Lactobacillus plantarum: amino acid utilization

by

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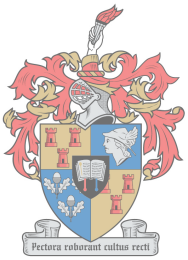

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Declaration

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Summary

Amino acid metabolism serves as a source of sulphur, carbon and nitrogen for bacteria growing in wine or grape juice. The metabolism of amino acids controls the rate of growth and malic acid degradation and also results in the formation of various aromatic compounds which may positively or negatively influence the aroma profile of wine. *L. plantarum*, a lactic acid bacterium (LAB), may be used as co-inoculant in high pH (≥ 3.5) grape juice for fast malic acid degradation and high aroma production.

Since the research on *L. plantarum* nitrogen metabolism is scarce, the overall goal of this study was to better understand it. The first aim was to determine the amino acid requirements in *L. plantarum* for growth and malic acid degradation, through single amino acid omissions. This entailed inoculation of nitrogen starved *L. plantarum* strains into chemically defined media (in this case synthetic grape juice) in which one amino acid is removed at a time. The data suggests that amino acid trophic requirements in *L. plantarum* are highly strain dependent, although Leu, Ile, Val, Glu and Met were shown under our conditions to be essential amino acids and Gln, Gly, His, Lys and Trp were non-essential amino acids. In a subsequent experiment, 5 single amino acid omissions (Ala, Arg, Gln, Trp and Val) were selected to evaluate their effect on growth and malic acid uptake in synthetic grape juice. During malolactic fermentation (MLF) the removal of Ala and Val had completely repressed MLF induced by *L. plantarum* while the removal of Trp and Arg had somewhat repressed MLF. Only the removal Gln did not hinder MLF for at least one strain.

The second aim was to determine the order of amino acid uptake by *L. plantarum* in synthetic grape juice using HPLC. Asp, Thr, Ser and Ala tends to be assimilated at a high rate within the first 72 h while the branched chain amino acids, aromatic amino acids (AAA) and Met are assimilated after 72 h.

The third aim determined the amino acid uptake in Chardonnay grape juice. The assimilation pattern differed considerably between synthetic grape juice and Chardonnay grape juice. In contrast to synthetic grape juice Arg, Leu, Phe and Ala were preferred amino acid sources. It is thought that the differences could be attributed to mainly two factors: initial nitrogen concentration (40 mg N/L in SGJ vs 240 mg N/L in grape juice) and the pre-culture conditions.

This study confirmed that higher nitrogen concentrations resulted in higher growth and quicker malic acid degradation. The high nitrogen requirement of certain amino acids combined with the harsh wine parameters experienced in sequential MLF might explain why *L. plantarum* struggles with MLF in this scenario. Further research should be directed towards identifying the preferred amino acids in dried and fresh *L. plantarum* starter cultures to assess if there is a difference. If nitrogen requirements continues to be investigated in *L. plantarum* successful tailored supplements can be created to aid the growth of *L. plantarum* in wine or grape juice.

Opsomming

Aminosuur metabolisme dien as ‘n bron van swawel, stikstof en koolstof vir bakterieë wat in wyn en druiwesap groei. Die metabolisme van aminosure beheer die tempo van groei, appelsuur afbraak asook die vorming van verskeie aromatiese verbindings wat die wyn aromaprofiel positief of negatief mag beïnvloed. *L. plantarum*, ‘n melksuurbakterium, mag gebruik word as ko-inokulant in hoë pH (≥ 3.5) druiwesap vir vinnige afbraak van appelsuur en hoë aroma produksie.

Aangesien navorsing op die stikstof metabolisme van *L. plantarum* seldsaam is, was dit die algehele doelstelling van hierdie studie om dit beter te verstaan. Die eerste doelwit was om die aminosuur vereistes in *L. plantarum* te bepaal in terme van groei en appelsuur afbraak d. m. v. aminosuur weglatings. Dit behels die inokulasie van ‘n stikstof-uitgehongerde *L. plantarum* ras in chemiese gedefinieerde media (in hierdie geval sintetiese druiwesap) waarin een aminosuur op ‘n keer weggelaat is. Die data stel voor dat aminosuur trofiese vereistes in *L. plantarum* baie sterk afhanklik is van die ras wat gebruik word. Algeheel toon Leu, Ile, Val Glu en Met om essensiële aminosure te wees terwyl Gln, Gly, His, Lys en Trp toon om nie-essensiële aminosure te wees. In ‘n daaropvolgende eksperiment is 5 enkele aminosuur weglatings (Ala, Arg, Gln, Trp en Val) gekies om die effek op groei en appelsuur afbraak in sintetiese druiwesap te evalueer. Gedurende appelmelksuurgisting (AMG) het die weglating van Ala en Val die proses heeltemal onderdruk terwyl die weglating van Trp en Arg AMG slegs gedeeltelik onderdruk het. Slegs die weglating van Gln het glad nie AMG verhinder nie vir ten minste een ras.

Die tweede doelwit het die volgorde van aminosuur opname deur *L. plantarum* in sintetiese druiwesap bepaal deur gebruik te maak van HPLC. Gevolglik, is bepaal dat Asp, Thr, Ser en Ala geneig is om eerste opgeneem te word teen ‘n hoë tempo binne die eerste 72 h van AMG terwyl Met, die vertakte ketting en aromatiese aminosure na 72 h geassimileer word.

Die derde doelwit het die aminosuur opname in Chardonnay druiwesap bepaal. Die patroon van aminosuur assimilasie verskil heelwat tussen sintetiese druiwesap en Chardonnay druiwesap. In teenstelling met die sintetiese druiwesap, is Arg, Leu, Phe en Ala verkies as voorkeur bronne van aminosure in Chardonnay druiwesap. Die verskil tussen die resultate kan heelwaarskynlik toegeskryf word aan hoofsaaklik 2 faktore: die aanvanklike stikstof konsentrasie (40 mg N/L in sintetiese druiwesap en 240 mg N/L in druiwesap) en die vooraf kultiverings toestande.

Hierdie studie bevestig dat hoër stikstof konsentrasies tot hoër groei en vinniger appelsuur afbraak lei. Die hoë stikstof vereistes tesame met die stresvolle wynkondisies wat verband hou met na alkoholiese fermentasie inokulasie mag verder verduidelik waarom *L. plantarum* sukkel onder hierdie toestande. Verdere navorsing behoort gerig te word om voorkeur aminosure in droë en vars aanvangskulture van *L. plantarum* te identifiseer, om te bepaal of daar ‘n verskil is. As stikstof vereistes in *L. plantarum* verder noukeurig ondersoek word kan stikstof aanvullings vervaardig word om *L. plantarum* te help met groei in wyn of druiwesap.

This thesis is dedicated to

My family

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Preface

This thesis is presented as a compilation of 4 chapters.

Chapter 1 **General introduction and project aims**

Chapter 2 **Literature review**

The factors influencing the amino acid catabolism in lactic acid bacteria

Chapter 3 **Research results**

The amino acid requirements and usage of *Lactobacillus plantarum*

Chapter 4 **General discussion and conclusions**

Table of Contents

List of Abbreviations

Ala	**Alanine**
Arg	**Arginine**
Asp	**Aspartic acid**
Asn	**Asparagine**
Cys	**Cysteine**
Cy2	**Cystine**
Glu	**Glutamic acid**
Gln	**Glutamine**
Gly	**Glycine**
His	**Histidine**
Ile	**Isoleucine**
Leu	**Leucine**
Lys	**Lysine**
Met	**Methionine**
Orn	**Ornithine**
Phe	**Phenylalanine**
Pro	**Proline**
Ser	**Serine**
Thr	**Threonine**
Trp	**Tryptophan**
Tyr	**Tyrosine**
Val	**Valine**
AAA	**Aromatic amino acids**
ADI	**Arginine deiminase**
BCAA	**Branched-chain amino acids**
HPLC	**High Performance Liquid Chromatography**
MLF	**Malolactic fermentation**
SGJ	**Synthetic grape juice**
VSC	**Volatile sulphur compounds**

Chapter 1

General introduction and project aims

Chapter 1 – General introduction and project aims

1.1 Introduction

Lactic acid bacteria (LAB) are non-sporulation, non-motile, low G+C Gram-positive bacteria (Dicks and Endo, 2009) that occupy a wide variety of ecological niches and have been unconsciously used for thousands of years in the fermentation of food and foodstuffs such as wine and cheese. LAB is an economically important group of microorganisms as they play a crucial role in the fermentation of many food and beverage products. They contribute to the flavour and aroma profile, texture and preservation of the final product. For example in wine, LAB's association with wine leads to the decarboxylation of L-malic acid to L-lactic acid and carbon dioxide and concomitant deacidification of wine in a process known as malolactic fermentation (MLF). MLF has a three-fold benefit for consumers and winemakers: firstly, LAB decreases the perceived acidity, secondly LAB provides microbial stability to the wine by degrading malic acid and thus prevents spoilage by other LAB, and thirdly it adds aromatic complexity to the wine (Bartowsky and Henschke, 2004).

In order to gain standardized and consistent quality in fermented products such as cheese and wine, selected pure starter cultures are often used. The success of a LAB starter culture is based on whether they can overcome the internal hostile environment and finish the fermentation in a relative short period with limited production of undesirable compounds (Sun et al., 2016; Torriani et al., 2011). For instance *Lactococcus lactis* is the preferred starter culture in cheese as this species thrive at pH 5, high osmolarity ($\leq$ 4%), in anaerobic environments and produces bacteriocins (Fox et al., 1998). Whereas wine has a pH of 3-3.4, an alcohol content 12-15%, therefore *Oenococcus. oeni* is the most commonly used starter culture as this species is most tolerant to the wine conditions. Noticeably, lactobacilli are not preferred starter bacteria in both cheese and wine but may nevertheless partake in cheese ripening and MLF (Du Toit et al., 2011; Fox et al., 1998). Of course, in wine, various factors such as pH, ethanol, fermentation temperature, yeast, the content of phenolic acids, sulphur dioxide, antimicrobial peptides, amino acids and sugars will determine the extent to which *Lactobacillus* and other non-starter bacteria will survive in wine (Du Toit et al., 2011). There is some risk associated with the inhabitation of *Lactobacillus* species in wine. *Lactobacillus brevis, L. fermentum* and *L. hilgardii* species are implicated in production tetrahydropyridine, a compound with aroma described to be similar to acetamide or mouse urine and is commonly referred to as a 'mousy' taint (Du Toit and Pretorius, 2000). However not all non-starter lactobacilli cultures are undesirable to the wine-industry. Aside from negative characteristics of high diacetyl (Pretorius, 2016) and acetic acid production from tartaric acid degradation (Du Toit and Pretorius, 2000), *L. plantarum* can positively contribute to wine production.

L. plantarum contains enzymes such as proteases, β-glucosidases, esterases and enzymes of the citrate lyase pathway (Mtshali et al., 2010). Equally important, *L. plantarum*'s extra- and intracellular

enzymatic fractions are active under wine-making conditions (Matthews et al., 2007; Pérez-Martín et al., 2013). These enzymes add or modulate the aroma in wine. A common and well-known contribution is the formation of diacetyl, the catabolic product of citrate degradation, which is responsible for the buttery aroma of cheese and wine (Bartowsky and Henschke, 2004; Malherbe et al., 2013). But some of the most important precursors to the production of aroma compounds are amino acids.

Amino acids provide the carbon skeleton for the production of carbonyls, higher alcohols, and esters. More specifically, the degradation of branched-chain amino acids (BCAA) which comprise of Leu, Ile and Val are responsible for the production of isoamyl alcohol (3-methylbutanol) (Dickinson et al., 1997; Smit et al., 2004), active amyl alcohol (2-methylbutanol) (Dickinson et al., 2000) and isobutyl alcohol (2-methylpropanol) (Dickinson et al., 1998), all well-known aroma compounds. The production of higher alcohols and esters, impart a floral and/or fruity note, while the production of volatile sulphur compounds provide for a cabbage aroma in cheese (Cheng, 2010; Smit et al., 2009). The same compounds are also noted to be influenced by *L. plantarum* in wine after MLF (Knoll et al., 2011; Lee et al., 2009; Maicas et al., 1999; Pozo-Bayon et al., 2005).

LAB amino acid metabolism is also linked to the health aspects of wine. Biogenic amines are the corresponding products of amino acid decarboxylation and are toxic to humans. Histamine and tyramine, for example causes dilation of blood vessels leading to headaches and high blood pressure (Mete et al., 2017; Silla Santos, 1996; Smit et al., 2008). In *L. plantarum* however the production of histamine, tyramine and phenylethylamine are noted to be absent (Landete et al., 2007; Lerm, 2010; Moreno-Arribas et al., 2000). Arg is one of the major amino acid found in grape juice. The catabolism through the Arginine deiminase (ADI) pathway leads to the production of ornithine, NH_4, ATP and most importantly citrulline. Extruded citrulline may react spontaneously with the abundant ethanol in wine medium to produce ethyl carbamate, a possible carcinogen (Schlatter and Lutz, 1990). The ADI pathway is absent in wine *L. plantarum* (Liu et al., 1995) due to the absence of Arg deiminase gene/enzyme (Lerm et al., 2011).

Amino acids also play a significant role in the growth of LAB in wine as limited quantity of nitrogen severely hamper the growth of LAB (Saguir and de Nadra, 2007; Terrade et al., 2009; Wegkamp et al., 2010). In turn adequate growth of LAB in wine would prevent 'stuck' MLF; a common problem related to MLF. Furthermore, the absence of certain amino acids completely supresses the growth of wine LAB. In *O. oeni* 14-16 amino acid result in zero growth and wine lactobacilli (*L. hilgardii* and *L. buchneri*) have 4-5 amino acid auxotrophies (Terrade and Mira de Orduña, 2009). However, *L. plantarum* being an alternative inducer of MLF in high pH grape juice has not been adequately investigated.

1.2 Project Aims

L. plantarum has to emerged as an alternative starter culture for MLF in the last decade (Du Toit et al., 2011). However, little is known at this stage about *L. plantarum* nutritional requirements with regards to nitrogen, microelements and vitamins and especially related to the wine matrix. This study focused on the amino acid nutritional requirements. Amino acid availability and uptake not only directly impacts the growth of *L. plantarum* (Saguir and de Nadra, 2007; Wegkamp et al., 2010) but also influences the aroma and health aspects of wine.

Therefore the aims of this study were as follows:

i) To determine the amino acid requirements of red wine isolated *L. plantarum* strains in a chemically defined medium;

ii) To determine the order of single-amino acid uptake in a chemically defined medium; and

iii) To determine the difference between amino acid uptake in grape juice and chemically defined medium.

1.3 References

Bartowsky, E.J., Henschke, P.A., 2004. The "buttery" attribute of wine - diacetyl - desirability, spoilage and beyond. Int. J. Food Microbiol. 96, 235–252.

Cheng, H., 2010. Volatile flavor compounds in yogurt: a review. Crit. Rev. Food Sci. Nutr. 50, 938–950.

Dickinson, J.R., Harrison, S.J., Dickinson, J.A., Hewlins, M.J.E., 2000. An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae* . J. Biol. Chem. 275, 10937–10942.

Dickinson, J.R., Harrison, S.J., Hewlins, M.J.E., 1998. An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae* . J. Biol. Chem. 273, 25751–25756.

Dickinson, J.R., Lanterman, M., Danner, B.J., Pearson, B.M., Sanz, P., Harrison, S.J., Hewlins, J.E., 1997. A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae*. J. Biol. Chem. 272, 26871–26878.

Dicks, L.M.T., Endo, A., 2009. Taxonomic status of lactic acid bacteria in wine and key characteristics to differentiate species. South African J. Enol. Vitic. 30, 72–90.

Du Toit, M., Engelbrecht, L., Lerm, E., Krieger-Weber, S., 2011. *Lactobacillus*: the next generation of malolactic fermentation starter cultures-an overview. Food Bioprocess Technol. 4, 876–906.

Du Toit, M., Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: using weapons from nature's own arsenal - a review. South African J. Enol. Vitic. 21, 74–96.

Fox, P.F., McSweeney, P.L.H., Lynch, C.M., 1998. Significance of non-starter lactic acid bacteria in Cheddar cheese. Aust. J. Dairy Technolgy 52, 83–89.

Knoll, C., Fritsch, S., Schnell, S., Grossmann, M., Rauhut, D., Du Toit, M., 2011. Influence of pH and

ethanol on malolactic fermentation and volatile aroma compound composition in white wines. LWT - Food Sci. Technol. 44, 2077–2086.

Landete, J.M., Pardo, I., Ferrer, S., 2007. Tyramine and phenylethylamine production among lactic acid bacteria isolated from wine. Int. J. Food Microbiol. 115, 364–368.

Lee, J.-E., Hwang, G.-S., Lee, C.-H., Hong, Y.-S., 2009. Metabolomics reveals alterations in both primary and secondary metabolites by wine bacteria. J. Agric. Food Chem. 57, 10772–10783.

Lerm, E., 2010. The selection and characterisation of lactic acid bacteria to be used as a mixed starter culture for malolactic fermentation. MSc thesis, Inst. Wine Biotechnol. Stellenbosch Univ. South Africa.

Lerm, E., Engelbrecht, L., du Toit, M., 2011. Selection and characterisation of *Oenococcus oeni* and *Lactobacillus plantarum* South African wine isolates for use as malolactic fermentation starter cultures. South African J. Enol. Vitic. 32, 280–295.

Liu, S., Pritchard, G.G., Hardman, M.J., Pilone, G.J., 1995. Occurrence of arginine deiminase pathway enzymes in arginine catabolism by wine lactic acid bacteria. Appl. Environ. Microbiol. 61, 310–316.

Maicas, S., Gil, J.V., Pardo, I., Ferrer, S., 1999. Improvement of volatile composition of wines by controlled addition of malolactic bacteria. Food Res. Int. 32, 491–496.

Malherbe, S., Menichelli, E., du Toit, M., Tredoux, A., Muller, N., Næs, T., Nieuwoudt, H., 2013. The relationships between consumer liking, sensory and chemical attributes of *Vitis vinifera* L. cv. Pinotage wines elaborated with different *Oenococcus oeni* starter cultures. J. Sci. Food Agric. 93, 2829–2840.

Matthews, A., Grbin, P.R., Jiranek, V., 2007. Biochemical characterisation of the esterase activities of wine lactic acid bacteria. Appl. Microbiol. Biotechnol. 77, 329–337. doi:10.1007/s00253-007-1173-8

Mete, A., Coşansu, S., Demirkol, O., Ayhan, K., 2017. Amino acid decarboxylase activities and biogenic amine formation abilities of lactic acid bacteria isolated from shalgam. Int. J. Food Prop. 20, 171–178.

Moreno-Arribas, V., Torlois, S., Joyeux, A., Bertrand, A., Lonvaud-Funel, A., 2000. Isolation, properties and behavior of tyramine-producing lactic acid bacteria from wine. J. Appl. Microbiol. 88, 584–593.

Mtshali, P.S., Divol, B., Van Rensburg, P., Du Toit, M., 2010. Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines. J. Appl. Microbiol. 108, 1389–1397.

Pérez-Martín, F., Seseña, S., Izquierdo, P.M., Palop, M.L., 2013. Esterase activity of lactic acid bacteria isolated from malolactic fermentation of red wines. Int. J. Food Microbiol. 163, 153–158.

Pozo-Bayon, M.A., Alegría, E.G., Polo, M.C., Tenorio, C., Martion-Álvarez, P.J., Calvo De La Banda, M., Ruiz-Larrea, F., Moreno-Arribas, M. V, 2005. Wine volatile and amino acid composition after malolactic fermentation: effect of *Oenococcus oeni* and *Lactobacillus plantarum* starter cultures. J. Agric. Food Chem. 53, 8729–8735.

Pretorius, N., 2016. Evaluation of citrate metabolism in *Oenococcus oeni* and *Lactobacillus plantarum* 96–99.

Saguir, F.M., de Nadra, M.C.M., 2007. Improvement of a chemically defined medium for the sustained growth of *Lactobacillus plantarum*: nutritional requirements. Curr. Microbiol. 54, 414–418.

Schlatter, J., Lutz, W.K., 1990. The carcinogenic potential ethyl carbamate (urethane): risk assesment at human dietary exposure levels. Food Chem. Toxicol. 28, 205–211.

Silla Santos, M.H., 1996. Biogenic amines: Their importance in foods. Int. J. Food Microbiol. 29, 213–231.

Smit, A.Y., Du Toit, W.J., Du Toit, M., 2008. Biogenic amines in wine: understanding the headache. South African J. Enol. Vitic. 29, 109–127.

Smit, B.A., Engels, W.J.M., Smit, G., 2009. Branched chain aldehydes: production and breakdown pathways and relevance for flavour in foods 987–999.

Smit, B.A., Engels, W.J.M., Wouters, J.T.M., Smit, G., 2004. Diversity of L-leucine catabolism in various microorganisms involved in dairy fermentations, and identification of the rate-controlling step in the formation of the potent flavour component 3-methylbutanal. Appl. Microbiol. Biotechnol. 64, 396–402.

Sun, S.Y., Gong, H.S., Liu, W.L., Jin, C.W., 2016. Application and validation of autochthonous *Lactobacillus plantarum* starter cultures for controlled malolactic fermentation and its influence on the aromatic profile of cherry wines. Food Microbiol. 55, 16–24.

Terrade, N., Mira de Orduña, R., 2009. Determination of the essential nutrient requirements of wine-related bacteria from the genera *Oenococcus* and *Lactobacillus* . Int. J. Food Microbiol. 133, 8–13.

Terrade, N., Noël, R., Couillaud, R., de Orduña, R.M., 2009. A new chemically defined medium for wine lactic acid bacteria. Food Res. Int. 42, 363–367.

Torriani, S., Felis, G.E., Fracchetti, F., 2011. Selection criteria and tools for malolactic starters development: An update. Ann. Microbiol. 61, 33–39.

Wegkamp, A., Teusink, B., de Vos, W.M., Smid, E.J., 2010. Development of a minimal growth medium for *Lactobacillus plantarum* . Lett. Appl. Microbiol. 50, 57–64.

Chapter 2

Literature Review:

The factors influencing the amino acid catabolism in lactic acid bacteria

Chapter 2- The factors influencing the amino acid catabolism in lactic acid bacteria

2.1 Introduction

Lactic acid bacteria (LAB) is responsible for fermentation of various food and food stuffs such as cheese and wine. In cheese, LAB is responsible for its aging which entails enzymatic degradation of lactose, fatty acids and proteins originating from milk (Engels, 1997). Similarly, LAB induces malolactic fermentation (MLF) in wine which results in the degradation of L-malic acid in grape must to L-lactic acid, resulting in a wine with a softer mouthfeel. With the inhabitation of LAB in these environments, the aroma and flavour profile is altered through a diverse number of enzymes and pathways (Mtshali et al., 2010).

The pathways that govern flavour and aroma formation are carbohydrate, lipolysis, organic acid, phenolic acid and nitrogen metabolism. The products of carbohydrate metabolism are highly dependent upon the genus and strain undertaking the catabolism as homofermentative bacteria produces lactic acid from glucose through glycolysis and heterofermentative bacteria produces additionally to lactic acid also acetic acid and ethanol through the pentose phosphate pathway. Malic acid, tartaric acid and citric acid are readily disseminated to lactic acid, acetic acid and diacetyl. Diacetyl is known for its characteristic butter aroma (Bartowsky and Henschke, 2004; Malherbe et al., 2013). Through the mechanism of β-oxidation, fatty acids are converted to secondary alcohols and lactones (Hassan et al., 2013). Several wine LAB strains possess the hydroxycinnamic acid decarboxylase capable of catabolising the grape derived hydroxycinnamic acid to volatile phenols (Cavin et al., 1997; Esteban-Torres et al., 2013; Rodríguez et al., 2009). These compounds have odours reminiscent of medicinal, barn yard and leather aromas and are quite detrimental to wine quality. Nitrogen metabolism plays an essential role in wine aroma as a significant portion of the volatile fraction originates from amino acid metabolism. Depending upon the composition of the media, this may be first initiated by protein hydrolysis since the amino acids in dairy products and wine is relatively scarce (most amino acids in grape must is taken up by yeast during alcoholic fermentation). LAB have an extensive network of proteinases and peptidases to hydrolyse casein in milk and cheese (Christensen et al., 1999) and mannoproteins in wine (Remize et al., 2006) to yield peptides and free amino acids. Once transported into the cell the catabolism of amino acids can pass through four pathways: decarboxylation, transamination, lyase and oxidative deamination.

LAB can also undergo MLF in grape juice. Contrary to wine and milk, grape juice contains a lot of amino acids of which Arg and Pro constitutes the largest composition. Depending upon the cultivar, vintage, agricultural practises, Val, Ala, His and Leu might be high as well (Monteiro and Bisson, 1991; Spayd and Andersen-Bagge, 1996). Without these amino acids it would be impossible for LAB to complete MLF in wine. It is therefore of interest to answer how these compounds are broken down to aid in LAB growth and what controls its metabolism.

Amino acid catabolism is reliant on the presence, functionality and expression of enzymes presiding over the reaction (Godon et al., 1993). Strict regulatory control is maintained over the gene's expression to resist the synthesis of unnecessary and wasteful metabolites. A good example of excellent regulatory control are genes clustered together in the same operon under the control of a single promoter and regulatory protein e.g. the His-operon (Delorme et al., 1999, 1993), BCAA operon (Godon et al., 1993) and Arg-operon (Divol et al., 2003; Zúñiga et al., 2002). The exogenous environment (pH, temperature, substrates availability) and the intracellular environment (intracellular pH, toxic substrate/product accumulation will determine the induction or repression of a gene. A pleiotropic gene e.g. CodY also controls either repression or induction of both catabolic and biosynthetic enzyme (Den Hengst et al., 2005b).

To better grasp the amino acid metabolism of LAB, regulation and factors inducing or repressing a response will be investigated for most of the amino acids. Analysis of the pathways will also shine light on their physiological significance.

2.2 Branched-chain and aromatic amino acid catabolism

2.2.1 Ehrlich pathway

More than 100 years ago it was suggested that a pathway exist in which branched chain amino acids (BCAA) are degraded into fusel alcohols. This was based upon the astute observation between the similarities of the carbon skeletons. Only much later was it confirmed through magnetic resonance and knock-out experiments that BCAA are indeed responsible for fusel alcohol formation in yeast (Dickinson et al., 2000, 1998, 1997). In this pathway, BCAA and aromatic amino acids (AAA) are transaminated to an α-keto acid, decarboxylated to an aldehyde and then reduced by an alcohol dehydrogenase to produce the corresponding fusel alcohol (**Fig. 2.1**). Likewise these compounds are also noted to be produced in LAB during food fermentations.

2.2.1.1 Transamination

The respective first and last step in the catabolism and anabolism of BCAA and AAA is transamination, catalysed by a transaminase enzyme (Chambellon and Yvon, 2003). The transaminase enzymes are pyridoxal 5'-phosphate (PLP) dependent and metal ion-independent enzymes composed of a homodimer. It transfers amino groups from an amino donor (amino acid) to an amino acceptor (α-keto acid). The most preferred α-keto acid is α-keto glutamate even though oxaloacetate and pyruvate is also shown to participate in transamination (Pudlik and Lolkema, 2012). In *Lc. lactis*, BCAA and AAA is catalysed only by the branched-chain amino acid transferase (BcaT) and the aromatic amino acid transferase (AraT) (Chambellon and Yvon, 2003; Rijnen et al., 2003). These transaminases catalyse all transaminase reactions (with exception of Asp) and works to complement one another. BcaT displays the highest activity towards Val, Ile and Met and decreased activity towards Leu and AAA (Yvon et al., 2000) while AraT on the other hand displays the highest activity toward all AAA and Leu but has very weak activity towards Met, Ile and Val (Chambellon and Yvon, 2003; Rijnen et al., 2003).

Transamination reaction in LAB is a major obstacle (bottleneck) in flavour formation. This is mainly due to the lack of α-keto glutamate supply (Kieronczyk et al., 2004; Rijnen et al., 2003; Yvon et al., 1998). Supply is generated by only two means: regeneration of α-keto glutamate or transportation of. α-keto glutamate. α-keto glutamate cannot be synthesized by lactobacilli and the majority of *Lc. lactis* due to an incomplete TCA cycle as the isocitrate dehydrogenase enzyme mediating the conversion of isocitrate toward α-keto glutamate is absent (Morishita and Yajima, 1995; Tanous et al., 2005). Glutamate dehydrogenase (GDH) is an enzyme that is responsible for the recycling of α-keto glutamate. It catalyse glutamate through oxidative deamination to α-keto glutamate but the activity is always moderate to low. Alternatively α-keto glutamate can be transported across the cell through citrate permease (CitP), however this transporter is promiscuous having affinity for all compounds containing $X\text{-}CR_2\text{-}COO^-$, in which X is either OH, O, or H (Pudlik and Lolkema, 2012). As the name suggests CitP is the transporter of citrate. The promiscuous nature of the enzyme results in competition between different metabolites resulting in decreased level of uptake of α-keto glutamate as observed with *Lc. lactis* in high citrate concentrations (Pudlik and Lolkema, 2013).

On the other hand high citric acid concentration might also be beneficial towards BCAA and AAA degradation. In an attempt to detoxify the media of excess citric acid, *Lc. lactis* will convert citrate to Asp. The last step in this pathway requires a transamination reaction to convert oxaloacetate to Asp. In this case oxaloacetate acts as the keto donor and BCAA and AAA acts as the amino donors to yield keto acids (Pudlik and Lolkema, 2012).

A factor which may limit BCAA and AAA conversion is high concentration of Asp as Asp will compete with BCAA and AAA for the limited α-keto glutamate. Asp can also compete for α-keto acid and is transaminate by Asp transaminase (EC 2.6.1.1). Unlike the other transaminases Asp transaminase has only affinity for Asp allowing faster catabolism of Asp and quicker diminishing of α-keto acids supply (Kieronczyk et al., 2004; Peralta et al., 2016)

Taking enzymatic kinetics into account, enzyme characterization reveals BcaT functions optimally in the presence of PLP at more neutral pH (7 and 8) (Pudlik and Lolkema, 2013; Thage et al., 2004; Yvon et al., 2000). However an activity assay revealed the transamination activity to be unaffected by slightly acidic pH (5 and 6). It is thought that purification of the enzyme may alter its properties and in this way makes the enzymatic (Pudlik and Lolkema, 2013). Increases in transport rate of BCAA in *Streptococcus cremoris* of BCAA from acidic intracellular pH to neutral emphasizes the underlying importance of pH in the catabolism of BCAA in LAB (Driessen et al., 1987).

2.2.1.2 α-Keto acid decarboxylation

From the preceding section it is clear that the conversion of BCAA and AAA to α-keto acid is theoretically very low. The conversion to aroma compounds is further hindered as α-keto acid becomes a centralised metabolite that can enter into four different reactions namely reverse transamination, CoA addition, hydroxyacid dehydrogenase and decarboxylation. Thus decarboxylation must compete with reverse

transamination, a CoA addition reaction and the more favourable hydroxyacid dehydrogenase to produce an aldehyde (Smit et al., 2009, 2004). It is for this reason that decarboxylation is the rate limiting step in the flavour formation (Smit et al., 2004). The latter reaction is the one that contributes to the aroma profile of either cheese or wine producing aldehydes, carboxylic acids, higher alcohols and ethyl and acetate esters. In a *Lc. lactis* isolate containing both decarboxylation and hydroxyacid dehydrogenase activity, Smit et al. (2004) showed that competitiveness between the four pathways exists and the prevailing condition will depend upon the reduction potential. In the absence of NADH, the NADH-dependent hydroxyacid dehydrogenase activity will cease and faster and higher production of aldehydes will be gained. When NADH is present hydroxyacid dehydrogenase will outcompete decarboxylation resulting in much higher levels of hydroxyacids than aldehydes.

Two decarboxylase enzymes have been isolated and characterised in *Lc. lactis*. α-Ketoisovalerate decarboxylase (Kidv) and branched-chain α-keto acid decarboxylase (KdcA). Both are thiamin diphosphate (ThDP)-dependent, has an optimal pH at 6.3 to 6.5 with KdcA having broader pH activity profile and is found to have the highest activity towards α-ketoisovalerate (derivative of Val), with much lower activity towards α-ketoisocaproate (derivative of Leu) and α-keto-β-methyl valerate (derivative of Ile) (De la Plaza et al., 2004; Smit et al., 2005).

A further obstruction in aroma formation is the absence of decarboxylation activity in LAB (Smit et al., 2004). Screening of 156 bacteria belonging to genera *Lactococcus*, *Lactobacillus* and *Leuconostoc* revealed only 16% of *Lactococcus* species possessed the decarboxylation activity (Fernández de Palencia et al., 2006).

2.2.1.3 Global BCAA regulation

CodY is a pleiotropic regulator of amino acids in Gram-positive bacteria in response to nitrogen availability (Den Hengst et al., 2005a; Guédon et al., 2001; Petranovic et al., 2004). The strength of CodY repression is modulated only by BCAA that acts as cofactors and directly stimulate CodY binding to the regulatory sites of the target genes (Petranovic et al., 2004). All the cofactors do not have the exact same effect on the CodY repression system. For example when Ile binds to CodY the global effect on repression is higher than when either Val or Leu is bound to CodY (Chambellon and Yvon, 2003). The CodY binds to a conserved high affinity binding site known as the CodY-box which is situated 80bp upstream of the first codon (Den Hengst et al., 2005b). Several molecules of CodY binds to the CodY-box preventing the RNA polymerase from binding to the target site and preventing transcription (Den Hengst et al., 2005a, 2005b). As already mentioned, only BCAA can actively interact with the CodY to repress catabolic enzyme formation but the exact mechanism by which the BCAA influences CodY to modulate gene expression is unknown (Den Hengst et al., 2005a). When the intracellular pool of BCAA are low, the co-regulated genes of the CodY regulon (Prtp proteinase, Opp transporter, PepN, PepC, PepO1 peptidases) are expressed. Protein degradation, peptide transport and cleavage increases the intracellular content of the amino acids. Increased concentration of amino acids allows BCAA to act as cofactors binding to CodY and represses the genes of the CodY operon (Guédon

et al., 2001). During stationary phase CodY-mediated repression of peptide and amino acid transport systems is relieved to maintain the intracellular nitrogen balance (Den Hengst et al., 2005b).

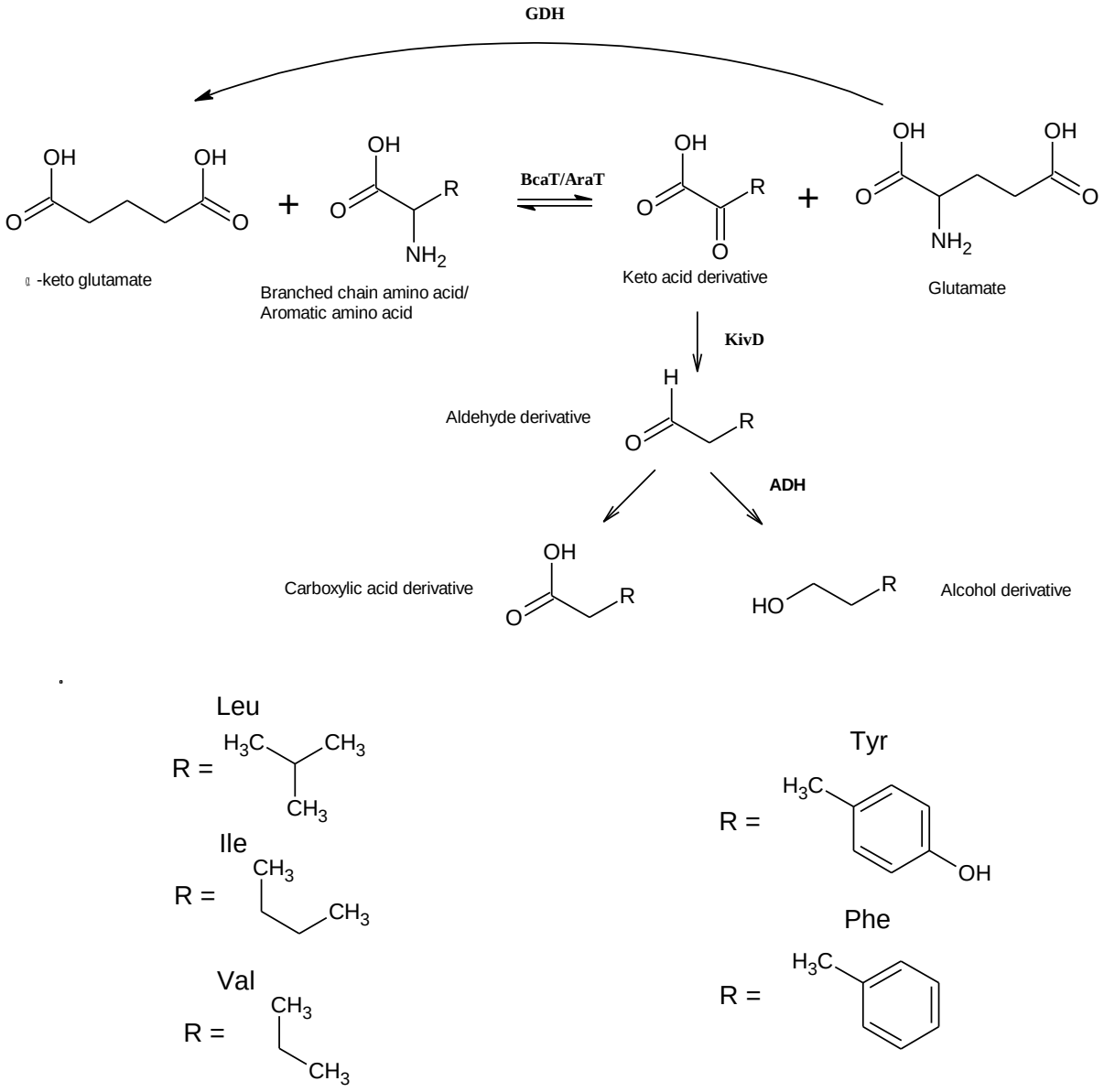

Fig. 2.1. Reaction scheme of simplified branched-chain amino acid and aromatic amino acid degradation pathway. GDH: glutamate dehydrogenase, BcaT: branched-chain amino acid, AraT: aromatic amino acid transaminase, KivD: Keto acid decarboxylase and ADH: alcohol dehydrogenase.

2.3 Arginine catabolism

2.3.1 Arginine deiminase pathway

Of all amino acids Arg is the most studied in LAB and is one of the major amino acids in grape must and wine. Arginine is degraded by LAB through the arginine deiminase (ADI) pathway. Not all LAB possess the ADI pathway and it seems to be genus specific. This pathway benefits the LAB through energy provision (under sugar limiting conditions) and increasing of intracellular pH by producing ATP and ammonium respectively.

Degradation of arginine entails 3 consecutive steps. The first reaction is a deamination reaction in which Arg is degraded to L-citrulline. The second step is the transfer of a carbamoyl (NH_2-CO) group from L-citrulline to a phosphate group to produce L-ornithine and carbamoyl phosphate. The L-citrulline may also be extruded and spontaneously react with the ethanol in the medium to produce the carcinogen, ethyl carbamate. In the final step a phosphate is transferred from carbamoyl phosphate to ADP to form ATP, carbon dioxide and ammonia. The cycle is self-sustaining as the intracellular ornithine (a product of arginine catabolism) is expelled for extracellular arginine effectively trading product for reactant and thus ensuring the cycle continuous (Tonon and Lonvaud-Funel, 2002).

In *L. sakei* the genes of the ADI path are arranged in a cluster (*argABCTDR*). The following genes *arcA*, *arcB*, *arcC arcT arcD* and *argR* codes for the expression of the arginine deiminase, ornithine transcarbomoylase, carbamate kinase, ornithine-arginine antiporter, transferase and the regulatory protein of the Crp/Fnr family respectively (Zúñiga et al., 2002, 1998). The organization of genes differ in *O. oeni*. The arcR lies upstream of argA, there are 2 arcD genes ($arcD_1$ and $arcD_2$). Thus the operon is organised as follows: *arcRABCD1D2* (Divol et al., 2003). Contrary to *L. sakei* the expression of *arc*D1 and *arg*D2 are constitutively expressed and are not influenced by the presence Arg (Divol et al., 2003).

Literature have identified several key aspects which could play a role in Arg catabolism regulation namely the LAB presiding over the fermentation, catabolic repression, pH of the medium and Arg supplementation. The ADI pathway is most commonly observed in obligate heterofermentative lactobacilli (*L. sanfranciscensis*, *L. hilgardii*, *L. brevis* and *L. fructivorans*) (De Angelis et al., 2002). The only homofermentative lactobacilli, *L. plantarum* is not often associated with ADI degradation (Lerm et al., 2011; Liu et al., 1995). Some *L. plantarum* stains have ADI activity but are sometimes noted to be deficient in one of the 3 enzymes. For example in a sourdough isolated *L. plantarum* strain, carbamate kinase activity was absent and a study into South African wine-isolated *L. plantarum* strains revealed an absence of the *argA* gene (Lerm et al., 2011). However *L. plantarum* strains isolated from Italian red must is seen to degrade Arg through the ADI pathway (Spano et al., 2004). This is also seen in *L. plantarum* isolated from orange juice (Arena et al., 1999). Therefore, the ability for LAB to degrade Arg through the ADI pathway is highly strain specific.

In *L. sake* the presence of glucose (0.1 g/L) is seen to exert repression upon the *argA* gene and citrulline accumulation (Montel and Champomier, 1992; Zúñiga et al., 1998). More energy is generated through substrate-level phosphorylation than chemiosmosis (through which the ADI pathway generates its energy) (Tonon and Lonvaud-Funel, 2002). Therefore, the need for chemiosmosis becomes unnecessary in the presence of sugars (Konings et al., 1997; Molenaar et al., 1993). However, catabolite repression is not reflected in all LAB strains as neither *L. plantarum* nor *O. oeni*, loses activity at high glucose concentrations (Spano et al., 2004; Tonon et al., 2001).

As mentioned before the ADI pathway leads toward the production of ammonia which causes intracellular pH to increase. LAB uses this pathway to overcome the acidic pH in media to ensure their survival (Tonon and Lonvaud-Funel, 2002). For this reason high expression of the *arc* gene is seen in *L. plantarum* at pH 3.6 and

4.5. The rise in pH has been shown to play a significant role in LAB survival in wine and improve metabolic turnover. In fact *O. oeni* can completely degrade Arg at pH of 3.9, partially at 3.6 and nothing at pH 3.3 due to the acidic environment (de Orduña et al., 2001). pH also influences enzymatic activity of the arginine deiminase, ornithine transcarbamoylase and carbamate kinase functions optimally at pH 5.0, 6.5 and 6.0 respectively(Champomier Vergès et al., 1999; De Angelis et al., 2002).

Lastly, the presence of Arg is a major inducer of ADI activity. Without the presence of Arg, basal level of *arc* gene are expressed but when Arg is added, the arc gene expression is significantly expressed in *L. plantarum* (Spano et al., 2004). In *O. oeni* there the presence of Arg does not influence the expression of the of the *arc* genes (Divol et al., 2003).

2.4 Biogenic amine formation

Biogenic amines are low molecular weight nitrogenous compounds commonly found in wine at low concentrations. This subject has been under investigation for decades since these molecules are found commonly in wine and the intake of these compounds are associated with adverse health defects in humans such as heart palpitations, headaches, high blood pressure and several allergic disorders in humans (Mete et al., 2017; Silla Santos, 1996). All the amino acid precursors of biogenic amines frequently found in wine are summarised in **Table 2.1**. Of all the biogenic amines histamine and tyramine is of the highest relevance since these two amines are the most toxic and their concentration generally increases during MLF (Marcobal et al., 2006). The concern for the toxic nature of these compounds has resulted in an embargo on wines containing histamine above a specific threshold of 10 mg/L from several European countries (Austria, Belgium, France, Germany and Switzerland) (Polo et al., 2011). Another prevalent biogenic amine is putrescine which smells reminiscent of rotten meat. Histamine, tryptamine and putrescine represent the majority of biogenic amines in wine (Moreno-Arribas et al., 2000).

Table 2.1.The amino acid precursors and the resulting product through decarboxylation [adapted from Silla Santos, (1996)]

Amino acid (substrate)	Biogenic amine (product)
Histidine	Histamine
Tyrosine	Tyramine
Tryptophan	Tryptamine
Phenylalanine	Phenylethylamine
Lysine	Cadaverine
Arginine/ Ornithine	Spermidine and spermine/Putrescine

There can be no doubt that spontaneous MLF causes biogenic amines to increase in wine (Marcobal et al., 2006; Wang et al., 2014). There are many factors that influences this increase but it can be summarised in 2 principal factors namely wine composition and the strain(s) used to conduct MLF. Several parameters of wine are identified to play a role in biogenic amine formation namely pH, SO_2, ethanol, amino acids, sugar and organic acid concentration.

Wine pH influences the viability of the LAB and the enzymatic activity of the decarboxylases. In the latter case enzymatic characterization of the *tdc* from *L. brevis* revealed the enzyme to be active at pH 3 -7 with optimal activity at pH 5 (Moreno-Arribas and Lonvaud-Funel, 1999). Wine pH lower than 3.4 are unfavourable towards the growth of LAB (Guerzoni et al., 1995). Polo et al. (2011) showed that the longer natural LAB remained viable, the more biogenic amines are produced. Thus higher pH are more favourable for biogenic amine accumulation as it has a larger diversity of LAB and the LAB also higher in number.

High concentrations of SO_2 and ethanol (11-13% v/v) will repress LAB growth and subsequently prevent amino acid decarboxylation (Mazzoli et al., 2009). However, ethanol is also a repressor of diamine oxidases responsible for oxidative deamination of biogenic amines (Silla Santos, 1996).

There are contradicting information on whether biogenic amine production is effected by the initial quantity of the amino acids prior to MLF. Many studies has observed an increase in histamine concentration with supplementation of His (Lorenzo et al., 2017; Mazzoli et al., 2009; Molenaar et al., 1993). In support of this evidence, Landete et al. (2006), found higher expression of the His decarboxylase (*hdc*) gene resulting in higher histamine concentration. However, in other studies, no correlation is seen with the availability of His and histamine accumulation (Bauza et al., 1995; Martínez-Pinilla et al., 2013). But when Arg and His was supplied together in synthetic medium, Mazzoli et al. (2009) found histamine concentration to decrease together with ornithine. This study concluded that histamine production was repressed through the division of metabolic flux between His decarboxylation and the ADI pathway. Therefore repression of an individual biogenic amine is mediated through the lack of amino acid supplementation and higher diversity in amino acid composition. Similar data is also seen with tyrosine supplementation and tyramine accumulation. Of all the factors analysed by Moreno-Arribas et al. (2000) only Tyr is seen to highly stimulate tyramine production. In contrast high tyramine concentration is seen to repress TDC activity.

LAB occupy various ecological niches. Not all of these niches are abundant in energy rich carbon sources. Wine, for example, contain 2-5 g/L of sugars and the generation of energy through substrate level phosphorylation in this environment is limited. Therefore, LAB must compensate by gathering energy via chemiosmosis. L-malic acid, the principal substrate in MLF, is decarboxylated to lactic acid, a milder acid. L-lactic acid is released by membrane vesicles outside the cell into the wine matrix in exchange for L-malic acid through an antiporter transport system. This exchange provides for a change in the transmembrane pH gradient and membrane potential for the synthesis of ATP. The intracellular environment becomes acidic while the pH of the wine matrix slightly increases while at the same time the membrane vesicle become negatively charged on account of the accumulation of deprotonated L-malic acid (Konings et al., 1997). ATP synthesis is driven in the exact same fashion with the decarboxylation of amino acids except the precursor (amino acids) have a pKa value higher than the product (biogenic amine) (Konings et al., 1997; Molenaar et al., 1993). It is possible the decarboxylation is activated under energy limiting conditions when ATP cannot be generated through substrate level phosphorylation. Also Landete et al. (2006) found that the *hdc* was activated during exponential phase possibly to provide energy to match the demand for energy demand during this phase. When the cells

reached stationary phase the *hdc* was repressed. In support of this expression study Moreno-Arribas et al. (2000) found increased HDC activity at the end of the exponential phase. Higher concentrations of D-glucose, D-fructose, L citric acid, L-malic acid and L-lactic acid is shown to repress the formation of histamine (Landete et al., 2006). L-citric acid has been shown to exert some repression on TDC activity. In contrast, other studies reported malate and citrate concentration have no effect on histamine accumulation and glucose is seen to enhance histamine formation (Mazzoli et al., 2009; Moreno-Arribas et al., 2000).

The most important criterion for limiting biogenic amine formation is to conduct MLF with a commercial strain of LAB that have been selected not to possess the genes responsible for biogenic amine formation. The distribution of decarboxylase-positive LAB are quite low in wine. LAB has only a few strains capable of producing biogenic amines (Lerm et al., 2011; Moreno-Arribas et al., 2000, 2003). Spontaneous MLF fermentations usually leads to higher increases in biogenic amines compared to MLF induced by a carefully selected commercial strain of *O. oeni* (Polo et al., 2011). This is because spontaneous fermentations have a wide variety of LAB genera and species which may carry the undesirable decarboxylase genes. Strains of *L. brevis* and *L. hilgardii* are more frequently associated with the presence of the *tdc* gene than other species (Coton et al., 2010; Downing, 2003; Lucas and Lonvaud-Funel, 2002) and are the most frequent producers of tyramine (Landete et al., 2007; Moreno-Arribas et al., 2000). In other lactobacilli, tyramine synthesis is rarely observed or completely absent (Guerrini et al., 2002; Landete et al., 2007; Lerm, 2010; Moreno-Arribas et al., 2000). *O. oeni* on the other hand has been associated with the production of histamine more frequently (Guerrini et al., 2002; Landete et al., 2005). Data also exist that shows *O. oeni* to be devoid of HDC activity (Moreno-Arribas et al., 2003). However the histamine production is not characteristic of *O. oeni* as the *hdc* gene is frequently remarked to be absent but when present, the production of histamine is quiet low when compared to other LAB strains such as *Pediococcus parvalus* and *L. hilgardii* (Guerrini et al., 2002; Landete et al., 2005; Moreno-Arribas et al., 2003).

Some winemaking factors such as the addition of pectolytic enzymes, aging with lees, longer skin maceration time and fermentation temperature are shown to influence the biogenic amine concentration (Lorenzo et al., 2017; Martín-Álvarez et al., 2006; Rosi et al., 2009). With aging the yeast autolyse and releases vitamins and amino acids that favour the growth of LAB, skin maceration releases phenolic compounds, amino acids, proteins and polysaccharides and increased fermentation temperatures increases the metabolic rate of LAB (Smit et al., 2008).

2.5 Threonine catabolism

Acetaldehyde is an important wine component and plays a role in the catabolism of Thr. Thr aldolase (EC 4.1.2.48), is the enzyme responsible for this reaction (Ott et al., 2000). Gly is also produced as a result. Enzyme assays on Thr aldolase revealed Gly might inhibit the enzyme depending on the organism. Numerous studies have shown in *Streptococcus thermophilus* and *Lactobacillus bulgaricus* that Gly have a feed-back inhibition on Thr aldolase i.e. a greater concentration of Gly would reduce the concentration of acetaldehyde. On the other hand increased concentration of Thr would stimulate acetaldehyde production (Marranzine et al., 1989;

Rysstad et al., 1990; Wilkins et al., 1986). Marranzine et al. (1989) pointed out that the stimulation of Thr aldolase by Thr may be greater than the inhibitory effect of Gly. Thus the Thr aldolase exist with the intension of creating and maintaining the Gly balance for growth.

2.6 Aspartate catabolism

The catabolism of Asp has already been extensively covered in another review (Fernández and Zúñiga, 2006). Briefly, Asp can be degraded by 1 of 3 pathways. Transamination catalysed by an Asp transferase (EC 2.6.1.1), decarboxylation via an Asp decarboxylase (EC 4.1.1.12) and elimination through the action of the aspartate lyase (EC 4.3.1.1).

Enzyme characterization of an Asp transferase from *Lactobacillus brevis* has shown the enzyme operates at maximal efficiency at 25ºC and has high affinity towards its substrates α-ketoglutarate and Asp (Hu et al., 2017). In contrast an Asp aminotransferase from *L. munnis* had optimum temperature of 40ºC and had a greater affinity for Asp than α-ketoglutarate. Asp seem to be the most preferred source of amino acid transferase activity than the BCAA, AAA and Met (Kieronczyk et al., 2004; Peralta et al., 2016). Subsequently, glutamate dehydrogenase activity (responsible for deamination of glutamate to α-keto glutamate) has been observed to favour the transamination of Asp. As a result more acetoin and diacetyl is produced (Kieronczyk et al., 2004).

2.7 Sulphur amino acid catabolism

Met and Cys are the sulphur-containing amino acids. The catabolism of Met is mainly responsible for the production of volatile sulphur compounds (VSC) like dimethylsulphide (DMS), dimethyldisulphide (DMDS), dimethytrisulphide (DMTS) and methional. Generally the formation of VSC above the perception threshold is quite detrimental to the aroma profile of wine but beneficial to the ripening of cheese as it adds the characteristic cheese aroma. Cysteine catabolism on the other hand produces hydrogen sulphide (H_2S), an odour reminiscent of rotten egg.

Met degradation can take place through 2 pathways: transamination pathway and elimination pathway (**Fig. 2.2**). In the transamination pathway, Met is exposed to the same pathway and enzymes as previously described for BCAA and AAA (see **section 2.2**) although the activity towards Met is markedly lower (Rijnen et al., 2003; Yvon et al., 2000). Therefore, Met degradation through the transaminase pathway is subjected to the same regulatory control. The final products of this pathway are methionol and 3-methylthiopropionic acid which are the alcohol and carboxylic acid derivatives respectively. *O. oeni* and *Lactobacillus* is capable of producing both of these compounds in red wine during MLF with *O. oeni* being the highest producer of all wine LAB (Pripis-Nicolau et al., 2004). Furthermore, the initial concentration of Met in wine before MLF is reported to affect the production of methionol. Any grape variety with higher concentration of Met in the grape must may result in higher concentration of methionol in the wine after MLF (Moreira et al., 2002; Ugliano and Moio, 2005).

Met elimination proceeds through a C-5 lyase catalysed by cystathionine-γ-lyase (EC 4.4.1.1) through α,γ-elimination producing methanethiol and ammonia (Hanniffy et al., 2009). The centralised metabolite in VSC

synthesis is methanetiol. DMS, DMDS and DMTS are oxidised chemically from methanethiol. Also, thioesters can be produced through the addition of fatty acids. The production of methanethiol can proceed through 2 pathways: either indirectly through transamination and decarboxylation or directly through C-5 lyase. In the transamination pathway, Met lead to formation of keto-γ-methylthiobutyric acid (KMBA) and is subsequently either chemically oxidised or decarboxylated to methanethiol (Hanniffy et al., 2009). Furthermore there exist a negative correlation with increase Met addition and aminotransferase activity (Dias and Weimer, 1998). Low decarboxylation activity in *Lc. lactis* is another impediment of methanethiol. Nevertheless *Lc. lactis* has high transaminase activity towards Met despite the above-mentioned obstacles (Hanniffy et al., 2009). However the subsequent decarboxylase activity is very low in *Lc. lactis*.

Both Cys and Met are substrates for elimination by cystathionine-γ-lyase (EC 4.4.1.1). The mechanism of action for this enzyme is an α,γ- elimination of Met resulting in methanethiol and ammonia and α,β-elimination of Cys resulting in H_2S and ammonia and pyruvate (Bruinenberg et al., 1997; Knoll et al., 2011). Enzyme characterization of cystathionine-γ-lyase in *O. oeni* and *Lc lactis* indicated that the enzyme greatly prefers Cys over Met as substrate and has optimal activity at alkaline pH (Bruinenberg et al., 1997; Bustos et al., 2011; Hanniffy et al., 2009; Knoll et al., 2011). All sulphur amino acid degradation enzymes (transaminases and lyases) are pyridoxyl-5-phosphate dependent (Bruinenberg et al., 1997; Knoll et al., 2011). Incubation with higher pyridoxyl-5-phosphate concentration increased production of VSC at cheese pH and temperature (Wolle et al., 2006). In addition enzymatic activity may increase with extended aging of cheese and degrade substrates at faster rates (Burbank and Qian, 2008). A C-5 lyase (YtjE) is shown also to be under control of the CodY repressor. The relative expression of YtjE only increases exponentially after stationary phase (García-Cayuela et al., 2012).

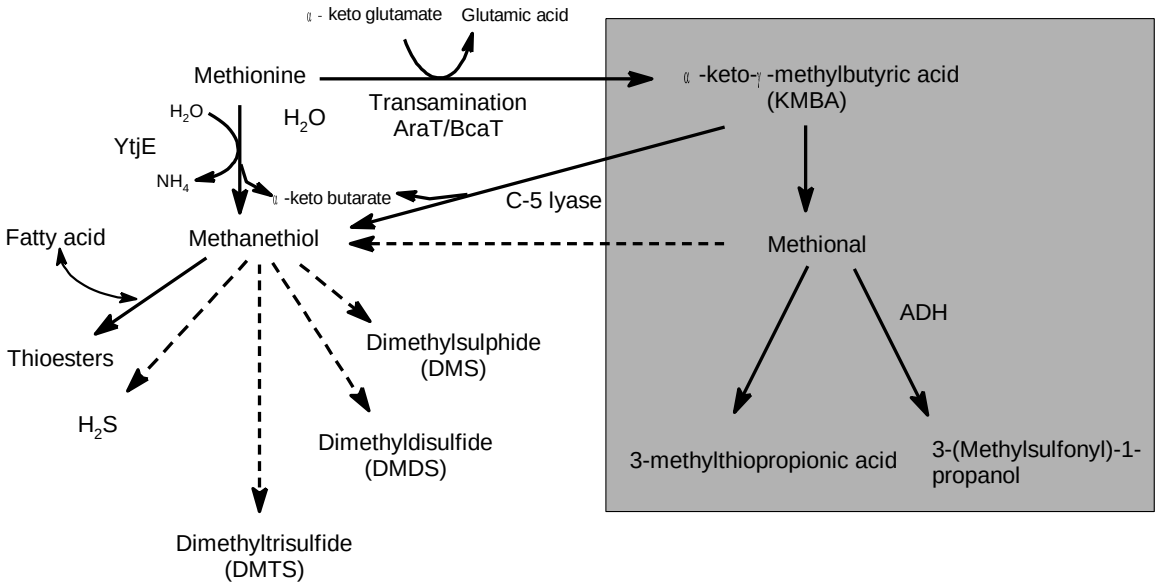

Fig. 2.2. Methionine catabolism in lactic acid bacteria (Adapted from Hanniffy et al., 2009).

2.8 Lysine catabolism

LAB metabolism of lysine has been implicated in the formation of 2-acetyltetrahydropyridine, a N-heterocycle compound. This is a spoilage odour compound responsible for the mousy taint of wine. 2-Lysine is probably degraded via a 2,3,4,5-tetrahydropyridine intermediate whereby acetylation occurs to produce 2-acetyltertrahydropyridine (Costello and Henschke, 2002). It seems the production of 2-acetyltetrahydropyridine seems only to be reserved for heterofermentative LAB (*Lactobacillus*, *Leuconostoc* and *Oenococcus*) (Costello et al., 2001). In heterofermentative LAB, fermentable carbon sources are degraded via the phosphoketolase pathway. Acetate and ethanol are synthesized and can be utilized in an acylation reaction with 2,3,4,5-tetrahydropyridine to synthesize 2-acetyltetrahydropyridine (**Fig. 2.3**). In contrast no N-heterocyclic production is seen in *L. plantarum* and *Pediococcus* which are homofermentative LAB (Costello et al., 2001; Zúñiga et al., 1993). It is for this reason substantial higher production of 2-acetyltertrahydropyridine is observed when fructose is available in excess. Furthermore, the presence of ethanol, Fe^{2+} ions and Lys increases the production of 2-acetyltetrahydropyridine (Costello and Henschke, 2002).

2.9 Conclusion

LAB occupy a variety of ecological niches with low pH, high osmolarity and anaerobiosis, which are uninhabitable to most other microorganisms. This is achievable due to LAB's frugal control over its metabolism. The catabolism of amino acids provides for energy through chemiosmosis, increase in the intracellular pH, redox balance and a source of nitrogen, sulphur and carbon.

Generally speaking, the degradation of amino acids were mostly influenced by 2 factors namely the growth of the LAB in question and the carbohydrate metabolism. Firstly if LAB favours the medium in which it resides there will be a corresponding higher growth and higher amino acid degradation since these conditions favour higher enzymatic activity. Secondly, in terms of carbohydrate metabolism, homofermentative bacteria are less associated with biogenic amine, ethyl carbamate and 2-acetyltetrahydropyridine formation than their heterofermentative counterparts.

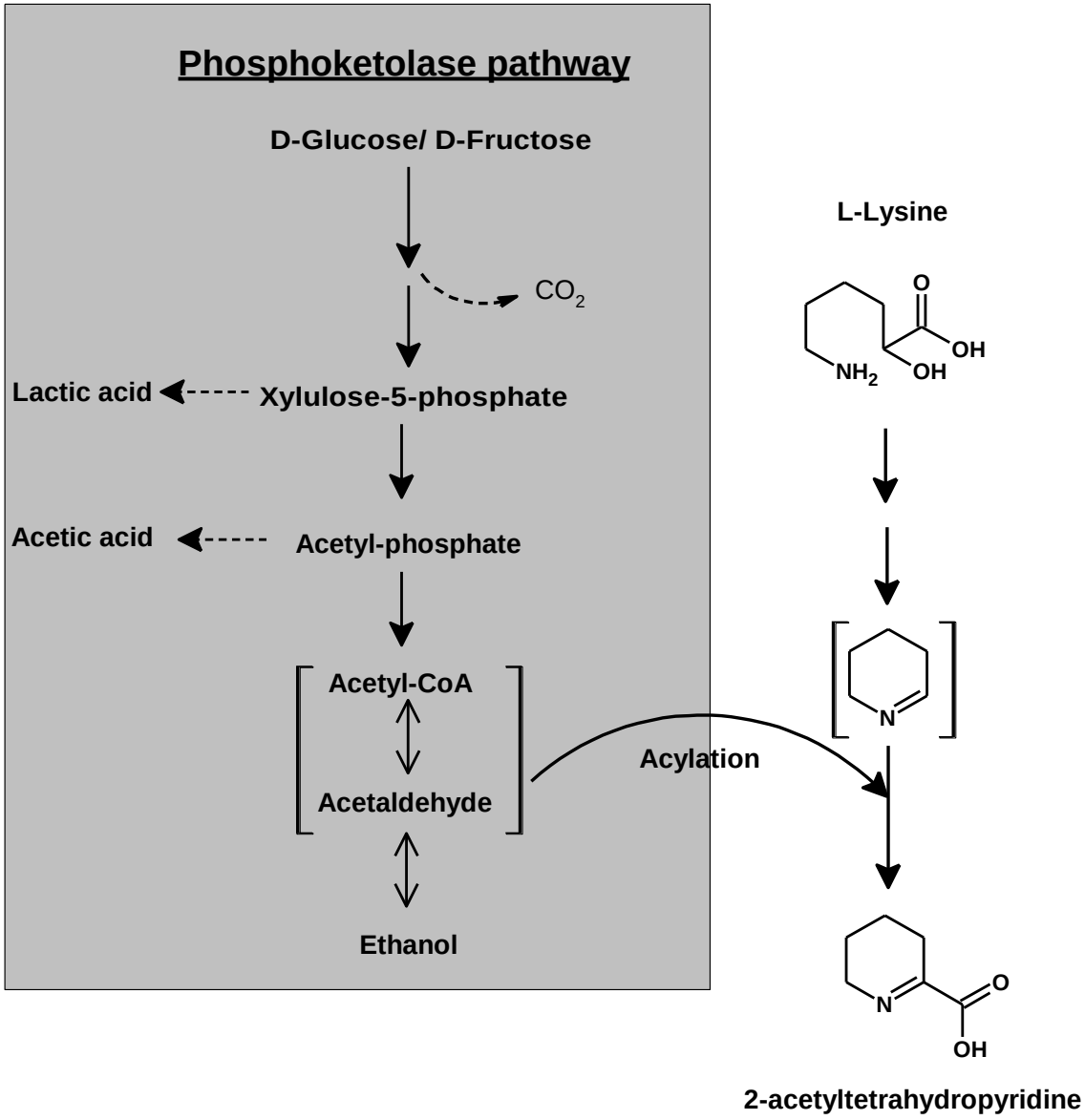

Fig. 2.3. Lysine degradation and the formation of 2-acetyltetrahydrpyridine (Adapted from Costello and Henschke, 2002)

2.10 References

Arena, M.E., Saguir, F.M., Manca De Nadra, M.C., 1999. Arginine dihydrolase pathway in *Lactobacillus plantarum* from orange. Int. J. Food Microbiol. 47, 203–209.

Bartowsky, E.J., Henschke, P.A., 2004. The "buttery" attribute of wine - diacetyl - desirability, spoilage and beyond. Int. J. Food Microbiol. 96, 235–252.

Bauza, T., Blaise, A., Daumas, F., Cabanis, J.C., 1995. Determination of biogenic amines and their precursor amino acids in wines of the Vallée du Rhône by high-performance liquid chromatography with precolumn derivatization and fluorimetric detection. J. Chromatogr. A 707, 373–379.

Bruinenberg, P.G., De Roo, G., Limsowtin, G.K.Y., 1997. Purification and characterization of cystathionine γ-lyase from *Lactococcus lactis* subsp. *cremoris* SK11: Possible role in flavor compound formation during cheese maturation. Appl. Environ. Microbiol. 63, 561–566.

Burbank, H., Qian, M.C., 2008. Development of volatile sulfur compounds in heat-shocked and pasteurized milk cheese. Int. Dairy J. 18, 811–818.

Bustos, I., Martínez-Bartolomé, M.A., Achemchem, F., Peláez, C., Requena, T., Martínez-Cuesta, M.C., 2011. Volatile sulphur compounds-forming abilities of lactic acid bacteria: C-S lyase activities. Int. J. Food Microbiol. 148, 121–127.

Cavin, J.-F., Barthelmebs, L., Divies, C., 1997. Molecular characterization of an inducible *p*-coumaric acid decarboxylase from *Lactobacillus plantarum*: gene cloning, transcriptional analysis, overexpression in *Escherichia coli*, purification, and characterization. Appl. Environ. Microbiol. 63, 1939–1944.

Chambellon, E., Yvon, M., 2003. CodY-regulated aminotransferases AraT and BcaT play a major role in the growth of *Lactococcus lactis* in milk by regulating the intracellular pool of amino acids. Appl. Environ. Microbiol. 69, 3061–3068.

Champomier Vergès, M.C., Zuñiga, M., Morel-Deville, F., Pérez-Martínez, G., Zagorec, M., Ehrlich, S.D., 1999. Relationships between arginine degradation, pH and survival in *Lactobacillus sakei* . FEMS Microbiol. Lett. 180, 297–304.

Christensen, J.E., Dudley, E.G., Pederson, J.A., Steele, J.L., 1999. Peptidases and amino acid catabolism in lactic acid bacteria. Antoine van Leeuwenhoek 76, 217–246.

Costello, P.J., Henschke, P.A., 2002. Mousy off-flavor of wine: Precursors and biosynthesis of the causative N-Heterocycles 2-ethyltetrahydropyridine, 2-acetyltetrahydropyridine, and 2-acetyl-1-pyrroline by *Lactobacillus hilgardii* DSM 20176. J. Agric. Food Chem. 50, 7079–7087.

Costello, P.J., Lee, T.H., Henschke, P., 2001. Ability of lactic acid bacteria to produce N-heterocycles causing mousy off-flavour in wine. Aust. J. Grape Wine Res. 7, 160–167.

Coton, M., Romano, A., Spano, G., Ziegler, K., Vetrana, C., Desmarais, C., Lonvaud-Funel, A., Lucas, P., Coton, E., 2010. Occurrence of biogenic amine-forming lactic acid bacteria in wine and cider. Food Microbiol. 27, 1078–1085.

De Angelis, M., Mariotti, L., Rossi, J., Servili, M., Fox, P.F., Rollán, G., Gobbetti, M., 2002. Arginine catabolism by sourdough lactic acid bacteria: Purification and characterization of the arginine deiminase pathway enzymes from *Lactobacillus sanfranciscensis* CB1. Appl. Environ. Microbiol. 68, 6193–6201.

De la Plaza, M., Fernández de Palencia, P., Carmen, P., Requena, T., 2004. Biochemical and molecular characterization of α-ketoisovalerate decarboxylase, an enzyme involved in the formation of aldehydes from amino acids by Lactococcus lactis. FEBS Lett. 238, 367–374.

de Orduña, R.M., Patchett, M., Lui, S.-Q., Pilone, G.J., 2001. Growth and arginine metabolism of the wine lactic acid bacteria *Lactobacillus buchneri* and *Oenococcus oeni* at different pH values and arginine concentrations. Appl. Environ. Microbiol. 67, 1657–1662.

Delorme, C., Ehrlich, S.D., Renault, P., 1999. Regulation of expression of the *Lactococcus lactis* histidine operon. J. Bacteriol. 181, 2026–2037.

Delorme, C., Godon, J.-J., Ehrlich, S.D., Renault, P., 1993. Gene inactivation in *Lactococcus lactis* : histidine biosynthesis. J. Bacteriol. 175, 4391–4399.

Den Hengst, C.D., Curley, P., Larsen, R., Buist, G., Nauta, A., van Sinderen, D., Kuipers, O., Kok, J., 2005a. Probing Direct Interactions between CodY and the oppD Promoter of *Lactococcus lactis*. J. Bacteriol. 187, 512–521. doi:10.1128/JB.187.2.512

Den Hengst, C.D., Van Hijum, S.A.F.T., Geurts, J.M.W., Nauta, A., Kok, J., Kuipers, O.P., 2005b. The *Lactococcus lactis* CodY regulon: Identification of a conserved cis-regulatory element. J. Biol. Chem. 280, 34332–34342.

Dias, B., Weimer, B., 1998. Conversion of methionine to thiols by Lactococci, Lactobacilli, and Brevibacteria.

Appl. Environ. Microbiol. 64, 3320–3326.

Dickinson, J.R., Harrison, S.J., Dickinson, J.A., Hewlins, M.J.E., 2000. An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae* . J. Biol. Chem. 275, 10937–10942.

Dickinson, J.R., Harrison, S.J., Hewlins, M.J.E., 1998. An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae* . J. Biol. Chem. 273, 25751–25756.

Dickinson, J.R., Lanterman, M.M., Danner, D.J., Pearson, B.M., Sanz, P., Harrison, S.J., Hewlins, M.J.E., 1997. A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae* 272, 26871–26878.

Divol, B., Tonon, T., Morichon, S., Gindreau, E., Lonvaud-Funel, A., 2003. Molecular characterization of *Oenococcus oeni* genes encoding proteins involved in arginine transport. J. Appl. Microbiol. 94, 738–746.

Downing, L., 2003. Characterisation of biogenic amine genes in lactic acid bacteria isolated from wine. Inst. Wine Biotechnol. Stellenbosch Univ. South Africa.

Driessen, A.J., de Jong, S., Konings, W.N., 1987. Transport of branched-chain amino acids in membrane vesicles of *Streptococcus cremoris*. J. Bacteriol. 169, 5193–5200.

Engels, W., 1997. Volatile and non-volatile compounds in ripened cheese: their formation and their contribution to flavour.

Esteban-Torres, M., Reverón, I., Mancheño, J.M., De las Rivas, B., Muñoz, R., 2013. Characterization of a feruloyl esterase from *Lactobacillus plantarum* . Appl. Environ. Microbiol. 79, 5130–5136.

Fernández, M., Zúñiga, M., 2006. Amino acid catabolic pathways of lactic acid bacteria. Crit. Rev. Microbiol. 32, 155–83.

Fernández de Palencia, P., De la Plaza, M., Amarita, F., Requena, T., Pelaez, C., 2006. Diversity of amino acid converting enzymes in wild lactic acid bacteria. Enzyme Microb. Technol. 38, 88–93.

García-Cayuela, T., Gómez de Cadiñanos, L.P., Peláez, C., Requena, T., 2012. Expression in *Lactococcus lactis* of functional genes related to amino acid catabolism and cheese aroma formation is influenced by branched chain amino acids. Int. J. Food Microbiol. 159, 207–213.

Godon, J., Delorme, C., Bardowski, J., Chopin, M., Ehrlich, S., Renault, P., 1993. Gene inactivation in *Lactococcus lactis*: Branched-chain amino acid biosynthesis. J. Bacteriol. 175, 4383–4390.

Guédon, E., Serror, P., Ehrlich, S.D., Renault, P., Delorme, C., 2001. Pleiotropic transcriptional repressor CodY senses the intracellular pool of branched-chain amino acids in *Lactococcus lactis* . Mol. Microbiol. 40, 1227–1239.

Guerrini, S., Mangani, S., Granchi, L., Vincenzini, M., 2002. Biogenic amine production by *Oenococcus oeni*. Curr. Microbiol. 44, 374–378.

Guerzoni, M.E., Sinigaglia, M., Gardini, F., Ferruzzi, M., Torriani, S., 1995. Effects of pH, temperature, ethanol, and malate concentration on *Lactobacillus plantarum* and *Leuconostoc oenos* : Modelling of the malolactic activity. Am. J. Enol. Vitic. 46, 368–374.

Hanniffy, S.B., Peláez, C., Martínez-Bartolomé, M.A., Requena, T., Martínez-Cuesta, M.C., 2009. Key enzymes involved in methionine catabolism by cheese lactic acid bacteria. Int. J. Food Microbiol. 135, 223–230.

Hassan, F., El-Gawad, M.A., Enab, A., 2013. Flavour Compounds in Cheese (Review). Res. Precis. Instrum. Mach. 2, 15–29.

Hu, S., Zhang, X., Lu, Y., Lin, Y.-C., Xie, D.-F., Fang, H., Huang, J., Mei, L.-H., 2017. Cloning, expression

and characterization of an aspartate aminotransferase gene from *Lactobacillus brevis* CGMCC 1306. Biotechnol. Biotechnol. Equip. 31, 544–553.

Kieronczyk, A., Skeie, S., Langsrud, T., Le Bars, D., Yvon, M., 2004. The nature of aroma compounds produced in a cheese model by glutamate dehydrogenase positive *Lactobacillus* INF15D depends on its relative aminotransferase activities towards the different amino acids. Int. Dairy J. 14, 227–235.

Knoll, C., Du Toit, M., Schnell, S., Rauhut, D., Irmler, S., 2011. Cloning and characterisation of a cystathionine β/γ-lyase from two *Oenococcus oeni* oenological strains. Appl. Microbiol. Biotechnol. 89, 1051–1060.

Konings, W.N.N., Lolkema, J.S.S., Bolhuis, H., Veen, H.W. van W. Van, Poolman, B., Driessen, A.J.M., 1997. Energy transduction and multidrug resistance F pH mv. Antonie Van Leeuwenhoek 71, 117–128.

Landete, J.M., Ferrer, S., Pardo, I., 2005. Which lactic acid bacteria are responsible for histamine production in wine? J. Appl. Microbiol. 99, 580–586.

Landete, J.M., Pardo, I., Ferrer, S., 2007. Tyramine and phenylethylamine production among lactic acid bacteria isolated from wine. Int. J. Food Microbiol. 115, 364–368.

Landete, J.M., Pardo, I., Ferrer, S., 2006. Histamine, histidine, and growth-phase mediated regulation of the histidine decarboxylase gene in lactic acid bacteria isolated from wine. FEMS Microbiol. Lett. 260, 84–90.

Lerm, E., 2010. The selection and characterisation of lactic acid bacteria to be used as a mixed starter culture for malolactic fermentation. MSc thesis, Inst. Wine Biotechnol. Stellenbosch Univ. South Africa.

Lerm, E., Engelbrecht, L., du Toit, M., 2011. Selection and characterisation of *Oenococcus oeni* and *Lactobacillus plantarum* South African wine isolates for use as malolactic fermentation starter cultures. South African J. Enol. Vitic. 32, 280–295.

Liu, S., Pritchard, G.G., Hardman, M.J., Pilone, G.J., 1995. Occurrence of arginine deiminase pathway enzymes in arginine catabolism by wine lactic acid bacteria. Appl. Environ. Microbiol. 61, 310–316.

Lorenzo, C., Bordiga, M., Pérez-Álvarez, E.P., Travaglia, F., Arlorio, M., Salinas, M.R., Coïsson, J.D., Garde-Cerdán, T., 2017. The impacts of temperature, alcoholic degree and amino acids content on biogenic amines and their precursor amino acids content in red wine. Food Res. Int. 99, 328–335.

Lucas, P., Lonvaud-Funel, A., 2002. Purification and partial gene sequence of the tyrosine decarboxylase of *Lactobacillus brevis* IOEB 9809. FEMS Microbiol. Lett. 211, 85–89.

Malherbe, S., Menichelli, E., du Toit, M., Tredoux, A., Muller, N., Næs, T., Nieuwoudt, H., 2013. The relationships between consumer liking, sensory and chemical attributes of *Vitis vinifera* L. cv. Pinotage wines elaborated with different *Oenococcus oeni* starter cultures. J. Sci. Food Agric. 93, 2829–2840.

Marcobal, Á., Martín-Álvarez, P.J., Polo, M.C., Muñoz, R., Moreno-Arribas, M.V., 2006. Formation of biogenic amines throughout the industrial manufacture of red wine. J. Food Prot. 69, 397–404.

Marranzine, R.M., Schmidt, R.H., Shireman, R.B., Marshall, M.R., Cornell, John, A., 1989. Effect of threonine and glycine concentration on threonine aldolase activity of yogurt microorganisms during growth in a modified milk prepared by ultrafiltration. J. Dairy Sci. 72, 1142–1148.

Martín-Álvarez, P.J., Marcobal, Á., Polo, C., Moreno-Arribas, M.V., 2006. Influence of technological practices on biogenic amine contents in red wines. Eur. Food Res. Technol. 222, 420–424.

Martínez-Pinilla, O., Guadalupe, Z., Hernández, Z., Ayestarán, B., 2013. Amino acids and biogenic amines in red varietal wines: The role of grape variety, malolactic fermentation and vintage. Eur. Food Res. Technol. 237, 887–895.

Mazzoli, R., Lamberti, C., Coisson, J.D., Purrotti, M., Arlorio, M., Giuffrida, M.G., Giunta, C., Pessione, E., 2009. Influence of ethanol, malate and arginine on histamine production of *Lactobacillus hilgardii*

isolated from an Italian red wine. Amino Acids 36, 81–89.

Mete, A., Coşansu, S., Demirkol, O., Ayhan, K., 2017. Amino acid decarboxylase activities and biogenic amine formation abilities of lactic acid bacteria isolated from shalgam. Int. J. Food Prop. 20, 171–178.

Molenaar, D., Bosscher, J.S., Ten Brink, B., Driessen, A.J.M., Konings, W.N., 1993. Generation of a proton motive force by histidine decarboxylatioin and electrogenic histidine/histamine antiport in *Lactobacllus buchneri*. J. Bacteriol. 175, 2864–2870.

Monteiro, F.F., Bisson, L.F., 1991. Biological assay of nitrogen content of grape juice and prediction of sluggish fermentations. Am. J. Enol. Vitic. 42, 47–57.

Montel, M.-C., Champomier, M.-C., 1992. Arginine catabolism in *Lactobacillus sake* isolated from meat. Am. Soceity Microbiol. 53, 2683–2685.

Moreira, N., Mendes, F., Pereira, O., Guedes De Pinho, P., Hogg, T., Vasconcelos, I., 2002. Volatile sulphur compounds in wines related to yeast metabolism and nitrogen composition of grape musts. Anal. Chim. Acta 458, 157–167.

Moreno-Arribas, M.V., Polo, M.C., Jorganes, F., Muñoz, R., 2003. Screening of biogenic amine production by lactic acid bacteria isolated from grape must and wine. Int. J. Food Microbiol. 84, 117–123.

Moreno-Arribas, V., Lonvaud-Funel, A., 1999. Tyrosine decarboxylase activity of *Lactobacillus brevis* IOEB 9809 isolated from wine and *L. brevis* ATCC 367. FEMS Microbiol. Lett. 180, 55–60.

Moreno-Arribas, V., Torlois, S., Joyeux, A., Bertrand, A., Lonvaud-Funel, A., 2000. Isolation, properties and behavior of tyramine-producing lactic acid bacteria from wine. J. Appl. Microbiol. 88, 584–593.

Morishita, T., Yajima, M., 1995. Incomplete operation of biosynthetic and bioenergetic functions of the citric acid cycle in multiple auxotrophic lactobacilli. Biosci. Biotechnol. Biochem. 59, 251–255.

Mtshali, P.S., Divol, B., Van Rensburg, P., Du Toit, M., 2010. Genetic screening of wine-related enzymes in *Lactobacillus* species isolated from South African wines. J. Appl. Microbiol. 108, 1389–1397.

Ott, A., Germond, J.E., Chaintreau, A., 2000. Origin of acetaldehyde during milk fermentation using 13C-labeled precursors. J. Agric. Food Chem. 48, 1512–1517.

Peralta, G.H., Bergamini, C.V., Hynes, E.R., 2016. Aminotransferase and glutamate dehydrogenase activities in *lactobacilli* and *streptococci* . Brazilian J. Microbiol. 47, 741–748.

Petranovic, D., Guédon, E., Sperandio, B., Delorme, C., Ehrlich, D., Renault, P., 2004. Intracellular effectors regulating the activity of the *Lactococcus lactis* CodY pleiotropic transaction regulator. Mol. Microbiol. 53, 613–621.

Polo, L., Ferrer, S., Peña-Gallego, A., Hernández-Orte, P., Pardo, I., 2011. Biogenic amine synthesis in high quality Tempranillo wines. Relationship with lactic acid bacteria and vinification conditions. Ann. Microbiol. 61, 191–198.

Pripis-Nicolau, L., De Revel, G., Bertrand, A., Lonvaud-Funel, A., 2004. Methionine catabolism and production of volatile sulphur compounds by *Oenococcus oeni*. J. Appl. Microbiol. 96, 1176–1184.

Pudlik, A.M., Lolkema, J.S., 2013. Uptake of α-ketoglutarate by citrate transporter CitP drives transamination in *Lactococcus lactis* . Appl. Environ. Microbiol. 79, 1095–1101.

Pudlik, A.M., Lolkema, J.S., 2012. Rerouting citrate metabolism in *Lactococcus lactis* to citrate-driven transamination. Appl. Environ. Microbiol. 78, 6665–6673.

Remize, F., Gaudin, A., Kong, Y., Guzzo, J., Alexandre, H., Krieger, S., Guilloux-Benatier, M., 2006. *Oenococcus oeni* preference for peptides: qualitative and quantitative analysis of nitrogen assimilation. Arch. Microbiol. 185, 459–469.

Rijnen, L., Yvon, M., Van Kranenburg, R., Courtin, P., Verheul, A., Chambellon, E., Smit, G., 2003. *Lactococcal* aminotransferases AraT and BcaT are key enzymes for the formation of aroma compounds from amino acids in cheese. Int. Dairy J. 13, 805–812.

Rodríguez, H., Curiel, J.A., Landete, J.M., de las Rivas, B., de Felipe, F.L., Gómez-Cordovés, C., Mancheño, J.M., Muñoz, R., 2009. Food phenolics and lactic acid bacteria. Int. J. Food Microbiol. 132, 79–90.

Rosi, I., Nannelli, F., Giovani, G., 2009. Biogenic amine production by *Oenococcus oeni* during malolactic fermentation of wines obtained using different strains of *Saccharomyces cerevisiae* . LWT - Food Sci. Technol. 42, 525–530.

Rysstad, G., Knutsen, W.J., Abrahamsen, R.K., 1990. Effect of threonine and glycine on acetaldehyde formation in goats' milk yogurt. J. Dairy Res. 3, 401–411.

Silla Santos, M.H., 1996. Biogenic amines: Their importance in foods. Int. J. Food Microbiol. 29, 213–231.

Smit, A.Y., Du Toit, W.J., Du Toit, M., 2008. Biogenic amines in wine: understanding the headache. South African J. Enol. Vitic. 29, 109–127.

Smit, B.A., Engels, W.J.M., Smit, G., 2009. Branched chain aldehydes: production and breakdown pathways and relevance for flavour in foods 987–999.

Smit, B.A., Engels, W.J.M., Wouters, J.T.M., Smit, G., 2004. Diversity of L-leucine catabolism in various microorganisms involved in dairy fermentations, and identification of the rate-controlling step in the formation of the potent flavour component 3-methylbutanal. Appl. Microbiol. Biotechnol. 64, 396–402.

Smit, B.A., van Hylckama Vlieg, J.E., Engels, W.J., Meijer, L., Wouters, J.T.M., Smit, G., 2005. Identification, cloning, and characterization of a *Lactococcus lactis* branched-chain α-keto acid decarboxylase involved in flavor formation. Appl. Environ. Microbiol. 71, 303–311.

Spano, G., Chieppa, G., Beneduce, L., Massa, S., 2004. Expression analysis of putative *arc*A, *arc*B and *arc*C genes partially cloned from *Lactobacillus plantarum* isolated from wine. J. Appl. Microbiol. 96, 185–193.

Spayd, S.E., Andersen-Bagge, J., 1996. Free amino acid composition of grape juice from 12 *Vitis vinifera cultivars* in Washington. Am. J. Enol. Vitic. 47, 389–402.

Tanous, C., Gori, A., Rijnen, L., Chambellon, E., Yvon, M., 2005. Pathways for α-ketoglutarate formation by *Lactococcus lactis* and their role in amino acid catabolism. Int. Dairy J. 15, 759–770.

Thage, B. V., Rattray, F.P., Laustsen, M.W., Ardö, Y., Barkholt, V., Houlberg, U., 2004. Purification and characterization of a branched-chain amino acid aminotransferase from *Lactobacillus paracasei* subsp. *paracasei* CHCC 2115. J. Appl. Microbiol. 96, 593–602.

Tonon, T., Bourdineaud, J.P., Lonvaud-Funel, A., 2001. The *arcABC* gene cluster encoding the arginine deiminase pathway of *Oenococcus oeni*, and arginine induction of a CRP-like gene. Res. Microbiol. 152, 653–661.

Tonon, T., Lonvaud-Funel, A., 2002. Arginine metabolism by wine - *Lactobacilli* isolated from wine. Food Microbiol. 19, 451–461.

Ugliano, M., Moio, L., 2005. Changes in the concentration of yeast-derived volatile compounds of red wine during malolactic fermentation with four commercial starter cultures of *Oenococcus oeni*. J. Agric. Food Chem. 53, 10134–10139.

Wang, Y.Q., Ye, D.Q., Zhu, B.Q., Wu, G.F., Duan, C.Q., 2014. Rapid HPLC analysis of amino acids and biogenic amines in wines during fermentation and evaluation of matrix effect. Food Chem. 163, 6–15.

Wilkins, D.W.., Schmidt, R.H., Kennedy, L.B., 1986. Threonine aldolase activity in yogurt bacteria as determined by headspace gas chromatography. J. Agric. Food Chem. 34, 150–152.

Wolle, D.D., Banavara, D.S., Rankin, S.A., 2006. Short communication: empirical and mechanistic evidence for the role of pyridoxal-5'-phosphate in the generation of methanethiol from methionine. J. Dairy Sci. 89, 4545–50.

Yvon, M., Berthelot, S., Gripon, J.C., 1998. Adding ??-ketoglutarate to semi-hard cheese curd highly enhances the conversion of amino acids to aroma compounds. Int. Dairy J. 8, 889–898.

Yvon, M., Chambellon, E., Bolotin, A., Roudot-Algaron, F., 2000. Characterization and Role of the Branched-Chain Aminotransferase (BcaT) Isolated from *Lactococcus lactis* subsp. *cremoris* NCDO 763. Appl. Environ. Microbiol. 66, 571–577.

Zúñiga, M., Champomier-verges, M., Pérez-martínez, G., 1998. Structural and functional analysis of the gene cluster encoding the enzymes of the arginine deiminase pathway of *Lactobacillus sakei*. J. Appl. Microbiol. 180, 4154–4159.

Zúñiga, M., del Carmen Miralles, M., Pérez-martínez, G., 2002. The product of *arc*R, the sixth gene of the *arc* operon of *Lactobacillus sakei*,is essential for expression of the arginine deiminase pathway. Appl. Environ. Microbiol. 68, 6051–6058.

Zúñiga, M., Pardo, I., Ferrer, S., 1993. An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. Int. J. Food Microbiol. 18, 37–42.

Chapter 3

Research Results

The amino acid requirements and usage of *Lactobacillus plantarum*

Chapter 3 – The amino acid requirements and usage of *L. plantarum*

3.1 Abstract

Lactobacillus plantarum is a lactic acid bacteria (LAB) capable of performing malolactic fermentation (MLF) in grape juice and wine. During MLF, LAB deacidify and alter the aroma composition of the wine. A major contributor to the aroma profile of wine is the metabolism of amino acids. This study aimed to better understand the amino acid requirements of *L. plantarum* strains, isolated from red wines, by identifying essential and preferable amino acids. Using single amino acid omissions, *L. plantarum* amino acid requirements was determined in solid and liquid chemically defined media. The trophic status of four amino acids is identical for all strains, namely auxotrophic for Glu and Val and prototrophic for Gln and Trp. All other amino acids trophic requirements are highly strain and sometimes media dependent. Subsequently, the order of amino acid uptake was determined during MLF over the course of 6 days in synthetic grape juice (SGJ). In addition, the quantity of amino acid uptake was also determined in Chardonnay grape juice. In SGJ, Asp, Thr, Ser and Ala tends to be taken up first, while branch chain amino acids, aromatic amino acid and Met are taken up last. In Chardonnay grape juice *L. plantarum* assimilates Lys and Leu at higher quantities than all other amino acids. This evidence suggests that *L. plantarum*'s nutrient requirements are strain dependent and certain amino acids will be assimilated faster depending upon the media composition and the pre-culture conditions.

3.2 Introduction

Lactobacillus plantarum is a facultative heterofermentative lactic acid bacteria (LAB) that is able to survive in an array of ecological niches such as plant surfaces, human gastrointestinal tract and food products such as cheese and wine (Siezen and van Hylckama Vlieg, 2011). In wine, *L. plantarum* will decarboxylate L-malic acid to L- lactic acid in the process known as malolactic fermentation (MLF). This decarboxylation reaction results in wine with enhanced microbial stability and aroma complexity (Malherbe et al., 2013). *L. plantarum* has been recognized to be the predominant LAB species in the early stages of spontaneous MLF in wine and brandy base wines (Cañas et al., 2009; Du Plessis et al., 2004; González-Arenzana et al., 2012; López et al., 2008; Ruiz et al., 2010). However their numbers eventually decline during MLF and are subsequently replaced by *Oenococcus oeni*, which is much better adapted to the major parameters associated with the wine environment (such as pH, SO_2, ethanol) (Du Plessis et al., 2004; G-Alegría et al., 2004; González-Arenzana et al., 2012). The ability of *L. plantarum* to survive in wine and to complete MLF is highly strain-dependent (G-Alegría et al., 2004; López et al., 2008; Maicas et al., 1999; Pozo-Bayon et al., 2005) as well as dependent upon the pH of wine as *L. plantarum* are more viable in slightly higher pH (≥3.5-vs 3.2) (Du Toit et al., 2011; G-Alegría et al., 2004; Guerzoni et al., 1995). A *L. plantarum* strain (*L. plantarum* V22) isolated from an Italian red wine has the ability to complete MLF faster than *O. oeni* when inoculated after alcoholic fermentation (AF) in a red wine with a pH of 3.6. Alternatively, *L. plantarum* can be co-inoculated with yeast in order for MLF to proceed with AF. In this way, *L. plantarum* is introduced

to the grape must when the ethanol and yeast antimicrobial metabolites concentrations are low (Du Toit et al., 2011) and the nutrient content is high. Under these conditions the growth and metabolic rate is substantially higher than the conditions in sequential inoculation (Guerzoni et al., 1995; Maarman, 2014).

Although *L. plantarum* may actively partake in MLF and its growth relative to *O. oeni* in wine is fairly well characterized, *L. plantarum*'s nitrogen metabolism in wine is inadequately researched. Amino acids are arguably one of the most important metabolites in terms of LAB metabolism in wine. Not only does the amino acids requirements impact the growth of the organism, the amino acid catabolism may influence the aroma and health aspects of wine. With regard to aroma formation, the degradation of branched-chain amino acids (BCAA) and Phe increases the fusel alcohol (higher alcohol) and ester content and adds to fruity and floral aromas to wine (Dickinson et al., 2000, 1998, 1997; Smit et al., 2005). Sulphur containing amino acids are the precursors for the volatile sulphur compounds (VSC), methional and hydrogen sulphide (H_2S) (McSweeney and Sousa, 2000). The VSC and methional smells reminiscent of fecal, cabbage and sulphur and H_2S smells of rotten egg (Friedrich and Acree, 1998; Molimard and Spinnler, 1996; Ott et al., 1997) and these sulphur aroma compounds are detrimental to the wine quality. Glu, Asp and Ala acts as keto donors in the degradation of BCAA, aromatic amino acids (AAA) and Met to aroma compounds. Decarboxylation of several amino acids such as the AAA, His, Glu, Lys and Arg leads to the formations of biogenic amines. Some of these compounds such as histamine tyramine and phenylethylamine are associated with adverse health defects such as migraines and high blood pressure while other biogenic amine such as cadaverine and putrescine not only enhance toxicity of other biogenic amines it also impart off-flavours to the aroma of wine (Lerm et al., 2010; Pessione and Cirrincione, 2016; Silla Santos, 1996; Smit et al., 2008).

Since *L. plantarum* can occupy a number of ecological niches, studies surrounding its amino acid requirements are not limited to a single environment or strain. Studies have been conducted in reconstituted skim milk and chemically defined media using strains that have been isolated from dairy environments (Ma et al., 2016; Morishita et al., 1981; Teusink et al., 2005) or orange skin peels (Saguir and de Nadra, 2007). Overall these studies have revealed that some amino acid trophic requirements appears to be common among all *L. plantarum* strains while other trophic requirements are highly strain dependent. These studies also revealed that amino acids with longer biosynthetic pathways are more likely to become auxotrophic (eg. His vs. Gly, Ala) (Morishita et al., 1981), feed-back inhibition may influence common biosynthetic pathways (eg. AAA repression of Shikimate pathway) (Teusink et al., 2005) and mutations will emerge after long term adaptation to a specific media e.g. BCAA (Godon et al., 1993).

The methods to date to determine the amino acid requirements in LAB used quantitative means for its characterization. In this way each amino acid requirement can either be classified as essential, stimulatory or non-essential (Garvie, 1967; Ma et al., 2016; Morishita et al., 1981; Osborne and Edwards, 2007; Saguir and de Nadra, 2007; Terrade and Mira de Orduña, 2009; Teusink et al., 2005). However not all of these studies ensured that the intracellular nitrogen content was depleted prior transfer to an omission media. In

fact only Terrade and Mira de Orduña (2009) applied and argued the case of depletion of intracellular nitrogen content prior to transfer into single-omission media. This was accomplished through three sub-cultivations of low cellular concentration (10^4 CFU/ml) in single amino acid omission media. Another point of contention is the omission of Gln and Asn (in some cases) from the chemically defined media. With these omissions, Glu and Asp cannot be supplemented and added pressure is placed upon the metabolic flux to generate free amino acid for protein synthesis and this could yield lower growth.

The aim of this study was to optimize the method for determination of amino acid auxotrophies and to determine the nitrogen requirements of red wine isolated strains of *L. plantarum*. The nitrogen requirements was further evaluated by determining the order of amino acid uptake for each amino acids in synthetic grape juice during MLF and to determine the overall amino acid uptake in Chardonnay grape juice after MLF.

3.3 Materials and methods

3.3.1 Bacterial strains, media and cultivation

The *Lactobacillus plantarum* strains used in this study were *L. plantarum* 65.1, *L. plantarum* 73.1, *L. plantarum* 75, *L. plantarum* 83 and *L. plantarum* 85.1. These strains were isolated from South African red wine fermentations in commercial cellars and form part of the culture collection of the Institute for Wine Biotechnology (IWBT). The LAB strains were all maintained as culture stocks at -80ºC in 50% (v/v) glycerol (Saarchem, Merck) solution.

The chemically defined media used for testing *L. plantarum*'s trophic requirements and amino acid utilization was a synthetic grape juice (SGJ) based on the media of Henschke and Jiranek (1993) with slight adjustments (**Table 3.1.**). I) The sugar concentration of glucose and fructose was reduced from 250 g/L to 10 g/L. II) All 20 proteogenic amino acids were added at the same nitrogen concentration (**Table 3.2.)** to the synthetic grape juice (SGJ).

The *L. plantarum* strains were cultivated routinely on De Man Rogosa and Sharp (MRS) plates containing 50 g/L MRS broth (Biolab, Merck) and 15 g/L bacteriological agar (Biolab, Merck). Upon incubation the MRS plate cultures were placed in anaerobic chambers with anaerobic sheets (Anaerocult® A, Merck). *L. plantarum* was cultivated at 30ºC for 2 days.

A single inoculated colony of the *L. plantarum* strains was cultured for 24 h in 5 ml MRS broth (Biolab, Merck) at 30ºC under microaerophilic (no agitation) conditions. Before inoculation into SGJ, the cultures were subjected to starvation: after the growth phase, the cells (from the 5 ml cultures) were harvested by centrifugation (5000 rpm, 7 min), washed thrice with 0.85% saline solution, and transferred to 100 ml starvation media (SGJ without amino acids). After a 48 h starvation period at 30ºC, cells were harvested by centrifugation (5 000 rpm, 7 min), washed twice with 0.85% saline solution and resuspended in 10 ml of the same solution.

Before inoculation into Chardonnay grape juice the strains were cultured in an activation media which consisted of 50 g/L MRS broth (Biolab, Merck), 40 g/L D (-) fructose (Biolab, Merck), 4.0 g/L L (-) malic acid (Biolab, Merck) and 1.0 g/L Tween 80. The pH of the activation media was adjusted to 3.5 with 37% HCl prior to autoclaving. *L. plantarum* was inoculated and cultured in the activation media to a final OD_{600} of 0.05 under microaerophilic conditions (no stirring) at 30ºC.

All fermentations in SGJ and Chardonnay grape juice were carried out 20ºC until the end of MLF (malic acid concentration ≤ 0.3 g/L). Initial OD_{600} values after inoculation of *L. plantarum* were 0.05. This value corresponded to approximately 5 -10 x 10^6 CFU/ml.

Growth in omission media were carried out at 30ºC. Growth continued in the solid omission media until single colonies emerged in the no amino acid omission control. In the liquid omission media growth was stopped after 4 days.

3.3.2 Species-specific PCR of LAB

To confirm that all strains belonged to *L. plantarum*, species-specific primers were used to amplify conserved genes via PCR. Genomic DNA was extracted according to the method of Lewington et al. (1987) with a slight adjustment as mutanolysin was added at a final concentration of 1000 U/ml to aid with the degradation of the bacterial cell wall. Nanodrop® ND-1000 (NanoDrop Technologies, Inc., Wilmington, USA) was used to measure the quantity and quality of the DNA. The *recA* gene was amplified via PCR since this gene is conserved in *L. plantarum* (Torriani et al., 2001). The PCR cycling parameters are summarized in **Table 3.3.**

3.3.3 Growth experiments on single amino acid omission chemically defined plate-based and liquid media

The amino acid auxotrophies were evaluated by using single amino acid omissions in chemically defined solid and liquid media. The solid SGJ was adjusted to pH 5.2 and 2% granulated agar (Difco™, Dickinson and Co.) was added prior to autoclaving. The vitamins, trace elements, lipids, amino acids and antibiotics [100 mg/L Delvocid (DSM Food Specialist, Netherlands), 25 mg/L Kanamycin (Sigma-Aldrich)] were filter sterilized through 0.22 µm filters (Whatman®) together prior to addition to the hot (60-70ºC) SGJ. Antibiotics were used to supress the growth of fungi and acetic acid bacteria on this nutrient rich media, which may appear several days after inoculation. When the amino acids were added to the SGJ, each amino acid was added at a concentration of 1 mg N/L (**Table 3.2.**). Two criteria are used for the acceptance of a prototrophic result, namely colonies must appear within the same time as the positive control (6 days) and colonies must be the approximate size of the positive control (Godon et al., 1993). Growth were classified as partial when smaller colonies emerged within 6 days of incubation.

Table 3.1. The composition of synthetic grape juice (SGJ) media.

	Substrate	In 1 L
Carbon Sources	Glucose	10 g
	Fructose	10 g
Acids	KH Tartrate	2.5 g
	L-Malic acid	3 g
	Citric acid	0.2 g
Salts	K_2HPO_4	1.14 g
	$MgSO_4.7H_2O$	1.23 g
	$CaCl_2.2H_2O$	0.44 g
Vitamins	Myo-inositol	100 mg
	Pyridoxine	2 mg
	Nicotinic acid	2 mg
	Calcium pantothenate	1 mg
	Thiamin.HCl	0.5 mg
	p-Aminobenzoic acid	0.2 mg
	Riboflavin	0.2 mg
	D-Biotin	0.125 mg
	Folic acid	0.2 mg
Trace elements	$MnCl_2.4H_2O$	200 µg
	$ZnCl_2$	135 µg
	$FeCl_2$	30 µg
	$CuCl_2$	15 µg
	H_3BO_3	5 µg
	$Co(NO_3)_2.6H_2O$	30 µg
	$NaMoO_4.2H_2O$	25 µg
	KIO_3	10 µg
Lipids	Ergosterol	10 mg
	Tween 80	0.5 ml
Nitrogen source		See **Table 3.2.**

The amino acid solution and SGJ was adjusted to pH 3.5 [as *L. plantarum* prefer a slightly higher wine pH (Guerzoni et al., 1995)] and 5 ml SGJ was aseptically transferred to 10 ml tubes. Anoxic gas was not added to the culture prior to incubation and the cultures incubated semi-aerobically upon inoculation.

Table 3.2. The amino acid composition of the synthetic grape juice to yield a final concentration of either 1 mg N/L or 2 mg N/L.

Amino acid	mg amino acid added to achieve 2 mg N/L	mg amino acid added to achieve 1 mg N/L
L-Alanine	12.72	6.36
L-Arginine	6.22	3.11
L-Aspartic acid	19.02	9.51
L-Asparagine	9.44	4.72
L-Cysteine	17.3	8.65
L-Glutamic acid	21.02	10.51
L-Glutamine	10.44	5.22
L-Glycine	10.72	5.36
L-Histidine	7.38	3.69
L-Isoleucine	18.74	9.37
L-Leucine	18.74	9.37
L-Lysine	10.44	5.22
L-Methionine	21.32	10.66
L-Phenylalanine	23.6	11.8
L-Proline	16.44	8.22
L-Serine	15	7.5
L-Threonine	17.02	8.51
L-Tryptophan	14.58	7.29
L-Tyrosine	25.88	12.94
L-Valine	16.74	8.37

Table 3.3. The species-specific primers and PCR parameters to confirm *L. plantarum* identity.

		Main cycling parameters					Reference
Primer pair	T_{DI}.	T_D.	T_A.	T_E	No. of cycles	T_{EF}	
plan F/pREV	94ºC (1)	94ºC (1)	55ºC (1)	72ºC (1)	30	72ºC (10)	Torriani et al., 2001

T_{DI}: initial denaturation temperature, T_D: denaturation temperature, T_A: annealing temperature, T_E extension temperature, T_{ET}: final extension temperature. Numbers in parenthesis indicates time in minutes.

Before inoculation the starved culture was serially diluted to 10^{-6}. The 10^{-6} dilution typically contained between 300-1000 colony forming units (CFU)/ml. The solid and liquid synthetic grape juice was plated and inoculated respectively into single-omission amino acid media with 100 µl of the 10^{-6} starved culture dilution. In the solid media trophic requirements were scored as prototrophic when single colonies had emerged within the same time as the no-omission control (i.e. within 144 h days of incubation). In the liquid-based approach a prototrophic result was scored when the strain displayed an OD_{600} value above 0.1 (limit of detection) which roughly corresponded with $1x10^8$ CFU/ml within the same time as the respective no-amino acid control. Results were mean of three biological repeats.

3.3.4 Fermentations in synthetic grape juice

Fermentations were carried out in 100 ml spice flasks capped with a rubber stopper and CO_2 outlet. The spice-flasks contained 80 ml of SGJ with all amino acids at 2 mg N/L (**Table 3.2**). The starved bacterial cultures were inoculated into SGJ at an approximate cell-concentration of $1x10^7$ CFU/mL. Fermentations preceded at 20ºC until malic acid was depleted. In each case stationary-phase was reached long before the depletion of malic acid. Samples were taken every 24 h for determination of malic acid, amino acid and CFU/mL. The supernatant was extracted by centrifugation (5000 rpm, 7 min) and stored at -20ºC for the quantification of malic acid and amino acid concentrations. The fermentations were carried out in triplicate.

3.3.5 Vinification of grape juice

Grape juice fermentations were carried out using clarified free run juice (cv. Chardonnay) received from Neethlingshof, Western Cape, South Africa. Prior to MLF, thermovinification was applied to the grape juice to eliminate the indigenous yeast and bacterial species. The juice was heated to 70-80ºC for 15 min. Hot grape juice were dispensed and capped into clean 2 L fermentation bottles and stored at -4ºC until further use.

The bottles were capped with rubber stopper and CO_2 outlet. Fermentation in grape juice was carried out at 20ºC for 144 h. The strains *L. plantarum* 73.1, 83 and 85.1 were chosen according to results obtained for auxotrophic analysis to conduct MLF in Chardonnay grape juice. Each strain was inoculated at approximately $1x10^7$ CFU/mL. MLF was monitored by the measurement of the degradation of malic acid using Arena 20XT. Samples were taken for the determination of cell counts (CFU/ml), malic acid and amino acid concentrations every 24 h. The parameters of the grape juices after thermovinification and before inoculation of *L. plantarum* are summarized in **Table 3.4.**

Table 3.4. The parameters of the grape juice (cv. Chardonnay) before inoculation.

	YAN (mg/L)	pH	Malic acid (g/L)	Total SO_2 (mg/L)
Neethlingshof	198.8	3.42	3.12	32

3.3.6 High Performance Liquid Chromatography for amino acid quantification

The high performance liquid chromatography (HPLC) was used to quantify the amino acids by pre-column derivatisation and fluorescence detection using an Agilent 1100 system. The method of Henderson and Brooks, (2010) was used with modification to the derivatisation and injection. Column Poroshell HPH-C18 (4.6x150mm, 2.7 µm) (Chemetrix (Pty) Ltd) was used for derivatisation of the amino acids and was fitted to a Guard column (UHPLC Guard, Poroshell HPH-C18 4.6mm) (Chemetrix (Pty) Ltd) to preserve the service life of the column. The temperature of the column apparatus were 40ºC during derivitisation. O-phthaldialdehyde (Sigma Aldrich) was used for the derivatisation of the primary amino acid, iodoacetic acid (Sigma Aldrich) was used for the derivatisation of cysteine, while fluorenylmethyloxycarbonyl chloride (Sigma Aldrich) was used for the derivatisation of the secondary amino acids. Norvaline and sarcosine were used as internal standards for primary and secondary amino acids respectively. Agilent Chemstation 32 was used as the software for the integration of the data.

3.3.7 Malic acid quantification

An Arena 20XT (Thermo Electron Corporation, Finland) automated enzymatic kit robot was used to quantify L-malic acid with an enzymatic kit (Enzytec™ Fluid L -malate Id-No: E5280, Roche, R-Biopharm).

3.4 Results

3.4.1 Strain identification

The *recA* gene of all 5 strains were amplified using PCR and the resulting amplicon length was 300 bp. The strains that were used in this study, therefore, belong to the species, *L. plantarum*, as the expected band size was obtained (Torriani et al., 2001).

3.4.2 Essential amino acid determination in *L. plantarum*

3.4.2.1 Solid omission media

Formation of colonies within 6 days at 30ºC was scored as growth, whereas formation of small colonies within the same time was scored as partial growth and no formation of colonies was scored as no growth (**Table 3.5**). To assess the viability of the starved culture cells 100 µl were plated on MRS-agar and all starved cultures could grow after 2 days on MRS-agar, indicating the cells were viable at the time of inoculation. All strains were unable to grow in the absence of all amino acid, while supplementation of

all 20 amino acids to SGJ enabled all 5 strains to grow. The data show a significant variation in amino acid auxotrophies: *L. plantarum* 65.1 and 73.1 were auxotrophic for 14 amino acids, *L. plantarum* 75 for 6 amino acids and *L. plantarum* 83 and 85.1 for 4 amino acids**.** Furthermore every single strain could grow in the presence of all amino acids and in the absence of Gln, Thr and Trp, while no strain could grow in the absence of Val, Asp, and Glu. Collectively the BCAA were nutritionally essential. For the rest of the amino acids, the strains showed great variability.

Table 3.5. Impact of single amino acid omission on the growth of *L. plantarum* on solid media.

Amino acid omitted	*L. plantarum* strain				
	65.1	73.1	75	83	85.1
Ala	−	+	±	+	+
Arg	±	−	±	±	±
Asn	+	+	−	+	+
Asp	−	−	−	−	−
Cys	−	−	+	+	+
Glu	−	−	−	−	−
Gln	+	+	+	+	+
Gly	−	−	±	+	+
His	−	−	±	+	+
Ile	−	−	±	±	±
Leu	−	−	−	+	−
Lys	−	+	+	+	+
Met	−	−	±	+	−
Phe	±	±	±	−	±
Pro	−	−	±	±	+
Ser	−	−	−	+	+
Thr	+	+	+	+	+
Trp	+	+	±	+	+
Tyr	−	−	±	+	+
Val	−	−	−	−	−
No omission	+	+	+	+	+
All AA omitted	−	−	−	−	−

(+) indicates growth, (±) indicates partial growth and (-) indicates an absence of growth on single amino acid omission solid media

3.4.2.2 Liquid omission media

The solid media growth assays were not always conclusive, as indicated by the partial growth, therefore interpretation of auxotrophy vs prototrophy required further investigation. To verify the data, the *L. plantarum* strains were subjected to the same auxotrophies in SGJ liquid media. No growth was observed when Ala, Cys, Leu and Val were omitted from the media (**Fig. 3.1**). Ile and Met were also essential in most of the strains. Conversely growth was observed when Gln, Gly, His, Lys, Trp and Tyr was omitted from the media. Additionally, Pro and Phe could be synthesized by all but one strain. In most cases a single omission of an amino acid lead neither to a nullification of growth nor a complete independence thereof, leading as in the case of solid media to intermediary growth phenotypes as well.

For example in most cases of an omission of Arg, Asn, Asp, Lys and Tyr the optical density were significantly lower than the zero omission control. In almost all cases the omission of Gln, Gly, His, Phe, Pro, Ser and Trp from the media did not influence the growth of the prototrophic strains significantly.

3.4.2.3 Impact of amino acid omission on MLF

In order to further evaluate the amino acid trophic requirements of *L. plantarum*, MLF was carried out in SGJ by two strains (73.1 and 83) (**Figs. 3.2 and 3.3)**. Five amino acid omissions were chosen for further investigation: Ala, Val, Trp, Gln and Arg based on the results obtained from the trophic studies. These amino acids present different trophic requirements, with 2 being essential (Ala and Val), 1 stimulatory (Arg) and 2 non-essential (Gln and Trp). *L. plantarum* 73.1 completely degraded malic acid only when Gln was omitted from SGJ. *L. plantarum* 73.1 degraded half of the malic acid when Arg was omitted, while only slight decreases in malic acid concentration were observed when Val and Ala were omitted. With the omission Ala and Val both strains grew very slowly, reaching an OD_{600} slightly beyond 0.100 after 144 h. Unexpectedly, with regards to *L. plantarum* 83, not a single omission had resulted in a complete degradation of malic acid. With the omission of Gln, Arg and Trp more than half of the malic acid was degraded. On the other hand, similarly to *L. plantarum* 73.1, the omission of Ala and Val led small degradation of malic acid and growth inhibition.

The growth results aligned with the malic acid degradation results. A total degradation of malic acid lead to an overall higher optical density in comparison to smaller malic acid uptake. In the case both of Ala and Val, both strains' optical density at OD_{600} only showed a slight increase beyond the limit of detection of 0.100 (OD_{600}) after MLF while at the same time the OD_{600} had increased greatly for Gln, Arg and the no omission control. Overall this data shows that amino acids are crucial for the completion of MLF.

3.4.3 Amino acid assimilation of *L. plantarum* in synthetic grape juice

3.4.3.1 Growth kinetics of *L. plantarum* in synthetic grape juice

Three strains were chosen form the liquid assays dataset to investigate the amino acid uptake in *L. plantarum*. During MLF in SGJ the strains all reached stationary phase at 72 h (**Fig. 3.4**) but MLF was only completed ([malic acid] $\leq$ 0.3 g/L) at 144 h. All strains reached approximately $1x10^8$ CFU/ml at 48 h and further increased till 60 h (*L. plantarum* 73.1) and 72 h (*L. plantarum* 83 and 85.1) where after cell counts began declining slightly over a 72 h period.

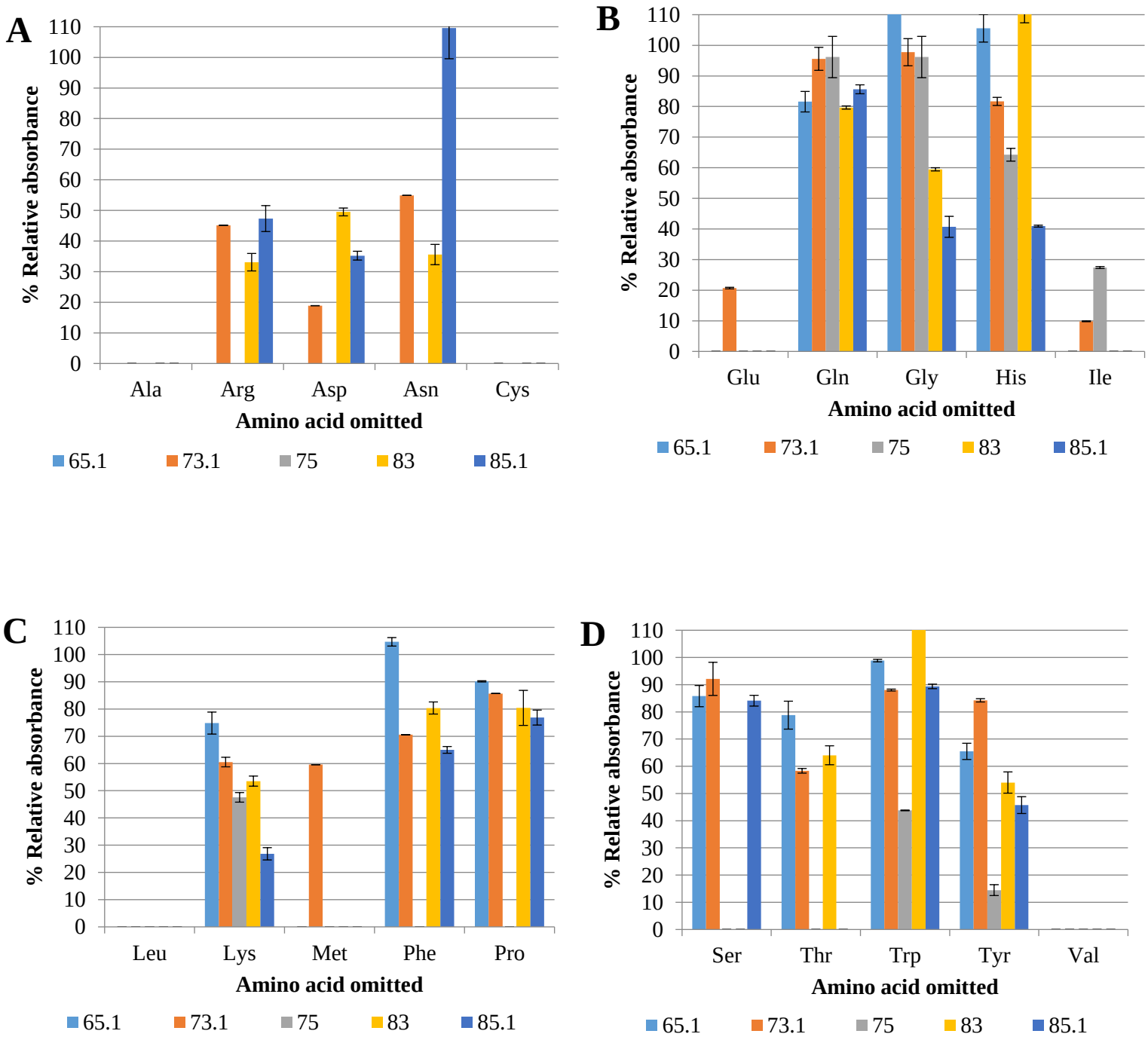

Fig. 3.1. The % relative optical density OD_{600} of *L. plantarum* grown in single amino acid omission SGJ relative to a zero amino acid SGJ control. The % relative optical densities in amino acid omission growth results are represented in (A) Ala, Arg, Asp, Asn and Cys, (B) Glu, Gln, Gly, His and Ile (C) Leu, Lys Met, Phe and Pro and (D) Ser, Thr, Trp, Tyr and Val.

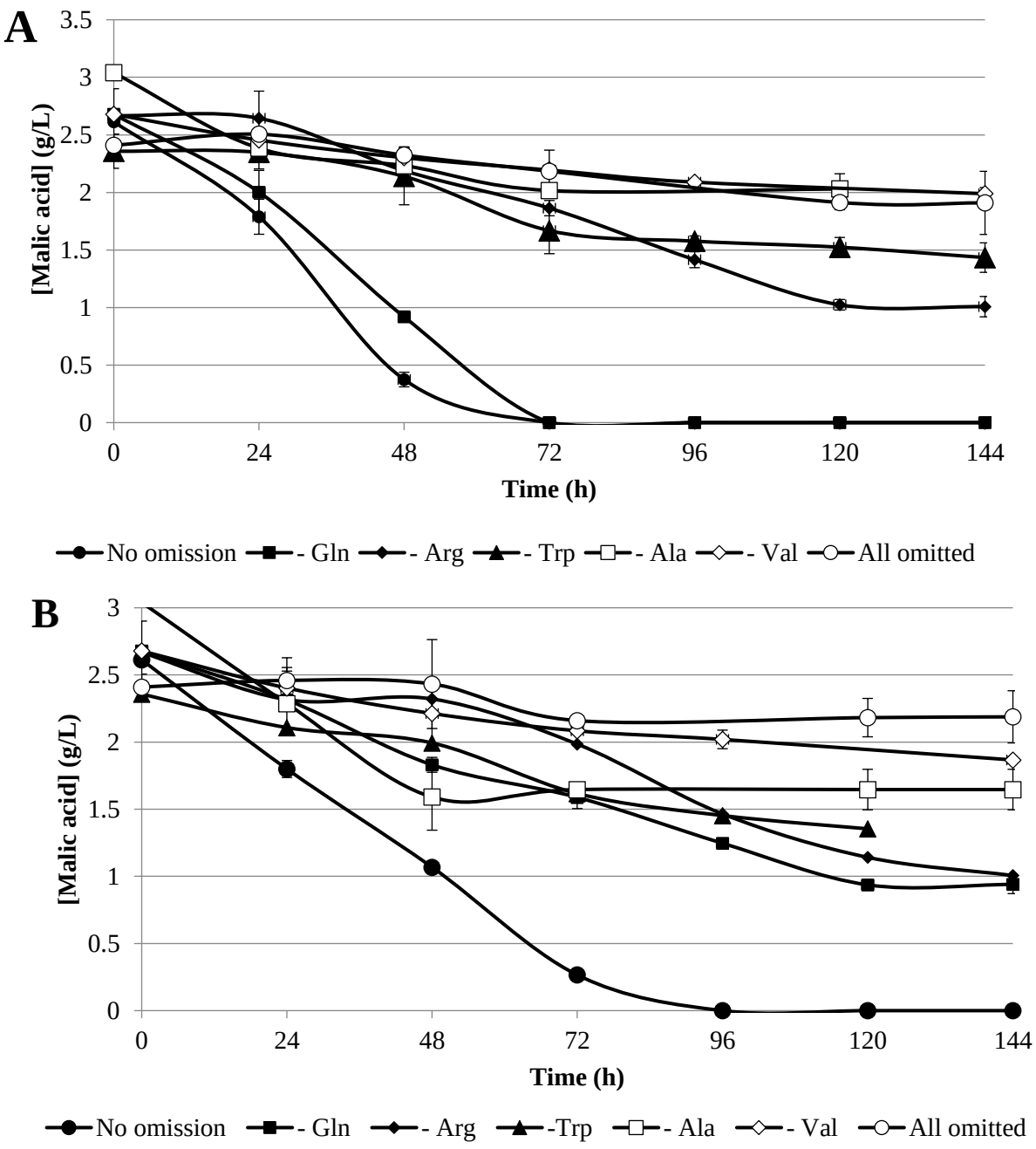

Fig. 3.2. The uptake of malic acid in *L. plantarum* 73.1 (A) and *L. plantarum* 83 (B) when the amino acids Val, Ala, Trp, Arg and Gln are omitted individually from the SGJ media. The fermentations included a no omission control and a control in which all amino acids were omitted.

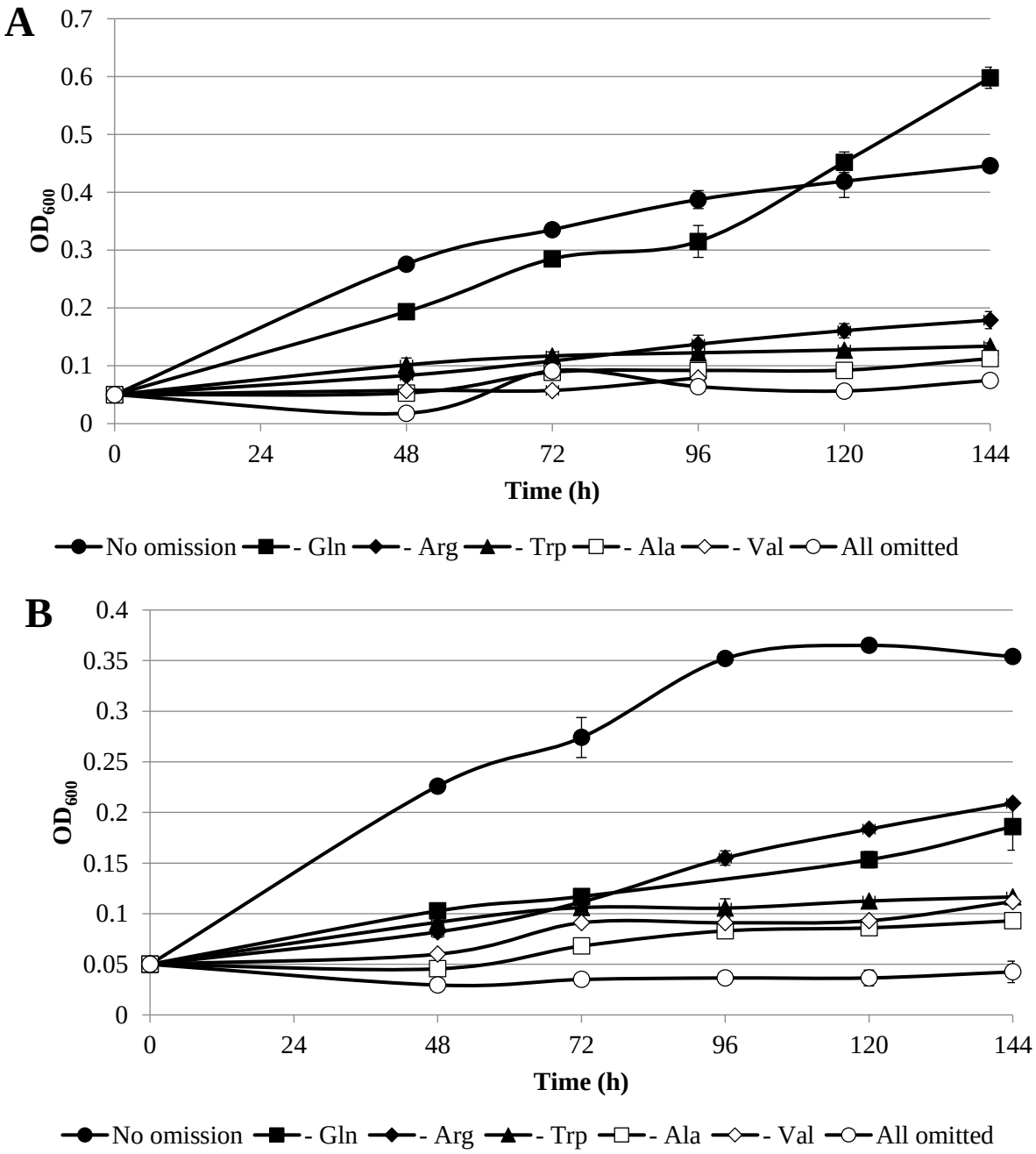

Fig. 3.3. The optical density at 600 nm for *L. plantarum* 73.1 (A) and *L. plantarum* 83 (B) when the amino acid Ala, Val, Trp, Arg and Gln was individually excluded from the SGJ. Controls included a zero omission and a control in which all amino acids were removed.

3.4.3.2 The order of uptake of amino acids by *L. plantarum* during MLF

In the media in which the concentration of each amino acid had been adjusted to the same amount of nitrogen (i.e. 2 mg N/L) each *L. plantarum* strain showed the same amino acids assimilation pattern and order of amino acid uptake, independently of the amino acid trophic requirements (**Figs. 3.5-3.8 and Table 3.6**). The quantity of single-amino acid uptake however differed in some cases. Asp is rapidly assimilated compared to any other amino acid and completely consumed within 72 h. 72 h coincides with the time that the bacteria entered stationary phase (**Fig. 3.4**). No other amino acid is completely consumed at this time point. In the first 36 h Asp, Lys and Ala are the most preferred amino acids representing at least over 50% of the amino acids absorbed at this time point. Only 12 amino acids are consumed in the first 36 h: Asp, Lys, Ala, Thr, His, Gly, Asn, Arg, Ser, Pro, Trp and Leu. Less preferred (in terms of uptake) amino acids like Glu, Ser, Thr, Arg, Tyr, Val, Met, Phe, Ile, Leu represents less than 20% of the cumulative uptake during MLF. Noticeably, the essential amino acids Glu, Ile and Val are absent from the assimilation despite the strains showing no growth impairment within 36 h. In the first 72 h the total amino acid assimilation of Asp, Thr, and Ser accounted for 40-50% of consumption. Glu consumption only sharply increased between 36-72 h with 0.5-1.1 mg N/L of uptake (**B**). BCAA and AAA assimilation was limited before 72 h with total BCAA uptake representing only 5% of total amino acid uptake. However after 72 h, BCAA assimilation represents 19.37-24.25% of total amino acid uptake (**D**). Moreover 70% of Leu assimilation, 70-75% of Ile assimilation, and 65-70% of Val assimilation had taken place after 72 h. Tyr and Phe assimilation after 72 h had accounted for 72-95% and 85-95% (**C**). The uptake of His, Arg and Pro (83 and 85.1) was also 50% and higher after 72 h. Ala, Thr and Asn assimilation was much higher at 36 – 72 h. representing more than 40% of their respective total assimilation. A decrease in Ser corresponded with an increase in Gly (**E**).

Overall Thr, Asp, Ser were completely assimilated at 144 h by all 3 strains (**Fig. 3.5**). For each strain there was a high assimilation of Ala, Glu, Arg, Lys and Leu while Ile, Tyr, Asn and Val always have moderate uptake. Among the BCAA, Leu always have the highest preference, followed by Ile and Val. Among the AAA, Phe was the most preferred amino acid, next to Tyr. The concentration of Cys could not be measured possibly due to oxygenation and spontaneous reaction with other metabolites.

3.4.4 Amino acid assimilation of *L. plantarum* in Chardonnay grape juice

3.4.4.1 Growth kinetics of *L. plantarum* in Chardonnay grape juice

The same strains used in MLF of SGJ were used to conduct MLF of Chardonnay grape juice. In Chardonnay, both *L. plantarum* 83 and *L. plantarum* 85.1 completed MLF at 48 h while *L. plantarum* 73.1 completed MLF at 72 h (**Fig. 3.9**). The CFU/ml of *L. plantarum* 73.1, 83 and 85.1 at the end of

MLF is $3.0x10^8$, $1.5x10^8$ and $4x10^8$ respectively. While CFU/ml of *L. plantarum* 83 and 85.1 remains constant after MLF. Only the CFU/ml of *L. plantarum* 73.1 increased to $1x10^9$ at 144 h.

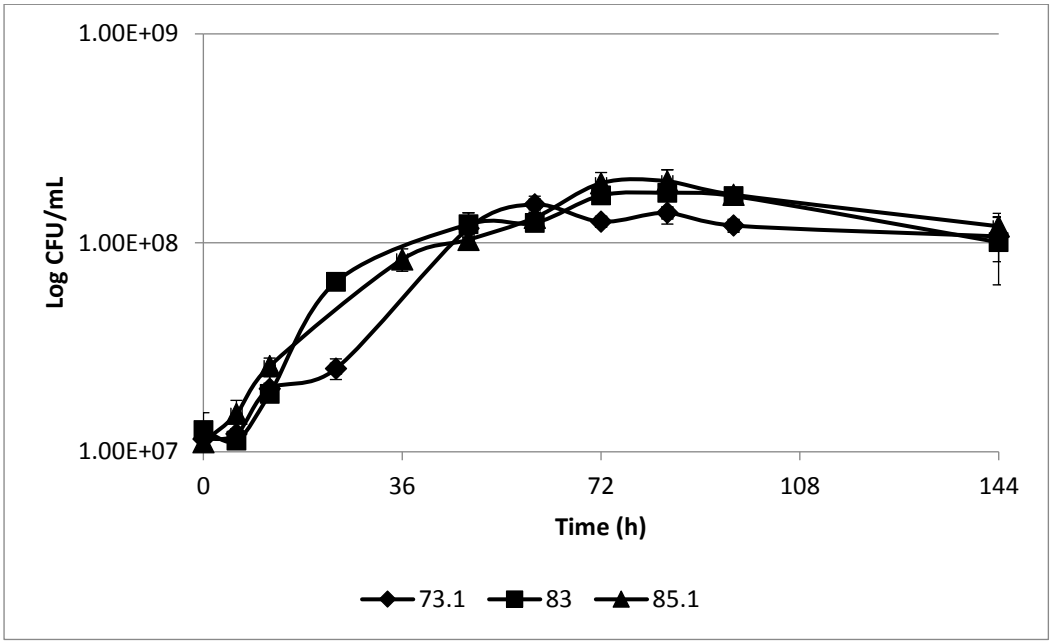

Fig. 3.4. The growth of *L. plantarum* 73.1◆, *L. plantarum* 83.1 ■ and *L. plantarum* 85.1▲in synthetic grape juice media containing all amino acids. Data points represents mean of triplicates.

3.4.4.2 Amino acid assimilation of *L. plantarum* in Chardonnay grape juice

A uniform pattern of amino acid assimilation was also observed with the MLF in Chardonnay grape juice (**Fig. 3.11**), although the specific uptake of single amino acids during MLF in natural grape juice after 72 h is different to the MLF in SGJ (**Fig. 3.10**). The total amino acid assimilation ranges from 190-290 mg/L (**Supplementary Table 4**). No amino acid was completely assimilated at the end of MLF. Overall, *L. plantarum* mainly consumed Leu, Arg Lys, Ala and Phe in grape juice. Although the % uptake of Ala and Arg is relatively low (> 20%) the absolute uptake of Ala and Arg was high (Ala: 17-27 mg; Arg 20-38 mg/L) since these amino acids were present in high concentrations in the juice. The % uptake of Leu, Lys and Phe is also high (60-85%) and accounts for more than 30 mg/L. 75-80% of Lys was taken up (20-22.38 mg/L) **(Fig. 3.10. A)**. In terms of the BCAA and AAA, Leu was taken up more than Ile and Val (32-36 mg/L vs 10 mg/L) and Phe was taken up more than Trp and Tyr (18-21 mg/L vs 4-11 mg/L). The uptake of all α-keto glutamic family of amino acids (Gln, Glu, Pro and Arg) was very low with less than 20 % of uptake (**Fig. 3.10. B**).

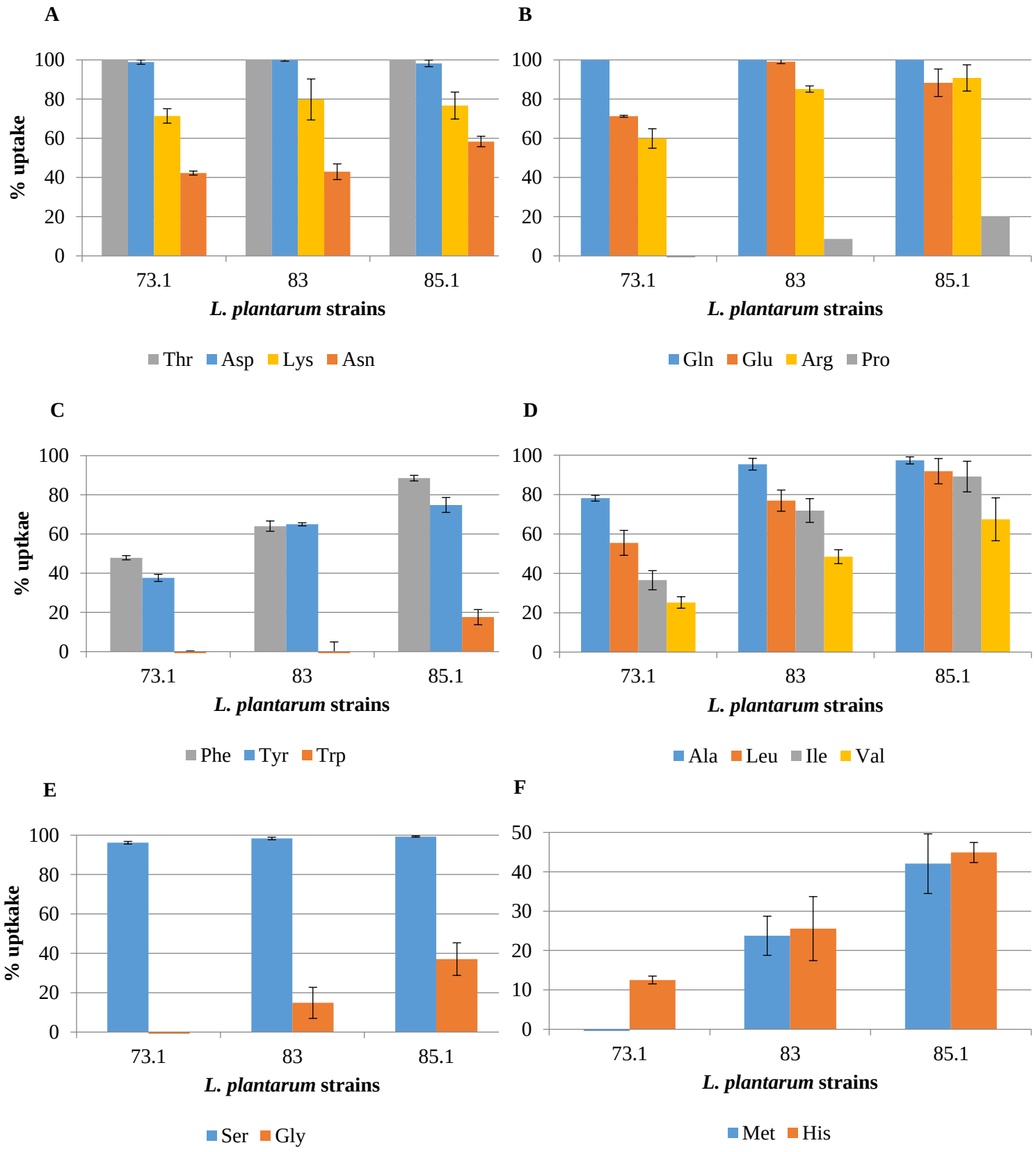

Fig. 3.5. Amino acid uptake by *L. plantarum* 73.1, *L. plantarum* 83 and *L. plantarum* 85.1 at 144 h for the amino acids Thr, Asp, Lys, Asn (A); Gln, Glu Arg, Pro (B); Phe, Tyr, Trp (C) and Ala, Leu, Ile and Val (D); Ser, Gly (E); Met, His (F).

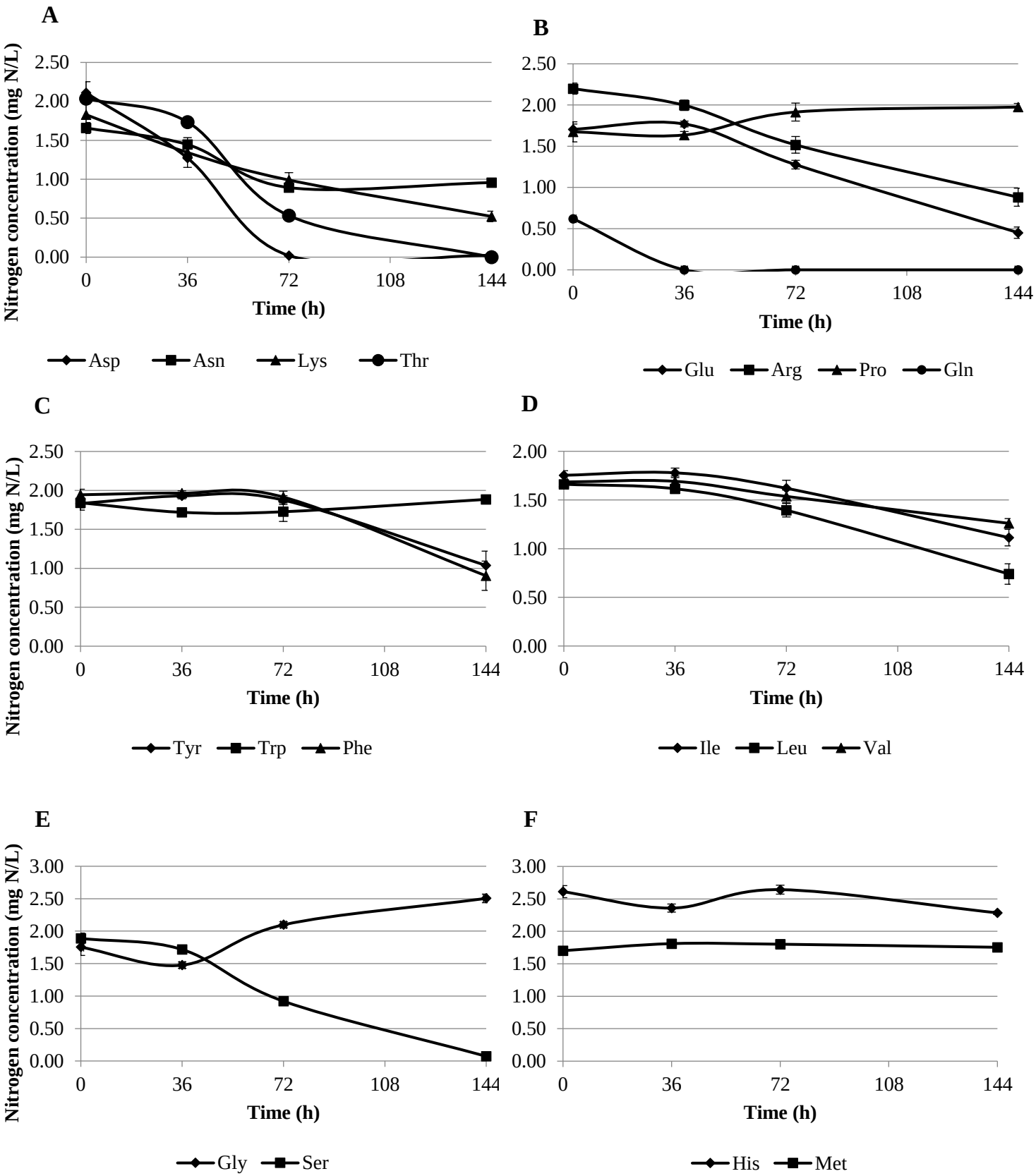

Fig. 3.6. The uptake of single amino acids during pure culture MLF by *L. plantarum* 73.1 (in SGJ) from the beginning to the end of MLF.

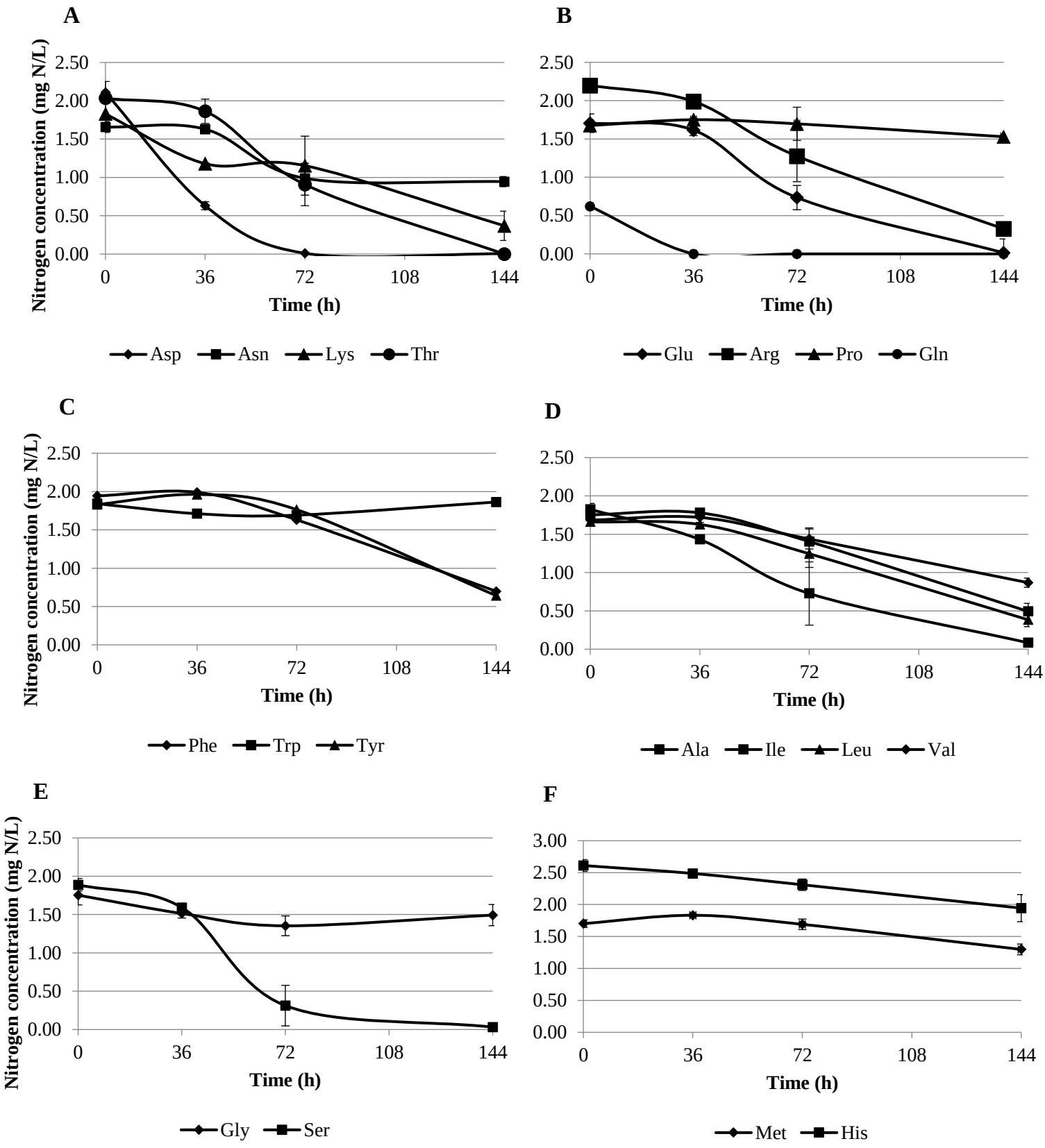

Fig. 3.7. The uptake of single amino acids during pure culture MLF by *L. plantarum* 83 (in SGJ) from the beginning to the end of MLF.

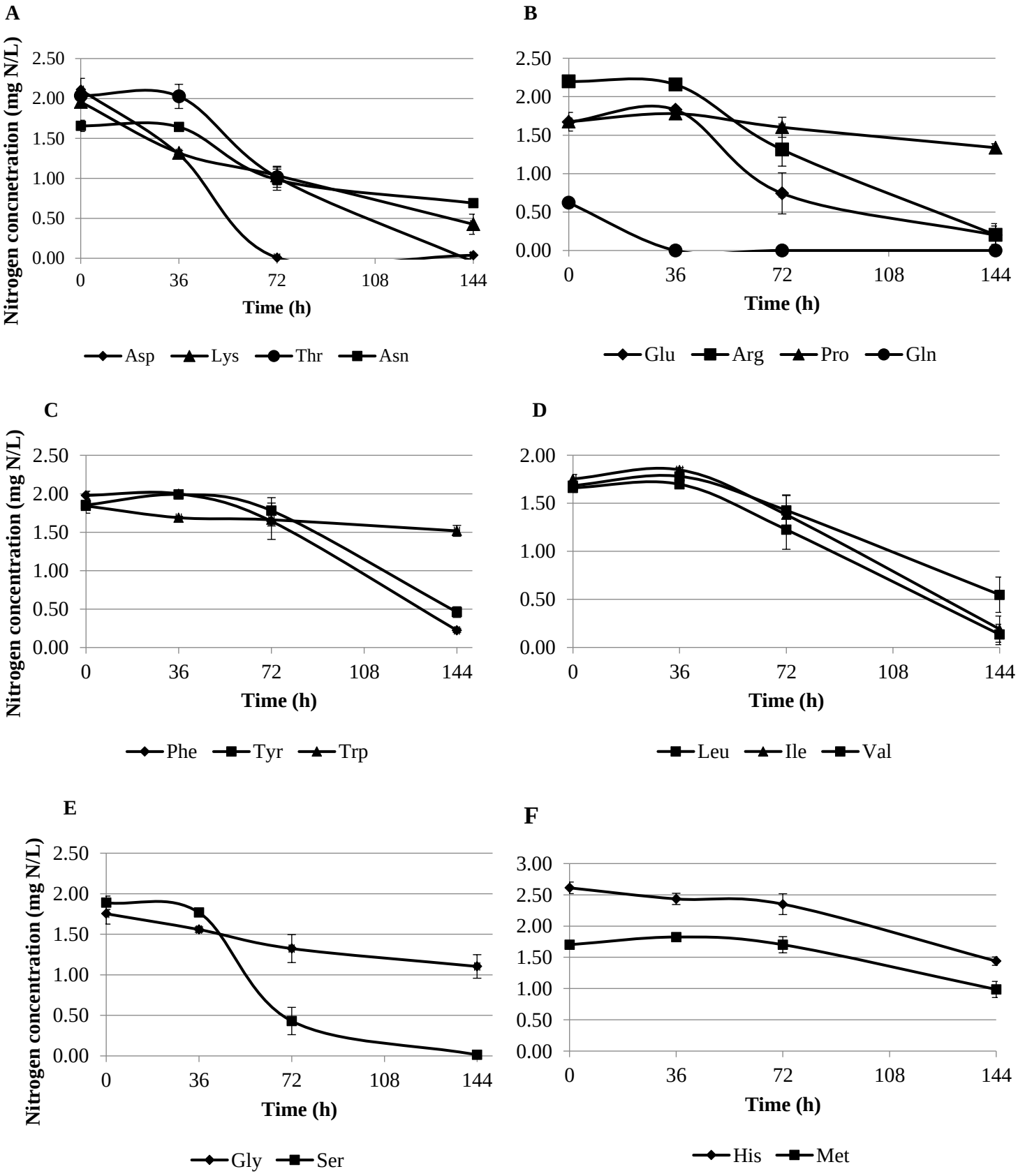

Fig. 3.8. The uptake of single amino acids during pure culture MLF by *L. plantarum* 85.1 (in SGJ) from the beginning to the end of MLF.

Table 3.6. The order of amino acid uptake by *L. plantarum* in synthetic grape juice (SGJ) after MLF (144 h).

Order of amino acid uptake	*L. plantarum* strain		
	73.1	83	85.1
1	Asp	Asp	Asp
2	Ser	Ser	Ser
3	Thr	Thr	Thr
4	Ala	Ala	Ala
5	Leu	Glu	Glu
6	Arg	Arg	Arg
7	Ile	Lys	Lys
8	Phe	Leu	Leu
9	Glu	Ile	Phe
10	Lys	Phe	Tyr
11	Tyr	Tyr	Asn
12	Val	Asn	Ile
13	Asn	Val	Val
14	His	Gly	His
15	Met	His	Trp
16	Gly	Met	Met
17	Pro	Pro	Pro
18	Trp	Trp	Gly

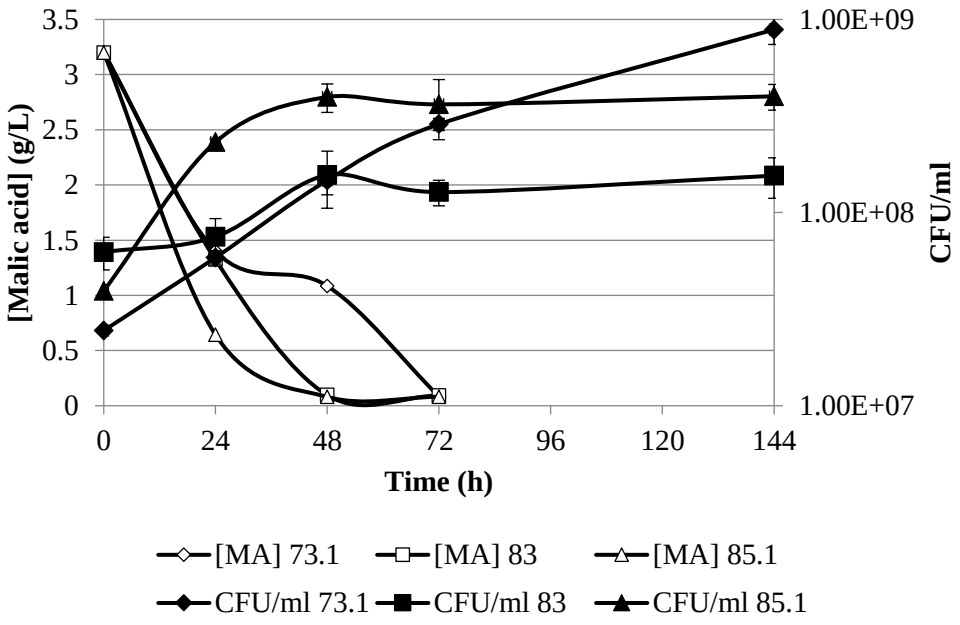

Fig. 3.9. The cell count and uptake of malic acid of *L. plantarum* 73.1, *L. plantarum* 85.1 and *L. plantarum* 83 throughout and after MLF in Chardonnay.

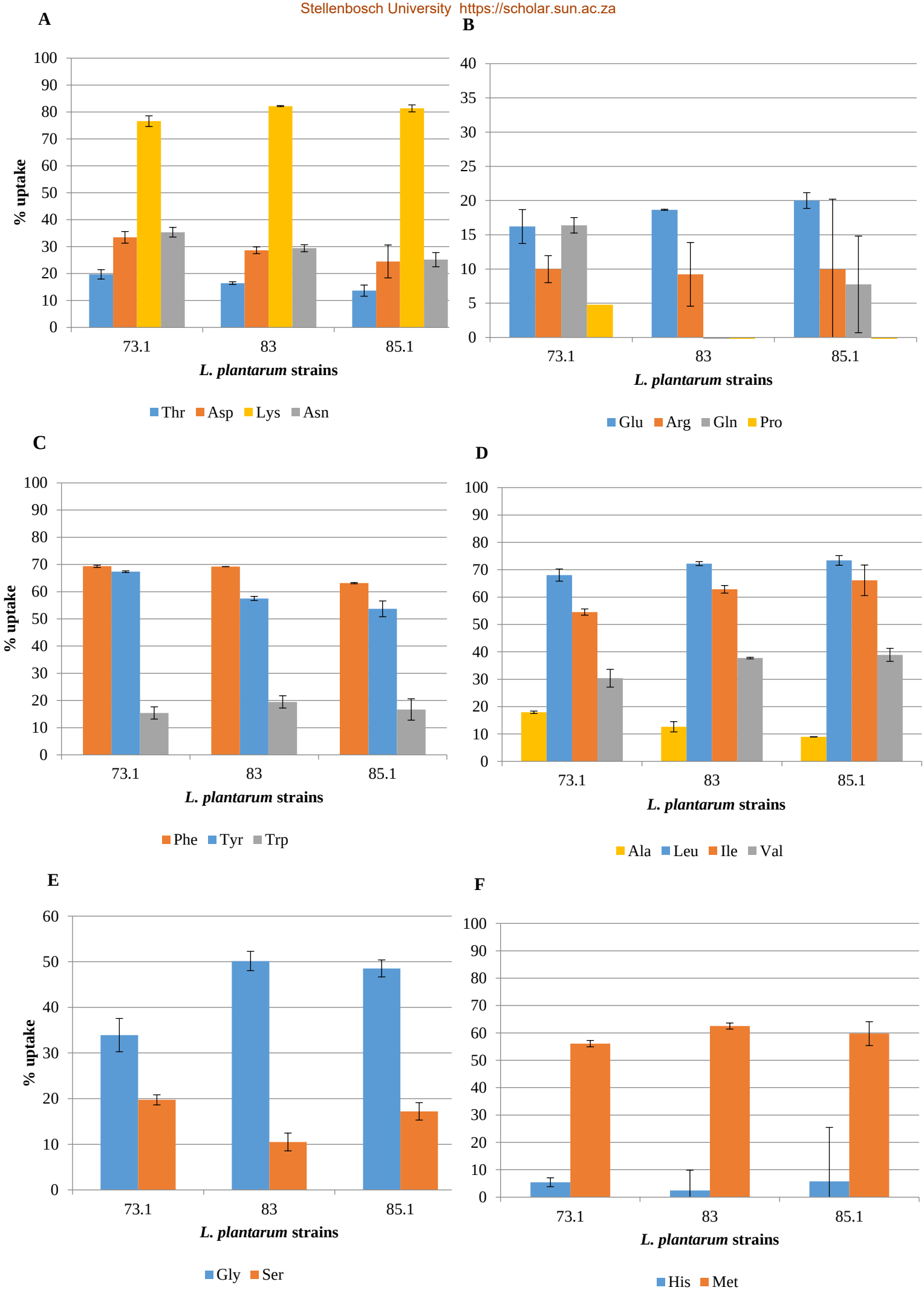

Fig. 3.10. Total uptake of amino acids by *L. plantarum* in Chardonnay 72 h after inoculation.

3.5 Discussion

3.5.1 Amino acid requirements in *L. plantarum*

This study investigated the effect of specific amino acid requirements on the growth of 5 *L. plantarum* strains. The single-omission methodology proved to be somewhat difficult to implement with *L. plantarum* since in many cases no clear yes/no response was apparent. This issue was addressed in both solid and liquid single-omission methodologies by placing strong emphasis on elimination of the intra- and extracellular nitrogen content in the cells through nitrogen starvation-induced pre-culture medium and inoculating low numbers of cells. This made results more reliable but did not fully address the problem. Both of these observations have also been emphasized by Terrada and Mira de Orduña, (2009). Contrary to other published studies on this subject, the amino acids Gln and Asn were added to the medium as these amino acids are also present in grape juice (Bely et al., 1990) and could potentially aid to overcome an amino acid auxotrophy. The only nitrogen source which was not provided in SGJ was NH_4 as this is a non-utilised in *L. plantarum*.

In this study, two single-omission methodologies were used to determine the effect of amino acid nutrient requirements on the growth of *L. plantarum*. A follow-up experiment evaluated the impact of specific amino acid omission on MLF. The strains' point of origin is important when discussing trophic requirements. Since these strains were isolated from spontaneous fermentation in Pinotage wines, these strains likely originated from the surface of grape berries as previously suggested in literature (Cañas et al., 2009; Ruiz et al., 2010). It is often suggested that LAB isolated from dairy-based environments have more nitrogen requirements than LAB isolated from plant environments as dairy LAB have evolved to possess an extensive network of proteolytic enzymes to liberate essential amino acids from the protein-rich environments. As a result the need for biosynthetic enzymes becomes unnecessary and mutations arise in the genes that encode them (Godon et al., 1993). Since plant-based environments do not provide the same support, LAB require more biosynthetic enzymes and have less amino acid requirements (Morishita et al., 1981). However the data in this study align well with the evidence in literature as the absolute amino acid requirements in this study ranged from 5-8 amino acids in the liquid assays while the amino acid requirements in dairy related environments ranged from 6- 7 (Ma et al., 2016; Morishita et al., 1981; Teusink et al., 2005).

Even with the significant variability between the two methods, the Val and Glu auxotrophies stood out and were shared by all strains. The amino acids found to be essential in the liquid-assay and in most published studies are the BCAA (Leu, Ile, Val), Met and Glu (Ma et al., 2016; Morishita et al., 1981; Saguir and de Nadra, 2007; Teusink et al., 2005). The requirement of the BCAA in the liquid assays is most likely due to an absence of the BCAA pathway in *L. plantarum* WCFS1 (Teusink et al., 2005). The Glu auxotroph stems from *L. plantarum*'s inability to generate α-keto glutamate due the absence of the isocitrate dehydrogenase enzyme in the citric acid cycle (Morishita et al., 1981). A curious

observation in this regard was the small accretion of growth when inoculated with *L. plantarum* 73.1. This growth was most likely due to a deamination of Gln to produce Glu through use of the glutamine synthetize-GOGAT activity as seen with *Lc. lactis* subsp. *lactis* NCDO 2118 (Lapujade and Loubiere, 1998). Ala biosynthesis requires only a single enzymatic reaction from pyruvate (a central metabolite) which can be catalysed by more than one enzyme (BcaT and AraT). His biosynthesis on the other hand requires 11 enzymatic reactions which is catalysed by eight enzymes of which three have bifunctional activity (Delorme et al., 1999, 1993; Umbarger, 1978). The more enzymes required in the biosynthesis of an amino acid, the higher the probability that the amino acid pathway may become disrupted by a genetic lesion(s) in one or more of the genes encoding the biosynthetic enzyme(s) (Morishita et al., 1981). We would thus expect a complete nutritional dependence upon the presence of His and not Ala, however the data from the liquid assay suggests an opposite narrative. This may be explained by the very low activity towards directed towards Ala biosynthesis in *Lc. lactis* (Pudlik and Lolkema, 2012) and the low and high concentration of His and Ala in grape juice respectively .

The low to intermediate growth in the liquid culture assays and fermentation trials with the omission of an amino acid are described by many studies as 'stimulatory'. For example, in *O. oeni*, growth in terms of optical density in omission media between 20% and 80% relative to the positive control is considered stimulatory (Garvie, 1967; Remize et al., 2006; Terrade and Mira de Orduña, 2009) as there is neither a complete reliance nor independence for growth on the particular amino acid. Of course, there is no set 'rule' on declaring certain values as stimulatory as it is based upon the judgement and preference of the author. Based upon the unilateral low growth in the omission of Arg, Asp, Lys and Tyr, these amino acids requirements are stimulatory to the *L. plantarum* strains in this study. This reduction in growth is related to diversion of energy and metabolites towards the synthesis of an amino acid which could place a metabolic strain upon *L. plantarum* (Wegkamp et al., 2010).

Not only was the trophic status strain dependent but media dependent as well. All conditions between the liquid and solid media assays were kept the same aside from pH, aeration and water activity. Clearly one or all of these factors may be responsible for Arg formation but the exact mechanism of repression is unknown. Only Bringel (1998) has reported the CPSasearginine (an enzyme responsible for Arg formation) to be inhibited by oxygen.

3.5.2 Amino acid assimilation in *L. plantarum*

L. plantarum amino acid assimilation was investigated in SGJ and grape juice using HPLC. All *L. plantarum* strains have a higher preference for Asp than any other amino acid in SGJ. Asp uptake might be related to the synthesis of other amino acids such as Asn, Lys, Met and Thr (Umbarger, 1978). Asp is also precursor to oxaloacetate which may also feed and sustain the citric acid cycle. Ser, Thr and Ala are also highly preferred amino acid sources. Ser and Thr catabolism are both responsible for Gly production in *Lc. lactis* (Ardö, 2006; Trip et al., 2013; Umbarger, 1978). Gly, in turn, can easily be

incorporated into the cell material (Saguir and de Nadra, 2007). It is interesting to note that the high assimilation of Ser, Ala and Asp in this study were also noted to be highly assimilated in propionic bacteria (Thierry and Maillard, 2002).

There is an association between peptidoglycan and the highest assimilated amino acids. The peptidoglycan of Gram-positive bacteria is made-up of cross-linked glycan strands which, in turn, is composed of alternating sugars of N-acetlyglucamic acid and N-acetyl N-acetylmuramic acid. N-acetyl muramic acid has an attached pentapeptide composed of L-Ala, D-Glu, L-Lys or meso-aminopimelic acid (L-Lys derivative), and 2 D-Ala. The cross-linking of the pentapeptide can either be direct or could take place by way of a cross bridge of one (D-Asp) or several (Gly, L-Ala and L-Ser) amino acids (Delcour et al., 1999). These amino acid all needs incorporation into the cell wall. It might be the reason for the higher assimilation during the exponential phase.

Generally, there is no higher consumption of essential amino acids although some correlation has been established before in *L. plantarum* (Saguir and de Nadra, 2007). BCAA and Met and other essential amino acids from the liquid-assays, were assimilated after 72 h at moderate to low levels (Ile, Val and Met). On the other hand, non-essential amino acids like Asp, Ser and Thr are taken up faster and at higher concentrations. Moreover with the omission of Tyr, *L. plantarum* grew more poorly than an omission of Phe yet Phe was assimilated at a slightly higher rate. The amino acid assimilation profile of *L. plantarum* in SGJ and propionic bacteria align. A review of the metabolism of propionic bacteria found Ser, Ala and Asp to be assimilated at very high levels than all other amino acids (Thierry and Maillard, 2002).

In this study the concentrations of amino acid undergoing transamination such as the BCAA, AAA and Met decreases rapidly after 72 h. This observation concurs with expression studies which revealed high expression of transaminases (*araT*, *bcaT*) and decarboxylase (*kivD*) encoding genes in *Lc. lactis* after stationary phase (García-Cayuela et al., 2012). Gram-positive bacteria is under strong regulation by a pleiotropic regulatory protein named CodY. This protein controls the expression both of *araT*, *bcaT* and *kivD* (decarboxylase gene) at transcriptional level (Chambellon and Yvon, 2003). The expression of CodY is only dependent upon the presence of intracellular BCAA (especially Ile) (Chambellon and Yvon, 2003; Petranovic et al., 2004). During stationary phase the BCAA concertation decreases and regulatory effect of CodY is less active. Since *L. plantarum* is a LAB and the amino acid assimilation behaves in the fashion just described, this species may be under CodY repression however further research is required to support this hypothesis

3.5.3 Differences in amino acid assimilation between synthetic grape juice and Chardonnay

The same strains of *L. plantarum* exhibited a different pattern of amino acid assimilation between SGJ (chemically defined media) and grape juice (differential media). The amino acid composition between

the SGJ used in this study and grape juice is different (**Supplementary Tables 1 - 4)**. In SGJ, the initial concentration of all amino acids are 2 mg N/L, whereas the starting concentrations of amino acids in grape juice are higher than 2 mg N/L with some amino acids as high as 97 mg N/L (Arg), 26 mg N/L (Ala), 24 mg N/L (Pro) and 22 mg N/L (Gln). Furthermore, as the pH was relatively high (pH 3.42) and SO_2 was below 60 mg/L adaptation to grape juice, through pre-culturing in activation media (**See Materials and Methods**), was a prudent and preventative measure to ensure *L. plantarum* would finish MLF prior to inhabitation of yeast, other LAB and acetic acid bacteria after several days, in this nutritious rich media. Notwithstanding higher amino acid concentrations and pre-culture conditions there was a few other factors which could have influenced the amino acid uptake pattern such as sugar concentration and MLF temperature. In SGJ, the sugar concentrations of glucose and fructose was each added at 10 mg/L. During optimization it was learned that *L. plantarum* take up very little sugar. For example in SGJ in which both glucose and fructose was added at 50 g/L only 2 g/L was taken up by *L. plantarum* over a 6 day period. When *L. plantarum* was inoculated into SGJ with 50 g/L of glucose and 50 g/L of fructose sugar quantification revealed *L. plantarum* to take up 2 mg/L of each sugar. Excessive additions was thus unnecessary to stimulate growth. However excessive sugar concentrations might influence amino acid uptake as the amino acid catabolism is under catabolite repression. The arginine deiminase pathway (ADI) for example is under catabolite repression (Bringel, 1998) Fermentation temperature in Chardonnay grape juice (20ºC) differed from the SGJ temperature (30ºC) to mimic alcoholic fermentation conditions used in the wine-industry namely between 20-25ºC for red wine and 12-16ºC for white wine. All these varying factors, might be responsible for the discrepancy observed between assimilation in SGJ and 'real' grape juice.

The same preferred amino acid (i.e. amino acid most likely to be assimilated) from SGJ and 'real' grape juice differed indicating that *L. plantarum* has no consistent demand for the same amino acids under different conditions. For instance the most assimilated amino acids were Phe, Leu, Arg and Lys while Asp, Ala, Ser and Thr was assimilated at a higher rate. This phenomenon is also apparent when comparing this study on amino acid assimilation with several others. Lee et al. (2009) reported increases in the levels of BCAA in MLF wines. In turn, Pozo-Bayon et al. (2005) reported decreases in Arg, Met, Thr, Trp and Tyr and increases in all other amino acids due to protein degradation. Consistency in amino acid assimilation in this study only existed in the non-preferred amino acids sources as His, Met, Gly was always taken up at low concentrations.

3.6 Conclusions

This study increased our knowledge surrounding *L. plantarum*'s amino acid metabolism. *L. plantarum* are unable to grow to its full extent or complete MLF without the availability of certain amino acids. *L. plantarum*, isolated from spontaneous red wine had slightly more essential amino acids as *L. plantarum* that reside in cheese and dairy products. It is well known and acknowledged by many that culturing

species in the same ecological niche for extended periods, may result in certain genes not being essential, increasing the likelihood of accumulation of mutations.

This was the first time the assimilation of non-essential and essential amino acids were investigated in chemically defined media for *L. plantarum*. Although Saguir and de Nadra (2007) also investigated amino acid requirements and uptake in synthetic media, amino acid assimilation was only investigated for the amino acids which were found to be essential.

Asp appears to be a limiting factor in the biomass formation. Asp was the only amino acid totally consumed in all bacteria when the bacteria reached stationary phase. 72 h coincides with the time Asp was totally consumed. This study highlighted the complexity of amino acid metabolism and suggested all wine-isolated *L. plantarum* have the same affinity for the same amino acid when cultured under the same conditions. When changing the conditions the assimilation changes. The factors conditions that have been identified to play a role in this study: nitrogen composition (relative ratios and quantity) and pre-culturing conditions. The development of fruity aroma from the catabolism of BCAA and Phe can only take place after stationary phase in *L. plantarum* if the subsequent decarboxylase and reductase are expressed. Although the BCAA are essential nutrient requirements in *L. plantarum* they do not play a major role in the growth of *L. plantarum*.

From an aromatic perspective the amino acids of interest are the amino acids capable of donating an amino group (Leu, Ile, Val, Phe, Tyr and Met) and donating a keto group (Ala, Asp and Glu) in a transamination reaction. The BCAA and AAA and Met are only assimilated after 72 h during stationary phase.

3.7 Acknowledgements

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3.8 References

Ardö, Y., 2006. Flavour formation by amino acid catabolism. Biotechnol. Adv. 24, 238–242.

Bely, M., Sablayrolles, J.-M., Barre, P., 1990. Automatic detection of assimilable nitogen deficiencies during alcoholic fermentation in oenological conditions. J. Ferment. Bioeng. 70, 246–252.

Bringle, F., 1998. Carbamoylphosphate and natural auxotrophies in lactic acid bacteria. Lait 78, 31-37.

Cañas, P.M.I., Pérez, P.R., Prieto, S.S., Herreros, M.L.P., 2009. Ecological study of lactic acid microbiota isolated from Tempranillo wines of Castilla-La Mancha. J. Biosci. Bioeng. 108, 220–224.

Chambellon, E., Yvon, M., 2003. CodY-regulated aminotransferases AraT and BcaT play a major role in the growth of *Lactococcus lactis* in milk by regulating the intracellular pool of amino acids. Appl. Environ. Microbiol. 69, 3061–3068.

Delcour, J., Ferain, T., Deghorain, M., Palumbo, E., Hols, P., 1999. The biosynthesis and functionality of the cell-wall of lactic acid bacteria. Antonie Van Leeuwenhoek 76, 159–184.

Delorme, C., Ehrlich, S.D., Renault, P., 1999. Regulation of expression of the *Lactococcus lactis* histidine operon. J. Bacteriol. 181, 2026–2037.

Delorme, C., Godon, J.-J., Ehrlich, S.D., Renault, P., 1993. Gene inactivation in *Lactococcus lactis* : histidine biosynthesis. J. Bacteriol. 175, 4391–4399.

Dickinson, J.R., Harrison, S.J., Dickinson, J.A., Hewlins, M.J.E., 2000. An investigation of the metabolism of isoleucine to active amyl alcohol in *Saccharomyces cerevisiae* . J. Biol. Chem. 275, 10937–10942.

Dickinson, J.R., Harrison, S.J., Hewlins, M.J.E., 1998. An investigation of the metabolism of valine to isobutyl alcohol in *Saccharomyces cerevisiae* . J. Biol. Chem. 273, 25751–25756.

Dickinson, J.R., Lanterman, M., Danner, B.J., Pearson, B.M., Sanz, P., Harrison, S.J., Hewlins, J.E., 1997. A 13C nuclear magnetic resonance investigation of the metabolism of leucine to isoamyl alcohol in *Saccharomyces cerevisiae* . J. Biol. Chem. 272, 26871–26878.

Du Plessis, H.W., Dicks, L.M.T., Pretorius, I.S., Lambrechts, M.G., Du Toit, M., 2004. Identification of lactic acid bacteria isolated from South African brandy base wines. Int. J. Food Microbiol. 91, 19–29. doi:10.1016/S0168-1605(03)00335-0

Du Toit, M., Engelbrecht, L., Lerm, E., Krieger-Weber, S., 2011. *Lactobacillus*: the next generation of malolactic fermentation starter cultures-an overview. Food Bioprocess Technol. 4, 876–906. doi:10.1007/s11947-010-0448-8

Friedrich, J.E., Acree, T.E., 1998. Gas chromatography olfactometry (GC/O) of dairy products. Int. Dairy J. 8, 235–241.

G-Alegría, E., López, I., Ruiz, J.I., Sáenz, J., Fernández, E., Zarazaga, M., Dizy, M., Torres, C., Ruiz-Larrea, F., 2004. High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiol. Lett. 230, 53–61.

García-Cayuela, T., Gómez de Cadiñanos, L.P., Peláez, C., Requena, T., 2012. Expression in *Lactococcus lactis* of functional genes related to amino acid catabolism and cheese aroma formation is influenced by branched chain amino acids. Int. J. Food Microbiol. 159, 207–213.

Garvie, E.I., 1967. The growth factor and amino acid requirements of species of the genus *Leuconostoc* , including *Leuconostoc paramesenteroides* (sp. nov.) and *Leuconostoc oenos* . J. Gen. Microbiol. 48, 439–447.

Godon, J., Delorme, C., Bardowski, J., Chopin, M., Ehrlich, S., Renault, P., 1993. Gene inactivation in *Lactococcus lactis*: Branched-chain amino acid biosynthesis. J. Bacteriol. 175, 4383–4390.

González-Arenzana, L., López, R., Santamaría, P., Tenorio, C., López-Alfaro, I., 2012. Dynamics of Indigenous Lactic Acid Bacteria Populations in Wine Fermentations from La Rioja (Spain) During Three Vintages. Microb. Ecol. 63, 12–19.

Guerzoni, M.E., Sinigaglia, M., Gardini, F., Ferruzzi, M., Torriani, S., 1995. Effects of pH, temperature, ethanol, and malate concentration on *Lactobacillus plantarum* and *Leuconostoc oenos* : Modelling of the malolactic activity. Am. J. Enol. Vitic. 46, 368–374.

Henderson, J., Brooks, A., 2010. Improved amino acid methods using Agilent ZORBAX Eclipse Plus C18 columns for a variety of Agilent LC instrumentation and separation goals.

Henschke, P., Jiranek, V., 1993. Yeast - Metabolism of nitrogen compounds. Wine Microbiol Biotechnol 77–164.

Lapujade, P., Loubiere, P., 1998. Glutamate Biosynthesis in *Lactococcus lactis* subsp. *lactis* NCDO 2118. Appl. Environ. Microbiol. 64, 2485–2489.

Lerm, E., Engelbrecht, L., du Toit, M., 2010. Malolactic Fermentation: The ABC's of MLF. South African J. Enol. Vitic. 31, 186–212.

Lewington, J., Greenaway, S.D., Spillane, B.J., 1987. Repid small scale preparation of bacterial genomic DNA, suitable for cloning and hybridization analysis. Lett. Appl. Microbiol. 5, 51–53.

López, I., López, R., Santamaría, P., Torres, C., Ruiz-Larrea, F., 2008. Performance of malolactic fermentation by inoculation of selected *Lactobacillus plantarum* and *Oenococcus oeni* strains isolated from Rioja red wines. Vitis - J. Grapevine Res. 47, 123–129.

Ma, C., Cheng, G., Liu, Z., Gong, G., Chen, Z., 2016. Determination of the essential nutrients required for milk fermentation by *Lactobacillus plantarum* . LWT - Food Sci. Technol. 65, 884–889.

Maarman, B.C., 2014. Interaction between wine yeast and malolactic bacteria and the impact on wine aroma and flavour. MSc thesis, Inst. Wine Biotechnol. Stellenbosch Univ. South Africa.

Maicas, S., Gil, J.V., Pardo, I., Ferrer, S., 1999. Improvement of volatile composition of wines by controlled addition of malolactic bacteria. Food Res. Int. 32, 491–496.

Malherbe, S., Menichelli, E., du Toit, M., Tredoux, A., Muller, N., Næs, T., Nieuwoudt, H., 2013. The relationships between consumer liking, sensory and chemical attributes of *Vitis vinifera* L. cv. Pinotage wines elaborated with different *Oenococcus oeni* starter cultures. J. Sci. Food Agric. 93, 2829–2840.

McSweeney, P.L.H., Sousa, M.J., 2000. Biochemical pathways for the production of flavour compounds in cheeses during ripening: A review. Lait 80, 293–324.

Molimard, P., Spinnler, H.E., 1996. Review: compounds involved in the flavor of surface mold-ripened cheeses: origins and properties. J. Dairy Sci. 79, 169–184.

Morishita, T., Deguchi, Y., Yajima, M., Sakurai, T., Yura, T., 1981. Multiple nutritional requirements of lactobacilli: genetic lesions affecting amino acid biosynthetic pathways. J. Bacteriol. 148, 64–71.

Osborne, J.P., Edwards, C.G., 2007. Inhibition of malolactic fermentation by a peptide produced by *Saccharomyces cerevisiae* during alcoholic fermentation. Int. J. Food Microbiol. 118, 27–34.

Ott, A., Fay, L.B., Chaintreau, A., 1997. Determination and origin of the aroma impact compounds of yogurt flavor. J. Agric. Food Chem. 45, 850–858.

Pessione, E., Cirrincione, S., 2016. Bioactive molecules released in food by lactic acid bacteria: encrypted peptides and biogenic amines. Front. Microbiol. 7, 1–19.

Petranovic, D., Guédon, E., Sperandio, B., Delorme, C., Ehrlich, D., Renault, P., 2004. Intracellular effectors regulating the activity of the *Lactococcus lactis* CodY pleiotropic transaction regulator. Mol. Microbiol. 53, 613–621.

Pozo-Bayon, M.A., Alegría, E.G., Polo, M.C., Tenorio, C., Martion-Álvarez, P.J., Calvo De La Banda, M., Ruiz-Larrea, F., Moreno-Arribas, M. V, 2005. Wine volatile and amino acid composition after

malolactic fermentation: effect of *Oenococcus oeni* and *Lactobacillus plantarum* starter cultures. J. Agric. Food Chem. 53, 8729–8735.

Pudlik, A.M., Lolkema, J.S., 2012. Rerouting citrate metabolism in *Lactococcus lactis* to citrate-driven transamination. Appl. Environ. Microbiol. 78, 6665–6673.

Remize, F., Gaudin, A., Kong, Y., Guzzo, J., Alexandre, H., Krieger, S., Guilloux-Benatier, M., 2006. *Oenococcus oeni* preference for peptides: qualitative and quantitative analysis of nitrogen assimilation. Arch. Microbiol. 185, 459–469.

Ruiz, P., Izquierdo, P.M., Seseña, S., Palop, M.L., 2010. Analysis of lactic acid bacteria populations during spontaneous malolactic fermentation of Tempranillo wines at five wineries during two consecutive vintages. Food Control 21, 70–75.

Saguir, F.M., de Nadra, M.C.M., 2007. Improvement of a chemically defined medium for the sustained growth of *Lactobacillus plantarum*: nutritional requirements. Curr. Microbiol. 54, 414–418.

Siezen, R.J., van Hylckama Vlieg, J.E., 2011. Genomic diversity and versatility of *Lactobacillus plantarum* , a natural metabolic engineer. Microb. Cell Fact. 10, S3.

Silla Santos, M.H., 1996. Biogenic amines: Their importance in foods. Int. J. Food Microbiol. 29, 213–231.

Smit, A.Y., Du Toit, W.J., Du Toit, M., 2008. Biogenic amines in wine: understanding the headache. South African J. Enol. Vitic. 29, 109–127.

Smit, Smit, B.A., Engels, W.J.M., 2005. Flavour formation by lactic acid bacteria and biochemical flavour profiling of cheese products. FEMS Microbiol. Rev. 29, 591–610.

Terrade, N., Mira de Orduña, R., 2009. Determination of the essential nutrient requirements of wine-related bacteria from the genera *Oenococcus* and *Lactobacillus* . Int. J. Food Microbiol. 133, 8–13.

Teusink, B., van Enckevort, F.H.J., Francke, C., Wiersma, A., Wegkamp, A., Smid, E.J., Siezen, R.J., 2005. In Silico reconstruction of the metabolic pathways of *Lactobacillus plantarum* : comparing predictions of nutrient requirements with those from growth experiments. Appl. Environ. Microbiol. 71, 7253–7262.

Thierry, A., Maillard, M., 2002. Production of cheese flavour compounds derived from amino acid catabolism by *Propionibacterium freudenreichii* . Lait 82, 17–32.

Torriani, S., Giovanna, E., Dellaglio, F., 2001. Differentiation of *Lactobacillus plantarum, L. pentosus* and *L. paraplantarum* by *rec*A gene sequence analysis and multiplex PCR assay with *rec* a gene-derived primers. Appl. Environ. Microbiol. 67, 3450–3454.

Trip, H., Mulder, N.L., Lolkema, J.S., 2013. Cloning, expression, and functional characterization of secondary amino acid transporters of *Lactococcus lactis* . J. Bacteriol. 195, 340–350.

Umbarger, H.E., 1978. Amino acid biosynthesis and its regulation. Annu. Rev. Biochem. 47, 533–606.

Wegkamp, A., Teusink, B., de Vos, W.M., Smid, E.J., 2010. Development of a minimal growth medium for *Lactobacillus plantarum* . Lett. Appl. Microbiol. 50, 57–64.

3.9 Supplementary data

Supplementary Table 1. The concentration of each amino acid (mg/L) at the start and 36 h after inoculation of *L. plantarum* in synthetic grape juice (SGJ).

	Initial concentration	concentration at t = 36 h								
		L. plantarum 73.1			*L. plantarum* 83			*L. plantarum* 85.1		
Asp	20.05	12.09	±	0.08	6.00	±	0.49	12.37	±	0.14
Glu	17.92	18.60	±	0.37	16.97	±	0.18	19.23	±	0.31
Cys	2.84	2.90	±	0.08	3.18	±	0.52	2.95	±	0.04
Asn	7.82	6.82	±	0.04	7.69	±	0.03	7.76	±	0.24
Ser	14.17	12.88	±	0.10	11.95	±	0.14	13.27	±	0.21
Gln	4.65	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
His	9.65	8.71	±	0.23	9.19	±	0.22	8.99	±	0.33
Gly	9.40	7.92	±	0.26	8.12	±	0.32	8.36	±	0.18
Thr	17.31	14.76	±	0.20	15.85	±	1.35	17.24	±	1.29
Arg	6.84	6.21	±	0.20	6.19	±	0.08	6.71	±	0.16
Ala	11.61	9.36	±	0.12	9.11	±	0.06	10.56	±	0.33
Tyr	23.71	24.97	±	0.48	25.40	±	0.31	25.76	±	0.74
Val	14.09	14.15	±	0.46	14.39	±	0.09	14.89	±	0.25
Met	18.14	19.30	±	0.42	19.53	±	0.20	19.45	±	0.16
Trp	13.43	12.54	±	0.26	12.48	±	0.32	12.32	±	0.14
Phe	22.94	23.18	±	0.36	23.48	±	0.61	23.59	±	0.30
Ile	16.43	16.66	±	0.46	16.68	±	0.28	17.31	±	0.23
Leu	15.56	15.13	±	0.36	15.25	±	0.25	15.90	±	0.10
Lys	9.55	7.01	±	1.00	6.16	±	0.06	6.89	±	0.15
Pro	13.77	13.46	±	0.35	14.40	±	0.59	14.63	±	0.42
Total amino acids	269.89	246.66			242.02			258.17		

Supplementary Table 2. The concentration of each amino acid (mg/L) at the start and 72 h after inoculation of *L. plantarum* in SGJ.

	Initial concentration	concentration at t = 72 h								
		L. plantarum 73.1			*L. plantarum* 83			*L. plantarum* 85.1		
Asp	20.05	0.19	±	0.10	0.10	±	0.07	0.08	±	0.05
Glu	17.92	13.42	±	0.55	7.72	±	2.28	7.80	±	2.81
Cys	2.84	4.36	±	1.90	3.18	±	0.78	2.76	±	0.22
Asn	7.82	4.21	±	0.11	4.67	±	0.45	4.64	±	0.62
Ser	14.17	6.91	±	0.23	2.34	±	1.99	3.23	±	1.26
Gln	4.65	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
His	9.65	9.76	±	0.25	8.54	±	0.33	8.68	±	0.61
Gly	9.40	11.25	±	0.25	7.26	±	0.69	7.10	±	0.93
Thr	17.31	4.54	±	0.40	7.73	±	2.36	8.63	±	1.07
Arg	6.84	4.72	±	0.31	3.97	±	1.04	4.08	±	0.67
Ala	11.61	4.99	±	0.32	4.63	±	2.63	5.47	±	1.45
Tyr	23.71	24.27	±	0.74	22.83	±	1.95	23.03	±	2.20
Val	14.09	12.86	±	0.55	12.03	±	1.09	11.93	±	1.35
Met	18.14	19.20	±	0.74	18.02	±	0.88	18.14	±	1.38
Trp	13.43	12.59	±	0.91	12.34	±	0.59	12.12	±	0.58
Phe	22.94	22.59	±	0.90	19.25	±	3.35	19.38	±	2.79
Ile	16.43	15.18	±	0.77	13.16	±	1.68	12.94	±	1.87
Leu	15.56	13.06	±	0.65	11.67	±	1.67	11.49	±	1.92
Lys	9.55	5.17	±	0.50	6.03	±	2.01	5.41	±	0.61
Pro	13.77	15.74	±	0.90	13.96	±	1.30	13.18	±	1.07
Total amino acids	269.89	205.02			179.43			180.09		

Supplementary Table 3. The concentration of each amino acid (mg/L) at the start and 144 h after inoculation of *L. plantarum* in SGJ.

	Initial concentration	concentration at t = 144 h								
		L. plantarum 73.1			*L. plantarum* 83			*L. plantarum* 85.1		
Asp	20.05	0.22	±	0.22	0.05	±	0.08	0.36	±	0.34
Glu	17.55	4.74	±	0.72	0.14	±	0.20	2.09	±	1.26
Cys	2.84	4.80	±	0.66	3.40	±	1.58	3.11	±	1.57
Asn	7.82	4.52	±	0.08	4.46	±	0.31	3.26	±	0.21
Ser	14.17	0.54	±	0.10	0.24	±	0.09	0.10	±	0.05
Gln	4.65	0.00	±	0.00	0.00	±	0.00	0.00	±	0.00
His	9.65	8.44	±	0.10	7.18	±	0.78	5.32	±	0.25
Gly	9.40	13.43	±	0.36	8.01	±	0.74	5.92	±	0.78
Thr	17.31	0.00	±	0.00	0.00	±	0.00	-0.36	±	0.01
Arg	6.84	2.74	±	0.34	1.02	±	0.11	0.63	±	0.46
Ala	11.84	2.31	±	0.42	0.53	±	0.34	0.30	±	0.21
Tyr	23.99	13.42	±	2.38	8.30	±	0.19	5.96	±	0.90
Val	14.09	10.54	±	0.41	7.26	±	0.50	4.59	±	1.54
Met	18.14	18.70	±	0.50	13.83	±	0.90	10.51	±	1.37
Trp	13.43	13.74	±	0.36	13.59	±	0.83	11.07	±	0.52
Phe	23.35	10.67	±	2.22	8.25	±	0.60	2.63	±	0.33
Ile	16.43	10.43	±	0.80	4.62	±	0.99	1.78	±	1.28
Leu	15.56	6.93	±	0.99	3.59	±	0.84	1.26	±	1.00
Lys	10.22	2.73	±	0.35	1.93	±	1.00	2.22	±	0.66
Pro	13.77	16.24	±	0.36	12.57	±	1.50	10.99	±	0.42
Total amino acids	271.10	145.15			98.97			71.73		

Supplementary Table 4. The concentration of each amino acid (mg/L) at the start and 72 h after inoculation of *L. plantarum* in Chardonnay grape juice.

	Initial concentration			concentration at t = 72 h								
				L. plantarum 73.1			*L. plantarum* 83			*L. plantarum* 85.1		
Asp	20.42	±	1.25	13.72	±	0.45	15.01	±	0.27	14.79	±	1.20
Glu	59.05	±	2.74	49.23	±	1.46	48.63	±	0.05	46.89	±	0.68
Cys	10.41	±	1.99	6.78	±	0.23	8.37	±	0.59	7.83	±	1.64
Asn	13.38	±	1.86	7.94	±	0.22	10.13	±	0.19	10.11	±	0.36
Ser	87.39	±	2.47	71.09	±	0.98	77.33	±	1.69	72.19	±	1.66
Gln	77.08	±	3.79	65.89	±	0.89	73.71	±	0.45	73.26	±	5.60
His	52.52	±	4.43	47.55	±	0.82	51.55	±	3.91	51.26	±	10.70
Gly	13.75	±	4.26	7.31	±	0.40	7.44	±	0.32	7.84	±	0.28
Thr	63.81	±	3.03	51.72	±	1.13	53.91	±	0.32	53.93	±	1.29
Arg	292.17	±	15.58	253.53	±	5.56	265.13	±	13.58	272.70	±	31.03
Ala	166.73	±	3.88	139.63	±	0.75	144.62	±	3.11	149.71	±	0.06
GABA	164.74	±	6.81	157.06	±	3.54	163.14	±	8.08	168.92	±	15.45
Tyr	15.44	±	1.41	4.84	±	0.05	6.81	±	0.12	7.15	±	0.45
CY2	26.57	±	5.81	14.08	±	0.72	14.63	±	0.34	15.88	±	3.58
Val	28.55	±	4.32	17.85	±	0.83	18.99	±	0.09	18.03	±	0.70
Met	9.86	±	3.02	3.58	±	0.09	4.34	±	0.13	3.97	±	0.43
Trp	26.27	±	2.01	21.01	±	0.56	21.68	±	0.61	22.55	±	1.06
Phe	28.68	±	7.50	7.28	±	0.10	10.05	±	0.00	10.92	±	0.07
Ile	15.76	±	3.75	6.10	±	0.15	6.39	±	0.24	5.64	±	0.93
Orn	7.94	±	1.16	7.67	±	0.35	8.20	±	1.25	8.90	±	1.65
Leu	47.57	±	17.00	11.39	±	0.79	15.16	±	0.39	13.97	±	0.93
Lys	26.23	±	12.93	4.15	±	0.35	5.85	±	0.07	5.23	±	0.36
Pro	223.59	±	42.19	219.39	±	23.66	255.52	±	31.86	203.46	±	35.43
Total amino acids	1477.90			1188.79			1286.59			1245.13		

Chapter 4

General discussion and conclusions

Chapter 4 – General discussion and conclusions

4.1 General discussion

Malolactic fermentation (MLF) can occur at the start, middle or end of alcoholic fermentation in grape juice or wine. MLF is defined as the process in which L-malic acid is converted to L-lactic acid. This reaction results in wine with a softer mouthfeel from less perceived acidity. Additionally, the LAB provides microbial stability to the wine and can alter the aroma profile of wine. *Oenococcus oeni* is predominantly used at the end of alcoholic fermentation for MLF, since most LAB species cannot tolerate the various combined factors of wine to finish MLF. Additionally, *O. oeni* produces limited quantities of spoilage and undesirable metabolites. When MLF is induced with other species of LAB it often leads to stuck or sluggish fermentation and the formation of metabolites associated with spoilage (Du Toit and Pretorius, 2000). However, one species of LAB may compete with *O. oeni* as commercial starter culture in the MLF of high pH grape juice, namely *Lactobacillus plantarum*. (Du Toit et al., 2011).

There are many advantages in using *L. plantarum* as commercial starter culture for MLF instead of *O. oeni*. Firstly, the decarboxylation genes involved in the formation of biogenic amines (e.g. histamine and tryptamine) are absent for the selected commercial strains but this phenomenon is strain dependent therefore the selection of their absence is important (Iorizzo et al., 2016; Lerm et al., 2011; López et al., 2008; Moreno-Arribas et al., 2000). Secondly, PCR screening of *L. plantarum* strains isolated from South African spontaneous red wine MLF revealed the Arg deiminase in the arginine deiminase (ADI) pathway to be absent (Lerm et al., 2011) although recently a single study have linked wine *L. plantarum* strains to the ADI degradation (Spano et al., 2004). Although this pathway increases energy production and intracellular pH through the release of ammonium it also synthesises citrulline which, when bound to ethanol in the wine, produces ethyl carbamate, a carcinogen (Schlatter and Lutz, 1990). In contrast to *L. plantarum*, *O oeni* has been observed to produce small quantities of histamine and tryptamine and is known to possess the ADI pathway (Divol et al., 2003; Lerm et al., 2011; Zúñiga et al., 2002, 1998). Thirdly, *L. plantarum* is homofermentative and as a result it does not use the pentose phosphate pathway for the degradation of glucose and fructose to produce and acetic acid which contributes to the volatile acidity of wine (Costello et al., 2001; Zúñiga et al., 1993). Fourthly, *L. plantarum* has been shown in some cases to produce wine with more fruitier aromas than *O. oeni* when co-inoculation is done (Lee et al., 2009; Pozo-Bayon et al., 2005; Sun et al., 2016). Lastly, some strains of *L. plantarum* is just as successful as *O. oeni* to induce and complete MLF in grape juice (Bravo-Ferrada et al., 2013; Fumi et al., 2010; G-Alegría et al., 2004; Guerzoni et al., 1995; Lerm et al., 2011; Maarman, 2014; Sun et al., 2016) .

There are many factors that influence the growth of *L. plantarum* in grape must and wine. To obtain successful MLF the grape must should comprise of a higher pH (3.5-4.0), and low initial SO_2 and ethanol concentrations (G-Alegría et al., 2004; Guerzoni et al., 1995). But even these factors which are considered by most studies as

important to prevent incomplete MLF, does not assure a successful MLF (Lerena et al., 2016). The other factor that might play a crucial role that is not easy to measure is the amino acid composition.

Therefore, this study focussed on amino acid usage to ascertain the amino acid requirements of *L. plantarum*. According to literature, all facets of amino acid that influences growth in an organism can be classified into three categories. The findings of this study and its relevance to winemaking will be discussed according to these categories.

The first category consist of amino acid trophic requirements in terms of essential and non-essential amino acids. This category defines whether the pathways for the biosynthesis of amino acids are present, functional and expressed (Godon et al., 1993). For example, *in silico* analysis of the genome of *L. plantarum* WCFS1 revealed the pathways for the biosynthesis of branched chain amino acids (BCAA) to be absent, while the pathways for the aromatic amino acids (AAA) are present but could remain unexpressed due to feed-back inhibition (Teusink et al., 2005). In our study, single amino acid growth experiments revealed amino acid requirements to be highly strain dependent. Overall, the 5 *L. plantarum* strains tested in this study had 6- 8 amino acid requirements. This in turn concurs with literature which consistently finds 6-8 amino acids to be essential in *L. plantarum* (Garvie, 1967; Ma et al., 2016; Saguir and de Nadra, 2007; Teusink et al., 2005). From a MLF perspective *O. oeni*, is a more nutritionally fastidious species than *L. plantarum* having 13-16 requirements (Terrade and Mira de Orduña, 2009). From a wine making perspective, a commercial strain with less nutrient requirements are more beneficial for the completion of MLF since *Saccharomyces cerevisiae* (primary yeast responsible for alcoholic fermentation) is known to readily assimilate various amino acids during its exponential phase which, in many cases, overlaps with the essential amino acids needs of *L. plantarum* strains observed in this study (Met, Ile, Leu) (Barrajón-Simancas et al., 2011; De Koker, 2015; Gobert et al., 2017). Additionally, since the amino acid requirements are highly strain dependent, the commercial *L. plantarum* strains' essential amino acids must be identified in order to assess the compatibility of these strains with *S. cerevisiae* during co-inoculation or to ensure that complex nutrients applied for MLF contains these essential amino acids as part of their composition. If these essential amino acids are assimilated before the completion of MLF, *L. plantaru*m must rely on its proteolytic system which could extend the time of MLF or lead to a 'stuck' MLF as the *de novo* synthesis of proteolytic enzymes requires more energy than simple uptake of single amino acids (Wegkamp et al., 2010). This provides one more reason why *L. plantarum* survives exceptionally well in grape juice for the first few days (González-Arenzana et al., 2012) and poorly in wine.

The second category are preferred and non-preferred amino acid sources and refers to amino acids that are either taken-up or remains unassimilated. Limitations to growth, with regards to amino acid composition in wine or grape juice, might not extend to the supply of essential amino acids alone, but also whether the demand for preferred amino acids (i.e. amino acid most likely to be assimilated) are adequately supplied. As observed in this study, the lack of the amino acid Asp in SGJ was the only identifiable factor that could have terminated the growth of *L. plantarum.* Furthermore, in this study all strains of *L. plantarum* had the same preference for

amino acids under the same conditions. When the composition of the media shifts from synthetic grape juice to Chardonnay grape juice and the *L. plantarum* strains are pre-cultured in a different media, different preferences were observed. All 3 strains for example had taken-up all Asp, Ala and Ser during MLF in synthetic grape juice while in clarified Chardonnay grape juice all strains had taken up a large quantity of Lys, Phe, Leu and Arg. This is also supported in literature as there is no case between different studies in which the uptake of amino acids in *L. plantarum* were uniform (Lee et al., 2009; Pozo-Bayon et al., 2005). This study identified that the preferred amino acid of *S. cerevisiae* (De Koker, 2015) overlaps with the preference of *L. plantarum*.

The third category which was not investigated in this study is efficient and non-efficient amino acid sources. Efficient nitrogen sources are characterised as sources which stimulates growth much faster than inefficient nitrogen sources. *L. plantarum* have preference for the same amino acids that is considered in *S cerevisiae* to be efficient nitrogen sources (Ala, Asp, Glu, Arg and Phe) (Mckinnon, 2013). In theory it would therefore stand to reason that co-inoculation with *L. plantarum* would severely reduce the rate of alcoholic fermentation and *S. cerevisiae* would reduce the rate of MLF. One study had discovered that simultaneous inoculation of *S. cerevisiae* and *L. plantarum* had disrupted both malic acid and citric acid uptake (Onetto and Bordeu, 2015). Then again, many other studies report *L. plantarum* and *S. cerevisiae* co-inoculation in grape juice to result in completion of both alcoholic fermentation and MLF (Fumi et al., 2010; Lerena et al., 2016; Lerm, 2010; Sun et al., 2016). Thus, there is a strong possibility that *L. plantarum* would not hinder alcoholic fermentation. Similarly, several studies have investigated simultaneous alcoholic fermentation with *S. cerevisiae* and MLF with *O. oeni* and discovered no significant retardation on the alcoholic fermentation kinetics or yeast growth (Abrahamse and Bartowsky, 2012; Guzzon et al., 2016; Lasik-Kurdyś et al., 2017; Muñoz et al., 2014; Taniasuri et al., 2016; Tristezza et al., 2016; Zapparoli et al., 2009).

Though it was not the aim of this study to investigate *L. plantarum* growth in grape juice some interesting results have emerged that has not been previously found. The Chardonnay grape juice used in this study had been clarified and was devoid of its initial natural microflora. The grape juice had a pH of 3.4 and sulphur dioxide concentration of 32 mg/L. In this grape juice, activated *L. plantarum* took 2-3 days to finish MLF. In sterile high pH wines *L. plantarum* is also noted to take-up all malic acid within 3-4 days (Bravo-Ferrada et al., 2013). Therefore the success of simultaneous inoculation is not solely dependent upon the composition of the juice but also upon the winemaking practises for instance application of thermovinification, flotation prior to inoculation are applied.

4.2 Future prospects

Future prospects should be directed to identifying the cause of disparity in data between the liquid and plate assay as this deserves some consideration. Although the principal of the omission technique is consistent, some factors between the solid and liquid assays were different. To illustrate an example, in the plate-based method the cultures were incubated anaerobically, while the liquid cultures were incubated micro-aerophylically. The absence of oxygen is seen to play a role in the biosynthesis of Arg through inhibition of the CPSaseArginine (Bringel, 1998). The liquid culture has one more auxotrophy for Arg than the plate-based method. Therefore,

the effect of oxygen should be investigated toward amino acid biosynthesis. Similarly other factors such as pH, temperature and amino acid composition might influence the activity of a biosynthetic enzyme, and should be investigated as well.

The uptake of amino acids were only investigated using inoculated starved and activated culture of *L. plantarum*, neither of which are standard practise in the wine industry. A subsequent investigation should examine the effect on amino acid assimilation using a freeze-dried culture of *L. plantarum*.

It was proven in this study that the fusel alcohol precursors may be the preferred amino acid sources in grape juice. It then becomes necessary to characterize the enzymes responsible for their formation such as the keto-acid isovalerate decarboxylase (KivD), the branched-chain amino acid transferase (BcaT), the aromatic amino acid transferase (AraT) and the glutamate dehydrogenase (GDH) as it would prove useful to assess whether a single pure culture of *L. plantarum* can add to the aroma profile through the Ehrlich pathway. The same characterized strains should also undergo MLF in grape juice and the aroma should subsequently be quantified to assess their influence on wine aroma.

If a *L. plantarum* amino acid usage is well-defined under various conditions a tailored nutrient may be developed to ensure the survival of *L. plantarum* and successful MLF in grape juice but one critical piece is missing. Amino acid requirements must be classified as efficient, inefficient and non-utilized in *L. plantarum*. When classification is done it would be known which efficient amino acids should be added at higher concentrations.

4.3 References

Abrahamse, C.E., Bartowsky, E.J., 2012. Timing of malolactic fermentation inoculation in Shiraz grape must and wine: influence on chemical composition. World J. Microbiol. Biotechnol. 28, 255–265.

Barrajón-Simancas, N., Giese, E., Arévalo-Villena, M., Úbeda, J., Briones, A., 2011. Amino acid uptake by wild and commercial yeasts in single fermentations and co-fermentations. Food Chem. 127, 441–446.

Bravo-Ferrada, B.M., Hollmann, A., Delfederico, L., Valdés La Hens, D., Caballero, A., Semorile, L., 2013. Patagonian red wines: Selection of *Lactobacillus plantarum* isolates as potential starter cultures for malolactic fermentation. World J. Microbiol. Biotechnol. 29, 1537–1549.

Bringel, F., 1998. Carbamoylphosphate and natural auxotrophies in lactic acid bacteria. Lait 78, 31–37.

Costello, P.J., Lee, T.H., Henschke, P., 2001. Ability of lactic acid bacteria to produce N-heterocycles causing mousy off-flavour in wine. Aust. J. Grape Wine Res. 7, 160–167.

De Koker, S., 2015. Nitrogen utilisation of selected non-*Saccharomyces* yeasts and the impact on volatile compound production. MSc thesis, Inst. Wine Biotechnol. Stellenbosch Univ. South Africa MSc Thesis.

Divol, B., Tonon, T., Morichon, S., Gindreau, E., Lonvaud-Funel, A., 2003. Molecular characterization of *Oenococcus oeni* genes encoding proteins involved in arginine transport. J. Appl. Microbiol. 94, 738–746.

Du Toit, M., Engelbrecht, L., Lerm, E., Krieger-Weber, S., 2011. *Lactobacillus*: the next generation of malolactic fermentation starter cultures-an overview. Food Bioprocess Technol. 4, 876–906.

Du Toit, M., Pretorius, I.S., 2000. Microbial spoilage and preservation of wine: using weapons from nature's

own arsenal - a review. South African J. Enol. Vitic. 21, 74–96.

Fumi, M.D., Krieger-Weber, S., Déléris-Bou, M., Angela, S., du Toit, M., 2010. Una nueva generación de bacterias malolácticas para vinos con pH elevado. Enoviticultura 6, 34–38.

G-Alegría, E., López, I., Ruiz, J.I., Sáenz, J., Fernández, E., Zarazaga, M., Dizy, M., Torres, C., Ruiz-Larrea, F., 2004. High tolerance of wild *Lactobacillus plantarum* and *Oenococcus oeni* strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiol. Lett. 230, 53–61.

Garvie, E.I., 1967. The growth factor and amino acid requirements of species of the genus *Leuconostoc* , including *Leuconostoc paramesenteroides* (sp. nov.) and *Leuconostoc oenos* . J. Gen. Microbiol. 48, 439–447.

Gobert, A., Tourdot-Maréchal, R., Morge, C., Sparrow, C., Liu, Y., Quintanilla-Casas, B., Vichi, S., Alexandre, H., 2017. Non-*Saccharomyces* yeasts nitrogen source preferences: impact on sequential fermentation and wine volatile compounds profile. Front. Microbiol. 8, 1–13.

Godon, J., Delorme, C., Bardowski, J., Chopin, M., Ehrlich, S., Renault, P., 1993. Gene inactivation in *Lactococcus lactis*: Branched-chain amino acid biosynthesis. J. Bacteriol. 175, 4383–4390.

González-Arenzana, L., López, R., Santamaría, P., Tenorio, C., López-Alfaro, I., 2012. Dynamics of Indigenous Lactic Acid Bacteria Populations in Wine Fermentations from La Rioja (Spain) During Three Vintages. Microb. Ecol. 63, 12–19.

Guerzoni, M.E., Sinigaglia, M., Gardini, F., Ferruzzi, M., Torriani, S., 1995. Effects of pH, temperature, ethanol, and malate concentration on *Lactobacillus plantarum* and *Leuconostoc oenos* : Modelling of the malolactic activity. Am. J. Enol. Vitic. 46, 368–374.

Guzzon, R., Moser, S., Davide, S., Villegas, T.R., Malacarne, M., Larcher, R., Nardi, T., Vagnoli, P., Krieger-Weber, S., 2016. Exploitation of simultaneous alcoholic and malolactic fermentation of Incrocio Manzoni, a traditional Italian white wine. South African J. Enol. Vitic. 37, 124–131.

Iorizzo, M., Testa, B., Lombardi, S.J., García-Ruiz, A., Muñoz-González, C., Bartolomé, B., Moreno-Arribas, M.V., 2016. Selection and technological potential of *Lactobacillus plantarum* bacteria suitable for wine malolactic fermentation and grape aroma release. LWT - Food Sci. Technol. 73, 557–566.

Lasik-Kurdyś, M., Gumienna, M., Nowak, J., 2017. Influence of malolactic bacteria inoculation scenarios on the efficiency of the vinification process and the quality of grape wine from the Central European region. Eur. Food Res. Technol. 243, 2163–2173.

Lee, J.-E., Hwang, G.-S., Lee, C.-H., Hong, Y.-S., 2009. Metabolomics reveals alterations in both primary and secondary metabolites by wine bacteria. J. Agric. Food Chem. 57, 10772–10783.

Lerena, M.C., Rojo, M.C., Sari, S., Mercado, L.A., Krieger-Weber, S., Combina, M., 2016. Malolactic fermentation induced by *Lactobacillus plantarum* in Malbec wines from Argentina. South African J. Enol. Vitic. 37, 115–123.

Lerm, E., 2010. The selection and characterisation of lactic acid bacteria to be used as a mixed starter culture for malolactic fermentation. MSc thesis, Inst. Wine Biotechnol. Stellenbosch Univ. South Africa.

Lerm, E., Engelbrecht, L., du Toit, M., 2011. Selection and characterisation of *Oenococcus oeni* and *Lactobacillus plantarum* South African wine isolates for use as malolactic fermentation starter cultures. South African J. Enol. Vitic. 32, 280–295.

López, I., López, R., Santamaría, P., Torres, C., Ruiz-Larrea, F., 2008. Performance of malolactic fermentation by inoculation of selected *Lactobacillus plantarum* and *Oenococcus oeni* strains isolated from Rioja red wines. Vitis - J. Grapevine Res. 47, 123–129.

Ma, C., Cheng, G., Liu, Z., Gong, G., Chen, Z., 2016. Determination of the essential nutrients required for milk fermentation by *Lactobacillus plantarum* . LWT - Food Sci. Technol. 65, 884–889.

Maarman, B.C., 2014. Interaction between wine yeast and malolactic bacteria and the impact on wine aroma and flavour. MSc thesis, Inst. Wine Biotechnol. Stellenbosch Univ. South Africa.

Mckinnon, A., 2013. The impact of amino acids on growth performance and major volatile compound formation by industrial wine yeast. MSc thesis, Inst. Wine Biotechnol. Stellenbosch Univ. South Africa.

Moreno-Arribas, V., Torlois, S., Joyeux, A., Bertrand, A., Lonvaud-Funel, A., 2000. Isolation, properties and behavior of tyramine-producing lactic acid bacteria from wine. J. Appl. Microbiol. 88, 584–593.

Muñoz, V., Beccaria, B., Abreo, E., 2014. Simultaneous and successive inoculations of yeasts and lactic acid bacteria on the fermentation of an unsulfited Tannat grape must. Brazilian J. Microbiol. 45, 59–66.

Onetto, C.A., Bordeu, E., 2015. Pre-alcoholic fermentation acidification of red grape must using *Lactobacillus plantarum* . Antonie van Leeuwenhoek, Int. J. Gen. Mol. Microbiol. 108, 1469–1475.

Pozo-Bayon, M.A., Alegría, E.G., Polo, M.C., Tenorio, C., Martion-Álvarez, P.J., Calvo De La Banda, M., Ruiz-Larrea, F., Moreno-Arribas, M. V, 2005. Wine volatile and amino acid composition after malolactic fermentation: effect of *Oenococcus oeni* and *Lactobacillus plantarum* starter cultures. J. Agric. Food Chem. 53, 8729–8735.

Saguir, F.M., de Nadra, M.C.M., 2007. Improvement of a chemically defined medium for the sustained growth of *Lactobacillus plantarum*: nutritional requirements. Curr. Microbiol. 54, 414–418.

Schlatter, J., Lutz, W.K., 1990. The carcinogenic potential ethyl carbamate (urethane): risk assesment at human dietary exposure levels. Food Chem. Toxicol. 28, 205–211.

Spano, G., Chieppa, G., Beneduce, L., Massa, S., 2004. Expression analysis of putative arcA, arcB and arcC genes partially cloned from *Lactobacillus plantarum* isolated from wine. J. Appl. Microbiol. 96, 185–193.

Sun, S.Y., Gong, H.S., Liu, W.L., Jin, C.W., 2016. Application and validation of autochthonous *Lactobacillus plantarum* starter cultures for controlled malolactic fermentation and its influence on the aromatic profile of cherry wines. Food Microbiol. 55, 16–24.

Taniasuri, F., Lee, P.R., Liu, S.Q., 2016. Induction of simultaneous and sequential malolactic fermentation in durian wine. Int. J. Food Microbiol. 230, 1–9.

Terrade, N., Mira de Orduña, R., 2009. Determination of the essential nutrient requirements of wine-related bacteria from the genera *Oenococcus* and *Lactobacillus* . Int. J. Food Microbiol. 133, 8–13.

Teusink, B., van Enckevort, F.H.J., Francke, C., Wiersma, A., Wegkamp, A., Smid, E.J., Siezen, R.J., 2005. In Silico reconstruction of the metabolic pathways of *Lactobacillus plantarum* : comparing predictions of nutrient requirements with those from growth experiments. Appl. Environ. Microbiol. 71, 7253–7262.

Tristezza, M., di Feo, L., Tufariello, M., Grieco, F., Capozzi, V., Spano, G., Mita, G., Grieco, F., 2016. Simultaneous inoculation of yeasts and lactic acid bacteria: Effects on fermentation dynamics and chemical composition of Negroamaro wine. LWT - Food Sci. Technol. 66, 406–412.

Wegkamp, A., Teusink, B., de Vos, W.M., Smid, E.J., 2010. Development of a minimal growth medium for *Lactobacillus plantarum* . Lett. Appl. Microbiol. 50, 57–64. Zapparoli, G., Tosi, E., Azzolini, M., Vagnoli, P., Krieger, S., 2009. Bacterial inoculation strategies for the achievement of malolactic fermentation in high-alcohol wines. South African J. Enol. Vitic. 30, 49–55.

Zúñiga, M., Champomier-verges, M., Pérez-martínez, G., 1998. Structural and Functional Analysis of the Gene Cluster Encoding the Enzymes of the Arginine Deiminase Pathway of Lactobacillus sakei Structural and Functional Analysis of the Gene Cluster Encoding the Enzymes of the Arginine Deiminase Pathway of La. J. Appl. Microbiol. 180, 4154–4159.

Zúñiga, M., del Carmen Miralles, M., Pérez-martínez, G., 2002. The Product of arcR, the sixth gene of the arc Operon of *Lactobacillus sakei*,is essential for expression of the arginnine deiminase pathway. Appl.

Environ. Microbiol. 68, 6051–6058.

Zúñiga, M., Pardo, I., Ferrer, S., 1993. An improved medium for distinguishing between homofermentative and heterofermentative lactic acid bacteria. Int. J. Food Microbiol. 18, 37–42.