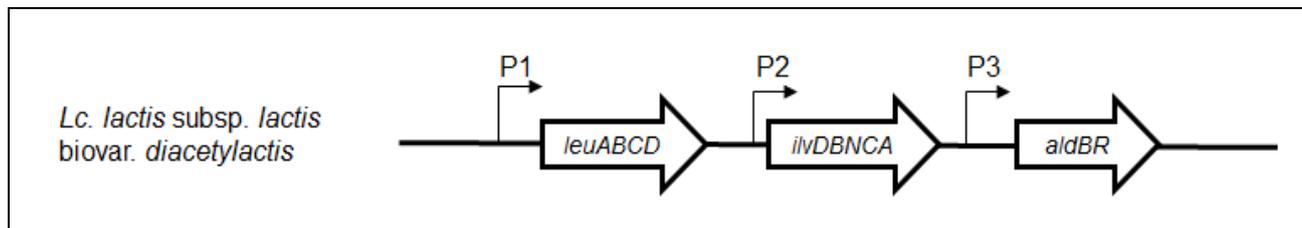
### 2.2.3 Conversion of pyruvate to flavour compounds

Pyruvate is produced as an intermediate during citrate metabolism or glycolysis and can be degraded via three different pathways catalysed by several enzymes (see **Figure 2.1**). Pyruvate can be converted to acetate, D-lactate or to C<sub>4</sub> flavour compounds, specifically diacetyl, acetoin and 2,3-butanediol (Ramos *et al.*, 1995; Bartowsky and Henschke, 2004). The redox potential of the cell as well as the intra- and extracellular pH are the main factors that determine which of these metabolites are formed from pyruvate.

The production of either D-lactate or acetate from pyruvate is regulated by the redox potential of the cell (**Figure 2.1**). Acetate is produced when the cell needs to regenerate the reduced form of nicotinamide adenine dinucleotide (NADH) by a three-step metabolic reaction. Firstly, pyruvate is oxidised to acetyl-CoA by pyruvate dehydrogenase, which consist of four subunits, namely E1 $\alpha$ , E1 $\beta$ , E2 and E3 (Cocaign-Bousquet *et al.*, 2002; Wagner *et al.*, 2005). These four subunits of pyruvate dehydrogenase are encoded by *pdhA*, *pdhB*, *pdhC* and *pdhD*, respectively (Cocaign-Bousquet *et al.*, 2002; Wagner *et al.*, 2005). Secondly, acetyl-CoA is phosphorylated to form acetyl-phosphate, which is finally converted to acetate by acetate kinase encoded by *ackA* (Wagner *et al.*, 2005; Kang *et al.*, 2013). If the cell has sufficient amounts of NADH, D-lactate would rather be produced from pyruvate in a single reduction reaction (**Figure 2.1**). This reduction reaction is catalysed by lactate dehydrogenase encoded by the *ldhD* gene (Goffin *et al.*, 2004; Wagner *et al.*, 2005).

Under acidic conditions, when the cell needs to maintain its intracellular pH, pyruvate would preferentially be converted to the neutral C<sub>4</sub> flavour compounds. Pyruvate is first converted to  $\alpha$ -acetolactate during a decarboxylation reaction catalysed by  $\alpha$ -acetolactate synthase (**Figure 2.1**). This enzyme is encoded by *alsS* in *O. oeni* (Garmyn *et al.*, 1996) and *E. faecalis* (Repizo *et al.*, 2011). However, in *Lc. lactis* (Swindell *et al.*, 1996; Goupil-Feuillerat *et al.*, 1997; Zuljan *et al.*, 2014) and *Lb. plantarum* (Illeghems *et al.*, 2015),  $\alpha$ -acetolactate synthase enzyme is encoded by the *als* gene. The  $\alpha$ -acetolactate can then either be decarboxylated to diacetyl or acetoin (**Figure 2.1**). Diacetyl is produced through a spontaneous reaction under aerobic conditions, whereas the production of acetoin is catalysed by  $\alpha$ -acetolactate decarboxylase and usually occurs under anaerobic conditions. This enzyme is encoded by *alsD* in *O. oeni* (Garmyn *et al.*, 1996) and *E. faecalis* (Repizo *et al.*, 2011). These studies have indicated that *alsS* and *alsD* form a single operon. However, recent studies concluded that the *alsS* and *alsD* genes do not form a single operon and are regulated independently in *O. oeni* (Olguín *et al.*, 2009; Mink *et al.*, 2014a; 2014b). The  $\alpha$ -acetolactate decarboxylase is encoded by the *aldB* gene in various other LAB such as *Lactobacillus* (Medina de Figueroa *et al.*, 1996; Wallenius *et al.*, 2011; Siezen *et al.*, 2012; Kaneko *et al.*, 2014; Illeghems *et al.*, 2015;), *Streptococcus* (Kaneko *et al.*, 2014) and *Lc. lactis* (Goupil-Feuillerat *et al.*, 1997; 2000; García-Quintáns *et al.*, 2008b). The *aldB* gene in *Lc. lactis* is clustered together with genes involved in the synthesis of branched-chain amino acids as indicated in **Figure**

**2.3.** The *alsS*, *alsD* and *aldB* genes are constitutively expressed, but their expression levels can be enhanced by certain factors such as acidic pH and citrate (Ramos *et al.*, 1995; Garmyn *et al.*, 1996; Goupil-Feuillerat *et al.*, 1997; Palles *et al.*, 1998; Repizo *et al.*, 2011).



**Figure 2.3** Genetic organisation of *leu-ilv-ald* operon in *Lc. lactis* subspecies *lactis* biovar *diacetylactis*, which contains the *aldB* gene encoding  $\alpha$ -acetolactate decarboxylase. Arrows indicate the transcription direction, while P1, P2 and P3 are promoters that control the expression of the genes represented by the arrows. Figure modified from García-Quintáns *et al.* (2008b).

The diacetyl produced from  $\alpha$ -acetolactate can then lastly be reduced to form acetoin, which can be further reduced to 2,3-butanediol (Ratray *et al.*, 2003). Diacetyl-acetoin reductase, encoded by *butA*, catalyses both the reduction of diacetyl to acetoin and the reduction of acetoin to 2,3-butanediol in *Lc. lactis* (Zuljan *et al.*, 2014), *W. pseudomesenteroides* (Ratray *et al.*, 2003) and *Lc. cremoris* (García-Quintáns *et al.*, 2008b; Karakas-Sen and Akyol, 2012). The conversion of 2,3-butanediol back to acetoin is catalysed by 2,3-butanediol dehydrogenase, encoded by the *butB* gene (Zuljan *et al.*, 2014). Both *butA* and *butB* is carried on chromosomal DNA in *Lactococcus* species and on plasmid DNA in *W. pseudomesenteroides* (Ratray *et al.*, 2003). Several studies use *dar* when referring to both the *butA* and *butB* genes (García-Quintáns *et al.*, 2008b; Karakas-Sen and Akyol, 2012; Zuljan *et al.*, 2014). In other LAB such as *O. oeni* (Ramos *et al.*, 1995) the irreversible reduction of diacetyl to acetoin is catalysed by diacetyl reductase, whereas the reversible reduction of acetoin to 2,3-butanediol is catalysed by acetoin reductase. Ramos *et al.* (1995) suggested that the genes encoding these enzymes are constitutively expressed, since they observed no major differences in the activity of these enzymes under different experimental conditions. However, there is limited information about the genes that encode these enzymes in *O. oeni*.

### **2.3 Regulation of citrate metabolism and factors influencing the concentrations of metabolites associated with this metabolism**

The expression of the genes involved in citrate metabolism is regulated on a transcriptional level. According to literature the presence of citrate and the pH of the media play an important role in the regulation of these genes. These factors together with several other factors, such as the presence of certain sugars, oxygen and temperature, influence the concentration of the metabolites produced during citrate metabolism (**Table 2.1**).

### 2.3.1 Citrate concentration

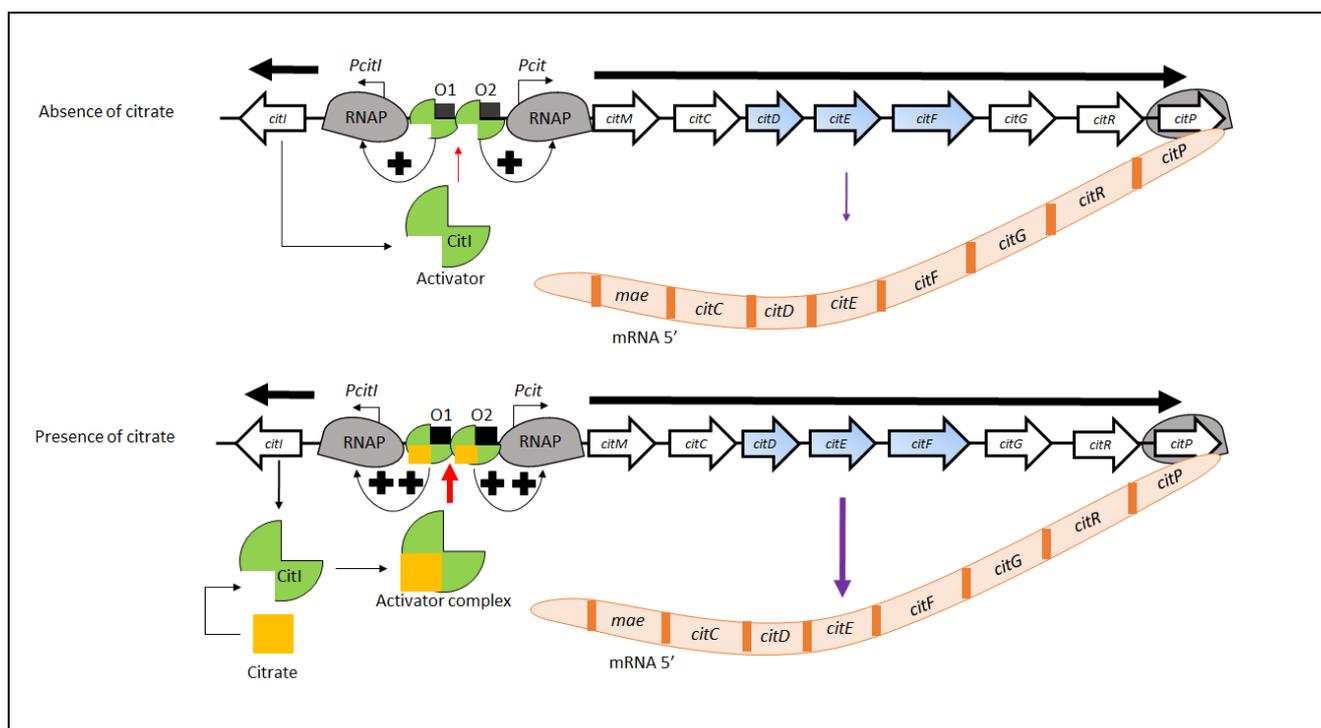
Citrate concentration is a crucial factor that determines the concentration of the metabolites associated with citrate metabolism. Acetate and D-lactate are the metabolites that are most frequently influenced by citrate. Several studies performed with various LAB isolated from different matrices, including dairy products (Boumerdassi *et al.*, 1997; Hache *et al.*, 1999; Magni *et al.*, 1999) and wine (Augagneur *et al.*, 2007) observed that the final concentration of acetate and D-lactate increased when the initial citrate concentration was increased. However, tremendous amounts of citrate are toxic to the growth of LAB due to over acidification in the cell caused by excess acetate and D-lactate (Magni *et al.*, 1999; Augagneur *et al.*, 2007). Furthermore, Branen and Keenan (1971) reported changes in the diacetyl concentrations when adding citrate to the growth medium of *Lb. casei*. During this study they observed increased activity of diacetyl reductase resulting in decreased diacetyl and increased acetoin concentrations.

Besides affecting the final concentration of the metabolites, citrate also regulates the expression levels of genes involved in citrate metabolism. The *citP* gene (Marty-Teyssset *et al.*, 1995) and *mae-citCDEF* operon (Bekal-Si Ali *et al.*, 1999) in *Leuc. mesenteroides* as well as the *citMCDEFGRP* operon of *W. paramesenteroides* (Martín *et al.*, 2005) are regulated by the absence or presence of citrate under anaerobic conditions. Citrate acts as an inducer in *Leuc. mesenteroides* that leads to the transcription of *citP* (Marty-Teyssset *et al.*, 1995) and *clyR* regulating the expression of genes in the *mae-citCDEF* operon (Bekal-Si Ali *et al.*, 1999). However, the regulatory gene (*citI*) of *W. paramesenteroides* is constitutively expressed irrespective of the citrate concentration. The presence or absence of citrate only influences the binding affinity of the CitI activator to the two DNA operator sites found upstream of the *citMCDEFGRP* operon (Martín *et al.*, 2005; **Figure 2.3**).

In the absence of citrate the CitI activator binds the O1 and O2 operator sites (**Figure 2.4**). The bound operator sites stimulate RNA polymerase to bind to the *PcitI* and *Pcit* promoters, resulting in the transcription of the genes in the *citMCDEFGRP* operon as indicated in **Figure 2.4** (Martín *et al.*, 2005). Thus the enzymes encoded by these genes, namely oxaloacetate decarboxylase, citrate lyase and citrate permease are only present at low basal levels when citrate is absent. As soon as citrate enters the cell, citrate binds the CitI activator, which enhances the binding affinity of this activator to the operator sites (Martín *et al.*, 2005; **Figure 2.3**). Therefore, in the presence of citrate the CitI activator binds more strongly to the operator sites, resulting in an increased stimulation of the RNA polymerase. This leads to increased RNA polymerase recruitment on the promoters, resulting in increased expression of the genes encoded by the *citMCDEFGRP* operon (Martín *et al.*, 2005; **Figure 2.3**). Thus the expression levels of the genes in the *citMCDEFGRP* operon are influenced by the presence or absence of citrate.

Studies have indicated that the activity of citrate lyase in *O. oeni* (Ramos *et al.*, 1995) and *Lb. plantarum* (Palles *et al.*, 1998) are also influenced by citrate and tends to increase in the

presence of citrate. This as well as the similar genetic organisation that these bacteria have in common with the *citMCDEFGRP* operon of *W. paramesenteroides* could suggest that the *citGT-mae-citCDEFX* operon of *Lb. plantarum* and the *mae-maeP-citCDEFXG* operon of *O. oeni* are regulated in a similar manner. However, a study by Augagneur *et al.* (2007) indicated that *maeP* and *citF* of *O. oeni* was not regulated by citrate or pH. Other factors might play a role in the regulation of these genes in *O. oeni* and more research is needed to better understand their regulation.



**Figure 2.4** Regulation of *citMCDEFGRP* operon in *Weissella paramesenteroides* by the absence or presence of citrate. Depicting the transcription of *citI* gene encoding the activator protein CitI, which stimulates the recruitment of RNA polymerase (RNAP) on the *PcitI* and *Pcit* promoters. This stimulation is indicated by the plus signs and the level of stimulation is represented by the number of plus signs. Once RNAP has bind to the promoters, the *citMCDEFGRP* operon is transcribed into mRNA. Red arrows indicate the binding of CitI activator to the two operator sites and purple arrows indicate transcription of genes into mRNA. The thickness of arrows indicates either binding affinity of the activator or the levels of expression.

### 2.3.2 pH

Another important factor that influences citrate metabolism is pH. Several studies have indicated that dairy LAB, such as *Leuc. lactis* (Cogan *et al.*, 1981), *E. faecalis* (Rea and Cogan, 2003a) and *Lc. lactis* (Starrenburg and Hugenholtz, 1991; García-Quintáns *et al.*, 1998) utilise citrate at an optimum rate when the medium has a pH of 5.0 to 6.0. However, *Lb. casei* (Brannen and Keenan, 1971; Palles *et al.*, 1998; Díaz-Muñiz and Steele, 2006) and *Lb. plantarum* (Montville *et al.*, 1987; Kennes *et al.*, 1991; Palles *et al.*, 1998) have a slightly lower optimum pH range of 4.5 to 5.0. Studies have also indicated that at higher pH levels citrate was not utilised and that citrate inhibits cell growth at lower pH levels (Montville *et al.*, 1987; Rea and Cogan, 2003a).

The pH of the medium affects the ionic species of citrate and therefore the citrate transport. At a pH below 3.13, citric acid is the ionic species present as indicated in **Figure 2.5**. Monovalent

citrate is the dominant ionic species within a pH range of 3.13 to 4.76. Divalent citrate is the next ionic species that is present within a pH range of 4.76 to 6.39. Above pH 6.39, the dominant ionic species is trivalent citrate. These ionic species are transported by specific transporters. Díaz-Muñiz and Steele (2006) observed that the citrate permease of *Lb. casei* was not able to transport monovalent citrate and thus citrate utilisation was slightly suppressed at pH values below 5. The citrate permease of these bacteria showed the highest activity at a pH of 6.0. Another study by Marty-Teyssset *et al.* (1995) also suggested that the citrate permease of LAB transports different citrate ionic species depending on their optimal growth pH. The citrate permease of LAB with a higher optimum pH such as *Lc. lactis* transports divalent citrate ( $\text{Hcit}^{2-}$ ), whereas LAB with a lower optimum pH such *O. oeni* transport monovalent citrate ( $\text{H}_2\text{cit}^-$ ).

The pH of the medium also plays a role in the regulation of some genes involved in citrate metabolism. Studies performed with *Lc. lactis* in a dairy matrix indicated that the P1 and *Pcit* promoters are sensitive to and activated by acidic pH levels rather than by the presence or absence of citrate (García-Quintáns *et al.*, 1998; Martín *et al.*, 2004; García-Quintáns *et al.*, 2008b). The activation of these promoters under acidic conditions increases the expression levels of *citQRP* and *citMCDEFXG* operon in *Lc. lactis* (García-Quintáns *et al.*, 1998; Martín *et al.*, 2004; García-Quintáns *et al.*, 2008b). Citrate is thus transported into the cell at a faster rate due to the citrate permease having an optimal activity when the pH is in a range of 4.5 to 5.5 when  $\text{Hcit}^{2-}$  is the predominant ionic species (Magni *et al.*, 1996). The regulation of the *citMCDEFXG* operon in *Lc. lactis* by pH rather than by citrate might be due to the absence of the O2 operator site as indicated in **Figure 2.2**.

The transcription of other genes involved in the conversion of pyruvate to carbonyl flavour compounds are also influenced by pH. One of these include the gene encoding  $\alpha$ -acetolactate decarboxylase which is stimulated by pH values below 4 resulting in acetoin accumulation in *Leuc. lactis* (Cogan *et al.*, 1981), *O. oeni* (Ramos *et al.*, 1995), *Lc. lactis* (García-Quintáns *et al.*, 2008b) and *Lb. casei* (Branen and Keenan, 1971). A study by Olguín *et al.* (2009) determined the influence of pH and ethanol on the expression of *citI*, *citE*, *maeP*, *alsS* and *alsD* genes in synthetic wine. They observed that in the absence of ethanol, pH only affected the expression of *maeP* and *alsD* after 7 days of inoculation. The highest relative expression was observed at pH 4.0 for *maeP* and at pH 3.5 for *alsD*. Thus citrate would be transport faster at a higher pH, but less acetoin could potentially be produced at pH 3.5 which is in agreement with the study performed by Ramos *et al.* (1995) that observed acetoin accumulation at pH values below 4.0.

Acetate and D-lactate concentrations are also influenced by pH. Ramos *et al.* (1995) tested the effect of different pH values ranging from 5.0 to 7.0 on the production of acetate and D-lactate by *O. oeni* in synthetic wine. During this study they observed increased acetate concentrations with decreasing pH levels and increased D-lactate concentrations with increasing pH values. However, Kennes *et al.* (1991) observed increased acetate concentrations with increasing pH levels when

they used *Lb. plantarum* in a synthetic orange juice with pH values of 3.0, 4.2 and 5.0. This contradiction between the studies may be due to the different LAB species and the different pH ranges that were tested.

### 2.3.3 Sugar

Various studies have reported that citrate metabolism is affected by glucose and that this affect is dependent on the LAB species (Rea and Cogan, 2003a, 2003b; Cabral *et al.*, 2007). Studies have indicated that *Enterococcus* species (Rea and Cogan, 2003a), some *Lactobacillus* species such as *Lb. rhamnosus* (Medina de Figueroa *et al.*, 2000; Jyoti *et al.*, 2004) and *Leuc. mesenteroides* (Levata-Jovanovic and Sandine, 1996) exhibits a diauxic growth when grown in the presence of glucose and citrate. These bacteria only utilise citrate when glucose has been depleted, since the activity of citrate permease (Levata-Jovanovic and Sandine, 1996; Medina de Figueroa *et al.*, 2000; Rea and Cogan, 2003b) or citrate lyase (Palles *et al.*, 1998) is suppressed by glucose usually through catabolite repression. However, some LAB such as *O. oeni* (Ramos and Santos, 1996), *Lb. plantarum* (Palles *et al.*, 1998), *Lb. casei* (Palles *et al.*, 1998; Díaz-Muñiz and Steele, 2006) and *Leuc. lactis* (Cogan *et al.*, 1981) are still able to degrade a small portion of citrate when glucose is present. This co-metabolism is usually seen when the bacteria are pre-cultured in a medium that contains both citrate and glucose (Palles *et al.*, 1998; Jyoti *et al.*, 2004). Pre-culturing the bacteria can also increase the production of acetate, D-lactate, diacetyl and acetoin during fermentation (Jyoti *et al.*, 2004).

The amount of glucose present during fermentation also influences the diacetyl and acetoin concentrations. In a study by Tsau *et al.* (1992) they observed that *Lb. plantarum* did not produce any acetoin when grown in a medium without glucose. Another study by Ramos and Santos (1996) observed that low glucose concentrations stimulated the production of diacetyl and acetoin. They also indicated that high glucose concentrations stimulated the reduction of diacetyl and acetoin to 2,3-butanediol by *O. oeni* in synthetic wine. This could suggest that high diacetyl and acetoin concentrations could be produced when a LAB starter culture is inoculated after the completion of alcoholic fermentation, known as sequential inoculation, as opposed to co-inoculation where the malolactic fermentation (MLF) starter culture is inoculated simultaneously with the yeast starter culture. A recent study by Versari *et al.* (2015) supported this statement in that they observed that co-inoculation wines were described as being more fruity due to the lower diacetyl concentrations present in these wines. They explained that the lower diacetyl concentrations were due to an increased reduction rate of diacetyl to acetoin by either the LAB or by the yeast starter culture. Furthermore, Antalick *et al.* (2013) also observed that co-inoculation of *O. oeni* with *Saccharomyces cerevisiae* resulted in increased acetoin and 2,3-butanediol concentrations in most of their Merlot wines.

Other sugars such as lactose and galactose can also influence the utilisation of citrate by LAB. Palles *et al.* (1998) observed that *Lb. plantarum* and *Lb. casei* are able to co-metabolise citrate and lactose as well as citrate and galactose. They observed that citrate was utilised at a faster rate and more acetoin and acetate were produced when citrate was co-metabolised with galactose, instead of glucose or lactose. However, the bacteria grew slower when incubated with galactose instead of glucose and lactose. Palles *et al.* (1998) therefore speculated that the faster citrate utilisation was linked to the increased intracellular pH obtained during the slower growth of these bacteria. Another study by Hache *et al.* (1999) also indicated that *Leuc. lactis* and *Leuc. mesenteroides* were able to co-metabolise citrate and lactose, which increased the D-lactate and acetate concentrations.

#### 2.3.4 Oxygen

Oxygen is an important culture condition that influence the concentrations of metabolites produced via citrate metabolism. Oxygen is a key component in the spontaneous formation of diacetyl from  $\alpha$ -acetolactate (**Figure 2.1**). Nielsen and Richelieu (1999) observed that in semi-aerobic conditions the diacetyl concentrations can be up to 6 times more than in anaerobic conditions. However, diacetyl concentrations decreases over a period of time when it is further reduced to acetoin which can finally be reduced to 2,3-butanediol (Kaneko *et al.*, 1991; Bassit *et al.*, 1993; Ramos *et al.*, 1995; Boumerdassi *et al.*, 1996; Elena *et al.*, 2006). In a study by Cretenet *et al.* (2014) they observed that the genes encoding the E1 $\alpha$  subunit of pyruvate dehydrogenase,  $\alpha$ -acetolactate synthase and  $\alpha$ -acetolactate decarboxylase were overexpressed under aerobic conditions. This resulted in increased acetoin and 2,3-butanediol under aerobic concentrations. The increase of diacetyl, acetoin and 2,3-butanediol under aerobic conditions can also be explained by a study performed by Ramos *et al.* (1995) which observed that *O. oeni* and *Lb. plantarum* consume pyruvate faster in the presence of oxygen and thus produce more flavour compounds.

Oxygen can also influence the production of acetate and D-lactate from pyruvate. Under aerobic conditions, the activity of lactate dehydrogenase decreases and less D-lactate is produced (Bobillo and Marshall, 1991; Bassit *et al.*, 1993; Boumerdassi *et al.*, 1996; De Felipe *et al.*, 1997). The decrease of D-lactate is coupled to an increase in acetate formation that can be seen when LAB are incubated under aerobic conditions (Bobillo and Marshall, 1991; Boumerdassi *et al.*, 1996; De Felipe *et al.*, 1997; Wang *et al.*, 2011; Cretenet *et al.*, 2014).

#### 2.3.5 Temperature

Temperature is another culture condition factor that influences the final concentration of flavour compounds usually by affecting the activity of enzymes involved in their production (Bassit *et al.*, 1995; Medina de Figueroa *et al.*, 2001). When incubating *Lb. rhamnosus* at temperatures ranging from 20°C to 45°C, Medina de Figueroa *et al.* (2001) observed maximum diacetyl and acetoin concentrations at 37°C and minimum concentrations at 20°C. These increased concentrations

were linked to the increased activity of citrate lyase and  $\alpha$ -acetolactate synthase at 37°C reported during this study. The highest activity of diacetyl reductase, responsible for the reduction of diacetyl to acetoin, was observed at 20°C which corresponded to the lowest diacetyl concentration at that temperature. In contrast, after testing 18°C, 22°C, 26°C and 30°C, Bassit *et al.* (1995) observed maximum diacetyl concentrations when *Lc. lactis* was incubated at 18°C which corresponded to a decrease in diacetyl reductase activity at that temperature. However, they did observe maximum acetoin concentrations when *Lc. lactis* was incubated at 30°C which could be linked to an increase in  $\alpha$ -acetolactate synthase activity at that temperature. In both of these studies they observed increased lactate dehydrogenase activity with increasing temperatures (Bassit *et al.*, 1995; Medina de Figueroa *et al.*, 2001). Other studies also observed that lactate concentrations increased with increasing temperatures, but that lactate production decreased again when the temperature reached 50°C (Trontel *et al.*, 2010; Wang *et al.*, 2011).

Several studies were also performed to evaluate the influence of temperature on the diacetyl concentrations in beer and wine. After testing various temperatures ranging from 12°C to 23°C, Portno (1966) observed that maximum diacetyl concentrations were achieved with a higher fermentation temperature. This is in agreement with several other studies such as the study performed by García *et al.* (1994) and Elena *et al.* (2006). Although, these studies observed increased diacetyl concentrations at higher temperatures, the diacetyl was reduced faster to acetoin at higher temperatures resulting in an overall decrease in diacetyl concentrations at the end of the fermentation. Ough and Amerine (1967) also observed that acetoin and 2,3-butanediol concentrations increased with increasing fermentation temperatures in wine.

### 2.3.6 Type of starter culture, inoculation dosage and duration of fermentation

Another major factor that can play a role in determining the final concentration of flavour compounds is the use of different LAB starter cultures. As mentioned, LAB can be divided into two groups regarding their ability to utilise citrate which is either cit<sup>+</sup> or cit<sup>-</sup> strains (Farkye and Vedamuthu, 2002; Drider *et al.*, 2004). Citrate positive LAB degrade citrate and therefore might produce higher concentrations of the C<sub>4</sub> flavour compounds than cit<sup>-</sup> LAB. However, cit<sup>-</sup> LAB might still be able to produce small amounts of the C<sub>4</sub> flavour compounds from pyruvate generated during glycolysis as indicated in **Figure 2.1**. Citrate negative LAB would therefore be the preferred malolactic bacterial starter cultures for winemakers when creating a fruity style wine. Chr. Hansen recognised the need for a cit<sup>-</sup> malolactic bacterial starter culture when they commercialised Viniflora® CiNe™, a cit<sup>-</sup> LAB strain unable to utilise citrate, in 2010 (Chr. Hansen, 2010).

Lactic acid bacteria are also divided into three major groups based on their sugar metabolism, as previously mentioned. These bacteria do not only use different pathways for the breakdown of sugars, but also differ from one another regarding their citrate utilisation and production of acetoin and diacetyl. In a study performed by Drinan *et al.* (1976) they observed that

both homo- and heterofermentative LAB were able to utilise citrate, but these two groups produced different amounts of acetoin. The obligate heterofermentative LAB, specifically *W. viridescens* (previously known as *Lb. viridescens*), *Lb. fermenti*, *Leuc. lactis* and *W. paramesenteroides*, produced little or no acetoin regardless of the presence or absence of citrate. However, the homofermentative and/or facultative heterofermentative LAB, specifically *Lb. plantarum* and *Streptococcus lactis* var. *daicetylactis*, produced large amounts of acetoin ( $\geq 100$  mg/l) only when citrate was present. These results were in contrast with the results from Medina de Figueroa *et al.* (2000) in that they did not observe specific differences between homo- and heterofermentative LAB regarding their ability to produce C<sub>4</sub> flavour compounds. In this study they did however observe that different LAB species produced different amounts of diacetyl and acetoin.

Several other studies have also observed that LAB strains within in the same species produce different concentrations of diacetyl and acetoin. Starrenburg and Hugenholtz (1991) indicated that two *Lc. lactis* subsp. *lactis* var. *diacetylactis* strains, Ru4 and C17, produced different amounts of acetoin under acidic conditions, but no diacetyl was detected. The Ru4 strain produced almost 29% less acetoin than C17 (Starrenburg and Hugenholtz, 1991) which might indicate that Ru4 were able to reduce acetoin faster to 2,3-butanediol. Unfortunately, 2,3-butanediol was not measured in this study to confirm the hypothesis.

Another study by Malherbe *et al.* (2012) investigated the different aroma profiles of four commercial *O. oeni* starter cultures, namely Enoferm alpha® (Lallemand), Lalvin VP41® (Lallemand), Viniflora® oenos (Chr Hansen) and Viniflora® Ch16 (Chr Hansen) in Pinotage and Shiraz wines. During this study they observed that the different strains produced different amounts of diacetyl and acetoin. Diacetyl and acetoin concentrations were also different between the two Pinotage vintages (2008 versus 2009) as well as between Pinotage and Shiraz. The strain that produced the highest diacetyl concentrations in the one cultivar did not necessary produce the highest concentrations in the other cultivar. These results emphasise the influence that different factors might have on the production of diacetyl and acetoin. Another study by Levata-Jovanovic and Sandine (1996) also observed that different *Leuconostoc* strains produced different concentrations of diacetyl and acetoin. These and several other studies (Martineau and Henickling, 1995a; Antalick *et al.*, 2012) provide evidence that different LAB strains produce different amounts of C<sub>4</sub> flavour compounds and have different reduction abilities.

Diacetyl concentrations can also be influenced by the type of yeast culture used during beer brewing and winemaking. After testing the diacetyl production ability of four different *Saccharomyces cerevisiae* strains and one *Saccharomyces pastorianus* strain, Portno (1966) observed that all the strains produced different diacetyl concentrations. The *Saccharomyces pastorianus* (NCYC No. 1057) strain produced the highest diacetyl concentration of 1.51 mg/L in this study. They also observed that when the yeast is inoculated in a high dosage they produce higher diacetyl concentrations and reduce diacetyl to acetoin more rapidly during the fermentation.

Mink *et al.* (2014c) also reported that *S. cerevisiae* CY3079 and Siha Cryarome were able to reduce most of the diacetyl treated Pinot blanc grape must, within the first two days of alcoholic fermentation. The diacetyl concentrations at the end of alcoholic fermentation would thus be less when one of these strains is used as a starter culture.

Diacetyl concentrations are also influenced by the inoculation dosage and the duration of the fermentation. In a review by Bartowsky and Henschke (2004) they explained that a lower inoculation dosage of bacteria and longer MLF favours diacetyl production. This increased diacetyl concentration might be due to the bacteria struggling during the fermentation and therefore degrading a larger portion of citrate to provide them with more energy. However, diacetyl concentrations might decrease at the end of the fermentation due to the ability of LAB and yeast to reduce diacetyl to acetoin and/or 2,3-butanediol.

### 2.3.7 Presence of sulphur dioxide and other compounds

Fermented products are stabilised upon the completion of fermentation. One of the most common preservative is sulphur dioxide (SO<sub>2</sub>). The addition of SO<sub>2</sub> to fermented products such as wine influences the sensory perception of diacetyl. Diacetyl becomes sensory inactive when it is bound by SO<sub>2</sub> and the perceived buttery aroma decreases (Nielsen and Richelieu 1999; Bartowsky *et al.*, 2002). This exothermic reaction is reversible and the diacetyl can become sensory active again as the SO<sub>2</sub> concentration decreases, although Martineau and Henick-Kling (1995b) observed unchanged diacetyl concentrations in aged wines. Several factors such as pH, aeration and the presence of other compounds that can bind SO<sub>2</sub> play a role in this reaction between SO<sub>2</sub> and diacetyl which can compensate for the differences seen between studies. At low pH levels, more active SO<sub>2</sub> is present in wine (Du Toit and Pretorius, 2000) which can bind more diacetyl and therefore decrease the buttery aroma. Furthermore, higher diacetyl concentrations can occur when the wine is stabilised before diacetyl is converted to the less sensory active acetoin and 2,3-butanediol by LAB or yeast cultures (Nielsen and Richelieu, 1999). Wines should therefore rather be stabilised when both the malate and citrate has been completely degraded to avoid a buttery aroma.

Other compounds present in the fermenting matrix can also influence the metabolites produced via citrate metabolism. Several studies have observed that diacetyl concentrations in red wines are less stable than in white wines (Castagnino and Vercauteren, 1996; Bartowsky *et al.*, 2002 Campos *et al.* 2009a). This is most likely due to the presence of phenolic compounds in red wines. Castagnino and Vercauteren (1996) observed that malvidin-3-glucoside present in red wine can bind to diacetyl and lower its sensory perception. Furthermore, Campos *et al.* (2009a) observed that the presence of p-coumaric acid and ferrulic acid delayed citrate utilisation and resulted in decreased acetate concentrations. These phenolic compounds increase the cell membrane permeability which resulted in decrease LAB cell viability during fermentation and

therefore delayed citrate consumption (Campos *et al.*, 2009b). However, Rozés *et al.* (2003) observed that citrate consumption was stimulated by phenolic compounds such as malvidin-3-diglucoside which resulted in increased acetate and decreased D-lactate concentrations. This contradiction between studies emphasise that different phenolic compounds have a different effect on the citrate metabolism and must be considered when inducing MLF. Rozés *et al.* (2003) suggested using a cit<sup>-</sup> strain to induce MLF in red wines, since the presence of some phenolic compounds can stimulate the production of acetate which results in increased volatile acidity.

**Table 2.1** Summary of factors influencing the concentrations of metabolites associated with citrate metabolism (Bartowsky and Henschke, 2004; Lerm, 2010).

Influencing factors	Effect on metabolites produced via citrate metabolism
<b>Citrate concentration</b>	Concentration of metabolites increases with increasing citrate.
<b>pH</b>	Lower pH favours diacetyl production and acetoin accumulation, while higher pH favours lactate production.
<b>Fermentable sugar content</b>	Glucose can inhibit citrate utilisation in some bacterial strains.  Several studies have also indicated that co-inoculated wines were described as being fruitier than sequential wines, with lower sugar concentrations.
<b>Oxygen</b>	Diacetyl, acetoin, 2,3-butanediol and acetate concentrations increases, while lactate concentrations decreases when oxygen is present.
<b>Fermentation temperature</b>	Acetoin, 2,3-butanediol and lactate concentrations increases with increasing temperatures.  Some studies indicated that maximum diacetyl concentrations were reached at lower temperatures, whereas others observed that diacetyl concentrations were the highest at higher temperatures.
<b>LAB strain</b>	Amount of metabolites produced varies greatly amongst LAB strains in that some strains are citrate positive and able to degrade citrate, whereas others are unable to degrade citrate.
<b>Inoculation rate of MLF bacteria</b>	Diacetyl production is favoured by a lower inoculation dosage such as $10^4$ - $10^5$ cfu/ml.
<b>Contact with actual yeast culture and lees</b>	Diacetyl concentrations decreases, while acetoin and 2,3-butanediol concentrations increases the longer the contact with the yeast culture or lees.
<b>Presence of SO<sub>2</sub> or other compounds</b>	Initial addition of SO <sub>2</sub> upon the completion of MLF and citrate metabolism decreases the amount of diacetyl.  The presence of other compounds such as phenolic compounds must also be considered since they can influence the aroma profile of fermented products such as wine.

## 2.4 Function of citrate metabolism

When degrading citrate, cit<sup>+</sup> LAB are provided with an extra carbon and energy source as well as precursors for the synthesis of certain amino acids which enhance their growth (Starrenburg and Hugenholtz, 1991; Hugenholtz *et al.*, 1993; Marty-Teyssset *et al.*, 1996; Medina de Figueroa *et al.*, 2000; Jyoti *et al.*, 2004; Sánchez *et al.*, 2008). Citrate metabolism also plays a role in pH homeostasis and the diacetyl produced during this metabolism exhibits anti-microbial activity further enhancing the growth of cit<sup>+</sup> LAB.

### 2.4.1 Bioenergetics

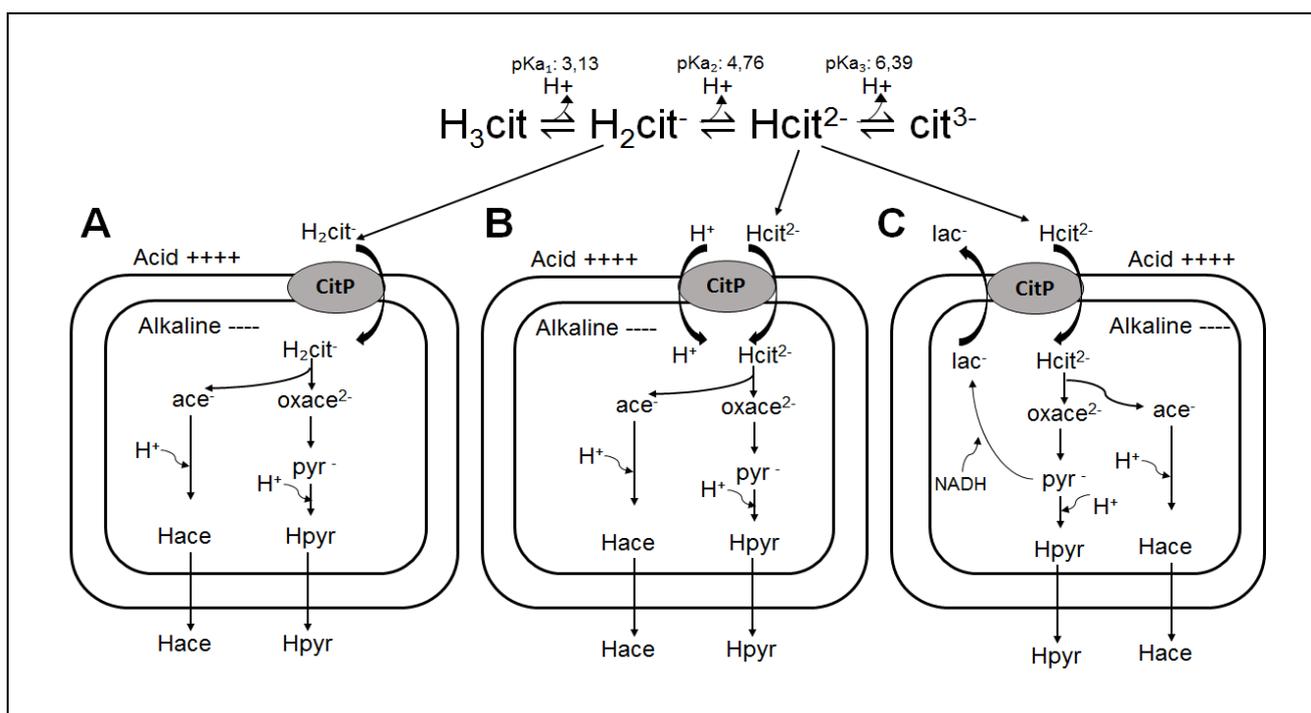
Studies have indicated that citrate utilising LAB display enhanced growth in the presence of citrate, since energy is released when these bacteria degrade citrate (Starrenburg and Hugenholtz, 1991; Hugenholtz *et al.*, 1993; Marty-Teyssset *et al.*, 1996; Medina de Figueroa *et al.*, 2000; Jyoti *et al.*, 2004; Sánchez *et al.*, 2008). However, some LAB such as *Lb. plantarum* (Kennes *et al.*, 1991) are not able to utilise citrate as the only energy source and require a carbohydrate as co-substrate, whereas LAB such as *Lc. lactis* (Starrenburg and Hugenholtz, 1991; Hugenholtz *et al.*, 1993) are able to utilise citrate as the only energy source.

Energy can be generated in the form of ATP during citrate metabolism through substrate phosphorylation when pyruvate is converted to acetate. Although, only 1 ATP is generated through this metabolism it is difficult to explain the enhanced growth of cit<sup>+</sup> LAB in the presence of citrate (Starrenburg and Hugenholtz, 1991; Hugenholtz *et al.*, 1993). The enhanced growth might be explained by the energy released in the form of a proton motive force (PMF) that is generated during citrate metabolism. The proton motive force consists of a membrane potential and a pH gradient.

Two mechanisms linked to citrate transport have been proposed for the generation of a PMF. The type of mechanism incorporated by LAB is dependent on the species and their optimum growth pH. In the first mechanism a membrane potential is generated by the electrogenic transport of H<sub>2</sub>cit<sup>-</sup> via an uniport system as indicated in **Figure 2.5A** (Hugenholtz *et al.*, 1993; Ramos *et al.*, 1994; Konings, 2002). This transport system is coupled to the translocation of charges and driven by the citrate gradient (high outside versus low inside). The citrate gradient determines the magnitude of the membrane potential (Hugenholtz *et al.*, 1993; Ramos *et al.*, 1994). Therefore, a greater membrane potential and thus a PMF is generated when the extracellular citrate concentration is high. This mechanism is mainly used by the wine-associated *O. oeni*, since H<sub>2</sub>cit<sup>-</sup> is the most abundant citrate ionic species at wine pH levels, which is usually between 2.9 to 3.8 (Ramos *et al.*, 1994; Augagneur *et al.*, 2007). However, no membrane potential is generated when *O. oeni* is incubated in a medium with a pH equal to or below 3.2 (Augagneur *et al.*, 2007). Other LAB such as *Leuc. mesenteroides* generates a PMF through a similar mechanism, however these bacteria transports Hcit<sup>2-</sup> together with a proton via a symport transport system as indicated in

**Figure 2.5B** (Marty-Teyssset *et al.*, 1995; Konings, 2002). This symport transport of  $\text{Hcit}^{2-}$  is also coupled to the translocation of charges, which generates a membrane potential and ultimately a PMF. In the study performed by Marty-Teyssset *et al.* (1995) they observed that *Leuc. mesenteroides* is able to exchange  $\text{Hcit}^{2-}$  for lactate under certain physiological conditions similar to the transport system used by *Lc. lactis* indicated in **Figure 2.5C**. This observation was supported by several other studies that used *Leuc. mesenteroides* (Vaughan *et al.*, 1995; Bandell *et al.*, 1997).

The PMF in the second mechanism is generated by the uptake of citrate via an antiport transport system (Hugenholtz *et al.*, 1993; Bandell *et al.*, 1998). This mechanism is mainly used by *Lc. lactis* and is indicated in **Figure 2.5C**. In this mechanism  $\text{Hcit}^{2-}$  is exchanged for D-lactate, pyruvate or acetate (Hugenholtz *et al.*, 1993; Bandell *et al.*, 1998). During this exchange the growth medium becomes more positively charged, while the intracellular area of the bacteria becomes more negative (Hugenholtz *et al.*, 1993). This antiport transport system is much faster than the uni- or symport systems described in the first mechanism (Konings, 2002) and thus the PMF is generated much faster. However, this mechanism cannot be incorporated by LAB at low pH values where  $\text{H}_2\text{cit}$  is the predominant species present.



**Figure 2.5** Proton motive force generated through different citrate transport systems. A) Uniport transport system; B) symport transport system; and C) antiport transport system.  $\text{H}_3\text{cit}$ , citric acid;  $\text{H}_2\text{cit}^-$ , monovalent citrate;  $\text{Hcit}^{2-}$ , divalent citrate;  $\text{cit}^{3-}$ , trivalent citrate;  $\text{ace}^-$ , monovalent acetate;  $\text{Hace}$ , acetate;  $\text{oxace}^{2-}$ , oxaloacetate;  $\text{pyr}^-$ , monovalent pyruvate,  $\text{Hpyr}$ , pyruvate;  $\text{lac}^-$ , monovalent lactate. Modified from Magni *et al.* (1996), Konings (2002) and Ramos *et al.* (1994).

### 2.4.2 pH homeostasis

Lactic acid bacteria expose themselves to acid stress during sugar fermentation due to the production of lactate<sup>1-</sup> via the EMP or pentose phosphate pathway briefly described in the introduction. Lactate<sup>1-</sup> accumulates in the cell and decreases the intracellular pH (Martín *et al.*, 2004). These bacteria therefore require a mechanism to detoxify the accumulated lactate<sup>1-</sup>.

Citrate metabolism is such a mechanism incorporated by LAB to detoxify the accumulated lactate<sup>1-</sup> (Hutkins and Nannen, 1993; García-Quintáns *et al.*, 1998; Martín *et al.*, 2004; Sánchez *et al.*, 2008). This detoxification is linked to the Hcit<sup>2-</sup>/lactate<sup>1-</sup> antiport transport system described in the previous section and leads to the alkalisation of the medium. Citrate metabolism also counteract the acidic pH as a result of glycolysis when oxaloacetate is decarboxylated during an exergonic reaction consuming one cytoplasmic proton which increases the intracellular pH (Hugenholtz *et al.*, 1993; Sánchez *et al.*, 2008). Citrate positive LAB are therefore more likely to co-metabolise glucose and citrate in acidic pH environmental conditions and survive longer than cit<sup>-</sup> LAB.

Lactic acid bacteria also attempt to limit lactate production under acidic conditions. In a study by Montville *et al.* (1987) they observed that the expression of *ldh*, encoding lactate dehydrogenase, in *Lb. plantarum* decreased at low pH values such as 4.5. This resulted in less lactate being produced from pyruvate. Augagneur *et al.* (2007) found similar results in that they observed that *O. oeni* does not produce D-lactate from citrate when the pH is below 4.5, although they did not focus on the gene expression of *ldh*. These studies and several other studies also observed that LAB such as *Lc. lactis* (Zuljan *et al.*, 2014), *Lb. plantarum* (Montville *et al.*, 1987), *O. oeni* (Ramos *et al.*, 1995; Augagneur *et al.*, 2007) and *E. faecalis* (Repizo *et al.*, 2011) produced more acetoin under acidic conditions than lactate from pyruvate. This shift towards acetoin production helps to maintain the intracellular pH under acidic conditions, since acetoin is a neutral compound unlike the acidic lactate.

### 2.4.3 Amino acid synthesis

Several studies have indicated that cit<sup>+</sup> LAB are able to synthesise certain amino acids from precursors produced during citrate metabolism (Marty-Teyssset *et al.*, 1996; Goupil-Feuillerat *et al.*, 1997; Magni *et al.*, 1999). These amino acids include leucine, valine, aspartate and asparagine (Marty-Teyssset *et al.*, 1996; Goupil-Feuillerat *et al.*, 1997; Pudlik and Lolkema, 2012).

Leucine and valine are synthesised from  $\alpha$ -keto-isovalerate produced from acetolactate, an intermediate in citrate metabolism (Goupil-Feuillerat *et al.*, 1997). Aspartate is produced via a transamination reaction from oxaloacetate as a means to get rid of excess oxaloacetate that might have built up in the cell (Ramos *et al.*, 1995; Marty-Teyssset *et al.*, 1996; Pudlik and Lolkema, 2012). Other amino acids such as asparagine, methionine and threonine are then synthesised from aspartate. The ability of cit<sup>+</sup> LAB to use certain metabolites produced during citrate metabolism to

synthesise certain amino acids allow these bacteria to grow better in environments where these amino acids are limited.

#### 2.4.4 Microbial advantage

Citrate positive LAB are also able to outgrow other micro-organisms by producing metabolites with anti-microbial properties during citrate metabolism. Lactate and diacetyl are two such metabolites. Lactate inhibits the growth of other micro-organisms by lowering the intracellular pH of those micro-organisms. The undissociated form of this organic acid is able to passively diffuse across the cell membrane (Laëtitia *et al.*, 2014). Once across the cell membrane, lactate dissociates to form a salt and a proton, which is responsible for lowering the intracellular pH. At high concentrations, lactate is also able to inhibit other micro-organisms by lowering the water activity in the growth environment (Laëtitia *et al.*, 2014). The hydroxyl group of lactate binds to the available water in the matrix and therefore lower the water activity in the media. Thus less water is available for the growth of other micro-organism. Studies have indicated that lactate effectively inhibits the growth of *Clostridium*, *Listeria*, *Staphylococcus* and *Salmonella* (De Wit & Rombouts, 1990; Stekelenburg, 2003; Hayman *et al.*, 2008).

Jay (1982) determined the anti-microbial properties of diacetyl by testing the efficiency of diacetyl concentrations within a range of 100 mg/L to 400 mg/L against various bacteria. In this study they observed that 200 mg/L diacetyl was sufficient to inhibit most bacteria. The inhibitory properties of diacetyl was tested against various strains of the following genera: *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Bacillus*, *Corynebacterium*, *Brevibacterium*, *Micrococcus*, *Sathylococcus*, *Acinetobacter*, *Citrobacter*, *Escherichia*, *Moraxella*, *Serratia*, *Salmonella*, *Pseudomonas*, *Yersinia*, *Candida lipolytica*, *Debaryomyces cantarellii*, *Rhodoturula rubra* and *Torulopsis candida*. Of the bacteria tested, the Gram-negative *Pseudomonas* and the Gram-positive *Bacillus*, *Micrococcus* and *Corynebacterium* were the most sensitive to diacetyl. Jay (1982) also observed that a diacetyl concentration of 300 mg/L was effective against all four yeast species tested, but that *Rhodoturula rubra* was the most sensitive. Only 200 mg/L diacetyl was required to successfully inhibit this yeast species. Another study performed by Olasupo *et al.* (2003) observed that a diacetyl concentration of 1076.12 mg/L can also effectively inhibit the growth of *Escherichia coli* and *Salmonella enterica*.

Diacetyl inhibits the growth of micro-organisms by reacting with the arginine-binding protein causing arginine to be unusable to these organisms. The inhibitory effect of diacetyl is most effective within a pH range of 5 to 7 and can be either stimulated or inhibited by certain compounds. Antagonistic compounds that can decrease the activity of diacetyl include glucose, acetate and Tween 80 (Jay, 1982; Olasupo *et al.*, 2003), while nisin can act as a stimulator enhancing the activity of diacetyl (Lee and Jin, 2008). When these antagonistic compounds are present, higher diacetyl concentrations are required to successfully inhibit various micro-

organisms. Diacetyl produced by LAB during MLF will not be sufficient to inhibit any micro-organisms, since a concentration of 0.05 mg/L to 4.10 mg/L would be too low. Furthermore, the inhibitory effect will be inhibited by glucose and acetate present in either the grape must or wine as well as by the low wine pH of 2.9 to 3.8. Thus there is no relevance of the anti-microbial properties of diacetyl in the wine industry.

## 2.5 Sensory perception/impact of flavour compounds produced during citrate metabolism

Acetate, D-lactate, diacetyl, acetoin and 2,3-butanediol are metabolites produced via citrate metabolism that contribute to the aroma profile of several fermented products. Of these metabolites diacetyl has the most important influence on the aroma profile, since the other metabolites are usually never above their specific odour/ sensory threshold values.

When present at low concentrations, acetate, D-lactate and diacetyl contribute to the complexity of wines, whereas high concentrations could potentially be regarded as off-flavours. Acetate which can contribute to volatile acidity has an odour threshold of 200 mg/L and can result in a vinegar off-flavour in wine when present above 600 mg/L, although its concentrations in wine is usually around 400 mg/L (Guth, 1997; Ferreira *et al.*, 2000). High D-lactate concentrations usually above 200 mg/L are used as an indicator of wine spoilage micro-organisms (Ribereau-Gayon *et al.*, 2006). Diacetyl concentrations of 0.2 mg/L is often found in white wines, whereas in red wines its concentrations are usually higher and ranges from 0.9 to 2.8 mg/L (Bartowsky and Henschke, 2004). Concentrations of 5 mg/L or higher are considered as an off-flavour, since the buttery aroma of diacetyl mask the fruity and/or vegetative aromas in wine (Martineau and Henick-Kling, 1995a; Malherbe *et al.*, 2012). The reduction of diacetyl to acetoin and 2,3-butanediol is often encouraged during fermentation due to their higher odour threshold values of 150 mg/L and 600 mg/L, respectively. Although, these compounds are rarely present in concentrations exceeding their odour threshold, they can contribute to a buttery, creamy aroma in wine. Acetoin concentrations in wine varies from 3 mg/L to 31.8 mg/L (Romano and Suzzi, 1996; Du Toit and Pretorius, 2000; Malherbe *et al.*, 2012), while 2,3-butanediol is usually present at a range of 0.08 mg/L to 3.2 mg/L (Romano *et al.*, 1998; Ehsani *et al.*, 2009; Tao and Li, 2009).

Beer is more likely to be spoiled by metabolites associated with citrate metabolism, since the odour thresholds of these compounds are much lower than those present in dairy products and wine (**Table 2.2**). Acetate concentrations in beer ranges from 30 mg/L to 200 mg/L and produces a vinegar aroma when above 130 mg/L (Barnes, 2011). When present at concentrations above 0.04 mg/L, D-lactate could influence the taste of beer by increasing its sourness (Barnes, 2011). Diacetyl concentrations in beer vary between different beer types as was seen for different wines. Some larger beers can have up to 2 mg/L diacetyl, whereas top-fermented beers have concentrations as low as 0.05 mg/L (NPCS Board of Consultants and Engineers, 2011). The odour threshold of diacetyl is 0.04 mg/L to 0.08 mg/L in larger beer and 0.08 mg/L to 0.4 mg/L in most

other beer types (Elena *et al.*, 2006). Diacetyl concentrations differ mainly due to the different wort and yeast that are used during brewing. The use of coloured malt wort instead of pale malt wort (Portno, 1966) and a wort with a free amino nitrogen lower than 120 mg/L or higher than 210 mg/L (Krogerus and Gibsn, 2013; Pires *et al.*, 2015) could result in higher diacetyl concentrations. The high diacetyl concentrations in beer can also be prevented by choosing a yeast strain that are able to reduce diacetyl to the less odour active acetoin that has an odour threshold of 10 mg/L (Portno, 1966; Barnes, 2011). Furthermore, diacetyl content in beer can be reduced by balancing the wort with the right amount of amino acids and fermentable sugars (Portno, 1966; Barnes, 2011).

**Table 2.2** Diacetyl concentrations present in various foods and beverages.

Food/Beverage	Diacetyl concentration (mg/L)	Reference
<b>Wine</b>	0.05-4.10	Martineau and Henick-Kling, 1995b; Ramos <i>et al.</i> , 1995; Bartowsky and Henschke, 2004
<b>Beer</b>	0.03-1.00	Krogerus and Gibson, 2013
<b>Cheese</b>	1.00-6.00	Ratray <i>et al.</i> , 2003; Weimer, 2007
<b>Buttermilk</b>	2.00-4.00	Ratray <i>et al.</i> , 2003; Schrader, 2007
<b>Yoghurt</b>	0.09-12.87	Tamine and Robinson, 1999; Baranowska, 2006
<b>Fermented raw milk</b>	48.0-133.0	Macciola <i>et al.</i> 2008

As can be seen in **Table 2.2**, dairy products require higher diacetyl concentrations to provide them with their characteristic buttery aroma. Several research studies have therefore been performed to genetically modify *Lc. lactis* to enhance diacetyl production in dairy products. Recently, Guo *et al.* (2012) genetically modified *Lc. lactis* by inserting a constitutive promoter upstream of the *noxE* gene. This gene, encoding NADH oxidase, converts oxygen to water and regenerates NAD<sup>+</sup>. When this gene is overexpressed it results in a shortage of NADH which is necessary for lactate production. The modified strains therefore produced less lactate and more

diacetyl. Furthermore, De Felipe *et al.* (1998) observed increased diacetyl and acetoin production by NADH oxidase-overproducing *Lc. lactis* strains that was constructed by cloning the *Streptococcus mutans nox-2* gene on a plasmid under the control of the *nisA* promoter. However, in both of these studies they observed increased acetoin concentrations suggesting that diacetyl were reduced to acetoin by  $\alpha$ -acetolactate decarboxylase. Thus, to generate a strain that only produce high diacetyl concentrations the *aldB* gene, encoding  $\alpha$ -acetolactate decarboxylase in *Lc. lactis*, has to be deleted in addition to the overexpressed *nox* genes.

Several studies have been performed where *aldB* as well as other genes involved in the production of metabolites other than diacetyl were deleted. Swindell *et al.* (1996) deleted the *aldB* gene of *Lc. lactis* by performing a double crossover homologous recombination. This *aldB* deletion increased the diacetyl concentration, since diacetyl could no longer be reduced to acetoin. Furthermore, Swindell *et al.* (1996) also observed that when *als*, encoding  $\alpha$ -acetolactate synthase, was overexpressed in *Lc. lactis* it resulted in increased concentrations of  $\alpha$ -acetolactate. The accumulation of  $\alpha$ -acetolactate led to the formation of high diacetyl concentrations. These modified *Lc. lactis* strains, where the *aldB* gene was deleted and the *als* gene overexpressed, were also able to produce tremendous amounts of diacetyl without utilising citrate (Swindell *et al.*, 1996). The dairy industry would prefer these modified strains that produce high diacetyl concentrations without utilising citrate. The reason for this being that less acetate is produced, which could give rise to a potential off-flavour.

## 2.6 Conclusions

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Citrate positive LAB are able to utilise citrate under anaerobic conditions via citrate metabolism. Several genes are involved in this metabolism, of which some genes are constitutively expressed and others induced. The expression levels of genes such as *ldh*, *ack*, *alsS* and *alsD* determine the amount of flavour compounds produced during this metabolism and are influenced by various factors. However, there is limited research that indicates how the expression levels of these genes are influenced by various factors, especially in a wine matrix. From the information gathered in this review, it seems as though high diacetyl concentrations can be obtained when a strain with a high potential for diacetyl production is used as starter culture, as well as when the fermentation is carried out at high temperatures under aerobic conditions. Since, the production of metabolites associated with citrate utilisation, especially acetate, D-lactate and diacetyl compounds can mask the fruity and/or vegetative aromas of wine and beer their formation is often avoided in these products. However, in dairy products the formation of diacetyl is encouraged to give these fermented products such as yoghurt and buttermilk their characteristic buttery aroma.

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# Chapter 3

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## Research results

Evaluation of citrate metabolism in *Oenococcus oeni*  
and *Lactobacillus plantarum* strains under different  
conditions

## 3 Research results

### Evaluation of citrate metabolism in *Oenococcus oeni* and *Lactobacillus plantarum* strains under different conditions

#### 3.1 Abstract

Citrate positive *Oenococcus oeni* and *Lactobacillus plantarum* are able to degrade citrate. These bacteria have the genes encoding the citrate permease and citrate lyase. Citrate permease transports citrate into the cell and citrate lyase degrades citrate. Citrate metabolism results in the production of compounds such as acetate, D-lactate, diacetyl, acetoin and 2,3-butanediol, which can influence the aroma profile of wine. In this study the effects of glucose, fructose and pH on citrate metabolism in citrate positive and negative *O. oeni* and *Lb. plantarum* strains were investigated. Different concentrations of glucose and fructose (115 g/L, 50 g/L and 2.5 g/L) and different pH levels (3.0, 3.5, 4.0 and 5.0) were used to determine their influence on citrate consumption, citrate lyase expression and on the production of D-lactate, acetate, diacetyl and acetoin. The relative expression of *citE* in *O. oeni* was linked to both the malate and citrate consumption. The highest relative expression was seen in the treatments where malate and citrate consumption by the cit<sup>+</sup> *O. oeni* strain was slightly delayed like in the 115 g/L fructose treatment and in the pH 3.0 treatment. The lowest relative expression was seen in the 115g/L glucose treatment where the malate and citrate were consumed the fastest. A high fructose concentration seemed to have increased the citrate consumption by the cit<sup>+</sup> *Lb. plantarum* strain and the production of diacetyl and acetoin by the cit<sup>+</sup> strains of both species. A low pH ranging from 3.0 to 4.0 increased the production of diacetyl and acetoin by the cit<sup>+</sup> *O. oeni* strain, while a high pH ranging from 4.0 to 5.0 increased the citrate consumption and thus the production of D-lactate, acetate, diacetyl and acetoin by the cit<sup>+</sup> *Lb. plantarum* strains. This study shows that different sugar concentrations, pH levels and the lactic acid bacteria strain used to induce malolactic fermentation can influence citrate metabolism, which may ultimately influence the final diacetyl and acetoin concentration and therefore the wine style.

#### 3.2 Introduction

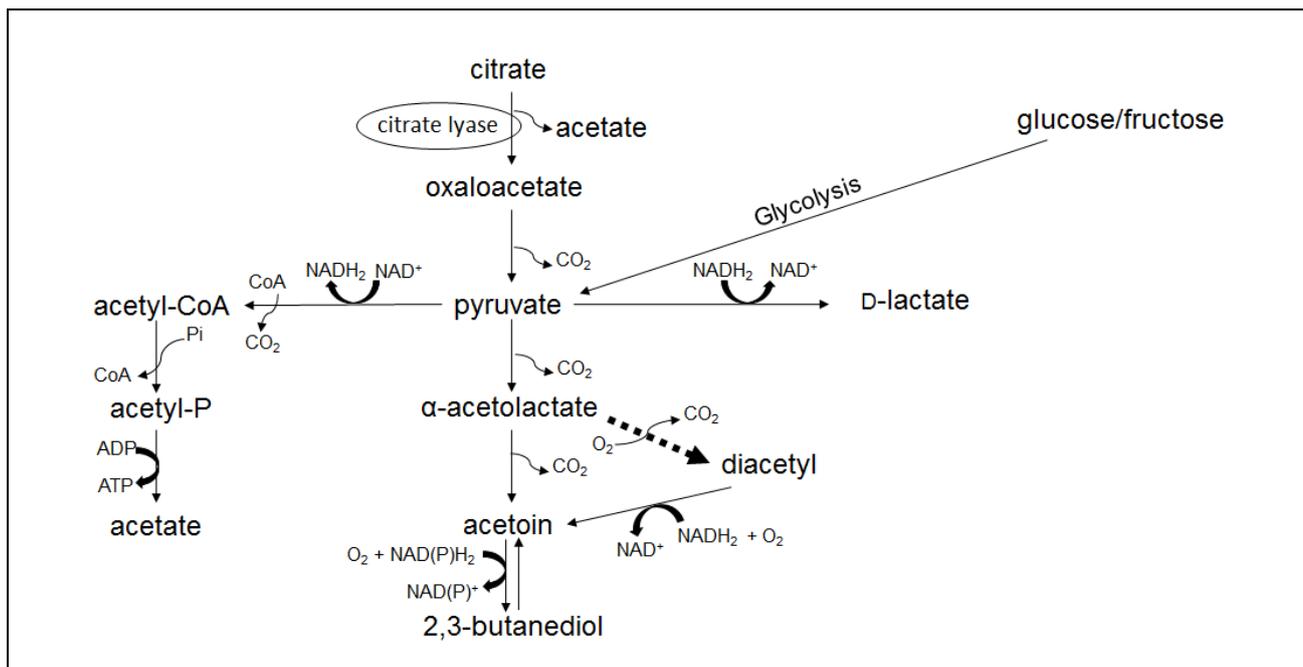
Lactic acid bacteria (LAB) are able to utilise citrate, an organic acid naturally present in grape must, during malolactic fermentation (MLF). Citrate metabolism occurs inside the cell and therefore citrate has to be transported across the cell membrane before this compound can be utilised by LAB (Ramos *et al.*, 1995; Ramos and Santos, 1996; Magni *et al.*, 1999; Bartowsky and Henschke, 2004; Olguín *et al.*, 2009). This transport is facilitated by a citrate or malate permease encoded by *citP* and *maeP*, respectively (Marty-Teyssset *et al.*, 1995; Vaughan *et al.*, 1995; Bandell *et al.*, 1997; Bandell and Lolkema, 2000; Olguín *et al.*, 2009).

Once transported into the cell citrate is degraded to acetate and oxaloacetate (**Figure 3.1**) by citrate lyase, which consists of three subunits. These subunits are  $\alpha$ ,  $\beta$  and  $\gamma$  and are encoded by *citD*, *citE* and *citF*, respectively (Bekal-Si Ali *et al.*, 1999; Martín *et al.*, 1999; Martín *et al.*, 2000; Olguín *et al.*, 2009). The transport and degradation of citrate is only carried out by citrate positive ( $\text{cit}^+$ ) LAB, since citrate negative ( $\text{cit}^-$ ) LAB lack either one or all of the genes encoding the permeases and citrate lyase subunits (Drider *et al.*, 2004). Other studies have also indicated that a few lactobacilli and *O. oeni* strains isolated from wine screened positive for the *citE* gene but negative for the *citD* and *citF* genes (Mtshali *et al.*, 2010; Lerm *et al.*, 2011). Citrate negative LAB are however still able to produce diacetyl, acetoin and 2,3-butanediol from pyruvate formed during glycolysis (**Figure 3.1**; Wagner *et al.*, 2005; Du Toit *et al.*, 2010). Oxaloacetate is converted to pyruvate, which is then converted to D-lactate, acetyl-coA (leading to acetate production) or  $\alpha$ -acetolactate (**Figure 3.1**). Several genes are involved in this conversion of pyruvate to the different metabolites such as *alsS* which encodes the  $\alpha$ -acetolactate synthase that catalyse the conversion of pyruvate to  $\alpha$ -acetolactate. Diacetyl is produced from  $\alpha$ -acetolactate through a decarboxylation reaction, while acetoin is produced either through a decarboxylation reaction from  $\alpha$ -acetolactate or from the reduction of diacetyl (**Figure 3.1**). The production of acetoin from  $\alpha$ -acetolactate is catalysed by the  $\alpha$ -acetolactate decarboxylase encoded by the *alsD* gene, while the reduction of diacetyl to acetoin is catalysed by diacetyl reductase encoded by *butA*. Acetoin can then also be reduced to 2,3-butanediol (**Figure 3.1**).

Diacetyl, acetoin and 2,3-butanediol produced via citrate metabolism are  $\text{C}_4$  flavour compounds that influences wine aroma. These flavour compounds are associated with buttery, creamy, nutty and toasty aromas when above their sensory threshold levels and may be regarded as off-flavours when produced above certain concentrations in wine (Bartowsky and Henschke, 1995; Martineau and Henick-Kling, 1995; Ramos *et al.*, 1995). Diacetyl has a sensory threshold of 0.2 mg/L to 2.8 mg/L depending on the wine type and are usually regarded as an off-flavour when above 5 mg/L (Martineau and Henick-Kling, 1995). The sensory thresholds for acetoin and 2,3-butanediol are 150 mg/L and 600 mg/L, respectively. The conversion of diacetyl to acetoin and 2,3-butanediol is usually encouraged by winemakers to create a less buttery wine style since diacetyl has a much lower sensory threshold than acetoin and 2,3-butanediol.

The conversion of diacetyl to acetoin and 2,3-butanediol is influenced by several wine-related factors such as citrate and sugar concentration, temperature, oxygen, sulphur dioxide content, pH, the MLF inoculation strategy and the strain used to induce MLF. Several studies have investigated a few of these factors previously. Olguín *et al.* (2009) investigated the effect of ethanol combined with a pH of 3.5 or 4.0, Nielsen and Richelieu (1999) determined the effect of oxygen, Martineau and Henick-Kling (1995) investigated the production of diacetyl and acetoin by different LAB strains and lastly a few studies determined the effect of the different inoculation scenarios on wine aroma profile in terms of being either more or less fruitier (Malherbe *et al.*, 2012; Antalick *et*

*al.*, 2013; Versari *et al.*, 2015). These studies, however, did not indicate the correlation between citrate consumption, gene expression and the change in diacetyl and acetoin concentration. A study is thus needed to indicate what the correlation is between the gene expression levels and the change in diacetyl concentrations under different winemaking conditions. This study therefore aims to improve the current knowledge of diacetyl and acetoin formation during fermentation by focusing on wine-related factors (sugar, pH and LAB strain) that may play a role in the formation of these compounds. This is also the first study to evaluate the citrate metabolism of *Lb. plantarum* strains in a wine matrix.



**Figure 3.1** Overview of citrate metabolism in lactic acid bacteria. Modified from Ramos *et al.* (1995) and Olguín *et al.* (2009).

### 3.3 Materials and methods

#### 3.3.1 Bacterial strains, medium and growth conditions

The four LAB strains used in this study were *O. oeni* IWBT B065, Viniflora® CiNe™, *Lb. plantarum* IWBT B205 and *Lb. plantarum* IWBT B382 (**Table 3.1**). *O. oeni* IWBT B065 and *Lb. plantarum* IWBT B382 were citrate positive, while Viniflora® CiNe™ and *Lb. plantarum* IWBT B205 were citrate negative. The *Lb. plantarum* strains were grown in MRS broth (Biolab diagnostics, Wadenville, South Africa) and plated out on MRS agar (50 g/L MRS broth with 15 g/L bacteriological agar (Biolab diagnostics)). The *Lb. plantarum* strains were anaerobically grown in broth for 2 days and on agar plates for 4 days. The *O. oeni* strains were anaerobically grown in MRSA broth (MRS broth with 20% preservative free apple juice (Ceres fruit juices (Pty) Limited, Paarl, South Africa), pH 5.2) and plated out on MRST agar (MRS broth with 20 g/L bacteriological agar and 10% preservative free tomato juice (Tiger Food Brands Limited, Sandton, South Africa), pH 5.0). The *O. oeni* strains were grown in the broth for 4 days and on agar plates for 7 days. All

cultures grown on agar plates were anaerobically incubated at 30°C using anaerobic containers with Anaerocult A (Merck, Darmstadt, Germany).

**Table 3.1** Source and characteristics of the four LAB strains.

Treatment	Bacteria	Strain	Characteristic	Source
1	<i>O. oeni</i>	IWBT B065	Citrate positive	IWBT collection <sup>a</sup>
2	<i>O. oeni</i>	Viniflora <sup>®</sup> CiNe <sup>™</sup>	Citrate negative	Chr Hansen, Denmark
3	<i>Lb. plantarum</i>	IWBT B205	Citrate negative	IWBT collection
4	<i>Lb. plantarum</i>	IWBT B382	Citrate positive	IWBT collection

<sup>a</sup>Institute for Wine biotechnology culture collection, Stellenbosch University, South-Africa

### 3.3.2 DNA extraction and detection of genes involved in citrate metabolism

Genomic DNA from the four LAB strains was isolated following a method previously described by Lewington *et al.* (1987). The integrity and quality of the DNA was assessed spectrophotometrically using a Nanodrop<sup>®</sup> ND-1000 (NanoDrop Technologies, Inc., Wilmington, USA) at 280 nm. To confirm the identity of the LAB strains, a polymerase chain reaction (PCR) was performed with species specific primers (**Table 3.2**). Each PCR reaction mixture (25 µL) contained 200 µM dNTP's, 0.5 µM of each primer, 1.5 mM magnesium chloride, 1.25 U of Taq DNA polymerase (Promega), Taq buffer and 100 ng DNA template. The thermal cycling conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 56°C for 10 s and 72°C for 30 s; and lastly 72°C for 5 min.

The four LAB strains were screened for *citP*, *maeP*, *citD*, *citE*, *citF*, *alsS* and *alsD*. The primers used for the different genes are shown in **Table 3.3**. Each PCR reaction mixture (25 µL) contained 250 µM dNTP's, 0.4 µM of each primer, 0.75 mM magnesium chloride, 2.0 U of Taq DNA polymerase (Promega), Taq buffer and 100 ng of the DNA template. The thermal cycling conditions were as follows: 94°C for 5 min; 35 cycles of 94°C for 1 min, 49°C for 45 s and 72°C for 1 min; and lastly 72°C for 10 min. All PCR products were separated on a 2% agarose gel stained with Gelred Nucleic Acid Gel stain<sup>®</sup> (Biotium, Hayward, CA, USA.).

### 3.3.3 DNA sequencing

To determine the difference between the genes encoding the citrate transporter in *O. oeni* and *Lb. plantarum* the PCR products amplified with the *citP* and *maeP* primers were selected for sequencing. A PCR was performed as described previously. The PCR products were then purified with the Zymoclean<sup>™</sup> Gel DNA Recovery Kit (Zymo Research, USA) following the manufacturer's instructions. Purified products were then evaluated by running 2 µL on a 1% agarose gel stained with Gelred Nucleic Acid stain for 1 hour at 80V. The purified products were then sequenced by the Central DNA Sequencing Facility (Stellenbosch, South Africa).

### 3.3.4 Comparative sequence analysis and phylogenetic tree

Once the sequence data were obtained, a BLASTN algorithm of the National Centre for Biotechnology Information (NCBI; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) was performed to confirm the genes. The genes were then aligned and a phylogenetic tree was constructed as previously described by Mtshali (2011) with a few modifications: MEGA v. 6 software (Tamura *et al.*, 2013) was used instead of v4.1 and p-distance correction model was used to construct the tree instead of the Kimura's two-parameter distance correction model.

### 3.3.5 Preparation of bacterial strains for microvinifications

The bacterial strains were first streaked out on agar plates from wet stock freeze cultures maintained at -80°C. After growth on the agar plates, single colonies were inoculated into broth. The cultures were then transferred to an adaptation medium (Miller, 2010) at a concentration of 1.5%. The *Lb. plantarum* strains were incubated at 30°C for 2 days in this medium, while the *O. oeni* strains were incubated for 3 days.

To obtain Viniflora® CiNe™ as a pure culture, this strain was first rehydrated following the protocol of the manufacturer. The rehydrated culture was then streaked out on agar. After the incubation period, a single colony of the culture was transferred to broth. A wet stock freeze culture was then prepared from the culture grown in broth by adding 1 ml 80% glycerol to 1 ml culture. The wet stock freeze culture was then stored at -80°C in order to pre-culture all the bacterial strains in the same manner.

### 3.3.6 Microvinifications

Synthetic wine medium (cFT80) adapted from Olguín *et al.* (2009) was used for the fermentations. The composition of this medium is indicated in **Table 3.4**. Some changes were made to the glucose, fructose and pH levels to evaluate the effect of these factors on citrate metabolism. The effect of glucose was determined by using 115 g/L, 50 g/L and 2.5 g/L glucose as the different treatments. No fructose and ethanol were added as to eliminate the effect of these components. For the fructose treatments, 115 g/L, 50 g/L and 2.5 g/L fructose were used and no glucose and ethanol were added. Lastly, to determine the effect of pH on citrate metabolism, the cFT80 medium contained 3.5 g/L fructose, 5 g/L glucose and 14% ethanol. The pH levels used as different treatments were 3.0, 3.5, 4.0 and 5.0.

Malolactic fermentation was induced in the different treatments by inoculating the four pre-cultured bacterial strains (**Table 3.1**) to a final concentration of  $10^7$  cfu/ml. All treatments were performed in triplicate and the fermentations were carried out at 20°C. An uninoculated control treatment was also included to observe if any changes occurred in the medium and to monitor the sterility of each fermentation set.

**Table 3.2** Species specific primers to identify the species used in this study.

Primer name	Organism	Gene	Primer sequence (5'3')	Amplicon size (bp)	Reference
planF (fwd)	<i>Lb. plantarum</i>	<i>recA</i>	CCGTTTATGCGGAACACCTAA	318	Torriani <i>et al.</i> (2001)
pRev (rev)			TCGGGATTACCAAACATCAC		
on1 (fwd)	<i>O. oeni</i>	<i>mle</i>	TAATGTGGTTCTTGAGGAGAAAAAT	1025	Zapparoli <i>et al.</i> (1998)
on2 (rev)			ATCATCGTCAAACAAGAGGCCTT		

**Table 3.3** Primers used to screen for genes involved in citrate metabolism in the lactic acid bacteria strains.

Primer name	Organism	Gene	Enzyme	Primer sequence (5'3')	Amplicon size (bp)	Reference
citP-1 (fwd)	<i>Lb. plantarum</i>	<i>citP</i>	citrate transporter	GGGATTGTCCCAGGACTTATTT	646	This study
citP-2 (rev)				CATACCCAGAACCGAAGAGAAC		
maeP-1 (fwd)	<i>O. oeni</i>	<i>maeP</i>	malate transporter	ATGGGTGTTTTTTGGACATCG	984	Mtshali (2011)
maeP-2 (rev)				TCAAATAAAGTTGATGATACTCATTA		
citD-f (fwd)	<i>O. oeni</i>	<i>citD</i>	citrate lyase $\alpha$ subunit	ATGGAAATTAARAMAACKGCAKTMGC	245	Mtshali <i>et al.</i> (2010)
citD-r (rev)	<i>Lb. plantarum</i>			GCYGCGYGTAAATRGTYGKYGCYTTWAT		
clase1 (fwd)	<i>O. oeni</i>	<i>citE</i>	citrate lyase $\beta$ subunit	TTACGBCGSACRATGATGTTTGT	897	Mtshali <i>et al.</i> (2010)
clase2 (rev)	<i>Lb. plantarum</i>			TATTTTTCAATGTAATDCCCTCC		
citF-a (fwd)	<i>O. oeni</i>	<i>citF</i>	citrate lyase $\gamma$ subunit	ATGGYATGACRATTTTCWTTYCAYCAYCA	1331	Mtshali <i>et al.</i> (2010)
citF-b (rev)	<i>Lb. plantarum</i>			ATCAATVAHBSWRCRTRCRGRTAYTC		
alsS-deg1 (fwd)	<i>O. oeni</i>	<i>alsS/aldB</i>	$\alpha$ -acetolactate synthase	GGTTAYGAYSCSRTYGAATATGARCCNCG	620	Mtshali (2011)
alsS-deg2 (rev)	<i>Lb. plantarum</i>			ATTTCTCTTGRAAYTTRACCATRTRCGTA		
alsD-Oe1 (fwd)	<i>O. oeni</i>	<i>alsD</i>	$\alpha$ -acetolactate decarboxylase	ATGAAAGATTTAACAAAAGCTTATC	717	Mtshali (2011)
alsD-Oe2 (rev)				TTATTCTGTCTTTTCAATCGCTT		

**Table 3.4** Composition of the synthetic wine medium (cFT80) (adapted from Olguín *et al.* (2009)).

Component	Content (g/L)
Meat extract (Saarchem, Merck)	5.0
Yeast extract (Saarchem, Merck)	4.0
KH <sub>2</sub> PO <sub>4</sub> (Sigma)	0.6
KCl (Saarchem, Merck)	0.45
CaCl <sub>2</sub> ·2H <sub>2</sub> O (Saarchem, Merck)	0.13
MgSO <sub>4</sub> ·7H <sub>2</sub> O (Saarchem, Merck)	0.13
MnSO <sub>4</sub> ·H <sub>2</sub> O (Saarchem, Merck)	0.003
D(-) fructose (Saarchem, Merck)	2.5/ 50.0/ 115.0
D(+) glucose (Saarchem, Merck)	2.5/ 50.0/ 115.0
L(-) malate (Saarchem, Merck)	3.0
Citrate (Sigma)	0.5
Tween 80 (Saarchem, Merck)	1.07
Absolute ethanol (% v/v) (Sigma)	0.0/ 12.0
pH	3.0/ 3.5/ 4.0/ 5.0*

\*pH was adjusted with either NaOH or HCl and then autoclaved.

### 3.3.7 Analysis of fermentations

The concentrations of L-malate (Enzytec™ Fluid L -malate Id-No: E5280, Roche, R-Biopharm), citrate (Roche yellow line citrate Roche Id-No. 10139076035, Roche, R-Biopharm), D-lactate (Enzytec™ Fluid D-lactate Id-No: E5240, Roche, R-Biopharm), glucose (Enzytec™ Fluid D-glucose Id-No: E5140, Roche, R-Biopharm) and fructose (Enzytec™ Fluid D-fructose Id-No: E5120, Roche, R-Biopharm) were measured enzymatically with an automated analyser (Konelab Arena 20XT, Thermo Electron Corporation, Finland). The acetate concentrations were measured enzymatically with the K-ACETRM Megazyme kit (Megazyme, Bray, Ireland) using the microplate assay procedure. It is important to note that only trends can be used from the acetate results and not absolute values due to the volatility of acetate which made it difficult to measure since the standard included in the K-ACETRM kit had a standard deviation of 0.14 g/L.

Malolactic fermentation was monitored by measuring the concentration of L-malate every 12 hours for the first three days and then every day until the end of MLF or until day 21. Malolactic fermentation was considered to be completed when the L-malate concentration was equal to or less than 0.3 g/L. The viability of the inoculated LAB strains was monitored by preparing tenfold serial dilutions of the fermentations every second day until day 4 and then only once a week until day 21. Bacterial colonies were counted and were reported as cfu/mL.

The citrate concentrations were monitored every day until day 5 and thereafter only once a week until completion or until day 21. The concentrations of glucose, fructose and D-lactate were also measured. The sample points for these analyses were day 0, 2, 5 and 21, but with a few exceptions. In the glucose treatments the last sampling point for the *O. oeni* strains was day 5 and in the fructose and pH treatments the last sampling point for the cit<sup>+</sup> *O. oeni* strain was day 8. These different end points were due to the bacterial strains degrading the citrate at different rates. Acetate concentrations were only measured on the last sampling day.

### 3.3.8 Solid-phase micro extraction gas chromatography-mass spectrometry analysis for carbonyl compounds

The samples that were used for the glucose, fructose and D-lactate analyses were also used for the carbonyl compound analysis. The carbonyl compounds, diacetyl and acetoin, were analysed and quantified by using headspace solid-phase micro extraction gas chromatography-mass spectrometry as described by Malherbe (2011). However, the following modifications were made: samples were prepared as described by Malherbe (2011), except that 0.01 µg/L anisole-d8 (Toronto Research Chemicals Inc, Canada) was used as the internal standard. Extraction of the carbonyl compounds from the headspace was performed by using an 85 µm carboxen/polydimethylsiloxane solid-phase micro extraction fiber (Supelco, Bellefonte, PA). Extraction of the carbonyl compounds from the headspace was performed at 40°C for 15 min, instead of at 50°C for 10 min. The fiber was then desorbed in the hot injection port of the gas chromatography-mass spectrometry at 220°C for 5 min. The injector was operated in split mode with the split ratio being 5 to 1. The flow of the helium carrier gas through the gas-chromatography column was 1.0 mL/min and the oven was programmed from 35°C for 5 min, then ramped at 5°C to 110°C and ramped at 10°C/min to 240°C for 2 min. Data analysis was performed in selected reaction monitoring mode. Separation was performed on the same column as described by Malherbe (2011), but a Thermo Trace 1300 gas chromatography coupled to a TSQ 8000 mass spectrometer (Thermo Fischer Scientific Oy, Finland) was used. Data were analysed with XCalibur software version 2.2. Calibration curves were prepared and established for each carbonyl compound using standard solutions and the levels ranged from 0.1 mg/L to 25 mg/L.

### 3.3.9 RNA extraction and reverse transcription

Samples (20 mL) were collected every 12 hours for the first three days and thereafter every day. The cells were harvested by centrifuging the samples for 10 min at 8500 rpm. All samples were stabilised by using RNeasy Protect<sup>®</sup> bacterial reagent (Qiagen) following the instructions of the manufacturer and stored at -80°C until RNA extraction. Total RNA was extracted from synthetic wine as previously described by Miller (2010). RNA was treated with 10 U of DNase (Roche) as described by the manufacturer.

RNA concentrations were determined spectrophotometrically using a NanoDrop ND-1000 at 260 nm. RNA integrity and the absence of chromosomal DNA were determined by ethidium bromide stained agarose gel electrophoresis (1.5% agarose gel, 1 hr at 100V). DNase treated RNA was diluted to 100 ng/μL and used for cDNA synthesis. The cDNA was synthesised using the ImProm-II™ Reverse Transcription System (Promega, Madison, USA) with the included random primers.

### 3.3.10 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was carried out on an Applied Biosystems 7500 Real-Time PCR System with 7500 Software version 2.3 (Life Technologies, United States). The Kapa SYBR® FAST qPCR Master Mix Universal kit (Kapa Biosystems, MA) was used for gene detection. The qPCR reaction was set up as proposed by the manufacturer. The internal control gene was *ldhD* in *O. oeni* and 16S rRNA in *Lb. plantarum* as these genes showed a stable expression in the different treatments used in this study. These genes were also used as internal control genes in previous studies with similar experimental conditions (Olguín *et al.*, 2009; Miller 2010; Nielsen *et al.*, 2010; Mink *et al.*, 2014). The target gene (*citE*) and internal control genes were amplified with the primers listed in **Table 3.5**. A negative control was included in each run. Thermal cycling conditions for amplification were as follows: initial denaturation at 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 58°C for 30 s and 72°C for 32 s. Fluorescence data were collected during each elongation step. The specificity of the qPCR for each primer pair was verified by a melting curve, which was established by an additional step starting from 95°C for 15 s to 58°C for 1 min. The qPCR efficiency (*E*) was calculated for each condition by the formula  $E = [10^{(1/s)} - 1] * 100$ , where *s* is the slope of a standard curve prepared from serial dilutions of cDNA.

The relative expression (RE) was calculated from the threshold cycle value ( $C_t$ ) data using the comparative critical threshold ( $\Delta\Delta C_t$ ) method (Livak and Schmittgen, 2001). The following equations were used to determine the relative expression:

$$\Delta C_T = C_T \text{ of housekeeping gene} - C_T \text{ of target gene}$$

$$\Delta\Delta C_T = \Delta C_T \text{ of control condition} - \Delta C_T \text{ of treated condition}$$

$$RE = 2^{-\Delta\Delta C_T}$$

In the second equation the control condition was day 0 of all the individual treatments, whereas the treated condition was any other day of that specific treatment. The average  $C_T$  of three technical repeats was used in the equations above.

### 3.3.11 Statistical analysis

All data obtained were analysed by performing a one-way analysis of variance (ANOVA) followed by a Fisher LSD test using XLSTAT (version 2016.05.33324, Addinsoft). Differences between treatments were regarded as significant when the p-values were below 0.05.

**Table 3.5** Primer sequences used for quantitative real-time PCR during this study for *O. oeni* and *Lb. plantarum*.

Organism	Gene	Enzyme	Primer name	Primer sequence (5'3')	Amplicon size (bp)	Reference
<i>O. oeni</i>	<i>citE</i>	Citrate lyase $\beta$ subunit	citEqF (fwd)	CGGACTTGATACGCCTTTTTTC	63	This work
			citEqR (rev)	ACATCGACGCCGGCTTT		
	<i>ldhD</i>	Lactate dehydrogenase	ldh-1 (fwd)	GCCGCAGTAAAGAACTTGATG	102	Desroche <i>et al.</i> (2005)
			ldh-2 (rev)	TGCCGACAACACCAACTGTTT		
<i>Lb. plantarum</i>	<i>citE</i>	Citrate lyase $\beta$ subunit	RTcitE-F (fwd)	GGTCAACCCTCGACAGATT	127	This work
			RTcitE-R (rev)	GGAAATAACACCAGAACCCTTG		
	16S rRNA	Small subunit of prokaryotic ribosome	16S qRT-f (fwd)	TCATGATTTACATTTGAGTG	121	Nielsen <i>et al.</i> (2010)
			16S qRT-r (rev)	GACCATGCGGTCCAAGTTGTT		

### 3.4 Results and discussion

#### 3.4.1 Screening of LAB strains for genes involved in citrate metabolism

The four LAB strains were screened for the presence of various genes involved in citrate metabolism, namely *citP*, *maeP*, *citD*, *citE*, *citF*, *alsS* and *alsD* (**Table 3.6**). Although, *Lb. plantarum* species are not known to have the *maeP* and *alsD* genes (Illegheems *et al.*, 2015) and *O. oeni* do not have the *citP* gene (Mills *et al.*, 2005), these strains were still screened for these genes using the primers listed in **Table 3.3**. The *citP* primers, designed specifically for *Lb. plantarum*, did amplify a region in *O. oeni*, but when the product was sent for sequencing and comparative sequence analysis was performed, the product aligned with a hypothetical protein and not with a citrate transporter. The *maeP* and *alsD* primers did not amplify any region in the *Lb. plantarum* strains which confirm the results of Illegheems *et al.* (2015).

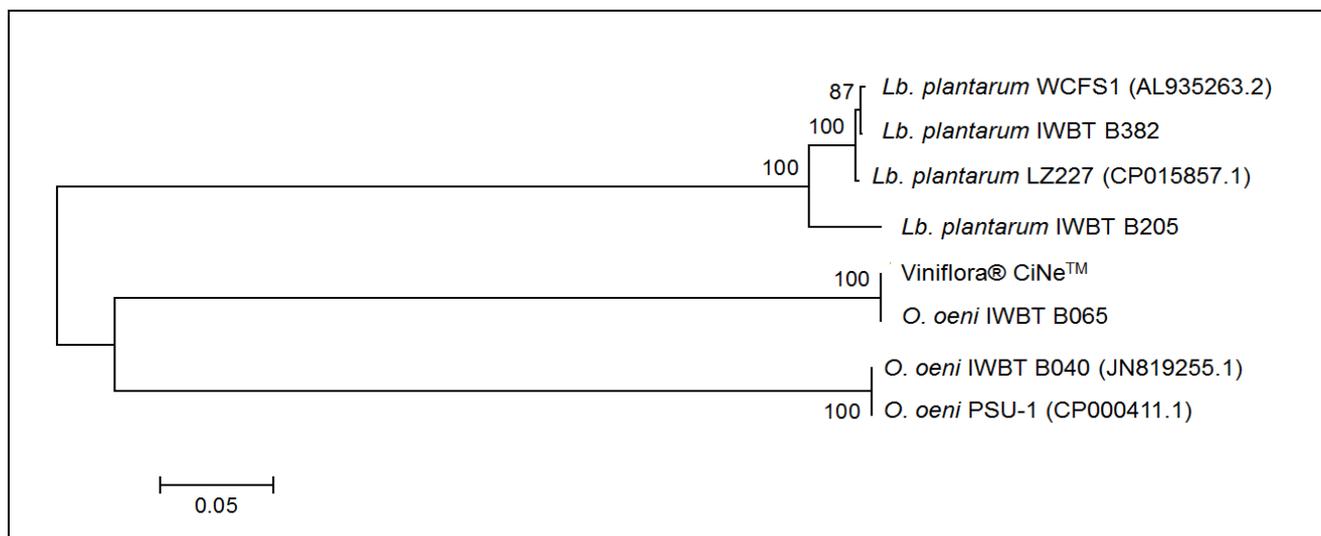
The rest of the primers used amplified a single product that was the same size as indicated in previous studies (**Table 3.6**). The *cit*<sup>+</sup> *O. oeni* strain screened positive for all the genes and the *cit*<sup>-</sup> *O. oeni* strain screened negative for only the *citF* gene. This gene encodes the  $\gamma$  subunit of citrate lyase, which plays an important part in the activation of citrate lyase for citrate degradation (Martín *et al.*, 1999; 2000). Thus this *cit*<sup>-</sup> *O. oeni* strain would not be able to degrade citrate as stated on the specification sheet provided by the company (<http://www.chr-hansen.com>). The *Lb. plantarum* strains screened positive for all the genes and there were no difference between the strains. However, *Lb. plantarum* IWBT B205 was selected as the *cit*<sup>-</sup> strain since this strain screened negative for the *citE* gene and was unable to degrade citrate in a preliminary study performed in synthetic juice (0.3 g/L citrate, 100 g/L glucose, 100 g/L fructose, 3.5 g/L malate, pH 3.1).

**Table 3.6** Results obtained from the genetic screening of the genes involved in citrate metabolism of *O. oeni* and *Lb. plantarum* strains used in this study.

Genes	LAB strain				Amplified product size (bp)
	<i>Lb. plantarum</i> IWBT B382	<i>Lb. plantarum</i> IWBT B205	<i>O. oeni</i> IWBT B065	Viniflora® CiNe™	
<i>citP</i>	+	+	na	na	646
<i>maeP</i>	na	na	+	+	984
<i>citD</i>	+	+	+	+	245
<i>citE</i>	+	+	+	+	897
<i>citF</i>	+	+	+	-	1331
<i>alsS/aldB</i>	+	+	+	+	620
<i>alsD</i>	na	na	+	+	717

na- not applicable

The phylogenetic tree that was constructed through the neighbour-joining tree method based on the nucleotide sequences of the *citP* and *maeP* genes is indicated in **Figure 3.2**. The closest relatives found during the comparative sequence analysis were also included in the tree to compare the gene sequences obtained in this study to the existing sequences on the NCBI database. From the phylogenetic tree, it is clear that there is a difference between the *citP* and *maeP* genes of *Lb. plantarum* and *O. oeni* as these species are clustered separately. The *O. oeni* strains used in this study grouped together but not with the other *O. oeni* strains obtained from the NCBI database. However, these strains still shared a common ancestor. The *Lb. plantarum* strains used in this study grouped together with the *Lb. plantarum* strains (Siezen *et al.*, 2012; Li *et al.*, 2016) obtained from the NCBI database. The *citP* gene sequence of the *cit*<sup>+</sup> *Lb. plantarum* strain had more similarities with the *Lb. plantarum* strains obtained from the NCBI database than the *cit*<sup>-</sup> *Lb. plantarum* strain. This strain clustered separately, but shared a common ancestor with the other *Lb. plantarum* strains. These results might explain the differences related to citrate consumption between the different strains.



**Figure 3.2** Phylogenetic tree indicating the relationship between the citrate transporter of *Lb. plantarum* and *O. oeni* based on the nucleotide sequences of the *citP* and *maeP* genes. The tree was constructed using the neighbour-joining method and the results of the bootstrap analysis (expressed as percentages of 1000 replicates) are represented by the numbers at the branching points. The scale bar represents the number of base substitutions per site.

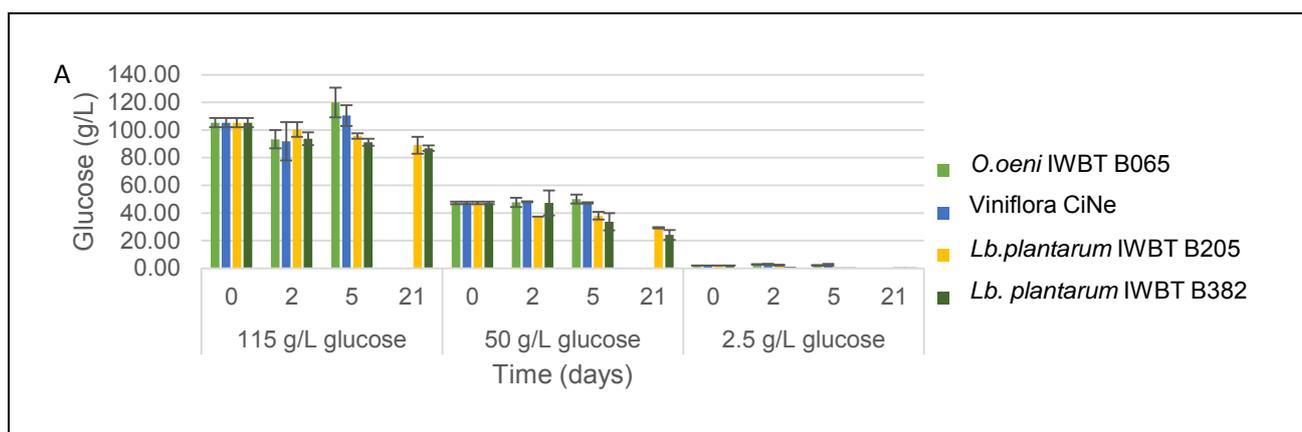
### 3.4.2 Microvinifications in synthetic wine media

#### 3.4.2.1 Effect of glucose on citrate metabolism

##### 3.4.2.1.1 Glucose, malate and citrate consumption

Lactic acid bacteria are able to utilise a number of carbon sources during fermentation. Sugars such as glucose and fructose are utilised either through the Embden-Meyerhof pathway (EMP) or the pentose phosphate pathway. Malate and citrate are other carbon sources that LAB can utilise during fermentation.

The glucose consumption by the four LAB strains during the fermentation is indicated in **Figure 3.3**. The *O. oeni* strains did not consume glucose in any of the treatments. The glucose concentrations of the *O. oeni* strains were only measured until day 5, since the cit<sup>+</sup> *O. oeni* strain completely consumed citrate on day 5 (**Figure 3.4**). The *Lb. plantarum* strains started to consume glucose two days after inoculation until the last sampling point which was day 21. In the 115 g/L treatment, the cit<sup>-</sup> *Lb. plantarum* strain consumed 15% of the glucose and the cit<sup>+</sup> *Lb. plantarum* strain consumed 17% on day 21. In the 50 g/L treatment, the cit<sup>-</sup> *Lb. plantarum* strain consumed 38% of the glucose and the cit<sup>+</sup> *Lb. plantarum* strain consumed 48% on day 21. In the 2.5 g/L treatment, the *Lb. plantarum* strains completely consumed glucose by day 5.



**Figure 3.3** Glucose consumption in the different treatments inoculated with the four LAB strains. Day 5 was the last sampling day for the *O. oeni* strains and day 21 for the *Lb. plantarum* strains. Data shown are averages of triplicates with error bars representing the standard deviation.

The cell counts and malate degradation duration by the four different LAB strains in the glucose treatments are indicated in **Table 3.7**. All the strains were inoculated between  $1 \times 10^7$  cfu/mL to  $8.5 \times 10^7$  cfu/mL. This variation between the initial cell counts of the different strains could potentially have influenced malate and citrate consumption and the concentrations of metabolites produced by the different strains. The initial cell counts of all the strains increased as soon as the strains have been inoculated. The cell counts of the cit<sup>+</sup> *O. oeni* strain in the 115 g/L and 50 g/L treatments and of the *Lb. plantarum* strains in all the treatments rapidly increased from  $10^7$  cfu/mL on day 0 to  $10^8$  cfu/mL on day 2. The cell counts of the cit<sup>+</sup> *O. oeni* strain in the 2.5 g/L treatment and of the cit<sup>-</sup> *O. oeni* strain in all the treatments also increased from day 0 to day 2, but the increase was slightly less than what was seen in the other treatments. The *Lb. plantarum* strains were able to survive longer in the fermentations than the *O. oeni* strains which might be coupled to the *Lb. plantarum* strains that were able to consume more glucose than the *O. oeni* strains in all the treatments.

Malate was completely degraded by all the LAB strains ranging from one to two days. The cit<sup>+</sup> *O. oeni* strain completely consumed malate after one and a half days in the 115 g/L treatment and after two days in the 50 g/L and 2.5 g/L treatments. The cit<sup>-</sup> *O. oeni* strain completely

consumed malate one and a half days after inoculation in the 115 g/L and 2.5 g/L treatments, while this strain took two days to completely consume malate in the 50 g/L treatment. The cit<sup>-</sup> *Lb. plantarum* strain completely consumed malate within one day after inoculation in the 115 g/L and 2.5 g/L treatments, but took two days in the 50 g/L treatment. The cit<sup>+</sup> *Lb. plantarum* strain completely consumed malate one day after inoculation in all the treatments. The *Lb. plantarum* strains consumed malate faster than the *O. oeni* strains in most of the treatments. This might be related to the ability of the *Lb. plantarum* strains to consume more glucose to maintain their cell counts.

Citrate consumption by the four LAB strains in the glucose treatments are indicated in **Figure 3.4**. Citrate was completely consumed by the cit<sup>+</sup> *O. oeni* strain within the first five days after inoculation in the glucose treatments. This is in agreement with the genetic screening results where the cit<sup>+</sup> *O. oeni* strain had all the necessary genes for citrate degradation. The fastest citrate utilisation period for this strain was seen in the 115 g/L treatment which is linked to the malate degradation duration that was also faster in this treatment (**Figure 3.4A**). On day 3, citrate was completely consumed (0.5 g/L) in the 115 g/L treatment, whereas only 0.3 g/L citrate was consumed in the other two treatments (**Figure 3.4A**). In the 50 g/L and 2.5 g/L treatments, citrate was completely consumed on day 5. The commercial cit<sup>-</sup> *O. oeni* strain did not degrade any citrate in the glucose treatments (**Figure 3.4B**), as expected since this strain lack the *citF* gene (**Table 3.6**). This gene plays a crucial part in the activation of citrate lyase for citrate degradation as previously mentioned (Martín et al., 1999; 2000).

The two *Lb. plantarum* strains had the same citrate consumption trend in the glucose treatments. In the 115 g/L and 50 g/L treatments, these strains consumed 36% and 42% of the initial citrate on day 21, respectively (**Figure 3.4C and D**). In the 2.5 g/L treatment, the cit<sup>-</sup> *Lb. plantarum* strain consumed 14% and the cit<sup>+</sup> *Lb. plantarum* strain consumed 24% of the initial citrate on day 21 (**Figure 3.4C and D**). The cell counts of these two strains also decreased more rapidly in the 2.5 g/L glucose treatment as opposed to the 50 g/L and 115 g/L treatments (**Table 3.7**). On the last sampling day (day 21), the cell counts in the 2.5 g/L treatment were 10<sup>5</sup> cfu/mL for the cit<sup>-</sup> *Lb. plantarum* strain and 10<sup>6</sup> cfu/mL for the cit<sup>+</sup> *Lb. plantarum* strain, compared to the 10<sup>8</sup> cfu/mL in the 115 g/L and 50 g/L treatments inoculated with these strains (**Table 3.7**). These bacteria seem to need high sugar concentrations to maintain their cell counts and metabolic activities. In contrast, the *O. oeni* strains were not able to use the high sugar present in the medium to maintain their cell counts as there was a rapid decrease from 10<sup>7</sup> cfu/mL to 10<sup>5</sup> cfu/mL observed in all the treatments inoculated with the cit<sup>+</sup> *O. oeni* strain (**Table 3.7**). The cell counts of the cit<sup>-</sup> *O. oeni* strain also decreased, but to a lesser extent than the cit<sup>+</sup> *O. oeni* strain, since their cell counts only decreased from 10<sup>7</sup> cfu/mL to 10<sup>6</sup> cfu/mL.

The citrate consumption by the *Lb. plantarum* strains was only seen from day 8, which corresponds to the results found by Drinan *et al.* (1976) who observed that lactobacilli only utilise

citrate during the late exponential phase. However, a previous study by Palles *et al.* (1998) indicated that glucose inhibits the synthesis of citrate lyase in *Lb. plantarum* and thus delay citrate consumption by these strains.

#### 3.4.2.1.2 Relative expression of *citE*

The effect of glucose on citrate metabolism was further determined by examining the RE of the *citE* gene throughout the fermentations. The efficiencies of all the primer pairs were close to 100% making them suitable for analysis by the comparative  $\Delta\Delta C_T$  method (Livak and Schmittgen, 2001). The RE levels of *citE* were determined on day 0.5, 2 and 5 in the *cit*<sup>+</sup> *O. oeni* strain, since this bacterial strain was the only strain that completely utilised citrate. The RE levels of *citE* in the rest of the bacterial strains were only determined on day 5, since these bacteria only partially consumed citrate (**Figure 3.4**). Day 0 was used as the control condition, as previously mentioned.

The RE of *citE* throughout the fermentations induced with the *cit*<sup>+</sup> *O. oeni* strain in the glucose treatments is indicated in **Figure 3.5**. In all the glucose treatments the RE first increase and then remained consistent until the end of the fermentation. In a previous study by Desroche *et al.* (2005), they suggested that a relevant transcription response only occurs when the RE level of the target gene is at least twofold lower or higher than the control condition. Thus a relevant transcription response was first seen on day 0.5 in the 115 g/L and 50 g/L treatments, but on day 2 in the 2.5 g/L treatment. The RE rapidly increased from day 0.5 to day 2. The RE on day 2 was 4.5 fold higher than on day 0.5 in the 50 g/L treatment, whereas in the 115 g/L and 2.5 g/L treatments the RE on day 2 was only 1.55 and 1.20 fold higher than on day 0.5, respectively. However, malate and citrate were consumed faster in the 115 g/L glucose treatment and the maximum RE could have been on day 1 which was not measured (**Table 3.7 and Figure 3.4**). Once the citrate was completely consumed or the maximum RE was reached, the RE remained consistent until the end of the fermentation since there were no significant differences between day 2 and 5 in all the treatments.

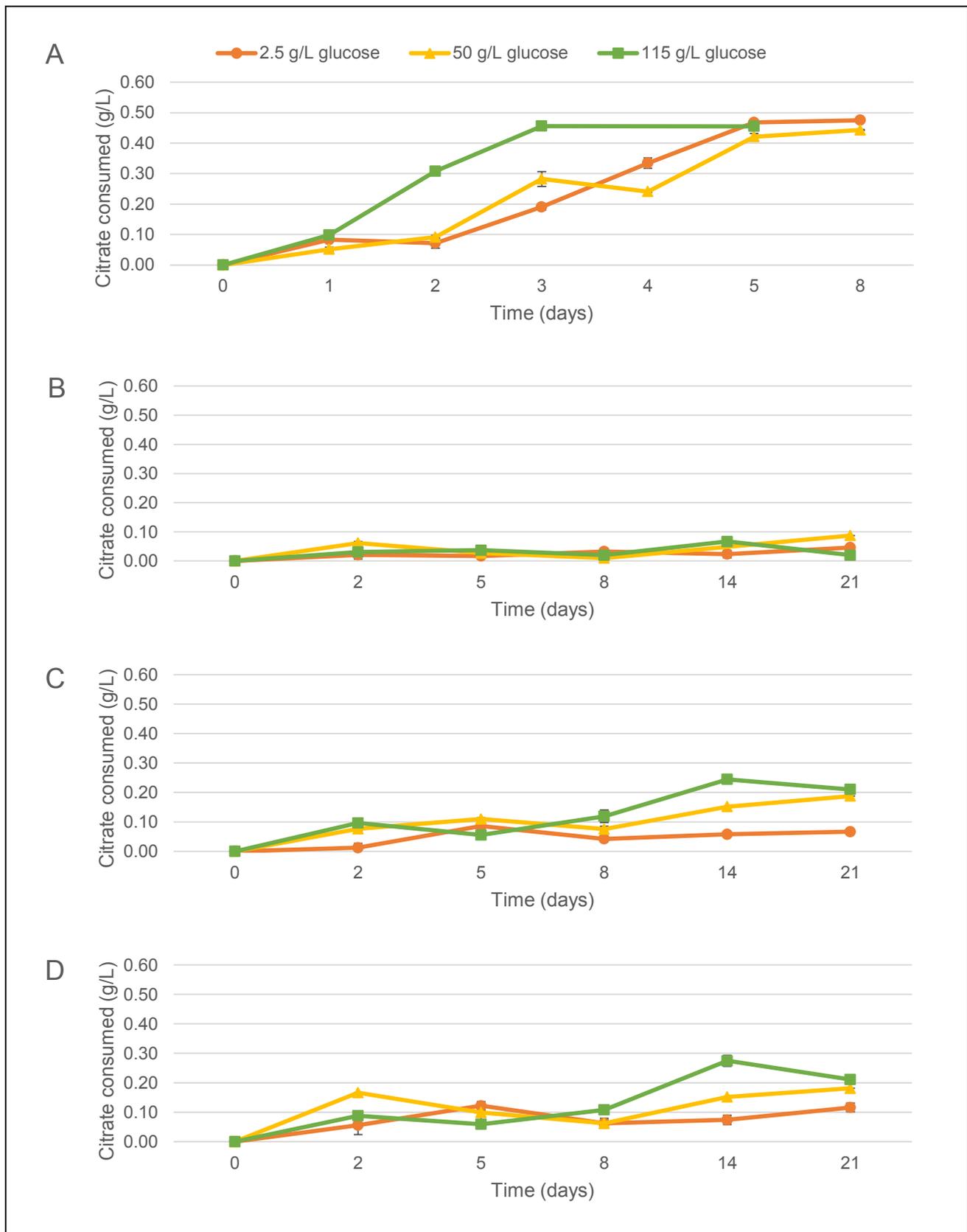
The RE was in most cases more than twofold lower in the 2.5 g/L glucose treatment inoculated with the *cit*<sup>+</sup> *O. oeni* strain than in the other treatments (**Figure 3.5**). This was unexpected, since the cell counts of this strain in all the glucose treatments were similar (**Table 3.7**) and the time period for malate and citrate consumption was the same in the 50 g/L and 2.5 g/L treatments.

A RE was observed for the rest of the bacterial strains, but the RE was not twofold lower or higher than the control condition and is therefore not shown. This correlates to the other strains not being able to completely consume citrate (**Figure 3.4**).

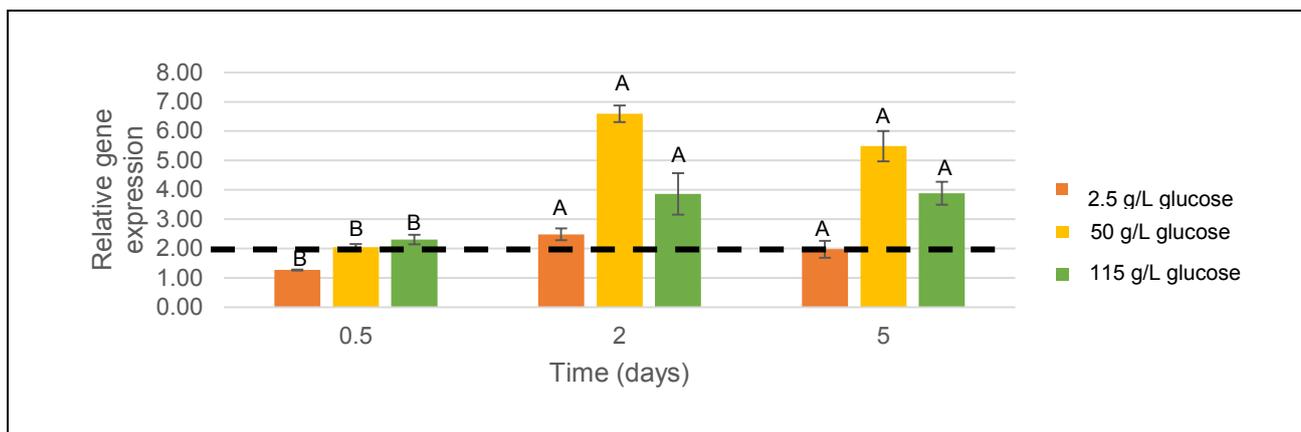
**Table 3.7** Overview of cell counts (cfu/mL) and malate degradation (days) in the glucose treatments inoculated with the different LAB strains.

Strain	Days						Malate degradation duration
	0	2	4	8	14	21	
<b>115 g/L glucose</b>							
<i>O. oeni</i> IWBT B065	8.58E+07 ± 1.40E+07	1.69E+08 ± 1.20E+07	1.82E+08 ± 1.00E+07	<1.00E+05			1.5
Viniflora® CiNe™	2.50E+07 ± 5.10E+06	5.50E+07 ± 4.50E+06	4.95E+07 ± 3.50E+07	1.75E+06 ± 6.36E+05	1.00E+06 ± 2.01E+05	<1.00E+05	1.5
<i>Lb. plantarum</i> IWBT B205	1.88E+07 ± 1.06E+06	5.40E+08 ± 1.40E+08	7.30E+08 ± 5.00E+08	8.05E+08 ± 4.95E+07	8.67E+08 ± 6.11E+07	1.24E+08 ± 1.40E+07	1.0
<i>Lb. plantarum</i> IWBT B382	2.67E+07 ± 6.04E+06	5.87E+07 ± 4.95E+07	9.20E+08 ± 8.70E+08	6.65E+08 ± 4.95E+07	6.20E+08 ± 2.83E+07	3.70E+08 ± 3.61E+07	1.0
<b>50 g/L glucose</b>							
<i>O. oeni</i> IWBT B065	8.05E+07 ± 7.78E+06	1.38E+08 ± 2.09E+07	1.58E+08 ± 2.83E+06	<1.00E+05			2.0
Viniflora® CiNe™	1.70E+07 ± 1.77E+06	9.80E+07 ± 2.65E+06	9.37E+07 ± 1.12E+07	8.20E+07 ± 2.55E+07	1.66E+06 ± 2.11E+05	<1.00E+05	2.0
<i>Lb. plantarum</i> IWBT B205	2.30E+07 ± 1.26E+06	6.20E+08 ± 3.61E+07	8.75E+08 ± 4.95E+07	4.75E+08 ± 1.06E+08	6.30E+08 ± 6.08E+07	5.07E+08 ± 9.07E+07	2.0
<i>Lb. plantarum</i> IWBT B382	2.64E+07 ± 1.91E+06	5.47E+08 ± 8.96E+07	8.40E+08 ± 1.27E+08	4.90E+08 ± 5.66E+07	5.23E+08 ± 1.53E+07	4.23E+08 ± 7.57E+07	1.0
<b>2.5 g/L glucose</b>							
<i>O. oeni</i> IWBT B065	5.55E+07 ± 7.78E+06	8.70E+07 ± 1.84E+07	9.47E+07 ± 1.77E+07	<1.00E+05			2.0
Viniflora® CiNe™	4.45E+07 ± 3.54E+06	6.97E+07 ± 6.81E+06	4.90E+07 ± 7.07E+05	7.10E+06 ± 2.15E+06	6.60E+06 ± 2.26E+06	<1.00E+05	1.5
<i>Lb. plantarum</i> IWBT B205	3.50E+07 ± 4.36E+06	2.55E+08 ± 4.95E+07	5.60E+08 ± 1.41E+07	6.75E+07 ± 1.06E+07	1.30E+07 ± 9.90E+05	3.88E+06 ± 1.70E+05	1.0
<i>Lb. plantarum</i> IWBT B382	2.30E+07 ± 1.73E+06	6.45E+08 ± 2.12E+07	4.05E+08 ± 2.12E+07	3.03E+07 ± 2.52E+06	1.75E+06 ± 2.83E+04	4.68E+05 ± 6.22E+04	1.0

Data shown are averages of triplicates with standard deviation.



**Figure 3.4** Monitoring citrate consumption by A) *O. oeni* IWBT B065, B) *Viniflora*® CiNe™, C) *Lb. plantarum* IWBT B205 and D) *Lb. plantarum* IWBT B382 in the 2.5 g/L (orange), 50 g/L (yellow) and 115 g/L (green) glucose treatments. Data shown are mean values of triplicates and error bars represent the standard deviations.



**Figure 3.5** Relative gene expression of the *citE* gene in *O. oeni* IWBT B065 in the glucose treatments. Data shown are averages of triplicates with error bars representing the standard deviations. Dashed line indicates a twofold expression level. Different alphabetical letters indicate significant differences ( $p < 0.05$ ) within a specific treatment.

### 3.4.2.1.3 Production of metabolites associated with citrate metabolism

In most of the glucose treatments the D-lactate concentrations gradually increased as the fermentations proceeded (**Figure 3.6**). This was expected as D-lactate is one of the metabolites produced during citrate and sugar metabolism. However, when an ANOVA followed by a Fisher LSD test was performed between the different days within a specific treatment it was observed that the D-lactate concentrations did not increase in the 115 g/L and 2.5 g/L treatments inoculated with the *cit<sup>-</sup>* *O. oeni* strain, as well as in the 2.5 g/L treatment inoculated with the *Lb. plantarum* strains (data not shown). The D-lactate concentrations in the 2.5 g/L treatment inoculated with the *Lb. plantarum* strains did not increase from day 5 to day 21, since these strains completely consumed glucose by day 5 (**Figure 3.5**).

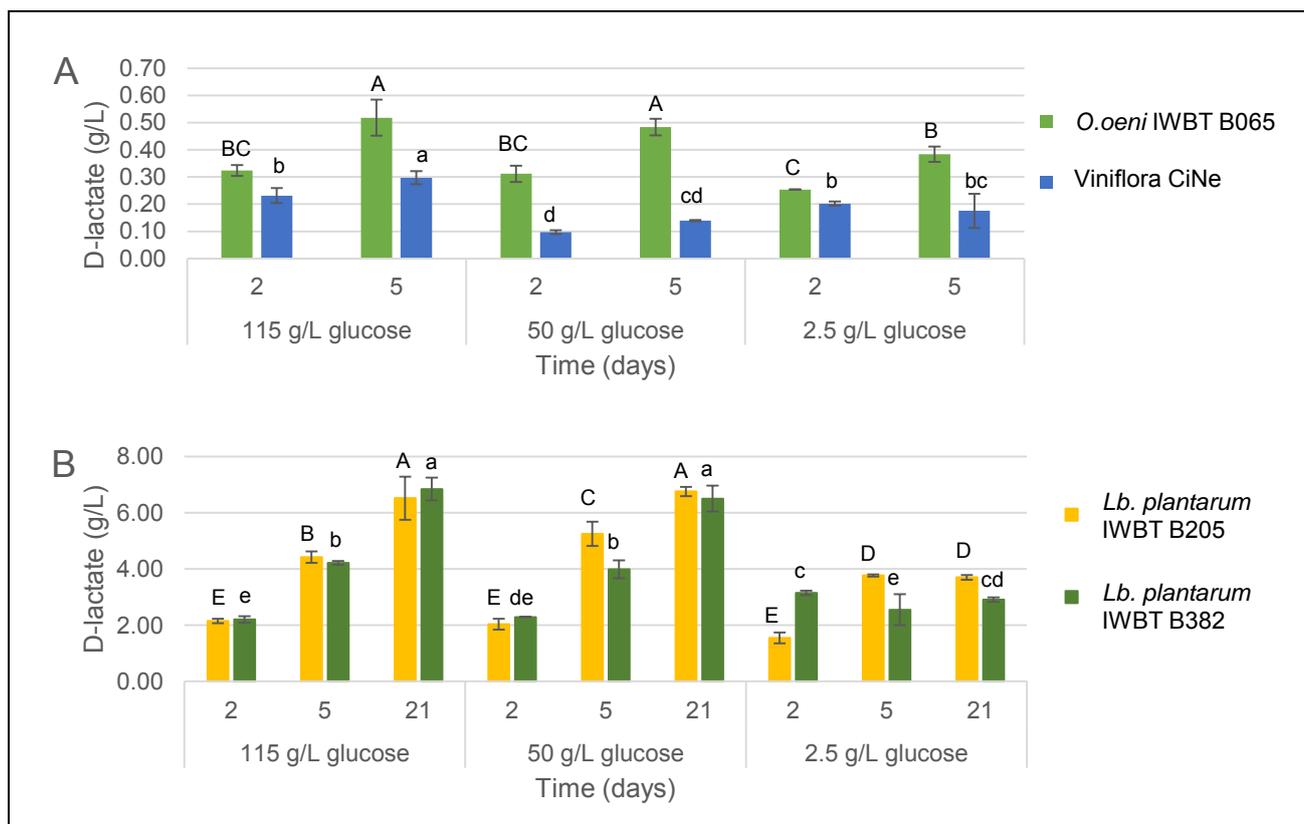
The same trend was observed for the *cit<sup>+</sup>* *O. oeni* strain and the *Lb. plantarum* strains regarding their D-lactate production. In the 115 g/L and 50 g/L treatments inoculated with these strains, there were no differences with regards to the final D-lactate concentrations which was day 5 for the *O. oeni* strains and day 21 for the *Lb. plantarum* strains (**Figure 3.6**). In the 2.5 g/L glucose treatment, the final D-lactate concentrations produced by the *cit<sup>+</sup>* *O. oeni* strain and the *Lb. plantarum* strains were lower than in the 50 g/L and 115 g/L treatments inoculated with these strains (**Figure 3.6**). The *cit<sup>-</sup>* *Lb. plantarum* strain produced 2.8 g/L and 3.0 g/L less D-lactate in the 2.5 g/L treatment than in the 115 g/L and 50 g/L treatments on day 21, respectively. The *cit<sup>+</sup>* *Lb. plantarum* strain produced more than 3.0 g/L less D-lactate in the 2.5 g/L treatment than in the other two treatments on day 21. The *cit<sup>+</sup>* *O. oeni* strain produced roughly 0.1 g/L less D-lactate in the 2.5 g/L treatment than in the other two treatments on day 5. The *cit<sup>-</sup>* *O. oeni* strain produced more D-lactate in the 115 g/L treatment than in the other two treatments inoculated with this strain on day 5. The D-lactate concentration on day 5 in the 115 g/L treatment was 0.30 g/L as opposed to the 0.14 g/L and 0.18 g/L concentrations in the 50 g/L and 2.5 g/L glucose treatments,

respectively. The lower D-lactate concentrations in the 2.5 g/L treatments might be due to the LAB not being able to produce as much D-lactate as in the other treatments since there was less glucose available for production (**Table 3.7**). According to Krieger-Weber (2016) lactate concentrations above 3 g/L can cause a loss in *O. oeni* cell viability. There is no information generated for *Lb. plantarum* on their sensitivity to lactic acid. There was not a loss in the cell counts of *Lb. plantarum* observed in this study, even though the lactate concentrations produced by these strains were above 3 g/L and the results obtained is therefore contradictory and may indicate that the lactic acid level necessary to inhibit *Lb. plantarum* might be higher.

Significant differences were obtained when performing an ANOVA followed by a Fisher LSD test between the various strains within a specific treatment with the data obtained at the last sampling point, which was day 5 for the *O. oeni* strains and day 21 for *Lb. plantarum* strains (data not shown). The cit<sup>+</sup> *O. oeni* strain had significantly higher D-lactate concentrations than the cit<sup>-</sup> *O. oeni* strain on day 5 in the 115 g/L and 50 g/L treatments. This corresponds to the citrate consumption rather than glucose consumption, as both strains did not consume glucose, but the cit<sup>+</sup> *O. oeni* strain consumed more citrate than cit<sup>-</sup> *O. oeni* strain (**Figure 3.3 and 3.4**). There was no significant difference between the two *O. oeni* strains in the 2.5 g/L treatment with regards to the D-lactate concentrations on day 5. In the 115 g/L and 50 g/L treatments inoculated with the *Lb. plantarum* strains, there were no significant differences between the two strains on day 21. In the 2.5 g/L treatment, the cit<sup>-</sup> *Lb. plantarum* strain produced significantly more D-lactate than the cit<sup>+</sup> *Lb. plantarum* strain on day 21. These strains consumed similar amounts of citrate and glucose and therefore it was not surprising that the two strains produced similar amounts of D-lactate in most of the treatments.

The D-lactate concentrations in the treatments induced with the *O. oeni* strains were less than in those induced with the *Lb. plantarum* strains (**Figure 3.6**). In the 115 g/L and 50 g/L treatments, the cit<sup>+</sup> *Lb. plantarum* strain produced more than 12 times more D-lactate than the cit<sup>+</sup> *O. oeni* strain at the last sampling point which was day 5 for the *O. oeni* strains and day 21 for the *Lb. plantarum* strains. The cit<sup>+</sup> *Lb. plantarum* strain produced more than nine times more D-lactate than the cit<sup>+</sup> *O. oeni* strain in the 2.5 g/L treatment at the last sampling point. The cit<sup>-</sup> *Lb. plantarum* strain produced more than 23 times more D-lactate than the cit<sup>-</sup> *O. oeni* strain in the 115 g/L and 50 g/L treatments. The cit<sup>-</sup> *Lb. plantarum* strain produced more than 16 times more D-lactate than the cit<sup>-</sup> *O. oeni* strain in the 2.5 g/L treatment at the last sampling point. These differences might be attributed to glucose consumption rather than citrate consumption, since the *Lb. plantarum* strains consumed more glucose and less citrate than the cit<sup>+</sup> *O. oeni* strain. The differences seen between the species might also be related to their different sugar metabolism. *Lb. plantarum* is facultative heterofermentative that use the EMP to metabolise hexose sugars and thus produce lactate, acetate or ethanol as the end products from these sugars. *Lb. plantarum* produces only lactate from sugars under wine conditions with a low pH (Tseng *et al.*, 1990). *O. oeni* is obligate

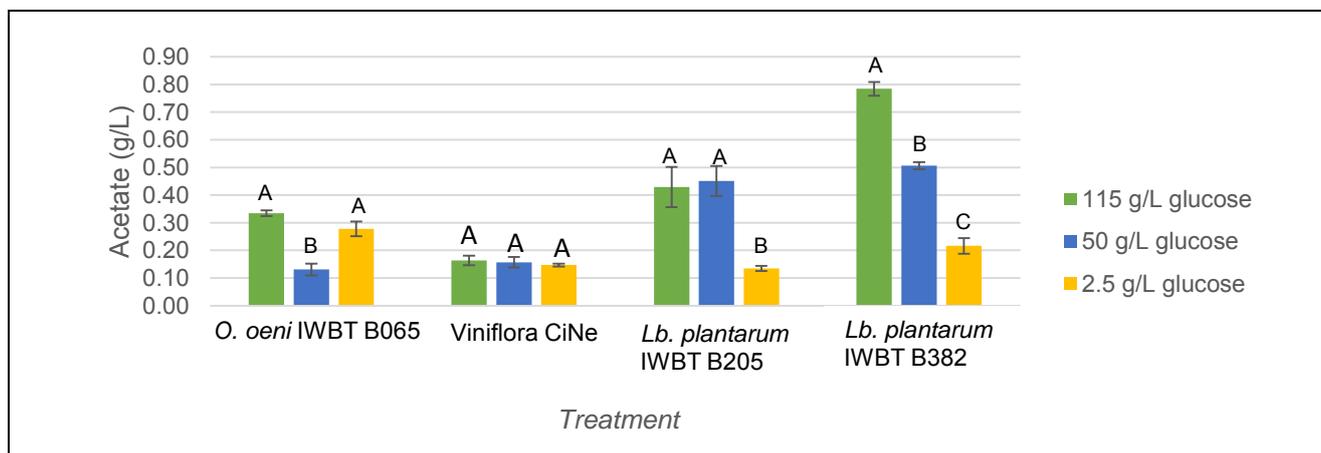
heterofermentative that produces lactate and acetate or ethanol via the pentose phosphate pathway (Dicks and Endo, 2009; Papadimitriou *et al.*, 2016).



**Figure 3.6** Concentrations of D-lactate produced by the *O. oeni* strains (A) and the *Lb. plantarum* strains (B) in the glucose treatments. Day 5 was the last sampling day for the *O. oeni* strains and day 21 for the *Lb. plantarum* strains. Data shown are averages of triplicates with error bars representing the standard deviation. Different alphabetical letters indicate significant differences between the different treatments of an individual strain ( $p < 0.05$ ).

**Figure 3.7** indicates the acetate concentrations at the last sampling point in the glucose treatments inoculated with the different LAB strains. As mentioned previously only trends can be used from these data and not absolute values. In the 115 g/L and 2.5 g/L treatments, the cit<sup>+</sup> *O. oeni* strain produced similar amounts of acetate. In the 50 g/L treatment inoculated with this cit<sup>+</sup> *O. oeni* strain, the acetate concentration was lower than in the 115 g/L and 2.5 g/L treatments. The treatments did not affect the acetate production by the cit<sup>-</sup> *O. oeni* strain which is in agreement with the glucose and citrate consumption by this strain that was similar in all the treatments. The 115 g/L and 50 g/L treatments inoculated with the cit<sup>-</sup> *Lb. plantarum* strain had similar acetate concentrations on day 21. In the 2.5 g/L treatment, the cit<sup>-</sup> *Lb. plantarum* strain produced less acetate than in the 115 g/L and 50 g/L treatments. The cit<sup>+</sup> *Lb. plantarum* strain produced more acetate in the 115 g/L treatment than in the 50 g/L and 2.5 g/L treatments, even though this strain consumed more sugar in the 50 g/L treatment than in the other treatments (**Figure 3.3**). This strain was the only strain that produced an acetate concentration that could have caused wine spoilage by producing a vinegar aroma since the acetate concentration produced in the 115 g/L treatment was above 0.6 g/L which is regarded as spoilage (Guth, 1997; Ferreira *et al.*, 2000).

Significant differences were determined by performing an ANOVA followed by a Fischer LSD test between the various strains within a specific treatment at the last sampling point (data not shown). The cit<sup>+</sup> *O. oeni* strain had higher acetate concentrations than the cit<sup>-</sup> *O. oeni* strain on day 5. This was expected since the cit<sup>+</sup> *O. oeni* strain completely consumed citrate and the cit<sup>-</sup> *O. oeni* strain did not and both strains did not consume glucose (**Figure 3.3 and 3.4**). However, no significant difference was observed between the *O. oeni* strains in the 50 g/L treatment. The same trend was observed for the *Lb. plantarum* strains in that the cit<sup>+</sup> strain produced more acetate than the cit<sup>-</sup> *Lb. plantarum* strain in the 115 g/L and 2.5 g/L treatments, but more or less the same amount of acetate in the 50 g/L treatment. The *O. oeni* strains produced less acetate than the *Lb. plantarum* strains in the 115 g/L and 50 g/L treatments. This was unexpected, since the cit<sup>+</sup> *O. oeni* strain consumed more citrate than the cit<sup>+</sup> *Lb. plantarum* strain and acetate is only produced by *O. oeni* during sugar fermentation (Dicks and Endo, 2009; Papadimitriou *et al.*, 2016). In the 2.5 g/L treatment, all the LAB strains produced more or less the same amount of acetate which might be attributed to the limited substrate available in this treatment.



**Figure 3.7** Acetate concentrations at last sampling point in the glucose treatments. Day 5 was the last sampling day for the *O. oeni* strains and day 21 for the *Lb. plantarum* strains. Data shown are averages of triplicates with error bars representing the standard deviation. Different alphabetical letters indicate significant differences ( $p < 0.05$ ) between the different treatments of an individual strain.

The diacetyl concentrations produced during the fermentations are indicated in **Table 3.8**. Diacetyl is one of the metabolites produced from pyruvate formed during sugar or citrate metabolism and could explain the increase that was seen as the LAB strains consumed glucose and citrate. Diacetyl can then be further reduced to acetoin and 2,3-butanediol which could explain the decrease in diacetyl concentrations at the end of the fermentation in the treatments inoculated with the *Lb. plantarum* strains. However, the 2.5 g/L treatment inoculated with the cit<sup>+</sup> *Lb. plantarum* strain was the only treatment that had a diacetyl concentration above the minimum quantification limit on day 21. The diacetyl concentration in this treatment could have contributed to a desirable buttery flavour as the sensory threshold of diacetyl ranges from 0.2 mg/L to 2.8 mg/L (Martineau and Henick-Kling, 1995). All the other treatments had only trace amounts of diacetyl.

Significant differences were determined by performing an ANOVA followed by a Fischer LSD test between the various strains within a specific treatment with the data obtained from the last sampling day (data not shown). The diacetyl concentrations produced by the *O. oeni* strains was below the minimum quantification limit. There were no significant differences between the concentrations produced by the *Lb. plantarum* strains in the 115 g/L and 50 g/L treatments on day 21. In the 2.5 g/L treatment inoculated with the cit<sup>+</sup> *Lb. plantarum* strain, the final diacetyl concentration of 4.46 mg/L was significantly more than the 0.39 mg/L produced by the cit<sup>-</sup> *Lb. plantarum* strain.

The acetoin concentrations produced in the glucose treatments inoculated with the four LAB strains are indicated in **Table 3.9**. The acetoin concentrations in the treatments inoculated with the *Lb. plantarum* strains increased as the diacetyl concentrations decreased, except in the 2.5 g/L treatment inoculated with the cit<sup>-</sup> *Lb. plantarum* strain (**Table 3.8**). This indicates that diacetyl was further reduced to acetoin via the citrate metabolism as previously stated by several other studies (Ramos *et al.*, 1995; Bartowsky and Henshke, 2004).

No significant difference was observed between the 115 g/L and 2.5 g/L treatments inoculated with the cit<sup>+</sup> *O. oeni* strain. In the 50 g/L treatment, the acetoin concentrations were significantly higher on day 5 than in the other two treatments inoculated with this cit<sup>+</sup> *O. oeni* strain. The cit<sup>-</sup> *O. oeni* strain did not produce any acetoin since this strain did not consume any glucose or citrate (**Figure 3.3 and 3.4**). The same trend was observed for the *Lb. plantarum* strains in the 115 g/L and 50 g/L treatments. In these treatments, there were no significant differences between the two treatments with regards to the final acetoin concentrations on day 21. In the 2.5 g/L treatment inoculated with the cit<sup>-</sup> *Lb. plantarum* strain, the acetoin concentration was 64% less than in the 115 g/L and 50 g/L treatments. The opposite was seen in the 2.5 g/L treatment inoculated with the cit<sup>+</sup> *Lb. plantarum* strain since the acetoin concentration was 27% and 48% more at the last sampling point than in the 115 g/L and 50 g/L treatments, respectively. The acetoin concentration of 175 mg/L in the 2.5 g/L treatment was also above the 150 mg/L sensory threshold and could have given rise to a creamy aroma (Malherbe *et al.*, 2012). The higher acetoin concentration in the 2.5 g/L treatment inoculated with the cit<sup>+</sup> *Lb. plantarum* strain is in agreement with the diacetyl data, since the highest diacetyl was also produced in this treatment. This could have been due to the lower acetate and D-lactate concentrations in the 2.5 g/L treatment, which might have provided more pyruvate for the production of diacetyl and acetoin.

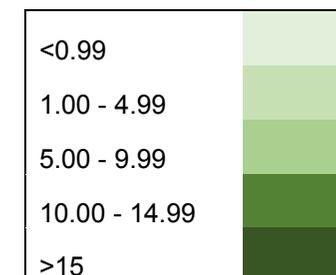
Significant differences were determined by performing an ANOVA followed by a Fischer LSD test between the various strains within a specific treatment with the data obtained from the last sampling day (data not shown). The same trend as for diacetyl concentrations was observed for the acetoin concentrations. The *O. oeni* strains produced acetoin concentrations below the minimum quantification limit. The cit<sup>+</sup> *Lb. plantarum* strain produced significantly more acetoin than

the cit<sup>-</sup> strain in the 115 g/L and 2.5 g/L treatments on day 21. In the 50 g/L treatment, no significant difference was observed between the *Lb. plantarum* strains on day 21.

The *Lb. plantarum* strains produced more acetoin than the *O. oeni* strains, since the *O. oeni* strains produced trace amounts of acetoin. This can be correlated to the *Lb. plantarum* strains that consumed more glucose than the *O. oeni* strains. Furthermore, it is unclear from literature whether *Lb. plantarum* can further reduce acetoin to 2,3-butanediol, since a few genome annotation studies have not yet indicated that this species have the gene encoding the necessary reductase enzyme (Illegheems, 2015). The *Lb. plantarum* strains could have produced more acetoin than the *O. oeni* strains in this study, since the *O. oeni* strains could have further reduced the acetoin to 2,3-butanediol. However, 2,3-butanediol could not be measured in this study to confirm this hypothesis.

**Table 3.8** Diacetyl concentrations (mg/L) in the glucose treatments inoculated with the four LAB strains. The last sampling day for *O. oeni* IWBT B065 and Viniflora® CiNe™ was day 5 and day 21 for the *Lb. plantarum* strains in all the treatments.

Treatment	Lactic acid bacteria							
	<i>O. oeni</i> IWBT B065		Viniflora® CiNe™		<i>Lb. plantarum</i> IWBT B205		<i>Lb. plantarum</i> IWBT B382	
<b>115 g/L glucose</b>								
Day 2	0.13	± 0.04	0.06	± 0.02	4.52	± 0.46	4.57	± 0.4
Day 5	0.00	± 0	0.02	± 0.01	1.21	± 0.07	1.08	± 0.14
Day 21					0.25	± 0.01	0.30	± 0.03
<b>50 g/L glucose</b>								
Day 2	0.07	± 0.03	0.23	± 0.04	3.59	± 0.08	2.58	± 0.4
Day 5	0.09	± 0.04	0.02	± 0.01	1.21	± 0.02	2.72	± 0.29
Day 21					0.37	± 0.06	0.43	± 0
<b>2.5 g/L glucose</b>								
Day 2	0.02	± 0.01	0.03	± 0	0.91	± 0.01	6.87	± 0.28
Day 5	0.09	± 0.02	0.08	± 0.01	0.24	± 0.03	2.12	± 0.16
Day 21					0.39	± 0.04	4.46	± 0.41



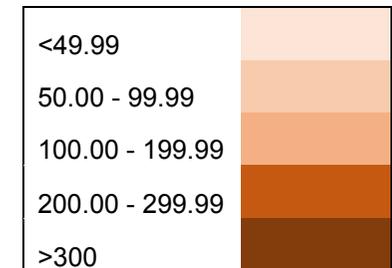
Values on last sampling day with different alphabetical letters are significantly different ( $p < 0.05$ ) between different treatments inoculated with a specific strain.

Significant differences were only calculated on values above the minimum quantification limit.

Minimum quantification limit for diacetyl was 0.5 mg/L.

**Table 3.9** Acetoin concentrations (mg/L) in the glucose treatments inoculated with the four LAB strains. The last sampling day for *O. oeni* IWBT B065 and Viniflora® CiNe™ was day 5 and day 21 for the *Lb. plantarum* strains in all the treatments.

Treatment	Lactic acid bacteria			
	<i>O. oeni</i> IWBT B065	Viniflora® CiNe™	<i>Lb. plantarum</i> IWBT B205	<i>Lb. plantarum</i> IWBT B382
<b>115 g/L glucose</b>				
Day 2	0.16 ± 0.05	0.00 ± 0.00	83.42 ± 2.42	87.66 ± 4.82
Day 5	0.00 ± 0.00	0.00 ± 0.00	90.93 ± 11.92	91.17 ± 11.90
Day 21			109.5 <sup>A</sup> ± 5.60	137.88 <sup>B</sup> ± 12.51
<b>50 g/L glucose</b>				
Day 2	0.00 ± 0.00	0.00 ± 0.00	94.35 ± 6.83	80.04 ± 13.47
Day 5	0.65 ± 0.00	0.00 ± 0.00	173.12 ± 21.18	132.20 ± 21.96
Day 21			121.19 <sup>A</sup> ± 16.33	118.01 <sup>B</sup> ± 14.78
<b>2.5 g/L glucose</b>				
Day 2	0.00 ± 0.00	0.00 ± 0.00	59.38 ± 16.83	153.71 ± 8.81
Day 5	0.00 ± 0.00	0.00 ± 0.00	38.24 ± 1.61	166.4 ± 11.82
Day 21			39.21 <sup>B</sup> ± 5.25	175.31 <sup>A</sup> ± 6.10



Values on last sampling day with different alphabetical letters are significantly different ( $p < 0.05$ ) between different treatments inoculated with a specific strain.

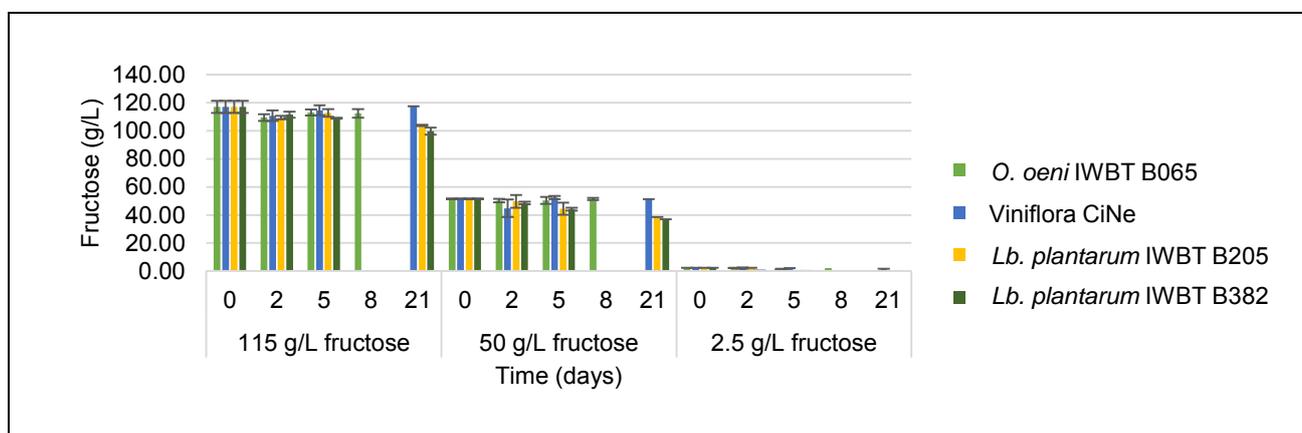
Significant differences were only calculated on values above the minimum quantification limit.

Minimum quantification limit for acetoin was 0.5 mg/L.

### 3.4.2.2 Effect of fructose on citrate metabolism

#### 3.4.2.2.1 Fructose, malate and citrate consumption

The fructose consumption by the LAB in the fructose treatments is indicated in **Figure 3.8**. The fructose treatments showed similar trends to the glucose treatments. The initial fructose added to the medium was not consumed by the *O. oeni* strains in the 115 g/L and 50 g/L treatments. However, in the 2.5 g/L treatment the cit<sup>+</sup> *O. oeni* strain consumed 13% of the fructose after eight days and the cit<sup>-</sup> *O. oeni* strain consumed 32% after 21 days. The *Lb. plantarum* strains consumed more fructose than the *O. oeni* strains. In the 115 g/L and 50 g/L treatments, the cit<sup>-</sup> *Lb. plantarum* strain consumed 11% and 24% of the initial fructose on day 21, respectively. The cit<sup>+</sup> *Lb. plantarum* strain consumed 14% of the fructose in the 115 g/L treatment and 28% in the 50 g/L treatments on day 21. In the 2.5 g/L treatment, the *Lb. plantarum* strains completely consumed fructose by day 5.



**Figure 3.8** Fructose consumption in the different treatments inoculated with the four LAB strains. Day 8 was the last sampling day for the *O. oeni* IWBT B065 and day 21 for the rest of the strains. Data shown are averages of triplicates with error bars representing the standard deviation.

The cell counts and malate degradation duration by the four different LAB strains in the fructose treatments are indicated in **Table 3.10**. All the strains were inoculated between  $1 \times 10^7$  cfu/mL to  $8.5 \times 10^7$  cfu/mL. This variation between the initial cell counts of the different strains could potentially have influenced malate and citrate consumption and the concentrations of metabolites produced by the different strains. The same trend was observed regarding the cell counts and malate degradation than what was observed in the glucose treatments. The initial cell counts of all the strains increased as soon as the strains have been inoculated. The *Lb. plantarum* strains were able to survive longer in the fermentations than the *O. oeni* strains which might be coupled to the *Lb. plantarum* strains that were able to consume more fructose than the *O. oeni* strains in all the treatments. The LAB strains were able to better maintain their cell counts in the fructose treatments than in the glucose treatments, since the cell counts on the last sampling day of all the strains were higher in the fructose treatments than in the glucose treatments. This was unexpected, since the *Lb. plantarum* strains consumed more glucose than fructose.

Malate was completely degraded by all the LAB strains ranging from one to three days. The *O. oeni* strains completely consumed malate after two days in the 50 g/L and 2.5 g/L treatments and after three days in the 115 g/L treatment. The cit<sup>+</sup> *Lb. plantarum* strain completely consumed malate within one and a half days after inoculation in the 115 g/L and 2.5 g/L treatments, but took only one day in the 50 g/L treatment. The cit<sup>-</sup> *Lb. plantarum* strain completely consumed malate one day after inoculation in the 50 g/L and 2.5 g/L treatments, but took one and a half days in the 115 g/L treatment. The *Lb. plantarum* strains consumed malate faster than the *O. oeni* strains in all the treatments. This might be related to the ability of the *Lb. plantarum* strains to consume more fructose to maintain their cell counts.

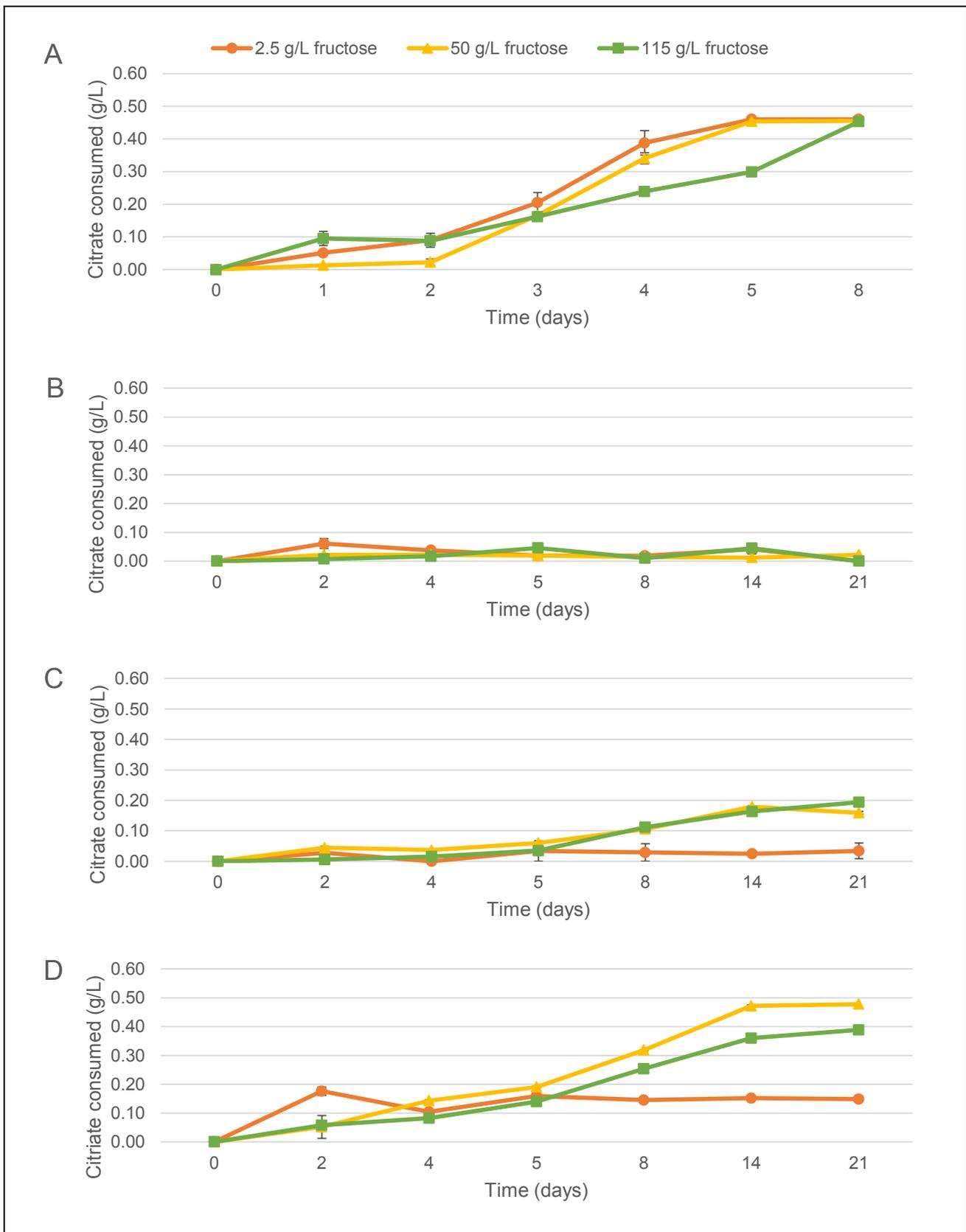
Citrate consumption by the four LAB strains in the fructose treatments are indicated in **Figure 3.9**. Citrate degradation by the cit<sup>+</sup> and cit<sup>-</sup> *O. oeni* strains and the cit<sup>-</sup> *Lb. plantarum* in the fructose treatments exhibit a similar trend as in the glucose treatments (**Figure 3.9**). The cit<sup>+</sup> *O. oeni* strain completely consumed citrate in all the treatments. However, complete citrate consumption in the 115 g/L fructose treatment took longer than in the 50 g/L and 2.5 g/L treatments (**Figure 3.9A**). On day 5, citrate was completely consumed (0.5 g/L) in the 50 g/L and 2.5 g/L treatments as compared to 0.3 g/L citrate that was consumed in the 115 g/L treatment. In the 115 g/L treatment, citrate was completely consumed by the cit<sup>+</sup> *O. oeni* strain on day 8. This might be due to the longer malate degradation period seen in this treatment, since citrate consumption is linked to malate degradation (**Table 3.10**). The cit<sup>-</sup> *O. oeni* strain did not utilise any of the citrate, as expected (**Figure 3.9B**). The cit<sup>-</sup> *Lb. plantarum* strain partially consumed citrate in all the fructose treatments as was seen in the glucose treatments (**Figure 3.9C**). In the 115 g/L and 50 g/L treatments, this strain consumed 32% and 38% of the citrate on day 21, respectively. However, in the 2.5 g/L treatment this strain did not consume any citrate after 21 days. This might be due to the decrease in cell viability that was seen in this treatment, which decreased from 10<sup>7</sup> cfu/mL on day 0 to 10<sup>5</sup> cfu/mL on day 21 (**Table 3.10**).

The cit<sup>+</sup> *Lb. plantarum* strain was able to completely consume citrate in the 50 g/L fructose treatment after 14 days (**Figure 3.9D**). However, in the 115 g/L and 2.5 g/L fructose treatments, the cit<sup>+</sup> *Lb. plantarum* strain consumed only 76% and 15% of citrate on day 21, respectively (**Figure 3.9D**). The cell counts of this strain in the 2.5 g/L treatment decreased faster than in the other two treatments. These results further support the hypothesis that the *Lb. plantarum* strains seem to need high sugar concentrations to maintain their cell counts and metabolic activities. The cit<sup>+</sup> *Lb. plantarum* strain was able to consume more citrate in the fructose treatments than in the glucose treatments, which corresponds with the results found by Palles *et al.* (1998) that indicated that glucose inhibits the synthesis of citrate lyase. The cit<sup>+</sup> *Lb. plantarum* strain was therefore unable to completely degrade citrate in the presence of glucose.

**Table 3.10** Overview of cell counts (cfu/mL) and malate degradation duration (days) of the four LAB strains in the fructose treatments.

Strain	Days						Malate degradation duration
	0	2	4	8	14	21	
<b>115 g/L fructose</b>							
<i>O. oeni</i> IWBT B065	8.55E+07 ± 2.19E+07	3.55E+07 ± 3.54E+06	1.90E+07 ± 5.66E+06	2.10E+07 ± 4.24E+06			3.0
Viniflora® CiNe™	3.30E+07 ± 2.83E+06	3.70E+07 ± 1.41E+07	2.63E+07 ± 3.06E+06	1.38E+07 ± 6.36E+05	1.20E+06 ± 9.90E+05	6.80E+05 ± 1.41E+04	3.0
<i>Lb. plantarum</i> IWBT B205	2.13E+07 ± 9.90E+05	7.70E+07 ± 2.12E+07	6.77E+08 ± 6.66E+07	4.21E+08 ± 5.54E+07	3.12E+08 ± 1.11E+07	2.95E+07 ± 1.91E+07	1.5
<i>Lb. plantarum</i> IWBT B382	1.21E+07 ± 1.00E+05	4.10E+08 ± 9.90E+07	8.50E+08 ± 1.41E+07	4.05E+08 ± 6.24E+06	2.96E+08 ± 2.15E+07	3.15E+07 ± 1.63E+07	1.5
<b>50 g/L fructose</b>							
<i>O. oeni</i> IWBT B065	6.80E+07 ± 8.49E+06	4.72E+07 ± 9.61E+06	8.90E+07 ± 4.00E+06	7.95E+06 ± 2.05E+06			2.0
Viniflora® CiNe™	2.90E+07 ± 3.61E+06	8.17E+07 ± 3.06E+06	7.27E+07 ± 8.50E+06	2.53E+07 ± 5.03E+06	1.63E+07 ± 2.61E+06	1.15E+07 ± 2.36E+06	2.0
<i>Lb. plantarum</i> IWBT B205	2.58E+07 ± 1.13E+06	2.77E+08 ± 5.13E+07	7.45E+08 ± 2.12E+07	7.97E+08 ± 1.41E+07	7.97E+08 ± 1.53E+08	3.09E+08 ± 3.58E+07	1.0
<i>Lb. plantarum</i> IWBT B382	1.68E+07 ± 1.62E+06	7.10E+08 ± 9.17E+07	5.70E+08 ± 7.07E+07	6.37E+08 ± 7.64E+07	6.37E+08 ± 1.21E+08	3.73E+08 ± 2.10E+07	1.0
<b>2.5 g/L fructose</b>							
<i>O. oeni</i> IWBT B065	5.03E+07 ± 1.10E+07	8.30E+07 ± 1.01E+07	1.21E+08 ± 2.12E+05	8.35E+06 ± 2.12E+06			2.0
Viniflora® CiNe™	5.93E+07 ± 1.18E+07	1.35E+08 ± 4.36E+06	1.40E+08 ± 9.90E+05	8.80E+06 ± 1.21E+07	9.15E+06 ± 6.36E+05	2.22E+07 ± 2.26E+06	2.0
<i>Lb. plantarum</i> IWBT B205	1.93E+07 ± 7.55E+05	3.93E+08 ± 1.12E+08	8.90E+08 ± 3.21E+06	1.97E+07 ± 2.26E+08	1.49E+06 ± 1.15E+05	7.00E+05 ± 1.27E+05	1.5
<i>Lb. plantarum</i> IWBT B382	1.86E+07 ± 1.94E+06	7.30E+08 ± 1.21E+08	4.73E+08 ± 1.53E+06	2.83E+07 ± 5.77E+06	1.69E+06 ± 1.50E+05	1.07E+06 ± 2.05E+05	1.0

Data shown are averages of triplicates with standard deviation.



**Figure 3.9** Monitoring citrate consumption by A) *O. oeni* IWBT B065, B) *Viniflora*® CiNe™, C) *Lb. plantarum* IWBT B205 and D) *Lb. plantarum* IWBT B382 in the 2.5 g/L (orange), 50 g/L (yellow) and 115 g/L (green) fructose treatments. Data shown are mean values of triplicates and error bars represent the standard deviations.

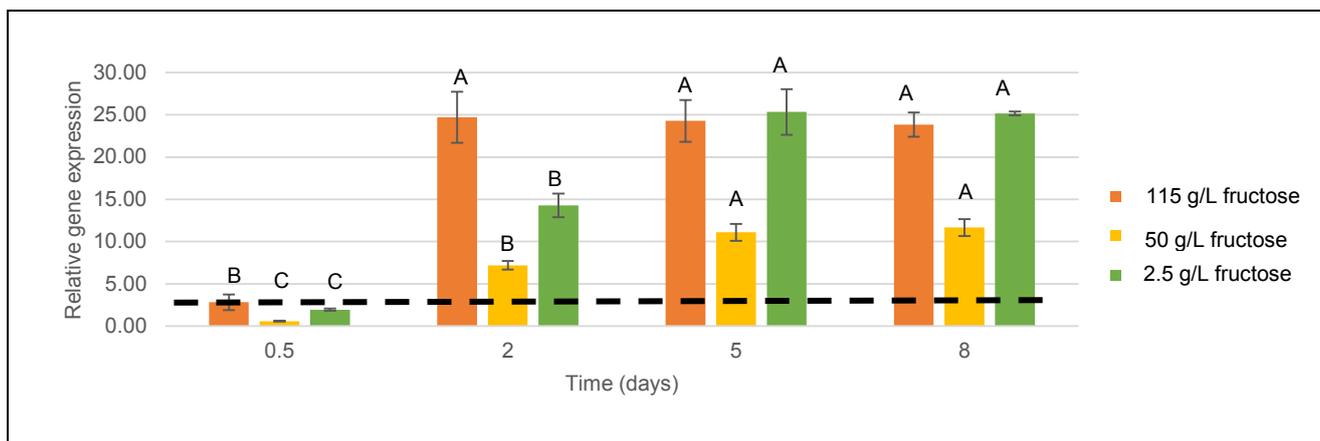
#### 3.4.2.2.2 Relative expression of *citE*

The citrate utilisation by the LAB strains were further investigated by determining the RE of *citE*. The RE levels of *citE* were determined on day 0.5, 2, 5 and 8 in the *cit*<sup>+</sup> *O. oeni* strain, since this bacterial strain completely utilised citrate in all the treatments within eight days. Samples taken on day 2, 5 and 21 were selected to determine the RE of *citE* in the *cit*<sup>+</sup> *Lb. plantarum* strain, whereas only the samples from day 5 were used for the *cit*<sup>-</sup> strains.

The RE of the *cit*<sup>+</sup> *O. oeni* strain in the fructose treatments is indicated in **Figure 3.10**. A relative transcription response was first observed on day 0.5 in the 115g/L treatment inoculated with this *cit*<sup>+</sup> *O. oeni* strain. Thereafter the RE increased with 21.8 fold on day 2. There were no significant differences observed in the RE after day 2 until the end of the fermentation. The 50 g/L and 2.5 g/L treatments inoculated with the *cit*<sup>+</sup> *O. oeni* strain, displayed a similar trend with regards to the RE. A relevant transcriptional response was first observed on day 2. Thereafter the RE increased from day 2 to day 5. The RE in the 50 g/L treatment was only 3.9 fold higher than the RE on day 2. In the 2.5 g/L treatment, the RE on day 5 was 11.1 fold more than on day 2. Thereafter the RE remained unchanged until the end of the fermentation.

The RE in the 50 g/L treatment was in most cases more than twofold lower than in the 115 g/L and 2.5 g/L treatments, which was unexpected since the cell counts in all the treatments were similar (**Table 3.10**) and the duration of malate and citrate consumption was the same in the 50 g/L and 2.5 g/L treatments. It is important to note that the RE was higher in the 115 g/L treatment where the malate and citrate were consumed over a longer period. Olguín *et al.* (2010) also observed that *citE* had a higher RE in the treatments where the *O. oeni* degraded malate over a longer period. They suggested that the bacteria delay citrate consumption as a mechanism for cell survival for when malate has been completely consumed.

A RE was observed for the other bacterial strains in all the fructose treatments, but there was not a relevant transcriptional response. This was unexpected since the *cit*<sup>+</sup> *Lb. plantarum* strain completely consumed citrate in the 50 g/L treatment. A relevant transcriptional response of the *cit*<sup>+</sup> *Lb. plantarum* strain could have been on day 8 or day 14 which was not measured in this study.



**Figure 3.10** Relative gene expression of the *citE* gene in *O. oeni* IWBT B065 in the fructose treatments. Data shown are averages of triplicates with error bars representing the standard deviations. Dashed line indicates a twofold expression level. Different alphabetical letters indicate significant differences ( $p < 0.05$ ) within a specific treatment.

### 3.4.2.2.3 Production of metabolites associated with citrate metabolism

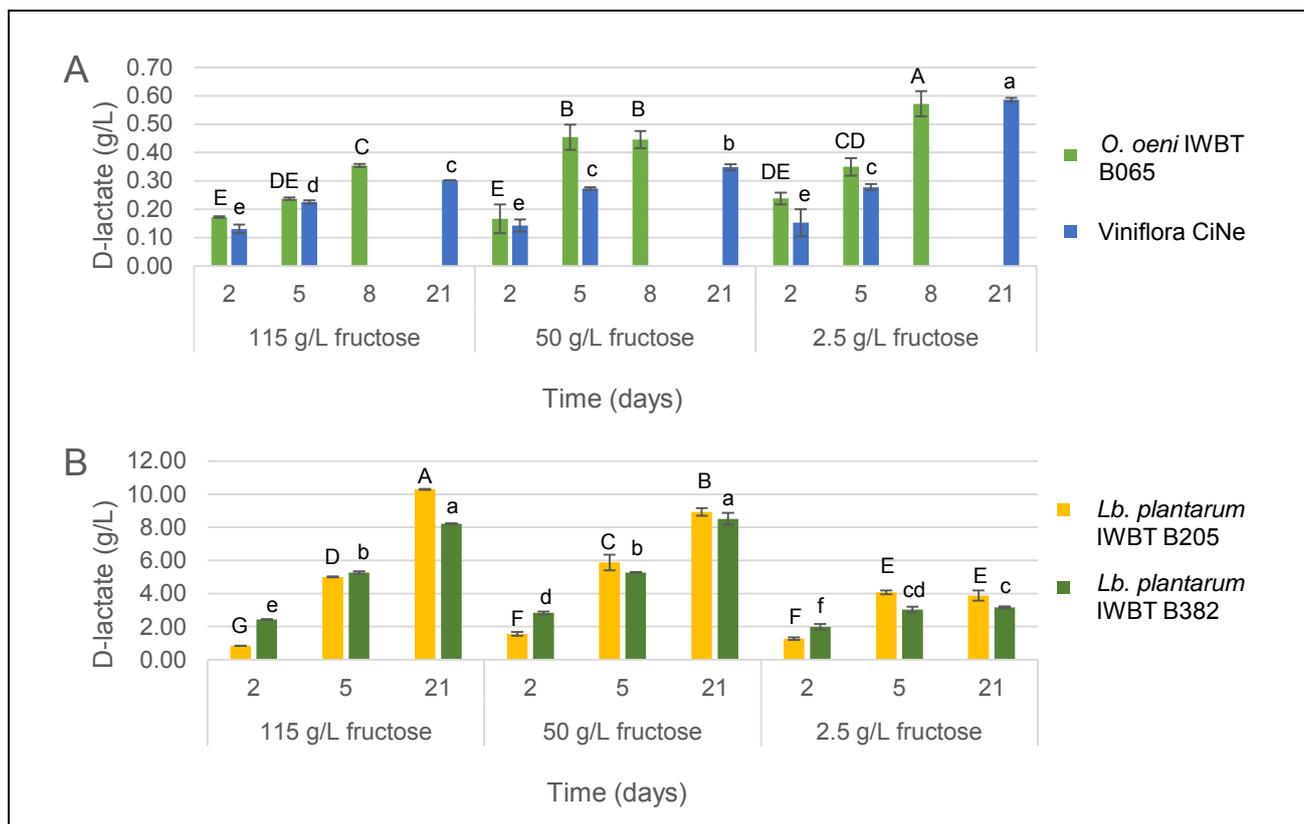
The D-lactate concentrations produced by the LAB strains in the fructose treatments are indicated in **Figure 3.11**. The D-lactate concentrations increased as the fermentation proceeded in all the treatments, except in the 2.5 g/L treatment inoculated with the *Lb. plantarum* strains (**Figure 3.11**). In the 2.5 g/L treatment inoculated with the *Lb. plantarum* strains, the D-lactate concentrations only increased from day 2 to day 5, but remained unchanged from day 5 until the end of the fermentation. This is mainly due to the *Lb. plantarum* strains that completely consumed the fructose by day 5 (**Figure 3.8**).

The different fructose treatments had a significant effect on the D-lactate production by the LAB strains. On day 8, the  $\text{cit}^+$  *O. oeni* strain produced 62% and 26% more D-lactate in the 2.5 g/L treatment than in the 115 g/L and 50 g/L treatments inoculated with this strain, respectively. The  $\text{cit}^-$  *O. oeni* strain produced 96% and 68% more D-lactate in the 2.5 g/L treatment than in the 115 g/L and 50 g/L treatments on day 21, respectively. In contrast, the D-lactate concentration in the 2.5 g/L treatment induced with the  $\text{cit}^-$  *Lb. plantarum* strain was 62% less than in the 115 g/L treatment and 56% less than in the 50 g/L treatment inoculated with this strain on day 21. The  $\text{cit}^+$  *Lb. plantarum* strain also produced 61% less D-lactate in the 2.5 g/L treatment than in the 115 g/L and 50 g/L treatments on day 21. The lower D-lactate concentrations in the 2.5 g/L fructose treatment might be due to the fructose being 20-46 times less in this treatment than in the other two treatments. However, the *O. oeni* strains consumed more fructose in the 2.5 g/L treatment than in the 115 g/L and 50 g/L treatments and therefore produced more D-lactate in the 2.5 g/L treatment than in the other two treatments.

An ANOVA followed by a Fischer LSD test was performed to determine significant differences between the various strains within a specific treatment with the data obtained on the last sampling day which was day 8 for the  $\text{cit}^+$  *O. oeni* strain and day 21 for the other strains (data

not shown). At the end of the fermentation, the cit<sup>+</sup> *O. oeni* strain had significantly higher D-lactate concentrations than the cit<sup>-</sup> *O. oeni* strain in the 115 g/L and 50 g/L treatments. In the 115 g/L treatment, the cit<sup>+</sup> *O. oeni* strain produced 16% more D-lactate than the cit<sup>-</sup> *O. oeni* strain. This difference was slightly more in the 50 g/L treatment where the cit<sup>+</sup> *O. oeni* strain produced 28% more D-lactate than the cit<sup>-</sup> *O. oeni* strain. The cit<sup>+</sup> *O. oeni* strain could have produced more D-lactate from the citrate consumed, since the cit<sup>-</sup> *O. oeni* strain did not consume any citrate and both strains did not consume fructose in the 115 g/L and 50 g/L treatments. No significant difference was observed between the *O. oeni* strains on the last sampling day in the 2.5 g/L treatment. This could have been due to the cit<sup>-</sup> *O. oeni* strain that consumed more fructose than the cit<sup>+</sup> *O. oeni* strain in the 2.5 g/L treatment, but the cit<sup>+</sup> *O. oeni* strain consumed more citrate than the cit<sup>-</sup> *O. oeni* strain. In the 50 g/L and 2.5 g/L treatments, there were no significant differences between the *Lb. plantarum* strains regarding the D-lactate concentrations produced on day 21. On the last sampling day in the 115 g/L treatment, the cit<sup>-</sup> *Lb. plantarum* strain produced 25% more D-lactate than the cit<sup>+</sup> strain. This was unexpected since the cit<sup>+</sup> *Lb. plantarum* strain consumed more fructose and citrate than the cit<sup>-</sup> *Lb. plantarum* strain.

The *Lb. plantarum* strains produced more D-lactate than the *O. oeni* strains. At the last sampling point, the cit<sup>+</sup> *Lb. plantarum* strain produced 29 times more D-lactate than the cit<sup>+</sup> *O. oeni* strain in the 115 g/L treatment. At the last sampling point in the 50 g/L and 2.5 g/L treatments, the cit<sup>+</sup> *Lb. plantarum* strain produced 20 and six times more D-lactate than the cit<sup>+</sup> *O. oeni* strain, respectively. This difference between the two species is mainly due to the *Lb. plantarum* strains that consumed more fructose than the *O. oeni* strains in all the fructose treatments. As previously mentioned, *Lb. plantarum* produces only lactate from their sugar metabolism whereas *O. oeni* produces lactate and acetate or ethanol (Dicks and Endo, 2009; Papadimitriou *et al.*, 2016).



**Figure 3.11** The D-lactate concentrations produced by the *O. oeni* strains (A) and by the *Lb. plantarum* strains (B) in the fructose treatments. The last sampling day was day 8 for *O. oeni* IWBT B065 and day 21 for the other strains. Data shown are averages of triplicates with error bars representing the standard deviation. Different alphabetical letters indicate significant differences ( $p < 0.05$ ) between the different treatments of an individual strain.

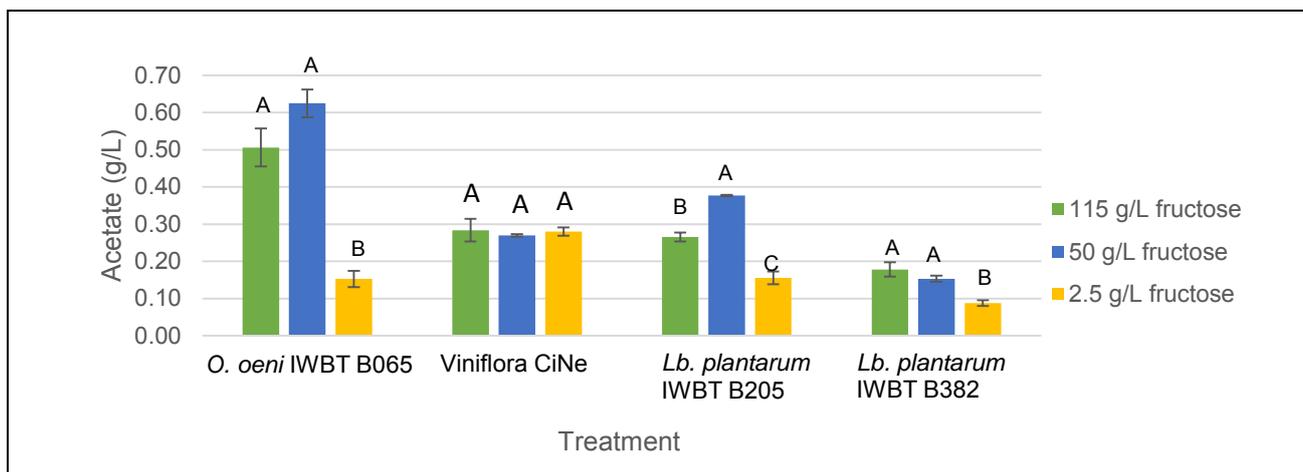
The acetate concentrations on the last sampling day in the fructose treatments are indicated in **Figure 3.12**. Only the trends can be used and not absolute values as previously mentioned due to the volatility of acetate. The 115 g/L and 50 g/L treatments inoculated with the  $\text{cit}^+$  *O. oeni* strain had more or less the same acetate concentrations. The acetate concentrations in these treatments were above 0.6 g/L which might have caused wine spoilage (Guth, 1997; Ferreira *et al.*, 2000). In the 2.5 g/L treatment, the  $\text{cit}^+$  *O. oeni* strain produced less acetate than in the 115 g/L and 50 g/L treatments. This was unforeseen since the  $\text{cit}^+$  *O. oeni* strain consumed more sugar in the 2.5 g/L treatment than in the 115 g/L and 50 g/L treatments and the same amount of citrate (**Figure 3.8 and 3.9**). This could indicate that the  $\text{cit}^+$  *O. oeni* strain produces ethanol rather than acetate from the sugars consumed (Dicks and Endo, 2009). The fructose treatments did not affect the acetate production by the  $\text{cit}^-$  *O. oeni* strain, as was seen in the glucose treatments.

The fructose treatments had a significant effect on the acetate concentrations produced by the  $\text{cit}^-$  *Lb. plantarum* strain (**Figure 3.12**). The maximum acetate concentration was observed in the 50 g/L treatment and the lowest concentration in the 2.5 g/L treatment inoculated with this strain. In the 115 g/L treatment, the acetate concentration was less than in the 50 g/L treatment inoculated with this  $\text{cit}^-$  *Lb. plantarum* strain. In the 2.5 g/L treatment, this strain produced less acetate than in the 50 g/L treatment. This corresponds to the fructose consumption by this strain,

since this strain consumed more fructose in the 50 g/L treatment than in the other two treatments (**Figure 3.8**). The fructose treatment also had an effect on the acetate concentrations produced by the *cit*<sup>+</sup> *Lb. plantarum* strain but to a lesser extent than what was observed for the *cit*<sup>-</sup> *Lb. plantarum* strain (**Figure 3.12**). The 115 g/L and 50 g/L treatments inoculated with the *cit*<sup>+</sup> *Lb. plantarum* strain had more or less the same amount of acetate. In the 2.5 g/L treatment, less acetate was produced by the *cit*<sup>+</sup> *Lb. plantarum* strain than in the other two treatments inoculated with this strain. As has been noted with the *cit*<sup>-</sup> *Lb. plantarum* strain, the *cit*<sup>+</sup> *Lb. plantarum* strain produced the lowest acetate concentration in the 2.5 g/L treatment which could be due to the limited fructose in this treatment (**Figure 3.8**).

An ANOVA followed by a Fischer LSD test was performed to determine significant differences between the various strains within a specific treatment (data not shown). On the last sampling day, the acetate concentrations in the 115 g/L and 50 g/L treatments inoculated with the *cit*<sup>+</sup> *O. oeni* strain were more in the same treatments inoculated with the *cit*<sup>-</sup> *O. oeni* strains. This was expected since both strains did not consume fructose and the *cit*<sup>+</sup> *O. oeni* strain completely consumed citrate while the *cit*<sup>-</sup> *O. oeni* strain did not (**Figure 3.8 and 3.9**). In the 2.5 g/L treatment, the *cit*<sup>-</sup> *O. oeni* strain produced more acetate than the *cit*<sup>+</sup> *O. oeni* strain, even though the *cit*<sup>+</sup> *O. oeni* strain completely consumed citrate. However, the *cit*<sup>-</sup> *O. oeni* strain consumed more fructose than the *cit*<sup>+</sup> *O. oeni* strain in the 2.5 g/L treatment (**Figure 3.8**). The *cit*<sup>+</sup> *Lb. plantarum* strain produced less acetate than the *cit*<sup>-</sup> *Lb. plantarum* strain in all the treatments. This was unexpected since the *cit*<sup>+</sup> *Lb. plantarum* strain consumed more citrate and fructose than the *cit*<sup>-</sup> *Lb. plantarum* strain in the 115 g/L and 50 g/L treatments.

Differences between the species regarding acetate concentrations were also observed. The *cit*<sup>+</sup> *Lb. plantarum* strain produced less acetate in all the treatments than the *cit*<sup>+</sup> *O. oeni* strain on the last sampling day which was day 8 for the *cit*<sup>+</sup> *O. oeni* strain and day 21 for the other strains. This was expected, since the *cit*<sup>+</sup> *O. oeni* strain consumed more citrate than the *cit*<sup>+</sup> *Lb. plantarum* strain, although the *cit*<sup>+</sup> *Lb. plantarum* strain consumed more fructose than the *cit*<sup>+</sup> *O. oeni* strain. In contrast, the *cit*<sup>-</sup> *Lb. plantarum* strain produced more acetate than the *cit*<sup>-</sup> *O. oeni* strain in the 50 g/L treatment. On the other hand, there was no significant difference between the *cit*<sup>-</sup> strains in the 115 g/L and 2.5 g/L treatments. The higher acetate concentration in the 50 g/L treatment inoculated with the *cit*<sup>-</sup> *Lb. plantarum* strain could have been due to this strain that consumed more fructose and citrate than the *cit*<sup>-</sup> *O. oeni* strain.



**Figure 3.12** Acetate concentrations on last sampling day in the fructose treatments. Day 5 was the last sampling day for *O. oeni* IWBT B065 and day 21 for the rest of the strains. Data shown are averages of triplicates with error bars representing the standard deviation. Different alphabetical letters indicate significant differences between the different treatments of an individual strain ( $p < 0.05$ ).

The diacetyl concentrations produced by the four LAB strains in the fructose treatments are indicated in **Table 3.11**. The diacetyl concentrations initially increased as the strains consumed fructose and citrate in most treatments. Once the maximum diacetyl levels were reached the diacetyl concentrations decreased in a few of the treatments. This decrease might be due to diacetyl that can be further reduced to acetoin and/or 2,3-butanediol. However, in the 115 g/L and 2.5 g/L treatments inoculated with the *Lb. plantarum* strains the diacetyl concentrations did not decrease. This might be due to the delayed citrate consumption by these bacteria (**Figure 3.9**).

The fructose treatments had a significant effect on the final diacetyl concentrations produced by the *O. oeni* strains. The cit<sup>+</sup> *O. oeni* strain produced significantly more diacetyl in the 115 g/L treatment than in the other two treatments on day 8. The diacetyl concentrations produced by this strain in the 115 g/L treatment could have potentially given rise to a buttery off-flavour, since the final concentration of 19.67 mg/L on day 8 was above the 5 mg/L limit indicated by previous studies (Martineau and Henick-Kling, 1995; Bartowsky and Henschke, 2004). In the 2.5 g/L treatment, the cit<sup>+</sup> *O. oeni* strain only produced trace amounts of diacetyl. The cit<sup>-</sup> *O. oeni* strain produced trace amounts of diacetyl in all the treatments, as was seen in the glucose treatments.

The fructose treatments also had an effect on the diacetyl concentrations produced by the *Lb. plantarum* strains. In the 50 g/L and 2.5 g/L treatments inoculated with the cit<sup>-</sup> *Lb. plantarum* strain, there was no significant difference on day 21. In the 115 g/L treatment, this strain produced 6.39 mg/L diacetyl on day 21, compared to 2.5 mg/L diacetyl in the other two treatments. This was surprising since the cit<sup>-</sup> *Lb. plantarum* strain consumed more fructose in the 50 g/L treatment and the same amount of citrate in the 115 g/L and 50 g/L treatments. This could indicate that the pyruvate produced during glycolysis or citrate metabolism was converted to acetate rather than to the C<sub>4</sub> flavour compounds, since the highest acetate concentration was seen in the 50 g/L treatment (**Figure 3.12**). The final diacetyl concentration produced by this strain in the 115 g/L

treatment could have given rise to a desirable buttery aroma, since the concentration was below the 5 mg/L limit (Martineau and Henick-Kling, 1995; Bartowsky and Henschke, 2004).

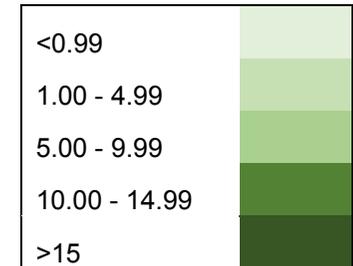
The diacetyl concentrations in the treatments inoculated with the cit<sup>+</sup> *Lb. plantarum* strain were significantly different in all the treatments. The maximum final diacetyl concentration of 22.02 mg/L was seen in the 115 g/L treatment and the lowest concentration of 7.41 mg/L was seen in the 50 g/L treatment. The diacetyl concentrations on day 21 in the 50 g/L treatment were 66% less than in the 115 g/L treatment. This was unexpected as this strain consumed more fructose and completely consumed citrate in the 50 g/L compared to the other two treatments (**Figure 3.8 and 3.9D**). The final diacetyl concentration in the 2.5 g/L treatment was significantly less than in the 115 g/L treatment, but significantly more than the 50 g/L treatment inoculated with this strain on day 21. The final diacetyl concentrations in all the treatments inoculated with the cit<sup>+</sup> *Lb. plantarum* strain were above the 5 mg/L limit of desirable diacetyl and could therefore have contributed to a buttery off-flavour (Martineau and Henick-Kling, 1995; Bartowsky and Henschke, 2004).

Statistical differences were determined by performing an ANOVA followed by a Fischer LSD test between the various strains within a specific treatment with the data obtained from the last sampling day which was day 8 for the cit<sup>+</sup> *O. oeni* strain and day 21 for the other strains (data not shown). The cit<sup>+</sup> *O. oeni* strain produced significantly more diacetyl in the 115 g/L and 50 g/L treatments than the cit<sup>-</sup> *O. oeni* strain, since this cit<sup>-</sup> *O. oeni* strain only produced trace amounts of diacetyl. This was not surprising as both strains did not consume fructose and the cit<sup>+</sup> *O. oeni* strain completely consumed citrate while the cit<sup>-</sup> *O. oeni* strain did not (**Figure 3.8 and 3.9**). In the 2.5 g/L treatment, both the *O. oeni* strains produced trace amounts of diacetyl. The cit<sup>+</sup> *Lb. plantarum* strain produced significantly more diacetyl than the cit<sup>-</sup> *Lb. plantarum* strain in all the treatments on day 21. The cit<sup>+</sup> *Lb. plantarum* strain consumed more fructose and citrate than the cit<sup>-</sup> strain during the fermentation and could therefore produce more diacetyl.

Differences between the species were also observed regarding the final diacetyl concentrations in the different treatments. The *Lb. plantarum* strains produced more diacetyl than the *O. oeni* strains at the last sampling point in all the treatments. In the 115 g/L treatments, the cit<sup>+</sup> *Lb. plantarum* strain produced 12% more diacetyl than the cit<sup>+</sup> *O. oeni* strain at the last sampling point, even though the cit<sup>+</sup> *Lb. plantarum* strain produced 53% less diacetyl on day 5 than the cit<sup>+</sup> *O. oeni* strain. This might be due to the *Lb. plantarum* strains that only started to utilise citrate during the late exponential phase as discovered by Drinan *et al.* (1976). The same trend was observed in the 50 g/L treatment than in the 115 g/L treatment. The cit<sup>+</sup> *O. oeni* strain produced 79% less diacetyl than the cit<sup>+</sup> *Lb. plantarum* strain on the last sampling day and 8% more diacetyl on day 5 in the 115 g/L treatment. The cit<sup>-</sup> *Lb. plantarum* strain also produced more diacetyl than the cit<sup>-</sup> *O. oeni* strain since this cit<sup>-</sup> *O. oeni* strain only produced trace amounts of diacetyl. The *Lb. plantarum* strains consumed more fructose than the *O. oeni* strains and could therefore have produced more diacetyl from the pyruvate formed during glycolysis.

**Table 3.11** Diacetyl concentrations (g/L) in the fructose treatments inoculated with the four LAB strains. The last sampling day for *O. oeni* IWBT B065 was day 8 and day 21 for the rest of the strains in all the treatments.

Treatment	Lactic acid bacteria			
	<i>O. oeni</i> IWBT B065	Viniflora® CiNe™	<i>Lb. plantarum</i> IWBT B205	<i>Lb. plantarum</i> IWBT B382
<b>115 g/L fructose</b>				
Day 2	16.75 ± 0.96	0.12 ± 0.02	3.86 ± 0.60	8.68 ± 0.84
Day 5	25.05 ± 0.46	0.10 ± 0.04	2.59 ± 0.12	11.77 ± 1.34
Day 8	19.67 <sup>A</sup> ± 0.69			
Day 21		0.00 ± 0.00	6.39 <sup>A</sup> ± 0.68	22.02 <sup>A</sup> ± 0.98
<b>50 g/L fructose</b>				
Day 2	5.33 ± 0.62	0.14 ± 0.05	3.61 ± 0.17	10.48 ± 0.55
Day 5	12.93 ± 0.46	0.14 ± 0.00	3.92 ± 0.36	11.35 ± 0.84
Day 8	1.55 <sup>B</sup> ± 0.51			
Day 21		0.15 ± 0.17	2.54 <sup>B</sup> ± 0.21	7.41 <sup>C</sup> ± 0.53
<b>2.5 g/L fructose</b>				
Day 2	0.04 ± 0.02	0.06 ± 0.00	1.13 ± 0.07	3.63 ± 0.39
Day 5	0.02 ± 0.01	0.10 ± 0.06	0.93 ± 0.06	6.74 ± 0.06
Day 8	0.03 ± 0.00			
Day 21		0.15 ± 0.06	2.51 <sup>B</sup> ± 0.15	11.08 <sup>B</sup> ± 1.11



Values on last sampling day with different alphabetical letters are significantly different ( $p < 0.05$ ) between different treatments inoculated with a specific strain.

Significant differences were only calculated on values above the minimum quantification limit.

Minimum quantification limit for diacetyl was 0.5 mg/L.

The acetoin concentrations produced in the fructose treatments inoculated with the four LAB strains are indicated in **Table 3.12**. As was seen in the glucose treatments, the acetoin concentrations increased as the diacetyl concentrations decreased during the fermentation in most treatments. However, in the 115 g/L fructose treatment inoculated with the cit<sup>-</sup> *O. oeni* strain the acetoin concentrations decreased over time. This might be due to the acetoin that can be further reduced to 2,3-butanediol. Furthermore, there was no significant difference in acetoin concentrations between day 5 and day 21 in the 50 g/L treatment inoculated with the cit<sup>-</sup> *Lb. plantarum* strain, which suggest that this strain produced the maximum acetoin concentrations on day 5 in this treatment.

The fructose treatments had a significant effect on the acetoin concentrations produced by the cit<sup>+</sup> *O. oeni* strain in all the treatments and in the 2.5 g/L treatment inoculated with the cit<sup>-</sup> *O. oeni* strain. On day 8, the cit<sup>+</sup> *O. oeni* strain produced 37% less acetoin in the 50 g/L treatment than in the 115 g/L treatment and only trace amounts of acetoin in the 2.5 g/L treatment. This trend was also observed for the diacetyl concentrations produced by this cit<sup>+</sup> strain in the fructose treatments, which could indicate that high fructose concentrations might stimulate the production of diacetyl and acetoin by this strain. The cit<sup>-</sup> *O. oeni* strain produced trace amounts of acetoin in the 115 g/L and 50 g/L treatments on day 21. However, in the 2.5 g/L treatment this strain produced significantly more acetoin than in the other two treatments. This significant higher acetoin concentration at the end of the fermentation could be related to the fructose consumption as this strain consumed more fructose in the 2.5 g/L treatment than in the other two treatments (**Figure 3.8**).

The *Lb. plantarum* strains also produced significantly different amounts of acetoin in the different fructose treatments. Both, *Lb. plantarum* strains produced the maximum acetoin concentration in the 115 g/L treatments. The cit<sup>+</sup> *Lb. plantarum* strain produced 41% and 49% less acetoin in the 50 g/L and 2.5 g/L treatments than in the 115 g/L treatment on day 21, respectively. The cit<sup>-</sup> *Lb. plantarum* strain produced 38% and 27% less acetoin in the 50 g/L and 2.5 g/L treatments than in the 115 g/L treatment on day 21, respectively. This trend was also observed in the treatments inoculated with the cit<sup>+</sup> *O. oeni* strain and further supports the hypothesis that high fructose concentrations could stimulate the production of diacetyl and acetoin. The acetoin concentrations produced by the cit<sup>-</sup> *Lb. plantarum* strain in the 115 g/L treatment and by cit<sup>+</sup> *Lb. plantarum* strain in all the treatments were above the 150 mg/L sensory threshold level and could potentially have given rise to an overwhelming buttery, creamy aroma (Malherbe *et al.*, 2012).

An ANOVA followed by a Fischer LSD test was performed to determine significant differences between the various strains within a specific treatment with the data collected from the last sampling day which was day 8 for the cit<sup>+</sup> *O. oeni* strain and day 21 for the other strains (data not shown). As was seen for the diacetyl concentrations, the cit<sup>+</sup> strains produced significantly

more acetoin than the cit<sup>-</sup> strains, except in the 2.5 g/L treatment inoculated with the *O. oeni* strains. In this 2.5 g/L treatment, the cit<sup>-</sup> *O. oeni* strain produced significantly more acetoin than the cit<sup>+</sup> *O. oeni* strain, even though the cit<sup>+</sup> strain completely consumed citrate. The cit<sup>-</sup> *O. oeni* strain consumed 0.46 g/L more fructose than the cit<sup>+</sup> *O. oeni* strain towards the end of the fermentation in the 2.5 g/L treatment and could therefore produce more acetoin from the pyruvate formed during glycolysis.

The *Lb. plantarum* strains also produced more acetoin than the *O. oeni* strains as has been noted for the diacetyl concentrations. This difference was more pronounced for the acetoin concentrations, since the cit<sup>+</sup> *O. oeni* strain produced less acetoin than the cit<sup>+</sup> *Lb. plantarum* strain in all the treatments on the last sampling day. The largest difference between the cit<sup>+</sup> strains were observed in the 2.5 g/L treatment where the cit<sup>+</sup> *O. oeni* strain produced 0.77 mg/L acetoin compared to the 362.68 mg/L acetoin produced by the cit<sup>+</sup> *Lb. plantarum* strain at the last sampling point. The treatments inoculated with cit<sup>-</sup> *O. oeni* strain had only trace amounts of acetoin and was therefore less than the cit<sup>-</sup> *Lb. plantarum* strain at the end of the fermentation.

**Table 3.12** Acetoin concentrations (g/L) in the fructose treatments inoculated with the four LAB strains. The last sampling day for *O. oeni* IWBT B065 was day 8 and day 21 for the rest of the strains in all the treatments.

Treatment	Lactic acid bacteria							
	<i>O. oeni</i> IWBT B065		Viniflora® CiNe™		<i>Lb. plantarum</i> IWBT B205		<i>Lb. plantarum</i> IWBT B382	
<b>115 g/L fructose</b>								
Day 2	12.29	± 0.26	3.06	± 0.20	64.12	± 2.74	155.68	± 11.45
Day 5	45.09	± 0.61	0.00	± 0.00	89.15	± 0.66	340.34	± 14.28
Day 8	37.85 <sup>A</sup>	± 0.36						
Day 21			0.00	± 0.00	225.61 <sup>A</sup>	± 7.00	499.32 <sup>A</sup>	± 11.57
<b>50 g/L fructose</b>								
Day 2	8.96	± 0.47	0.00	± 0.00	58.78	± 9.17	185.24	± 14.52
Day 5	17.04	± 1.97	0.00	± 0.00	134.23	± 1.49	285.74	± 13.20
Day 8	23.79 <sup>B</sup>	± 7.97						
Day 21			0.00	± 0.00	132.75 <sup>B</sup>	± 16.18	308.11 <sup>C</sup>	± 9.91
<b>2.5 g/L fructose</b>								
Day 2	0.53	± 0.02	0.00	± 0.00	22.15	± 0.85	121.07	± 9.51
Day 5	0.59	± 0.00	0.00	± 0.00	58.67	± 16.72	90.89	± 7.91
Day 8	0.77 <sup>C</sup>	± 0.00						
Day 21			11.43	± 1.92	113.7 <sup>B</sup>	± 5.95	362.68 <sup>B</sup>	± 11.22

<49.99	
50.00 - 99.99	
100.00 - 199.99	
200.00 - 299.99	
>300	

Values on last sampling day with different alphabetical letters are significantly different ( $p < 0.05$ ) between different treatments inoculated with a specific strain.

Significant differences were only calculated on values above the minimum quantification limit.

Minimum quantification limit for acetoin was 0.5 mg/L.

### 3.4.2.3 Effect of pH on citrate metabolism

#### 3.4.2.3.1 Malate and citrate consumption

The cell counts and malate degradation duration by the four different LAB strains in the pH treatments are indicated in **Table 3.13**. All the strains were inoculated between  $1 \times 10^7$  cfu/mL to  $8.5 \times 10^7$  cfu/mL. This variation between the initial cell counts of the different strains could potentially have influenced malate and citrate consumption and the concentrations of metabolites produced by the different strains. The cell counts of all the strains decreased two days after inoculation. This might be due to the 14% ethanol that was present in the synthetic wine medium. The cell counts of the *O. oeni* strains decreased faster in the pH 5.0 treatment, whereas the cell counts of the *Lb. plantarum* strains decreased faster in the pH 3.0 treatment. This is in agreement with the optimum growth pH of the different species. *O. oeni* is known to be better adapted to harsh wine conditions such as low pH and high ethanol concentrations (Drici-Cachon *et al.*, 1996), whereas *Lb. plantarum* prefers to grow in high pH and low ethanol environments (Sedewitz *et al.*, 1984).

Malate was completely degraded by the cit<sup>+</sup> *O. oeni* strain in all the treatments ranging from two to five days (**Table 3.13**). In the pH 5.0 treatment, malate was completely degraded after five days, whereas malate was completely consumed after two days in the pH 3.5 and 4.0 treatments and after two and a half days in the pH 3.0 treatment. The cit<sup>-</sup> *O. oeni* strain completely degraded malate after two and a half days in the pH 3.5 and 4.0 treatments and after eight days in the pH 3.0 treatment. However, in the pH 5.0 treatment this strain only degraded 80% of the malate after 21 days. The cit<sup>+</sup> *O. oeni* strain degraded malate over a shorter period than the cit<sup>-</sup> *O. oeni* and were able to sustain its cell counts for longer than the cit<sup>-</sup> *O. oeni* in most of the treatments. This might be related to the citrate consumption as the cit<sup>+</sup> strain consumed more citrate than the cit<sup>-</sup> strain (**Figure 3.13A and B**) as previous studies have indicated that citrate can be a carbon and energy source for cit<sup>+</sup> bacteria (Starrenburg and Hugenholtz, 1991; Hugenholtz *et al.*, 1993; Marty-Teyssset *et al.*, 1996; Medina de Figueroa *et al.*, 2000; Jyoti *et al.*, 2004; Sánchez *et al.*, 2008). The *Lb. plantarum* strains displayed a similar trend with regards to the malate degradation. In the pH 3.5, 4.0 and 5.0 treatments, the *Lb. plantarum* strains completely degraded malate ranging from one to two and a half days. The fastest malate degradation period by these strains was seen in the pH 5.0 treatment. In the pH 3.0 treatment, the cit<sup>-</sup> and cit<sup>+</sup> *Lb. plantarum* strains only degraded 66% and 53% in the pH 3.0 treatment after 21 days, respectively. This is in agreement with the cell counts of the different strains as well as their optimum growth pH, as previously mentioned.

**Table 3.13** Overview of cell counts (cfu/mL) and malate degradation duration (days) of the four LAB strains in the pH treatments.

Strain	Days												Malate degradation duration	
	0	2	4	8	14	21								
pH 3.0														
<i>O. oeni</i> IWBT B065	6.20E+07	± 7.07E+06	2.55E+07	± 4.95E+06	2.02E+07	± 3.25E+06	1.11E+07	± 1.41E+06					2.5	
Viniflora® CiNe™	3.00E+07	± 2.83E+06	1.64E+07	± 1.85E+06	1.41E+07	± 4.36E+05	1.20E+06	± 2.76E+06	1.15E+05	± 1.00E+05	<1.00E+05			8.0
<i>Lb. plantarum</i> IWBT B205	3.07E+07	± 3.51E+06	5.60E+06	± 5.66E+05	1.74E+06	± 1.63E+05	8.20E+04	± 4.24E+03	<1.00E+03				sf	
<i>Lb. plantarum</i> IWBT B382	1.83E+07	± 2.47E+06	1.45E+06	± 7.07E+04	1.60E+06	± 1.41E+05	3.57E+04	± 3.79E+03	<1.00E+03				sf	
pH 3.5														
<i>O. oeni</i> IWBT B065	5.70E+07	± 1.41E+06	2.30E+07	± 2.83E+06	1.88E+07	± 4.95E+05	3.30E+06	± 2.83E+05					2.0	
Viniflora® CiNe™	5.70E+07	± 8.49E+06	5.65E+07	± 9.19E+06	2.56E+07	± 3.39E+06	2.40E+06	± 1.05E+05	1.06E+06	± 3.82E+05	8.00E+05	± 5.56E+06	2.5	
<i>Lb. plantarum</i> IWBT B205	4.20E+07	± 9.90E+06	1.47E+07	± 1.91E+06	1.53E+07	± 2.76E+06	1.34E+07	± 2.21E+06	4.70E+06	± 2.83E+05	3.20E+06	± 3.61E+05	2.5	
<i>Lb. plantarum</i> IWBT B382	4.20E+07	± 4.24E+06	1.34E+07	± 7.07E+05	1.32E+07	± 2.83E+06	1.19E+07	± 2.12E+06	6.15E+06	± 9.19E+05	2.65E+06	± 7.78E+05	2.5	
pH 4.0														
<i>O. oeni</i> IWBT B065	8.25E+07	± 9.19E+06	6.10E+07	± 7.07E+06	3.65E+06	± 1.34E+06	4.00E+05	± 2.25E+05					2.0	
Viniflora® CiNe™	3.30E+07	± 1.41E+06	5.03E+07	± 7.77E+06	3.85E+07	± 1.06E+07	3.95E+06	± 7.07E+04	7.25E+06	± 7.07E+04	6.50E+06	± 7.70E+05	2.5	
<i>Lb. plantarum</i> IWBT B205	2.35E+07	± 2.10E+06	1.40E+07	± 2.83E+06	2.75E+07	± 2.12E+06	4.90E+07	± 2.83E+06	2.11E+07	± 3.11E+06	8.57E+06	± 3.21E+05	2.0	
<i>Lb. plantarum</i> IWBT B382	1.66E+07	± 1.06E+06	9.30E+06	± 1.25E+06	1.43E+07	± 1.34E+06	2.11E+07	± 2.69E+06	1.77E+07	± 2.55E+06	7.30E+06	± 1.84E+06	2.0	
pH 5.0														
<i>O. oeni</i> IWBT B065	8.45E+07	± 2.12E+06	7.55E+07	± 7.78E+06	4.45E+07	± 1.48E+06	3.47E+06	± 1.20E+06					5.0	
Viniflora® CiNe™	3.55E+07	± 3.54E+06	4.50E+06	± 2.83E+05	6.03E+05	± 7.77E+04	<1.00E+04					sf		
<i>Lb. plantarum</i> IWBT B205	4.05E+07	± 4.95E+06	3.75E+07	± 7.07E+05	6.75E+07	± 7.07E+05	5.84E+07	± 2.26E+06	3.03E+07	± 4.78E+06	4.75E+06	± 3.54E+05	1.5	
<i>Lb. plantarum</i> IWBT B382	3.95E+07	± 7.07E+05	2.85E+07	± 6.36E+06	3.75E+07	± 3.54E+06	3.31E+07	± 2.97E+06	1.43E+07	± 1.32E+06	1.85E+06	± 2.12E+05	1.0	

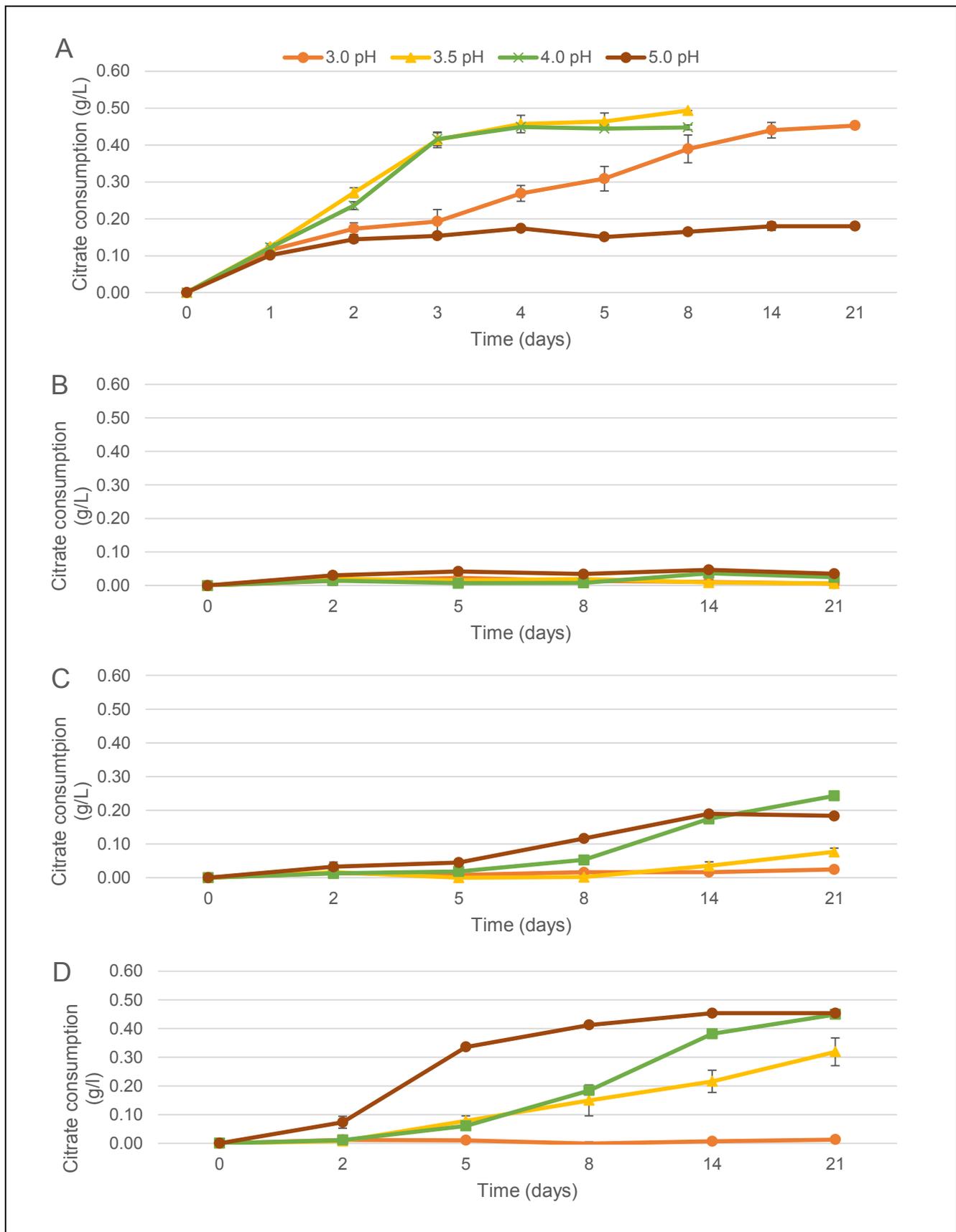
sf- stuck malate degradation

Data shown are averages of triplicates with standard deviation.

Citrate consumption by the four LAB in the different pH treatments are indicated in **Figure 3.13**. Citrate was completely consumed by the cit<sup>+</sup> *O. oeni* strain in the pH 3.0, 3.5 and 4.0 treatments (**Figure 3.13A**). Citrate consumption was delayed in the pH 3.0 treatment. On day 8, citrate was completely consumed in the pH 3.5 and 4.0 treatments compared to 0.39 g/L in the pH 3.0 treatment. Citrate was completely consumed on day 21 in the pH 3.0 treatment. In the pH 5.0 treatment, this cit<sup>+</sup> *O. oeni* strain only consumed 34% of the initial citrate after 21 days. The cell counts also rapidly decreased from 10<sup>7</sup> cfu/mL on day 0 to 10<sup>4</sup> cfu/mL on day 8 in the pH 5.0 treatment which provides a possible reason for the partial citrate consumption in this treatment (**Table 3.13**). These results indicate that this strain was not able to survive in the pH 5.0 treatment, which corresponds with previous results that indicated that *O. oeni* is better adapted to low pH environments (Drici-Cachon *et al.*, 1996). The cit<sup>-</sup> *O. oeni* strain did not consume any of the citrate as was seen in the sugar treatments (**Figure 3.13B**).

As has been noted in the sugar treatments, the cit<sup>-</sup> *Lb. plantarum* strain only partially consumed citrate (**Figure 3.13C**). This strain consumed 36% and 48% citrate in the pH 5.0 and 4.0 treatments after 21 days, respectively. It is important to note that this strain was selected as a cit<sup>-</sup> *Lb. plantarum* strain since it did not consume any citrate in the preliminary studies performed in synthetic juice with a pH of 3.1. In the pH 3.0 and 3.5 treatments, this cit<sup>-</sup> *Lb. plantarum* strain consumed trace amounts of citrate which is in agreement with the preliminary results. The lower citrate consumption in the lower pH treatments might be related to either the optimum growth pH of this strain or to the citrate transporter. Since, the cell counts in the pH 3.5 treatment was the same as in the pH 4.0 and 5.0 treatment, the lower citrate consumption might rather be related to the citrate transporter that only transports divalent citrate present above a pH of 4 (Konings, 2002).

The cit<sup>+</sup> *Lb. plantarum* strain completely consumed citrate in the pH 4.0 and 5.0 treatments. The citrate consumption was delayed in the pH 4.0 treatment, since citrate was completely consumed (0.5 g/L) in the pH 5.0 treatment after 14 days compared to 0.38 g/L citrate that was consumed in the pH 4.0 treatment on day 14. In the pH 4.0 treatment, citrate was completely consumed after 21 days. This can be attributed to the slightly higher cell counts in the pH 5.0 treatment (**Table 3.13**). In the pH 3.5 treatment, only 64% of the citrate was consumed by this cit<sup>+</sup> *Lb. plantarum* strain after 21 days. These results are in agreement with previous studies that indicated that *Lb. plantarum* utilise citrate at an optimum rate when the pH of the medium is within a range of 4.5 to 5.0 (Montville *et al.*, 1987; Kennes *et al.*, 1991; Palles *et al.*, 1998). This cit<sup>+</sup> *Lb. plantarum* strain did not consume any citrate in the pH 3.0 treatment, since this strain was not able to maintain its cell counts in this treatment (**Table 3.13**). The cell counts of this strain rapidly decreased from 10<sup>7</sup> cfu/mL on day 0 to less than 10<sup>3</sup> cfu/mL on day 21. A previous study by Sedewitz *et al.* (1984) also indicated that *Lb. plantarum* are not able to survive in a medium with a low pH and high ethanol concentration like the medium used in this study.



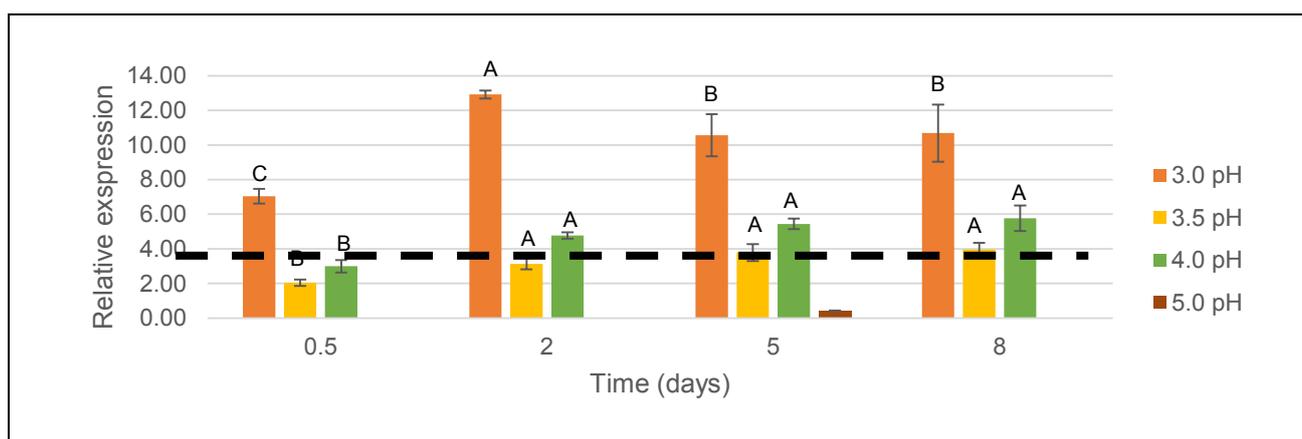
**Figure 3.13** Monitoring citrate consumption by A) *O. oeni* IWBT B065, B) *Viniflora*® CiNe™, C) *Lb. plantarum* IWBT B205 and D) *Lb. plantarum* IWBT B382 in the 3.0 (orange), 3.5 (yellow), 4.0 (green) and 5.0 (dark red) pH treatments. Data shown are mean values of triplicates and error bars represent the standard deviations.

### 3.4.2.3.2 Relative expression of *citE*

The influence of pH on citrate metabolism was further investigated by determining the RE of *citE* throughout the fermentation as was described for the glucose and fructose treatments. However, the RE for the *cit*<sup>+</sup> *O. oeni* strain was only determined on day 5 in the pH 5.0 treatment, since citrate was only partially consumed in this treatment (**Figure 3.13A**).

The RE of the *citE* gene in the *cit*<sup>+</sup> *O. oeni* strain is indicated in **Figure 3.14**. A transcriptional response was first observed on day 0.5 in the pH 3.0, 3.5 and 4.0 treatments. Thereafter the RE increased on day 2 in these pH treatments. The RE on day 2 was 5.8 fold more than on day 0.5 in the pH 3.0 treatment. In the pH 3.5 and 4.0 treatments, the RE on day 2 was more than one fold higher than on day 0.5. The RE slightly decreased from day 2 to day 5 in the pH 3.0 treatment and thereafter remained unchanged until the end of the fermentation. In the pH 3.5 and 4.0 treatments, the RE was stable from day 2 onwards until the end of the fermentation. There was no transcriptional response in the pH 5.0 treatment on day 5. The highest RE was seen in the pH 3.0 treatment inoculated with the *cit*<sup>+</sup> *O. oeni* strain. This could be related to the delayed citrate consumption and longer malate degradation period in this treatment. This was also observed previously in the fructose treatments and by Olguín *et al.* (2010) which suggested that the bacteria incorporate the higher RE and delay citrate as a survival mechanism for when malate has been depleted.

A RE was observed for the other bacterial strains in all the treatments, but there was not a relevant transcriptional response. This was unexpected, since the *cit*<sup>+</sup> *Lb. plantarum* strain completely consumed citrate in the pH 4.0 and 5.0 treatments. A relevant transcriptional response might have been on day 8 or day 14 which was not measured in this study.



**Figure 3.14** Relative gene expression of the *citE* gene in *O. oeni* IWBT B065 in the different pH treatments. Data shown are averages of triplicates with the error bars representing the standard deviations. Dashed line indicates a twofold expression level. Different alphabetical letters indicate significant differences ( $p < 0.05$ ) within a specific treatment.

### 3.4.2.3.3 Production of metabolites associated with citrate metabolism

The production of D-lactate by the different bacterial strains in the pH treatments is indicated in **Figure 3.15**. A similar trend was observed regarding the D-lactate production by the *Lb. plantarum* strains as was seen in the sugar treatments since the D-lactate production increased during the fermentation in the pH 3.5, 4.0 and 5.0 treatments (**Figure 3.15B**). The D-lactate concentrations in the pH 3.0 treatment did not increase during the fermentation since the *Lb. plantarum* strains were not able to survive for long in this treatment. In the treatments inoculated with the *O. oeni* strains the D-lactate concentrations also increased as the fermentation proceeded, but there were no significant differences observed between the days in the pH 3.0, 3.5 and 5.0 treatments inoculated with the cit<sup>+</sup> *O. oeni* strain and in the pH 3.5 and 5.0 treatments inoculated with the cit<sup>-</sup> *O. oeni* strain. The D-lactate concentrations increased from day 2 to day 5 in the pH 4.0 treatment inoculated with the cit<sup>+</sup> *O. oeni* strain and from day 5 to day 21 in the pH 3.0 treatment inoculated with the cit<sup>-</sup> *O. oeni* strain.

The different pH levels had a significant effect on the D-lactate concentrations produced by the different LAB strains (**Figure 3.15**). There was no significant difference in the pH 3.0 and 3.5 treatments inoculated with the cit<sup>+</sup> *O. oeni* strain (**Figure 3.15A**). There was also no significant difference between the pH 4.0 and 5.0 treatments inoculated with this cit<sup>+</sup> *O. oeni* strain. On day 8, the final D-lactate concentrations of 0.4 g/L in the pH 4.0 and 5.0 treatments were higher than the 0.3 g/L in the pH 3.0 and 3.5 treatments. In the pH 3.5 and 4.0 treatments inoculated with the cit<sup>-</sup> *O. oeni* strain, there was no significant difference between these treatments. In the pH 3.0 treatment inoculated with this strain the final D-lactate concentration on day 21 was 30% lower than in the pH 3.5 and 4.0 treatments. The final D-lactate concentration of 0.12 g/L in the pH 5.0 treatment inoculated with the cit<sup>+</sup> *O. oeni* strain was 53% lower than in the pH 3.5 and 4.0 treatments. The lower D-lactate concentration in the pH 5.0 treatment might be coupled to the decrease in cell counts of this strain from 10<sup>7</sup> cfu/mL on day 0 to less than 10<sup>4</sup> cfu/mL on day 21 (**Table 3.13**).

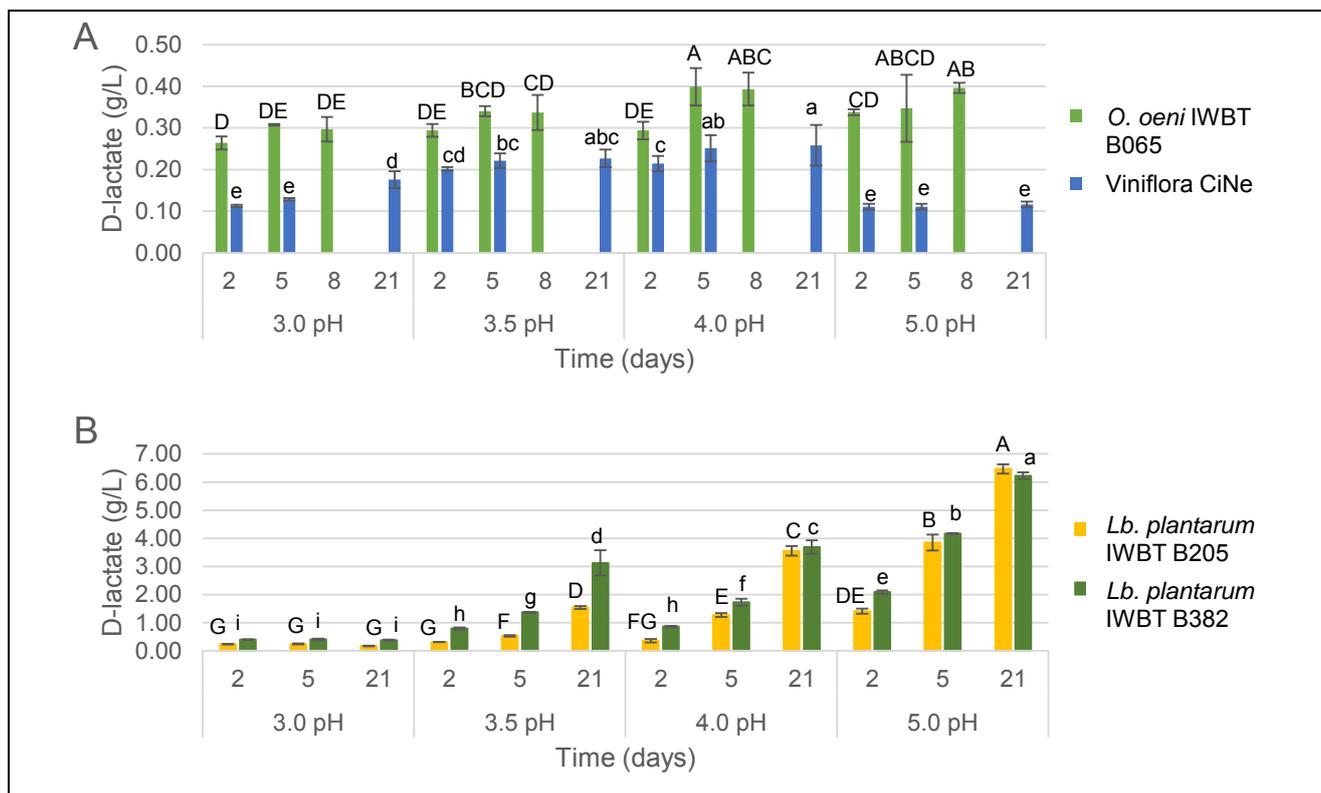
The *Lb. plantarum* strains displayed a similar trend regarding their D-lactate concentrations. The D-lactate concentrations produced by these strains increased with the increasing pH treatments (**Figure 3.15B**). The final D-lactate concentration of 0.18 g/L produced by the cit<sup>-</sup> *Lb. plantarum* strain in the pH 3.0 treatment was 97% less than the 6.47 g/L produced in the pH 5.0 treatment on day 21. In the pH 3.5 and 4.0 treatments inoculated with the cit<sup>-</sup> *Lb. plantarum* strain, the final D-lactate concentrations were also less than in the pH 5.0 treatment, but higher than the pH 3.0 treatment.

In the pH 3.0 treatment inoculated with the cit<sup>-</sup> *Lb. plantarum* strain, the final D-lactate concentration of 0.38 g/L was 93% less than the 6.47 g/L produced in the pH 5.0 treatment on day 21. The final D-lactate concentrations in the pH 3.5 and 4.0 treatments were only 49% and 40% less than in the pH 5.0 treatment inoculated with the cit<sup>+</sup> *Lb. plantarum* strain, but still higher than in

the pH 3.0 treatment. The lower D-lactate concentrations in the pH 3.0 treatments might be due to the *Lb. plantarum* strains not being able to survive in these harsh environments as the cell counts of these bacteria decreased from  $10^7$  cfu/mL on day 0 to less than  $10^3$  cfu/mL on day 21 (**Table 3.13**). These results also correlates with the results from Montville *et al.* (1987) which indicated that D-lactate production was inhibited by pH values below 4.5 as a mechanism to prevent over acidification in LAB.

An ANOVA followed by a Fischer LSD test was performed to determine significant differences between the various strains within a specific treatment with the data obtained from the last sampling day which was day 8 for the cit<sup>+</sup> *O. oeni* strain and day 21 for the other strains (data not shown). The cit<sup>+</sup> *O. oeni* strain had significantly higher final D-lactate concentrations than the cit<sup>-</sup> *O. oeni* strain in all the treatments. This was expected as the cit<sup>+</sup> *O. oeni* strain consumed more citrate than the cit<sup>-</sup> *O. oeni* strain in all the treatments. The cit<sup>+</sup> *Lb. plantarum* strain had significantly more D-lactate than the cit<sup>-</sup> *Lb. plantarum* strain in the pH 3.0 and 3.5 treatments on day 21. In the pH 3.0 treatment, the cit<sup>-</sup> *Lb. plantarum* strain produced 52% less D-lactate than the cit<sup>+</sup> *Lb. plantarum* strain. The difference between these two strains was slightly less in the pH 3.5 treatment. However, there was no significant difference between the *Lb. plantarum* strains in the pH 4.0 and 5.0 treatments despite the fact that the cit<sup>+</sup> *Lb. plantarum* strain completely consumed citrate and the cit<sup>-</sup> *Lb. plantarum* strain only partially consumed citrate in these treatments.

On the last sampling day, the *Lb. plantarum* strains produced more D-lactate than the *O. oeni* strains in the pH 3.5, 4.0 and 5.0 treatments as was seen for the sugar treatments. The maximum D-lactate concentration of 6.23 g/L produced by the cit<sup>+</sup> *Lb. plantarum* strain in the pH 5.0 treatment on day 21 was 16 times more than the maximum D-lactate concentration of 0.40 g/L produced by the cit<sup>+</sup> *O. oeni* strain in the pH 5.0 treatment on day 8. The maximum D-lactate concentration of 0.26 g/L produced by the cit<sup>-</sup> *O. oeni* strain in the pH 4.0 treatment on day 21 was 24 times less than the maximum of 6.47 g/L produced by the cit<sup>-</sup> *Lb. plantarum* strain in the pH 5.0 treatment on day 21. However, the cit<sup>-</sup> strains produced the same D-lactate concentration of 0.18 g/L in the 3.0 pH treatment. These differences between the LAB strains are in agreement with the cell counts and citrate consumption by the individual species.



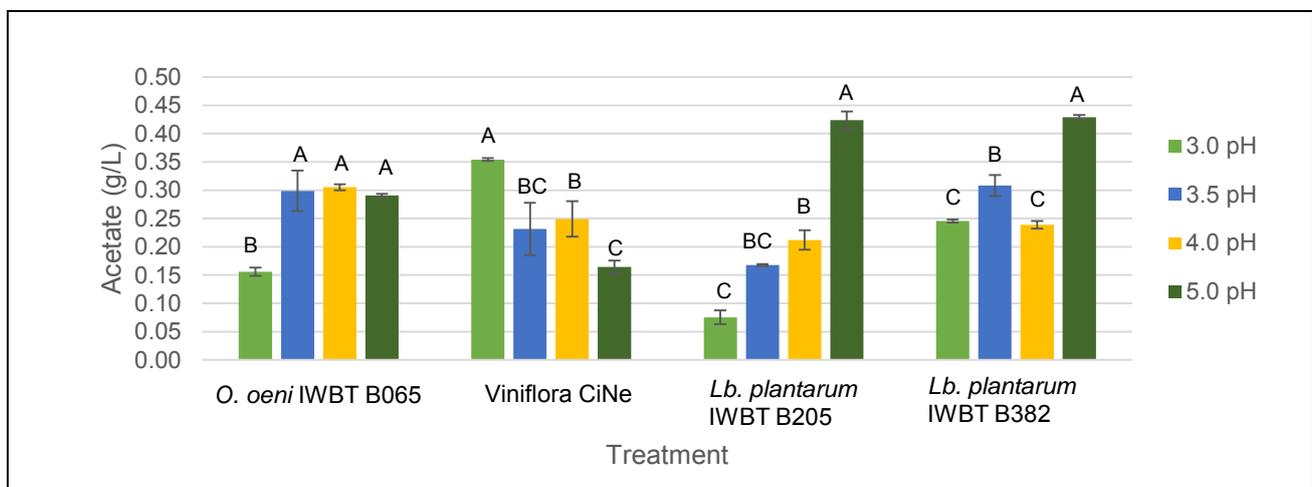
**Figure 3.15** Concentrations of D-lactate produced by the *O. oeni* strains (A) and by the *Lb. plantarum* strains (B) in the pH treatments. The last sampling day was day 8 for *O. oeni* IWBT B065 and day 21 for the other bacterial strains. Data shown are averages of triplicates with error bars representing the standard deviation. Different alphabetical letters indicate significant differences ( $p < 0.05$ ) between treatments of an individual strain.

The acetate concentrations produced in the pH treatments are indicated in **Figure 3.16**. Only trends can be used as mentioned previously. The pH 3.5, 4.0 and 5.0 treatments inoculated with the  $\text{cit}^+$  *O. oeni* strain had approximately the same amount of acetate. The acetate concentration in the pH 3.0 treatment inoculated with this  $\text{cit}^+$  *O. oeni* strain was less than in the rest of the pH treatments inoculated with this strain. This strain could have produced less acetate in the lower pH treatment as a mechanism to prevent over acidification of the cell. A previous study performed by Ramos *et al.* (1995) observed that acetate concentrations increased with decreasing pH levels as was seen in the treatments inoculated with the  $\text{cit}^-$  *O. oeni* strain. This might indicate that the  $\text{cit}^+$  and  $\text{cit}^-$  *O. oeni* strains react differently to the different treatments. The  $\text{cit}^-$  *O. oeni* strain produced more acetate in the pH 3.0 treatment than in the pH 3.5 and 4.0 treatments. This might be due to this strain that could have consumed more sugar in the lower pH treatments to produce more acetate.

The two *Lb. plantarum* strains displayed a similar trend regarding their acetate concentrations in the different treatments. These strains produced more acetate with the increasing pH treatments. The acetate concentration produced by the  $\text{cit}^-$  *Lb. plantarum* strain in the pH 3.0 treatment was less than in the other pH treatments. The  $\text{cit}^+$  *Lb. plantarum* strain produced less acetate in the pH 3.0 and 4.0 treatments than in the pH 3.5 and 5.0 treatments. The pH 3.5

treatment inoculated with this strain had an acetate concentration slightly higher than in the pH 3.5 and 4.0 treatments, but lower than the pH 5.0 treatment. These results are in agreement with a previous study by Kennes *et al.* (1991) that also observed that *Lb. plantarum* produces more acetate in a medium with a higher pH, which is in contrast with what was observed by Ramos *et al.* (1995) for *O. oeni* strains.

The cit<sup>+</sup> *O. oeni* strain produced more acetate than the cit<sup>-</sup> *O. oeni* strain in the pH 3.5, 4.0 and 5.0 treatments. This correlates with the citrate consumption results since the cit<sup>+</sup> *O. oeni* strain consumed more citrate than the cit<sup>-</sup> *O. oeni* strain. In contrast, the cit<sup>+</sup> *O. oeni* strain produced less acetate than cit<sup>-</sup> *O. oeni* strain in the pH 3.0 treatment, which could be due to the cit<sup>-</sup> *O. oeni* strain that might have consumed more sugar in this treatment. The cit<sup>+</sup> *Lb. plantarum* strain also produced more acetate than the cit<sup>-</sup> *Lb. plantarum* strain in the pH 3.0 and 3.5 treatments. In the pH 4.0 and 5.0 treatments the strains produced similar amounts of acetate, even though the cit<sup>+</sup> *Lb. plantarum* strain completely consumed citrate in these treatments. The cit<sup>+</sup> *Lb. plantarum* strain produced more acetate than the cit<sup>+</sup> *O. oeni* strain in the pH 3.0 and 5.0 treatment, but the cit<sup>+</sup> *O. oeni* strain produced more acetate than the cit<sup>+</sup> *Lb. plantarum* strain in the 4.0 pH treatments. The cit<sup>+</sup> strains produced similar amounts of acetate in the pH 3.5 treatment. The cit<sup>-</sup> *O. oeni* strain produced more acetate in the pH 3.0, 3.5 and 4.0 treatments, but less in the pH 5.0 treatment than the cit<sup>-</sup> *Lb. plantarum* strain.



**Figure 3.16** Acetate concentrations in the pH treatments at the last sampling point. The last sampling day was day 8 for *O. oeni* IWBT B065 and day 21 for the rest of the bacterial strains. Data shown are averages of triplicates with error bars representing the standard deviation. Different alphabetical letters indicate significant differences ( $p < 0.05$ ) between the treatments inoculated with a specific strain.

The diacetyl concentrations produced by the four LAB strains in the different pH treatments are indicated in **Table 3.14**. The diacetyl concentrations increased as the LAB consumed citrate in the pH 3.0 and 3.5 treatments inoculated with the cit<sup>+</sup> *O. oeni* strain as well as in the pH 3.5 and 4.0 treatments inoculated with the *Lb. plantarum* strains. In the pH 5.0 treatment inoculated with the cit<sup>-</sup> *Lb. plantarum* strain, the diacetyl concentrations only increased from day 2 to day 5, but decreased on day 21. The same trend was observed in the pH 5.0 treatment inoculated with the

cit<sup>+</sup> *Lb. plantarum* strain where the diacetyl concentrations increased from 1.91 mg/L on day 2 to 8.55 mg/L on day 5, but decreased from 8.55 mg/L on day 5 to 5.77 mg/L on day 21. This decrease might be due to the diacetyl that can be further reduced to acetoin by the LAB strains as an increase in acetoin concentrations was seen in these treatments (**Table 3.15**). In the pH 3.0 treatment inoculated with the *Lb. plantarum* strains the diacetyl concentrations were approximately the same between the different days. The cit<sup>-</sup> *O. oeni* strain only produced trace amounts of diacetyl in all the treatments, which is in agreement with this strain not being able to consume citrate.

The pH treatments had a significant effect on the final diacetyl concentrations produced by the LAB strains. The maximum diacetyl concentration of 25.09 mg/L produced by the cit<sup>+</sup> *O. oeni* strain was in the pH 3.0 treatment on day 8. In the pH 3.5 treatment inoculated with this strain, the final diacetyl concentration of 18.52 mg/L was 26% less than in the pH 3.0 treatment. The diacetyl concentrations produced in the pH 3.0 and 3.5 treatments could have potentially given rise to an overwhelming buttery aroma that could have mask the fruity and/or vegetative aromas in wine, since the concentrations were higher than the 5 mg/L limit of desirable diacetyl suggested by previous studies (Martineau and Henick-Kling, 1995; Bartowsky and Henschke, 2004). In the pH 4.0 and 5.0 treatments, the cit<sup>+</sup> *O. oeni* strain only produced trace amounts of diacetyl which was below the minimum quantification limit. These results might be linked to the acetate concentrations as this cit<sup>+</sup> *O. oeni* strain produced less acetate in the pH 3.0 treatment (**Figure 3.16**) and could have produced more diacetyl in this treatment from pyruvate. In a review by Bartowsky and Henschke (2004) they also indicated that diacetyl concentrations usually increases as the pH decreases as was seen in this study.

The *Lb. plantarum* strains also produced different amounts of diacetyl in the different pH treatments. The cit<sup>-</sup> *Lb. plantarum* strain produced a maximum diacetyl concentration of 3.79 mg/L in the pH 3.5 treatment. The final diacetyl concentration of 1.79 mg/L in the pH 4.0 treatment inoculated with this strain was 52% less than the maximum diacetyl produced by this strain in the pH 3.5 treatment. In the pH 3.0 and 5.0 treatments, the cit<sup>-</sup> *Lb. plantarum* strain only produced trace amounts of diacetyl. The cit<sup>+</sup> *Lb. plantarum* strain completely consumed citrate in the pH 4.0 and 5.0 treatments (**Figure 3.13**) and thus produced significantly more diacetyl in these treatments than in the pH 3.0 and 3.5 treatments. The maximum diacetyl concentration of 7.39 mg/L was produced by this strain in the pH 4.0 treatment on day 21. The final diacetyl concentration of 5.77 mg/L and 5.32 mg/L in the pH 5.0 and 3.5 treatments, respectively, was 21% and 22% less than the maximum diacetyl produced by the cit<sup>+</sup> *Lb. plantarum* strain on day 21 in the pH 4.0 treatment. The final diacetyl concentration of 1.02 mg/L produced by this strain in the pH 3.0 treatment was significantly lower than in the other three treatments. This might be due to the strain not being able to maintain its cell counts in this treatment as there was a decrease in the cell counts of this strain (**Table 3.13**). The final diacetyl concentrations in the pH 3.5, 4.0 and 5.0

treatments inoculated with the cit<sup>+</sup> *Lb. plantarum* strain were within the range of desirable diacetyl and could have contributed to a desirable buttery aroma.

A one-way ANOVA followed by a Fischer LSD test was performed to determine significant differences between the various strains within a specific treatment with the data obtained from the last sampling day which was day 8 for the cit<sup>+</sup> *O. oeni* strain and day 21 for the other strains (data not shown). The cit<sup>+</sup> *O. oeni* strain produced significantly more diacetyl than the cit<sup>-</sup> *O. oeni* strain in the pH 3.0 and 3.5 treatments, since the cit<sup>-</sup> *O. oeni* strain only produced trace amounts of diacetyl. The difference between these strains was expected, since the cit<sup>+</sup> strain completely consumed citrate and the cit<sup>-</sup> strain did not (**Figure 3.13**). However, there was no significant difference between the *O. oeni* strains at the last sampling point in the pH 4.0 and 5.0 treatments, since both of these strains produced only trace amounts of diacetyl. The cit<sup>+</sup> *Lb. plantarum* strain also produced significantly more diacetyl than the cit<sup>-</sup> *Lb. plantarum* strain in all the pH treatments. This was not surprising as the cit<sup>+</sup> *Lb. plantarum* strain consumed more citrate than the cit<sup>-</sup> *Lb. plantarum* strain.

There were also differences between the LAB species regarding their final diacetyl concentrations in the treatments. In the pH 3.0 and 3.5 treatments, the cit<sup>+</sup> *O. oeni* strain produced 24 and 3.5 times more diacetyl than the cit<sup>+</sup> *Lb. plantarum* strain, respectively. The opposite was seen in the pH 4.0 and 5.0 treatments where the cit<sup>+</sup> *Lb. plantarum* strain produced more diacetyl than the cit<sup>+</sup> *O. oeni* strain, since the cit<sup>+</sup> *O. oeni* strain only produced trace amounts of diacetyl. These differences might be related to the optimum growth pH and citrate utilisation of the different LAB species as previously mentioned. Since, the cit<sup>-</sup> *O. oeni* strain consumed less citrate than the cit<sup>-</sup> *Lb. plantarum* strain, the diacetyl concentrations in the treatments inoculated with the cit<sup>-</sup> *O. oeni* strain were significantly less than in the treatments inoculated with the cit<sup>-</sup> *Lb. plantarum* strain.

The acetoin concentrations produced during the fermentations in the pH treatments are indicated in **Table 3.15**. The acetoin concentrations increased as the fermentation progressed in the pH 3.0 and 3.5 treatments inoculated with the cit<sup>+</sup> *O. oeni* strain as well as in the pH 3.5, 4.0 and 5.0 treatments inoculated with the *Lb. plantarum* strains. The acetoin concentrations did not increase in the pH 4.0 and 5.0 treatments inoculated with the cit<sup>+</sup> *O. oeni* strain and in the pH 3.0 treatment inoculated with the *Lb. plantarum* strains. The cit<sup>-</sup> *O. oeni* strain only produced trace amounts of acetoin as was seen for the sugar treatments.

The different pH treatments had a significant effect on the production of acetoin by the cit<sup>+</sup> *O. oeni* strain and the *Lb. plantarum* strains. Of the treatments inoculated with the cit<sup>+</sup> *O. oeni* strain, the maximum acetoin concentration of 30.93 mg/L was seen in the pH 3.5 treatment and the lowest concentration was seen in the pH 5.0 treatment. The final acetoin concentration in the pH 3.0 treatment was 28% less than the maximum concentration in the pH 3.5 treatment. In the pH 4.0 treatment inoculated with this strain, the final acetoin concentration of 10.24 mg/L was significantly

less than the 22.04 mg/L and 30.93 mg/L concentrations produced in the pH 3.0 and 3.5 treatments. These results are in agreement with previous studies that indicated that more acetoin is produced at low pH levels (Branen and Keenan, 1971; Cogan *et al.*, 1981; Ramos *et al.*, 1995; García-Quintáns *et al.*, 2008).

The acetoin concentrations produced by the *Lb. plantarum* strains were also influenced by the different pH treatments and displayed a similar trend. Both *Lb. plantarum* strains produced maximum acetoin concentrations in the pH 4.0 treatment and the lowest in the pH 3.0 treatment. These results were in agreement with the citrate consumption data since these strains consumed more citrate in the pH 4.0 treatment than in the pH 3.0 treatment (**Figure 3.13**). The acetoin concentrations produced by the cit<sup>+</sup> *Lb. plantarum* strain in the pH 3.5, 4.0 and 5.0 treatments were above the 150 mg/L sensory threshold of acetoin and could have spoiled the wine with a creamy, buttery off-flavour (Malherbe *et al.*, 2012).

The same trend as was seen for the diacetyl was observed with regards to the acetoin concentrations between the cit<sup>+</sup> and cit<sup>-</sup> strains. The cit<sup>-</sup> *O. oeni* strain produced less acetoin than the cit<sup>+</sup> *O. oeni* strain in the pH 3.0, 3.5 and 4.0 treatments on the last sampling day. In the pH 5.0 treatment both strains produced trace amounts of acetoin. The cit<sup>-</sup> *Lb. plantarum* strain also produced less acetoin than the cit<sup>+</sup> *Lb. plantarum* strain. In the pH 3.0 treatment, the cit<sup>-</sup> *Lb. plantarum* strain produced 39% less acetoin than the cit<sup>+</sup> *Lb. plantarum* strain. The difference between the *Lb. plantarum* strains was more pronounced in the pH 5.0 treatment where the cit<sup>-</sup> strain produced an acetoin concentration of 70.88 mg/L which was 78% less than the 334.95 mg/L produced by the cit<sup>+</sup> strain. In the pH 3.5 and 4.0 treatments, the cit<sup>-</sup> *Lb. plantarum* strain produced 68% and 59% less acetoin than the cit<sup>+</sup> *Lb. plantarum* strain, respectively. The *Lb. plantarum* strains produced more acetoin than the *O. oeni* strains, except in the pH 3.0 treatment where the cit<sup>+</sup> *O. oeni* strain produced more acetoin than the cit<sup>+</sup> *Lb. plantarum* strain. This further emphasise the hypothesis that *Lb. plantarum* might not be able to further reduce acetoin to 2,3-butanediol.

**Table 3.14** Diacetyl concentrations (mg/L) in the pH treatments inoculated with the four LAB strains. The last sampling day for *O. oeni* IWBT B065 was day 8 and day 21 for the other strains in all the treatments.

Treatment	Lactic acid bacteria			
	<i>O. oeni</i> IWBT B065	Viniflora® CiNe™	<i>Lb. plantarum</i> IWBT B205	<i>Lb. plantarum</i> IWBT B382
<b>pH 3.0</b>				
Day 2	4.35 ± 0.33	0.06 ± 0.03	0.82 ± 0.09	1.32 ± 0.08
Day 5	22.36 ± 1.95	0.02 ± 0.03	0.56 ± 0.03	1.57 ± 0.13
Day 8	25.09 <sup>A</sup> ± 2.54			
Day 21		0.00 ± 0.00	0.48 ± 0.04	1.02 <sup>D</sup> ± 0.05
<b>pH 3.5</b>				
Day 2	9.56 ± 0.04	0.06 ± 0.01	0.97 ± 0.02	2.39 ± 0.03
Day 5	11.34 ± 0.22	0.00 ± 0.00	0.88 ± 0.05	3.14 ± 0.40
Day 8	18.52 <sup>B</sup> ± 2.36			
Day 21		0.04 ± 0.03	3.79 <sup>A</sup> ± 0.46	5.32 <sup>C</sup> ± 0.10
<b>pH 4.0</b>				
Day 2	7.45 ± 1.98	0.12 ± 0.04	0.71 ± 0.07	1.10 ± 0.10
Day 5	0.21 ± 0.06	0.05 ± 0.05	1.22 ± 0.22	4.07 ± 0.26
Day 8	0.20 ± 0.11			
Day 21		0.03 ± 0.01	1.79 <sup>B</sup> ± 0.04	7.39 <sup>A</sup> ± 0.13
<b>pH 5.0</b>				
Day 2	0.07 ± 0.00	0.02 ± 0.01	0.44 ± 0.07	1.91 ± 0.22
Day 5	0.09 ± 0.01	0.04 ± 0.01	1.10 ± 0.15	8.55 ± 0.23
Day 8	0.06 ± 0.02			
Day 21		0.10 ± 0.03	0.66 <sup>C</sup> ± 0.09	5.77 <sup>B</sup> ± 0.28



Values on last sampling day with different alphabetical letters are significantly different ( $p < 0.05$ ) between different treatments inoculated with a specific strain.

Significant differences were only calculated on values above the minimum quantification limit.

Minimum quantification limit for diacetyl was 0.5 mg/L.

**Table 3.15** Acetoin concentrations (mg/L) in the pH treatments inoculated with the four LAB strains. The last sampling day for *O. oeni* IWBT B065 was day 8 and day 21 for the other strains in all the treatments.

Treatment	Lactic acid bacteria							
	<i>O. oeni</i> IWBT B065		Viniflora® CiNe™		<i>Lb. plantarum</i> IWBT B205		<i>Lb. plantarum</i> IWBT B382	
<b>pH 3.0</b>								
Day 2	11.23	± 1.11	2.77	± 0.25	13.69	± 0.61	20.98	± 0.88
Day 5	21.52	± 2.44	1.21	± 0.03	9.72	± 0.85	18.44	± 2.57
Day 8	22.04 <sup>B</sup>	± 1.96						
Day 21			0.00	± 0.68	11.21 <sup>C</sup>	± 1.50	18.62 <sup>C</sup>	± 0.76
<b>pH 3.5</b>								
Day 2	14.79	± 0.65	0.00	± 0.00	11.52	± 0.23	26.8	± 3.92
Day 5	25.20	± 5.86	0.00	± 0.00	16.32	± 2.57	47.55	± 0.71
Day 8	30.93 <sup>A</sup>	± 2.22						
Day 21			0.00	± 0.00	78.24 <sup>B</sup>	± 8.18	247.69 <sup>B</sup>	± 19.19
<b>pH 4.0</b>								
Day 2	11.77	± 0.70	0.00	± 0.00	10.30	± 1.10	17.5	± 0.48
Day 5	11.03	± 1.19	0.00	± 0.00	44.35	± 2.25	90.85	± 12.38
Day 8	10.24 <sup>C</sup>	± 0.11						
Day 21			0.00	± 0.00	143.69 <sup>A</sup>	± 15.24	351.32 <sup>A</sup>	± 41.48
<b>pH 5.0</b>								
Day 2	0.00	± 0.00	0.00	± 0.00	10.02	± 1.01	35.22 <sup>D</sup>	± 4.77
Day 5	0.00	± 0.00	0.00	± 0.00	46.89	± 4.33	223.36 <sup>B</sup>	± 10.03
Day 8	0.00	± 0.00						
Day 21			0.00	± 0.00	70.88 <sup>B</sup>	± 2.17	334.95 <sup>A</sup>	± 24.70

<49.99	
50.00 - 99.99	
100.00 - 199.99	
200.00 - 299.99	
>300	

Values on last sampling day with different alphabetical letters are significantly different ( $p < 0.05$ ) between treatments inoculated with a specific strain.

Significant differences were only calculated on values above the minimum quantification limit.

Minimum quantification limit for acetoin was 0.5 mg/L.

### 3.5 Conclusion

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As the inoculation of LAB starter cultures together with the yeast, known as co-inoculation, becomes a more common practise it is important to understand what the effect of high sugar concentrations will have on wine aroma. Several studies have previously investigated the effect of co-inoculation on the overall wine aroma, but not necessarily the impact of sugar on the buttery aroma. This study therefore focused on understanding the influence of sugar and pH on the citrate metabolism of LAB which can potentially determine the winemaking practises that can be used to obtain a buttery aroma or not.

The results obtained in this study indicated that glucose had the smallest impact on the citrate metabolism of *O. oeni* and *Lb. plantarum*, since the 115 g/L and 50 g/L glucose treatments had similar trends regarding the production of metabolites associated with citrate metabolism specifically D-lactate, acetate, diacetyl and acetoin. The 2.5 g/L glucose treatment inoculated with the different strains had overall less D-lactate and acetate which might be attributed to the lower glucose concentrations present in this treatment. However, the cit<sup>+</sup> *Lb. plantarum* strain produced more diacetyl and acetoin in the 2.5 g/L treatment, whereas the other strains used produced only trace amounts. The fructose and pH treatments had a more pronounced effect on the citrate metabolism of the strains tested. The *O. oeni* strains produced the highest D-lactate concentration in the 2.5 g/L fructose treatment. The cit<sup>+</sup> *O. oeni* strain produced the highest acetate concentration in the 115 g/L fructose treatment. The fructose treatments had no effect on the acetate produced by the cit<sup>-</sup> *O. oeni* strain. The *Lb. plantarum* strains produced the lowest D-lactate and acetate concentrations in the 2.5 g/L fructose treatments, but not necessarily the highest concentrations in the 115 g/L fructose treatment. The 115 g/L fructose treatment inoculated with the cit<sup>+</sup> strains had the highest diacetyl and acetoin concentrations, while the 2.5 g/L fructose treatment inoculated with these strains had the lowest concentrations. The *Lb. plantarum* strains consumed more citrate and produced more D-lactate, acetate, diacetyl and acetoin in the pH 3.5, 4.0 and 5.0 treatments than in the pH 3.0 treatment. The opposite was seen for the cit<sup>+</sup> *O. oeni* strain which consumed more citrate and produced more diacetyl and acetoin in the pH 3.0 and 3.5 treatments than in the pH 4.0 and 5.0 treatments. However, this cit<sup>+</sup> *O. oeni* strain did not necessarily produce more D-lactate and acetate in the lower pH treatments. The cit<sup>-</sup> *O. oeni* strain did not consume citrate and produced trace amounts of diacetyl and acetoin in all the treatments.

The cit<sup>+</sup> strains produced more diacetyl and acetoin than the cit<sup>-</sup> strains in most of the treatments, but no trend was observed in the acetate and D-lactate concentrations. The *Lb. plantarum* strains produced more D-lactate and acetoin than the *O. oeni* strains in most of the treatments, which might be linked to the different metabolic activities of these species. The diacetyl and acetate concentrations between the different species were different in the different treatments and there was no conclusive trend observed.

From the results obtained in this study, it seems as if a high fructose concentration stimulated the production of diacetyl and acetoin by cit<sup>+</sup> strains of the species *O. oeni* and *Lb. plantarum*. Furthermore, a low pH increased the diacetyl and acetoin concentrations by the cit<sup>+</sup> *O. oeni* strains, while a high pH increased the D-lactate, acetate, diacetyl and acetoin production by the cit<sup>+</sup> *Lb. plantarum* strain. Thus, grape must from warm climate regions with a higher pH must rather be inoculated with an *O. oeni* starter culture when a buttery aroma is not wanted. Lastly, the use of the cit<sup>+</sup> LAB strains would not be encouraged to induce MLF in wines where the buttery aroma is not desired as the cit<sup>+</sup> LAB strains used in this study produced much higher diacetyl and acetoin concentrations than the cit<sup>-</sup> strains.

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# Chapter 4

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## General discussion and conclusions

## 4 General discussion and conclusions

### 4.1 General discussion and conclusion

Malolactic fermentation (MLF) is a secondary fermentation that occurs either with or after the completion of alcoholic fermentation. This process is carried out by lactic acid bacteria (LAB) which can alter the aroma profile of wine. These bacteria can alter the organoleptic properties of wine through several mechanisms such as metabolising several compounds naturally present in grape must. Citrate is one of many compounds that LAB can utilise during vinification to produce flavour compounds such as D-lactate, acetate, diacetyl, acetoin and 2,3-butanediol. Only the LAB that contain the necessary genes encoding the citrate transporter and citrate lyase are able to degrade citrate. These bacteria are referred to as citrate positive ( $\text{cit}^+$ ) bacteria. Citrate negative ( $\text{cit}^-$ ) LAB are incapable of citrate degradation due to the absence of one or all the genes necessary for citrate transport and/or degradation. However, these bacteria are still able to produce the flavour compounds associated with citrate metabolism from pyruvate formed via sugar metabolism.

The flavour compounds produced during citrate metabolism can be beneficial or detrimental to the aroma and thus wine quality. An acetate concentration above 600 mg/L gives rise to a vinegar off-flavour and is therefore considered as detrimental to wine quality (Guth, 1997; Ferreira *et al.*, 2000). When within the range of 0.2 mg/L to 2.8 mg/L, diacetyl have a buttery, creamy and/or toasty aroma and contribute to wine complexity (Martineau and Henick-Kling, 1995; Bartowsky and Henschke, 2004). However, a diacetyl concentration above 5 mg/L is considered to be detrimental to wine quality, since the buttery aroma becomes overwhelming and masks the fruity and vegetative wine aromas. During the fermentation, diacetyl can be further reduced to the less sensory active acetoin and 2,3-butanediol. These compounds also have a buttery, creamy aroma when above 150 mg/L and 600 mg/L, respectively (Romano *et al.*, 1998; Ehsani *et al.*, 2009; Malherbe *et al.*, 2012). The reduction of diacetyl to acetoin and/or 2,3-butanediol is usually encouraged by winemakers to create a less buttery wine style.

Several factors can influence the production of D-lactate, acetate and diacetyl as well as the reduction of diacetyl to acetoin and/or 2,3-butanediol. A few of these factors include the LAB or yeast starter culture, sugar concentration, citrate concentration, pH, oxygen, presence of yeast lees, sulphur dioxide concentration and temperature. The overall objective of this study was to better understand how sugar concentration and pH influence the citrate metabolism of *O. oeni* and *Lb. plantarum* and to provide more information on the winemaking practices which can contribute to a desirable or undesirable buttery aroma. This is also the first study to determine the influence of *Lb. plantarum* as a starter culture on the buttery aroma in wine.

In this study, it was shown that glucose, fructose, pH and the LAB strains used to induce MLF in synthetic wine can influence citrate consumption, the *citE* gene expression and the

production of metabolites associated with citrate metabolism, specifically D-lactate, acetate, diacetyl and acetoin. Of the factors tested, glucose had the smallest impact on citrate metabolism. The cit<sup>+</sup> *O. oeni* strain was the only strain that completely consumed citrate. This strain consumed citrate faster in the 115 g/L glucose treatment than in the 50 g/L and 2.5 g/L treatments. However, the lowest relative expression was seen in the 115 g/L glucose treatment where malate and citrate were consumed the fastest which the bacteria might have incorporated as a survival mechanism for when malate has been depleted (Olguín *et al.*, 2010). The 115 g/L and 50 g/L glucose treatments did not influence the D-lactate, acetate, diacetyl and acetoin concentrations produced by all the strains. The 2.5 g/L glucose treatment inoculated by most strains had the lowest D-lactate and acetate concentrations which might be due to the limited substrate in this treatment. The cit<sup>+</sup> *Lb. plantarum* strain was the only strain that produced diacetyl concentrations above the minimum quantification limit in the 2.5 g/L glucose treatment. This treatment inoculated with cit<sup>+</sup> *Lb. plantarum* strain had a final diacetyl concentration above the 0.2 mg/L to 2.8 mg/L sensory threshold value which could have contributed to a desirable buttery aroma (Martineau and Henick-Kling, 1995; Bartowsky and Henschke, 2004). The cit<sup>+</sup> *Lb. plantarum* strain produced more acetate, diacetyl and acetoin than the cit<sup>-</sup> *Lb. plantarum* strain, but similar amounts of D-lactate. The cit<sup>+</sup> *O. oeni* strain produced more D-lactate and acetate than the cit<sup>-</sup> *O. oeni* strain, but both strains produced trace amounts of diacetyl and acetoin. The *Lb. plantarum* strains produced more D-lactate, acetate, diacetyl and acetoin than the *O. oeni* strains in all the treatments.

The fructose treatments had a more pronounced effect on the citrate metabolism of LAB than the glucose treatments. The fructose treatments only influenced the citrate consumption by the cit<sup>+</sup> strains. The cit<sup>+</sup> *Lb. plantarum* strain was able to consume more citrate in the 115 g/L and 50 g/L fructose treatments than in the glucose treatments, but there was still not a relative transcriptional response. The citrate consumption by the cit<sup>+</sup> *O. oeni* strain was slightly delayed in the 115 g/L fructose treatment which had the highest relative expression of *citE*. As mentioned previously, it seems as if the relative expression of *citE* might be linked to the malate degradation period. Furthermore, it seems as if high fructose concentrations stimulated the production of diacetyl and acetoin by the cit<sup>+</sup> strains, since the highest diacetyl and acetoin concentrations were produced in the 115 g/L treatment. The diacetyl concentrations in the 115 g/L fructose treatment inoculated with the cit<sup>+</sup> strains were above the 5 mg/L limit of desirable diacetyl and could have spoiled the wine by giving rise to an overwhelming buttery aroma which could have masked the fruity and vegetative aromas in wine (Martineau and Henick-Kling, 1995; Bartowsky and Henschke, 2004). These results indicate that co-inoculated wines with a cit<sup>+</sup> strain might cause wine spoilage due to the high diacetyl and acetoin concentrations produced. This is however in contrast with previous studies which indicated that co-inoculated wines, where LAB are inoculated simultaneous with the yeast, have been described as being more fruitier than sequential wines, where the LAB are inoculated after the completion of alcoholic fermentation (Antalick *et al.*, 2013; Versari *et al.*, 2015). This contradiction might be due to the fact that during this study there were no yeast

present to reduce the diacetyl produced by the LAB strains to the less sensory active acetoin and 2,3-butanediol. The *cit*<sup>+</sup> *O. oeni* strain produced more D-lactate, acetate, diacetyl and acetoin than the *cit*<sup>-</sup> *O. oeni* strain. The *cit*<sup>+</sup> *Lb. plantarum* strain produced more diacetyl and acetoin than the *cit*<sup>-</sup> *Lb. plantarum* strain, but similar amounts of D-lactate and less acetate. As was seen for the glucose treatment, the *Lb. plantarum* strains produced more D-lactate, diacetyl and acetoin than the *O. oeni* strains. However, the *O. oeni* strains produced more acetate than the *Lb. plantarum* strains in most of the treatments.

Lastly, the pH treatments influenced the LAB species differently which is mainly coupled to the optimum growth pH of the individual species. *Lb. plantarum* has a growth optimum pH above 3.5 and the *Lb. plantarum* strains were therefore able to survive longer, consume more citrate and thus produced more D-lactate, acetate, diacetyl and acetoin in the pH 3.5, 4.0 and 5.0 treatments (Sedewitz *et al.*, 1984). On the other hand, *O. oeni* is better adapted than *Lb. plantarum* to a low pH and are able to survive in wines with a pH equal to or below 3.5 (Drici-Cachon *et al.*, 1996). The *cit*<sup>+</sup> *O. oeni* strain was therefore able to consume more citrate in the lower pH treatments, pH 3.0 and 3.5 treatments, and produced more diacetyl and acetoin than in the pH 4.0 and 5.0 treatments. The relative expression of *citE* in the *cit*<sup>+</sup> *O. oeni* strain was also the highest in the pH 3.0 treatment where the malate and citrate were consumed over a longer period as was seen in the sugar treatments. The D-lactate and acetate concentrations produced by this *cit*<sup>+</sup> *O. oeni* strain were however not more in the lower pH treatments than in the higher pH treatments. The *cit*<sup>+</sup> *O. oeni* strain produced diacetyl above the 5 mg/L limit in the pH 3.0 and 3.5 treatments and could have contributed to a buttery off-flavour in wine (Martineau and Henick-Kling, 1995; Bartowsky and Henschke, 2004). The *cit*<sup>+</sup> *Lb. plantarum* strain produced diacetyl concentrations below 5 mg/L but higher than the 0.2 mg/L to 2.8 mg/L diacetyl sensory threshold value in the pH 3.5, 4.0 and 5.0 treatments and could have contributed to a desirable buttery aroma and thus wine complexity (Martineau and Henick-Kling, 1995; Bartowsky and Henschke, 2004). This *cit*<sup>+</sup> *Lb. plantarum* strain was the only strain that produced acetoin concentrations above the 150 mg/L threshold value (Malherbe *et al.*, 2012) in the pH 3.5, 4.0 and 5.0 treatments and could have caused an overwhelming creamy aroma. The *cit*<sup>+</sup> strains only produced more diacetyl and acetoin than the *cit*<sup>-</sup> strains, but similar amounts of D-lactate and acetate. As was seen in the sugar treatments, the *Lb. plantarum* strains produced more D-lactate and acetoin than the *O. oeni* strains in all the pH treatments, except in the pH 3.0 treatment where the *O. oeni* strains produced more D-lactate than the *Lb. plantarum* strains. However, the *O. oeni* strains produced more acetate than the *Lb. plantarum* strains in all the pH treatments, except in the pH 5.0 treatment where the *Lb. plantarum* strains produced more acetate than the *O. oeni* strains.

From the results obtained in this study, it was evident that diacetyl and acetoin concentrations increased with a low glucose and a high fructose concentration. A low pH stimulated the production of diacetyl and acetoin by the *cit*<sup>+</sup> *O. oeni* strain, whereas a high pH

stimulated the diacetyl production of these compounds by the cit<sup>+</sup> *Lb. plantarum* strain. To avoid the production of an overwhelming buttery, creamy aroma in wine, winemakers can induce MLF with a cit<sup>-</sup> LAB strain such as Viniflora® CiNe™ from Chr. Hansen. This strain was the only strain that did not consume any citrate and therefore produced the lowest D-lactate, acetate, diacetyl and acetoin concentrations in most of the treatments. Although the factors tested during this study influenced the concentrations of these compounds produced by this strain, the concentrations were never above their sensory threshold values and could have only contributed to wine complexity.

## 4.2 Future work

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Future research is needed to better understand why the cit<sup>+</sup> *Lb. plantarum* strain only consume citrate under certain conditions and why this strain produced more D-lactate, diacetyl and acetoin than the cit<sup>+</sup> *O. oeni* strain in most treatments. This can be achieved by evaluating the relative expression of more genes involved in the citrate metabolism, especially the genes encoding the citrate transporter. Furthermore, a transport assay can be used to further understand why several LAB species can only partially consume citrate and others can completely consume citrate. Moreover, a study must be performed to evaluate the combination of glucose and fructose on citrate metabolism, since the results obtained for the individual sugar treatments were in contrast with one another with regards to the production of diacetyl and acetoin. A study must also be performed to include 2,3-butanediol analysis to confirm the hypothesis that *Lb. plantarum* might not be able to further reduce acetoin to 2,3-butanediol. Lastly, the fructose and pH treatments should be performed in grape must with RNA sequencing and sensory evaluation to determine if similar results will be obtained in a grape and/or wine matrix and to get an holistic overview of all the genes involved in citrate metabolism.

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