# Phenolic Acid Metabolism in Lactic Acid Bacteria and its Ecological Relevance

by

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# Abstract

Phenolic compounds are common and structurally diverse plant secondary metabolites. Epidemiological studies have associated their consumption to health benefits such as reduction of chronic inflammation and risk of colorectal cancer. Phenolic acids are a major class of dietary phenolic compounds possessing antioxidant, antimicrobial, and bioactive properties. The metabolism of phenolic compounds by food-fermenting lactic acid bacteria is a significant contributor to the safety, nutritional content, and quality of many plant-based fermented foods. *Lactobacillaceae* possess enzymes responsible for hydrolysis, decarboxylation, and reduction of phenolic acids; however, several genetic determinants responsible for these activities remain uncharacterized.

In this work, screening of the strain-specific phenolic acid metabolism of a diverse set of *Lactobacillaceae* guided a comparative genomic analysis, and enabled identification of 3 novel phenolic acid reductases and esterases. The function of *par1* and *hcrF* from *Furfurilactobacillus milii* and *Limosilactobacillus fermentum* in reduction of hydroxycinnamic acids was confirmed via construction of deletion mutants and biochemical characterization of heterologously expressed enzyme respectively. Hydroxycinnamic acid esterase *hceP* was identified in *Lactiplantibacillus plantarum* TMW1.460; it hydrolyses chlorogenic acid and methyl ferulate. Model food fermentations with isogenic mutants of *Lp. plantarum* lacking phenolic acid esterases provided evidence for differential regulation of enzymes based on substrate composition, highlighting the need for confirmation of enzymatic activity in food systems for usage of strains in targeted metabolite production.

To assess the role of genetic determinants in production of various phenolic acid metabolites during food fermentations, 5 isogenic mutants of *Ff. milii* FUA3583 lacking genes involved in hydroxycinnamic acid metabolism were created: Δ*est* (esterase), Δ*pad* (decarboxylase), ΔΔ*par1/par2* (reductases), ΔΔΔ*par1/par2/pad* and ΔΔΔΔ*par1/par2/pad/est*. Wild type and mutant strains were used to ferment different sorghum cultivars with differences in strain behaviour and metabolite production observed upon quantification of free phenolic acid metabolites using HPLC. Competition experiments performed between mutant and wild type strains in sorghum sourdoughs indicated the contribution of phenolic acid metabolism genes to ecological fitness in a phenolic acid rich environment. Bioinformatic analysis revealed insights into the association of lifestyle and ecology to the presence of phenolic acid metabolism genes in *Lactobacillaceae,* with nomadic strains possessing an extensive genetic toolkit compared to almost complete lack of phenolic metabolism genes in insect-adapted lifestyles.

This study also identified enzymes with unconfirmed activity homologous to characterized phenolic acid enzymes, along with documentation of metabolic activity of *Lactobacillaceae* on phenolic acids and patulin without known genetic determinants. These findings indicate the potential of *Lactobacillaceae* in metabolizing other plant and fungal secondary metabolites. Taken together, this research expands our knowledge on the behaviour and phenolic acid metabolism of *Lactobacillaceae* in food fermentations. The information on distribution of phenolic metabolism genes across *Lactobacillaceae* also allows for easier selection of strains to better control fermentation outcomes, while also documenting the potential of cereal fermentations for production of bioactive phenolics and functional foods by selectively metabolizing phenolic acids to different end products.

# Preface

This thesis is an original work by Gautam Gaur.

Chapter 2 of this thesis is a literature review being prepared as a manuscript for submission as Gautam Gaur and Michael G. Gänzle, "Conversion of phenolic compounds in food fermentations by lactic acid bacteria: Novel insights into metabolic pathways and functional metabolites" to Current Opinion in Food Science. I reviewd the literature and collected the information, performed bioinformatics analysis and prepared the manuscript. Dr. Gänzle was the supervisory author guiding me in data interpretation and manuscript editing.

Chapter 3 of this thesis has been published as Gautam Gaur, Jee-Hwan Oh, Pasquale Filannino, Marco Gobbetti, Jan-Peter van Pijkeren and Michael G. Gänzle (2020), "Genetic Determinants of Hydroxycinnamic Acid Metabolism in Heterofermentative Lactobacilli" Applied and Environmental Microbiology. 86(5): e02461-19. I conducted all the experiments, analyzed the data, and wrote the manuscript. Dr. Jee-Hwan Oh and Dr. Jan-Peter van Pijkeren provided the methodology and guided me in the construction of *par1* and *par2* deletion mutants and provided feedback on the manuscript. Dr. Pasquale Filannino and Dr. Marco Gobbetti provided feedback on the manuscript. Dr. Gänzle was the supervisory author guiding the whole study and revised the manuscript.

Chapter 4 of this thesis has been prepared as a manuscript ready for submission as Gautam Gaur, Hiu Kwan Lo and Michael G. Gänzle, "Contribution of phenolic acid metabolism of *Furfurilactobacillus milii* to ecological fitness in cereal fermentations" to Food Microbiology. Hui Kwan Lo prepared brown sorghum sourdough for 5 treatments in duplicates under my mentorship.

I conducted all the remaining experiments, analyzed the data, and wrote the manuscript. Dr. Gänzle was the supervisory author guiding the whole study and revised the manuscript.

Chapter 5 of this thesis is titled "Biochemical characterization of phenolic acid reductase (HcrF) from *Limosilactobacillus fermentum"*. A version of this chapter is under preparation as a manuscript for submission to a peer-reviewed journal. I conducted all the experiments and analyzed the data with Dr. Gänzle guiding the study.

Chapter 6 of this thesis has been prepared as a manuscript ready for submission as Gautam Gaur, Chen Chen and Michael G. Gänzle, "Characterization of phenolic acid esterases in *Lactiplantibacillus plantarum* TMW1.460" to Applied and Environmental Microbiology. Dr. Chen Chen contributed to the study design, constructed all the deletion mutant strains and prepared samples for characterization of strain phenotypes, and performed the food fermentations. I contributed to the study design and experimental troubleshooting, prepared samples for HPLC analysis, performed the bioinformatics analysis, analyzed the experimental data and wrote the manuscript. Dr. Gänzle was the supervisory author guiding the whole study and revised the manuscript. Dr. Chen Chen and I contributed equally to the prepared manuscript.

Chapter 7 of this thesis has been prepared as a manuscript ready for submission as Gautam Gaur and Michael G. Gänzle, "Role of thiols and ascladiol production in patulin degradation by *Lactobacillaceae*" to Letters in Applied Microbiology. I conducted all the remaining experiments, analyzed the data, and wrote the manuscript. Dr. Gänzle was the supervisory author guiding the whole study and revised the manuscript.

*This thesis is dedicated to my parents Lata Gaur and Chandresh Gaur. Thank you for always being there for me. I owe my success to you.*

# Acknowledgements

I would like to express my deepest gratitude to Dr. Michael G. Gänzle for accepting me as your student to pursue my graduate studies. Thank you for your patience and trust in me even while things weren't going well, and for all the invaluable advice and guidance. Your quote 'Nothing is rocket science' always resonated and pushed me to learn and understand things which I never thought I would have been able to. It was truly a privilege to learn from a person of your intelligence, caliber, and work ethic who is truly devoted to his students and science.

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My special thanks to Dr. Yalu Yan and Kaixing Tang for helping me getting started in the lab and motivating me and reinforcing my confidence. I also thank Dr. Qing Li, Dr. Yuan Fang and Dr. David J. Simpson for their time and commitment for always being available to help and give

advice, no matter how busy they were themselves. Special thanks to Dr. Chen Chen for his significant contribution to this research and for sharing his advice and experience. Thanks to Nanzhen Qiao for providing scripts and advice for bioinformatic analysis. I would also like to thank all the other amazing colleagues and friends, I met in the Food Microbiology Lab 2-50 and the AFNS department, especially Luis E. Rojas Tovar, Dr. Zhiying Wang, Shaelyn Xu, Zheng Zhao, Vi Pham, Dr. Felicitas Pswarayi, Dr. Nuanyi Liang, Dr. Justina Zhang, Dr. Ying Xu, Jin Xie, Oanh Nguyen, Chandre Van De Merwe, Savanna Won, Yuqi Shao, Tongbo Xu, Dr. Xin Zhao, Dr. Yuanyao Chen, Dr. Hongbing Fan, Brasathe Jeganathan and Dr. Barun Yadav, for their help and support throughout my graduate studies. Special thanks to Heather Vandertol-Vanier for her guidance, support and sharing her expertise and experiences which taught me a lot.

My sincerest acknowledgment goes to my friends- Dr. Anjal Shah, Shreyance Jain, Shubham Gupta, Mayank Modi, Vidha Nigam, Abhishek Singh, Surabhi Ingle, Ann Maria Vincent, Shubham Sharma and Piyush Agrawal. While our conversations may have been brief over the last few years, I would not have been here without their encouragement and support throughout my life. Last but not the least, I would like to thank my parents and girlfriend Dr. Amritha J. Prasad for their unconditional support, belief, patience, and constructive criticism for my betterment.

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# List of Abbreviations

| | |
|---|---|
| ANOVA | Analysis of Variance |
| ATP | Adenosine triphosphate |
| BLAST | Basic Local Alignment Search Tool |
| CFU | Colony Forming Unit |
| Cm | Chloramphenicol |
| Cy | Cysteine |
| ddPCR | Droplet Digital Polymerase Chain Reaction |
| DNA | Deoxyribonucleic acid |
| EGCG | Epigallocatechin gallate |
| Em | Erythromycin |
| FAD | Flavin adenine dinucleotide |
| FMN | Flavin mononucleotide |
| GSH-PAT | Glutathione-Patulin |
| HCL | Hydrochloric acid |
| HPLC | High Performance Liquid Chromatography |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| LB | Luria–Bertani |

| | |
|---|---|
| LC-MS | Liquid Chromatography-Mass Spectrometry |
| LCR | Ligase Cycling Reaction |
| mMRS | Modified de Man, Rogosa & Sharpe |
| mRNA | Messenger Ribonucleic acid |
| NADH | Nicotinamide adenine dinucleotide |
| NCBI | National Center for Biotechnology Information |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| RT-qPCR | Quantitative Reverse Transcription Polymerase Chain Reaction |
| SCO/DCO | Single Crossover/ Double Crossover |
| SDS-PAGE | Sodium dodecyl sulfate–Polyacrylamide Gel Electrophoresis |
| UV | Ultraviolet |
| WT | Wild Type |

# Chapter 1- General introduction and thesis objectives

## 1.1. Introduction

Phenolic compounds are secondary metabolites that are widely distributed across the plant kingdom. Their production by plants is thought to be the result of environmental adaptation by amphibious plants on land in response to solar UV radiation (Cheynier et al., 2013). They display a large structural diversity and varied abundance across various edible plants, likely reflective of the functional requirements of their producers in response to various biotic and abiotic stresses (Quideau et al., 2011). Phenolic compounds impart bitter taste and/or astringency in many foods, and possess antinutritive properties because they inhibit digestive enzymes and reduce the utilization of vitamins and minerals (Chung et al., 1998). However, over the last 2 decades, various epidemiological and intervention studies have indicated the beneficial role of a diet rich in phenolic compounds in human health. Health benefits are linked to reduced chronic inflammation, and a reduced risk of colon cancer, type 2 diabetes, neurodegenerative diseases, and cardiovascular diseases (Bonaccio et al., 2017; Cardona et al., 2013; De Filippis et al., 2016; Kim et al., 2016; Klinder et al., 2016; Shahidi and Yeo, 2018; Vitaglione et al., 2015). In many cases, however, it is unclear whether these health benefits are solely related to the presence of phenolic compounds or on as combination of phenolics with other plant bioactives including non-starch polysaccharides.

Phenolic acids, flavonoids and polymeric phenols including tannins are the major classes of dietary phenolics abundant in edible parts of plants (González-Sarrías et al., 2020). Phenolic acids are further classified as hydroxybenzoic acids ($C_6$-$C_1$) and hydroxycinnamic acids ($C_6$-$C_3$). Phenolic acids possess antimicrobial activity (Sánchez-Maldonado et al., 2011), while their aromatic metabolites are approved food additives and flavouring compounds (JECFA, 2001; Muñoz et al.,

2017). In addition, free hydroxycinnamic acids including caffeic acid and ferulic acid, and their metabolites display anti-inflammatory and anticancer properties in *in-vitro* and animal model studies (Chao et al., 2010; Janicke et al., 2011; Leonard et al., 2021b).

Most of the total phenolic content in raw foods comprises of bound phenolics, limiting their bioavailability (Acosta-Estrada et al., 2014; Zhang et al., 2020). Phenolic acids mostly exist as esters or glycosides bound to different cell wall polysaccharides in various cereals, fruits, and vegetables (Shahidi and Yeo, 2018; Zhang et al., 2020). Fermentation of plant material can increase the total free phenolic content by activity of microbial esterases and glycosidases along with enhancing their extractability (Bhanja Dey et al., 2016; Gänzle, 2019).

Fermented foods have been an integral part of human food culture with evidence dating cereal fermentations for production of bread and alcoholic beverages to more than 14,000 years ago (Arranz-Otaegui et al., 2018; Hayden et al., 2013). Fermentation increases the shelf life and alters the sensory profile of foods. In addition, it can also contribute to reduction of non-nutritive and toxic compounds while increasing the palatability via microbial and enzymatic conversions (Filannino et al., 2018; Gänzle, 2019, 2020). *Lactobacillaceae* are among major fermenting organisms frequently isolated from spontaneous and traditional fermentations with safe use of hundreds of strains documented in fermented foods (Bourdichon et al., 2022- IDF N°514/2022). *Lactiplantibacillus plantarum* and *Levilactobacillus brevis* generally dominate towards the end of spontaneous vegetable fermentations. Dominant microbiota in cereal fermentations include strains of *Limosilactobacillus, Lactobacillus, Companilactobacillus* and *Pediococcus* in addition to *Lactiplantibacillus* and *Levilactobacillus* (Gänzle and Zheng, 2019; Van Kerrebroeck et al., 2017). *Fructilactobacillus sanfranciscensis* is the most dominant species in type I wheat and rye sourdough (Gänzle and Zheng, 2019), however, it was competently outcompeted in model

sorghum fermentations by other ting isolates (traditional fermented sorghum product) with the high antimicrobial activity of sorghum phenolic compounds likely responsible (Sekwati-Monang et al., 2012). Sorghum is rich in phenolic acids and their glycerol esters, 3-deoxyanthocyanidins, condensed tannins and flavonoids (Svensson et al., 2010) and has a higher phenolic content than other cereals such as wheat and rye (Awika and Rooney, 2004; Dykes and Rooney, 2006). This indicates that fermented foods rich in phenolic compounds may select for strains capable of metabolizing phenolic compounds with metabolites of phenolic acids having lower antimicrobial activity (Sánchez-Maldonado et al., 2011).

Release of free phenolic acids via esterase, glycosidase and tannase enzymes is the first step for metabolism of conjugated/bound phenolic acids. Hydroxycinnamic acids can undergo decarboxylation and/or reduction while hydroxybenzoic acids can only be decarboxylated by *Lactobacillaceae* (Sánchez-Maldonado et al., 2011). The decarboxylated vinyl-derivatives of hydroxycinnamic acids can be further reduced to ethyl-derivatives (Sánchez-Maldonado et al., 2011). Until the last decade, knowledge of the genetic determinants involved in metabolism of phenolic acids was limited. Phenolic acid decarboxylase from *Lp. plantarum* was the first enzyme characterized for its activity on hydroxycinnamic acids (Cavin et al., 1997; Rodríguez et al., 2008). Most enzymes related to phenolic acids have been characterized in homofermentative *Lp. plantarum* namely, intracellular tannase (Iwamoto et al., 2008; Rodríguez et al., 2008) and extracellular tannase (Jiménez et al., 2014) that are active on hydroxybenzoic acid esters, gallate decarboxylase (Jiménez et al., 2013), phenolic acid esterases that are active on hydroxycinnamic acids esters (Esteban-Torres et al., 2015, 2013), phenolic acid reductase (Santamaría et al., 2018a) and vinyl phenol reductase that are active (Santamaría et al., 2018b) on hydroxycinnamic acids and their vinyl-derivatives. Among other *Lactobacillaceae,* two cinnomyl esterases were identified

in *Lactobacillus johnsonii* (Lai et al., 2009) while the homolog of *Lp. plantarum* phenolic acid decarboxylase was also characterized in *Lv. brevis* (Landete et al., 2010). Information on phenolic acid glycosidases remains limited.

Homofermentative and heterofermentative *Lactobacillaceae* differ phylogenetically and physiologically (Zheng et al., 2015). Heterofermentative strains of *Weissella cibaria* (Filannino et al., 2014), *Lm. fermentum* (Filannino et al., 2015; Sánchez-Maldonado et al., 2011) and *Furfurilactobacillus rossiae* (Filannino et al., 2014; Ripari et al., 2019) displayed hydroxycinnamic acid reductase activity despite lacking the phenolic acid reductase that was identified in *Lp. plantarum.* Thus, indicates the presence of novel uncharacterized genes and enzymes related to phenolic acid metabolism in other *Lactobacillaceae.*

Metabolism of phenolic acids in lactic acid bacteria is considered strain specific (Filannino et al., 2015; Ripari et al., 2019) while presence of multiple enzymes with overlapping substrate specificities in a single strain makes accurate prediction of metabolites difficult. Metabolite production in laboratory media does not always match strain behaviour in food fermentations (Filannino et al., 2015), with the regulation of phenolic acid genes and enzymatic activity likely affected by the substrate composition of a food matrix (Pswarayi et al., 2022; Ripari et al., 2019). Therefore, in addition to the identification of genes involved in phenolic acid metabolism, confirmation of their activity and expression in food substrates is necessary to optimize the fermentation technology for targeted production of various bioactive metabolites.

### 1.2. Hypothesis

1) Heterofermentative *Lactobacillaceae* possess novel genes for metabolizing phenolic acids.

2) Hydroxycinnamic and hydroxybenzoic acids are metabolized by distinct metabolic pathways.
3) Phenolic acid metabolism genes provide *Lactobacillaceae* with an ecological advantage in a phenolic rich environment.
4) Strain specific phenolic acid metabolism of *Lactobacillaceae* allows for production of targeted metabolites in plant fermentations.

### 1.3. Objectives

1) To investigate the presence of phenolic acid metabolism genes in *Lactobacillaceae* and to assess possible links to phylogeny and lifestyles (Chapter 2 and 3).
2) To identify hydroxycinnamic acid reductases in heterofermentative *Lactobacillaceae* (Chapter 3).
3) To characterize phenolic acid metabolism of *Ff. milii* in sorghum sourdough and its contribution to ecological fitness (Chapter 4).
4) To characterize phenolic acid reductase (HcrF) of *Lm. fermentum* (Chapter 5).
5) To characterize phenolic acid esterases in *Lp. plantarum* and their *in-situ* activity in food fermentations (Chapter 6).
6) To assess the contribution of uncharacterized reductase Par2, thiols and ascladiol production in patulin detoxification (Chapter 7).

# Chapter 2- Conversion of phenolic compounds in food fermentations by lactic acid bacteria: Novel insights into metabolic pathways and functional metabolites

## 2.1. Introduction

Phenolic compounds include a variety of secondary plant metabolites diverse in their chemical structure and function. They can be classified based on their carbon backbone structure. Major phenolic compounds abundant in edible plants include flavonoids ($C_6$-$C_3$-$C_6$), phenolic acids ($C_6$-$C_3$ or $C_6$-$C_1$), and polymeric phenols including tannins (Tsimogiannis and Oreopoulou, 2019). Plants have evolved to accumulate diverse phenolic compounds in response to environmental stresses aiding in adaptation via both structural and non-structural functions (Lattanzio et al., 2012). They possess antinutritive properties, ability to precipitate proteins, inhibit digestive enzymes and are responsible for imparting astringency and/or bitter taste in many foods (Chung et al., 1998). Various epidemiological and intervention studies point towards their beneficial role in human health (Bordenave et al., 2014; Kim et al., 2016; Shahidi and Yeo, 2018). Bound phenolics make up the majority of total phenolic composition in raw foods limiting their bioavailability (Zhang et al., 2020).

Fermentation has been an integral part of human food culture for thousands of years with production of alcoholic beverages and bread possibly predating neolithic period (Arranz-Otaegui et al., 2018; Hayden et al., 2013). Fermentation can increase the total free phenolic content in foods while also altering their technological and sensory properties by production of various metabolites via microbial conversions.

*Lactobacillaceae* have a long history of association and safe usage in fermented foods (Bourdichon et al., 2022- IDF N°514/2022) and are major fermenting organisms frequently found in traditional fermentations of phenolic rich foods such as cereals and vegetables. The past decade has seen significant progress in determining genetic determinants of phenolic acid metabolism and biochemical characterization of enzymes active on phenolic compounds. Recent comprehensive reviews have summarized the benefits of fermentation for modification of phenolic composition and focusing on their influence on gut microbiota and health (Leonard et al., 2021b, 2021a; Loo et al., 2020). This review explores the metabolic pathways utilized by *Lactobacillaceae* to alter major phenolic compounds present in vegetables, fruits, and cereals during fermentation. This study identified the distribution of genetic determinants for metabolism of phenolic compounds to identify associations between ecological niche, taxonomy and metabolism of *Lactobacillaceae*.

## 2.2. Overview of fermentable phenolic compounds present in foods

### 2.2.1. Phenolic acids

Phenolic acids (Table 2.1) account for almost one-third of the total dietary phenolics (Haminiuk et al., 2012) in fruits and grains. They comprise hydroxybenzoic acids ($C_6$–$C_1$) and the more abundant hydroxycinnamic acids ($C_6$–$C_3$). Concentrations of free phenolic acids in raw foods varies based on the matrix composition but is generally very low in comparison to conjugated and bound phenolic acids. They are frequently found linked to arabinoxylans and other cell wall polysaccharides via ester and ether linkages (Vitaglione et al., 2008). The hydroxyl and carboxylic groups can also form covalent linkages with other molecules such as monosaccharides and alcohols with phenolic acid esters showing a high diversity (Acosta-Estrada et al., 2014; Shahidi and Yeo, 2018).

**Table 2.1. Overview of common phenolic compounds metabolized by *Lactobacillaceae*.**

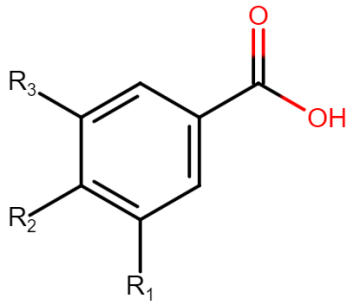
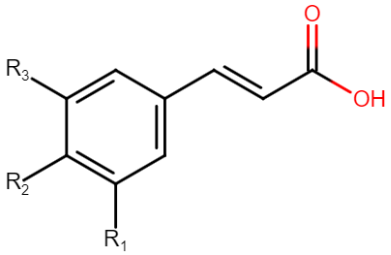
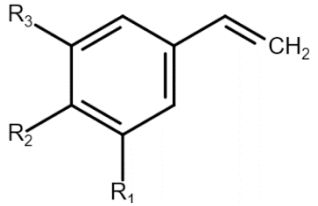
| Class of phenolic compounds | Basic chemical structure | Active microbial enzymes |
|---|---|---|
| Hydroxybenzoic acids ($C_1$-$C_6$)<br>R= H, OH, $OCH_3$ |  | -Esterase (Tannase)<br>-Decarboxylase |
| Hydroxycinnamic acids<br>($C_6$–$C_3$)<br>R= H, OH, $OCH_3$ |  | -Esterase<br>-Decarboxylase<br>-Reductase |
| Vinyl-derivatives of hydroxyl-cinnamic acids |  | -Reductase |

**Table 2.1.** (continued)

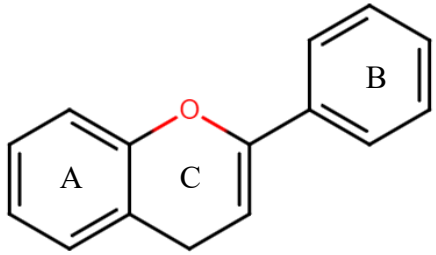
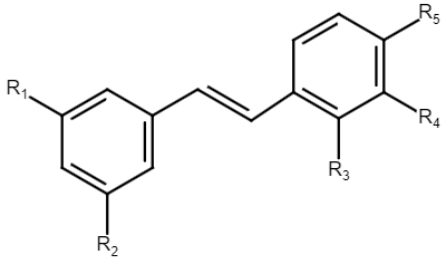
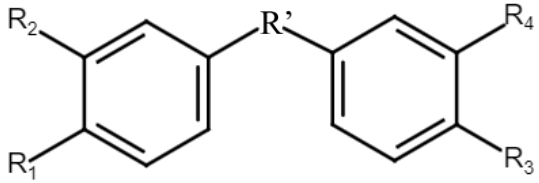
| Class of phenolic compounds | Basic chemical structure | Active microbial enzymes |
|---|---|---|
| Flavonoids ($C_6$-$C_3$-$C_6$)<br>C ring substitutions- Different flavonoid classes<br>A and B ring substitutions- Different compounds |  | -Glycosyl hydrolase |
| Stilbenes ($C_6$–$C_2$–$C_6$)<br>R= H, OH, $OCH_3$ |  | -Glycosyl hydrolase |
| Lignans ($C_6$–$C_3$)$_2$<br>R= OH, $OCH_3$ |  | -Glycosyl hydrolase |

**Table 2.1.** (continued)

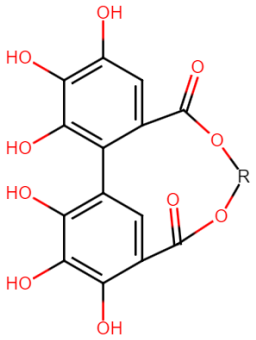
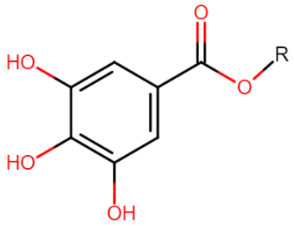
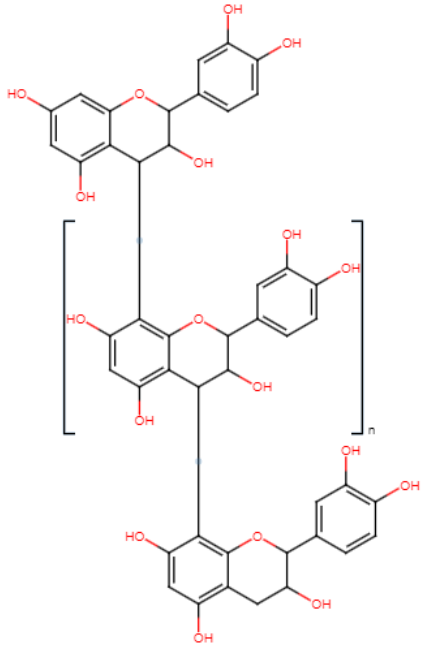
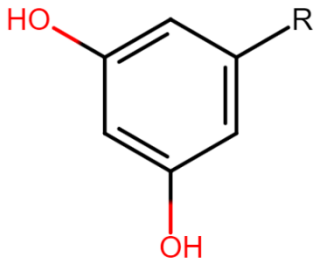
| Class of phenolic compounds | Basic chemical structure | Active microbial enzymes |
|---|---|---|
| Hydrolysable tannins<br>A-Gallotannins<br>B- Ellagitannins | A  B  | -Tannase |
| Proanthocyanidins<br>$(C_6\text{-}C_3\text{-}C_6)n$ |  | -Esterase/Tannase |

**Table 2.1.** (continued)

| Class of phenolic compounds | Basic chemical structure | Active microbial enzymes |
|---|---|---|
| Alkylresorcinols<br>R= Aliphatic carbon chain |  | -Conversion not reported |

Major dietary phenolic acids include ferulic acid, *p*-coumaric acid, caffeic acid, sinapic acid, gallic acid, vanillic acid and syringic acid found in fruits, vegetables, and cereals (Nayak et al 2015; Martinez et al 2017). Sinapic acid derivatives and hydroxybenzoic acids have lower abundance in fruits and vegetables compared to other hydroxycinnamic acids and their esterified derivatives (Rashmi and Negi, 2020; Septembre-Malaterre et al., 2018). Chlorogenic acid is the most abundant caffeic acid ester in edible plants with potatoes, coffee, eggplant, kiwi, carrot, leafy greens, pear, and blueberry being among the common sources. (Mattila and Hellström, 2007; Santana-Gálvez et al., 2017; Santos et al., 2014). Sinapic acid derivatives are particularly abundant in plants of the *Brassicaceae* family (Martínez-Sánchez et al., 2008). Cereals such as rye and barley are rich in hydroxycinnamic acids with ferulic acid esterified to arabinoxylans making up the majority of phenolic acid content in wheat (Rosa-Sibakov et al., 2015). Sorghum and oats also contain glycerol esters of ferulic, *p*-coumaric and caffeic acid. (Svensson et al., 2010; Varga et al., 2018). The abundance of phenolic acid glycosides is low compared to other polyphenol glycosides but are frequently found in sinapic acid rich flaxseed, canola, and mustard (Engels et al., 2012; Khattab et al., 2010; Materska et al., 2003).

### 2.2.2. Flavonoids

Flavonoids is the most diverse class of phenolic compounds that encompasses more than 6000 known compounds (Vuolo et al., 2019). Alterations in the heterocyclic C ring of their basic $C_6$-$C_3$-$C_6$ carbon framework is responsible for the flavonoid subclasses such as flavonols, flavanones and anthocyanidins. Flavonoids are found mainly as glycosides. Among flavones, apigenin and luteolin glycosides are the common in a variety of herbs and spices, peppers, watermelon, and Chinese cabbage (de la Rosa et al., 2019). The three major flavanones are hesperetin, naringenin and eriodictyol; these are most abundant in citrus fruits and juices such as orange, lemon, and

grapefruit. Citrus fruits are also rich in polymethoxylated flavones such tangeretin and nobiletin (Ho and Kuo, 2014). Cereals such as sorghum also contain flavanones as 7-O-glucosides along with apigenin and 3-deoxyanthocyanidins (Bai et al., 2014; Svensson et al., 2010). Isoflavones differ from flavones as the B ring is attached to C3 and they are also known as phytoestrogens due to structural resemblance with estrogen. Legumes are typically rich in isoflavones with soybeans, being its most abundant source having high genistein and daidzein content (Liggins et al., 2000). Flavonols is one of the most widespread classes of flavonoids with kaempferol, myricetin and quercetin along with their glycosidic derivatives found in a variety of fruits and vegetables (Aherne and O'Brien, 2002; Barreca et al., 2021). Flavan-3-ols are abundant in tea, wine, cereals, chocolates apart from various fruits and vegetables (de la Rosa et al., 2019). Monomeric flavan-3-ols including catechins, gallocatechin, their isomers and gallic acid esters along with oligomeric flavan-3-ols (proanthocyanidins) rarely exist as glycosides. The last major flavonoid group consists of anthocyanidins whose glycosylated derivatives (anthocyanins) are particularly rich in coloured fruits including grapes, berries, cherry, and vegetables such as red cabbage, rhubarb and red onions (Manach et al., 2004).

### 2.2.3. Tannins

Tannins are polymeric phenolic compounds with the ability to form strong complexes with carbohydrates and proteins (Serrano et al., 2009). Based on the monomeric units, they can be further classified as water insoluble condensed tannins (proanthocyanidins), hydrolysable tannins and complex tannins. Gallotannins (esterified gallic acid) and ellagitannins are the most common hydrolysable tannins found in fruits such as berries, mangoes and grapes with a few legumes, vegetables and nuts being minor sources (Serrano et al., 2009). Proanthocyanidins tend to accumulate in peels of fruits with grapes the biggest source of condensed tannins in our diet

(Haminiuk et al., 2012). Legumes, nuts, and other certain cereals such as sorghum and barley can also be sources condensed tannins (Gu et al., 2004; Saura-Calixto et al., 2007).

### 2.2.4. Stilbenes, lignans and alkylresorcinols

Resveratrol is the only stilbene compound of interest in human diet with grape skins and subsequently wine being its primary source. Lignans are composed of two phenylpropanoid (C6–C3) units and are widespread in edible plants with low abundance in fruits and vegetables (de la Rosa et al., 2019). Oilseeds, nuts, whole grain cereals and legumes are typically rich sources of lignans (Rodríguez-García et al., 2019). Flaxseed and carob bean are the most abundant sources of common dietary lignans including secoisolariciresinol, lariciresinol, pinoresinol and syringaresinol (Moreno-Franco et al., 2011). Alkylresorcinols are phenolic compounds generally containing 15-25 carbon chain attached to a hydroxybenzene ring. Whole grain cereals such as rye followed by wheat are particularly rich dietary sources of alkylresorcinols (Mattila et al., 2005).

## 2.3. Enzymes involved in conversion of phenolic compounds by lactic acid bacteria

*Lactobacillaceae* possess various enzymatic activities for biotransformation of bioactive dietary phenolic compounds (Muñoz et al., 2017). The choice of microbiota and understanding of its metabolic pathways is critical to control the outcomes of a fermentation process. The recent taxonomic reclassification of the family *Lactobacillaceae* (J. Zheng et al., 2020) along with the identification of lifestyles of many lactobacilli (Duar et al., 2017) enables an opportunity to explore the phenolic biotransformation potential of different genera. All the type strains in the *Lactobacillaceae* family (February 2022) were screened for characterized genetic determinants (Table 2.2) responsible for metabolism of various phenolic compounds and evaluated for the association of lifestyles and phylogeny to specific enzymatic activities (Figure 2.1).

**Table 2.2. Summary of characterized phenolic metabolism enzymes in *Lactobacillaceae***

| Enzyme | Query sequence ID | Known phenolic substrates | Source | Reference |
|---|---|---|---|---|
| | | **Phenolic acid estearses** | | |
| Lp_0796 | YP_004888771.1 | Hydroxycinnamic acid esters- Methyl ferulate, methyl caffeate, methyl p-coumarate, methyl sinapinante | *Lactiplantibacillus plantarum* WCFS1 | (Esteban-Torres et al., 2013) |
| Est_1092 | WP_015825406.1 | Hydroxycinnamic acid esters- Methyl ferulate, methyl caffeate, methyl p-coumarate, methyl sinapinante<br>Hydroxybenzoic acid esters- Methyl gallate, methyl vanillate, ethyl gallate, ethyl protcatechuate, epigallocatechin gallate | *Lp. plantarum* DSM 1055 | (Esteban-Torres et al., 2015) |
| TanA | WP_003640628.1 | Hydroxybenzoic acid esters- Methyl gallate, ethyl gallate, propyl gallate, ethyl protcatechuate, gallocatechin gallate, epigallocatechin gallate, tannic acid | *Lp. plantarum* ATCC 14917 | (Jiménez et al., 2014) |
| TanB | YP_004890536.1 | Hydroxybenzoic acid esters- Methyl gallate, ethyl gallate, propyl gallate, lauryl gallate, ethyl protcatechuate, gallocatechin gallate epigallocatechin gallate, tannic acid | *Lp. plantarum* WCFS1 | (Curiel et al., 2009; Iwamoto et al., 2008) |
| Lj0536 | WP_004898050.1 | Chlorogenic acid, ethyl ferulate, rosmarinic acid | *Lactobacillus johnsonii* N6.2 | (Lai et al., 2009) |
| Lj1228 | WP_011162057.1 | Chlorogenic acid, ethyl ferulate, rosmarinic acid | | |
| HceP | WP_011101978.1 | Chlorogenic acid, methyl ferulate | *Lp. plantarum* TMW1.460 | Chapter 6 |

**Table 2.2.** (continued)

| Enzyme | Query sequence ID | Known phenolic substrates | Source | Reference |
|---|---|---|---|---|
| | | **Hydroxycinnamic acid reductases** | | |
| HcrB | YP_004889276.1 | *m-, o-* and *p*-Coumaric acid, sinapic acid, ferulic acid, caffeic acid | *Lp. plantarum* WCFS1 | (Santamaría et al., 2018a) |
| Par1 | WP_161000921.1 | *p*-Coumaric acid, sinapic acid, ferulic acid, caffeic acid | *Furfurilactobacillus milii* FUA3583 | Chapter 3 |
| Par2 | WP_161002483.1 | - | | |
| HcrF | WP_003682980.1 | *p*-Coumaric acid, sinapic acid, ferulic acid, caffeic acid | *Limosilactobacillus fermentum* FUA3589 | Chapter 5 |
| | | **Vinyl phenol reductase** | | |
| VrpA | YP_004890680.1 | Vinyl catechol, vinyl phenol, vinyl guaiacol | *Lp. plantarum* WCFS1 | (Santamaría et al., 2018b) |
| | | **Phenolic acid decarboxylases** | | |
| Pad | YP_004891133.1 | *p*-Coumaric acid, ferulic acid, caffeic acid | *Lp. plantarum* ATCC 14917, *Levilactobacillus brevis* RM84 | (Landete et al., 2010; Rodríguez et al., 2008) |
| PadG | WP_003644796.1 | Gallic acid, protocatechuic acid | *Lp. plantarum* WCFS1 | (Jiménez et al., 2013) |

**Table 2.2.** (continued)

| Enzyme | Query sequence ID | Known phenolic substrates | Source | Reference |
|---|---|---|---|---|
| | | **Flavonoid glycosidases** | | |
| Ram1 | WP_011102176.1 | Rutin, Nicotiflorin, Narirutin, Hesperidin, *p*NP-α-l-rhamnopyranoside | *Lp. plantarum* WCFS1, DSM 20205, *Pediococcus acidilactici* DSM 20284 | (Ávila et al., 2009b; Beekwilder et al., 2009; Michlmayr et al., 2011) |
| Ram2 | WP_011102178.1 | | | |
| $RamA_{La}$ | WP_003548204.1 | Naringin, Rutin, Nicotiflorin, Narirutin | *Lactobacillus acidophilus* NCFM | (Beekwilder et al., 2009) |
| rBGLa | WP_007123550.1 | Geniposide, *p*NP-β-D-glucopyranoside, Daidzin, Genistin, Secoisolariciresinol diglucoside | *Limosilactobacillus antri* DSM 16041, *Limosilactobacillus mucosae* INIA P508 | (Gaya et al., 2020; Kim et al., 2017) |
| LcGUS30 | BAO73305.1 | Baicalin, Wogonoside, *p*NP-β-D-glucuronide | *Lv. brevis* FERM BP-4693 | (Sakurama et al., 2014) |

| Genus name | Hydroxycinnamic acid | | | | | | | | | Hydroxybenzoic acid | | | Glycosyl hydrolasaes | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Lp_0796 | Est_1092 | HceP | Par1 | Par2 | HcrF | HcrB | Pad | VprA | TanA | TanB | PadG | Ram1 | Ram2 | RamALa | rBGLa | LcGUS30 |
| *Lactobacillus* | 0 | 78 | 68 | 37 | 44 | 78 | 0 | 12 | 24 | 2 | 0 | 7 | 0 | 2 | 22 | 7 | 0 |
| *Amylolactobacillus* | 100 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Holzapfelia* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Bombilactobacillus* | 100 | 0 | 0 | 0 | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 50 | 0 | 100 | 0 |
| *Companilactobacillus* | 100 | 40 | 60 | 88* | | 0 | 26 | 74 | 2 | 0 | 25 | 71 | 0 | 11 | 26 | 100 | 0 |
| *Lapidilactobacillus* | 42 | 42 | 28 | 0 | 0 | 0 | 29 | 14 | 0 | 0 | 0 | 28 | 0 | 0 | 0 | 0 | 0 |
| *Agrilactobacillus* | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 50 | 0 | 50 | 50 | 100 | 50 |
| *Schleiferilactobacillus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 |
| *Lacticaseibacillus* | 48 | 4 | 0 | 20 | 24 | 4 | 0 | 36 | 12 | 0 | 0 | 52 | 0 | 4 | 0 | 56 | 12 |
| *Paralactobacillus* | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 |
| *Latilactobacillus* | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 80 | 0 | 0 | 0 | 20 | 20 | 20 | 0 | 0 | 0 |
| *Loigolactobacillus* | 0 | 11 | 11 | 78* | | 0 | 0 | 66 | 44 | 0 | 0 | 66 | 11 | 0 | 22 | 11 | 0 |
| *Dellaglioa* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Ligilactobacillus* | 0 | 20 | 27 | 20 | 20 | 13 | 27 | 27 | 13 | 0 | 7 | 7 | 0 | 0 | 0 | 7 | 0 |
| *Liquorilactobacillus* | 0 | 42 | 8 | 25 | 17 | 0 | 33 | 0 | 0 | 0 | 0 | 17 | 0 | 33 | 17 | 17 | 0 |
| *Pediococcus* | 0 | 36 | 36 | 0 | 0 | 0 | 0 | 73 | 0 | 0 | 0 | 27 | 45 | 55 | 0 | 0 | 0 |
| *Lactiplantibacillus* | 100 | 29 | 52 | 59 | 82 | 6 | 65 | 100 | 58 | 23 | 64 | 94 | 29 | 47 | 35 | 6 | 0 |
| *Fructilactobacillus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Acetilactobacillus* | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Apilactobacillus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Lentilactobacillus* | 100 | 0 | 0 | 29 | 12 | 0 | 6 | 0 | 0 | 5 | 0 | 11 | 29 | 29 | 0 | 65 | 29 |
| *Secundilactobacillus* | 100 | 16 | 16 | 33 | 33 | 67 | 25 | 83 | 58 | 16 | 0 | 58 | 16 | 25 | 0 | 42 | 17 |
| *Levilactobacillus* | 95 | 0 | 70 | 0 | 0 | 0 | 4 | 83 | 12 | 12 | 62 | 50 | 0 | 16 | 4 | 96 | 25 |
| *Paucilactobacillus* | 100 | 0 | 14 | 0 | 0 | 0 | 14 | 71 | 14 | 0 | 0 | 85 | 14 | 14 | 14 | 43 | 43 |
| *Limosilactobacillus* | 68 | 7 | 89 | 0 | 7 | 11 | 0 | 36 | 21 | 0 | 4 | 4 | 4 | 4 | 0 | 39 | 18 |
| *Furfurilactobacillus* | 67 | 0 | 0 | 67 | 67 | 0 | 0 | 33 | 33 | 0 | 0 | 0 | 0 | 0 | 0 | 33 | 0 |
| *Periweisella* | 60 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 20 | 0 | 0 | 0 | 0 | 0 |
| *Weissella* | 0 | 26 | 0 | 0 | 0 | 0 | 0 | 21 | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 5 | 0 |
| *Oenococcus* | 0 | 50 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 25 | 25 | 0 | 0 | 0 | 25 | 0 |
| *Convivina* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Fructobacillus* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| *Leuconostoc* | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 13 | 0 |

**Figure 2.1. In silico identification of presence/absence of phenolic metabolism genes across *Lactobacillaceae.***

Query sequences listed in Table 2.2 were used to perform protein BLAST on all *Lactobacillaceae* type strains (336) available in the NCBI database (January 2022), with cutoff values of 75% query cover and 35% amino acid identity. The heatmap represents the percentage of type species with a positive hit in each genus with white colour representing absence in all type strains of a genus. The colours in genus name column represent lifetyles; red- Vertebrate-adapted, orange- Insect-adapted, blue- Free-living, green- Nomadic, white- Unassigned (Duar et al., 2017; J. Zheng et al., 2020). *Blast hits cannot be differentitalted for Par1 and Par2 (amino acid identites within 2% of each other).

The metabolism of hydroxycinnamic acids is well documented with two metabolic pathways of decarboxylation and reduction available for free phenolic acids. Currently, three hydroxycinnamic acid esterases (Esteban-Torres et al., 2015, 2013; Lai et al., 2009; Chapter 6), three hydroxycinnamic acid reductases (Santamaría et al., 2018; Chapter 3, 5) and one hydroxycinnamic acid decarboxylase (Cavin et al., 1997; Landete et al., 2010) have been characterized. The decarboxylated vinyl derivatives of phenolic acids can further be converted into ethyl derivatives via a vinyl phenol reductase (Santamaría et al., 2018b).

Lp_0796 was the first biochemically characterized hydroxycinnamic acid esterase (Esteban-Torres et al., 2013) and is the most widespread esterase among the *Lactobacillaceae* type strains. Est_1092 (Esteban-Torres et al., 2015) and HceP (Lai et al., 2009) show an association to lifestyle with a high prevalence in vertebrate host adapted strains and are sparsely distributed in environmental type strains of *Lapidilactobacillus, Loigolactobacillus, Liquorilactobacillus* and *Secundilactobacillus*. HceP occurs frequently in *Levilactobacillus* type strains. It is the only hydroxycinnamic acid esterase whose activity has also been confirmed via a deletion mutant unlike other biochemically characterized enzymes (Chapter 6).

Among hydroxycinnamic acid reductases, Par1 and its uncharacterized homolog Par2 were first identified in *Furfurilactobacillus milii* (Chapter 3) while HcrB and its variant HcrF were characterized in *Lactiplantibacillus plantarum* (Santamaría et al., 2018a) and *Limosilactobacillus fermentum* (Chapter 3, 6), respectively. HcrF occurs frequently in *Lactobacillus* and *Secundilactobacillus,* while HcrB is mostly associated with *Lactiplantibacillus*. *Secundilactobacillus* and *Ligilactobacillus* genera possess all three hydroxycinnamic acid reductases while type strains of *Loigolactobacillus* and *Furfurilactobacillus* encode for Par1/Par2 exclusively. *Companilactobacillus* also has a very high prevalence for Par1/Par2 with some type

strains also encoding for HcrB. Identification of the presence of Par1 and Par2 is confounded because the high amino acid identity between their sequences (51%) makes an accurate distinction between the homologs of two enzymes difficult. Par1 has shown activity as hydroxycinnamic acid reductase while substrates for Par2 remain to be identified (Chapter 3).

Hydroxycinnamic acid decarboxylase *pad* is frequently encoded by *Lactobacillaceae*. *Schleiferilactobacillus* and *Agrilactobacillus* are the only environmental associated genera lacking both decarboxylase and reductase enzymes specific for hydroxycinnamic acids. Strains of *Companilactobacillus* and *Pediococcus* have a high occurrence of decarboxylases. Pad activity has been associated with formation of vinylphenol adducts of deoxyanthocyanidins and pyranoanthocyanidins (Bai et al., 2014).

Vinyl phenol reductase VprA characterized in *Lp. plantarum* (Santamaría et al., 2018b) displays low occurrence in vertebrate host adapted type strains. Strains of *Loigolactobacillus, Secundilactobacillus* and *Lactiplantibacillus* are most likely to possess VprA (44-58%).

Two hydroxybenzoic acid esterases have been characterized commonly referred as tannase (Curiel et al., 2009; Iwamoto et al., 2008; Jiménez et al., 2014). Tannase activity is prevalent in *Levilactobacillus* and *Lactiplantibacillus* strains. Extracellular tannase TanA is extremely rare and highly strain specific (Jiménez et al., 2014). *Lactobacillus* strains are best suited for hydroxycinnamic acid esterase activity while strains of *Levilactobacillus* and *Lactiplantibacillus* most likely candidates for broad range phenolic acid esterase activity (Table 2.1).

Gallate decarboxylase PadG is active on hydroxybenzoic acids (Jiménez et al., 2013) and is fairly abundant in type strains belonging to environmental and nomadic lifestyles, except for *Schleiferilactobacillus.* The free-living *Agrilactobacillus, Liquorilactobacillus* and

*Lentilactobacillus* genera exclusively encode for PadG while other free-living lactobacilli also encode for hydroxycinnamic acid decarboxylases.

Multiple studies report glycosyl hydrolase activity in *Lactobacillaceae* but literature on their activity on glycosylated flavonoids and phenolic acids is limited. Glycosidases usually have specificity towards glycosidic linkages and one of the two sugar moieties rather than the phenolic substrates, in contrast to phenolic acid esterases, which are likely specific towards the phenolic acids. Thus, multiple glycosyl hydrolase families that are present in numerous *Lactobacillaceae* are likely to also be active on flavonoid glycosides despite being uncharacterized for specific phenolic substrates (Michlmayr and Kneifel, 2014). β-Glucosidase and α-rhamnosidase activities observed in *Lp. plantarum, Lacticaseibacillus rhamnosus* and *Lactiplantibacillus pentosus* have been associated with production of flavonoid aglycones such as naringenin, hesperetin and quercetin in synthetic growth media (Park et al., 2021; Pereira-Caro et al., 2018; Tsui and Yang, 2021).

Among the five characterized flavonoid glycosidases, α-rhamnosidases Ram1 and Ram2 have been characterized in *Lp. plantarum* and *Pediococcus acidilactici* (Ávila et al., 2009b; Beekwilder et al., 2009; Michlmayr et al., 2011). Vertebrate host adapted type strains rarely encode for α-rhamnosidases despite characterization of $RamA_{La}$ in *Lactobacillus johnsonii* (Beekwilder et al., 2009). Apart from the genera *Lactiplantibacillus* and *Pediococcus,* α-rhamnosidases are sporadically present in environmental associated strains. β-Glucosidase rBGLa characterized in *Limosilactobacillus antri* (Kim et al., 2017) is the most widespread flavonoid glycosidase across *Lactobacillaceae* type strains. Strains of *Furfurilactobacillus*, *Oneococcus*, *Leuconostoc* have low frequency for β-glucosidase rBGLa. Insect associated type strains of *Bombilactobacillus* also encode for Ram2 and rBGLa flavonoid glycosidases along with *Companilactobacillus.* β-

Glucuronidase characterized in *Levilactobacillus brevis* (Sakurama et al., 2014) occurs occasionally in *Limosilactobacillus, Lacticaseibacillus* and several other environmental genera. *Lapidilactobacillus* is the only free-living genus lacking all of the characterized flavonoid glycosidases.

Heterologous overexpression of a β-glucosidase (Glu913) from *Limosilactobacillus mucosae* INIA P508 in several other bacterial hosts such as *Lactococcus lactis* and *Lm. fermentum* displayed glucosidase activity on isoflavones, flavanones and flavonols glucosides along with lignan glycosides of matairesinol and pinoresinol (Gaya et al., 2020). β-Glucosidases Glu913 and rBGLa both belong to glycosyl hydrolase family 3 and share a 69% amino acid identity with each other. A β-glucosidase active on stilbene glucosides was purified from *Companilactobacillus kimchi* JB301 and is capable of converting polydatin to resveratrol (Ko et al., 2014).

Insect associated strains mostly lack genes involved in metabolism of phenolic compounds which can be correlated to their highly specialized lifestyle and small genome sizes. On the opposite end of the spectrum, nomadic *Lp. plantarum* shows highest diversity and frequency with respect to genes involved in metabolism of phenolic compounds. Among the strains with unknown lifestyles, *Companilactobacillus* and *Pediococcus* show the most potential for biotransformation of phenolic compounds.

Low occurrence of phenolic acid esterases and β-glucosidase in *Oenococcus* type strains matches the literature with presence of Est_1092 homolog in some *Oenococcus oeni* strains associated with hydroxycinnamic acid esterase activity (Collombel et al., 2019). Strains of *O. oeni* also displayed β-glucosidase activity associated with deglycosylation of anthocyanin glucosides, such as malvidin-3-glucoside in model wine solution (Devi et al., 2020). Strains of the *Weissella* genus have displayed hydroxycinnamic acid reductase (Filannino et al., 2014; Chapter 3) and gallate

decarboxylase (Sáez et al., 2017) activities in modified growth media. Despite their very close association to *Furfurilactobacillus* (Qiao et al., 2022), they lack the genes responsible for these enzymatic activities suggesting presence of uncharacterized enzymes different from already known *Lactobacillaceae* enzymes. *Dellagolia, Convivina, Fructobacillus* and *Leuconostoc* genus had no hits except rare presence of rBGLa in *Leuconostoc,* likely indicating the lack of phenolic metabolism genes in these genera.

*Lactobacillaceae* have been reported to produce equol from daidzein via reductase enzymes (Heng et al., 2019). Conversion of anthocyanidins to chalcones has also been proposed (Ávila et al., 2009a), with similar enzymatic activity reported for conversion of patulin to ascladiol (Hawar et al., 2013; Wei et al., 2020; Chapter 7). While the role of uncharacterized enzymes can not be discounted, characterized proteins displaying moonlighting activity (Jeffery, 2018) may be responsible for various bioconversions including degradation metabolites of various phenolic compounds and glycosyl hydrolase activities.

### 2.4. Transformation of phenolic compounds in food fermentations

Biotransformation of phenolic compounds during fermentation by *Lactobacillaceae* does not always match with the presence/absence of enzymes and metabolic activity observed in laboratory media. *Limosilactobacillus fermentum* preferentially reduced hydroxycinnamic acid substrates despite encoding phenolic acid decarboxylase Pad (Chapter 3). Conversely strains of *Lactiplantibacillus plantarum* almost exclusively decarboxylated hydroxycinnamic acids in laboratory media while they preferred phenylpropionic acid production in cherry juice and broccoli fermentations (Filannino et al., 2015). Decarboxylation of hydroxybenzoic acids by *Levilactobacillus hammesii* was observed in whole wheat sourdoughs but not in rye malt sourdoughs (Ripari et al., 2019). The substrate composition of individual foods may be responsible

for altering gene expression and enzyme production, affecting the behaviour of fermenting microbiota. The expression of genes coding for phenolic metabolism in *Lactiplantibacillus pentosus* was strain specific, in addition, differences in processing steps of olive extracts also led to differential expression of genes (Carrasco et al., 2018). Similarly, hydroxycinnamic acid esterase and reductase genes also had altered gene expression among millet malt and sorghum fermentations relative to gene expression in broth (Pswarayi et al., 2022). Thus, it is advisable to combine information of characterized enzymes and strain behaviours in laboratory media along with model food fermentations to better predict the fate of phenolic compounds in fermented foods.

### 2.4.1. Fruits and fruit products

Strains of *Lactiplantibacillus* and *Lacticaseibacillus* are most frequently used for fermentation of various fruit substrates (Table 2.3). Fermentation frequently results in increased concentrations of free phenolic compounds including gallic acid, syringic acid, caffeic acid and catechins. Grape juice fermented with co-culture of *Lp. plantarum* and *Levilactobacillus brevis* had significantly increased concentrations of procyanidin B1, B2, catechin and epicatechin after 12 h (Wu et al., 2021). Increases in anthocyanin and flavonoid glycoside content has also been reported (Ricci et al., 2019a, 2019b). However, many studies lack proper controls to account for the effect of inherent food enzymes and enhanced extractability after fermentation. This makes it difficult to assess the contribution of bacterial esterases and glycosidases to increasing the total free phenolic content during fermentation.

Strains of *Lp. plantarum* increased kaempferol and quercetin concentrations in fermented apple and mulberry substrates likely via flavonoid glycosidase activity (Kwaw et al., 2018; N. K. Lee et al., 2015; Z. Li et al., 2018), while this activity was strain specific in elderberry juice fermentation (Ricci et al., 2019a, 2019b). Fermentation of mulberry pomace decreased cyanadin-3-O-glucoside

concentrations with a corresponding increase in cyanidin levels giving evidence of anthocyanin glycosidase activity of *Lp. plantarum* CICC 20265 (Tang et al., 2021).

Strains of *Lp. plantarum* and *Lc. casei* reduced chlorogenic acid concentration during fermentation (Lizardo et al., 2020; Zhou et al., 2020). Similarly, papaya puree fermented using strains of *Leuconostoc pseudomesenteroids*, *Weissella cibaria* and *Lp. plantarum,* decreased gallocatechin gallate and chlorogenic acid content after a 48 h fermentation and 7 days of storage at 4°C (Mashitoa et al., 2021a). Tannase and esterase enzymes that are responsible for these conversions are frequently encoded by *Lp. plantarum* but are rarely present in *Leuconostoc* and *Weissella. Lactobacillus* genus on the other hand frequently encodes hydroxycinnamic acid esterases with activity observed in fermented jujube and mulberry juice using strains of *Lactobacillus helveticus* and *Lactobacillus acidophilus* (Kwaw et al., 2018; Li et al., 2021). Strong evidence of hydroxycinnamic acid esterase activity was observed in fermented avocado puree with *Lp. plantarum* AVEF17 significantly increasing caffeic acid with a corresponding decrease of rosmarinic acid concentration compared to a chemically acidified control (Filannino et al., 2020). *Oenococcus* strains typically lack phenolic metabolism genes; however, one out of six *Oenococcus oeni* strains tested significantly increased the hydroxycinnamic acid concentrations during model wine malolactic fermentation (Diez-Ozaeta et al., 2021).

The content of ellagitannins such as β-punicalagin and punicalin was reduced after fermentation with *Lp. plantarum* and *L. acidophilus* which is attributable to tannase or uncharacterized esterases (Valero-Cases et al., 2017). There was no corresponding increase in ellagic acid concentration which may indicate enzymatic conversions to unidentified ellagic acid derivatives (Valero-Cases et al., 2017).

**Table 2.3. Studies with biotransformation of phenolic compounds during fermentation of food substrates via *Lactobacillaceae***

| Food Matrix | Microorganisms | Microbial enzymes | Substrates converted | Metabolites detected | Reference |
|---|---|---|---|---|---|
| **Fruits and juices** | | | | | |
| Cherry juice | *Lactiplantibacillus plantarum, Limosilactobacillus fermentum, Limosilactobacillus reuteri, Levilactobacillus spicheri* | Phenolic acid decarboxylase, hydroxycinnamic acid reductase, vinyl phenol reductase | Protocatechuic acid, caffeic acid, *p*-coumaric acid | Catechol, dihydrocaffeic acid, phloretic acid, ethyl phenol | (Filannino et al., 2015; Ricci et al., 2019b) |
| Cloudy apple juice | Co fermentation- *Lp. plantarum*, *Lm fermentum*, *Lactobacillus acidophilus* | Flavonoid glycosidase | Rutin | Quercitrin | (Han et al., 2021) |
| Apple juice | *Lp. plantarum, Lacticaseibacillus* spp., *Lactobacillus* spp. | Chlorogenic acid esterase, flavonoid glycosidase | Chlorogenic acid, quercetin-3-O-galactoside, phlorizin | Caffeic acid, quercetin, phloretin | (Z. Li et al., 2018; Wu et al., 2020) |
| Apple pomace | *Lacticaseibacillus rhamnosus* | β-glucosidase | Quercitrin, phlorizin | Quercetin, phloretin | (Liu et al., 2021) |
| Mulberry juice and pomace | *Lp. plantarum, Lacticaseibacillus paracasei, L. acidophilus* | Flavonoid glycosidase | Cyanidin-3-O-glucoside, peonidin-3-O-glucoside/peonidin-3-O-galactcoside, quercetin-3-O-rhamnoside | Cyanidin, petunidin, quercetin, kaempferol | (Kwaw et al., 2018; Tang et al., 2021) |

**Table 2.3.** (continued)

| Food Matrix | Microorganisms | Microbial enzymes | Substrates converted | Metabolites detected | Reference |
|---|---|---|---|---|---|
| Mango and Papaya puree | *Lp. plantarum, Weissella cibaria* | Flavonoid glycosidase, tannase, chlorogenic acid esterase | Gallocatechin gallate, chlorogenic acid | Quercetin, ellagic acid | (Mashitoa et al., 2021a, 2021b) |
| Acerola cherry puree | *Lacticaseibacillus casei, L. acidophilus* | Flavonoid glycosidase, chlorogenic acid esterase | Hesperidin, rutin, chlorogenic acid | Caffeic acid | (de Assis et al., 2021) |
| Avocado puree | *Lp. plantarum* | Phenolic acid esterase, tannase, hydroxycinnamic acid reductase, hydroxycinnamic acid decarboxylase, vinyl phenol reductase | Rosmarinic acid, caffeic acid, ferulic acid, *p*-coumaric acid, sinapic acid | Caffeic acid, epicatechin, dihydroferulic acid, ellagic acid, ethyl catechol | (Filannino et al., 2020) |
| Pomegranate juice | *Lp. plantarum, L. acidophilus* | Tannase | β-punicalagin, punicalin | - | (Valero-Cases et al., 2017) |
| Bitter melon juice | *Lp. plantarum* | Hydroxybenzoic acid decarboxylase, hydroxycinnamic acid decarboxylase, hydroxycinnamic acid reductase | Gallic acid, caffeic acid, protocatechuic acid, *p*-coumaric acid | Pyrogallol, vinyl catechol, catechol, vinyl phenol, dihydrocaffeic acid phloretic acid, ellagic acid | (Gao et al., 2019) |
| Cactus cladodes | *Lp. plantarum, Levilactobacillus brevis* | Flavonoid glycosidase | - | Kaemferol, isorhamnetin | (Filannino et al., 2016a) |

**Table 2.3.** (continued)

| Food Matrix | Microorganisms | Microbial enzymes | Substrates converted | Metabolites detected | Reference |
|---|---|---|---|---|---|
| **Cereals and pseudocereals** | | | | | |
| Whole wheat and rye sourdough | *Lp. plantarum, Lv. brevis, Levilactobacillus hammesii* | Phenolic acid esterase, hydroxycinnamic acid decarboxylase, hydroxycinnamic acid reductase, viny phenol reductase | Ferulic acid | Vinyl guaiacol, dihydroferulic acid, ethyl guaiacol | (Ripari et al., 2019) |
| Wheat and rye sourdough | *Lp. plantarum, Lv. brevis* | Flavonoid glycosidase, hydroxycinnamic acid reductase | Isorhamnetin-3-O-hexoside, ferulic acid, caffeic acid, sinapic acid | Isorhamnetin, dihydroferulic acid, dihydrocaffeic acid, dihydrosinapic acid | (Koistinen et al., 2018) |
| Red sorghum sourdough | *Lc. casei, Lp. plantarum, Lm. reuteri, Lm. fermentum* | Flavonoid and phenolic acid glycosidase, hydroxycinnamic acid esterase, hydroxycinnamic acid reductase, hydroxycinnamic acid decarboxylase, viny phenol reductase | Naringenin-7-O-glucoside, eriodictyol-7-O-glucoside, coumaroyl-caffeoylglycerol, coumaroyl-feruloylglycerol, coumaroylglycerol, ferulic acid, caffeic acid, 3-deoxyanthocyanidin (Apigeninidin, methoxyapigeninidin) | Naringenin, eriodictyol, caffeic, *p*-coumaric acid, ferulic acid, dihydroferulic acid, vinyl catechol, ethyl catechol, pyrano-3-deoxyanthocyanidins, 3-deoxyanthocyanidin−vinylphenol adducts | (Bai et al., 2014; Svensson et al., 2010) |

**Table 2.3.** (continued)

| Food Matrix | Microorganisms | Microbial enzymes | Substrates converted | Metabolites detected | Reference |
|---|---|---|---|---|---|
| Red quinoa | Co fermentation- *L. acidophilus, Lc. casei, Lc. paracasei* | Flavonoid glycosidase | - | Quercetin, kaempferol | (Zhang et al., 2021) |
| **Vegetables and leafy/plant substrates** | | | | | |
| Kale | *Lc. paracasei* | Flavonoid glycosidase | Kaempferol-3-o-sophoroside | Kaempferol | (Shimojo et al., 2018) |
| Curly kale juice | *Lp. plantarum, Latilactobacillus sakei* | Chlorogenic acid esterase | Chlorogenic acid, | Caffeic acid | (Szutowska et al., 2021) |
| Mulberry leaves | *Lp. plantarum* | Flavonoid glycosidase | - | Quercetin, kaempferol | (N. K. Lee et al., 2015) |
| Broccoli puree | *Lp. plantarum, Lm. fermentum, Lm. reuteri, Lv. spicheri* | Chlorogenic acid esterase, hydroxycinnamic acid reductase | Chlorogenic acid | Caffeic acid, quinic acid, dihydrocaffeic acid | (Filannino et al., 2015) |
| Sweet Potato | *L. acidophilus* | Hydroxycinnamic acid esterase | 4,5-dicaffeoylquinic acid | Caffeic acid, *p*-coumaric acid, ferulic acid | (Shen et al., 2018) |
| African nightshade | *Lp. plantarum, W. cibaria* | Flavonoid glycosidase, tannase | - | Quercetin, luteolin, ellagic acid | (Degrain et al., 2020) |
| Chinese skullcap | *Lv. brevis* | β-glucuronidase | Baicalin, wogonoside | Baicalein, wogonin | (Xu and Ji, 2013) |
| Jussara pulp | *Lactobacillus* spp.*, Lv. brevis, Lm. fermentum* | Flavonoid glycosidase, hydroxycinnamic acid reductase | Cyanidin 3-glucoside, cyanidin 3-rutinoside, pelargonidin 3-glucoside | Dihydrocaffeic acid | (Braga et al., 2018) |

**Table 2.3.** (continued)

| Food Matrix | Microorganisms | Microbial enzymes | Substrates converted | Metabolites detected | Reference |
|---|---|---|---|---|---|
| *Cudrania tricuspidata* leaves | *Lp. plantarum* | Flavonoid glycosidase | Quercetin-7-O-beta-glucopyranoside, kaempferol-3-O-beta-glucopyranoside, kaempferol-7-O-beta-glucopyranoside | Quercetin, kaempferol | (Y. Lee et al., 2015) |
| **Legumes and oilseeds** | | | | | |
| Soybean (Cheonggukjang) | *Lactobacillus intestinalis* | Isoflavone reductase | Daidzein | Equol | (Heng et al., 2019) |
| Soymilk | *Lm. fermentum, Lp. plantarum, Lc. rhamnosus, L. bulgaricus, L. acidophilus, Lc. casei, Lentilactobacillus kefiri* | Flavonoid glycosidase | Daidzin, genistin, glycitin | Daidzein, genistein, glycitein | (de Queirós et al., 2020; Lodha et al., 2021) |
| Soybean flour | *Lc. casei* | Flavonoid glycosidase | Daidzin, genistin, glycitin and their malonyl and acetyl derivatives | Daidzein, genistein, glycitein | (S. Li et al., 2020) |
| Sunflower substrates | *L. gasseri* | Chlorogenic acid esterase | Chlorogenic acid | Caffeic acid | (Fritsch et al., 2016) |

Fermentation with *L. helveticus* greatly increased the gallic acid concentration in apple juice while hydroxycinnamic acids levels dropped (Wu et al., 2020) likely due to further metabolism (decarboxylation and/or reduction). Hydroxybenzoic acids can be decarboxylated as evident with catechol formation from protocatechuic acid during elderberry and cherry juice fermentation (Filannino et al., 2015; Ricci et al., 2019a). Decarboxylation of hydroxycinnamic acids is also observed during fermentation but strains of *Lp. plantarum*, *Lc. rhamnosus* and *Lm. fermentum* predominantly reduced caffeic acid and *p*-coumaric acid to their respective dihydro-derivatives (Filannino et al., 2015; Gao et al., 2019; Ricci et al., 2019a). Strains of *Lp. plantarum* can also produce ethyl derivatives of phenolic acids such as ethyl phenol and ethyl catechol detected in fermented cherry juice and avocado puree by action of vinylphenol reductase on decarboxylated phenolic acids (Filannino et al., 2020; Ricci et al., 2019b). *Limosilactobacillus reuteri* did not metabolize any of the phenolic acids present in cherry juice likely due to absence of requisite enzymes (Filannino et al., 2015).

### 2.4.2. Cereal/legume fermentations

Increased free phenolic acids such as gallic acid, vanillic acid, *p*-coumaric acid and ferulic acid, epicatechin and procyanidin A2 were also observed in fermented cereals (Guan et al., 2021; Ripari et al., 2019; Zhang et al., 2022, 2017). Fermentation can also be used to enhance effect of enzymatic treatments on free phenolic content. Fermentation of quinoa using a mixed culture of *L. acidophilus*, *Lc. casei* and *Lc. paracasei* after enzymatic hydrolysis significantly increased the concentrations of procyanidin B2, quercetin and kaempferol compared to an unfermented enzymatically hydrolyzed control (Zhang et al., 2021).

Fermentation plays a major role in converting flavonoid glycosides to corresponding aglycones in cereals and legumes. Dual species cultures of *Lc. casei*: *Lp. plantarum* and *Lm. reuteri*: *Lm.*

*fermentum* displayed glucosidase activity on naringenin-7-O-glucoside and eriodictyol-7-O-glucoside releasing their respective flavanone aglycones in red sorghum sourdough (Svensson et al., 2010). Conversion of isoflavone glycosides to corresponding aglycones has been attributed to β-glucosidase activity of *Lactobacillaceae.* Fermentation of soymilk using a wide variety of strains including *Lp. plantarum*, *Lm. fermentum*, *Lc. casei*, *Lc. rhamnosus*, *Lentilactobacillus kefiri*, *L. acidophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* significantly increased concentrations of daidzein and genistein marked with corresponding decrease of daidzin and genistin (de Queirós et al., 2020; Lodha et al., 2021). Fermentation of whole soybean flour with *Lc. casei* also showed glucosidase activity on glycitin along with malonylglucosides and acetylglucosides of isoflavones (S. Li et al., 2020).

Strains of *Pediococcus acidilactici* increased the secoisolariciresinol content in fermented cereal bran while also altering alkylresorcinols content based on length of carbon chain (Bartkiene et al., 2020, 2018). While contribution of bacterial glycosidase remains possible, further research is required to differentiate between the contribution of cereal and microbial enzymes to these bioconversions.

Strains of *L. johnsonii*, *L. acidophilus* and *Lm. reuteri* increased free phenolic acid content in whole grain barley and oat groat displaying hydroxycinnamic acid esterase activity (Hole et al., 2012). Glycerol esters of phenolic acids such as coumaroyl-caffeoylglycerol were also hydrolyzed by strains of *Lactiplantibacillus*, *Lacticaseibacillus* and *Limosilactobacillus* in sorghum sourdough (Svensson et al., 2010). On the other hand, strains of *Lc. rhamnosus* decreased ferulic acid concentration in fermented wheat bran likely via further bioconversion to metabolites (Bertsch et al., 2020).

Co-fermentation using strains displaying complementary activities can further increase free phenolic acid and their metabolite concentrations. Addition of *Lv. hammesii* along with *Lp. plantarum* increased the ferulic acid esterase activity in whole wheat sourdough (Ripari et al., 2019). *Lp. plantarum* produced dihydrocaffeic acid, vinyl guaiacol and ethyl guaiacol via phenolic acid reductase, decarboxylase, and vinyl phenol reductase enzymes respectively, while *Lv. hammesii* only contributed to vinyl guaiacol production due to the absence of enzymes responsible for other two conversions (Ripari et al., 2019). Decarboxylase activity of *Lactobacillaceae* strains can also contribute to formation of vinyl phenol adducts of 3-deoxyanthocyanidins and pyrano-3-deoxyanthocyanidins during sourdough fermentation in sorghum (Bai et al., 2014).

### 2.4.3. Vegetables and miscellaneous plants

Hyroxycinnamic acid esterase activity is frequently observed in a variety of vegetable and plant fermentations. *L. acidophilus* hydrolyzed 4,5-dicaffeoylquinic acid in sweet potato fermentation and displayed ferulic acid esterase activity in sugarcane bagasse (Pattnaik et al., 2022; Shen et al., 2018). Strains of *Lp. plantarum, Latilactobacillus sakei*, *Lactobacillus gasseri* and *Limosilactobacillus* displayed chlorogenic acid esterase activity during fermentation of substrates such as kale, sunflower, and broccoli (Filannino et al., 2015; Fritsch et al., 2016; Szutowska et al., 2021). However, a decrease in the released caffeic acid concentrations have also been reported, likely caused by further bioconversion to reduced and/or decarboxylated metabolites as observed in fermented broccoli puree (Filannino et al., 2015). Strain specific increase in ellagic acid concentrations were also reported after fermentation, which can be attributed to tannase activity releasing hydroxybenzoic acids from gallotannins and ellagitannins (Degrain et al., 2020).

Fermentation of *Cudrania tricuspidata* leaves using *Lp. plantarum* SDL 1413 deglycosylated flavonol-7-O-beta-glucopyranosides and kaempferol-3-O-beta-glucopyranoside into their

respective aglycones (Y. Lee et al., 2015). β-Glucuronidase and β-glucosidase activities have also been reported by strains of *Lv. brevis* and *Lc. paracasei* during fermentation releasing baicalein and wogonin, and kaempferol from their glycosides in *Scutellaria baicalensis* (Xu and Ji, 2013) and kale (Shimojo et al., 2018) extracts respectively.

Detection of phenolic acid metabolites after fermentation of a food matrix provides strong evidence of bacterial enzymatic activity. On the other hand, associating specific bacterial enzymes to esterase and glycosidase activities during a food fermentation is much more challenging. Most studies rely on quantification of a decrease in concentration of specific compounds with corresponding increase in expected metabolites, which provides reasonable evidence when combined with presence/absence of genotype in fermenting microbiota. However, lack of studies utilizing isogenic mutants to confirm the activity of specific enzymes in food fermentations along with limited characterization of expression of genes in complex food substrates remain limiting factors in strain selection and optimizing fermentation outcomes.

### 2.5. Influence of phenolic metabolites on health and food quality

Consumption of plant-based foods has been associated with improved gut health (De Filippis et al., 2016) with epidemiological studies correlating consumption of polyphenol rich dietary fibre with anti-inflammatory, anti-diabetic effects along with reduction in risk factors of cardiovascular diseases and cancer (Cardona et al., 2013; Shahidi and Yeo, 2018; Vitaglione et al., 2015). Fermentation can release the bioactive phenolics from their precursors via esterase and glycosidase activities and significantly alter the phenolic constituents of the food matrix. Flavonoids, phenolic acids, and their metabolites have been studied extensively with numerous health benefits reported *in-vitro* and in animal models (Lei et al., 2020; Leonard et al., 2021b). Phenolic compounds can

also modulate the gut microbiota and enabling production of various bioactive metabolites during colonic fermentation (Loo et al., 2020).

In addition to their antioxidant activity, phenolic acids and flavonoids also possess antimicrobial activity (Górniak et al., 2019; Sánchez-Maldonado et al., 2011) which can contribute to enhanced food safety when used in combination with existing preservatives and safety technologies (Wu and Zhou, 2021). Fermentation can also enhance shelf life with delayed staling caused by the activity of esterases and glycosidases, increasing non-starch polysaccharides such as arabinoxylans. *Lactobacillaceae* may be targeting the sugar molecules attached to phenolic compounds for growth releasing free phenolic compounds in the process (Brochet et al., 2021). Reduction of phenolic acids also has a similar advantage to heterofermentative *Lactobacillaceae* with co-factor recycling indirectly aiding in growth via phosphoketolase pathway (Filannino et al., 2016b, 2014).

Phenolic compounds have the potential to alter the digestibility of foods, lowering the glycemic index. Flavonoids and proanthocyanidins are effective in inhibiting digesting enzymes such as α -amylase and α-glucosidase and can also affect regulation of glucose transporters *in-vivo* (Sun and Miao, 2020). Phenolic acids can also form complexes with starch reducing its digestibility and altering its rheological properties (M. Li et al., 2020, 2018; Zheng et al., 2020).

Fermentation can increase the palatability of food and animal feed by reducing the content of bitter tasting phenolics and antinutritive compounds. Fermented beverages and porridges made using traditional fermentation of phenolic rich cereals such as sorghum and millet have improved flavour, with biotransformation of phenolic compounds likely playing a role (Gänzle, 2019). Vinyl and ethyl derivatives produced by metabolism of hydroxycinnamic acids are considered as flavour volatiles (Muñoz et al., 2017). Glycosidase activity of *Lactobacillaceae* may contribute to production of aromatic aglycones from odourless precursors (Iorizzo et al., 2016) and convert

bitter phenolic compounds to more palatable aglycones such as such as oleuropein in olives (Gänzle, 2019; Heperkan, 2013). Bitter tasting free phenolics may also provide an opportunity for regulation of health via taste receptors in the gut (Tarragon and Moreno, 2020).

In conclusion, significant progress has been made over the last two decades in characterization of genetic determinants responsible for biotransformation of phenolic compounds. Pathways for metabolism of phenolic acids is mostly complete with distinct enzymes responsible for conversion of hydroxybenzoic acids and hydroxycinnamic acids. The genetic determinants and substrate specificities for esterases and glycosidases still remain unknown with evidence mostly limited to quantification of generic enzymatic activity and/or increase in concentrations of limited phenolic metabolites. Nomadic and insect-adapted *Lactobacillaceae* display clear association between their ecology and presence of phenolic metabolism genes. The distribution of phenolic metabolism genes provided in this study enables better strain selection and prediction of fermentation metabolites. Lack of information on the regulation and expression of *Lactobacillaceae* enzymes in food systems remains a limiting factor in optimizing fermentation outcomes, with further research needed to better understand the influence of substrate composition on strain behavior.

# Chapter 3- Genetic determinants of hydroxycinnamic acid metabolism in heterofermentative *Lactobacillaceae*

## 3.1. Introduction

Phenolic acids are a class of phenolic compounds that are abundant in edible parts of plants. In plants, hydroxycinnamic acids occur mainly bound to cell wall polysaccharides, as glycosides or as esters (Acosta-Estrada et al., 2014; Andreasen et al., 2000; Dabrowski and Sosulski, 1984; Svensson et al., 2010). Phenolic acids can be distinguished as two types: hydroxybenzoic acids and the more abundant hydroxycinnamic acids. Epidemiological studies have associated consumption of dietary fibers rich in phenolic acids with reduction in chronic inflammation and a reduced risk of colon cancer, type 2 diabetes, neurodegenerative diseases, and cardiovascular diseases (Kim et al., 2016; Shahidi and Yeo, 2018). The free hydroxycinnamic acids such as caffeic acid and ferulic acid display anti-inflammatory and anticancer properties with reduction in tumor multiplicity (Chao et al., 2010; Janicke et al., 2011). Hydroxycinnamic acids also have antimicrobial activity at concentrations that correlate to their abundance in plants (Sánchez-Maldonado et al., 2011).

During fermentation of plants, esterase activities of lactic acid bacteria release bound hydroxycinnamic acids. In addition, lactic metabolism converts hydroxycinnamic acids via decarboxylation and reduction reactions (Sánchez Maldonado et al., 2014; Svensson et al., 2010). The decarboxylation as well as the reduction of hydroxycinnamic acids by microbial metabolism reduces their antimicrobial activity (Sánchez-Maldonado et al., 2011). Vinyl as well as ethyl derivatives that result from microbial metabolism are flavor volatiles that impact the aroma of fermented foods (Shahidi and Yeo, 2018). Vinyl derivatives also react with anthocyanins and 3-

deoxyanthocyanins that are present in many fruits and sorghum, respectively, to form pyranoanthocyanins and 3- deoxypyranoanthocyanins (Azevedo et al., 2014; Bai et al., 2014). The conversion of phenolic acids during food fermentations thus impacts quality, safety, and nutritional properties of fermented foods. Metabolism of phenolic acids, however, is strain specific and relatively poorly characterized compared to other metabolic pathways of lactic acid bacteria. Enzymes responsible for metabolism of hydroxycinnamic acids have been identified primarily in homofermentative lactic acid bacteria. Phenolic acid esterases were characterized in *Lactobacillus johnsonii* (Lai et al., 2009) and *Lactiplantibacillus plantarum* (Esteban-Torres et al., 2015, 2013). Phenolic acid decarboxylases were characterized only in *Lp. plantarum* (Cavin et al., 1997; Rodríguez et al., 2008). Until recently, Clostridium was the closest relative to *Lactobacillaceae* for which enzymes capable of reducing hydroxycinnamates were characterized (Mordaka et al., 2018; Sun et al., 2016). In 2018, phenolic acid reductases reducing hydroxycinnamic acids to substituted phenylpropionic acids (HcrB) or reducing the decarboxylated vinyl derivatives to ethyl derivatives (VprA) were characterized in *Lp. plantarum* (Santamaría et al., 2018b, 2018a). Heterofermentative lactic acid bacteria, including *Limosilactobacillus fermentum* (Filannino et al., 2015; Sánchez-Maldonado et al., 2011), *Furfurilactobacillus rossiae* (Filannino et al., 2014; Ripari et al., 2019), *Apilactobacillus kunkeei* (Filannino et al., 2016b), and *Weissella cibaria* (Filannino et al., 2014) also reduce hydroxycinnamic acids to the corresponding phenylpropionic acids; however, enzymes involved in phenolic acid metabolism of heterofermentative lactic acid bacteria have not been characterized (Muñoz et al., 2017). In heterofermentative *Lactobacillaceae*, the reduction of phenolic acids reduces NADH; this conversion increases the energy yield in the phosphoketolase pathway and thus has a high priority in heterolactic metabolism (Filannino et al., 2014; Gänzle, 2015). Because heterofermentative *Lactobacillaceae* differ phylogenetically and

physiologically from homofermentative *Lactobacillaceae* (Zheng et al., 2015), these organisms may harbor novel genes and enzymes responsible for reduction of hydroxycinnamic acids. This work aimed to study hydroxycinnamic metabolism of eight heterofermentative strains of lactic acid bacteria, using two *Lp. plantarum* strains as a reference. The strain-specific phenolic acid metabolism guided a comparative genomic analyses to identify homologs to HcrB as putative phenolic acid reductases. Two novel phenolic acid reductases were identified in heterofermentative *Lactobacillaceae*. Further bioinformatics analyses also determined the presence of different phenolic acid reductases, phenolic acid decarboxylase and vinyl phenol reductase, across *Lactobacillaceae*.

## 3.2. Materials and Methods

### 3.2.1. Bacterial strains and growth conditions

*Lactiplantibacillus plantarum* TMW1.460 (Ulmer et al., 2000) and *Levilactobacillus brevis* TMW 1.465 (Behr et al., 2006) isolated from spoiled beer, *Levilactobacillus hammesii* DSM 16381 (Valcheva et al., 2005) and *Furfurilactobacillus milii* C5 isolated from sourdough (Ripari et al., 2019), *Ff. milii* FUA3583 (previously classified as *Ff. rossiae* (Simpson et al., 2022)), *Lp. plantarum* FUA3584, and *Limosilactobacillus fermentum* FUA3589 isolated from Mahewu (Pswarayi and Gänzle, 2019) as well as *Limosilactobacillus reuteri* DSM 20016, *Apilactobacillus kunkeei* DSM 12361, and *Weissella cibaria* 10M (Schwab et al., 2008) were used in this study. Strains were subcultured from – 80°C stock and grown in modified De Man, Rogosa and Sharpe (mMRS) medium (Zhao and Gänzle, 2018) at 30°C under microaerophilic conditions.

### 3.2.2. Chemicals

Ferulic acid and caffeic acid were obtained from Extrasynthèse (Genay, France). Sinapic acid, dihydrosinapic acid, dihydrocaffeic acid, 4-vinylguaiacol, 4-ethylguaiacol, and 4-ethylcatechol were obtained from Sigma-Aldrich (St. Louis, MO, USA Canada). Dihydroferulic acid was purchased from MP Biomedicals (Illkirch, France) and components for mMRS media were purchased from BD (Sparks, MD, USA) and Sigma-Aldrich (St. Louis, MO, USA).

### 3.2.3. Hydroxycinnamic acid metabolism of lactic acid bacterial strains

Sinapic acid, ferulic acid, or caffeic acid was added to mMRS medium, and supplemented media were inoculated with 10% overnight culture of bacterial strains and incubated for 24 h at 30°C (Svensson et al., 2010). Uninoculated mMRS media containing corresponding hydroxycinnamic acids were used as controls. Following the incubation, cells were removed by centrifugation; the supernatant was acidified to pH 1.5 using HCl and extracted twice with ethyl acetate (Sánchez-Maldonado et al., 2011). The extracts were combined and analyzed with an Agilent 1200 series HPLC system equipped with an Agilent Eclipse XDB C18 column (4.6 by 150 mm; 5 m) coupled to an UV detector. Ten-microliter samples were injected and eluted at a flow rate of 0.7 ml/min using mobile phase consisting of (A) 0·1% (v/v) formic acid in water and (B) 0·1% formic acid in water/acetonitrile (10:90, v/v). The gradient applied on phase B was as follows: 10 to 15% (0-6 min); 15 to 100% (6-14 min); isocratic at 100% (21-24 min); 100 to 10% (24–30 min). Quantification was performed using external standards at 280 nm, with duplicate independent experiments performed. Due to the low stability of vinylcatechol, relative peak areas were calculated as a ratio of peak area out of 1, with vinylcatechol peak area divided by total peak area subtracted by interference peak area.

### 3.2.4. Comparative genomics and sequence analysis

Genome sequences of *Lv. hammesii* DSM 16381, *Lv. brevis* TMW 1.465, *Lm. reuteri* DSM 20016, *Ap. kunkeei* DSM12361, and *Lm. fermentum* FUA3589 were retrieved from NCBI. Whole genome shotgun sequences of *Lp. plantarum* TMW 1.460, *Ff. milii* C5, *W. cibaria* 10M, *Ff. milii* FUA3583, and *Lp. plantarum* FUA3584 were obtained in this study. DNA was isolated using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The quantity and quality of DNA was verified by gel electrophoresis, and with a NanoDrop® one spectrophotometer (Thermo Scientific, Madison, WI, USA). Sequencing was performed using Illumina TruSeq on a HiSeq2500 platform with high-output run mode by McGill University and Génome Québec Innovation Centre (Montreal, QC, Canada). The quality check of 125 bp paired end reads was done using FastQC tool (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Sequence assembly was performed using SPAdes (Bankevich et al., 2012) and MeDuSa (Bosi et al., 2015). Genomes were then annotated using the RAST server (Aziz et al., 2008). Genes involved in the hydroxycinnamate reductase operon of *Lp. plantarum* WCFS1 (Santamaría et al., 2018a) were used as query sequences to search for homologous protein sequences in the sample genomes using BLAST+. Protein sequence analysis was performed using InterProScan (Mitchell et al., 2014) and InterPro tools (Mitchell et al., 2019).

### 3.2.5. RNA isolation and quantification of relative gene expression by RT-qPCR

mMRS media containing 1 mM sinapic acid, caffeic acid, and ferulic acid were inoculated with overnight cultures of *Lactobacillaceae*. mMRS medium without addition of hydroxycinnamic acids was used as a reference condition for each strain. Strains were grown to early exponential phase corresponding to an optical density at 600 nm (OD600) of 0.3 to 0.4. RNA was stabilized by adding 2 volumes of RNAprotect bacterial reagent (Qiagen, Hilden, Germany) to 1 volume of

bacterial cultures. RNA was isolated with the RNeasy minikit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA quantification and quality were measured using a NanoDrop One spectrophotometer (Thermo Scientific, Madison, WI, USA), and DNA was hydrolyzed with RQ1 RNasefree DNase (Promega) according to the manufacturer's instructions. cDNA was synthesized with the QuantiTect reverse transcription kit (Qiagen, Hilden, Germany); 1 g of RNA was used as the template, and reverse transcription was done according to the manufacturer's instructions.

qPCR was performed in a 7500 fast realtime PCR instrument (Applied Biosystems, Life Technologies, Burlington, ON, Canada) with a QuantiFast SYBR green PCR kit (Qiagen, Hilden, Germany). Primers were designed using the Integrated DNA Technologies (IDT- Coralville, IA, USA) PrimerQuest Tool (Table 3.1). Gene expression relative to the reference conditions was calculated according to the method by Pfaffl, 2001. The phosphoketolase gene was used as the housekeeping gene for heterofermentative *Lactobacillaceae*, and the phosphofructokinase gene was used as the housekeeping gene for strains of L. plantarum (Teixeira et al., 2013). mRNA abundance under experimental conditions were normalized to the mRNA abundance under reference conditions, i.e., during growth of *Lactobacillaceae* in the absence of phenolic acids. Triplicate independent experiments were conducted (replicate bacterial cultures), and statistical analysis was performed using one-way analysis of variance (ANOVA) with Holm-Sidak post hoc analysis.

**Table 3.1. Primer sequences for qPCR.**

| Strain Name | Primer (forward, F; reverse, R) | Primer Sequences (5'→3') |
|---|---|---|
| *Lp. plantarum* TMW 1.460 and *Lp. plantarum* FUA3584 | *hcrR* -F/R | CGGTGAGCTGGACTTCTTAAT/GTTGACGGTGTTCGGGATAA |
| | *hcrA* -F/R | GGGACGAATGCAACCAAATC/TCGGTCTTCCGGTTCATTAAA |
| | *hcrB* -F/R | CGCATACCTGACTGCCAATA/ CAGTCCGTTGACCACCTAAA |
| | *hcrC* -F/R | TGGATCACCGACTTTCATCTTC/TGGATCACCGACTTTCATCTTC |
| | *parR* -F/R | GCATGCAACCCGCAATTATC/ATCCGTCAAATCAGCCAAGAA |
| | *hcrA2* -F/R | TGGCGGATAAGATCGAACAAG/AGGGCTGGATATGGAATGATAAC |
| | *par1* -F/R | CAGTATCAAGGTGGCGGTAAT/CTAGTATTCGCATCCGGCTTAG |
| | *par2* -F/R | GTGAACTGGCACGGTAACT/CTAGTACGTGGGCACCATTATC |
| | *fccA* -F/R | CTGGTGCCTACTTGATCTTTGA/CCAAGTCAGTCCCAGTCTTTAC |
| | *padR* -F/R | TGAAGCGACTAGAAGAACAAGG/ GCGGTGATGTGGTAGAGTTT |
| | *pad*- F/R | CATGTTGACCGAAGGCATTTAC/CGTACCGTGTAGTTTCTTCTCAT |
| | *vrpA* -F/R | ACCGGTGGTTACCTCAATAATC/ACCGTCACCAGTTCCTTTAC |
| | *pfk* (housekeeping) -F/R | CAATTACGGCTTTGCTGGATTAG/CTGGATAACGTGCGGAGTATAG |
| *Ff. milii C5* | *parR* -F/R | ATTGGTGCCGAGGATGTAAG/TTCGCTGAAAGCCGGAAT |
| | *hcrA2* -F/R | GATGGGTTGTTTGCCGATTTC/CTTGCTTCTTGGCGATTGATG |
| | *hcrA3* -F/R | ATGCTGCCTGCCGATAAA/CCAGTCCGTAACATGTGGATAA |
| | *par1* -F/R | ATCGTGGTTCGGGTAACTTATG/GAATCTCCTCCACGGCTTTAC |
| | *par2* -F/R | GCCAAACAGGGCAGAGATTAT/GTGGACCGCCTTGTGTATTT |
| | *fccA* -F/R | CCGGACCATTCTTCGCTATT/CTAGGATGATCTGACCGCTTTC |
| | *phosphoketolase* (housekeeping) -F/R | GAGCGATGCTGACTTGACTAA/CCATGTCTTGATGAGCCTTCT |

**Table 3.1.** (continued)

| Strain Name | Primer (forward, F; reverse, R) | Primer Sequences (5'→3') |
|---|---|---|
| *Ff. milii* FUA3583 | *parR* -F/R | GTTACCACTACACAGGCGTTTA/CGGTGAAATGCTGGCAATAAG |
| | *hcrA2* -F/R | CGATGACGACGGAAATTTGTTAG/CTTGCTTCTTGGCGATTGATG |
| | *hcrA3* -F/R | CGATGCGTGACAGTGAGAATA/CTAACTTCTGACCGGTCTTAGC |
| | *par1* -F/R | GCGGATGATGGTAGTCGTTATG/AGATGAGGATGCGCGTAAAC |
| | *par2* -F/R | GGAATACGAAGGTGTCCATTCA/TTATCAGCGCGGTCCATAAC |
| | *fccA* -F/R | AGACACAGGTATTGGACGAAAG/AGCCTGACGACCAAAGATTAC |
| | *padR* -F/R | GATGTTGACTGATGGCTGGA/TCGTGACCATCTGCTGTAATC |
| | *pad* -F/R | GGGTTGAGGAACATCCAGAAA/GCAAATTCTGGCACCACTAAC |
| | *phosphoketolase* (housekeeping) -F/R | GAGCGATGCTGACTTGACTAA/TGTCTTGGTGAGCCTTCTTG |
| *Lm. fermentum* FUA3589 | *hcrR* -F/R | CAAGGTGGTGATGGTCTCAA/GTTGATCGGCGTTTCCTTAATC |
| | *hcrA2* -F/R | CGATCGAATGGCTGTCCTATAA/GAAGAACCCTGGGTGAAGTAAG |
| | *hcrA3* -F/R | ATCATGCCATCATCCGAATACA/GTGTCGAAGTCGGCGAATAA |
| | *hcrF* -F/R | GTGACCGTCCGTGCTAAAT/TCGTCAATGTGCTCCCAATAG |
| | *fccA* -F/R | CAGTGCTAAGGCAGTGATTCT/GGTTGGTTGGTCGTCTTGTA |
| | *padR* -F/R | CCTACGTCATCTTAGGGATCATTG/TTGGCTGTGCGAGGATTT |
| | *pad* -F/R | GGGAATACGAATGGTACGCTAAA/TGTGGGCTTCTTGGTCATTC |
| | *phosphoketolase* (housekeeping) -F/R | TGGCTGCTTCATGGTTCTC/CGGGAAAGGATAGTTGGGTTAG |
| *Lm. reuteri* DSM 20016 | *hcrA2* -F/R | CCTGAGGCTGTTGCTGATATT/CAGAGGTAACAGAGTGGTTGTATT |
| | *hcrA3* -F/R | GCGGCCTTAAAGAGTACAGTAG/GAAGCTTGGCGTCCATAAGA |
| | *fccA* -F/R | GTGTTCTGGAAGGGCAGTAATC/GTGGTGAAGGTGCAATCTTAGT |
| | *phosphoketolase*(housekeeping) -F/R | CAGAACACCAAGCTGAAGGA/GAATCAACAACACGACCGAATG |
| *Ap. kunkeei* DSM 12361 | *fccA* -F/R | GGTCGAGTTACCACCTAACTTATC/GTTCTGGTGCCACAGGATTA |
| | *phosphoketolase* (housekeeping) -F/R | TCAGCAAACACACCGAATAGA/ACTGGTTAGGTGCCGTTATG |

### 3.2.6. Construction of *Ff. milii* Δ*par1* and Δ*par2* mutants

Upstream and downstream flanking regions of target genes (800 to 1,000 bp) were PCR amplified and cloned into counterselection plasmid pVPL3002 (Zhang et al., 2018) using the ligation cycling reaction (LCR) (Kok et al., 2014). Primers for mutant construction are shown in Table 3.2. The resulting recombinant plasmids, pVPL3002/Δpar1 and pVPL3002/Δpar2, were transformed into *Escherichia coli* EC1000 (Leenhouts et al., 1996) and plated on Luria-Bertani (LB) medium containing 300 mg/L of erythromycin for selection. Electrocompetent *Ff. milii* FUA3583 cells were first transformed at 2.5 kV, 25 F, and 400 Ω with 4 g of plasmid pVE6007 (Sanders et al., 1998). After 3 h of recovery, cells were plated in the presence of 5 mg/L of chloramphenicol for selection of transformants. *Ff. milii* FUA3583 harboring pVE6007 was then transformed with 2 to 3 g of plasmid DNA (pVPL3002/Δpar1 or pVPL3002/Δpar2) using the same conditions as mentioned above. After recovery in medium with 5 mg/L of chloramphenicol, a fast-track genome editing approach was followed (Zhang et al., 2018). Briefly, the recovered cells were transferred into 40 ml of medium containing 2.5 mg/L of erythromycin and 5 mg/L of chloramphenicol. Cells were washed with medium not containing any antibiotics after 48 to 60 h of incubation. Cells were then subcultured 2 or 3 times in medium containing 2.5 mg/L of erythromycin. Cells were once again washed and subcultured into medium without any antibiotics for one passage, followed by plating on medium containing 500 mg/L of vancomycin for selection of double-crossover (DCO) mutants. *Ff. milii* was grown in MRS medium plus cysteine (0.5 g/L) at 37°C under anaerobic conditions during mutant construction. Mutants with deletions in *par1* and *par2* were confirmed using colony PCR, while the phenotype was tested using HPLC.

**Table 3.2. Primers for mutant construction.**

| Primer (forward, F; reverse, R) | Description | Primer Sequences (5'→3') |
|---|---|---|
| oVPL 188 F (Zhang et al., 2018) | amplifies pVPL3002 backbone | ATCCTCTAGAGTCGACCTGC |
| oVPL 187 R | | TACCGAGCTCGAATTCACTGG |
| par1 U/S F | upstream flanking region of *par1* in *Ff. milii* FUA3583 | GCAGCCAGATAGCCTGAAAC |
| par1 U/S R | | CGACTGGCAGTTGCGCCAGCTGCGC |
| par1 D/S F | downstream flanking region of *par1* in *Ff. milii* FUA3583 | AAGACGTTGGTCGTAAGGCCGTG |
| par1 D/S R | | CATAGCGGCAGTGAACTTGA |
| par1 BO1 | LCR bridging oligo for pVPL3002/Δpar1 | AAACGACGGCCAGTGAATTCGAGCT CGGTAGCAGCCAGATAGCCTGAAAC AATTCGTTGG |
| par1 BO2 | LCR bridging oligo for pVPL3002/Δpar1 | CTTTGGCGCAGCTGGCGCAACTGCC AGTCGAAGACGTTGGTCGTAAGGCC GTGGAGGAGA |
| par1 BO3 | LCR bridging oligo for pVPL3002/Δpar1 | GAATCCTTCATCAAGTTCACTGCCG CTATGATCCTCTAGAGTCGACCTGC AGGCATGCAA |
| par1 DCO F | DCO screening for *Δpar1* in *Ff. milii* FUA3583 | AATCGTTGATCCGGCATTAC |
| par1 DCO R | | TCACACGCGATAGGTCTGAG |
| par2 U/S F | upstream flanking region of *par2* in *Ff. milii* FUA3583 | ACGCATGGTCTACCAGTTCC |
| par2 U/S R | | TAACGGGTGTTACCACCTTCATG |
| par2 D/S F | downstream flanking region of *par2* in *Ff. milii* FUA3583 | ATCAGTATCTAGCCGCGCTATT |
| par2 D/S R | | GCAGTTGTCAGCAAGGAACA |
| par2 BO1 | LCR bridging oligo for pVPL3002/Δpar2 | AAACGACGGCCAGTGAATTCGAGCT CGGTAACGCATGGTCTACCAGTTCC TGAAACCGTG |
| par2 BO2 | LCR bridging oligo for pVPL3002/Δpar2 | AAACGGACATGAAGGTGGTAACAC CCGTTAATCAGTATCTAGCCGCGCT ATTAAAGACGC |
| par2 BO3 | LCR bridging oligo for pVPL3002/Δpar2 | TGCCAACGGATGTTCCTTGCTGACA ACTGCATCCTCTAGAGTCGACCTGC AGGCATGCAA |
| par2 DCO F | DCO screening for *Δpar2* in *Ff. milii* FUA3583 | GATTCCAATCGCCATAATGC |
| par2 DCO R | | CCATTAATTGCAGGCCAGTT |

### 3.2.7. Phylogenetic analysis

HcrB, Par1, and HcrF protein sequences were used as query sequences to search for homologs using BLAST on NCBI with default parameters across all species of *Lactobacillus* (old genus name before reclassification by Zheng et al., 2020) and *Pediococcus* for which genome sequences were available in July 2018. Sequences with greater than 80% query cover and 40% amino acid identity were retrieved for each species. Sequences differing by more than 10% in amino acid identity with respect to the query sequence within a species were also retrieved. Multiple-sequence alignment was performed using MUSCLE (Edgar, 2004). A maximum likelihood tree was constructed using IQ TREE web server (Trifinopoulos et al., 2016) using the best fit model predicted by ModelFinder (Kalyaanamoorthy et al., 2017). Bootstrap values for 1000 replicates were calculated with ultrafast bootstrap (UFBoot) (Hoang et al., 2018). Phylogenetic analysis for phenolic acid decarboxylase and vinylphenol reductase was also performed using the same process with protein sequences of Pad (YP_004891133.1) and VprA (YP_004890680.1) from *Lp. plantarum* WCFS1 used as query sequences for respective tree constructions. Tree visualization was done using the iTOL online tool (Letunic and Bork, 2016).

### 3.2.8. Data availability

The NCBI genome accession numbers for strains studied are as follows: NZ_AZFS00000000.1 (*Lv. hammesii* DSM 16381), GCA_000833395.1 (*Lv. brevis* TMW1.465), NC_009513.1 (*Lm. reuteri* DMS 20016), NZ_AZCK00000000.1 (*Ap. kunkeei* DSM 12361), NZ_SMZH00000000.1 (*Lm. fermentum* FUA3589), WEZQ00000000 (*Ff. milii* C5), WEZT00000000 (*Ff. milii* FUA3583), WEZR00000000 (*Lp. plantarum* TMW1.460), WEZU00000000 (*Lp. plantarum* FUA3584), and WEZS00000000 (*W. cibaria* 10M).

## 3.3. Results

### 3.3.1. Hydroxycinnamic acid metabolism of heterofermentative *Lactobacillaceae*

To assess the metabolism of hydroxycinnamic acids by heterofermentative *Lactobacillaceae*, strains were grown in the presence of sinapic acid, ferulic acid, or caffeic acid and metabolites were analyzed by reverse-phase high-performance liquid chromatography (HPLC) (Figure 3.1). Sinapic acid was metabolized exclusively by reduction, while ferulic and caffeic acids were converted by strain- or species-specific reduction and/or decarboxylation reactions (Figure 3.1). *Lp. plantarum* TMW1.460 and *Lp. plantarum* FUA3584 reduced sinapic acid to dihydrosinapic acid but differed with respect to the metabolism of ferulic and caffeic acids. *Lp. plantarum* TMW1.460 metabolized ferulic acid to dihydroferulic acid and vinylguaiacol by reduction and decarboxylation reactions, respectively, whereas the decarboxylation product vinylcatechol was the only metabolite from caffeic acid. *Lp. plantarum* FUA3584 reduced ferulic acid but decarboxylated caffeic acid. Vinylcatechol was not detected and its reduced form, ethylcatechol, was the only product (data not shown). *Ff. milii* strains also differed in metabolism of caffeic acid. *Ff. milii* C5 reduced all three substrates, while *Ff. milii* FUA3583 reduced sinapic and ferulic acids but metabolized caffeic acid mainly by decarboxylation. Metabolites observed in cultures of *Lm. fermentum* FUA3589 and *W. cibaria* 10M were similar to those for *Ff. milii* C5, showing exclusive reduction of all three substrates. *Lv. hammesii* and *Lv. brevis* strains solely displayed decarboxylation activity toward ferulic and caffeic acids and did not metabolize sinapic acid. *Lm. reuteri* DSM 20016 and *Lm. kunkeei* DSM 12361 showed no activity against any of the three substrates.

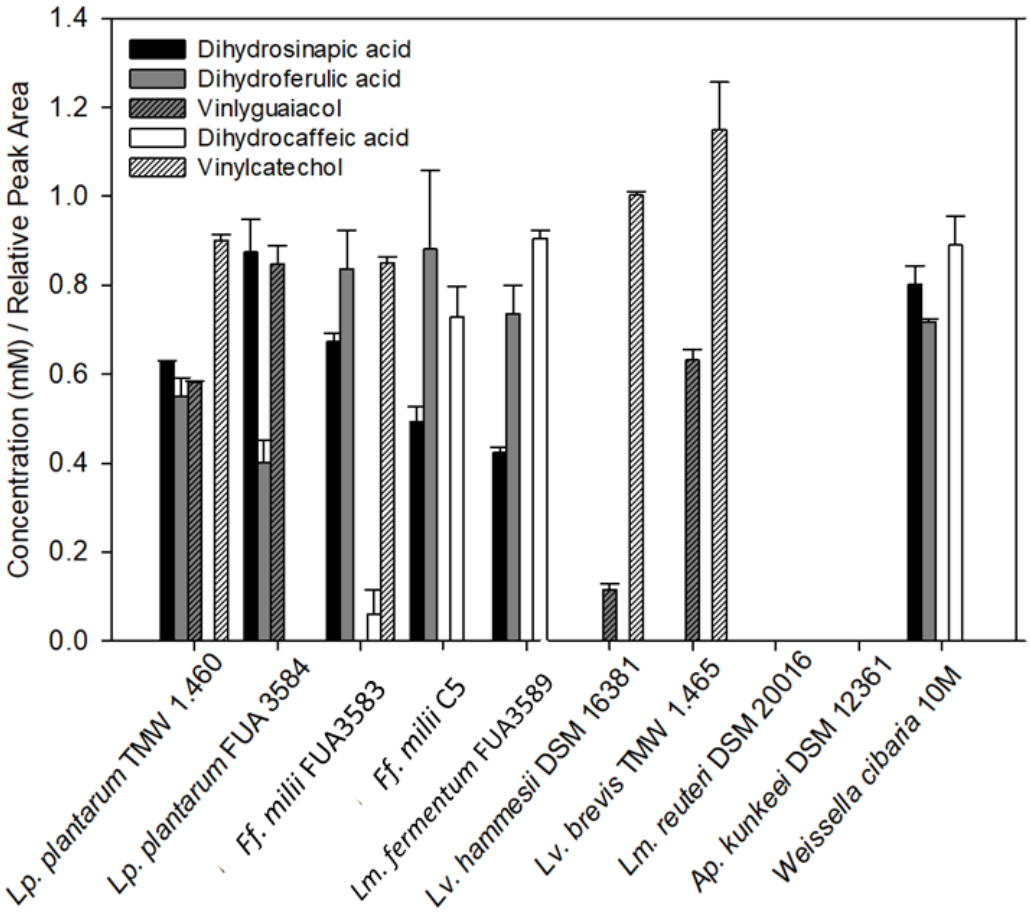



**Figure 3.1. Metabolites of phenolic acid conversion by strains of *Lactobacillaceae.***

Strains were incubated with 1 mM of different hydroxycinnamic acids for 24 h. Data are shown as means ± SDs from two independent experiments.

### 3.3.2. Identification of putative phenolic acid reductases

Putative phenolic acid reductases in *Ff. milii* and *Lm. fermentum* strains were identified by searching for homologs of phenolic acid reductase HcrB in *Lp. plantarum* WCFS1 (Santamaría et al., 2018a); other proteins that were encoded in the proposed operon, including HcrR, HcrA, and HcrC, were additionally used as query sequences (Table 3.3). *Lp. plantarum* TMW1.460 and FUA3584 contained the complete operon including the genes encoding HcrR, HcrA, HcrB, and HcrC; amino acid similarities to the protein sequences in *Lp. plantarum* WCFS1 were 98% or

higher. *Lm. fermentum* also harbored an HcrR homolog (Table 3.3). In *Ff. milii* C5 and FUA3583, a LysR-type transcriptional regulator encoded by parR with 28% amino acid identity to HcrR was present (Table 3.3). HcrR homologs in *Ff. milii* and *Lm. fermentum* were encoded in proximity of putative phenolic acid reductases. Homologs of HcrA were present in all reductase-positive strains as well in *Lm. reuteri* DSM 20016 and *W. cibaria* 10M. Two HrcB homologs, Par1 and Par2, were identified in *Ff. milii* C5 and *Ff. milii* FUA3583, but these proteins exhibited an amino acid identity of only 25 to 26% to HcrB. Of note, Par1 and Par2 homologs were also present in both *Lp. plantarum* strains. An HcrB homolog in *Lm. fermentum* FUA3589, termed HcrF, is 63% identical to HcrB but has the same size as Par1/Par2 (Table 3.3). A protein annotated as flavocytochrome c containing the fumarate reductase flavoprotein subunit FccA (Pealing et al., 1999) was present in all strains except *Lv. hammesii* and *Lv. brevis*. The amino acid identity of all of the FccA proteins to HcrB was less than 34%, and the protein length was only 462 to 464 amino acids. HcrC was present only in *Lp. plantarum* (Table 3.3).

A potential role of HcrB homologs in phenolic acid metabolism was further assessed by comparison of the domain architecture of the proteins. HcrB consists of 3 domains, an NADH binding domain, a flavin mononucleotide (FMN) binding domain, and a flavin adenine dinucleotide (FAD) binding domain (Figure 3.2). Par1/Par2 and HcrF contained two of the HcrB domains; the domain organization in HcrF matches that of HcrB, but the domain organization in Par1 and Par2 differs from HcrB. The fumarate reductase flavoprotein subunit FccA contained only one FAD binding protein domain. HcrR and ParR proteins belong to the same LysR transcriptional regulator family.

**Table 3.3. *In silico* identification of putative phenolic acid reductases using *Lp. plantarum* WCFS1 protein sequences as query.**

a) Protein accession numbers for HcrR, HrcA, and HcrB are YP_004889274.1, YP_004889275.1, and YP_004889276.1, respectively. aa, amino acids. b) Similar results were obtained for *Ff. milii* C5 and FUA3583; only results for *Ff. milii* C5 are shown.

| **Bacterial Strains** | ***L. plantarum* WCFS1 query proteins** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **HcrR[a], – 315 aa** | | | **HcrA[a]– 204 aa** | | | **HcrB[a]– 812 aa** | | |
| | Protein Name | Amino acid identity (%) | Protein length (aa) | Protein Name | Amino acid identity (%) | Protein length (aa) | Protein Name | Amino acid identity (%) | Protein length (aa) |
| *Ff. milii* C5[b] | ParR | 28 | 304 | HcrA2 / HcrA3 | 52 / 47 | 202 / 452 | Par1 / Par2<br>FccA | 25 / 26<br>33 | 614 / 616<br>465 |
| *Lm. fermentum* FUA 3589 | HcrR | 43 | 308 | HcrA2 / HcrA3 | 52 / 49 | 203 / 450 | HcrF<br>FccA | 63<br>33 | 617<br>464 |
| *Lv. hammesii* DSM 16381 | - | - | - | - | - | - | - | - | - |
| *Lv. brevis* TMW 1.465 | - | - | - | - | - | - | - | - | - |
| *Lm. reuteri* DSM 20016 | - | - | - | HcrA2<br>HcrA3 | 39<br>46 | 190<br>416 | FccA | 34 | 464 |
| *Ap. kunkeei* DSM 12361 | - | - | - | - | - | - | FccA | 30 | 462 |
| *W. cibaria* 10M | - | - | - | HcrA2 | 50 | 283 | FccA | 32 | 464 |

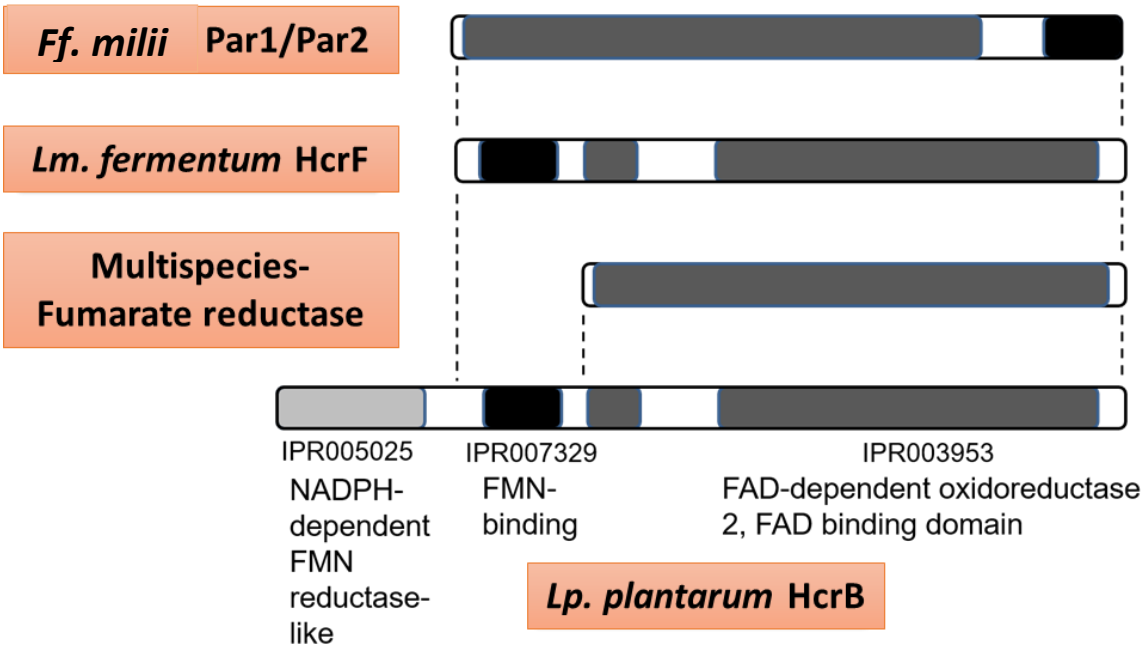



**Figure 3.2. Domain architecture of protein sequences of putative phenolic acid reductases.**

HcrB from *Lp. plantarum* WCFS1 was used as reference for comparison. InterPro domain names along with their InterPro identifiers are provided. The same colors represent conserved domains across sequences.

### 3.3.3. Expression profile of putative phenolic acid reductases

To further elucidate a potential role of putative phenolic acid reductases role in hydroxycinnamic metabolism, the gene expression by exponentially growing cells in response to phenolic acids was quantified by reverse transcription-quantitative PCR (RT-qPCR). *Lp. plantarum* TMW1.460 and FUA3584 overexpressed ($P < 0.05$) *hcrA*, *hcrB*, and *hcrC* in the presence of hydroxycinnamic acids at early exponential phase (Figure 3.3). The genes coding for phenolic acid decarboxylase activity were overexpressed in response to ferulic and caffeic acids; other genes, including the homologs of *par1* and *par2* (both strains) and *vprA* in *Lp. plantarum* FUA3584, were not overexpressed (Figure 3.3).

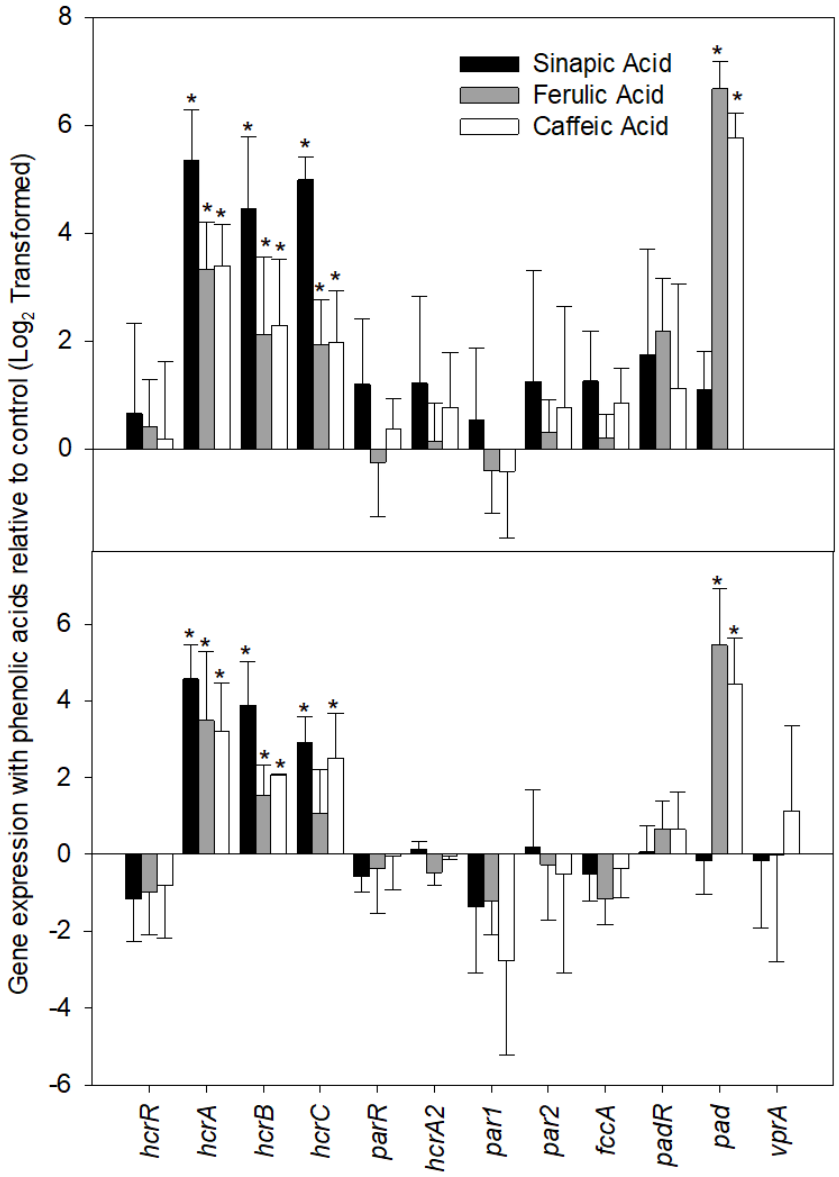



**Figure 3.3. Relative expression of phenolic acid metabolism genes by strains of *Lp. plantarum*.**

*Lp. plantarum* TMW1.460 (top panel); *Lp. plantarum* FUA3584 (bottom panel). Strains were incubated with 1 mM concentrations of different hydroxycinnamic acids in mMRS broth, and broth without any hydroxycinnamic acids was used as the reference condition. An asterisk indicates that the gene is significantly overexpressed ($P < 0.05$) with respect to its expression under the reference condition. Genes and their corresponding proteins or other descriptions are as follows: *hcrR*- regulator of *hcr* operon; *hcrABC*- phenolic acid reductase genes; *parR*- putative regulators of phenolic acid reductase; *par1* and *par2*- putative phenolic acid reductase; *hcrA2*- homolog of *hcrA* that is not part of a phenolic acid reductase operon; *fccA*- subunit of fumarate reductase; *padR*- phenolic acid decarboxylase transcriptional regulator; *pad*- phenolic acid decarboxylase, and *vprA*- vinyl phenol reductase.

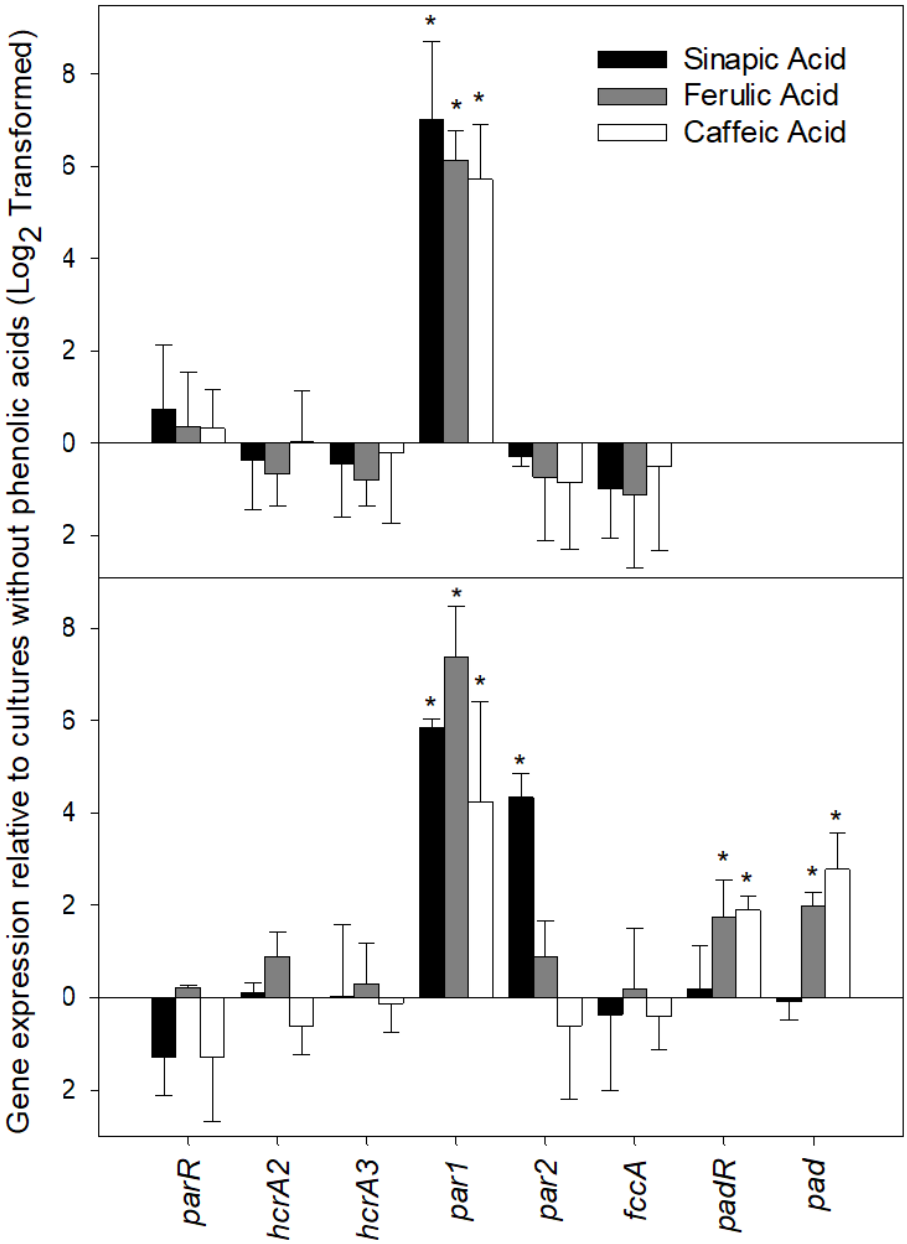



**Figure 3.4. Relative expression of phenolic acid metabolism genes by strains of *Ff. milii.***

*Ff. milii* C5 (top panel); *Ff. milii* FUA3583 (bottom panel). Strains were incubated with 1 mM concentrations of different hydroxycinnamic acids in mMRS broth, and broth without any hydroxycinnamic acids was used as the reference condition. An asterisk indicates that the gene is significantly overexpressed ($P < 0.05$) with respect to its expression under the reference condition. *hcrA3* is a homolog of *hcrA* that is not part of a phenolic acid reductase operon; for descriptions of the rest of the genes, see the legend to Figure 3.3.

*Ff. milii* C5 and FUA3583 both overexpressed *par1* in the presence of all three substrates ($P < 0.05$) (Figure 3.4). Interestingly, its homolog *par2* was overexpressed 20-fold ($P < 0.05$) in *Ff. milii* FUA3583 when cultured with sinapic acid but not in *Ff. milii* C5. The *padR* and *pad* genes of *Ff. milii* FUA3583 were overexpressed in the presence of ferulic and caffeic acids.

*Lm. fermentum* FUA3589 overexpressed ($P < 0.05$) *hcrF* in the presence of caffeic acid (Figure 3.5). Surprisingly, *pad* was not overexpressed in the presence of any of the three substrates. The genes *hcrA2, hcrA3,* and *fccA* were never overexpressed under any condition, and differentially expressed genes were not found in *W. cibaria* 10M or in reductase-negative strains when cultured with hydroxycinnamic acids.

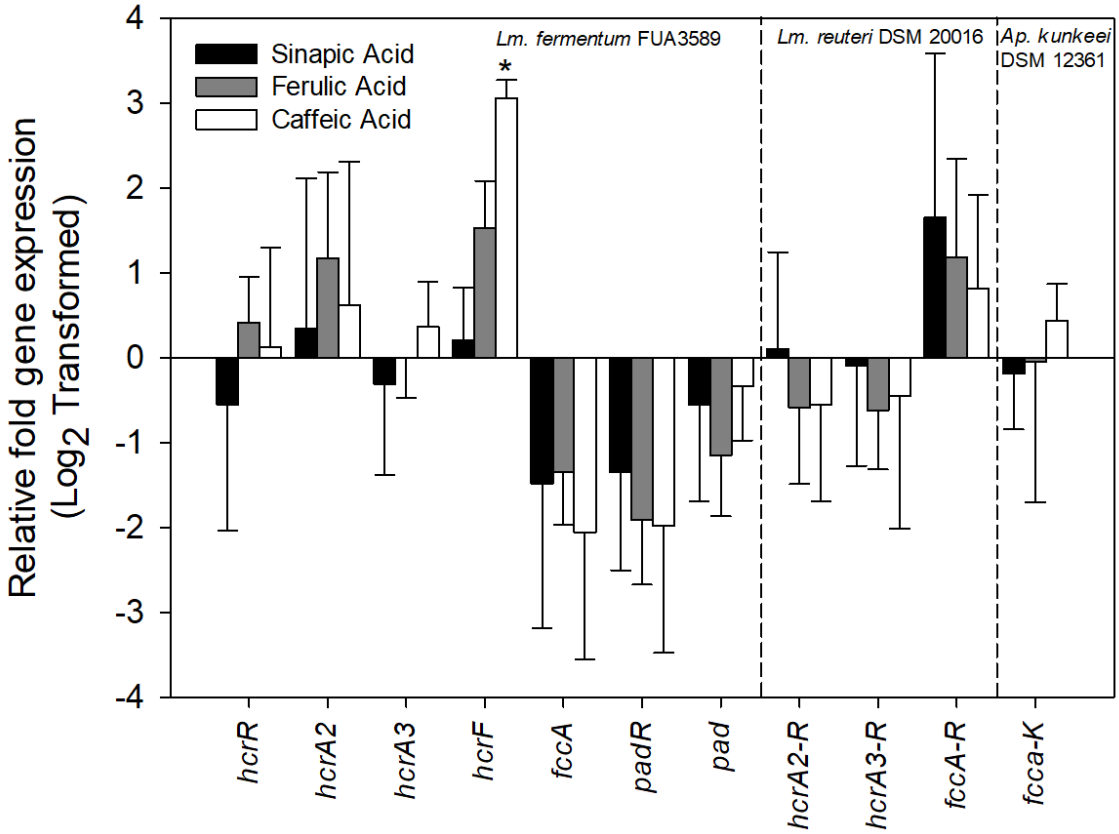



**Figure 3.5. Relative expression of phenolic acid metabolism genes by strains of *Lm. fermentum, Lm. reuteri* and *Ap. kunkeei.***

Strains were incubated with 1 mM concentrations of different hydroxycinnamic acids in mMRS broth, and broth without any hydroxycinnamic acids was used as the reference condition. An asterisk indicates that

the gene is significantly overexpressed ($P < 0.05$) with respect to is expression under the reference condition. *hcrF* encodes a putative phenolic acid reductase (HcrB homolog) in *Lm. fermentum*; for descriptions of the rest of the genes, see the legend to Figure 3.

### 3.3.4. Phylogenetic analysis of major genes involved in hydroxycinnamic acid metabolism

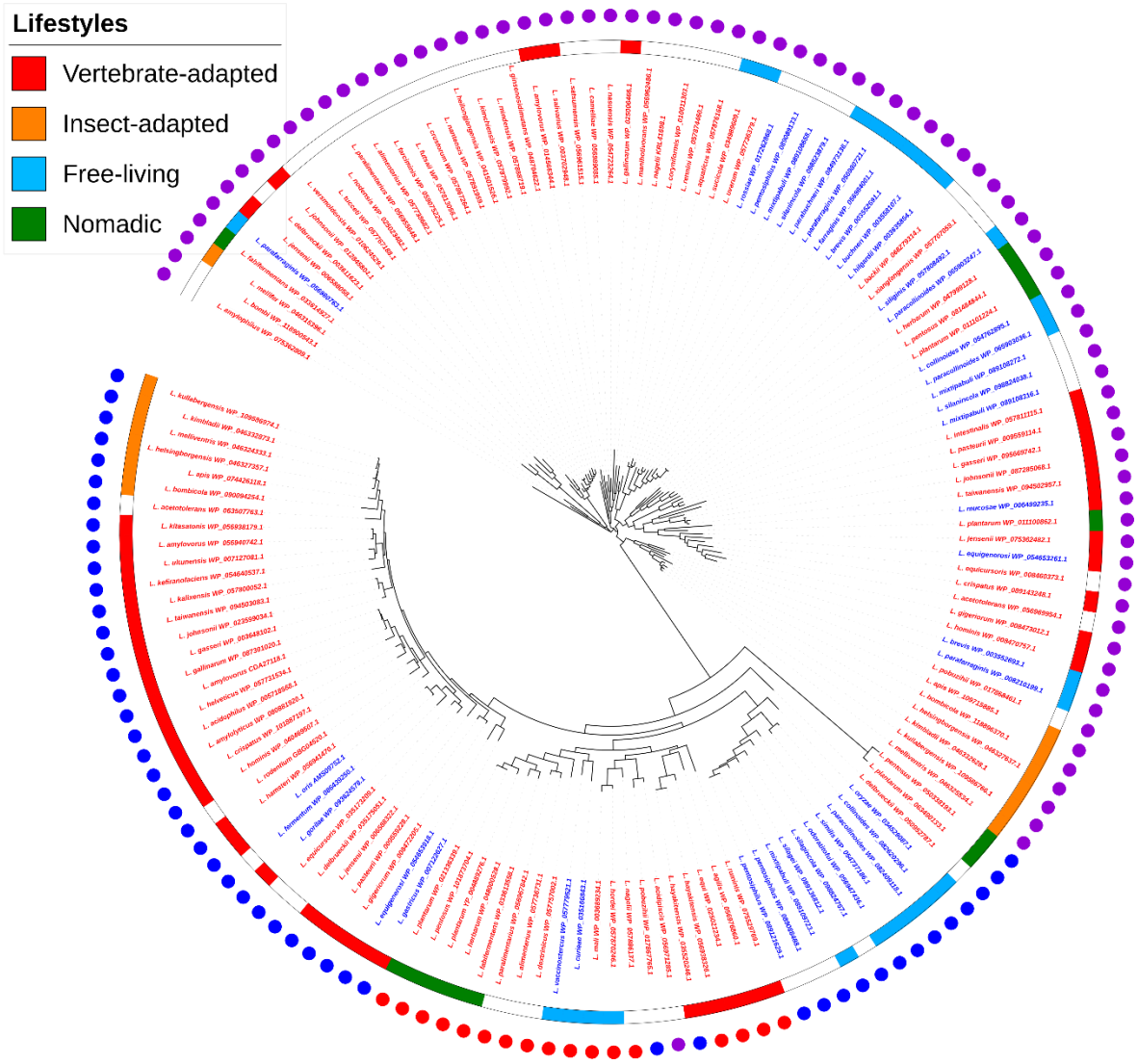
The presence of 3 types of phenolic acid reductases were analyzed, those corresponding to *hcrB, hcrF*, and *par1*/*par2*, along with the only known phenolic acid decarboxylase (corresponding to *pad*) and the recently characterized vinylphenol reductase (corresponding to *vprA*), across all the sequenced *Lactobacillus* spp. (old nomenclature prior to reclassification by Zheng et al., 2020) and *Pediococcus* spp. Out of the 196 species analyzed, 98 *Lactobacillaceae* spp. contained at least one phenolic acid reductase (Figure 3.6a). The most abundant phenolic acid reductase was that corresponding to *par1/par2*, which is present in 68 *Lactobacillaceae* spp., while that corresponding to *hcrB* was the least abundant and present in only 16 *Lactobacillaceae* spp. Phenolic acid reductases were not identified in any of the *Pediococcus* spp.

Thirty-four *Lactobacillaceae* spp. contained more than one type of phenolic acid reductase. Two species, *Lp. plantarum* and *Lp. pentosus*, contained homologs for all three types of phenolic acid reductases. *Ligilactobacillus hayakitensis* was the only species which had both *hcrB* and *hcrF* phenolic acid reductases, while 5 species encoded both *hcrB* and *par1/par2* phenolic acid reductases. The most abundant combination in species having more than one type of phenolic acid reductase was *hcrF* and *par1/par2*, with 26 *Lactobacillaceae* spp. having strains with both the genes.

Sixty-nine *Lactobacillus* spp. (old nomenclature) and 7 *Pediococcus* spp. carried *pad* (Figure 3.6b). The corresponding protein sequence had a very high degree of conservation; proteins identified in *Lactobacillaceae* were more than 76% identical to Pad in *Lp. plantarum* WCFS1. The

only exception is Pad from *Fructilactobacillus florum*, which had 60% amino acid identity with the query sequence (data not shown). VprA was present in 34 *Lactobacillaceae* spp. (Figure 3.6c). Interestingly, 15 *Lactobacillaceae* spp. encoded VprA but not Pad. Only 19 *Lactobacillaceae* spp. possess genes responsible for both decarboxylation of hydroxycinnamic acids to vinyl derivatives and for further reducing them to their ethyl derivatives.

a)



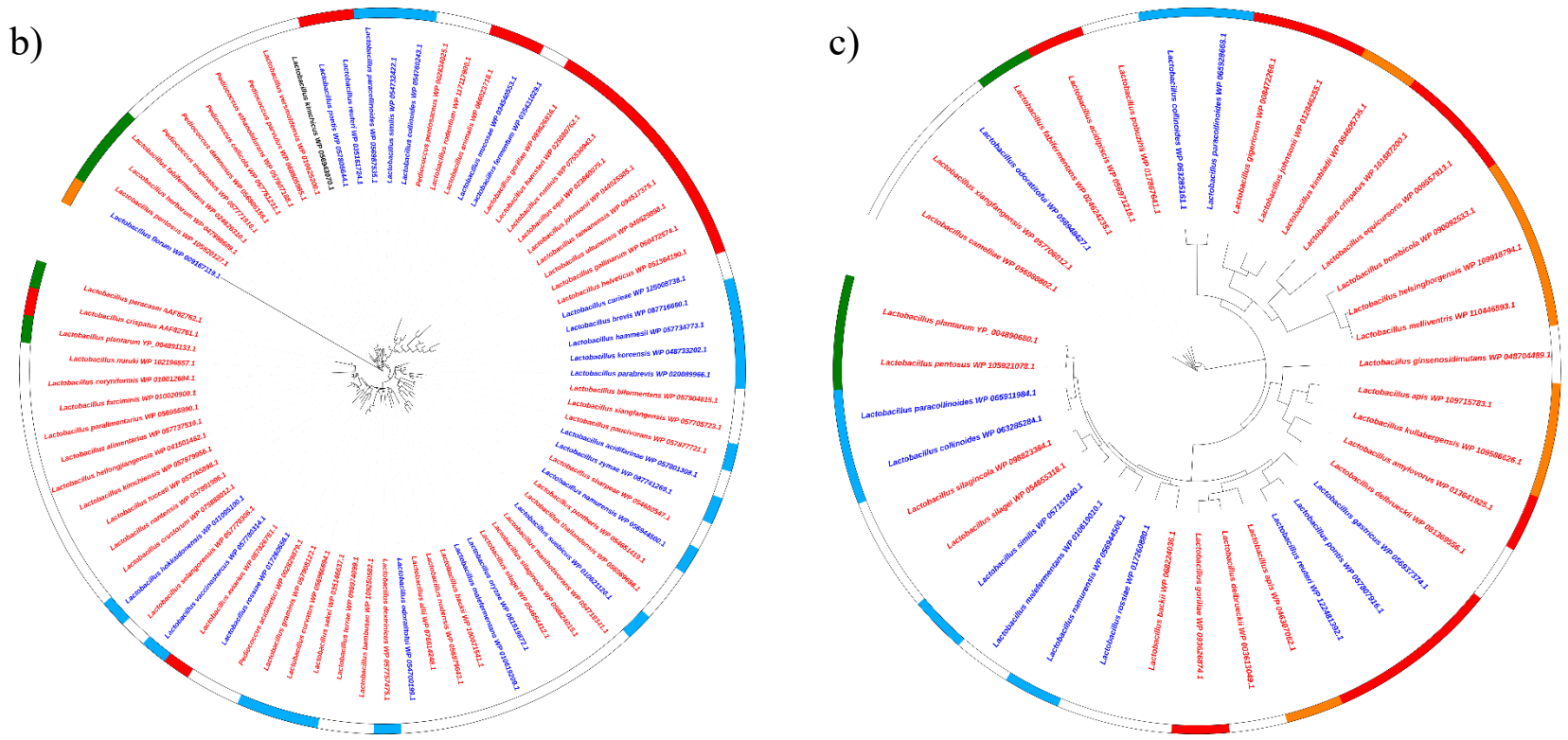



**Figure 3.6. Phylogenetic analysis of phenolic acid metabolism genes across *Lactobacillaceae* strains.**

Phylogenetic analysis was performed on 196 *Lactobacillus* (old genus name before reclassification by J. Zheng et al., 2020) and *Pediococcus* spp. The name of the species followed by the NCBI protein accession number is provided. Homofermentative and heterofermentative species are represented by red and blue colors, respectively. The color strip represents the lifestyle for species from the work of Duar et al., 2017. (A) Phylogenetic analysis of HcrB, HcrF, and Par1/Par2. Color of the solid circles indicates the phenolic acid reductase the given sequence is homologous to, as follows: red, HcrB; blue, HcrF; and purple, Par1/Par2. (B) Phylogenetic analysis of Pad. (C) Phylogenetic analysis of VprA. Strains names represented on the tree follow the nomenclature prior the recent reclassification of *Lactobacillus* genus by J. Zheng et al., 2020.

### 3.3.5. Comparison of genotype and phenotype in *Lactobacillaceae*

To determine whether the three putative novel phenolic acid reductases explain phenolic acid metabolism in the *Lactobacillaceae* species observed in this study, genotype and phenotype are compared in Table 3.4. In *Lactobacillaceae*, genotype and phenotype always matched, i.e., the metabolism of a certain compound was accurately predicted by the presence or absence of the metabolic genes (Table 3.4). Exceptions pertained only to the alternative metabolism by decarboxylation or reduction of hydroxycinnamic acids. *Ff. milii* FUA3583 and *Lm. fermentum* FUA3589 possessed decarboxylase and reductase genes with activity on caffeic and ferulic acids; for ferulic acid, however, only the reduced dihydroferulic acid was observed. This may indicate that the decarboxylase is not active or not expressed, or it may reflect the strong preference of heterofermentative *Lactobacillaceae* for cofactor regeneration (Filannino et al., 2014). *Lm. fermentum* FUA3589 exclusively reduced all substrates, but *pad* was not overexpressed in the presence of hydroxycinnamic acids. Of note, the *vprA*-positive strain *Lp. plantarum* FUA3584 reduced vinylcatechol but not vinylguaicol, suggesting a differential regulation of the enzyme and/or higher specificity toward vinylphenol and vinylcatechol (Reverón et al., 2012; Santamaría et al., 2018b). The *Ff. milii* FUA3583 Δ*par1* mutant did not reduce any of the substrates but decarboxylated ferulic acid to produce vinyguaiacol, which was not detected in the wild-type strain. The metabolism of hydroxycinnamic acids by the *Ff. milii* FUA3583 Δ*par2* mutant was identical to that of the wild-type strain. The present study did not identify any of the genes coding for reduction of hydroxycinnamic acids in *Weissella cibaria*.

**Table 3.4. Summary of hydroxycinnamic acid metabolism of *Lactobacillaceae* strains.**

(+) denotes presence of genotype with text representing the type of phenolic acid reductase present with overexpression under the influence of a specific substrate. (-) denotes absence of a genotype. Shaded and unshaded boxes represent presence and absence of phenotype respectively.

| **Strain Name** | **Sinapic Acid** | **Ferulic Acid** | | **Caffeic Acid** | | |
|---|---|---|---|---|---|---|
| | Dihydro-sinapic acid | Dihydroferulic acid | 4-Vinylguaiacol (Pad) | Dihydrocaffeic acid | 4-Vinylcatechol (Pad) | 4-Ethylcatechol (VprA) |
| *Lp. plantarum* TMW1.460 | + (*hcrB*) | + (*hcrB*) | + | + (*hcrB*) | + | - |
| *Lp. plantarum* FUA3584 | + (*hcrB*) | + (*hcrB*) | + | + (*hcrB*) | + | + |
| *Ff. milii* FUA3583 | + (*par1/ par2*) | + (*par1*) | + | + (*par1*) | + | - |
| *Ff. milii* FUA3583 Δ*par1* | - (*par2)* | - | + | - | + | - |
| *Ff. milii* FUA3583 Δ*par2* | + (*par1*) | + (*par1*) | + | + (*par1*) | + | - |
| *Ff. milii* C5 | + (*par1*) | + (*par1*) | - | + (*par1*) | - | - |
| *Lm. fermentum* FUA3589 | + (*hcrF*) | + (*hcrF*) | + | + (*hcrF*) | + | - |
| *Lv. hammesii* DSM 16381 | - | - | + | - | + | - |
| *Lv. brevis* TMW1.465 | - | - | + | - | + | - |
| *Lm. reuteri* DSM 20016 | - | - | - | - | - | - |
| *Ap. kunkeei* DSM 12361 | - | - | - | - | - | - |
| *Weissella cibaria* 10M | - | - | - | - | - | - |

### 3.4. Discussion

The pathway for hydroxycinnamic acid metabolism in lactic acid bacteria has been known for over a decade, with the first enzyme characterized being phenolic acid decarboxylase in *Lp. plantarum* (Cavin et al., 1997). The first hydroxycinnamic acid reductase was also characterized in *Lp. plantarum* (Santamaría et al., 2018a), but this phenolic acid reductase is absent in many species that reduce hydroxycinnamates (this study). The genomic diversity observed between homofermentative and heterofermentative *Lactobacillaceae* (Zheng et al., 2015) indicates that novel enzymes may contribute to hydroxycinnamic acid metabolism in heterofermentative *Lactobacillaceae*. This study identified several putative phenolic acid reductases in heterofermentative *Lactobacillaceae*. Evidence for the contribution of phenolic acid reductases to conversion of hydroxycinnamic acids was based on (i) characterization of the strain-specific metabolism of hydroxycinnamic acids, (ii) comparative genomic analyses to identify putative phenolic acid reductases, (iii) identification of those putative enzymes that were overexpressed in the presence of their substrates and (iv) characterization of the impact of deletion of *par1* or *par2* in *Ff. milii* on conversion of hydroxycinnamic acids.

HcrR is a regulator of phenolic acid reductase in *Lp. plantarum* (Santamaría et al., 2018a). An HcrR homolog or ParR, a LysR-type transcriptional regulator identified in *Ff. milii*, was present in all *Lactobacillaceae* that reduced hydroxycinnamic acids, suggesting that this metabolism is generally regulated (Santamaría et al., 2018a). An HcrR or ParR homolog was not identified in *W. cibaria* 10M, but the lack of any recognizable genes for phenolic acid metabolism in that strain prevents any further conclusions related to *Weissella*.

The *hcrA* homologs present in heterofermentative hydroxycinnamic acid reducers were not upregulated upon induction, nor are these genes part of operons related to phenolic acid

metabolism. They resemble the genes for previously characterized NADH-dependent flavin reductases in *L. johnsonii* that play a role in hydrogen peroxide production (Hertzberger et al., 2014). Moreover, HcrA homologs were also identified in *Lm. reuteri*, which does not metabolize hydroxycinnamic acids. HcrA in *Lp. plantarum* WCFS1 does not possess enzymatic activity, and the absence of reductase activity of *hcrA* knockout mutants might be explained by prevention of induction of hcrB, which is cotranscribed and present downstream of *hcrA* (Santamaría et al., 2018a).

Most *Lactobacillaceae*, with exception of the two species of the *Lv. brevis*, harbored *fccA* (Filannino et al., 2016b, 2015, 2014; Ripari et al., 2019; Sánchez-Maldonado et al., 2011). FccA is 30 to 34% identical to HcrB and particularly contains the same FAD binding domain. Its presence in reductase-negative strains *Lm. reuteri* DSM 20016 and *Ap. kunkeei* DSM 12361, lack of a significant upregulation upon induction with any of the substrates, and the high similarity to *Shewanella frigidimarina* FccA, which is active as fumarate reductase (Pealing et al., 1999), makes an involvement in phenolic acid metabolism unlikely.

HcrF in *Lm. fermentum* was more than 60% identical to HcrB in *Lp. plantarum* but lacked the NADH binding domain, suggesting that only FMN binding and FAD binding domains are essential for reduction of phenolic acids. The presence of *hcrR* upstream of *hcrF* suggests that the *hcrR* LysR family transcriptional regulator is responsible for transcriptional regulation of *hcrF* (Santamaría et al., 2018b, 2018a). High amino acid identity of HcrF with HcrB in the aligned region and significant overexpression indicate its likely role in hydroxycinnamic acid metabolism.

*Ff. milii* C5 and FUA3583 reduced all three hydroxycinnamic acids but lacked either *hcrB* or *hcrF*. Two genes coding for putative phenolic acid reductases, *par1* and *par2*, were identified in the genomes of *Ff. milii* strains. Despite the low amino acid identity, the presence of a domain

architecture similar to that of other phenolic acid reductases points toward their role in reduction of hydroxycinnamic acids in *Ff. milii*. In addition, both strains overexpressed *par1* in the presence of hydroxycinnamic acids, while only sinapic acid induced *par2* expression only in *Ff. milii* FUA3583. The lack of reductase activity observed in the *Ff. milii* FUA3583 Δ*par1* mutant confirms its contribution as a phenolic acid reductase active on hydroxycinnamic acids. Homologs of Par1 and Par2 are also present in *Lp. plantarum* but do not contribute to reduction of hydroxycinnamic acids (Santamaría et al., 2018a). This, with the lack of influence of the *Ff. milii* FUA3583 Δ*par2* mutant on the phenotype, further indicates the presence of an unknown spectrum of related compounds which can be potentially metabolized by lactic acid bacteria.

*W. cibaria* 10M showed phenolic acid reductase activity, comparable to other strains of *W. cibaria* that were evaluated with respect to phenolic acid metabolism but not genome sequenced (Filannino et al., 2014). Surprisingly, none of the three phenolic acid reductases were found in the sequenced genome or upon BLAST search against all genome sequenced strains of *Weissella* species in the NCBI database. These results indicate the presence of unidentified phenolic acid reductases in *Weissella* species.

Hydroxycinnamic acid metabolism in *Lactobacillaceae* is highly strain specific(Filannino et al., 2016b, 2015, 2014; Ripari et al., 2019). Heterofermentative *Lactobacillaceae* use hydroxycinnamic acids as external electron acceptors (Filannino et al., 2014); in addition, reduction or decarboxylation of phenolic acids reduces their antimicrobial activities (Sánchez-Maldonado et al., 2011). Three *pad* genes have been previously characterized from different *Lactobacillaceae* (Cavin et al., 1997; Landete et al., 2010; Rodríguez et al., 2008). The differences in their substrate specificity can be related to minor amino acid differences in the C-terminal region (Landete et al., 2010). There is also evidence of other unidentified phenolic acid decarboxylases

(Barthelmebs et al., 2000), suggesting differing genotypes among strains and/or a differential regulation. The *Clostridium* 2-enoate reductases also show strain-specific substrate specificity for reduction of hydroxycinnamic acids (Mordaka et al., 2018; Sun et al., 2016). In *Lactobacillaceae*, hydroxycinnamic acids are reduced by three different enzymes, HcrB, HcrF, and Par1, with different strains and species having different substrate specificities and/or regulation explaining their differential hydroxycinnamic acid metabolism (this study). Remarkably, Par1 contributes to the metabolism of hydroxycinnamic acids in *Ff. milii* (this study) but not in *Lp. plantarum* (Santamaría et al., 2018a), and deletion of Par2 did not alter hydroxycinnamic acid metabolism in *Ff. milii*. The presence of metabolic enzymes with unknown substrates suggests that the metabolic toolset of *Lactobacillaceae* to metabolize plant secondary metabolites is more extensive than currently known.

To relate the ecological relevance of phenolic acid metabolism in *Lactobacillaceae*, *Lactobacillaceae* genomes were screened for the presence or absence of genes related to phenolic acid metabolism. Species belonging to 11 of 24 *Lactobacillaceae* phylogenetic groups (Zheng et al., 2015) were represented on the tree. Species of the *Lp. plantarum* group are capable of metabolizing hydroxycinnamic acids, which can be attributed to their broad metabolic potential that relates to the nomadic lifestyle (Duar et al., 2017). Hydroxycinnamic acid metabolism was also frequently identified in species of the *Companilactobacillus alimentarius* group. Genomes of several species known to reduce hydroxycinnamic acids, including *Lactobacillus crispatus*, *Secundilactobacillus collinoides*, and *Lentilactobacillus hilgardii,* contained *par1/par2* sequences as sole genes for phenolic acid reductases (Buron et al., 2011; de las Rivas et al., 2009; van Beek and Priest, 2000). Heterofermentative *Lactobacillaceae* harboring *hcrF* exclusively belonged to the *Lm. reuteri* and *Secundilactobacillus collinoides* groups, while homofermentative species with

*hcrF* belonged to the Lactobacillus delbrueckii group, including *Lactobacillus gasseri*. Some *L. gasseri* strains also displayed reductase activity in the absence of *hcrB* (Oh et al., 2016). *Lm. reuteri* group organisms and *L. delbrueckii* group organisms share the same ecological niche in the intestine of humans and animals (Duar et al., 2017; Leser et al., 2002; Lin et al., 2018; Tannock et al., 2012); the almost exclusive presence of HrcF in these species may reflect a lifestyle adaptation. With the exception of *Lentilactobacillus curieae* and *Paucilactobacillus vaccinostercus*, the 16 species harboring HcrB were all homofermentative.

Metabolism of hydroxycinnamic acids among heterofermentative *Lactobacillaceae* was most frequent in species with a free-living lifestyle, particularly in organisms of the *S. collinoides* and *Lentilactobacillus buchneri* groups. These organisms were mostly isolated from plant or fermented plant products, where they regularly encounter phenolic acids (Duar et al., 2017; Gänzle, 2019). None of the heterofermentative insect-adapted *Lactobacillaceae* possessed genes related to phenolic acid metabolism. Vertebrate-adapted *Lactobacillaceae* of the *Lm. reuteri*, *L. delbrueckii*, and *Ligilactobacillus salivarius* groups also have a significant presence on the three trees, which may relate to their interaction with phenolic acids passing through the gut due to consumption of foods and dietary fibers rich in phenolic compounds by the host (Acosta-Estrada et al., 2014). Seven of the 13 species that belong to the *L. delbrueckii* group harbor *vprA* but not *pad* and are species with an insect-adapted lifestyle. Phenolic acid decarboxylase and vinyl reductase are thought to be part of the same metabolic pathway (Santamaría et al., 2018b), and the presence of only VprA may point to syntrophic interactions among insect gut microbiota. Alternatively, VprA homologs in insect-adapted *Lactobacillaceae* may be active on substrates that relate to the intestine of bees but are not related to vinyl derivatives of hydroxycinnamic acids (Kwong and Moran, 2015).

In conclusion, the current study expanded knowledge on the genetic determinants of the diverse metabolism of hydroxycinnamic acids by lactic acid bacteria that explain species- and strain-specific metabolic differences. Two novel phenolic acid reductases were identified, with Par1 being active in *Ff. milii* and HcrF being likely responsible in *Lm. fermentum* for reduction of hydroxycinnamic acids. The phenotypic analysis of strains suggests that the genotype, the substrate specificity of the enzymes, and the regulation of gene expression are responsible for the strain-specific differences in metabolism. This study also provided evidence for additional, yet-uncharacterized phenolic acid reductases in *Weissella* spp. In addition, Par1/Par2 enzymes with homologies to phenolic acid reductases in *Lp. plantarum* along with *par2* in *Ff. milii* appear to be inactive on hydroxycinnamic acids and thus may convert related secondary metabolites of plants or fungi expanding the utility of lactic acid bacteria.

# Chapter 4- Contribution of phenolic acid metabolism of *Furfurilactobacillus milii* to ecological fitness in cereal fermentations

## 4.1. Introduction

Fermentation of plant material with lactic acid bacteria reduces the level of antinutritive components including phenolic compounds (Filannino et al., 2018; Gänzle, 2020). Metabolism of phenolic acids has been considered strain specific and involves the release of phenolic acids from soluble or insoluble esters, followed by decarboxylation and / or reduction (Filannino et al., 2015; Ripari et al., 2019). The enzymes involved in metabolism of hydroxycinnamic and hydroxybenzoic acids have been identified and characterized (Chapter 3; Muñoz et al., 2017; Santamaría et al., 2018a) but their contribution to metabolite production in actual food systems has not been studied extensively. Hydroxybenzoic acids are decarboxylated by lactic acid bacteria while hydroxycinnamic acids are either reduced to the corresponding dihydro-derivatives, or decarboxylated to vinyl-derivatives, which can be further reduced to ethyl-derivatives (Sánchez-Maldonado et al., 2011). The antimicrobial activity of the phenolic acid metabolites is lower than the activity of free phenolic acids, thus the conversion was described as a means of detoxification (Sánchez-Maldonado et al., 2011). In heterofermentative *Lactobacillaceae*, the NADH-dependent reduction of hydroxycinnamic acids increases the ATP yield in the phosphoketolase pathway (Filannino et al., 2016b, 2014). Thus, the metabolism of phenolic acids may increase the ecological fitness of lactic acid bacteria during growth in substrates with high concentration of phenolic acids.

Sorghum has a higher content of diverse polyphenols when compared to other cereals including maize, wheat and rye (Awika and Rooney, 2004; Dykes and Rooney, 2006). Sorghum is a major crop popular in Africa and Asia, ranking 5$^{th}$ in the worldwide production among cereal crops

(Xiong et al., 2019). The diversity and concentration of phenolic compounds in sorghum vary among different cultivars of sorghum and is closely associated with colour (Xiong et al., 2019). Red sorghum is abundant in phenolic acids and their glycerol esters, 3-deoxyanthocyanidins, condensed tannins and flavonoids (Svensson et al., 2010). The high content of phenolic compounds in sorghum has been associated with health benefits (Hullings et al., 2020; Xiong et al., 2019). Sorghum phenolics, however, also impart bitterness and astringency and particularly red and black sorghum cultivars are generally consumed after fermentation or malting to reduce bitterness (Kobue-Lekalake et al., 2007). Phenolic compounds with antimicrobial activity also select for fermentation organisms that are resistant to the antimicrobial activity of sorghum phenolics and maintain an extensive toolset for conversion of phenolic compounds (Pswarayi et al., 2022; Sekwati-Monang et al., 2012).

Lactic acid bacteria in cereal fermentations include *Fructilactobacillus sanfranciscensis*, which occurs only in wheat and rye sourdoughs that are propagated as a leavening agent. It is often associated with *Levilactobacillus* and *Companilactobacillus* species, *Lactobacillus* and *Limosilactobacillus* species, which occur in back-slopped type II sourdoughs, and *Lactiplantibacillus* or *Pediococcus* species, which are characteristic for spontaneous cereal fermentations (Gänzle and Zheng, 2019; Van Kerrebroeck et al., 2017). *Furfurilactobacillus* species were isolated from back-slopped sourdoughs as well as spontaneous millet fermentations in Europe, Asia, and Africa and most isolates of furfurilactobacilli were obtained from cereal fermentations (Corsetti et al., 2005; Pswarayi and Gänzle, 2019; Ripari et al., 2016; J. Zheng et al., 2020). The metabolism of phenolic acids by *Furfurilactobacillus milii* is well characterized (Chapter 3); however, the phenolic acid metabolism in laboratory media does not necessarily match the metabolic pathways observed in food fermentations (Filannino et al., 2015). In addition,

the genotype of *Lactobacillaceae* does not always match the phenotype because alternative pathways of metabolism are available (Chapter 3; Ripari et al., 2019). This work therefore aimed to study the influence of genetic determinants of hydroxycinnamic acid metabolism on metabolite conversion in a complex phenolic rich food environment. Five isogenic mutants of *Furfurilactobacillus milii* FUA3583 were constructed lacking genes involved in hydroxycinnamic acid metabolism and quantified the free phenolic acid profile in different pure cultivar sorghum sourdoughs. The role of hydroxycinnamic acid metabolism in ecological fitness was also assessed by performing competition experiments with mutant and wild type (WT) strains in sorghum.

## 4.2. Materials and Methods

### 4.2.1. Bacterial strains and growth conditions

Bacterial strains and plasmids used in this study are listed in the Table 4.1. *Escherichia coli* EC1000 was grown in Luria-Bertani (LB) media (BD Difco, Sparks, MD, USA) with the addition of 300 mg/mL of erythromycin where applicable at 37°C. Strains of *Ff. milii* strains were subcultured twice after being streaked on agar plates from -80°C glycerol stocks. They were grown in modified de Man, Rogosa and Sharpe (mMRS) media (Zhao and Gänzle, 2018) or MRS (BD Difco, Sparks, MD, USA) with 5 g/L cysteine under microaerophilic conditions at 30° or 37°C.

### 4.2.2. Materials and chemicals

Pure sorghum cultivars Mahube, PAN 8609, Town (red) and Segaolane (white) were kindly provided by the National Food Technology Research Centre, Kanye, Botswana. Brown sorghum flour was obtained from a local supermarket. Sinapic acid, *p*-coumaric acid, dihydrosinapic acid, phloretic acid, dihydrocaffeic acid, 4-vinylphenol, 4-vinylguaicol, erythromycin (Em) and chloramphenicol (Cm) were all obtained from Sigma Aldrich (St. Louis, MO, USA). Caffeic acid

and ferulic acid were purchased from Extrasynthèse (Genay, France); dihydroferulic acid was obtained from MP Biomedicals (Illkirch, France). Vancomycin was purchased from Chem-Impex International, Inc (Wood Dale, IL, USA). Media components for mMRS media were obtained from BD (Sparks, MD, USA) and Millipore Sigma (St. Louis, MO, USA).

**Table 4.1. Bacterial strains and plasmids used in this study.**

| Strains | Description | Reference |
|---|---|---|
| *Ff. milii* FUA3583 | Mahewu Isolate, Wild type (WT) | (Pswarayi and Gänzle, 2019) |
| *Ff. milii* FUA3583 pVE6007 | WT strain harboring helper plasmid pVE6007, $Cm^R$ | (Chapter 3) |
| *Ff. milii* FUA3583 Δ*par2* | Single deletion mutant of *Ff. milii* FUA3583 lacking *par2* gene | (Chapter 3) |
| *Ff. milii* FUA3583 Δ*par1/Δpar2* | Double deletion mutant of *Ff. milii* FUA3583 lacking *par1* and *par2* genes | This study |
| *Ff. milii* FUA3583 Δ*pad* | Single deletion mutant of *Ff. milii* FUA3583 lacking *pad* gene | This study |
| *Ff. milii* FUA3583 Δ*estR* | Single deletion mutant of *Ff. milii* FUA3583 lacking *estR* gene | This study |
| *Ff. milii* FUA3583 Δ*par1/Δpar2/Δpad* | Tripe deletion mutant of *Ff. milii* FUA3583 lacking *par1*, *par2* and *pad* genes | This study |
| *Ff. milii* FUA3583 Δ*par1/Δpar2/Δpad/ΔestR* | Quadruple deletion mutant of *Ff. milii* FUA3583 lacking *par1*, *par2*, *pad* and *estR* genes | This study |
| *Escherichia coli* EC1000 | Cloning host for pVPL 3002 based plasmids, $RepA^+$, $Km^R$ | (Leenhouts et al., 1996) |

**Table 4.1.** (continued)

| Plasmids | Description | Reference |
|---|---|---|
| pVPL 3002 | Suicide vector encoding DdlF258Y as the counter selection marker, $Em^R$ | (Zhang et al., 2018) |
| pVE6007 | Helper plasmid $RepA^+$, $Cm^R$, | (Sanders et al., 1998) |
| pVPL3002/Δpar1 | pVPL 3002 containing *par1* flanking regions, $Em^R$ | (Chapter 3) |
| pVPL3002/Δpad | pVPL 3002 containing *pad* flanking regions, $Em^R$ | This study |
| pVPL3002/ΔestR | pVPL 3002 containing *estR* flanking regions, $Em^R$ | This study |

### 4.2.3. Construction of *Ff. milii* FUA3583 isogenic mutants lacking genes involved in phenolic acid metabolism

Five new isogenic mutants of *Ff. milii* FUA3583, namely Δ*par1*Δ*par2*, Δ*pad*, Δ*estR*, Δ*par1*Δ*par2*Δ*pad* and Δ*par1*Δ*par2*Δ*pad*Δ*estR*, were constructed in this study. Single deletion mutants for *pad* (phenolic acid decarboxylase) and *estR* (esterase) genes were made using the protocol published previously (Chapter 3) using a vancomycin counter-selection plasmid pVPL 3002 (Zhang et al., 2018). Briefly, 700-1000 bp of upstream and downstream flanking regions of *pad* and *estR* were amplified by PCR and ligated yielding pVPL 3002/Δpad and pVPL 3002/ΔestR recombinant plasmids by LCR (Ligase Cycling Reaction) (Kok et al., 2014), followed by their transformation into *E. coli* EC1000. Electrocompetent *Ff. milii* FUA3583 WT harboring pVE6007 ($repA^+$ helper plasmid) were transformed with 2-4 µg of plasmid DNA (2.5 kV, 25 µF, and 400 Ω) and recovered for 3-4 h in media containing 5 mg/L chloramphenicol. After recovery, fast track genome editing approach by (Zhang et al., 2018) was followed to obtain the double cross over

(DCO) mutants that were selected by plating on vancomycin (500 mg/L) mMRS plates. For construction of double deletion mutant Δ*par1*Δ*par2, Ff. milii* FUA3583 Δ*par2* was first transformed with pVE6007 helper plasmid followed by transformation with pVPL3002/Δpar1 plasmid. Further successive deletions for *pad* and *estR* genes were made on *Ff. milii* Δ*par1*Δ*par2* using the same protocol as above to make triple Δ*par1*Δ*par2*Δ*pad* and quadruple Δ*par1*Δ*par2*Δ*pad*Δ*estR* mutants. Deletion mutants were confirmed using colony PCR and sequencing. *Ff. milii* strains were all grown in MRS + cysteine media during the construction of mutants at 37°C under anaerobic conditions. Primer sequences used for cloning and screening are listed in Table 4.2.

### 4.2.4. Phenotypic characterization of the constructed mutants using HPLC

Overnight cultures (10 %) were inoculated in mMRS broth containing 1mM of sinapic acid, ferulic acid, caffeic acid or *p*-coumaric acid followed by incubation at 30°C for 24h (Svensson et al., 2010). Samples were centrifuged and the supernatant was acidified to pH 1.5 using hydrochloric acid, followed by solvent extraction using ethyl acetate (twice). Extracted samples were eluted on Agilent Eclipse XDB C18 column (4.6 x 150mm; 5µm) using an Agilent 1200 series HPLC system and the following gradient of 0.1% (vol/vol) formic acid in water (buffer A) and 0.1% formic acid in 90% acetonitrile (buffer B) at 0.7 mL/min: 0 min, 10%; 6 min, 15%; 14 min, 100%. Compounds were analyzed using a UV detector at 280 nm and substrates and metabolites were quantified with external standards (Chapter 3).

**Table 4.2. Primers used for genetic manipulations.**

| Primer (forward, F; reverse, R) | Description | Primer Sequences (5'→3') |
|---|---|---|
| oVPL 188 F (Zhang et al., 2018) | amplifies pVPL3002 backbone | ATCCTCTAGAGTCGACCTGC |
| oVPL 187 R | | TACCGAGCTCGAATTCACTGG |
| par1 U/S F (Chapter 3) | upstream flanking region of *par1* in *Ff. milii* FUA3583 | GCAGCCAGATAGCCTGAAAC |
| par1 U/S R | | CGACTGGCAGTTGCGCCAGCTGCGC |
| par1 D/S F | downstream flanking region of *par1* in *Ff. milii* FUA3583 | AAGACGTTGGTCGTAAGGCCGTG |
| par1 D/S R | | CATAGCGGCAGTGAACTTGA |
| par1 BO1 | LCR bridging oligo for pVPL3002/Δpar1 | AAACGACGGCCAGTGAATTCGAGCTCGG TAGCAGCCAGATAGCCTGAAACAATTCGT TGG |
| par1 BO2 | LCR bridging oligo for pVPL3002/Δpar1 | CTTTGGCGCAGCTGGCGCAACTGCCAGTC GAAGACGTTGGTCGTAAGGCCGTGGAGG AGA |
| par1 BO3 | LCR bridging oligo for pVPL3002/Δpar1 | GAATCCTTCATCAAGTTCACTGCCGCTAT GATCCTCTAGAGTCGACCTGCAGGCATGC AA |
| par1 DCO F | DCO screening for Δ*par1* in *Ff. milii* FUA3583 | AATCGTTGATCCGGCATTAC |
| par1 DCO R | | TCACACGCGATAGGTCTGAG |
| pad U/S F | upstream flanking region of *pad* in *Ff. milii* FUA3583 | GTTGATTCTGGACGGACGAT |
| pad U/S R | | CAGCCATTGTCGTACGTGTAA |
| pad D/S F | downstream flanking region of *pad* in *Ff. milii* FUA3583 | CCATACGATGGGATGACTGA |
| pad D/S R | | AACGACAGGCTCGTAAGCAG |
| pad BO1 | LCR bridging oligo for pVPL3002/Δpad | AAACGACGGCCAGTGAATTCGAGCTCGG TAGTTGATTCTGGACGGACGATTTACCAA AAC |

**Table 4.2.** (continued)

| Primer (forward, F; reverse, R) | Description | Primer Sequences (5'→3') |
|---|---|---|
| pad BO2 | LCR bridging oligo for pVPL3002/Δpad | TCACTTTATTTACACGTACGACAATGGCTGCCATACGATGGGATGACTGATGATATTCGC |
| pad BO3 | LCR bridging oligo for pVPL3002/Δpad | ACCTCTATTTCTGCTTACGAGCCTGTCGTTATCCTCTAGAGTCGACCTGCAGGCATGCAA |
| pad DCO F | DCO screening for Δ*pad* in *Ff. milii* FUA3583 | CCGCGATCCTAGAAGGATTAAA |
| pad DCO R | | GCATAACGCACACTCACAATC |
| estR U/S F | upstream flanking region of *estR* in *Ff. milii* FUA3583 | GGCCGACCAATGCTCTATTA |
| estR U/S R | | TACAGTGGTTCTGGTTGACGA |
| estR D/S F | downstream flanking region of *estR* in *Ff. milii* FUA3583 | GCATCACCAATTGCAAACAG |
| estR D/S R | | GGAATTGCATTGGCTTCATC |
| estR BO1 | LCR bridging oligo for pVPL3002/ΔestR | AAACGACGGCCAGTGAATTCGAGCTCGGTAGGCCGACCAATGCTCTATTAATTGGTGTTC |
| estR BO2 | LCR bridging oligo for pVPL3002/ΔestR | AATTCATTTTCGTCAACCAGAACCACTGTAGCATCACCAATTGCAAACAGACGTTGAAGC |
| estR BO3 | LCR bridging oligo for pVPL3002/ΔestR | GGAAGTATTGGATGAAGCCAATGCAATTCCATCCTCTAGAGTCGACCTGCAGGCATGCAA |
| estR DCO F | DCO screening for Δ*estR* in *Ff. milii* FUA3583 | GGCGATTCTCTTGATTACGG |
| estR DCO R | | CGCGGTCAGTCAGATAAACA |

### 4.2.5. Sorghum sourdough fermentation and determination of pH and cell counts

Sourdoughs were prepared in biological triplicates using two pure cultivars of sorghum grains- Mahube (red) and Segaolane (white) along with one commercially available brown sorghum flour for comparison. Overnight cultures of *Ff. milii* FUA3583 WT and five mutant strains were harvested by centrifugation, followed by washing and resuspension in 10 mL of sterile tap water. Resuspended cultures were then mixed with 10 g of grounded pure cultivars and commercial flour and incubated at 30°C for 24 h. Initial cell counts in the inoculum for all the cultures was $10^8$ log CFU/g. Chemically acidified dough was also prepared for all varieties of sorghum by addition of acids (lactic acid : acetic acid- 4:1) and sterile tap water for a total volume of 10 mL and incubated at same conditions.

Measurements of pH and viable cell counts were performed for all sourdough samples and unfermented controls. Fermented samples (1 g) were diluted 10 times using milli-Q water for pH measurements. Viable cell counts were obtained by plating 10-fold serial dilutions prepared in 0.1% peptone water on mMRS agar plates incubated anaerobically at 30°C for 48-72 h. Colony morphology and pH were used as indicators of quality control and contamination. Remaining fermented sourdoughs were freeze dried and stored at -20°C for further analysis.

### 4.2.6. Quantification of free phenolic acids and metabolites in fermented sorghum sourdoughs

Freeze dried sourdough samples were used for the extraction of free phenolics using the protocol by Ripari et al., 2019. Briefly, 250 mg of samples were extracted twice using 1 mL of 80% ethanol each time followed by centrifugation and collection of supernatants. Ethanol was evaporated under nitrogen and remaining solids were dissolved in 500 µl of 2% acetic acid adjusted to pH 2 using

12 M hydrochloric acid. Samples were extracted twice using 500 µl of ethyl acetate followed by evaporation under nitrogen and addition of 200 µl of methanol containing 0.1%formic acid. Extracted free phenolic samples were then analyzed using the same column and HPLC system as mentioned above. Solvent system consisted of 0.1% (v/v) formic acid in water (phase A) and 0.1% formic acid in acetonitrile/water (90:10 v/v) (phase B). Injection volume was 10µl and samples were eluted at a flow rate of 0.3 mL/min using the following gradient: 0 min, 10%; 35 min, 42%; 50 min, 48%. Quantification was performed at 280 and 330 nm using external standards for 3 independent biological replicates. Vinyl catechol was quantified using % of relative peak area as an external standard was unavailable.

Quantification of acetate, lactate and ethanol was performed using Aminex HPX-87H column (300 x 7.8mm, 9µm) (Bio-Rad Laboratories Inc., Redmond, WA, USA). Samples were prepared according to the protocol by Ripari et al., 2019 with isocratic elution at 70°C with 5mM sulphuric acid on the same HPLC system connected to a refractive index (RI) detector at a flow rate of 0.4 mL/min.

### 4.2.7. Competition experiments and DNA isolation

The red sorghum cultivars PAN 8609 and Town were used for the competition experiments. Overnight cultures of *Ff. milii* FUA3583 WT and mutant strains were washed with sterile tap water and their optical density (OD) was measured at 600 nm. Each mutant culture was individually mixed with the WT strain in equal amounts using OD to a final volume of 1mL. The five pairs of WT vs mutant cultures were inoculated into 1 g of sorghum flours and mixed thoroughly. Samples were incubated at 30°C for 24 h and back-slopped with 5% inoculum in fresh sorghum flours and sterile water for ten 24 h fermentation cycles. Fermentations were performed for 3 independent biological replicates.

Sourdough samples (1.9-2 g) from cycles 1, 4, 7 and 10 were homogenized with 25mL of 0.8% (Wt/V) saline (NaCl) solution and centrifuged at 500rcf for 6min to remove the solids. Cells were harvested by centrifugation at 5300rcf for 20 min, followed by DNA extraction with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions (Lin and Gänzle, 2014). DNA concentrations were measured using NanoDrop one spectroctrophotometer (Thermo Scientific, Madison, WI, USA) and samples were diluted as required using nuclease free water.

### 4.2.8. Probe design and quantification of gene copies by droplet digital polymerase chain reaction (ddPCR)

Four sets of primers and probes listed in Table 4.3 were designed for the analysis of competition experiments samples using ddPCR. Par2-fam, pad-hex and estR-hex sets were designed to target a site in the deleted region of *par2, pad* and *estR* respectively. Par2-hex set was designed by targeting flanking regions close to the site of deletion for *par2.* All primers and probes were designed using Primer3Plus (Untergasser et al., 2007) online tool and ordered via Integrated DNA technologies Inc. (IDT – Coralville, IA, USA).

A QX200 ddPCR system with an automated droplet generator (Bio-Rad Laboratories Inc., USA) was used for the analysis of samples. All DNA samples were diluted 100 times and 1 µl of 100-600 pg of template DNA was added to the reaction mixture. Each 20 µl reaction contained 10µl of 2X ddPCR supermix for probes (Bio-Rad Laboratories Inc., USA) along with 500 nM of each probe and 460 nM of each forward and reverse primer. After the reaction set up in 96 well plates, 20 µl of droplet generation oil for probes was added to a microfluidic DG8 cartridge (Bio-Rad Laboratories Inc., Germany), enabling mixing of the sample and oil for the generation of around 20,000 droplets for each sample using automated droplet generator module.

**Table 4.3. Primers and probes used for ddPCR.**

| Primer/probe (forward, F; reverse, R) | Description | Primer/probe Sequences (5'→3') |
|---|---|---|
| par2_famF | Primers and FAM dye labelled probe targeting the deletion region of *par2* | CACTGGCGATGATTTTGACG |
| par2_famR | | TAGCACTGACTGGTTCAACG |
| par2_fam Probe | | /6-FAM/TGTCGACGG/ZEN/ TATGGTCCACATGCGTGAC/IABkFQ/ |
| par2_hexF | Primers and HEX dye labelled probe targeting flanking regions close to the site of deletion of *par2* | TGGTGCCAGAGTTTTGCTAA |
| par2_hexR | | AGTATTGCGGCGGTCTTTTT |
| par2 hex Probe | | /HEX/AGCCGCGCT/ZEN/ ATTAAAGACGCCGTGAAGG/IABkFQ/ |
| pad_hexF | Primers and HEX dye labelled probe targeting the deletion region of *pad* | AACGACCACACCGTTGATTA |
| pad_hexR | | TTTGTAAACGCCTGGCACTA |
| pad_hexProbe | | /HEX/TGGCGGAAT/ZEN/ GGTTGCAGGCCGTTGG/IABkFQ/ |
| estR_hexF | Primers and HEX dye labelled probe targeting the deletion region of *estR* | GCATACTCTGGCAGTAGCAA |
| estR_hexR | | CGAACATTGGGGCATAGACT |
| estR_hex Probe | | /HEX/TTGGGCGTG/ZEN/ GCTTGGCGCGT/IABkFQ/ |

The plates with droplets were subjected to PCR amplification using a C1000 Touch™ thermal cycler (Bio-Rad Laboratories Inc., Singapore) with the following reaction set up- enzyme activation at 95°C (10 min), 40 cycles at 94°C (30 sec) and 60°C (50 sec) followed by 1 cycle of enzyme inactivation at 98°C (10 min). Droplet reader was used to measure fluorescence of each individual droplet in each sample. Experiments comparing the WT vs double, triple and quadruple mutants were done using par2-fam and par2-hex set probe system with quantification of WT strain

and mutant strains in FAM (6-carboxyfluorescein) and HEX (6-carboxy-2,4,4,5,7,7-hexachlorofluorescein) channels, respectively. WT vs Δ*pad* and Δ*estR* experiments were performed using par2-fam/pad-hex and par2-fam/estR-hex sets respectively. WT strain was quantified using signals in both FAM and HEX channels while the mutant strains were quantified by signals only in the FAM channel. Fluorescence data for all the droplets of each sample was analyzed QuantaSoft software version 1.3.2 (Bio-Rad Laboratories Inc.). Positive and negative controls were analyzed for both channels of each competition experiment and data points were represented as mean ± standard deviation of the copy number ratio of WT/mutant for 3 independent experiments.

#### 4.2.9. Statistical analysis

The slope of the ratio of wild type to mutants over the # fermentation cycles was determined by linear regression for each of the triplicate independent experiments. Significant differences among the different slopes were assessed by one-way ANOVA with Holm-Sidak post hoc analysis using SigmaPlot 13.5 (Systat software Inc.). Two-way ANOVA was performed on the organic acid data using SAS version 5.1.26 (SAS Institute Inc., NC, USA) followed by Tukey's LSD test.

### 4.3. Results

#### 4.3.1. Phenotypic characterization of the phenolic acid metabolism in isogenic mutants

Phenolic acid reductases (*par1* and *par2*) and phenolic acid decarboxylase (*pad*) were previously identified in *Ff. milii* FUA3583 (Chapter 3). Protein BLAST was performed on the genome of *Ff. milii* FUA3583 to search for the presence of other genes related to phenolic acid metabolism. The search revealed presence of an alpha/beta fold hydrolase (locus tag- GB992_RS06035) showing

42% amino acid identity with Lp_0796 (YP_004888771.1) from *Lp. plantarum* WCFS1 (Esteban-Torres et al., 2013), hereafter referred as EstR (Figure 4.1)

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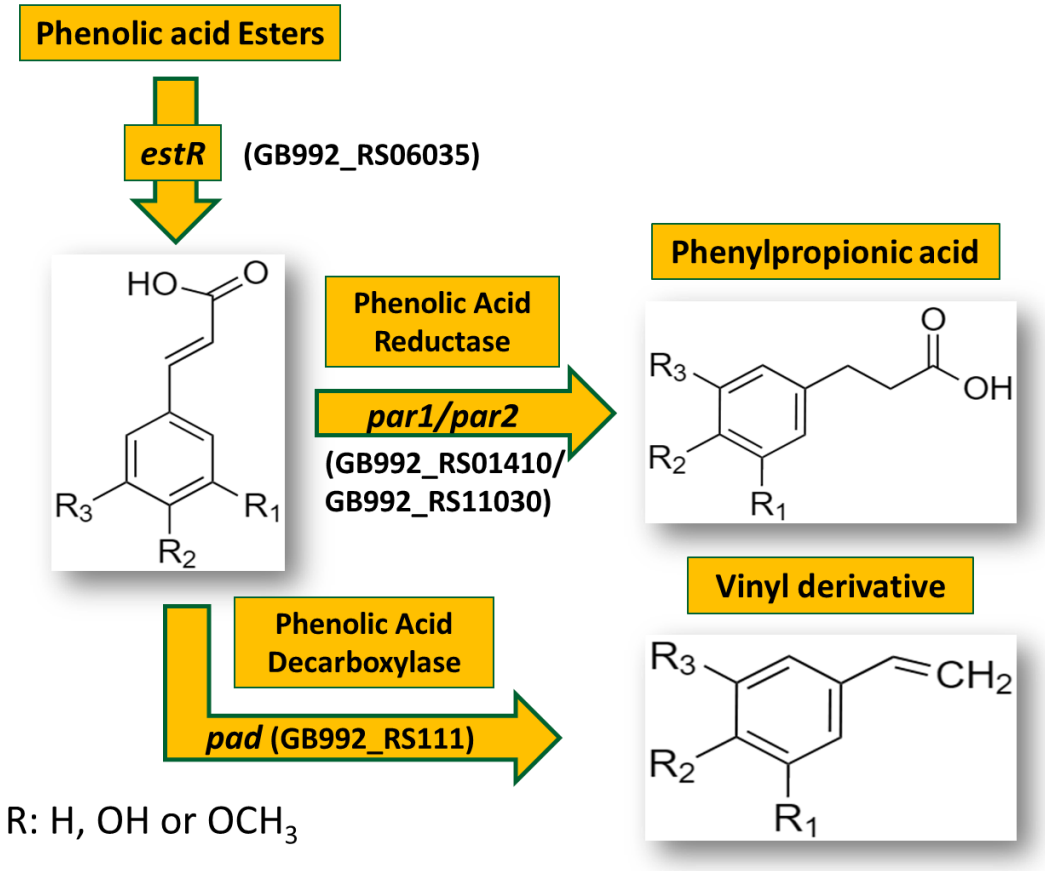



**Figure 4.1. Hydroxycinnamic acid metabolism of *Ff. milii* FUA3583.**

*estR*- putative phenolic acid esterase, homolog of Lp_0796 from *Lp. plantarum* WCFS1 (Esteban-Torres et al., 2013); *par1*- phenolic acid reductase (Chapter 3); *par2* – homolog of *par1*; *pad*- phenolic acid decarboxylase (Rodríguez et al., 2008). Gene locus tag numbers from NCBI are shown in the parenthesis.

To confirm the phenotype of genetic determinants of hydroxycinnamic acid metabolism, five isogenic mutants of *Ff. milii* FUA3583 were created in this study. Single deletion mutants were made for decarboxylase (Δ*pad*) and esterase (Δ*estR*). Successive deletions of *pad* and *estR* on the double deletion reductase mutant Δ*par1*Δ*par2* resulted in the triple mutant Δ*par1*Δ*par2*Δ*pad* and the quadruple mutant Δ*par1*Δ*par2*Δ*pad*Δ*estR*. Phenotypic characterization was performed by

incubating the strains with 1 mM of different hydroxycinnamic acids in mMRS for 24 h, followed by analysis of extracts using HPLC. The WT strain reduced all of the substrates tested to the corresponding phenylpropionic acid derivatives but only decarboxylated caffeic and *p*-coumaric acids (Table 4.4). The reductase mutant Δ*par1*Δ*par2* decarboxylated all of the substrates except sinapic acid, which remained unmetabolized. The Δ*pad* decarboxylase mutant reduced all the tested compounds to the corresponding phenylpropionic acid derivatives while the triple and quadruple mutants did not metabolize any of the phenolic acids tested. To confirm the phenotype of the esterase mutants, methyl ferulate and chlorogenic acid were used as additional substrates but no difference in the metabolism was observed between WT and Δ*estR* mutant (Data not shown).

### 4.3.2. General characteristics of sorghum sourdoughs

Fermentation reduced the pH of red sorghum sourdoughs from 6.38 to 4.3 with the final cell counts for all the strains being around 9.5 log CFU/g (Table 4.5). Fermentation of white and commercial sorghum flour sourdoughs reduced pH to around 4.3 and 3.4 respectively, with final cell counts ranging from 9.4 to 9.9 log CFU/g. The concentrations of acetate, lactate, and ethanol were not different ($P>0.05$) in sourdoughs fermented with different strains. Acetate production was significantly higher in red sorghum in comparison to white sorghum sourdoughs ($P=0.001$). Lactate concentrations were higher in white ($P=0.029$) and commercial sorghum sourdoughs ($P<0.001$). Ethanol production was significantly higher in white sorghum sourdoughs in comparison to red and commercial sourdough with white having the least amounts ($P<0.001$).

**Table 4.4. Phenotypic characterization of hydroxycinnamic acid metabolism of *Ff. rossiase* FUA3583 strains.**

Wild type and isogenic mutant strains were incubated with 1 mM of different hydroxycinnamic acid substrates. Shaded (+) and unshaded (-) boxes represent presence and absence of the phenotype for the metabolites as detected by the HPLC, respectively.

| Strain Name | Sinapic Acid | Ferulic Acid | | Caffeic Acid | | *p*-Coumaric acid | |
|---|---|---|---|---|---|---|---|
| | Dihydro-sinapic acid | Dihydro-ferulic acid | 4-Vinyl-guaiacol | Dihydro-caffeic acid | 4-Vinyl-catechol | Phloretic acid | 4-Vinyl-phenol |
| *Ff. milii* FUA3583 | + | + | - | + | + | + | + |
| *Ff. milii* FUA3583 Δ*par1/Δpar2* | - | - | + | - | + | - | + |
| *Ff. milii* FUA3583 Δ*pad* | + | + | - | + | - | + | - |
| *Ff. milii* FUA3583 Δ*estR* | + | + | - | + | + | + | + |
| *Ff. milii* FUA3583 Δ*par1/Δpar2/Δpad* | - | - | - | - | - | - | - |
| *Ff. milii* FUA3583 Δ*par1/Δpar2/Δpad/ ΔestR* | - | - | - | - | - | - | - |

**Table 4.5. Metabolite concentrations, pH and cell counts in sorghum sourdoughs.**

Sorghum sourdoughs were fermented for 24h at 30°C using single strains of *Ff. milii* FUA3583. Data are shown as mean ± standard deviation (n=3). Nd- Not determined.

| Sourdough sample | Acetate (mM) | Lactate (mM) | Ethanol (mM) | pH | Cell Counts (Log cfu/g) |
|---|---|---|---|---|---|
| **Red sorghum sourdoughs** | | | | | |
| Unfermented control | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 6.38 ± 0.06 | <5 |
| Chemically acidified | Nd | Nd | Nd | 3.35 ± 0.03 | <4 |
| *Ff. milii* FUA3583 WT | 31.25 ± 5.34 | 96.54 ± 9.55 | 52.59 ± 8.58 | 4.30 ± 0.05 | 9.49 |
| *Ff. milii* FUA3583 Δ*par1*/Δ*par2* | 37.17 ±5.01 | 98.79 ±10.21 | 54.41 ± 7.29 | 4.31 ± 0.02 | 9.37 |
| *Ff. milii* FUA3583 Δ*pad* | 37.46 ± 6.70 | 103.31 ± 19.89 | 59.20 ± 9.88 | 4.28 ± 0.02 | 9.58 |
| *Ff. milii* FUA3583 Δ*estR* | 33.25 ± 3.85 | 101.68 ± 14.50 | 59.22 ± 2.62 | 4.24 ± 0.04 | 9.66 |
| *Ff. milii* FUA3583 Δ*par1*/Δ*par2*/Δ*pad* | 37.29 ± 8.80 | 113.64 ± 19.66 | 63.33 ± 4.70 | 4.26 ± 0.05 | 9.54 |
| *Ff. milii* FUA3583 Δ*par1*/Δ*par2*/Δ*pad*/Δ*estR* | 35.89 ± 3.42 | 98.76 ± 14.48 | 61.21 ± 11.27 | 4.28 ± 0.07 | 9.68 |
| **White sorghum sourdoughs** | | | | | |
| Unfermented control | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 6.6 ± 0.31 | <5 |
| Chemically acidified | Nd | Nd | Nd | 3.40 ± 0.06 | <4 |
| *Ff. milii* FUA3583 WT | 29.62 ± 4.46 | 128.40 ± 16.00 | 72.92 ± 4.49 | 4.32 ± 0.10 | 9.50 |
| *Ff. milii* FUA3583 Δ*par1*/Δ*par2* | 28.26 ±3.46 | 109.18 ±6.05 | 69.99 ± 2.71 | 4.34 ± 0.01 | 9.52 |
| *Ff. milii* FUA3583 Δ*pad* | 30.20 ± 2.92 | 126.82 ± 9.24 | 73.70 ± 3.04 | 4.28 ± 0.07 | 9.76 |
| *Ff. milii* FUA3583 Δ*estR* | 24.49 ± 3.91 | 107.74 ± 15.89 | 71.55 ± 1.20 | 4.22 ± 0.07 | 9.54 |

**Table 4.5.** (continued)

| Sourdough samples | Acetate (mM) | Lactate (mM) | Ethanol (mM) | pH | Cell Counts (Log cfu/g) |
|---|---|---|---|---|---|
| **White sorghum sourdoughs** | | | | | |
| *Ff. milii* FUA3583 Δ*par1*/Δ*par2*/Δ*pad* | 30.94 ± 4.58 | 124.83 ± 22.42 | 71.35 ± 10.65 | 4.30 ± 0.10 | 9.72 |
| *Ff. milii* FUA3583 Δ*par1*/Δ*par2*/Δ*pad*/Δ*estR* | 31.06 ± 5.36 | 121.68 ± 23.01 | 62.50 ± 14.41 | 4.27 ± 0.09 | 9.82 |
| **Commercial (brown) sorghum sourdoughs** | | | | | |
| Unfermented control | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 5.58 ± 0.15 | <4 |
| Chemically acidified | Nd | Nd | Nd | 3.49 ± 0.06 | <4 |
| *Ff. milii* FUA3583WT | 30.45 ± 5.36 | 207.13 ± 44.88 | 41.09 ± 7.52 | 3.4 ± 0.21 | 9.42 |
| *Ff. milii* FUA3583 Δ*par1*/Δ*par2* | 35.79 ±4.34 | 221.93 ± 33.76 | 45.92 ± 4.44 | 3.48 ± 0.16 | 9.60 |
| *Ff. milii* FUA3583 Δ*pad* | 30.54 ± 5.64 | 229.69 ± 19.02 | 45.94 ± 7.29 | 3.47 ± 0.18 | 9.50 |
| *Ff. milii* FUA3583 Δ*estR* | 26.40 ± 6.39 | 183.21 ± 45.28 | 36.88 ± 5.23 | 3.44 ± 0.13 | 9.73 |
| *Ff. milii* FUA3583 Δ*par1*/Δ*par2*/Δ*pad* | 38.93 ± 6.21 | 248.00 ± 16.02 | 58.09 ± 3.81 | 3.42 ± 0.11 | 9.88 |
| *Ff. milii* FUA3583 Δ*par1*/Δ*par2*/Δ*pad*/Δ*estR* | 29.04 ± 4.71 | 223.54 ± 30.41 | 49.47 ± 4.78 | 3.43 ± 0.10 | 9.64 |

### 4.3.3. Hydroxycinnamic acid metabolism in sorghum sourdoughs

Sorghum sourdoughs fermented with WT and mutant strains were characterized using reverse phase HPLC. The concentration of phenolic acids and metabolites in red, and white and commercial brown flour are shown in Figures 4.2 and 4.3, respectively. Overall, the concentration of phenolic acids and their metabolites was highest in the red cultivar Mahube and lowest in the white cultivar Segaolane (Figures 4.2 and 4.3a). Low concentrations of dihydrocaffeic and phloretic acid were detected in unfermented sorghum and in chemically acidified controls (Figures 4.2 and 4.3). Because these microbial metabolites are not present in intact seeds, these peaks are likely attributable with other aromatic compounds that co-elude with the analytes. The concentrations of sinapic acid, 4-vinylguicol and dihydrosinapic acid were not quantified due to their concentrations being below their respective detection limits (4-8 mg/kg), and interference from other compounds. Fermentation with the wild type strain significantly ($P$<0.001) increased the total concentration of free phenolic acids; the highest concentration was determined for dihydrocaffeic acid (Figures 4.2 and 4.3). *Ff. milii* FUA3583 metabolised phenolic acids almost exclusively by reduction to the corresponding dihydro-derivatives. Products of decarboxylation were usually below the detection limit of 1.5 mg/kg (4-vinylphenol) and 8 mg/kg (4-vinylguaiacol). Deletion of reductase genes abolished the production of dihydro-derivatives; the concentration of dihydrocaffeic acid in all sourdoughs fermented with *par1* and *par2* reductase mutant was comparable to the chemically acidified control while the concentration of 4-vinylcatechol increased ($P$<0.001) relative to sourdoughs fermented with the wild type strain. Despite the deletion of the phenolic acid reductases, decarboxylated metabolites from substrates other than caffeic acid and *p*-coumaric acid were not detected. Deletion of the hydroxycinnamic acid decarboxylase Pad in the wild type had no impact on the spectrum of metabolites; the same

deletion in a Δ*par1*Δ*par2* background abolished formation of 4-vinylcatechol. The deletion of the esterase Δ*estR* in either a wild type or Δ*par1*Δ*par2*Δ*pad* background had little impact on the metabolite spectrum in red or white sorghum sourdoughs (Figure 4.2 and 4.3). In sourdoughs fermented with Δ*par1*Δ*par2*Δ*pad* or Δ*par1*Δ*par2*Δ*pad*Δ*estR* mutants, the concentration of ferulic acid, *p*-coumaric acid and particularly of caffeic acid was higher ($P$<0.001) than in unfermented or chemically acidified controls (Figures 4.2 and 4.3).

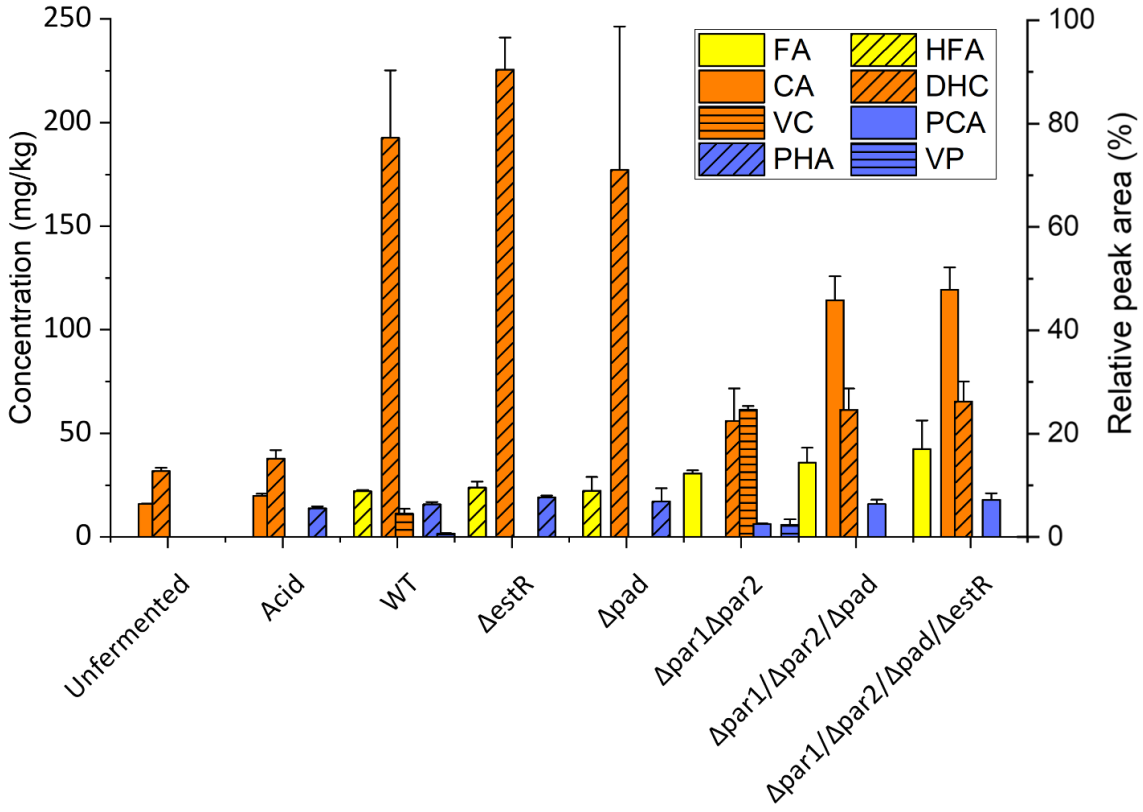



**Figure 4.2. Concentration of free hydroxycinnamic acids and their metabolites in pure cultivar red sorghum (Mahube variety) sourdough.**

Sourdoughs were fermented for 24 h at 30°C with single strains of *Ff. milii* FUA3583. Extracted samples were analyzed using HPLC. FA- Ferulic acid, HFA- Dihydroferulic acid, CA- Caffeic acid, DHC- Dihydrocaffeic acid, VC- Vinylcatechol, PCA- *p*-Coumaric acid, PHA- Phloretic acid, VP- Vinylphenol. VC represented as % of relative peak area. Data are shown as mean + standard deviation of three independent experiments.

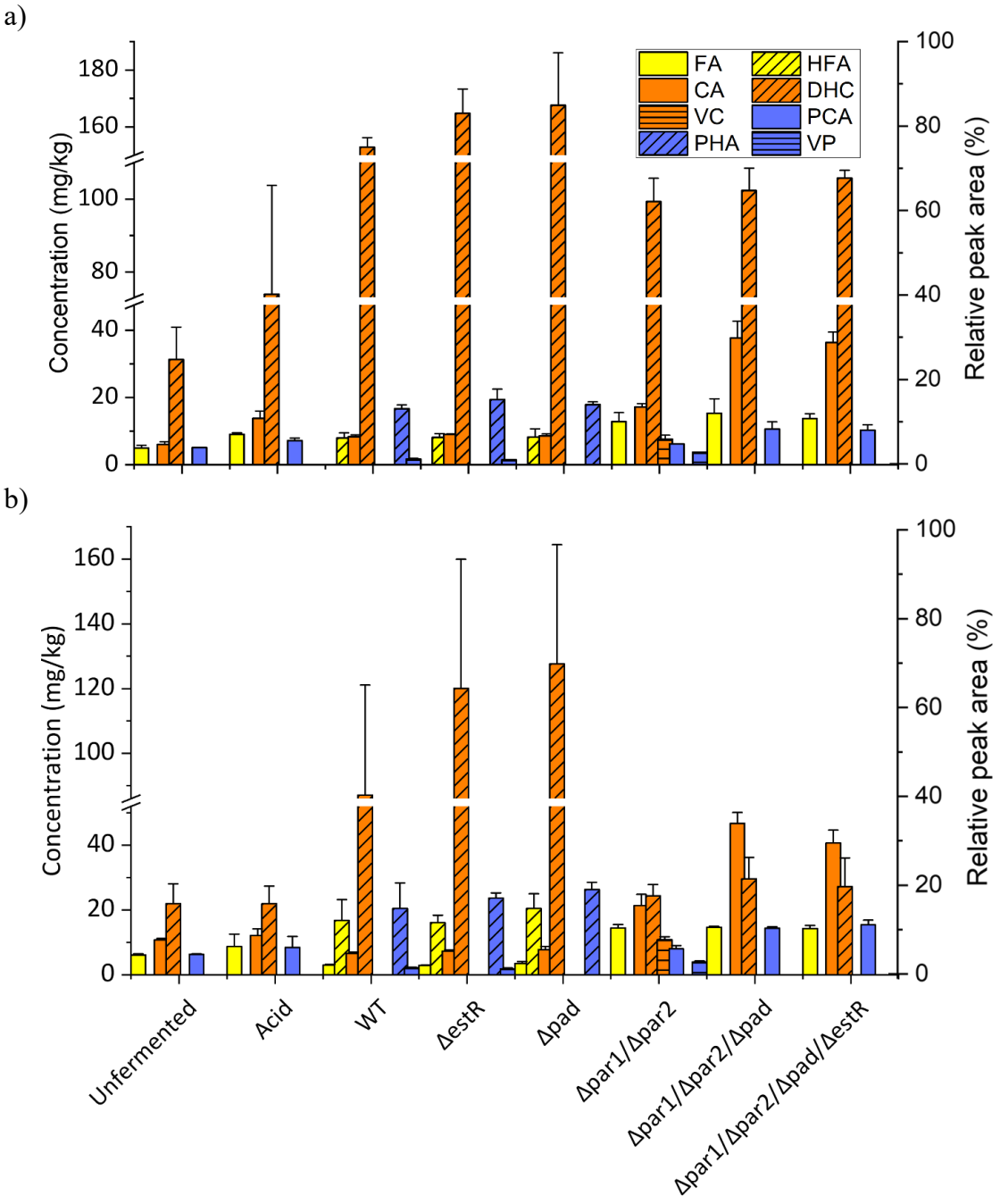



**Figure 4.3. Concentration of free hydroxycinnamic acids and their metabolites in pure cultivar and commercial sorghum sourdoughs.**

Sourdoughs were fermented for 24h at 30°C with single strains of *Ff. milii* FUA3583. a) Cultivar Segaolane (White) b) Commercial flour (Brown). Extracted samples were analyzed using HPLC. FA- Ferulic acid, HFA- Dihydroferulic acid, CA- Caffeic acid, DHC- Dihydrocaffeic acid, VC- Vinylcatechol, PCA- *p*-Coumaric acid, PHA- Phloretic acid, VP- Vinylphenol. VC represented as % of relative peak area Data are shown as mean + standard deviation for three independent experiments.

### 4.3.4. Role of phenolic acid metabolism genes in ecological fitness

To assess the role of genes encoding for phenolic acid metabolism to the ecological fitness in phenolic rich environments, competition experiments were performed between the WT and the isogenic mutant strains in the red sorghum varieties PAN 8609 and Town (Figure 4.4). The ratio of the wild type to the mutant strains was quantified by ddPCR. The plots of the log-transformed ratio of wild type strain to mutant strains were linear in all of the 10 binary competition experiments; therefore, the slopes of the linear regression lines were used as a measure of the ecological fitness (Figure 4.4). The choice of the sorghum cultivar did not impact the competitiveness of the mutant strains relative to the wild type strain and the data obtained in sourdoughs produced from cultivars PAN 8609 and Town essentially overlapped (Figure 4.4). The wild type strain outcompeted the Δ*par1*Δ*par2*Δ*pad* and Δ*par1*Δ*par2*Δ*pad*Δ*estR* mutants with a slope of about 0.1 / fermentation cycle (Figure 4.4). Deletion of only hydroxycinnamic acid esterase, decarboxylase, or reductase genes did not affect the ecological fitness of mutant strains in sorghum and the ratio of wild type to mutant strains remained unchanged over 10 fermentation cycles (Figure 4.4). This indicates that presence of just one of the two metabolic pathways suffice for ecological fitness in sorghum sourdoughs. While the deletion of *estR* in a wild type background did not impact the ecological fitness, deletion of the same gene in a Δ*par1*Δ*par2*Δ*pad* background significantly improved the ecological fitness compared to the Δ*par1*Δ*par2*Δ*pad* mutant strain, suggesting *in situ* activity of the *estR* gene product.

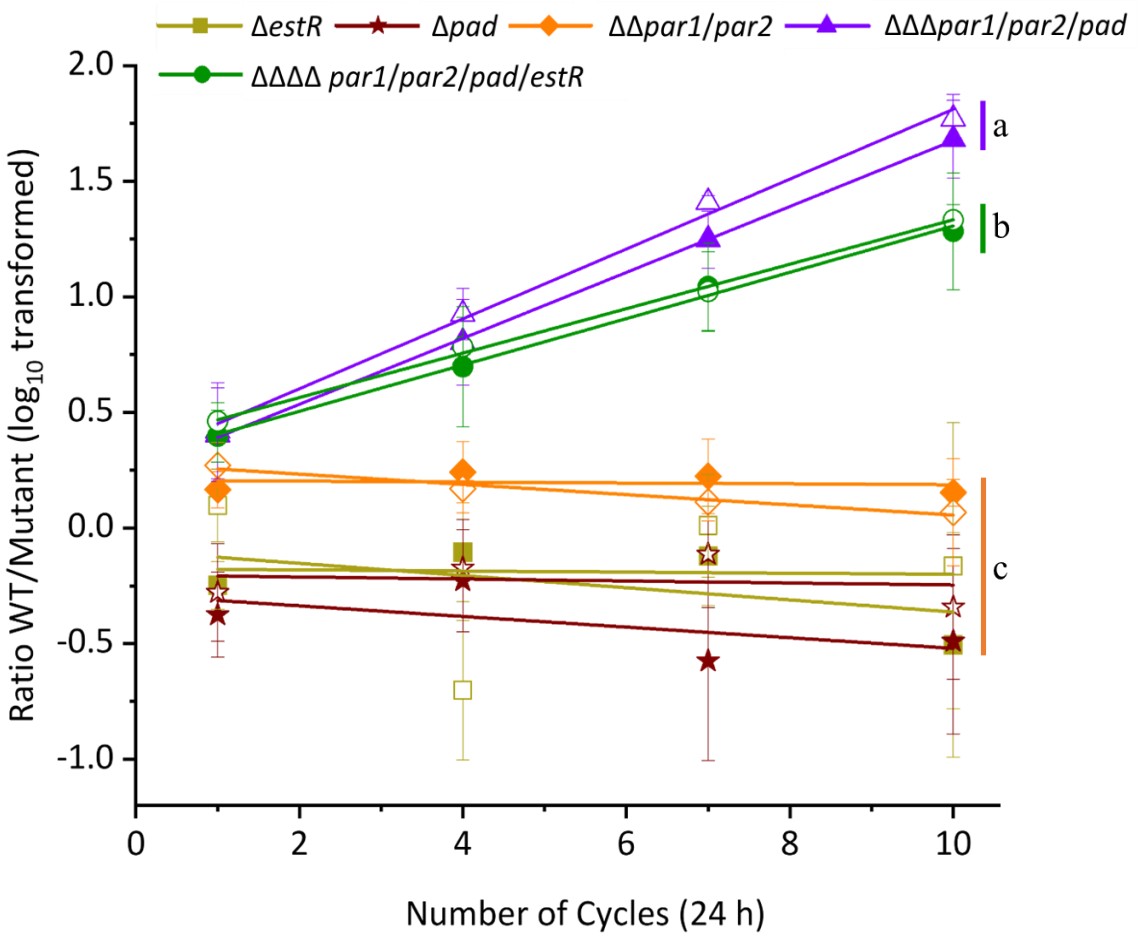



**Figure 4.4. Effect of phenolic acid metabolism genes on the ecological fitness of *Ff. milii* FUA3583 WT in red sorghum fermentations.**

Competition experiments were performed by addition of equal amounts of WT and isogenic mutant strains in two different pure cultivar red sorghum varieties (PAN 8609 and Town). After 24 h fermentation, 5% of sourdoughs were back-slopped into fresh dough for 10 cycles. The copy number of wild type and mutant genomes were quantified by droplet digital polymerase chain reaction (ddPCR). Shown is the ratio of gene copies of the wild type to the mutant genomes over a period of 10 refreshment cycles. Solid lines show the average of the linear regressions for each experiment. Filled symbols, cultivar PAN 8609; open symbols, cultivar Town. Data are shown as means ± standard deviation of three independent experiments. Linear regression fit was performed for each independent sourdough and statistical analysis was done on the slopes obtained for each replicate. Curves that do not share a common lowercase letter differ ($P$<0.05).

## 4.4. Discussion

Phenolic acid metabolism by lactic acid bacteria has been previously characterized in some food fermentations with decarboxylation and reduction being the two pathways of bioconversion for phenolic acids. Unlike hydroxybenzoic acids, which are only decarboxylated, hydroxycinnamic acids can be metabolized by both pathways but the metabolism is highly strain specific (Filannino et al., 2015; Chapter 3; Ripari et al., 2019). Genetic determinants involved in the reduction and decarboxylation of hydroxycinnamic acids have been characterized in some *Lactobacillaceae* (Cavin et al., 1997; Rodríguez et al., 2008; Santamaría et al., 2018a) but their presence/absence does not always match the phenotype (Filannino et al., 2015; Chapter 3; Ripari et al., 2019). This study looked at the role of hydroxycinnamic acid metabolism genes in sorghum fermentations to better understand their role in metabolite production and ecological fitness in phenolic rich fermented foods by, i) construction of isogenic mutants of *Ff. milii* FUA3583 lacking genes involved in phenolic acid metabolism, ii) quantifying free phenolic acids and metabolites in 3 different sorghum sourdoughs and, iii) assessing the effect of phenolic acid metabolism genes on ecological fitness.

Strains of *Ff. milii* have been isolated from pineapple (Di Cagno et al., 2010), spontaneous jalapeño peppers fermentation (Medeiros et al., 2021), human feces (Di Cagno et al., 2009) and swine gut (De Angelis et al., 2006) but most isolates were obtained from cereal fermentations (Pswarayi and Gänzle, 2019; Schneiderbanger et al., 2019; J. Zheng et al., 2020). *Ff. milii* FUA3583 was isolated from *mahewu*, a fermented beverage from Zimbabwe and likely originates from millet malt, which is the main source of fermentation organisms in *mahewu* (Pswarayi and Gänzle, 2019). Studies on the ecology of the genus are lacking but the available evidence suggests that furfurilactobacilli

have a nomadic lifestyle similar to *Lactiplantibacillus plantarum* (Duar et al., 2017; J. Zheng et al., 2020).

Sorghum is a rich source of phenolic compounds with the composition varying based on different cultivars (Awika and Rooney, 2004; Sekwati-Monang et al., 2012). Fermentation greatly increased the total free hydroxycinnamic acid content in sorghum sourdoughs which is consistent with previous studies that characterized phenolic profiles in rye and wheat sourdoughs (Ripari et al., 2019; Skrajda-Brdak et al., 2019) and spontaneous sorghum sourdough (Ravisankar et al., 2021). Red sorghum had showed the highest free phenolic content after fermentation followed by brown and white sorghum sourdoughs.

Phenolic acids can also act as external electron acceptors in heterofermentative *Lactobacillaceae* resulting in higher acetate production and ATP generation via the phosphoketolase pathway (Filannino et al., 2016b, 2014). The higher concentration of phenolic acids in red sorghum that acting as external electron acceptors may explain the significantly higher concentration of acetate in red sorghum sourdoughs when compared to white sorghum sourdoughs (this study). Despite the high concentrations of hydroxycinnamic acids in sorghum, deletion of reductase and/or decarboxylase genes did not result in any significant differences in acetate production; however, the experimental error for quantification of acetate, ranging from 3 to 10 mmol/kg, is larger than the concentration of hydroxycinnamic acids, ranging from 1 to 2 mmol/kg (Figure 4.2, 4.3 and Table 4.5).

Many *Lactobacillaceae* including furfurilactobacilli, possess alternative pathways for metabolism of hydroxycinnamic acids, decarboxylation or reduction. NADH-dependent reduction of hydroxycinnamic acids leads to production of phenylpropionic acids while decarboxylation to vinyl derivatives consumes intracellular protons (Gänzle, 2015; Sánchez-Maldonado et al., 2011).

Both phenylpropionic acid and vinyl derivatives have a lower antimicrobial activity than the corresponding substrates (Sánchez-Maldonado et al., 2011). A vinylphenol reductase (VprA) that generates ethyl derivatives has been characterized in *Lp. plantarum* WCFS1 (Santamaría et al., 2018b) but this enzyme is less frequent in *Lactobacillaceae* than phenolic acid decarboxylases (Chapter 3). This chapter could not identify a phenotype for the hydroxycinnamic esterase mutant of *Ff. milii* either *in vitro* or *in situ*; however, the diverse hydroxycinnamic acid esters in sorghum (Svensson et al., 2010) were not identified or quantified by the analytical setup used in the present study. Hydroxycinnamic acid esterases that were characterized in *Lp. plantarum* and *Lactobacillus johnsonii* have also shown a diverse substrate specificity (Esteban-Torres et al., 2015, 2013; Lai et al., 2009).

The metabolism of hydroxycinnamic acid in laboratory media and in food fermentations can differ substantially (Filannino et al., 2015; Ripari et al., 2019). Decarboxylation was the primary route for caffeic acid metabolism by *Ff. milii* FUA3583 in mMRS (Chapter 3), but reduction was by far major pathway observed in sorghum fermentations (Figure 4.2 and 4.3). *Ff. milii* FUA3583 over-expresses both *par1* and *pad* in response to challenge with 1mM of the substrates (Chapter 3) but the quantification of gene expression in *Lp. plantarum* and *Lm. fermentum* in cereal substrates demonstrated that some but not all enzymes involved in metabolism of hydroxycinnamic acids are over-expressed in cereal substrates relative to mMRS (Pswarayi et al., 2022). The concentration of free hydroxycinnamic acids in sorghum is relatively low (Awika and Rooney, 2004; Svensson et al., 2010) and it is unclear how esters of hydroxycinnamic acids or other phytochemicals that are associated with free and conjugated phenolic acids impact gene expression in *Lactobacillaceae*.

Competition experiments quantified the ecological fitness of strains with deletions of genes coding for metabolism of hydroxycinnamic acids relative to the wild type in sorghum fermentations. Antimicrobial activity of phenolic acids can act as a selective pressure in phenolic rich environments (Gänzle, 2014). *Fl. sanfranciscensis* is a predominant organisms in wheat and rye sourdoughs and handily outcompetes other *Lactobacillaceae* in wheat and rye sourdoughs that are back-slopped frequently at ambient conditions (Dinardo et al., 2019; Meroth et al., 2003; Ripari et al., 2016). In wheat and rye, the concentrations of phenolic acid are well below their minimum inhibitory concentrations against *Fl. sanfranciscensis* (Boskov Hansen et al., 2002; Sekwati-Monang et al., 2012). Conversely, *Fl. sanfranciscensis* is outcompeted by other *Lactobacillaceae* in sorghum sourdoughs or in wheat sourdough with addition of ferulic acid (Dinardo et al., 2019; Sekwati-Monang et al., 2012) and has not been isolated from sourdoughs produced with sorghum, millet or other gluten free flours (Van Kerrebroeck et al., 2017). The present study is the first to document the role of individual genes related to metabolism of phenolic compounds on the competitiveness of *Lactobacillaceae* in food fermentations. Any one of the two pathways for converting hydroxycinnamic acids is sufficient to provide strains with a competitive advantage in sorghum sourdoughs. Strains that maintained only the reductase pathway were as competitive as strains that maintained only the decarboxylase pathway, indicating that this contribution to ecological fitness is not dependent on $NADH^{+}$ recycling (Filannino et al., 2015) or proton consumption through decarboxylation (Gänzle, 2015). The ecological relevance of these genes may not be limited to phenolic acid metabolism, extending towards providing resistance against other plant secondary metabolites as a result of the adaptation of *Lactobacillaceae* adaptation to plants including cereals (Pswarayi et al., 2022). The competition experiments also suggested an ecological role for the hydroxycinnamic acid esterase EstP. Deletion of *estP* in a wild type

background did not impact ecological fitness, indicating that the energy expenditure for *estP* expression did not impact ecological fitness (Fig. 4). Deletion of *estP* in a *Δpar1Δpar2Δpad* background increased ecological fitness (Fig. 4). The mechanisms for this difference are unclear; however, because the esterase expressed by *Ff. milii* FUA3583*Δpar1Δpar2Δpad* - remained present in these fermentations and the antimicrobial activity of free phenolic acids is expected to impact the *Δpar1Δpar2Δpad* mutant as much as the *Δpar1Δpar2ΔpadΔestR* mutant, unless the esterase products remain trapped in the cytoplasm of the strain expressing the intracellular enzyme (Esteban-Torres et al., 2013).

The isogenic mutants of *Ff. milii* that were generated in this study confirm the role of hydroxycinnamic acids as antimicrobial compounds in red and white sorghum sourdoughs (Sekwati-Monang et al., 2012; Svensson et al., 2010). Deletion of phenolic acid reductase genes leads to an increased vinyl derivative production that can impact the aroma and flavour of fermented foods (Muñoz et al., 2017; Shahidi and Yeo, 2018). These vinyl derivatives also react with anthocyanidins and 3-deoxyanthocyanidins to pyranoanthocyanidins and pyrano-3-deoxyanthocyanidins (Bai et al., 2014). Free phenolic acids and their phenylpropionic acid metabolites have also been shown to supress or inhibit cell proliferation *in vitro* in human Caco-2 and SW480 carcinoma cell lines (Ekbatan et al., 2018; Martini et al., 2019). The mutant strains may also be useful to explore other biological activities of hydroxycinnamic acids, which have been shown to suppress inflammatory cytokines related to IBD (inflammatory bowel disease) in BALB/c mice models (Katayama et al., 2017; Lee, 2018) and exerted anti-inflammatory effects on colitis induced C57BL/6J mice (Zhang et al., 2016).

In conclusion, this study further expands on the behavior and phenolic acid metabolism of heterofermentative *Lactobacillaceae* in food fermentations. Use of isogenic mutants unlike

previous studies provides higher confidence in the influence of genetic determinants on metabolite production and insights into their ecological relevance for *Lactobacillaceae*. The putative esterase EstR remains uncharacterized but indicates towards the presence of other unidentified phenolic esters with diverse substrate specificities involved in release of bound phenolic compounds. Cereal fermentations are an attractive proposition for production of bioactive phenolics and functional foods by selectively metabolizing hydroxycinnamic acids to different end products.

# Chapter 5- Biochemical characterization of phenolic acid reductase (HcrF) from *Limosilactobacillus fermentum*

## 5.1. Introduction

Phenolic acids are major phenolic constituents in edible plants with whole grain cereals being abundant in hydroxycinnamic acids such as ferulic acid, *p*-coumaric acid and caffeic acid (Călinoiu and Vodnar, 2018). Phenolic acids and their metabolites including dihydro-derivatives are considered bioactive compounds whose consumption has been associated with various health benefits (Leonard et al., 2021b; Martini et al., 2019).

*Limosilactobacillus fermentum* is frequently isolated from plant materials and is a dominant microorganism along with *Lactiplantibacillus plantarum* in traditional cereal fermentations (Gänzle, 2019). Fermentation increases the concentration of free phenolic acids with cereals such as sorghum shown to select for *Lactobacillaceae* capable metabolizing antimicrobial phenolic acids (Sekwati-Monang et al., 2012), with phenolic acid metabolites having lower antimicrobial activity than their precursors (Sánchez-Maldonado et al., 2011). Hydroxycinnamic acids can be metabolized into dihydro- and vinyl derivatives via reduction and decarboxylation reactions respectively, while hydroxybenzoic acids are exclusively decarboxylated by lactic acid bacteria (Sánchez-Maldonado et al., 2011). Genetic determinants responsible for these bioconversions have primarily been characterized in *Lp. plantarum* including the first phenolic acid reductase HcrB, capable of reducing hydroxycinnamic acids (Santamaría et al., 2018a). However, several other *Lactobacillaceae* strains lacking HcrB with reductase activity have also been reported (Chapter 3) including strains of *Lm. fermentum* (Koval et al., 2022; Svensson et al., 2010). Two additional putative phenolic acid reductases Par1 and HcrF were recently identified in heterofermentative

*Lactobacillaceae,* with the role of Par1 confirmed in *Furfurilactobacillus milii* (Chapter 3). This work aimed to confirm the involvement of HcrF in reduction of hydroxycinnamic acids by heterologous production and biochemical characterization of the expressed enzyme.

## 5.2. Materials and methods

### 5.2.1. Bacterial strains and materials

*Limosilactobacillus fermentum* FUA3589 was streaked on modified deMan, Rogosa and Sharpe (mMRS) (Zhao and Gänzle, 2018) agar plates from -80°C glycerol stocks, and incubated at 30°C for 24 h. Isolated colonies were further subcultured into mMRS broth and grown overnight at 30°C under microaerophilic conditions. *Escherichia coli* BL21 star (DE3) was used as the host strain for heterologous expression of recombinant protein with pET-28a(+) used as the expression vector. *E. coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) medium with media containing kanamycin (50 mg/L) used for growing *E. coli* harbouring pET-28a(+) plasmids.

### 5.2.2. Chemicals

LB media and granulated agar were purchased from Becton, Dickinson and Company (BD Difco Sparks, MD, USA) while media components and chemicals required to make mMRS medium were obtained from BD (Sparks, MD, USA) and Millipore Sigma (St. Louis, MO, USA). Sinapic acid, *trans*-cinnamic acid, dihydrosinapic acid and dihydrocaffeic acid were purchased from Millipore Sigma (St. Louis, MO, USA) while dihydroferulic acid was purchased from MP biomedicals (Illkirch, France). Ferulic acid and caffeic acid were obtained from Extrasynthèse (Genay, France). Isopropyl-β-D-thiogalactopyranoside (IPTG) and kanamycin, flavin mononucleotide (FMN) and nicotinamide adenine dinucleotide (NADH) salts were also purchased from Millipore sigma (St.

Louis, MO, USA) while flavin adenine dinucleotide (FAD) salt was obtained from Thermo Scientific (Geel, Belgium).

### 5.2.3. Cloning, expression, and purification of HcrF

Genomic DNA and plasmid DNA were isolated using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and GeneJET Plasmid Miniprep Kit (Thermo Scientific, Mississauga, ON, Canada) respectively according to manufacturer instructions. Gene encoding putative phenolic acid reductase HcrF (WP_135293431.1) in *Lm. fermentum* FUA3589 was PCR amplified using oligonucleotides- F-*hcrF* (5'-GAAGGATCCATGAAAGCAGGAACGTACAA-3′) and R-*hcrF* (5'-TATTCTCGAGGCCATTTTCTTGTGCGGCGG-3') containing BamHI and XhoI restriction sites respectively. GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Scientific, Mississauga, ON, Canada) was used for the purification of *hcrF* PCR fragment followed by its digestion using restriction endonucleases (BamHI/ XhoI) along with purified pET-28a(+) expression vector. The digested products were ligated to form plasmid pET-28a(+)/*hcrF* using T4 DNA ligase (Thermo Scientific) and transformed into chemically competent *E. coli* BL21 star (DE3) cells. Insert was confirmed using colony PCR and sequencing.

Overnight cultures of *E. coli* BL21 harbouring pET-28a(+)/*hcrF* were inoculated into fresh LB media and grown at 37°C until they reached an optical density of 0.4-0.6, followed by the addition of IPTG to a final concentration of 0.22 mM for inducting the expression of recombinant protein. Induced cells were then incubated overnight at 22°C followed centrifugation at 4°C to harvest the cells. Harvested cells were washed and stored at -80°C until further use. Uninduced and induced *E. coli* BL21 harbouring pET-28a(+)/*hcrF* and empty vector lacking the insert, respectively, were used as negative controls.

Cells were lysed using Mini-Beadbeater-16 (Biospec products, Bartlesville, OK, USA) and the soluble His-tagged HcrF was purified using HisPur Ni-NTA spin column (Thermo Scientific, Rockford, IL, USA) following the manufacturer instructions. Purified enzyme was confirmed using SDS-PAGE and concentrated using Amicon® Ultra Centrifugal Filters (cut off- 30kDa) to achieve a final concentration of 10 mg/mL in phosphate buffer saline (PBS pH- 7.4).

### 5.2.4. Determination of substrate specificity and cofactors

To determine the substrate specificity, 10 µg of protein was added to the reaction mixture containing FMN (5 mM), FAD (5 mM) and NADH (10 mM) as cofactors for a final reaction volume of 500 µL. Sinapic acid, ferulic acid, caffeic acid, *p*-coumaric acid and *trans*-cinnamic acid were used as substrates having a final concentration of 1 mM in their respective samples.

For determination of cofactors required by the enzyme, FMN, FAD and NADH were added alone for a final concentration of 10 mM, or in combinations of two at a time with final concentrations of 5 mM for FMN and/or FAD and 10 mM for NADH. Ferulic acid (1 mM) was used as the substrate and 10 µg of protein was added to the reaction mixture. All samples were incubated overnight at 30°C with samples lacking co-factors used as negative control.

After incubation, solvent extraction using ethyl acetate was performed using the protocol described by Svensson et al., 2010. Extracts were analyzed on an Agilent 1200 high performance liquid chromatography (HPLC) system coupled to a UV detector, with Agilent eclipse XDB-C18 column as the stationary phase. Quantification of reduced dihydro-derivatives was performed at 280 nm with external standards using the previously described HPLC protocol (Chapter 3).

### 5.2.5. Enzyme activity assay and biochemical characterization

Enzyme activity was measured by quantification of dihydroferulic acid production using HPLC method described in the above section. The reaction mixture (500 µL) consisted of 1.5 mM of ferulic acid as substrate, 20 µg/mL of purified enzyme and the cofactor mixture of FMN (5 mM) and NADH (10 mM).

To determine the effect of pH on enzyme activity, reactions were performed in the pH range of 3 to 10 and incubated at 30°C. The following buffers were used for the reactions at different pH values- citrate phosphate buffer (3-6), PBS (7) and glycine-NaOH buffer (8-10). Optimum temperature was determined by incubating reactions in the range of 4 to 70°C at pH 7.4. The reaction time was 5 min, and reaction was stopped by addition of hydrochloric acid and ethyl acetate extraction (Svensson et al., 2010).

### 5.2.6. Determination of kinetic parameters

Estimation of kinetic constants $K_m$ and $V_{max}$ was performed for four different hydroxycinnamic acid substrates, namely ferulic acid, caffeic acid, *p*-coumaric acid and sinapic acid. Reactions were performed by varying the substrate concentrations in the range of 0.047 to 10 mM. Samples were incubated at 30°C and reactions were terminated by acidification followed by ethyl acetate extraction after 10 min. Reaction rate (V) was calculated using the experimental data and plotted against the substrate concentration (S) according to Michaelis-Menten equation. Kinetic parameters were estimated using a non-linear curve fit in SigmaPlot 12.5 (Systat Software, Inc.) (Xie and Gänzle, 2021).

## 5.3. Results

### 5.3.1. Purification of HcrF and confirmation of enzymatic activity

To confirm if the putative phenolic acid reductase gene *hcrF* present in strains of *Lm. fermentum* is responsible for the reduction of hydroxycinnamic acids, HcrF was purified after successful heterologous expression in *E. coli* BL21. SDS-PAGE analysis showed a very prominent band at around 70 kDa for the purified sample and crude cell lysate, which is extremely faint in negative controls comprising of *E. coli* harbouring an empty vector and uninduced strain with pET-28a(+)/*hcrF* plasmid (Figure 5.1). The estimated protein mass is slightly lower than the predicted mass of 66.3 (https://web.expasy.org/compute_pi/).

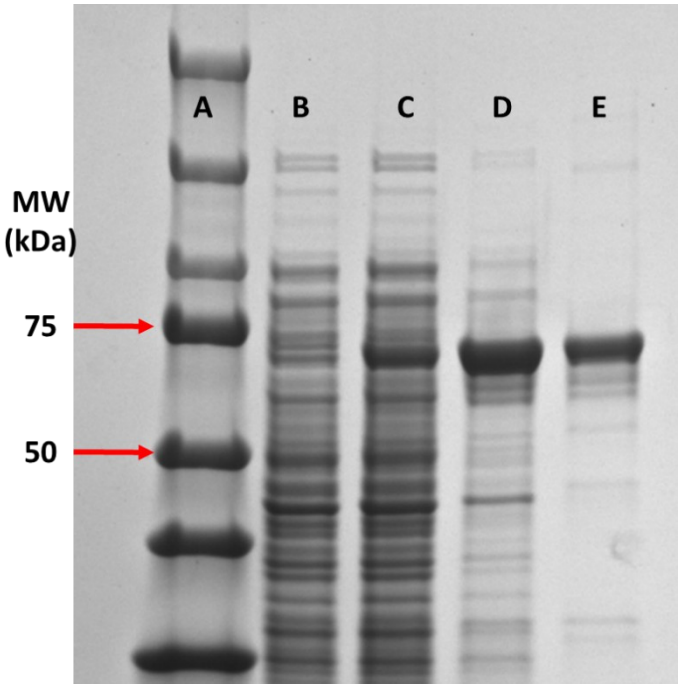



**Figure 5.1. SDS-PAGE analysis of putative phenolic acid reductase HcrF heterologously overexpressed in *E. coli* BL21 (DE3).**

Lane A- molecular weight marker; B- induced cell lysate of *E. coli* with empty vector; C- uninduced cell lysate of *E. coli* pET-28a(+)/*hcrF*; D- induced cell lysate of *E. coli* pET-28a(+)/*hcrF*; E- purified HcrF.

Purified enzyme was added to a reaction mixture containing FMN, FAD and NADH as cofactors and incubated overnight at 30°C with different hydroxycinnamic acid substrates. Dihydro-derivatives of hydroxycinnamic acids were quantified using HPLC. HcrF successfully reduced all the tested substrates except *trans*-cinnamic acid suggesting the presence of a hydroxyl substitution in the phenyl group being essential for activity (Figure 5.2). Concentrations of dihydrocaffeic and dihydrosinapic acid were significantly lower than dihydroferulic acid and phloretic acid ($P<0.001$) when incubated under similar conditions, displaying a higher preference of HcrF towards ferulic acid and *p*-coumaric acid.

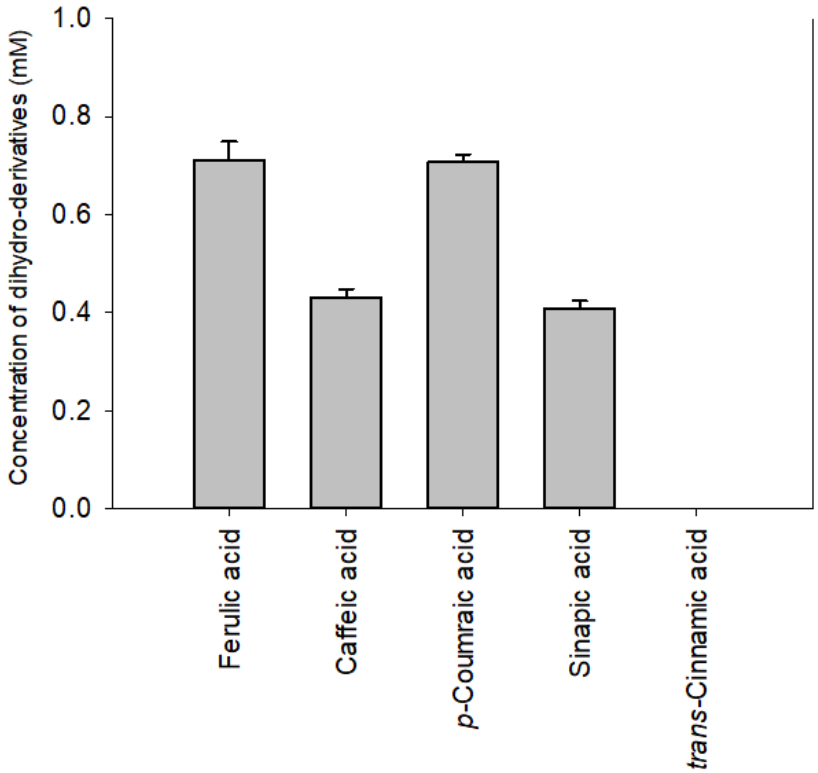



**Figure 5.2. Reductase activity of purified HcrF on different phenolic acid substrates.**

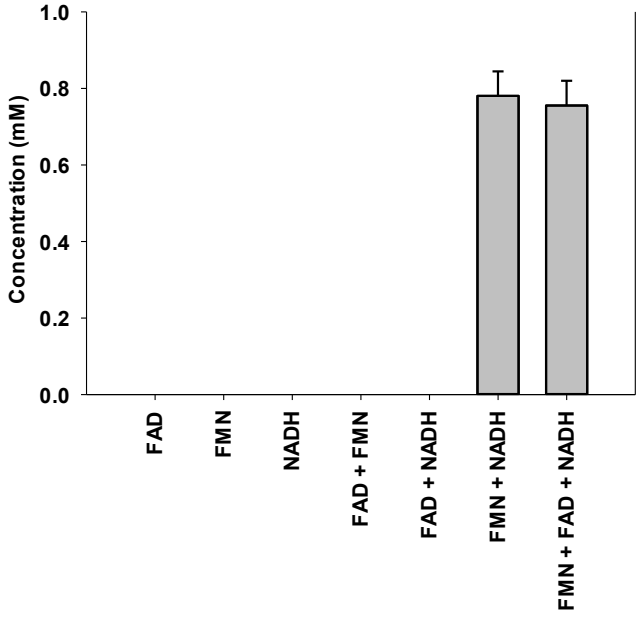
Enzymatic activity of HcrF on 1 mM of different phenolic acid substrates was determined by quantification of dihydro-derivatives produced by the reduction of respective substrates using HPLC. Data shown as mean ± standard deviation (n=3).

### 5.3.2. Biochemical characterization of HcrF

To confirm if all three cofactors used are essential for enzymatic activity, FAD, FMN and NADH were added alone or in combination in the reaction mixture with ferulic acid as the substrate. HcrF displayed reductase activity only in the samples containing FMN + NADH and FMN + FAD + NADH; however, there were no significant differences between dihydroferulic acid concentrations between the two conditions (Figure 5.3a). Therefore, presence of both FMN and NADH as cofactors is essential for activity of purified HcrF with addition of FAD likely not playing any role to enzymatic activity.

Enzymatic activity of HcrF was quantified in the pH range of 3-10 and temperature range of 4-70°C. Optimum activity was observed at a pH of 7 while the HcrF displayed >60% relative activity at pH values of 6 and 8 (Figure 5.3b). Trace activity was observed at pH 4 and 10 while enzyme was inactivated at pH 3. HcrF displayed optimal activity in the range of 30-40°C with >60% relative activity observed at 50°C (Figure 5.3c). HcrF had no enzymatic activity at the end point temperatures of 4 and 70°C used in the study.

a)



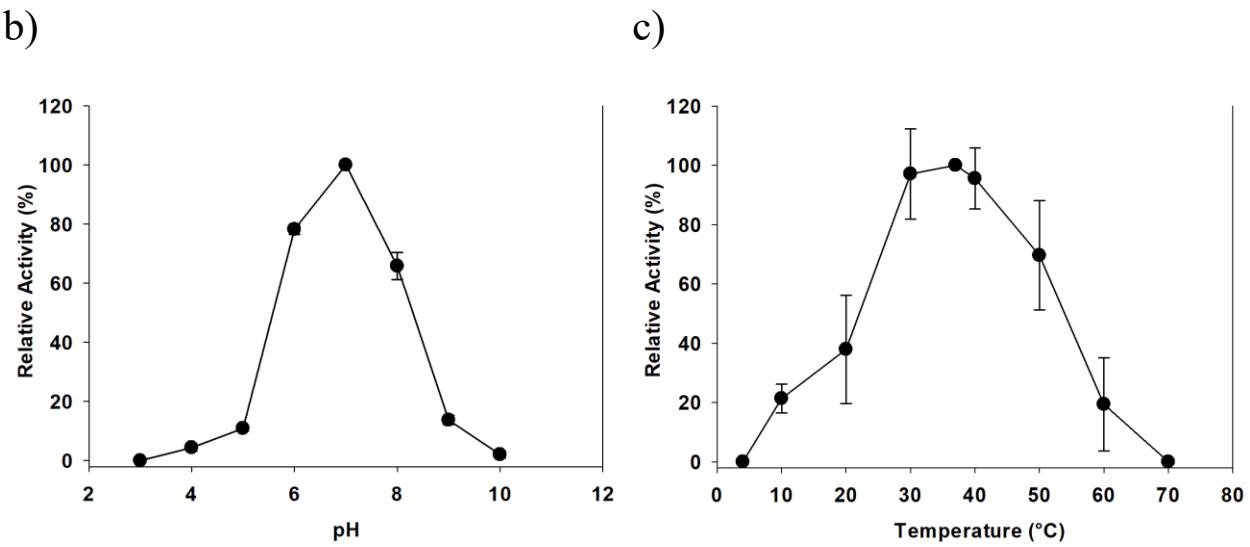



**Figure 5.3. Biochemical characteristics of purified HcrF from *Lm. fermentum* FUA3589.**

Activity of HcrF was measured by quantification of dihydrocaffeic acid produced with 1.5 mM ferulic acid used as the substrate. a) Samples were incubated in the presence of different cofactors at 30ºC and reaction pH of 7.4; b) relative activity at different reaction pH at 30ºC; c) relative activity at different temperatures at reaction pH of 7.4.

### 5.3.3. Kinetic characteristics of HcrF for different hydroxycinnamic acid substrates

HcrF displayed reductase activity on all the hydroxycinnamic acid substrates tested. The kinetic constants $K_m$ and $V_{max}$ were determined by fitting experimental data according to the Michaelis-Menten equation. The concentration of dihydro-derivatives produced by HcrF behaved linearly for at least 14 min of reaction time (data not shown), with 10 min chosen as maximum the reaction time for samples incubated with varying substrate concentration. The $K_m$ values for ferulic acid, *p*-coumaric acid and caffeic acid were 0.37, 0.33 and 0.17 mM, respectively. $V_{max}$ values were highest for ferulic acid followed by *p*-coumaric acid and caffeic acid (Figure 5.4). HcrF displayed very low reaction rates towards sinapic acid in comparison to other substrates. The available data points were not sufficient for a curve fit for the maximum substrate concentration used in this study leaving $K_m$ and $V_{max}$ values undetermined for sinapic acid (data not shown). Use of higher substrate

concentrations was avoided due to low solubility of sinapic acid while limiting the amount of ethanol in the reaction mixture.

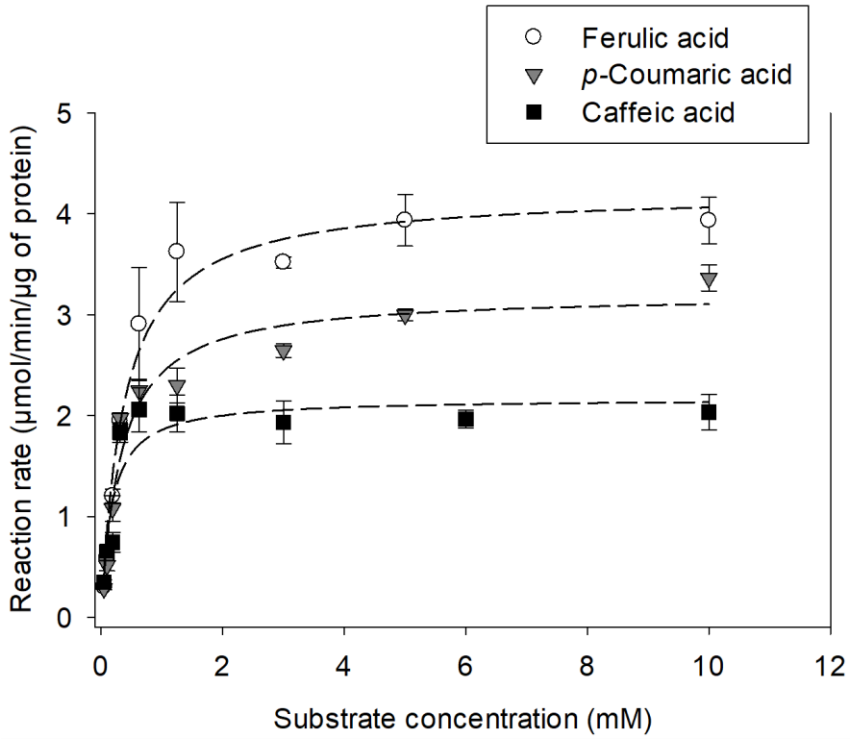



**Figure 5.4. Kinetic characterises of HcrF with different hydroxycinnamic acid substrates.**

Reactions were performed at a reaction pH of 7.4 with samples incubated at 30ºC. Data are the means of 3 replicates ± standard deviation.

## 5.4. Discussion

Phenolic acid reductases responsible for the reduction of hydroxycinnamic acids by *Lactobacillaceae* remained elusive for decades until the recent characterization of HcrB in *Lactiplantibacillus plantarum* (Santamaría et al., 2018a). Another putative phenolic acid reductase was later identified in *Lm. fermentum* FUA3589, which produced dihydro metabolites during growth in mMRS media containing phenolic acids. The gene encoding HcrF was significantly

overexpressed by *Lm. fermentum* in the presence of caffeic acid (Chapter 3). This study provides the confirmation for the role of HcrF in the reduction of hydroxycinnamic acids to their respective phenylpropionic acid derivatives by biochemical characterization of purified enzyme, and determination of its substrate specificity and kinetic characteristics.

The enzyme HcrF is annotated as a FAD-dependent oxidoreductase (NCBI) and is a multi-domain protein predicted to possess both FMN-binding (InterPro ID- IPR007329) and FAD-binding (IPR003953) domains (Chapter 3). HcrF only shares a 24% amino acid identity with phenolic acid reductase Par1 from *Ff. milii* FUA3583 despite containing the same predicted domains (Chapter 3). HcrF (617 bp) also shares a 62% amino acid identity with HcrB (812 bp) from *Lp. plantarum* WCFS1 (Santamaría et al., 2018a); however, HcrB contains an additional NADPH-dependent FMN reductase domain (IPR005025) at the N-terminus.

HcrF was successfully purified with its observed 70 kDa molecular mass in agreement with the predicted value of 66.3 kDa, while it was significantly smaller than HcrB (90 kDa). FAD was not a required cofactor for the enzymatic activity of HcrF despite the presence of predicted FAD-binding domain. Addition of both FMN and NADH is essential with no enzymatic activity observed in samples containing FMN or NADH alone. Soluble HcrF also exhibited a yellow colour after purification, a characteristic of flavin cofactor dependent enzymes also observed with HcrB and a vinyl phenol reductase- VprA (Santamaría et al., 2018b, 2018a).

*Lm. fermentum* FUA3589 converted all the hydroxycinnamic acid substrates tested to their respective dihydro-derivatives in broth but did not significantly overexpress *hcrF* in the presence of ferulic acid and sinapic acid (Chapter 3). However, both substrates were reduced by the purified enzyme indicating its role as a hydroxycinnamic acid reductase. The absence of enzymatic activity on *trans*-cinnamic acid is in agreement with results observed by Santamaría et al., 2018b,

suggesting that the presence of a hydroxyl substitution on the phenyl ring may be essential for enzymatic activity of HcrF. *Lp. plantarum* WCFS1 significantly overexpressed *hcrB* in the presence of *trans*-cinnamic acid but not in presence of gallic acid, with the purified HcrB displaying no enzymatic activity on either substrate (Santamaría et al., 2018a).

HcrF likely shows the highest affinity towards caffeic acid ($K_m$ = 0.17 mM) among the tested substrates, due to the indirect inverse relationship of $K_m$ and enzyme affinity. HcrF reduced ferulic acid and *p*-coumaric acid with significantly higher efficiency compared to caffeic acid and sinapic acid. This may be due to the high concentration of substrates used ($S >> K_m$) combined with higher $V_{max}$ values of ferulic acid (4.20 µmol/min*µg) and *p*-coumaric acid (3.21 µmol/ min*µg) compared to caffeic acid (2.17 µmol/min*µg). Lower enzymatic efficiency towards sinapic acid may be due to steric hindrance caused by the highest number of substitutions (2: -$OCH_3$ and 1: -OH) in the phenyl ring among hydroxycinnamic acids.

HcrF is more widespread across *Lactobacillaceae* in comparison to HcrB (Chapter 3). It is frequently encoded by strains of *Lactobacillus* and *Secundilactobacillus* while also being present in the strains of *Limosilactobacillus* and *Ligilactobacillus* (Chapter 2). Phenolic acid reductases may provide an additional ecological advantage to *Lactobacillaceae* with production of dihydro-derivatives associated with cofactor recycling providing a growth advantage, especially to heterofermentative organisms by increasing metabolic energy (Filannino et al., 2016b, 2014). In food fermentations, production of dihydro-derivatives was found to be the preferred pathway by *Lactobacillaceae* encoding for both phenolic acid reductase and phenolic acid decarboxylase (Filannino et al., 2015; Ripari et al., 2019; Chapter 4). *Lp. plantarum* and *Lm. fermentum* both significantly overexpressed genes encoding phenolic acid reductase HcrB and HcrF respectively, but not phenolic acid decarboxylase Pad during mahewu fermentation (Pswarayi et al., 2022).

The genetic organization for phenolic acid reductases differs between *Lp. plantarum* and *Lm. fermentum,* with an additional gene *hcrA* encoding a NADPH-dependent FMN reductase co-expressed in *Lp. plantarum* WCFS1 along with HcrB (Santamaría et al., 2018a). While HcrB is the only enzyme required for the reductase activity, the purified protein was found to be relatively unstable compared to the stability of the heterodimeric complex of co-expressed and purified HcrAB (Santamaría et al., 2018a). In addition, Liu et al., 2022 found that *E. coli* expressing HcrB were unable to efficiently reduce *p*-coumaric acid to phloretic acid, suggesting that HcrF might be better suited for industrial production of dihydro-derivatives.

Another FAD-dependent oxidoreductase VprA has been characterized in *Lactobacillaceae* responsible for reduction of vinyl derivatives of hydroxycinnamic acids such as vinyl phenol, vinyl guaiacol and vinyl catechol to respective ethyl derivatives (Santamaría et al., 2018b). It contains a single FAD-binding domain also present in HcrF, with about 30% amino acid identity. Several other NADH and flavin dependent 2-enoate reductases have been identified in *Clostridium* and *Eubacterium* which also displayed activity on phenolic compounds (Gall et al., 2014; Mordaka et al., 2018). Heterologously expressed enzymes from *Clostridium acetobutylicum* and *Clostridium sporogenes* were capable of reducing cinnamic acid and *p*-coumaric acid (Mordaka et al., 2018; Sun et al., 2016). Fcr from *Eubacterium ramulus* can convert flavanone and flavanonol chalcones to their respective dihydrochalcones derivatives via reduction (Braune et al., 2019). While these enzymes show great potential for production of reduced phenolic derivatives via heterologous expression, their use in targeted metabolite production from food/feed substrates may be limited due to their generic broad range substrate specificity and oxygen sensitivity (Gao et al., 2012; Rohdich et al., 2001). Therefore, *Lactobacillaceae* phenolic acid reductases may be better suited

for targeted production of dihydro-derivatives via heterologous expression and during fermentation.

In conclusion, this study confirmed the function of HcrF as phenolic acid reductase active on hydroxycinnamic acids. The hydroxyl substitution in phenyl ring likely plays a role in the specificity of the enzyme with FMN and NADH being the required cofactors for activity, however a wider substrate panel may be required to gain more insights into substrate specificity. Kinetic analysis revealed greater affinity of HcrF towards caffeic acid compared to other hydroxycinnamic acids. It can effectively reduce ferulic acid and *p*-coumaric acid at high substrate concentrations and has the potential to be used for production bioactive dihydro-derivatives from phenolic acids.

# Chapter 6- Characterization of phenolic acid esterases in *Lactiplantibacillus plantarum* TMW1.460

## 6.1. Introduction

The abundance of phenolic compounds in plant-based foods plays a significant role in their sensory, nutritive properties and bioactive potential. Though their composition varies greatly among different foods, phenolic acids are among the major constituent in fruits, vegetables, and cereals (Acosta-Estrada et al., 2014). Phenolic acids are antimicrobials, and their metabolism can alter the sensory properties of fermented foods (Sánchez-Maldonado et al., 2011). They mostly occur in conjugated or bound form covalently linked to different cell wall structural components and molecules (Acosta-Estrada et al., 2014). They are bound to cell wall polysaccharides in various cereals and plants such as wheat, rye, kidney beans and cruciferous vegetables (Shahidi and Yeo, 2018; Zhang et al., 2020). Esters of phenolic acids with monosaccharides and alcohols, and ether linkages are common (Shahidi and Yeo, 2018). Consumption of dietary phenols has been linked with beneficial effects on gut health with free phenolic acids been shown to have anti-inflammatory and anti-obesity effects in animal models (Leonard et al., 2021b).

*Lactiplantibacillus plantarum* has been isolated from a variety of food fermentations including vegetables, cereals, and dairy along with human intestinal tract and insect associated habitats (Gänzle, 2019; Martino et al., 2016). This nomadic lifestyle is thought to contribute towards the metabolic flexibility of the species making it a popular candidate for use as starter cultures and probiotics (Duar et al., 2017; J. Zheng et al., 2020).

The free phenolic content can be increased by fermentation of plant materials with lactic acid bacteria by the action of phenolic acid esterases, tannases and glycosyl hydrolases (Gänzle, 2019).

Two cinnomyl estreraes (Lj0536 and Lj1228) were identified in *Lactobacillus johnsonii* (Lai et al., 2009), whose homologs in other lactobacilli such as *L. helveticus* (Song and Baik, 2017), *L. acidophilus* (Kim and Baik, 2015) and *Lm. fermentum* (Liu et al., 2016) are also biochemically characterized primarily as ferulic acid esterases.

The phenolic acid metabolism of *Lp. plantarum* has been studied quite extensively in comparison to other *Lactobacillaceae*, with many enzymes being biochemically characterized from this species including phenolic acid decarboxylases, reductases, tannases and esterases (Muñoz et al., 2017; Santamaría et al., 2018a, 2018b). A total of four phenolic acid esterases have been identified in *Lp. plantarum* with two each among them characterized as hydroxycinnamic acid and hydroxybenzoic acid esterase (Esteban-Torres et al., 2015, 2013; Iwamoto et al., 2008; Jiménez et al., 2014). The biochemically characterized enzymes display overlapping substrate specificities. This when coupled with concurrent occurrence of multiple genes encoding different esterase enzymes in a single strain, makes it harder to ascertain their contribution to *in situ* activity in fermentations.

This work aimed to identify putative phenolic acid esterases in *Lp. plantarum* TMW1.460 and study their role in release of phenolic acids. Deletion mutants lacking genes coding for phenolic acid esterases were constructed and tested for their esterase activity on different phenolic acid esters. Selected mutants were further used in model food fermentations to characterize their esterase activity and its influence on phenolic acid metabolite production.

## 6.2. Materials and Methods

### 6.2.1. Bacterial strains and growth conditions

*Lactiplantibacillus plantarum* and *Escherichia coli* EC1000 were streaked onto modified De Man, Rogosa and Sharpe (mMRS) (Zhao and Gänzle, 2018) and Luria–Bertani (LB) agar plates respectively from -80°C glycerol, followed by subculturing in their respective liquid medium. Strains of *Lp. plantarum* were grown at 30°C under microaerophilic conditions, while *E. coli* strains were grown at 37°C under aerobic conditions. MRS and LB media containing 5 µg/mL and 300 µg/mL erythromycin respectively were used where applicable. All the strains and plasmids used in this study are listed in Table 6.1.

### 6.2.2. Chemicals

Chlorogenic acid, methyl ferulate, methyl gallate, tannic acid, epigallocatechin gallate (EGCG), pyrogallol, gallic acid and erythromycin (Em) were purchased from Millipore Sigma (St. Louis, MO, USA). Ferulic acid and caffeic acid were purchased from Extrasynthèse (Genay, France), while dihydroferulic acid and dihydrocaffeic acid were obtained from MP Biomedicals (Illkrich, France). Polyethylene glycol (PEG) 1500 was obtained from Thermo Scientific (Mississauga, ON, Canada). MRS, LB media and agar were purchased from BD (Sparks, MD, USA). Ingredients required for making mMRS media were obtained from BD (Sparks, MD, USA) or Millipore Sigma (St. Louis, MO, USA).

### 6.2.3. *In silico* identification of phenolic acid esterases in strains of *Lp. plantarum*

Genome sequence and protein fasta files of *Lp. plantarum* TMW1.460 (GCA_009864015.1) and query sequences listed in Table 6.2 were downloaded from NCBI. Protein BLAST was performed using query sequences with cut-off values of 80% query cover and 40% amino acid (aa) identity.

Nucleotide sequences of best match hit for each query were then used for primer design and genetic manipulations Table 6.3.

**Table 6.1. Strains and plasmids used in this study.**

| Strains | Description | Reference |
|---|---|---|
| *Lactiplantibacillus plantarum* TMW1.460 | Isolated from spoiled beer | (Ulmer et al., 2000) |
| *Lp. plantarum* TMW1.460 Δ*lp_0796* | Single deletion mutant of *Lp. plantarum* TMW1.460 lacking *lp_0796* gene | This study |
| *Lp. plantarum* TMW1.460 Δ*est_1092* | Single deletion mutant of *Lp. plantarum* TMW1.460 lacking *est_1092* gene | This study |
| *Lp. plantarum* TMW1.460 Δ*tanB* | Single deletion mutant of *Lp. plantarum* TMW1.460 lacking *tanB* gene | This study |
| *Lp. plantarum* TMW1.460 Δ*hceP* | Single deletion mutant of *Lp. plantarum* TMW1.460 lacking *hceP* gene | This study |
| *Lp. plantarum* TMW1.460 Δ*est_1092* / Δ*hceP* | Double deletion mutant of *Lp. plantarum* TMW1.460 lacking *est_1092* and *hceP* genes | This study |
| *Escherichia coli* EC1000 | Cloning host for pVPL 3002 based plasmids, $RepA^{+}$, $Km^{R}$ | (Leenhouts et al., 1996) |
| | **Plasmids** | |
| pVPL 3002 | pORI19 derived suicide vector, $Em^{R}$ | (Zhang et al., 2018) |
| pVPL3002/ΔLp_0796 | pVPL 3002 containing *lp_0796* flanking regions, $Em^{R}$ | This study |
| pVPL3002/ΔEst_1092 | pVPL 3002 containing *est_1092* flanking regions, $Em^{R}$ | This study |
| pVPL3002/ΔTanB | pVPL 3002 containing *tanB* flanking regions, $Em^{R}$ | This study |
| pVPL3002/ΔHceP | pVPL 3002 containing *hceP* flanking regions, $Em^{R}$ | This study |

Whole genome nucleotide sequences of all *Lp. plantarum* strains (328) assembled at chromosomal level (as of January 2022) were downloaded from NCBI database and reannotated using Prokka 1.13.7 (Seemann, 2014). In addition, genome sequences of *Lp. plantarum* TMW1.460 (Chapter 3), *Lp. plantarum* FUA3584 and FUA3590 (Pswarayi and Gänzle, 2019), and 15 previously sequenced lab strains were also used in the analysis. Presence/ absence of genes were determined by performing protein BLAST on each genome with cut-off values of 75% query cover and 70% amino acid (aa) identity. Protein sequence of Lp_2953 from *Lp. plantarum* WCFS1 (YP_004890534.1) was used as a query sequence in addition to the ones listed in Table 6.2.

### 6.2.4. Construction of phenolic acid esterase knockout mutants

Upstream and downstream flanking regions (800-1000 bp) of identified esterases were amplified by PCR along the plasmid backbone of pVPL3002 (Zhang et al., 2018). Ligation and plasmid assembly was performed using Gibson assembly (Gibson et al., 2009). Plasmid constructs were transformed into electrocompetent *E. coli* EC1000 host cells (Leenhouts et al., 1996) and plated on LB plates containing erythromycin (300 µg/mL) after recovery. Transformants were confirmed using colony PCR and subcultured overnight. pVPL3002 plasmid constructs were isolated and purified using GeneJET Plasmid Miniprep Kit (Thermo Scientific) following the manufacturers instructions.

**Table 6.2. *In silico* identification of biochemically characterized phenolic acid esterases in *Lp. plantarum* TMW1.460.**

| Esterase | Query Sequence ID | Known phenolic acid ester substrates | Locus tag in TMW1.460 | Amino acid Identity (%) | Protein length (aa) | Reference |
|---|---|---|---|---|---|---|
| Lp_0796 | YP_004888771.1 | Hydroxycinnamic acid esters- Methyl ferulate, methyl caffeate, methyl *p*-coumarate, methyl sinapinante. | GB998_RS03015 | 100 | 249 | (Esteban-Torres et al., 2013) |
| Est_1092 | WP_015825406.1 | Hydroxycinnamic acid esters- Methyl ferulate, methyl caffeate, methyl *p*-coumarate, methyl sinapinante. Hydroxybenzoic acid esters- Methyl gallate, methyl vanillate, ethyl gallate, ethyl protcatechuate, epigallocatechin gallate. | GB998_RS05085 | 100 | 295 | (Esteban-Torres et al., 2015) |
| TanA | WP_003640628.1 | Hydroxybenzoic acid esters- Methyl gallate, ethyl gallate, propyl gallate, ethyl protcatechuate, gallocatechin gallate epigallocatechin gallate, tannic acid. | - | - | - | (Jiménez et al., 2014) |
| TanB | YP_004890536.1 | Hydroxybenzoic acid esters- Methyl gallate, ethyl gallate, propyl gallate, lauryl gallate, ethyl protcatechuate, gallocatechin gallate epigallocatechin gallate, tannic acid. | GB998_RS11910 | 99 | 469 | (Curiel et al., 2009; Iwamoto et al., 2008) |
| Lj0536 | WP_004898050.1 | Chlorogenic acid, ethyl ferulate. | GB998_RS11900 | 53 | 249 | (Lai et al., 2009) |
| Lj1228 | WP_011162057.1 | | | | | |

**Table 6.3. Primers used in this study for construction of mutants.**

| Primer (forward, F; reverse, R) | Description | Primer Sequences (5'→3') |
|---|---|---|
| oVPL 188 F | amplifies pVPL3002 backbone (Zhang et al., 2018) | ATCCTCTAGAGTCGACCTGC |
| oVPL 187 R | | TACCGAGCTCGAATTCACTGG |
| oVPL97 F | insert check in pVPL3002 plasmids (Zhang et al., 2018) | CCCCCATTAAGTGCCGAGTGC |
| oVPL49 R | | ACAATTTCACACAGGAAACAGC |
| Lp_0796 U/S F | upstream flanking region of *lp_0796* in *Lp. plantarum* TMW1.460 | CAGTGAATTCGAGCTCGGTAGGCATCTTC TTGCCAATC |
| Lp_0796 U/S R | | CCAATTGGTGGGCATGTTGGCCATGTTC |
| Lp_0796 D/S F | downstream flanking region of *lp_0796* in *Lp. plantarum* TMW1.460 | CCAACATGCCCACCAATTGGAACAAGATG |
| Lp_0796 D/S R | | GCAGGTCGACTCTAGAGGATCGTGGCAAC ATTGGAATC |
| Lp_0796 DCO F | DCO screening for Δ*lp_0796* in *Lp. plantarum* TMW1.460 | CGGCAATGCTGTTATTTTGAATTTG |
| Lp_0796 DCO R | | TGTGACCGAATCACACTTTGGAAA |
| Est_1092 U/S F | upstream flanking region of *est_1092* in *Lp. plantarum* TMW1.460 | CAGTGAATTCGAGCTCGGTAGCATGTTAA ACGGATGAAT |
| Est_1092 U/S R | | TTCCTCCAGACCTCCATTTATTGGCTCTAT C |
| Est_1092 D/S F | downstream flanking region of *est_1092* in *Lp. plantarum* TMW1.460 | TAAATGGAGGTCTGGAGGAAAATATGATG AAG |
| Est_1092 D/S R | | GCAGGTCGACTCTAGAGGATGTCGTTGCC AAGTTCAAG |
| Est_1092 DCO F | DCO screening for Δ*est_1092* in *Lp. plantarum* TMW1.460 | CCTTGCGATAATCACGGTTTTTATTTTACC |
| Est_1092 DCO R | | CCAGCACATCCATAATGGTTGGTGA |
| TanB U/S F | upstream flanking region of *tanB* in *Lp. plantarum* TMW1.460 | CAGTGAATTCGAGCTCGGTAATAACCGCA GCAACCATTG |
| TanB U/S R | | AGGTCACAAGTGAATACGATGAGTGAAA GC |
| TanB D/S F | downstream flanking region of *tanB* in *Lp. plantarum* TMW1.460 | ATCGTATTCACTTGTGACCTCCATTTCTAT C |
| TanB D/S R | | GCAGGTCGACTCTAGAGGATTTCCGACGA TTCTAGTTC |

**Table 6.3.** (continued)

| Primer (forward, F; reverse, R) | Description | Primer Sequences (5'→3') |
|---|---|---|
| TanB DCO F | DCO screening for Δ*tanB* in *Lp. plantarum* TMW1.460 | AAAACTGTTAAAGTTCGTCGATGCT |
| TanB DCO R | | AAATAATTCGAGTGACGTCGATTCC |
| HceP U/S F | upstream flanking region of *hceP* in *Lp. plantarum* TMW1.460 | CAGTGAATTCGAGCTCGGTACGGCTTTACG ACCTATATG |
| HceP U/S R | | TGAGATGACATGACTTACGCCAGTTAATAT AATG |
| HceP D/S F | downstream flanking region of *hceP* in *Lp. plantarum* TMW1.460 | GCGTAAGTCATGTCATCTCACTTATTCATTA TTCACAC |
| HceP D/S R | | GCAGGTCGACTCTAGAGGATGTGTTATTGG GACCGGCATTTG |
| HceP DCO F | DCO screening for Δ*hceP* in *Lp. plantarum* TMW1.460 | GCTTTTTCCACCGACTTAAAGATTTTC |
| HceP DCO R | | TTTCGGCAGGTGTTTCTAATGCTAT |

*Lp. plantarum* TMW1.460 electrocompetent cells were prepared by washing overnight cultures 2-3 times with ice cold 10% PEG 1500 and transformed with 2 μg of plasmid DNA (2.5 kV, 400 Ω, 25 μF). Cells were recovered for 2-3 h in MRS medium and plated on MRS containing erythromycin (5 μg/mL) and incubated for 48-72 h. $Em^R$ colonies were then cultured for a minimum of 2 passages at 42°C, followed by plating on Em MRS plates to obtain single crossover colonies (SCO). To obtain double cross over (DCO), washed cells were cultured in MRS medium containing no antibiotics for 10-20 passages. Mutants were screened by replica plating on MRS and MRS with Em agar. $Em^S$ colonies were screened, and mutants were confirmed via colony PCR and sequencing.

### 6.2.5. Characterization of phenolic acid esterase activity using HPLC

Samples for HPLC were prepared using the protocol by Svensson et al., 2010. Briefly, mMRS media was supplemented with 1 mM of chlorogenic acid, methyl ferulate, methyl gallate, tannic acid and EGCG. Overnight cultures of wild type and mutant strains (10%) were inoculated into supplemented mMRS broth and incubated at 30°C for 24 h or 10 days. Samples were centrifuged, and supernatant was acidified using hydrochloric acid to pH 1.5. Solvent extraction (2X) was performed using half volume ethyl acetate followed by filtration.

Samples were analyzed on an Agilent 1200 series HPLC system equipped with a multi-wavelength UV detector and Eclipse XDB C-18 column (4.6 by 150 mm; 5 µm). Mobile phase consisted of 0.1% formic acid in water (phase A) and 0.1% formic acid in 90% acetonitrile-water (v/v) (phase B). The HPLC method and conditions used have been described previously in Chapter 3.

### 6.2.6. Fermentation of whole wheat sourdough and broccoli

Whole wheat flour and fresh broccoli were purchased from a local supermarket. Overnight cultures of *Lp. plantarum* TMW1.460 wild type, TMW1.460 Δ*tanB* and TMW1.460 ΔΔ*hceP/est_1092* were washed and resuspended in sterile tap water. Blended broccoli and whole wheat flour (10 g) were mixed with 10 mL of $10^8$ CFU/mL of washed cells and incubated at 30°C for 24 h along with chemically acidified controls. They were prepared by adding sterile tap water and adjusting the pH using lactic acid for final volume of 10 mL.

After 24 h of fermentation, 1g of fermented samples were mixed with 9 mL of sterile deionized water for pH measurements along with unfermented controls. mMRS plates were plated with 10-fold serial dilutions of fermented and unfermented samples prepared in 0.1% peptone water and

incubated for 24-48 h for cell counts. Remaining samples were freeze dried and stored at -20°C for further analysis.

### 6.2.7. Quantification of free phenolic metabolites in fermentation samples

Extraction of free phenolics was performed using the protocol described by Ripari et al., 2019. Briefly, solvent extraction was performed twice on freeze dried fermented samples (250 mg) using 1 mL of 80% ethanol. Collected supernatants were mixed and evaporated under nitrogen. Precipitate obtained was dissolved in acetic acid (2%) with pH of the solution adjusted to 2 for a final volume of 500 µL, followed by solvent extraction using ethyl acetate. The Extract was once again evaporated under nitrogen followed resuspension of solids in 200-400 µL of 100% methanol (0.1% formic acid).

Extracted free phenolics samples were run using the same HPLC, column and solvent system as mentioned in section 6.2.5. The gradient used was as follows- 10% to 42% B (35 min), 42%-48% B (15 min), 48% to 10% B (1 min) and isocratic with 10% B (14 min), described in Chapter 4. Quantification was performed using external standards at 280 and 330 nm. Injection volume was 10 µL with a flow rate of 0.3 mL/min.

## 6.3. Results

### 6.3.1. Phenolic acid esterases in *Lp. plantarum* TMW1.460

Four phenolic esterases have been characterized in *Lp. plantarum* including 3 intracellular esterases and one extracellular tannase. Protein BLAST results revealed the presence of only intracellular esterases in this strain with extracellular TanA absent in *Lp. plantarum* TMW1.460 (Table 6.3). Lp_0796 and Est_1092 were characterized as hydroxycinnamic acid esterases with the latter also shown to be active on hydroxybenzoic acids. TanB is characterized as an intracellular

tannase. All the three enzymes showed almost 100% amino acid identity to the query sequences from other strains of *Lp. plantarum*. *Lp. plantarum* TMW1.460 also has another uncharacterized phenolic acid esterase that shares 53% amino acid identity with 2 homologous phenolic acid esterases characterized in *Lactobacillus johnsonii,* hereafter referred as HceP (Lai et al., 2009).

### 6.3.2. Phenotypic characterization of phenolic acid esterase activity and metabolism

To confirm which of the phenolic acid esterases present in *Lp plantarum* TMW1.460 are responsible for the esterase activity, different hydroxycinnamic and hydroxybenzoic acid esters were added to mMRS broth inoculated with wild type and mutant strains lacking genes for different phenolic acid esterases and incubated at 30°C for 24 h or 10 d. Wild type strain had esterase activity on both types of phenolic acid esters (Table 6.4). Chlorogenic acid, methyl ferulate and methyl gallate were all hydrolyzed to release caffeic acid, ferulic acid and gallic acid, respectively.

*Lp. plantarum* TMW1.460 Δ*lp_0796* and TMW1.460 Δ*est_1092* had the same phenotype as the wild type strain with respect to phenolic acid esterase activity. *Lp. plantarum* TMW1.460 Δ*tanB* also hydrolysed hydroxycinnamic acid esters but did not hydrolyze methyl gallate to gallic acid. Conversely, the strain *Lp. plantarum* TMW1.460 Δ*hceP* only hydrolyzed methyl gallate but did not hydrolyse chlorogenic acid or methyl ferulate. Pyrogallol, the decarboxylation metabolite of gallic acid was also detected for all the samples in which methyl gallate was hydrolyzed to gallic acid. However, no metabolites of released hydroxycinnamic acids were detected for any of the strains after a 24 h incubation. None of the strains hydrolyzed epigallocatechin gallate and tannic acid. Methyl gallate was the only hydroxybenzoic acid substrate that was hydrolyzed in this study.

**Table 6.4. Characterization of phenolic acid esterase activity and metabolite production of *Lp. plantarum* TMW1.460 and its knockout mutants.**

WT and its isogenic mutant strains were incubated with 1 mM of different substrates for 24 h or 10 days at 30ºC. Shaded and unshaded boxes represent presence and absence of the phenotype for the metabolites as detected by the HPLC.

| Strain Name | Incubation Time (days) | Chlorogenic Acid | Methyl Ferulate | | Methyl Gallate | | Tannic Acid | Epigallocatechin-3-gallate |
|---|---|---|---|---|---|---|---|---|
| | | Caffeic Acid | Ferulic Acid | Dihydroferulic Acid | Gallic Acid | Pyrogallol | Gallic Acid | |
| *Lp. plantarum* TMW1.460 WT | 1 | + | + | - | + | + | - | - |
| | 10 | + | + | + | + | + | - | - |
| *Lp. plantarum* TMW1.460 Δ*lp_0796* | 1 | + | + | - | + | + | - | - |
| | 10 | + | + | + | + | + | - | - |
| *Lp. plantarum* TMW1.460 Δ*est_1092* | 1 | + | + | - | + | + | - | - |
| | 10 | + | + | + | + | + | - | - |
| *Lp. plantarum* TMW1.460 Δ*tanB* | 1 | + | + | - | - | - | - | - |
| | 10 | + | + | + | - | - | - | - |
| *Lp. plantarum* TMW1.460 Δ*hceP* | 1 | - | - | - | + | + | - | - |
| | 10 | - | - | - | + | + | - | - |
| *Lp. plantarum* TMW1.460 Δ*hceP/* Δ*est_1092* | 1 | - | - | - | + | + | - | - |
| | 10 | - | - | - | + | + | - | - |

Since the all the phenolic acid esterases present in *Lp. plantarum* TMW1.460 have been previously characterized as intracellular enzymes, strains were also incubated for a period of 10 days to observe any possible differences in metabolic activity. None of the strains showed any change in the metabolism after extended incubation for any of the substrates except ferulic acid. Dihydroferulic acid was detected in all the strains that had showed esterase activity on methyl ferulate after 10-day incubation. The double mutant *Lp. plantarum* TMW1.460 Δ*hceP*/Δ*est_1092* had the same phenotype as the TMW1.460 Δ*hceP* mutant strain under all the conditions tested.

### 6.3.3. Characterization of esterase activity and phenolic acid metabolism in food fermentations

To determine the influence of phenolic acid esterases on metabolite formation during food fermentations, the wild type strain, and the mutants *Lp. plantarum* TMW1.460 Δ*tanB* and TMW1.460 Δ*hceP/* Δe*st_1092* were used for fermentation of broccoli and whole wheat flour. The cell counts after fermentation were 9-9.5 log CFU/mL with pH value dropping to 3.75 and 3.45 for broccoli puree and whole wheat sourdough respectively (Table 6.5).

The major phenolic acid metabolites detected in broccoli fermentations were dihydrocaffeic acid and dihydrosinapic acid. Dihydroferulic acid was the major metabolite detected in whole wheat sourdoughs (Figure 6.1). No significant differences were observed among the wild type and mutant strains for both fermentation substrates used in this study.

**Table 6.5. Cell counts and pH of samples fermented with *Lp. plantarum* TMW1.460 and its mutant strains.**

Samples were incubated at 30ºC for 24 h (n=3).

| Sample | Cell count (Log CFU/ml) | | pH | |
|---|---|---|---|---|
| | Broccoli puree | Whole wheat sourdough | Broccoli puree | Whole wheat sourdough |
| Unfermented control | 5.50 ± 0.25 | 4.54± 0.11 | 6.75± 0.03 | 5.88± 0.02 |
| Chemically acidified | <4 | <4 | 3.50± 0.02 | 3.50± 0.02 |
| *Lp. plantarum* TMW1.460 WT | 8.94± 0.23 | 9.40 ± 0.28 | 3.75± 0.02 | 3.45± 0.04 |
| *Lp. plantarum* TMW1.460 Δ*tanB* | 9.04 ± 0.22 | 9.32 ± 0.13 | 3.73± 0.03 | 3.40± 0.03 |
| *Lp. plantarum* TMW1.460 Δ*hceP/* Δe*st_1092* | 9.11 ± 0.20 | 9.25 ± 0.11 | 3.76± 0.02 | 3.43± 0.05 |

## 6.4. Discussion

The first phenolic acid esterase identified in *Lactobacillaceae* was a tannase (TanB) of *Lp. plantarum* ATCC 14917; in addition, the extracellular tannase TanA and two hydroxycinnamic acid esterases, Lp_0796 and Est_1092, have been characterized in strains of *Lp. plantarum* (Curiel et al., 2009; Esteban-Torres et al., 2015, 2013; Iwamoto et al., 2008; Jiménez et al., 2014). *Lp. plantarum* stains also possess an uncharacterized homolog of cinnamoyl esterases identified in *L. johnsonii* with >50% amino acid identity (this study). The presence of multiple phenolic acid esterases with potentially overlapping substrate specificity in a single strain makes the accurate estimation of genotype and phenotype relationships challenging; in addition, the characterization

of heterologously expressed proteins may not relate to the *in situ* activity of enzymes in food fermentations. This study therefore characterized the role of different phenolic acid esterases in *Lp. plantarum* TMW1.460 by characterization of isogenic mutants lacking one or more of the esterases and confirmed the activity of a novel hydroxycinnamic acid esterase and its role in two different food fermentations.

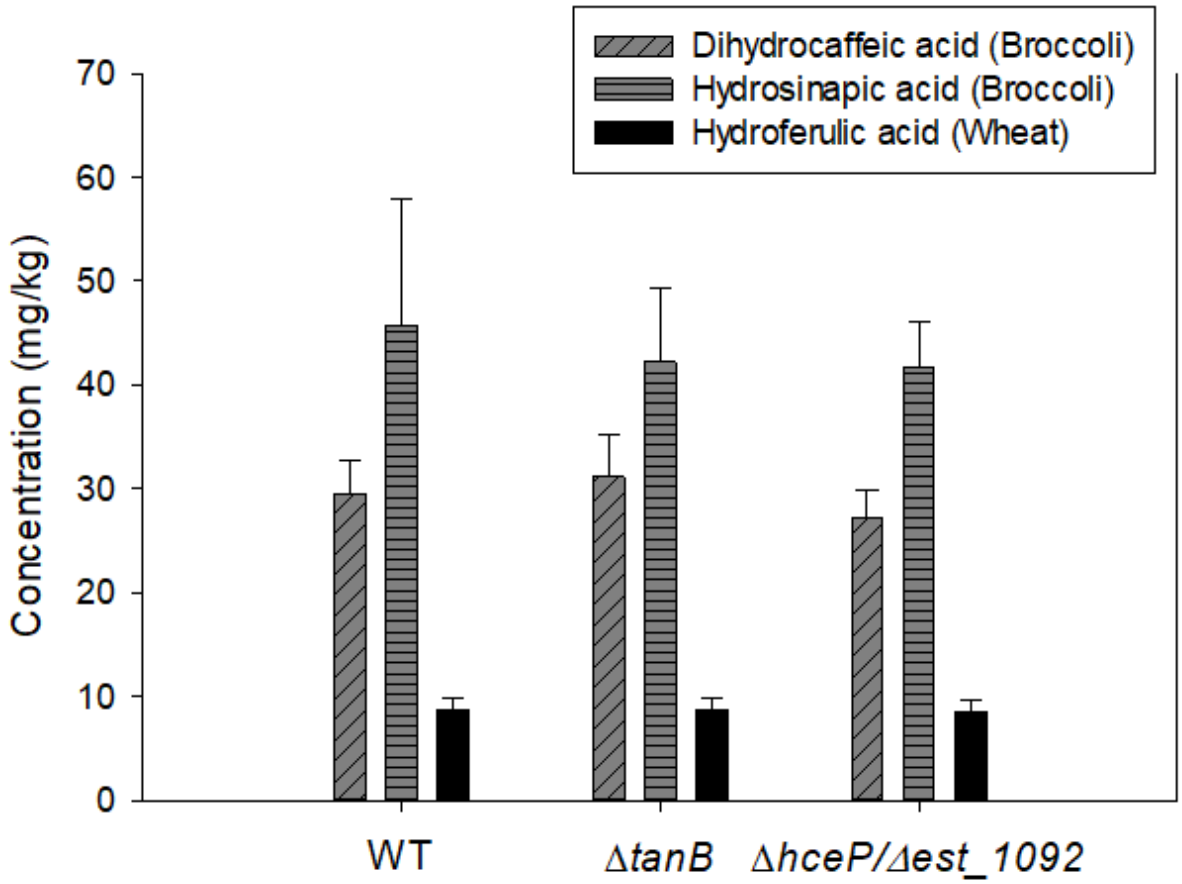



**Figure 6.1. Characterization of esterase activity via quantification of free phenylpropionic acid metabolites in broccoli and whole wheat fermented samples.**

Both food matrices were fermented with strains of *Lp. plantarum* TMW1.460 for 24 h at 30°C. WT- Wild type, *tanB*- tannase, *hceP* and e*st_1092*- phenolic acid esterases (n=3).

The hydroxycinnamic acid esterase Lp_0796 was characterized in *Lp. plantarum* WCFS1. Cell free extracts from strain exhibited a very low activity on methyl esters of ferulic and *p*-coumaric acid; however, whole cells of this strain did not hydrolyze these substrates (Esteban-Torres et al., 2013). Biochemical characterization of Est_1092 revealed that it is also active on methyl esters of hydroxycinnamic acids and was suggested to be the esterase that mediate ester hydrolysis in *Lp. plantarum* WCFS1 (Esteban-Torres et al., 2015). The genome of *Lp. plantarum* TMW1.460 encodes for both Lp_0796 and Est_1092 and hydrolysed methyl ferulate and methyl gallate. *Lp. plantarum* TMW1.460 Δ*lp_0796* and TMW1.460 Δ*est_1092* mutants showed the same phenotype as the wild type strain, pointing towards the presence of another esterase that is active independent of Est_1092.

*In silico* analysis revealed *Lp. plantarum* TMW1.460 also contains a homolog of the two *L. johnsonii* cinnamoyl esterases (Lj0536 and Lj1228), which is hereafter referred as HceP. Lj0536 exhibited high activity on ethyl ferulate only while Lj1228 was also active with other esters of hydroxycinnamic acids (Lai et al., 2009)**.** These two enzymes are 50% identical to each other and to HceP (this study). The deletion mutant *Lp. plantarum* TMW1.460 Δ*hceP* lost esterase activity with methyl ferulate and chlorogenic acid as substrates. This enzyme is widespread in homofermentative vertebrate adapted lactobacilli with homologs characterized in *L. helveticus* (Song and Baik, 2017)*, L. acidophilus* (Kim and Baik, 2015)*,* and *L. gasseri* (Fritsch et al., 2017). The genomes of some heterofermentative *Lactobacillaceae* such as *Lv. hammesii* and *Lm. reuteri* also encode for homologs of HceP. HceP homologues have also been biochemically characterized in *Lm. fermentum* (Deng et al., 2019; Liu et al., 2016). *L. gasseri* and *Lm. reuteri* have also displayed chlorogenic acid esterase activity in food substrates such as sunflower flour and broccoli puree respectively (Filannino et al., 2015; Fritsch et al., 2016).

*Lp. plantarum* TMW1.460 thus possess three hydroxycinnamic acid esterases but only deletion of *hceP* resulted in the loss of esterase activity, demonstrating that the esterases Lp_0796 and Est_1092 are not responsible for the activity of cultures of *Lp. plantarum* TMW1.460. In *Lp. plantarum* WCFS1, *est_1092* and *hceP* was over-expressed in response to methyl-ferulate while *lp_0796* was down-regulated (Esteban-Torres et al., 2015). However, LP_0796 was not over-expressed during growth of *Lp. plantarum* in cereal substrates while HceF, the homologue to HceP in *Lm. fermentum*, was over-expressed during growth in millet malt (Pswarayi et al., 2022). Current data on the expression of phenolic acid esterases is too limited to differentiate whether these differences in gene expression relate to differences between strains, or between different substrates.

The intracellular tannase TanB was characterized in *Lp. plantarum* ATCC 14917 and showed activity on tannic acid, EGCG and other simple gallic acid esters (Curiel et al., 2009; Iwamoto et al., 2008). The extracellular tannase TanA was required for esterase activity by whole cells (Jiménez et al., 2014). *Lp. plantarum* TMW1.460 only encodes for TanB which explains the absence of activity on tannic acid and EGCG in growing cultures. The wild type strain but not its TanB-negative mutant released gallic acid from methyl gallate, confirming its role as a hydroxybenzoic acid esterase (Reverón et al., 2017).

*Lp. plantarum* TMW1.460 converts hydroxycinnamic acids to their respective metabolites (Chapter 3). Despite the presence of both phenolic acid decarboxylase and phenolic acid reductase, the strains of *Lp. plantarum* TMW1.460 did not further metabolize caffeic acid and ferulic acid that were released from chlorogenic acid and methyl ferulate, respectively, after 24 h of incubation. It may be due to the low concentration of antimicrobial free phenolics present since their metabolism is considered a detoxification method (Sánchez-Maldonado et al., 2011, Chapter 4).

Gallic acid was always further metabolized to pyrogallol, which is the decarboxylated metabolite of gallic acid via the action of gallic acid decarboxylase (Jiménez et al., 2013).

Fermentation of food substrates with strains of *Lp. plantarum* demonstrated phenolic acid metabolism including esterase activities on chlorogenic acid and ferulic acid esters (Filannino et al., 2015; Hole et al., 2012; Ripari et al., 2019). To confirm that phenolic acid esterases characterized in this study are responsible for *in situ* activity, strains were fermented in broccoli puree and whole wheat sourdough. Fermentation with wild type and mutant strains equally increased the concentration of metabolites from free phenolic acid. Phenyl propionic acids produced by the reductase HrcB (Santamaría et al., 2018a) was the major metabolite detected in both fermented food matrices (this study). Unfermented broccoli is rich in sinapic acid esters and chlorogenic acid while whole wheat flour is rich in ferulic acid (Filannino et al., 2015; Ripari et al., 2019). Our results match the data of previous studies with high concentrations of dihydrocaffeic acid and dihydrosinapic acid detected in broccoli fermentations and dihydroferulic acid in whole wheat sourdoughs. Decarboxylated metabolites for *p*-coumaric acid and caffeic acid were below detection limit, while vinyl catechol was not identified due to lack of the external standard.

*Levilactobacillus hammesii* DSM16341, metabolized hydroxybenzoic acids in wheat sourdoughs but not in rye malt sourdoughs (Ripari et al., 2019). Similarly, fermentation of cherry juice with strains of *Lp. plantarum* resulted in higher concentrations of phenylpropionic acid, while vinyl/ethyl derivatives were the major metabolites when the strain was cultured in mMRS broth (Filannino et al., 2015). In sorghum fermentation *Ff. milii* FUA3583 converted caffeic acid to predominantly dihydrocaffeic acid while vinyl catechol was the major product upon growth in

mMRS media (Chapter 4). This indicates that phenolic acid metabolism enzymes are differentially expressed in different food systems.

Strains of *Lp. plantarum* displayed strain specific activity in altering quinic acid in broccoli fermentation, but chlorogenic acid concentrations remained unchanged (Filannino et al., 2015; Ye et al., 2019). Concentrations of caffeic acid and its metabolites also remained unchanged between broccoli samples fermented with *Lp. plantarum* TMW1.460 and its Δ*lp_0796*/Δ*hceP* esterase mutant strain (this study). On the other hand, *Lm. reuteri* FUA3168, which also encodes for an HceP homolog (51% amino acid identity) like the *Lp. plantarum* strains, significantly reduced chlorogenic acid concentrations in broccoli fermentation (Filannino et al., 2015). This variation in metabolic activity may be attributed to the effect of substrate composition on the expression of phenolic acid esterases. The substrates used to quantify gene expression such as free hydroxycinnamic acids and their methyl esters are absent or present in low concentrations in foods. Thus, the regulation of gene expression of phenolic acid esterases remains unclear.

The presence of multiple enzymes with overlapping substrate specificities likely reflects the diversity of phenolic compounds including esters of phenolic acids in plants. Accumulation of phenolic compounds helps in survival and adaptation of plants to different environments (Lattanzio et al., 2012). Chlorogenic acid is abundant in foods such as coffee and broccoli while also being widespread across different edible plants (Santana-Gálvez et al., 2017). Phenolic acid glycosides are less common compared to flavonoid glycoside esters but are found in flaxseeds, mustard, and canola, and in red peppers (Alu'datt et al., 2017; Engels et al., 2012; Materska et al., 2003). Cereals such sorghum and oats are rich in glycerol esters of phenolic acids (Svensson et al., 2010; Varga et al., 2018).

Bioinformatic analysis revealed that Lp_0796, HceP and TanB esterases are present in > 99% of 345 strains analyzed. On the other hand, the extracellular tannase TanA and Est_1092 are accessory genes present in only 14% and 16% of *Lp. plantarum* strains respectively (Table 6.6). Phenolic compounds can act as substrates for co-factor recycling by *Lactobacillaceae* (Filannino et al., 2019), while the -CHO part of the ester such as glucose may be available as substrate for consumption by the action of phenolic acid esterases. Ability to release various free phenolic acids via hydrolysis might be ecologically relevant for plant associated *Lactobacillaceae* which is further supported by the presence of two esterases and one tannase in the core genome (Brochet et al., 2021). The concentration of individual phenolic acid esters in cereals and fruits is generally low, except for chlorogenic acid. In addition, multiple esters likely hydrolyzed by different esterases contribute to high concentrations of phenolic acids and their metabolites, targeted in this study. Therefore, to ascertain the effect of individual enzymes in food substrates, quantification of phenolic acid esters by LC-MS/MS is required.

**Table 6.6. Distribution of phenolic acid esterases across *Lactiplantibacillus plantarum* strains.**

Data shown as positive hits for protein BLAST with cutoff values of 75% query cover and 70% amino acid identity.

| Esterase | Number and percentage of positive strains |
|---|---|
| Lp_0796 | 344 (100 %) |
| Est_1092 | 55 (16 %) |
| TanA | 48 (14 %) |
| TanB | 344 (100 %) |
| HceP | 341 (99 %) |

In conclusion, this study expanded on the role of different phenolic acid esterases present in *Lp. plantarum* TMW1.460 along with characterization of a novel hydroxycinnamic acid esterase HceP responsible for hydrolysis of chlorogenic acid and methyl ferulate. While direct use of enzymes or heterologous overexpression can be routes for targeted metabolite production as shown by Landete et al., 2021 for *est_1092* and *tanA*, the biochemically characterized enzymes are not concrete evidence of *in situ* activity in food fermentations. This may be due to the complementing substrate specificities of enzymes and differential gene expression in varied phenolic substrate compositions. This study also provided evidence of differential metabolism between laboratory media and food fermentations while confirming the role of different esterases by generation of isogenic mutants.

# Chapter 7- Role of thiols and ascladiol production in patulin degradation by *Lactobacillaceae*

## 7.1. Introduction

Patulin is a heat stable mycotoxin produced by over 60 species of fungi including *Penicillium*, *Aspergillus* and *Byssochlamys*. *Penicillium expansum* is regarded as its main producer with respect to patulin contamination in food (Moake et al., 2005; Wright, 2015). The European Food Safety Authority (EFSA) allows for a maximum of 50 µg/L patulin in fruit juices, ciders and fermented drinks containing apple juice and 25 µg/kg for solid apple products and puree (EFSA 2006). Patulin levels as high as 2.7 mg/L have been reported in apple juice compared to 35.2 µg/L in commercial apple cider, which may indicate patulin degradation during fermentation (Harris et al., 2009; Ioi et al., 2017; Stinson et al., 1978). Apart from pome fruits, patulin contamination can also occur in vegetables, cereals, cheeses, and shellfish, but their contribution to chronic toxicity may be insignificant compared to patulin intake via apple products (Wright, 2015).

Chronic patulin intake has been associated with various gastrointestinal symptoms such as nausea, abdominal pain, and diarrhea (Fung and Clark, 2004; Mandappa et al., 2018). Animal studies have shown DNA damage in liver, hippocampus, and kidneys along with increased cardiotoxicity due to acute consumption of patulin at a level of 1.0–3.75 mg/kg) (Boussabbeh et al., 2015; de Melo et al., 2012). Patulin forms adducts with thiols resulting in reduction of cellular glutathione and generating oxidative stress (Fliege and Metzler, 2000). It also reacts with nucleotide bases leading to DNA damage and genotoxicity (Pfenning et al., 2016). Its possible presence in several foods and agricultural products necessitates its accurate detection and detoxification to keep its concentration below the thresholds set by regulatory agencies.

Implementation of stringent controls in post harvest processing can reduce the risk of mycotoxin accumulation (Errampalli, 2014; Ioi et al., 2017). Pasteurization, microfiltration, clarification and radiation have been shown to reduce patulin levels effectively alone or in combination with other processing steps (Diao et al., 2018). The use of food fermenting yeasts and lactic acid bacteria has been proposed as an alternative for reduction of patulin levels, with most studies focussed on adsorption to microbial cells as a means of reducing the mycotoxin levels. Live and heat inactivated cells reduced the patulin content by more than 90% but the efficacy varied for different initial concentrations of patulin and the biomass of the microbial strains (Bahati et al., 2021; Lai et al., 2022; Zheng et al., 2020; Zoghi et al., 2017).

Reduction in patulin levels via biodegradation has also been reported by many yeasts including *Saccharomyces cerevisiae, Candida guilliermondii* and *Rhodosporidium kratochvilovae*, which produce ascladiol and / or desoxypatulinic acid from patulin (Castoria et al., 2011; Luo et al., 2022; Zhong et al., 2021). Among lactic acid bacteria, only ascladiol production by few strains of *Lp. plantarum* has been reported (Hawar et al., 2013; Wei et al., 2020). The role of thiol accumulation by lactic acid bacteria in reduction of patulin levels remains unknown. This study aimed to better understand the mechanisms of patulin reduction of *Lactobacillaceae* and in particular to assess the relevance of bioconversion, formation of thiol-adducts, and absorption to the cell wall. Therefore, 11 strains of *Lactobacillaceae* belonging to 6 different genera were screened for patulin absorption and biodegradation in apple juice. The contribution of thiols in reduction of patulin levels was assayed by comparison of cultures of *Fructilactobacillus sanfranciscensis* DMS 20451 and its glutathione reductase (Δ*gshR*) negative mutant.

### 7.2. Materials and Methods

### 7.2.1. Bacterial strains and growth conditions

All the strains used in this study were subcultured from -80°C stocks and grown in modified de Man, Rogosa and Sharpe (mMRS) media (Zhao and Gänzle, 2018). *Lactiplantibacillus plantarum* TMW1.460 (Ulmer et al., 2000), *Lp. plantarum* LA1 (Ripari et al., 2019), *Apilactobacillus kunkeei* DSM 12361, *Levilactobacillus brevis* TMW1.465 (Behr et al., 2006), *Levilactobacillus hammesii* DSM 16381(Valcheva et al., 2005), *Furfurilactobacillus milii* C5 (Ripari et al., 2019), *Ff. milii* FUA3583 (Pswarayi and Gänzle, 2019), *Fructilactobacillus sanfranciscensis* DSM 20451 and *Limosilactobacillus fermentum* FUA3590 (Pswarayi and Gänzle, 2019) were grown under microaerophilic conditions at 30°C while *Limosilactoabcillus reuteri* DSM 20016 was incubated at 37°C. *Fl. sanfranciscensis* DSM 20451 Δ*gshR* (Jänsch et al., 2007) lacking glutathione reductase activity was also used having identical growth conditions as the wild type strain with the addition of erythromycin (10 µg/mL).

### 7.2.2. Chemicals

Patulin and erythromycin (Em) were purchased from Millipore Sigma (St. Louis, MO, USA). Apple juice was purchased from a local supermarket. Media components used to make mMRS were obtained from BD (Sparks, MD, USA) while remaining components were obtained from Millipore Sigma (St. Louis, MO, USA).

### 7.2.3. Fermentations in mMRS and apple juice in the presence of patulin

Overnight cultures were washed with sterile water and resuspended in fresh mMRS and mMRS without cysteine (Cy) media. Patulin was added to mMRS and mMRS w/o Cy for a final concentration of 50 mg/L and inoculated with 10% of respective resuspended overnight cultures.

Samples were incubated at 30°C for 24 h. Uninoculated media with and without patulin were used as controls.

Apple juice was adjusted to a pH of 5.8 using of 5M sodium hydroxide (NaOH) followed by addition of patulin. Apple juice (900 µL) was inoculated with 100 µL of washed overnight cultures resuspended in sterile water for a final patulin concentration of 50 mg/L. For preparation of concentrated inoculums, 1 mL of cells were centrifuged at 8600 x *g* for 10 min and resuspended in 100 µL of sterile water. Heat inactivated controls were prepared by treating washed cells at 60°C for 30 min followed by addition to apple juice patulin samples. Uninoculated apple juice with and without patulin were used as controls. All apple juice samples were incubated at 30°C for 48 h except *Lm. reuteri* DSM 20016 samples which were incubated at 37°C.

### 7.2.4. Quantification of patulin and its metabolites using HPLC and LC-MS

Samples were centrifuged at 8000 x *g* for 10 min for removal of cells. The Supernatant was acidified using hydrochloric acid (HCl) followed by addition of 500 µL ethyl acetate. Solvent extraction was performed twice, and extracts were mixed for analysis (Ripari et al., 2019). Samples were analyzed on an Agilent 1200 series HPLC system equipped with a reverse phase XDB C-18 column (4.6 by 150 mm; 5 µm) using the protocol established in Chapter 3. Quantification was performed using a UV detector at 280 nm along with a patulin external standard.

Reverse phase HPLC-MS was performed using an Agilent 1200 SL HPLC system for the identification of patulin degradation product. Samples were analzyed on a Phenomenex Luna omega C18 column (50 by 2.1 mm; 1.6 µm) with trap cartridge at 50°C. Solvents consisted of 0.1% formic acid in water (A) and 0.1% formic acid in 100% acetonitrile. The following gradient was used at a flow rate of 0.5 mL/min- isocratic at 1% B (0-1.5 min), 1% to 95% B (1.5-5.5 min),

isocratic at 95% B (5.5-6.5 min) and 95 to 1% B (6.5-7 min). Analytes were detected at 280 nm using a diode array detector. Mass spectra were acquired using an Agilent 6220 Accurate-Mass time-of-flight HPLC-MS system (Santa Clara, CA, USA) equipped with a dual sprayer electrospray ionization source.

### 7.2.5. Statistical Analysis

Data was analyzed using one-way analysis of variance (ANOVA) followed by Holm-Sidak or Tukey post hoc test. Apple juice data between live and heat inactivated cultures for each strain was analyzed using t-test in SigmaPlot 12.5 (Systat Software Inc.).

## 7.3. Results

### 7.3.1. Reduction of patulin levels and metabolite formation by *Lactobacillaceae*

Strains of *Lactobacillaceae* were incubated in mMRS broth containing patulin for 24 h and the patulin concentration was determined by HPLC. *Lp. plantarum*, *Lv. brevis*, *Lv. hammesii*, *Ff. milii*, *Lm. reuteri* and *Lm. fermentum* reduced the patulin concentration (data not shown). Reduced levels of patulin were associated with increased intensity of an unidentified peak.

The conversion of patulin by *Lactobacillaceae* was also assayed in apple juice. To account for the reduction of patulin levels via adsorption, heat inactivated cultures of *Lactobacillaceae* were also incubated in similar conditions and compared to viable cells. Patulin levels in live and heat-inactivated cultures of *Lv. brevis*, *Ff. milii* C5, and *Lm. fermentum* differed significantly but the strains did not reduce patulin levels relative to the uninoculated control (Figure 7.1). *Lp. plantarum* TMW1.460 was the only strain that had a very high patulin reduction for live cultures in comparison to both uninoculated controls and heat inactivated culture samples.

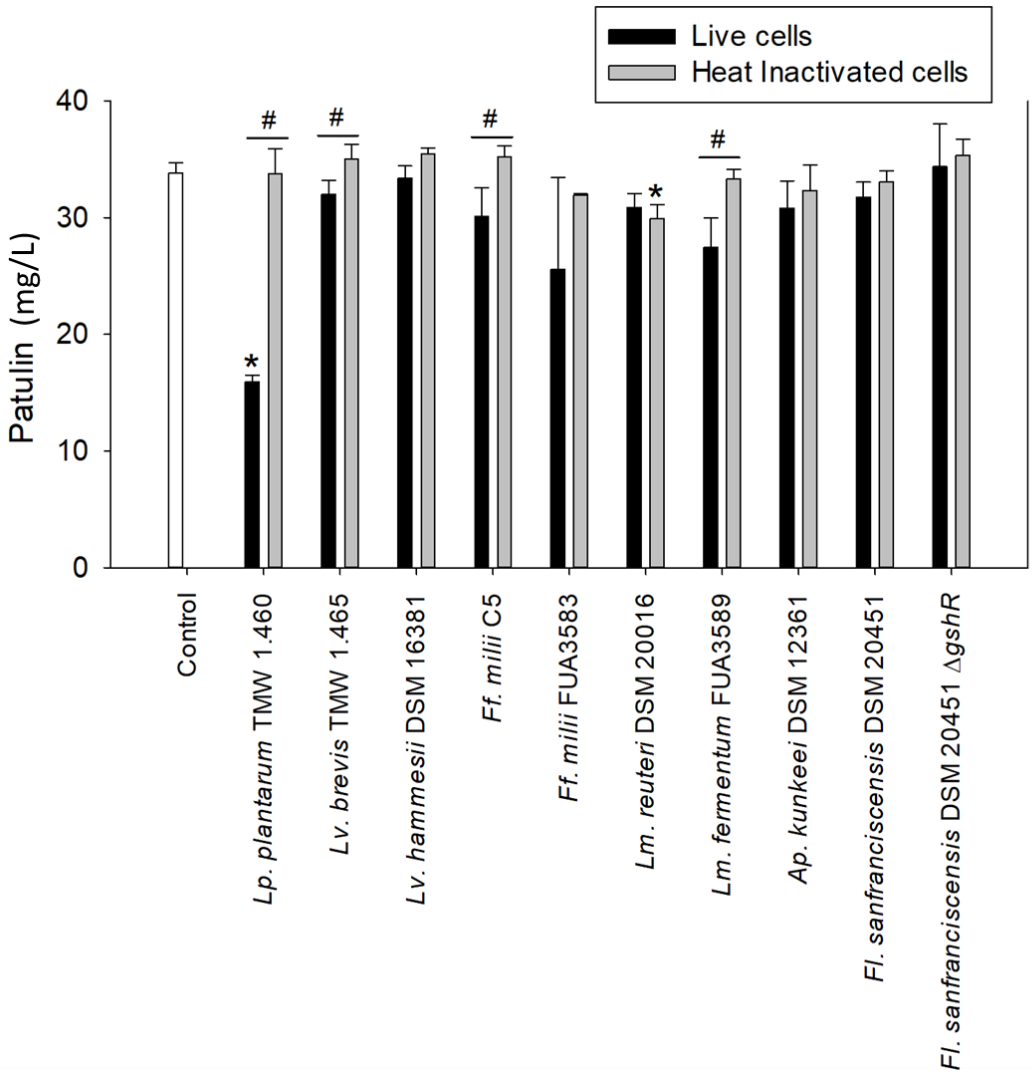



**Figure 7.1. Mean patulin concentration in apple juice inoculated with live and heat inactivated cells of different strains of *Lactobacillaceae*.**

Apple juice fermentation samples were incubated for 48 h at 30°C. Bars with * represents significant difference ($P < 0.05$) from the control. Bars with # represent significant differences between live and heat inactivated cultures for each strain. Data are means ± SD of three independent experiments.

### 7.3.2. Identification of ascladiol production by *Lactobacillaceae*

For identification of the degradation peak observed in several *Lactobacillaceae* samples in both mMRS and apple juice media, *Lp. plantarum* samples were analyzed by LC-MS. The peak at *m/z*

157.0494 in positive ion mode matches the molecular ion peak of ascladiol + $H^+$. In addition, the characteristic fragments at *m/z* 131 and *m/z* 114 were also present (Hawar et al., 2013; Wei et al., 2020) (Figure 7.2).

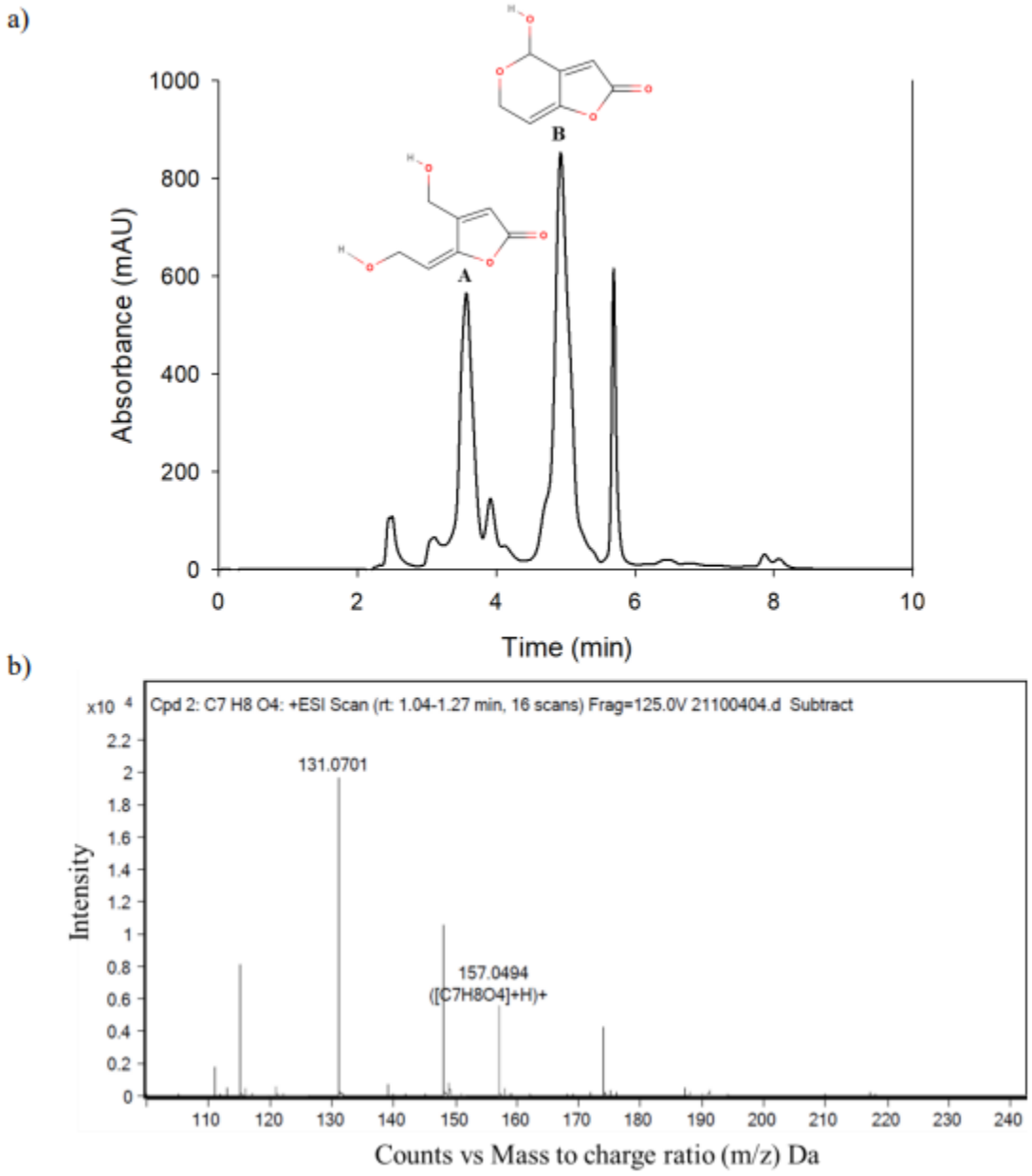



**Figure 7.2. HPLC chromatogram of apple juice spiked with patulin and fermented with *Lp. plantarum* TMW1.460.**

a) HPLC-UV chromatogram (280 nm) of apple juice spiked with patulin and fermented with *Lp. plantarum* TMW1.460 for 48 h at 30°C. Peak A) ascladiol; peak B) Patulin.; b) LC-MS spectrum of peak A) identified as ascladiol (m/z- 157.0494).

Apple juice samples fermented by other *Lactobacillaceae* were also analyzed to confirm if any other strains had the ability to produce ascladiol. Ascladiol was detected in culture supernatants of all strains except *Ap. kunkeei* DSM 16341 and *Fl. sanfranciscensis* DSM 20451 after 48 h of incubation (Table 7.1).

**Table 7.1. Summary of ascladiol production by *Lactobacillaceae* strains.**

Different symbols represent values of relative peak area obtained for ascladiol after 48 h fermentation in apple juice. Presence of ascladiol was confirmed using LC-MS. ++ : >25 %, + : >10 %, ± : >2 %, - : Not detected, X: Not determined.

| Strains | Inoculum Concentration | |
|---|---|---|
| | 1X | 10X |
| *Lactiplantibacillus plantarum* TMW1.460 | ++ | |
| *Lactiplantibacillus plantarum* LA1 | ++ | |
| *Levilactobacillus brevis* TMW1.465 | ± | + |
| *Levilactobacillus hammesii* DMS 12361 | ± | |
| *Furfurilactobacillus milii* C5 | ± | |
| *Furfurilactobacillus milii* FUA3583 | ± | |
| *Limosilactobacillus reuteri* DSM 20016 | X | ± |
| *Limosilactobacillus fermentum* FUA3589 | ± | |
| *Apilactobacillus kunkeei* DSM 16341 | - | - |
| *Fructilactobacillus sanfranciscensis* DSM 20451 | - | ± |
| *Fructilactobacillus sanfranciscensis* DSM 20451 Δ*gshR* | - | - |

To check the influence of higher initial microbial concentration on ascladiol production, apple juice medium inoculated with 10 times (10X) concentrated cell suspensions were also analyzed. The relative peak area of the ascladiol peak increased for all the strains except *Lv. hammesii* DSM 12361. Interestingly, ascladiol formation was also detected in *Lm. reuteri* DSM 20016 and *F. sanfranciscensis* DSM 20451 when the cell density was increased. The peak area corresponding to ascladiol was highest in strains of *Lp. plantarum*, followed by *Lv. brevis* TMW1.465.

### 7.3.3. Effect of glutathione reductase and thiols in reduction of patulin levels

To assess the role of thiols in reduction of patulin levels, *Fl. sanfranciscensis* DSM 20451 and its glutathione reductase mutant strain Δ*gshR* were incubated in the presence/ absence of cysteine in mMRS broth containing patulin (50 mg/L) for 24 h at 30°C. In the presence of cysteine, the wild type strain significantly reduced the patulin concentration in comparison to the Δ*gshR* mutant (Figure 7.3). The presence of cysteine in uninoculated controls did not affect the patulin concentrations significantly. Addition of cysteine resulted in reduced patulin concentrations in media inoculated with *Fl. sanfranciscensis* when compared to the cultures without addition of cysteine. The wild type strain reduced patulin concentrations to a greater extent than the Δ*gshR* mutant irrespective of cysteine addition. No significant differences were observed between Δ*gshR* mutant and its corresponding controls in both conditions. In contrast, the wild type strain significantly reduced the patulin concentration in the presence of cysteine compared to the uninoculated control.

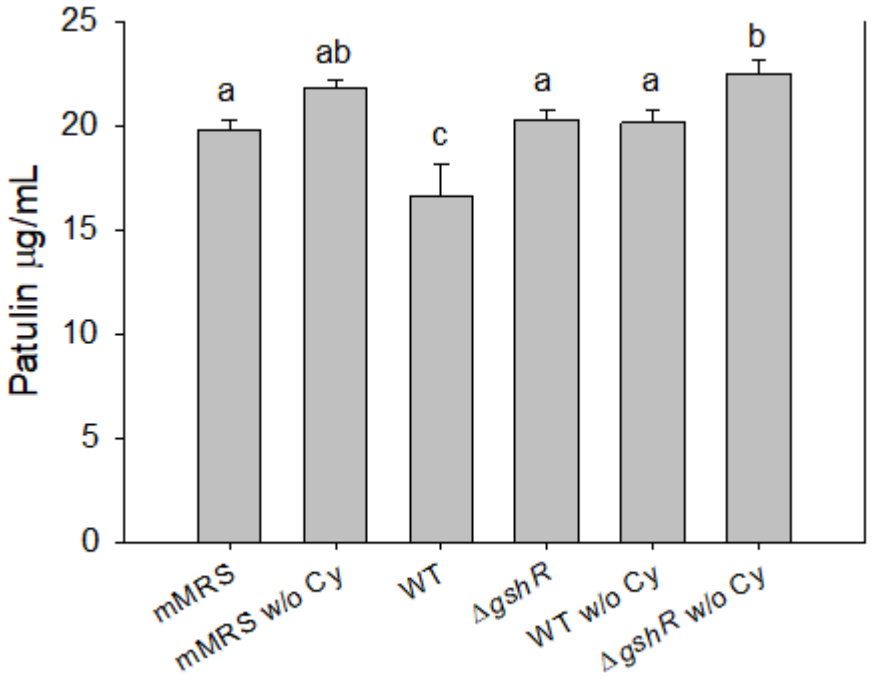



**Figure 7.3. Reduction in mean patulin concentration by *Fl. sanfranciscensis* DSM 20451 and effect of thiols.**

Strains were incubated for 24 h in mMRS medium containing patulin with or without cysteine (Cy- 0.5 g/L) at 30°C. WT- Wild type strain; Δ*gshR*- Mutant strain with inactivated glutathione reductase gene. Bars with different letters are significantly different ($P < 0.05$). Data are means ± SD for three replicates.

Strains of *Fl. sanfranciscensis* were unable to alter patulin concentrations relative to uninoculated controls in apple juice fermentations unlike mMRS (Figure 7.1). *Fl. sanfranciscensis* does not perform well in apple juice in the absence of appropriate energy source for metabolism, which was evident by Δ*gshR* mutant's struggle for growth with high final pH (5.56 ± 0.09) obtained after incubation. To overcome the growth deficit, samples inoculated with concentrated (10X) cultures were also analyzed but increasing the initial microbial counts also didn't show any influence on patulin concentration reducing ability for *Fl. sanfranciscensis* strains in apple juice (Figure 7.4).

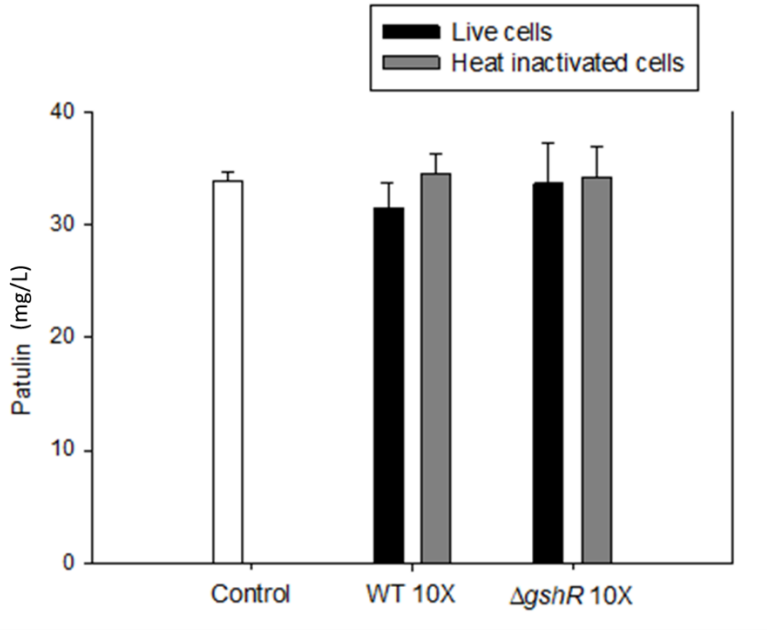



**Figure 7.4. Influence of concentrated inoculums (10X) of live and heat inactivated *Fl. sanfranciscensis* DSM 20451 on patulin concentration in apple juice.**

Apple juice fermentation samples were incubated for 48 h at 30°C. WT- Wild type strain; Δ*gshR*- Mutant strain with inactivated glutathione reductase gene. Data is represented as means ± SD for three independent experiments.

## 7.4. Discussion

Fermentation can reduce the patulin levels in foods with biotransformation by yeast and role of thiol-compounds reported as detoxification mechanisms (Diao et al., 2018). Lactic acid bacteria also have the potential to reduce levels of patulin in foods, but the mechanisms for this activity remain poorly understood. Most studies focus on the patulin adsorption ability of the *Lactobacillaceae* cells while reports of detoxification via biotransformation remain limited. This study explores the potential of various *Lactobacillaceae* for reduction of patulin levels by confirmation of ascladiol production and providing evidence for contribution of thiols.

Several *Lactobacillaceae* strains reduced patulin levels in mMRS broth which was associated with increased intensity of an unidentified peak, highest for *Lp. plantarum* and *Lv. brevis*. In apple juice fermentation, heat inactivated cultures were also used as control to assess the impact of cell adsorption in patulin reduction. *Lm. reuteri* DSM 20016 was the only strain showing significant reduction in patulin levels among heat inactivated cultures, indicating high adsorption of patulin. Reduction in patulin levels by adsorption is dependent on initial concentration of toxin, cell density and pH of the solution (Fuchs et al., 2008). *Lp. plantarum* ATCC 8014 and *L. kefiranofaciens* JKSP109 were shown to remove >90% of 100 µg/L patulin from supplemented apple juice inoculated with > log 10 CFU/mL cells at pH <4 (Bahati et al., 2021; Zoghi et al., 2019). The lower cell density (7-8 log CFU/mL) and high concentration of patulin (50 mg/L) used in this study can explain the lack of patulin removal by heat inactivated cultures via adsorption. Increasing the microbial load by 10 times had a positive influence on patulin reduction via adsorption (Data not shown).

Biotransformation of patulin to ascladiol was first reported in *Saccharomyces cerevisiae* with both isomers *(E)*-ascladiol and *(Z)*-ascladiol found in anerobic conditions (Moss and Long, 2002). *(E)*-Ascladiol was the predominant product while its conversion to *(Z)*-ascladiol is thought to be the result of a non-enzymatic reaction (Moss and Long, 2002). *Gluconobacter oxydans* and *Lp. plantarum* also degraded patulin by conversion to ascladiol (Hawar et al., 2013; Ricelli et al., 2007). *Lp. plantarum* TMW1.460 was the only strain in this study which significantly reduced the patulin concentration in apple juice with respect to both controls, likely via ascladiol production. To confirm the identity of degradation peak, *Lp. plantarum* samples were analyzed via LC-MS with the degradation peak tentatively identified as ascladiol.

To confirm which other strains had the ability to produce ascladiol, apple juice fermentation samples for remaining *Lactobacillaceae* were also analyzed using LC-MS. To date, the conversion of patulin to ascladiol in *Lactobacillaceae* has been reported only for a few strains of *Lp. plantarum* (Hawar et al., 2013; Wei et al., 2020). The present study detected low levels of ascladiol also in culture supernatants of *Lv. brevis* TMW1.465, *Lv. hammesii* DMS12361, *Ff. milii* C5, *Ff. milii* FUA3583, *Lm. reuteri* DSM 20016, *Lm. fermentum* FUA3589 and *Fl. sanfranciscensis* DSM 20451. Interestingly, both hop resistant beer spoiling strains used in this study namely, *Lp. plantarum* TMW1.460 and *Lv. brevis* TMW1.465 showed high ascladiol production. Hawar *et al.* 2013 also reported the presence of hydroascladiol after >10 d of incubation in *Lp. plantarum* samples. Par2 (an uncharacterized homolog of phenolic acid reductase) was hypothesized to be involved in reduction of ascladiol to hydroascladiol. However, incubation with *Ff. milii* FUA3583 and its isogenic mutants lacking genes coding for Par enzymes showed identical patulin profile with presence of hydroascladiol unconfirmed (Table 7.2).

**Table 7.2. Patulin metabolism by *Furfurilactobacillus milii* FUA3583 and isogenic mutants in mMRS broth.**

*par1*- Phenolic acid reductase, *par2*- uncharacterized homolog of Par1. (±) represents presence in trace amounts; (-): not detected.

| Strain | Ascladiol | Hydro-ascladiol |
|---|---|---|
| *Furfurilactobacillus milii* FUA3583 | ± | - |
| *Furfurilactobacillus milii* FUA3583 Δ*par2* | ± | - |
| *Furfurilactobacillus milii* FUA3583 Δ*par1*Δ *par2* | ± | - |

Ascladiol production by other yeast species such as *Meyerozyma guilliermondii*, *Sporobolomyces* sp. and *Candida guilliermondii* has been attributed to an inducible short-chain dehydrogenase (Chen et al., 2017; Ianiri et al., 2017; Luo et al., 2022). Recently, Xing *et al.*, 2021 heterologously expressed and characterized CgSDR from *C. guilliermondii* 2.63 responsible for converting patulin to ascladiol. *Lactobacillaceae* possess numerous putative alcohol dehydrogenases with unknown substrate specificities (Liu et al., 2008) which, in analogy to yeasts, also may contribute to ascladiol. The production of ascladiol might also be result of dehydrogenase enzymes displaying moonlighting activity on patulin (Jeffery, 2018). Another possible patulin metabolite includes desoxypatulinic acid, which is produced by some yeasts such as *Rhodosporidium kratochvilovae* and *Rhodotorula mucilaginosa* (Castoria et al., 2011; Li et al., 2019), but its production by *Lactobacillaceae* has not been reported.

It is generally believed that patulin conversion to ascladiol reduces toxicity but the data on the toxicity of dietary ascladiol in humans remains limited (Tannous et al., 2017). A histological study on pig intestinal tissue showed ascladiol to be non-toxic (Maidana et al., 2016). Ascladiol has low to no toxicity at concentrations of less than 5 mg/L on some human cell lines including human colon carcinoma (Caco-2), human hepatoma (HepG2) and human esophageal epithelial cells (Het-1a) (Tannous et al., 2017; Yang et al., 2021; Zheng et al., 2018). The conversion of patulin to ascladiol by *Lactobacillaceae* thus likely reduces the toxicity.

To assess the role of thiols in reduction of patulin levels, *Fl. sanfranciscensis* and its glutathione reductase mutant (Δ*gshR*) were incubated in patulin containing mMRS broth along with the presence/ absence of cysteine. Patulin can undergo electrophilic reactions and has been shown to form a variety of glutathione adducts in the presence of glutathione (Fliege and Metzler, 2000; Schebb et al., 2009). Concentrations of glutathione and cysteine were found to be significantly

reduced in patulin treated *Saccharomyces cerevisiae* in comparison to untreated control (Shao et al., 2012). In this study, wild type strain significantly reduced the patulin levels in the presence of cysteine compared to Δ*gshR* mutant. Higher patulin reduction obtained in the presence of glutathione reductase and cysteine provides evidence for role of thiols in patulin reduction by lactic acid bacteria. Similar activity by *Fl. sanfranciscensis* strains was not observed in apple juice likely due to poor bacterial growth and high pH obtained after fermentation Presence of cysteine in acidic conditions has been shown to reduce patulin levels at temperatures of 90°C and above where patulin is shown to have high relative stability (Diao et al., 2021). Liu *et al.*, 2019 created a cysteine based synthetic metal-organic framework adsorbent which effectively removed 4.38 μg of patulin per mg of adsorbent from apple juice.

Can *Lactobacillaceae* effectively reduce patulin levels in apple products? Liquorilactobacilli such as *Lq. mali* and *Lq. sicerae* are frequently isolated from fermented apple ciders (Carr and Davies, 1970; Puertas et al., 2014). Other *Lactobacillaceae* such as *Lp. plantarum* and *Lv. brevis* are also part of dominant microbiota in traditional cider production (Dueñas et al., 1994). In this study, both *Lp. plantarum* and *Lv. brevis* strains converted significant amounts of patulin to ascladiol in apple juice. Many other *Lactobacillaceae* strains tested also produced trace amounts of ascladiol. Concentration of patulin used in this study is 1000 times more than the maximum permitted limit which is unlikely to be present in actual apple fermentations in lieu of proper pre-harvest handling practices (EFSA 2006). This might enable the small significant differences observed due to thiols and trace ascladiol production being relevant in patulin reduction.

The low risk of patulin contamination in cider production can be attributed to fermentation, with yeast being the major contributor in comparison to *Lactobacillaceae* (Cousin et al., 2017). Fermentation with yeast lead to a 99% reduction of 15 mg/L patulin in spiked apple juice (Stinson

et al., 1978). A recent study by Zhong *et al.*, 2021 showed that ascladiol producing *Saccharomyces cerevisiae* S288C also converts patulin into other metabolites including glutathione adducts such as c-GSH-PAT and l-GSH-PAT. The concentrations of ascladiol and desoxypatulinic acid obtained (<10 %) do not correspond with the concentration of patulin reduced by yeasts (Ianiri et al., 2013; Zhong et al., 2021). Also, ascladiol formation was limited to fermentation, with cell lysate predominantly reducing paulin levels via GSH adduct formation with heat treatment having no significant effect in patulin reduction (Zhong et al., 2021). The production of other unknown metabolites remains possible, but in yeasts, thiol adduct formation likely is the predominant route of detoxification. Patulin thiol adducts have been shown to be significantly less toxic than their parent mycotoxin but studies regarding effects of GSH-patulin adducts consumption and safety remain limited (Diao et al., 2022; Lindroth and Von Wright, 1978).

Patulin contamination in other foods remains possible. A survey of semi-hard cheeses revealed the presence of 15 to 460 µg/kg of patulin, but most of it was limited to the rind (Pattono et al., 2013). Based on quantity of dietary intake and contamination levels present, the contribution of other foods to chronic patulin toxicity in humans remains unlikely (Cunha et al., 2014; Dobson, 2017; Ji et al., 2017). Furthermore, *Lactobacillaceae* may contribute to removal of patulin during food fermentations especially in the absence of yeasts. Based on limited ascladiol production observed in this study, thiol adduct formation may be the main contributor except for strains showing very high ascladiol production such as strains of *Lp. plantarum*.

Application of *Lactobacillaceae* as adsorbents may not be a practical alternative to existing physical and processing steps involved in the production of apple products. They possess a risk of producing metabolites which can alter sensory attributes, are required in large amounts for use in industrial scale and their efficiency is highly sensitive to environmental factors (Diao et al., 2018).

Use of well characterized synthetic adsorbents and clarifying agents might be more promising given their consistent performance along with easier integration in the processing plants (Liu et al., 2019a).

In conclusion, *Lactobacillaceae* have the potential to effectively reduce patulin levels in pome fruit products and fermentations. They can do this by enzymatic conversion of patulin to ascladiol and/or by maintaining a supply of thiols facilitating patulin adduct formation.

# Chapter 8- General discussion and conclusion

## 8.1. Is there a clear link between lifestyle and ecology on the presence of phenolic metabolism genes in *Lactobacillaceae*?

A clear association with lifestyle can be made for nomadic and insect-adapted lifestyles. Insect-adapted strains rarely possess any phenolic acid metabolism genes (Chapter 2) reflective of their small genome sizes and limited carbohydrate metabolism (Ellegaard et al., 2015; Kwong and Moran, 2016; Zheng et al., 2015). Based on these criteria, strains from *Holzapfelia* and *Amylolactobacillus* genera are also likely to follow an insect-adapted lifestyle. Insect associated strains of *Lactobacillus melliventris* clade in *L. delbrueckii* group had hits for some hydroxycinnamic acid metabolism genes (Chapter 3). However, they can also metabolize a wider range of carbohydrates than insect-adapted genera of *Apilactobacillu*s and *Bombilactobacillus* (J. Zheng et al., 2020), and remain a part of vertebrate-adapted *Lactobacillus* genus.

Nomadic genera of *Lactiplantibacillus* and *Lacticaseibacillus* frequently possess a diverse toolkit for metabolism of phenolic compounds (Chapter 2) owing to large genome size and varied isolation sources reflective of their lifestyle (J. Zheng et al., 2020). *Lp. plantarum* and *Lc. casei,* and their closely related species have been firmly assessed in the literature for their metabolic activity on phenolic compounds. Other nomadic *Lactobacillaceae* also likely possess huge potential, however their ability to transform phenolic compounds remains to be assessed.

Vertebrate-host adapted *Lactobacillaceae* seem to specialize in hydroxycinnamic acid metabolism with very high frequency of reductase and esterase genes in *Lactobacillus* and *Limosilactobacillus* strains respectively (Chapter 2). *L. gasseri*, *L. acidophilus*, *L. helveticus* and *Lm. reuteri* are the most common vertebrate gut isolates studied for their metabolic activity on phenolic compounds in model food fermentations.

Most *Lactobacillaceae* genera with an assigned lifestyle are categorized as free-living microorganisms and frequently include environmental and plant isolates (Duar et al., 2017). Except for *Schleiferilactobacillus,* type strains of all the genera following this lifestyle had the presence of at least one phenolic acid metabolism gene with genetic determinants for decarboxylation occurring frequently (Chapter 2). Decarboxylation and/or reduction of phenolic acids is considered a detoxification mechanism (Sánchez-Maldonado et al., 2011), which may indicate a lifestyle adaptation in these organisms. In addition, hydroxycinnamic acids and their vinyl derivatives can act as external electron acceptors, increasing energy yield for heterofermentative *Lactobacillaceae* via reduction reactions (Filannino et al., 2014; Santamaría et al., 2018b). Presence of at least one of the pathways is sufficient to confer a selective advantage in a phenolic rich environment (Chapter 4). However, the extensive diversity of the lifestyle (Duar et al., 2017) and sparse distribution of known enzymes (Chapter 2) does not allow for a clear association.

The type strains of *Secundilactobacillus* genus seem to possess a diverse toolkit of multiple phenolic metabolism genes which may be reflective of their role as secondary fermenters or spoilage organism found in nutrient deficient plant fermentations (J. Zheng et al., 2020), using phenolic substrates, and attached glycosides for aid in growth. Further classification of free-living *Lactobacillaceae* into plant associated, secondary fermenters and truly free-living organisms may allow for better insights into the association of phenolic metabolism potential and ecology.

*Companilactobacillus* and *Pediococcus* are the largest genera with unassigned lifestyle and while not dominant, they are frequently present in a variety of food fermentations including vegetable and cereals. Type strains of both genera also frequently encode for phenolic metabolism genes (Chapter 2). Information on ecology and lifestyle remains limited for these genera (J. Zheng et al.,

2020), and while some strains of *Companilactobacillus* may display a nomadic behaviour, majority of *Companilactobacillus* and *Pediococcus* species possess small genome sizes uncharacteristic of this lifestyle (Duar et al., 2017; J. Zheng et al., 2020).

The dominance of *Lp. plantarum* and *Lv. brevis* in spontaneous plant and cereal fermentations (Gänzle, 2019) may have a correlation with frequent occurrence of phenolic acid metabolism genes present in these genera (Chapter 2). Cereals substrates with high phenolic content such as sorghum and millet select for strains resistant to the antimicrobial activity of phenolic compounds (Sekwati-Monang et al., 2012). Strains of *Lp. plantarum*, *Lm. fermentum, Ff. milii* and *Lc. casei* isolated from sorghum fermented products (Pswarayi and Gänzle, 2019; Sekwati-Monang et al., 2012) frequently possess phenolic metabolism genes suggesting ecological relevance (Chapter 2, 3 and 4). Strains of *Weissella cibaria* are also frequent isolates (Pswarayi and Gänzle, 2019) with documented reductase/ decarboxylase activity against phenolic acids (Filannino et al., 2014; Chapter 3).

**8.2. Is the current knowledge on genotype and characterized enzymes sufficient to select strains for food fermentations?**

*Lactobacillaceae* possess separate genetic determinants responsible for decarboxylation of hydroxycinnamic and hydroxybenzoic acids. Phenolic acid decarboxylase active on hydroxycinnamic acids (Pad) has been biochemically characterized in strains of *Lp. plantarum* (Cavin et al., 1997) and *Lv. brevis* (Landete et al., 2010) along with confirmation of its activity via construction of isogenic mutant in *Ff. milii* (Chapter 4). Gallate decarboxylase active on hydroxybenzoic acids has been confirmed via both mutant construction and biochemical characterization in *Lp. plantarum* (Jiménez et al., 2013; Reverón et al., 2017). Reductase enzymes are active only on hydroxycinnamic acids with activity of HcrB and Par1 confirmed via inactivity

of mutant strains in *Lp. plantarum* (Santamaría et al., 2018a) and *Ff. milii* (Chapter 3) respectively, while HcrF from *Lm. fermentum* confirmed via biochemical characterization of purified protein (Chapter 5). An additional reductase VprA active on decarboxylated vinyl-derivatives of hydroxycinnamic acids has also been confirmed in *Lp. plantarum* (Santamaría et al., 2018b).

Confirmation of most phenolic acid decarboxylases and reductases via isogenic mutants likely allows for prediction of metabolites when fermenting using strains possessing the genotype, except for *Weissella* genera possessing uncharacterized hydroxycinnamic acid reductases. However, strains specific activity is observed in strains possessing both decarboxylase and reductase enzymes for hydroxycinnamic acid metabolism (Filannino et al., 2015, 2014; Ripari et al., 2019; Sánchez-Maldonado et al., 2011; Chapter 3). *Lactobacillaceae* have been reported to prefer the reductase pathway in certain food fermentations (Filannino et al., 2015; Chapter 4); however, further research is required to better understand the regulation of these competing pathways in food substrates.

Hydroxybenzoic acid esterases (Tannase- EC 3.1.1.20) have been identified only in *Lp. plantarum* with an extracellular tannase TanA biochemically characterized (Jiménez et al., 2014), while activity of intracellular tannase TanB confirmed by deletion mutant (Reverón et al., 2017; Chapter 6). Tannase positive strains can be selected based on the presence of TanB, with TanA rarely encoded by *Lactobacillaceae* (Chapter 2). In case of hydroxycinnamic acid esterases, presence of biochemically characterized enzymes is not sufficient to predict strain behaviour. Only HceP was responsible for the esterase activity in *Lp. plantarum,* with Δ*hceP* strain lacking esterase activity despite possessing two additional hydroxycinnamic acid esterases (Chapter 6). The simple substrates such as methyl esters of hydroxycinnamic acids used for biochemical characterization of enzymes are not reflective of the structural diversity of hydroxycinnamic acid esters in plants.

Also, it remains unknown which plant secondary metabolites are sensed to regulate gene expression in edible plants during fermentation, making accurate prediction of hydroxycinnamic acid esterase activity based on genotype difficult. HceP remains the most likely candidate for hydrolysis with documented activity on hydroxycinnamic acid esters such as chlorogenic acid and rosmarinic acid (Lai et al., 2009; Chapter 6), while enzymes responsible for hydrolysis of glycerol esters of phenolic acids remain unknown. Further studies using isogenic mutants lacking genes encoding different esterases for food fermentations, coupled with proper quantification of diverse hydroxycinnamic acid esters using LC-/MS/MS is required to enable selection of strains based on expected metabolites.

*Lactobacillaceae* encode for multiple glycosyl hydrolase families which likely show specificity towards glycosidic linkages and the attached sugars (Michlmayr and Kneifel, 2014). The few flavonoid glycosidases identified have been tested on limited phenolic substrates. The characterized and uncharacterized glycosidase enzymes may exhibit activity on wide range of phenolic substrates, however, further research is required to confirm their role.

The current literature seems mostly complete for genetic determinants of decarboxylation and reduction of phenolic acids, with separate enzymes specific for hydroxycinnamic acids and hydroxybenzoic acids. Phenolic acid esterases may display overexpression in the presence of both hydroxycinnamic acids and hydroxybenzoic acids (Esteban-Torres et al., 2015; Reverón et al., 2017), but their enzymatic activities are also likely specific for either hydroxycinnamic acids or hydroxybenzoic acids. For other enzymes including esterases and glycosidases, current literature allows for narrowing down to genus and/or species level, but confirmation of activity in actual food systems is still essential.

## 8.3. Metabolic potential of *Lactobacillaceae* in conversion of other plant secondary metabolites

Genes encoding hydroxycinnamic acid esterase Lp_0796 and Est_1092 were downregulated by *Lp. plantarum* in the presence of methyl ferulate and methyl gallate respectively. However, the purified enzyme successfully hydrolyzed those substrates (Esteban-Torres et al., 2015, 2013). In addition, Lp_0796 is also widespread among strains lacking hydroxycinnamic acid esterase activity (Esteban-Torres et al., 2015; Chapter 2). These observations when combined with the result that they are not responsible for hydroxycinnamic acid esterase activity of *Lp. plantarum* TMW1.460 in mMRS broth, indicates towards their potential in hydrolysis of other phenolic esters and plant secondary metabolites.

Similarly, inactive homologs of Par1/Par2 and VprA may also be involved in reduction of other plant secondary metabolites. NADH-flavin dependent reductase Par2 is inactive on hydroxycinnamic acids despite being overexpressed in the presence of sinapic acid (Chapter 3). Homologs of vinyl phenol reductase VprA are also present in several *Lactobacillus* strains which lack phenolic acid decarboxylase despite being considered part of the same pathway (Sánchez-Maldonado et al., 2011; Santamaría et al., 2018a; Chapter 3).

Strains of *Lm. fermentum* reduced cinnamic acid to dihydrocinnamic acid (Koval et al., 2022) despite hydroxycinnamic acid reductases HcrB and HcrF not displaying activity on cinnamic acid (Santamaría et al., 2018b; Chapter 6). Deletion of phenolic acid decarboxylase (Pad) in *Lp. plantarum* NC8 provided evidence of another enzyme displaying a very low decarboxylase activity on hydroxycinnamic acids (Barthelmebs et al., 2000). These activities may be the result of moonlighting enzymes specific for other similar compounds.

Bioconversion of several phenolic compounds without known enzymes has been documented in *Lactobacillaceae. Lactobacillus intestinalis* was reported to produce equol by reduction of diadzein and its metabolites (Heng et al., 2019). Ávila et al. 2009a reported conversion of anthocyanidin to monophenolic metabolites by *Lactobacillaceae* via flavonoid chalcone formation. Similar activity has been documented in *Eubacterium ramulus,* where an NADH-dependent reductase (Fcr) converted flavanones to corresponding dihydrochalcones (Braune et al., 2019). Conversion of patulin to ascladiol by *Lp. plantarum* (Hawar et al., 2013; Wei et al., 2020; Chapter 7) likely also involves a similar reductive cleavage of cyclic ether bonds. While the role of uncharacterized enzymes can not be discounted, known enzymes displaying moonlighting activity may also be responsible for these bioconversion (Jeffery, 2018).

### 8.4. Conclusion and future directions

This research expanded the knowledge on the genetic determinants responsible for hydroxycinnamic acid metabolism by *Lactobacillaceae* explaining the species- and strain-specific metabolic differences. The hypothesis that heterofermentative *Lactobacillaceae* possess novel genes for metabolizing phenolic acids was proved with identification of two novel phenolic acid reductases Par1 in *Ff. milii* and HcrF in *Lm. fermentum*. Use of isogenic mutants lacking hydroxycinnamic acid metabolism genes provides higher confidence on the influence of genetic determinants in metabolite production in food fermentations. Competition experiments between the wild type and mutant strains confirmed the role of phenolic acid metabolism genes to ecological fitness, proving the hypothesis that phenolic acid metabolism genes provide *Lactobacillaceae* with an ecological advantage in a phenolic rich environment.

This research also expanded on the role of different phenolic esterases in *Lp. plantarum* with characterization of a novel hydroxycinnamic acid esterase HceP. Absence of esterase activity in

food fermentations indicates differential regulation of phenolic metabolism genes based on substrate composition and/or substrate specificities. Assessment of the distribution and frequency of characterized phenolic acid metabolism enzymes across all *Lactobacillaceae* genera allowed for determination of ecology and lifestyle-adapted traits. *Lactobacillaceae* also possess a lot of potential for metabolizing various other plant and fungal secondary metabolites owing to numerous bioconversions with uncharacterized enzymes during fermentations, and presence of genes encoding enzymes with unknown activity that are similar to the ones active on phenolic compounds.

The information on distribution of phenolic metabolism genes across *Lactobacillaceae* also allows for easier selection of strains for food fermentations. Cereal fermentations are an attractive proposition for production of bioactive phenolics and functional foods by selectively metabolizing phenolic acids to different end products, proving the hypothesis that strain-specific phenolic acid metabolism of *Lactobacillaceae* allows for production of targeted metabolites in plant fermentations. The isogenic mutants constructed in this study allow for an opportunity to study *in vivo* health benefits of phenolic metabolites in intervention studies. By altering the phenolic concentrations of targeted metabolites in fermented foods, influence of specific compounds can be assessed on gut health independent or in conjunction with the complex dietary composition of fermented foods.

Heterologous expression and biochemical characterization of genetic determinants responsible for metabolism of various phenolic compounds do not always translate to enzymatic activity in food fermentations. In addition, metabolism studied in the laboratory media is not always reflective of the strain behaviour in food fermentations. Therefore, it is recommended that future studies focus on confirming the role of genetic determinants in metabolite production by construction of deletion

mutants and studying them in actual plant fermentations. To better understand the behaviour of strains possessing genes with complementary or overlapping substrate specificities, quantification of gene expression in food substrates is also essential. Due to the diverse composition of phenolic compounds in food substrates, monitoring of a wider range of phenolic metabolites during fermentaion using sensitive analytical tools such as LC-MS/MS is also recommended to pinpoint substrate specificities of esterases and glycosidases.

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